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The effects of prolactin on prolactin receptor gene expression and wool growth in Romney ewes.

Doctor of Philosophy

in
Animal Science at
Massey University

Renata Montenegro
2003
Abstract
The effect of exogenous prolactin on prolactin receptor (PRLR) gene expression and wool growth in pregnant and non-pregnant Romney ewes was assessed. Three experiments were performed where exogenous prolactin was administered by subcutaneous injection (daily for 18 days) or constant infusions (for 3, 9 or 18 days) and endogenous prolactin secretion was altered by exposing ewes to long day or short day photoperiods. Prolactin administration started a week after mating (in autumn), or in non pregnant ewes in mid-spring. Blood samples were collected for measurement of circulating prolactin by radioimmunoassay, skin biopsies were collected for the quantification of PRLR long (PRLR-L) and PRLR short form (PRLR-S) mRNA expression using real-time PCR assay. Wool patch samples were clipped monthly for assessing wool growth.

Constant prolactin infusion of more than 3 days activated a positive feedback mechanism for PRLR-L synthesis, resulting in a sustained elevation PRLR-L mRNA expression for up to 38 days after infusion was over. This was associated with short- and long-term stimulation of wool growth in the pregnant Romney ewe. The main increase in wool production happened after parturition. This positive effect on wool growth by prolactin treatment was related to the length of prolactin treatment. A 3 day infusion resulted in a smaller degree of enhancement compared to the 9 days and 18 days. The biggest impact on wool growth was observed in one of the 18 days infused group, which resulted in a 25% increase in clean fibre production when compared to the pregnant group. The expression of PRLR-S mRNA was not associated with an elevation of prolactin levels. Daily injections neither increased PRLR-L mRNA expression nor increased wool growth, demonstrating that a constant and moderate increase in prolactin levels is necessary to stimulate PRLR synthesis.

Data obtained in these trials also suggests that other reproductive hormones may influence PRLR expression and wool growth. The non-pregnant groups showed steady
levels of PRLR-L mRNA expression, which could be associated with changes in hormonal levels due to the reproductive cycle. Seasonal molecules could also interfere with the system, as prolactin manipulation in non-pregnant ewes exposed to an artificial short day environment during spring time showed a different pattern of PRLR-L and PRLR-S mRNA expression and no wool growth effect.

A mathematical model of prolactin/PRLR interaction was shown to be a good predictor of short-term PRLR gene expression, as its simulations agreed with our biological data. However, the inclusion of other gestational and seasonal hormones may be necessary if the model is to be used for simulations of long-term PRLR expression and wool growth during pregnancy and lactation.

Overall, these results suggest that seasonal wool growth can be manipulated via prolactin, which increases PRLR-L mRNA expression resulting in enhancement of wool growth. However, there is a minimum period of constant prolactin elevation necessary to activate this positive feedback mechanism. Also there is a window of opportunity where this mechanism can be manipulated. This window is most likely associated with the animals interpretation of photoperiod, which also regulates the reproductive seasonality and therefore, could as well interact with prolactin in the regulation of PRLR mRNA expression and seasonal wool growth. This observation could lead to the development of products, suitable for on farm conditions, to enhance wool production.
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List of Abbreviations:

aa  Amino acids
BSA  Bovine serum albumin
CFW  Clean fleece weights
CIDR  Controlled internal drug release
CT  Cycle threshold
DEPC  Diethyl Pyrocarbonate
DP  Dermal papilla
DPAR  Pre-development assay reagent
DTT  Dithiothreitol
ECD  Extracellular domain
EDTA  Ethylenediaminetetraacetic acid
FFD  Fleece fibre diameter
FW  High fleece weight Romney
FY  Fleece yield
GABA  Gamma aminobutyric acid
GAP  Gonadotrophin releasing hormone-associated peptide
GAPDH  Glyceroldehyde-3-phosphate dehydrogenase
GFW  Greasy fleece weight
GH  Growth hormone
GHR  Growth hormone receptor
hCG  Human chorionic gonadotropin
ICD  Intracellular domain
IRS  Inner root sheath
JAK 2  Janus kinase 2
LD  Long photoperiod
LIC  Livestock Improvement Corporation
MAPK  Mitogen-activated protein kinase
MCR  Metabolic clearance rate
ND  Natural photoperiod
NRS  Normal rabbit serum
ORS  Outer root sheath
PBS  Phosphate buffered saline
<table>
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<tr>
<td>PMSG</td>
<td>Pregnant mare serum gonadotropin</td>
</tr>
<tr>
<td>PRLR</td>
<td>Prolactin receptors</td>
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<tr>
<td>PRLR-L</td>
<td>Prolactin receptor long form</td>
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<tr>
<td>PRLR-S</td>
<td>Prolactin receptor short form</td>
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<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
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<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
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<tr>
<td>RT</td>
<td>Reverse transcriptase reaction</td>
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<tr>
<td>SAS</td>
<td>Sheep anti-rabbit serum</td>
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<tr>
<td>SCG</td>
<td>Superior cervical ganglion</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus</td>
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<tr>
<td>SD</td>
<td>Short photoperiod</td>
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<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TE</td>
<td>Tris/EDTA</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotropin-releasing hormone</td>
</tr>
<tr>
<td>UBEI</td>
<td>Ubiquitin activating enzyme 1</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>WRONZ</td>
<td>Wool research organization of New Zealand (Inc)</td>
</tr>
<tr>
<td>NIDDK</td>
<td>National institute of diabetics &amp; digestive &amp; kidney diseases</td>
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CHAPTER 1

GENERAL INTRODUCTION
The earth rotates around the sun with a 23-degree tilt from its plane of orbit. The resulting difference in sun exposure between the hemispheres causes seasonal changes in daylength and temperature. The greater the distance from the equator, the greater the difference in seasonal daylength and temperature. As a consequence, organisms at higher latitudes have to be able to survive severe environmental changes. Some adapt through a dormant phase in their life cycle (for example: hibernation in mammals, metamorphoses in insects, or parasite eggs that are resistant to climate changes). Other strategies include the adjustment of reproductive cycles and metabolic rate to seasonal climate changes.

Many species change their outer coat to survive the temperature variation and to blend in with the environment as camouflage from predators. The Mouflon sheep, believed to be a common ancestor of modern sheep breeds, sheds its fleece once a year in spring (Ryder, 1973). These adaptations are triggered by seasonal environmental cues. One of the most influential environmental cues is the change in the amount of light observed in a day, or photoperiod. While domestic sheep breeds do not shed, they exhibit a seasonal cycle of wool growth. Production is high during the summer months and low during the winter (Story and Ross, 1960; Ryder, 1973; Hawker and Littlejohn, 1986; Sumner et al., 1998).

Prolactin, a protein hormone produced by the pituitary gland, displays a seasonal pattern of secretion and has been associated with the seasonality of wool production (Lincoln and Ebling, 1985; Lincoln, 1990). The photoperiod-associated decrease in fibre growth observed during the winter months is further depressed by gestation (Stevens and Wright, 1951; Sumner and McCall, 1989; Parker et al., 1991). This seasonal depression in wool growth is accompanied by a decrease in fibre diameter (Pearson et al., 1999) which has a negative impact on fibre value by increasing susceptibility to breakage during processing.
For the last ten years, AgResearch Ruakura and Massey University have been studying the physiology of fibre growth in a number of species including the seasonal wool growth of domesticated sheep breeds. In the shedding Wiltshire sheep breed, Pearson et al (1996) and Pearson et al (1997) were able to demonstrate an association between short-term reductions in wool follicle growth activity with naturally and experimentally-induced surges of prolactin. As part of these studies, Kendall (1999) conducted a series of experiments with pregnant and lactating Romney ewes with the objective of determining if there was an association between the circulating prolactin profile and wool growth. His results suggested that the pre-partum surge in prolactin concentration was associated with a medium- to long-term positive effect on the subsequent wool growth. A high peak and a gradual decline of the pre-partum prolactin concentration seemed to be associated with this enhancement of fibre growth.

If natural circulating prolactin levels have a medium- to long-term effect on wool growth (Kendall, 1999) and if exogenous prolactin was able to influence follicle activity in a shedding breed (Pearson et al., 1997) it is possible that the seasonal wool growth of commercial wool breeds could be altered by using exogenous prolactin. For example, manipulating prolactin levels in early pregnancy might prevent the wool decline observed during mid- and late-gestation, thus enhancing overall wool growth. The ability to inhibit the decline in wool production of lambing ewes would be of major economic benefit to the farmers. The wool processing industry would also benefit, since increased wool growth is also associated with an increase in the minimum winter fibre diameter. However, hormonal manipulation is expensive and opposed by many consumers. By understanding the mechanism by which prolactin influences wool growth and wool characteristics it might be possible to manipulate this system through a non-hormonal product.

The biological effect of prolactin is caused by its interaction with a specific transmembrane receptor. Dr. Vern Choy, at Ruakura, has previously demonstrated the
existence of prolactin receptors (PRLR) in the dermal papilla (DP), germinal matrix (GM) and outer root sheath (ORS) of the wool follicle of shedding Wiltshire sheep (Choy et al., 1995; Choy et al., 1997). It has also been shown that changes in circulating prolactin levels regulate the expression of PRLR in the skin of Wiltshire sheep (Nixon et al., 2002). This relationship suggested that cyclic wool growth responses to the hormone signal is governed not so much by the concentration of circulating prolactin as by the abundance of receptors. A similar mechanism might also apply to seasonal variation in continuous wool growth of the Romney sheep; however no information in PRLR expression in this breed is available. Therefore, understanding the mechanisms of PRLR expression, regulation and signalling within wool follicles may suggest new options for the stimulation or inhibition of specific pathways within this system.

The study of receptor kinetics, regulation and signalling is complex. It involves “cross-talk” between a number of circulating and cytoplasmic molecules. To guide further experiments in this area, a mathematical model to predict PRLR gene expression in response to known physiological signals would be of major interest. Such a model is being developed by Dr. Tanya Soboleva and Dr. Kumar Vetharaniam of the Mathematical Modelling group at AgResearch Ruakura. But model predictions relevant to wool growth have to be verified by biological data, which were not available in the literature.

Building on this previous history of discovery outlined above, the aims of this research were to:

- Develop real-time (Polymerase chain reaction) PCR assay for measurement of PRLR gene expression in sheep skin;
- Determine the role of circulating prolactin levels on the regulation of PRLR expression and its association with wool growth patterns;
• Evaluate if prolactin manipulation in early pregnancy influences subsequent wool growth in the lambing ewe;

• Determine an appropriate delivery route and profile of exogenous prolactin to generate a wool growth response;

• Generate biological data for verifying a mathematical model of the prolactin-PRLR system in the skin.

The research program was conducted from April 1999 until November 2002 and consisted of three animal experiments, involving pregnant and non-pregnant Romney ewes. Circulating prolactin was manipulated using photoperiod, prolactin injections and continuous infusions. The experimentally determined changes in PRLR gene expression are contrasted and discussed in relation to the predictions of the mathematical model. The desired outcome was a new framework around which wool growth responses to prolactin can be interpreted and tested.
CHAPTER 2

REVIEW OF LITERATURE
Introduction

The sheep industry in New Zealand still plays an important role in the country's economy. The sheep population is around 40 million animals, generating 30 million lambs per year, which are mainly sold on international markets (Ministry of Agriculture and Forestry, 2003). New Zealand is the second largest wool exporter in the world, selling 150,000 tonnes of wool in 2002. However, despite wool having excellent properties, such as high insulation, breathability and being non-flammable, the industry has been declining, partly due to problems with processing. Being a natural fibre, wool growth and quality are not consistent, creating the need for costly and complex processing with significant losses resulting from fibre breakage. Synthetic fibres have a constant diameter and strength leading to efficiency during processing and lower costs to the consumer. Consequently, one of the areas of interest of wool physiology research is to minimise the wool breakage and enhance production efficiency in order for wool to compete more effectively with synthetics.

What underlies this variation in natural fibre growth and characteristics? Bissonnette (1935) noted that the timing of the pelage change of ferrets was related to the seasonal variation of daylength. Prolactin, a pituitary hormone, also has a regulated secretion in response to changes in the photoperiod (Tamanini et al., 1988; Grasselli et al., 1992). This seasonal change in prolactin secretion has been associated with the shedding of the pelage in mink (Martinet et al., 1984), hamsters (Duncan and Goldman, 1984a; Duncan and Goldman, 1984b), deer (Thomas et al., 1994), goats (Dicks et al., 1994), shedding sheep (Pearson et al., 1996; Pearson et al., 1997); and also the seasonal variation of wool growth of non-shedding sheep (Kendall, 1999).

The first associations of seasonal wool growth with photoperiodic hormonal changes were made by Coop and Hart (1953) and Hart (1955) at Lincoln College in New Zealand. The manipulation of wool growth using artificial changes in the daylength was
subsequently described by Hart (1961) and Hart et al (1963). Since then, the effect of seasonality and prolactin on wool growth on a number of sheep breeds has been established. More recently, the elevation of prolactin during pregnancy and at parturition was also shown to have a positive medium- to long-term effect on wool production and quality on Romney ewes (Kendall, 1999).

Accepting that prolactin is one of the factors responsible for variation in wool growth and quality, the question is how does this physiological system works? Choy et al (1995) demonstrated the existence of prolactin receptors (PRLR) in the wool follicle of the Wiltshire sheep; hence, allowing for a direct effect of prolactin on wool follicles. Prolactin receptors belong to the class 1-cytokine receptor family (Bole-Feyset et al., 1998; Freeman et al., 2000). Interestingly, prolactin itself has been shown to regulate the expression of its own receptor in many tissues (Rosa et al., 1982; Barash et al., 1986; Feng et al., 1998; Bowen et al., 2000) including the skin (Nixon et al., 2002). In the latter study, the changes in PRLR expression in response to prolactin were associated with the timing of follicle activity in Wiltshire sheep. Consequently, it is possible that PRLR regulation is also linked to the seasonal regulation of wool growth in the non-shedding sheep breeds.

With these concepts in mind, the hypothesis of this study was that the manipulation of circulating prolactin would modify PRLR population in the follicles, regulating its expression and signalling, resulting in changes in wool growth and wool characteristics.

This review will briefly describe the prolactin molecule, its production, seasonal secretion and its main biological effects, with emphasis on its role on fibre growth. The actions of prolactin through gestation and lactation and the relationship to other reproductive hormones will be described in domestic sheep breeds. The manipulation of circulating prolactin levels is summarised in a number of different species, including its kinetics, clearance rate and half-life. A review on the PRLR distribution, isoforms, expression and signalling in sheep and other species concludes this chapter.
2.1 Prolactin: its production and secretory regulation

2.1.1 Prolactin

Prolactin is a polypeptide hormone produced by the lactotrophic cells in the anterior pituitary gland. It was first isolated by Stricker and Grueter (1928) from a pituitary extract and identified as a substance capable of stimulating lactation in rabbits. Riddle et al (1933) confirmed the lactational role of prolactin and also described its ability to stimulate the growth of the crop sac of pigeons. Today, prolactin is known for having more than 300 biological activities in a variety of species (for reviews see (Bole-Feysot et al., 1998; Freeman et al., 2000).

The functional protein is composed of 197-199 amino acids with a molecular weight of ~ 23-kDa (Freeman et al., 2000). However, different molecular sizes exist due to alternative splicing, proteolytic cleavage (Corbacho et al., 2002; Romao et al., 2002) or post-translational modification, including glycosylation and phosphorylation (Gambino et al., 1999; Bollengier et al., 2001; Walker, 2001). The biological function of these variants is unknown but it appears that they generally have reduced bioactivity (Brooks and Saiduddin, 1998). Nevertheless, the ratio between the 23-kDa protein and these isoforms could play an important role in the modulation of prolactin actions.

The hypothalamus exerts an inhibitory effect on circulating prolactin (Ben-Jonathan, 1985; Lamberts and Macleod, 1990), as the secretion of prolactin from the anterior pituitary gland is spontaneous in the absence of hypothalamic influence. Dopamine, which is produced in the tuberoinfundibular neurones, has been shown to be the major inhibitory agent of prolactin synthesis and release (Birge et al., 1970; MacLeod et al., 1970; MacLeod and Lehmeyer, 1974; Donnelly and Dailey, 1991). Other inhibitory factors include gamma aminobutyric acid (GABA), gonadotrophin releasing hormone-associated peptide (GAP) and somatostatin (Schally et al., 1977; Kimura et al., 1986; Loeffler et al., 1986; Yu et al., 1988; Lamberts and Macleod, 1990).
In contrast, oestrogen stimulates the synthesis, storage and secretion of prolactin as well as the proliferation of lactotrophs (Augustine and Macleod, 1975; Kiino and Dannies, 1981; Takahashi, 1992). Seratonin, thyrotropin-releasing hormone (TRH) and vasoactive intestinal peptide (VIP) also increase prolactin synthesis and release (Grosvenor and Mena, 1980; Frawley and Neill, 1981; Abe et al., 1985; de Greef et al., 1987; Sinha and Jacobsen, 1987; Thomas et al., 1988; Robinson et al., 1996).

2.1.2 Seasonal secretion

Prolactin has a seasonal pattern of secretion, being high during the summer months and low during winter (Pelletier, 1973; Rhind et al., 1991). These seasonal changes in circulating prolactin levels are, to a greater or lesser extent, dependent on pineal melatonin secretion and responsible for some of the physiological adaptations many animals experience during the year. Melatonin secretion is also dependent on the light-dark cycle (Axelrod, 1974) and therefore important for the animal's recognition of the seasonal light changes (Kennaway et al., 1987). The secretion of melatonin is mediated by the light falling on the retina and generating a signal which travels via the retinohypothalamic tract to the suprachiasmatic nucleus (SCN). The signal then travels through the paraventricular nucleus (PVN) to the superior cervical ganglion (SCG). Neurones from the SCG project to the pineal gland stimulating the synthesis and release of melatonin from pinealocytes (Kennaway et al., 1987). The duration of melatonin secretion is important in regulating seasonal prolactin secretion (Ebling and Foster, 1989; Kusakari et al., 1991). Furthermore, Rose et al (1985) have found evidence that melatonin inhibits prolactin secretion; corroborating the hypothesis that melatonin is important for the seasonal variation of prolactin.

2.1.3 Extrapituitary production

Although the anterior pituitary is the main site for prolactin production, other tissues have been shown to produce prolactin-like hormones. These include the intestine,
pregnant uterus, lymphocytes, mammary gland, prostate, skin and sweat glands (Bigazzi et al., 1979; Walker et al., 1989; Swarlo-Santo, 1992; Tseng et al., 1992; Ben-Jonathan et al., 1996; Freeman et al., 2000; Craven et al., 2003b). The local production of prolactin indicates that prolactin could act as a paracrine and/or an autocrine-signalling molecule, modulating the numerous reported effects of pituitary-derived prolactin in different tissues. However, the mechanisms regulating extrapituitary prolactin synthesis are still not known.

2.2 Physiological concentrations of prolactin in sheep

In sheep, physiological prolactin levels fluctuate between 2 and 800 ng/ml. This variation is due to prolactin being a seasonal hormone that also varies widely with different physiological states such as pregnancy and lactation. In autumn, the prolactin levels of non-pregnant ewes fall reaching a winter nadir (10-30 ng/ml). Circulating prolactin then commences to rise, especially after the vernal equinox, reaching a peak in late spring (80-150 ng/ml) (Pearson et al., 1996; Kendall, 1999). This circannual pattern is due to alterations in the pulses and diurnal pattern of prolactin secretion (Rhind et al., 1991), regulated by changes in the photoperiod and temperature (Tamanini et al., 1988; Grasselli et al., 1992). Both high temperatures (Wettermann et al., 1982; Howland et al., 1983); and long days (16 h light : 8 h dark) elevate prolactin concentration, while low temperatures (Wettermann et al., 1982; Howland et al., 1983) and short days (8 h light : 16 h dark) reduce circulating levels (Pelletier, 1973; Poulton and Robinson, 1987).

In the pregnant ewe, seasonal variation of prolactin is present but is enhanced by gestation and lactation. During the first 100 days of gestation, plasma prolactin levels vary from 8 to 50 ng/ml (Kelly et al., 1974; Munro et al., 1980; Fitzgerald et al., 1981; Kendall, 1999). When compared to the non-pregnant ewe, prolactin concentration is slightly, but significantly, lower in the pregnant ewe suggesting that other reproductive
hormones, such as placental lactogen or progesterone, could be responsible for suppressing prolactin production and/or release (Kendall, 1999). In fact, in the pregnant rat, placental lactogen seems to suppress prolactin secretion (Grattan, 2002).

According to Kelly et al (1974), Peterson et al (1990) and Kendall (1999), prolactin level rises 1-2 days prior to lambing, reaching peaks of 350 to 600 ng/ml at parturition. Through lactation prolactin level ranges from 20-270 ng/ml (Lamming et al., 1974; Kendall, 1999). Suckling causes rises in prolactin levels of the lactating ewe reaching peaks of 800 ng/ml in early lactation and 400 ng/ml in mid-lactation (Lamming et al., 1974).

### 2.3 Physiological effects of prolactin

Since the first description of the lactogenic action of prolactin in rabbits (Stricker and Grueter, 1928), this hormone has been implicated in over 300 biological functions (for review see (Bole-Feysot et al., 1998; Freeman et al., 2000). By regulating the humoral and cellular responses (Buskila and Shoenfeld, 1996; Neidhart, 1998), prolactin plays an important part in the integration of the network formed by the endocrine, neurological and immune system, necessary for the maintenance of healthy individuals (Goffin et al., 1998).

In the luteal function, depending on the species and stage of the oestrus cycle, prolactin can either be luteotrophic or luteolytic. The luteotrophic effects occur following mating (Morishige and Rothchild, 1974) by inhibiting 20-α hydroxysteroid dehydrogenase, an enzyme that inactivates progesterone (Freeman, 1994). On the other hand, prolactin stimulates apoptosis in the corpus luteum when pregnancy is not established (Kiya et al., 1996; Kanuka et al., 1997).

Prolactin acts in the growth and development of the mammary gland by stimulating ductual side branching and lobuloalveolar development during pregnancy (Brisken et al., 1999). It has also been associated with milk synthesis stimulation (Nelson and
Gaunt, 1936) and maintenance of lactation in many species (Bintarnihsih et al., 1958; Cowie et al., 1969).

The major behavioural effect of prolactin is the onset of maternal instincts (Bridges et al., 1985), but it has also been implicated in the migration of birds (Silverin et al., 1989) and amphibians (Iwata et al., 2000), libido (Sumiya et al., 1991) and the sleep cycle (Roky et al., 1993).

Prolactin also has an osmoregulatory role in influencing the sodium and potassium transportation in the mammary gland (Falconer and Rowe, 1977; Falconer et al., 1983) or the transfer of sodium and chloride across the intestinal membranes (Mainoya, 1975a; Mainoya, 1975b).

2.4 The effect of prolactin on hair growth

2.4.1 Follicle structure

The hair follicle is composed of distinctive cell populations of mesenchymal and ectodermal origin. The mesenchymal cells constitute the DP at the base of the follicle and the connective tissue surrounding the follicle cells from the embryonic ectoderm form most of the structures in the interior of the follicle (For review see (Hardy, 1992). Figure 1 A illustrates the anatomy of the hair follicle with its different components. The bulb region contains undifferentiated proliferating cells, which migrate upwards as they differentiate into keratin producing cells. At the core of the follicle are the medulla, cortex and cuticle layers, forming the mature hair that extrudes from the skin. Outer concentric layers include the inner root sheath (IRS), composed of Huxley’s and Henle’s layers. The inner root sheath is believed to be important for the mould and shape of the hair. The ORS is the outermost layer of the follicle to which the arrector pili muscle is attached. Also, at this location, a bulge of slow cycling stem cells is present and thought to be important in follicle cycling (Cotsarelis et al., 1990; Wilson et al., 1994). PRLR have been identified in the Wiltshire wool follicle (Choy et al., 1995). Figure 1 B shows
PRLR expression in the ORS, DP and GM (Choy et al., 1997) suggesting that these cells could mediate changes in fibre growth related to seasonal changes in circulating prolactin.

Figure 1: (A) Anatomy of the hair follicle (Rufaut et al., 1999). (B) PRLR localization in the Wiltshire sheep follicle (Choy et al., 1997).

2.4.2 The hair cycle

Hair and wool follicles have a cycle of growth that can be divided into four periods (Figure 2): a growth initiation period or proanagen, a growth period or anagen, a regression period or catagen and a resting period or telogen (Chase et al., 1951; Ryder, 1969; Parry et al., 1995).
Each of these stages can be morphologically divided into sub-phases. For a detailed description of the morphological changes observed in the prolactin-induced wool follicle growth of the Wiltshire sheep see (Parry et al., 1995).

The fibre length produced by particular follicle populations, for example, scalp, eyebrow or body hair, depends on the rate of growth and the time the follicle stays in the anagen phase (Paus and Cotsarelis, 1999).

2.4.3 Influence of prolactin on follicle cycling

Prolactin is able to influence the follicle growth cycle. In shedding animals, prolactin seems to influence the cycle by synchronising the follicles into a specific growth phase, resulting in shedding. In the Wiltshire sheep, for example, changes in the gene expression of molecules important for the cell cycling, RNA splicing and structural
formation of the hair follicle are linked to growth cycling induced by changes in circulating prolactin (Rufaut et al., 1999). Nevertheless, the effect that prolactin might have on the hair follicle cycle can be associated with the reproductive status, such as pregnancy and lactation, resulting in non-seasonal shedding; or related to the seasonal prolactin fluctuation resulting in seasonal molts.

### 2.4.3.1 Non-seasonal animals

Mice show non-seasonal synchronised waves of hair growth, which are related to the age of the animal and reproductive status (Craven et al., 2001). The intervals between coat changes become longer as the animals mature (Ahmad et al., 1998). Depending on the strain of mice, the first pelage change occurs at 22 to 28 days of age. The following molts are also largely age-dependent but influenced by systemic or local stimuli (Stenn and Paus, 2001). The exact mechanism of fibre development and cycling is still unclear, however studies in mice have identified some molecules important to the process, for example, insulin-like growth factor-1 (Danilenko et al., 1996; Stenn et al., 1996), fibroblast growth factor-7 (Guo et al., 1996), fibroblast growth factor 5 (Hebert et al., 1994), androgens (Hibberts et al., 1998) and oestrogen (Oh and Smart, 1996).

Prolactin and PRLR are expressed in the IRS and ORS of the mice hair follicle and their mRNA expression change during the course of the hair cycle (Craven et al., 2001; Foitzik et al., 2003). Prolactin expression is up-regulated during anagen, when the length of the IRS is increasing. PRLR expression, on the other hand, is down-regulated at the start of anagen but by the end of this growth stage, its expression is up-regulated (Foitzik et al., 2003). In experiments using PRLR knockout mice, Craven et al (2001) demonstrated that the absence of prolactin signalling advanced the transition from telogen to anagen, inferring that prolactin has an inhibitory effect on fibre growth. This was confirmed by further knockout mice studies and skin organ culture, which also indicated that prolactin delays anagen. At high doses, prolactin induces catagen
accompanied by a down-regulation of keratinocyte proliferation (Foitzik et al., 2003). Placental lactogen is also locally produced in a hair cycle-dependent manner in mice, peaking during anagen and rapidly declining afterwards (Foitzik et al., 2003). Therefore, it is also a possible candidate for further investigation into the control of non-seasonal hair moulting.

Human hair growth is largely a non-seasonal and non-synchronised process, and moulting occurs in a mosaic pattern (Stenn and Paus, 2001). As in other species, there are numerous types of hair follicles, which have their own rhythms of growth and can be influenced by different factors. Known regulatory molecules include androgens, oestrogen, growth hormone (GH), corticosteroids, and prolactin (Paus and Cotsarelis, 1999; Stenn and Paus, 2001). Male subjects with hyperprolactinaemia can develop abnormal sparse body hair (Walsh and Pullan, 1997) and females can show hair loss (telogen effluvium, dystrophic hair and hypertrichosis) under circumstances where prolactin could be implicated (Orfanos and Hertel, 1988).

2.4.3.2 Seasonal animals

Shedding animals: To survive environmental changes, species like mink, fox, hares, small rodents, deer and goats produce two different coats: a thinner summer coat and a denser winter coat, sometimes of a different colour. Bissonnette (1935) noted that the timing of these pelage changes in ferrets was related to daylength. With the evidence that prolactin is secreted in a seasonal manner (Pelletier, 1973; Rhind et al., 1980; Rhind et al., 1991), the association between the pelage changes and alterations in circulating prolactin was proposed and today prolactin is thought to be the major hormone involved in the control of seasonal fibre moulting.

In mink, the Northern Hemisphere spring moults starts in mid-April in the rostral area working though to the caudal area. The autumn moults starts in late August and it has a caudal to rostral orientation (Martinet et al., 1984). In this species prolactin has a
primary role as the trigger to initiate the seasonal coat changes. Blocking the prolactin rise during spring delays, but does not prevent, the spring moult (Martinet et al., 1982; Rose et al., 1998) and a decline in prolactin levels is associated with the onset of winter coat growth (Duby and Travis, 1972; Rose et al., 1987). Melatonin, which suppresses prolactin secretion, is necessary for triggering the autumn shedding and onset of winter hair development, but is not necessary for further growth and maturation (Valtonen et al., 1995), giving further evidence of the timing role prolactin may have on the seasonal coat changes. Nixon et al (1995) showed the importance of daylength and melatonin in the seasonal fibre growth of ferrets and implicated the reproductive hormones in the divergent growth patterns of males and females, corroborating the findings by Rust et al (1965) of an induced out-of-season lactation resulting in an out-of-season moult.

In the Djungarian hamster prolactin is also the trigger for the changes from summer to winter coat. Duncan and Goldman (1984a) and Niklowitz and Hoffmann (1988) were able to manipulate the summer pelage growth by exogenous manipulation of prolactin. However, in male Djungarian hamsters, testosterone was shown to be essential for the complete change to the winter coat (Duncan and Goldman, 1984b), demonstrating the importance of hormonal interactions on the fibre growth process. In the Meadow vole, prolactin injections in animals maintained in short days blocked the winter pelage change. Furthermore, the changes in prolactin levels were linked to the seasonal coat replacement, rather than constantly elevated or depressed levels (Smale et al., 1990). This same regulatory effect was observed in the blue fox (Smith et al., 1987) and in the racoon (Xiao et al., 1996). Siberian hamsters also seem to have their hair cycle related to the seasonal changes in prolactin levels (Anchordoquy and Lynch, 2000).

Exogenous prolactin infusion into the epidermis of female red deer was responsible for an advance in summer coat development in the infused area (Thomas et al., 1994). Webster and Barrell (1985) also demonstrated that a decline in prolactin levels advanced the growth of the winter primary follicles. However, nutrition and lactation
influenced the sensitivity of the response to the prolactin variation (Heydon et al., 1995). Changes in prolactin concentration also influenced the timing of follicle activity and moulting of cashmere goats (Dicks et al., 1994) and the Saanen dairy goat (Gebbie et al., 1999).

A number of mammals moult once a year including seals (Berta and Sumich, 1999), squirrels, coyote, red fox, black bear (Ling, 1970) and sheep (Ryder, 1965; Lincoln, 1990). In the shedding sheep, the moult occurs in spring and is dependent on the increasing photoperiod (Lincoln, 1990). This allows the animals to grow their new coat during the summer, when sufficient forage is available prior to the onset of winter. The shedding sheep breeds mostly studied are the Mouflon, Soay and Wiltshire sheep. The Mouflon is thought to be one of the ancestors of the European domesticated sheep. It sheds during spring and after the moult, follicle activity reaches its highest during late summer and early autumn (Ryder, 1973). These changes are associated with the seasonal cycle in prolactin concentration (Lincoln, 1998). In the Soay sheep the seasonal horn and pelage growth are also related to the increase in prolactin levels during spring (Ryder and Lincoln, 1976; Lincoln, 1990). However, there is a difference in the timing of the new fibre growth between males and females, with rams commencing the fibre production one month earlier than the ewes (Ryder, 1971). This indicates that, as in some other mammals that have synchronised coat changes, reproductive hormones associated with pregnancy and lactation might interact with prolactin in the regulation of seasonal fibre growth.

The regulatory mechanisms of the spring moult of the Wiltshire sheep (Ryder, 1969) has been extensively studied by the Ruakura group. Like the other two shedding breeds, prolactin elevation due to increasing photoperiod is responsible for fleece shedding in late spring and subsequent coat replacement (Parry et al., 1995; Pearson et al., 1996). Using melatonin treatment to suppress the spring elevation of prolactin, researchers were able to prolong the follicle activity of Wiltshire (Parry et al., 1995) and Soay sheep
(Lincoln and Baker, 1995). Later confirmation of this theory came with the work by Pearson et al (1996) where elevation of plasma prolactin level was associated with the synchronisation of the follicle growth cycle. In the Wiltshire sheep, a rise in plasma prolactin concentration is linked to follicle regression (catagen) followed by a short resting phase (telogen). The late-spring “subsidiary follicle growth” cycle, originally noted by (Ryder, 1969) is in fact a direct consequence of the prolactin surge at this time (Pearson et al., 1996). Subsequently, the follicles enter a synchronised proanagen culminating with the shedding of the previous fleece.

Non-shedding sheep: Sheep were one of the first species to be domesticated by man. During the domestication process, animals were selected for high wool production. Over the centuries, sheep breeds selected for wool growth have lost their ability to synchronise their follicle activity in response to prolactin variation, thus they do not moult. Nevertheless, these breeds still show a seasonal pattern of wool growth, which has been associated with seasonal prolactin secretion (Ryder and Stephenson, 1968a; Wuliji et al., 1993; Woods et al., 1995; Kendall, 1999). Wool growth rates in the different breeds respond differently to the seasonal changes in photoperiod. The comparison of wool growth rates between Romney, Coopworth, Perendale, Corriedale and Merino shows that the Coopworth sheep have the greatest seasonal variation of wool growth and that the Merino shows the least variation (Sumner, 1979; Wilkinson et al., 1986; Lincoln, 1990).

The high fleece weight (FW) Romney line of the Massey University selection flocks display a production of greasy fleece weight 25% higher than the control line (Blair et al., 1985). However, wool production was only statistically higher in the FW line during the spring and summer months (Kelly et al., 1993). Similarly, during spring and summer, plasma prolactin levels were statistically higher in the FW line, suggestive of a potential involvement of prolactin in the seasonal fibre growth of non-shedding domesticated sheep breeds. However, there are conflicting data in this area. For
example, treatment of domestic sheep breeds with bromocryptine at different times of the year have shown no effect on wool growth (Dolling et al., 1986; Curlewis et al., 1991; McCloghry et al., 1993). Also, in Romney sheep, the administration of exogenous prolactin in hypophysectomised animals (Ferguson et al., 1965) or prolactin infusion into the skin (Kelly et al., 1993) had no effect on wool growth. On the other hand, Kendall (1999) manipulated circulating prolactin levels by using bromocryptine and/or artificial photoperiod and was able to depress or stimulate wool growth in Romney ewes at different times of the year and of differing physiological status. These discrepancies in the literature could be explained by the fact that prolactin only works as a trigger to some seasonal physiological effects, therefore, the timing and duration of prolactin manipulation is crucial for a biological response. Also, the hypophysectomised animals would have other hormonal system disrupted, which could also explain the lack of biological function of prolactin treatment.

2.5 Reproduction and wool growth

In commercial sheep breeds, pregnancy has a further negative impact on winter wool growth (Stevens and Wright, 1951; Seebeck and Tribe, 1963; Ross, 1965; Armstrong and O'Rourke, 1976). The reduction in annual fleece production of the pregnant ewe ranges from 11 to 24% in relation to non-pregnant animals (Turner et al., 1968; Reid, 1978; Sumner and McCall, 1989; Masters et al., 1993). However, during lactation, wool production increases (Betteridge et al., 1992; Masters et al., 1993) and at weaning there is no difference in the wool growth rate between the lambed and the non-pregnant ewe (Parker et al., 1991). Furthermore, after lactation, ewes that produced a single lamb grow more wool than the ewes that were barren (Lee and Atkins, 1995).

Dividing the period of gestation into early, mid and late pregnancy, there is little evidence of wool depression in the first 50 days of gestation (Reid, 1978; Lee and Atkins, 1995). These data were obtained by measuring wool weights, which do not
closely reflect follicle growth rates because of the time lag necessary for the fibre to be extruded from the skin surface. Pearson et al (1999), using autoradiography, showed that the wool growth depression occurs as early as days 21-35 of pregnancy while the wool weight difference was only observed on day 60.

In early pregnancy, prolactin (Kelly et al., 1974; Lamming et al., 1974; Fitzgerald et al., 1981; Kendall, 1999), progesterone (Bassett et al., 1969; Stabenfeldt et al., 1972; Boulfekhar and Brudieux, 1980), oestrogen (Challis, 1971), growth hormone (Blom et al., 1976; McMillen et al., 1987) and cortisol (Cox, 1975) are all at low levels and are unlikely to be involved in this early gestational depression of wool growth. From day 50 onwards, in mid-pregnancy, the decline in wool production in comparison to non-pregnant ewes becomes obvious. Parker et al (1991) showed that ewes kept in grazing conditions produced 14% less wool during days 57-90 of gestation than the non-pregnant control ewes. In pen-feeding conditions wool growth declined from the third month of gestation (Henderson et al., 1970). At this time, progesterone levels are increasing (Bassett et al., 1969; Tsang and Hackett, 1979), placental lactogen is about to peak (Kelly et al., 1974; Butler et al., 1981; Kappes et al., 1992) and prolactin levels became slightly, but statistically lower than in non-pregnant ewes (Kendall, 1999). Therefore, at this stage, these hormones could have an impact on the wool growth of the pregnant ewe. Progesterone, however, does not appear to have any influence on the wool growth of sheep (Slen and Connell, 1958) or hair growth of rats (Mohn, 1958) and mice (Davis, 1963). Placental lactogen, on the other hand does influence the wool follicle formation of new born lambs by suppressing the development of secondary follicles (Wickham et al., 1992). As placental lactogen can act via the growth hormone (Emane et al., 1986) or prolactin (Noel et al., 2003) receptors, it is therefore a candidate for the wool depression. The lower prolactin levels at this stage might also contribute to the wool depression, since pregnant animals kept in long day photoperiod had a higher
prolactin profile than the pregnant ewes kept in natural days and showed less decline in wool growth rate during winter lambing (Kendall, 1999).

The largest reduction in wool production in the gestating ewe occurs during late pregnancy (Reid, 1978; Sumner and McCall, 1989; Betteridge et al., 1992; Kendall, 1999). At this stage oestrogen (Challis, 1971), growth hormone (Blom et al., 1976; McMillen et al., 1987) and cortisol (Bassett and Thorburn, 1969; McNatty et al., 1972; Chamley et al., 1973) increase. All three hormones have been shown to depress wool growth (Slen and Connell, 1958; Wallace, 1979; Wynn et al., 1988).

During early lactation, wool production rate is lower when compared to non-lactating ewes (Story and Ross, 1960; Oddy, 1985; Betteridge et al., 1992); although radiolabelled-fibre data suggests that wool growth increases rapidly from parturition (Pearson et al., 1999). As lactation advances, wool growth increases (Masters et al., 1993). Prolactin (Lamming et al., 1974) and growth hormone (Gow et al., 1983) are elevated during lactation, and both could be involved in the increase in wool production at this time.

Kendall (1999) extensively studied the effects of manipulating prolactin levels during pregnancy on wool growth, and his findings were important to the rationale behind this thesis program. His first experiment was performed with pregnant (spring-lambing) and non-pregnant ewes under natural day (ND) and short day (SD) photoperiod. It was thought that the prolactin peak observed at parturition could be responsible for reduced diameter and weakening of the fibre observed close to parturition ("lambing break"). Therefore, two SD pregnant groups had their parturition and lactational or their lactational prolactin rise blocked by bromocryptine. The next trial was composed of pregnant (winter-lambing) and non-pregnant ewes, which were separated into groups exposed to long days (LD) and ND. The pregnant ND group was further divided into a group that had their parturition plus lactational prolactin surge blocked and another where just the lactational rise was blocked. He also studied the timing of wool growth rate depression using a radiolabelling technique and described in detail the prolactin
profile of pregnant ewes before, during and after parturition. His main findings were that:

- Photoperiod was the primary determinant of prolactin concentration in the non-pregnant ewe;
- Higher prolactin concentrations in non-pregnant ewes from mid-pregnancy until parturition, suggested that pregnancy inhibits endogenous prolactin secretion;
- A significant reduction in wool growth rate was measured within the first 60 days of pregnancy which was not associated with prolactin concentrations, feed intake or live weight;
- Gestational inhibition of wool growth ceases following lambing, which could partially account for the increase in wool production during lactation;
- Winter prolactin concentration in non-pregnant ewes were positively associated with higher wool growth rates over spring months in SD ewes and prevented the winter wool growth decline in LD ewes;
- Prepartum changes in prolactin concentration were associated with medium- to long-term increases in wool growth. Within experiments higher levels of prepartum and periparturient prolactin were associated with larger wool growth responses. Peak prolactin concentration, rather than the magnitude of the increase from prepartum levels, appeared to be more significantly associated with subsequent wool growth;
- High plasma prolactin concentrations in the lactating ewe were associated with increased medium-term wool growth. This stimulatory effect appears to be enhanced if the postpartum decline in prolactin concentration was gradual;
• In all experiments, the abolition of endogenous pituitary prolactin as a result of bromocriptine treatment was associated with lower rates of long-term wool growth compared to control sheep;

• The suppression of postpartum plasma prolactin secretion was associated with reduced levels of long-term wool growth;

• The rise in prolactin concentration at parturition appeared to provide some stimulatory signal. Wool growth rates were higher in ewes whose prolactin was suppressed 3 days after parturition compared to ewes whose prepartum peak was suppressed;

• Changes in wool growth rate in response to increased plasma prolactin concentration followed with a lag of 2-3 months.

Kendall’s results showed that prolactin manipulation does have a medium-to long-term effect on wool growth of the pregnant Romney ewe, and the timing and magnitude of this manipulation are important in determining the biological effect.

2.6 Manipulation of plasma prolactin

Experimentally, circulating prolactin levels can be manipulated by a variety of means.

2.6.1 Light manipulation

Plasma prolactin levels increase with the length of the experimental photoperiod. This is a simple, non-invasive way to manipulate prolactin levels. The disadvantages of this technique are:

• Animal variation - In some cases animals may show relatively small changes in the prolactin profile in response to the light treatment.

• The need for expensive and labour-intensive indoor animal housing to allow light: dark ratio manipulation.
• Not suitable for controlling prolactin levels over short periods of time.

2.6.2 **Drugs**

Drugs are available that can both suppress and stimulate pituitary prolactin secretion. Bromocriptine is a potent dopamine agonist and has been used extensively in experiments and for clinical reasons in human medicine. Treatment is effected through intramuscular or subcutaneous injections or by infusion. Its efficacy in reducing prolactin secretion has been extensively reported in many species (Brue *et al.*, 1992; Tsagarakis *et al.*, 1995; Ciccarelli and Camanni, 1996; Picazo *et al.*, 2000). Other dopamine agonists such as Cabergoline (Abs *et al.*, 1998), Quinagolide (Trouillas *et al.*, 1994) Roxindol (Jaspers *et al.*, 1994) and Terguride (Mizokawa *et al.*, 1993) have also been used with success in reducing prolactin levels in patients with acromegaly and prolactinoma.

The disadvantages of drugs are:

- May involve a relatively invasive administration procedure, such as multiple serial injections.
- Difficult to control prolactin levels over short periods of time.
- Most drugs are relatively expensive.

2.6.3 **Exogenous prolactin**

Exogenous prolactin has been used in a number of experiments, which resulted in biological responses. A major advantage is the ability to control circulating prolactin levels to a high degree, especially when endogenous prolactin secretion is suppressed with bromocryptine. However, there is variation in reported prolactin sources, doses, routes and duration of administration and the biological effects caused by these treatments. The disadvantages of this methodology are:

- Intravenous administration is an invasive procedure.
• Indoor housing usually required.
• Availability and high cost of bioactive prolactin.

2.7 Prolactin administration regimes and physiological effects

Prolactin has been delivered to experimental animals in 6 different ways: intravenously and by subcutaneous, intramuscular, peritoneal, intraductal and intracerebral ventricular injections (see Tables 1, 2, 3 and 4).

In rats, experimental prolactin doses have varied from 30 to 4000 µg per animal. Different prolactin doses increased plasma levels of vitamin D and consequently enhanced intestinal calcium absorption (Mainoya, 1975a; James et al., 1977; Robinson et al., 1982). In other experiments different prolactin doses stimulated lipid metabolism (Kadim et al., 1996); fluid and sodium absorption (Mainoya, 1975b); food intake (Gerardo-Gettens et al., 1989; Noel and Woodside, 1993); lactation (Hebert et al., 1993; Yoneda et al., 1995); maternal behaviour (Moltz et al., 1970; Bridges et al., 1985); bile secretory rates (Liu et al., 1992) and reproduction (Bartke, 1971a; Bartke, 1971b; Hafiez et al., 1972). The effects on lactation (Hebert et al., 1993), food intake (Gerardo-Gettens et al., 1989) and bile secretory rates (Liu et al., 1992) were dose dependent. Treatment duration ranged from single injections to 5-day constant infusions. Hebert et al (1993) showed that constant infusion of prolactin was more effective in restoring lactation in rats when compared to infusion pulses.

In rabbits, the manipulation of plasma prolactin level was achieved with 10 µg to 1 mg of exogenous prolactin per animal. Fortun-Lamothe et al (1996) and Falconer and Rowe (1977) reported that elevation of plasma prolactin increased lipid mobilisation and ion metabolism in the mammary gland, respectively.

In hamsters, mink and meadow voles, prolactin doses ranged between 100 and 300 µg per animal. Duncan and Goldman (1984a), Duncan and Goldman (1984b), Martinet et al
(1984) and Smale et al (1990) demonstrated that rises in circulating prolactin were associated with changes of the winter and summer pelage in those species. Increased prolactin level in Siberian hamsters was also responsible for a decrease of the winter torpor bouts (hibernation) characteristic of these animals (Ruby et al., 1993).

In lactating pigs, 15 to 30 mg per animal of prolactin had no effect on milk production in early lactation (King et al., 1996). When prolactin was administered before parturition, milk production was advanced, suggesting that this hormone is important for the onset of lactation rather than milk yield in this species (King et al., 1996).

In cattle, the range of prolactin dose was 3 to 540 mg per animal per day, the lower doses being given during a 2.5-h infusion. Tucker et al (1973) and Plaut et al (1987) showed no positive effect of elevated prolactin levels on milk production. Liesman et al (1988), manipulated plasma prolactin levels at the time of parturition and reported that the prepartum prolactin increase was responsible for stimulating lipoprotein lipase activity, which catalyses 40% of the synthesis of bovine milk fat. When studying the influence of prolactin in the reproductive system, Forrest et al (1980) found no increase of luteinizing hormone (LH) levels, when infusing ovariectomized cows with exogenous prolactin.

In sheep, the range of doses used to manipulate prolactin levels have varied between 4 µg to 50 mg per animal per day (Manns and Boda, 1965; Burstyn et al., 1972; Horrobin et al., 1973; Hooley, 1979; Brinklow and Forbes, 1983; Barlet, 1985; Pearson et al., 1997). Calcium absorption was stimulated in the pregnant ewe using daily injections of very low amounts of prolactin (Barlet, 1985). The elevated prolactin levels also effected biological changes in sodium and potassium metabolism by restoring the saluretic actions of aldosterone (Burstyn et al., 1972; Horrobin et al., 1973), nitrogen retention (Brinklow and Forbes, 1983) and wool follicle activity (Pearson et al., 1997). In agreement with the lactational studies performed with bovines, Hooley (1979) found no effect on milk production with elevated plasma prolactin levels. Manns and Boda (1965)
found no physiological effect on lipid metabolism when a weekly injection of exogenous prolactin was given to wethers. This may not indicate that prolactin lacks effect on fat metabolism in sheep, but rather that a catabolic effect of prolactin requires more than a weekly treatment.

The administration of 2 mg of prolactin twice a day to cashmere goats altered follicle activity (Dicks et al., 1994). Jacquemet and Prigge (1990) and Jacquemet and Prigge (1991) infused 4 to 12 mg of prolactin into lactating goats and showed that prolactin elevation did not enhance milk production, compared to untreated animals. The same researchers reported no increase in nitrogen retention in lactating goats treated with prolactin, in contrast to Brinklow and Forbes (1983) who showed an increase in nitrogen retention in growing lambs. This could be due to species differences or the fact that the animals in these experiments differed in physiological status.

As it has been shown, different tissues respond differently to prolactin manipulation, for example the mammary gland and reproductive system. Dose-related differences in the biological response may also be due to different modes or duration of treatment or physiological status. Therefore, it is essential to consider such variables when attempting to use prolactin manipulation to influence the physiology of a specific organ.

2.8 Kinetics of circulating prolactin

2.8.1 Steady state prolactin concentration

As prolactin is infused into the animal it is distributed throughout the different tissues. The circulating concentration of prolactin rises until equilibrium dependent on animal size is reached (Table 5). After infusion is finished, plasma prolactin concentration decreases due to clearance mechanisms.

When male rats were infused with radiolabelled prolactin, organ uptake from maximum to minimum was: kidneys, liver, lungs, blood vessels, hair follicles, adrenal
glands, thyroid gland, stomach, testes, epididymis and seminal vesicle (Rajaniemi et al., 1974). In the pregnant female rat, the kidneys, liver and lungs showed the same strong radiolabelling as occurred in the male. Labelling of the ovaries and mammary gland was weak.

The weak labelling observed in some target organs might be due to low levels of receptors already occupied by endogenous prolactin. In this case experimental suppression of endogenous prolactin secretion might be required to reveal the binding sites.

2.8.2 Prolactin clearance

The kidneys, liver and the mammary gland are considered to be the main organs responsible for prolactin degradation and excretion (Sgouris and Meites, 1952; Rajaniemi et al., 1974; Lim et al., 1979; Emmanouel et al., 1981). In an early experiment by (Sgouris and Meites, 1952) kidney homogenate broke down 76% of the prolactin added to the culture. Humans with renal disease frequently have elevated prolactin levels, which are corrected after successful renal transplantation (Nagel et al., 1973; Czernichow et al., 1976; Lim et al., 1979; Saha et al., 2002). This is supported by experiments using nephrectomized or ureteral-ligated animals, which show an increase in circulating prolactin compared to control animals (Emmanouel et al., 1981; Martin-Oar et al., 1981; Falconer and Vacek, 1983).

Rajaniemi et al (1974) and Donatsch and Richardson (1975) found that prolactin was accumulated in the proximal tube cells. Also, a small amount of precipitated radioactivity was found in the urine, showing that prolactin was degraded by the kidneys before it was excreted (Birkinshaw and Falconer, 1972). Further evidence that prolactin is voided by glomerular filtration and tubular reabsorption and degradation comes from studies by Rustom et al (1992) and Osicka and Comper (1997) who
concluded that the degraded proteins observed in the urine were associated with the proteolytic activity of the proximal tubes.

The mammary gland is also responsible for prolactin clearance. When prolactin was infused or injected in lactating animals, a rise in the levels of prolactin in both blood and milk was observed (McMurtry and Malven, 1974b; Grosvenor and Whitworth, 1976; Akers and Kaplan, 1989). Furthermore, the metabolic clearance rate (MCR) of lactating animals is higher than in non-lactating controls (Davis and Borger, 1973; Akers et al., 1980). This rapid transfer of prolactin from the blood to the mammary gland occurred in direct proportion to plasma prolactin levels (McMurtry and Malven, 1974b; Grosvenor and Whitworth, 1976). After plasma prolactin levels declined, milk prolactin levels remained high for a longer period of time, indicating that prolactin was accumulated in the mammary gland (Birkinshaw and Falconer, 1972; McMurtry and Malven, 1974a).

The liver also contributes to prolactin degradation. Sgouris and Meites (1952) showed that liver homogenates broke down 70% of available prolactin within 1 h. Livers of nephrectomized animals had twice the amount of radiolabelled prolactin compared to control animals, suggesting a capacity of the liver to replace renal degradation (Falconer and Vacek, 1983). Strong and diffuse labelling over the parenchymal and Kupffer cells in the liver of male and female rats suggested that both cell types may be involved in prolactin metabolism (Rajaniemi et al., 1974).
Table 1: Intravenous administration of prolactin - Dose, time and outcome

<table>
<thead>
<tr>
<th>Author</th>
<th>Species</th>
<th>Dose and time of administration</th>
<th>Rise in prolactin levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manns and Boda (1965)</td>
<td>Sheep</td>
<td>1 mg/kg once a week</td>
<td>Not measured</td>
</tr>
<tr>
<td>Winkler et al (1971)</td>
<td>Dog</td>
<td>1 mg/kg single injection</td>
<td>Not measured</td>
</tr>
<tr>
<td>Tucker et al (1973)</td>
<td>Cattle</td>
<td>1 mg/day followed by 2.5 h infusion (3 mg/h)</td>
<td>Control: 15 ng/ml Treated: 59 ng/ml</td>
</tr>
<tr>
<td>Hooley (1979)</td>
<td>Sheep</td>
<td>1 mg/hour 10 h infusion</td>
<td>343 mg/ml 5 h after start of infusion</td>
</tr>
<tr>
<td>Forrest et al (1980)</td>
<td>Cattle</td>
<td>10 mg/hour, 3 h infusion</td>
<td>200 ng/ml 30 min after start of infusion</td>
</tr>
<tr>
<td>Brinklow and Forbes (1983)</td>
<td>Sheep</td>
<td>Single injection 10 mg/day for 10 days</td>
<td>Control: 31 ng/ml Treated: 84 ng/ml</td>
</tr>
<tr>
<td>Dusza et al (1986)</td>
<td>Pig</td>
<td>0.5 mg every 2 h for 2 days</td>
<td>Control: 2 ng/ml Treated: 100 ng/ml</td>
</tr>
<tr>
<td>Liesman et al (1988)</td>
<td>Cattle</td>
<td>6.0, 8.0, 9.8, 20.8 and 22.4 mg/h for 24 h</td>
<td>Control: 29 ng/ml Treated: 350 ng/ml</td>
</tr>
<tr>
<td>Jacquemet and Prigge (1990)</td>
<td>Goats</td>
<td>12 mg/day infused in 14 h after milking for 8 days</td>
<td>Control: 355 ng/ml Treated: 605 ng/ml</td>
</tr>
<tr>
<td>Jacquemet and Prigge (1991)</td>
<td>Goats</td>
<td>2 or 4 mg/h after milking (2 h infusion)</td>
<td>2 mg: 575 ng/ml Treated: 808 ng/ml</td>
</tr>
<tr>
<td>Ruby et al (1993)</td>
<td>Hamsters</td>
<td>0.55 µg/h for 14 days</td>
<td>22 to 338 ng/ml</td>
</tr>
<tr>
<td>Hebert et al (1993)</td>
<td>Rats</td>
<td>50 to 300 µg/day in constant infusion or 8 pulses of 2 h</td>
<td>Not measured</td>
</tr>
<tr>
<td>Author</td>
<td>Species</td>
<td>Dose and time of administration</td>
<td>Rise in prolactin levels</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------</td>
<td>----------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Mainoya (1975a)</td>
<td>Rats</td>
<td>0.25 to 4.0 mg prior to culling</td>
<td>Not Measured</td>
</tr>
<tr>
<td>Fleming (1976)</td>
<td>Rats</td>
<td>30 IU 2x day for 7 days</td>
<td>Not Measured</td>
</tr>
<tr>
<td>Robinson et al (1982)</td>
<td>Rats</td>
<td>500 μg every 12 h for 3 days</td>
<td>Not Measured</td>
</tr>
<tr>
<td>Duncan and Goldman (1984a)</td>
<td>Hamsters</td>
<td>100 μg for 12 days (SD photoperiod)</td>
<td>Not Measured</td>
</tr>
<tr>
<td>Robinson et al (1982)</td>
<td>Rats</td>
<td>500 μg 2x day for 13 days</td>
<td>Not Measured</td>
</tr>
<tr>
<td>Barlet (1985)</td>
<td>Sheep</td>
<td>0.1 μg/kg live weight for 14 days before parturition</td>
<td>Not Measured</td>
</tr>
<tr>
<td>Gerardo-Gettens et al (1989)</td>
<td>Rats</td>
<td>3.0 μg/kg live weight 2x day for 10 days</td>
<td>Control: 8 ng/ml Treated: 71 ng/ml</td>
</tr>
<tr>
<td>Noel and Woodside (1993)</td>
<td>Rats</td>
<td>1 or 3.0 μg/kg live weight 2x day for 10 days</td>
<td>Not Measured</td>
</tr>
<tr>
<td>Dicks et al (1994)</td>
<td>Goats</td>
<td>2 mg 2x day for 7 days</td>
<td>Not Measured</td>
</tr>
<tr>
<td>Kadirn et al (1996)</td>
<td>Rats</td>
<td>0.2 or 0.4 μg/g live weight for 21 days</td>
<td>Not Measured</td>
</tr>
<tr>
<td>Fortun-Lamothe et al (1996)</td>
<td>Rabbits</td>
<td>1 mg single dose</td>
<td>Not Measured</td>
</tr>
<tr>
<td>Thompson et al (1997)</td>
<td>Horses</td>
<td>4 mg/day for 45 days</td>
<td>Before injection: 0.1 ng/ml After injection: 94 ng/ml</td>
</tr>
<tr>
<td>Pearson et al (1997)</td>
<td>Sheep</td>
<td>10, 20, 30, 40, 50 mg/day for 10 days</td>
<td>Before injection: 2 ng/ml After injection: 10mg:61 ng/ml; 50 mg: 270 ng/ml</td>
</tr>
</tbody>
</table>
Table 3: Intramuscular administration of prolactin - Dose, time and outcome

<table>
<thead>
<tr>
<th>Author</th>
<th>Species</th>
<th>Dose and time of administration</th>
<th>Rise in prolactin levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horrobin et al (1971)</td>
<td>Human</td>
<td>8 mg single dose</td>
<td>Not measured</td>
</tr>
<tr>
<td>Burstyn et al (1972)</td>
<td>Sheep</td>
<td>5 mg single injections for 4 days</td>
<td>Not measured</td>
</tr>
<tr>
<td>Horrobin et al (1973)</td>
<td>Sheep</td>
<td>5 mg + 50 mg cortisol for 4 days</td>
<td>Not measured</td>
</tr>
<tr>
<td>Martinet et al (1984)</td>
<td>Mink</td>
<td>0.3 mg once a day (SD photoperiod)</td>
<td>Not measured</td>
</tr>
<tr>
<td>Plaut et al (1987)</td>
<td>Cattle</td>
<td>120 mg 1x day for 14 days</td>
<td>Not measured</td>
</tr>
<tr>
<td>King et al (1996)</td>
<td>Pig</td>
<td>15 mg 2x day from day 102 gestation to weaning</td>
<td>Control: 28-51 ng/ml Treated: 1351-1963 ng/ml</td>
</tr>
</tbody>
</table>

Table 4: Other types of prolactin administration - Dose, time and outcome

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Author</th>
<th>Species</th>
<th>Dose and time of administration</th>
<th>Rise in prolactin levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraperitoneal</td>
<td>Bartke (1971a)</td>
<td>Rats</td>
<td>7.5 IU 2x days for 3 or 4 days</td>
<td>Not measured</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>Bartke (1971b)</td>
<td>Rats</td>
<td>12 IU</td>
<td>Not measured</td>
</tr>
<tr>
<td>Intracuticular</td>
<td>Falconer and Rowe (1977)</td>
<td>Rabbits</td>
<td>10 µg for 3 days</td>
<td>Not measured</td>
</tr>
<tr>
<td>Intracerebroventricular (ICV)</td>
<td>Buntin (1989)</td>
<td>Ring doves</td>
<td>1 µg at the beginning of dawn</td>
<td>Not measured</td>
</tr>
<tr>
<td>Epidermis infusion</td>
<td>Thomas et al (1994)</td>
<td>Deer</td>
<td>600 µg/ml for 28 days at a rate of 2.5 µl/h</td>
<td>Not measured</td>
</tr>
</tbody>
</table>
2.8.3 Metabolic clearance rate (MCR)

The MCR can be thought of as the volume of blood from which the substance of interest is completely cleared per unit of time. The MCR has a significant positive correlation with body surface area or metabolic body weight, therefore MCR is usually expressed as ml/min per m2 or ml/min per kg 0.75. MCR varies between lactating and non-lactating animals. Davis and Borger (1973) reported that lactating ewes had a higher prolactin MCR than ovariectomized, pregnant and anestrous ewes (6.09, 2.93, 4.56 and 3.84 ml/min per kg 0.75, respectively). Akers et al (1980) showed that cows in early lactation had a MCR of 5.6 ml/min/kg body weight and non-lactating cows had a MCR of 3.2 ml/min/kg body weight. This illustrates the importance of the mammary gland in the elimination of prolactin. Koch et al (1971) reported no difference in the MCR among intact male, intact female, ovariectomized and hypophysectomized rats. In this study, the mean MCR was 1.26 ml/min. In humans, the MCR varies between 71 and 78 ml/min per m2, showing no difference between males and non-pregnant females (Cooper et al., 1979; Molitch et al., 1987).

2.8.4 Half-life

The half-life is the period over which the concentration of a given drug or chemical falls to half of its original concentration and is correlated with the metabolic rate of the animals. Rats, rabbits and mice, for example have shorter half-lives compared to humans, cows and pigs (Table 6). In sheep, the half-life reported by Akbar et al (1974) was 23 min. However, Litherland (1996) reported a half-life of 42 min in sheep injected with a single dose of prolactin. In the same study, after ceasing a 2-day infusion, the measured half-life was 105 min, suggesting that the dose and duration of treatment could influence the half-life. Other factors influencing the half-life are lactational state (Falconer and Vacek, 1983), age (Brown-Borg et al., 1993) and renal function (Sievertsen et al., 1980).
2.9 Prolactin receptors

As with other peptide hormones, prolactin exerts its effects by coupling with a specific receptor in the cell membrane of the target organ. PRLR have been identified in numerous tissues and multiple isoforms have been identified in a number of species. The biological responses due to prolactin signalling in different tissues, and in the same tissue under different physiological states, can vary markedly. The mechanisms underlying this plasticity in prolactin response is still far from clear. However, the evidence that different tissues have varying isoform ratios, and that these ratios can change depending on the physiological status of the animal is providing new perspective to prolactin research.

2.9.1 Prolactin receptor distribution and measurement techniques

PRLR are expressed in a number of different tissues, including: central nervous system, retina, olfactory system, ganglia, pituitary gland, adrenal cortex, skin, bone tissue, gills, lung, heart, muscle, brown adipose tissue, liver, pancreas, gastrointestinal tract, kidneys, bladder, immune system cells, female and male reproductive system (for comprehensive reviews see (Bole-Feysot et al., 1998; Freeman et al., 2000). Early studies used techniques such as injection of radiolabelled prolactin to determine where prolactin was distributed, and by implication the existence of PRLR in that tissue. However, with the growing wish to understand the control of prolactin over so many physiological functions, quantification of the PRLR population has become increasingly important.

The receptor protein can be quantified by a number of techniques. The most cited ones are: (1) Receptor Binding Assay – Where the protein is extracted from the tissue of interest and incubated with the radiolabelled ligand with the presence or absence of excess unlabelled ligand. The sample is then centrifuged and the radioactivity of the precipitated pellet is determined; (2) Immunoblotting – The protein contents also have to be extracted from the tissue and the proteins are run on a gel and transferred to a
membrane. The membrane is incubated with specific labelled antibodies and the resulting bands measured by densitometry.

The receptor expression can also be quantified by measuring the amount of mRNA of the target protein. The mRNA quantification techniques are: (1) Ribonuclease Protection Assay, where RNA is extracted and hybridised with specific probes. After this, RNase is added to the solution and the single-stranded mRNA are digested leaving only the double-stranded RNA, which is then run on a gel and quantified with an imaging system. The gene expression of the target protein is expressed relative to an internal control gene; (2) Semi-quantitative RT-PCR – Extracted total RNA is transformed into cDNA by a reverse transcriptase reaction. The cDNA samples are amplified by PCR reaction using specific primers. The PCR product is then run on a gel, quantified and the amount of the specific target mRNA is expressed relative to the amount of an internal control mRNA. (3); Quantitative RT-PCR – Follows the same procedure as the Semi-quantitative RT-PCR but it has an internal control RNA (cRNA) of a known concentration, which allows a more precise estimate of the target mRNA.

While RT-PCR techniques are specific, their sensibility can be compromised by non-exponential amplification of the target gene at higher cycle numbers. In this PhD program the relatively new real-time PCR technique will be used. This technique enables the measurement of the amount of template at the end of every cycle; thus measurement of the target gene template can be restricted to the exponential phase of amplification. Hence the procedure is not only highly specific but also highly sensitive. This technique has not previously been used to measure the expression of PRLR in the sheep skin. The development and optimisation of a stable assay was part of this work, which will be described in the Materials and Methods (Chapter 3).
<table>
<thead>
<tr>
<th>Authors</th>
<th>Animal</th>
<th>Route of administration</th>
<th>Priming dose</th>
<th>Infusion rate</th>
<th>Steady state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akers et al (1980)</td>
<td>Lactating and pregnant cows</td>
<td>Intravenous constant infusion</td>
<td>No priming dose</td>
<td>4 or 8 mg prolactin/cow/h for 4 h</td>
<td>120-159 min</td>
</tr>
<tr>
<td>Akbar et al (1974)</td>
<td>Mature ewes</td>
<td>Intravenous constant and single infusion</td>
<td>20 μCi ¹²⁵I prolactin</td>
<td>50 μCi ¹²⁵I prolactin over 2 h a total of 1.7 μg of prolactin</td>
<td>60 min</td>
</tr>
<tr>
<td>Davis and Borger (1973)</td>
<td>Sheep</td>
<td>Intravenous constant and single infusion</td>
<td>1/3 of the total dose 30 min before infusion</td>
<td>0.2 - 0.5 ng prolactin/kg over 120 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Sievertsen et al (1980)</td>
<td>Humans</td>
<td>Intravenous constant and single infusion</td>
<td>20% of total dose</td>
<td>6.5 – 19 μCi ¹²⁵I prolactin over 3 h</td>
<td>120 min</td>
</tr>
<tr>
<td>Cooper, 1979 et al</td>
<td>Humans</td>
<td>Intravenous constant and single infusion</td>
<td>22-35% of the total dose</td>
<td>Rest of the buffer which contained 10-20 μCi ¹²⁵I prolactin over 3 h</td>
<td>90-120 min</td>
</tr>
<tr>
<td>Molitch et al (1987)</td>
<td>Humans</td>
<td>Intravenous constant and single infusion</td>
<td>90-70 μg of prolactin in a 5-min period</td>
<td>1.39 – 2.9 μg/min for 95 – 180 min</td>
<td>30-60 min</td>
</tr>
<tr>
<td>Grosvenor et al (1977)</td>
<td>Lactating and virgin rats</td>
<td>Intravenous constant infusion</td>
<td>No priming dose</td>
<td>100, 200 or 472 ng/prolactin/min for 35 min</td>
<td>25 min</td>
</tr>
<tr>
<td>Grosvenor and Whitworth (1976)</td>
<td>Lactating rats</td>
<td>Intravenous constant infusion</td>
<td>No priming dose</td>
<td>200 or 472 ng/prolactin/min for 35 min</td>
<td>20-25 min</td>
</tr>
</tbody>
</table>
Table 6: Routes of administration, dose and half-life of prolactin in different species treated with exogenous prolactin

<table>
<thead>
<tr>
<th>Author</th>
<th>Species</th>
<th>Route of administration</th>
<th>Dose</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birkinshaw and Falconer (1972)</td>
<td>Rabbits (pseudo-pregnant)</td>
<td>Single intravenous injection</td>
<td>1.0 mg</td>
<td>12 min</td>
</tr>
<tr>
<td>Koch et al (1971)</td>
<td>Rats</td>
<td>Single intravenous injection</td>
<td>Not shown</td>
<td>4-6 min</td>
</tr>
<tr>
<td>Goodman et al (1979)</td>
<td>Lactating cows</td>
<td>Endogenous prolactin</td>
<td>Not applicable</td>
<td>23 min</td>
</tr>
<tr>
<td>Tucker et al (1973)</td>
<td>Lactating cows</td>
<td>Single intravenous injection and constant infusion</td>
<td>Loading dose 1 mg</td>
<td>23-25 min</td>
</tr>
<tr>
<td>Forsyth et al (1995)</td>
<td>Lactating and pregnant goats</td>
<td>Constant intravenous infusion</td>
<td>Constant infusion 50 μCi 125 prolactin</td>
<td>20 min</td>
</tr>
<tr>
<td>Bryant and Greenwood (1968)</td>
<td>Goats</td>
<td>Single intravenous injection</td>
<td>10 mg</td>
<td>19 min</td>
</tr>
<tr>
<td>McMurtry and Malven (1974b)</td>
<td>Lactating goats</td>
<td>Single intravenous injection</td>
<td>10 mg</td>
<td>60 min</td>
</tr>
<tr>
<td>Molitch et al (1987)</td>
<td>Humans</td>
<td>Single intravenous injection and constant infusion</td>
<td>Loading dose: 90 μg</td>
<td>37 ± 10 min</td>
</tr>
<tr>
<td>Sievertsen et al (1980)</td>
<td>Humans</td>
<td>Single intravenous injection and constant infusion</td>
<td>6.5 – 19 μCi 125 prolactin 20% in bolus injection 80% constant infusion for 180 min</td>
<td>52 ± 4 min</td>
</tr>
</tbody>
</table>
2.9.2 Prolactin receptor structure and isoforms

The PRLR belongs to the family of class 1-cytokine receptors. They are single-pass transmembrane chains, with a highly conserved extracellular domain (ECD), a transmembrane domain (TMD) and an intracellular domain (ICD) (Kelly et al., 1991; Goffin et al., 1996b; Goffin and Kelly, 1997; Bole-Feyesot et al., 1998). There are a number of different isoforms of PRLR (Figure 3). They result from alternative splicing of the primary transcript and their main difference is in the length and composition of their ICD. Each of these forms is unique to a specific species.

The PRLR was first identified in the rat liver as a mature protein with 291 amino acids (aa), now known as the short form (PRLR-S). Later, another form was identified in the rat ovary. This protein comprised 591 aa and was called the long form (PRLR-L) (Kelly et al., 1991). Rats also have a mutant form of the receptor expressed in the Nb2 cell line, which express an intermediate form with 393 aa (Goffin and Kelly, 1997). The rabbit PRLR-L has 592 aa and the human 598 aa (Kelly et al., 1991). In mice there are one long and three short forms of the PRLR (Davis and Linzer, 1989; Tzeng and Linzer, 1997) (Figure 3). Recently, other isoforms of the receptor have been identified in humans, including a soluble ECD of the PRLR (Kline et al., 2002; Trott et al., 2003). The regulation of the expression of these different isoforms is complex and Ormandy et al (1998) proposed that the different isoforms of the PRLR in mouse liver are generated by usage of multiple promoters and 3'-exon splicing. Hu et al (2002) also stated that multiple promoters regulate the transcription of PRLR in human breast cells.

Sheep express a long (557 aa) and a short form (272 aa) of the receptor. The mRNA of the short form has a 39 base pair (bp) insert, derived from alternative splicing, with two stop codons at the 3'-end. Hence the translation of the latter transcript results in a receptor with a shorter ICD (Anthony et al., 1995; Bignon et al., 1997).
Despite these differences between species and isoforms, there is a highly conserved area in the ICD called the homology box-1, a membrane proximal region composed of 8 aa highly enriched with proline and hydrophobic residues (Clevenger et al., 1995; Bole-Feysot et al., 1998). This area is where JAK 2, a protein from the Janus Kinase family responsible for phosphorylating other proteins of the prolactin signalling pathway, associates with the PRLR (Pezet et al., 1997; Bole-Feysot et al., 1998; Davey et al., 1999; Kazansky et al., 1999; Lerant et al., 2001).

There are two major conserved sites in the ECD. The first comprises two pairs of disulfide-linked cysteines in the N-terminal subdomain (D1). The other is a pentapeptide WS motif in the membrane-proximal C-terminal subdomain (D2) (Bole-Feysot et al., 1998). Mutation of the conserved cysteines from D1 results in impaired function of the receptor (Rozakis-Adcock and Kelly, 1991; Rozakis-Adcock and Kelly, 1992). The WS motif on the C-terminal region is thought to be crucial for maintenance of the correct folding of the protein and cellular “trafficking” (Bole-Feysot et al., 1998).

2.9.3 Prolactin receptor expression

The regulation of PRLR expression is complex. Within the same tissue, but between different species, expression of PRLR isoforms is highly variable. Also, the PRLR population in different tissues of the same species responds differently to identical hormonal cues. Stimulatory and inhibitory effects on PRLR expression have been studied mostly in the mammary gland and liver of pregnant and lactating rats, mice, rabbits and sheep.

2.9.3.1 Rat

In the rat liver, the short form is the predominant PRLR variant (Jahn et al., 1991; Nagano and Kelly, 1994). The expression of PRLR-S in the male liver was low when compared with the expression in female where it was also influenced by the reproductive status (Moldrup et al., 1993). The mRNA expression of the PRLR-S form
was two-fold higher during proestrus when compared with diestrous expression (Nagano and Kelly, 1994). During pregnancy, the amount of PRLR-S mRNA was up-regulated followed by down-regulation at parturition and during lactation (Jahn et al., 1991; Telleria et al., 1997). In the mammary gland the expression of mRNA of PRLR-L was more abundant than PRLR-S (Jahn et al., 1991). In contrast to the liver, PRLR-L expression in the mammary gland was low and constant during pregnancy. Close to parturition and throughout lactation, PRLR-L mRNA levels were up-regulated (Jahn et al., 1991; Telleria et al., 1997).

The PRLR population in the ovaries also responded to fluctuations of the reproductive hormones. The expression of the PRLR-L was higher than the PRLR-S form; but the ratio between the two forms changed in different hormonal environments (Telleria et al., 1997). This variation was thought to be important for the luteotrophic and luteolytic effects of prolactin on the ovaries. When Kinoshita et al (2001) induced proestrus in the immature female rat with pregnant mare serum gonadotropin (PMSG), the PRLR mRNA expression was up-regulated significantly (688%) when compared to the control group. The increase in PRLR-S expression was much lower (184%). The effect of human chorionic gonadotropin (hCG) in immature female rats revealed a time-specific regulation of PRLR. The mRNA expression of the PRLR-L was rapidly down-regulated after hCG treatment followed by a gradual up-regulation, which lasted until 48 h after treatment. In contrast, PRLR-S expression levels declined and reached a nadir around 12 h after treatment. Subsequently, receptor levels increased significantly. This experiment clearly showed that the regulation of PRLR isoforms not only responded differently to hormonal stimuli but also in a distinctive time-specific manner. These authors suggested that the long form of the receptor was involved in the folliculogenesis and the short form with formation and maintenance of the corpus luteum. Bowen et al (2000) also pointed out that prolactin could have both trophic and lytic effects on the rat corpus luteum and that this phenomenon could be explained by the difference in
the PRLR expression of the two isoforms in the distinct reproductive stages. The immune system (Gunes and Mastro, 1997), pancreas (Moldrup et al., 1993) and brain (Sugiyama et al., 1994; Shamgochian et al., 1995) also show differences in PRLR gene expression due to changes in the hormonal status during the oestrus cycle.

It is clear that the reproductive status of the animal can influence the expression of PRLR in different tissues. However, in some cases, the reproductive hormones that influence this expression may exert their effect through prolactin itself. Prolactin has been shown to be an important regulator of the expression of its own receptors (Bole-Feysot et al., 1998) (Section 2.9.4). For example, oestrogen is a positive regulator of prolactin production and release (Lamberts and Macleod, 1990; Pasolli et al., 1992) and has been implicated in enhancing PRLR numbers in the liver (Posner et al., 1975). Short-term increases in oestrogen level enhance hypothalamic dopamine concentrations, which suppress prolactin secretion. On the other hand, long-term elevation of oestrogen reduce dopamine concentration in the portal vessels, resulting in a higher basal level of circulating prolactin in the females compared with the males (Sanford and Robaire, 1990). Therefore, the changes in PRLR concentration during the oestrus cycle could be related to changes in oestrogen secretion, via modulation of prolactin production and release. These findings could be implicated in the gender differences found in the PRLR population of some target organs.
Figure 3: PRLR isoforms in different species. The purple and green colours represent areas of more than 68% of similarity between the extracellular domain of the isoforms. The blue represents the transmembrane domain and the red illustrates the area of more than 68% similarity of the intracellular domain of the receptors. The non-coloured strips represent areas of 46 to 68% similarities among isoforms. Figure adapted from (Kelly et al., 1991; Bignon et al., 1994; Jabbour and Kelly, 1997).
Progesterone also influences the regulation of PRLR gene expression. It has been reported that this hormone is responsible for the inhibition of PRLR mRNA expression in rat mammary gland cell culture (Nishikawa et al., 1994). Djiane and Durand (1977) also reported that prolactin and progesterone had antagonistic effects on PRLR expression regulation. Teyssot and Houdebine (1981) suggested that progesterone inhibited PRLR mRNA accumulation and transcription rate, leading to a decrease in casein protein synthesis. However, the progesterone inhibition is tissue specific, since in the liver, progesterone appears to up-regulate PRLR levels during pregnancy.

2.9.3.2 Mice

In the mouse embryo, PRLR mRNA levels were relatively constant, except for a sudden drop observed on day 14 of gestation. At this time placental lactogen peaks, suggesting that it could be involved with the down-regulation of PRLR levels. In the embryo liver, the PRLR-L was more abundant than the PRLR-S before day 18 of pregnancy. Subsequently, the PRLR-S became more abundant and this was maintained until after birth (Tzeng and Linzer, 1997). As observed in rats, PRLR expression is highly influenced by the reproductive hormones. Female mice with diabetes had higher basal testosterone and lower 17-β oestradiol levels. In the diabetic male, testosterone levels were lower, but there was no difference in 17-β oestradiol (Yasui et al., 1999). These authors also noted that in the diabetic female PRLR mRNA levels were lower than in the control group. In the diabetic male, receptor expression was up-regulated in comparison to the non-diabetic males. Therefore, under these conditions, testosterone may have an inhibitory impact on the expression of PRLR in the liver while oestrogen has a stimulatory effect. During pregnancy, the expression of PRLR mRNA in the liver, showed a 3.4-fold increase when compared to virgin and lactating mice (Ling et al., 2000). This corroborates the findings in rats where progesterone has a positive effect on the expression of liver PRLR.
In the mammary gland, Mizoguchi et al. (1997a) and Hovey et al. (2001) confirmed the importance of the reproductive hormones on the regulation of PRLR mRNA population. Four isoforms of the PRLR (one long and three short forms—S1, S2, S3) were identified in the mammary gland. The PRLR-L, PRLR-S1 and PRLR-S3 levels increased during puberty, reaching a peak at late pubescence and during early pregnancy. Treatment of ovariectomized mice with 17-β oestradiol increased PRLR mRNA, while progesterone decreased its expression (Mizoguchi et al., 1997a). These findings, together with the data from PRLR expression in the mammary gland and liver of rats, establish the tissue specificity of PRLR expression regulation under a specific hormonal environment. In the mammary gland, Mizoguchi et al. (1997b) also reported that peripartum corticosterone up-regulated the gene expression of PRLR-L mRNA.

During lactation, mRNA expression of PRLR-L was up-regulated in the adipose tissue when compared to virgin and pregnant mice. There was no difference in the PRLR-L mRNA population between virgin females and male mice in this tissue (Ling et al., 2000).

2.9.3.3 Rabbits

As in rats, PRLR levels in the mammary gland were low during gestation and were up-regulated during lactation. It is believed that the hormonal environment at parturition, with high prolactin levels, low progesterone levels plus a peak of cortisol combine to produce this effect (Djiane and Durand, 1977).

In the gastrointestinal tract of the adult rabbit, PRLR numbers were higher in the duodenal and jejunal epithelium when compared to the colon (Lobie et al., 1993). In the neonatal rabbit, the PRLR population in the digestive system was very low and receptor numbers began to rise after birth, suggesting that the milk-borne prolactin could be responsible for this regulation (Dusanter-Fourt et al., 1992). A similar observation that prolactin derived from the mother’s milk can regulate PRLR expression in the neonate
was observed in the spleen and thymus of rat pups (Gunes and Mastro, 1996). Hence, we can now observe the importance of the capability of the mammary gland to store prolactin, as it has significant effects on the immune system and brown fat metabolism, both of which are crucial for neonate survival.

2.9.3.4 Sheep

The presence of mRNA for the PRLR-L and PRLR-S has been identified in the sheep foetal liver and adult ovaries (Anthony et al., 1995). The mRNA expression of PRLR in the foetal liver was manipulated using cortisol treatment, indicating that the prepartum cortisol surge could be important in the regulation of PRLR expression in this tissue (Phillips et al., 1999).

In the neonate, the long and the short form of the PRLR protein was found in the brown adipose tissue and their expression showed a positive correlation with the food intake of the mother during pregnancy (Budge et al., 2000; Stephenson et al., 2001; Budge et al., 2003). Foetuses from mothers that had their caruncles removed, consequently deprived of oxygen and nutrients, had depressed PRLR-L expression in perirenal adipose tissue. No change in expression of the PRLR-S was observed. These foetuses were also presented with a substantial depression of their plasma prolactin levels (Symonds et al., 1998). However, it seems that the relationship between nutritional status and PRLR expression is a tissue specific response, since caruncle removal did not effect mRNA levels of either form of the PRLR in the foetal liver or kidneys (Phillips et al., 2001).

In contrast to the regulation of PRLR expression in the mammary gland of rats and rabbits, which were up-regulated at parturition and lactation, a binding study in sheep showed that PRLR numbers in the mammary gland were up-regulated approximately 50 days prior to parturition and then stabilised until lambing (N'Guema Emane et al.,
This suggests that prolactin could be responsible for the growth and differentiation of the ovine mammary gland rather than for galactopoiesis.

According to Cassy et al (1998) mRNA expression of PRLR followed a similar pattern to that observed in the binding studies. In their work, PRLR mRNA remained stable in the first half of pregnancy and then sharply increased during the second half. At parturition levels of mRNA for the PRLR decreased and remained low throughout lactation. During early pregnancy, oestrogen and progesterone are present at low concentrations and commenced to increase from day 50 of gestation (Bassett et al., 1969; Carnegie and Robertson, 1978). This coincides with the up-regulation of mRNA expression of the PRLR. Therefore, in the mammary gland of sheep, in contrast to rats, progesterone does not appear to exhibit an inhibitory effect on the regulation of PRLR expression. On the contrary, according to Cassy et al (2000) a combination of oestrogen and progesterone was required for up-regulating the expression of both forms of the PRLR in the ovine mammary gland.

The ratio between the long and short form of the PRLR may play an important role in the biological function of the sheep mammary gland. This ratio was constant during the first half of pregnancy and increased in the second half (Cassy et al., 2000). This rise was also observed when animals were treated with glucocorticoid and GH. These hormones could be responsible for changing the alternative splicing pattern, in favour of the PRLR-L. The long form of the PRLR was associated with the up-regulation of the expression of milk proteins (Djiane et al., 1982; Bignon et al., 1999), while the short form of PRLR has been shown to induce cell proliferation (Das and Vonderhaar, 1995). Hence, the shift in the expression of PRLR isoforms could explain the physiological change of the mammary gland from growth and differentiation to milk production after parturition.

In the endometrium, PRLR expression was stimulated during late pregnancy coinciding with the parturition surge in prolactin and decline of progesterone (Cassy et al., 1999).
In conclusion, it is clear that regulation of PRLR expression is not only species-specific but also tissue specific. Oestrogen and progesterone play an important role in this regulation. Also, the prepartum surge in cortisol seems to stimulate up-regulation of the receptors in more than one tissue and is probably important for modulating the differential effects of prolactin, by changing the ratio of short to long form of the receptor.

2.9.4 Prolactin regulation of its own receptor

In most organs prolactin up- or down-regulates the level of its own receptor (Djiane et al., 1979; Djiane et al., 1982; Barash et al., 1986; Klemcke et al., 1990; Rose and Stormshak, 1993; Feng et al., 1998; Galsgaard et al., 1999; Bowen et al., 2000). The duration of the exposure and concentration of the hormone are important to this regulation. An initial down-regulation of receptor protein was rapidly reversible and followed by up-regulation (Djiane et al., 1982; Barash et al., 1983; Bole-Feysot et al., 1998). The initial reduction in PRLR numbers occurs even when exogenous prolactin was at concentrations as low as 25 ng/ml. Conversely, extreme elevation of circulating prolactin caused a massive down-regulation of the existing receptors (Barash et al., 1983), most likely due to increased internalisation of hormone-receptor complex and degradation in the lysosomes (Costlow et al., 1975; Djiane et al., 1982). The mRNA expression of PRLR has also been reported to show this initial down-regulation in response to plasma prolactin levels. When sheep were shifted from a SD environment to LD, a decline in PRLR mRNA expression in the skin was observed 5 days after the transition. At this stage, circulating prolactin had only increased by 10 ng/ml (Nixon et al., 2002). Up-regulation of the receptors occurs after a few days of moderate prolactin elevation (Costlow et al., 1975; Djiane and Durand, 1977; Bole-Feysot et al., 1998), and the mRNA expression was also up-regulated after a constant moderate elevation of circulating prolactin (Asfari et al., 1995; Nixon et al., 2002).
It appears that the PRLR-L is the isoform that responds to the prolactin stimulus. In studies where the PRLR isoforms were measured separately after prolactin treatment, only the long form of the receptor is significantly changed (Asfari et al., 1995; Feng et al., 1998; Galsgaard et al., 1999).

In vitro, these responses occur more rapidly. Once prolactin is added to a culture system, down-regulation of PRLR numbers is observed in 15-30 min and the following up-regulation is seen after 6-24 h depending on the cell culture and prolactin dose (Barash et al., 1986). High doses of prolactin, however, may cause a refractory state. In this circumstance massive down-regulation occurs and could be affecting the rate of gene transcription (Djiane et al., 1979; Djiane et al., 1982). Alternatively, high levels of prolactin can result in the formation of monomers (1 prolactin molecule bound to 1 receptor molecule – 1H:1R), which are unable to activate down-stream signalling (Goffin et al., 1994) (Section 2.9.5).

2.9.5 Prolactin receptor signalling

Prolactin performs its biological functions by binding to a specific transmembrane receptor. Signalling, however, requires the formation of a dimer (1 prolactin molecule that binds to 2 receptor molecules- 1H:2R) for receptor activation (Goffin et al., 1996a; Goffin and Kelly, 1997; Clevenger et al., 1998) (Figure 4).
Growth hormone receptor (GHR) also belongs to the class 1-cytokine receptors (Bazan, 1990; Cosman et al., 1990) and the study of the kinetics of GH/GHR binding demonstrated that GH has two binding sites (Wells, 1996). The receptors have a higher affinity to the binding site 1 of the hormone. Because dimerization of the receptors is necessary for signal transduction, if GH concentration is high, the receptors will bind first to the site 1 of the receptor, forming a 1H:1R inactive complex. When GH concentration is lower, a second receptor will bind to the site 2 of the inactive complex forming the active dimer 1H:2R (Goffin et al., 1994). The prolactin molecule also has 2 binding sites and it has been demonstrated that the prolactin/PRLR behaves in a similar manner as the GHR (Kinet et al., 1999). Thus, the biological activity of prolactin and GH can be controlled by the availability of the hormone through the formation of the active dimer.
Once a 1H:2R complex is established, tyrosine phosphorylation of a number of cellular proteins, including the receptor itself, occurs and activation of target genes follows. However, if the dimer formed is a heterodimer (PRLR-L + PRLR-S), signal transduction does not occur (Goffin et al., 1994; Clevenger et al., 1998). In this manner the ratio between the different isoforms of the receptors is important in modulating the intensity of the biological response to prolactin stimulation.

The phosphorylation of JAK 2, which is constitutively associated with the PRLR, is required to initiate the tyrosine phosphorylation cascade of down-stream signalling proteins (Lebrun et al., 1994; Rui et al., 1994; Han et al., 1997; Bole-Feysot et al., 1998). The interaction between the PRLR and JAK 2 involves the homology box 1, present in both isoforms of the PRLR. Thus, both the short and the long form of the PRLR are capable of activating JAK 2 (Bole-Feysot et al., 1998).

The Signal Transducer and Activator of Transcription (STAT) family is another cytoplasmatic protein group capable of tyrosine phosphorylation, which participates in the prolactin signalling pathway. STAT 5 is important to PRLR signalling (Han et al., 1997; Hynes et al., 1997; Jahn et al., 1997; Pezet et al., 1997; Bole-Feysot et al., 1998; Davey et al., 1999). The JAK2/receptor complex phosphorylates STAT 5, which forms a dimer with another phosphorylated STAT 5. These dimers translocate to the nucleus and activate promoters of the target gene (Bole-Feysot et al., 1998). The phosphorylated tyrosines of the receptor are also able to activate STAT 5 (DaSilva et al., 1996; Pezet et al., 1997).

The JAK/STAT signalling cascade is down regulated by tyrosine phosphatases, which include Cytokine-inducible SH2 protein (CIS), Suppressor of Cytokine Signalling protein (SOCS), and JAK2 binding protein (JAB) (Naka et al., 1997; Bole-Feysot et al., 1998; Tomic et al., 1999; Barkai et al., 2000). A number of authors state that the short form of the PRLR acts as an inhibitor on the prolactin signalling, since the formation of a homodimer of the short form of the PRLR is unable to activate the JAK/STAT-
signaling pathway in the mammary gland of mice (Saunier et al., 2003). However, this dimer has been shown to activate the mitogen-activated protein kinase (MAPK) cascade, through the recruitment of Fyn and Raf 1 phosphorylated proteins, resulting in cell proliferation (Das and Vonderhaar, 1995; Bole-Feysot et al., 1998; Pearson et al., 2001).

2.10 Conclusion

This review shows that prolactin is important to fibre growth physiology in many mammals. In the domesticated sheep breeds, prolactin elevation is linked to stimulation of wool growth. However, many questions remain, including the ability of exogenous prolactin manipulation to alter wool growth and quality. It is also clear from this review, although not usually appreciated, that different routes, duration and timing of prolactin manipulation are important in the biological responses. While prolactin is able to regulate the expression of its own receptor, this is influenced by physiological status, the duration and dose of prolactin elevation, as well as other hormones, especially reproductive steroids.

Although the research of Kendall (1999) gave us a broad knowledge of the effects of prolactin on the wool production of lambing Romney ewes, some aspects of his findings remain unexplained and further experiments were suggested. For example, the knowledge that elevation of circulating prolactin had a medium- to long-term effect on wool growth suggest that an experimentally-induced hyper-prolactinaemia early in pregnancy could reduce or eliminate the large wool depression observed in mid-to late-gestation.

Furthermore, although Kendall's study revealed that seasonal, parturitional and lactational rises in prolactin were associated with subsequent wool growth, the mechanism by which this hormone influences fibre production is still unclear. Further studies on the elements of prolactin signalling pathway rather than prolactin itself are
necessary to explain how prolactin controls wool growth rate in domesticated sheep breeds.

With this information, a PhD program was designed to study the influence of prolactin manipulation on wool growth of Romney ewes, using different routes and duration of prolactin administration, and to animals of different physiological status. The ability of prolactin to regulate the expression of PRLR in the skin of this non-shedding sheep breed will also be investigated, given the lack of such data in the literature. The expression of PRLR was measured by real-time PCR, a new technique, which was optimised for specifically measuring the long and the short forms of the ovine PRLR in skin.
CHAPTER 3

MATERIALS AND METHODS
3.1 Introduction

For the fulfilment of this PhD program, a series of three animal experiments were undertaken at Ruakura Agricultural Research Centre, Hamilton, New Zealand. The animals used consisted of pregnant and non-pregnant Romney ewes of 3-4 years of age. The ewes were kept indoors (latitude 37° 46 S, longitude 175° 19 E), under controlled lighting and nutrition. In all of the trials the ewes were weighed regularly, underwent regular jugular blood sampling, mid-side patch wool clipping and skin biopsies. Some sheep were cannulated via the jugular vein and treated with exogenous ovine prolactin. Wool, blood and skin samples collected during the experiments were stored and analysed once the trial was finished. The laboratory procedures included:

- Radioimmunoassay (RIA) for determining the prolactin levels;
- RNA extraction, reverse transcriptase (RT) reaction, Ribogreen (RNA quantification) and real-time PCR. All these techniques are necessary for the relative quantification of PRLR expression;
- Wool metrology for evaluating fibre growth and quality.

These procedures will be described in detail in this chapter. Additional methodologies restricted to a particular trial will be described in the experimental chapters.

Data from animals exhibiting health problems or who died during the experimental period were excluded in the results. All the animal experiments were approved by the Ruakura Animal Ethics Committee.

3.2 Animal Husbandry

3.2.1 Animals

Romney ewes of 3-4 years of age, from the main Ruakura sheep flock, were used in all the experiments.
3.2.2 Basic Health

On arrival at the indoor facility, the station veterinarian inspected the sheep and they were treated against internal parasites (Ivomec ®; Ivermectin, Merial, USA). Their vaccination status was checked and, if necessary, they received immunisations against Salmonella (Salvexin ®; Pitman Moore, Upper Hutt, NZ) and clostridial diseases (Vetivax 5 plus ®; Bomac Laboratories Ltd., Manukau City, NZ) Pregnant ewes were vaccinated against campylobacter (Campylovexin ® Pitman-Moore; Upper Hutt, NZ).

To prevent the lambs from developing goitre, an oral dose of 4 ml of potassium iodine was administered to ewes at 90 to 120 days of gestation. The ewes were also dosed weekly with 10 ml of a 10% zinc sulphate solution to prevent copper toxicity, which is associated with maintaining sheep on a pelleted feed (Radostits et al., 1994; Lewis et al., 1997).

3.2.3 Housing

For a period of two weeks before the selection of experimental animals, ewes were acclimatised to the indoor environment. They were maintained in individual pens (1.4 x 0.8 m) and fed commercial pellets. Their food consumption and weight were monitored during this period and any sheep that did not adapt to these conditions were discharged from the selection process. The husbandry routine consisted of cleaning the pens early in the morning followed by the daily feeding. Water was offered ad libitum.

3.2.4 Diet

Animals were fed a commercial pelleted diet (Country Harvest Stock Feed, Cambridge, NZ) (Table 7).
Table 7: Diet composition

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>%</th>
<th>Composition</th>
<th>%</th>
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<tbody>
<tr>
<td>Lucerne</td>
<td>60</td>
<td>Dry Matter</td>
<td>95.1</td>
</tr>
<tr>
<td>Barley</td>
<td>30</td>
<td>Protein</td>
<td>14.6</td>
</tr>
<tr>
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<td>Ash</td>
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<td>5</td>
<td>Digestible fibre</td>
<td>33.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-digestible fibre</td>
<td>58.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carbohydrate</td>
<td>15.9</td>
</tr>
</tbody>
</table>

The feeding regime was based on the protocol developed by Kendall (1999) for maintaining the ewes at a constant maternal live weight. The objective of this was to remove, as far as possible, any effect that nutrition could exert on wool follicle growth.

Non-pregnant ewes and pregnant ewes (until day 100 of gestation) were fed approximately 900 g pellet feed per day adjusted to their initial live weight. After day 100, pregnant ewes were fed an increasing ration based on their individual weights over the last 6 weeks of gestation. A larger ration (20% more) was provided for twin-bearing ewes. During this period daily feed intake was measured by weighing the dry refusal.

### 3.2.5 Reproduction

The oestrus cycle was synchronised using progesterone-implanted EAZI-breed CIDR ® type G device (InterAg, Hamilton, NZ), implanted for 14 days. After CIDR removal ewes were joined with Romney rams with crayon-fitted harnesses. Ewes that were not marked by the rams were removed from the selection process. At approximately day 60 of pregnancy, ewes were scanned for pregnancy confirmation and determination of single- or twin-carrying ewes in order to adjust their feed consumption.
3.2.6 Light Treatment

Three different light treatments were used in different trials:

Natural Days: The animals were exposed to normal seasonal daylength. No artificial lights used.

Short Days: Eight h of light and 16 h of dark (8L:16D). This was achieved by closing window shutters at 1600 h and opening then at 0800 h every day.

Long Days: Sixteen h of light and 8 h of dark (16L:8D). An electric timer was used to control fluorescent lights (turned on at 0500 h and turned off at 2100 h every day).

The light intensity from the fluorescent lights alone, measured at 1 m above the ground and at 5 different locations in the room, ranged between 245 and 275 lux in the LD conditions. In the SD environment, with the shutters closed, light intensity was below 0.5 lux.

3.3 Cannulation

The jugular vein of selected experimental animals was cannulated before the beginning of each trial. Following surgical procedures, the ewes had their necks shaved and cleaned with soap and water. A solution of 10% iodine was then applied and a subcutaneous local anaesthetic (Lopain; Ethical Agents Ltd., NZ) was administered using the local infiltration/field block method. A disposable cannula (Cavafix, B.Braun; Melsungen, Germany) was then inserted into the jugular vein and held in place by adhesive tape and elastic netting (Setonet, Seton Healthcare Group, UK). Once cannulation was over, 5 ml of a long-acting broad-spectrum antibiotic (Duplocillin®, LA, USA) was injected intramuscularly in each ewe. After infusions were terminated, the cannulae were removed and the same dose of antibiotic was provided.
3.4 Prolactin administration

The prolactin used in the trials was derived from NZ sheep pituitaries at AgResearch Wallaceville (Dr. Lloyd Moore, batches # o 399 and o 499). Both batches were tested in a Radioimmunoassay against the NIDDK-oprolactin, and also showed no cross reactivity with growth hormone. The AgResearch Wallaceville prolactin was also used in cell culture experiments as a positive control in bioactivity assays used to test the activity of prolactin receptor antibodies in prolactin sensitive cells. Prolactin was dissolved in a saline solution containing 0.025 M NaHCO3 (2.38%) + 0.025 M Na2CO3 (10.13%) (final pH 9.2). Ovine albumin (0.05%) was added as a carrier protein to the infusion solution to prevent loss of prolactin by adsorption to containers and plastic tubing. The solution was filtered using a 0.2 μm filter before being administered to the animals. All the containers and tips were sterilised prior to use.

In Chapter 4, the prolactin dose to each ewe was 50mg/day. In Chapters 5 and 6 this was either prepared as a single injection volume (50 mg/5 ml), or diluted into an infusion bag. Bags were of 250 ml volume (for a 5 day infusion), 200 ml (4 day) and 150 ml (3 day). Every infusion bag was prepared on the day it was to be used.

3.5 Sample collection

3.5.1 Wool Sampling

Before commencing each trial all ewes were shorn. Using a template a standardised 100 by 100 mm area was marked on the right mid-side of each animal. This area was clipped at a monthly interval using a small animal clipper (Oster ®- Milwaukee, WI, USA). The wool collected from each patch clipping was identified and stored. At the end of the trial ewes were shorn again and the greasy fleece weighed. At this time a full length mid-side wool sample was collected.
3.5.2 Live Weight

All sheep were weighed regularly using electronic scales (Howard’s Weigh, Napier, NZ).

3.5.3 Blood Sampling

Blood samples were taken from the jugular vein using 10 ml vacuum blood collection tubes containing 0.34 M ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (Vacutainer®, Becton Dickinson, Rutherford, NJ, USA). The blood samples were centrifuged at 1200 g for 15 min at 4°C. Plasma was then separated using transfer tips and stored at -20°C until analysed.

3.5.4 Skin Biopsies

Skin biopsies were taken from all animals at various intervals, alternating between the right and left mid-sides. The area of the skin to be sampled was trimmed of excess fibre and disinfected with ethanol. The area was then anaesthetised (Lopaine; Ethical Agents Ltd., NZ) using an “L” shape surgical method (Tufvesson, 1963). A snip biopsy was collected and the area stitched using standard surgical procedure. The skin samples were labelled and preserved in liquid nitrogen. Subsequently, they were stored at -70°C until assayed.

3.6 Wool Processing

3.6.1 Fibre parameters – Wool quality and quantity

The patch clip samples and the full length mid-side wool sample were sent to WRONZ Development Ltd., Christchurch, NZ for analysis. The steps are briefly described below.

3.6.1.1 Scouring

The wool samples were weighed (greasy weight) and then washed sequentially with a non-ionic detergent (Teepol) at a controlled temperature and time according to the protocol in table 8.
Table 8: Wool washing protocol

<table>
<thead>
<tr>
<th>Order</th>
<th>Washing condition</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.20% w/v detergent</td>
<td>75°C</td>
<td>4 min</td>
</tr>
<tr>
<td>2</td>
<td>0.10% w/v detergent</td>
<td>60°C</td>
<td>4 min</td>
</tr>
<tr>
<td>3</td>
<td>water</td>
<td>55°C</td>
<td>4 min</td>
</tr>
</tbody>
</table>

3.6.1.2 Conditioning

After the wash step the samples were oven-dried at 50°C for 10 min in a forced-fan oven. To determine the washing yields, the dried samples were held for 48 h under standard atmospheric conditions (20°C, 65% relative humidity) and then weighed.

3.6.1.3 Measurement of fibre parameters

The mean fibre diameter, fibre diameter variation and mean fibre curvature of the wool samples were measured using the automated Optical Fibre Diameter Analyser as described by Brims (1993) and Edmunds (1995).

3.6.1.4 Fleece growth estimation

At each collection period, the mean rate of growth of clean wool (g per day) of each individual ewe was estimated by proportioning the ewe’s total clean fleece weight using the relative weight of the clean patch clip sample during that period (Sumner, 1977).

3.7 Prolactin Radioimmunoassay – RIA

The concentration of plasma prolactin was measured by a double-antibody radioimmunoassay, based on the NIDDK reagents separation of antibody, which has
been optimised to our conditions by Janet Wildermoth, AgResearch Ruakura, Hamilton, NZ.

3.7.1 Buffers

The laboratory chemicals used were supplied by BDH Laboratory supplies, Palmerston North, NZ, unless stated otherwise.

0.5 M sodium phosphate (pH 7.4)

Solution A 0.5 M Na₂HPO₄·12H₂O
Solution B 0.5 M NaH₂PO₄·2H₂O

0.01 M phosphate buffered saline (PBS) (pH 7.6)

Solution A 0.5 M Na₂HPO₄·12H₂O
Solution B 0.5 M NaH₂PO₄·2H₂O

NaCl
Distilled water

0.05 M sodium phosphate (pH 7.5)

0.5 M sodium phosphate

0.01 M PBS

1% Bovine Serum Albumin (BSA, Sigma, St. Louis, MO, USA)

0.01 M PBS

BSA

Assay diluent (EDTA) (pH 7.6)

0.01 M PBS

1% BSA

0.01% sodium azide (Riedel-de-Haën, Seelze, Germany)

0.05 M EDTA disodium salt
4% polyethylene glycol (PEG)

PEG 6000

0.01 M PBS

3.7.2 Prolactin iodination

Prolactin (NIH batch # AFP 10789 B) was iodinated using the lactoperoxidase method (Thorell and Johansson, 1971). Twenty to fifty μg of ovine prolactin (NIDDK-oprolactin-1-2, 500 μg/ampoule) was weighed using a micro scale and dissolved in 0.05 M sodium phosphate buffer (pH 7.5) to a final concentration of 0.5 μg/μl. The prolactin antigen solution was stored at −20 °C in 11 μl aliquots for up to two months. The ovine prolactin antiserum (rabbit - NIDDK-anti-oprolactin-2, 1 ml) was stored at −20 °C as a 1:30 dilution stock using 0.01 M PBS. Lactoperoxidase enzyme (Sigma L8257, EC 1.11.1.7) was dissolved in 0.5 M sodium phosphate buffer (pH 7.4) to a 0.025 nmoles solution. Twenty microlitres aliquots were stored at −20°C for up to 6 months. Hydrogen peroxide was diluted using distilled water to a 6.2 nmole/20 μl (1:60,000 dilution) solution. This solution was made up fresh for each iodination.

A total of 0.5 mCi of 125 I-iodide (New England Nuclear, Wilmington, DE, USA) was used in each iodination, which was performed in the high-level radioactivity laboratory at AgResearch, Ruakura, under protective equipment. In a 1.5 ml eppendorf tube the solutions were added in the following order:

20 μl lactoperoxidase
10 μl ovine prolactin
5 μl 125 I-iodide (0.5 mCi)
20 μl hydrogen peroxide

Once the solutions were added, the eppendorf was gently vortexed 3 times and the reaction was allowed to proceed for 3 min. Next, 300 μl of 0.05 M sodium phosphate (pH 7.5) was quickly added to stop the reaction and the iodinated solution was
transferred to a prepared Sephadex column (G 50). The separation of labelled prolactin from the excess 125 I -iodide was done by fraction collection from the sephadex column, flushed with 1% BSA. The radioactivity of these fractions was then measured in a 1261 Multigamma counter (LKB Wallac, Turku, Finland). The fractions that formed the first peak of radioactivity were mixed with glycerol and stored at -20 °C.

3.7.3 Radioimmunoassay procedure

3.7.3.1 Prolactin standards

Prolactin (NIH batch # AFP 10789 B) was weighed and dissolved in 0.01 M NaHCO3 to a final concentration of 100 μg/ml and used to make the prolactin standards diluted in the EDTA assay diluent (Table 9). All standards were stored at -20°C in 500 μl aliquots.

<table>
<thead>
<tr>
<th>pg/100 μl</th>
<th>ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>10000</td>
<td>100</td>
</tr>
<tr>
<td>5000</td>
<td>50</td>
</tr>
<tr>
<td>2500</td>
<td>25</td>
</tr>
<tr>
<td>1250</td>
<td>12.5</td>
</tr>
<tr>
<td>500</td>
<td>5</td>
</tr>
<tr>
<td>250</td>
<td>2.5</td>
</tr>
<tr>
<td>125</td>
<td>1.25</td>
</tr>
<tr>
<td>62.5</td>
<td>0.625</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

3.7.3.2 Prolactin controls

Using sheep plasma of a known prolactin concentration together with horse plasma, which has no ovine prolactin, three plasma standards were prepared and stored at -20°C. These were high (2500 pg) Medium (1000 pg) and Low (200 pg).

The medium control was used to determine the intra-assay and the inter-assay coefficients of variation.
3.7.3.3 Antiserum

The stock antiserum (1:30) was diluted with the assay diluent EDTA to a final assay dilution of 1:800,000.

3.7.3.4 Secondary antibody

In the first experiment (Chapter 4) Solid Phase Secondary Antibody Coated Cellulose Sustention (SAC-CEL) (IDS, Boldon, UK) were used as a secondary antibody, according to the manufacturer's instructions. The following experiment (Chapters 5 and 6) used normal rabbit serum (NRS) and sheep anti-rabbit serum (SAS) combined in the following ratio in 4% PEG/PBS solution.

NRS 1:300 (dilution)
SAS (#521) 1:150 (dilution)

3.7.3.5 Assay protocol

Assay samples, controls, standards, blanks (B) and total counts (T) were assayed in duplicate using 10 x 75 polystyrene tubes (Galantai Manufacturing Ltd., Auckland, NZ). The solutions were added in the following order.

- Sample 100 μl (or 10 μl if high concentrations were expected) or 100 μl of standard or control.
- Appropriate volume of assay diluent EDTA so that final volume was 200 μl. Tubes were vortexed at this stage.
- Antiserum 100 μl.
- Ovine prolactin tracer (antigen) 100 μl = approximately 15000 cpm.

Tubes were vortexed and the reaction incubated at room temperature for 40 h. At the conclusion of this time, 200 μl of the secondary antibody was added. Tubes were vortexed again and the reaction was incubated at room temperature for another 2 h. To
stop the reaction 1 ml of 4% PEG was added and tubes were vortexed once again. The tubes were then centrifuged at 2300 g at 4°C for 20 min. The supernatant was decanted and the radioactivity of the pellet was measured with the 1261 Multigamma counter and counts were analysed using the RIA CALC program. Inter-and intra-assay variations will be given in each experimental chapter.

3.8 PRLR gene expression

3.8.1 Products and buffers

TRIzol® reagent (Gibco BRL, Rockville, MD, USA)
Plastic Tubes (Global Science and Technology Ltd., Auckland, NZ)
Chloroform
Isopropyl ethanol
Ethanol
Formaldehyde
Agarose (Gibco BRL- Life Technologies NY, USA)
Dietethyl Pyrocarbonate-DEPC (Amersham Bioscience, UK)
RNA loading buffer (Sigma)
Ethidium bromide
RiboGreen Molecular probes (Invitrogen, Auckland, NZ)
RT kit (Invitrogen)
Primers and probes
TaqMan reagent
18s DPAR
PCR optical plates and covers

DBH Laboratory Supplies, Palmerston North, NZ
Applied Biosystem, Melbourne, VIC, Australia
3.8.2 RNA extraction

Frozen skin samples were ground to powder in a freezer mill (SPEX 7700, Glen Creston Ltd, Middlesex, UK). Frozen skin samples were weighed (200 to 400 mg) and immediately transferred to pre-chilled tubes and into the freezer mill containing liquid nitrogen. Samples were ground for 3 min and the powder was emptied into tubes containing 1 ml of TRIzol® reagent for every 100 mg of tissue. Between each sample, the grinding tubes and accessories were washed and chilled in liquid nitrogen. Homogenized samples were incubated for 5 min at room temperature.

0.2 ml of chloroform per 1 ml of TRIzol® reagent was added to the homogenized mix. Samples were capped securely and shaken vigorously. The mix was then incubated at room temperature for 3 min. Then, the tubes were centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase from the centrifuged tube was collected and transferred to a fresh tube. RNA was precipitated by adding 0.5 ml of isopropyl alcohol per 1 ml of TRIzol® reagent. The resulting solution was mixed by inverting the tubes and incubated at room temperature for 10 min. Tubes were then centrifuged at 12,000 g for 10 min at 4°C.

A visible gel-like pellet was formed after centrifugation. The supernatant was removed and the RNA pellet was washed twice with 1 ml of 75% DEPC ethanol per 1 ml of TRIzol® reagent. The samples were vortexed and centrifuged at 7,500 g for 5 min at 4°C. The 75% DEPC ethanol solution was discarded, the pellets were allowed to air-dry and then re-suspended in DEPC water. The samples were stored at -70°C.

3.8.3 RNA agarose/formaldehyde gel

To check the quality of the RNA, samples were run on a 1.5% agarose/formaldehyde gel (Table 10).
Table 10: 1.5% Agarose gel recipe

<table>
<thead>
<tr>
<th>Components</th>
<th>For a 30 ml gel (ml)</th>
<th>For a 100 ml gel (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>0.36</td>
<td>1.2</td>
</tr>
<tr>
<td>DEPC water</td>
<td>79.9</td>
<td>93.2</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>0.54</td>
<td>1.8</td>
</tr>
<tr>
<td>20 x MOPS</td>
<td>1.5</td>
<td>5</td>
</tr>
</tbody>
</table>

The gels were allowed to set and were loaded with 5 μl of RNA, 2 μl of RNA loading buffer and ethidium bromide. Electrophoresis was carried out for 45 min at 80 V.

3.8.4 RNA Quantification – Ribogreen method

An aliquot of the mRNA samples were diluted 1:50 so as to fall within the high range of the Ribogreen assay. The assay was set up in a 96 well plate at a final reaction volume of 200 μl. The Ribogreen dye was diluted down to 1:200 with 1x TE, and the standard RNA from the kit was diluted to produce standards of 0, 2, 20, 25, 50 and 100 ng/μl. One μl of the 1:50 diluted mRNA was added to 99 μl of 1x TE in each well together with 100 μl of the diluted Ribogreen dye. The plates were read in a fluorescence plate reader (Microplate fluorescence reader FL 500, Bio-Tek instruments, Winooski, USA), using an excitation of 480 nm and an emission of 520 nm. The data were then analysed using an Excel spreadsheet and the mRNA samples were standardized to a concentration of 0.5 μg/μl and stored at -70°C.

3.8.5 Reverse Transcriptase reaction

Previous experience with skin samples from Wiltshire sheep showed that a concentration of 0.5 μg/μl of total mRNA resulted in more efficient RT reactions. This was also valid for the Romney skin samples, therefore, all RT reactions were performed at this total mRNA concentration.
3.8.5.1 RT Reaction using Oligo dT

mRNA mix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA (0.5μg/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>10nM dNTP</td>
<td>1 μl</td>
</tr>
<tr>
<td>Oligo dT primer</td>
<td>1 μl</td>
</tr>
<tr>
<td>DEPC water</td>
<td>7.0 μl</td>
</tr>
</tbody>
</table>

The mRNA samples were dispensed into labelled eppendorf tubes and 9 μl of the above mix was added. The solution was mixed and incubated at 65°C for 5 min and then chilled on ice.

RT mix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x RT buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>25 nM MgCl₂</td>
<td>4 μl</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>2 μl</td>
</tr>
<tr>
<td>RNase out</td>
<td>1 μl</td>
</tr>
<tr>
<td>Superscript II</td>
<td>1 μl</td>
</tr>
</tbody>
</table>

Aliquots of 10 μl of the RT mix were added to each tube, mixed and incubated at 42°C for 50 min. The reaction was terminated by incubating the samples at 70°C for 15 min and then chilled on ice. One μl of RNase H was added to each tube (2 units/μl). The resulting solution was mixed and incubated at 37°C for 20 min. Samples were then stored at -20°C. No RT enzyme (no RT) and no template (no RNA) negative controls were performed at each RT reaction.

3.8.6 Real-time PCR

The real-time PCR technique for measuring mRNA levels detects and quantifies fluorescence emission at the end of each PCR cycle (real time as opposed to end point detection). Two methods are available for amplicon quantification: DNA-binding agents
(SYBR Green system) and fluorescence probes (TaqMan system). In both systems, the concepts of baseline cycle threshold (CT) and \( \Delta R_n \) are the basis of quantification of gene expression. The CT is defined by the number of cycles at which there is a significant raise in the fluorescence signal and is associated with the exponential increase of the PCR product during the log-linear phase. This phase of the reaction correlates to the initial amount of the target template. Lower CT values indicate higher initial amounts of target gene. The \( \Delta R_n \), which corresponds to the slope of the amplification curve, shows the amplification efficiency. To normalize the differences in the amounts of total cDNA added to each reaction requires the use of a gene which is more abundant than the target gene and whose expression does not alter between samples. Such gene is called an endogenous control and for this experimental program GAPDH (glyceroldehyde-3-phosphate dehydrogenase) and 18S ribosomal RNA were used.

3.8.6.1 The SYBR Green System

The initial approach for the quantification of PRLR was to use the SYBR Green technology, mainly because of its affordability. The SYBR Green detection system measures the increase in fluorescence caused by the binding of the SYBR Green dye to the double stranded DNA at the end of each cycle. The major problem with this system is that it does not distinguish fluorescence generated by the formation of specific and non-specific amplification. The real-time PCR master mix includes a passive reference dye (ROX), which does not participate in the PCR reaction. Nevertheless, it is used to normalize the SYBR Green/double stranded DNA complex signal to correct for the well to well fluorescence variation.

The primers for this method were designed using the Applied Biosystem software Primer Express and are described in Table 11.

The amounts of each component were (for a 25 \( \mu l \) final volume):
The assay was performed in triplicate dispensing 1 μl of the cDNA from the RT reaction and 24 μl of the PCR mix into separate wells. The assay was performed in a 96 well plate. In each plate, a standard curve for each target gene was set up. During the development of this assay, an inhibitory effect at high concentrations of cDNA was found for all target genes (Figure 5).

The reasons for this inhibition are unclear. However, it was noticed that at the part of the standard curve where samples were more diluted the inhibition did not occur. Therefore, a serial dilution of the cDNA sample with each specific primer was performed to determine the range of cDNA concentrations where the amplification was efficient. The starting point for this standard curve was a 1:10 dilution of total cDNA and this process appeared to solve the inhibition problem (Figure 6).

The next step was to run a number of samples at this dilution and select a sample that gave the lowest CT values in order to establish the standard curve for the entire assay. During this procedure, it was noticed that there was a significant variation in the GAPDH assay, making it unsuitable as an endogenous control. Samples from three different animas at different collection dates were run together in the same plate (Figure 7).
<table>
<thead>
<tr>
<th>Primer identification</th>
<th>Sequence</th>
<th>Amplicon size</th>
<th>Technology</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRLR common forward (PRLR F)</td>
<td>5'-CCAGATACCTAATGACTTCCC-3'</td>
<td>200 bp</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>PRLR-long reverse (PRLR L)</td>
<td>5'-TCTTCCGACTTGCCCTTCTCC-3'</td>
<td>229 bp</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>PRLR-short reverse (PRLR S)</td>
<td>5'GCCTTCTATATTAAAAACACAGAC-3'</td>
<td>424 bp</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>GAPDH forward (GAPDH F)</td>
<td>5'-GTGGCGCGCAAGGGGTCTCA-3'</td>
<td>63 bp</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>GAPDH reverse (GAPDH R)</td>
<td>5'GTGGCAGGCAGGTCGCTCTCC-3'</td>
<td>52 bp</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>β-Actin forward</td>
<td>5'-GCACGGCCGGGTCTGTGGTG-3'</td>
<td>83 bp</td>
<td>TaqMan</td>
</tr>
<tr>
<td>β-Actin reverse</td>
<td>5'-GACCGCCGGCCGGCTATGTG-3'</td>
<td>168 bp</td>
<td>TaqMan</td>
</tr>
<tr>
<td>UBE-1 forward</td>
<td>5'-TCAAGCAGCCGGCAGAA-3'</td>
<td>52 bp</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>UBE-1 reverse</td>
<td>5'ACAAACTTGGAGTCTGTGAGTATTG-3'</td>
<td>63 bp</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>PRLR-long forward</td>
<td>5'-GCATGGTGACCTGCATCT-3'</td>
<td>83 bp</td>
<td>TaqMan</td>
</tr>
<tr>
<td>PRLR-long reverse</td>
<td>5'-CGGCTTGCCCTTCTCC-3'</td>
<td>83 bp</td>
<td>TaqMan</td>
</tr>
<tr>
<td>PRLR-short forward</td>
<td>5'-TCGGACTTGCTCTCTATTTAACC-3'</td>
<td>168 bp</td>
<td>TaqMan</td>
</tr>
<tr>
<td>PRLR-short reverse</td>
<td>5'-TATAGCATGTGGCTGCTCTCC-3'</td>
<td>83 bp</td>
<td>TaqMan</td>
</tr>
<tr>
<td>PRLR-long probe</td>
<td>5'CACCCAGTTCCAGGGCCCAAAAATAAAGC-3'</td>
<td>200 bp</td>
<td>TaqMan</td>
</tr>
<tr>
<td>PRLR-short probe</td>
<td>5'-ACAAGGCGAGAAAGCTGTAATCTCCAG-3'</td>
<td>200 bp</td>
<td>TaqMan</td>
</tr>
</tbody>
</table>
Figure 5: Inhibition of the real-time PCR reaction: (A) GAPDH; (B) PRLR-L and (C) PRLR-S
Figure 6: cDNA dilution series (1:10): (A) GAPDH; (B) PRLR-L and (C) PRLR-S

A

\[ y = -1.4946 \ln(x) + 11.276 \]
\[ R^2 = 0.9936 \]

cDNA concentration

B

\[ y = -1.9318 \ln(x) + 24.942 \]
\[ R^2 = 0.9906 \]

cDNA concentration

C

\[ y = -1.4831 \ln(x) + 26.398 \]
\[ R^2 = 0.9972 \]

cDNA concentration
Such variation in the expression of GAPDH was not expected. Therefore, two alternative endogenous control genes were investigated. Ovine primers for β-actin and ubiquitin activating enzyme 1 (UBE1) were used and similar patterns of expression were evident for all three genes (Figure 8).

The endogenous control that showed the least variation was the β-actin, however, it was still not sufficiently stable for use as an endogenous control. At this point, the strategy for the relative quantification of mRNA was changed from the SYBR Green to the TaqMan technology. This allowed the use of the Applied Biosystem 18S ribosomal RNA pre-developed assay reagents (PDAR) as the endogenous control.

Figure 8: Internal control mRNA expression during the 6 sampling periods.
3.8.6.2 The TaqMan system

In the TaqMan system, a probe is designed that specifically anneals to an internal region of the PCR product. The assay is very specific, since a reporter dye is located at the 5'-end of the probe and a quencher dye, which suppresses fluorescence emission, is at the 3'-end. The polymerisation of the new strand starts and as it reaches the 5'-end of the probe it cleaves the probe dislocating the reporter dye that, when separated from the quencher dye, emits fluorescence. Hence, non-specific amplification is not detected.

3.8.6.3 RT Reaction using Random hexamers

The use of the 18S RNA PDAR required random hexamers as the primer for the RT reaction. Therefore, new RT reactions were conducted following the manufacturer's recommendations.

RNA/Primer mix:

- RNA (total of 0.5 µg/µl) 1 µl
- Random hexamers (50 ng/µl) 1 µl
- 10 nM dNTP1 1 µl
- DEPC water 7 µl

RNA/primer solution was mixed gently, incubated at 65°C for 5 min, placed on ice for at least 1 min and briefly spun down.

Reaction mix:

- 10x RT buffer 2 µl
- 25 nM MgCl2 4 µl
- 0.1 M DTT 2 µl
- RNaseOUT 1 µl

The reaction mix was added to the RNA/Primer, mixed gently and briefly centrifuged. Tubes were then incubated at 25°C for 2 min. One µl of Superscript II enzyme was
added to each tube, mixed and incubated at 25°C for 10 min. The tubes were then incubated at 42°C for 50 min. To terminate the reaction, tubes were transferred to a 70°C water bath for 15 min. The tubes were then chilled on ice and briefly centrifuged. To remove any remaining RNA, 1 μl of RNase H was added to each tube and incubated at 37°C for 20 min. The cDNA was stored at -20°C. No RT enzyme (No RT) and no template (no RNA) negative controls were performed at each RT reaction.

3.8.6.4  **Real-time PCR using TaqMan technology**

The relative quantification of PRLR expression was measured using the TaqMan real-time PCR system according to the manufacturer’s specifications (Applied Biosystems, Melbourne, Australia). Primers and probes for the PRLR-L and PRLR-S were designed using the Primer Express software (Applied Biosystems) (Table 11).

The reactions were run in a ABI Prism ® 7700 Sequence Detection System according to the universal cycling parameters determined by (Applied Biosystems) (Table 12).

<table>
<thead>
<tr>
<th>Times and Temperatures</th>
<th>Initial Steps</th>
<th>Melt</th>
<th>Anneal/Extend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>Hold</td>
<td>15 s</td>
<td>1 min</td>
</tr>
<tr>
<td>2 min 50°C</td>
<td>10 min 95°C</td>
<td>95°C</td>
<td>60°C</td>
</tr>
</tbody>
</table>

Similar inhibition problems as in the SYBR Green system were observed using TaqMan. Applied Biosystems had identified this problem and recommended a serial dilution of the cDNA to identify concentration ranges where the inhibition was not present. The concentration of cDNA chosen for the assay was a 1:20 dilution of the RT reaction.
3.8.6.5 Primer optimization

Optimal assay performance is achieved by independently varying the forward and reverse primer concentrations and using the combination of primers (Table 13) that provide the lowest CT value and the highest ΔRn.

Table 13: Primer optimisation.

<table>
<thead>
<tr>
<th>Reverse Primer (nM)</th>
<th>Forward Primer (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>100/100</td>
</tr>
<tr>
<td>100</td>
<td>100/300</td>
</tr>
<tr>
<td>100</td>
<td>100/600</td>
</tr>
<tr>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>300</td>
<td>300/100</td>
</tr>
<tr>
<td>300</td>
<td>300/300</td>
</tr>
<tr>
<td>300</td>
<td>300/600</td>
</tr>
<tr>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>600</td>
<td>600/100</td>
</tr>
<tr>
<td>600</td>
<td>600/300</td>
</tr>
<tr>
<td>600</td>
<td>600/600</td>
</tr>
</tbody>
</table>

The chosen primer combinations were:

- **PRLR-L**: Forward 100 nM
  - Reverse 300 nM
- **PRLR-S**: Forward 300 nM
  - Reverse 600 nM

3.8.6.6 Probe optimisation

Using the primer concentrations defined above a probe concentration optimization was also performed using 50, 100 or 250 nM of probe in each PCR reaction. Results indicated that the 250 nM probe concentration gave the best ΔRn results.

3.8.6.7 Assay

The assay was initially run on the ABI Prism ® 7700 Sequence Detection System at the Waikato University Gene Sequency Lab, with 96 well plates. Because of the high number of samples to be analysed a multiplex assay, where the endogenous control and the target gene are amplified in the same well, was attempted. However, the
amplification of both genes did not have similar efficiency and further optimizing steps were necessary. As an ABI Prism ® 7720 Sequence Detection System became available at the Livestock Improvement Corporation (LIC), which takes 384 well plates. The standard curve method for separate relative quantification of PRLR and endogenous control expression was undertaken using the LIC facilities.

3.8.6.8 Setting up the assay

All the plates incorporated samples to generate a standard curve for the target genes and an endogenous control (18S PDAR). Three negative controls (No RT, No template and water) for each target gene were also included on all plates. Samples were done in triplicate and run on the ABI Prism ® 7720 Sequence Detection System. Data were analysed using the software provided (Applied Biosystems) (Figure 9).

3.8.6.9 Data analysis

For relative quantification using the real-time PCR, the quantity of the target gene are determined by a standard curve, normalized by an endogenous control and divided by a calibrator. Thus, the quantity of the target gene is expressed as an n-fold difference relative to this calibrator. A calibrator is an experimental sample chosen to be a basis for the relative quantification. Each target gene plus the endogenous control (18S ribosomal mRNA) have in each plate a standard curve (a two fold dilution of total RNA preparation) from the same stock, which allows for comparison across plates.

The output of interest from the real-time PCR run is the CT value. Results from the run were plotted in Excel with the X-axis having the log transformed total RNA input and the Y-axis the CT values of target or endogenous control. A trend line was plotted and a linear regression was fitted through the data point.
Figure 9: Real-time PCR output. Panel A shows the amplification curve for the PRLR and panel B for the 18S RNA using the TaqMan system. The red line perpendicular to the exponential curve represents the CT value and the Y axis represents the ΔRn.
The log input amount of target and endogenous control genes were calculated by using the following formula: \( (\text{CT value} - b)/m \), where \( b \) = intercept of standard curve and \( m \) = slope of standard curve. The input amount of the target genes were then normalized by the corresponding amount of the endogenous control (PRLR/18S) of each experimental sample. This normalized amount of target gene is unitless and the relative amount of this target gene through the different experimental samples was achieved by comparing the amount of the normalized target gene with a designated sample from the experiment, known as a calibrator. In this experiment, because the objective was to verify if prolactin could change the expression of its own receptor in the sheep skin, the sample from the start of the treatment, before any prolactin manipulation was done, was chosen as the calibrator. Therefore, the results from the PRLR-L and PRLR-S mRNA expression are shown in n-fold difference relative to samples from the start of the experiment.

3.9 Statistical analysis

Live weight, feed intake, fleece weight and fibre diameter data were analysed by analysis of variance (Anova) at each sampling time to determine differences between treatments, using the computer statistical package Data Desk v. 6.0.2 (Ithaca, NY, USA). Means between treatments were compared using the LSD Post Hoc Test. Results are expressed as means ± SEM.

Wool growth rate and fibre diameter changes (Patch clip data) were adjusted to the initial value (Patch clip 1) and an analysis of covariance was performed using the computer statistical package GenStat v. 6.0 (Lawes Agricultural Trust, UK). Results are shown as adjusted values of the means and SED.

Prolactin levels and the expression of PRLR mRNA data were log transformed to remove the skewness and stabilise the variance. For the prolactin levels, the data were analysed by Anova and means are shown as back transformed values and SED. The
PRLR mRNA expression was analysed by Anova measuring the change from the pre-treatment values in the log receptors. Results were back transformed and error bars show the SED. The analysis was done using the computer statistical package GenStat.

3.10 The mathematical model of the prolactin-prolactin receptor interaction

3.10.1 Background

The endocrine system regulates the function of many tissues through the interaction of hormones and their specific receptors. However, these interactions are not simple and the biological response generated by the hormone-receptor interaction is dependent on interactions with other hormones (Geisinger et al., 1990; Ormandy et al., 1997; Suh and Rechler, 1997), different receptor isoforms (Buck et al., 1992; Anthony et al., 1995; Ratajczak, 2001; Culler et al., 2002) and the dynamics of the receptor population of the target tissue (Sarkar et al., 2003). The membrane receptor population is regulated by receptor synthesis, internalisation, degradation, recycling, and the dissociation process. These depend on pH, ion concentrations, temperature and interactions with other molecular structures associated with the plasma membrane (Lauffenburger and Lindermann, 1993) (Figure 10).

Mathematical models were introduced to the biological field mostly through epidemiological studies. However, lately, other biological systems have been represented by model simulations to predict clinical progression of diseases (Laptev and Nikulin, 2003), dynamics of viral infections (Bocharov et al., 2003; Layden et al., 2003), pharmacokinetics of drugs (Sanaka et al., 2002), tumor growth (Newman and Lazareff, 2003) and receptor-ligand simulations (Romiszowski and Sikorski, 2002; Kovyazina et al., 2003).
Prolactin is an important regulator of seasonal wool growth (Ryder and Lincoln, 1976; Montgomery and Hawker, 1987; Wuliji et al., 1993), and PRLR have been identified in the wool follicle of Wiltshire sheep (Choy et al., 1995; Choy et al., 1997). Therefore, understanding the interaction between prolactin and its receptor in the wool follicle may provide the means to effectively manipulate wool growth. A mathematical model that would enable the prediction of prolactin manipulation and its effect on receptor-ligand binding and signalling for different physiological states could be very helpful. Furthermore, animal experiments are costly, due to the need for animal housing, feeding and prolactin administration and are time consuming. For this reason also, a mathematical tool to assist in designing such experiments would be of major interest.
The development of the prolactin model by Dr. Tanya Soboleva in collaboration with Dr. Allan Pearson, Dr. Allan Nixon and Renata Montenegro, commenced in 1998 with the assumption that signalling was proportional to the amount of bound receptors and that receptors could only be in one of the two states, bound or unbound (Figure 11). The model was based on the theory that prolactin activates its receptor and the following signalling triggers a positive feedback mechanism to up-regulate PRLR expression. (Section 2.9.4).

*Figure 11: Diagram of the 1998 prolactin model.*

As an example, a simulation using this model is shown in Figure 12. Eighteen days of constant prolactin infusion up-regulated the amount of bound receptors (enhancing signalling), and down-regulated the number of unbound receptors. The decrease of unbound receptors is due to binding to prolactin, ultimately leading to up-regulation of the number of bound receptors. Once infusion is over, the number of bound receptors decreases through degradation, dissociation and internalisation.
The prolactin model was subsequently further developed with the assistance of Dr Kumar Vetharaniam. At this stage, it was taken into account that prolactin receptors belong to the class 1-cytokine family of receptors which require the formation of a dimer (1 prolactin hormone and 2 receptors) for initiation of the signalling process (Goffin and Kelly, 1997) (Chapter 2). There are 3 receptor states in the 2003 prolactin model: unbound (U), one receptor bound to a prolactin molecule (B1) and two receptors bound to a prolactin molecule forming a dimer (B2). The number of receptors in these different stages depends on prolactin concentration and parameters such as binding affinity, dissociation, internalization and degradation rates. Also, in the 1998 prolactin model, the rates of production and degradation of the receptors were arbitrary. However, prolactin administration changes these rates in different ways. Gertler et al (1986), reported that degradation and dissociation rates of PRLR in beef cattle were much faster than the rates used in the 1998 prolactin model. Once these adjustments were made the model proved to be more stable and robust (Figure 13).
This prolactin model was verified using results from the prolactin receptor expression of the first two experiments of this thesis (Chapters 4 and 5). Data obtained from the animal experiments were also used to characterise the elevation and decline of plasma prolactin levels and establish the duration of prolactin treatment for simulation purposes.

### 3.10.2 Description of the model

The mathematical model was based on the available knowledge of PRLR and general receptor dynamics (Posner et al., 1975; Barash et al., 1983; Barash et al., 1986; Gertler et al., 1986; Bolander, 1998). The state variables included in current prolactin model (Figure 13) are described by the equations below.

#### 3.10.2.1 Plasma prolactin concentration (P)

Prolactin concentration is increased by endogenous secretion (for example, lactation, stress and photoperiod) or exogenous manipulation (by infusion or injection). The
concentration decreases through clearance. The differential equation for the rate of change in plasma prolactin plasma level is:

\[ \frac{dP}{dt} = -GP(t) + k(t) \]  

(1)

Where \( k(t) \) is the exogenous and endogenous input of prolactin and \( G \) is prolactin degradation rate. However, for fitting the data set (the corresponding prolactin data from each experimental group), as prolactin is not dependent on the receptor dynamics, it was treated as an independent external input (smoothed data to produce a continuous curve) in equations 2, 3 and 4.

### 3.10.2.2 Numbers of unbound receptors (\( U \))

The number of unbound receptors decrease due to prolactin binding, internalisation and degradation; and increases due to dissociation, synthesis and recycling. The differential equation for the rate of change in number of unbound receptors is:

\[ \frac{dU}{dt} = k_a - k_n U(t) - \alpha P(t) U(t) - \alpha_1 B_1(t) U(t) + d B_1(t) + d_{12} B_2(t) + \frac{a B_2(t)}{b_0 + B_2(t)} \]  

(2)

Where:

- \( a \) = rate of receptor synthesis (at a constant rate)
- \( k_n U(t) \) = rate of unbound receptor degradation
- \( \alpha P(t) U(t) \) = rate of dimer formation
- \( \alpha_1 B_1(t) U(t) \) = rate of trimer formation
- \( d B_1(t) \) = rate of dimer dissociation
- \( d_{12} B_2(t) \) = rate of trimer dissociation
- \( \frac{a B_2(t)}{b_0 + B_2(t)} \) = induced receptor synthesis
3.10.2.3 Number of bound receptor (B₁ or B₂)

The number of bound receptors increases due to binding of prolactin to the unbound receptor and decreases due to degradation and dissociation. They can be in the form of receptor dimers (B₁) or trimers (B₂). Assuming that the binding and the dissociation rates obey a mass-action law and degradation of receptors as a first order process, the differential equations for each class of bound receptors are:

\[
\frac{dB_1}{dt} = \alpha P(t)U(t) - dB_1(t) - g_{b1}B_1(t) - \alpha_1B_1(t)U(t) + d_{12}B_2(t) \tag{3}
\]

\[
\frac{dB_2}{dt} = \alpha_1B_1(t)U(t) - d_{12}B_2(t) - g_{b2}B_2(t) \tag{4}
\]

Where \( g_{b1}B_1(t) \) = rate of dimer degradation and \( g_{b2}B_2(t) \) = rate of trimer degradation.

Table 14 shows the parameters used in the simulations of PRLR population in response to the prolactin profile of each experimental group.

Table 14: Parameters values used in the simulation of PRLR population in response to prolactin profiles (based on Bole-Feyt et al., 1998 and Gertler et al., 1996).

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
<th>Parameter value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )</td>
<td>Binding affinity of prolactin to PRLR</td>
<td>4.1472 L nmol⁻¹ day⁻¹</td>
</tr>
<tr>
<td>( \alpha_1 )</td>
<td>Binding affinity of bound PRLR to other PRLR</td>
<td>3.0240 L nmol⁻¹ day⁻¹</td>
</tr>
<tr>
<td>( d )</td>
<td>Dissociation rate of bound PRLR</td>
<td>12.9600 day⁻¹</td>
</tr>
<tr>
<td>( d_{12} )</td>
<td>Dissociation rate of dimers</td>
<td>4.7174 e⁻³ day⁻¹</td>
</tr>
<tr>
<td>( B_0 )</td>
<td>Formation of new unbound PRLR</td>
<td>25000 nM</td>
</tr>
<tr>
<td>( G )</td>
<td>Degradation rate of prolactin</td>
<td>35.6 day⁻¹</td>
</tr>
<tr>
<td>( \alpha )</td>
<td>Induced PRLR synthesis</td>
<td>187500 nM/h</td>
</tr>
<tr>
<td>( g_{b1} )</td>
<td>Degradation rate of bound PRLR</td>
<td>0.0625 day⁻¹</td>
</tr>
<tr>
<td>( g_{b2} )</td>
<td>Degradation rate of dimers</td>
<td>4.375 day⁻¹</td>
</tr>
<tr>
<td>( g_u )</td>
<td>Degradation rate of unbound PRLR</td>
<td>0.9375 day⁻¹</td>
</tr>
</tbody>
</table>
The simulations were run using the computer package MatLab v.6 R12 and results are expressed as the concentration of PRLR-L protein in the different binding states. Under the assumption that the initial conditions are in equilibrium, the model was used to predict the effect of each of the prolactin profiles in the different treatment groups of the 3 experimental trials. These results will be discussed separately in Chapters 4, 5 and 6.
CHAPTER 4

THE INFLUENCE OF PHOTOPERIOD, PROLACTIN AND PREGNANCY ON WOOL GROWTH AND PROLACTIN RECEPTOR GENE EXPRESSION IN ROMNEY EWES

INFUSION VS INJECTIONS
4.1 Abstract

The effects of prolactin manipulation during early pregnancy in Romney ewes on prolactin receptors (PRLR) mRNA expression and seasonal wool growth were investigated using long day (LD) photoperiod (n=11) and daily subcutaneous injection (50 mg/day) (n=11) or constant infusion of prolactin (50 mg/day) (n=2). Non-pregnant (n=8) and pregnant (n=11) control groups were also monitored. The ewes were maintained indoors from April to September 1999. A week after mating, prolactin treatment commenced and continued for 18 days. The LD treatment started at the same time and was carried out until September 29, when the trial finished. Blood samples were collected regularly and prolactin levels were analysed by radioimmunoassay. Six skin biopsies were taken from the start of the trial until June 26 and PRLR mRNA expression was measured using real-time PCR. Monthly patch clip data were analysed to estimate wool growth and fibre diameter during the course of the trial.

Constant infusion of prolactin during early pregnancy resulted in a short- to long-term positive effect (p<0.001), which resulted in a 24% increase on wool growth. This enhancement in production was associated with an increase (p<0.05) in PRLR expression during the infusion period. Daily injection of prolactin showed no effect on wool production or PRLR mRNA expression. Ewes maintained in LD, had elevated prolactin levels and, after parturition, wool growth was enhanced in this group. No changes in PRLR mRNA expression were noted in the LD group.

These results indicated that prolactin up-regulates the expression of its own receptor in the skin of Romney sheep. However, the prolactin profile was shown to be critical to this response. A constant infusion of prolactin was effective while daily injections were not. PRLR up-regulation is likely to be linked to the observed short- and long term wool growth responses.
4.2 Introduction

Domesticated sheep breeds selected for wool growth still exhibit a seasonal pattern of wool growth. In summer, production is higher than in the winter months (Story and Ross, 1960; Ryder, 1973; Hawker and Littlejohn, 1986; Sumner et al., 1998), and is associated with increased fibre diameter (Bigham et al., 1978; Scobie et al., 1993; Woods et al., 1995; Kendall, 1999). Prolactin is a protein hormone produced by the pituitary gland which also has a seasonal pattern of secretion, being high in the summer and low in the winter (Lincoln and Ebling, 1985; Lincoln, 1990). The rise in prolactin levels during spring has been associated with the seasonal moult observed in some domesticated and wild sheep breeds (Lincoln and Ebling, 1985; Allain et al., 1986; Pearson et al., 1996), and with the annual cycle of wool growth in Romney ewes (Kendall, 1999). PRLR have been identified in the skin of Wiltshire sheep (Choy et al., 1995) and their expression is, in part, regulated by changes in circulating levels of prolactin (Nixon et al., 2002). Hence, prolactin could have a local effect on fibre growth through a specific receptor.

Much of the data on the influence of prolactin on wool growth comes from shedding breeds such as the Wiltshire, Soay and Mouflon. However, Kendall (1999) extensively studied the effects of prolactin on the seasonal wool growth of Romney sheep. These trials demonstrated that it was possible to manipulate wool growth of the pregnant ewe through changes in circulating prolactin. This is an important finding considering that pregnancy is responsible for a major depression in winter wool growth (Stevens and Wright, 1951; Sumner and McCall, 1989; Parker et al., 1991).

The purpose of this study was to investigate the manipulation of plasma prolactin levels in early pregnancy on the seasonal winter decline in wool production. To maintain better control over plasma prolactin levels exogenous prolactin was used instead of artificial photoperiod to elevate circulating levels. Nevertheless, exogenous
manipulation of a hormone is not a simple task. Reproductive hormones, including prolactin, are secreted in a pulsatile manner (Ben-Jonathan, 1985) and these hormones interact with complex dose and temporal relationships to cause their biological effects. In reproduction, for example, it is critical that the timing and duration of LH, Follicle-Stimulating Hormone (FSH), oestradiol, progesterone and prostaglandin treatment are correct for successful control of the oestrus cycle (Wright and Malmo, 1992; Driancourt, 2001; Diskin et al., 2002). Also, Furuya and Issacs (1993) showed that manipulation of circulating androgen can induce apoptosis or cell proliferation depending on the status of the cell cycle at the time. Hebert et al (1993) were able to restore lactation in rats using constant infusion of prolactin, however, pulses of small duration were not effective. These examples illustrate the importance of the dosage, timing and route of hormonal administration to cause a biological response. Hence in this trial prolactin was administered both via daily injections and as constant prolactin infusion. PRLR were quantified to ascertain whether prolactin regulates the expression of its own receptor in the skin of Romney sheep and for any association with wool growth.

4.3 Material and Methods

4.3.1 Experimental Animals

Forty-three Romney ewes were transferred indoors to individual pens on March 2, 1999. Thirty-five ewes were randomly selected and induced to cycle by treatment with progesterone-impregnated CIDRs, from April 8-20. These ewes were joined to Romney rams with crayon-fitted harnesses on April 21-22 (day 0 of gestation). Marked ewes were transferred to individual pens in three separate rooms in the Ruakura Physiology Building. The prolactin administration commenced a week after mating on April 29 and continued for 18 days. The trial finished on September 29.
4.3.2 Experimental Groups

The 35 induced ewes were randomly allocated into 4 groups and the remaining 8 non-pregnant ewes formed Group 1 as shown in Table 15.

Table 15: Experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Reproductive status</th>
<th>Photoperiod</th>
<th>Prolactin treatment</th>
<th>n</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Non-pregnant</td>
<td>Natural days</td>
<td>None</td>
<td>8</td>
<td>Non-preg</td>
</tr>
<tr>
<td>2</td>
<td>Pregnant</td>
<td>Natural days</td>
<td>None</td>
<td>11</td>
<td>Preg</td>
</tr>
<tr>
<td>3</td>
<td>Pregnant</td>
<td>Long days</td>
<td>None</td>
<td>11</td>
<td>LD</td>
</tr>
<tr>
<td>4</td>
<td>Pregnant</td>
<td>Natural days</td>
<td>Constant infusion</td>
<td>2</td>
<td>Inf-18</td>
</tr>
<tr>
<td>5</td>
<td>Pregnant</td>
<td>Natural days</td>
<td>Daily S/C. injection</td>
<td>11</td>
<td>S/C-18</td>
</tr>
</tbody>
</table>

The prolactin treatments lasted for 18 days. Group 5 received 50 mg of prolactin as single daily subcutaneous injections (S/C) at 0900. Group 4 was cannulated and the 50 mg of prolactin was delivered daily as a constant infusion over the 18 day treatment period. The imbalance in the number of ewes in Group 4 (n=2) was based on results from previous experiments that manipulated fibre growth using daily prolactin injections (Duncan and Goldman, 1984a; Dicks et al., 1994). Furthermore, maintaining patent cannulae for 18 days in sheep is not a simple task, compared to daily injections. Daily injections were also thought to mimic, to some extent, the circadian rhythm of prolactin secretion (Chamley et al., 1974; Lincoln et al., 1982; McMillen et al., 1987). For all these reasons, it was decided to base the trial on injections of prolactin, but also to include 2 infused ewes given the possibility that the prolactin profile could be important (Hebert et al., 1993). Animals in Group 3 were exposed to artificial LD lighting from April 29 until the end of the trial. The ovine prolactin used in the experiment was sourced and formulated as described in Section 3.4.
4.3.3 Live weight
All sheep were weighed at fortnightly intervals for the duration of the trial to monitor changes in live weight.

4.3.4 Feeding
The daily feeding regime for pregnant and non-pregnant ewes was based on live weights as described in the Section 3.2.4.

4.3.5 Wool sampling
The ewes were shorn on March 24 and a patch clip established on April 1. Patch clip samples were subsequently collected monthly until the end of the experiment, when the ewes were again shorn on September 29.

4.3.6 Blood sampling
Jugular blood was collected at least once per week from all ewes. More frequent samples were collected twice daily from sheep in the S/C-18 and Inf-18 groups, starting 2 days prior to prolactin administration until the last day of the treatment. The first sample was collected immediately prior to the prolactin injection and the second sample 90 min later. Circulating prolactin levels were measured by RIA (Section 3.7). The inter-assay and intra-assay coefficients of variation for the assay at 10 ng/ml were 4.7% and 5.8%, respectively.

4.3.7 Skin sampling
Six skin biopsies were taken from all animals at intervals over the first 6 weeks of the trial. The first sample was collected immediately prior to mating (April 20), and then on days 2, 7, 18, 28 and 56 relative to the first day of prolactin treatment.
4.3.8 **PRLR relative quantification**

RNA from the skin samples was extracted using the TRIzol ® method. cDNA was then obtained by reverse transcriptase reaction and PRLR-L was quantified using the real-time PCR assay. See Section 3.8 for details.

4.3.9 **Lamb data**

Lambs born during the trial were weighed at two weeks of age and then weekly until they were weaned at 45 days of age.

4.3.10 **Statistical analysis**

Analysis of variance was used to test the effect of prolactin manipulation, pregnancy and their interaction on feed intake, live weights, plasma prolactin concentration, wool growth, fibre diameter and expression of PRLR. Analysis of covariance was used for the data from the patch clips. Results are presented as means ± SEM or SED.

4.4 **Results**

4.4.1 **Lambing data**

A total of 49 lambs were delivered between September 9-19. Three were dead at birth and two died in the perinatal period. The surviving lambs were comprised of 24 females and 20 males with a mean weight at two weeks of age of 8.5 kg. No difference in birth weight and weight gain at 2 weeks of age due to maternal treatment group was observed (Figure 14).
4.4.2 Feed intake

Table 16 shows cumulative and average daily feed intake (dry matter) during the trial. During the 145 days, total feed intake showed no difference between treatments among the pregnant ewes. The Non-preg group received maintenance feeding only, explaining the significantly lower intake when compared to the pregnant ewes.

Table 16: Total feed intake (kg DM/ewe) and average daily feed intake (g DM/ewe/day) during the trial (mean±SEM).

<table>
<thead>
<tr>
<th></th>
<th>Non-preg</th>
<th>Preg</th>
<th>LD</th>
<th>Inf-18</th>
<th>S/C-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total intake</td>
<td>117.3±4.3b</td>
<td>162.4±3.9a</td>
<td>156.1±4.0a</td>
<td>151.8±7.4a</td>
<td>152.4±4.0a</td>
</tr>
<tr>
<td>Av.daily intake</td>
<td>809±29b</td>
<td>1120±26a</td>
<td>1077±27a</td>
<td>1047±51a</td>
<td>105±27a</td>
</tr>
</tbody>
</table>

Different superscript letters represent a statistical difference (p<0.001)

4.4.3 Live weight

The live weights of the animals at the beginning of the trial did not differ (Table 17). There was also no difference in the weights of the pregnant ewes under the different treatments. Pregnant ewe live weight increased from 48.1±1.4 kg at the time they were mated, to 65.0±1.4 kg two weeks before parturition (P< 0.001) (Figure 15).
The mean post-lambing (1-2 weeks after parturition) weight was significantly lower (p<0.001) than the pre-lambing weight (1-2 weeks before parturition). However, ewes were still 10.6 kg heavier than their pre-mating weight. This is partially due to the weight of the functioning mammary gland, changes in fluid retention due to gestation and lactation, and to fleece growth (average 2.0 kg). After allowing for these factors, the ewes still gained some weight during the trial and after shearing. The Non-preg group, which were under a maintenance feeding regime, also gained 6.7 kg during the course of the experiment.

**Table 17:** Live weight (kg): Pre-mating (2 weeks prior to the start of the trial), pre-lambing (one week before start of parturition) and post-lambing (one week after lambing). Mean ±SEM.

<table>
<thead>
<tr>
<th></th>
<th>Non-preg</th>
<th>Preg</th>
<th>LD</th>
<th>Inf-18</th>
<th>S/C-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-mating</td>
<td>48.1±1.6 a</td>
<td>48.2±1.4 a</td>
<td>47.6±1.5 a</td>
<td>49.0±2.7 a</td>
<td>48.3±1.5 a</td>
</tr>
<tr>
<td>Pre-lamb</td>
<td>53.2±2.4 b</td>
<td>66.2±2.1 a</td>
<td>64.5±2.2 a</td>
<td>63.8±4.1 a</td>
<td>64.3±2.2 a</td>
</tr>
<tr>
<td>Post-lamb*</td>
<td>55.0±1.9 a</td>
<td>60.2±1.7 a</td>
<td>59.2±1.8 a</td>
<td>60.6±3.3 a</td>
<td>56.1±1.8 a</td>
</tr>
</tbody>
</table>

Different letters in the same row represent a statistical difference among groups (p<0.001)
* represent also the weight of the ewes at the end of the trial after shearing.

**Figure 15** Live weight of the different treatment groups. Arrow shows the pre-lambing weight. Error bar represents the pooled SEM.
4.4.4 Plasma prolactin concentration

Plasma prolactin levels were low at the beginning of the trial and showed no difference among the treatment groups. The Non-preg group plasma prolactin level of the start of the trial was 48 ± 16 ng/ml (Figure 16). Concentrations dropped to a nadir of 2.4 ± 4.0 ng/ml at the beginning of May and fluctuated between 10 and 80 ng/ml until the end of the trial. From the beginning of July until mid August, the plasma prolactin level of the Non-preg group was higher (p<0.05) than levels in the Preg and S/C-18 groups. Significant differences over this period were not detected between the Inf-18 and Non-preg groups, because there were only 2 ewes in the Inf-18 group. However, average plasma prolactin levels of the Inf-18 group were around 30 ng lower than the Non-preg group.

At the beginning of the experiment, the plasma prolactin level of the LD group was 42 ± 15 ng/ml (Figure 16). By May, levels declined to 4.7 ± 3.8 ng/ml, and then increased, reaching a peak on September 21 of 464 ± 71 ng/ml. From the beginning of June until the end of the trial, plasma prolactin levels were higher in the LD group compared to the Preg, Inf-18 and S/C-18 groups (p<0.01).

Figure 16: Mean plasma prolactin levels for the LD, Preg and Non-Preg group during the experimental period. Error bars indicate the pooled SEDs.
During prolactin infusion, quantification of the prolactin concentration in the blood of the infused sheep was not possible by withdrawing blood from the infusion cannula because of contamination by the infusate. However, an estimation was possible in one sheep when it became necessary to sample by venipuncture after occlusion to blood withdrawal via the cannula occurred on day 3 of treatment. In this ewe, prolactin levels were elevated throughout the infusion period and the averaged $730 \pm 4$ ng/ml (Figure 17).

*Figure 17 Plasma prolactin profile of the Inf-18 group (mean ± pooled SEM). The shaded bar indicates the prolactin manipulation period (values from one ewe during this time) and the arrow indicates the time of parturition.*

Plasma levels of the S/C-18 group (Figure 18) rose from a mean of $25.0 \pm 17.0$ ng/ml before sub-cutaneous injection to $1017 \pm 31$ ng/ml two h later. ($p<0.001$). These levels declined during the subsequent 22 h, and by the next day blood levels were back to pre-injection values.
One week after treatment, plasma prolactin levels of both constantly infused and subcutaneously injected animals had declined to 5 ± 21 and 8 ± 17 ng/ml, respectively. These levels fluctuated between 4 and 47 ng/ml until September 7, when levels started to increase prior to parturition. The Inf-18 group reached a parturition peak of 395 ng/ml on September 21 and the S/C-18 group peaked at 379 ng/ml on September 14.

4.4.5 Prolactin receptor relative quantification

The PRLR mRNA expression measurements are represented as log fold differences relative to values at the start of the experiment (Section 3.8.6.9). Despite having only 2 animals in this group, constant infusion of ovine prolactin was associated with a substantial elevation (p<0.05) of PRLR-L mRNA expression in pregnant Romney ewes (Figure 19). Thirty eight days from the end of infusion, PRLR-L mRNA expression was still up-regulated (p<0.05) compared to pre-treatment values. The Non-preg group also exhibited up-regulation of PRLR mRNA expression in relation to values at the start of the experiment and compared to the trial averages of the Preg (p<0.05), LD (p<0.01) and 18-S/C (p<0.01) groups. The other pregnant groups (LD, Preg and S/C-18) demonstrated very little change in the expression of PRLR mRNA. In the Preg group there was a peak in expression 18 days after initiation of the experiment, but this was attributable to an individual animal.
Figure 19: The effect of prolactin manipulation and reproductive status on PRLR-L mRNA expression (log fold change). Error bars represent the pooled SED between groups of the fold change. A bar represents the treatment period.

4.4.6 Fleece-wool growth

Using the patch wool growth data, the wool growth of the whole fleece was estimated for each patch sampling period (Section 3.5). The results show that wool growth declined from May, reaching a minimum in July and rising subsequently (Figure 20, Table 18). There were no group differences in wool growth during April or May. In June, the Inf-18 group showed a tendency (p<0.10) to produce more wool than the LD, Preg and Non-Preg groups. In July (p<0.01), August and September (p<0.001), inf-18 ewes grew more wool than the other treatment groups (Figure 20). Even though the Inf-18 group had only 2 animals, the statistical significance between the wool growth of this group and the other treatments is substantial and deserving of further consideration.

Subcutaneous injections of prolactin had no initial influence on wool growth. Until August, there was no difference between the S/C-18 and the Preg groups. However, in September S/C-18 ewes grew less (p<0.05) wool when compared to the Preg group. Until August there was also no difference in wool growth rate between the LD and Preg
group. However, between August and September LD wool growth was significantly higher (p<0.05) than in the Preg group.

**Figure 20: Effect of prolactin manipulation and reproductive status on wool growth. Adjusted means and the error bars represent the average SEDs between treatments. The open bar represents the treatment period, and the arrow represents the time of parturition.**

![Figure 20](image)

**Table 18: Effect of prolactin manipulation and reproductive status on the wool growth (g/day) of Romney ewes (adjusted means ± average SEDs).**

<table>
<thead>
<tr>
<th></th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-preg</strong></td>
<td>8.4±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.4±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.1±0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.4±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.3±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Preg</strong></td>
<td>8.5±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.5±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.2±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.0±0.95&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>LD</strong></td>
<td>9.2±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.5±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.5±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.9±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.7±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Inf-18</strong></td>
<td>8.8±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.0±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.7±0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.6±0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.8±0.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>S/C-18</strong></td>
<td>9.0±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.8±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.2±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.8±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.7±0.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscript letters in the same column represent a statistical difference (p<0.01) * (p<0.001).

### 4.4.7 Patch fibre diameter

Fibre diameter followed a similar pattern to wool growth (Figure 21, Table 19). The fibre diameter of the Inf-18 and the LD groups showed an overall smaller decline in fibre diameter than the S/C-18, Preg, and Non-preg groups throughout the experiment. A statistical difference (p<0.05) between the fibre diameter of the treatment groups was observed in June. Which could be attributable to the higher fibre diameter of the LD,
S/C-18 and Inf-18 groups in relation to the Preg and Non-preg groups. In September, a statistical difference (p<0.01) between treatments was once more observed. On this occasion the difference was attributable to the higher fibre diameter in the LD and Inf-18 groups when compared to the S/C-18, Preg and Non-preg groups.

Figure 21: Effect of prolactin manipulation and reproductive status on mean fibre diameter. Adjusted means and error bars represent the average SEDs between treatments. The open bar represents the treatment period and the arrow the time of parturition.

Table 19: Effect of prolactin manipulation and reproductive status on the mean wool mean fibre diameter (μm) of Romney ewes (adjusted means ± average SEDs)

<table>
<thead>
<tr>
<th></th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-preg</td>
<td>31.7±0.9a</td>
<td>29.7±1.0a</td>
<td>30.7±1.1a</td>
<td>29.5±1.1a</td>
<td>29.7±1.3a</td>
</tr>
<tr>
<td>Preg</td>
<td>33.0±0.9a</td>
<td>30.5±1.0a</td>
<td>30.5±1.1a</td>
<td>29.4±1.1a</td>
<td>29.3±1.3a</td>
</tr>
<tr>
<td>LD</td>
<td>34.1±0.9a</td>
<td>32.3±1.0b</td>
<td>31.7±1.1a</td>
<td>32.0±1.1a</td>
<td>34.0±1.3b'</td>
</tr>
<tr>
<td>Inf-18</td>
<td>34.2±0.9a</td>
<td>34.1±1.0b</td>
<td>33.5±1.1a</td>
<td>33.4±1.1a</td>
<td>34.9±1.3b'</td>
</tr>
<tr>
<td>S/C-18</td>
<td>34.8±0.9a</td>
<td>32.7±1.0b</td>
<td>30.6±1.1a</td>
<td>31.5±1.1a</td>
<td>29.6±1.3a</td>
</tr>
</tbody>
</table>

Different superscript letters in the same column represent a statistical difference (p<0.05) *(p<0.01).

4.4.8 Greasy and clean fleece weight

The fleece data spans wool growth for the duration of the trial. Constant infusion of prolactin had a significant positive effect on the greasy and clean wool growth from April to September (Table 20). The Inf-18 group had higher greasy fleece weight (GFW)
when compared to the LD (p<0.05), Non-preg (p<0.05), Preg (p<0.01) and S/C-18 (p<0.001) groups. The same statistical differences were observed with the clean fleece weight (CFW). GFW and CFW were statistically lower in the S/C-18 group when compared to the Inf-18 (P<0.001), Non-preg (p<0.01) and LD (p<0.05) groups.

Table 20 Effect of prolactin manipulation and reproductive status on the greasy fleece weight (GFW) (kg), clean fleece weight (CFW) (kg), fleece fibre diameter (FFD) (microns) and fleece yield (FY) (%) (means ±SEM).

<table>
<thead>
<tr>
<th></th>
<th>Non-preg</th>
<th>Preg</th>
<th>LD</th>
<th>Inf-18</th>
<th>S/C-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFW</td>
<td>2.23±0.1b</td>
<td>2.09±0.1x</td>
<td>2.16±0.1b</td>
<td>2.63±0.2a</td>
<td>1.90±0.1c</td>
</tr>
<tr>
<td>CFW</td>
<td>1.38±0.1b</td>
<td>1.25±0.1x</td>
<td>1.39±0.1x</td>
<td>1.64±0.1a</td>
<td>1.22±0.1c</td>
</tr>
<tr>
<td>FFD</td>
<td>32.1±0.9ab</td>
<td>31.1±0.8a</td>
<td>32.7±0.9ab</td>
<td>34.0±1.6ab</td>
<td>33.1±0.9b</td>
</tr>
<tr>
<td>FY</td>
<td>68.9±1.5b</td>
<td>67.1±1.3a</td>
<td>70.4±1.4b</td>
<td>70.8±2.6ab</td>
<td>71.9±1.4b</td>
</tr>
</tbody>
</table>

Different letters in the same row represent statistical a difference among groups (p<0.05)

4.4.9 Fleece mean fibre diameter

The fleece fibre diameter (FFD) of the Inf-18 and the LD groups showed a tendency (p<0.10) to be greater than in the Preg group. Although there was a difference of 2.9 µm between the Inf-18 and Preg group, this was not statistically significant, due to the small number of ewes in the Inf-18 group.

4.4.10 Mathematical model simulations of PRLR-L population

The prolactin profiles measured in the different experimental groups from April until September were used in the mathematical model to predict changes in PRLR population throughout the trial (Figure 22). This not only served to verify the model, by checking simulation predictions against the biological data, but it also served as a framework to predict the response of the PRLR population to the different prolactin profiles once skin sampling was completed on June 25.

The simulations corresponding to the Non-preg group (Figure 22 A) shows little change in the concentration of total PRLR protein throughout the experiment in response to
relatively constant levels of circulating prolactin. According to the model, PRLR protein concentration in the Preg group (Figure 22 B) and LD group (Figure 22 C) show similar patterns, in the first 60 days of the experiment, given that their prolactin profiles were similar at this time. Subsequently, the prolactin profile of the LD group increases and the total receptor concentration in this group reaches levels above 2000 nmol/l, while in the Preg group these levels remain below 2000 nmol/l. The parturition rise in prolactin levels also increases the concentration of PRLR protein. The higher prolactin levels observed in the LD group at this time also results in a higher peak of PRLR protein.

The simulation predicts that the prolactin profile achieved by daily injection has no effect on PRLR population (Figure 22 D). The transient elevation of prolactin in the S/C-18 group results in only transient modification of the different receptor states, which does not activate the positive feedback mechanism of PRLR synthesis by prolactin itself. On the other hand, PRLR is up-regulated in the infused group through an increased concentration of PRLR in the dimerised state, responsible for prolactin signalling. This results in an increase in the concentration of total PRLR due to a rise in PRLR synthesis (Figure 22 E). The predicted population of PRLR in the Preg, Inf-18 and S/C 18 groups was similar from June until the end of the trial.
Figure 22: Mathematical simulation of the PRLR population in using the prolactin profile from the experimental groups. First panel illustrates the smoothed prolactin data; the middle panel shows the concentration of bound (B₁) and dimerised (B₂) receptors and the last panel shows the concentration of unbound (U) and total receptor. (A: Non-preg; B: Preg; C: LD; D: S/C-18 and E: Inf-18).
4.5 Discussion

Pregnancy is responsible for a major reduction in wool production and quality (Turner et al., 1968; Reid, 1978; Corbett, 1979; Sumner and McCall, 1989; Masters et al., 1993; Kendall, 1999). The loss in clean fleece production ranges between 7%-24% per lambing season. In this trial the Preg ewes grew 10% less clean fleece, during the 6 months of the experiment, when compared to the Non-preg group, corroborating existing data. The results from this experiment also confirmed the seasonal wool growth pattern reported in Romney sheep and their crosses (Story and Ross, 1960; Geenty et al., 1984; Woods et al., 1995; Sumner et al., 1998). Although these intrinsic mechanisms were still present in this experiment, the manipulation of prolactin levels during early pregnancy was demonstrated to influence both the seasonal and gestational wool growth patterns. In the Inf-18 group the winter wool growth reduction was eliminated.

4.5.1 Prolactin levels

From the winter solstice on June 21, photoperiod slowly increases to a maximum on December 22 (summer solstice). Hence, this transition from SD to LD is the cue for enhanced prolactin secretion as seen in the Non-preg group. In an experimental environment, where sheep were shifted from 6 h into 18 h of light, circulating prolactin levels rapidly increased from 6 to 80 ng/ml in a period of 20 days (Nixon et al., 2002). This seasonal increase after June was somewhat inhibited by pregnancy. The mean plasma prolactin concentration in the Non-preg group was slightly, but statistically higher (p<0.05) than the Preg and S/C-18 groups from July until parturition (September), corroborating the findings of Kendall (1999). In the pregnant ewe, levels of ovine placental lactogen commence to rise around day 60 of gestation (Kelly et al., 1974; Chan et al., 1978; Bazer and First, 1983), coinciding with the time when the prolactin profile of the pregnant and Non-preg groups begin to differ. In ruminants, placental lactogen can bind and activate PRLR via the formation of homodimers or by
forming heterodimers of one PRLR molecule and one GHR molecule (Gertler and Djiane, 2002). Grattan (2002) proposed that in pregnant rats, the increase in circulating levels of placental lactogen activates PRLR signalling in the hypothalamus, inhibiting prolactin secretion. This regulation of prolactin secretion by placental lactogen (PL) could also occur in the pregnant ewe, explaining the different prolactin profiles.

The difference in circulating prolactin between the LD and Preg groups from June until the end of the trial suggests that LD treatment can override the reproductive suppression of prolactin secretion. The parturition prolactin peak was also higher in the LD group when compared to the other pregnant ewes, corroborating existing data (Kendall, 1999)

Daily injection of prolactin resulted in high transient peaks with considerable variation. Average concentrations fell from 1400 ng/ml to 25 ng/ml in a 24-h period, in accordance with the short prolactin half-life (Akbar et al., 1974; Litherland, 1996). On the other hand, constant infusion resulted in a stable elevation of prolactin. Once prolactin treatment was over, prolactin levels did not differ from the levels exhibited by the untreated Preg group until the end of the trial. This suggests that prolactin injections or constant infusions did not influence pituitary prolactin secretion once the treatment was concluded.

4.5.2 Prolactin receptor mRNA expression

There are many factors that can influence the expression of PRLR which are species and tissue specific. One of the known regulators of PRLR expression is prolactin itself (Barash et al., 1983; Kelly et al., 1991; Bole-Feysot et al., 1998; Nixon et al., 2002). PRLR have been identified in the wool follicle (Choy et al., 1995). Nixon et al (2002) demonstrated that the expression of PRLR mRNA was also influenced by changes in circulating prolactin, which were associated with the wool cycle of the Wiltshire sheep. However, in this study, PRLR mRNA expression in the skin of pregnant and non-
pregnant Romney ewes is described for the first time. This experiment showed that the PRLR regulation at the commencement of pregnancy appeared to be influenced not only by prolactin treatment but also by changes in the reproductive hormonal environment.

During the skin sampling period from April 20 to June 25, the Non-preg group had a slight up-regulation of the expression of PRLR mRNA in relation to pre-treatment values. The Preg and LD groups, on the other hand, showed no difference in PRLR mRNA expression during the sampling period.

There was no difference between the prolactin profile of the Preg, Non-preg and LD groups during most of the skin sampling period. Therefore, it is unlikely that the slight up-regulation of PRLR mRNA expression in the Non-preg group was dependent on circulating prolactin levels. This suggests that some other endocrine signal associated with gestation could be responsible for this difference. It has been demonstrated that in the ovaries, immune system, pancreas and brain of non-pregnant rats, PRLR mRNA expression is influenced by the oestrus cycle (Moldrup et al., 1993; Sugiyama et al., 1994; Shamgochian et al., 1995; Gunes and Mastro, 1997; Kinoshita et al., 2001). The non-pregnant Romney ewes were likely to be cycling until June. Hence, this hormonal environment could be influencing PRLR expression. Furthermore, 17β-oestradiol is the main oestrogen in the non-pregnant ewe (Baird et al., 1973), while oestrone sulphate and oestradiol sulphate are predominant in the pregnant ewe (Carnegie and Robertson, 1978; Tsang et al., 1978). These different forms of estrogens can have different effects (Tice et al., 1978). Indeed, Leondires et al (2002) showed that 17β-oestradiol stimulated the expression of two isoforms of the human PRLR. Therefore the endocrine changes during the oestrus cycle and the differences in circulating oestrogen between pregnant and non-pregnant ewes could explain the divergence in PRLR mRNA expression.

One of the main purposes of this trial was to investigate if PRLR expression in skin of Romney ewes is regulated by prolactin and if this regulation is linked to wool growth.
The results showed that constant infusion of prolactin in early pregnancy was responsible for an up-regulation of PRLR mRNA expression, corroborating the existing data in different tissues (Djiane et al., 1979; Djiane et al., 1982; Rosa et al., 1982; Barash et al., 1986; Klemcke et al., 1990; Rose and Stormshak, 1993; Galsgaard et al., 1999; Bowen et al., 2000). This treatment also significantly enhanced the overall wool production.

Daily injections, on the other hand, had no impact on PRLR mRNA expression or wool growth. This disagrees with Dicks et al (1994) and Duncan and Goldman (1984a) who gave daily prolactin injections and were able to influence the follicle cycle in goats and hamsters, respectively. However, the species used in the above trials undergo synchronized hair growth cycles, while the Romney sheep do not; possibly influencing the responses to prolactin injections.

The duration and concentration of plasma prolactin levels are important in the regulation of PRLR population (Costlow et al., 1975; Djiane et al., 1977; Asfari et al., 1995; Bole-Feysot et al., 1998). The higher, but transient, prolactin levels achieved with daily injections were unable to induce receptor up-regulation. Indeed, Djiane et al (1982) and Costlow et al (1975) reported that high prolactin concentrations accelerate receptor internalization, causing a reduction in the receptor population in the plasma membrane, with consequent changes in the degradation, recycling, production and trafficking of the PRLR.

Another explanation for the lack of injection response is that the prolactin molecule has two binding sites with different affinities to the PRLR (Kinet et al., 1999). If prolactin is available in high concentrations, as was seen following the injections, there is a tendency for the formation of an inactive complex (1H: 1PRLR), which does not transmit a down-stream signal (Goffin et al., 1996a; Goffin and Kelly, 1997; Goffin et al., 1998) and has a lower internalization rate thus becoming more abundant in the cell.
surface. This could also lead to an inability of the prolactin injection treatment to influence the PRLR population.

Aside from the PRLR dynamics in the plasma membrane, down-stream factors may also influence prolactin signalling. The formation of a homodimer between a prolactin molecule and two receptors activates the JAK2/STAT signalling pathway (Bole-Feysot et al., 1998; Davey et al., 1999). This pathway is inhibited by tyrosine phosphatases, which include CIS, SOCS, JAB and SSI proteins (Naka et al., 1997; Tomic et al., 1999; Barkai et al., 2000). Therefore, an intensive signalling provoked by high prolactin availability, might also invoke the tyrosine phosphatase down-regulation mechanism, inhibiting the positive feedback on PRLR expression (Figure 4).

In some tissues, elevation of prolactin levels cause an initial rapid and reversible down-regulation of the receptor, followed by up-regulation when prolactin levels are sustained (Matsuda and Mori, 1996; Bole-Feysot et al., 1998). Nixon et al (2002) also demonstrated that elevation of circulating prolactin by increasing photoperiod induced the same pattern in skin PRLR mRNA expression, which was temporally related to the wool follicle cycle of the shedding Wiltshire sheep.

The results from the Inf-18 group did not show an initial down-regulation caused by prolactin elevation. The steady stage of plasma prolactin level was reached between 30 and 60 min after the initiation of infusion, as also reported by Akbar et al (1974) and Davis and Borger (1973). The initial down-regulation of PRLR mRNA expression is observed even with modest elevations of plasma prolactin levels (Nixon et al., 2002). However, a decline in PRLR mRNA expression could have occurred within the initial 48 h, prior to the first post-infusion skin sample.

The up-regulation of PRLR mRNA levels in the Inf-18 group was sustained after treatment ceased. This finding differs from the results of Nixon et al (2002) where PRLR mRNA expression fell as prolactin levels declined with the shortening of the
photoperiod, after a two week lag period. This difference could have various explanations. Nixon et al (2002) used Wiltshire sheep that shed their wool fibres annually, and may have different responses to environmental cues compared to Romney sheep, which have been selected for wool growth. These authors also used a photoperiod manipulation to increase prolactin levels, which produced a slower and smaller rise, and a slower decrease in circulating levels than in the present study. Finally, they used males and non-pregnant ewes in their trial. It is clear in the literature that reproductive status has a major impact on PRLR regulation. However, any speculation on the influence of reproductive hormones in the regulation of PRLR expression in the skin must be treated with caution, since in different species and tissues the effects of such hormones are different. For example, in rats and mice progesterone has an inhibitory effect on PRLR expression (Djiane and Durand, 1977; Nishikawa et al., 1994; Mizoguchi et al., 1997a); while in sheep, progesterone plays a stimulatory role in the mammary gland (Cassy et al., 1998; Cassy et al., 2000).

4.5.3 Mathematical model simulations

The mathematical model was used to predict changes in the PRLR protein expression in response to the prolactin profile of each treatment. The results are expressed as the concentrations of the different binding states of the PRLR-L protein (Section 3.10.2).

During the skin sampling period the results of the simulation from the LD and Preg groups agree with the biological data assuming the relative concentrations of PRLR-L mRNA are linked to protein concentrations. The simulation of the Non-preg group was similar to the LD and Preg group, showing no change in receptor protein expression from mid-April to mid-June based on the prolactin profile of that group. However, the biological data showed that the Non-preg group had an slight up-regulation of PRLR mRNA expression during this time. The mathematical model does not take into account the presence of oestrus cycle hormones which could have an important role in the
regulation of PRLR expression (Moldrup et al., 1993; Sugiyama et al., 1994; Kinoshita et al., 2001).

Once the skin sampling period was over, the simulations predicted that the concentration of total receptors and dimer formation would remain constant until the end of the experiment in the Non-preg group. In the Preg group, as prolactin levels increased close to parturition, so did the PRLR-L protein expression. The model also predicts an increase in the concentration of total receptors and dimer formation during lactation. In the LD group, from mid-pregnancy until the end of the trial, prolactin levels were higher compared to the other groups. The simulation of this prolactin profile results in an increase in the concentration of total PRLR and dimer formation when compared to the Preg group. This suggests enhanced overall prolactin signalling, possibly explaining the increased postpartum wool production observed in the LD group when compared to the Preg group. However, this change in PRLR population could be underestimated by the model. PRLR mRNA expression in the sheep mammary gland has been shown to be up-regulated by progesterone, oestrogen and cortisol (Cassy et al., 1999; Cassy et al., 2000). If these hormones also increase PRLR mRNA expression in the skin, the PRLR system would be primed and could respond with more efficiency to the higher prolactin parturition peak observed in the LD group. However, the model does not take into consideration the effect other of reproductive hormones on the PRLR population.

During the skin sampling period, the prediction of PRLR protein expression of the Inf-18 and S/C-18 groups agree with the biological data. In the Inf-18 group, the concentration of dimerized receptors and total receptor increased, reaching a plateau and then remaining constant until the end of infusion. The enhancement of the dimerized receptor concentration would allow for increased signalling and therefore a positive feedback on PRLR expression. Once prolactin treatment was over, the predicted total receptor concentration and receptor in the dimerized state showed a
sharp decline, whereas, the measured mRNA expression of PRLR displayed a sustained up-regulation until the last skin sampling date. This model does not include the interaction of PRLR with other reproductive hormones such as placental lactogen, which is also known to bind and activate PRLR (Gertler and Djiane, 2002), or progesterone, which is known to up-regulate PRLR mRNA expression in different tissues (Cassy et al., 2000; Ling et al., 2000) and which could be responsible for this sustained elevation in the expression of PRLR.

In the S/C-18 group, the mathematical model predicts no change in the concentration of total PRLR and receptors in the dimerized state from April to June, agreeing with the biological data on mRNA expression. This corroborates the concept that the transient elevation of prolactin levels is not sufficient to activate the positive feedback mechanism of PRLR synthesis.

Both Inf-18 and S/C-18 groups had similar prolactin profiles to the Preg group, once prolactin manipulation was over, hence the model predictions do not differ for the remainder of the experiment.

4.5.4 Wool growth

The wool growth in the Non-preg, Preg and LD groups showed a similar decline from April to June. In July, however, wool growth rate in the Non-preg group was higher than the Preg ewes. Pearson et al (1999) used an accurate radiolabelling method to track wool follicle output. Their results showed that in pregnant ewes fibre length growth was depressed as early as week 6 of pregnancy, but changes in the patch clip data were only observed a month later, indicating that there was a lag between the fibre elongation and quantifiable wool growth. Therefore, the wool growth difference observed between the Preg and Non-preg group in July, could be due to the PRLR mRNA regulation, which, was up-regulated in May in the Non-preg ewes compared to the Preg ewes.
After parturition, wool growth rate increased in the Preg and LD groups, supporting the finding of Kendall (1999), that the parturition peak of prolactin has a positive effect on subsequent wool growth. Despite higher prolactin levels in the LD group from July until the end of the experiment, differences in wool growth between LD and Preg groups were only observed in September. The rise in wool growth after parturition was responsible for an 11% increase in clean fleece production at the end of the trial. This stimulatory effect was also observed by Kendall (1999), who found that LD treatment enhanced clean fleece production of winter lambing ewes by 26% when compared with ND treatment. This higher wool production could be due to the difference in the time of pregnancy (winter lambing) and due to the wool data being collected not only during gestation but the whole of lactation, when the largest increase in wool growth was observed.

Although there were only 2 animals the infused group, this treatment appeared to have both a short- and a long-term effect on wool growth, eliminating the winter decline and enhancing the post-lambing wool growth. This resulted in an overall increase in clean fleece production of 23.5%, 25.4%, 15.4% and 15.9% compared to the Preg, S/C-18, LD and Non-preg groups respectively. This positive effect on post-lambing wool growth due to a prior prolactin treatment has not been described before. It is speculated that the earlier prolactin manipulation may have enhanced the response of the postpartum wool growth by increasing the PRLR population. This would allow a greater response to the prepartum surge of prolactin. Since there were no biological data to confirm this, simulations of the mathematical model were used.

The model prediction does not show any enhancement effect to PRLR protein expression of the Inf-18 ewes in comparison to the Preg group that could explain the boost in wool production at this time. However, factors like storage of PRLR in intracellular compartments (Posner et al., 1979; Sakai et al., 1994; Bolander et al., 1997),
which could be externalized in the cell membrane in response to a prolactin elevation (Knazek et al., 1978) may be involved.

Prolactin injection at the beginning of pregnancy had no effect on wool growth. Wool production in this group did not differ from the Preg group. This group also showed a significantly lower (p<0.05) live weight at the end of the trial compared to the Preg group. However, there was no association between the individual live weight changes and wool growth at that time. As predicted by the mathematical model, transient elevations of prolactin did not up-regulate PRLR mRNA expression; consequently, there was no biological effect. This is consistent with Hebert et al (1993) who reported that in the lactating rat, constant infusion of prolactin was effective in restoring lactation. However, when prolactin was given in pulses, lactation was not re-established.

The depression in wool growth in the S/C-18 group after parturition could be related to the fact that PRLR mRNA expression in this group showed no statistical difference until the last day of skin sampling when compared to the pre-treatment values. If at the time of the prolactin peak the PRLR population was suppressed, the amplitude of the signalling would be lower, depressing wool growth. However, the mathematical model, in this present formation does not predict this outcome.

4.5.5 Mechanism of the prolactin influence on wool growth

The mechanisms by which prolactin stimulates wool growth in domesticated sheep breeds are still unknown. However, prolactin is known to stimulate protein expression (Lesueur et al., 1991; Djiane et al., 1994; Goupille et al., 1997; Han et al., 1997; Stewart et al., 2000; Chughtai et al., 2002) and cell proliferation (Das and Vonderhaar, 1997; Clevenger et al., 1998; Olazabal et al., 2000; Friedrichsen et al., 2001) in other tissues which could be reflected in wool follicle keratinocytes.
In summary, prolactin manipulation up-regulates the expression of PRLR in the skin of early pregnant Romney ewes. The constant prolactin infusion was also responsible for an increase in the winter and postpartum wool growth. This increase could be a result of priming the intrinsic system of postpartum wool growth, which is most likely linked with the PRLR. However, the route of administration was important as injections were unable to influence either PRLR expression or wool production. Higher prolactin levels observed in the LD group during pregnancy and at parturition also resulted in an increase in wool growth. Other reproductive hormones are also likely to effect the regulation of PRLR expression and consequent wool growth.

4.5.6 Conclusions

- Constant infusion of prolactin during the early stages of pregnancy up-regulated the expression of PRLR.
- Daily injection over the same period had no effect on PRLR, or on wool growth.
- Exposing ewes to long days during pregnancy had no effect on circulating prolactin or on PRLR in the first 60 days of pregnancy.
- Increased PRLR expression in response to elevated prolactin by constant infusion or by LD photoperiod was related to increases in wool growth during late pregnancy and an increase in post-lambing wool growth.
- The mathematical model is a useful tool for predicting changes in the PRLR population in response to circulating prolactin. However, it does not predict long-term changes. Further studies on the effect of reproductive hormones on PRLR population are needed with new information incorporated into the model structure.
CHAPTER 5

THE EFFECT OF PROLACTIN INFUSION AND ITS DURATION ON PROLACTIN RECEPTOR GENE EXPRESSION AND WOOL GROWTH
5.1 Abstract

Romney ewes were maintained indoors from March 2000 until March 2001. Constant prolactin intravenous infusions of 3 (n=6), 9 (n=6) and 18 days (n=6) duration were carried out to investigate whether the length of prolactin treatment plays a role in the up-regulation of prolactin receptor (PRLR) and subsequent wool growth. Prolactin treatment commenced one week after mating. A non-pregnant group (n=6) was also included for comparison.

Blood samples were collected regularly throughout the trial. At the start and end of each infusion, hourly 24-h blood samples were taken to determine the steady state concentration and half-life of circulating prolactin. The samples were analysed by radioimmunoassay (RIA). Six skin biopsies were taken from the start of the trial until June 26 and PRLR mRNA expression was measured by real-time PCR. Monthly patch clips were collected to estimate wool growth rate and fibre diameter during the course of the trial. A mathematical model was used to simulate PRLR protein expression using the prolactin profiles of each treatment group.

The results showed that the length of prolactin infusion was important in the up-regulation of PRLR mRNA expression, with a maximal response to the duration of treatment of between 3 and 9 days (p<0.05). Prolactin infusion during early pregnancy had a positive impact on the post lambing wool growth, with the 9 day infusion showing the greatest effect (p<0.05). This supports the hypothesis that prolactin treatment undertaken early in gestation regulates PRLR mRNA expression, and influences the post lambing wool growth. Prolactin half-life differed between treatments with the 3 day infused ewes showing a half-live of 58±4 min, which was statistically longer (p<0.001) when compared to 30±3 and 29±2 min of the Inf-9 and Inf-18 respectively. This implies that the length of prolactin infusion alters the dynamics of
prolactin uptake and/or degradation. The mathematical simulations correctly predicted PRLR protein population over the duration of the skin sampling period.

5.2 Introduction

From the previous trial (Chapter 4) it was concluded that the route of prolactin administration, which results in distinct plasma profiles, is important in regulating the PRLR population and wool growth. Constant infusion with ovine prolactin for 18 days up-regulated the expression of PRLR-L and caused a significant increase in wool growth. In comparison to an 18 day infusion of prolactin, pulses of prolactin lasting a few hours were ineffective in altering either PRLR gene expression or wool growth (Chapter 4). Between these limits it is likely that there is a threshold and a saturation treatment duration. Hence this experiment was designed to further explore the system response dynamics by undertaking prolactin infusions of intermediate durations (3 and 9 days) and repeating the 18-day infusion.

Various durations of prolactin treatment have been previously reported. According to Young et al (1990) in the endometrium of pregnant gilts, the increase in PRLR levels only occurred after 3 to 4 days of constant infusion of prolactin. To increase nitrogen retention in growing lambs, Brinklow and Forbes (1983) treated the animals with a constant prolactin infusion for 10 days. Liesman et al (1988) increased lipase activity of lactating cows using constant infusion of prolactin for 5 days. On the other hand, Thomas et al (1994) infused prolactin locally for 28 days before noticeable changes in the summer coat of the red deer were observed.

In sheep there are two isoforms of the PRLR, which have been described in different tissues. These are the PRLR-L of 557 aa and the PRLR-S of 272 aa (Anthony et al., 1995; Bignon et al., 1997; Cassy et al., 1998; Phillips et al., 1999; Cassy et al., 2000; Stephenson et al., 2001). In different tissues, the expression of these isoforms respond in distinct ways to specific hormonal environments. (Cassy et al., 1999; Budge et al., 2000; Cassy et
This tissue-specific expression regulation has also been reported in other species (Jahn et al., 1991; Nagano and Kelly, 1994; Mizoguchi et al., 1997a; Mizoguchi et al., 1997b; Bowen et al., 2000; Hovey et al., 2000; Kinoshita et al., 2001). PRLR-L and PRLR-S have also been implicated in different physiological roles. The PRLR-L is known to up-regulate milk protein synthesis (Lesueur et al., 1990; Lesueur et al., 1991; Goupille et al., 1997). The PRLR-S, on the other hand, has an inhibitory role in prolactin signal transduction by forming an inactive heterodimer with the PRLR-L (Berlanga et al., 1997; Goffin et al., 1999; Saunier et al., 2003). In contrast, experiments performed in different species showed that PRLR-S signalling directly stimulated cell proliferation (Das and Vonderhaar, 1995; Das and Vonderhaar, 1997; Olazabal et al., 2000). This background raises questions on the functions of the PRLR variant populations in the skin.

For these reasons, the aim of this trial was to investigate different durations of prolactin infusion in early pregnancy, and assess the effects on the expression of PRLR isoforms in the skin and on subsequent wool growth in Romney ewes.

5.3 Materials and methods

5.3.1 Experimental animals

Thirty-five Romney ewes from the Ruakura farm were transferred to indoor facilities and induced to cycle using progesterone-implanted CIDRs from April 5-19, 2000. They were then joined with Romney rams with crayon-fitted harnesses from April 20-26. Eighteen ewes that had been heavily marked by the rams were then placed in individual pens, under ND photoperiod in the Ruakura Physiology Building until March 2001.

5.3.2 Experimental groups

The 24 marked ewes were randomly allocated into 4 groups as shown in Table 21:
## Table 21: Experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Reproductive status</th>
<th>Treatment</th>
<th>n</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pregnant</td>
<td>3 day constant prolactin infusion</td>
<td>6</td>
<td>Inf-3</td>
</tr>
<tr>
<td>2</td>
<td>Pregnant</td>
<td>9 day constant prolactin infusion</td>
<td>6</td>
<td>Inf-9</td>
</tr>
<tr>
<td>3</td>
<td>Pregnant</td>
<td>18 day constant prolactin infusion</td>
<td>6</td>
<td>Inf-18</td>
</tr>
<tr>
<td>4</td>
<td>Non-pregnant</td>
<td>18 day constant saline infusion</td>
<td>6</td>
<td>Non-preg</td>
</tr>
</tbody>
</table>

A pregnant control group was also planned but these ewes were not mated due to a miscommunication. Nevertheless, they were retained in the experiment as non-pregnant comparison. Prolactin treatment (1 mg/kg/day) started on May 4 and finished on May 7 (Inf-3), May 13 (Inf-9) and May 22 (Inf-18), respectively.

### 5.3.3 Live weight

All sheep were weighed at fortnightly intervals for the duration of the trial to monitor changes in live weight.

### 5.3.4 Feeding

The daily feeding regime for pregnant and non-pregnant ewes were based on their live weight as described in Section 3.2.4.

### 5.3.5 Wool sampling

The ewes were shorn on March 1, 2000 and a patch clip established on March 8, 2000. Patch clip samples were collected monthly until the end of the experiment, when ewes were again shorn on March 13, 2001.
5.3.6 Blood sampling

Both jugular veins were cannulated, so that one vein received prolactin and blood samples could be withdrawn from the other. Twenty-four hour blood sampling was performed at the beginning, and at the end of the prolactin infusion for each group. These samplings sessions commenced 2 h before prolactin treatment began and 12 h prior to the end of the treatment (Figure 23). For the first 2 h after beginning or finishing the infusion, blood samples were taken every 15 min. Otherwise, samples were collected every hour. Blood samples were also collected daily from all the ewes during the course of the prolactin infusions. After the completion of the infusion period, blood samples were collected at weekly intervals.

Circulating prolactin levels were measured by RIA (Section 3.7). The inter-assay and intra-assay coefficients of variation for the assay at 10 ng/ml were 8.9% and 9.4%, respectively.

Figure 23: Blood sampling regime

5.3.7 Skin sampling

Six skin biopsies were taken from all animals at intervals over the first 6 weeks of the trial. Samples were collected on days -2, 1, 7, 18, 28 and 56 in relation to the first day of prolactin infusion. See Section 3.5.4 for details.
5.3.8  **PRLR relative quantification**

RNA from the skin samples was extracted using TRizol ® method. cDNA was obtained by reverse transcriptase reaction and PRLR long and short forms were quantified using the real-time PCR assay. See Section 3.8 for details.

5.3.9  **Lamb data**

Lambs were weighed 12 to 24 h after birth and weekly until weaning at 45 days of age.

5.3.10  **Statistics**

Analysis of variance was used to test the effect of prolactin manipulation, pregnancy and their interaction on feed intake, live weights, plasma prolactin concentration, wool growth, fibre diameter and expression of PRLR. Analysis of covariance was used for the patch clip data. For more detail see Section 3.9.

A two compartment model was fitted to the prolactin concentration decay curve at the end of each infusion to estimate the prolactin half-life for individual ewes.

\[ \text{PRL} = \log(a_2 \exp(-b_2 \text{time}) + c_2 \exp(-d_2 - \text{time})) \]

The differences between treatments were measured using a weighted Anova with the computer statistical package GenStat v. 6.0.

5.4  **Results**

5.4.1  **Animal health and behaviour**

Two animals, one from the Inf-9 and the other from the Inf-18 groups, died from cardiac abscess probably caused by the cannulation. Also, two animals from the Inf-18 group aborted their lambs early in their gestation. These abortions may also have been the result of infections due to the cannulation procedure. When the first feverish ewe was observed, all of the animals were treated with a long lasting antibiotic and received
a boost of the Campylobacter vaccine. Data from the animals that died or aborted are not reported or used in the statistical analysis.

Another problem observed during this trial was wool chewing, either self-inflicted or by ewes in neighbouring pens. To avoid wool chewing, the lambs were weaned at 45 days. However, after weaning some wool chewing by the ewes was still observed. After separation by an empty pen, some ewes continued to pull their own fleece. Hence, the total fleece weight was compromised and consequently the estimation of the fleece wool growth during the experiment, calculated as described in Section 3.6.1.4, could not be made. Hence, the wool growth data presented here is based on the growth of the patch clip area only. This still allows experimental effects on wool growth rates to be reported but prevents an accurate comparison between trials.

5.4.2 Lambing data

A total of 18 lambs were delivered (11 females and 7 males) between September 14-21, 2000. There were no stillborn or peri-natal deaths and 4 ewes had twins. One of each twin pair was removed from the dam. The mean weight at birth was 5.0 kg and at weaning it was 17.0 kg. There was no difference in the weight gain of lambs among the different treatments (Figure 24). Only one lamb from the Inf-9 group showed a significantly lower weight gain. This ewe had a rupture in her udder ligaments, making it hard for the lamb to suckle properly.
5.4.3 Live weight

There was no difference in live weights between treatment groups at the beginning of the trial (Table 23, Figure 25). The Non-preg group maintained a constant weight until August, when it started to show a slow and consistent increase until the end of the trial. The average weight of the pregnant groups began to rise in June. From August until lambing, live weights increased sharply, reaching a peak of 69.2±3.0, 67.5±4.7 and 70.4±4.6 kg for the Inf-3, Inf-9 and Inf-18 days groups respectively. After lambing live weights declined and, by the end of the trial (after shearing), the Non-preg ewes were heavier than the ewes that had lambed.

Table 23: Live weight (kg) at the start of the trial, at 100 days of pregnancy (100 preg), one week before parturition (prepartum), 24 h postpartum, at weaning, and at the end of the trial after shearing (mean ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>Start</th>
<th>100 preg</th>
<th>Pre-partum</th>
<th>Weaning</th>
<th>End trial *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inf-3</td>
<td>50.5±3.0</td>
<td>56.0±3.0</td>
<td>69.2±3.0</td>
<td>58.4±3.5</td>
<td>47.4±4.3</td>
</tr>
<tr>
<td>Inf-9</td>
<td>47.5±4.8</td>
<td>55.2±3.9</td>
<td>67.5±4.7</td>
<td>57.9±4.1</td>
<td>47.0±2.9</td>
</tr>
<tr>
<td>Inf-18</td>
<td>51.3±2.1</td>
<td>55.5±2.9</td>
<td>70.4±4.6</td>
<td>55.6±4.0</td>
<td>47.3±4.5</td>
</tr>
<tr>
<td>Non-preg</td>
<td>50.6±1.5</td>
<td>52.9±1.7</td>
<td>56.9±2.4</td>
<td>59.7±2.7</td>
<td>53.9±3.9</td>
</tr>
</tbody>
</table>

* Different superscript letters represent a statistical difference (p<0.05) post-shearing weight
5.4.4 Plasma prolactin levels

At the start of the trial prolactin levels were relatively low and ranged from 20 to 50 ng/ml. Once prolactin infusion commenced plasma levels rapidly increased, reaching a plateau after 2 h of infusion. During the infusion period prolactin levels remained elevated with no difference between the infused groups (594.5±85.7, 500.1±78.9 and 460.5±83.8 ng/ml for Inf-3, Inf-9 and Inf-18, respectively), and were all higher (p<0.001) than the Non-preg group (8.45±4.21 ng/ml) (Figures 26, 27, 28).

In all treated groups, plasma prolactin levels quickly declined once the infusion ended. Two hours after the cessation of infusion prolactin levels were 99.9±25.3 ng/ml, 63.1±12.2 ng/ml and 40.9±7.1 ng/ml for the Inf-3, Inf-9 and Inf-18 groups, respectively.

After infusion until the beginning of September, prolactin levels remained low and showed no difference between the infused pregnant groups. However, the Non-preg group had significantly higher (p<0.001) prolactin levels during July and August compared to the pregnant groups (Figure 29).

The seasonal rise in prolactin in the Non-preg group started at the end of June, reaching a peak in early November of 117±73 ng/ml. Prolactin levels were maintained around
100 ng/ml until mid-January, when levels began to decline. By the end of the trial, in March, endogenous prolactin levels in this group were 1.4±0.8 ng/ml.

In the treated groups, a second rise in prolactin concentration was observed at parturition and throughout lactation. Parturition occurred from September 14-21 and prolactin peaks at this time can be seen in Figures 26, 27 and 28. The parturition peak of prolactin in the Inf-18 group (79±14 ng/ml) was significantly lower (p<0.05) than the Inf-9 group (316±108 ng/ml).

During lactation, mean plasma prolactin levels values did not differ between the pregnant animals, but were higher (p<0.001) than the Non-preg group. A week after weaning, prolactin concentration declined and by the end of the trial in March 2001, endogenous levels among the experimental groups were the same with values of 2.5±1.5, 3.0±2.3, 3.4±2.6 and 1.4±0.8 ng/ml for the Inf-3, Inf-9, Inf-18 and Non-preg, respectively.

*Figure 26: Mean plasma prolactin profile (ng/ml) of the Inf-3 group. Arrow indicates the time of parturition and * indicates the 3 day infusion period.
Figure 27: Mean plasma prolactin profile (ng/ml) of the Inf-9 group. Arrow indicates the time of parturition and ** indicates the 9 day infusion period.

Figure 28: Mean plasma prolactin profile (ng/ml) of the Inf-18 group Arrow indicates the time of parturition and *** indicates the 18 day infusion period.
Figure 29: Plasma prolactin profile of the pregnant groups (Inf-3, Inf-9 and Inf-18) and the Non-preg group from the end of the prolactin infusion period. Arrow represents time of parturition and error bars represent the SEM for the Non-preg group.

5.4.5 Prolactin half-life

The half-life of prolactin differed depending on the length of the infusion treatment. The half-life of the Inf-3 group was of 58±4 min, which was higher (p<0.001) than the Inf-9 and Inf-18 groups (30±3 and 29±2 min, respectively) (Figure 30).

Figure 30: Effects of prolactin infusion duration on prolactin half-life (mean ± SEM)
5.4.6  *PRLR relative quantification*

The infused groups demonstrated a similar pattern of PRLR-L expression, with an initial down-regulation (p<0.05) followed by an up-regulation and stabilisation at pre-treatment values once infusion was terminated (Figure 31). The Non-preg group exhibited a tendency for up-regulation (p<0.10) at the beginning of the trial but expression levels returned to pre-treatment levels on trial day 19 and remained the same until the end of the sampling period.

Although the up-regulation of PRLR-L expression in the Inf-3 group on day 7 was not significant, the increase in PRLR-L mRNA occurred once the infusion was over and was similar to that observed in the other infused groups. In relation to sampling date 2, the Inf-9 and Inf-18 groups showed a further increase (p<0.05) in the PRLR-L expression on sampling day 18. PRLR expression in these groups was then down-regulated to pre-treatment levels on days 28 and 56.

*Figure 31: The effect of prolactin manipulation and reproductive status on PRLR-L mRNA expression (log fold change). Error bars represent the pooled SED between groups of the fold change. The bar represents the infusion periods.*

The changes in PRLR-S expression through the sampling period are shown in Figure 32. The samples from the sampling day 28 (June 1) did not perform well in the real-time PCR reaction and are not reported. The lower abundance of PRLR-S mRNA relative to
the PRLR-L variant may increase susceptibility to degradation during sampling and/or RNA storage.

In the Non-preg group PRLR-S expression was up-regulated between sampling days 1 and 18. By June 29 PRLR-S expression in this group was 4.5 times higher than the mean initial value.

The prolactin infused groups only showed changes in their PRLR-S expression on day 7, when they were all up-regulated. In contrast to PRLR-L, the expression of PRLR-S in the Inf-3 group on day 18 was down-regulated, stabilised in the Inf-9 group and in the Inf-18 group, where prolactin infusion had just ceased, expression of PRLR-S peaked.

The main contrast in the expression of PRLR-S is that even though its expression was up-regulated in the infused ewes, the expression levels declined after treatment had stopped. PRLR-S expression in the Non-preg group, on the other hand, remained elevated.

*Figure 32: The effect of prolactin manipulation and reproductive status on PRLR-S mRNA expression (log fold change). Error bars represent the pooled SED between groups of the fold change. The bar represents the infusion periods.*
5.4.7 Patch clip data - wool growth

The monthly wool growth data of the four experimental groups exhibited the expected seasonal variation in wool growth. The production declined in the winter and started to increase in early spring (Figure 33). The pattern of wool growth in the Non-preg group was slightly different. Wool growth declined until June and increased in September. The other pregnant groups showed depressed wool production until August. After parturition, wool growth sharply increased. From July until November, wool growth was statistically higher (p<0.001) in the Inf-9 group when compared to the Inf-3 and Inf-18 groups and on January Inf-18 grew less (p<0.05) wool than the Inf-9 group. In October, November and January wool production in the Inf-9 group was significantly higher (p<0.05) than the Non-preg group.

Figure 33: Effect of prolactin manipulation and reproductive status on wool growth. Data presented as adjusted means and the error bars represent the average SEDs between treatments. The arrow represents the time of parturition, and the bar represents the 18-day infusion period.

5.4.8 Patch clip data - fibre diameter

Mean fibre diameter values (Figure 34) followed a similar pattern as the clean wool growth. A statistical difference occurred in June, when fibre diameter in the Inf-9 and
Inf-18 groups was greater (p<0.05) than in the Inf-3 and Non-preg groups. In July fibre diameter in the Inf-9 group had larger (p<0.05) fibre diameter than the other experimental groups. In August and September, mean fibre diameter in the Inf-3 and Inf-18 groups were lower (p<0.05) than in the Inf-9 and Non-preg ewes. From January until March, all groups experienced a decline in fibre diameter, but with no differences between groups.

*Figure 34: Effect of prolactin manipulation and reproductive status on fibre diameter. Data presented as adjusted means and the error bars represent the average SEDs between treatments. The arrow represents the time of parturition, and the bar represents the 18 day infusion period.*

5.4.9 **Mathematical model**

The results from the simulations of the PRLR-L protein population in response to the different prolactin profiles observed in each group during the trial are shown in Figure 35. In the Non-preg group (Figure 35 A) as the spring prolactin elevation occurs, the number of total receptor and dimer formation also increases, reaching a maximum in December. Once prolactin starts its seasonal decline, so does the number of total receptors and dimers. However, the decline of the receptor population was somewhat delayed relative to the prolactin decrease, especially the dimer population, which declines sharply in February.
The simulation of the PRLR population on the Inf-3 group (Figure 35 B) shows that the number of total receptor and dimers increases at the commencement of the simulated infusion, reaching levels close to 3000 nmol/litre for the total receptor and around 500 nmol/litre for the dimers. When compared to the Inf-9 (Figure 35 C) and Inf-18 (Figure 35 D) groups it is clear that in the inf-3 the group maximum numbers of receptor were not achieved, since in the other infused groups the total receptors reach a plateau of 3200 nmol/litre and dimers plateau at approximately 600 nmol/litre after 6 days of constant infusion. After infusion ceases, total receptors and dimers decline in all the treated groups to values similar to those at the start of the infusion.

Between prolactin treatment and parturition, the predicted receptor population among the infused groups is similar. At parturition and lactation the number of total receptors and dimer formation increase in comparison to the Non-preg group. After weaning, the receptor population profile follows the seasonal variation in circulating prolactin.
Figure 35: Mathematical simulation of the PRLR population using the prolactin profile from the experimental groups. First panel illustrates the smoothed prolactin data; the middle panel shows the concentration of bound (B1) and dimerised (B2) receptors and the last panel shows the concentration of unbound (U) and total receptor. (A: Non-preg; B: Inf-3; C: Inf-9; and D: Inf-18).
5.5 Discussion

In Chapter 4, it was demonstrated that an 18 day infusion of prolactin up-regulated the expression of PRLR mRNA in the skin of Romney ewes. This treatment also had a short- to long-term positive effect on the subsequent wool growth. In the current experiment, the ability of prolactin to regulate PRLR mRNA expression and stimulate wool growth was confirmed and the duration of the prolactin treatment required for maximum wool growth stimulation appears to be between 3 and 9 days.

5.5.1 Prolactin profile

The seasonal decline in circulating prolactin levels differed among groups. From July to August, the Non-preg ewes had significantly higher prolactin levels than the pregnant ewes. This was first described by Kendall (1999) and confirmed in the previous experiment. At parturition prolactin levels rose significantly, however the parturition peak of the Inf-9 group was significantly higher than the Inf-18 group. This finding is probably related to differences in the timing of blood sampling in relation to parturition. Prolactin levels start to increase 1-3 days before parturition (Kelly et al., 1974; Lamming et al., 1974; Kendall, 1999). Some authors state that the prepartum peak of prolactin starts to rise at about 3-6 h before labour, with bursts of release resulting in plasma concentrations of 100 to 700 ng/ml in the final stages of labour (Kelly et al., 1974; Koritnik et al., 1981; Peterson et al., 1990). In this experiment, blood samples were taken once a week at different times in relation to lambing in individual ewes. Hence there was considerable variation in the prolactin elevation among pregnant ewes close to parturition. After the parturition peak, prolactin levels declined agreeing with the finding of Lamming et al (1974) and Kendall (1999). The prolactin oscillation observed over lactation may also be attributed to time of sampling, since suckling can increase prolactin levels up to 800 ng/ml while pre-suckling levels can be as low as 100 ng/ml in early lactation (Lamming et al., 1974). As lactation progressed, prolactin
concentration decreased (Rhind et al., 1980) and by late lactation, levels were similar to those in the Non-preg group. In mid-January, after the summer solstice, prolactin levels in all groups declined with photoperiod (Munro et al., 1980; Webster and Haresign, 1983; Buys et al., 1990).

5.5.1.1 Prolactin half-life

The prolactin half-life of the Inf-3 group (58±4 min) was statistically higher than those calculated in the Inf-9 (30±3 min) and Inf-18 (29±2 min) groups. These results differ from the 23 min observed by Akbar et al (1974) and the 42 (injection bolus) and 105 min (2 days constant infusion) observed by Litherland (1996) in sheep. The discrepancy among these results could reside in the different doses administered, routes of prolactin administration as well as variation in live weight, age and physiological status of the animals (Section 2.9).

Half-life is related to the MCR, which is associated with the size/weight of the animal (Davis and Borger, 1973). Smaller animals have shorter prolactin half-lives. Although Litherland (1996) used a 42 kg ewe against the average 50 kg ewes used in this trial, the estimated half-life after infusion was still much longer than those calculated in the Inf-3, Inf-9 and Inf-18 groups. According to Grosvenor et al. (1977) the MCR is enhanced significantly, when the prolactin dose is increased. Hence higher doses of prolactin should result in a faster clearance rate and therefore a shorter half-life. Litherland (1996) infused 7.56 mg of prolactin per day. The shorter half-life observed by Akbar et al (1974) was derived using a very small prolactin dose; however there is no mention of the weight, age or physiological status of the animals.

The ewes in this experiment had similar weight, were of the same age, same physiological status and received 50 mg/day of prolactin at the same infusion rate. Therefore, the likely explanation for the difference in the half-life between the infused groups is the different lengths of prolactin infusion. Costlow and Hample (1982a)
suggested that prolactin treatment of cultured rat mammary tumor cells would recruit cryptic PRLR to the cell membrane and therefore enhance 125I prolactin internalization and degradation. The different half-life calculated in the Inf-9 and Inf-18 groups in comparison to the Inf-3 group could be explained by such a mechanism in the many tissues that express PRLR. In the Inf-9 and Inf-18 group, the infusion may have been long enough to stimulate maximum recruitment of PRLR resulting in a faster uptake of circulating prolactin. Degradation pathways via the kidney and liver (Section 2.8.2) may also become more efficient under conditions of sustained hyperprolactineamia.

5.5.2 PRLR mRNA expression

In the Non-preg group, the expression of PRLR-L mRNA showed the tendency (p<0.10) to increase one week after the experiment had started, followed by a decline in expression until the end of the sampling period. This data is similar to that reported in the previous experiment (Chapter 4).

The expression of PRLR-S mRNA in the Non-preg group, showed a steady increase from one week after the start of the experiment until the end of skin sampling. In the rat liver, where PRLR-S is the most abundant form of the receptor (Jahn et al., 1991; Nagano and Kelly, 1994) reproductive hormones appear to be the main regulator of PRLR expression. The liver of female rats has more PRLR-S than males (Moldrup et al., 1993), while during proestrus the amount of PRLR-S mRNA is twice that during diestrus (Jahn et al., 1991; Telleria et al., 1997). In these cycling ewes, which did not receive prolactin, expression of PRLR-S mRNA was constantly up-regulated suggesting, that in the skin, this form of the receptor is more likely to be regulated by reproductive hormones rather than by prolactin itself, which remained low until spring. This agrees with a number of studies which have concluded that prolactin is only able to up-
regulate the expression of PRLR-L in certain species and tissues (Asfari et al., 1995; Feng et al., 1998; Galsgaard et al., 1999; Stephenson et al., 2001).

In all of the infused groups, after the first day of infusion, PRLR-L mRNA expression was down-regulated in relation to its pre-treatment values and was then followed by an up-regulation, in agreement with Nixon et al (2002). While down-regulation was not detected in the previous experiment (Chapter 4), this is likely to be attributable to the different skin sampling intervals adopted relative to the commencement of prolactin infusion.

In the Inf-3 group PRLR-L mRNA levels were up-regulated on day 7 even though prolactin treatment had finished 4 days earlier. Similar to the Inf-3 group, up-regulation of PRLR-L mRNA expression in the Inf-9 group was seen 9 days after prolactin treatment had ended. This agrees with the findings of the experiment in Chapter 4, which showed a sustained up-regulation of PRLR-L mRNA expression after prolactin treatment had finished. This longer-term increase in PRLR-L mRNA expression, specially in the Inf-3 group, needs further investigation. At this early stage of pregnancy, progesterone and oestrogen, which have been shown to up-regulate PRLR mRNA levels (Cassy et al., 2000), are present in low levels, and therefore, are less likely to be involved. An alternative candidate is cortisol, which has been shown to be necessary for PRLR-L mRNA expression in the adipose tissue of the sheep foetus (Mostyn et al., 2003). With cannulation, blood sampling and handling by different people, these ewes could have had transitory higher circulating cortisol levels, which may have maintained the PRLR-L mRNA expression.

However, when comparing the effects of prolactin on PRLR-L mRNA levels, it is clear that, in the trial described in Chapter 4, the sustained effect was maintained for longer. The difference between trials could be explained by individual variation, since there were only two animals in that experiment, or by slightly different times of the infusion period (late April versus early May).
Of interest, was that in the 3 infused groups PRLR-L mRNA expression was similar on day 7, but an increase from a 3-day infusion to a 9-day infusion resulted in further up-regulation of PRLR-L mRNA levels. However, at day 18, PRLR-L mRNA expression in the Inf-9 and Inf-18 groups were the same. A week after prolactin treatment had finished in the Inf-18 group, PRLR-L mRNA expression did not differ between the infused groups. This suggests the existence of a minimum treatment duration necessary for PRLR-L mRNA expression to reach maximal levels. Extending the duration of the prolactin treatment beyond this time did not further enhance PRLR-L expression level.

The expression of PRLR-S mRNA in the infused groups appeared not to respond directly to circulating prolactin. Many authors state that the major function of the PRLR-S is to be a negative regulator of prolactin signalling, since the formation of heterodimers do not activate the JAK/STAT pathway (Clevenger et al., 1998; Goffin et al., 1999; Saunier et al., 2003) (Section 2.9.5). Therefore, it is possible to speculate that PRLR-L was responsible for the regulation of PRLR-S mRNA expression in the infused groups. The expression of PRLR-S mRNA rose after the initial increase in PRLR-L mRNA expression. Hence the PRLR-L down-stream signalling system may act as a negative feedback loop in the form of increased PRLR-S production. This could be mediated by enzymes that require the accumulation of specific activated proteins in the cytoplasm to induce transcription of PRLR-S via its multiple promoters (Moldrup et al., 1996; Ormandy et al., 1998; Hu et al., 2002). Once prolactin infusion is over, accumulation of these molecules ceases due to diminished prolactin signalling, and the PRLR-S mRNA expression is down-regulated.

5.5.3 Mathematical model

The mathematical model predictions for PRLR protein expression during the initial skin sampling period (April 19–June 29) in the Non-preg group agree in part with the biological data. The simulation does not predict the short-term up-regulation observed
in the PRLR-L mRNA levels, but conforms to the measured outcomes over the remainder of the infusion duration.

In the Inf-3 group simulation, the concentration of dimerized receptors and total receptors increase. However, at day 3, dimerized receptor concentration has not reached 500 nmol/l and the concentration of total receptor is below 3000 nmol/l. Therefore the 3-day elevation of prolactin is not sufficient for activation of the positive feedback mechanism to its full potential, resulting in a smaller biological effect. The simulations of the Inf-9 and Inf-18 groups, on the other hand, show that under similar circulating levels of prolactin, the concentration of dimerized PRLR-L reaches a plateau around 650 nmol/l and the total receptor peaks close to 3500 nmol/litre after 6 days of constant infusion. Hence, the mathematical model agrees with the biological data, which places the maximal effective exposure duration to between 3 and 9 days of constant infusion.

Once the skin sampling period was completed, the simulation of the PRLR protein expression shows little difference among the infused groups. In the Non-preg group, higher circulating prolactin results in a larger concentration of total receptors and dimers, which could be related to the higher wool production of non-pregnant ewes during July and August when compared to the Inf-3 and Inf-18 groups.

During parturition and lactation, when circulating levels of prolactin increase, the model predicts an increase in the concentration of total receptor and dimer formation in the pregnant groups. The predicted lowest formation of total receptor and dimer observed at parturition in the Inf-18 group results from a lower parturition prolactin peak, which may have been influenced by sampling date.

5.5.4 Wool Growth

In this trial, all experimental groups demonstrated the seasonal decline in wool production as previously observed by many authors (Story and Ross, 1960; Ross, 1965;
Bigham et al., 1978; Geenty et al., 1984; Woods et al., 1995; Sumner et al., 1998; Kendall, 1999). The wool decline in the Non-preg ewes continued until June, while the pregnant ones demonstrated a further decline until August. This decline in production could have been established earlier, when prolactin levels start to decline due to diminishing daylength after the summer solstice. This agrees with a number of studies, which have shown that in many species, the seasonal coat changes occur in response to a change in photoperiod (Duby and Travis, 1972; Martinet et al., 1982; Duncan and Goldman, 1984a; Rose et al., 1987; Smith et al., 1987; Smale et al., 1990). After the winter solstice at June 21, it is possible that the small increments in circulating prolactin levels may stimulate spring wool growth in the non-pregnant ewe. Hence, circulating prolactin could be linked directly to the difference in wool production between pregnant and non-pregnant ewes during mid-pregnancy.

In the Chapter 4 experiment, prolactin infusion had a short- and a long-term stimulatory effect on wool growth of pregnant ewes. In the present trial, even though there was no statistical difference, there was a tendency (P<0.10) for the Inf-9 group to grow more wool than the other groups over the patch clip period that followed the prolactin infusion. Wool production was similar to the Non-preg group in July and August when the Inf-3 and Inf-18 groups exhibited declining wool production. A similar response was observed in the Chapter 4 experiment, when ewes were constantly infused with prolactin for 18 days. There is a discrepancy between the wool growth response between the Inf-18 groups of these two experiments. However, during this trial there were health problems associated with cannulation, particularly affecting the Inf-18 group. Therefore, although PRLR mRNA expression was up-regulated by prolactin treatment, the wool growth may not have responded as might have been anticipated in healthy sheep.

A comparison between the Inf-3 and Inf-9 groups suggest that increasing PRLR signalling could be associated with the long-term increase in wool growth. The wool
growth in the Inf-3 group was lower (p<0.05) than the Inf-9 group from July until November. The results of the measurements of PRLR-L mRNA expression suggest that a 3-day infusion was not sufficient for maximal signalling to occur. Taken together these results support the hypothesis that prolactin infusion in early pregnancy has a short-term effect on wool growth, but is dependent on the duration of the treatment. The treatment also appears to increase the mid-gestational and post-parturition wool production.

The mechanism underlying this long term effect on wool growth by a previous prolactin elevation is intriguing. It is possible that the expression of PRLR mRNA at parturition and early lactation is enhanced due to previous prolactin treatment. In the present experiment, the skin sampling did not encompass the period around parturition. The mathematical model shows no evidence of such behaviour, but the model does not take into account the existence of intracellular storage of PRLR (Posner et al., 1979; Costlow and Hample, 1982b; Sakai et al., 1994; Bolander et al., 1997), nor the actions of other reproductive hormones, (Djiane and Durand, 1977; Mizoguchi et al., 1997a; Mizoguchi et al., 1997b; Cassy et al., 1999; Yasui et al., 1999; Cassy et al., 2000; Ling et al., 2000), as discussed previously. Indeed, Ormandy et al (1997) showed that in human breast cell lines prolactin, progesterone and oestrogens are synergistic in regulating each others receptor expression. So, the gestational increase of these hormones could be recruiting intracellular PRLR. Therefore once prolactin peaks at parturition, enhanced prolactin signalling may result from an increased PRLR population leading to greater wool production.

The fibre diameter followed the same trend as the wool growth rate. This has already been described by Kendall (1999) and also observed in the previous experiment (Chapter 4). This is an encouraging result as fibre diameter variability was significantly reduced during gestation by prolactin manipulation in early pregnancy. However, further studies would need to be performed in the period of mid-lactation and post-
weaning. Kendall (1999) showed that there is a lag between the effects on wool growth and fibre diameter. However, in the current experiment, fibre diameter in all pregnant groups declined during lactation and post weaning when wool grew at fairly constant rate.

5.5.5 Conclusions

- Seasonal and gestational declines in wool growth were confirmed.
- Prolactin treatment in early pregnancy was associated with short- and long-term stimulation of wool growth.
- Constant infusion of ovine prolactin in early pregnancy altered the expression of PRLR which was dependent on the duration of prolactin treatment.
- Further PRLR protein quantification and localization is necessary to establish the mechanism of the long-term effect of prolactin on wool growth.
- The down-stream effects of PRLR stimulation in the wool follicle have yet to be elucidated.
- Studies on the influence of reproductive hormones on PRLR expression in sheep skin are necessary to establish the cause of the decline in wool production in the pregnant ewe.
CHAPTER 6

THE EFFECT OF TWO PERIODS OF PROLACTIN ELEVATION ON PROLACTIN RECEPTOR GENE EXPRESSION AND WOOL GROWTH OF NON-PREGNANT EWES
6.1 Abstract

The hypothesis that earlier prolactin treatment may influence the response of a subsequent prolactin elevation was tested using non-pregnant ewes, who received two 9-day infusions of prolactin (n=7) or saline (n=7). The experiment was carried out from October 2001 to March 2002 and treated animals were maintained indoors under short day (SD) photoperiod to suppress the endogenous seasonal prolactin rise. The first infusion commenced on October 9 and the second on November 20. A third untreated group of ewes (n=7) were maintained under natural days (ND).

Blood samples were collected throughout the trial and analysed by radioimmunoassay. Ten skin biopsies were taken from the start of the trial until December 18 and prolactin receptors (PRLR) mRNA expression was measured using real-time PCR. Monthly patch clips were collected to estimate wool growth and fibre diameter. A mathematical model was used to simulate PRLR protein expression using the prolactin profiles of each treatment group.

PRLR expression was up-regulated during both infusions; however, a sustained effect on mRNA expression was not observed. There was no evidence of an enhancement in PRLR expression in response to a previous elevation of circulating prolactin. The PRLR expression in the saline group was consistently lower (p<0.05) compared to the infused and ND groups. Wool production did not differ between groups. The mathematical model needs to incorporate the influence of other hormonal changes that also regulate PRLR expression in order to be used under the different hormonal environments, especially cycling and pregnancy.
6.2 Introduction

The previous two trials (Chapters 4 and 5) demonstrated that constant elevation of plasma prolactin levels in early pregnant ewes was responsible for an up-regulation of PRLR-L and PRLR-S, and a short- and a long-term enhancement in wool production.

These results also suggested that reproductive hormones could have an important role in regulating PRLR mRNA expression. Therefore, with the aim of separating the effects of prolactin from the changes in the hormonal environment of the cycling and the lambing ewe, this trial involved non-pregnant and non-cycling ewes studied during spring and early summer.

The seasonal secretion of prolactin is important to the regulation of its biological effects. Prolactin is critical for the timing of fibre growth in species that have a strong seasonal variation in prolactin production and secretion, for example, mink (Martinet et al., 1982), blue fox (Smith et al., 1985), goat (Grasselli et al., 1992) and sheep (Lincoln, 1990; Pearson et al., 1996; Kendall, 1999). Other vertebrates, such as mouse (Michael, 1976), rat (Wong et al., 1983) and human (Maes et al., 1997) have a small or a non-existent seasonal prolactin variation, and their fibre growth appears to be more influenced by age or reproductive hormones (Paus and Cotsarelis, 1999; Craven et al., 2001). Therefore, the animals in this trial were subjected to an artificial SD light environment in order to suppress endogenous prolactin.

A further aim of this trial was to investigate the hypothesis that a previous prolactin treatment may be able to influence PRLR gene expression at a subsequent prolactin elevation and therefore enhance wool production. Hence prolactin was infused for two periods of 9 days separated by 42 days. The expression of PRLR-L and PRLR-S mRNA and wool growth were monitored throughout the trial.
6.3 Materials and Methods

6.3.1 Experimental Animals

Twenty-one non-pregnant Romney ewes were transferred to the AgResearch Ruakura Animal Physiology Building in September 2001. The ewes were maintained indoors in individual pens throughout the trial, which started on October 4 and finished on February 13, 2002.

6.3.2 Experimental Groups

The twenty-one ewes were randomly allocated into three groups (Table 24).

<table>
<thead>
<tr>
<th>Group</th>
<th>Reproductive status</th>
<th>Treatment</th>
<th>Photoperiod</th>
<th>n</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Non-pregnant</td>
<td>2x 9 day prolactin infusion</td>
<td>Short days</td>
<td>7</td>
<td>Inf-9</td>
</tr>
<tr>
<td>2</td>
<td>Non-pregnant</td>
<td>2x 9 day saline infusion</td>
<td>Short days</td>
<td>7</td>
<td>Saline</td>
</tr>
<tr>
<td>3</td>
<td>Non-pregnant</td>
<td>No treatment</td>
<td>Natural days</td>
<td>7</td>
<td>ND</td>
</tr>
</tbody>
</table>

The ewes kept in SD (Groups 1 and 2) received either an infusion of ovine prolactin (1 mg/kg/day) for 9 days (commencing on October 9 = day 0) or were infused with saline buffer for the same period of time. The treatment was repeated 42 days after the beginning of the first infusion on November 20 (day 40). The ewes in ND (Group 3) received no treatment throughout the trial. The prolactin and the preparation of the infusate are described in Chapter 3.

6.3.3 Live weight

All ewes were weighed at fortnightly intervals for the duration of the trial.
6.3.4 **Feeding**

The non-pregnant ewes feeding regime was based on their live weight as described in Chapter 3.

6.3.5 **Wool sampling**

Ewes were shorn on September 4 and a patch area established on September 7. Monthly patch clip samples were collected until the end of the experiment in February 2002, when the ewes were shorn again.

6.3.6 **Blood sampling**

A blood sample was collected daily from all animals during the 9-day infusions. Otherwise, blood was collected at weekly intervals. Circulating prolactin levels were measured by RIA (Section 3.7). The inter-assay and intra-assay coefficients of variation for the assay at 10 ng/ml were 7.9% and 11.3%, respectively.

6.3.7 **Skin sampling**

Skin biopsies were taken from all animals at intervals over the first 8 weeks of the trial. Biopsies were taken on days -1, 1, 3, 6, 10, 20, 40, 45, 50 and 75 in relation to the first infusion (from October 9 to December 18).

6.3.8 **PRLR relative quantification**

RNA from the skin samples was extracted using TRizol ®. cDNA was then obtained by reverse transcriptase reaction and PRLR-L and PRLR-S were quantified using real-time PCR assay.

6.3.9 **Statistical analysis**

Analysis of variance was used to test the effect of prolactin manipulation, pregnancy and their interaction on feed intake, live weights, plasma prolactin concentration, wool
growth, fibre diameter and PRLR expression. The data from the first patch clip were used as a covariate in the analysis of the subsequent patches. Means between treatments were compared using LSD Post Hoc Test.

6.4 Results

6.4.1 Live weight

Live weight during the trial showed no differences between groups and was fairly constant until the end of December, when in 20 days all groups gained around 5 kg (p<0.05). After this gain, the weights stabilised until the end of the trial (Figure 36).

Figure 36: Live weight (kg) during the experimental period (means)

6.4.2 Plasma prolactin concentration

At the start of the trial the ewes kept in SD had lower plasma prolactin levels than the ewes in ND (Figure 37).

Prolactin concentrations of the Inf-9 ewes rose sharply as the infusion began. The mean plasma level through the first infusion was 467±38. ng/ml, which was statistically higher (p<0.001) than the Saline (27±10 ng/ml) and the ND groups (84± 1.6 ng/ml).
During this time, prolactin levels of the ND group were significantly higher (p<0.01) than the Saline group.

Once infusion was terminated, the prolactin level of the Inf-9 group fell and averaged 11± 4 ng/ml until the next infusion. The prolactin concentration in the Saline group during this time averaged 15± 3 ng/ml. Both groups exposed to SD had significantly (p<0.001) lower prolactin levels than the animals in the ND group (68±22 ng/ml). During the second infusion period mean prolactin concentration in the Inf-9 group was 457± 61 ng/ml, while levels in the Saline group were 29± 13 ng/ml and 91± 14 ng/ml in the ND group.

After the second prolactin infusion ewes exposed to SD had a slight, though significant (p<0.05), elevation of circulating prolactin. By January 2002, the Inf-9 and the Saline groups exhibited average prolactin levels of 28±17 and 26±15 ng/ml, respectively. The circulating prolactin in the ND group exhibited the expected seasonal decline in January and February 2002, when plasma prolactin levels did not differ between groups.

Figure 37: Mean plasma prolactin profile (ng/ml) of the experimental groups.
6.4.3 Prolactin receptor relative quantification

The mRNA expression of PRLR-L in the ND group showed very little change during September to October (Figure 38). From November until the end of the sampling regime it declined slightly reaching its lowest level of a 0.6-fold decrease.

During both infusion periods, PRLR-L mRNA expression in the Inf-9 group was up-regulated (p<0.01). Both infusion periods showed similar increases relative to pre-infusion levels (2.9-fold change). The PRLR-L mRNA expression declined sharply on day 7 of the first infusion period, however, by the start of the second infusion PRLR-L expression levels were at pre-treatment values again. After the up-regulation of the PRLR-L mRNA expression during the second infusion, the expression was down-regulated on the last skin sample.

Throughout the sampling period, the expression of PRLR-L mRNA in the Saline group was significantly (p<0.05) down-regulated, reaching a nadir a day after the first infusion period had ceased (0.6-fold decrease). The average expression of PRLR-L throughout the sampling period was statistically lower (p<0.01) than the expression of the Inf-9 group. During the second infusion PRLR-L mRNA expression was up-regulated in relation to the pre-infusion value on day 40 and similar to its value at the start of the experiment. From November to December PRLR-L mRNA expression in the Saline group increased reaching pre-treatment values by the last day of sampling.

Figure 38: The effect of prolactin manipulation on PRLR-L mRNA expression (log fold change). Error bars represent the pooled SED between groups of the fold change. The open bars represents the treatment periods.
In the Inf-9 group, the expression of the PRLR-S mRNA was up-regulated throughout the trial, reaching a peak of a 2.5-fold increase \((p<0.05)\) on day 17 (Figure 39). In contrast to the first infusion, PRLR-S declined during the second infusion reaching pre-treatment values on day 45.

The PRLR-S mRNA expression in the ND and Saline groups was down-regulated in relation to pre-treatment values throughout the trial. The average expression of PRLR-S in the Saline group was statistically lower \((p<0.001)\) when compared to the expression in the Inf-9 group. The average PRLR-S expression of the ND group showed the tendency \((p<0.10)\) to be lower than the expression measured in the Inf-9 group.

*Figure 39: The effect of prolactin manipulation on PRLR-S mRNA expression (log fold change). Error bars represent the pooled SED between groups of the fold change. The open bars represents the treatment periods.*

6.4.4 *Greasy and clean fleece weight*

During the 6 months of the trial there was no statistical difference in the greasy or clean fleece production among the different treatments (Figure 40).
6.4.5 Fleece fibre diameter

There was no difference in the fleece fibre diameter among the treatment groups (Figure 41).

Figure 41: Effect of prolactin manipulation on the fleece fibre diameter of non-pregnant ewes (means ± SEM).
6.4.6 *Patch clip data-wool growth*

The daily wool growth rate was estimated using the wool production of the monthly patch clips and the whole fleece weight (Figure 42). All groups showed a similar pattern of wool growth with no statistical difference between them. In February, all groups experienced a sharp decline in wool production, finishing the trial at 8.5±1.1, 9.0±1.5 and 9.7±1.5 g/day for the Inf-9, Saline and ND groups, respectively.

*Figure 42: The effect of prolactin manipulation on wool growth of non-pregnant ewes. Data presented as adjusted means and error bars represent the SEDs.*

6.4.7 *Patch clip data-fibre diameter*

At the start of the trial the mean fibre diameter did not differ between the 3 experimental groups (34.4±4.2, 33.7±4.3 and 33.8±3.1 μm for the Inf-9, Saline and ND groups, respectively. Throughout the trial, no statistical difference between groups was observed (Figure 43). Mean fibre diameter of the ewes in SD photoperiod declined from October to December. The fall in the ND group was smaller, but not statistically different.
Figure 43: The effect of prolactin manipulation on fibre diameter of non-pregnant ewes. Data presented as adjusted means an, error bars represent the SEDs.

6.4.8 Mathematical model

Simulations of the PRLR population in response to changes in the prolactin profile of each experimental group during the trial were carried out (Figure 44).

In the ND group, the number of total receptors and dimers remain constant while prolactin levels are at their spring/summer plateau (Figure 44 A). Once prolactin levels start to decline after January, the number of dimers follow the same trend, although total receptor numbers continue to remain relatively constant.

For the Saline group, with prolactin levels suppressed by SD photoperiod, the amount of total receptors and dimer formation is lower when compared to the ND group (Figure 44 B). When prolactin rises slightly after January, both total receptors and dimer formation increase.

In the Inf-9 group, the number of total receptors and dimers prior to and between infusion treatments is lower than the ND group due to lower prolactin levels at these times (Figure 44 C). However, once prolactin treatment is simulated the number of total
receptors increase above the 3000 nmol/litre level observed for the ND group. Dimer formation also increases but levels do not exceed the approximately 800 nmol/litre value observed in the ND group. After January, total receptor and dimer formation increase similarly to the Saline group.

Figure 44: Mathematical simulation of the PRLR population in using the prolactin profile from the experimental groups. First panel illustrates the smoothed prolactin data; the middle panel shows the concentration of bound (E₁) and dimerised (E₂) receptors and the last panel shows the concentration of unbound (U) and total receptor. (A: ND; B: Saline; and C: Inf-9).
6.5 Discussion

The main objective of this experiment was to investigate the hypothesis that an elevation of plasma prolactin levels may influence the biological response to a subsequent increase in prolactin, via changes in PRLR expression. To determine whether prolactin itself was a major determinant of PRLR expression, non-cycling and non-pregnant ewes were used and maintained under SD photoperiod to suppress endogenous prolactin secretion. Although PRLR did respond to the prolactin treatments, there was no effect on wool growth in the Inf-9 group relative to the untreated groups. Nevertheless, a number of interesting results and pertinent questions arose from the findings of this trial.

6.5.1 Prolactin profile

Plasma prolactin in the ND group was already elevated at the commencement of the experiment in September and remained at approximately 100 ng/ml until declining in February. These concentrations are similar to those previously reported by Kendall (1999) under matching conditions.

As expected, maintaining the ewes in a constant SD environment decreased circulating levels of prolactin when compared to the ewes in the ND environment. However, from December onwards, the prolactin concentration started to increase slowly, approximately doubling by February (p<0.01). It could be speculated that these ewes entered a refractory phase, as reported by (Poulton and Robinson, 1987; Lynch and Russel, 1989; Robinson et al., 1991). These authors concluded that constant treatment with melatonin, which mimics a SD environment, initially suppressed circulating prolactin levels. However, after a period of time the animals became refractory to this treatment and prolactin levels started to rise.

Another possibility is that the higher temperatures observed from December until February stimulated prolactin production and release in the ewes kept in SD
(Wettermann et al., 1982; Howland et al., 1983). In support of this is the fact that prolactin concentration in the ewes kept in ND, started to decline in January as daylength shortened. Therefore, the photoperiod cue (declining daylengths) appeared to override the influence of temperature (Howland et al., 1983) in the ND group.

In the Saline group, prolactin levels were slightly elevated during the second infusion, with concentrations increasing from 10 to 25 ng/ml. Prolactin is a hormone associated with stress responses (Neill, 1970) and the fact that these animals were cannulated, restrained in head bails and bled daily, could have contributed to elevated prolactin levels. During the first infusion, prolactin levels were high at the start (50 ng/ml), declined in the middle (15 ng/ml) and increased again by the end of the saline infusion period (50 ng/ml). These relatively small fluctuations, although not statistically different, are described because it is possible that they could have impacted on the expression of PRLR-L expression (Nixon et al., 2002).

6.5.2 Prolactin receptor mRNA expression

The expression of PRLR-L mRNA in the ND group was essentially constant until November 20, when it started to decline. This supports the concept that changes in circulating prolactin are necessary to modulate PRLR-L mRNA expression.

In the Saline group, expression of PRLR-L mRNA was depressed throughout the trial when compared to its initial value on October 9. In the previous trial (Chapter 5), where a group of non-pregnant ewes received saline infusion during late autumn, the expression of PRLR-L mRNA was not depressed. Prolactin levels in the first 30 days of each trial were similar; both groups were non-pregnant and received the same diet. One difference is that in ewes are likely to be cycling in the autumn. Therefore, the reproductive hormones could have had a positive effect on PRLR-L mRNA expression (Moldrup et al., 1993; Sugiyama et al., 1994; Shamgochian et al., 1995; Gunes and Mastro, 1997; Gunes et al., 1997; Kinoshita et al., 2001).
A major finding in this trial was that the overall PRLR-L expression of the Saline group was significantly lower than the expression of the Inf-9. These ewes were maintained in an artificial constant SD environment starting in mid-spring. This environment could activate a system that inhibits PRLR-L expression, but the presence of prolactin via infusion could override the effect. For example, 1α, 25-dihydroxyvitamin D (Vitamin D), is formed in the skin through a photochemical reaction catalyzed by sunlight. Seasonal variation of Vitamin D has been demonstrated in sheep (Smith and Wright, 1984), llamas and alpacas (Smith and Van Saun, 2001; Parker et al., 2002). These levels are higher in the spring/summer (LD) and lower in autumn/winter (SD). The Vitamin D receptor (VDR) belongs to the Nuclear Receptor Superfamily and acts as a transcriptional regulator by forming heterodimers with the retinoid X receptors (RXR) and binding to the promoters regions of their target genes (for review see Barsony and Prufer, 2002). These VDRs are essential for normal hair cycling (Sakai et al., 2001; Kong et al., 2002) and are highly expressed in the DP and ORS (Reichrath et al., 1994; Kong et al., 2002). The actions of the VDR in the hair cycle are most likely ligand-independent. Panda et al (2001) generated mice that do not produce 25(OH)D-1alpha-hydroxylase (1alpha(OH)ase), an enzyme necessary for Vitamin D formation, and no abnormalities were observed in the whiskers and hair growth, despite the low levels of circulating Vitamin D. Another interesting association is that in the presence of the transcription factor Ets-1 (E26 transformation-specific), VDR stimulates the prolactin promoter in a ligand-independent manner. Craven et al (2003b) observed that prolactin was synthesised in mice skin in association with the hair cycle. The question arises of why the receptor of a seasonal molecule but not necessarily its ligand is important to hair growth? One possibility is that these studies were performed in mice or humans, which do not show a distinct seasonal pattern of fibre growth. It is possible, however, that more seasonal animals, like the Romney ewes in this study, still rely on the seasonal variation of Vitamin D to influence the seasonal changes in fibre growth. In
support of this concept, Bataille-Simoneau et al (1996) showed that Vitamin D enhanced PRLR mRNA levels in two human osteosarcoma cell lines. Therefore, further investigation on the role of Vitamin D in the control of hair growth could be a fruitful area of investigation.

During the second prolactin infusion, PRLR-L mRNA expression of the Saline ewes were up-regulated (0.9 fold) in relation to PRLR-L expression on day 42, coinciding with a doubling of the circulating prolactin levels (Figure 37). Neill (1970) reported that the stress caused by blood sampling can elevate prolactin levels. Fujikawa et al (1995) also reported that stress can up-regulate PRLR-L expression in rats. Hence, the prolactin elevation caused by the stress of the blood sampling period may have been sufficient to up-regulate PRLR-L mRNA expression. This concurs with the findings of Barash et al (1983), Djiane et al (1982) and Nixon et al (2002) who demonstrated that small increments in the circulating levels of prolactin are sufficient to modify PRLR mRNA expression. In contrast, when the first saline infusion started, circulating prolactin declined and no effect on PRLR-L expression was observed.

Infusion of ovine prolactin resulted in an up-regulation of PRLR-L mRNA expression in the first 3 days of infusion. This enhancement in expression at the beginning of infusion was also observed in the experiment described in Chapter 4. The results from this experiment conflict to some extent with the trial results described in Chapter 5 and those reported by Nixon et al (2002), where a very rapid down-regulation of PRLR-L mRNA expression was observed as prolactin levels started to increase. However, because the down-regulation is only a transient response, the nadir in PRLR mRNA expression could have been missed in the skin sample collected one day after the start of the infusion.

The most striking difference between the current trial and the previous trials performed in early gestation (Chapters 4 and 5) is that there was a sharp down-regulation of PRLR-L mRNA expression after the peak of expression while prolactin was still being
administered. On the contrary, early pregnant ewes showed increased PRLR-L mRNA expression which was sustained during and after prolactin treatment. Recently, studies on the mechanism of mRNA stabilization have demonstrated the existence of AU-rich elements (AREs) in the 3'-untranslated region of mRNAs, which are responsible for a rapid decay of mRNA molecules (for review see Shim and Karin (2002)). The stabilization of mRNA is specific to cell type and physiological status. Bollig et al (2003) and Chen et al (2002) described the existence of proteins that bind specifically to the AREs resulting in mRNA stabilization. The p38 MAP kinase pathway seems to play a role in the activation of these proteins (Bollig et al., 2002; Tebo et al., 2003). Other types of mRNA stabilization have also been described. For example, UV light has been suggested to stabilize mRNA that lack AREs (Bollig et al., 2002). Mitchell and Ing (2003) demonstrated that oestrogen stabilizes the mRNA of its receptor in the sheep endometrium, while Kong et al (2003) showed that a complex formed by alpha-globulin mRNA and protein alphaCP extends the half-life of the mRNA. Therefore, the down-regulation of PRLR-L mRNA expression observed during the first prolactin infusion could be related to stabilization mechanisms being less effective in the non-pregnant ewe.

At the second infusion, PRLR-L mRNA expression was again up-regulated (sample collected at day 45) to the same extent as during the first infusion. At the end of the second infusion (day 50) PRLR-L expression returned to pre-treatment levels indicating that there was no sustained up-regulation of PRLR-L. This experiment demonstrated that under these conditions, a previous prolactin elevation did not prime the response of PRLR-L expression to a second prolactin elevation.

The expression of PRLR-S in the ND group was down-regulated throughout the trial when circulating prolactin was at a seasonal plateau. Similarly, in the Saline group PRLR-S expression was also depressed relative to the mean at the start of the trial, although prolactin levels were low in this group. In contrast, in the previous trial
(Chapter 5), PRLR-S expression increased in non-pregnant ewes during late autumn and early winter while plasma prolactin was also low. Either prolactin is not the major regulatory factor for PRLR-S expression or other complicating factors such as season or oestrous cycle hormones are involved. The expression of PRLR-S mRNA in ND ewes was down-regulated throughout the trial. The expression pattern of PRLR-S mRNA in the non-pregnant ewes from the previous trial (Chapter 5) was almost opposite to the results from this trial. In late autumn-early winter, when prolactin levels were low, PRLR-S mRNA showed a constant increase in expression. In this trial, when prolactin levels are at their seasonal peak, PRLR-S mRNA expression was depressed. Either prolactin is not the main regulatory factor for PRLR-S mRNA expression or it directly inhibits the expression PRLR-S mRNA in the non-pregnant ewe.

There was a striking difference in the pattern of PRLR-S mRNA expression of the Inf-9 group when compared to the Saline and ND groups. Throughout the experiment, PRLR-S mRNA expression in the Inf-9 groups was up-regulated in relation to its pre-treatment values, while expression was down-regulated in the other groups. In the experiment described in Chapter 5 the expression of the PRLR-S was up-regulated after 3 days of prolactin infusion and down-regulated once prolactin infusion was over. In the current experiment, PRLR-S was up-regulated during the first prolactin treatment; however, during the second infusion PRLR-S mRNA expression was down-regulated. Some authors suggest that prolactin is only able to up-regulate the expression of the PRLR-L (Asfari et al., 1995; Feng et al., 1998; Galsgaard et al., 1999). The results from Chapter 5 and the second infusion treatment agree with this suggestion. On the other hand, the data from the first infusion period agrees with Nixon et al (2002) who showed that in non-pregnant ewes and mature rams the expression of the PRLR-S and PRLR-L mRNA responded in a similar pattern to prolactin elevation. Consequently, there may be factors other than prolactin that regulate the expression of PRLR-S mRNA. Indeed, Hu et al (2002), Ormandy et al (1998) and Moldrup et al (1996) demonstrated that the
transcription of PRLR isoforms were under the control of multiple promoters. The fact that the same hormonal treatment at different times and physiological status resulted in distinct PRLR-S mRNA expression patterns tends to support this finding. Other possibilities lie in the presence or absence of molecules that stabilize the PRLR-S mRNA.

6.5.3 Mathematical model simulations

The predictions of the mathematical model agree with the biological data for the ND group, showing no change in the amount of total receptors and dimers until January. However, prolactin levels in the ND group ewes were higher when compared to the non-pregnant ewes in the previous winter/spring trials (Chapters 4 and 5), resulting in a higher predicted PRLR protein expression when compared to that predicted for the non-pregnant ewes in Chapters 4 and 5.

The mathematical model simulation for the Saline group also essentially agreed with the biological results. It predicted no change to the concentration of total receptors or receptor dimer until January. Nevertheless, during the second saline infusion (from November 20-29), PRLR-L mRNA expression increased. The prolactin increase during the second saline infusion was about 15 ng/ml in magnitude. According to Nixon et al. (2002) and Barash et al. (1983) small prolactin increments can up-regulate PRLR expression. This small transient elevation in plasma prolactin was not reflected in the fitted prolactin input function in the model, and hence no increase in prolactin protein is predicted.

PRLR-L mRNA expression was up-regulated during both prolactin infusion periods, and the mathematical model also predicted these events. However, the pattern of the up-regulation between biological data and mathematical model predictions did not entirely agree. The model is based on inputs of prolactin, rates of formation and degradation of the different states of the PRLR and assumes the same equilibrium state
at the start of the simulation. As a result, the predictions are similar to those obtained in the previous trials (Chapters 4 and 5). Therefore, the amounts of total receptors and receptor dimer reached a plateau at 3500 and 500 nmol/l, respectively, around day 6 while the observed PRLR-L mRNA expression was down regulated during the infusions. This may simply be ascribed to the differing expression profiles of PRLR mRNA and protein expression. But the discrepancy described in this Chapter accentuates the importance of developing studies on other mechanisms that could be influencing the signalling, transcription, translation and intracellular storage of PRLR in the skin of the breeding and non-breeding ewe.

6.5.4 Wool growth

Another aspect that became clear with this trial was that prolactin acts as a modulator of an intrinsic system. This agrees with Goffin et al. (2002), who suggests that the various actions of prolactin on different tissues are regulated by, but not dependent on, the hormone.

The trial was conducted from September to March, a period when daylength has been increasing since the winter solstice and wool growth is naturally at its highest (Ryder and Stephenson, 1968b; Wuliji et al., 1993; Woods et al., 1995). Therefore, the enhancement of wool growth would have already been triggered before the animals entered into SD. Additionally, elevated prolactin levels appear to have had no effect.

The inability of the wool follicle to respond to the prolactin treatment at this time could also reside in the fact that there are other seasonal hormones, which could have some influence. Because these ewes were kept in SD, the seasonal variation of these hormones could have been abolished, for example, alpha-melanocyte stimulating hormone (alpha-MSH). This hormone is produced in the pituitary gland and derives from the cleavage of a larger molecule, the pro-opiomelanocortin (POMC). Alpha-MSH is distributed through a number of tissues, including the skin (for review see Oktar and
Alican, 2002) and has been implicated in having anti-inflammatory/immunosuppressant properties (Gatti et al., 2002; Oktar and Alican, 2002; Scholzen et al., 2003). Alpha-MSH also acts as a neuromessenger in feed intake behaviour, playing an important role in the energy balance of the individual (Crowley et al., 2003; Zimanyi and Pelleymounter, 2003) and in seasonal body weight and feed intake changes. Further, the expression of alpha-MSH and its receptor fluctuate during a synchronized hair follicle (Paus et al., 1999). These authors also demonstrated that high doses of alpha-MSH to murine skin culture modulated epidermal and/or follicle keratinocyte proliferation (Paus et al., 1999). It has been established that this hormone also shows a seasonal pattern of secretion. In Siberian hamsters, circulating levels of alpha-MSH were described as being higher in the summer and lower in the winter (Logan and Weatherhead, 1980). However, in Soay rams, the alpha-MSH plasma concentration was basal during LD photoperiod. At the end of the 16 week exposure to LD, levels start to increase and peak in the first weeks after the shift to SD treatment (Lincoln et al., 2001). The shifting of the animals from an increasing daylength to a SD photoperiod could have disrupted the rhythm of circulating alpha-MSH, changing the energy balance of the experimental animals. As nutrition plays a major role in wool production (Sumner and Wickham, 1969; Sumner, 1979; Hawker, 1985; Betteridge et al., 1992) and alpha-MSH is important in regulating seasonal feed intake and body weight (Lincoln et al., 2001) together with the association of alpha-MSH with the hair cycle it is possible that seasonal wool growth may be mediated by the interaction of prolactin, alpha-MSH and other systems. Other hormones that have seasonal behaviour in sheep are beta-endorphin, LH, FSH, testosterone and insulin (Lincoln and Richardson, 1998). They could all, directly or indirectly, play a part on the regulation of wool growth.

Not only were the animals in this trial in a different season, but they were also different from the sheep in the previous trials in physiological status. Although there is extensive work showing that gestation is responsible for a major decline in wool production,
especially in late pregnancy (Stevens and Wright, 1951; Bigham et al., 1978; Betteridge et al., 1992; Kendall, 1999), the parturition peak of prolactin also plays an important role in stimulating the post-lambing wool growth (Kendall, 1999). It is possible that the parturition prolactin peak interacts with progesterone, oestrogen, cortisol and placental lactogen, which also increase during late pregnancy (Bassett et al., 1969; McNatty et al., 1972; Chamley et al., 1973; Carnegie and Robertson, 1978; Tsang and Hackett, 1979; Talamantes et al., 1980; Kappes et al., 1992). As has been reported by Cassy et al. (2000), a combination of progesterone and cortisol was responsible for an up-regulation of PRLR-L mRNA expression. This hormonal environment was not present at the second prolactin infusion. Therefore, this suggests that the reproductive hormones, together with elevated prolactin, could have an important role in the regulation of PRLR expression and consequent wool growth.

6.6 Conclusions:

- The manipulation of prolactin in non-pregnant ewes maintained in SD during spring and summer caused no short-term or long-term effects on wool production.

- Two periods of elevated plasma prolactin levels equally up-regulated the expression of PRLR-L mRNA.

- A priming treatment with prolactin did not enhance PRLR-L mRNA expression when animals were subjected to a subsequent rise in exogenous prolactin levels.

- The seasonal wool growth in the Romney sheep could be regulated by other factors such as Vitamin D and POMC in conjunction with prolactin. Research into the effect of other seasonal hormones should be integrated with prolactin manipulation.
• mRNA stabilizers could be involved in maintaining high levels of PRLR-L mRNA expression in pregnant ewes.

• The expression of PRLR-S in sheep skin does not seem to be directly regulated by circulating prolactin. Other reproductive hormones may be involved.
CHAPTER 7

GENERAL DISCUSSION
At the start of this PhD study the seasonal pattern of wool growth and the association of seasonal prolactin variation with this pattern were well documented (Coop and Hart, 1953; Geenty et al., 1984; Montgomery and Hawker, 1987; Lincoln, 1990; Clarke et al., 1993; Kelly et al., 1993; McClohgry et al., 1993; Wuliji et al., 1993; Lincoln, 1998; Kendall, 1999). The negative effects of reproduction on the annual pattern of wool growth were, also, well known (Stevens and Wright, 1951; Seebeck and Tribe, 1963; Sumner and McCall, 1989; Masters et al., 1992). Conversely, an association of the parturition prolactin peak with the increase on wool production observed after lambing had been recognized (Kendall, 1999), indicating that prolactin could also have a mid- to long-term positive effect on wool growth.

It was clear that prolactin could act directly on the hair follicle, since the PRLR was found to be present in the ORS, DP and GM (Choy et al., 1995; Choy et al., 1997), and their gene expression seemed to vary through the changes in the wool cycle of Wiltshire sheep (Nixon et al., 1998). In the skin of this shedding sheep breed, PRLR gene expression was up-regulated by endogenous elevation of circulating prolactin, which was, in turn, related to changes in the wool growth cycle (Nixon et al., 2002). With this in mind, the main aims of this program were to:

1. Investigate if PRLR expression in the skin of Romney ewes would be regulated by exogenous prolactin manipulation and its association with wool growth pattern;

2. Determine the appropriate delivery route and prolactin profile to generate a wool growth response;

3. To attenuate the winter/reproductive decline in wool growth by elevating prolactin levels in early pregnancy based on the positive long-term effect of prolactin on wool growth.
The development of a real-time PCR assay for quantifying ovine PRLR in the skin was required to fulfil the aims of this PhD program and was successfully achieved (Chapter 3). Biological data from the three animal experiments were also integrated with the development of a mathematical model of hormone-receptor interaction, conducted by the AgSystems modelling group at Ruakura.

7.1 Prolactin manipulation and PRLR expression

The results from a series of experiments reported in Chapters 4, 5 and 6 suggested that prolactin manipulation does modulate PRLR-L expression in skin. This regulation was dependent on the profile of circulating prolactin. Daily subcutaneous injection increased circulating prolactin above physiological levels. However, the duration of this elevation was short and unable to activate the proposed positive feedback mechanism responsible for the synthesis of new PRLR-L. On the other hand, steady prolactin elevation obtained by constant infusion was, for the first time, associated with an up-regulation of PRLR-L expression in the skin of Romney ewes. Moderate and constant elevation of prolactin has already been shown to increase PRLR in other tissues and species (Barash et al., 1986; Jahn et al., 1991; O'Neal et al., 1991; Koh and Phillips, 1993; Ormandy and Sutherland, 1993; Goffin and Kelly, 1997; Bole-Feysot et al., 1998).

Computer simulation based on a mathematical model incorporating this positive feedback loop also predicted that there would be no change in the concentration of total receptors when the subcutaneous injection prolactin profile was used. Consistent with the biological data, the constant infusion profile increased total receptor concentration. This agreement supports the hypothesis that prolactin's regulation of its receptor is pivotal to eliciting the wool growth response.

Different duration or frequency of prolactin elevation can generate different biological responses depending on the tissue, species and hormonal environment. For example, in rats that had their endogenous prolactin secretion blocked by bromocryptine, prolactin
injections were not able to restore lactation; however, constant infusion did restore the lactation of rats that received bromocryptine (Hebert et al., 1993). Twice-daily prolactin injection into the paraventricular nucleus was responsible for an increase in food intake (Sauve and Woodside, 2000), and single intracerebroventricular prolactin injections facilitated male sexual activity, whereas chronic manipulation (constant delivery for 5 days) decreased sexual behaviour (Cruz-Casallas et al., 1999). In mice, daily prolactin injections had an antiestrogenic effect in the uterus, preventing the formation of atypical hyperplasia (Gunin et al., 2002), and also promoted hematopoiesis (Woody et al., 1999). In early lactating sows, prolactin injections had no effect on milk yield, back-fat or live weight (Farmer et al., 1999). These different responses to both transient and constant prolactin elevation in target tissues, demonstrate that the over 300 biological functions (Bole-Feysot et al., 1998; Freeman et al., 2000) associated with prolactin are well regulated. It would be a physiological problem if all tissues responded equally to the same prolactin changes. Therefore, as a way of preventing physiological chaos, different tissues associate prolactin functions with other regulatory factors, which provides a tissue specific response to a systemic prolactin increase. Such regulatory factors could include oestrogen and progesterone, which are already shown to regulate each others receptor expression, together with prolactin (Ormandy et al., 1997). These hormones also share some of the signalling pathways, for example oestrogen activates the MAPK pathway (Treeck et al., 2003). Migliaccio et al (2002) and Castoria et al (1999) specifically demonstrated that sex steroids activate the Src/Ras/ERK signalling pathway. Furthermore, the STAT signalling pathway is also activated by progesterone (Lin et al., 2003) and oestrogen (Frasor et al., 2001).

The results demonstrating the importance of the duration of prolactin elevation for the activation of the positive feedback mechanism of PRLR-L synthesis (Chapter 4) were confirmed in the experiment with 3 different lengths of prolactin infusion (Chapter 5). The experiment described in chapter 5 demonstrated that 3 days of prolactin infusion
was not long enough for the feedback mechanism to reach its maximum. The biological data led to the conclusion that a constant prolactin elevation between 3 to 9 days was necessary for the maximum signalling to occur. Mathematical simulations of these prolactin profiles showed that after 6 days of constant infusion, concentration of receptors would reach a plateau agreeing with the biological data. The existence of such a threshold in the duration of prolactin treatment for maximum receptor synthesis in the skin has not been suggested before. This finding could explain the results from experiments which showed that long prolactin treatment periods were necessary for a noticeable effect on fibre growth in different species (Duncan and Goldman, 1984a; Thomas et al., 1994; Pearson et al., 1997; Thompson et al., 1997).

Prolactin manipulation at the beginning of pregnancy resulted in sustained up-regulation of PRLR-L expression once prolactin infusion was over. This effect could be based on mRNA stabilization already known to occur with other molecules (Bollig et al., 2002; Bollig et al., 2003; Kong et al., 2003; Tebo et al., 2003). However, this mRNA stabilization mechanism could be regulated by other seasonal factors and/or reproductive environment, since in the experiment described in Chapter 6 using non-pregnant ewes, this effect was not observed. Further research in mRNA stabilization should be developed, because it could provide an explanation for the long-term biological effect such as that observed in this study.

The regulation of PRLR-S expression in the sheep skin is still unclear. There is evidence that prolactin only up-regulates the expression of the PRLR-L in insulin producing cell lines and in the immune system of other species (Asfari et al., 1995; Feng et al., 1998; Galsgaard et al., 1999), inferring that, under these experimental conditions, prolactin does not regulate the expression of PRLR-S. The PRLR-S expression data generated in Chapter 5 and 6 similarly suggested that circulating prolactin did not directly regulate PRLR-S expression. Expression only increased after several days of prolactin elevation and after PRLR-L had been up-regulated, inferring that the down-stream signalling via
the PRLR-L could regulate PRLR-S expression. Results from Chapter 6 showed a difference in PRLR-S expression between the prolactin treated group and the groups that did not receive prolactin subjected to short or natural photoperiod. This finding supports the theory that PRLR-S expression can be indirectly influenced by prolactin via the signalling of PRLR-L. However, in the case of non-pregnant ewes with no prolactin treatment (Chapter 5), PRLR-S expression was up-regulated until the end of the skin sampling period (first 56 days of experiment), reinforcing the need for further studies on the effect of reproductive hormones in the regulation of PRLR-S expression.

7.2 PRLR regulation and reproductive hormones

The expression of PRLR-L in non-pregnant ewes was up-regulated in comparison to the pregnant ewes that did not receive prolactin treatment. This suggests that in the cycling sheep, reproductive hormones are also able to influence PRLR-L expression. Progesterone (Cassy et al., 2000; Ling et al., 2000), oestrogen (Posner et al., 1975; Yasui et al., 1999), and cortisol (Mizoguchi et al., 1997b; Phillips et al., 1999) have been shown to up-regulate both forms of PRLR expression in different tissues and species. However, progesterone (Teyssot and Houdebine, 1981; Nishikawa et al., 1994; Cassy et al., 1999) and testosterone (Yasui et al., 1999) can also have a negative effect on PRLR expression. Those opposing effects are tissue and species specific; therefore it would be useful to identify the role of these hormones in PRLR regulation in sheep skin at different physiological stages. To achieve this, animal experiments where these hormones are manipulated (either by enhancing or blocking their secretion or by delivering the hormones exogenously) or monitored during the different pregnancy stages could be performed. Then, the quantification of PRLR gene expression in response to these changes could be achieved by real-time PCR. If an interaction is established, cell culture experiments where hormonal manipulation can be undertaken in isolation from other systemic factors could improve the understanding of the system.
These hormones could also have an effect on the sustained up-regulation of PRLR-L expression observed in infused ewes in Chapters 4 and 5, since progesterone (Bassett et al., 1969; Stabenfeldt et al., 1972; Cox, 1975), placental lactogen (Kelly et al., 1974; Kappes et al., 1992) and oestrogen (Carnegie and Robertson, 1978) start to increase in early pregnancy to reach peaks at mid- or late-pregnancy. They are, therefore, all candidates for maintaining PRLR expression. The lack of this pregnant hormone environment could also explain the absence of sustained PRLR-L up-regulation observed in the infused ewes in Chapter 6.

7.3 Prolactin manipulation and wool growth

The constant prolactin infusion during the early stages of pregnancy was associated with a positive short- to long-term effect on wool growth and fibre diameter (Chapters 4 and 5), demonstrating that the seasonal/reproductive decline of wool growth could be attenuated via prolactin manipulation. The short- and medium-term effect could be partially explained by the up-regulation of PRLR expression, which by increasing its availability in the cell membrane, amplified signalling and therefore the biological response (Figure 45).

The association between prolactin, PRLR regulation and wool growth revealed by this study raises the question of how prolactin signalling might interact with the more immediate mechanisms controlling fibre growth within the follicle. As reviewed earlier (Section 2.4.3), the annual pattern of wool production reflects the seasonal hair replacement of ancestral sheep, and probably represents an attenuated hair cycle. One current theory of hair cycle control is the stem cell trafficking hypothesis. Oshima et al. (2001), suggested that multipotent stem cells migrate from the bulge region of the ORS to the GM where they divide and differentiate, initiating a new cycle of fibre growth. Intriguingly, this migration path in the ORS is also the location of the highest concentration of both PRLR mRNA (Nixon et al., 2002) and PRLR protein (Choy et al.,
and expression in this zone varies markedly with prolactin induced wool cycles in Wiltshire sheep (Nixon et al., 2002). It is highly likely that the variation in PRLR mRNA observed in this study occurs in this region of the follicle. Therefore, if stem cell trafficking holds for seasonal wool growth in Romney sheep, it is possible that prolactin acts within the ORS to alter the properties of migrating stem cells and thereby influence the lineage of proliferating keratinocytes in the follicle bulb. In fact, cell migration stimulated by prolactin has been described in the human mammary gland (Maus et al., 1999) and monocytes in rat ovaries (Olson and Townson, 2000).

It is known that PRLR-L is responsible for the expression of milk proteins by the JAK/STAT signalling pathway (Goupille et al., 1997; Jahn et al., 1997; Bole-Feysot et al., 1998); therefore, the up-regulation of the expression of PRLR-L in the skin due to prolactin treatment could enhance protein synthesis, which is necessary for wool production. The PRLR-S homodimers, on the other hand, were shown to have a mitogenic effect in other species mammary gland (Das and Vonderhaar, 1995; Das and Vonderhaar, 1997) and liver (Piccoletti et al., 1997) via the MAPK signalling pathway. Furthermore, prolactin itself has been shown to be internalised and translocated to the cell nucleus with the help of the chaperone protein, cyclophilin B (cypB) (Rycyzyn et al., 2000). The presence of prolactin in the nucleus seems to act as a mitogen co-factor (Clevenger and Rycyzyn, 2000; Rycyzyn and Clevenger, 2002). More recently, cyclophilin A (cypA) has also been shown to interact directly with the PRLR, suggesting that cypA could have a regulatory role on the PRLR signalling pathway (Syed et al., 2003). In rats, PRLR-S has also been reported to be internalized faster than the PRLR-L (Vincent et al., 1997). Utilizing the PRLR-S could be a means of enhancing prolactin nuclear retrotranslocation, which could affect the balance of protein expression and cell proliferation, both necessary for fibre growth.

The activation of PRLR transcription would be another interesting area for further research, especially considering that the two isoforms of the receptor can have different
biological effects. These isoforms are derived by alternative splicing, and in other species activation of multiple promoters regulate their expression (Hu et al., 1996; Moldrup et al., 1996; Hu et al., 2002). This different promoter usage could have an impact on the seasonal wool growth by changing the ratio of the different receptor isoforms.

Other factors such as ornithine decarboxylase (ODC) have also been shown to respond to prolactin treatment. ODC is a key enzyme in the biosynthesis of polyamines, which are associated with cell growth differentiation and proliferation (Gonzalez et al., 1991; Williams et al., 1995). This enzyme is activated by prolactin treatment (Oka and Perry, 1976; Rillema, 1985) and has been found in sheep skin (Williams et al., 1995), and to be localized in the follicle fraction. Nancarrow et al (1999) and Hynd and Nancarrow (1996) showed that blocking ODC activity altered fibre length and diameter in sheep, and eflornithine, an ODC inhibitor, is used with success in cases of hirsutism by slowing hair growth (Balfour and McClellan, 2001; Trueb, 2002). The mechanism of prolactin activation of ODC is not known, however PRLR are likely to be involved.

Another possibility could be that prolactin actually suppresses an inhibiting factor such as the polyamine oxidase (PAO). Ferioli et al (2000) showed that hydrogen peroxide is one of the products of PAO activity, which results in apoptosis. Prolactin inhibits, and glucocorticoids stimulate PAO activity. Therefore, by inhibiting PAO activity, prolactin diminishes the apoptosis process that is related to the regression of fibre growth (Hollis and Chapman, 1987; Su et al., 1999). Further research to investigate the presence and activation of these enzymes during prolactin treatment could also provide a better understanding seasonal wool growth.

It can be speculated that wool growth is activated after the winter solstice. The prolactin levels in non-pregnant ewes started to increase at this time and became statistically higher when compared to pregnant ewes until parturition, as previously reported (Kendall, 1999). The higher prolactin levels could be responsible for the
activation and maintenance of wool growth, which would explain the higher wool production of non-pregnant ewes compared to pregnant ewes at this time.

Grimm et al (2002) have demonstrated that the balance between progesterone and prolactin receptors is important for the regulation of cell proliferation in mice mammary gland. Considering that the balance between these receptors is most likely different between pregnant and non-pregnant ewes, this could also explain the difference in wool growth between these groups during mid-pregnancy. This reinforces the need of further research on the molecular mechanism of PRLR regulation and wool growth mediated by reproductive hormones.

The long-term positive effect of prolactin on wool growth and fibre diameter was first described by Kendall (1999). This series of experiments not only confirmed the long-term effect of prolactin elevation on wool production but also demonstrated that this effect also occurred when prolactin elevation took place during early pregnancy via exogenous manipulation (Chapters 4 and 5). However, the mechanism involved with this delayed positive effect of prolactin on wool growth is still unclear.

Goffin et al (2002) speculated that apart from its reproductive and lactational role, prolactin seems to have modulatory rather than a definitive role. The wool growth response to the prolactin infusion in Romney ewes corresponds to this idea because the pattern of wool growth observed among the experimental groups in Chapter 4 and 5 was similar, what changed was the rate of growth. From this observation, it was speculated that the previous prolactin manipulation could enhance the PRLR signalling during the parturition peak, therefore enhancing the postpartum wool growth. Figure 45 shows a possible mechanism behind this delayed effect of prolactin on wool growth. The first panel illustrates the positive feedback loop that stimulates PRLR expression. Through PRLR signalling, gene transcription of PRLR is activated, which increases PRLR synthesis. At this stage, prolactin signalling is also activating transcription of genes necessary for wool growth, resulting in the short-term wool growth. The second
panel (mid-pregnancy) suggests that the sustained PRLR expression once prolactin treatment ceased resulted in the translation of PRLR mRNA and storage of these newly formed receptors into intracellular compartments. Indeed, there have been indications that the majority of PRLR are localized intracellularly (Posner et al., 1981; Sakai et al., 1994; Bolander, 1999). The second panel also infers that the increase in other gestational hormones could be stimulating PRLR gene transcription and/or regulating the storage of PRLR. There is no evidence of how long these proteins are stored. Consequently, the possibility that they are still present when the parturition peak of prolactin occurs cannot be ruled out. The third panel (parturition) is basically an enhancement of the first panel. It suggests that the prolactin increase stimulates the externalization of stored receptors enhancing the overall prolactin signalling resulting in a boost of the already established seasonal wool growth mechanism. Therefore, although prolactin elevation of all the pregnant groups was similar, ewes that had PRLR levels up-regulated earlier responded more efficiently, resulting in the higher post-lambing wool production of the infused groups when compared to non-infused pregnant ewes (Chapter 4). Another possibility is that the progesterone, oestrogen and cortisol rise observed from mid-pregnancy until close to parturition could be responsible for regulating the externalization of these cryptic PRLR, since these hormones regulate each others receptors in other tissues (Ormandy et al., 1997). Further research in the quantification, intracellular localization, half-life and trafficking of the PRLR protein in the skin after prolactin manipulation is necessary for additional understanding of wool growth via PRLR signalling.

There is also a possibility that prolactin treatment up-regulated prolactin receptors at the time of infusion resulting in the short-term effect of wool growth, and at the same time it also down-regulated a wool growth inhibitor, such as the epidermal pentapeptide, described by Paus (1991). However, there is little recent evidence in the literature supporting this speculation.
The goal of the last experiment was to test the hypothesis that the parturition PRLR signalling can be enhanced by a previous prolactin infusion. Although this hypothesis was not confirmed, the results from this trial were still useful because they highlighted the importance of an intrinsic cycle of wool growth. The first important result was that although prolactin treatment up-regulated PRLR levels during both infusion times, regulation was different from that observed in the ewes from Chapter 4 and 5. It has been demonstrated that reproduction can have a significant effect on PRLR expression and wool growth. Therefore, the absence of progesterone, oestrogen and cortisol could be responsible for the findings in Chapter 6. An animal experiment similar to that in Chapter 4 and 5, but with an extended skin sampling regime until parturition, is necessary to verify whether a previous prolactin treatment is responsible for enhancing the postpartum wool growth.

The lack of a sustained up-regulation of PRLR-L expression could be one of the reasons for the absence of a boost in PRLR signalling at the second infusion. The mRNA stabilization mechanism might not be active due to the absence of the appropriate hormonal environment; therefore, numbers of cryptic receptors could have been low and unavailable to enhance the overall signalling of the second infusion. Also, the SD environment depressed PRLR expression of ewes which received saline. The depression of PRLR expression could be associated with other seasonal molecules such as Vitamin D which should be considered in future research.
Figure 45: Hypothesis of short- to long-term effect prolactin manipulation has on PRLR dynamics and wool growth of pregnant Romney ewes. Panel 1 shows short-term effect in response to the initial prolactin manipulation. Panel 2 shows the mid-pregnancy period and Panel 3 shows the enhanced of PRLR signalling and therefore enhanced wool growth (long-term) in response to the increased prolactin levels at parturition. Red homodimer indicate active receptors.
It has been long recognized that a change in circulating prolactin levels is the key for the triggering of seasonal coat changes and seasonal wool growth (Duby and Travis, 1972; Ryder and Lincoln, 1976; Allain et al., 1986; Smith et al., 1987; Lincoln, 1990; Smale et al., 1990; Pearson et al., 1996; Xiao et al., 1996). The wool growth mechanism in Chapter 6 could have been triggered earlier when prolactin levels start to increase after the winter solstice. This could explain the lack of wool growth response despite proper prolactin elevation and PRLR up-regulation in the Chapter 6 infused ewes. This confirms that fibre growth responds to external signals, such as prolactin (Orfanos and Hertel, 1988; Pearson et al., 1999; Craven et al., 2003a) and steroid hormones (Paus and Cotsarelis, 1999; Stenn and Paus, 2001); however, growth cycles are governed by intrinsic rhythms that could override these external regulators (Rougeot et al., 1984; Allain et al., 1994; Stenn and Paus, 2001). Therefore discovering the exact time of this triggering event and the physiological factors involved with this mechanism could enable the manipulation of wool growth throughout the year.

At the beginning of this study manipulation of wool growth on an agricultural scale was quite unfeasible because it is unpractical and uneconomic to keep ewes in a LD environment during the winter time. The idea of hormonal injection is still expensive, and also not totally accepted by the general public. The series of experiments of this thesis have demonstrated that enhanced wool production could be achieved by manipulation of PRLR, and suggests that a product could be developed to stimulate or inhibit a particular enzymatic part of the PRLR signalling pathway. Alternatively it may be possible to develop new genotypes based on a detailed understanding of the biochemical process involved. A better understanding of PRLR expression and signalling is still necessary, however, we are now a step closer to a viable wool growth manipulation suitable for a farm scale usage. Further studies in this area may also lead to pharmacological treatments to ameliorate pathological hair growth conditions observed in humans, especially those related to hyperprolactineamia.
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