INVESTIGATION OF FACTORS INFLUENCING LACTATION PERSISTENCY IN THE GOAT

JOANNA R BROWN

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT

This thesis examines some of the factors which play a role in determining lactation persistency in the goat with the overall aim of improving persistency.

The plasticity of mammary gland growth during pregnancy and lactation was investigated by treatments of growth hormone and stimulation of compensatory growth. Cell activities and differentiation could be altered but the effects were dependent on the physiological state of the animal with longer term responses in milk production associated with treatments given during late gestation.

The endocrine influence of prolactin and oxytocin were examined by attempting to elevate circulating prolactin by administering perphenazine and by giving exogenous oxytocin during lactation. The former treatment was successful in the short-term when given by intravenous infusion but oral and injected doses given during lactation were not. Oxytocin had a small and positive effect on persistency, most marked when combined with reduced milking frequency in late lactation.

The effects of once- and thrice-daily milking, suckling and continuous drainage of milk from the mammary gland for the first six weeks of lactation, followed by routine milking for the remainder of lactation were examined in conjunction with the influence of parity on the responses obtained. Drainage proved ineffective in increasing yields or improving persistency. Suckling and thrice-daily milking enhanced milk yields in the short-term but no long-term alteration of persistency was seen. Once-daily milking was tolerated well by multiparous but not primiparous animals, but there was a trend of improved persistency with this treatment, regardless of parity. This may reflect alterations in the partitioning of milk stored within the udder but measurements made 5 hours post-milking did not show any differences between these and contralateral thrice-daily milked glands.

Finally the effectiveness of caseins (milk proteins) in providing a measurement of lactation persistency by indirect measurement of cellular involution was studied. It was found that the production of caseins varied over lactation, and that γ-casein production was highly (negatively) correlated with milk production. However, the best measure of cellular function was to measure milk yield itself.

In conclusion, the timing of treatments can be crucial in determining the response obtained and alteration of lactation persistency was very difficult to achieve. The most promising route suggested by this work is reducing milking frequency during established lactation, in combination with early lactation treatments to enhance yields.

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LIST OF ABBREVIATIONS

The abbreviations used here are those described as 'accepted' in the instructions to authors of the Biochemical Journal (Biochem. J. (1994) **297** 1-15) with the following additions:

1X	- once-daily (milking)
3X	- thrice-daily (milking)
ACC	- acetyl CoA carboxylase
AE	- aryl esterase
ANOVAR	- statistical analysis of variance
AUC	- area under the curve
BrdU	- 5'-bromo-deoxyuridine
bST	- bovine somatotropin
cn	- casein
cortisol	- hydrocortisone-21-acetate
EGTA	- ethylene glycol-bis(β-amino-ethyl ether) N,N,N'N'-tetraacetic acid
Expt	- experiment
FAS	- fatty acid synthetase
FIL	- feedback inhibitor of lactation
FPLC	- fast protein liquid chromatography
GH	- growth hormone
GT	- galactosyl transferase
HPLC	- high-performance liquid chromatography
HX	- hemimastectomy
IGF-I	- insulin-like growth factor-I
IGF-II	- insulin-like growth factor-II
PA	- plasminogen activator
PFS	- particle-free supernatant
Prl	- prolactin
PZ	- perphenazine
RMYQ	- relative milk yield quotient
SD	- standard deviation of the mean
SEM	- standard error of the mean

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I wish to dedicate this thesis to my maternal grandmother, Muriel Collinson. She has seen more changes in her lifetime than any other generation before hers, and has learned, adapted and coped with it in a way which is an example to us all.

DECLARATION

The data presented was collected by myself between September 1991 and August 1994 with the exceptions of:

Determination of oxytocin concentrations in frequent blood samples in experiment 1 in Chapter 6 which were performed by Berit Samuelsson and Kerstin Svennersten, Sveriges Lantbruksuniversitet, Uppsala, Sweden.

Measurement of gland size, secretory tissue weights and BrdU incorporation in Chapter 3, and the statistical analysis were performed by Kris Sjersen and IL Sorensen, National Institute of Animal Science, Foulum, Denmark.

With these exceptions, I declare that the work contained in this thesis is my own, undertaken under the supervision and guidance of Dr Chris Knight. No part of this work has been submitted for consideration for any other degree or award.

Joanna R Brown

CHAPTER 1

CONTEXTS

1. INTRODUCTION

Lactation is a vital process for the development of young mammals. The milk produced is the primary, if not only, energy source for the offspring initially, it improves their immunity and the close contact of the young aids maternal bonding (Peaker, 1989; Grove *et al*, 1991). The development of the mammary gland to support the process of lactation occurs over a variable period depending on a variety of factors including the expected lifespan and reproductive life of the animal, and the lengths of the ovarian cycle and gestation. These contribute towards determining a general 'timetable' of development for a particular species and can be divided into several reasonably defined stages, most being hormonally directed: pre- and post-pubertal, pregnancy, lactation and non-lactating (dry) periods (Cowie *et al*, 1980). Lactation itself usually follows a distinct pattern in a species in terms of milk production. In the dairy industry this often reflects management techniques rather than the 'natural' rhythm of the species; in goats lactation commences after a gestation of approximately 5 months, milk yields increase to a peak 6-8 weeks *post partum*, steadily decline over the remaining period, and secretion stops after approximately nine months if the animal becomes pregnant again. However, continuing milking during pregnancy has no effect on milk yields after parturition (Fowler *et al*, 1991) and production can continue in the absence of pregnancy, with seasonal oscillations in yield, for several years (Linzell, 1973). Cow lactation shares many common features with that of goats; a 9 month gestation is followed by approximately 10 months of lactation, with peak production occurring about 6 weeks *post partum* (Linzell, 1973). However, in contrast to goats, cows require a *pre-partum* non-lactating period for normal milk production to occur in the following lactation (Swanson, 1965; Smith *et al*, 1967). When the gland is no longer lactating it is in a state broadly similar to that seen before pregnancy although this state varies between species. Rodent mammary glands regress to a state similar to that of the virgin (Strange *et al*, 1992) while goat mammary structures are more developed and the gland larger in animals which are between pregnancies in comparison to those in the mature virgin state (Knight & Wilde, 1993).

This chapter will firstly examine aspects of milk composition, production and subsequent processing, then will briefly summarise the growth and development of the mammary gland required to support this process. Endocrine and local control of milk

production and other influences on lactation (such as udder anatomy) will be considered, and finally lactation persistency and the potential for manipulation of lactation will be discussed.

2. MILK

2.1 Composition

Milk provides the primary source of nutrition for young mammals until they are able to digest more solid food. It contains protein (providing a source of amino acids), minerals (such as calcium, sodium, potassium, phosphorous, magnesium and chloride), trace elements, carbohydrates (mainly lactose which regulates milk volume) and lipids (including phospholipids, cholesterol and triglycerides) (for reviews see Parkash & Jenness, 1968; Jenness, 1980). Immunoglobulins present in the serum fraction of milk help to confer passive immunity upon the offspring with the highest levels found in colostrum (the 'first milk') (Dalgleish, 1992).

The proteins present in milk divide into two main categories, caseins (defined as the proteins which precipitate at pH 4.6 at 20°C) (Dalgleish, 1992) and those that remain in the serum, known as whey proteins. Ruminants produce six major proteins which represent 95% of output; the whey proteins β-lactoglobulin and α-lactalbumin, and α_{s1}-, α_{s2}-, β- and κ-caseins (Martin & Grosclaude, 1993). A major difference in nomenclature between ruminant caseins and those of some other species (humans or rodents, for example) is that ruminants do not have a gene coding for γ-casein (Vonderhaar & Ziska, 1989). Much work has been carried out in the analysis of cows' milk but there are distinct differences in the milk obtained from different species, indeed contamination of goats milk with that of cows can be detected by high-performance liquid chromatography (HPLC) (Kaminarides & Anifantakis, 1993). In general, comparing goats' milk to that of cows' shows that the former contains more fat and the globules tend to be smaller, but both milks have similar ratios of fatty acids. Total casein concentrations tend to be lower in goats milk but it has a much higher β-cn content and more non-protein nitrogen constituents (Juarez & Ramos, 1986).

Hormones are also present in milk and have been locally produced by the mammary gland or transported over the mammary epithelium from the maternal bloodstream. Some act on the mammary gland itself and affect the mother, but these do not necessarily play a biological role in the young. Hormones and growth factors

such as prolactin, prostaglandins, oestrogens, insulin, relaxin, epidermal growth factor and lactoferrin are all detected in milk (Peaker, 1991; Peaker & Neville, 1991) as well as a variety of enzymes, some of which are specific to certain fractions of milk (Brunner, 1981).

2.2 Casein genes and their expression

In the goat, casein accounts for 77% of the total protein present in milk and α_{s1}, α_{s2}-, β- and κ-caseins are present in a ratio of 1:2:5:1 with a typical protein concentration of 21 g/l (Jaubert & Martin, 1992). Cow and goat caseins are broadly similar, although a caprine homologue for bovine α_{s1}-cn has not been found. The nomenclature for the caseins can be confusing as caprine α_{s1}-cn is homologous to bovine α_{s2}-cn, not α_{s1}-cn as cited in the early literature (Jenness, 1980).

The four ruminant caseins are produced from closely linked genes with an overall genetic organisation conserved through evolution. Three of the major caseins (α_{s1}-, α_{s2}- and β-caseins) are calcium-sensitive with common motifs in their promoter, signal peptide and phosphorylation sites, while κ-casein has a different gene structure and is calcium-insensitive (Martin & Grosclaude, 1993; Mercier & Vilotte, 1993). Each casein gene has a variety of genetic polymorphs (for summary see Brunner, 1981). For example, there are two β-cn variants, β_1- and β_2-cn, differing by one phosphate per molecule (Richardson *et al*, 1974) and more variation is seen in α_{s1}-casein; seven polymorphs have so far been described, each giving different levels of expression of the protein (Grosclaude *et al*, 1987; Mahe & Grosclaude, 1989).

Lactogenic hormones can stimulate transcription of these genes; prolactin, insulin and hydrocortisone are necessary for maximal induction of casein synthesis *in vitro* and a close relationship between the proportion of caseins in milk and the relative amount of their mRNAs present has been found in bovine mammary explants. Prolactin and glucocorticoids can increase the stability of casein mRNA, increasing its transcription and half-life (Choi *et al*, 1988) while progesterone can antagonise the effect by preventing accumulation of casein mRNA, thereby reducing casein synthesis and secretion (Matusik & Rosen, 1978). These *in vitro* findings support the *in vivo* observations of rapidly dropping progesterone concentrations at parturition coinciding with the initiation of full milk secretion and lactogenic gene expression (Vonderhaar & Ziska, 1989).

2.3 Caseins and their modifications

Caseins, in combination with calcium, phosphate and small amounts of citrate, form spherical micelles, composed of probably between 1000 and 100 000 submicellar units (Dalgleish, 1992). Each unit is 10-15nm in diameter (Richardson *et al*, 1974) and they are held together by electrostatic and hydrophobic bonds (Walstra, 1990). Total micelle diameters range from 30-300nm while weights of 10^7 to 10^9 Da can be obtained (Brunner, 1981). Goat casein micelles have, in general, a lower maximum frequency distribution than those of cows (Ono & Creamer, 1986) but the micellar substructures are similar in goat, cow and sheep milk (Richardson *et al*, 1974). The ratio of α_s- to β-casein remains almost constant over a range of micellar diameters (Ono & Creamer, 1986) but α_s-cn is evenly distributed between the micelle interior and exterior, while β-cn can have up to 90% present in the interior. In contrast, the interior of the micelle contains only 0.6 to 1.2% of the total κ-casein present (Dalgleish *et al*, 1989), the remainder being on the surface. This asymmetrical distribution of hydrophobic κ-cn places a physical limit on the total surface area of a particular micelle (Brunner, 1981), indeed the relative amount of κ-cn present is inversely related to micellar diameter. This is in contrast to β-cn which decreases its relative amount proportionately with micellar diameter (Davies & Law, 1983). κ-Casein is also unique among the caseins in binding relatively little calcium (due to the presence of only one or two phosphoserine residues) and consequently it does not precipitate from milk as easily as α_s- and β-caseins do (Dalgleish, 1992).

Changes in micelle structure and composition alter the physico-chemical characteristics of milk, important in industrial processing as milk products provide one-quarter of the total dietary protein consumed in industrialised countries (Martin & Grosclaude, 1993). One major process is cheesemaking, in which the enzyme chymosin (present in rennet/rennin) is added to milk and proteolytically cleaves κ-casein, leaving para-κ-casein (caprine residues 1-105) on the micellar surface and a free caseinomacropeptide (corresponding to residues 106-171) (for review see Jenness, 1980). This alters micellar stability and causes aggregation and formation of a curd (mainly casein and fat) which is further processed to make cheese. Changes in concentration and the ratio of protein to fat determines the texture and optimum yield of the cheese but changing one component without alteration of the other can also be detrimental to production (Dalgleish, 1992). Breakdown of other caseins by proteolysis is desirable in certain types of cheesemaking, for example, during maturation Swiss-

type cheese decreases its β-cn content, dependent on plasmin activity. Variations in proteolysis between cheeses are probably due to the different water, salt and pH levels, providing more or less favourable conditions for plasmin activity (Richardson & Pearce, 1981).

2.4 Degradation of caseins

Proteolysis of proteins in milk produces a variety of products known as proteose-peptones, of which approximately two-thirds are believed to be produced by plasmin degradation of caseins (Andrews & Alichanidis, 1983). When purified bovine plasmin is incubated with milk, β-casein is rapidly degraded, producing γ-cn fragments, each corresponding to different sections of the β-cn polypeptide chain (Eigel, 1977b). α_{s1}-Cn has an intermediate resistance to plasmin attack while κ-cn is even more resistant (Eigel, 1977a). Active plasmin is produced by cleavage of its zymogen, plasminogen, by plasminogen activators associated with the casein fraction (Politis *et al*, 1992b). Inhibitors of plasminogen activators and plasmin are found in the serum fraction of milk (Korycka-Dahl *et al*, 1983) and the major whey proteins (α-lactalbumin, β-lactoglobulin and serum albumin) inhibit both plasminogen activators and plasmin. This can cause underestimation of their activities when they are measured in milk (Politis *et al*, 1993).

In vivo, plasmin activity has been significantly correlated with increases in the relative amounts of γ- and α_s-caseins and decreases in β-cn (Aaltonen *et al*, 1988; Politis *et al*, 1989b) and seasonal increases in plasmin and plasminogen concentrations have been well documented (Richardson, 1983; Politis *et al*, 1989a & b). These observations can be explained by increasing activation of plasminogen and/or by leakage through the mammary epithelium from blood (often seen during mastitis). This may, in turn, be related to an increase in mammary involution (Politis *et al*, 1989a). However, these components are not only involved with involution and regression of the gland. The presence and activity of plasmin during the period of mammary gland growth and development is also vital for the process of tissue reorganisation (remodelling) and for the facilitation of cellular proliferation (Turner & Huynh, 1991), especially during phases of rapid growth such as pregnancy.

3. GROWTH AND DEVELOPMENT OF THE MAMMARY GLAND

3.1 Overview

The process of growth and development of the mammary gland determines its potential secretory capacity, and the secretion of milk during lactation is itself influenced by a variety of other factors. These include the extent of neonatal milk demand, maternal nutritional status and the hormonal environment experienced by the mammary gland. The main development of the mammary gland commences at puberty; pre-pubertal cows have few mammary ducts but these increase in size, number and complexity after puberty (Delouis *et al*, 1980). The hormonal balance changes again when mature virgin animals become pregnant, stimulating further mammary growth and development. Oestrogens stimulate the development of ductular tissue and, in combination with progesterone, increase the proliferation of secretory epithelial cells. The rough endoplasmic reticulum content of these cells also increases in response to glucocorticoid action, again increasing the potential for the production of proteins. As this growth of secretory tissue progresses, the 'fat pads' of connective and adipose tissue account for an ever-decreasing proportion of tissue mass. Vascularisation and cell numbers increase (hyperplasia) and as cells mature the degree of cellular differentiation also increases (hypertrophy) (for review see Erb, 1977).

Mammary growth is also influenced by the foetus during pregnancy via placental lactogen, signalling to the mother that milk demand will be from one or more offspring *post partum*. Placental lactogen and placental mass during pregnancy (day 77 to term) correlate with milk production over the first fifty days of lactation and foetal number is positively related to the rate of growth and mass of secretory tissue. This maximises the production potential of the animal and goats with twins or triplets produce 27% and 46% more milk, respectively, than those with single kids (Hayden *et al*, 1979).

Initially it was believed that the majority of mammary development occurred during pregnancy (Convey, 1974) but it is now recognised that it continues beyond this point: mice double their secretory cell population approximately every six days during pregnancy and into early lactation (Knight & Peaker, 1982), in spite of the major changes in the hormonal environment of the gland. Goat mammary tissue also proliferates exponentially during pregnancy and growth continues *post partum*, albeit at a reduced rate (Knight & Peaker, 1984). Predictive equations for goat mammary growth during pregnancy have been tested and the best fit was achieved using an

exponential model, $Y = A.e^{bX}$ where Y=index of mammary growth, A=parameter measured, e=base for natural logarithms, b=rate constant for growth and X=day or month of pregnancy (Anderson *et al*, 1981). In early lactation, mammary growth and milk production can be modified by post-natal demand for milk and milking frequency, most probably through local, rather than endocrine, control (Bar-Peled *et al*, 1995). In goats, both secretory cell number and differentiation increased between parturition and week 3 of lactation and the subsequent rise in milk yield to its peak was supported by continuing differentiation, indicated by increased secretory cell enzyme activities (Knight & Wilde, 1993). However in rodents 75% of the increase in milk production between parturition and peak lactation was supported by increased cell numbers, and the remainder by increased differentiation (Knight *et al*, 1984).

Changes in mammary growth after parturition could have longer-term effects on milk production as this is determined by the summation of secretory cell numbers and the activity of those cells. Indeed, if these parameters could be altered during pregnancy or early lactation, then it could potentially alter the magnitude, and/or the profile, of subsequent milk production.

3.2 Measurements of mammary growth

Growth measurements usually involve a compromise between obtaining a representative sample and minimising the disturbance to the animal. Samples can be obtained by a variety of methods, one of which is by biopsy. The advantages of this technique include it being a relatively straightforward surgical procedure, glands remain *in situ*, recovery is rapid and there is usually only a temporary reduction in milk yield (Knight & Peaker, 1984). Disadvantages are that the tissue sample can be unrepresentative of the whole mammary gland, especially if there has been a previous biopsy and scar tissue is present. There may also be some internal bleeding but this can be minimised by using electrocautery to remove the sample.

Removal of the whole gland allows an accurate assessment of gland size, composition and differentiation but this is a complex procedure and raises the problem of stimulation of 'compensatory growth' in the remaining gland(s). This phenomenon is observed in cows (Turner *et al*, 1963) and goats (Linzell, 1963; Knight & Peaker, 1982a; Knight, 1987) and raises the question of the extent to which mammary growth can be influenced by loss of a gland during pregnancy or early lactation when growth is still occurring.

A non-invasive method that can be used is magnetic resonance imaging. This accurately determines the amount of secretory tissue present within a gland. Unfortunately it is expensive, the size of the animal that can be measured is restricted (goats being the largest practicable) and the degree of cellular differentiation of the secretory cell population cannot be determined (Knight & Wilde, 1993).

The level of differentiation of mature secretory cells can be measured by assaying key enzymes involved in the synthesis of the major components of milk; caseins, lactose and fat. *In vitro* measurement of synthesis rates of casein and lactose can also be used and more imprecise estimates of cellular differentiation can be made by measurement of RNA/DNA ratios (Wilde *et al*, 1987).

Cell DNA content remains relatively constant regardless of the animal's physiological status, allowing estimation of the total cell population from measurement of total glandular DNA content, but this does not distinguish between different cell types (Knight & Peaker, 1982b). If DNA concentration is determined from a tissue sample of known weight, and the size of the udder determined by, for example, water displacement (Linzell, 1966), then total DNA, and therefore cell number, can be estimated. However, this method measures the gross size of the gland which is dependent on the mass of secretory and non-secretory tissue, on the thickness of the skin covering the udder and on teat size (Knight & Peaker, 1982b).

3.3 Declining lactation and involution

The decline in milk yield after peak lactation has been attributed, in the goat, to decreasing cell numbers, rather than a loss in synthetic capacity due to de-differentiation of those cells already present (Wilde & Knight, 1989). If this loss could be slowed somehow it would provide a potential route for reducing the rate of decline in milk production. When milking stops, the cessation of milk secretion is mainly due to increased intramammary pressure and other factors attributable to a lack of milk removal (Fleet & Peaker, 1978). In cows, within two weeks, synthetic and secretory capacities drop to very low levels and alveolar surface area (both epithelial and lumenal) decreases. Within about three weeks the gland has regressed to a 'resting state' but some synthetic and secretory capacity still remains (Sordillo & Nickerson, 1988). This is in contrast to many other species and probably reflects the small loss of alveolar epithelial cells and the maintenance of organelle integrity, despite major changes in cell histology (Holst *et al*, 1987). Rodent milk secretion stops

when suckling stimuli stop, mainly due to the reduction in the release of lactogenic hormones (Grosvenor & Turner, 1959). Cell activity drops rapidly, followed by a substantial loss of secretory epithelial cells which separate from the basement membrane. Myoepithelial cells increase in size, filling the gaps and the gland becomes fully involuted after approximately two weeks (Knight & Peaker, 1982b; Holst *et al*, 1987).

The apparent differences between ruminant and rodent involution may also reflect the different 'timetable' of pregnancy and lactation rather than mechanistic differences in the process of involution. As a result of general farming practice, cows usually become pregnant while they are still producing milk, while laboratory rodents are generally either pregnant, or lactating. When mice were concurrently pregnant and lactating, a retardation of involution was observed, cell numbers were maintained, but they stopped lactating just prior to the second lactation despite continued lactogenic stimulation (Mizuno & Sensui, 1978). This requirement for a 'dry period' in concurrently pregnant and lactating animals is similar to that seen in cows which require a non-lactating period of between 40 and 60 days. If less it can be detrimental to milk production during the subsequent lactation (Smith *et al*, 1967) however the non-lactating period appears to be less essential to goats (Fowler *et al*, 1991).

Changes in gross cell number reflect the balance between the rates of cell death and cell replication (Stringer *et al*, 1990) and during late lactation and involution, cell death rates exceed those of replacement. Metabolic and local factors influence this process but there is also evidence for a process of apoptosis ('programmed cell death') in addition to necrosis in mammary tissue. Apoptosis is characterised by the induction and expression of specific genes which correlate closely with morphological changes that occur after the cessation of milking and DNA becomes fragmented giving a distinctive 'laddering' pattern when separated by (gel) electrophoresis (Gerschenson & Rotello, 1992). Apoptosis occurs at different rates in mice and rats, but in both it leaves the mammary gland with a degree of organisation in its cytoarchitecture (Walker *et al*, 1989). Apoptosis has also been detected in goat mammary tissue during declining lactation, at slower rate than rodents, although this was affected by milking frequency (Quarrie *et al*, 1994).

The gradual remodelling of mammary tissue during involution also involves activation of serine proteases, notably plasmin (Ossowski *et al*, 1979). Plasmin is sensitive to changes in the hormonal environment of the mammary gland, for example,

its activity can be rapidly induced by early removal of rat pups (cessation of the suckling stimulus) (Ossowski *et al*, 1979). Both plasmin and plasminogen are present in milk and the breed of animal, udder health and individual variations between animals also influence their presence (Richardson, 1983). In cows, concentrations of both plasmin and plasminogen increase as stage of lactation increases and Politis *et al* (1989a) found a strong correlation between plasmin, stage of lactation and involution. They postulated that the sharp rise in plasmin concentrations immediately before involution was directly due to the increased activation of plasmin from plasminogen, and that the positive relationship between increasing somatic cell counts with lactation stage was also indicative of this process. However, it has been estimated that only 12% of the total protease activity in milk is due to plasmin activation and correlations reflect the increased permeability of the mammary epithelium, transferring plasmin and plasminogen from the blood to milk (Korycka-Dahl *et al*, 1983; Richardson, 1983). Similar changes are seen during mastitis when somatic cell counts and mammary permeability increase (Politis *et al*, 1989b). So correlations between the various factors must be treated with care, especially when looking at lactational trends. Subsequent work by Politis *et al* (1989b and 1993) have also found that the assays for plasmin and plasminogen can be influenced by the presence of other milk proteins and that the assay conditions have to be carefully standardised to ensure accurate and reproducible results.

4. ENDOCRINE ASPECTS OF LACTATION

4.1 Hormonal control of mammary function

The mammary gland is a target organ for many hormones (Figure 1.1) which have various roles including the regulation of parturition and the onset of copious milk secretion (for review see Tucker, 1988). Briefly the major endocrine changes around parturition are increases in the concentrations of prostaglandins and glucocorticoids, and steadily increasing oestrogen and growth hormone levels. Progesterone levels drop very sharply before birth, allowing prolactin concentrations to increase rapidly and triggering the onset of milk secretion, lactogenesis (Erb *et al*, 1976). The alteration in maternal glucocorticoid concentrations partly reflect the effects of labour, but fetal glucocorticoids also increase as parturition nears, and the blocking action of progesterone on lactogenesis is overcome. Indeed, they compete with progesterone for binding to glucocorticoid receptors and synergise with prolactin to enhance casein and lactose production (Convey, 1974) and may well play a role in

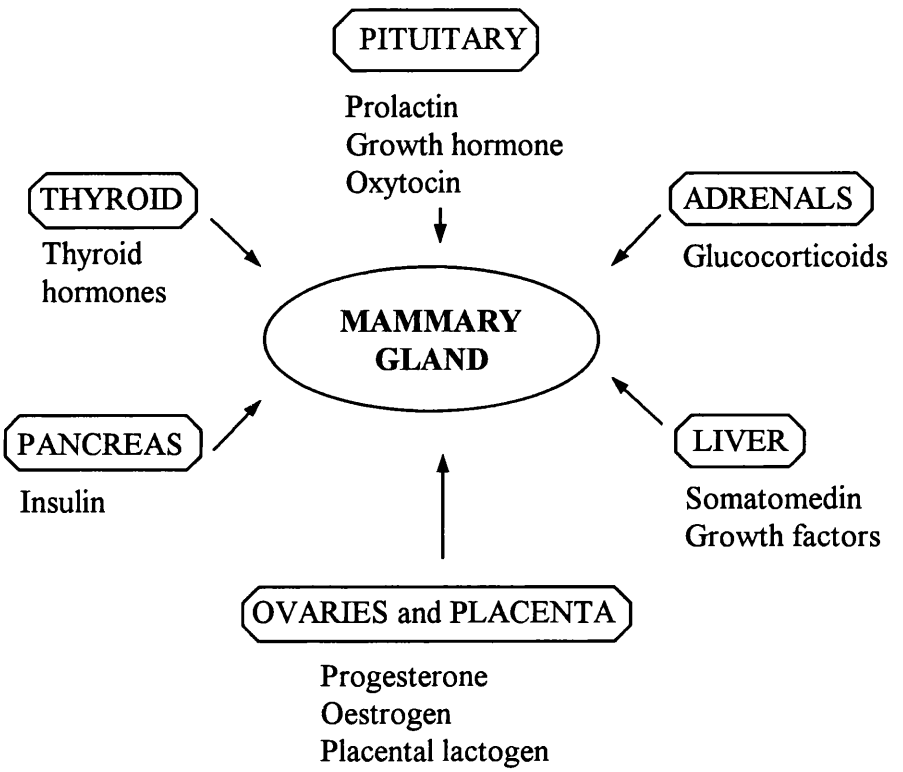


Figure 1.1 Summary of the major hormones which act on the mammary gland

initiating milk secretion.

Once lactogenesis has commenced the hormonal requirements of the mammary gland change and prolactin, growth hormone and glucocorticoids, insulin, parathyroid and thyroid hormones are all required, to varying degrees, for continuing milk production, termed galactopoiesis. Some are released in response to suckling by the young (and often in response to machine milking of commercial dairy animals), while others, insulin and growth hormone in particular, vary with nutritional status and food intake. Fertility and environmental factors such as temperature and photoperiod can also play a role in the complex hormonal interactions controlling lactation. The roles of prolactin, growth hormone and a neuropeptide, oxytocin, will be considered in more detail in the following sections.

4.2 Evolution of prolactin and growth hormone

Prolactin and growth hormone are present in most vertebrates and probably originated from a single gene duplication during the early stages of vertebrate evolution, 60-70 million years ago (Sinha, 1992). They are members of the growth hormone/prolactin family of peptide hormones, related phylogenically, and have common structural features at primary to tertiary levels, and in their receptors. Bovine growth hormone and prolactin have 37% homology in their amino acid sequences (allowing for conservative replacements) (Vonderhaar, 1987). Other members of the family show variable degrees of prolactin and growth hormone-like characteristics, for example, ruminant placental lactogens have many prolactin-like features and appear to have arisen from duplication of a prolactin gene (Wallis, 1992).

4.3 PROLACTIN

Prolactin and its receptor

Prolactin molecules show heterogeneity in their amino acid sequence and have variable molecular weights depending on both sequence length and post-translational modifications. Generally prolactin comprises of 197-199 amino acids with a molecular weight of 23 to 24KDa. The human pituitary gland contains many variants, the most abundant forms being 23K, 21K and 25KDa respectively, and glycosylation is the most common modification. These variants have differing biological potencies and specific types may possibly carry out particular biological functions (Sinha, 1992). This may go some way to explaining how prolactin can modulate such a wide

variety of biological functions without losing specificity. Discrepancies in determination of *in vivo* prolactin levels using bioassay (for example the pigeon crop sac assay) and radioimmunoassay methods have been attributed to the different systems picking up different molecular variants. A further complication in determining 'prolactin' is that there is no apparent relationship between *in vivo* activity and the detectability of variants (Klindt *et al*, 1982) so direct comparison of results obtained using different assay methods and antibodies should be regarded cautiously, although strong correlations between bioassay and radioimmunoassays have been derived (Leung *et al*, 1978).

The prolactin receptor is a member of the cytokine-growth hormone-prolactin receptor family with characteristic features of a conserved region near the C-terminal containing a WSXWS motif and two pairs of conserved cysteines in the N-terminal region. A wide range of tissues have prolactin binding sites (sizes range from 28 to 320 KDa) but some 'cryptic' sites do not elicit biological effects (Vonderhaar, 1987). Two classes of prolactin receptor isoforms have been determined- short and long- probably generated by alternative splicing of a single gene (Kelly *et al*, 1992). The two forms of receptor in rabbit mammary gland have similar binding affinities for prolactin, but only the long-form could stimulate milk protein gene transcription. In rats the short-form can represent up to 70% of the total receptor number (Lesueur *et al*, 1991) and although it cannot initiate milk protein transcription, it can still bind prolactin, dimerise, carry out signal transduction and is involved in receptor turnover (Rozakis-Adcock & Kelly, 1991). Differential expression of four distinct molecular forms of the prolactin receptor has been observed in the rodent mammary gland, dependent on physiological state (Guillamot & Cohen, 1994) and a 33K caprine receptor has been found in the mammary gland throughout pregnancy and lactation (Smith *et al*, 1993). The prolactin response is further modified by alteration of receptor numbers and hence the sensitivity of the target tissue can be varied. For example, receptor numbers increase during pregnancy and more so during lactation, and are 'fine-tuned' by up- or down-regulation (Delouis *et al*, 1980). Negative and positive co-operativity between prolactin and its receptor also occurs and this further modulates the binding process, possibly causing further stimulation of the mammary gland (Sakai, 1994).

There have been some recent advances in the elucidation of the cytokine-growth hormone-prolactin receptor family signal transduction cascade. None of these receptors

contain intrinsic enzyme activity (Wakao *et al*, 1994), however Janus kinases (JAKs) were identified as being closely associated with the receptor. These proteins recruit transcription factors (STAT proteins) which are subsequently phosphorylated. This triggers the formation of a complex which leads to transcriptional activation of the appropriate genes (for review see Finidori & Kelly, 1995).

Secretion and release

Evidence for a direct relationship between the amount of prolactin present in the pituitary and that present in the blood after its release is conflicting (Ben-David *et al*, 1970; Sinha *et al*, 1975). The observations could be explained if a two-stage release process occurs (Shani *et al*, 1976), consisting of an initial transformation phase followed by release into the circulation (Whitworth *et al*, 1981). Factors which increase prolactin, such as dopamine antagonists and perphenazine, are believed to act on the former rather than the latter stage (Grosvenor *et al*, 1980). Circulating prolactin concentrations increase with the stimulus of suckling or milking although the pituitary release of prolactin tends to decrease with lactation stage (Hart, 1975a; Beck *et al*, 1979), probably due to the influence of seasonal factors such as photoperiod and temperature (Hart, 1975b). The synthesis and storage of prolactin in the pituitary and alterations in tissue response will, in turn, modify the availability and effectiveness of prolactin's action (Johke, 1970). For example, the binding capacity of the mammary gland for prolactin increases with lactation stage (Beck *et al*, 1979), increasing the gland's sensitivity.

Its role in lactation

Prolactin is involved in many physiological processes (Figure 1.2) and the most common approaches to determining its role during lactation generally either reduce it to minimal levels by administering prolactin suppressants, or increase it, directly or otherwise. It became clear that prolactin played different roles in different species at different times.

Prolactin concentrations increase shortly before parturition and, in general, high levels of prolactin are essential for full lactogenesis to occur. When the periparturient prolactin surge is blocked in goats by bromocriptine, lactogenesis was delayed for several days (Forsyth & Lee, 1993). Other species, especially non-ruminants, can show

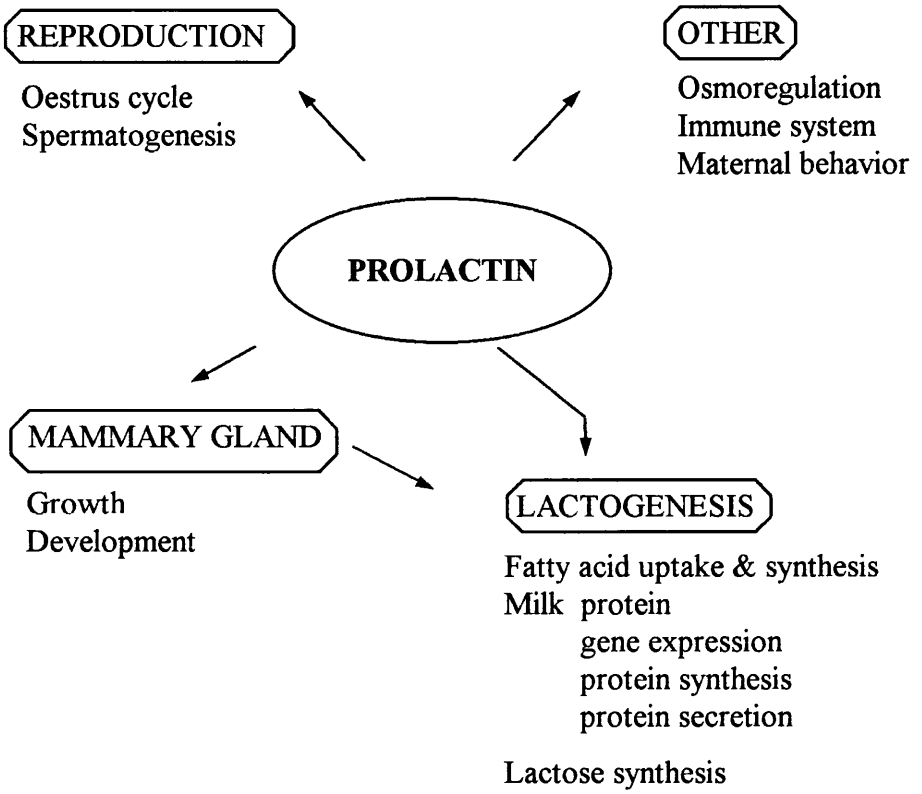


Figure 1.2 Summary of the main physiological roles of prolactin

more profound responses to prolactin restriction. For example, rabbit lactation is highly dependent on prolactin and yields could drop by 90% within 24 hours of prolactin suppression (Taylor & Peaker, 1975) and in rats milk production can be suppressed by bromocriptine treatment (Flint & Gardner, 1994). Unfortunately the precise role of prolactin during established lactation is less clear in ruminants and cows and goats, specifically bred for traits of high milk production, cannot always be compared with other ruminants such as sheep (Cowie *et al*, 1980). In the latter, bromocriptine does suppress established milk yield (Kann *et al*, 1978). In the former, data have been interpreted to indicate that it has no inhibitory effect, although individual cows (for instance) showed responses varying from 0 to 20% inhibition (Karg *et al*, 1972). Over the long-term, bromocriptine treatment of goats has little effect on gross milk production but secretory cell activity decreases (determined by measurement of RNA/DNA ratio) as does alveolar cell size. Lactotrophs (prolactin-producing cells in the pituitary gland) are probably also negatively affected by treatment, causing a decrease in pituitary response to prolactin-releasing stimuli (Forsyth & Lee, 1993). Exogenous administration of prolactin to increase basal concentrations also fails to influence milk secretion rates in goats (Jacquemet & Prigge, 1991) or improve total milk yields in cows (Plaut *et al*, 1987). These observations led to the conclusion that in established lactation, prolactin is not normally rate-limiting (Tucker, 1981). However there is much evidence to be found (even in the above citations) to suggest that this is not entirely true. Schams *et al* (1972) gave three cows bromocriptine over 9 days *pre-partum* and for 3 days *post partum*, and one out of three animals significantly reduced yields compared to the previous year. Post-treatment, prolactin concentrations and milk yields remained low, indicating a prolactin effect during established lactation. Bromocriptine given to goats during late lactation causes a drop in yield of 77% and increases the rate of decline of milk production (Gabai *et al*, 1992). So why are there conflicting results for the role of prolactin during established lactation? Although it has clearly been shown that bromocriptine suppresses prolactin to very low levels, prolactin may still be at an adequate level to sustain lactation. This depends on the threshold limit for prolactin to continue acting effectively on milk production and Smith *et al* (1974) calculated that a concentration of 1ng prolactin per millilitre of serum would still deliver 20mg of prolactin per day to a 'typical' cow mammary gland. Prolactin may also compensate for the bromocriptine suppression by increasing the production of high activity variants (not necessarily detectable by RIA), or it may be

produced locally in the mammary gland and not be detectable in the circulation (Forsyth & Lee, 1993).

So is there a direct relationship between prolactin and milk yields? There is evidence both to support and refute a link- basal prolactin correlates positively with milk yields over a 3 month *post partum* period in cows (Beck *et al*, 1979) and Walsh *et al* (1980) calculated that for the first sixty days of lactation an increase of 1 ng/ml serum prolactin was associated with an increase in total daily yield of 6kg. But over lactation as a whole they found no significant correlation between prolactin and milk yield. This latter finding supports the work of Hart (1974 & 1975a) and Koprowski & Tucker (1973) who found no correlations between basal or milking-induced release of prolactin in goats and cows over a lactation. These results could be explained on the basis that prolactin is important throughout lactation, but more so during early lactation when it helps maximise milk production. When yields are declining it is not a limiting factor but when concentrations are restricted (using bromocriptine), bringing it to negligible levels (Gabai *et al*, 1992), then it becomes limiting, showing that its presence is still required for the maintenance of lactation. Confirmation of its effect could possibly be determined by keeping prolactin at a high level throughout lactation although the relative importance of basal compared to milking-induced levels of prolactin are also yet to be elucidated.

Manipulation of endogenous prolactin

The mechanism by which prolactin is released from the pituitary gland was initially suggested to be through stimulation of prolactin-releasing factors (PRFs) (Mishkinsky *et al*, 1966). However, later work proposed that the increased prolactin release was due to inhibition of a tonic prolactin-inhibitory factor (PIF) (Ben-David *et al*, 1970) through tuberoinfundibular neurons regulated by prolactin, but the regulation of this process is still under debate (for review see Frawley, 1994). The regulation of these neurons at the level of the hypothalamus is independent of direct dopamine control (Ireland *et al*, 1991) although dopamine does have a role in influencing prolactin secretion at both the hypothalamic and pituitary level (Langer *et al*, 1977; Higuchi *et al*, 1983).

Prolactin release can be manipulated experimentally in a variety of ways. Direct methods include administration of exogenous prolactin to increase circulating concentrations of the hormone, or by interruption of the pituitary-hypothalamic

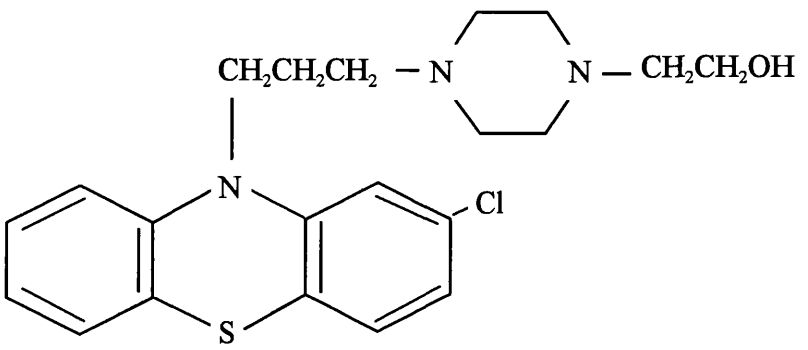
connection which increases prolactin as the pituitary inhibition by PIFs is lost (Ben-David *et al*, 1965). Indirect methods can use pharmacological agents such as bromocriptine, a dopamine antagonist (Karg *et al*, 1972) to reduce prolactin, or stimulate it with 'psychopharmocologic prolactin releasers', more commonly used for their sedative properties. Some of these have side-effects which include mammary development and/or enhancement of milk secretion, caused by a different active part of the molecule (Ben-David *et al*, 1965; Bass *et al*, 1974).

One of these agents, perphenazine, a phenothiazine derivative (Figure 1.3), causes a rapid release of prolactin in a variety of species including male and female mice, rats and rabbits (Mishkinsky *et al*, 1966; Blackwell *et al*, 1973; Sinha *et al*, 1975). It stimulates growth of rat mammary epithelial cells, particularly in alveolar regions (Stringer *et al*, 1990) while sheep show dose-dependent increases in the size of both ducts and alveoli (Morag *et al*, 1971). Administration of perphenazine to small animals and sheep is generally by intramuscular, intravenous or intraperitoneal injection (McNeilly & Lamming, 1971; Morag *et al*, 1971) but side-effects (particularly in ruminants) include loss of appetite, weight loss, lethargy and tenderness at the injection site (Shani *et al*, 1975). Implantation of perphenazine into the brain median eminence of rabbits (Mishkinsky *et al*, 1966) and goats (Vandeputte-Van Messom & Peeters, 1982) removed these side-effects but the surgical procedures used were complex and had inherent disadvantages. An alternative approach of oral dosing in mares (Ireland *et al*, 1991) and rats (Stringer *et al*, 1990) has also increased prolactin concentrations.

4.4 GROWTH HORMONE

Growth hormone and its receptor

Growth hormone is produced in the anterior pituitary and comprises up to 10% of the weight of a dried pituitary, far in excess of any other hormone present (Lewis, 1992). It has a single polypeptide chain (with 2 disulphide bridges) of about 200 amino acids with a typical molecular weight of 22KDa (Wallis *et al*, 1985). As with prolactin, variants are produced by alternative splicing of mRNA and there are a variety of post-translational modifications which alter its biological activity (Lewis, 1992). Growth hormone appears to be modified to an active form in the pituitary before release into the circulation where between 5 and 20% of the hormone exists in a relatively stable dimeric form (Wallis *et al*, 1985). Both bioassays and radioimmunoassays are used to determine its activity and concentrations but again, as with prolactin, the difference



4-[3-(2-Chlorophenothiazin-10-yl)propyl]-1-piperazineethanol

Figure 1.3 Structural formula of perphenazine

between detectability and activity of the hormone must be borne in mind. Growth hormone has certain features in common with prolactin and some cytokines; its tertiary structure contains four α-helices in an 'up-up-down-down' orientation (Wallis, 1992). Growth hormone receptors have not been detected on mammary gland epithelial cells using conventional receptor binding methods and
in vitro growth hormone was unable to bind directly to mammary epithelial cell homogenates or explants. These observations suggest that the stimulatory action of the hormone on milk production is not a direct effect of the hormone binding to the secretory epithelium (Gluckman & Breier, 1987), however, mRNA coding for the receptor has been detected by *in situ* hybridisation in bovine mammary tissue (Hauser *et al*, 1990), specifically on the alveolar epithelium (Glimm *et al*, 1990).

Regulation and secretion

In most mammals growth hormone release is pulsatile and circulating concentrations exhibit circadian periodicity- children and pubertal humans have maximal growth hormone secretion within the first hour of sleep. In cows hormonal release occurs episodically but there is no apparent circadian influence on secretion (Wallis *et al*, 1985) and no response to changes in photoperiod or ambient temperature (Peters & Tucker, 1978). Wide variations in hormonal release patterns are seen between individuals and are probably determined, in part, genetically (Wallis *et al*, 1985). Growth hormone release is inhibited by somatostatin and stimulated by growth hormone-releasing hormone, both of which are produced in the hypothalamus (Wehrenberg & Gaillard, 1989). They are, in turn, regulated by opioid peptides (Moore *et al*, 1992). These are transported via the hypothalamic-hypophyseal portal vessels to the pituitary gland where they act on growth hormone-secreting cells, somatotrophs (Gluckman & Breier, 1987). Acute or chronic growth hormone treatment causes negative feedback of its own release, via stimulation of somatostatin, but accumulation of growth hormone during inhibitory periods may increase a subsequent response to a stimulatory factor (Lanzi & Tannenbaum, 1992). Growth hormone acts directly on target tissues when receptors are present but many of its actions indirect and are mediated via insulin-like growth factors. For example, immunisation against growth hormone-releasing hormone reduces growth hormone and IGF-I concentrations also drop (Moore *et al*, 1992). IGF's are produced mainly in the liver and, as they are not stored to any great extent, are present predominantly in the circulation.

But when associated with binding proteins, their half-lives can be increased up to 18-fold. Receptors for these factors are present in a variety of tissues and mRNA for IGF-I has been detected at high levels in stromal and blood fractions from bovine mammary gland. IGF-I receptors are structurally similar to insulin and both have protein kinase activity (Wallis *et al*, 1985).

Physiological roles and mode of action

A major role for growth hormone is to promote growth by acting on many metabolic processes and balancing the demands of this with nutritional supply. It simulates protein synthesis (*de novo* and increases amino acid uptake and transport mechanisms), and increases lipid catabolism at the expense of carbohydrate catabolism (antagonising insulin's action) (Wallis *et al*, 1985). Anabolic processes such as cell division, skeletal growth (especially cartilage and muscle) and galactopoietic activity are also modulated by changes in growth hormone (Gluckman & Breier, 1987). In humans a lack of growth hormone, or of factors mediating its action, can lead to a variety of medical conditions which can have significant and negative effects on growth, dwarfism for example (Wallis *et al*, 1985). The plane of nutrition of the animal and the level of feeding are important as they also determine the amount of circulating growth hormone (Gluckman *et al*, 1987) and in times of nutritional constraint growth hormone concentrations will increase (Breier & Gluckman, 1991), directing energy towards conservation of tissue mass. Genetic and seasonal factors also play a role in determining basal growth hormone levels (Hart, 1973b & 1983).

However, there are still many questions to be answered about the mechanics of how growth hormone actually acts on the mammary gland. The body of experimental evidence suggests that growth hormone cannot bind directly to mammary secretory cells so it must therefore be acting through some sort of mediator system. In an attempt to elucidate the pathway of growth hormone's action in rats, endogenous growth hormone and prolactin were supressed to such an extent that milk production virtually ceased. Various test substances or hormones were then given exogenously in an attempt to restore milk secretion to pre-treatment levels. Both growth hormone and prolactin partially restored yields and IGF-I concentrations increased when growth hormone was administered systemically. However IGF-I alone, or when pre-complexed to binding proteins, was unable to mimic this effect (Flint *et al*, 1994). Follow-up studies found that locally administered growth hormone could partially restore milk production in

treated glands, without affecting IGF-I levels (Flint & Gardner, 1994), suggesting that growth hormone was able to act directly on the mammary gland. In goats, growth hormone introduced into the mammary gland via the teat canal (intraductal administration) with EGTA to allow paracellular movement through to the basal surface, had no effect on milk production (Sejrsen & Knight, 1994). This again supports the case for growth hormone being unable to bind to secretory cells but it could also be due to the growth hormone not being successfully delivered by this route.

So is IGF-I involved in the modulation of growth hormone's action? Systemic growth hormone increases IGF-1 concentrations and milk production in ruminants (Davis *et al*, 1987) supporting the observations made in rodents. The elevation of IGF-I concentrations in response to growth hormone was measured in goats then reproduced by giving IGF-I systemically. However this had no effect on milk production while growth hormone gave increases of up to 30% (Davis *et al*, 1989). Direct infusion of IGF-I into the mammary gland increased milk yields but unfortunately this was combined with increased milking frequency (2 hourly) and oxytocin administration which themselves have stimulatory effects on milk yields (Prosser *et al*, 1990). So in conclusion, growth hormone appears to act locally, but not necessarily directly, on the secretory cell. A mediator is probably involved, possibly IGF-I, but more evidence is required and growth hormone's action could be mediated through a factor which has, as yet, not been identified.

4.5 OXYTOCIN

Physiological roles

Oxytocin is a neuropeptide synthesised in the neural lobe of the posterior pituitary and has an amino acid sequence sequentially similar to mammalian vasopressin (Benson & Folley, 1957; Richard *et al*, 1991):

Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-(NH_2)

It's physiological roles include eliciting milk ejection, it is involved in the regulation of ruminant oestrus cycles (Wathes *et al*, 1992) and it induces uterine contractions which lead to foetal expulsion during parturition (Folley & Knaggs, 1965). In a lactating cow approximately 3% of the total oxytocin present is in the circulation, but milking (which provides a potent stimulus for oxytocin release) can cause up to one third of the stored oxytocin to be released in a pulsatile manner. The magnitude of release tends to decrease with time of stimulation (Gorewit *et al*, 1983) although high

sustained levels are also observed, probably due to inadequate sampling frequencies (see Wakerley *et al*, 1988). Estimates of oxytocin's half life in the circulation generally range between 60 and 216 seconds (Thompson *et al*, 1973; Gorewit, 1979). However, when measured during a steady state infusion of oxytocin, two half lives have been determined-fast (about 4 minutes) and slow (25 minutes) and these are likely to be closer to the normal physiological values (Gorewit *et al*, 1983).

Oxytocin acts in concert with α-adrenergic and β-adrenergic receptor systems to control, respectively, milk ejection and milk flow (Bruckmaier *et al*, 1991) and exogenous oxytocin decreases the time to milk ejection and increases the duration of maximal milk flow (Graf, 1968). Cleverly & Folley (1970) calculated that exogenous oxytocin reaches the mammary gland within 50 seconds of intravenous injection and *in vivo* the milk ejection response can be elicited in cows within 0.4 minutes of giving iv oxytocin. An intramuscular administration route is also frequently used and the time to milk ejection increases to an average time of approximately 4 minutes, although response times vary with individuals and with dose levels (Graf, 1968). The effect of oxytocin on the mammary gland is modulated by its receptors (present on myoepithelial cells) whose numbers vary with physiological state; in the rat mammary gland they increase exponentially during pregnancy, continue to increase at a slower rate during lactation and once mammary regression (involution) begins, receptor numbers drop off sharply (Soloff & Wieder, 1983).

Role in milk removal

Milk removal from secretory epithelial cells is facilitated by contraction of myoepithelial cells which form a network over alveolar epithelium. When oxytocin binds the contraction of the myoepithelium forces milk out of the alveolar lumenal spaces and small ducts into the gland cistern (Sagi *et al*, 1980). This process of milk ejection is the natural response to the vigorous teat stimulation of the suckling offspring (Benson & Folley, 1957) and maximises the milk available to them. Other types of physical contact with the gland, for example washing the udder before milking (Cleverley & Folley, 1970) or placing teat cups on the teats (Folley & Knaggs, 1966) provide potent stimuli. Conditioned stimuli, generally associated with a milking routine, elicit milk ejection, the recognition of auditory cues such as the milking machine being switched on, for example. Cows moved to an unfamiliar milking environment show a depression in peak oxytocin concentrations attained during milking

(Bruckmaier *et al*, 1993) but within ten days of a new milking routine commencing, a full conditioned reflex release is seen (Cleverley & Folley, 1970). Presence of the calf without physical contact can be stimulatory but can also depress the ejection response (and milk yield) if it is subsequently removed from sight and/or sound of the mother (Bar-Peled *et al*, 1995).

The movement of milk from the alveolar to the cisternal areas causes an increase in intramammary pressure, usually within 44-126 seconds of increased oxytocin being detected in the blood (Cleverly & Folley, 1970). The increased pressure can be sustained for about 10 minutes if milk is not removed (Bruckmaier *et al*, 1991) although observations of increased pressure as long as 60 minutes later have been reported in cows (Cleverly & Folley, 1970). This overestimate was probably due to the technique employed to measure the pressure changes: after oxytocin administration three glands were emptied while the measured gland remained full and the physical effects of 'one full, three empty' probably accounted for the result. Measurement of intramammary pressure in goats using an intraductal pressure transducer shows a rapid and characteristic peak in pressure in response to exogenous oxytocin administration (at a physiological level). Pressure then drops to a level similar, or slightly above, that measured pre-treatment (CH Knight & JR Brown, unpublished results). Some milk remains in the alveoli and small ducts of the gland after machine milking (residual milk) which is only removed if oxytocin levels increase to above those experienced at a normal milking (Thompson *et al*, 1973). In goats, if milk is removed and the milk ejection reflex avoided (by, for example, cannulation of the teat allowing passive drainage of milk through a catheter) then 86% of the milk obtained by hand milking can be removed. If passive drainage is combined with adminstration of exogenous oxytocin then 97% of the milk can be obtained (Henderson & Peaker, 1987), illustrating the importance of oxytocin for efficient milk removal.

Effects on milk production

Administration of high, non-physiological concentrations of oxytocin has detrimental effects in several species including rabbits (Linzell, 1975), goats (Linzell & Peaker, 1971a) and cows (Allen, 1990). The excessive contraction of the myoepithelial cells is thought to cause damage and loss of integrity of secretory cells, milk yields drop and milk composition changes. The latter effect is probably due to increased permeability of the mammary epithelium allowing sodium and other

components to leak from the blood into milk through previously 'tight' cell-cell junctions (Linzell & Peaker, 1971b). This process is detectable by alteration of milk Na:K ratios and is also seen during mastitis, often providing an early indication of mammary infection in apparently healthy cows (Allen, 1990).

In contrast, giving smaller, physiological doses of oxytocin over variable periods often has a positive effect on milk yields, although responses vary between individuals and studies. When oxytocin is given before milking the improvement in yield can be explained by the more efficient removal of milk. However, debate has arisen as to whether or not this is its only effect as oxytocin has been claimed to increase milk yields when given after milking. Ballou *et al* (1993) designed an experiment in which cows were given oxytocin or control saline injections before or after milking. Their summary stated that oxytocin significantly increased milk production by 3% given either before or after milking. However, these increases were equivalent to a kilogram, or less, of milk and absolute levels of significance were $p < 0.08$. So their suggestion that oxytocin was not only acting by 'emptying' but by positively altering the production, or by maintaining secretory cell activity requires further substantiation. Recent work in cows (Knight, 1994) shows that oxytocin adminstration increases milk yields only if milk is removed from the gland at the time of treatment, challenging the proposal that oxytocin was acting at a metabolic or hormonal level.

It has been suggested that oxytocin's effects are due to a direct relationship between oxytocin and prolactin (Benson *et al*, 1960), however, subsequent evidence is equivocal. Cultured anterior pituitary cells release prolactin specifically in response to oxytocin addition but are probably not significantly involved in modulating basal concentrations of prolactin or in regulating acute prolactin release *in vivo* (Johnston & Negro-Villar, 1988). Peaks in circulating oxytocin concentrations in response to suckling occur at prolactin minima in rats (Higuchi *et al*, 1983) but administration of oxytocin antagonists and anti-oxytocin antibodies reduce the magnitude and duration of the prolactin response to suckling (Samson *et al*, 1986). It is probable that any link between the two is probably at the neuroendocrine level, although they are temporally related to each other (Higuchi *et al*, 1983; Johnston & Negro-Villar, 1988). In conclusion, it appears that the direct effects of oxytocin are mainly confined to local effects associated with improved milk removal around the alveoli and small ducts in particular (Sagi *et al*, 1980), but the mammary gland must be in a favourable hormonal

environment for a full realisation of oxytocin's potential.

5. LOCAL CONTROL OF MILK PRODUCTION

5.1 Alterations in milking frequency

In goats, the rate of milk secretion is negatively related to the interval since milking with a curvilinear relationship seen between them (Wheelock *et al* 1967). Accumulation of secreted milk in the goat udder is relatively constant for approximately 6 hours after emptying, slows considerably after this period and eventually reaches a plateau (Peaker & Blatchford, 1988). In cows, milk accumulation is fairly constant for about 12 hours before markedly decreasing (Wheelock *et al*, 1966; Knight *et al*, 1994). Normal milking practices generally operate on a twice daily regime and it would be predicted that a switch from the usual 8 and 16 hour milking intervals to two 12 hour intervals would increase yields by virtue of extending the time of maximal milk production. The response to more frequent milking (shortening intervals between milkings) has been investigated and well documented- it increases milk yields. When goats are milked thrice-daily, at equal 8 hour intervals, milk yields increase by between 4.7 and 10.7%, depending on lactation stage (Henderson *et al*, 1983). Hourly milking of glands enhances yields even more (up to 20%) although the effect is abolished in fasted animals (Linzell, 1967), probably due to nutritional constraints. Other researchers have applied different milking frequencies to individual glands in the same animal. For example, twice-daily milking two udder quarters of a cow, and thrice-daily milking of the contralateral quarters increases milk yield in the more frequently milked gland alone (Shinde, 1978). Similar results are obtained in goats and the increased yield curve is parallel to that of normally milked gland (Henderson *et al*, 1983) illustrating the local nature of the effect and in both studies yields rapidly returned to twice-daily levels on the cessation of the extra milking. The enhancement of milk yield achieved by increased milking frequency is restricted to the period of treatment, is reversible and confined to those glands receiving more frequent milking. However if a more frequent milking regime is continued over longer periods of time there is evidence to suggest that there are longer term changes to the mammary gland secretory cell population (Shinde, 1978; Knight *et al*, 1990).

5.2 The evidence for local control by FIL

Milk secretion and its subsequent accumulation of milk in the mammary gland

does not occur at a constant rate but tends to slow (at varying times) as time after milking increases. This natural constraint on milk production was thought to reflect the limit of the physical stretching of the gland, with increasing intramammary pressure inhibiting further milk production (Turner, 1955). However, it was later found that pressure was not playing a major role as the enhanced rate of milk secretion continues after removed milk is replaced with an equal volume of isosmotic sucrose solution at the extra milking, maintaining intramammary pressure (Henderson & Peaker, 1984). It seemed as though a local control process associated with milk removal was acting in the mammary gland. Hourly milking a denervated, autotransplanted mammary gland which had no neural connections and could therefore not respond to, or release into the circulation, hormones associated with milking still showed an increase in milk yield. This shows that it is milk removal that is essential for the effect rather than the hormonal factors associated with milking (Linzell & Peaker, 1971a). The close positive correlation between mammary blood flow and milk secretion led to suggestions that the increased supply of metabolites to the gland might enhance milk production. The vasoconstrictory properties of certain prostaglandins and the decrease in milk secretion with decreased blood flow led to the identification of $PGE_{2\alpha}$ as being a potential inhibitor of milk production. However, doubt was shed on its importance when intraductal adminstration initially stimulated milk secretion, then caused a period of inhibition (Maule Walker, 1984). The action of milk removal seemed to provide the key.

It has been noted that changes in milk secretion rates during lactation when milk was diluted or replaced with an isosmotic solution are consistent with the presence of a chemical inhibitor in milk (Henderson & Peaker, 1987). The field was narrowed by finding that the whey fraction of goat milk inhibited casein and lactose synthesis in rabbit mammary gland explants (Wilde *et al*, 1987). Subsequent separation and purification of whey proteins into definable fractions resulted in identification of a single protein (M_r 7600) which had an inhibitory action in rabbit mammary cell cultures. It was named FIL- the feedback inhibitor of lactation. *In vivo*, various doses of FIL intraductally administered to goats cause a local inhibition of milk secretion up to a maximum of 17.4% at the highest dose of 750 μg (Wilde *et al*, 1995).

In conclusion, the rapid and reversible alterations in milk yields with changing milking frequency are consistent with the hypothesis of local control of milk secretion by FIL; more frequent removal of milk, hence FIL, reduces the level of autocrine

inhibition on milk secretion, allowing cells to secrete maximally for longer periods as FIL concentrations are reduced (Wilde & Knight, 1990). The positive effects of oxytocin on milk yields can also be explained by improved removal of milk from the secretory areas. Although the component responsible for the local control of milk secretion has been identified it must be remembered that milk secretion is not only under local control. It represents a complex systemic interaction between many hormones and other factors, for example, increased milking frequency has increased prolactin receptor numbers on secretory cell surfaces (McKinnon *et al*, 1988) and can increase the size of the secretory cell population (Henderson *et al*, 1985).

5.3 The importance of the sites of milk storage

The compartmentalisation of secreted milk within the udder plays an important role in determining the productivity of an animal and this can be, in part, related to the presence of FIL in the mammary gland. Milk is stored in alveolar and cisternal areas (see Figure 1.4). Cisternal milk is that which is stored in large ducts and the cistern and can be removed by catheter drainage (thereby avoiding the milk ejection reflex). Alveolar milk is stored in small ducts and the alveolar lumenal spaces and is removable after drainage by milking with simultaneous administration of oxytocin (Knight *et al*, 1994). These descriptions of the milk fractions will be used in this thesis. There will also be a negligible amount of milk which remains in the gland which is not removable by milking or giving exogenous oxytocin (Henderson & Peaker, 1987). However, it must be noted that not all authors define cisternal and alveolar fractions in this way and care should be taken when making comparisons between studies. Stelwagen *et al* (1994), for example, define alveolar milk as that being obtained after hand-milking, by a further milking with administration of oxytocin. The initial hand-milking would stimulate endogenous oxytocin release and milk ejection, resulting in an overestimation of 'cisternal milk' and the second milking with exogenous oxytocin would therefore, by the present definition, underestimate 'alveolar milk'. The milk remaining after a normal milking is also called residual milk by some authors (for example, Peaker & Blatchford, 1988).

Milk accumulation in the udder is not evenly balanced between alveolar and cisternal areas; in goats, catheter plus hand milk increases linearly over a 16 hour period while residual milk increases steadily over 6 hours, then remains constant (Peaker & Blatchford, 1988). Filling in the cow follows a similar trend, but on a

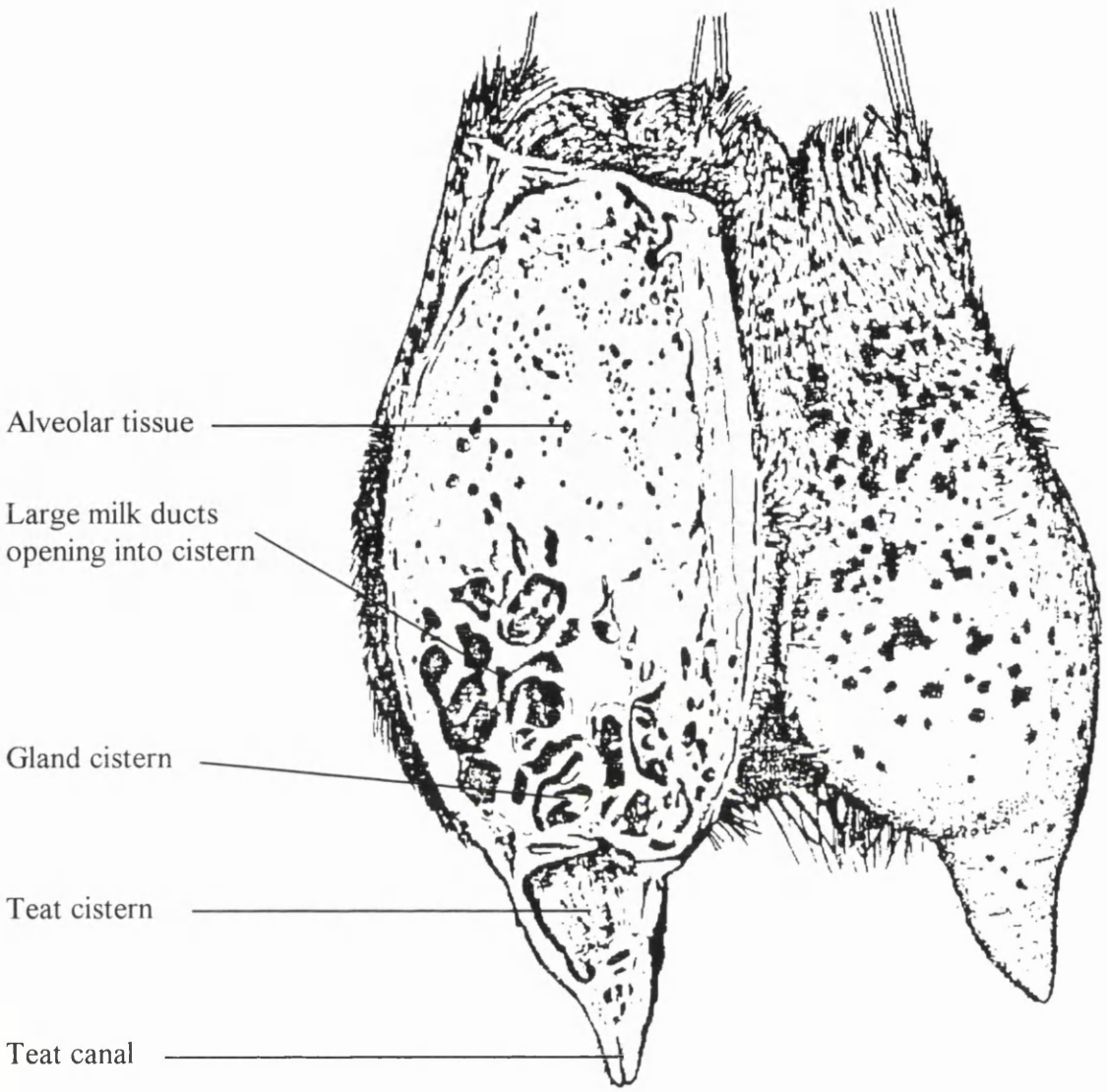


Figure 1.4 Illustration of a goat udder with one gland shown in partial cross-section. Adapted from Mepham (1984).

different timescale; shortly after milking some milk moves into the cistern but the main accumulation occurs more than 6 hours later. Meanwhile alveolar milk volume steadily increases for at least 12 hours (Knight *et al*, 1994). The greater resistance to cistern filling in cows reflects the relative amount of milk stored cisternally (approximately 30%) (Dewhurst & Knight, 1994) compared to goats which have cisternal percentages of about 80% (Peaker & Blatchford, 1988). Wide variations in storage characteristics are seen between individuals, between species, and both age and parity tend to increase cistern capacity in cows (Dewhurst & Knight, 1994).

So what is the relationship between FIL and the site of milk storage? Milk must be removed specifically from the alveolar areas of the mammary gland to relieve FIL inhibition of milk secretion- secretion rate increases by 11% only if oxytocin is administered during frequent catheter milking, ensuring emptying of alveolar areas (Henderson & Peaker, 1987) as catheter drainage alone avoids the milk ejection reflex. Conversely, FIL must be in contact with secretory cells to exert its effects. So once milk has moved away from the alveolar secretory cells and begins to accumulate in the cistern, FIL effectively becomes inactive (Dewhurst & Knight, 1993). An inverse relationship between milk secretion and the proportional amount of milk present in the alveolar areas has been found in goats (Peaker & Blatchford, 1988). Animals storing proportionately more milk in the alveolar areas have lower rates of milk secretion. The responses in milk production to alterations in milking frequency have also been related to cistern proportion in goats (Wilde & Peaker, 1990) and cows (Dewhurst & Knight, 1994). Cows with large cisterns are more tolerant of infrequent milking (Knight & Dewhurst, 1994), while those with small cisterns (and therefore with relatively more milk present in alveolar areas) respond best to increases in milking frequency, due to the increased removal of FIL. It was suggested thirty years ago that udder morphology played a more important role in determining the tolerance of infrequent milking than factors such as secretion rate, milk yield and intramammary pressure (Turner, 1955). The latest findings support a role for udder morphology in this process but there are complex inter-relationships between the various factors which inextricably link them together. For example, udder morphology determines the relative sites of milk storage which in turn influence milk secretion rates and hence milk production. But the level of milk production can subsequently modify udder morphology by stretching or shrinking the gland.

6. LACTATION PERSISTENCY

6.1 Definition

There are two main factors which determine the shape of the lactation curve for an animal, the peak yield achieved and the rate of decline in yield after this point. The latter, or both, can be used in calculations to quantify 'lactation persistency' (for summary see Broster & Broster, 1984). A variety of methods are used to measure persistency; some use the yield obtained at mid-lactation to compare the productivity of animals, while others look at lactation as a whole using, for example, the number of days that milk is produced, or the proportion of the total milk yield produced over a particular defined period of lactation. Two animals which have identical rates of decline in milk yields but different levels of peak production will have different total yields, different lengths of lactation and possibly different 'persistencies' depending on the terminology used. Other authors have used algebraic models to evaluate lactational trends in milk composition and yields (Wood, 1976) or complex statistical models have been fitted to the data; in some cases treatment animals have been excluded as they do not fit the model applied (Nostrand *et al*, 1991). In this thesis, persistency is measured and defined as the rate of decline in milk yield with time after peak lactation has been achieved. The rate of decline in yield was determined over defined periods and was calculated by regression analysis of average daily milk yield against lactation week. This method was applicable to all animals used in the various studies, and by monitoring yield losses after peak lactation rather than, for example, total production over a lactation, it gave a more accurate reflection of the changing status of the mammary gland during declining lactation as milk yields themselves reflect secretory cell activity and number.

6.2 Biological and imposed constraints on lactation

There are natural constraints on lactation as its main purpose is to provide a source of nutrients for the neonate. Once weaned there is little point, biologically, in production continuing as lactation is an energy-draining metabolic process for the mother. The level (or plane) of nutrition and body condition influences milk yields-well-fed goats generally produce more milk than those given restricted diets although there is some species variation (Sachdeva *et al*, 1974) and the yield-enhancing effects seen with frequent milking are abolished when dietary intake is reduced (Blatchford & Peaker, 1983). In India the food supply is so scarce and nutritional constraints so high

that the indigenous species (*bos indicus*) lactate only while calves are present and regularly suckling (Crotty, 1980).

The amount of secretory tissue in the mammary gland and its activity also determine the level of milk production and consequently affect lactation persistency. Once peak lactation has been achieved cell numbers decline, secretion drops and most, or all, of the secretory tissue involutes and lactation ceases. A favourable hormonal environment is required for continued cell survival and maintenance of secretion. A reduction in, for example, the amount of suckling as the young mature will affect persistency as prolactin levels change in response to changes in the physical stimulus of suckling and the pattern of milk removal from the gland. The maintenance of functional secretory cells is also important, particularly so during declining lactation, and the process of apoptosis has been detected in goat mammary tissue during involution (Quarrie *et al*, 1994). If this process of 'programmed cell death' could be delayed by, for example, application of appropriate stimuli, then there could be a potential route for improved persistency by virtue of increasing the longevity of cells - reducing cell death and maintaining milk yields (Knight & Wilde, 1993). Seasonal factors such as photoperiod and temperature can also alter hormone secretion; reduced daylight and lower ambient temperatures are temporally correlated with decreasing prolactin concentrations in cows (Peters & Tucker, 1978). Photoperiodicity controls fertility in goats as they exhibit seasonal anoestrus, usually coinciding with the period of peak milk production (Shelton, 1978), and they 'come into season' in the autumn when yields are dropping. Fertility and subsequent pregnancy both influence milk production during established lactation and this situation of concurrent pregnancy and lactation usually has a negative effect on milk production during the later stages of pregnancy, especially immediately *pre partum*. It can also reduce milk production during the subsequent lactation (Mizuno & Sensui, 1978; Knight & Wilde, 1988). The resumption of oestrus in rats, even without recurring pregnancy, causes yields to decline markedly (Flint *et al*, 1984b).

Imposed management effects are particularly important in breeds which have been selected for high, commercial milk production, particularly cows. Dairy herd calving is generally designed (and regulated by artificial insemination) to be concentrated over a few months, or more evenly spread through the year. Animals calving between January and July tend to produce less milk than those calving between August and December but the spring-calving cows may still be profitable for the

farmer, dependent on the economic conditions at that time (Castle & Watkins, 1984). Cows are generally fed in slight excess during early lactation to minimise any nutritional constraints on peak lactation and they tend to be remated when yields are still relatively high (weeks 12-24). This pattern of concurrent pregnancy and lactation allows a reduction in the time that the animal is not lactating and consequently a 'calving index' of 360 to 380 days becomes possible (although a non-lactating period is required for optimising subsequent production) (Morris, 1976). This intensive and artificially regulated production system results in very high metabolic demands being placed on animals and even during the short non-lactating period they are providing for a rapidly growing foetus which places ever-increasing demands on them.

6.3 The potential for manipulation of lactation persistency

The secretory cell population

So to what degree are animal management techniques restricting lactation? If goats are not remated at the end of lactation and continue to be milked, milk secretion continues at a low level for several years with small seasonal oscillations, notably an increase in production in springtime and a nadir at midwinter. Total milk production gradually drops and lactation curves show decreasing variability in maximum and minimum yields (Linzell, 1973). Similar results are obtained in ovariectomised animals and the pattern could therefore reflect seasonal or nutritional influences. In rodents, lactation can be extended by continuing suckling; replacing rat litters every 10 days with a new-born litter can extend lactation from the usual 21 days to 70, although milk production is at a reduced level from day 20 onwards. This extension of lactation is possible due to a combination of events, for example, maintenance of a favourable hormonal environment and delayed involution of the mammary glands (Nicoll & Meites, 1959) and provides an avenue for manipulation of lactation. Closer study of secretory cell function during extended lactation in rodents shows that cell numbers are maintained and the gradual loss in secretory capacity is attributable to reduced cell activity (Nagasawa & Yanai, 1976) in contrast to the normal situation when there is a substantial loss of cells after peak lactation (Knight & Peaker, 1982c). In the former case there may be an increase in the amount of cell replacement or a reduction in the rate of cell death (extending cell life) but the overall effect is to substantially alter the lactation profile and change persistency. Reduction of cell loss has been achieved experimentally by a long-term increase in milking frequency (from twice to thrice-

daily) in goats by a combination of increased cell proliferation and improved cell longevity (Wilde *et al*, 1987b). The latter effect is also seen in mice when litter swapping extends lactation (Shipman *et al*, 1987). There is some carry-over of secretory cells from one lactation to the next in rats (Pitkow *et al*, 1972), ruminant mammary gland size generally increases with parity (although this includes non-secretory tissue (Knight *et al*, 1994) and cows which do not have a dry period retain more cells than those which do (Pitkow *et al*, 1972). However, cows respond poorly to a shortening of the non-lactating period, possibly reflecting overall nutritional constraints rather than limitations on the secretory cells (Knight, 1989). If more cells can be carried over then there would be an increased stock of cells, increasing the overall production potential of the animal (Knight, 1989). So manipulation of the secretory cell population will potentially affect subsequent lactations, as well as the current lactation.

Prolactin

Decreasing prolactin concentrations are clearly a factor in reducing milk yields in rats (Flint *et al*, 1984) but direct administration of prolactin to elevate circulating concentrations has little effect on milk yield in cows (Plaut *et al*, 1987). Indirect elevation of prolactin can be successful; hypothalamically implanted perphenazine stimulates mammary development and initiates lactogenesis in rabbits (Mishkinsky *et al*, 1966), oestradiol-primed rats (Ben-David *et al*, 1965) and virgin goats (Vandeputte-Van Messom *et al*, 1976). Intravenous injection of perphenazine also stimulates mammogenesis, but does not affect milk yields during established lactation (Morag *et al*, 1971). Indications seem to be that perphenazine, hence prolactin, was stimulating mammary growth but not elevating milk production directly. However much of this work was carried out during early or mid-lactation when prolactin may not have been the limiting factor on secretion. If this was the case then increasing prolactin concentrations would not necessarily have had any effect. In late lactation, prolactin manipulation seems to be more effective in altering production, perhaps reflecting that this is the time when endogenous prolactin levels are dropping (Hart, 1972). Perphenazine sulphoxide given to late lactation ewes significantly reduces the rate of decline in yields (Morag *et al*, 1971) and a similar pattern is seen with hypothalamic perphenazine in goats, although there was an initial drop in yields due to the side-effects of surgery (Vandeputte-Van Messom & Peeters, 1982). Unfortunately in the

studies cited above, no blood samples were collected and analysed for prolactin content, although Danon *et al* (1963) and Bryant *et al* (1968a,b) clearly show that perphenazine stimulates prolactin release both *in vitro* and *in vivo*. Hence, the full effect of perphenazine on circulating prolactin concentrations throughout a lactation, and any subsequent effects on milk production are yet to be determined.

Growth hormone

Growth hormone has a proven galactopoietic action and high yielding cows generally have higher growth hormone levels then lower yielding cows of the same species. Changes in milk yield are positively related to fluctuations in growth hormone (Hart & Morant, 1980) although both high- and low-yielding cows have similar insulin concentrations at peak lactation (Hart, 1983). When exogenous growth hormone is administered (often referred to as bST, bovine somatotropin) milk yields can be increased substantially and significantly and, in general, increases of between 10 and 15% are commonly obtained in ruminants (Bines & Hart, 1982).

So how is growth hormone achieving this effect? It acts on metabolism in a co-ordinated manner by redirecting ('repartitioning') nutrients, rather than increasing the absolute nutrient supply of the animal in the short term. The effects of bST on milk production can be explained by more energy being directed towards the mammary gland, increasing milk production, with no overall increase in the gross energy intake of the animal. However this improvement in the 'efficiency of milk production' is at the expense of other metabolic processes (Bauman *et al*, 1985). The repartitioning process is facilitated, in part, by increased blood flow delivering more substrates to the mammary gland (Mepham *et al*, 1984) and can be amplified by increasing milking frequency to further elevate milk yields in goats. For example, increases in milk yields of 18% (bST only) and 36% (bST and thrice-daily milking) are possible, contrasting with a rise of 8% with thrice-daily milking only (Knight *et al*, 1990). However growth hormone's effects seem to be confined to influencing the plane of milk production and it does not appear to affect lactation persistency (the rate of decline in milk yields) (Bauman *et al*, 1985). However, other definitions of 'persistency' may produce an apparent improvement, for example if persistency is measured as days of milk production before yield drops to a standard value, because peak yields are higher with growth hormone treatment.

Oxytocin

The question of whether or not oxytocin affects lactation persistency and if oxytocin's effects are due merely to milk removal has given rise to debate. The first indications of a positive effect on persistency were seen in rats when mammary involution was delayed for up to nine days after litters were prematurely removed and mothers given oxytocin thrice-daily (Benson & Folley, 1957). Oxytocin was synergising with pituitary hormones as there was no effect when hypophysectomised animals were used but hormone replacement with a complex of prolactin, cortisol and oxytocin restored the delay in involution (Meites & Hopkins, 1961). The clearest evidence for an effect on persistency in ruminants is provided by Nostrand *et al* (1991) who administered oxytocin to cows immediately before every milking over a ten month period. Treated animals produced significantly more milk than controls and most of the increase was obtained by improved yields during declining lactation, improving persistency. In contrast, oxytocin given on alternate four day periods through lactation increased milk yields but did not significantly alter persistency (Sprain *et al*, 1954). However the statistics were carried out using group means and the authors noted that the best responses were obtained in animals with poor persistency records. A closer examination of the data may show that there was a persistency effect in this sub group. Normal milking was probably providing an inadequate stimulation of the milk ejection reflex, causing a more rapid decline in milk production due to increased involution (Knodt & Petersen, 1942). Incomplete milking of goats during late lactation also reduced persistency, probably due to short-term and local inhibitory effects on secretory cells (Wilde *et al*, 1989b) and longer term cell loss (Knight, 1989). If the sole effect of oxytocin was to increase the efficiency of milk removal from the gland there would be an absolute yield increase at the first oxytocin-assisted milking (as the gland was completely emptied for the first time) but no further effects. So how can oxytocin delay involution at the cellular level? It clearly improves the efficiency of milk removal from the alveoli and small ducts (Sagi *et al*, 1980b) and this may extend cell longevity and maintain activity, possibly due to local effects, as long as physiological doses are administered. Much of the data is equivocal about oxytocin's effects, particularly on persistency, and more long-term studies are needed.

7. AIMS OF THIS WORK

This thesis examined some of the influences on mammary gland function in goats. The overall aim was to investigate a variety of approaches which might allow lactation to be manipulated, and in particular to improve lactation persistency, the rate of decline in yield after peak lactation is achieved.

The potential for manipulation of mammary growth during either late gestation or immediately *post partum* (ie early lactation) was investigated. Two treatments were applied- exogenous growth hormone was given, and an attempt was made to stimulate compensatory growth by removing a single mammary gland at the start of the experimental period and monitoring the subsequent effects on the remaining gland. Milk yields obtained during lactation were recorded and various parameters were measured to determine if there had been mammary cell proliferation and differentiation, facilitating measurement of 'mammary function' (Chapter 3).

An endocrine approach to manipulation of persistency was also attempted. A pharmacological agent, perphenazine, was used to stimulate prolactin's circulating concentrations (Chapter 4) and exogenous oxytocin was given at milking to supplement endogenous release at that time (Chapter 5) in the hope that they would influence the duration of lactation and increase or maintain milk production, thereby altering persistency.

The local effects of altering the frequency and method of milk removal during early lactation and the consequences of this for later lactation and persistency were studied, with some groups being comprised of primiparous or multiparous animals to investigate the effect of parity on the response to treatment. Methods of suckling, draining, once- and thrice-daily milking were applied (Chapter 6).

Finally a comprehensive study of the lactational changes in casein composition in goat's milk was carried out with the aim of elucidating whether or not this could provide an indirect marker for involution in the mammary gland, providing another method for the measurement of lactation persistency (Chapter 7).

CHAPTER 2

MATERIALS AND METHODS

1. INTRODUCTION

This chapter describes the methods commonly used in the experiments detailed in this thesis. Techniques used in specific experiments are detailed in the chapter describing that work.

All chemicals used, unless stated otherwise, were supplied by Sigma Chemical Company Ltd, by BDH (Merck Ltd), both Poole, UK or by Fisons Scientific Equipment, Loughborough, UK.

Radiochemicals were supplied by ICN Flow, High Wycombe, UK (^{125}I-sodium iodide and ^{14}C-sodium bicarbonate) and by DuPont (UK) Ltd, Stevenage, UK (UDP-[^{14}C]-galactose).

Veterinary products and medications were supplied by Veterinary Drug Company, York, UK.

Single-distilled tap water was used throughout with the exception of the galactosyl transferase assay detailed in Chapter 3, when double-distilled water was used to re-equilibrate DOWEX resin filtration columns.

2. COLLECTION AND PREPARATION OF PLASMA SAMPLES

Individual blood samples of approximately 5ml were collected by jugular venepuncture into a plastic syringe containing heparin (1000 IU/ml mucous injection). Blood and heparin were mixed by inverting the syringe several times, cooled to 4°C and plasma prepared by centrifuging the mixture (1800g, 15 minutes, 4°C). The resulting supernatant (plasma) was removed and stored at -20°C until analysis for hormone content.

When a series of samples was required the same procedure was followed with the exception of blood being removed via an indwelling jugular cannula. Catheterisation was carried out at least one hour before the commencement of sampling; a short length of sterile medical grade polyethylene tubing (id 1.0mm, od 1.5mm; Dural Plastics and Engineering Pty Ltd, Auburn, NSW, Australia) was introduced into a jugular vein using an intravenous catheterisation set (Medicut 14g; Sherwood Medical, Tullamore, Ireland) and sealed with a 3-way tap. After each withdrawal of blood, an approximately equal volume of sterile saline solution (0.9% NaCl) containing heparin (1000IU/100ml) was used to flush the cannula.

3. HORMONE DETERMINATIONS

Heterologous double-antibody radio-immunoassays (antibodies raised against ovine hormones) were used for the quantification of caprine prolactin and growth hormone based on the method described by Vernon *et al* (1981).

3.1 Hormone standards

Ovine prolactin (AFP-9221A) and growth hormone (AFP-9220A) for biological studies (donated by the National Institute of Health, Bethesda, Maryland, USA) were used to prepare hormone standard solutions of 10μg/ml in radio-immunoassay (RIA) buffer and stored at -20°C until use. Where undiluted plasma samples were assayed these standards were prepared in human low activity plasma (donated by the West of Scotland Blood Transfusion Service, Carluke, UK), otherwise RIA buffer was used. Standard curves were prepared by serial dilution of the highest standard in plasma or buffer, as appropriate, with concentrations typically ranging from 1.56-200 ng/ml for prolactin and 0.62-40 ng/ml for growth hormone.

3.2 Radio-immunoassay buffer

RIA buffer (pH 7.4) containing 0.05M sodium phosphate, 0.15M sodium chloride, 0.5% w/v bovine serum albumin (fraction V, RIA grade) and 0.05% w/v sodium azide was prepared for use in RIAs.

3.3 First antibody

Anti-ovine prolactin (AFP-C358109611 and AFP-973260) and anti-ovine growth hormone (AFP-C0123080) for radio-immunoassay (donated by NIH) were stored at 1:100 dilution in 7.5% sodium bicarbonate solution and diluted in RIA buffer immediately before use. An effective concentration was determined by experimentation and optimally bound 30% of the total added radio-labelled hormone. Typical antibody dilutions of 1:60,000-75,000 for prolactin and 1:15,000-25,000 for growth hormone were used, 100μl contributing to a total assay volume of 300μl.

3.4 Second antibody

49.57% v/v RIA buffer, 49.57% v/v polyethylene glycol (using 16% PEG solution) and 0.012M ethylenediamine tetraacetic acid were mixed and pH adjusted to 7.4. Then 0.83% v/v anti-rabbit (donkey) antibody and 0.03% v/v normal rabbit serum were added (both donated by the Scottish Antibody Production Unit, Carluke, UK).

3.5 Radiolabelling of hormone

Radiolabelling was carried out using a method based on that described by Fraker and Speck (1978). Radioactive iodine (^{125}I in sodium iodide) was incorporated into ovine prolactin (AFP-7150B), growth hormone (AFP-8758C) (both NIH) for iodination or bovine recombinant growth hormone (bST, M11505178; Monsanto, St Louis, Missouri, USA) by use of Iodogen^{Tm} (Pierce Europe BV, Oud-Beijerland, Netherlands). Approximately 5μg protein (10μl at 0.5mg/ml in 7.5% sodium bicarbonate), 10μl phosphate buffer (0.5M, pH7.4) and 500 μCi ^{125}I were placed in an iodogen-coated microtube (30μl iodogen in 5% w/v chloroform, evaporated to dryness) and incubated for 20 minutes. 100μl phosphate buffer was added and the mixture transferred to the top of a gel filtration column (Sephadex G-10 pre-soaked in RIA buffer; Pharmacia, Uppsala, Sweden). 200μl potassium iodide solution (2% w/v) was washed through the microtube and added to the column.

Iodinated hormone was eluted by addition of RIA buffer and the eluate fractionally collected. The radioactivity of each fraction was determined by monitoring with a Geiger counter (Mini-Instruments Ltd, Essex, UK). Percentage incorporation of ^{125}I into prolactin or growth hormone was determined by precipitation with trichloroacetic acid solution (10%) of a fraction of known radioactivity as follows:

acid precipitable radioactivity (protein-associated ^{125}I)

= [pellet counts/initial counts] x 100

Fractions were pooled if necessary and diluted in RIA buffer until stock solutions varied from 100 000 to 300 000 cps per 100μl volume. Aliquots were stored at -20°C, behind lead, until use.

3.6 Radio-immunoassay of prolactin and growth hormone

Samples or standards (100μl) were incubated with first antibody (1st Ab, 100μl) for a minimum of 4 hours when assaying for prolactin and for approximately 24 hours when assaying for growth hormone. A tube containing no sample (B_o) or 1st Ab (to determine non-specific binding), one containing only ^{125}I-oPrl ('total counts') and a 'zero' which contained no hormone were included in every assay. 100μl ^{125}I-oPrl containing approximately 500cps/100μl, or ^{125}I-oGH of approximately 300cps/100μl in RIA buffer were added, mixed and incubated overnight at room temperature. Second antibody was added (300μl), mixed, and further incubated for a minimum of 2 hours. All tubes were centrifuged (1800g, 30 minutes, room temperature) and the resulting

supernatant decanted prior to counting (Gamma counter, 74% efficiency; Packard, Meriden, USA).

Inter-assay coefficients of variation were typically 10.3% for prolactin, and 8.7% for growth hormone.

4. MILK COMPOSITIONAL ANALYSIS

4.1 Total solids

The solid content of a milk sample was determined by taking duplicate aliquots of freshly collected milk of known weight (approximately 1ml) and drying at 70°C until reaching a constant weight (after approximately 36 hours). The resulting milk solids were weighed, the difference representing the amount of liquid which had evaporated. Total solids content was calculated by dividing the total weight of the milk sample by the weight of solids present after drying, meaning duplicate samples, and multiplying by 100 to express total solids as a percentage of the whole milk sample.

4.2 Fat

The assay was carried out using the rapid fat method of Fleet and Linzell (1964) based on the separation of whole milk into a cream layer (white) and a serum layer (opaque) by centrifugation. The length of the cream fraction was expressed as a percentage of the total sample length and a correction factor applied to determine fat content as only 75% of the cream layer was attributable to fat.

Freshly collected samples were incubated at 37°C for a minimum of 30 minutes and thoroughly mixed before drawing into duplicate capillary tubes (Hawksley, UK) and sealing with wax (Radiometer, Denmark). Tubes were centrifuged (10000g, 15 minutes, room temperature) and read within 15 minutes (Microhaematocrit centrifuge and reader, Hawksley). Duplicate measurements were made for every sample, readings averaged, and corrected by multiplying by 0.75 to determine percentage fat content.

4.3 Bradford protein

The method of Bradford (1976) was used to determine protein content by measuring the change in absorbance produced by a dye binding to protein. A protein standard curve was obtained by dilution of stock bovine serum albumin solution (containing 0.1mg protein/ml) to give standards ranging from 1 to 10μg protein.

Freshly collected milk samples were diluted so that their protein concentrations could be determined from the standard curve. Dilutions of 1:350, 1:700 or 1:1400 milk in distilled water were used. The assay was carried out in 96-well micro-titration plates (Dynatec, Billinghurst, UK).

50μl aliquots of standards and samples were placed in duplicate wells, 240μl Bradford reagent added (20%, Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and incubated at room temperature for between 5 and 15 minutes. Absorbance of the coloured complex formed was monitored at 620nm (Titertek Multiskan MCC340 MkII type 347, Labsystems, Basingstoke, UK) and protein concentration expressed as grammes of protein per 100ml milk.

4.4 Lactose

Lactose determinations were carried out using a Sigma diagnostic kit (510A) for measuring glucose. Milk samples were pre-incubated with β-galactosidase, degrading lactose into glucose and galactose, allowing glucose to be assayed (see Figure 2.1).

Lactose standard solution (10mM α-lactose in 0.1M sodium phosphate buffer, pH 7.3) was used to prepare a standard curve with concentrations ranging from 1 to 5mM. Quality controls containing 0.5, 2,5 and 5mM lactose were prepared, subaliquotted and stored at -20°C until use, and run in every assay. Milk samples were diluted 1:40 in 0.1M sodium phosphate buffer.

The following were placed in a microtube, vortexed and incubated in the dark at 37°C for exactly 30 minutes:

400μl diluted milk sample, lactose standard or quality control

70μl 0.1M Potassium phosphate buffer (pH 7.3)

20μl 0.1M Magnesium sulphate

10μl β-galactosidase

The reaction was stopped by addition of perchloric acid (100μl 4.2% v/v). 10μl aliquots of reaction mixture were placed in a 96-well micro-titration plate, 300μl Peroxidase/glucose oxidase/o-dianisidine solution added to each well and incubated for 10 minutes at 37°C. Absorbances were read at 450nm and lactose content determined by reading from the standard curve after correction for dilution of the original milk sample.

5. PHYSIOLOGICAL MEASUREMENTS

5.1 Total udder volume determination

Udder volumes were determined by water displacement based on the method of Linzell (1966). Goats were given exogenous oxytocin (400mU Oxytocin-S, Intervet, Cambridge, UK) by intravenous injection and immediately milked. They were then stood with their front legs elevated while the whole udder was gently immersed in a bucket of luke-warm water of known weight. The bucket was reweighed after this procedure, the weight of water displaced representing the udder's volume. This was repeated (usually three times) until replicates were within 100g of each other.

A second method of udder 'casting' was also used (Dewhurst *et al*, 1993). Udders were shaved to remove excess hair, udder cream ('Starcare', Lever Industrial, Runcorn, UK) applied liberally and the midline between the glands marked (Sprayline stock marker, Ritchley Tagg Ltd, Masham, UK). The udder was emptied by milking with oxytocin as before and was immediately casted in quick-set foam (Froth-Pak^{R}, Foampax Scotland, Newmilns, UK). Gross udder volumes were determined with material of known specific gravity by filling the cast to the position of the body wall. Individual gland volumes were measured by splitting the cast along the midline and remeasuring.

5.2 Cisternal and alveolar storage capacities

Cisternal milk volume was determined first (by catheter drainage), followed by alveolar milk volume, measured using the method described by Knight *et al* (1994). Teats were swabbed with 70% alcohol-2% hibitane solution and locally anaesthetised (xylocaine gel 2%, Astra Pharmaceuticals, Kings Langley, UK). A sterile sheath from an intravenous cannulation set (Medicut 14g; Sherwood Medical, Tullamore, Ireland) was carefully introduced into the teat duct and canal until approximately 10mm of the cannula protruded. This allowed milk to flow freely from the gland without any further teat contact. Milk was collected and weighed to determine the volume removed. This procedure was carried out in a quiet environment and glands drained simultaneously, when possible, to minimise the chance of milk ejection. The milk flowing from the cannula was visually monitored for signs of milk ejection, characterised by a transient increase in flow rate and volume. Alveolar milk volume was determined immediately after cisternal drainage by administering iv oxytocin (400mU), hand-milking to remove the remaining milk and weighing the amount obtained. Cisternal percentage was calculated by dividing cisternal volume by the total amount of milk collected from the gland and multiplying x100.

CHAPTER 3

STIMULATION OF MAMMARY GROWTH IN PREGNANT AND LACTATING GOATS

1. INTRODUCTION

The mammary gland grows during pregnancy, exponentially so during late gestation, in preparation for the demands of the newborn. However mammary growth continues during early lactation, albeit at a slower rate (Knight *et al*, 1987). If this growth process could be enhanced either during pregnancy or early lactation then it might be possible to improve the production potential of an animal by increasing secretory cell numbers and their differentiation, resulting in a greater capacity for milk secretion.

The factors controlling and restricting mammary growth have not yet been fully elucidated; systemic hormones can be modulated by factors such as receptor numbers, the production of variants and the presence of circulatory binding proteins. Mediators of somatotrophic factors, for example, IGF, IGF-binding proteins and growth factors (eg. EGF, TGF and fibroblast GF) exert local paracrine and autocrine mitogenic effects (Collier *et al*, 1993). However other growth factors such as interferons and TGF-β exert negative influences. Chalones were identified as cell cycle inhibitors in the 1960s but specific negative regulators have only recently been identified (for review see Johnson, 1994). Mammastatin, found in the cytoplasm, inhibits mammary cell proliferation (Ervin *et al*, 1989) but many inhibitors are present at the cell surface and may respond to cell-cell contact, including mammary-derived growth inhibitor and sialoglycopeptide inhibitor.

Nutrition of the animal, not only during pregnancy and lactation, but throughout life, especially around puberty, will also influence subsequent mammary development (Sejrsen, 1994). Genetic inheritance also plays a role- identical twins had similar rate constants for mammary growth although the stage of gestation had the greatest influence on mammary development (Swanson & Poffenbarger, 1979). So there are many factors which are acting to determine mammary size. The gland's functional capacity can be estimated by determining the size and differentiative state of the cell population (by measuring DNA and specific enzyme activities, respectively). Incorporation of a thymidine analogue into DNA allows measurement of cell proliferation to be made and the monitoring of milk yields also indicates the extent of mammary function.

The normal pattern for mammary growth in goats during pregnancy is one of an exponential increase in parenchymal volume which slows post-partum, then drops, while differentiation increases around parturition and remains at a higher level during lactation (Knight & Wilde, 1993). Stimulation of mammary growth has been achieved using growth hormone; in pre-pubertal heifers 15 weeks of treatment resulted in a 46% increase in parenchymal volume (Sejrsen *et al*, 1986). However growth hormone administered during late gestation to goats did not give a growth response or increased milk yield during the subsequent lactation (Lee & Forsyth, 1988). This contrasts with treatment during lactation when parenchymal volumes were increased over control glands (probably due to hypertrophy as ^{3}H-TdR incorporation did not increase) and milk yields increased by 23% (Knight *et al*, 1990). It is still not clear whether these observations are due to hypertrophy, hyperplasia, or some combination of them both, and metabolic state also influences the effect of treatment.

The 'yield response' to exogenous growth hormone is well known in lactating ruminants with increases of up to 30% in cows and goats (Bauman *et al*, 1985; Knight *et al*, 1990). This has been ascribed to increased blood flow reducing the restriction of substrate supply to the mammary gland in goats (Mepham *et al*, 1984), to alteration of IGF-I levels (Davis *et al*, 1989) and to the mobilisation of tissue reserves towards milk production ('nutrient partitioning'), among others. However these effects of growth hormone are limited to the period of treatment with little or no carry-over (Knight, 1992) and a detailed investigation of its effects on mammary cell proliferation and differentiation has not been conducted in goats.

It had been recognised by Turner *et al* (1963) that gross udder size was not as good a measure for 'glandular tissue' as the determination of DNA content of the gland. They designed an experiment in which a cow udder half was removed before a 6 month treatment period, to serve as a control, and the remaining half removed post-treatment. In all animals tissue weights increased (130-347%) as did total DNA (113-376%), suggesting that the removal of glands might be having an effect on the remaining ones. Linzell (1963) transplanted mammary glands in lactating Saanen goats and found that if the transplanted gland failed, the remaining gland showed increased growth (up to 62%) and in virgin goats which subsequently lactated, as much milk was produced from one gland as control animals did from both glands. It was hypothesised that the loss of a gland was detected by interruption of major blood vessels and nerves and there was a rapid compensatory response. However, others (Benson *et al*, 1965;

Cowie *et al*, 1965) have argued that during lactation any 'compensatory growth' reflects the release of lactogenic and steroid hormones rather than a growth stimulus *per se*.

This study compared the potential growth stimuli of hemimastectomy and exogenous growth hormone using goats in two reproductive states- late gestation and early lactation.

2. ANIMALS

British Saanen goats from the Institute herd were used. Hay and water were available *ad libitum* and animals were offered 1.0kg (when pregnant) or 1.5kg (when lactating) of a proprietary concentrated feed (Goat mix no.1, Edinburgh School of Agriculture, Edinburgh, UK) per day.

3. EXPERIMENTAL DESIGN

3.1 Experimental groups

Eighteen primiparous goats were selected on the basis of the time of mating and confirmed pregnancy and were randomly assigned to six groups containing three animals in each. Kidding dates ranged from 18th March-10th April 1993.

3.2 Treatments

The experiment employed a 2x3 factorial design with factors of reproductive state (pregnant or lactating) and experimental treatment, namely growth hormone or hemimastectomy or no treatment (control). Treatment groups were abbreviated to PGH, PHX, LGH & LHX while two control groups were described as PC & LC.

Growth hormone (12 mg BST in sterile saline solution, gift of Monsanto, St Louis, USA) was given by subcutaneous injection every second day for 6 weeks, commencing 6 weeks before anticipated parturition in the PGH group, and immediately after parturition in the LGH group (referred to as the (GH) treatment period).

Hemimastectomies were performed 6 weeks pre-partum in the PHX group and within 4 days of parturition in lactating animals (LHX).

Bromodeoxyuridine (BrdU) (200mg in sterile saline) was given intravenously once every four days (a total of 7 doses) to all animals during the last 4 weeks of the treatment period to facilitate the measurement of cell proliferation.

3.3 Measurements

Mammary gland biopsies were taken within 4 days of parturition in pregnant groups, and during lactation week 7 (within 4 days of the end of the treatment period) in lactating groups. During lactation week 5 (pregnant groups) or week 11 (lactating groups) ie. four weeks after the end of the treatment period, animals were euthanised and mammary glands removed for the collection of tissue and the measurement of gross and parenchymal tissue weights.

When lactating, all groups were milked twice daily at 0800 and 1600h and milk yields from individual glands were recorded.

4. METHODS

4.1 Surgical procedures

On the afternoon prior to surgery hay and concentrates were withdrawn from animals and only water was available (*ad libitum*) until after the procedure was completed. Immediately before surgery animals received oxytocin (100mU i.v.) and milk removed from mammary glands by hand milking.

Mammary gland biopsies and hemimastectomies were performed as described by Knight & Peaker (1984) and Knight (1987), respectively. Briefly, anaesthesia was induced with sodium pentobarbitone (Sagatal, May & Baker, Dagenham, UK) and animals were maintained on this during biopsy; secretory tissue was exposed by incision and blunt dissection and a small portion removed by electrocautery. For hemimastectomy, anaesthesia was maintained by administration of halothane in oxygen for the duration of the procedure. Mammary glands were separated along the mid-line by an initial incision and blunt dissection to the body wall. A second incision was made to include the teat and some skin of the gland to be removed, and then blunt dissection was used to separate it from the body wall. Blood vessels were ligated or cauterised until the gland could be completely removed. The space remaining after biopsy or hemimastectomy was filled by layering surrounding tissue into it and closing by the application of sutures. External sutures were removed between 10 and 14 days post-operatively. Penicillin was applied locally at the time of skin closure and flunixin meglumine and oxytetracycline (Finabiotic, Schering-Plough, Mildenhall, UK) was given by daily i.m. injection for three days after surgery.

4.2 Preparation of tissue

Biopsy samples were trimmed until only secretory tissue unaffected by electrocautery was present. Hemimastectomised glands were weighed, dissected until only parenchymal tissue remained, then reweighed. Samples of secretory tissue obtained by both procedures were wrapped in silver foil, stored in liquid nitrogen as quickly as possible after removal and later assayed for enzyme activities and DNA content. A further portion was placed in fixative (4% paraformaldehyde solution diluted 1:1 with 0.1M phosphate buffer, pH 7.4) and subsequently processed for measurement of BrdU incorporation.

4.3 Determination of enzyme activities

Preparation of assay samples

Tissue samples were ground to a fine powder under liquid nitrogen and two portions accurately weighed out. Samples for galactosyl transferase assay were placed in 0.25M sucrose solution (1 part in 25) and homogenised (Polytron, 70% of maximum speed, 15 seconds). For other determinations, samples were placed in iso-osmotic Tris-sucrose buffer (1 part in 10) (containing 300mM sucrose, 30mM Tris-HCl, 1mM EDTA and 1mM reduced glutathione (GSH); pH 7.4), homogenised (45 seconds) and particle-free supernatant (PFS) prepared by centrifugation (11000rpm, 60 seconds). The homogenate was retained and frozen at -20°C without loss of activity (CJ Wilde, personal communication) for measurement of aryl esterase activity and DNA content and PFS was used for acetyl CoA carboxylase and fatty acid synthetase determinations (adapted from Wilde *et al*, 1986).

Details of the reactions catalysed by these enzymes are illustrated in Figure 3.1.

Acetyl CoA carboxylase (ACC)

The process of fatty acid synthesis starts with a 'committed step' reaction catalysed by acetyl CoA carboxylase [EC 6.4.1.2] (Scott & Eagleson, 1988). The enzyme's activity was determined by addition of a radioactively labelled precursor to a reaction mixture and measuring its incorporation over a fixed incubation time using a modified version of the method described by Ingle *et al* (1973). Its activity was expressed as the number of nanomoles of bicarbonate incorporated per minute per gram of tissue.

Samples of PFS (150μl) were preincubated (30 minutes, 37°C) in 0.02ml distilled water and 0.33ml stock solution (200mM Tris/HCl, 100mM $MgCl_2$, 200mM

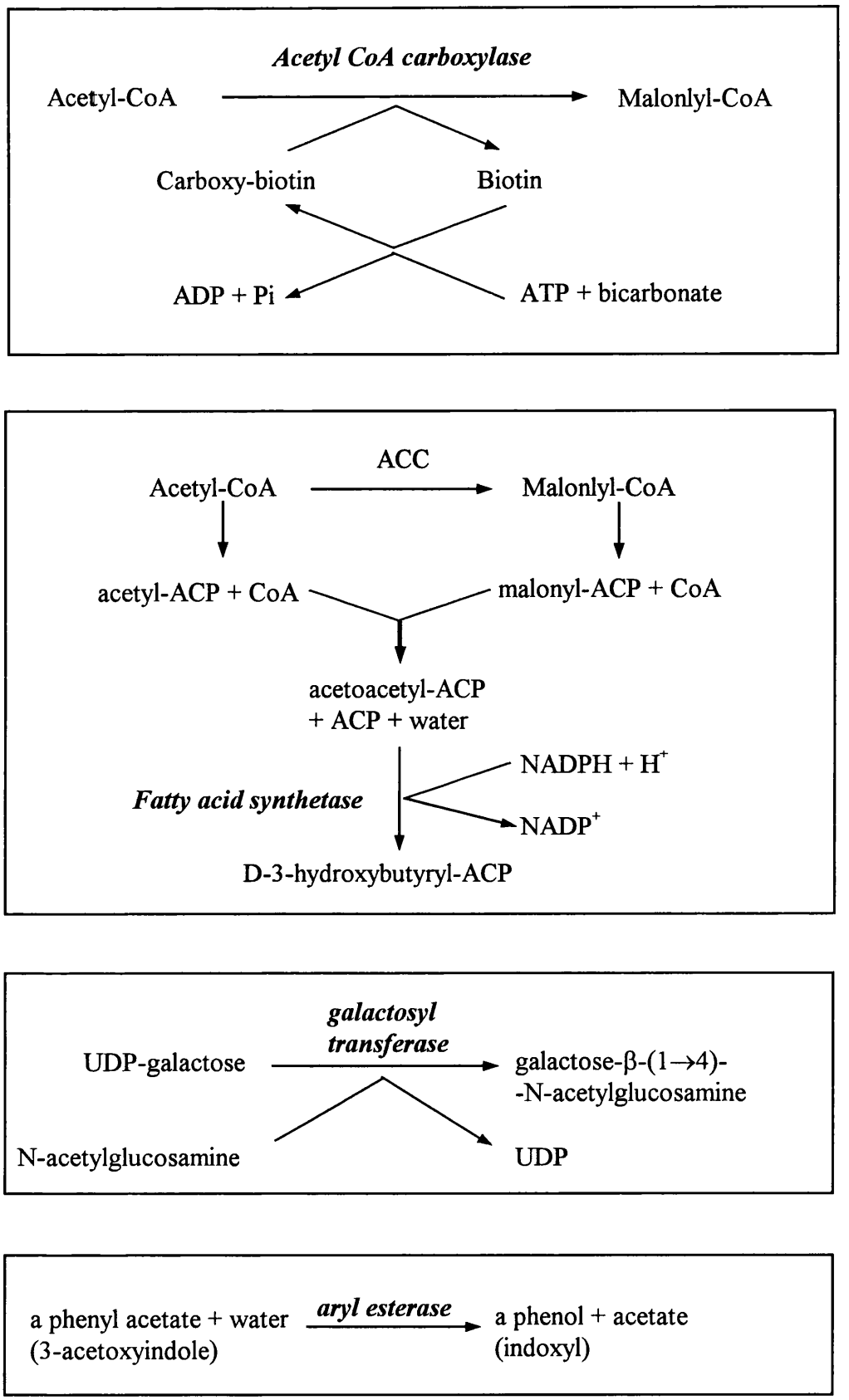


Figure 3.1 Summary of reactions catalysed by the enzymes:
(**A**) Acetyl CoA carboxylase (**B**) Fatty acid synthetase
(**C**) Galactosyl transferase & (**D**) Aryl esterase.

citrate, 1mM EDTA, 50 mg/ml BSA, 100mM GSH; pH 7.3) in sealed vials. Assay solution (0.33ml) containing acetyl CoA (0.05ml of 4.45mM acetyl CoA), ATP (0.1ml of 50mM ATP; pH 7.5) and sodium bicarbonate (1.35μCi $NaH^{14}CO_3$ made to 25μl with distilled water; specific activity 0.1-0.5 mCi/ml, ICN Flow, High Wycombe, UK) was warmed to 37°C with acetyl CoA omitted from blank reactions. The reaction was initiated by addition of 0.5ml assay solution to the pre-incubation solution, the mixture incubated (90 seconds, 37°C) and 5M HCl (0.2ml) added to stop the reaction.

Unreacted sodium bicarbonate was removed by evaporation; samples were warmed (60 minutes, 55°C), ground dry ice added and mixed in tubes, and further dry ice added. Finally, scintillation fluid (10ml Emulsifier Safe, Packard, Meriden, USA) was added, the mixture thoroughly shaken and counted for ^{14}C content (^{14}C 4 minute counts; Packard 1600TR liquid scintillation analyser).

Fatty acid synthetase (FAS)

Fatty acid synthetase is a complex multifunctional protein and carries out elongation of fatty acid chains in the process of fat synthesis (Speake *et al*, 1975; Scott & Eagleson, 1988). The first reduction step catalysed by the enzyme produces $NADP^+$ whose production can be monitored spectrophotometrically, giving a direct measurement of enzyme activity. However this reaction depends on the second reduction in the pathway being prevented by the absence of flavin. FAS activity was determined using a method based on that described by Speake *et al* (1975) and activity expressed as the number of micromoles of NADPH which were incorporated into product per minute per gram of tissue.

A solution containing PFS (25μl of PFS diluted 1 part in 5 with iso-osmotic Tris-sucrose buffer), NADPH (0.1ml of 1mM NADPH, Boehringer Mannheim, Lewes, UK) and phosphate buffer (0.795ml of 0.25M KH_2PO_4 containing 1.5mM EDTA and 1.5mM GSH; pH 6.6) was pre-incubated for 5 to 10 minutes at 30°C and monitored by measuring absorbance at 340nm (Cecil CE5501 double beam UV spectrophotometer, Ambridge, UK). Once a stable baseline was obtained acetyl CoA was added (50μl of 0.6mM acetyl CoA) and the reaction rate monitored. Once a steady rate was achieved, malonyl CoA was added (30μl of 1.3mM malonyl CoA) and absorbance measured.

Galactosyl transferase (GT)

Galactosyl transferase [EC 2.4.1.22] is composed of catalytic and modifier subunits, the latter being the whey protein α-lactalbumin, and it catalyses the production of lactose. However, in the absence of the modifier subunit GT adds carbohydrate to glycoproteins. Disruption of the Golgi membranes allows free access of substrates to the enzyme complex and its activity can be determined by incorporation of a radiolabelled precursor into the end-product (Kuhn & White, 1977; Stryer, 1988). GT activity was assayed using the method of Kuhn & White (1977) and expressed as the number of micromoles of galactose incorporated per minute per gram of tissue.

Reaction mixture (25μl of 2:2:1 ratio of 0.2M TES, 150mM $MnCl_2$ and 5% v/v Triton X-100), UDP-[^{14}C]-galactose (5μl of 5mM UDP-gal. containing approximately 25000dpm, DuPont Ltd, Stevenage, UK) and N-acetyl glucosamine (10μl of 0.1M NAG) were added together and incubated for 10 minutes at 37°C. The latter was omitted from reaction blanks. The reaction was initiated by the addition of sample (10μl homogenate), incubated (10 minutes, 37°C) and stopped by placing tubes in a boiling water bath for 90 seconds. The resulting solutions were placed on ice, distilled water added (50μl) and transferred to the top of a DOWEX resin column (formate form, 400 mesh, 4% crosslinking) with reaction tubes washed twice with further distilled water (2 x 100μl), the washings also being transferred to the column. Reaction product was eluted with 1ml distilled water, the eluate collected, 10 ml scintillation fluid added and the resulting solution counted for ^{14}C content.

Aryl Esterase (AE)

Aryl esterase [EC 3.1.1.2] is not involved in lactational metabolism (Knight *et al*, 1992) and originates from the rough endoplasmic reticulum of a cell. It is found in microsomal cell fractions and can be used as a specific marker for microsomes. Addition of substrate to the enzyme forms a product which can be measured spectophotometrically and hence enzyme activity determined. The method used was described by Shephard & Hubscher (1969) and AE activity expressed as the number of micromoles of indoxyl produced per minute per gram of tissue.

Homogenate (40μl), phosphate buffer (1.0ml of 0.1M KH_2PO_4; pH 6.8) and distilled water (120μl) were pre-incubated for 5-10 minutes at 37°C and absorbances measured at 386nm. Once a steady baseline was obtained 40μl indoxyl acetate was added (25mM in 50% ethanol) and the resulting change in absorbance measured.

4.4 Determination of DNA content

DNA was assayed using the method of Labarca and Paigen (1980) with concentrations determined from a standard curve and the DNA in samples expressed as milligrams per gram of tissue.

Homogenates in iso-osmotic Tris-sucrose buffer were further sonicated (20KHz Kontes microultrasonic cell disrupter, Burkard Scientific, Uxbridge, UK) in 20 second blocks, with cooling, for a total of one minute. Samples were diluted appropriately with assay buffer (2M NaCl, 0.1M NaH_2PO_4; pH 7.4, buffer filtered through 2μm membrane). DNA standards in assay buffer were prepared from bovine thymus DNA (1mg/ml in dH_2O, diluted to 10μg/ml with assay buffer). 1ml aliquots of sample or standard were taken in duplicate, 0.5ml fluor solution added (1mg/ml bisbenzimide in water, diluted to 3μg/ml with assay buffer) and incubated for 15-20 minutes at room temperature before determination of fluorescence (Fluorometer, Hoefer Scientific, San Fransisco, USA).

4.5 Determination of BrdU incorporation

Preparation of tissue

Tissue was fixed for a minimum of 2 hours in fixative then dehydrated in a graded series of ethanol and cleared using histoclear (National Diagnostics, Atlanta, USA). Samples were mounted in moulds using four changes of wax (RA Lamb, London, UK) and after solidifying they were removed from the moulds, cut and mounted on embedding stubs (Agar Scientific Ltd, Stansted, UK). Subsequent sectioning was carried out using a 2040 autocut microtome (Cambridge Instruments GmbH, Nussloch, Germany) and glass cutting blades (histology grade, Agar Scientific).

Visualisation of BrdU

The protocol used to measure BrdU incorporation was that recommended by DAKO a/s (Glostrup, Denmark) for fixation and staining of tissue, and detecting with monoclonal mouse anti-bromodeoxyuridine (clone Bu20a).

Poly-L-lysine-coated microscope slides were prepared by cleaning in acid solution (10% w/v potassium chromate, 10% concentrated sulphuric acid v/v) overnight, rinsing thoroughly in water and incubating in poly-L-lysine solution (10%).

Tissue sections were placed on poly-L-lysine-coated slides, pre-incubated (60°C) and circled with a 'PAP pen' (Agar Scientific Ltd). Samples were rehydrated by

incubation in histoclear followed by a graded series of ethanol.

Endogenous peroxidase activity was quenched (1% hydrogen peroxide in methanol) and the DNA present partially denatured with 2M HCl to facilitate antibody binding. The reaction was stopped by addition of sodium borate solution (0.1M, pH 8.5) and sections incubated with protease (0.25 mg/ml in TBS) to unmask antigenic binding sites by proteolysis. Incubation in avidin (0.05 mg/ml in 0.05M Tris/HCl, pH 7.4) followed by biotin (0.05 mg/ml in 0.05M Tris/HCl, pH 7.4) was carried out to block endogenous avidin-binding activity. A further incubation in normal rabbit serum (10% in 0.05M Tris/HCl, pH 7.2) was used to block any non-specific background binding.

BrdU incorporation was detected and visualised by sequential incubation, and TBS rinsing, in the following: mouse anti-BrdU antibody (2.5% in Tris/HCl, pH 7.2), biotinylated rabbit anti-mouse immunoglobulin (0.1% in 0.05M Tris/HCl, pH 7.2) and avidin-biotin-peroxidase DAKO ABComplex/HRP kit). Staining was carried out by application of 3,3-diaminobenzidine tetrahydrochloride (0.5mg/ml DAB in TBS containing 0.03% H_2O_2 v/v) followed by Mayer's hematoxylin. Tissue was destained (1% HCl in 95% ethanol), dehydrated through a graded series of ethanol then mounted with coverslips using DPX mounting medium and allowed to dry overnight before photographing under magnification (x660).

4.6 Statistical analysis

Data were analysed using the General Linear Model ANOVAR procedure (SASS). Factors in the model were liveweight, stage (pregnant or lactating) and treatment (control, hemimastectomy and growth hormone).

5. RESULTS

5.1 Milk yields

Average daily gland yields for pregnant (Figure 3.2) and lactating (Figure 3.3) treatment groups were determined during lactation until the end of the experiment. Both hemimastectomised groups increased gland yields to above 1.7 l/d *post partum*, in contrast to control and growth hormone groups which achieved a peak yield of 1.40 l/d. There was no difference between left and right glands of the latter groups so the average yield of both glands was calculated and used in statistical analyses. In lactating groups the analysis was based on yields obtained in the last 15 days of the treatment phase as earlier yields were compounded by the effect of surgery (LHX group).

There was a significant effect of treatment of milk yield but no effect of liveweight or stage, and no significant interaction between treatment and stage (Table 3.1). Hemimastectomy increased milk yields whether the gland was removed during pregnancy or lactation, with a peak yield of 1.71 l/d achieved in PHX compared to 1.05 l/d in controls. There was no apparent effect of growth hormone treatment on yield, which was contrary to our expectations. Examination of milk yields (Figure 3.2) indicated a decrease in the yield of LGH goats following the cessation of treatment. When this post-treatment yield difference between PC and PGH was included as a covariate in the analysis, the corrected mean yields were 1.23 (LC) and 1.42 (LGH) l/d, indicating a yield response to treatment. This difference, although numerically greater than uncorrected yields, did not achieve statistical significance.

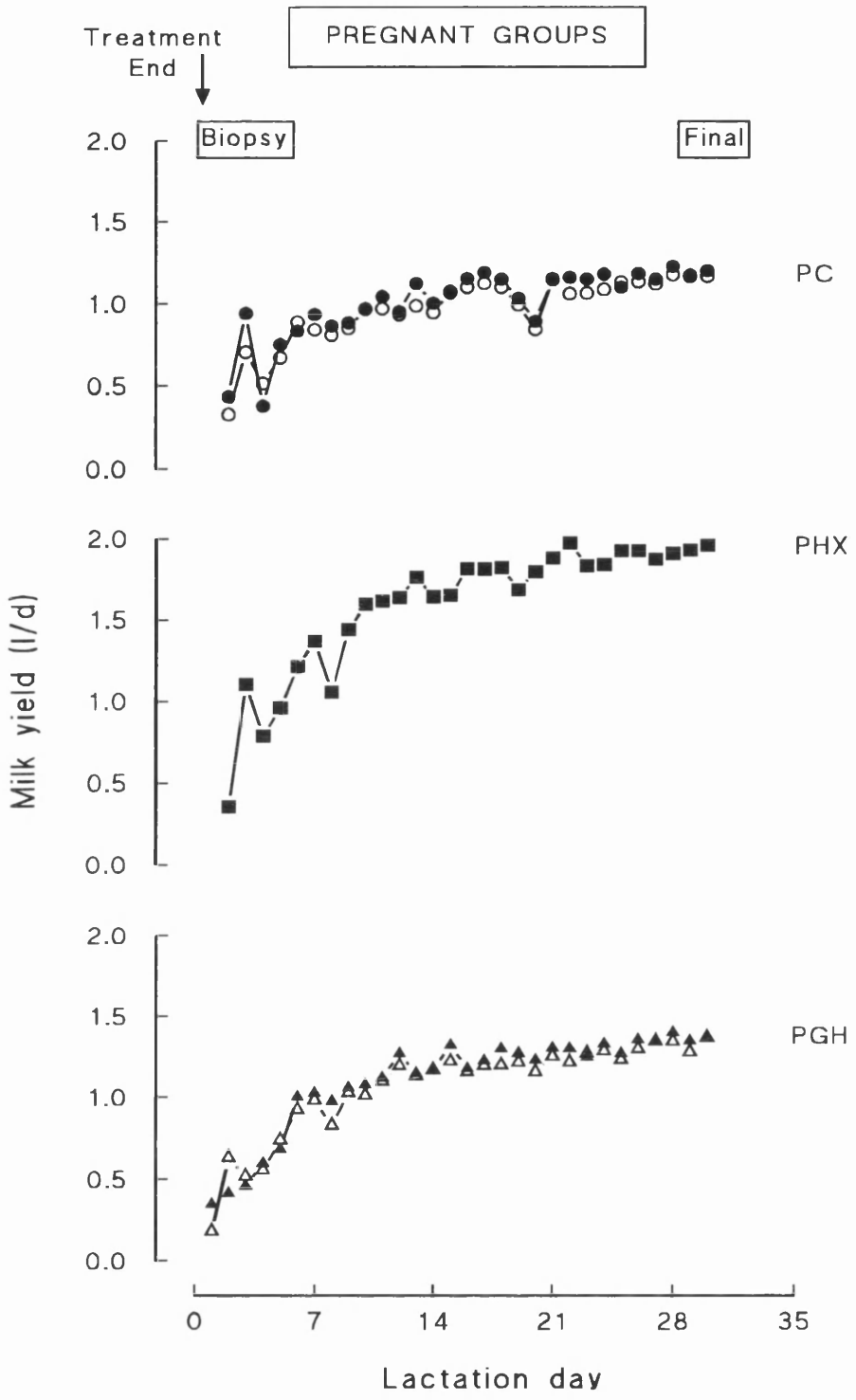


Figure 3.2 Average daily milk yields from individual glands in pregnant control (PC), pregnant hemimastectomised (PHX) and pregnant growth hormone-treated (PGH) groups. The GH treatment phase lasted from 42 days (6 weeks) pre-partum until parturition. Biopsies of mammary tissue were obtained within 4 days of parturition and final samples were obtained during lactation week 5, a minimum of 4 weeks post-treatment.

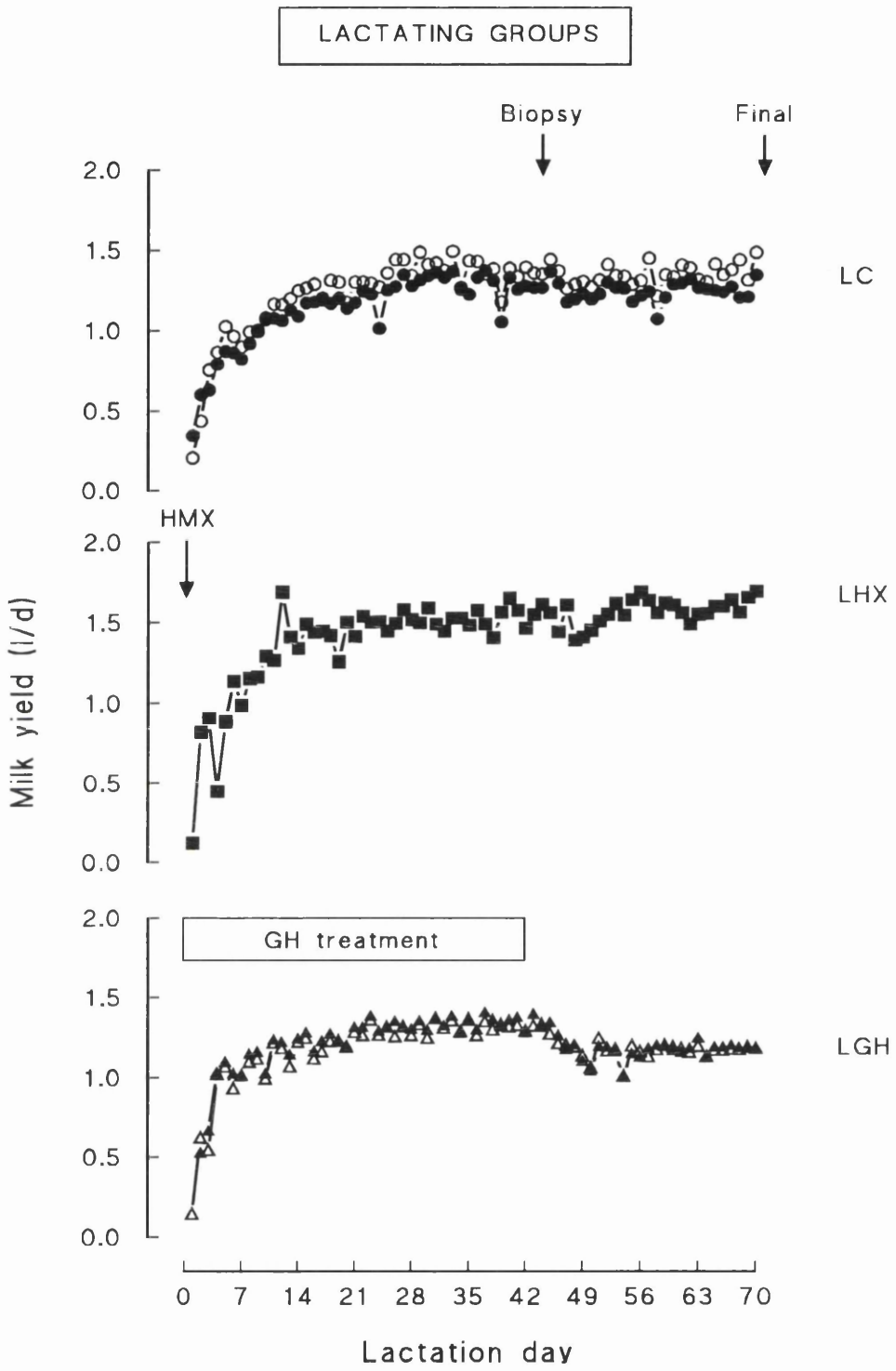


Figure 3.3 Average daily milk yields from glands in lactating control (LC), lactating hemimastectomised (LHX) and lactating growth hormone-treated (LGH) groups. The GH treatment phase lasted from parturition for 42 days (6 weeks of lactation). Biopsies of mammary tissue were obtained within 7 days of the cessation of treatment and final samples were obtained in lactation week 11 (a minimum of 4 weeks post-treatment).

Milk yield (l/d/gland)					
Source	**Stage**		**Treatment**		
Factor	*Pregnant*	*Lactating*	*Control*	*HX*	*GH*
mean (SEM)	1.32 (0.10)	1.27 (0.10)	1.13 (0.12)	1.56 (0.11)	1.19 (0.11)
p	n.s.		-	0.022	n.s.
PC	***PHX***	***PGH***	***LC***	***LHX***	***LGH***
1.05 (0.15)	1.71 (0.16)	1.19 (0.17)	1.21 (0.18)	1.41 (0.16)	1.20 (0.16)
-	0.012	n.s.	-	n.s.	n.s.

Table 3.1 Milk yields during lactation for control goats (PC/LC), for goats hemimastectomised during pregnancy (PHX) or during lactation (LHX), and for goats treated with growth hormone during pregnancy (PGH) or lactation (LGH). Values are least squares means and sems were calculated from pooled data. Significance levels are comparisons relative to the appropriate control group.

5.2 Liveweight

Gross body weights were measured at the end of the experimental period and meaned for each treatment group. Control goats had the highest weights (PC 40.2 $\pm$ 3.7, LC 46.0 $\pm$ 2.1 kg) when compared to the other treatment groups with hemimastectomised animals being of intermediate weight (PHX 38.8 $\pm$ 2.0, LHX 43.0 $\pm$ 2.3 kg). Growth hormone-treated groups had the lowest mean weights (PGH 37.2 $\pm$ 2.0, LGH 39.7 $\pm$ 0.4 kg) but none of these differences were statistically significant. The higher weights in the lactating groups compared to the equivalent pregnant groups reflects that their liveweight measurements were made 6 weeks later than the pregnant groups (in lactation weeks 11 & 5 respectively).

5.3 Mammary gland weight

Total gland weight and parenchymal tissue weight were determined in all 6 groups four weeks after the end of the treatment phase (Figure 3.4) and statistical analysis conducted (Table 3.2). Estimates of parenchymal DNA were also made (Table 3.2B).

Total gland weight was related to liveweight ($p<0.002$), confirming previous observations (Sejrsen, 1994). Effects of treatment on parenchymal tissue and parenchymal DNA were also found ($p\leq0.01$). Hemimastectomy provided the most potent stimulus for mammary growth with total gland, parenchymal tissue and DNA significantly increased over controls ($p\leq0.004$). The largest difference was seen during pregnancy, supporting the increased milk yield at this time, but there was no statistically significant interaction of treatment*stage indicating that surgery could be performed *pre-* or *post-partum* and elicit a similar effect.

Growth hormone during pregnancy stimulated parenchymal tissue growth and a positive effect of treatment was seen on parenchymal DNA levels ($p<0.05$). However this was not apparent when individual pregnant and lactating groups were compared. Growth hormone administered during lactation had little effect on the amount of mammary tissue with values similar to controls. It appeared to be having a limited effect on the secretory cells of the mammary gland, but there was a small stimulation during pregnancy, confirming the measurements of milk yield. However these changes were small in comparison to the stimulation achieved by hemimastectomy.

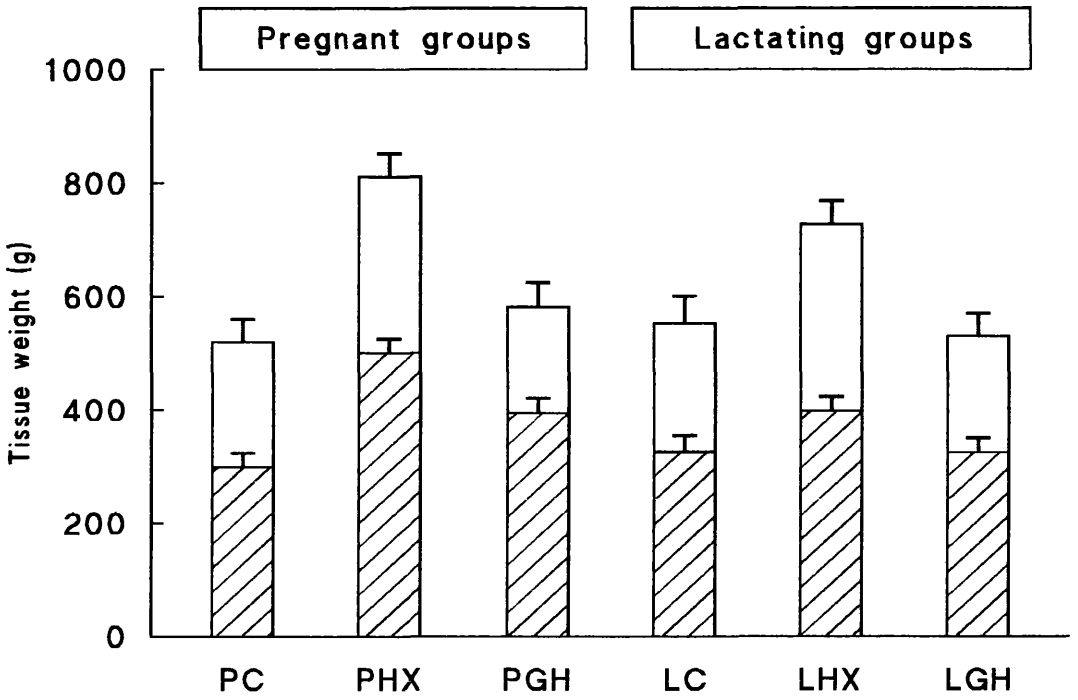


Figure 3.4 Least square mean values for gross gland weights (open bars) and parenchymal tissue weights (hatched bars). Goats were either controls, hemimastectomised or given growth hormone during pregnancy (PC,PHX, PGH) or during lactation (LC, LHX, LGH). Error bars indicate standard errors for each group. These measurements were made four weeks after the end of the treatment phase (10 weeks after hemimastectomy). Statistical analysis of this data is illustrated in Table 3.2 A.

A

Significance levels					
Gross gland weight (g)					
Source	**Stage**		**Treatment**		
Factor	*Pregnant*	*Lactating*	*Control*	*HX*	*GH*
p	n.s.		-	0.0002	n.s.
PC	***PHX***	***PGH***	***LC***	***LHX***	***LGH***
-	0.0003	n.s.	-	0.012	n.s.
Parenchymal tissue weight (g)					
Source	**Stage**		**Treatment**		
Factor	*Pregnant*	*Lactating*	*Control*	*HX*	*GH*
p	n.s.		-	0.0002	n.s.
PC	***PHX***	***PGH***	***LC***	***LHX***	***LGH***
-	0.0001	0.022	-	n.s.	n.s.

B

Parenchymal DNA (mg)					
Source	**Stage**		**Treatment**		
Factor	*Pregnant*	*Lactating*	*Control*	*HX*	*GH*
mean	1716.8	1972.1	1286.6	2236.4	2010.4
(SEM)	(156.1)	(156.1)	(189.0)	(175.0)	(190.4)
p	n.s.		-	0.004	0.029
PC	***PHX***	***PGH***	***LC***	***LHX***	***LGH***
1417.6	2295.5	2203.1	1155.5	2177.3	1817.8
(248.0)	(271.5)	(271.5)	(295.0)	(256.2)	(250.4)
-	0.030	n.s.	-	0.017	n.s.

Table 3.2 Statistical analysis of total gland and parenchymal tissue weights (**A**) and parenchymal DNA (**B**) in control (PC/LC), hemimastectomised or growth hormone during pregnancy (PHX, PGH) or lactation (LHX, LGH). Significance levels quoted are relative to the appropriate controls.

5.4 Cell proliferation

Cell number (per mm^2) was used as an estimate of cell size while the number of labelled cells (stained with BrdU, see Plate 3.1) and the percentage of labelled cells were used as markers for cellular proliferation. Measurements were made in samples obtained at biopsy (6 weeks after hemimastectomy / immediately after the cessation of GH treatment) and at the end of the experiment (final sample), 4 weeks after biopsies were taken . The results are shown in Tables 3.3 and 3.4.

There was a significant interaction between biopsy, biopsy*treatment and biopsy*stage ($p<0.01$) on total and labelled cell number.

The percentage of labelled cells was also affected by biopsy ($p=0.001$), stage ($p=0.001$) and biopsy*stage ($p<0.005$). The 'biopsy effect' reflected a general loss of cells (both unlabelled and BrdU labelled) in the period between biopsy and final samples while the stage interaction indicated a significant and positive effect of pregnancy on the degree of cellular proliferation compared to lactating tissue.

There was no apparent proliferative response to hemimastectomy with values similar to controls, surprising as there was a clear increase in mammary gland size and parenchymal tissue. This apparent discrepancy could be explained if there was an early proliferative response soon after the removal of the gland which occurred in the period before BrdU was administered. Growth hormone treatment increased total cell number ($p=0.059$), significantly so when PGH and PC biopsy samples were compared ($p=0.013$), but little effect was seen when it was administered during lactation. This again supports our measurements of gland size and milk yield.

5.5 Cell differentiation

Assessment of cellular differentiation was made by determining DNA concentrations in biopsy and final samples (Table 3.5), and measuring the enzyme activities of three 'key milk enzymes'- acetyl CoA carboxylase (ACC), fatty acid synthetase (FAS) (Table 3.6) and galactosyl transferase (GT), and one 'control' enzyme (aryl esterase, AE) (Table 3.7).

DNA concentrations were significantly affected by an interaction with biopsy ($p=0.005$)- higher concentrations being associated with final samples. An interaction with stage was also found for FAS ($p=0.03$) although there were no other significant interactions for the other enzymes.

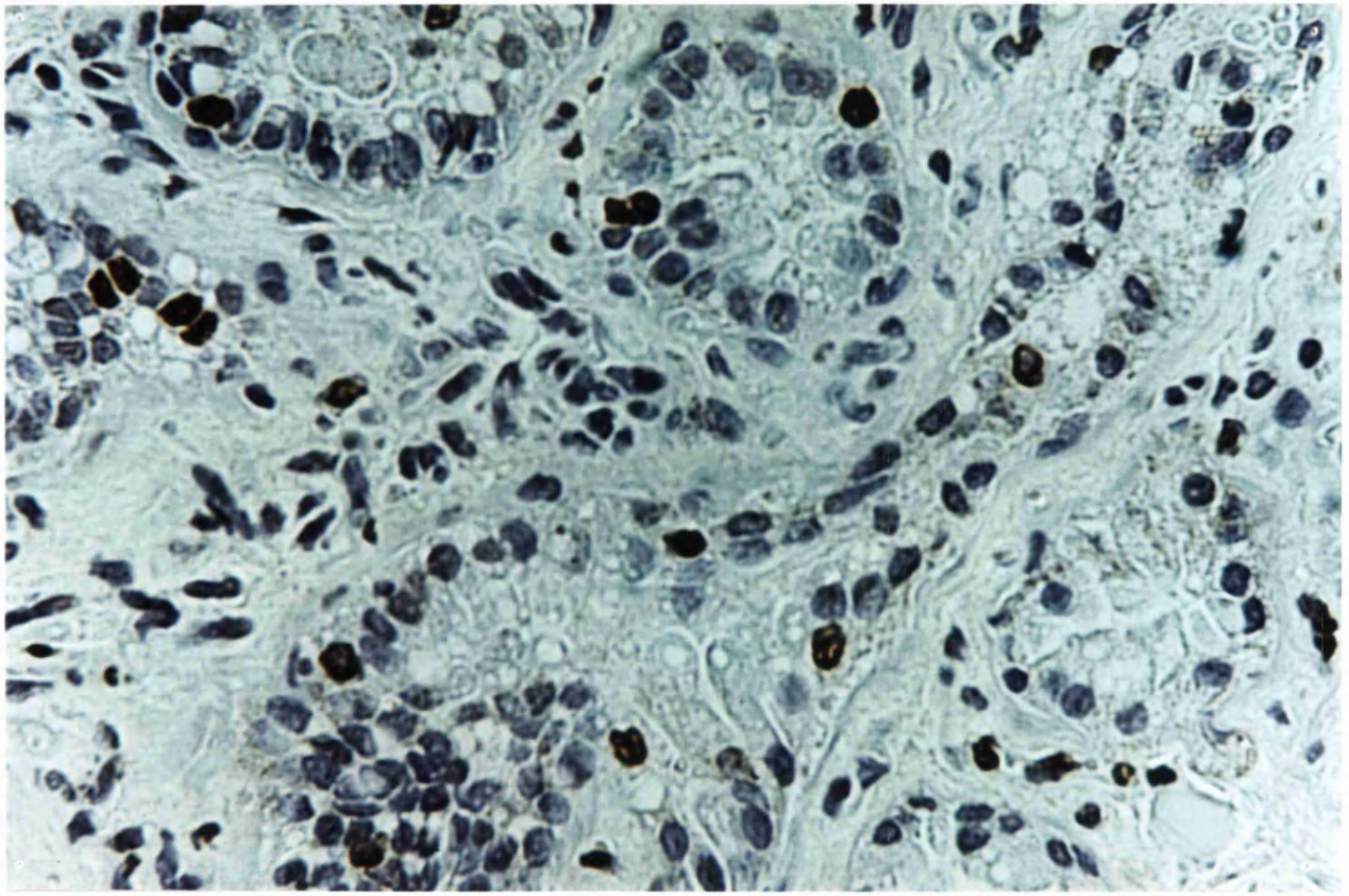


Plate: 3.1 Cell proliferation measured by incorporation of Brdu in vivo. Proliferating cells are stained dark brown.

A

Total cell number (per mm^2)						
Source:	**Time**	**Stage**		**Treatment**		
Factor:	*Sample*	*Pregnant*	*Lactating*	*Control*	*HX*	*GH*
Biopsy	417.6	478.0	357.2	415.9	321.4	515.4
Final	326.6	292.6	360.6	325.3	335.2	319.2
(SEM)	(19.5)		(27.6)			(33.8)
p biopsy	-	0.009		-	n.s.	n.s.
final		n.s.		-	n.s.	n.s.
Group:	***PC***	***PHX***	***PGH***	***LC***	***LHX***	***LGH***
Biopsy	452.8	331.8	649.3	379.0	311.0	381.5
Final	284.7	295.0	298.2	366.0	375.5	340.5
(SEM)						(47.8)
p biopsy	-	n.s.	0.013	-	n.s.	n.s.
final	-	n.s.	n.s.	-	n.s.	n.s.

B

BrdU-labelled cell number (per mm^2)						
Source:	**Time**	**Stage**		**Treatment**		
Factor:	*Sample*	*Pregnant*	*Lactating*	*Control*	*HX*	*GH*
Biopsy	28.08	48.89	7.28	24.42	22.17	37.67
Final	8.56	14.50	2.61	7.75	9.83	8.08
(SEM)	(0.92)		(1.6)			(1.60)
p biopsy	-	0.0001		-	n.s.	n.s.
final		0.0001		-	n.s.	n.s.
Group:	***PC***	***PHX***	***PGH***	***LC***	***LHX***	***LGH***
Biopsy	42.50	34.17	70.00	6.33	10.17	5.33
Final	11.83	18.67	13.00	3.67	1.00	3.17
(SEM)						(2.20)
p biopsy	-	0.0225	0.0001	-	n.s.	n.s.
final	-	n.s.	n.s.	-	n.s.	n.s.

Table 3.3 Total number of cells (**A**) and BrdU-labelled cells (**B**) in control (PC/LC), hemimastectomised or growth hormone groups during pregnancy (PHX, PGH) or lactation (LHX, LGH). Values are least square means and sems are for pooled data. Significance levels are comparisons relative to the appropriate control group.

Percentage labelled cells (%)						
Source:	**Time**	**Stage**		**Treatment**		
Factor:	*Sample*	*Pregnant*	*Lactating*	*Control*	*HX*	*GH*
Biopsy	6.44	10.54	2.34	5.69	7.00	6.63
Final	2.94	5.06	0.82	2.64	3.48	2.72
(SEM)	(0.40)		(0.60)			(0.69)
p biopsy	-	0.0001		-	n.s.	n.s.
final		0.0002		-	n.s.	n.s.
Group:	***PC***	***PHX***	***PGH***	***LC***	***LHX***	***LGH***
Biopsy	9.68	10.40	11.52	1.71	3.59	1.73
Final	4.10	6.67	4.43	1.18	0.29	1.00
(SEM)						(0.98)
p biopsy	-	n.s.	n.s.	-	n.s.	n.s.
final	-	n.s.	n.s.	-	n.s.	n.s.

Table 3.4 Percentage of BrdU-labelled cells in control goats (PC/LC), and those hemimastectomised or given growth hormone during pregnancy (PHX, PGH) or lactation (LHX, LGH). Values are least square means and sem's quoted are for pooled data. Significance levels are relative to the appropriate control groups.

DNA concentration (mg/g)						
Source:	**Time**	**Stage**		**Treatment**		
Factor:	*Sample*	*Pregnant*	*Lactating*	*Control*	*HX*	*GH*
Biopsy	3.48	3.37	3.60	3.10	3.28	4.07
Final	4.91	4.81	5.01	4.33	5.05	5.35
(SEM)	(0.29)		(0.41)			(0.51)
p biopsy	-	n.s.		-	n.s.	n.s.
final		n.s.		-	n.s.	n.s.
Group:	***PC***	***PHX***	***PGH***	***LC***	***LHX***	***LGH***
Biopsy	2.13	2.97	5.00	4.07	3.60	3.13
Final	4.63	4.50	5.30	4.03	5.60	5.40
(SEM)						(0.72)
p biopsy	-	n.s.	0.015	-	n.s.	n.s.
final	-	n.s.	n.s.	-	n.s.	n.s.

Table 3.5 DNA concentrations for control (PC/LC) goats, those hemimastectomised during pregnancy (PHX) or lactation (LHX), or given growth hormone during pregnancy (PGH) or lactation (LGH). Values are least square means and sem's for pooled data. Significance levels are comparisons to the appropriate sample of the control group

A

Acetyl CoA carboxylase activity (nmol/mg DNA)						
Source:	**Time**	**Stage**		**Treatment**		
Factor:	*Sample*	*Pregnant*	*Lactating*	*Control*	*HX*	*GH*
Biopsy	11.83	11.68	11.98	12.63	15.02	7.85
Final	11.42	13.73	9.10	9.87	13.87	10.52
(SEM)	(1.40)		(1.97)			(2.42)
p biopsy	-	n.s.		-	n.s.	n.s.
final		n.s.		-	n.s.	n.s.
Group:	***PC***	***PHX***	***PGH***	***LC***	***LHX***	***LGH***
Biopsy	16.87	15.40	2.80	8.40	14.63	12.90
Final	11.33	17.63	12.23	8.40	10.10	8.80
(SEM)						(3.42)
p biopsy	-	n.s.	0.013	-	n.s.	n.s.
final	-	n.s.	n.s.	-	n.s.	n.s.

B

Fatty acid synthetase activity (μmol/mg DNA)						
Source:	**Time**	**Stage**		**Treatment**		
Factor:	*Sample*	*Pregnant*	*Lactating*	*Control*	*HX*	*GH*
Biopsy	0.86	1.05	0.67	0.92	1.04	0.63
Final	0.98	1.35	0.62	0.95	1.06	0.94
(SEM)	(0.11)		(0.15)			(0.18)
p biopsy	-	n.s.		-	n.s.	n.s.
final						
Group:	***PC***	***PHX***	***PGH***	***LC***	***LHX***	***LGH***
Biopsy	1.36	1.45	0.35	0.47	0.64	0.91
Final	1.25	1.60	1.20	0.65	0.52	0.68
(SEM)						(0.26)
p biopsy	-	n.s.	0.018	-	n.s.	n.s.
final	-	n.s.	n.s.	-	n.s.	n.s.

Table 3.6 ACC (**A**) and FAS (**B**) enzyme activities for control goats (PC/LC) or those hemimastectomised or given growth hormone during pregnancy (PHX, PGH) or during lactation (LHX, LGH). Values are least square means. Significance levels are relative to the appropriate controls.

A

Galactosyl transferase activity (μmol/mg DNA)						
Source:	**Time**	**Stage**		**Treatment**		
Factor:	*Sample*	*Pregnant*	*Lactating*	*Control*	*HX*	*GH*
Biopsy	0.27	0.28	0.26	0.28	0.32	0.20
Final	0.33	0.37	0.28	0.34	0.32	0.31
(SEM)	(0.02)		(0.03)			(0.04)
p biopsy	-	n.s.		-	n.s.	n.s.
final		n.s.		-	n.s.	n.s.
Group:	***PC***	***PHX***	***PGH***	***LC***	***LHX***	***LGH***
Biopsy	0.39	0.33	0.11	0.18	0.31	0.28
Final	0.36	0.42	0.34	0.32	0.22	0.28
(SEM)						(0.06)
p biopsy	-	n.s.	0.006	-	n.s.	n.s.
final	-	n.s.	n.s.	-	n.s.	n.s.

B

Aryl esterase activity (μmol/mg DNA)						
Source:	**Time**	**Stage**		**Treatment**		
Factor:	*Sample*	*Pregnant*	*Lactating*	*Control*	*HX*	*GH*
Biopsy	0.73	0.97	0.49	1.06	0.63	0.50
Final	0.45	0.46	0.44	0.58	0.35	0.43
(SEM)	(0.10)		(0.14)			(0.18)
p biopsy	-	0.038		-	n.s.	0.045
final		n.s.		-	n.s.	n.s.
Group:	***PC***	***PHX***	***PGH***	***LC***	***LHX***	***LGH***
Biopsy	1.60	0.82	0.50	0.53	0.44	0.51
Final	0.56	0.39	0.43	0.59	0.31	0.42
(SEM)						(0.25)
p biopsy	-	0.048	0.009	-	n.s.	n.s.
final	-	n.s.	n.s.	-	n.s.	n.s.

Table 3.7 GT (**A**) and AE (**B**) enzyme activities for control goats (PC/LC) or those hemimastectomised or given growth hormone during pregnancy (PHX, PGH) or during lactation (LHX, LGH). Values are least square means and sem's were calculated from pooled data. Significance levels are relative to the appropriate controls.

Hemimastectomy had no apparent effect on the level of differentiation with values similar to controls throughout. The single exception was aryl esterase (which is not involved in milk synthesis) in the biopsy sample of the PHX group when it was lower than controls ($p=0.048$), this was also seen in PGH ($p=0.009$) so probably did not reflect a treatment effect *per se*. The similarity in results to control groups shows that the cell population in hemimastectomised animals was well differentiated and suggested that there had been no measurable proliferative response during the measurement period which would have resulted in new and relatively undifferentiated cells.

DNA concentrations increased when growth hormone was given during pregnancy ($p=0.015$) but this was only apparent in biopsy samples and no effect was seen during lactation. Enzyme activities were significantly lower in PGH compared to PC groups ($p \leq 0.018$), but these subsequently increased and were not significantly different to control levels in final samples, collected 4 weeks later. This suggested that there was a population of relatively undifferentiated cells present at biopsy (immediately after the cessation of treatment), also detected by the increased DNA concentration, and that the secretory cell population became more mature over the next four weeks, achieving a level of differentiation similar to those of the other groups.

6. DISCUSSION

Hemimastectomy in late pregnancy had a significant stimulatory effect on milk yield *post partum* with a peak yield of 1.96 l/d compared to 1.23 l/d in controls and 1.40 l/d in growth hormone-treated groups. When hemimastectomised shortly *post partum* yields again increased to a peak of 1.71 l/d while control and growth hormone groups achieved 1.50 and 1.40 l/d, respectively. This increased milk production was supported by increased mammary size and parenchymal tissue weight in both reproductive states although the effects were more pronounced during pregnancy, and consequently these groups had the highest gland DNA contents. HMX was associated with the lowest cell numbers per mm^2 in biopsy samples, indicating that cells tended to be larger in size and the percentage incorporation of BrdU was above that of controls showing that there had been a proliferative response. It must be borne in mind that in this group the biopsy sample was taken 6 weeks, and final samples 10 weeks, after surgery was performed, giving ample time for both proliferation and subsequent differentiation of cells to occur. These observations were supported by the measurements of enzyme activity; milk enzyme activities in PHX were higher than PC in final samples and LHX were also higher than controls in biopsy samples, indicating that cellular differentiation had indeed occurred. So hemimastectomy was successful in stimulating a 'compensatory growth' response, particularly so during late gestation, indicating a considerable plasticity in the growth pattern of the mammary gland. Restrictions on mammary size include the level of gland development during puberty, gross body weight, partly limited by the size of the mammary fat pad, by steroid hormones (particularly oestradiol) and modulated by tissue sensitivity (via receptors) to a variety of factors, among others (Sejrsen, 1994). The results presented here support the phenomenon of compensatory growth when the animal 'senses' damage or loss of the mammary gland, allowing lactation to proceed with a reduced but adequate supply of milk available for the new born, improving their chances of survival.

Growth hormone increased yields only during the treatment period in lactating goats, but when given during gestation there was a carryover effect of increased yield in the post-treatment period. The latter observation corresponded with increased mammary gland, parenchymal tissue weights, and therefore total DNA content (also confirmed by significantly increased DNA concentration in biopsy samples). BrdU incorporation and the percentage of labelled cells were also elevated showing that there had been a marked proliferative response. However these cells were relatively

undifferentiated in terms of milk synthesis, shown by depressed enzyme activities. However in final samples these had increased to control levels showing that there was a differentiative response, albeit delayed.

When growth hormone was given during lactation there were no significant stimulatory effects on mammary growth, either gross or on cell numbers, supporting the observation that the alteration in milk yield was short-term and restricted to the treatment period. However this raises the question of how the increase in milk yield during treatment was possible? Measurements of milk enzyme activity at biopsy showed an increase compared to controls, an effect absent in final samples- 4 weeks after treatment had stopped, suggesting that improved production was due to increased enzyme activity and cellular differentiation.

Growth hormone is mammogenic in pregnant goats and this is supported by *in vitro* observations of GH and somatomedins stimulating proliferation and differentiation, respectively, of certain cell types. The latter synergise with PDGF and EGF to promote fibroblast cell division and other cell types probably require other combinations of growth factors for mitogenesis (Wallis, 1985). The lactation response was not mammogenic and apparently reflected improved milk secretion in the existing cell population. This may have, in turn, be supported by improved substrate supply and nutrient partitioning towards the mammary gland but we have no evidence to support this hypothesis, although the general effect has been well documented (Bauman & Currie, 1980).

There was a clear difference in response to treatment in the two reproductive states investigated- late gestation and early lactation, most probably influenced by hormonal changes. One important hormonal difference is the presence of placental lactogen during pregnancy. This hormone is mammogenic in cows (Byatt *et al*, 1994) and sensitive to the requirements of the foetus (Hayden *et al*, 1979) and could synergise with other hormones and factors to enhance mammary growth, however it is absent during lactation. The rapid drop in progesterone at parturition and parturition itself, may also signal a change in mammary gland responsiveness to various hormones and factors and lactation is associated with the regular release of lactogenic hormones and oxytocin when suckling or milking occurs. A further factor in the different 'reproductive responses' will be the increasingly high level of foetal demand for nutrients in late gestation while in early lactation these are redirected towards the mother, facilitating lactogenesis.

Bines & Hart (1981) said that the "potential of the (bovine) mammary gland to synthesise milk probably is determined fully at parturition" and to an extent this is true for growth hormone. When given during lactation it improved the 'efficiency' of milk production rather than increasing the cell population so that could be seen as fulfilling the potential of the gland. However hemimastectomy shortly after parturition showed that the mammary gland still retains the capacity for growth and so application of appropriate stimuli could further enhance milk production *post partum*.

The long term effects of these treatments were not investigated in this study but effects on lactation persistency could be postulated, especially if the pattern of mammary growth has been altered, as seen with hemimasectomy and to a lesser extent with growth hormone during pregnancy. Growth hormone and IGF-1 suppress plasmin activity in milk, and the elevation of circulating IGF-1 by exogenous bST and IGF-1 reduces plasminogen activator secretion in bovine mammary cells (Turner, 1991). This suggests that there might be reduced proteolysis and remodelling of the mammary gland which in turn could maintain secretory function during declining lactation.

CHAPTER 4

THE INFLUENCE OF PROLACTIN ON LACTATION PERSISTENCY

1. INTRODUCTION

In ruminants, prolactin concentrations are high during early and peak lactation but subsequently decline over the remainder of lactation. This pattern correlates temporally with milk production peaking and gradually dropping as lactation progresses (Hart, 1975b). This raises the question of whether or not the association between the hormone and milk production is purely temporal, or if there is a closer, physiological link between high prolactin and high milk production. If they are indeed related then could maintaining prolactin at a high level throughout lactation also keep milk production near maximal levels?

Evidence to support the hypothesis of a role for prolactin during established lactation has been seen in some species. In goats, correlations are found between average (monthly) prolactin concentrations and milk yield (Hart, 1975a) but studies using bromocriptine to decrease endogenous prolactin dispute the relevance of this since this treatment has little effect on yield (Forsyth & Lee, 1993). In rats, lactation could be extended by maintaining the suckling stimulus, and therefore prolactin, by regularly swapping an older litter with a younger set (Flint *et al*, 1984). A variety of pharmacological agents which stimulate prolactin release have also been used *in vivo* to investigate their effects on milk production and mammary development. One agent, perphenazine (PZ), proved to be effective in inducing prolactin release in sheep and had positive mammogenic effects (Morag *et al*, 1971) although there was no apparent effect on milk production when given to sheep in mid-lactation (Bass *et al*, 1974). This drug has also been implanted in the brain median eminence of goats and had a significant and positive effect on prolactin levels and maintained milk production, ie. improved lactation persistency. However, this administration route required the use of a specialised surgical procedure and there were some negative post-operative side-effects (Vandeputte-Van Messom & Peeters, 1982). So there is some evidence to support the hypothesis that prolactin is not only involved in the establishment, but also in the subsequent maintenance of lactation *post partum*, and that the decreased prolactin concentrations observed as lactation progresses can contribute to the general decline in milk production in ruminants.

Unfortunately there are some side-effects associated with perphenazine treatment

but these vary between species, depending on dose level and the delivery route used. In general, ruminants are less amenable to intramuscular or interperitoneal injections with side effects of pain and tenderness at the injection site, behavioural problems (generally lethargy) and weight loss (Shani *et al*, 1975), and a bolus injection limits the longevity of the drug administered. Oral dosing has been used successfully without side-effects in rats (by doping drinking water and thereby facilitating small but frequent PZ dosing) (Stringer *et al*, 1990) and horses (Ireland *et al*, 1991) but has not been attempted in ruminants. Although surgical implantation of perphenazine is clearly successful in goats, it is a complex procedure to carry out and has inherent disadvantages. The development of a delivery route which did not cause significant side-effects and was relatively straightforward to administer would be desirable for further investigations in this area.

The overall aim of this work was to clarify the role of circulating prolactin on milk production by elevating and maintaining prolactin concentrations using perphenazine. An initial experiment was designed to determine a minimum effective perphenazine dose when administered by intravenous infusion. The second experiment attempted to maintain high prolactin concentrations over a lactation and monitor the effects of this on lactation persistency and a variety of other parameters. A change in milking frequency was incorporated into this experiment in an attempt to elucidate whether or not prolactin was acting locally or systemically on the mammary gland; one gland was switched to once-daily milking (1X) while the contralateral gland continued on routine twice-daily (2X) milking so both glands experienced similar systemic but different local endocrine environments.

2. ANIMALS

British Saanen goats from the Institute herd were used. Hay and water were available *ad libitum* and, in addition, a proprietary concentrate feed was offered twice daily, dependent on physiological state, as follows: non-pregnant 0.5 kg/d, pregnant 1.0 kg/d and lactating goats 1.5 kg/d.

3. EXPERIMENTAL DESIGN

Two experiments were carried out consecutively. Firstly, goats in various physiological states were infused for up to 4 days with varying doses of PZ and the effects on prolactin secretion were studied. In the second experiment lactating goats

were treated with PZ orally or by injection and were studied over 26 weeks during which time a variety of physiological parameters were measured.

3.1 Experimental groups

The first experiment utilised three groups of four goats which were (i) non-pregnant and non-lactating (December 1991- January 1992), (ii) pregnant with stages of gestation ranging from weeks 14 to 18 (February 1992) and (iii) lactating in lactation weeks 1-3 (March 1992); three goats appeared in both latter groups.

Two groups of four lactating goats matched for parity and body weight, with kidding dates ranging from 8th-24th March 1992, were used in the second experiment of treatment throughout lactation.

3.2 Treatments

For infusions, animals were housed singly in metabolic crates within sight and sound of other goats, and lactating animals were temporarily disconnected from the infusion apparatus when milked twice daily at 0800 and 1600h. PZ was dissolved in a minimal volume of 0.2N HCl and added to sterile 0.9% w/v sodium chloride solution to yield final concentrations of 82- 552mg PZ/l, depending on the rate of infusion, the weight of the animal, the dose level and duration of infusion required. Vehicle or PZ-containing solutions were infused through polyethylene tubing (Dural SP70/102 tubing, Critchley Electrical Products Pty Ltd, Auburn, Australia) at a constant rate via an indwelling jugular vein cannula using a peristaltic rocking pump (Frost Instruments Ltd, Cambridge, UK). Flow rates were constant during each infusion (range 5.1- 7.2 ml/h) while two of the non-pregnant, non-lactating group received infusate at rates of 17 and 19 ml/h due to the inner diameter of the peristaltic tubing used.

Vehicle alone was infused for a 24 hour pre-treatment period followed by PZ given at doses as follows: non-pregnant, non-lactating animals received 0.47mg PZ/kg/d for 48h, pregnant goats received 1.01mg PZ/kg/d for 96h while lactating animals were given 0.26mg PZ/kg/d for 56h followed by 0.44mg PZ/kg/d then 1.10mg PZ/kg/d for 24h each. PZ infusions were followed by a post-treatment infusion of vehicle alone for 8 or 8.5h. The amount of infusate administered was determined by weight difference at regular and noted times, allowing the exact dose to be calculated for each animal. In all groups, the switch between vehicle alone and PZ-containing infusions and changes in PZ dose were made between 0900 and 1000 hours.

In experiment 2, treatment through lactation, PZ was given as follows:

lactation week 13-15: oral dose of 25mg/goat/d
16-20: oral dose of 50mg/goat/d
21-22: daily s.c. injection of 25mg/goat/d
23-28: daily s.c. injection of 50mg/goat/d

For oral dosing, PZ was suspended in distilled water at a concentration of 5mg/ml and administered as a single bolus between 0900 and 1000h daily, using a commercial dosing gun. For injection, PZ was dissolved in a minimal volume of 0.2N HCl, added to 30% polyvinylpyrrolidone solution, pH adjusted to 7.0 and administered daily at 1400h.

Lactating goats in experiment 2 were milked twice daily at 0800 and 1600h from parturition until lactation week 25. From week 26 onwards, right glands were switched onto a regime of once-daily milking at 0800h only (1X), while left glands continued to be milked twice daily (2X).

3.3 Measurements

In experiment 1, during infusions, regular blood samples were collected from indwelling jugular vein catheters, plasma prepared and analysed for prolactin content. Sampling frequencies varied between groups: at hourly intervals between 0900 and 1700, and at 2000 and 2200 in the non-pregnant, non-lactating goats and at two hourly intervals from 0900-1700 and at 1945 and 2145 hours in the pregnant and lactating groups. All goats were monitored over an 8 or 8.5 hour period (0900-1700/1730h) following the cessation of PZ treatment.

In the second experiment milk yields obtained at each milking were recorded throughout. Mixed samples of bulk milk (the whole flow) were collected from both glands in weeks 11, 12, 13, 16, 18, 20, 22, 25, 28, 32 and 37 and analysed for protein, total solids, rapid fat and lactose contents. Additional samples were collected for casein analysis (see Chapter 7).

Blood samples were obtained twice weekly by jugular venepuncture at 1400h while more frequent profile sampling was carried out in weeks 11, 13, 16, 20, 25, 28, 32 and 37. For this, catheterisation of the jugular vein was carried out at approximately 1600h on the day preceding sampling. Samples were collected from indwelling cannula immediately before, and 20 minutes after, morning and afternoon milkings, and at 1000, 1200 and 1400h. Plasma was prepared and subsequently analysed for

prolactin and growth hormone content.

Empty udder volume was measured by water displacement in weeks 11, 12, 13, 16, 20, 25, 30, 32 and 37 of lactation. Alveolar and cisternal storage volumes were determined by catheter drainage 5 hours after a normal morning milking in weeks 13, 25 and 37.

General nutritional status was monitored by measuring body weights once-weekly and additional measurement of food refusals (uneaten concentrate feed) were made on weekdays by weighing the amount remaining prior to each milking.

4. RESULTS

4.1 Perphenazine infusions

Typical profiles for prolactin concentrations before, during and after PZ infusion from a representative individual in each group are illustrated in Figure 4.1. Concentrations for each animal were averaged over each treatment period and group means calculated (Figure 4.2). Comparisons between groups were made using Student's unpaired t-test.

Significant differences in plasma prolactin concentrations were found between non-pregnant/non-lactating, pregnant and lactating groups, during the pre-treatment infusion periods (9.6 ± 2.0; 49.6 ± 7.9 and 566 ± 129 ng/ml prolactin, respectively, $p<0.001$). PZ infusion significantly increased prolactin concentrations in all three groups; non-pregnant, non-lactating goats showed the greatest incremental response (from 9.6 ng/ml to 74.6 ng/ml during treatment, $p<0.0001$). Prolactin concentrations fluctuated over the day, and in response to treatment, with concentrations of up to 78 ng/ml pre-treatment, 300 ng/ml during PZ and to 88 ng/ml post-treatment.

Pregnant animals had an intermediate increase during treatment (49.6 to 293 ng/ml, $p<0.0001$) and there was some carryover of PZ's effect into the post-treatment period: prolactin dropped from 293 to 184 ng/ml ($p=0.014$) but this was still above pre-treatment levels ($p=0.0014$). Again there was variability across the day with concentrations of up to 134 ng/ml prolactin being seen pre-treatment, 1395 ng/ml during PZ and 48-701 ng/ml post-treatment.

Lactating goats approximately doubled their prolactin concentrations (556 to 1070 ng/ml) in response to the first dose of PZ ($p=0.0023$) but the next highest dose (0.44mg PZ/kg/d) was associated with a drop in prolactin ($p=0.011$) although concentrations remained above pre-treatment levels ($p=0.53$). When the dose was

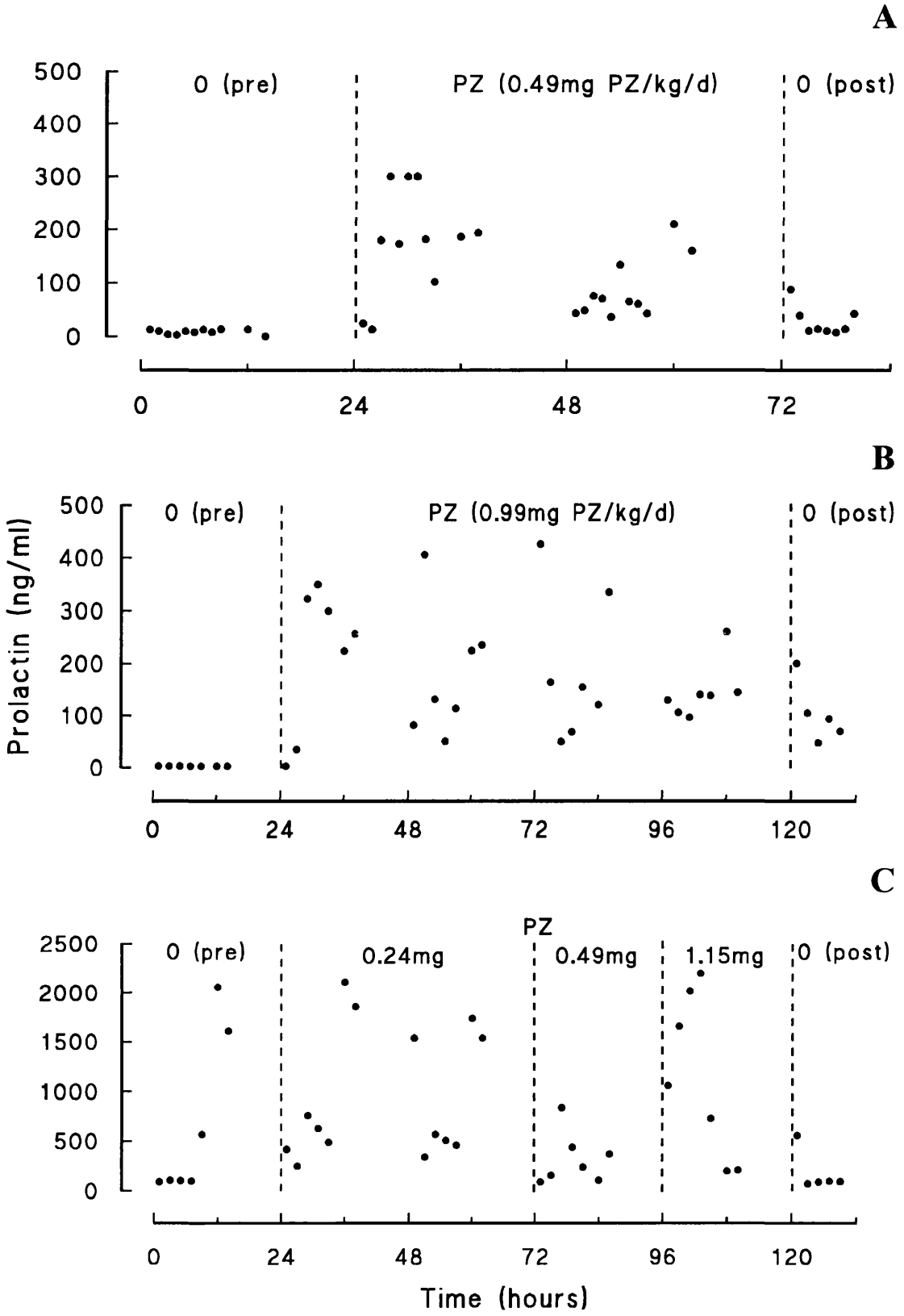


Figure 4.1 Prolactin responses to perphenazine (PZ) infusion in selected individuals in (**A**): non-pregnant, non-lactating (goat B18), (**B**) pregnant and (**C**) lactating animals (B & C represented by goat B19).

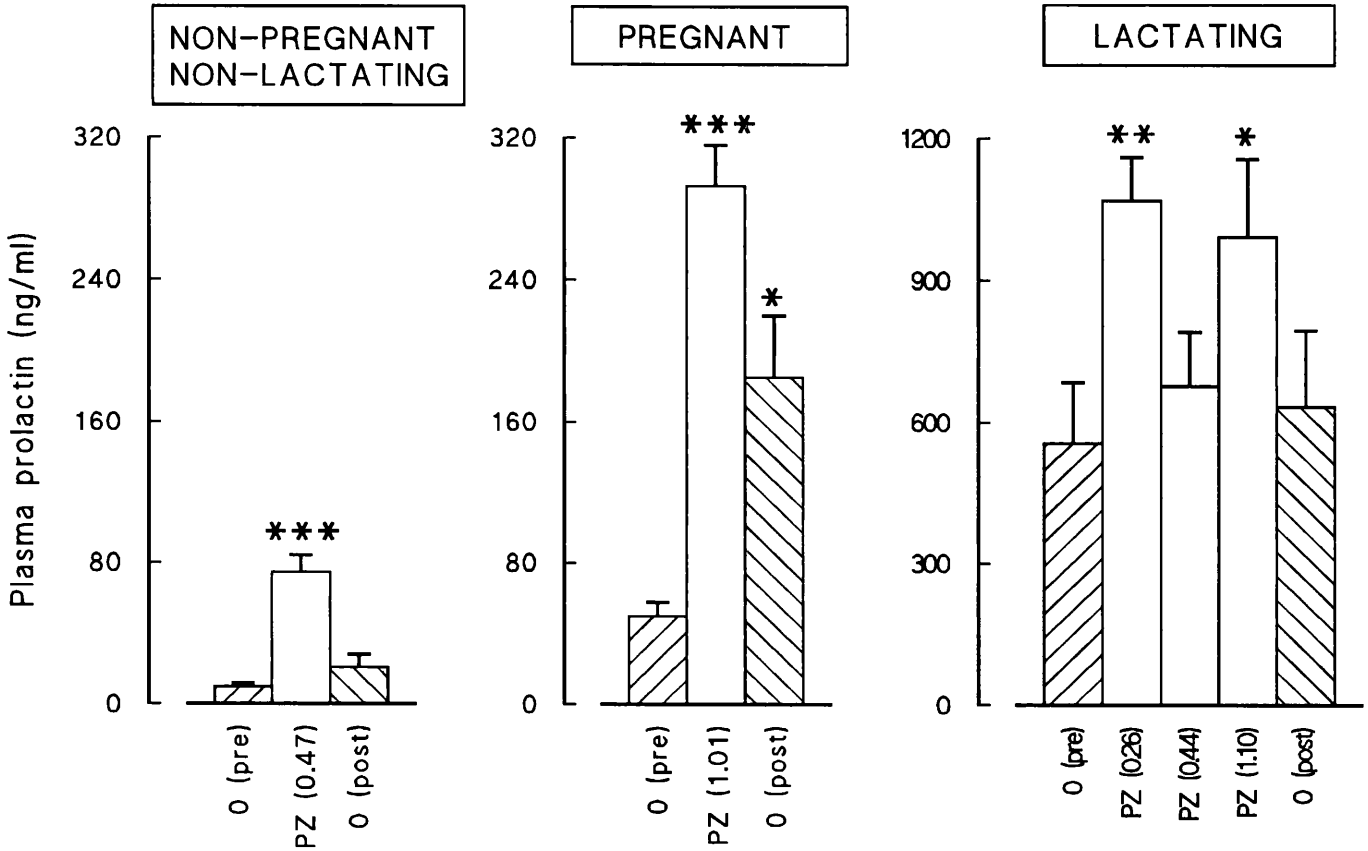


Figure 4.2 The effect of infusing perphenazine (PZ) on plasma prolactin levels in animals in a variety of physiological states. n=4 in each group. Open bars represent periods of PZ treatment, hatched represent control periods (saline infusion). Asterisks represent significance of the prolactin response as follows: *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

further increased to 1.10mg PZ/kg/d, 24 hours later, prolactin was again increased, relative to pre-treatment levels ($p=0.047$). This group showed the widest variation in prolactin concentrations (ng/ml); pre-treatment 46-1165, $PZ_{0.26}$ 588-2005, $PZ_{0.44}$ 482-1002, $PZ_{1.10}$ 231-1438 and post-treatment 84-2005. Samples were collected one hour after milking (morning and afternoon) in this group to avoid the transient prolactin surge associated with milking itself, to make conditions between the groups as similar as possible. The range in prolactin concentrations during each treatment phase are reflected in the standard errors calculated in Figure 4.2 for all groups.

4.2 Long-term perphenazine treatment

Milk yields and lactation persistency

Lactation curves showing the average daily yields for each group were plotted (Figure 4.3 A). Perphenazine was administered orally from weeks 13-20, by injection on weeks 21-27 and treatment was discontinued in week 28. Milk yields declined gradually but there was a more rapid decrease in the perphenazine group apparently associated with PZ injections. When single gland milk yields were compared between weeks 20 (last week of oral PZ dose) and 27 (last week of injections), a significant drop was found in both glands of the PZ group (2X: 0.58 $\pm$ 0.15 kg/d; 1X: 0.65 $\pm$ 0.08 kg/d, $p \leq 0.033$), and this drop was greater than that seen in controls over the same period (2X: 0.38 $\pm$ 0.12 kg/d; 1X: 0.42 $\pm$ 0.09 kg/d, $p \leq 0.051$). The rapid decline in yield in the PZ group corresponded temporally with decreased food intake and fluctuations in body weight (Figure 4.4). Treatment was stopped during week 28, resulting in a temporary recovery in yields, followed by a gradual decline. Statistical analysis of yields (ANOVA) indicated that there was no effect of treatment on yields ($p=0.266$) but the change in milking frequency and lactation period both affected yield ($p<0.001$).

There was no apparent effect of PZ treatment on lactation persistency, determined by regression analysis of individual yield profiles (Figure 4.3 B) and subsequent ANOVA, although again lactation period had a significant negative effect on persistency ($p=0.018$). There was a slight trend towards slightly improved persistency of glands which were milked once-daily in late lactation, compared to contralateral 2X milked glands (Table 4.1). This was observed in 5 out of 8 animals, independently of treatment, but differences between glands were not significant ($p=0.41$, paired t-test).

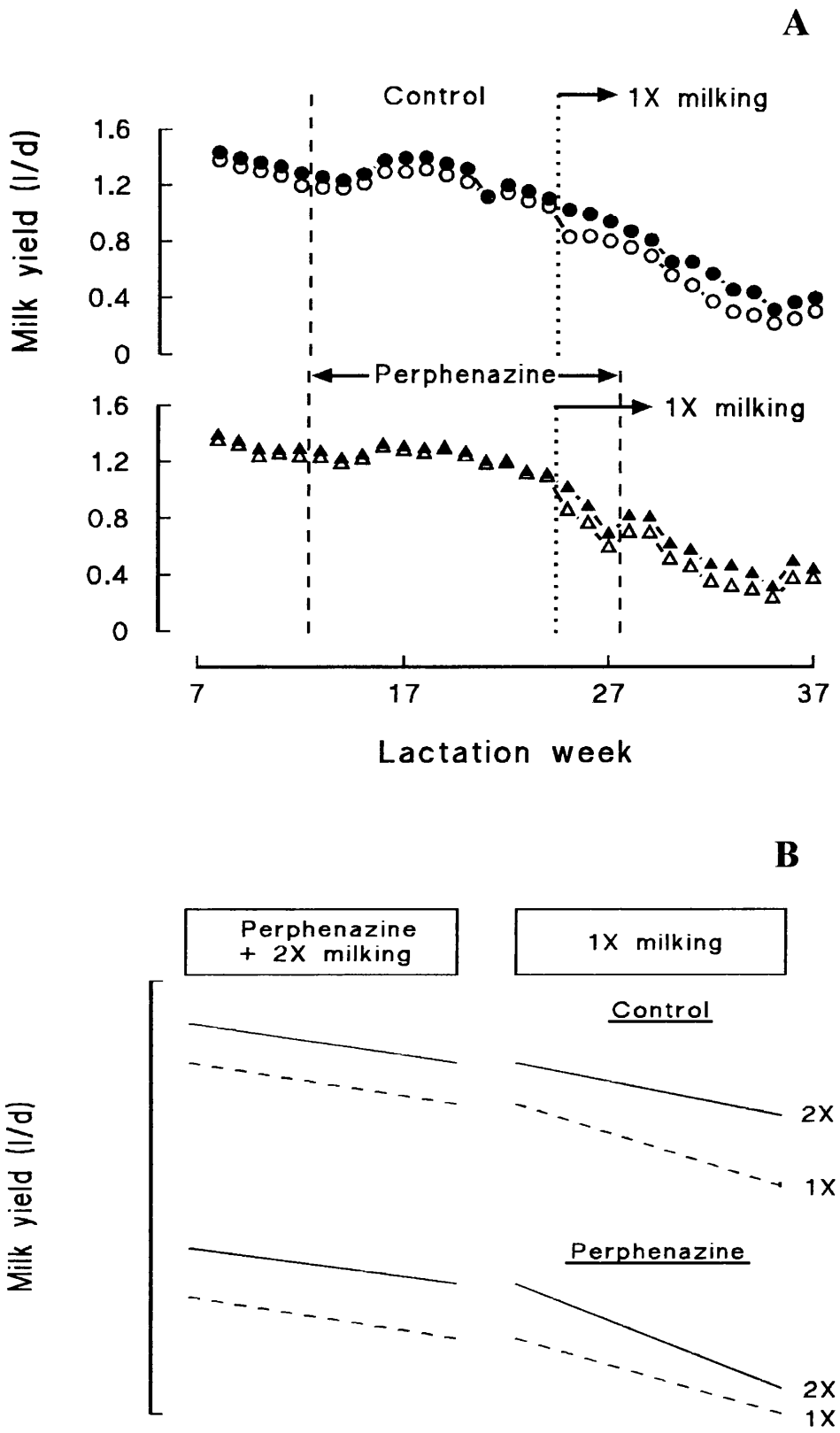


Figure 4.3 (**A**) Average daily milk yield from control 2X (●) and 1X (○) glands and perphenazine 2X (▲) and 1X (Δ) glands. Vertical lines represent changes in PZ treatment- orally on weeks 13-20 and by subcutaneous injection until week 28. (**B**) Regression analysis of milk yields was conducted (full analysis in Table 4.1), means obtained for each group in weeks 13-25 and weeks 29-37, and lines plotted with displacement for clarity.

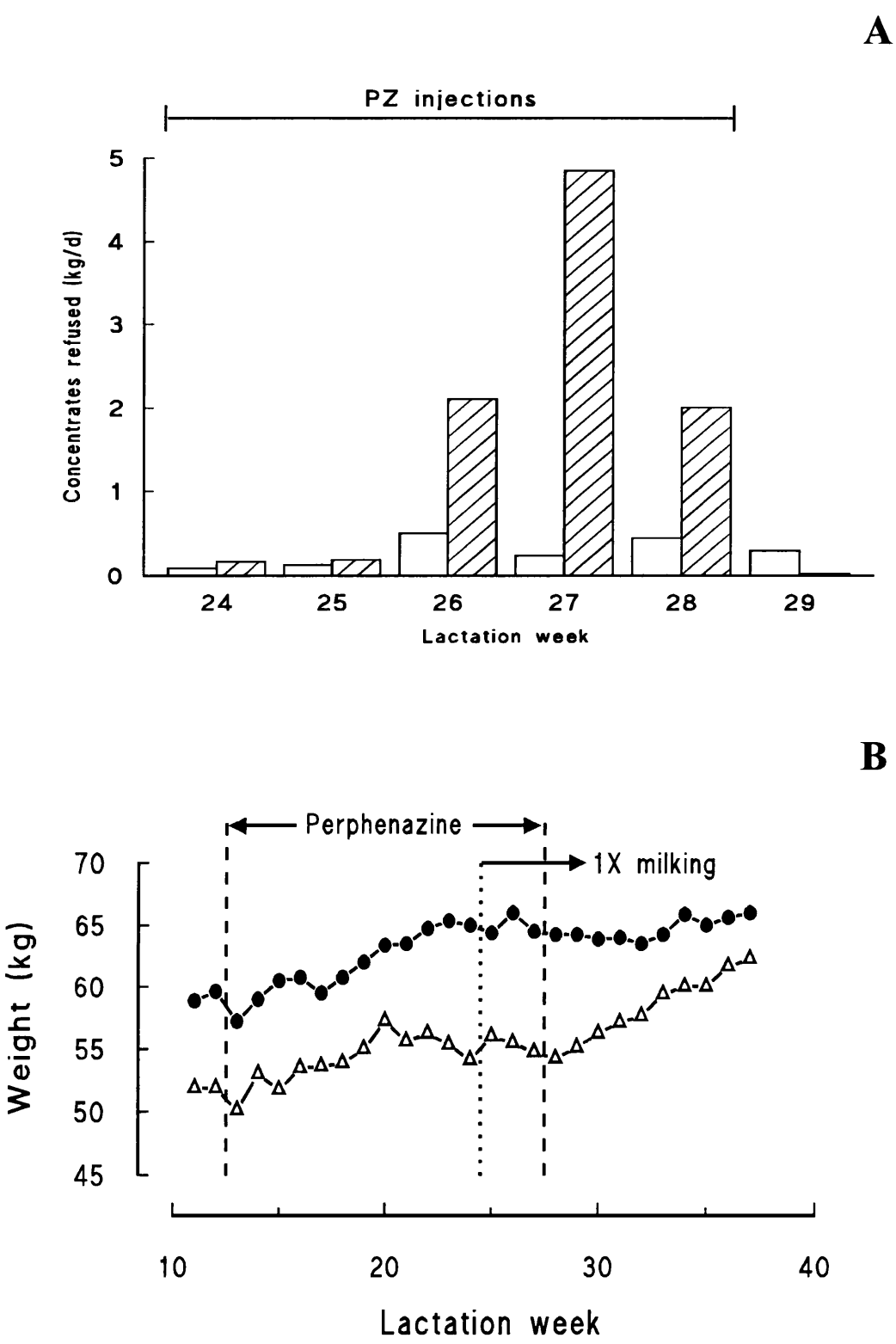


Figure 4.4 Measurements of nutritional status in experiment 2.
(**A**) Average daily concentrate refusals from weeks 24-29 in control (open bars) and PZ (hatched bars) groups when PZ was given by injection at a dose of 25mg PZ/d in week 23 and at 50mg PZ/d during weeks 24 to 28. Treatment was discontinued in week 28.
(**B**) Average body weights for control (●) and PZ (Δ) groups.

Group	Milked	Treatment only **m** (weeks 13-25)	Treatment + 1X **m** (weeks 29-37)
Control	**2X**	-47.7	-48.5
		10.8	-98.1
		-18.9	3.3
		-21.2	-101.0
	mean	-19.2	-61.1
		r^2 46.8%, p=0.050	r^2 67.8%, p=0.159
Control	**1X**	-46.4	-38.3
		13.9	-83.3
		-17.0	-19.0
		-30.6	-83.6
	mean	-20.0	-56.0
		r^2 49.6%, p=0.029	r^2 82.6%, p=0.047
Perphenazine	**2X**	-6.5	-83.0
		-12.4	-20.3
		-17.9	-80.1
		-31.6	20.8
	mean	-17.1	-40.6
		r^2 40.0, p=0.073	r^2 52.9, p=0.072
Perphenazine	**1X**	-13.7	-59.7
		-3.8	-23.5
		-29.2	-87.5
		-33.2	25.2
	mean	-20.0	-36.4
		r^2 36.0%, p=0.170	r^2 53.0, p=0.063

Table 4.1 Summary of regression lines fitted to individual milk yield profiles for each animal's glands. **m**=change in average daily yield, ml/week. r^2 and p indicate the accuracy and probability of the best line being fitted to the data.

Nutritional status

Orally administered PZ (given during weeks 13-20) had no apparent effect on appetite, in week 20 mean refusals were 26.2 ± 19 g/d in the control group and 43.1 ± 15 g/d in PZ-treated animals ($p=0.52$). However, within 2 to 3 weeks of PZ injections commencing, concentrate feed refusals began to steadily increase (Figure 4.4A) with all treatment animals refusing food (albeit to variable levels) during weeks 26 and 27. The highest levels of refusals in the PZ group were obtained during week 27 with control animals refusing 558 ± 239 g/d while PZ animals refused 4852 g/d ($p=0.12$). The increase in refusals over this period (week 20 compared to week 27) did not achieve significance in either group ($p=0.12$ and $p=0.073$ in controls and PZ, respectively, paired t-test), reflecting the variability between individuals. Appetites were rapidly regained when injections were stopped during week 28. Goat weights were determined weekly (Figure 4.4B) and the rate of weight gain between the groups was similar during the period of oral PZ dosing (CON: +0.549 kg/wk; PZ +0.518 kg/wk). From the cessation of treatment to the end of the study (weeks 28-37) the PZ group had a much higher rate of weight gain than controls (+0.889 & +0.227 kg/week, respectively).

Milk composition

There were general trends of increased protein, fat and solids content from lactation week 20 onwards. Lactose showed a different pattern with concentrations initially high then dropping to a lower level for the remainder of lactation (Figure 4.5). There were no significant differences in milk protein, lactose, fat or solids content either between glands within individuals, or between the experimental groups over the period studied.

Hormone concentrations

Lactational profiles for prolactin and growth hormone obtained from routine blood sampling are shown in Figures 4.6 and 4.7, respectively. Weekly concentrations were averaged over each treatment phase for each group and compared using Student's unpaired t-test. Prolactin levels were not significantly different between the groups except in the last phase of 1X milking (after PZ treatment was discontinued) when prolactin was lower in the control group. Growth hormone concentrations were similar during the pre-treatment phase but became lower in PZ-treated animals once treatment

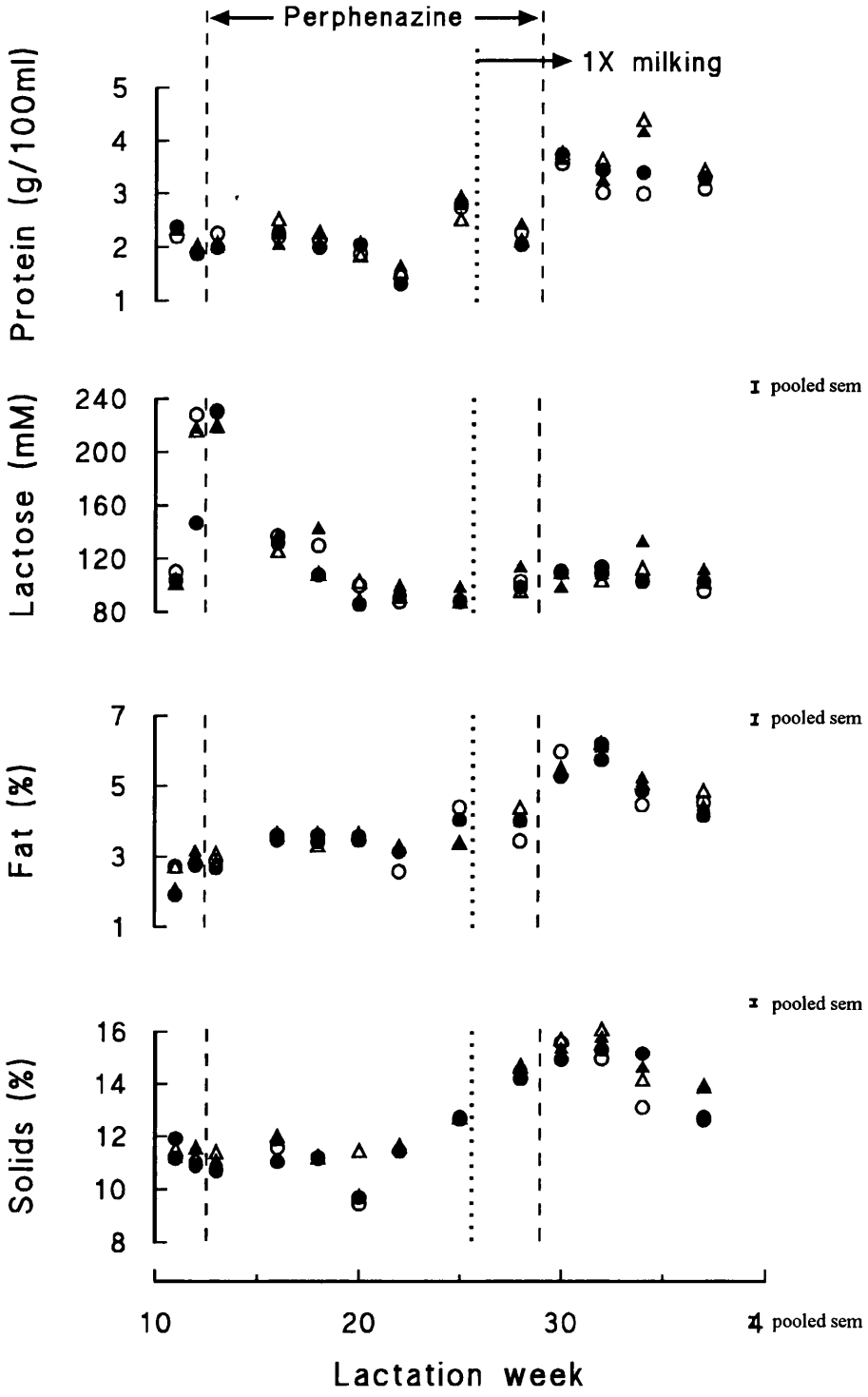


Figure 4.5 Milk compositional analysis determined in Experiment 2 in control 2X (●) and 1X (○) glands, and in perphenazine 2X (▲) and 1X (△) glands. Dashed lines show the period of treatment while the dotted line illustrates the commencement of 1X milking.

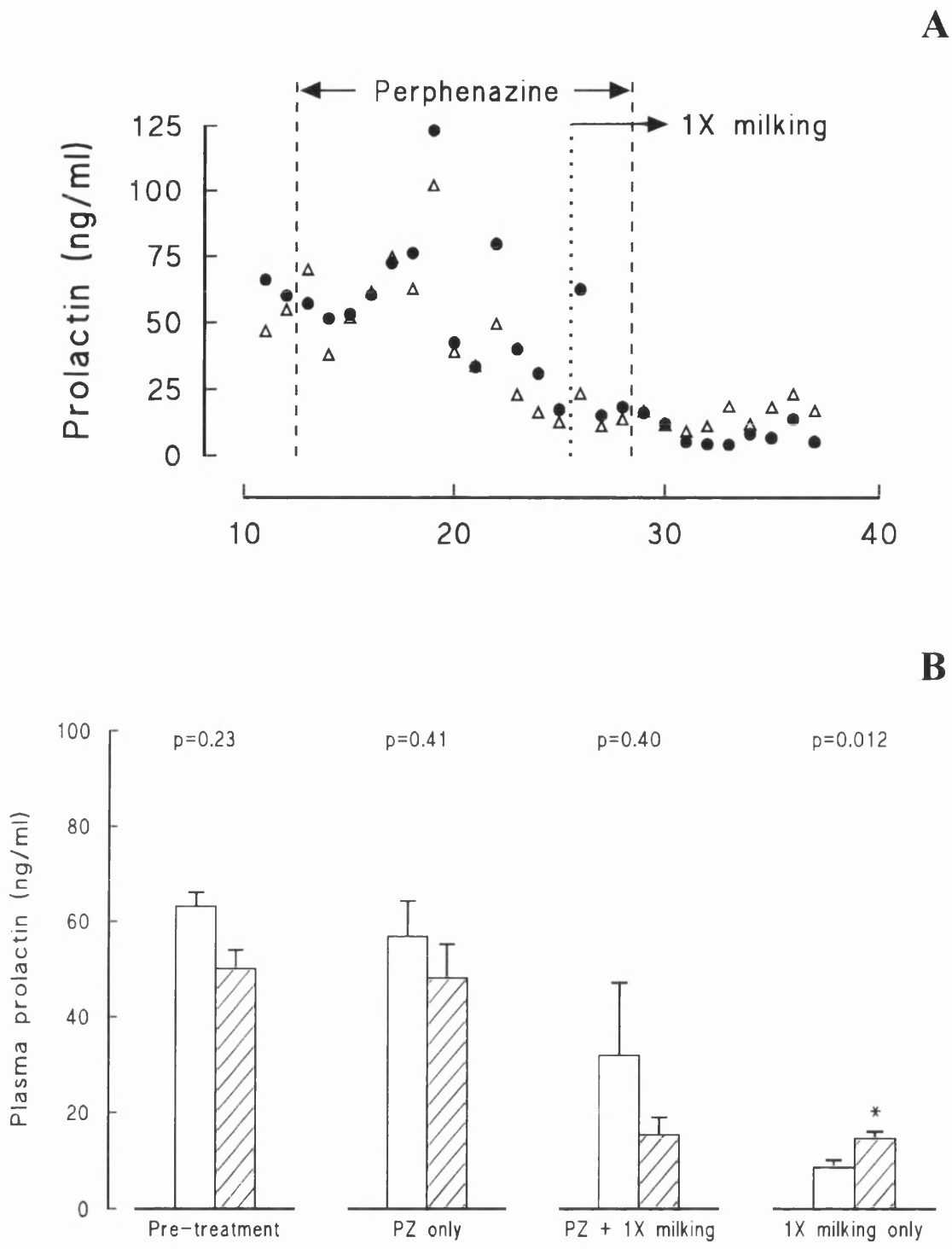


Figure 4.6 Effect of treatment on prolactin concentrations determined by routine blood sampling. (**A**) Lactational profile for control (●) and PZ (Δ) groups. (**B**) Statistical analysis of prolactin levels tested by Student's unpaired t-test. Open bars- controls, hatched bars- PZ treated, n=4 for both groups.

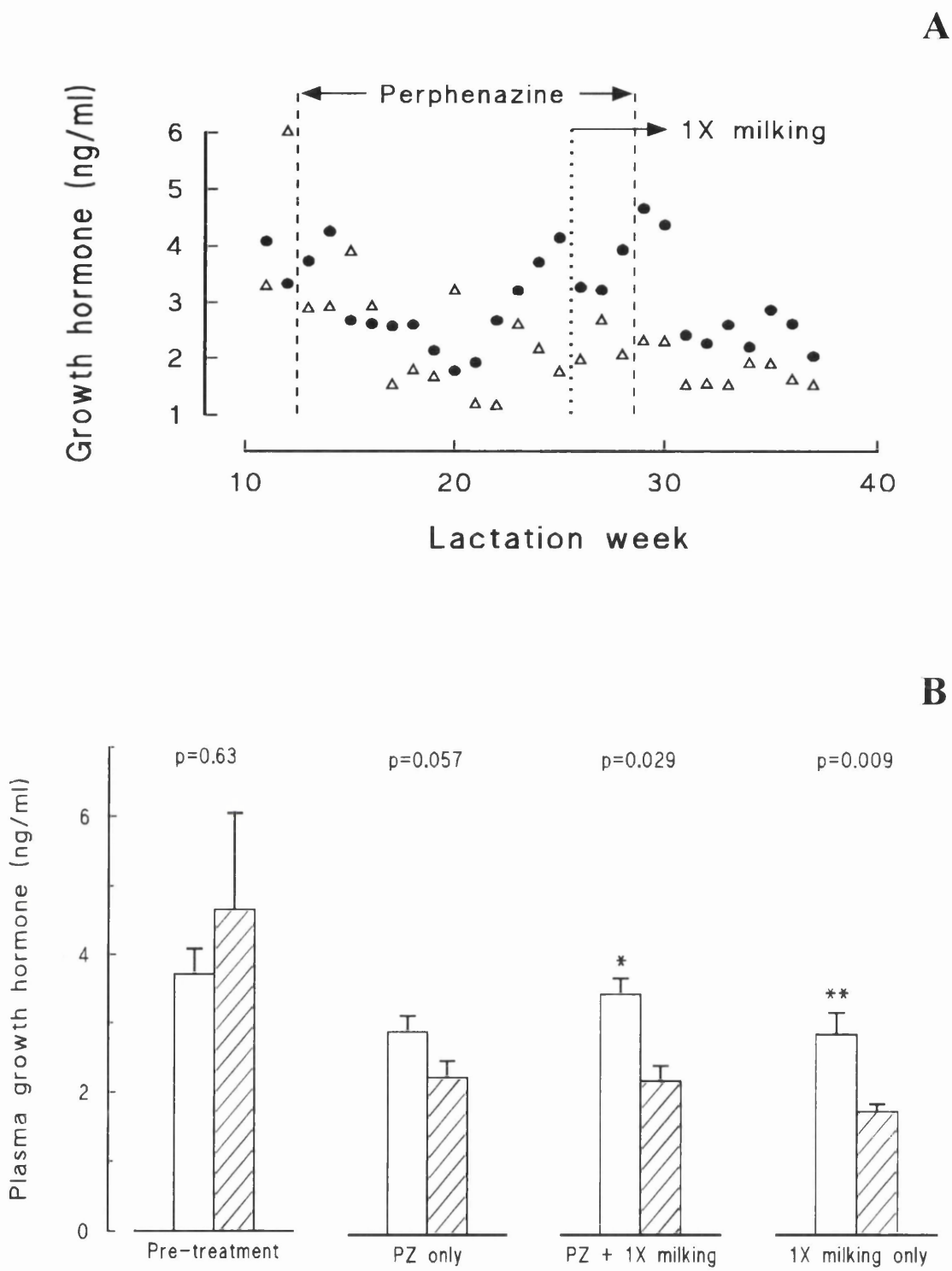


Figure 4.7 Effect of treatment on growth hormone levels determined by routine blood sampling. (**A**) Lactational profile for control (●) and perphenazine (Δ) groups. (**B**) Statistical analysis of growth hormone concentrations tested by Student's unpaired t-test. Open bars- controls, hatched bars- PZ treated, n=4 for both groups.

commenced. This became significant in later lactation even though treatment had been discontinued.

More frequent profile sampling was also conducted and mean prolactin and growth hormone concentrations were calculated for each group at each timepoint for every sampling week (Figures 4.8 & 4.9 respectively). Pre-treatment (week 13) prolactin concentrations were very similar between the groups and subsequent treatment had no significant effect on plasma prolactin. There was, however, a trend towards prolactin being slightly higher in the PZ group after treatment finished (weeks 28, 32 and 37), confirming the observations made in the routinely collected samples.

Growth hormone profiles showed a reduction in concentrations in PZ-treated animals from week 25 onwards. When hormone concentrations were averaged over the day for each group, and lactation weeks compared, the decrease in the PZ group was statistically significant ($p<0.001$) in weeks 25, 32 and 37 (CON: 4.0, 4.0 and 3.7 ng/ml GH; PZ: 2.4, 1.5 and 1.7 ng/ml GH, respectively), again confirming the trends seen in the routinely collected samples.

Udder characteristics

Treatment itself had no significant effect on gross udder volume with both groups showing an initial decrease in volume between pre-treatment and treatment phases (CON: $p=0.002$, PZ $p=0.056$), and subsequent maintainance at a lower level for the remainder of lactation (Table 4.2 & Figure 4.10).

The sites of milk storage were also determined in weeks 13 and 25 (Figure 4.11) and there were no significant differences between the groups in week 25 ($p \geq 0.36$). Alveolar capacity was initially higher in the PZ group compared to controls ($p=0.009$) but the former lost capacity by week 25 ($p=0.014$), in contrast to controls which increased volumes over the same period ($p=0.098$). Cistern volumes were similar between groups in week 13 ($p=0.71$) and both showed a decrease in volume over time (CON: $p=0.051$, PZ $p=0.036$). Cistern percentage was significantly higher in controls in week 13 ($p=0.013$), reflecting the disparate alveolar capacities of the groups, but percentages dropped significantly in both groups by week 25 ($p \leq 0.018$).

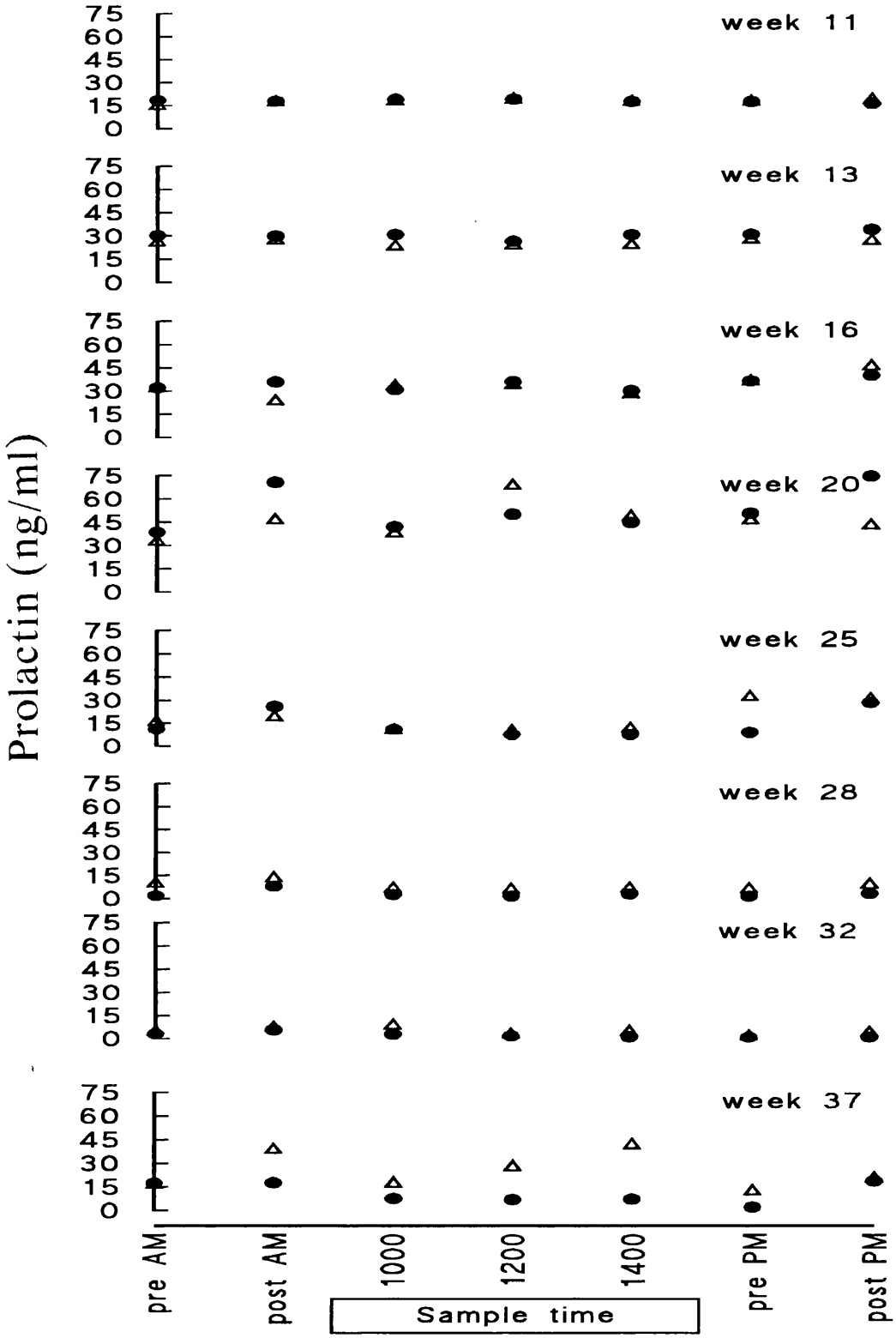


Figure 4.8 Prolactin concentrations determined by frequent sampling in experiment 2 in control (●) and PZ (Δ) groups. Treatments phases were as follows: pre-treatment - week 11. PZ treatment - weeks 13, 16, 20, 25 and 1X milking and no PZ- week 28 onwards. Pooled sem- 0.904 ng/ml.

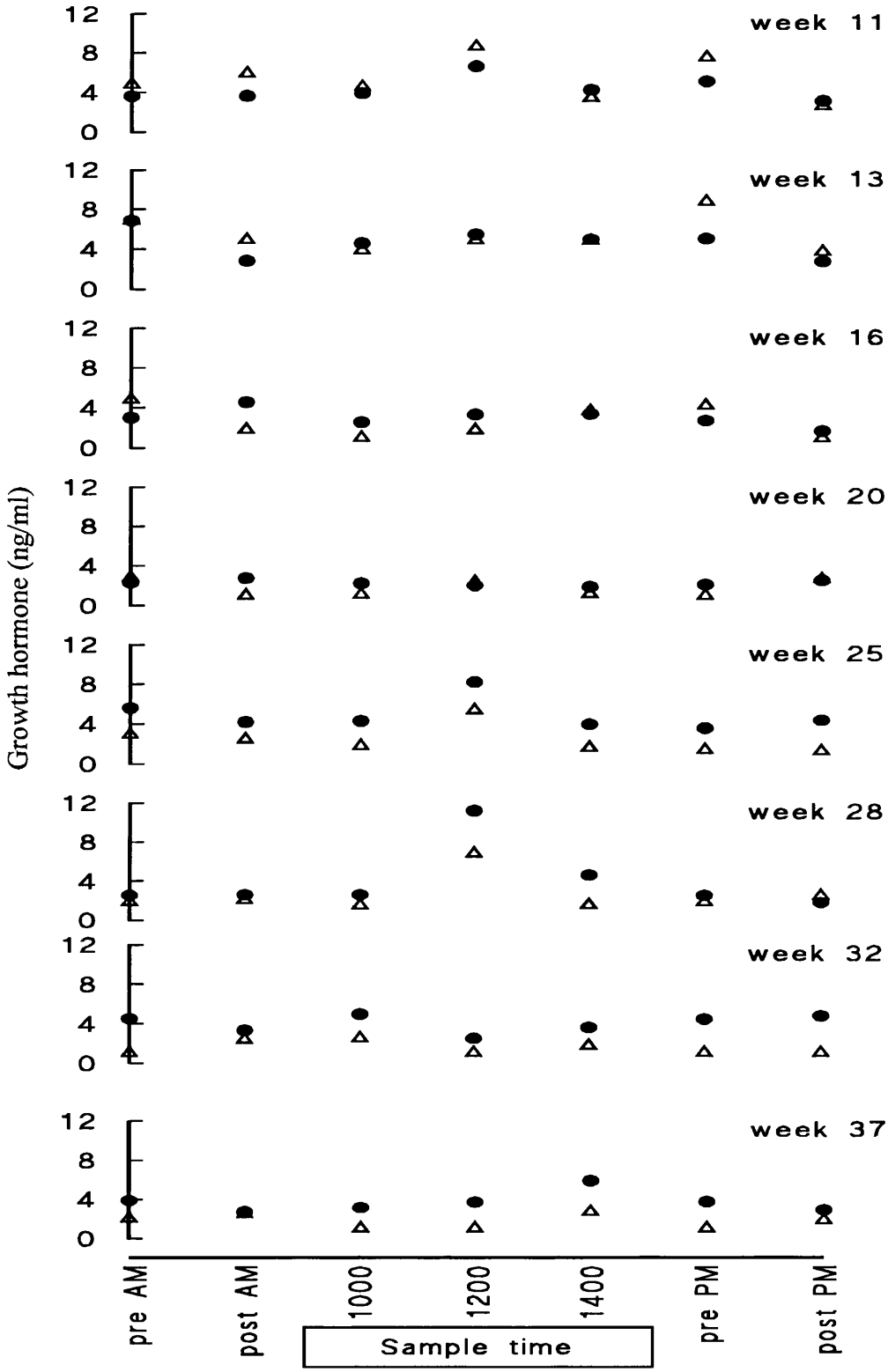


Figure 4.9 Growth hormone concentrations (ng/ml) determined by frequent sampling in experiment 2 in control (●) and perphenazine (Δ) groups. Treatment phases were as follows: pre-treatment - week 11. PZ treatment - weeks 13, 16, 20, 25, and 1X milking and no PZ- week 28 onwards. Pooled sem was 0.133 ng/ml.

Phase:	*Pre-treatment*	*PZ treatment*			*1X milking only*		
Lactation week:	11 + 12	13	20	25	30	32	37
Control	1542	1525	1185	1085	1182	989	953
s.e.	102	112	160	96	96	137	32
PZ	1558	1362	1120	1082	996	1060	862
s.e.	111	106	56	150	86	81	93

Table 4.2 Udder volumes (ml) measured in experiment 2.

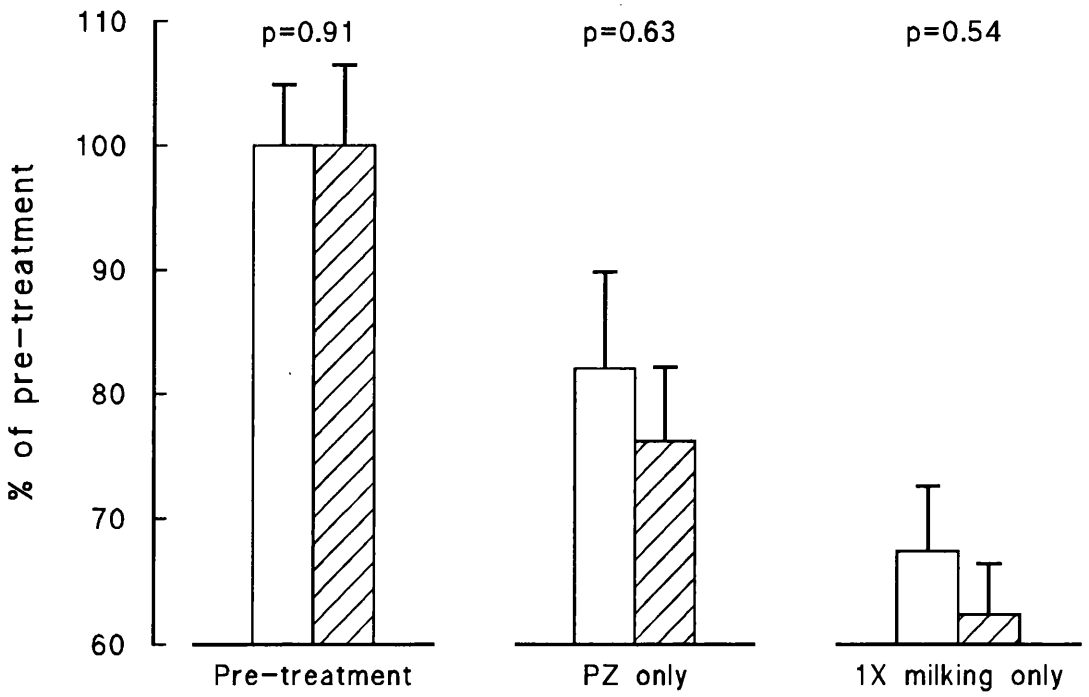


Figure 4.10 Statistical analysis of udder volume measurements in control (open bars) and PZ (hatched bars) groups. Volumes were averaged over each treatment phase and expressed as a percentage of the pre-treatment level.

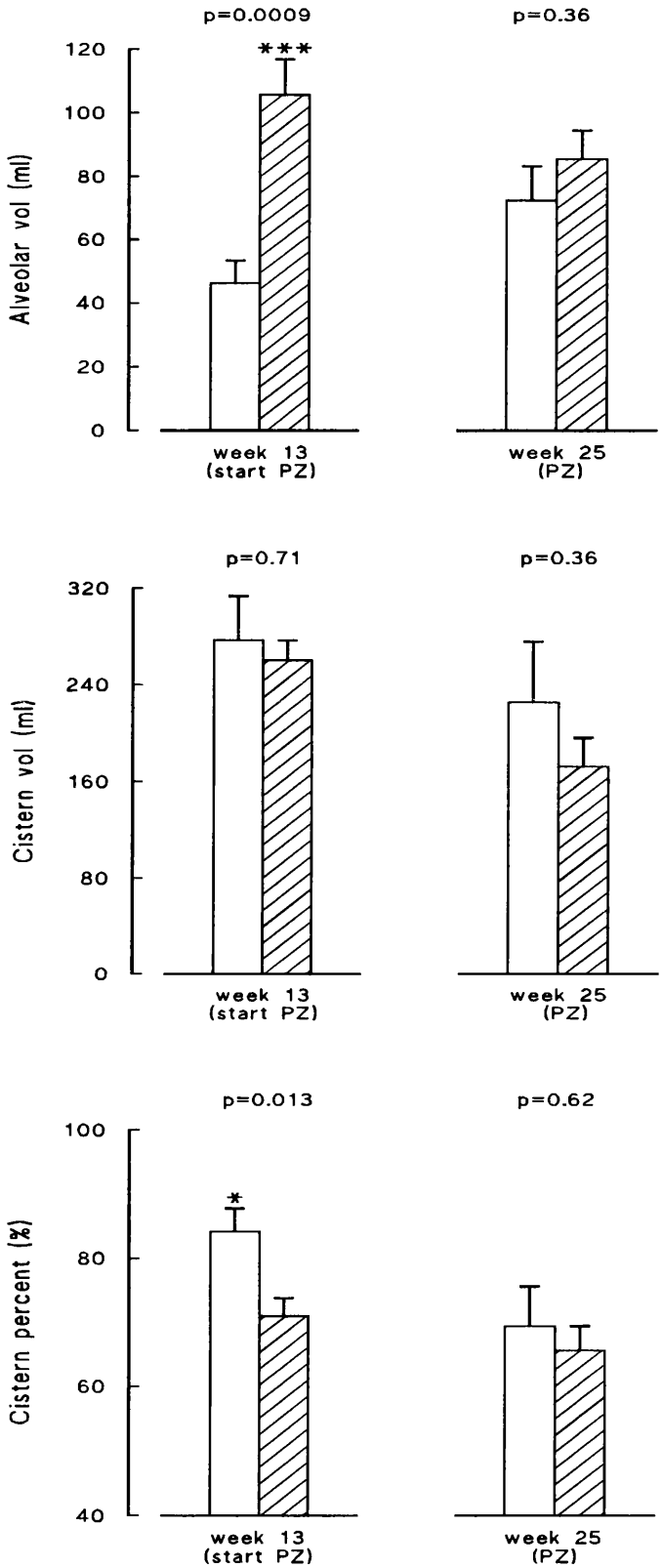


Figure 4.11 The sites of milk storage determined in experiment 2. Open bars represent control animals, hatched the perphenazine group. In weeks 13 & 25 both glands were being milked twice-daily and PZ was being given. n=8 for each measurement, p-values represent the difference between groups.

5. DISCUSSION

The main question addressed in this work was can increased prolactin concentrations improve lactation persistency? The first experiment tested the effects of perphenazine infusion, at a variety of doses, on prolactin concentrations in goats in various physiological states. PZ significantly increased prolactin with one exception- in lactating goats receiving 0.44mg PZ/kg over a 24h period. Close examination of individual prolactin profiles indicated a trend towards decreasing concentrations as time of infusion increased. This could reflect a decrease in sensitivity to PZ or a pituitary restriction on prolactin release. The subsequent elevation of prolactin with an increased dose may have overcome any desensitisation or there may have been sufficient synthesis and restoration of the pituitary stores of prolactin. PZ did elevate prolactin but it is not known whether absolute prolactin concentrations or the magnitude of the increase are physiologically relevant to the animal. Changes in prolactin concentrations with physiological state, determined before treatment, were consistent with those found in previous studies and reflected the increasing requirement for prolactin in pregnancy and lactation. Lactating goats have higher prolactin levels for up to 16h a day compared to non-lactating ones (Hart, 1974) and the mammary gland has an elevated prolactin binding capacity, supporting milk synthesis (Beck *et al*, 1979). Environmental influences of daylength and temperature will also modify prolactin levels; daylength and circulating prolactin are positively correlated (Hart, 1975a). Non-pregnant, non-lactating goats were studied between December and January, while the remainder (pregnant and lactating) were observed during February and March with daylight increasing from about 8 hours to 12 hours over this period.

The infusion method used in experiment 1 was not suitable for long-term PZ treatment due to welfare considerations- animals were housed in metabolic crates to restrict their movement, but they could still be quickly and easily disconnected from the apparatus for milking or other procedures. It had been envisaged that for the second experiment osmotic minipumps (Alza Corporation, Palo Alto, USA) would be used as a means of extended PZ administration. Two of these pumps could administer a total volume of 4ml solution over a period of 1 to 4 weeks, as appropriate. However, it was subsequently found that the minipump was incompatible with the solvent concentrations which could successfully dissolve the PZ dose required. PZ has a maximum solubility of 153 mg/ml in ethanol (ABPI data compendium, 1990) but the minipump could only tolerate ethanol to 5%, too dilute to dissolve PZ to 31 mg/ml, the required dose for an

average 70kg goat given 0.25mg PZ/kg/d over a one week period. Unfortunately the determination of a suitable PZ dose for lactating goats coincided with seasonal kidding and as the long-term experiment had to commence near peak lactation an alternative administration route had to be found quickly.

Consequently in experiment 2, PZ was initially given orally in the light of reports in rats (Stringer *et al*, 1990) and horses (Ireland *et al*, 1991). But after several weeks of treatment and having increased the daily dose from 25 to 50 mg PZ there was no still no apparent effect on prolactin concentrations. So why was PZ ineffective in lactating goats? Stringer *et al* used young, non-pregnant, non-lactating rats and gave them a syrupy form of PZ dissolved in drinking water, made fresh every 48 hours, elevating prolactin about 5-fold. Goats in a comparable physiological state responded to equivalent PZ infusions (8-fold increase in experiment 1) but a similar dose given orally had no effect. PZ was made fresh every day, so one would not predict inactivation prior to administration. Ireland *et al* (1991) gave PZ to late pregnant mares in combination with bromocriptine (to reduce prolactin). PZ prevented part of the prolactin decrease and the dose used was within the range used in these experiments. So could it be due to the administration route rather than the dose level used? The lack of effect in goats may reflect species variation in the digestive system; rats and horses have 'simple' stomachs which, in the latter, have adapted to cope with the digestion of fibre (horses have an enlarged caecum) while goats, and other ruminants, have an additional stomach, the rumen, which relies on a large microbial population of bacteria, fungi and protozoa to degrade cellulose and facilitates extended periods of regurgitation and chewing to improve digestion (Czerkawski, 1986). It could well be that PZ was inactivated and degraded in the rumen before it could be absorbed and exert its effects in the goat.

PZ administration was then switched to intramuscular injection but prolactin still remained lower in treated animals. This depression may be partly explained by decreased food intake, and increased lethargy which may have given them a measure of 'resistance' to stress, both factors which can increase prolactin (Hart, 1973b). The lack of response and side-effects observed supported previously published observations (Shani *et al*, 1975) and it appears to be a problem specific to localisation of PZ in body tissues. It is perhaps surprising that brain implantation of PZ (localising PZ in the brain over a prolonged period) proved successful, but it may have caused less tissue irritation in this region. Unfortunately this technique involves difficult surgery, more intensive

husbandry of animals due to post-operative weakness, and causes an initial reduction in milk yield (Vandeputte-Van Messom & Peeters, 1976 & 1982). Despite this, it appears to be the best approach towards long-term alteration of prolactin in goats by pharmacological means. A more direct approach may, however, now be possible. A recent abstract has reported that recombinant prolactin has been given to goats and milk yields were increased by 7% (Knight *et al*, 1995). Unfortunately the experiment was conducted over a short time period and there was no data provided to verify that the treatment elevated systemic levels of prolactin, but the increased yield indicates that there a stimulation of milk secretion, probably due to the increased availability of prolactin in the mammary gland. If recombinant prolactin could be used in conjunction with a subcutaneous osmotic minipump then it could be given over periods of several weeks. Alternatively it could be administered by injection, like BST, possibly in a delayed release vehicle which could increase the interval between injections up to fortnightly. The stability of prolactin given by this route has been verified (Hebert *et al*, 1991). Use of these methods could facilitate a long-term study on the effects of maintaining prolactin concentrations over a lactation.

Lactation persistency was altered in late lactation not by PZ treatment but by the change in milking frequency; 1X milking tended to improve persistency. The change from twice- to once-daily milking was predicted to decrease persistency as milk accumulated over a single 24 hour period rather than over 8 and 16h intervals, decreasing the period of maximal milk secretion (Peaker & Blatchford, 1988). Endocrine environments were similar in both glands as the milking-induced release of hormones continued twice-daily, although the 1X gland was only emptied at every second milking, suggesting that another local factor, other than hormones, was modulating the effect. Udder anatomy can influence milk production; cows with greater cisternal storage of milk had higher rates of milk secretion (Dewhurst & Knight, 1993), an effect thought to be modulated by FIL. This protein acts as an autocrine inhibitor of milk secretion and as such requires to be in contact with the secretory cells themselves. Once it leaves the alveolar areas of the gland it effectively becomes inactive and therefore a greater movement of milk from this area to the cistern would minimise its inhibitory effects on the secretory cells, partly relieving the local inhibition on secretion. Measurements of milk compartmentation in the udder were made at the start and near the end of PZ treatment but both glands were being milked twice-daily during this time. Further measurements were made in week 37 but measurable milk

was obtained from one animal in each group so the data proved inconclusive as to whether or not changes in the partitioning of milk stored in the udder could be the explanation for our observations. More frequent measurement of cisternal and alveolar ratios would be required for a closer study of this phenomenon.

The lactation curve showed a double peak at approximately weeks 8 and 16, the former as expected. The latter peak was not predicted and did not coincide with any change in treatment and the suppression of milk yields between the two was possibly due to a nutritional constraint such as the quality of the hay available to the goats.

Another observation made in experiment 2 was that the PZ group had generally lower growth hormone concentrations, becoming more marked later in lactation, despite discontinuation of treatment. This initial depression coincided with decreasing concentrate intake, however, when animals are in negative energy balance growth hormone levels increase, inhibiting proteolysis and stimulating incorporation of amino acids into muscle (Bauman & Currie, 1980). Simultaneously other metabolites are diverted away from storage by tissue deposition (Hart, 1983) and redirected towards energy utilisation. This allows protein reserves to be retained during times of high metabolic stress. So instead of the predicted increase in growth hormone as animals decreased their food intake (Breier & Gluckman, 1991), we saw a decrease in growth hormone. PZ could be having a direct or indirect effect, for example, growth hormone decreases when there is a restriction on growth hormone-releasing factor. In the latter case, IGF-I concentrations also drop, detectable in serum samples (Moore *et al*, 1992) but we did not assay samples for IGF. Alternatively, growth hormone could have directed these changes, and it was impossible to separate cause and effect in this case. Growth hormone remained at low levels while animals regained their appetites, the rapid rate of weigh gain supporting the measurement of low growth hormone, directing metabolites towards tissue deposition. Milk yields may also have reflected this- there were initially small but rapid recoveries in production which soon tailed off.

CHAPTER 5

THE INFLUENCE OF OXYTOCIN ON LACTATION PERSISTENCY

1. INTRODUCTION

The classical role of oxytocin is to promote the emptying of milk from the alveolar areas of mammary glands by facilitating the milk ejection reflex during suckling or milking. It can be used therapeutically to enable milk removal in animals with compromised milk ejection, but will also improve milk removal in those with normal milk ejection. Typically milk left in the udder goat or cow udder after a normal milking might constitute some 10% of the volume of the milk removed and this 'residual fraction' can be obtained by administering exogenous oxytocin (Bruckmaier *et al*, 1993; Sagi *et al*, 1980). This procedure can also be used to reduce yield losses when milking regimes are changed to ones of less frequent milking (Carruthers *et al*, 1993), attributed to the improved removal of the residual milk fraction. Similar positive effects have been seen in other studies (see Ballou *et al*, 1993; Sprain *et al*, 1954) but treatment periods have been generally quite short-term (up to 14 days). The immediate and short-term effect of oxytocin is to physically improve the removal of residual milk and therefore increased yield is not, in itself, evidence of increased milk secretion. However, other effects have been reported: oxytocin administered to rats has extended lactation by up to 10 days after litters have been removed (Benson & Folley, 1957) and some recent work has suggested that oxytocin can also have long term effects on lactation persistency in ruminants: Nostrand *et al* (1991) gave oxytocin to cows immediately before milking (twice-daily) over 305 days and found that oxytocin improved milk production by 11.6%, an average of 2.78 kg/d. The treatment had the greatest effect during declining lactation but there was a wide range in response within the treatment group (6.3 to 22.7%) and complex statistical evaluation of the data was required. Indeed some animals were excluded as their milk production was not adequate to fit the analysis. Consequently the positive effect of oxytocin on lactation persistency requires further investigation.

If we assume that oxytocin is indeed having a long-term effect, then how could it be acting? It could be either a direct metabolic action of oxytocin on the mammary gland or an indirect effect of milk removal. Support for the former hypothesis was produced when oxytocin was given to cows either before or after milking: milk yields were increased slightly irrespective of whether treatment was given before milking

(explicable by improved milk removal) or after milking, the latter clearly not an effect of milk removal as milking had already finished (Ballou *et al*, 1993). However absolute differences in yield between treatment animals were very small compared to controls ($\leq$ 1kg/d) and p-values of <0.08 were quoted (significance is usually assumed to be reached when $p \leq 0.05$) and further work carried out by Knight (1994) has not substantiated the initial findings. By milking the two halves of the same udder at different times and administering oxytocin at only one of these times it was shown that milk removal immediately after oxytocin administration was essential for enhanced milk production. Although the group size used was small, the effect was clearly significant ($p < 0.001$), since each animal served as its own control rather than comparing groups of individuals.

So could oxytocin be affecting milk production via milk removal? If so, its positive effect could be explained by a reduction in the concentration, or amount, of FIL around the secretory cells, allowing maximal rates of milk secretion to continue for longer periods (Wilde & Knight, 1990). Conversely, infrequent or incomplete milking during later lactation speeds up the process of involution (Wilde *et al*, 1989) which may reflect the lack of milk removal from the alveolar areas of the gland where FIL exerts its action. It would be therefore predicted that less frequent milking would reduce lactation persistency and oxytocin should help to minimise, or negate, this effect as it improves milk removal from these areas (Carruthers *et al*, 1993), although the results from the previous chapter suggest that once-daily milking can have a positive effect most probably due to alteration of milk storage characteristics.

The aim of this work was to determine the long-term effect of oxytocin treatment on lactation persistency and the experiments were designed to incorporate a change in milking regime while oxytocin treatment continued, to test whether or not oxytocin was acting directly ('metabolically') or indirectly (via milk removal). Consequently, treatment animals received oxytocin at both milkings throughout lactation and during the initial treatment phase both glands were milked twice-daily. Later in lactation one gland was switched to once-daily milking (1X) while the contralateral gland continued on twice-daily milking (2X), hence both glands continued to be subjected to oxytocin but 1X glands were emptied every second dose, while 2X ones were emptied every time a dose was given. If milk removal was required for oxytocin to exert an effect then a difference in lactation persistency between glands would be predicted during the second phase when milking frequencies were altered.

However, if oxytocin was having a general metabolic effect then there should be little difference between the glands' persistencies when milking frequencies were changed.

2. ANIMALS

British Saanen goats from the Institute herd were used. Hay and water were available *ad libitum* and, twice daily, animals were offered a total of 1.5kg of a proprietary concentrate feed.

3. EXPERIMENTAL DESIGN

Two long-term experiments were conducted over successive years in which treated animals received oxytocin prior to every milking while control animals were milked normally. All animals were initially milked twice-daily then, in later lactation, one gland was switched to once-daily milking. Various parameters were measured during the experiments including hormone concentrations, milk yield and composition, and udder capacities.

3.1 Experimental groups

The first experiment utilised lactating goats kidding between 8th-24th March, 1992. They were randomly divided into control and treatment groups (n=4, 3 primiparous & 1 multiparous, 3rd lactation). The control group was the same as that described in Chapter 4 (PZ treatment), and consequently vehicle alone was not given.

The second experiment used lactating goats kidding between 4th-15th May, 1993. These had been used in a study conducted between parturition and lactation week 8 (detailed in Chapter 6), consequently animals were assigned to oxytocin (n=5, all multiparous, 2-4th lactation) or control (n=4, all multiparous, 2nd lactation) groups on the basis of the treatment given in the previous study. Frequently milked and suckled animals were evenly divided between control and oxytocin groups, one drained animal was placed in the control group and the remaining two were assigned to the oxytocin group.

3.2 Treatments

Oxytocin (5 IU; Oxytocin-S, Intervet, Cambridge, UK) was administered intramuscularly immediately before milking at 0800 and 1600h every day from lactation week 12 (experiment 1) or week 13 (experiment 2) onwards. The oxytocin dose used was that recommended by the manufacturer. To minimise tissue inflammation and tenderness due to repeated injections, the site of injection was

swapped from left to right flanks on alternate weeks. After 12 or 13 weeks of twice-daily milking and oxytocin treatment, one gland was switched to once-daily milking (1600h) while milking of the contralateral gland and oxytocin treatment continued as before. Hence, in the first experiment oxytocin was administered during lactation weeks 13-37 and unilateral once-daily milking conducted on weeks 26-37. In experiment 2, treatments were given on weeks 12-31 and in conjunction with once-daily milking during weeks 24-31.

In experiment 1, all animals received intravaginal Chronogest sponges (30mg fluorogestone acetate, Intervet), changed every 10-14 days from lactation week 26 until the end of the experiment, to prevent the resumption of seasonal oestrus. In the second experiment the goats commenced natural oestrus cycles in the second week of October, lactation weeks 23-24.

3.3 Measurements

Blood samples were collected by jugular venepuncture twice-weekly at 1400h and analysed for prolactin (routine samples). In experiment 1, additional measurements of growth hormone and prolactin were made in blood samples taken at frequent intervals through a day, at timings detailed in Chapter 4, in lactation weeks 11, 13, 16, 20, 25, 28, 32 and 37.

Mammary gland cisternal and alveolar storage capacities were determined after 5 hours milk accumulation in both experiments: in weeks 13, 25 and 37 in experiment 1, and weeks 11, 14, 19, 23, 27 and 30 in experiment 2. Gross udder volumes were determined at the same time in experiment 1 only.

Mixed samples of bulk milk were collected for compositional analysis of protein, lactose, fat and solids content in weeks 11, 12, 13, 16, 18, 20, 22, 25, 28, 32 and 37 in experiment 1 and in weeks 14, 18, 21, 25, 29 and 31 in experiment 2.

4. RESULTS

4.1 Milk yields

Milk yields were calculated by determining average daily gland yields over one week periods for each goat and meaning according to treatment group (Figure 5.1). Statistical analysis of yields obtained during each treatment phase was performed (Figure 5.2). Left and right glands were evenly balanced in terms of milk production with the exception of control animals in experiment 1 whose 2X glands produced more

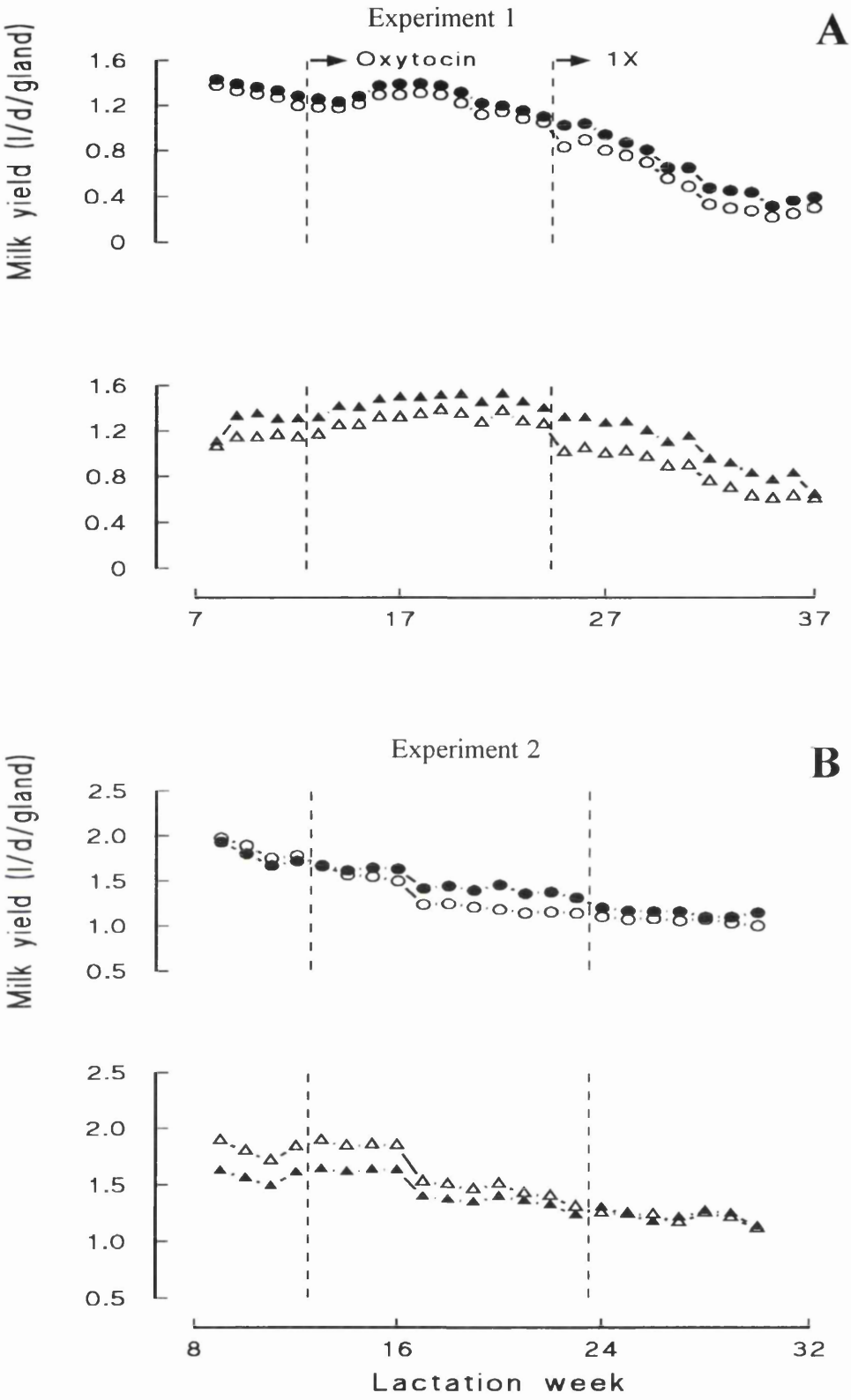


Figure 5.1 Average daily milk yields from control 2X (●) and 1X (❍) glands and oxytocin 2X (▲) and 1X (Δ) glands in experiments 1 (**A**) and 2 (**B**). Vertical dashed lines indicate commencement of appropriate treatments.

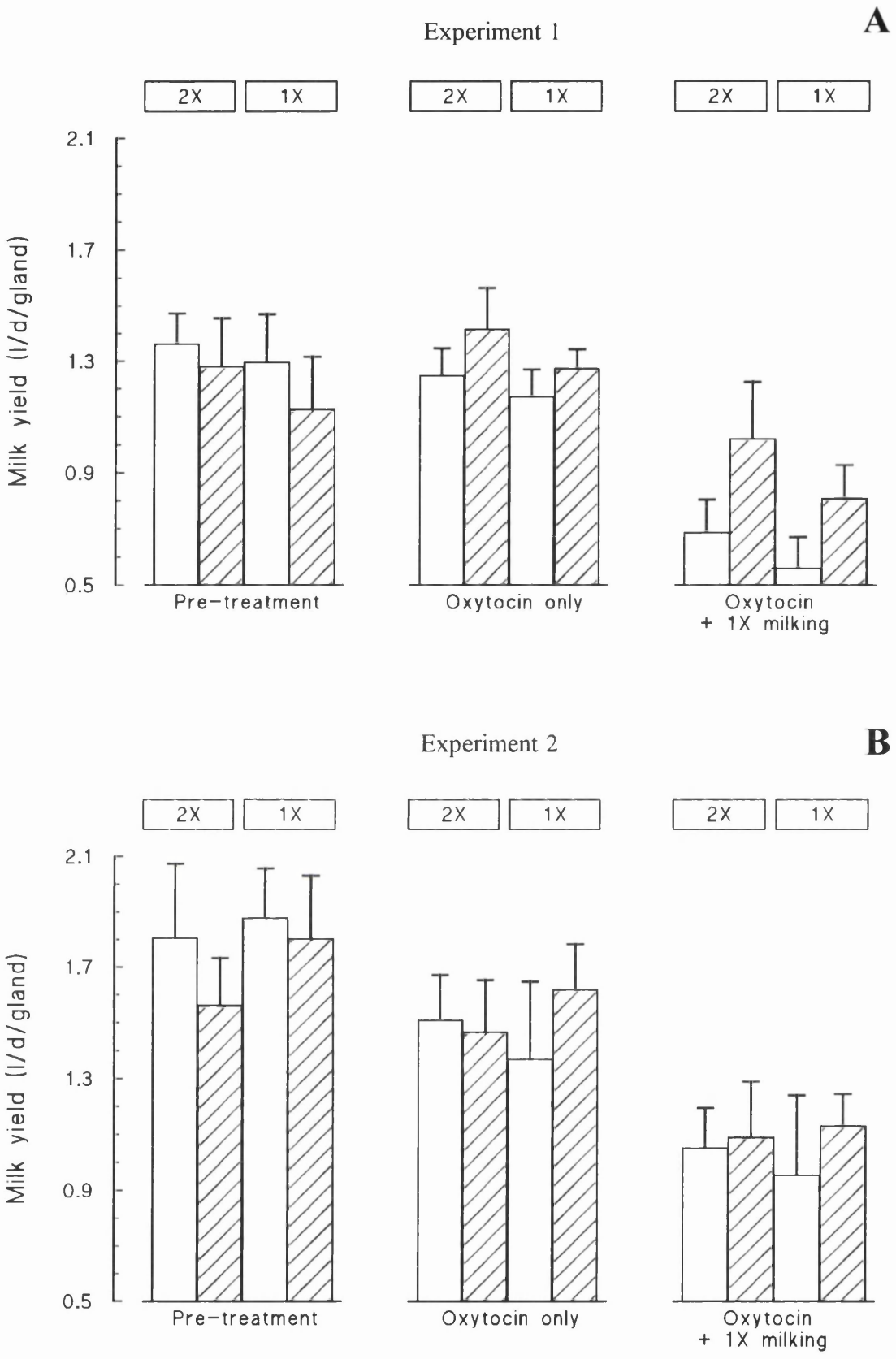


Figure 5.2 Mean values for milk yield in experiments 1 (**A**) and 2 (**B**) divided into treatment phases. Open bars represent control animals, hatched- oxytocin treated and glands were subjected to either once- or twice daily milking as indicated.

milk than 1X glands (determined by Student's paired t-test, $p \leq 0.02$) throughout the experiment. Statistical analysis of changes over time (ANOVA) found that in experiment 1 the pre-treatment period the oxytocin group were producing less milk than controls ($p=0.077$) but during treatment yields increased to above controls ($p<0.001$). The switch to 1X milking also led to a loss of yield ($p=0.001$) in these glands. In experiment 2, milk yields were not significantly different between groups either before or during treatment ($p \geq 0.092$) and there was little effect on yields after the switch to 1X milking ($p=0.795$).

4.2 Lactation persistency

To obtain a measurement of lactation persistency regression lines were fitted to milk yield curves (average daily gland yields) for individual animals in each treatment phase in experiments 1 (Table 5.1) and 2 (Table 5.2). In experiment 1, regression lines over the treatment phase (weeks 13-25) gave an average r^2 of 22.7%. This was increased to 64.7% when weeks 16-25 were analysed, due to fluctuations in yields over the intervening period, so the latter period was used in subsequent calculations. Group means were calculated (Figure 5.3) and statistical analysis by ANOVA conducted. Oxytocin treatment and milking frequency had no significant effect on lactation persistency ($p \leq 0.842$) but there was a difference between the two measurement periods with the second period having reduced persistency ($p \leq 0.056$).

4.3 Milk composition

Similar trends were apparent in both experiments (Figures 5.4 & 5.5), with all components showing an increase over lactation, with the exception of lactose. Lactose concentrations showed a large increase shortly before the start of oxytocin treatment in experiment 1 but this was not seen in the second experiment, and the biological significance of the initial observation is not known.

Statistical analyses for each milk component were carried out using Student's paired or unpaired t-test to compare glands within animals, and treatment groups, respectively over each treatment phase. Within individual goats, the two glands exhibited similar compositions throughout the experiment. There were no significant

Group	Milked	Treatment only **m** (weeks 16-25)	Treatment + 1X **m** (weeks 26-37)
Control	**2X**	-86.5	-57.6
		-8.5	-120.0
		-34.2	3.5
		-40.6	-71.8
	mean	-42.4 r^2 72.2%, p=0.092	-61.5 r^2 75.3%, p=0.037
Control	**1X**	-82.4	-44.8
		-21.2	-112.0
		-32.3	-10.4
		-47.8	-56.9
	mean	-45.9 r^2 81.0%, p=0.002	-56.0 r^2 79.6%, p=0.006
Oxytocin	**2X**	-38.3	23.9
		-19.8	-104.0
		-13.0	-87.1
		12.6	-66.1
	mean	-14.6 r^2 49.8, p=0.050	-58.3 r^2 86.6, p<0.001
Oxytocin	**1X**	-43.9	1.1
		-27.8	-69.3
		-23.8	-81.2
		27.2	-41.9
	mean	-17.1 r^2 55.8%, p=0.022	-47.8 r^2 69.0, p=0.205

Table 5.1 Summary of regression lines fitted to individual milk yield profiles for each animal's glands in experiment 1. **m** is the change in average daily yield, ml/week. r^2 and p indicate the accuracy and probability of the best line being fitted to the data.

Group	Milked	Treatment only **m** (weeks 12-23)	Treatment + 1X **m** (weeks 24-30)
Control	**2X**	-87.1 -14.3 -18.0 -38.8	-36.9 -16.8 -156.0 -113.0
	mean	-39.6 r^2 79.2%, p=0.002	-80.7 r^2 53.4%, p=0.116
Control	**1X**	-62.6 -28.4 -78.3 -60.9	-137.0 -147.0 -3.7 -43.5
	mean	-18.4 r^2 81.2%, p<0.001	-82.8 r^2 63.6%, p=0.052
Oxytocin	**2X**	-63.0 -41.9 -45.6 -23.3 -16.9	65.1 -104.0 -165.0 -169.0 -4.6
	mean	-38.1 r^2 72.4, p=0.003	-101.5 r^2 55.0, p=0.160
Oxytocin	**1X**	-47.6 -51.4 -142.0 -33.4 -12.2	-118.0 -20.0 -30.2 -66.2 -113.0
	mean	-57.3 r^2 76.0%, p=0.003	-69.5 r^2 46.0, p=0.133

Table 5.2 Summary of regression lines fitted to individual milk yield profiles for each animal's glands in experiment 2. **m** is the change in average daily yield, ml/week. r^2 and p indicate the accuracy and probability of the best line being fitted to the data.

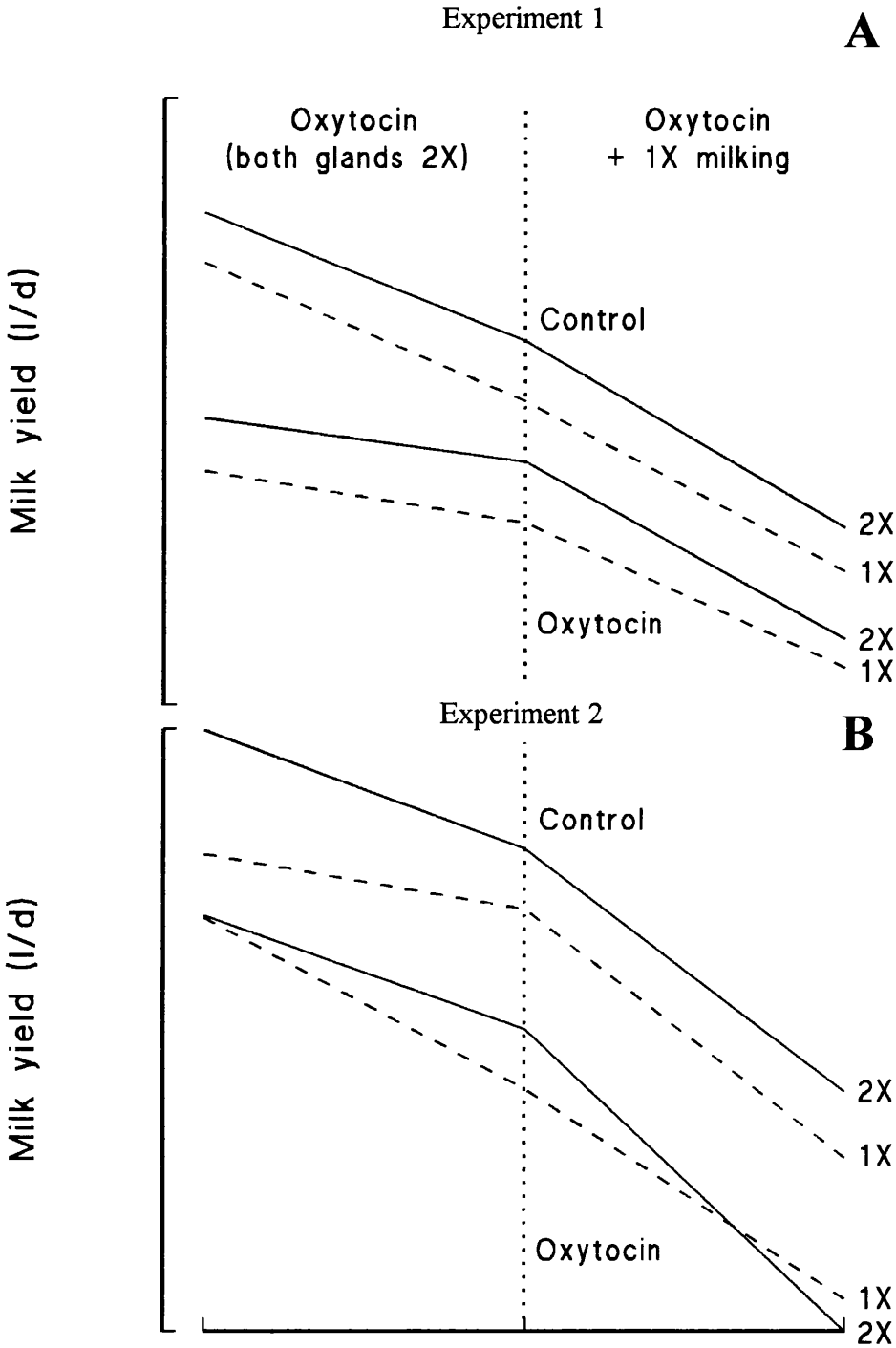


Figure 5.3 Summary of regression analysis for control and treatment groups in both experiments. In experiment 1 (**A**) both glands were milked 2X in weeks 16-25 and switched to unilateral 1X milking in weeks 26-37. In experiment 2 (**B**) these milking frequencies were applied in weeks 12-23 and 24-30, respectively. Lines have been plotted with displacement for clarity. Full analysis of this data is illustrated in (**A**) Tables 5.1 & (**B**) 5.2

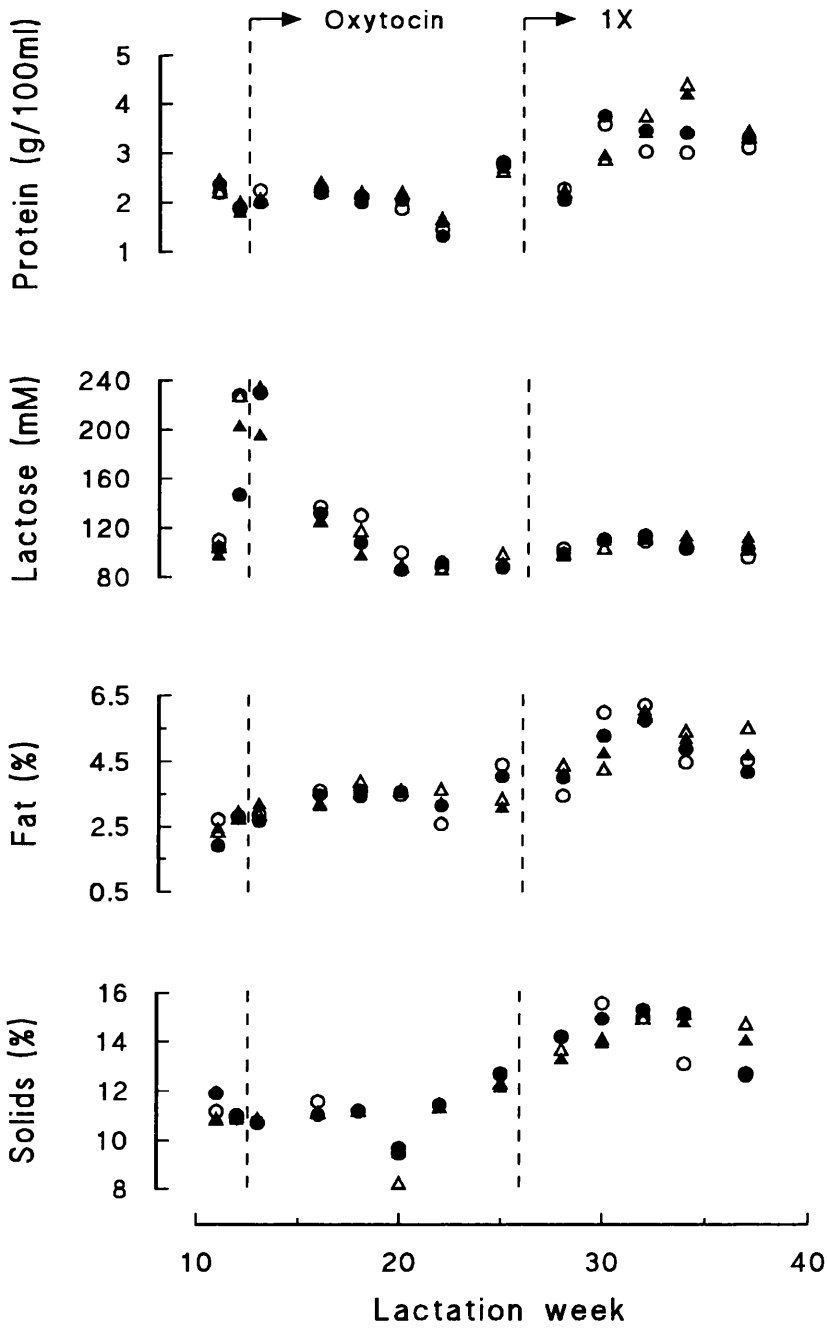


Figure 5.4 Milk compositional analysis determined in Experiment 1 in control 2X (●) and 1X (○) glands, and in oxytocin 2X (▲) and 1X (Δ) glands. Pooled sems were 0.06 g/100ml protein, 3.35mM lactose, 0.09 and 0.17% for fat and solids, respectively.

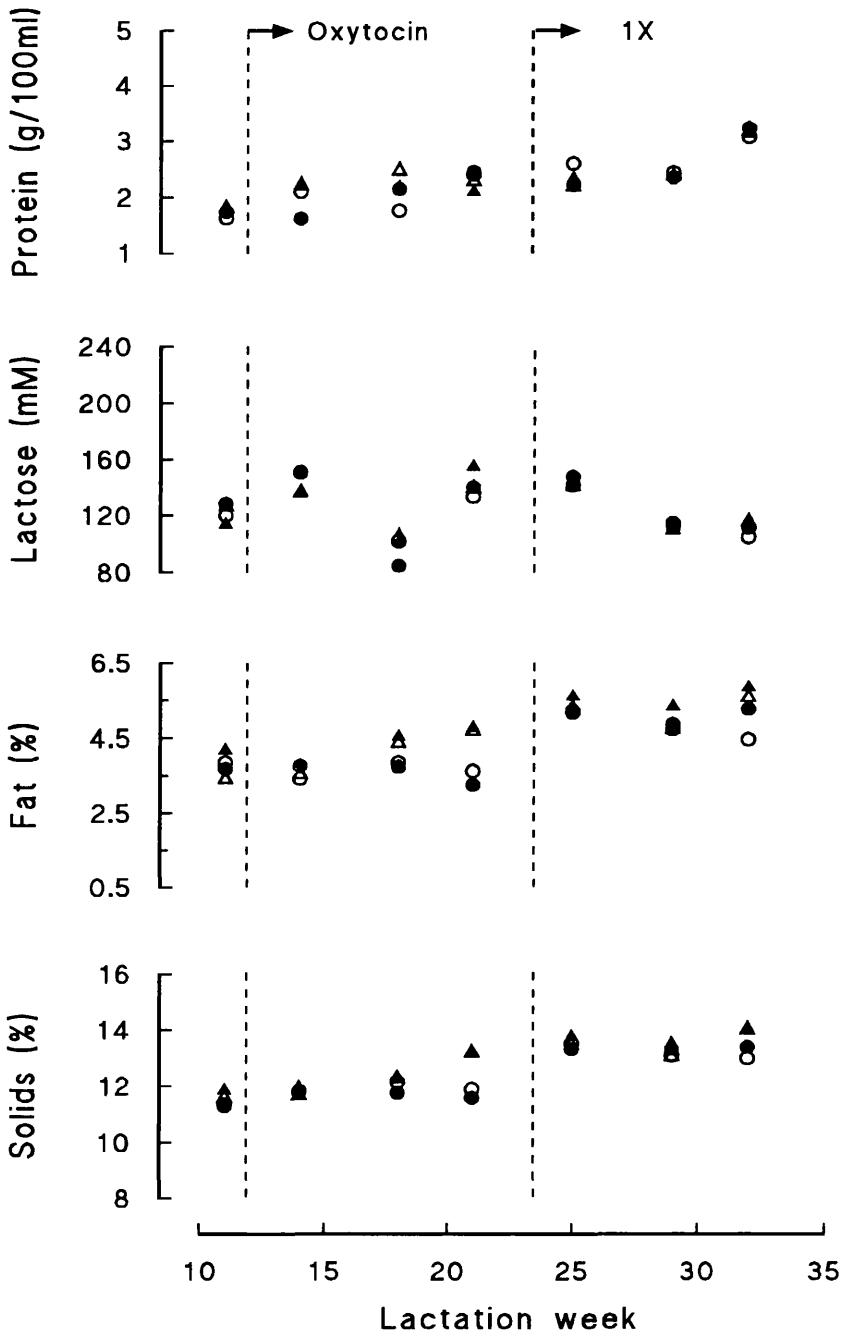


Figure 5.5 Milk compositional analysis determined in Experiment 2 in control 2X (●) and 1X (○) glands, and in oxytocin 2X (▲) and 1X (Δ) glands. Pooled sems were 0.06g/100ml protein, 2.26mM lactose, 0.10 and 0.14% fat and solids, respectively.

effects of oxytocin or of milking frequency on milk protein, lactose or solids content in either experiment. However, some differences in milk fat content apparent during the treatment phase of experiment 2. Higher fat percentages were obtained in oxytocin-treated animals, 2X glands being significantly higher than control 2X glands ($p=0.021$).

4.4 Udder characteristics

In experiment 1 milk storage capacities and distributions were measured 5 hours after milking at 3 timepoints. In the final week, measurable milk was obtained from only one control and three oxytocin goats (albeit from both glands) so statistical comparisons between groups were not possible. The frequency of measurements was increased in Experiment 2 in an attempt to monitor more closely any changes that occurred, and again milk storage was measured 5 hours post-milking.

Gross udder volumes

Changes in udder volumes were determined in experiment 1 only; the data is illustrated in Table 5.3. There was a significant reduction in gland size ($p<0.05$) between each phase (ANOVA), but no significant differences between the groups at any stage of lactation (Figure 5.6). There was, however, a trend towards oxytocin animals maintaining their udder volumes in late lactation: in weeks 32 and 37 volumes (as a proportion of that measured pre-treatment) were 14% and 11% higher than controls.

Sites of milk storage

The initial experiment indicated that oxytocin treatment could alter milk storage characteristics (Table 5.4). Alveolar volumes increased significantly between weeks 13 and 25 in oxytocin-treated animals ($p=0.0017$) and were higher than controls in week 25 ($p=0.0012$), resulting in a lower cistern proportion than controls ($p=0.055$). By lactation week 37, 3/4 oxytocin animals and 1/4 control animals were measurable 5 hours post-milking so statistical comparisons were not possible. However this again suggested that oxytocin was having a positive effect on milk production and the second experiment aimed to extend these findings.

The results for experiment 2 are illustrated in Figure 5.7 and statistical comparisons using ANOVA were conducted. There was a significant and positive effect of oxytocin treatment on alveolar storage of milk ($p=0.013$), pre-treatment the

Phase:	*Pre-treatment*	*Oxytocin treatment*			*Oxytocin + 1X milking*		
Lactation week:	11 + 12	13	20	25	30	32	37
Control	1542	1525	1185	1085	1182	989	953
s.e.	102	112	160	96	96	137	32
Oxytocin	1508	1263	1174	940	1145	1174	1106
s.e.	90	116	105	80	67	69	163

Table 5.3 Udder volumes (ml) measured in Experiment 1

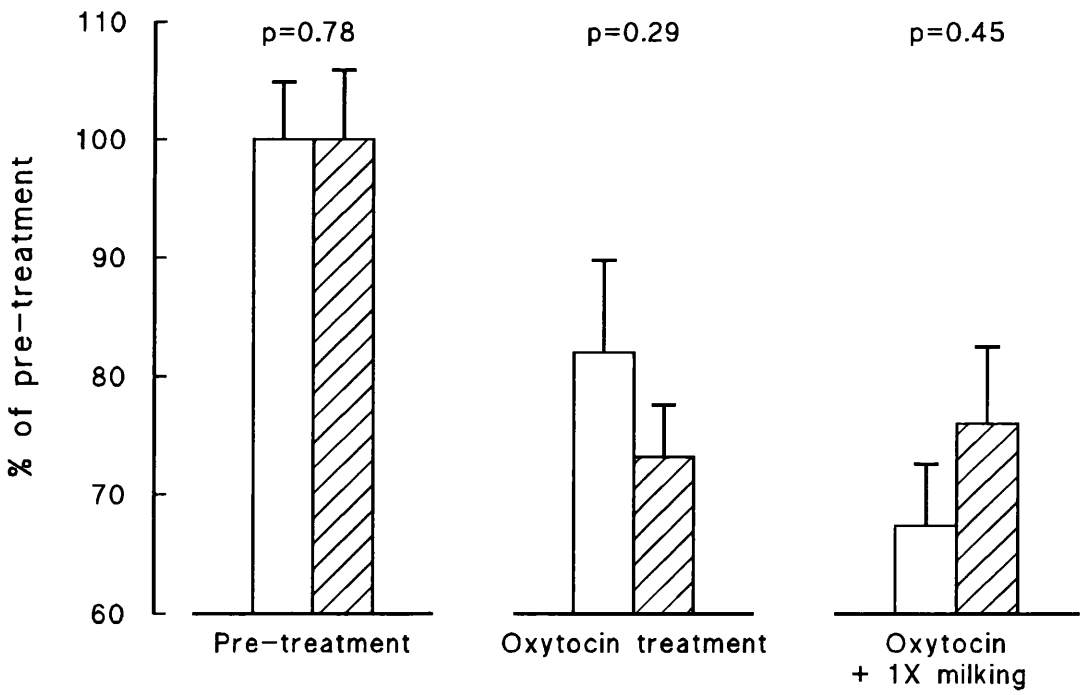


Figure 5.6 Statistical analysis of udder volume measurements in control (open bars) and oxytocin (hatched bars) groups in Experiment 1. Volumes were averaged over each treatment phase and expressed as a percentage of the pre-treatment level.

Treatment phase: (lactation week)	**Pre-treatment** (week 13)	**Oxytocin treatment** (week 25)	**Oxytocin + 1X milking** (week 37)
Alveolar volume (ml)			
Control	46.2 (7.1)	72.5 (10.8)	55.0 *
Oxytocin	52.5 (10.4)	145.0 (13.9)	109.2 (24.2) +
Cisternal volume (ml)			
Control	276.9 (36.6)	225.6 (50.0)	87.5 *
Oxytocin	244.4 (10.5)	173.7 (25.9)	97.6 (21.8) +
Cistern proportion (%)			
Control	84.1 (3.6)	69.4 (6.2)	61.4 *
Oxytocin	83.4 (2.6)	53.0 (4.6)	47.8 (2.8) +

Table 5.4 Effect of oxytocin treatment on milk storage characteristics in experiment 1. Each dataset represents n=8 with the following exceptions where * n=2 and + n=6.

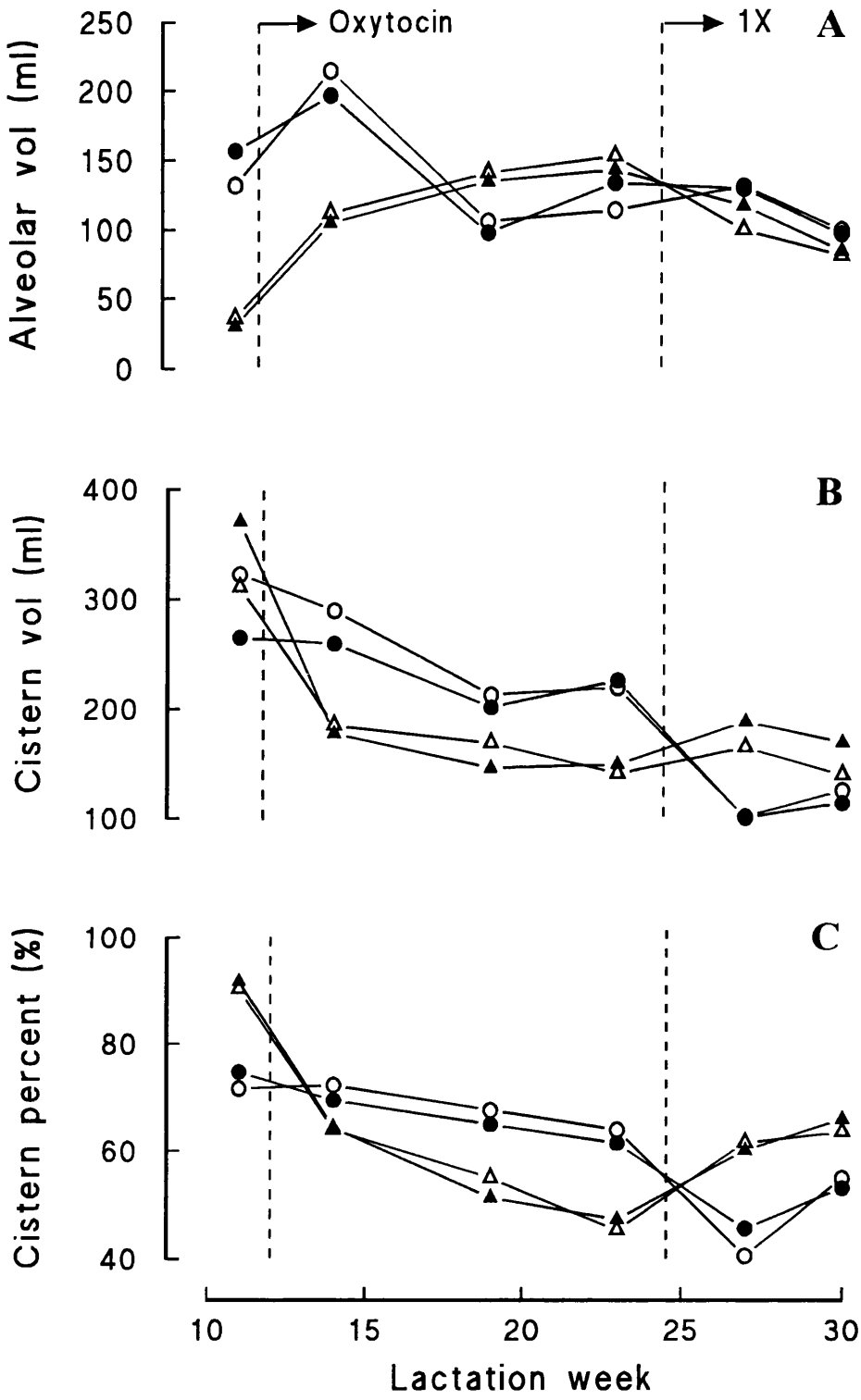


Figure 5.7 Changes in (**A**) alveolar volume, (**B**) cisternal volume and (**C**) cistern proportion in Experiment 2. Control glands were milked 2X (●) or 1X (○), as were oxytocin glands (2X ▲; 1X △).

oxytocin group had much lower capacities than controls but they steadily increased during the first treatment phase, then declined in the last period (+ 1X milking). All components (alveolar and cisternal volumes and cistern percent) were significantly affected by period with the lowest values being obtained in the last phase ($p < 0.001$). There was no apparent effect of milking frequency on any of the milk storage characteristics measured ($p \geq 0.845$) and within goats, glands had similar alveolar and cisternal volumes.

4.5 Prolactin

Overall, prolactin levels determined by routine sampling showed a general decline as lactation progressed (Figure 5.8). Oxytocin-treated animals were found to have lower mean prolactin concentrations in experiment 2, becoming significant during the last phase of treatment with 1X milking (Figure 5.9). However, there was no apparent difference detectable between the groups in the first experiment.

More frequent samples were also collected and prolactin concentration profiles were obtained for each group at stages of lactation (Figure 5.10). Student's unpaired t-test was used to determine any treatment effects but again both groups had very similar prolactin concentrations.

4.6 Growth hormone

Routinely collected blood samples were analysed for growth hormone content during experiment 1. There were indications of a slight downward trend as lactation progressed but concentrations were variable (Figure 5.11) and no significant differences were detected between treatment groups or in response to changed milking frequency. Frequently collected blood samples (Figure 5.12) also showed no differences between groups over time. Consequently, samples were not analysed for growth hormone content in the second experiment.

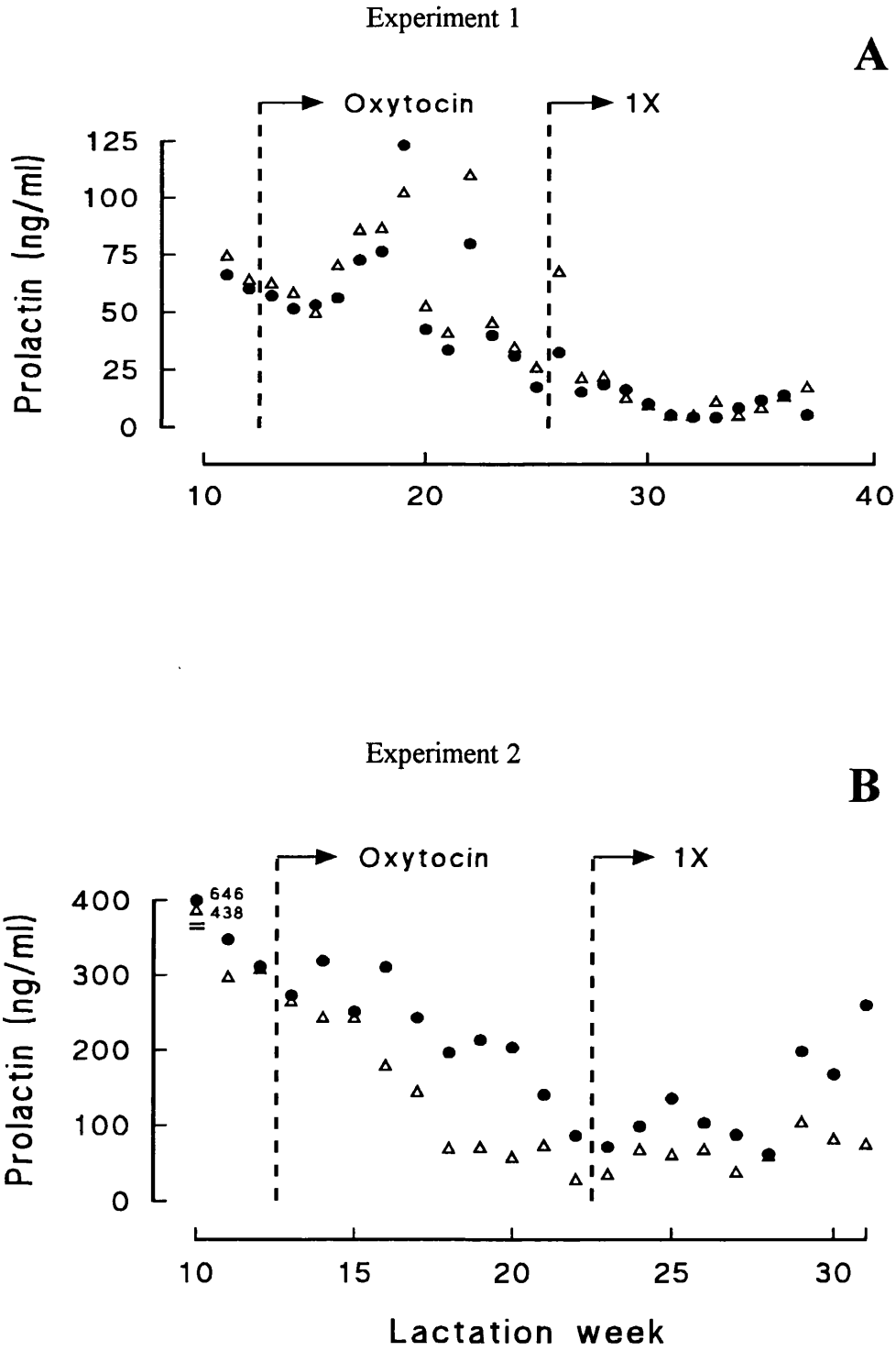


Figure 5.8 Prolactin concentrations in control (●) and oxytocin (Δ) groups determined by routine blood sampling in (**A**) Experiment 1 and (**B**) Experiment 2.

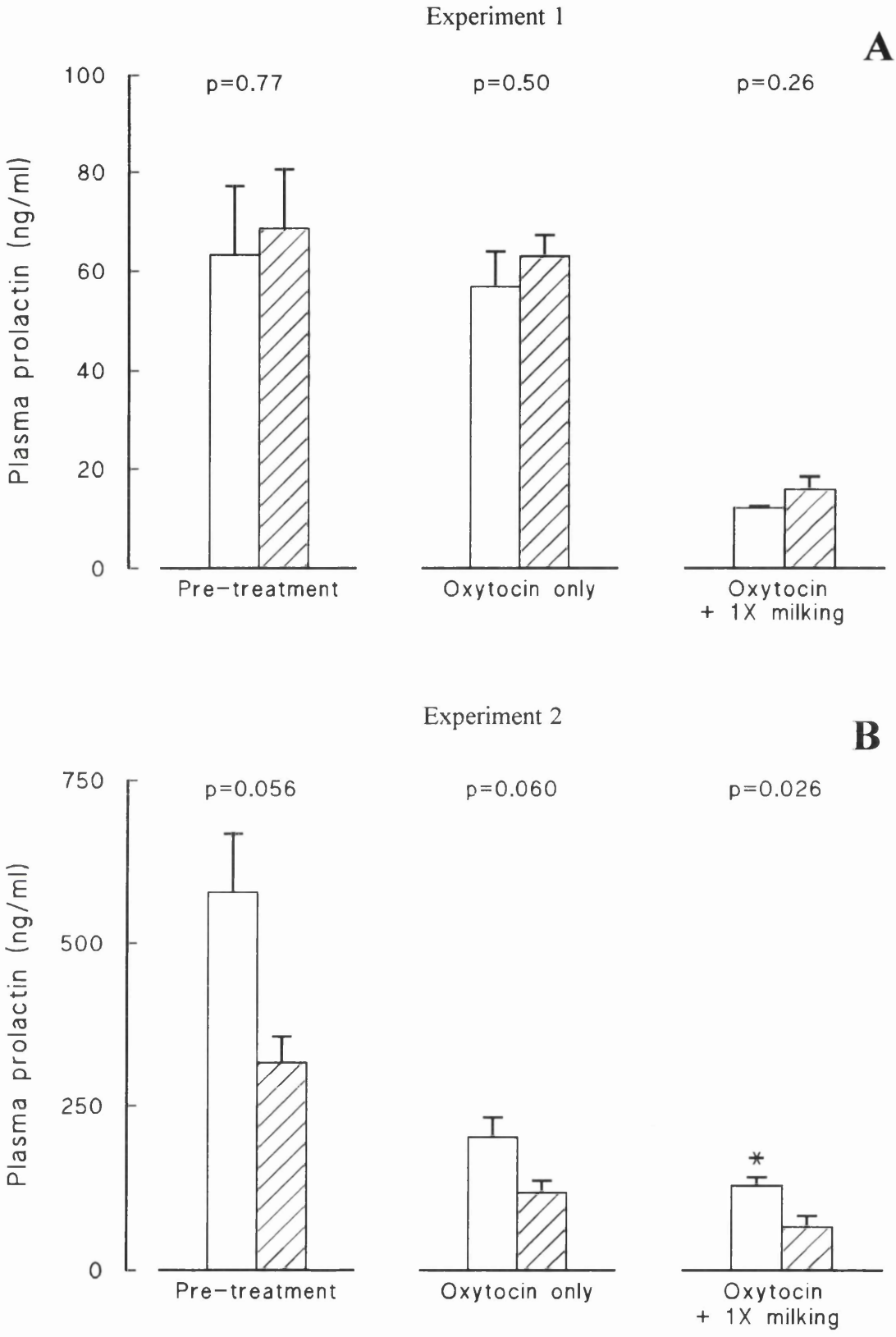


Figure 5.9 Statistical analysis of prolactin concentrations in experiments 1 (**A**) and 2 (**B**), divided into treatment phases in control (open bars) and oxytocin treated (hatched bars) groups. Differences between groups were tested by Student's unpaired t-test. n=4 for control groups and oxytocin in experiment 1, and n=5 in experiment 2.

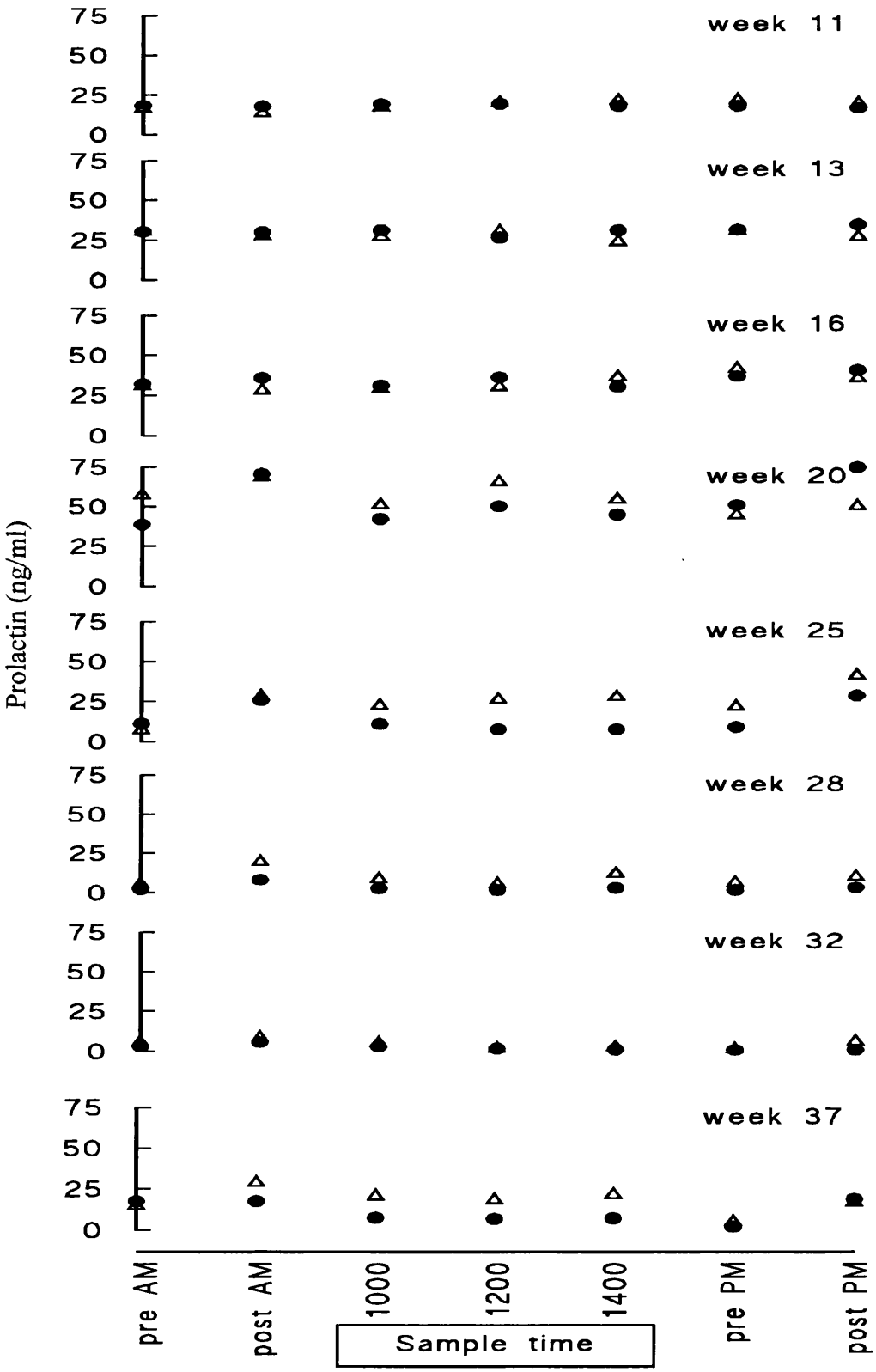


Figure 5.10 Prolactin concentrations determined by frequent sampling in experiment 1 in control (●) and oxytocin (Δ) groups. Treatments phases were as follows: pre-treatment - week 11, oxytocin treatment - weeks 13, 16, 20, 25 and oxytocin + 1X milking- week 28 on.

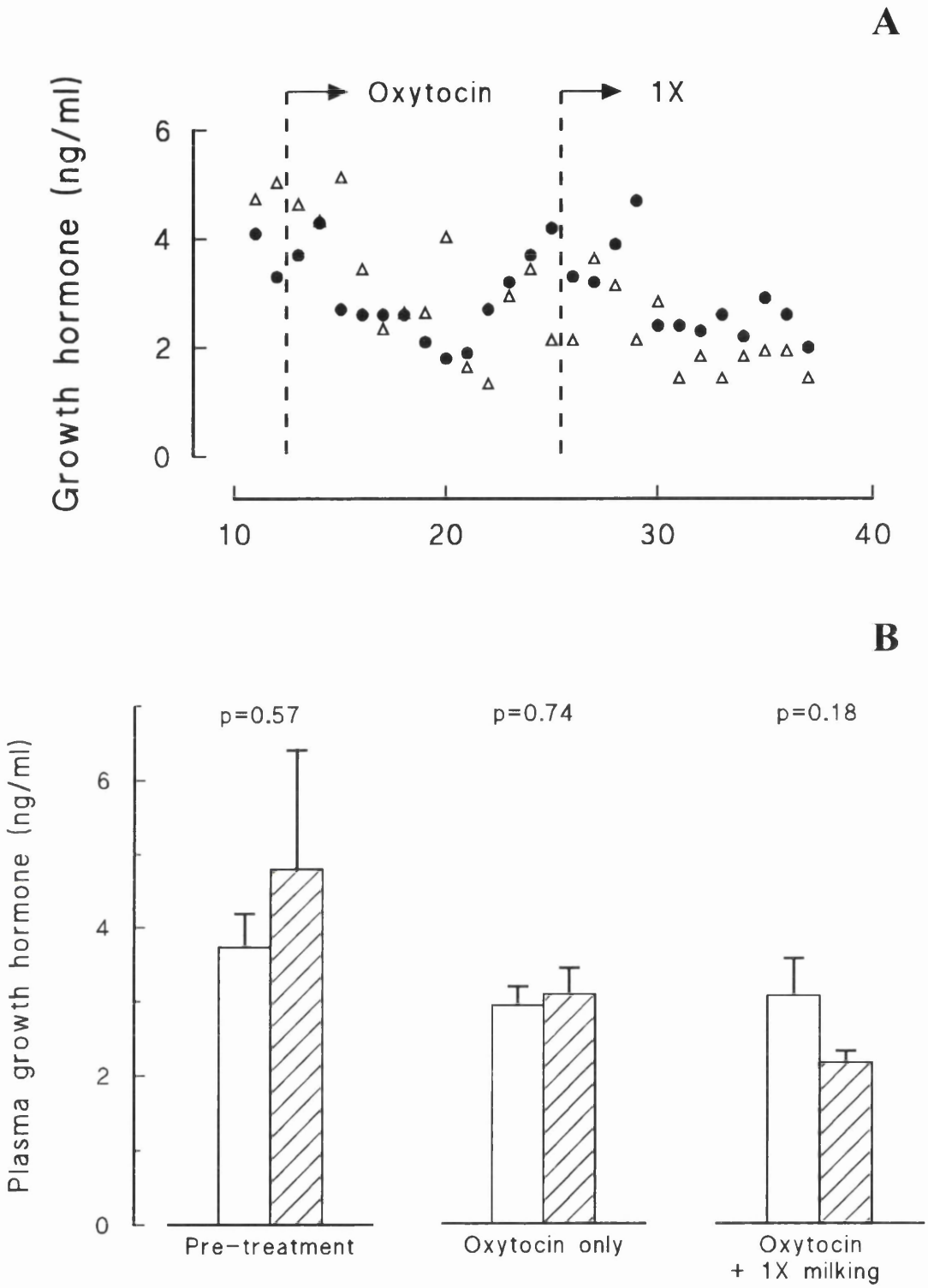


Figure 5.11 Effect of treatment on growth hormone levels determined in experiment 1 by routine blood sampling. (**A**) Lactational profile for control (●) and oxytocin (Δ) groups. (**B**) Statistical analysis of growth hormone concentrations tested by Student's unpaired t-test. Open bars- controls, hatched bars- oxytocin treated, n=4 for both groups.

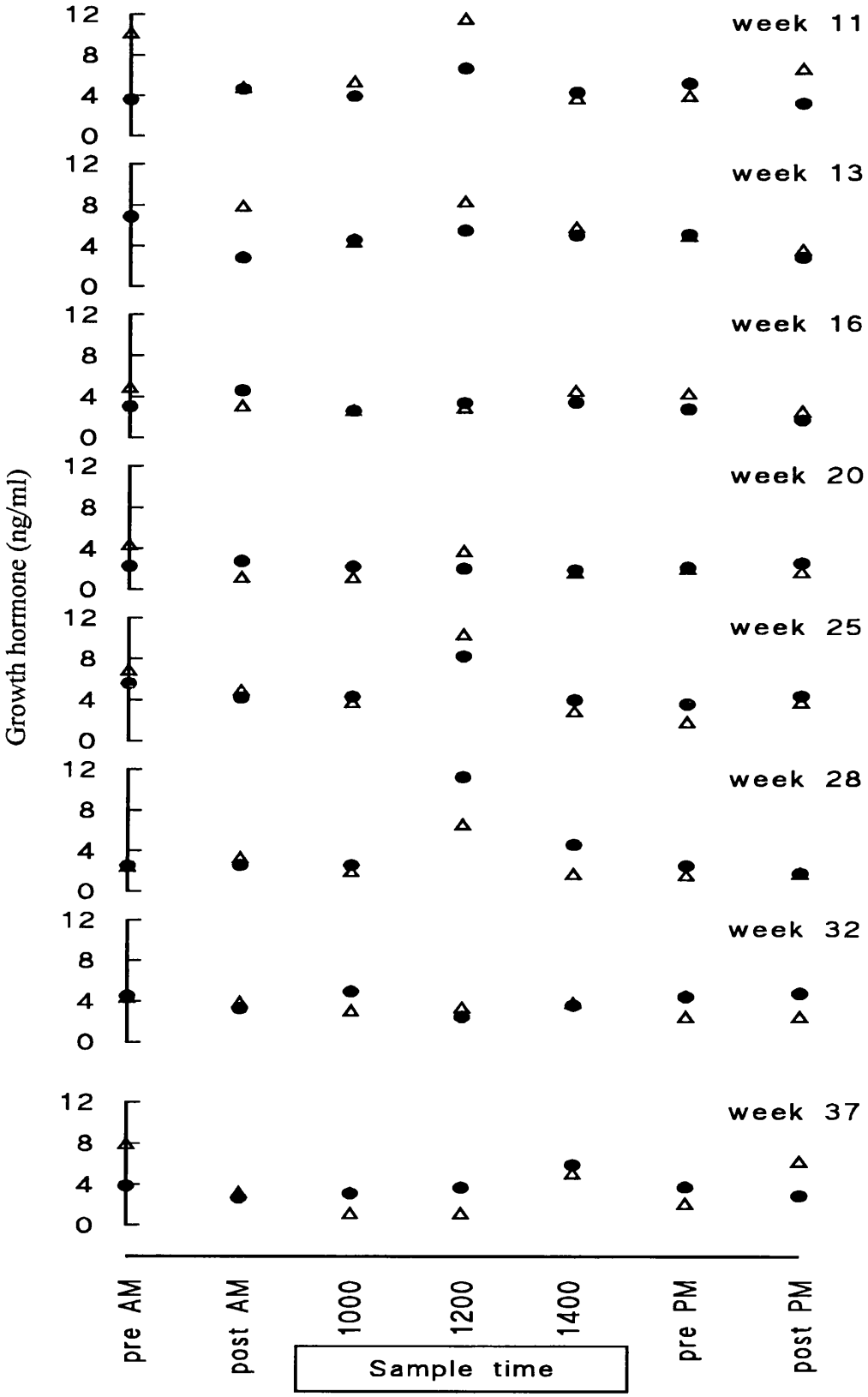


Figure 5.12 Growth hormone concentrations determined by frequent sampling in experiment 1 in control (●) and oxytocin (Δ) groups. Treatments phases were as follows: pre-treatment - week 11, oxytocin treatment - weeks 13, 16, 20, 25 and oxytocin + 1X milking- week 28 on.

5. DISCUSSION

The initial results of the effect of oxytocin on lactation persistency and milk production were encouraging; milk yields were maintained above controls during late lactation and lactation persistency was also positively affected. However the second experiment gave little response to treatment, confirming the observations of Nostrand *et al* (1991) of variable responses to treatment between animals. So how can this be explained? The main difference between the two experiments was that in the second the animals had been used for another study during the first six weeks of lactation and although these previous treatments were taken into account when control and oxytocin groups were assigned in this work, there may have been a general carry-over effect which reduced or prevented the possibility of an 'oxytocin response'. However, persistencies were generally similar in the control groups in both experiments suggesting that it did reflect a genuine difference in response to treatment. Another point to note is that both groups in the first experiment had a late peak in milk yields (around lactation weeks 17-20) which may indicate that they were subject to some nutritional constraint sometime after the initial peak in lactation, but again the similarity between the two control groups must be borne in mind. Parity might also have an effect; in experiment 1 3/4 primiparous animals were in each group while in experiment 2, 6/8 animals were on their 2nd lactation. If the main effect of oxytocin is to relieve the local inhibition of milk secretion then this would be expected to have more of an effect in animals with smaller cisterns and more alveolar storage of milk (Blatchford & Peaker, 1982), characteristics of primiparous animals.

To what extent could hormonal factors be responsible for influencing milk production? Prolactin is a lactogenic hormone and correlations have been derived between its release at milking and average monthly milk yields (Hart, 1974) but this is of debatable physiological significance because, in ruminants, artificial depression of circulating prolactin concentrations has little effect on milk yield during established lactation (Hart, 1974). It is possible that there is some interaction between oxytocin and prolactin beyond their temporal relationship (release at milking). However, a closer involvement is subject to debate; when suckling was studied in detail in rats it was found that prolactin release was pulsatile and that peak oxytocin concentrations occurred during prolactin minima (Higuchi *et al*, 1983). However, the magnitude and duration of prolactin peaks can be reduced by oxytocin antagonists and anti-oxytocin antibodies (Samson *et al*, 1986; Johnson & Negro-Villar, 1988). Anterior pituitary cells

in culture specifically release prolactin on the addition of exogenous oxytocin (Samson *et al*, 1986) but no such response has been seen *in vivo* and any association between the two is probably at a neuroendocrine level (Higuchi *et al*, 1983). The observations presented here do not support the hypothesis of a link between circulating prolactin and milk yield, or between prolactin and oxytocin in general. Prolactin profiles were similar for both experiments although gross milk yields were different, and oxytocin-treated animals had variable prolactin levels although in the second experiment prolactin was lower than controls. More detailed sampling at various stages of lactation with samples around milking times were also collected but no differences were found between groups. Overall this suggests that the reduction in prolactin in the second experiment was probably not a side-effect of oxytocin treatment. Indeed, giving oxytocin by repeated injection would be predicted to have increased the stress on the animal, and in retrospect comparison with control groups would have been improved if vehicle-alone injections had been given. However, an elevation in prolactin with treatment would be predicted if there was stress (Hart, 1973a) but this was not seen. It could also affect appetites but the rate of weight gain was similar in all groups.

Another question addressed was how could oxytocin elicit a long-term effect? If it was acting metabolically one would expect both 2X and 1X glands to have similar persistencies in the last treatment phase but if it was dependent on milk removal then poorer persistency would be predicted in 1X milked glands. Oxytocin increased yields in the first experiment but little change was seen the the second, but the switch to 1X milking did not cause a significant drop in yield in the latter. Regression analysis of yields indicated that 1X milking did not decrease persistency as predicted. When oxytocin-treated glands were switched to 1X milking they showed an improvement in persistency over contralateral 2X glands in 7 out of 9 animals. Of the remaining 2, one regression line gave a poor fit for the 1X gland so was not a good comparison for the 2X gland. A similar but less marked trend was also seen in the control group, suggesting that the observation might be ascribed to a change in milking frequency rather than an oxytocin effect *per se*. Due to this compounding factor the importance of removing milk at the time of oxytocin treatment could not be unequivocally determined, and the 'metabolic hypothesis' could not be confirmed or refuted.

So how can this tolerance for reduced milking frequency and less rapid loss of milk production in late lactation be explained? Oxytocin seemed to enhance the persistency effect and it altered some milk storage characteristics in the udder.

Treatment initially increased alveolar capacities, then maintained them, but trends were less clearly defined in the second experiment, probably reflecting the increased frequency of observations over a shorter time span. The observation of an increasing correlation between alveolar volume and milk secretion with lactation could indicate that absolute secretory tissue mass becomes more important as lactation progresses and as milk yields decline. If secretory cell number was maintained or improved in early lactation then there could be a carry-over into later lactation, potentially improving overall milk production without necessarily changing persistency. The oxytocin group did maintain their gross udder volumes above controls in late lactation, indicating a higher tissue mass although the increase could be attributed to, for example, thicker skin or more fat on the udder. Cisternal volumes were also higher in oxytocin animals in late lactation and this may partly explain their tolerance for infrequent milking. Local control of milk secretion is modulated through FIL but it must be in contact with secretory cells for autocrine inhibition to occur. If milk moves more readily into the cistern, then FIL essentially becomes inactive as it is distant from the secretory cells (Dewhurst & Knight, 1992). A change in milk storage dynamics due to, for example, a larger cistern, might promote or restrict the flow of milk away from its site of production, and could therefore alter milk yields via FIL action.

A further investigation of the influence of milking frequency on lactation persistency could be worthwhile as milking once a day would save on farming labour costs, although savings would be partly offset by increased milking times. Further experimental investigation could use three groups of animals which were milked twice a day until a specified time point and then either continued on twice-daily milking, switched to once-daily milking, or, as in this experiment, one gland switched to once-daily milking while the contralateral one continued on twice-daily milking. Comparison of milk yields could then indicate if it would be worthwhile reducing milking frequencies at this stage. Whether or not the regular use of oxytocin at milking is viable is questionable. The improvements in yield were at best variable and the administration route not suitable for widespread commercial use. However it may well be worth identifying animals which have poor persistency records and give them oxytocin at milkings. Sprain *et al* (1954) found that these animals responded well to treatment although there was no evidence for a persistency effect. Oxytocin may well work best with these animals whereas those which are already producing near their maximum potential will show little improvement in response to treatment. Further

investigation of the influence on parity on the response to treatment could also be conducted. Selective oxytocin treatment in combination with altered milking frequency may well be the best solution for improving production and lactation persistency.

CHAPTER 6

VARYING MODES OF MILK REMOVAL IN EARLY LACTATION AND THEIR EFFECTS ON LACTATION PERSISTENCY

1. INTRODUCTION

Mammary secretory tissue grows exponentially during late pregnancy and slower growth continues for several weeks after kidding, at least in primiparous goats (Knight, 1993) so if this growth pattern could be altered then it could affect overall milk production since this is dependent on secretory cell numbers and their activity. An alteration in growth could be stimulated by altering the method or frequency of milk removal *post partum*, for example, milking goats thrice-daily stimulated further growth and differentiation of the mammary glands (Wilde *et al*, 1987). So it might be expected that a short period of frequent milking in early lactation would have a long-term positive effect and conversely that infrequent milking early in lactation might have an unfavourable effect on development of the gland and on milk yields. Bar-Peled *et al* (1995) have provided some evidence for the former hypothesis in cows: increasing milking frequency from thrice- to six-times daily or milking with additional thrice-daily suckling, for six weeks *post partum* increased yields not only during treatment but significantly increased milk production when measured over an 18 week period.

The most natural method of increasing the frequency of milk removal is to keep goats with their kids after parturition, but there have been only a few reports on its long-term effects on gross milk production. Milk removal by suckling appears to be less efficient than machine milking as the latter can remove approximately 90% of the milk in the gland (Linzell & Peaker, 1971a) while two Damascus goat kids suckling thrice-daily (restricted to 15 minute intervals) for 38 days *post partum* removed, on average, 68% of the available milk (Louca *et al*, 1975). However, it must be remembered that the 'efficiency' of removal by kids will be restricted by their appetites and also by the time allowed for suckling, while there are no such constraints on machine milking. A more recent study, also in Damascus goats, found that kids allowed to suckle *ad lib* removed an average of 76% of the available milk over lactation days 3 to 38 and this regime had a significant and positive effect on lactational performance during the treatment period compared to goats milked twice daily (Hadjipanayiotou, 1986). In both studies no post-weaning carryover effect was found.

So why should there be differences in total milk yields between milked and

suckled animals? Machine milking removes most of the milk in the gland but the physical stimulus is confined to the area where the teat is within the machine's teat cup. This contrasts with suckling kids which vigorously push against and pummel the udder as well as sucking the teat. Both methods stimulate an acute reflexive release of prolactin (Johke, 1970) but cows can release significantly more prolactin and oxytocin in response to suckling and hand-milking rather than machine-milking; the magnitude and duration of peak hormone concentrations increases. So different methods of milk removal might result in subtly different hormone release profiles which, in turn, could influence the level of milk production. It has been suggested that the differential responses obtained by milking or suckling may be mediated through activation of specific sensory nerves in the teat involved in the neuroendocrine reflex (Svennerston, 1990a,b) which modify the response to the stimulus applied. Goat kids would also be expected to remove milk more frequently than that of the standard milking practice (twice-daily) but even when suckling was restricted to the same times as milking an increase in yield was seen (Hadjipanayiotou, 1986).

Udder anatomy can influence the response of an animal to an alteration in milking frequency: goats with relatively small cisterns have lower rates of milk secretion per gram of secretory tissue than those with larger cisterns who store proportionately more milk there (Peaker & Blatchford, 1988). These observations are probably due to the increased inhibition of milk secretion by FIL which occurs when milk is directly in contact with secretory cells (Wilde *et al*, 1995). If milk moves away from these areas into cisternal regions then FIL effectively becomes inactive (Dewhurst & Knight, 1993). Animals which store a greater proportion of their milk in alveolar areas would therefore be expected to respond well to increases in milking frequency as this would relieve the local inhibition of secretion at a milking. Udder anatomy, sites of milk storage and milk production of cows is also influenced by parity (Dewhurst & Knight, 1993) while in goats it can affect the plane of production (Peris, 1994) so one would predict that there could also be differences between primiparous and multiparous animals in response to different milking regimes.

The primary aim of this work was to manipulate the mammary gland during early lactation by applying various milking frequencies and methods of milk removal. The short- and long-term effects of treatment were investigated with the hypothesis that increased milk removal would increase yield and possibly udder size, and that this may, in turn, be modulated by milk storage characteristics of the udder and hormone

levels. Two studies were carried out over successive lactations to investigate the effects of once- and thrice-daily milking, suckling by kids, and continuous draining. All treatments in both experiments (with one exception) were unilateral ('treatment' compared to 1X milking) and this allowed a closer study of the local effects of milk removal as both glands experienced similar endocrine environments. The draining method was developed to examine the effect of allowing milk to continuously drain from the gland without accumulating in the udder and avoided the teat stimulation associated with conventional methods of milk removal. In the first experiment the groups were of mixed parity but in the second the response of primiparous and multiparous animals to unilateral once- and thrice-daily milking was tested while a third group (predominantly primiparous) were suckled bilaterally.

Prolactin and oxytocin responses to milking or suckling were measured and in the second experiment the suckling group were examined in more detail to investigate the influence of the presence of kids combined with the method of milk removal on prolactin profiles. The importance of the sites of milk storage within the udder in modifying responses to treatment was also considered. The majority of the observations were conducted during the treatment period (early lactation) however milk yields were recorded throughout lactation in both experiments. This allowed lactation persistency, that is the rate of decline in milk yields with time after peak lactation, to be measured and the long-term effects of early lactation treatments on milk production to be elucidated.

2. ANIMALS

British Saanen goats from the Institute herd were used and experiments commenced immediately post-partum. Animals had access to hay and water *ad libitum* and were offered 1.5kg of concentrated feed per day.

3. EXPERIMENTAL DESIGN

Two experiments were carried out over successive years with animals being selected on the basis of kidding date and parity (both the number of kids born and the number of previous lactations experienced). Treatments were milk removal by suckling, by milking thrice-daily (3X) or by continuous drainage. Animals giving birth to twins were preferentially assigned to suckled groups to ensure that suckled glands were adequately emptied during treatment. In the first experiment assignment to the

remaining treatment groups was random. In the second experiment animals were selected for inclusion in parity-based 3/1X milking or bilateral suckling groups. With the exception of the latter, all treatments were based on a within-animal design whereby comparison was made with the contralateral gland milked once-daily (1X). Treatments were applied during the first 6-8 weeks of lactation and for the remainder of lactation all glands were milked twice-daily.

3.1 Experimental groups

Experiment 1

Kidding dates ranged from 4th-15th May, 1993. Three goats were assigned to the suckled group (all twins), three to the drained group (2 singles, 1 triplet) and four to the thrice-daily milking (3X) group (all singles). All mothers were multiparous (2-4 lactations) with one exception in the suckle group (primiparous). Due to circumstances outwith the experiment the suckled group was reduced to 2 animals in the post-treatment period. From week 13 onwards these animals were used in the oxytocin study detailed in Chapter 5, experiment 2.

Experiment 2

Six goats were assigned to the suckled group (all twins; mothers- 5 primiparous, 1 multiparous), 6 primiparous goats were placed in the primiparous 3/1X group (3 singles, 3 twins) and kidding dates ranged from 1-13th March 1994. The first 6 multiparous animals to kid after this period were assigned to the multiparous 3/1X group (kidding dates ranged from 15th March-1st April, 1994; 4 singles, 2 twins).

3.2 Treatments

Kids were removed from goats within 24 hours of birth with the exception of the suckled group's kids who remained with their mothers throughout the treatment period. In the first experiment treatments commenced within 9 days of parturition and in the intervening period goats were milked twice-daily on both glands at 0800 and 1600h, allowing pre-treatment yields to be calculated for individual glands. In the second experiment treatments commenced within 24 hours of parturition so pre-treatment yields were not obtained.

Treatments were carried out for 6 weeks (Expt 1) or 6-8 weeks (Expt 2) and consisted of unilateral thrice-daily milking at 0000, 0800 and 1600h, suckling by kids (both experiments) or passive drainage (Expt 1 only). In the latter two groups treated

glands were hand- or machine-milked at 1600h to remove any remaining milk, allowing estimation of the effectiveness of the treatment. All contralateral glands were milked once-daily at 1600h (1X) with the exception of the suckled group in the second experiment when both glands were suckled. In experiment 1 this protocol was followed 6 days per week while the 7th day was designated as a 'measurement day' (see p.129). In experiment 2, the treatments were applied as detailed above for all but 3 days when additional measurements were made in the suckled group (weigh-suckle-weigh) (see p.129).

Post-treatment all goats were milked on both glands twice-daily at 0800 and 1600h.

Methods used to apply treatments

Suckled group

During the pre-treatment period in experiment 1 kids were kept with their mothers and a device designed to cover the goat's udder to prevent them suckling. This was further modified once treatments commenced to allow unilateral suckling. The 'unilateral suckling device' (Plate 6.1) was composed of a length of Tubigrip (Seton Healthcare Group, Oldham, UK) sewn into a bag and shaped to fit the udder. A small hole was formed and reinforced to allow protrusion of one teat, giving kids access to that teat only. An adjustable elastic band was sewn into the top of the device and fitted around the base of the udder against the body wall. A further piece of elastic formed a brace over the hips of the animal and led to the collar to prevent the device slipping or being pulled off the udder by the kids.

In the second experiment the treatment was modified to a bilateral one (allowing kids access to both teats) so the udder was not covered during the experiment.

Drained group

The drained group were housed in single pens with sawdust bedding changed on alternate days to ensure cleanliness. A sterilised teat cannula (Boviplug, Veterinary Drug Co, York, UK) was carefully inserted into the teat canal, allowing milk to drain continuously from the gland (Plate 6.2). Patency was checked regularly and teats cleaned frequently with a sterilising solution (2% hibitane in 70% alcohol) to reduce contamination and the risk of mastitis. The small but fairly constant flow of milk from the gland, seen especially when goats were standing up, also helped to minimise infection in the teat.

Plate 6.1 Unilateral suckling device *in situ*. Note that only one teat is accessible to the kid and is suckled, the other teat is covered and machine- milked once daily.

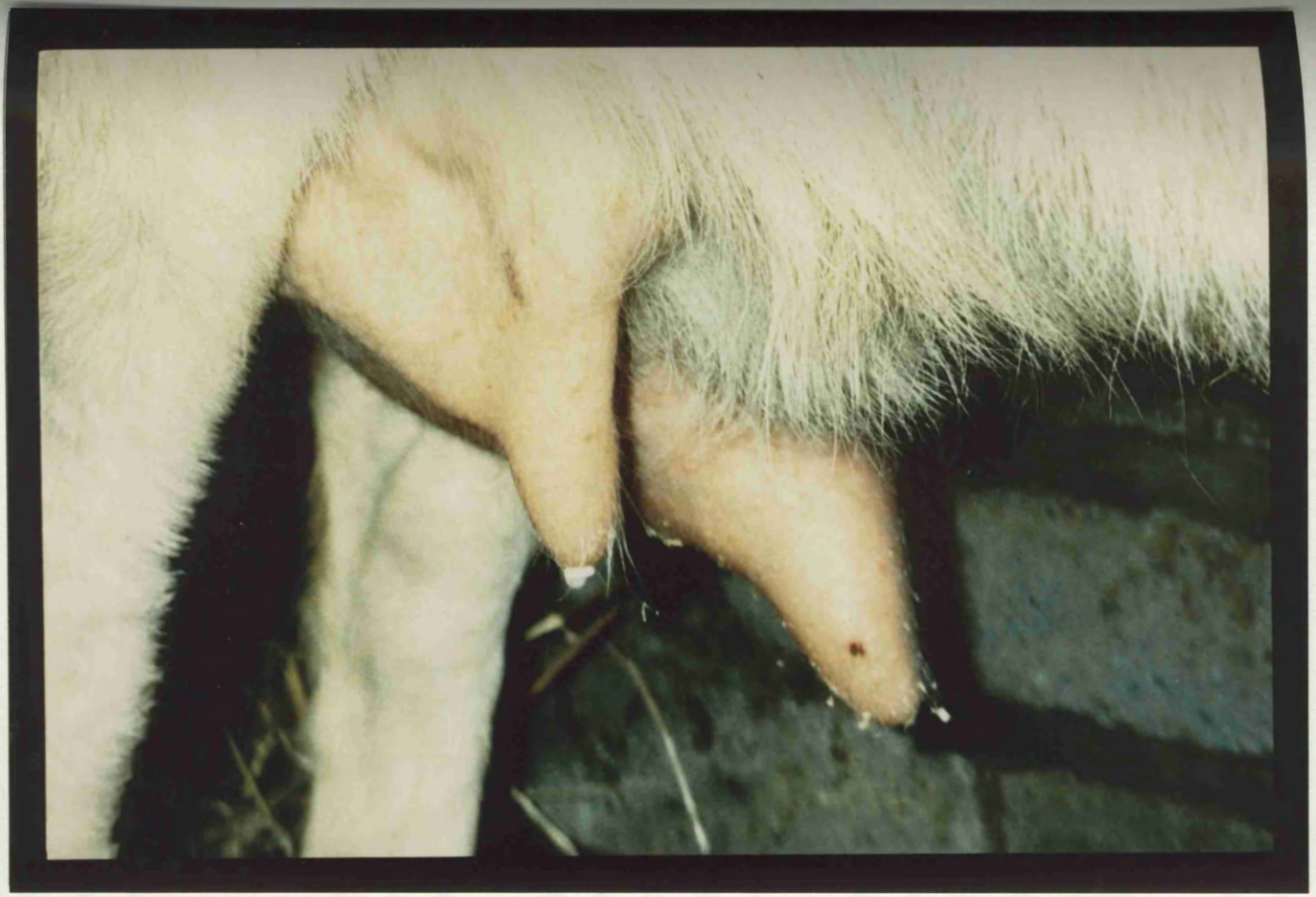


Plate 6.2 Teat cannula *in situ* (Drained group). Note the drop of milk forming on the tip of the cannula and the flaccid appearance of the gland, contrasting with the gland behind which is accumulating milk over 24 hour periods.

Measurement of milk yield in treated glands

In the first experiment, one day per week was designated as a 'measurement day' on which the milk yield of treated glands was determined by milking at 1600, 0000 and 0800h with exogenous oxytocin (0.4IU i.v.) to ensure complete milk removal. In the suckled group, kids were separated from their mothers, and the drained group had their teat cannulas either removed or closed during this period. Contralateral 1X glands were milked at the normal time of 1600h.

In the second experiment the suckled group were subjected to three periods of weigh-suckle-weigh measurements (adapted from the method described by Louca *et al*, 1975) made in weeks 1, 3 and 6 in which milk yields were estimated by measuring the amount of milk removed by kids. This improved the continuity of treatment but gave a gross yield for each animal rather than an individual gland yield. The technique used was as follows: kids were separated from their mothers after milking at 1600h and were weighed immediately prior to suckling bouts, each of which lasted for a maximum of 15 minutes, carried out at 0000, 0800 and 1600h. Once satiated, kids were reweighed, the weight difference representing their milk intake. While suckling they were carefully watched for signs of urination as this would cause underestimation of suckled milk. After each suckling bout mother and kids were again separated. After the 1600h measurement, goats were given oxytocin (0.4IU) and hand-milked to allow estimation of the efficiency of milk removal by the kids.

Both suckling and draining proved effective in keeping the gland relatively empty with approximately 250ml milk being removed each day by hand milking in experiment 1. Bilateral suckling in experiment 2 was also effective with an average of 190ml/d being obtained from each gland by hand-milking.

3.3 Measurements

A variety of measurements were made, summarised in Table 6.1. Briefly, individual gland milk yields were recorded throughout lactation and milk samples collected periodically for gross compositional analysis. Cisternal and alveolar milk storage characteristics were measured at the times indicated after 5 hours milk accumulation. An additional measurement of gross gland volume was made in experiment 2 immediately after milk storage measurements. A casting method was used, as described in Chapter 2, udder mid-lines were marked (Sprayline stock marker, Ritchey Tagg, Masham, UK) and some of this dye was transferred from the udder onto

Group/ Measurement	**Experiment 1** ***all groups***	***Primiparous***	**Experiment 2** ***Multiparous***	***Suckled***
Commencement of treatment	within 9 days post-partum	within 24h post-partum		
weigh-suckle-weigh	-	-	-	d5-9, d20-28 & d35-43
milk composition	weeks 1, 3 & 6	d6-9 & d38-42	d6-9 & d32-40	d6-10 & d36-43
milk storage	weeks 0 & 5	d2-7 & d39-41	d3-7 & d40-49	d2-5 & c39-43
blood samples routine	collected twice-weekly at 1400h			
blood samples frequents	weeks 0, 3-4 & 6	d26-35	-	d29-39
gross weights	once weekly			
biopsies	week 7	d13-15	-	d12-16
termination of treatment	week 7	d40-51	d43-60	d45-52

Table 6.1 Summary of measurements made and scheduling during experiments 1 and 2. Note that in the former measurements were based on experimental week (week 0= pre-treatment) whilst in the latter measurements were conducted with respect to lactation day.

the cast during casting. This allowed identification and measurement of individual gland volumes by extrapolation, assuming a vertical midline.

Routine blood samples were collected twice weekly at 1400h for the determination of prolactin by RIA (Chapter 2). More frequent blood sampling was also conducted in both experiments and prolactin measured, but the aims and timings of the frequent sampling varied slightly between the two experiments. Measurement of oxytocin concentrations (by RIA) in experiment 2 were kindly performed by Berit Samuelsson and Kerstin Svennersten, Sveriges Lantbruksuniversitet, Uppsala, Sweden.

In experiment 1 animals were cannulated at 0900 on the sampling day, or between 1600 and 1700 on the preceeding day, and hourly blood samples collected from 0900 until 1300h. This was followed by two windows of more frequent sampling at -30, -15, -10, -4, -2, zero, 2, 4, 6, 8, 10, 15, 30 and 60 minute intervals. At time zero (1400h) the suckled group's kids were allowed to suckle for several minutes, for the other groups this was a control sampling window with no teat stimulation. The sequence was repeated but at the second time zero (1600h) all goats were machine milked. A maximum of three goats were measured on any one day and suckled animals were separated from their kids between 1600 and 1700h on the preceeding day and reunited once the sampling day was completed. This design allowed a comparison within the suckle group of their responses to suckling and milking, and between them and the drain and 3X groups in their responses to machine milking, over the experimental period.

In experiment 2 the aim was to investigate in detail the response of suckled animals to the presence or absence of kids, in combination with the application of suckling or milking stimuli. The group was arbitrarily divided into two subgroups of 3 which were randomly tested with the following four combinations conducted out on successive days: (a) kids present throughout the day, suckling stimulus, (b) kids present, milking stimulus, (c) kids removed, replaced briefly for suckling stimulus and (d) kids removed, milking stimulus. As an additional comparison the 3/1X primiparous group was subdivided into two groups which were used as controls when the no kids, milking combination (d) was tested in the suckled group. Animals were cannulated at 0900 on the first day of the investigation. Samples were collected hourly from 0900-1300 then a single frequent sampling window was carried out at -60, -30, -20, -12, -8, -4, zero, 4, 8, 12, 20, 30 and 60 minute intervals. At time zero (1500h) goats were either suckled or machine milked for approximately 4 minutes. If kids were present

during the day they were removed one hour prior to time zero, if not they were removed at 1700h on the day preceeding the measurement. A maximum of nine goats were sampled on any one day.

Biopsies were carried out in both experiments using the method of Knight & Peaker (1984), providing samples for other experiments performed by colleagues (results not shown).

3.4 Calculation of relative milk yield quotients

Relative milk yield quotients (RMYQ) were used to measure the effect of treatment on opposing glands within animals (Linzell & Peaker, 1971). The following equation was used:

$$\mathbf{RMYQ} = (T_t \,.\, C_c) \,/\, (T_c \,.\, C_t)$$

T = treatment gland

C = control gland

t = treatment period

c = control period

A value greater than 1.00 indicates an increase in the yield of the treated gland relative to the control, while a RMYQ of less than 1.00 indicates a decrease relative to the control gland. In experiment 1, period 1 was before treatment commenced, in experiment 2 it was shortly after the start of treatment since treatment commenced at parturition. For both experiments period 2 was week 6 of treatment.

3.5 Statistical comparisons

Unless stated otherwise, statistical analysis was conducted using Student's paired or unpaired t-test, as appropriate, to compare between glands or between groups.

4. RESULTS

4.1 Milk yields

In experiment 1, treated glands were subjected to unilateral suckling, 3X milking or draining, while contralateral glands were milked 1X. Milk yields from treated glands were compared on measurement days and expressed as a percentage of the average daily yield obtained on 3 days immediately before treatment commenced (Figure 6.1). Lactational profiles of milk yields were also plotted (Figure 6.2) to illustrate the average daily yields obtained on both treatment and measurement days

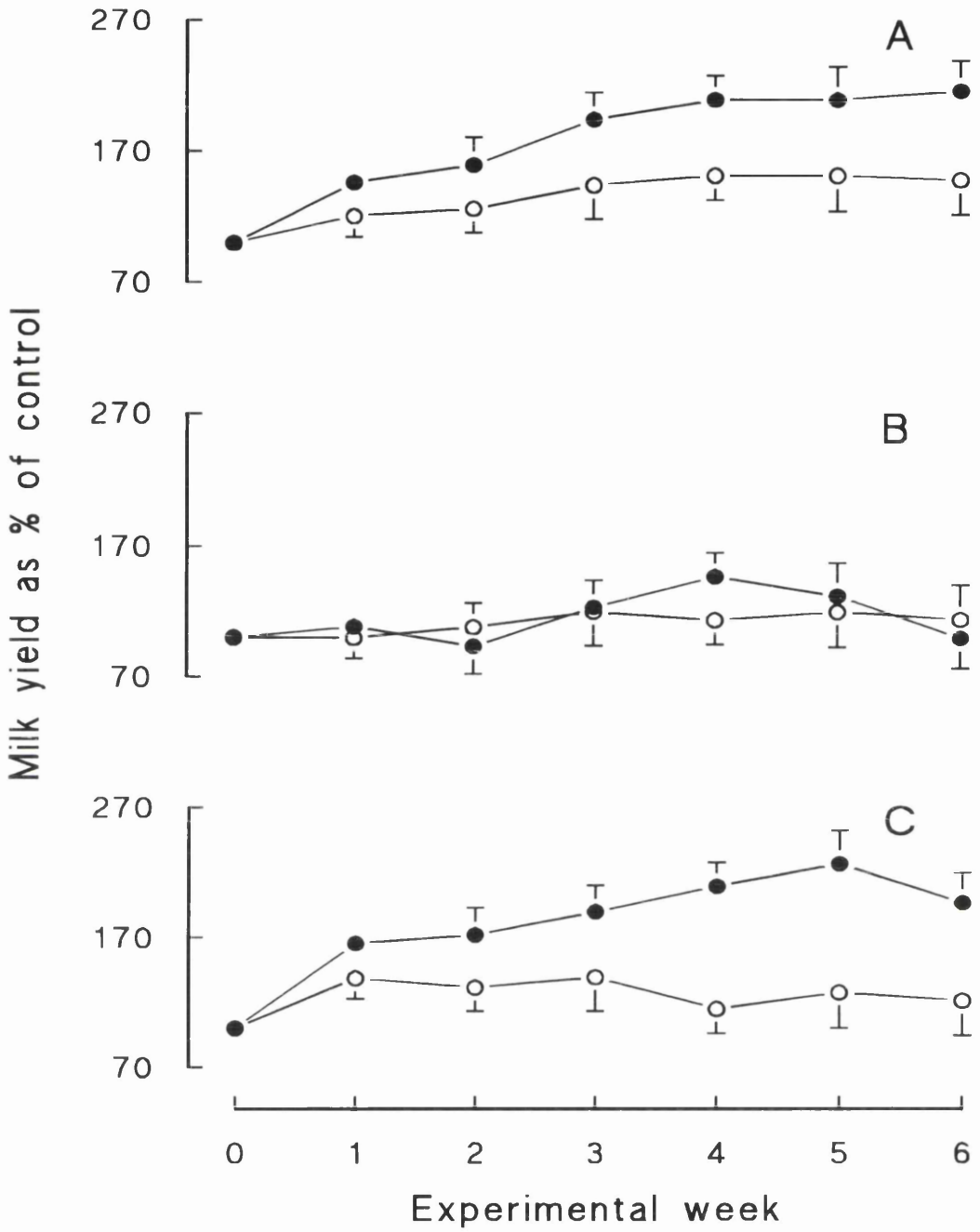


Figure 6.1 Milk yields determined on measurement days in Experiment 1, expressed as a percentage of the pre-treatment yield, determined during twice-daily milking.. Treatment glands (●) were (**A**) milked thrice-daily, (**B**) drained or (**C**) suckled while contralateral glands (❍) were milked once-daily.

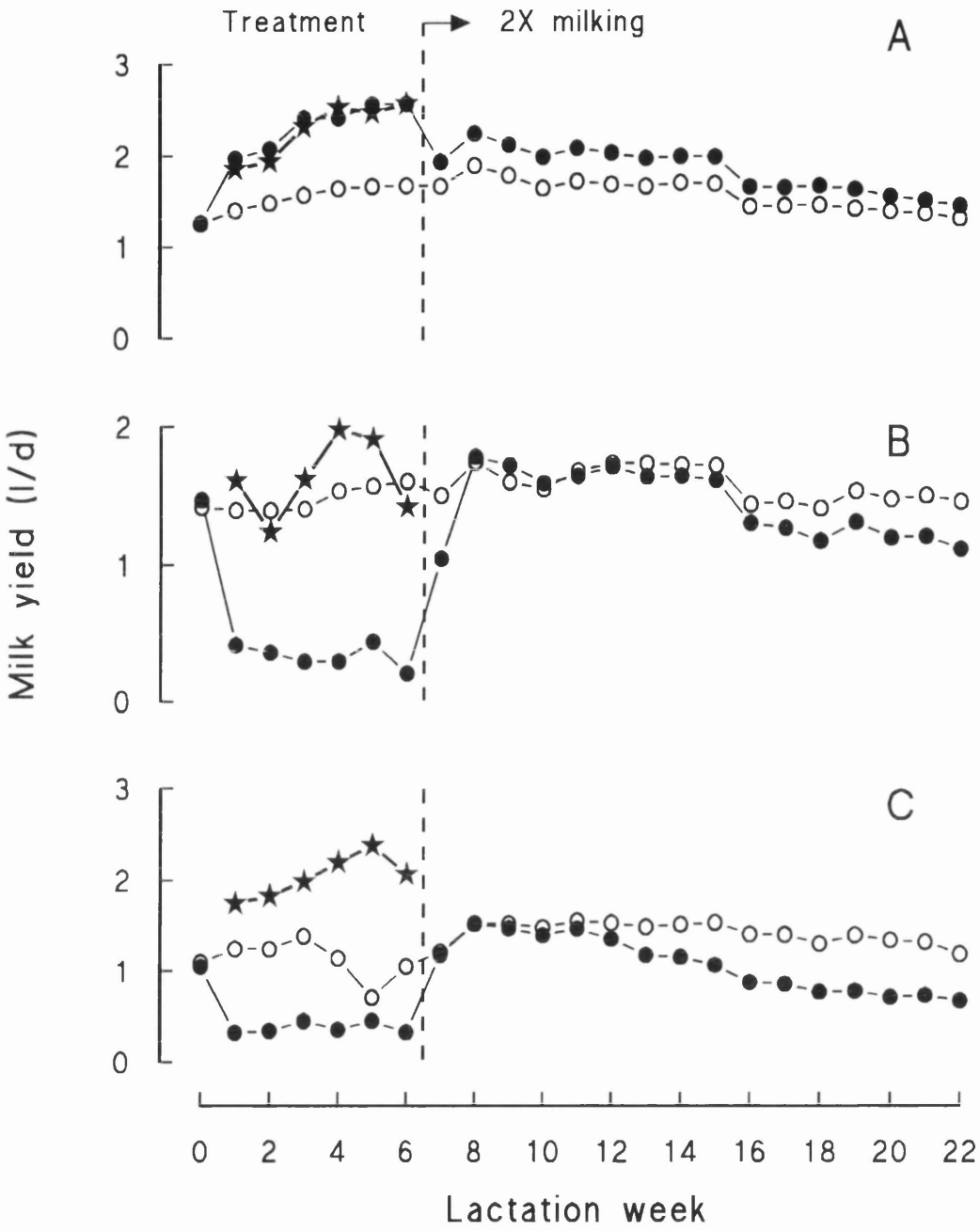


Figure 6.2 Average daily milk yields measured in Experiment 1. Treatment glands (●) were (**A**) milked thrice-daily, (**B**) drained or (**C**) suckled while contralateral glands (❍) were milked once-daily. On measurement days all treatment glands were milked thrice-daily, yields represented by (★). All glands were milked twice-daily from week 7 onwards.

during the treatment period, and during routine twice-daily milking after treatments had ceased. Finally the effectiveness of treatment within animals was assessed by calculation of relative milk yield quotients (RMYQ's).

Suckling significantly increased yields over opposing 1X glands when compared on measurement days both on a percentage basis, and on actual yields ($p<0.01$), confirmed by RMYQ values (1.611 ± 0.14, $p=0.048$). A similar pattern was seen in response to 3X milking ($p<0.004$) although the treatment effect measured by RMYQ was not as marked (1.439 ± 0.13, $p=0.074$). Draining did not stimulate milk production with percentages and yields similar to those of contralateral 1X glands and the RMYQ of 0.901 ± 0.18 ($p=0.63$) reflected this lack of response. There may have been some carryover of suckling and 3X milking onto contralateral 1X glands during treatment as they increased their percentage yields above those of drained group's 1X glands ($p<0.05$), but this was not apparent when absolute yields were compared, suggesting that any overall effect was small. In the post-treatment period 3X glands consistently produced more milk than contralateral 1X glands with an average difference of 0.265 ± 0.019 l/d ($n=15$, $p<0.0001$). This effect was reduced towards the end of the experimental period but this was after a prolonged period (16 weeks) of twice-daily milking. No significant effect of early lactation treatment on yield in the post-treatment period (weeks 7-22) was seen in the other groups; the improved yield in the suckled group 1X glands provided insufficient data for analysis as there were only 2 animals available for measurement.

In experiment 2 milk yields were determined over the lactational period (Figure 6.3) and statistical analysis conducted (Figure 6.4). During the treatment phase, yields obtained by weigh-suckle-weigh measurements from bilaterally suckled animals were compared to those obtained of the other groups; suckled glands produced more milk than primiparous 1X ones ($p=0.01$). In the post-treatment period a difference in yield between suckled glands was found ($p=0.0001$), despite receiving identical treatments throughout. This may reflect an inherent imbalance between the glands due to circumstances outwith the experiment, not apparent during the treatment phase as yields were measured on a whole animal basis then divided by 2 to obtain an average gland yield.

3X milking increased yields above contralateral 1X glands in the primiparous group ($p=0.0001$) and an RMYQ of 1.494 ± 0.21 ($p=0.062$) was calculated, confirming the results obtained in experiment 1. There was some carryover of

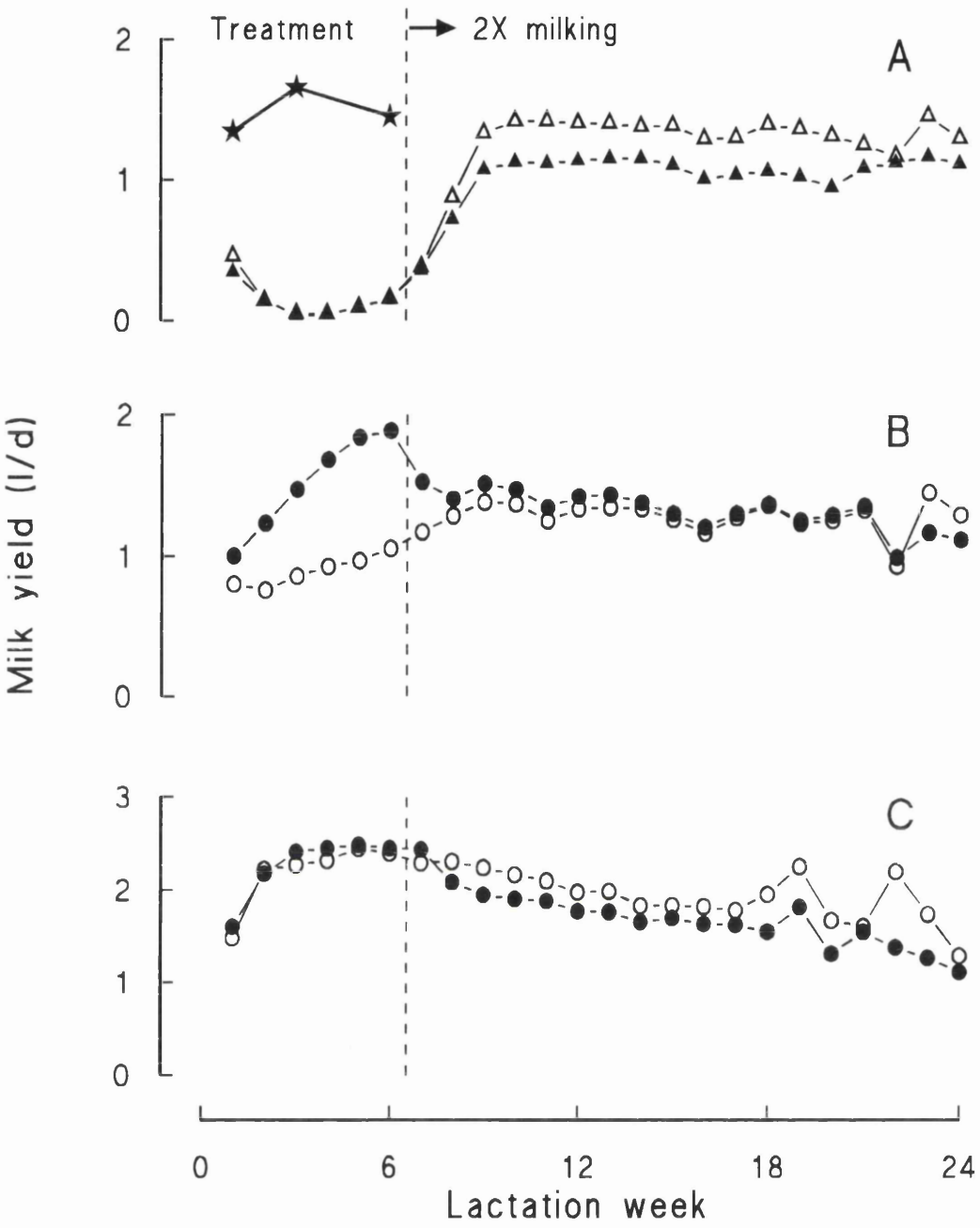


Figure 6.3 Average daily milk yields measured in Experiment 2. (**A**) During the treatment period both glands were suckled in the suckle group and (★) represent yields obtained from weigh-suckle-weigh measurements. (**B**) Primiparous and (**C**) multiparous 3/1X groups were milked thrice-daily (●) and once-daily (❍) on contralateral glands. All glands in all groups were milked twice-daily from week 7 onwards.

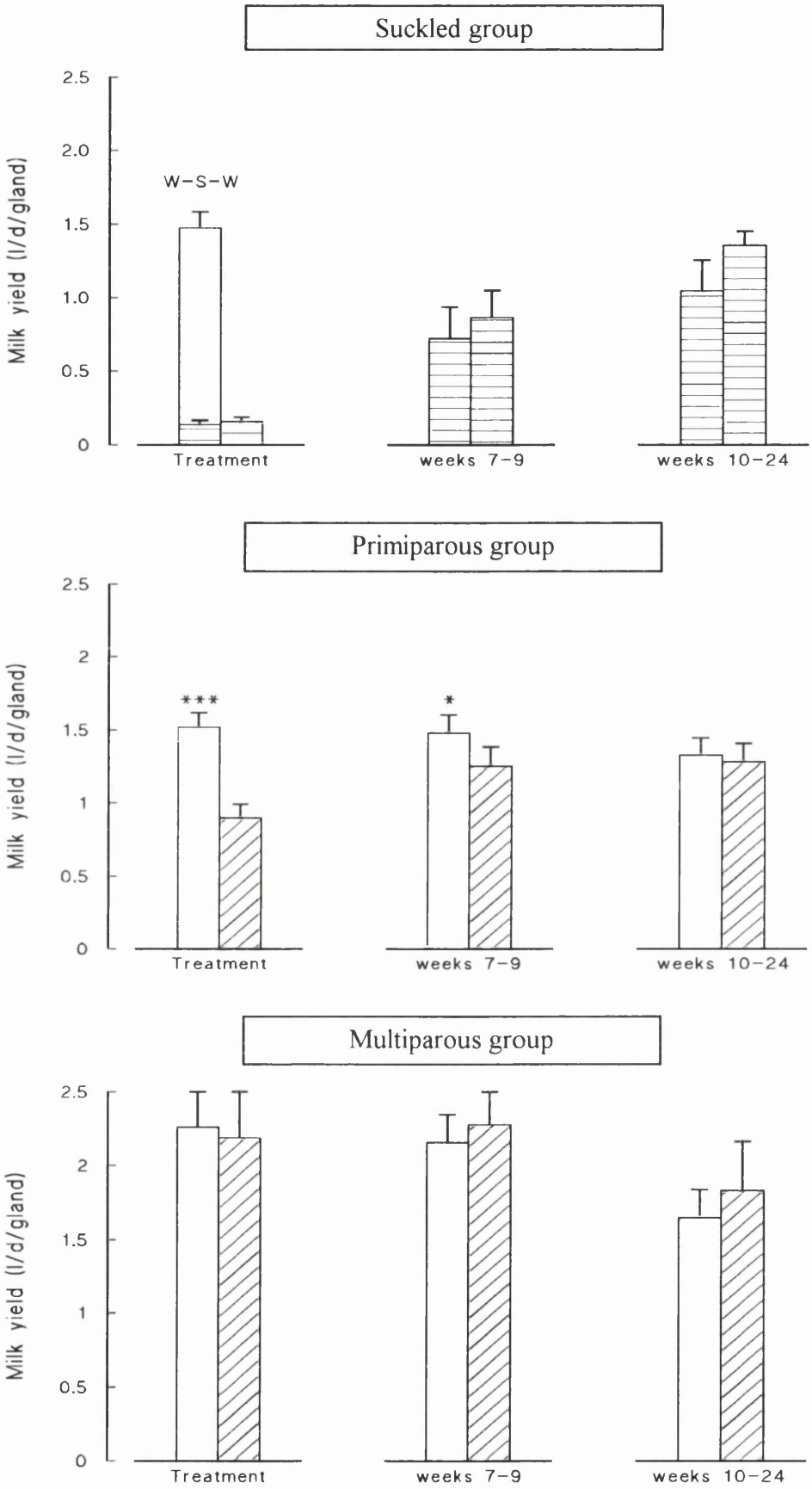


Figure 6.4 Statistical analysis of milk yields in Experiment 2, n=6 for each measurement. Horizontally-hatched bars represent suckled glands, open bars represent 3X glands and diagonal-hatched bars represent 1X glands.

treatment apparent in weeks 7-9 ($p=0.039$) but not thereafter. 3/1X Multiparous animals also increased yields in response to 3X milking but proved remarkably tolerant of 1X milking. Both glands had very similar yields during treatment, reflected in the RMYQ of 1.008 ± 0.14. Meaned data for this group (Figure 6.4) indicated that in the post-treatment period glands previously milked 1X produced more milk than contralateral 3X glands. When the data was examined more closely and Student's paired t-test used to calculate statistical significance, the average difference between 1X and 3X glands of each animal over lactation weeks 7-24 was calculated to be 0.174 ± 0.024 l/d. This was highly significant ($p<0.0001$), indicating a carryover effect of early lactation 1X milking on milk production in later lactation. This group also produced the largest volume of milk (an average of 3.69 l/d compared to 2.38 and 2.60 l/d in suckled and 3/1X primiparous groups respectively, $p<0.01$).

4.2 Lactation persistency

Lactation persistency was measured over weeks 8 to 22 in experiment 1 and weeks 10-24 in experiment 2 when all glands were milked twice daily (Table 6.2). A general trend of improved persistency in 1X glands was seen in all but the multiparous 3/1X group (15/20 1X glands had higher persistencies over contralateral 1X glands), but the effect was significant only in the 3/1X group in experiment 1 ($p=0.009$). However it must be remembered that contralateral glands were being emptied 2X throughout this period so there was twice-daily milking-induced hormone release, which probably influenced persistency in 1X glands. The bilaterally suckled and primiparous 3/1X groups had the highest persistencies, regardless of treatment; the former group contained 5/6 primiparous animals, suggesting that parity may also be a factor influencing persistency. When the regression lines for individual glands in experiment 2 were divided into whether animals were primi- or multi-parous, it was found that the former had significantly higher persistencies over the latter (an average drop of -17.3 ml/d for primiparous animals, -30.5 ml/d for multiparous, $p=0.043$).

4.3 Milk composition

Bulk milk samples were collected from all glands and all goats in both experiments. They were analysed for protein, lactose, fat and solids content but no significant effects of treatment within animals or between groups was found (Table 6.3).

	Treated gland			Contralateral (1X) gland		
Group	**m**	**r^2**	**p**	**m**	**r^2**	**p**
Mixed 3/1X	-43.7 (11.3)	77.2 (14.4)	0.001 (0.001)	-27.6 (9.0)	63.5 (22.0)	0.033 (0.033)
Primiparous 3/1X	-11.1 (2.9)	26.3 (9.5)	0.211 (0.130)	-4.5 (2.0)	12.3 (6.9)	0.500 (0.165)
Multiparous 3/1X	-28.8 (6.8)	51.1 (11.4)	0.026 (0.013)	-34.5 (3.8)	26.9 (12.8)	0.017 (0.012)
Drained	-48.9 (28.7)	63.6 (8.4)	0.000 (0.000)	-19.1 (7.6)	37.9 (21.5)	0.101 (0.062)
Suckled unilaterally	-75.0 (21.0)	84.5 (7.1)	0.000 (0.000)	-36.5 (16.6)	71.7 (11.6)	0.001 (0.001)
Suckled bilaterally	-16.4 (4.4)	41.8 (12.0)	0.101 (0.063)	-6.0 (9.6)	43.2 (15.2)	0.303 (0.189)

Table 6.2 Summary of regression lines (**m**) fitted to individual milk yield curves for each animal's glands in both experiments. The fit of the line (r^2) and probability (**p**) are as indicated. Numbers in brackets represent the sem with the exception of the suckled unilaterally group when it represented the sd. Group sizes were as follows:
Mixed 3/1X n=4; drained n=3; suckled n=2 (all expt 1); primiparous3/1X, multiparous 3/1X & suckled bilaterally n=6 (all expt 2).

Experiment 1	***3X***		***Drained***		***Suckled***	
	1X	3X	1X	Drained	1X	Suckled
Protein (g/100ml)	2.3 (0.1)	2.5 (0.2)	2.3 (0.1)	2.3 (0.2)	2.2 (0.1)	2.4 (0.0)
Lactose (mM)	101.0 (7.2)	100.3 (8.2)	104.1 (5.5)	104.6 (7.2)	103.0 (7.2)	99.8 (5.0)
Fat (%)	6.1 (1.2)	6.2 (1.1)	5.3 (0.3)	5.0 (0.3)	5.9 (0.5)	5.5 (0.4)
Solids (%)	14.1 (1.4)	14.2 (1.4)	13.4 (0.6)	13.3 (0.7)	14.0 (0.6)	13.8 (0.5)
Experiment 2	***Multiparous 3/1X***		***Primiparous 3/1X***		***Suckled***	
	1X	3X	1X	3X	Suckled (left)	Suckled (right)
Protein (g/100ml)	2.9 (0.5)	2.9 (0.5)	2.7 (0.3)	2.7 (0.3)	2.7 (0.3)	2.6 (0.2)
Lactose (mM)	145.2 (5.2)	135.6 (3.9)	137.4 (5.3)	136.5 (4.6)	122.8 (6.7)	135.4 (5.6)
Fat (%)	4.7 (0.2)	4.5 (0.4)	3.9 (0.6)	4.2 (0.5)	4.1 (0.4)	3.7 (0.4)
Solids (%)	13.1 (0.5)	13.1 (0.4)	12.9 (0.5)	13.4 (0.4)	12.2 (0.6)	11.5 (0.5)

Table 6.3 Milk compositional analysis of samples collected in experiments 1 and 2. Numbers in brackets represent sem and n=5 for each measurement with the exception of suckled and primiparous 3/1X glands in Expt 2.

4.4 Udder characteristics

Sites of milk storage

A summary of alveolar and cisternal storage volumes, and of cisternal percentages obtained in experiment 1 are shown in Figures 6.5 and 6.6. There were no statistically significant differences between groups in their alveolar and cisternal storage characteristics in week 0 (pre-treatment) or after 5 weeks of treatment, reflecting the variation between individuals. However, there were changes within glands, across time, in response to treatment. Alveolar volumes increased in suckled and 3X glands while cistern proportion dropped, suggesting a positive effect of treatment on the secretory cell population. Drained and 1X glands increased or maintained cisternal volumes, decreased alveolar volumes and consequently 1X milking increased cistern percentage in all three groups. The reduction in alveolar capacity suggests that this regime was not particularly effective in maintaining secretory cell function, most probably due to a lack of stimulation. Visual confirmation of the trend of cistern proportion increasing was obtained- after an identical period of milk accumulation more milk was seen in the cistern of 1X glands than in contralateral treatment glands (Plate 6.3).

The results obtained in experiment 2 are summarised in Figures 6.7 and 6.8. At the start of treatment (week 1) there were some significant differences between the groups in their storage characteristics. The 3/1X multiparous group had the highest cisternal capacity ($p < 0.05$), while the 3/1X primiparous group had the lowest alveolar capacities ($p < 0.05$). These observations most probably reflect parity effects as measurements were made shortly *post partum*. In all 3 groups cisternal volumes and cistern proportion increased over the treatment period, while alveolar volumes dropped, significantly so in primiparous 1X glands ($p < 0.001$), indicating a particular lack of tolerance for 1X milking in this group. In contrast, multiparous animals had the highest alveolar volumes at the end of the treatment period ($p < 0.05$), corresponding with their high levels of milk production from both glands.

Gross udder volumes

In experiment 2, gross udder volumes were determined by casting, then the cast was cut along the midline and individual gland volumes estimated. The 3/1X multiparous group had the largest glands throughout ($p < 0.01$), supporting their higher level of milk production, while 3/1X primiparous animals had the smallest (Figure 6.8). Treatment did not significantly affect gland volumes, all dropped over the six week period with the exception of 1X glands in the 3/1X multiparous group.

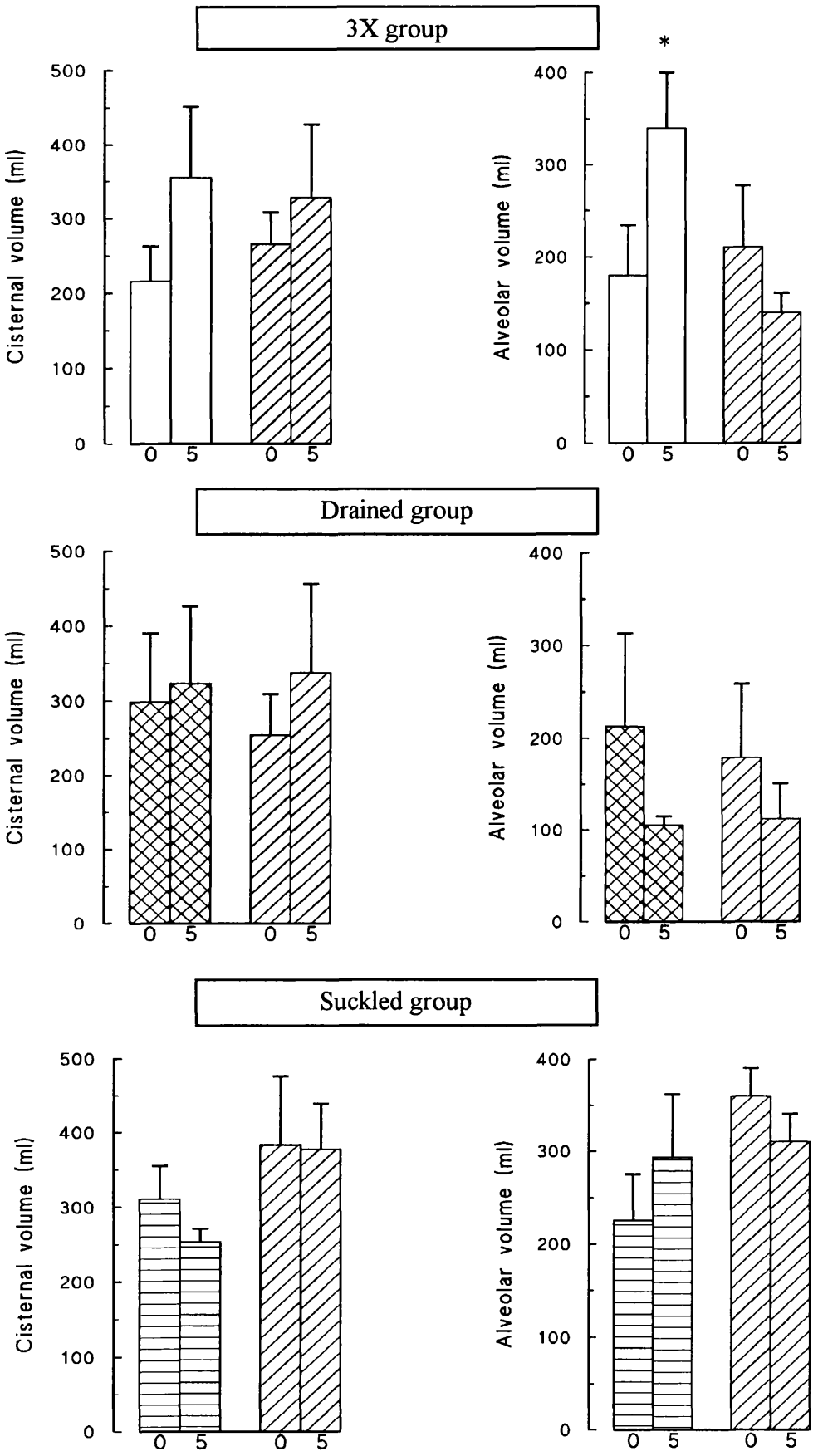


Figure 6.5 Capacity of cisternal and alveolar compartments in lactation weeks 0 (pre-treatment) and 5, in Experiment 1. Diagonal-hatched bars represent 1X glands, the others were treated as detailed.

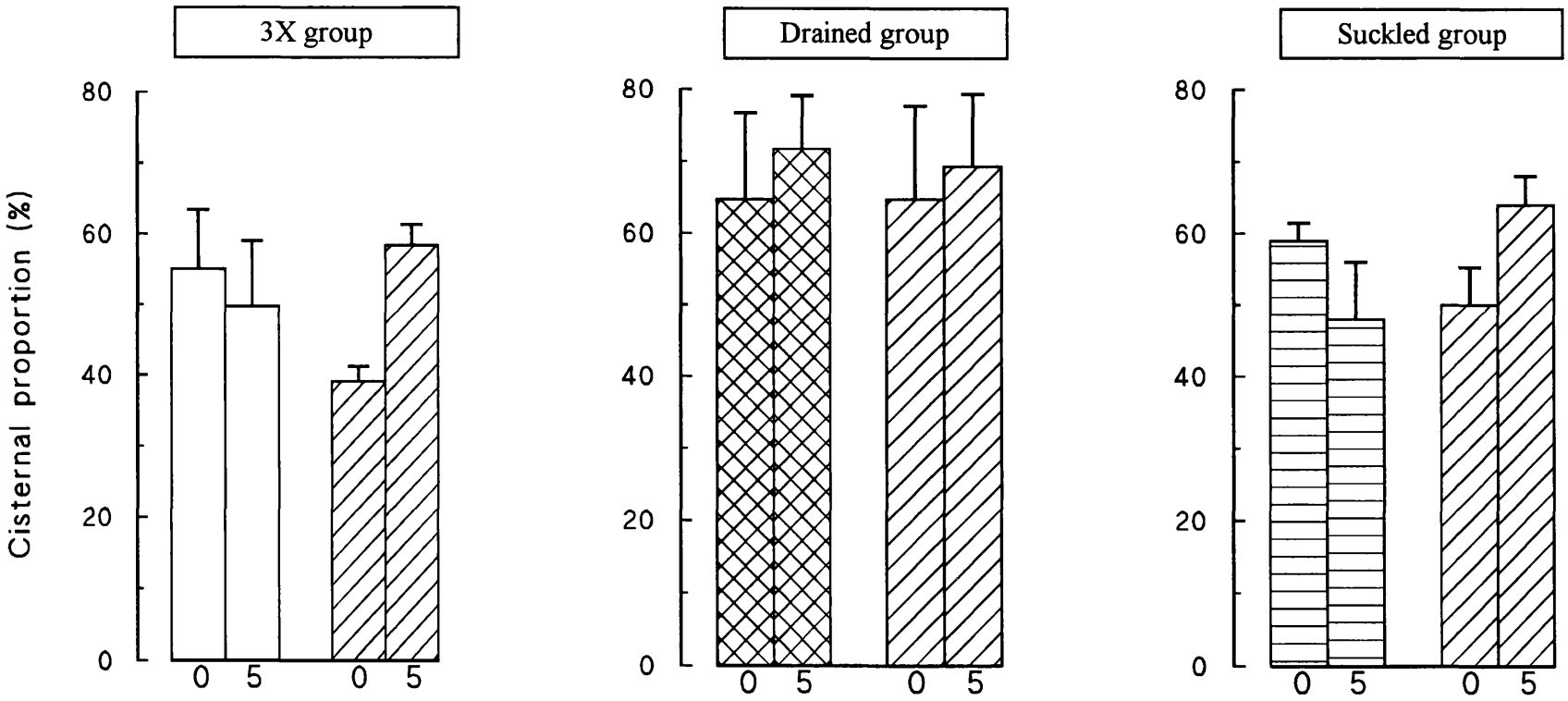


Figure 6.6 Cistern proportion measured in Experiment 1. Diagonally-hatched bars represent 1X glands, others glands were given the appropriate treatment.

Plate 6.3 One of the drained group just prior to the 1600h milking on a measurement day. Both glands were emptied at the last milking (0800h) and the teat cannula in the right gland plugged. Note the distended appearance of the left (once-daily milked) gland in the cisternal area, while the right gland appears more swollen in the alveolar area. After milking with oxytocin a similar yield was obtained from each gland.

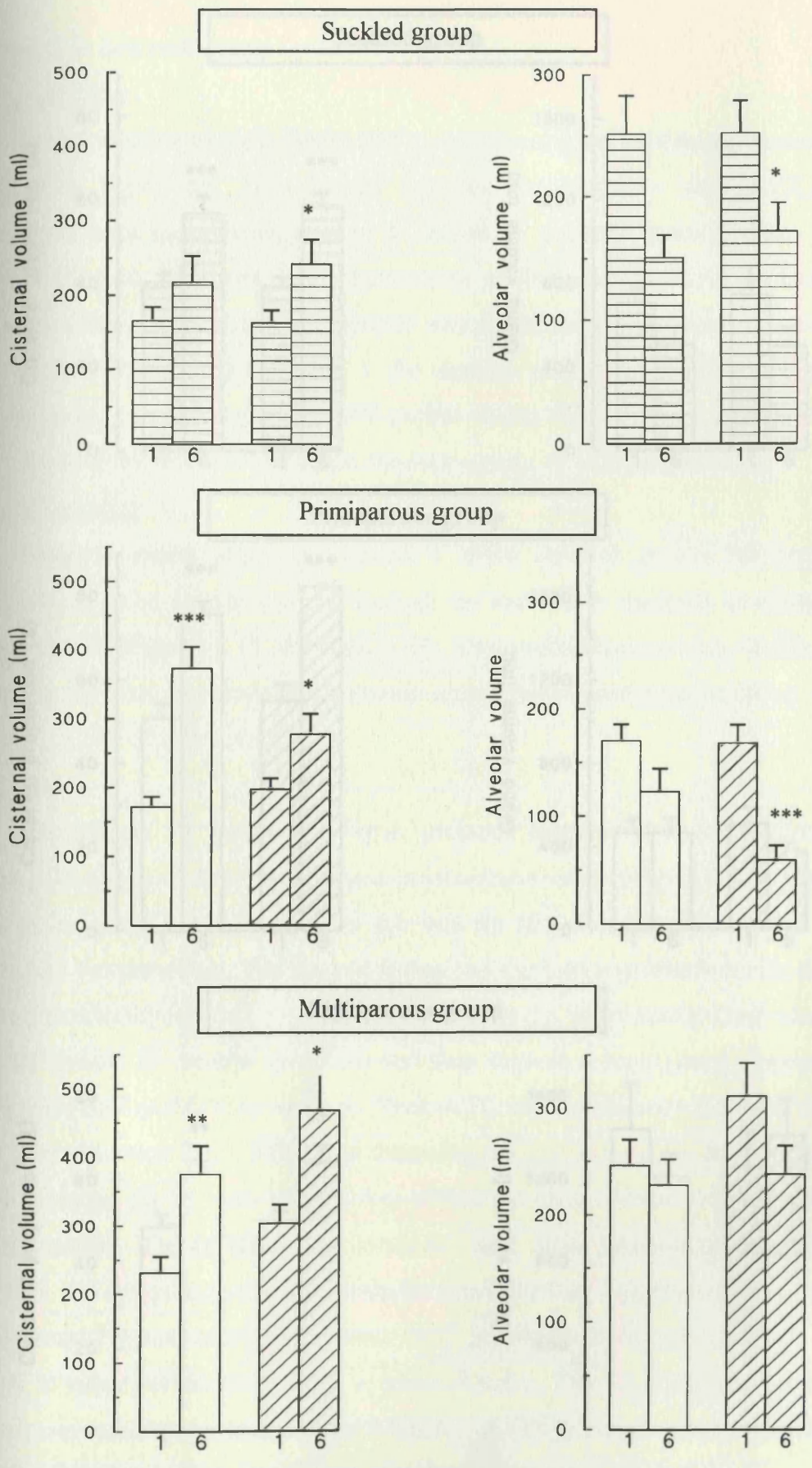


Figure 6.7 Capacity of cisternal and alveolar compartments in weeks 1 & 6 in Experiment 2. Horizontally-hatched bars represent suckled glands, open bars 3X glands and diagonal-hatched bars represent 1X glands.

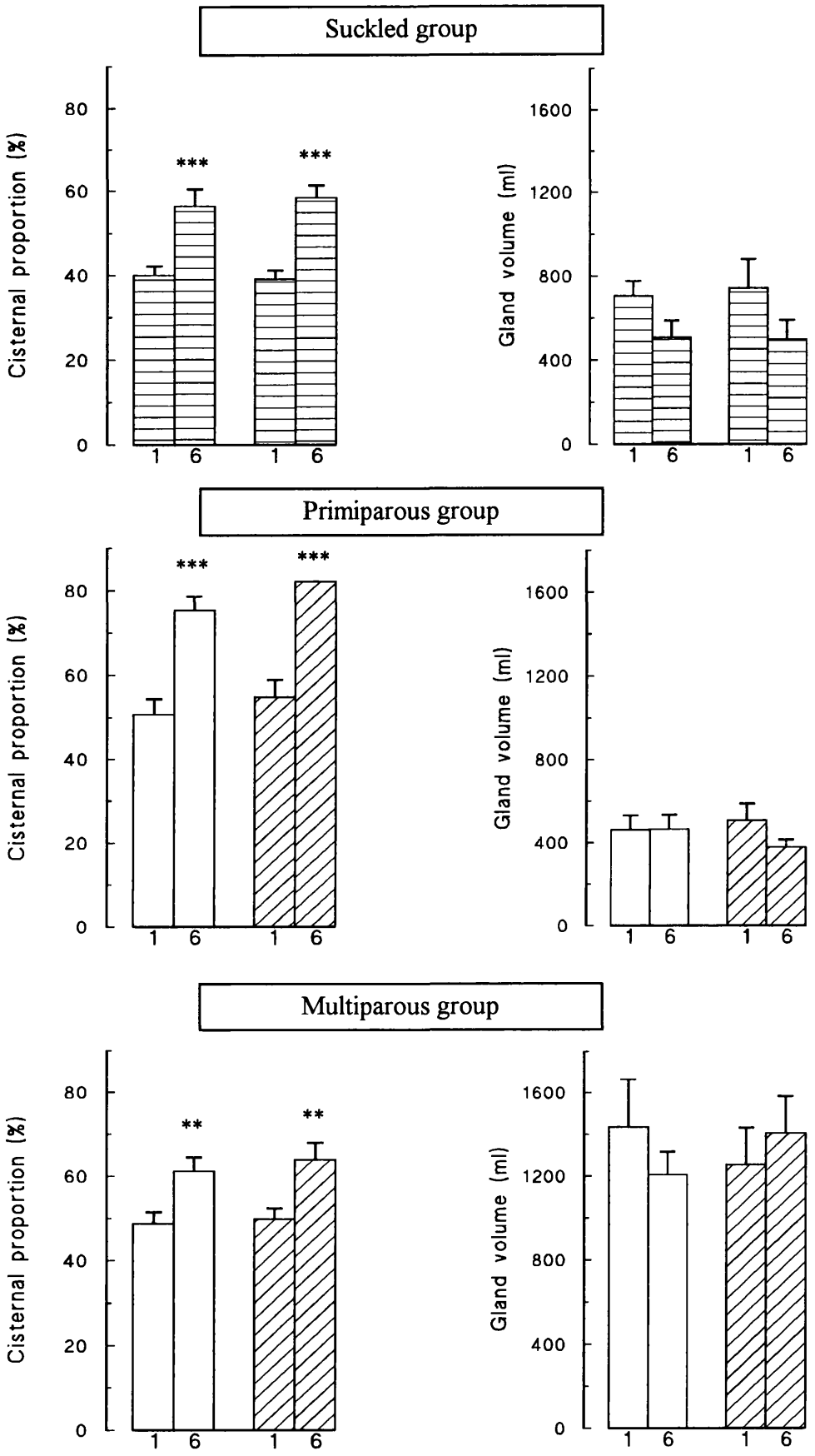


Figure 6.8 Cistern proportion and gross gland capacities in weeks 1 and 6 in Experiment 2. Horizontal-hatched bars- suckled glands; open bars- 3X and diagonal-hatched bars represent 1X glands.

4.5 Hormone determinations

Routine sampling

Prolactin concentrations determined by routine sampling in both experiments are illustrated in Figure 6.9. During early lactation concentrations were particularly variable but both experiments showed a decline in prolactin concentrations after treatment stopped, consistent with observations of declining prolactin as lactation progresses (Hart, 1972). Statistical analysis was conducted on the basis of treatment phase (Figure 6.10). In experiment 1 the drained group had significantly lower prolactin levels than the suckled and 3X groups during treatment ($p<0.006$), but no other statistical differences were found between groups in either experiment.

Frequent sampling

Frequent blood sampling allowed a more detailed profile of hormone concentrations to be determined both through the day and in response to milking or suckling stimuli (Figures 6.11, 6.12 and 6.13). The results obtained were analysed by calculating the total area under the hormone concentration profile curve (areas under the curve, AUC) (Table 6.4).

Experiment 1

Drained and 3X groups had higher prolactin concentrations in the first few samples collected in week 0 than was seen in subsequent weeks (Figure 6.11), possibly reflecting increased levels of stress as this was the first time the animals had been subjected to the procedure. The drained group had the lowest prolactin levels during treatment (excluding the first 3 measurements in week 0), significantly lower than the other two groups by week 6 ($p<0.01$) and they showed a diminishing response to milking with time ($p<0.01$ by week 6). Their AUC was significantly less than that of the 3X group in week 3 ($p=0.040$) and these observations support the hypothesis that passive drainage allows milk removal but avoids the characteristic milking-induced hormone release. The suckled group increased their AUC between weeks 3 and 6 ($p=0.042$) and comparison of actual prolactin concentrations supported this; increases were detected between each measurement week ($p<0.05$). However there was little response to either suckling or milking in weeks 3 and 6. The 3X group also increased prolactin concentrations between weeks 3 and 6 ($p<0.001$) but, in contrast to suckled animals, responded well to the milking stimulus throughout.

Oxytocin results are illustrated in Figure 6.12 and Table 6.4. Calculation of AUCs found no significant inter- or intra-group differences at any timepoint. Analysis

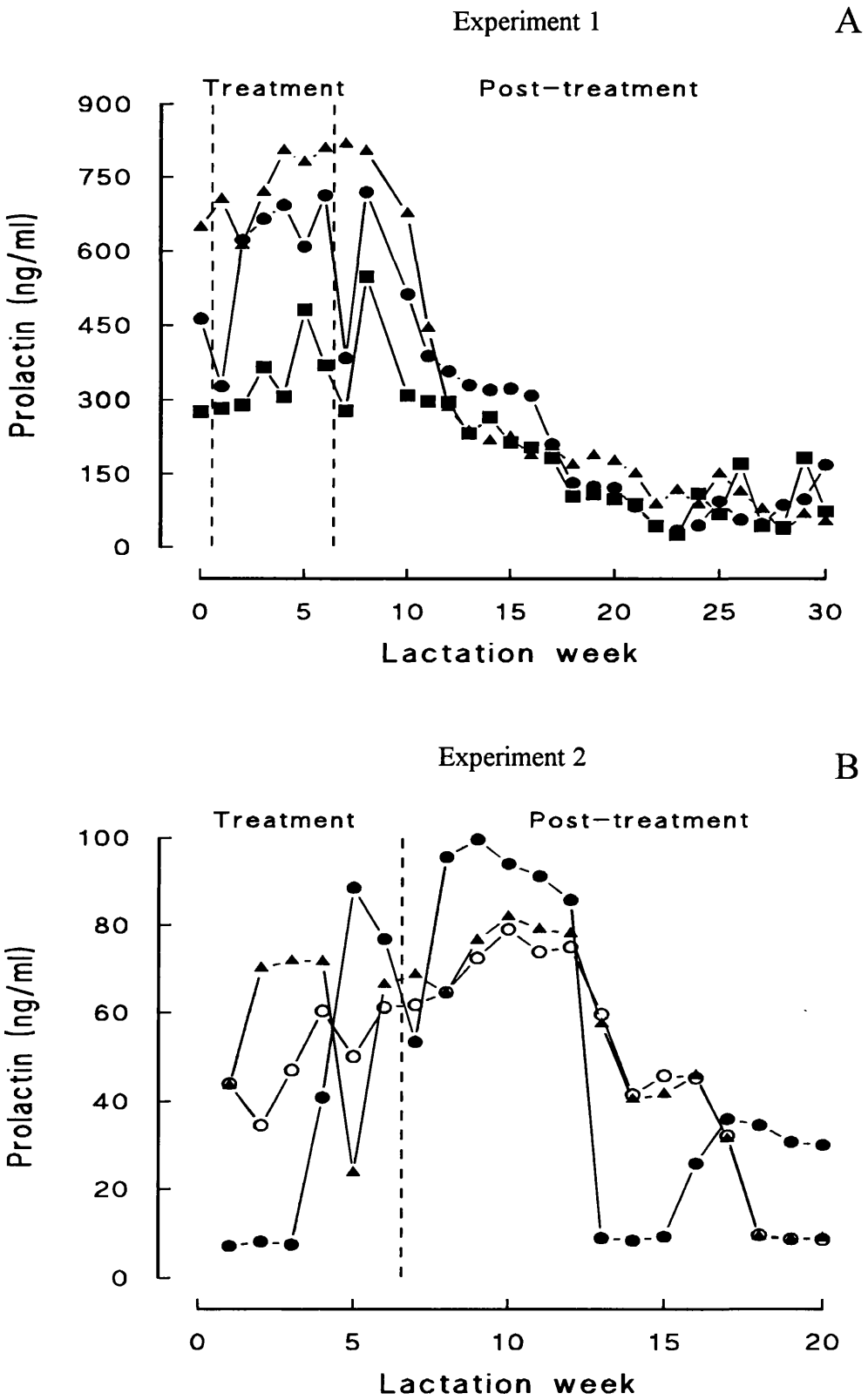


Figure 6.9 Plasma prolactin concentrations determined by routine blood sampling in (**A**) Experiment 1 where (●) represent mixed 3/1X, (■) drained and (▲) suckled groups, and (**B**) Experiment 2 where (●) represents multiparous 3/1X, (○) primiparous 3/1X and (▲) suckled groups.

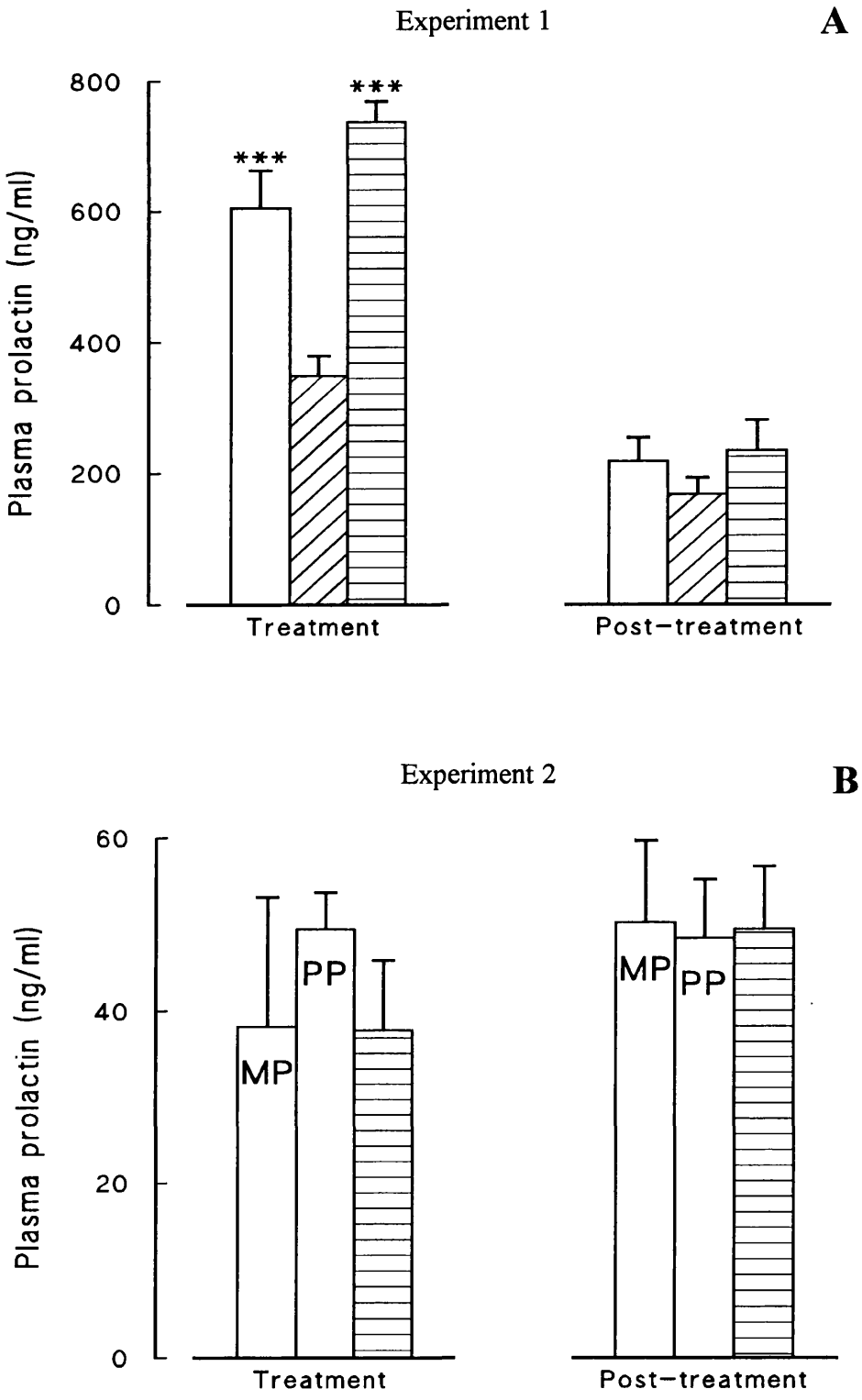


Figure 6.10 Statistical analysis of prolactin concentrations obtained by routine sampling in experiments 1 (**A**) and 2 (**B**), divided into treatment phases. Open bars represent 3/1X milked groups (MP= multiparous, PP= primiparous), diagonal hatched bars drained glands, and cross-hatched bars the suckled glands.

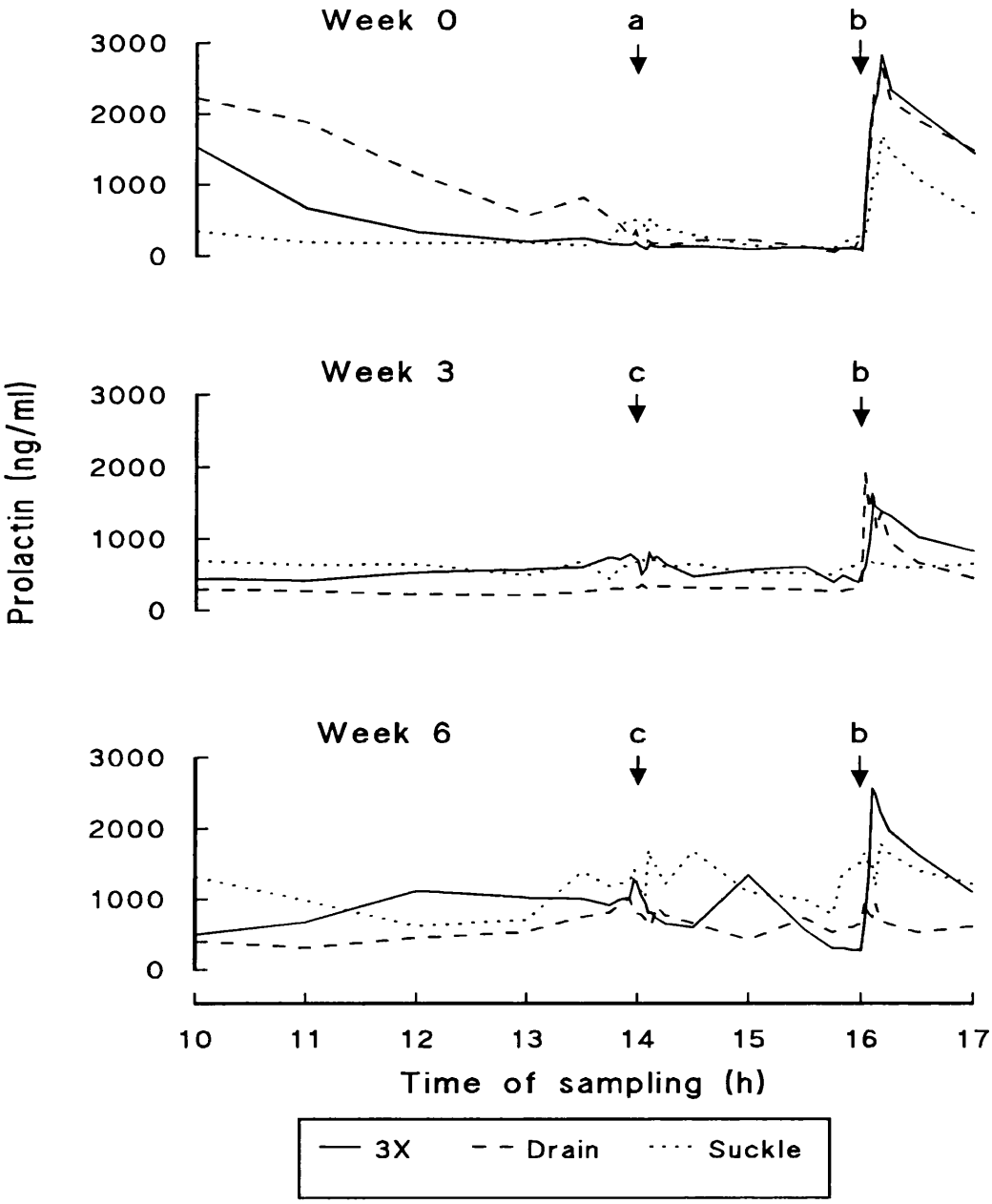


Figure 6.11 Prolactin concentrations determined by frequent blood sampling in Experiment 1. Arrows indicate **a**- control sampling for all groups, **b**- milking for all groups and **c**- suckling for suckled group. Pooled sem 19.8 ng/ml.

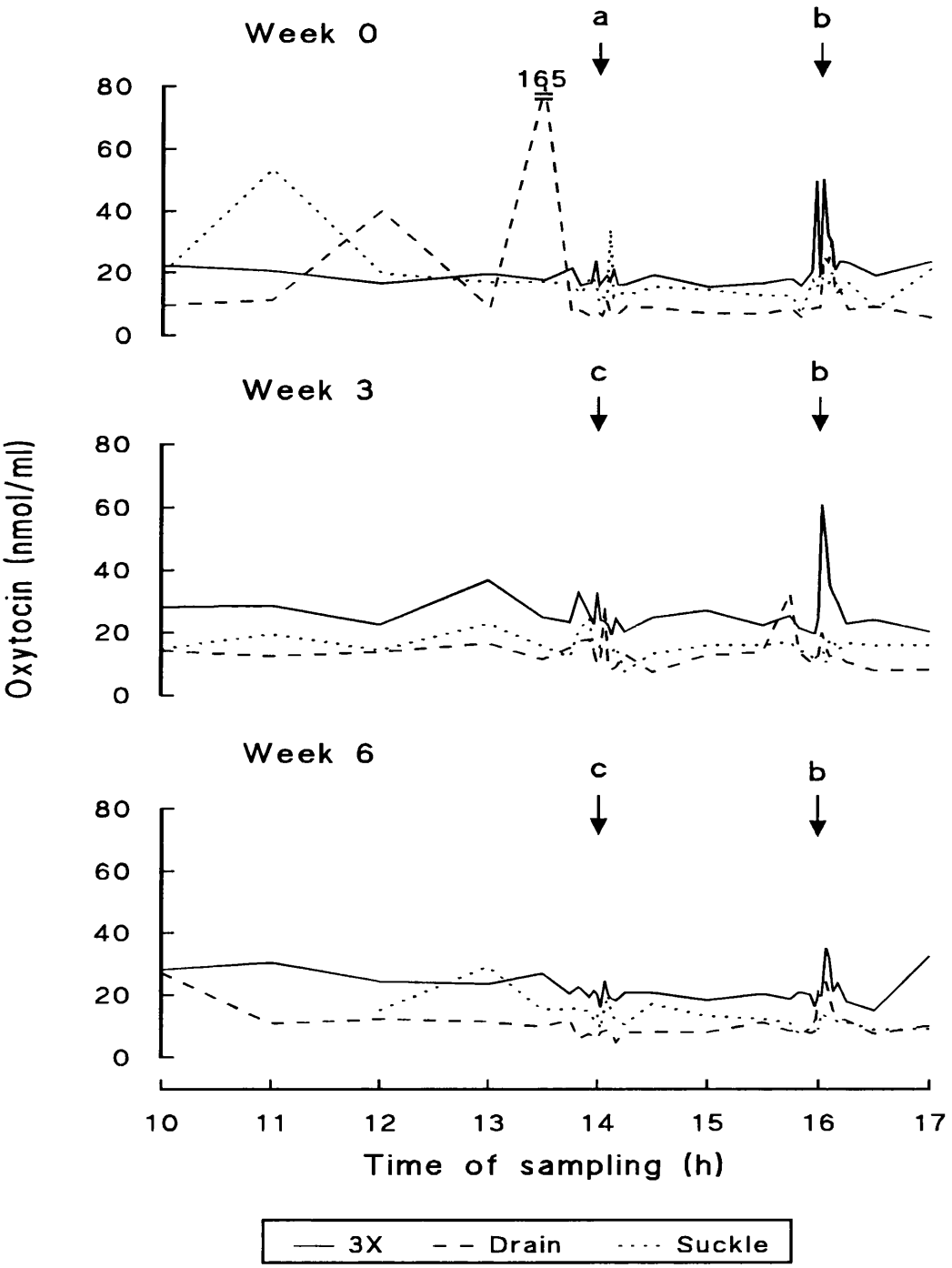


Figure 6.12 Oxytocin concentrations determined by frequent blood sampling in Experiment 1. Arrows indicate **a**- control sampling for all groups, **b**- milking for all groups and **c**- suckling for suckled group. Pooled sem 1.38 nmol/ml oxytocin.

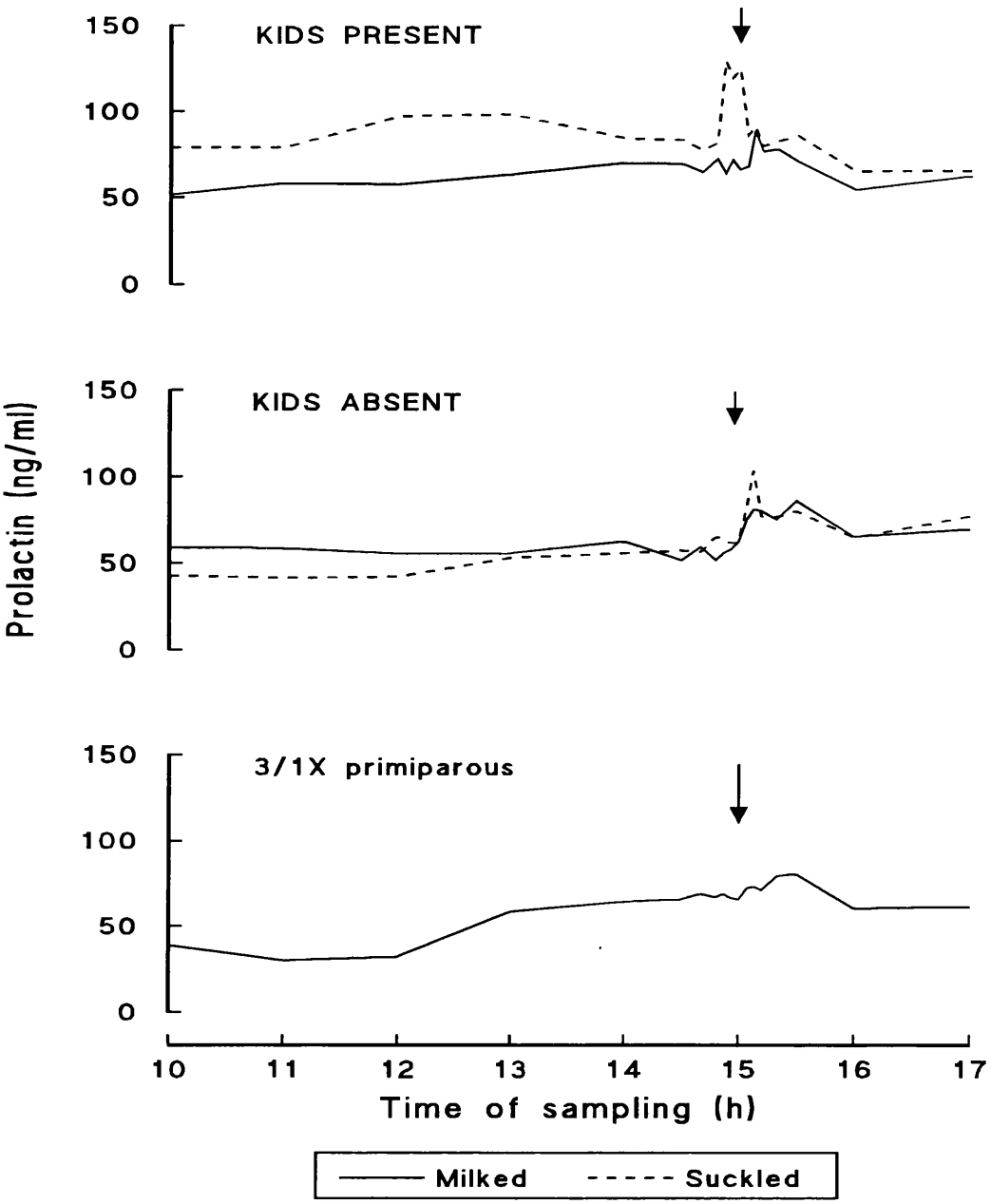


Figure 6.13 Prolactin concentrations determined in the suckled group by frequent blood sampling in Experiment 2. The primiparous 3/1X group were used as controls for the 'kids absent and milked' combination. Arrows indicate when milking or suckling stimuli were applied. Pooled sem was 12.6 ng/ml.

Measurement/ Group	Area under the curve		
	week 0	**week 3**	**week 6**
Prolactin			
Suckled	2323 (263)	4237 (364)	7689 (2110)
3X	4207 (568)	4232 (645)	6673 (2242)
Drained	7183 (1615)	2429 (371)	3845 (1750)
Oxytocin			
Suckled	121 (54)	107 (42)	197 (96)
3X	135 (14)	186 (30)	166 (43)
Drained	153 (63)	91 (29)	92 (19)

Table 6.4 Analysis of prolactin and oxytocin 'environments' experienced by animals during the day as determined by frequent blood sampling in experiment 1. Numbers in brackets represent sems and n=3 for each measurement. Statistical significance is indicated in the text .

of oxytocin concentrations found significant increases in the 3X group at each measurement week ($p<0.007$), above that of the suckled group in week 0 ($p<0.009$) and above suckled and drained groups in week 3 ($p<0.0001$). However on week 6 there were no significant differences between the groups. Comparison of oxytocin profiles found that in general there was a clear oxytocin response to machine milking in the 3X group which increased from week 0, a poor response in suckled animals and an improving response in the drained group.

The suckled group showed contrasting reactions to milking stimuli which varied between measurement weeks. When milked in week 0 they showed a characteristic prolactin peak, albeit of lesser magnitude than the other groups, but no detectable oxytocin release, by mid-treatment there was little response to either suckling or milking and by the end of treatment both stimuli elicited prolactin release. In both latter cases oxytocin release was greater in response to suckling than milking. It was therefore decided to look at this group in more detail in the follow-up work.

Experiment 2

This aimed to clarify the prolactin response to suckling and milking stimuli in the suckled group with kids being present throughout the day or absent except for a brief suckling period (Figure 6.13). The primiparous group was used as a control comparison for the 'kids absent, milked' combination. When AUC's were compared over the whole day (Table 6.5) the only significant difference found was the kids present, suckled resulted in higher AUC than the kids absent, suckled combination ($p=0.024$) and neared significance when compared to kids present, milked ($p=0.068$). This pattern was reinforced when concentrations were compared- kids present, suckled resulted in higher prolactin than the other treatments ($p<0.001$).

The response to teat stimulation was varied. In the control animals there was no clear prolactin peak at milking, although a small and sustained rise was detected. A similar pattern was seen when kids were absent and milked in the suckled group- their response was greater but of similar duration. A very clear prolactin peak was obtained when these animals were suckled and levels remained elevated over a similar time. When kids were present there was a reduced response to milking- the peak was of similar magnitude but of shorter duration. The kids present, suckled combination gave a much higher basal prolactin level throughout the day, probably reflecting intermittent suckling by the kids, and the highest peak was obtained with suckling although levels rapidly fell after the stimulus.

Suckled group kids	Stimulus	Area under curve
Present	Suckling Milking	5766 (571) 4357 (347)
Absent	Suckling Milking	3917 (344) 4312 (843)
Primiparous group *Absent*	Milking	4185 (647)

Table 6.5 Analysis of the prolactin environment experienced in specific conditions in experiment 2. Numbers in brackets represent sems and n=6 for each measurement.

5. DISCUSSION

The main aim of the experiment was to investigate the short- and long-term effects of different methods and frequencies of milk removal on milk yield and on lactation persistency. Suckling had a positive effect on milk yields compared to contralateral 1X glands, milk yields of suckled glands being similar to that of 3X milked glands (compared on measurement days and by weigh-suckle-weighing). Milk removal by kids was relatively efficient with an average of approximately 200ml remaining in the gland after a 24 hour suckling period (range 30-440ml). When residual milk was measured after 3 weigh-suckle-weigh episodes over 24 hours the efficiency of removal varied from 62 to 100% with an average of 91%. This contrasts with previous work (Louca *et al*, 1975; Hadjipanayiotou, 1986) which found that suckling was not particularly efficient in removing milk (68-78% suckled). Suckling was restricted to twice-daily in these studies while in the experiments described here kids were allowed to suckle freely. However on measurement or weigh-suckle-weigh days when access was restricted to thrice-daily there was still relatively complete milk removal. It may well be that the differences reflect the appetites of the kids rather than the effectiveness of the stimulation; indeed close examination of the weigh-suckle-weigh data illustrated that the poorest 'removal percentages' were associated with an 8 hour milk yield in excess of 1000ml and the converse was true for yields of under 1000ml. Combining milking with suckling will ensure regular emptying of glands and also provide the vigorous teat stimulation of suckling and this has resulted in an overall increase in production in cows, above that of those milked at identical intervals (Bar-Peled *et al*, 1995).

We found no long term positive effects of suckling on lactation persistency, confirming the results of Louca *et al* (1975) and Hadjipanayiotou (1986). Murciano-Granadina goats who suckled their kids for 7 weeks *post partum*, when switched to twice-daily milking also had very similar yields to those milked twice-daily throughout (Peris, 1994). In the first experiment the persistency profile was very poor for suckled glands but there were only 2 animals available for measurement in the post-treatment period and there may have been complications such as teat damage which contributed to the rate of decline. In the second experiment suckled group's persistency was comparable to that of the 3/1X primiparous group, suggesting that the effect might also be attributable to an effect of parity, as well as a treatment effect. In both of these groups there was little decline in milk yield with a loss of approximately 125ml milk

over the 14 week post-treatment period. It may be that the continued growth of the mammary gland in early lactation (Knight & Wilde, 1993) and the effect of milking/suckling combine to maintain mammary function. The benefits of suckling remain variable as during treatment yields are enhanced but milk is lost to the kids; advantageous to those investing in and rearing kids, but not to those relying economically on milk production itself. Perhaps a compromise of both suckling and milking may provide the key in not only allowing the rearing of kids and collection of marketable milk, but also having a potential long-term positive effect on lactational performance as indicated in cows (Bar-Peled *et al*, 1995).

Hormone levels will play a role in the modulation of treatments on milk yield and lactation persistency; analysis of routinely collected samples showed that during the treatment phase the drained group had significantly lower prolactin concentrations than the other two groups and they responded poorly to treatment in terms of enhancing milk yield, but no significant differences were detected in experiment 2 or post-treatment in either experiment. However it must be borne in mind that these samples provide a 'snapshot' of the plasma concentration at that particular time. More frequent sampling attempted to elucidate group differences in more detail. Draining had the least effect in enhancing milk yields, the treatment avoided teat stimulation and milk ejection occurred only once every 24 hours when animals were machine milked. Basal hormone concentrations reflected the lack of stimuli but at milking oxytocin was released. The magnitude of the response was not restricted by the treatment, reflecting the level of stimulus that milking provides. 3X milked animals had, in general, the highest hormone levels and responded well to milking, especially by the end of the treatment phase. They had higher milk yields and this may reflect a combination of frequent milk removal and teat stimulation maintaining favourable hormonal conditions and reducing local inhibition of milk secretion. Suckled animals showed variable responses to suckling and milking. The follow-up in experiment 2 found a more distinct release of prolactin with suckling, rather than milking and higher overall levels associated with the presence of kids. However the elevated basal level seen when kids were present and suckling at time 0, rather than milked, cannot be explained as one would predict similar levels for both stimuli. The response in the control group was also less than expected from the initial results and the overall differences may partly reflect the timing of frequent blood sampling which was carried out from week 3-4 onwards, a period in which hormonal fluctuations (determined in routine samples) were less

marked.

So how can the teat stimulation of suckling or milking produce different hormonal release patterns? The physical stimulus of kids suckling as opposed to milking by machine affects the magnitude and pattern of hormone release (Svennersten, 1990). Conditioned responses at milking will play a role in facilitating hormonal release and if animals are suckled when the environmental cues associated with milking are absent then this could alter the hormonal response. In experiment 1 prolactin release appeared to be inhibited by presence of kids, as has been seen in cows kept with their calves (Akers & Lefcourt, 1984). This restriction could be due to increased stimulation of the mammary gland due to intermittant suckling, increasing pituitary depletion of stored prolactin, or reducing the response to suckling by a process of down-regulation (Johke, 1970).

Increased milking frequency, to thrice-daily, improved milk yields during the treatment period in primiparous goats, but this response was lacking in multiparous animals receiving identical treatments. The response to increased milking frequency has been observed in Saanen goats by several authors; Henderson *et al* (1983) using animals on their 2nd to 5th lactations and Wilde *et al* (1987) whose group comprised of 6 primiparous and a multiparous (9th lactation) animal. So why did we find a lack of response in our multiparous group of goats on their 5th-7th lactations? One clear difference between our groups was that multiparous animals produced larger volumes of milk but showed poor lactation persistency. A similar effect has been seen in Murciano-Granadina goats; third lactation animals had significantly higher daily milk yields than primiparous ones, but reduced lactation persistency (Peris, 1994). Another difference between the groups was that multiparous animals had significantly larger udders than primiparous animals although cisternal and alveolar milk storage characteristics were not significantly different. This could reflect the short period allowed for milk accumulation before measurements of the sites of milk storage were made, and it is conceivable that differences were present but undetectable after five hours. This time period had been chosen to provide minimal interference to the treatments being applied in the experiments.

In experiment 1 a significant carryover effect of 3X milking on milk yields in the post-treatment period was found when compared to 1X glands. This indicated a local and long-term effect which could reflect an increase in secretory tissue or its differentiation in the 3X gland, or a physical alteration of milk storage which improves

the efficiency of milk production. The effect was not confirmed in the second experiment and our initial observations may reflect that the group were subsequently used in another experiment, while in experiment 2 animals received no additional treatments. It could also be due in part to parity effects as the 3/1X group in experiment 1 was of mixed parity.

A long-term carryover effect of 1X milking in early lactation on subsequent milk yields was found in the 3/1X multiparous group. The effect was apparent throughout the post-treatment period although the level of persistency between 1X and 3X glands was similar. The effect could well be due to the physical effect of milk accumulating in the gland over a 24 hour period which would continue beyond the treatment period. If cistern capacity was increased then the local inhibitory effects of FIL in milk could be reduced as milk left the alveolar areas and entered the cistern, improving the efficiency of milk production.

It was hypothesised that drainage would improve milk yields over once-daily milking as milk would not accumulate in the gland and there would be reduced feedback inhibition of secretion. However drained glands showed no stimulation of yield even though milk removal was fairly continuous and there was virtually no accumulation of milk in the cistern. These observations could be explained by the requirement for milk to be removed specifically from secretory cells for FIL inhibition to be reduced. During draining milk was merely overspilling from the alveolar areas and draining away without stimulating the teat there would be no stimulation of hormone release and consequently no milk ejection. The secretory cell environment was probably comparable to the gland being milked once-daily, the similarities in yields between 1X and drained glands suggested that this was indeed the case. All 1X glands showed an increase in yield over the treatment period. Multiparous animals were most tolerant of this regime and post-treatment there was a distinct trend of improved persistency in 1X glands, albeit not statistically significant.

So in conclusion, the sites of milk storage may also modulate the yield response of an animal to various modes of milk removal. Suckling was generally associated with increasing alveolar capacity and decreasing cisternal storage, resulting in a lower proportion of milk being stored in the cistern. These observations were consistent with frequent milk removal providing little opportunity for milk to accumulate and 'stretch' the cistern. Secretory capacity was probably maintained by a combination of more frequent and complete milk removal minimising FIL inhibition of secretion, and as a

response to the vigorous teat stimulation and massage of the udder by the kids. Once-daily milking in early lactation gave steady or slight increases in milk yield and was associated with increasing cisternal volumes, decreasing alveolar volumes and hence a greater proportion of milk was stored cisternally by the end of the treatment period. On the switch to twice-daily milking these glands showed greater lactation persistency than those on other treatments (with the exception of the 3/1X multiparous group) and was the most consistent effect seen in this work. The suggestion is that increased milk accumulation times caused a long-term alteration in milk storage sites, namely milk moving more readily into the cistern. However effects on udder storage were not as marked as the persistency profiles suggested. This may be due to the relatively short time after milking that the measurements were made (5 hours post-milking) and also that measurements were made during early lactation. Perhaps if measurements were made after 8 or even 12 hours milk accumulation and at various points in later lactation then more pronounced effects on the partitioning of milk in the udder could be detected.

CHAPTER 7

CAN CASEINS IN MILK INDICATE LACTATION PERSISTENCY?

1. INTRODUCTION

Various physiological and cellular changes occur within the mammary gland to support the process of lactation. Milk yields are determined predominantly by the number and activity of the secretory cells in the gland (Oliver & Sordillo, 1989) and as yields fall after peak lactation the gland gradually involutes. In goats the decline in yield is accounted for by decreased cell number without a corresponding reduction in their secretory activity (Wilde & Knight, 1989) whilst in rats cellular activity alone, (Knight *et al*, 1984), and a combination of activity and cell number in mice (Shipman *et al*, 1987) determine yield. Most studies investigating involution have used the acute stimulus of stopping milk removal entirely and observing the consequences of this, for example after the cessation of milking in cows (Holst *et al*, 1987) or after litter removal in rodents (Walker *et al*, 1989). However this does not accurately mimic the gradual process of involution which occurs during declining lactation and can be spread over a period of months in ruminants.

During declining lactation the mammary tissue is gradually remodelled until milk production ceases and the gland is fully involuted. This process is modulated by plasminogen activator (PA) (which also operates in a variety of other tissues and physiological processes) which is responsive to changes in hormone concentrations (Ossowski *et al*, 1979). PA cleaves plasminogen to form an active alkaline protease, plasmin [E.C. 3.4.21.7] whose activity correlates positively with involution. Plasmin activity also correlates with increasing somatic cell counts, often an early sign of mastitis, in dairy cows (Politis *et al*, 1989a). Hence determination of PA and plasmin activity, or their ratio, could provide an index of the involution occurring in the gland, and could also, potentially, be related to lactation persistency as the rate of decline in milk production will reflect the extent of involution and loss of secretory capacity.

Plasmin also proteolyses proteins present in milk; caseins (cn) have variable resistances to its action (β-cn < α_s-cn < κ-cn) (Eigel, 1977b). Indeed, plasmin proteolysis of β-casein produces heterologous and distinct fragments of 'γ-casein' (Eigel, 1977a). *In vivo*, plasmin activity negatively correlates with β- and α_{s1+2}-caseins and positively with γ-caseins (Aaltonen *et al*, 1988; Politis & Ng Kwai Hung, 1989) supporting the *in vitro* work of Eigel (1977a,b).

These observations led us to form the hypothesis that indirect measurement of involution could be made by measuring degradative casein products (ie γ-caseins) in milk, reflecting the level of activation of the plasmin system. This hypothesis was tested in two experiments conducted over successive lactations. The first aimed to establish the methodology for longitudinal measurement of caseins in milk while the second experiment investigated the effect of treatments predicted to alter lactation persistency on γ-casein production.

2. ANIMALS

British Saanen goats from the Institute herd were used. Hay and water were available *ad libitum* and, twice daily at milking, animals were offered a total of 1.5kg/d of a proprietary concentrated feed.

3. EXPERIMENTAL DESIGN

Two experiments were conducted over successive lactations and will be described separately.

3.1 Experiment 1

Five animals were milked twice-daily throughout lactation and milk yields from individual glands were recorded at each milking. Animals were not subject to any experimental treatments during this period. Bulk milk samples were collected once every two weeks from lactation weeks 2 to 18, at four-weekly intervals until week 34, and the final samples were collected in week 39.

Sample analysis was carried out using anion- and cation-exchange FPLC and on week 14 whole casein samples from each individual were subjected to SDS-, alkaline and acid PAGE. In addition, whole casein samples collected from one representative goat throughout lactation were separated by alkaline and SDS-PAGE.

3.2 Experiment 2

The methods developed in experiment 1 were subsequently applied to samples collected during the studies described in Chapters 4 and 5. In these, three groups of four animals received either no treatment, or perphenazine or oxytocin treatment, from lactation week 13 onwards, in an attempt to alter lactation persistency. All animals were milked twice-daily on both glands from parturition to lactation week 26, thereafter one gland was milked once-daily. Milk yields from individual glands were recorded

throughout.

Milk samples were collected from both glands in lactation weeks 12, 16, 20, 25, 28, 32 and 37 and all were separated by anion- and cation-exchange FPLC. In addition, samples of whole casein from one animal in the control group were separated by SDS- and alkaline PAGE.

4. METHODS

4.1 Casein preparation

Milk samples were collected at a morning milking (0800h, after 16 hours milk accumulation). In experiment 1 a bulk sample was obtained while in experiment 2 mid-flow milk was collected; goats were machine milked until milk flow was established, the machine switched off and the goat's teats swabbed with alcohol solution (70% alcohol-2% hibitane). Approximately 20ml milk was collected by hand-milking into a sterile container, the goat was reconnected to the milking machine and milking continued as normal.

Milk samples were defatted by centrifugation (1000g, 30 minutes, 20°C) and filtering through glass wool. The filtrate was collected, diluted 1:1 with distilled water and pH adjusted to 4.6 (1M HCl). The resulting suspensions were throughly mixed, centrifuged (200g, 60 seconds, 20°C) and the supernatant (which contained whey proteins) tipped off. In experiment 1 this was retained and used for an investigation of lactational changes in whey proteins (Law & Brown, 1994). The casein pellets were washed with acidified distilled water (pH 4.6), recentrifuged, lyophilised and stored at -20°C.

4.2 Anion-exchange chromatography (Mono-Q)

All buffers used in both anion- and cation-exchange chromatography were filtered through 0.2μm filters (supor-200, 45mm, Gelman Sciences Inc, Michigan, USA), stored at 4°C and warmed to 20°C immediately before use. The method used was similar to that described by Law & Tziboula (1993).

Sample preparation

Lyophilised casein was dissolved to 8mg/ml in sample buffer (3.3M urea containing 0.005M bis,tris-propane and 0.007M HCl), pH adjusted to 7.0 and 4μl/ml mercaptoethanol (50% in sample buffer) added. Samples were mixed (Variomag multipoint HP stirrer, Camlab, Cambridge, UK) for a minimum of one hour before

filtering (Supor-200, 13mm, Gelman Sciences Inc, Michigan, USA).

Column separation

A minimum sample volume of 1ml was applied to a Mono-Q column (Mono-Q HR5/5 anion exchange column, Pharmacia, Uppsala, Sweden) for fractionation of casein types by fast protein liquid chromatography (Pharmacia). Elution buffer (1M NaCl in 3.3M urea, 0.005M bis,tris-propane and 0.008M HCl) was at a concentration of 0.0M after 1ml, 0.17M after 5.0ml, 0.23M after 17.5ml and 0.30M after 43.5ml. A flow rate of 1ml per minute was used. Absorbances were monitored at 280nm and analysis carried out on the basis of the area obtained beneath the sample's absorbance curve.

4.3 Cation-exchange chromatography (Mono-S)

This was carried out using an adaptation of the method of Davies & Law (1987). Absorbance coefficients to determine the amounts of individual caseins as quoted by Law & Tziboula (1992) were used in calculations.

Sample preparation

Lyophilised casein was dissolved to 8mg/ml in sample buffer (6M urea containing 20mM acetate), pH adjusted to 7.0, 4μl/ml mercaptoethanol (50% in sample buffer) added, mixed and left overnight at 5°C. Immediately before use the pH was adjusted to 5.0 and the solutions filtered.

Column separation

A minimum sample volume of 1ml was applied to a Mono-S column (Mono-S HR 5/5 cation exchange column, Pharmacia) for fractionation of casein types with elution buffer (6M urea containing 20mM acetate and 1M sodium chloride) at a concentration of 0.0M after 1ml, 0.025M after 3.1ml, 0.075M after 17.5ml, 0.15M after 27.5ml and at 0.27M after 43.5ml. The flow rate was 1ml per minute and absorbances were monitored at 280nm.

Calculations

Results were analysed on the same basis as those separated by Mono-Q fractionation to obtain percentage areas for the different casein types. In addition, molar extinction coefficients at 280nm were used to calculate the relative amounts of casein present (α_{s1}- 9.9 ; α_{s2}- 9.9 ; β- 4.4 ; κ- 8.2; γ (minor)- 8.2).

4.4 SDS-PAGE analysis (Phast System)

Sample preparation

Microtubes containing 3mg or 6mg/ml casein in sample solvent (12.5% SDS, 5% mercaptoethanol, 6.25% bromophenol blue in 10mM tris/HCl buffer containing 1mM EDTA) were placed in boiling water for 5 minutes and allowed to cool to room temperature.

Electrophoresis and resolution of caseins

Approximately 1μl of sample solutions were loaded onto a 20% homogenous media Phastgel and electrophoresed over a 40 minute period. The gel was stained with stain solution (0.1% Phastgel Blue R in 60% methanol) then washed in a 1:1 mixture of stain and destain (30% methanol, 10% acetic acid) containing 0.1% w/v copper sulphate. Finally gels were washed in destain solution, soaked in preservative (5% glycerol, 10% acetic acid v/v) and photographed.

4.5 Alkaline PAGE analysis

The method used to separate whole casein was based on that described by Peterson & Kopfler (1966).

Sample preparation

Casein was dissolved to 40mg/ml casein in TEBarb buffer (0.025M tris/HCl, 0.0032M EDTA, 0.027M barbitone, pH 8) containing 7M urea. 1μl mercaptoethanol per mg protein was added, the mixture vortexed and 5μl bromophenol blue in buffer (saturated solution) added. Samples were left for a minimum of one hour before applying to a pre-formed gel.

Gel preparation

150ml gel solution, pH 8 (4.5% monomers, 4% crosslinking; acrylamide/ N,N'-methylenebisacrylamide at 96:4 ratio in TEBarb buffer containing 4.5M urea) was warmed to 20°C, 300mg ammonium persulphate and 450μl dimethylaminopropionitrile added, mixed and poured immediately into a gel former.

Electrophoresis

The gel was polymerised for 30 minutes in darkness, pre-run for 30 minutes at 300V and samples loaded (10μl per lane). They were electrophoresed in TEBarb buffer

containing 7M urea for approximately six hours at 200V.

Resolution of caseins

Gels were soaked in MWA solution (40% methanol, 8% acetic acid v/v in distilled water) for 10 minutes then immersed in stain solution (MWA solution containing 1% w/v Naphthalene black 12B) for 20 minutes. They were rinsed in deionised water, destained with several washes of MWA solution over a 48-72 hour period, and recorded by photographing.

4.6 Acid PAGE analysis

Sample preparation

Casein (25mg/ml) was dissolved in sample buffer, pH 1.9 (8.6% acetic acid, 2.25% formic acid v/v), 0.5μl mercaptoethanol per mg protein added, and vortexed together. 5μl methyl red in urea buffer (sample buffer containing 8M urea) was added, mixed and samples left for a minimum of one hour before applying to the gel.

Gel preparation

150ml gel solution (10% monomers in sample buffer containing 4.5M urea) was warmed to 20°C, 350mg ammonium persulphate and 1ml TEMED were added, mixed and poured immediately. The gel was left to polymerise (2 hours, 30°C) before use.

Electrophoresis and resolution of caseins

The gel was pre-run in electrophoresis buffer (7.7% glacial acetic acid v/v) for 20 minutes at 200V before sample loading (10μl per lane) and running for approximately seven hours at 200V. Both electrophoretic steps were carried out with reversed polarity electrodes. Casein bands were resolved by staining (1% Coomassie brilliant blue R in MWA solution).

4.7 Acid PAGE analysis (Phast System)

A 20% homogenous gel (Pharmacia) was equilibrated in urea buffer, with two changes of buffer, for approximately one hour, then dried. Samples of whole casein were dissolved in urea buffer to 6mg/ml and gel strips were prepared by mixing 2% agarose w/v in sample buffer, cooling, pouring into moulds and allowing to solidify for a minimum of thirty minutes.

Reverse-polarity electrophoresis was carried out using the Phast-System according to manufacturers instructions. The subsequent staining and preservation of the gel was carried out as detailed for SDS-PAGE analysis.

4.8 Photography

All gels were immersed in preservative, placed on a light box and photographed using a T2203 enlarger, black and white print film (type 665, Polaroid) and a Kodak wratten 15 filter (all supplied by Sigma Chemical Company Ltd). An aperture of f.8 and $^1/_8$th second exposure time were used. Photographs were left intact for 30-35 seconds, print and negative separated, and the latter incubated at 20°C in developer (22% w/v sodium sulphite solution) before washing thoroughly in cold water. Prints were dried at room temperature after coating with gloss.

4.9 Kjeldahl determination of total nitrogen

Casein precipitation was based on the original description of Rowland (1938). Colorimetrical determinations were made using the method of Reardon *et al* (1966) and protein content calculated using the method summarised by Barbano & Lynch (1992).

Casein preparation

Whole milk was skimmed, a standard volume diluted 1:1 with acetate buffer, pH 5.0 (2.5% w/v glacial acetic acid, 0.1M sodium acetate), mixed and casein precipitated by centrifugation at 200g for 15 minutes at 20°C. The supernatant was discarded, casein pellets washed and recentrifuged three times, then lyophilised.

Sample preparation

Casein samples were dissolved in distilled water, acidified (0.25M sodium hydroxide) and made to a standard volume with further distilled water. Portions of these test solutions were analysed for nitrogen content. Blanks and standards (0.005M ammonium sulphate solution of which 1ml contains 0.14mg nitrogen) were run in each assay.

Measurement of nitrogen content

Micro-kjeldahl flasks were prepared with boiling mixture (1:100 selenium: potassium sulphate mixture, ground to homogeneity) and 3:1 ratio of concentrated sulphuric acid to test solution added. Solutions were boiled until they became clear, cooled, and made to a standard volume with distilled water. They were analysed in triplicate as follows: Reagents A (0.75M sodium hydroxide), B (0.87×10^{-4}M sodium nitroprusside, 0.23M sodium salicylate) and C (2.4×10^{-3}M sodium dichloroisocyanurate) were added stepwise in a ratio of 1:2:1 to the test solution, mixed and incubated at room temperature for 30 minutes before absorbances were read at 667nm.

Calculations

Mean absorbances were obtained for each unknown, the assay blank subtracted and converted to mg protein present as follows:

$$\text{mg protein} = (A_U / A_S).\ a.\ b.\ c$$

where:

A_U= absorbance of the unknown sample at 667nm

A_S= absorbance of the standard at 667nm

a= 0.14; conversion factor for obtaining mg N/ml digest

b= 6.38; conversion factor for N to protein

c= 25; correction factor for dilution of milk

4.10 Expression of caseins in milk samples

The initial fractionation of whole casein by FPLC and conversion of areas under the curve using the appropriate absorbance coefficients (see 4.2/4.3) allowed caseins to be expressed as a fraction of the whole present ('relative amounts', %). Kjeldahl determination of total protein measured total casein in the milk and subsequent multiplication by the appropriate relative amounts of each casein in the sample allowed the concentrations of individual caseins to be calculated (g/l). These two measures allowed comparisons between animals to be made who may, for example, have similar relative distributions of the caseins, but secreted total casein at a higher or lower concentration in the milk. Also, changes in the relative amount of casein can be a more sensitive indicator of casein degradation than expressing it as a change in total concentration- a drop from 58% to 56% could equate to only 0.4 g/l in a total of 11g/l.

5. RESULTS

5.1 Experiment 1

Whole casein samples were separated by anion- and cation-exchange chromatography and selected elution profiles from early and late lactation are illustrated in Figures 7.1 to 7.4. The separation is based on the different weights and charges on the molecules using a +/- charged column under pressure to which caseins with the opposite charge bind with varying affinity and are then removed (eluted) by an increasing salt gradient. The peaks produced by separating milk casein using this method are well characterised and identification was conducted with reference to Law & Tziboula (1992 & 1993) who separated goat casein by cation- and anion-exchange FPLC respectively. It became apparent that one animal (goat 41) had a much higher level of α_{s1}-casein expression than other members of the group. Consequently, goat 41's results are presented separately from the remainder of the group whose data has been meaned (n=4).

Cation-exchange FPLC

The results obtained by this method were used to calculate the relative amounts of the caseins present and lactational changes in casein composition were observed; the proportion of α_{s1}-cn gradually increased between weeks 10 and 39 while α_{s2}-cn levels peaked in week 18, then gradually decreased. β-Casein increased slightly in the first 4 weeks, then showed a gradual downward trend while κ-cn increased over lactation, more rapidly so from week 18 onwards (Figure 7.5).

Casein concentrations

Determination of the total nitrogen present in whole casein samples allowed the relative amounts of the various caseins (determined by FPLC) to be converted into concentrations for each casein fraction (Figure 7.6). α_{s2}-Casein was the only component not to be produced at a consistently higher level in goat 41, and showed the greatest fluctuation through lactation. γ- and κ-Caseins increased from lactation week 18, as did α_{s1}- and β-cn, but to a lesser extent.

Anion-exchange FPLC

This method was the most effective in separating γ-cn from the other casein components and consequently calculation of the relative amount of γ-cn was derived

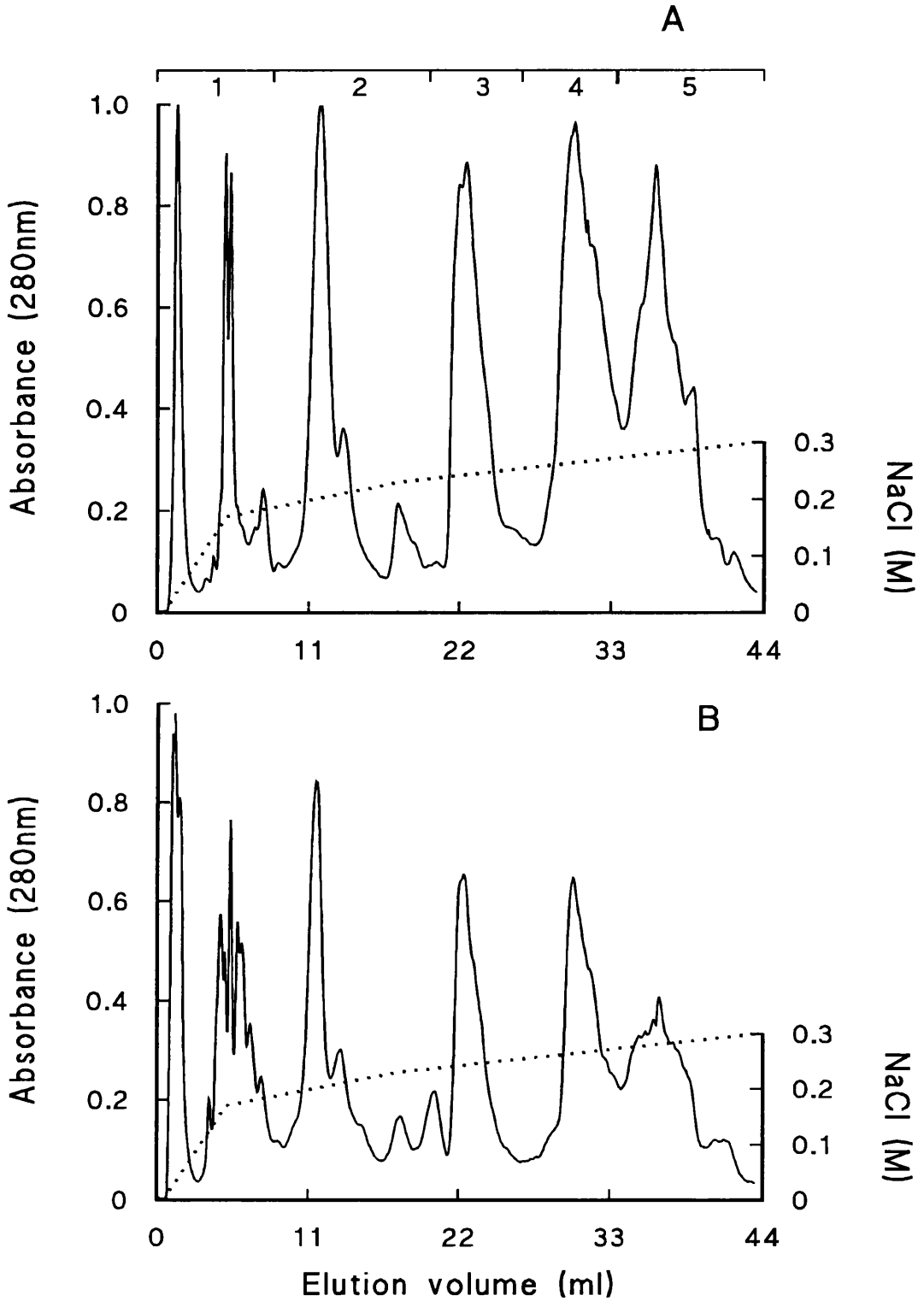


Figure 7.1 Elution profiles from anion-exchange fast protein liquid chromatography separation of whole casein collected from goat 227 (producing low α_{s1}-casein, representative of remainder of group) in lactation weeks 4 (**A**) and 39 (**B**). Casein fractions were identified as follows:
1. γ-cn and other breakdown products **2**. κ-cn **3**. β-cn **4**. β- and α_{s2}-cn **5**. α_{s2}- and α_{s1}-cn.

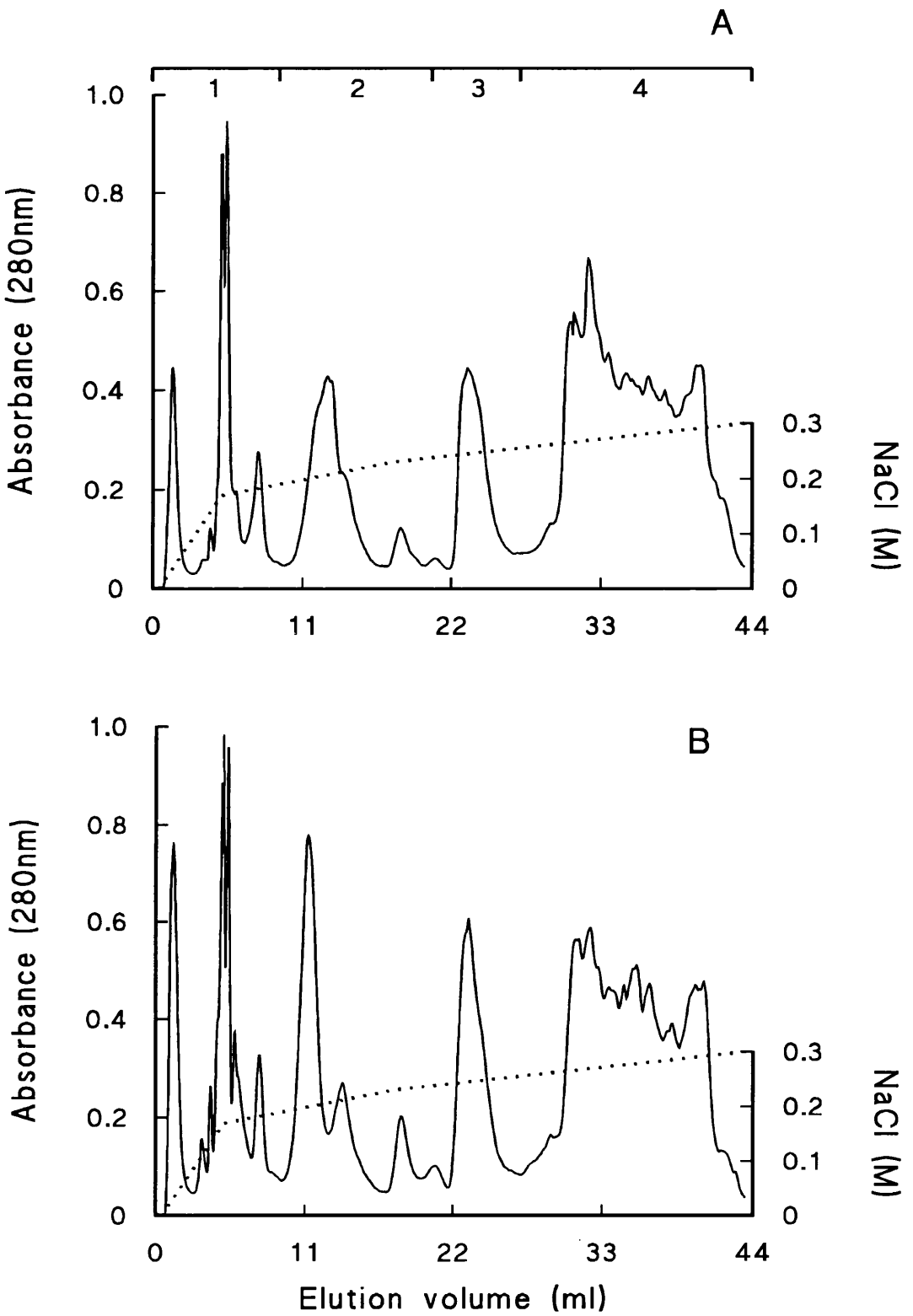


Figure 7.2 Elution profiles from anion-exchange fast protein liquid chromatography separation of whole casein collected from goat 41 (producing high α_{s1}-casein) in lactation weeks 4 (**A**) and 39 (**B**). Casein fractions were identified as follows:

1. γ-cn and other breakdown products **2**. κ-cn **3**. β-cn

4. α_{s1} - and α_{s2} -cn with some β-cn.

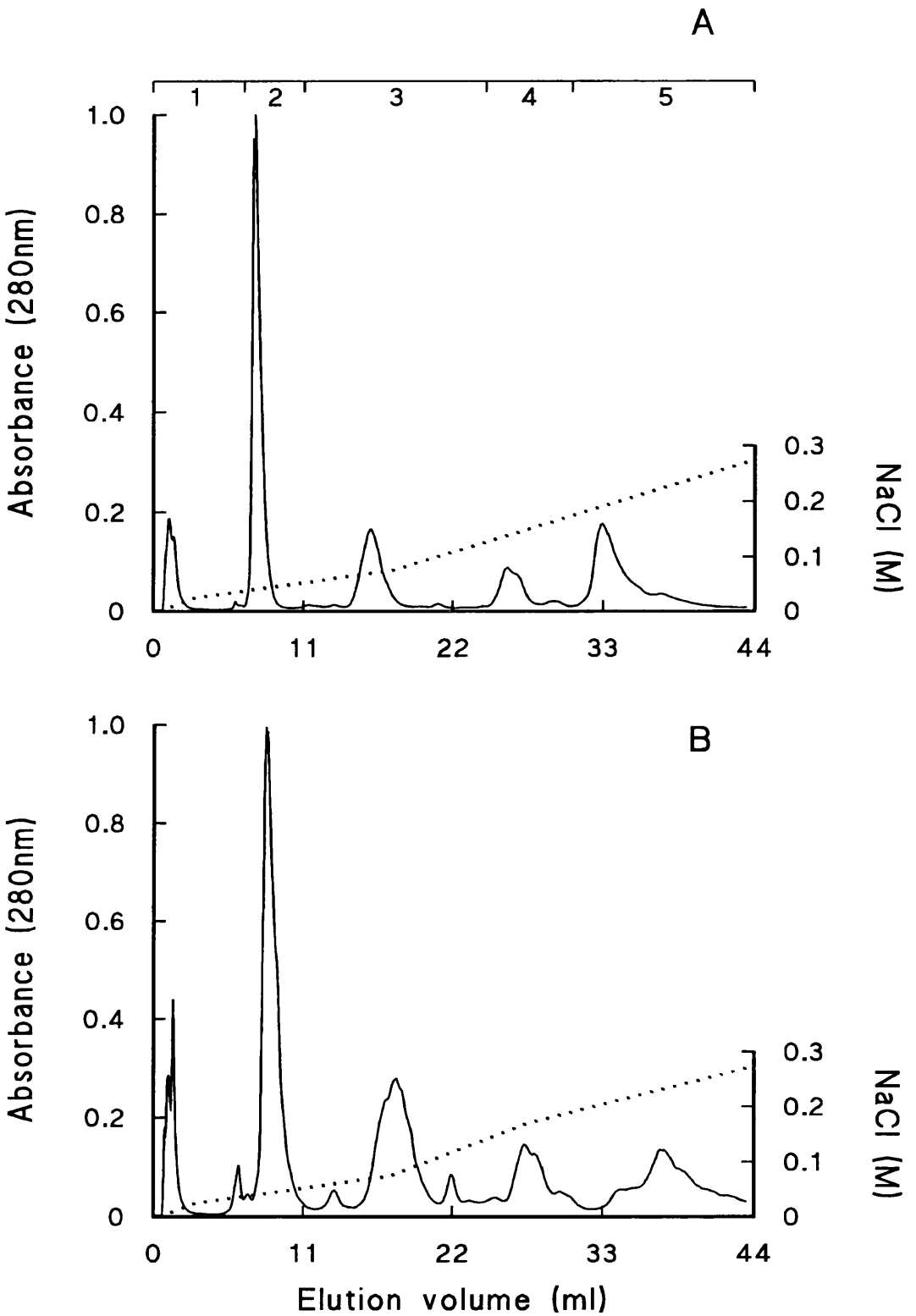


Figure 7.3 Elution profiles from cation-exchange fast protein liquid chromatography separation of whole casein collected from goat 227 (producing low α_{s1}-casein, representative of remainder of group) in lactation weeks 4 (**A**) and 39 (**B**). Casein fractions were identified as follows: **1**. γ-cn and other breakdown products **2**. β-cn **3**. κ-cn **4**. α_{s1}-cn **5**. α_{s2}-cn

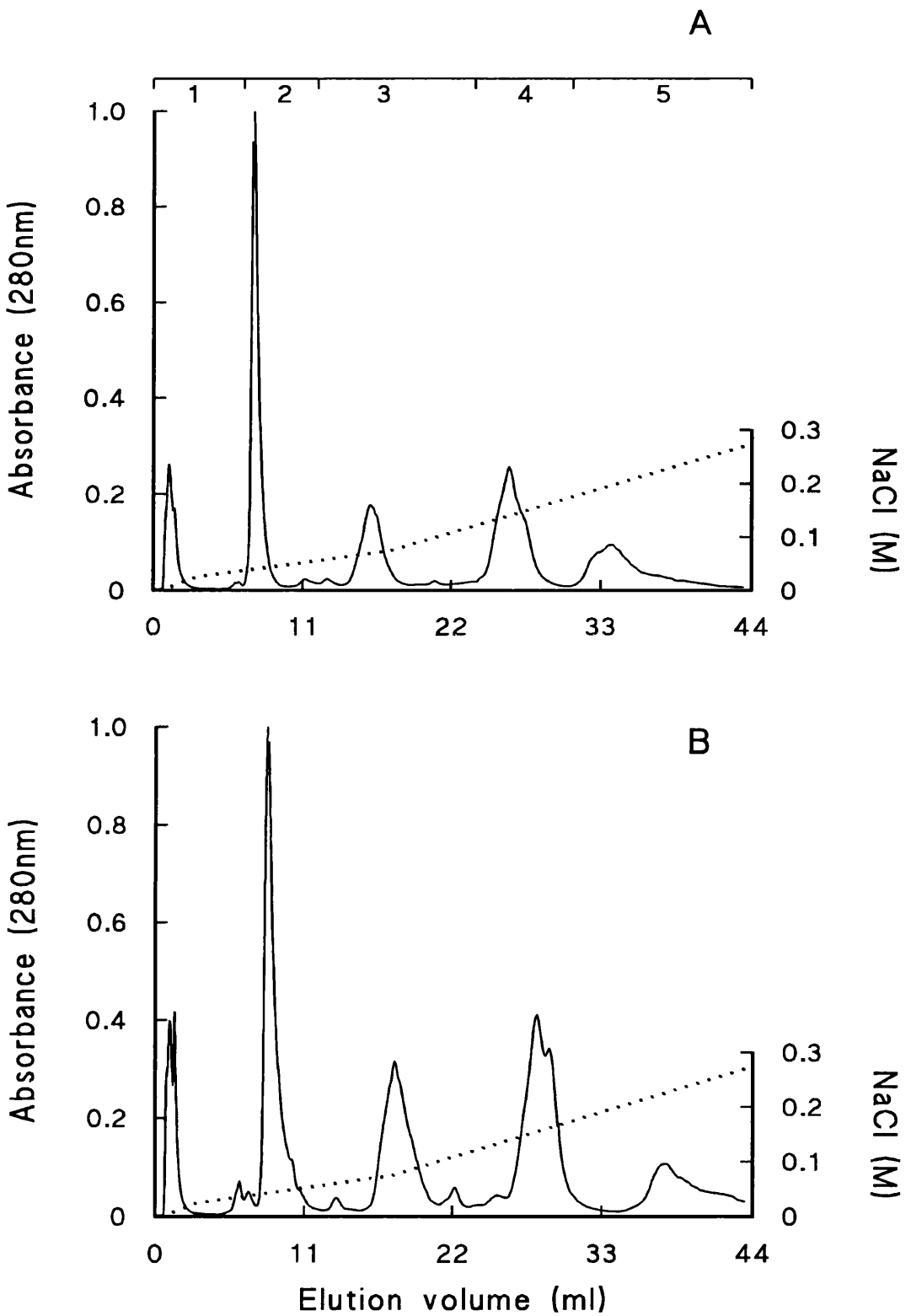


Figure 7.4 Elution profiles from cation-exchange fast protein liquid chromatography separation of whole casein collected from goat 41 (producing high α_{s1}-casein) in lactation weeks 4 (**A**) and 39 (**B**). Casein fractions were identified as follows:
1. γ-cn and other breakdown products **2**. β-cn **3**. κ-cn **4**. α_{s1}-cn **5**. α_{s2}-cn

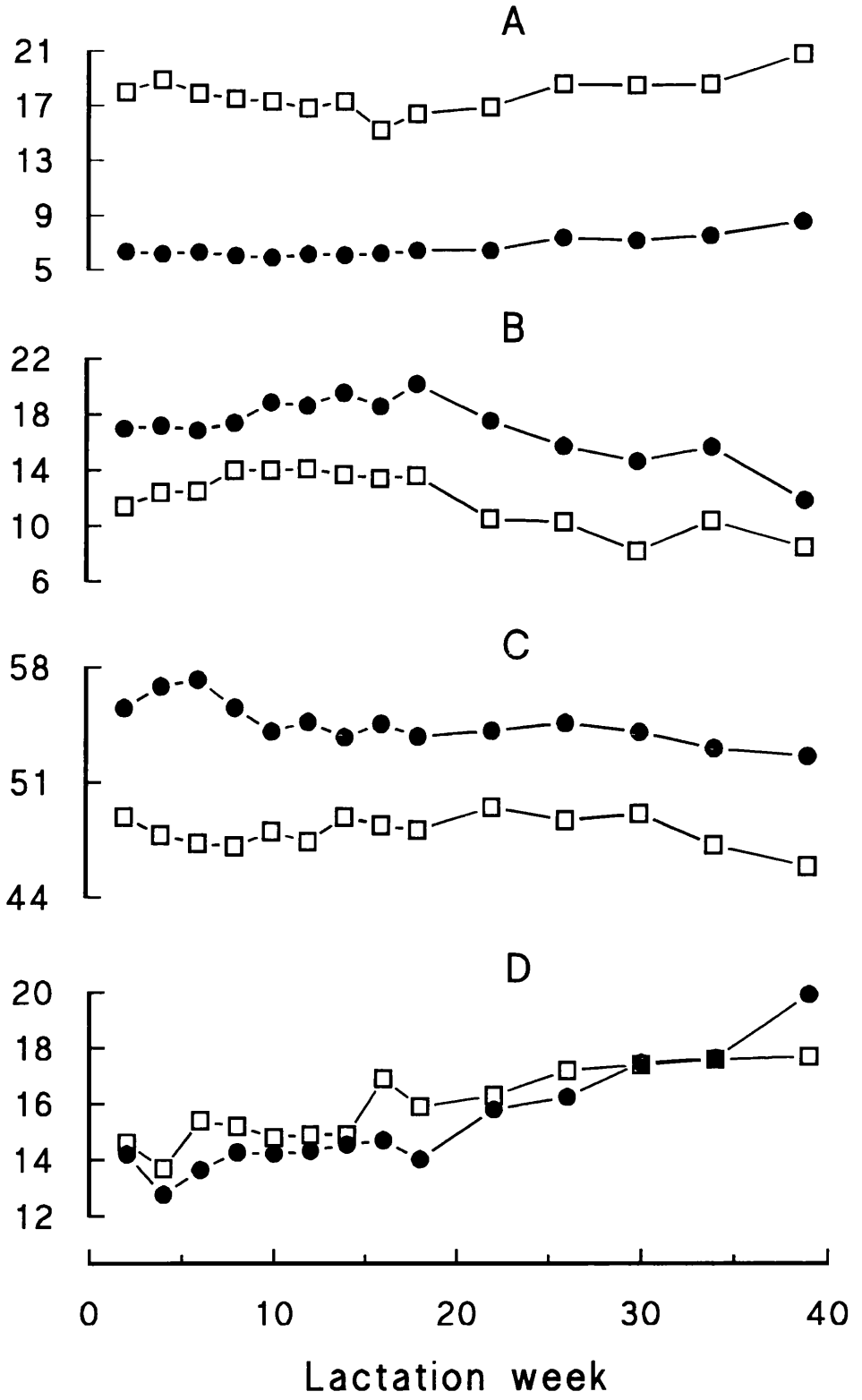


Figure 7.5 Changes in the relative amounts of casein with stage of lactation in goat 41 (❑) and the remainder of the group (●) (n=4). (**A**) α_{s1}-cn (**B**) α_{s2}-cn (**C**) β-cn and (**D**) κ-cn. Pooled sems for the latter were 0.12, 0.31, 0.24 & 0.30 % respectively. Units represent the percentage of total casein of which the particular casein comprises.

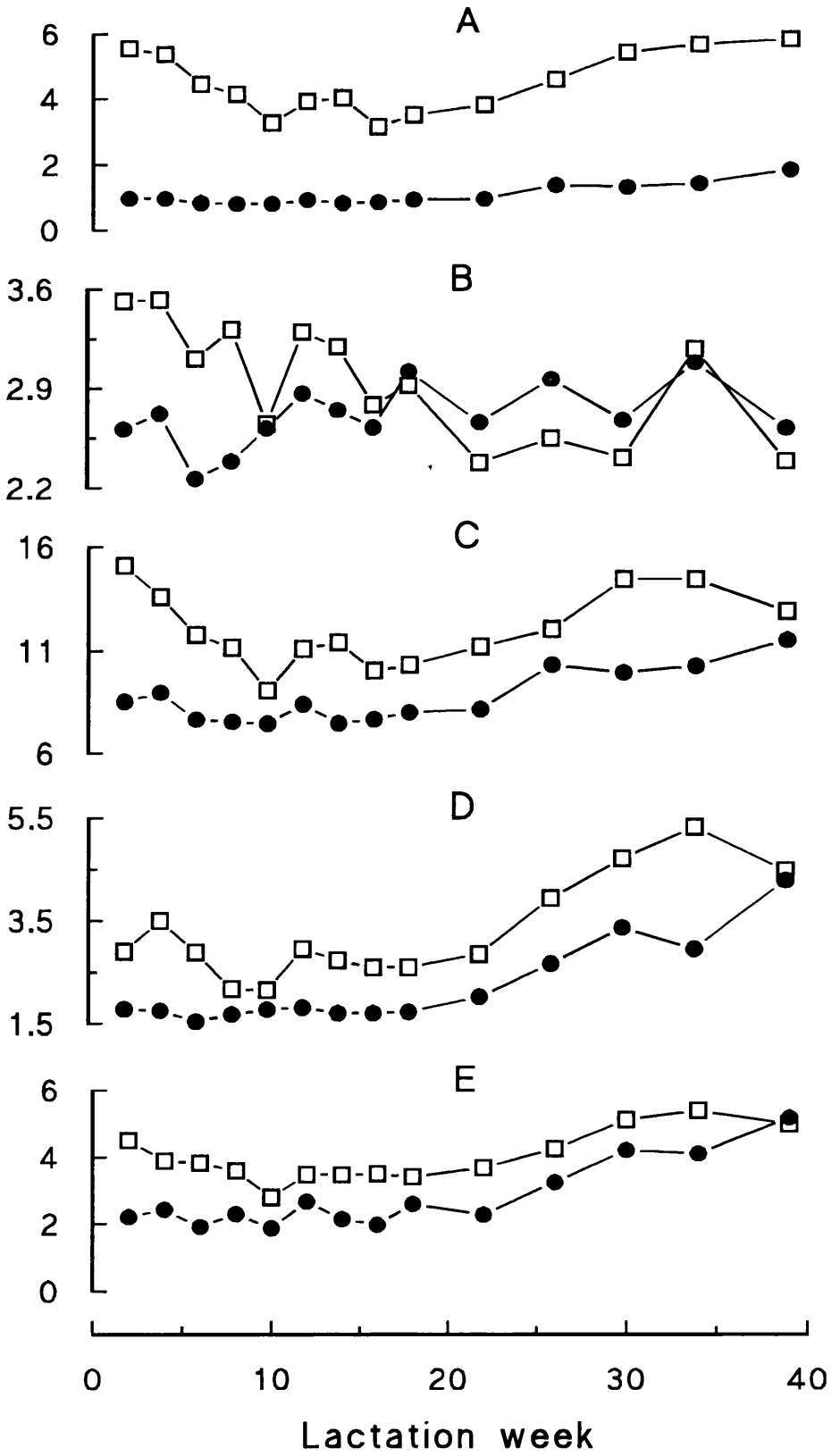


Figure 7.6 Changes in casein concentrations (g/l) with lactation stage in goat 41 (❑) and the remainder of the group (●) (n=4).
(A) α_{s1}-cn, **(B)** α_{s2}-cn, **(C)** β-cn, **(D)** γ-cn and **(E)** κ-cn. Pooled sems were 0.05, 0.09, 0.30, 0.13 & 0.11 g/l, respectively.

from this data. γ-Casein was determined for each individual and, as no significant difference was detected between goat 41 and the other animals in the relative amount of γ-cn present (Student's unpaired t-test, $p \geq 0.25$, data not shown), a group mean was calculated for each timepoint (Figure 7.7).

Relationship between γ-casein, milk yield and lactation persistency

Lactation persistency was measured from week 12 onwards as this represented the period when yields were steady then began to decline. Regression analysis was carried out for individual glands and values meaned; a reduction in yield of 0.0397 ± 0.004 litres/week ($r^2 = 94.0 \pm 2.1\%$, $p=0.000$) was obtained. The Pearson correlation coefficient for milk yield against lactation stage was calculated to be -0.969 ± 0.011.

Similar calculations were carried out for changes in the relative amount of γ-cn with time and it was found that γ-cn increased by 0.258 ± 0.035 percent per week ($r^2=71.1 \pm 4.5\%$, $p=0.006 \pm 0.003$) and a correlation of 0.841 ± 0.026 was calculated.

The relationship between γ-cn and average daily milk yield was also determined for each goat at each sampling point by correlation. Over the whole experimental period (weeks 2-39) a correlation of -0.684 ± 0.064 was obtained ($n=5$). However, when the analysis was restricted to lactation week 12 onwards (when yields began to decline and γ-cn to increase) the correlation coefficient improved to -0.824 ± 0.049. This was further increased to -0.940 when average milk yield was correlated with average γ-cn percentage for the group.

PAGE separations of caseins

Samples collected from goat 227 throughout lactation were separated by alkaline PAGE (Plate 7.1). The most notable change was a lactational decrease in α_{s2}-cn while α_{s1}- and β-cn appeared to remain relatively steady. Acid PAGE separation of whole casein from each animal in mid-lactation (Plate 7.2) allowed α-cn variants to be determined. When taken in combination with results obtained from the other PAGE separations it was found that all animals in the group were heterozygous α_{s2}-Cn^{AB}. The substantially higher levels of α_{s1}-cn in goat 41 were attributed to a genotype of α_{s1}-Cn^{AB}. The remainder of the group had a much lower level of expression of this casein, had no bands corresponding to A, and were therefore most probably homozygous for α_{s1}-Cn^{E}.

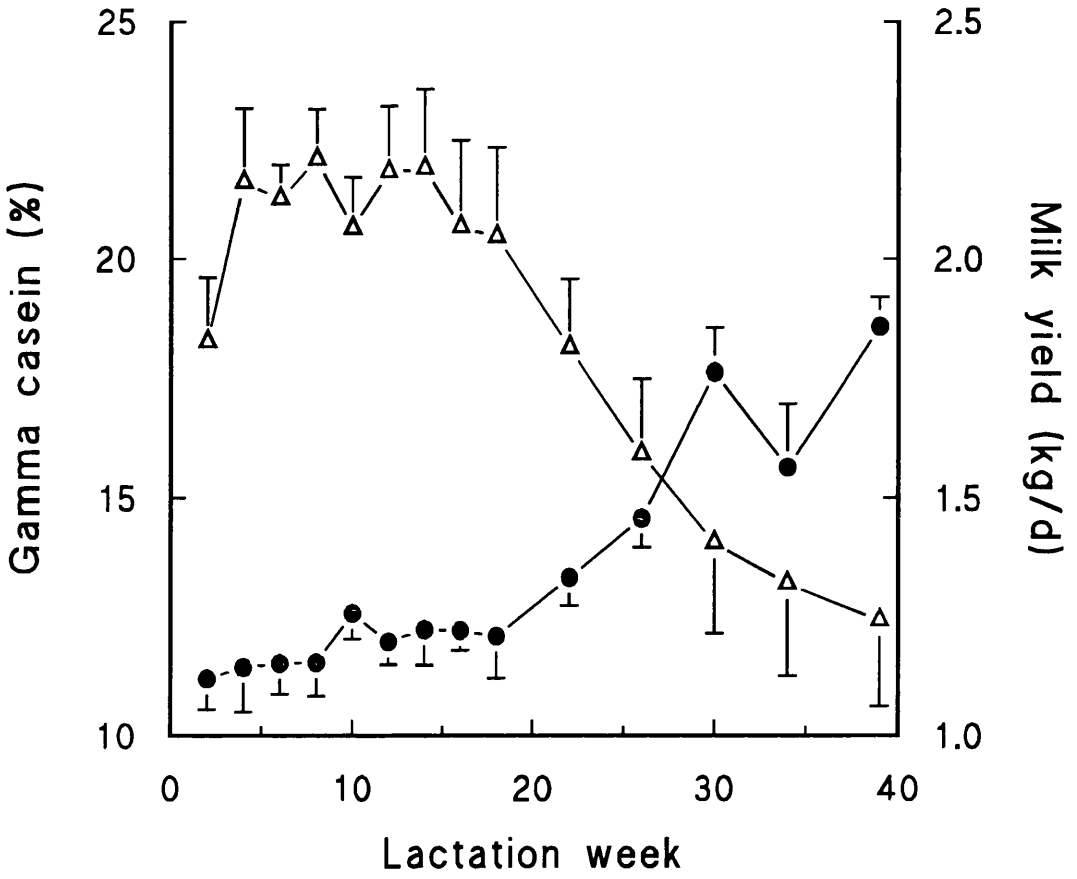


Figure 7.7 Changes in the relative amount of γ-casein (●) and in milk yields (Δ) over lactation in Experiment 1.
Values plotted are means ± SEM and n=5 for each measurement.

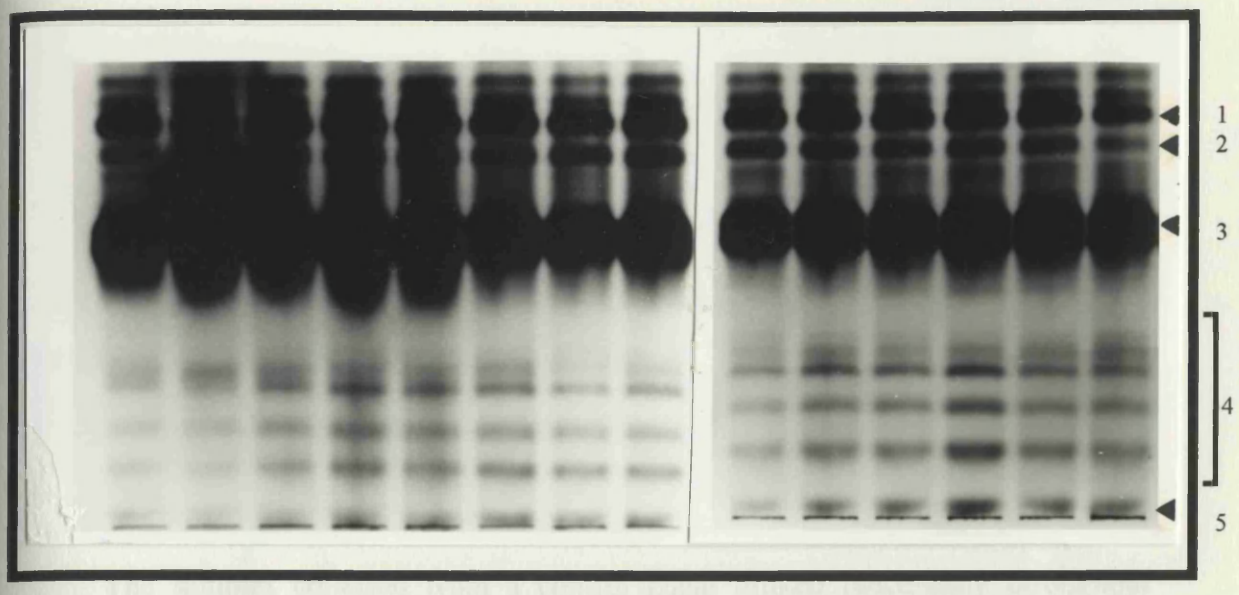


Plate 7.1 Alkaline PAGE separation of whole casein samples collected through lactation from goat 227 in Experiment 1
1: αs_1-cn **2**: αs_2-cn **3**: β + κ-cn **4**: γ- & minor cns **5**: para-κ-cn

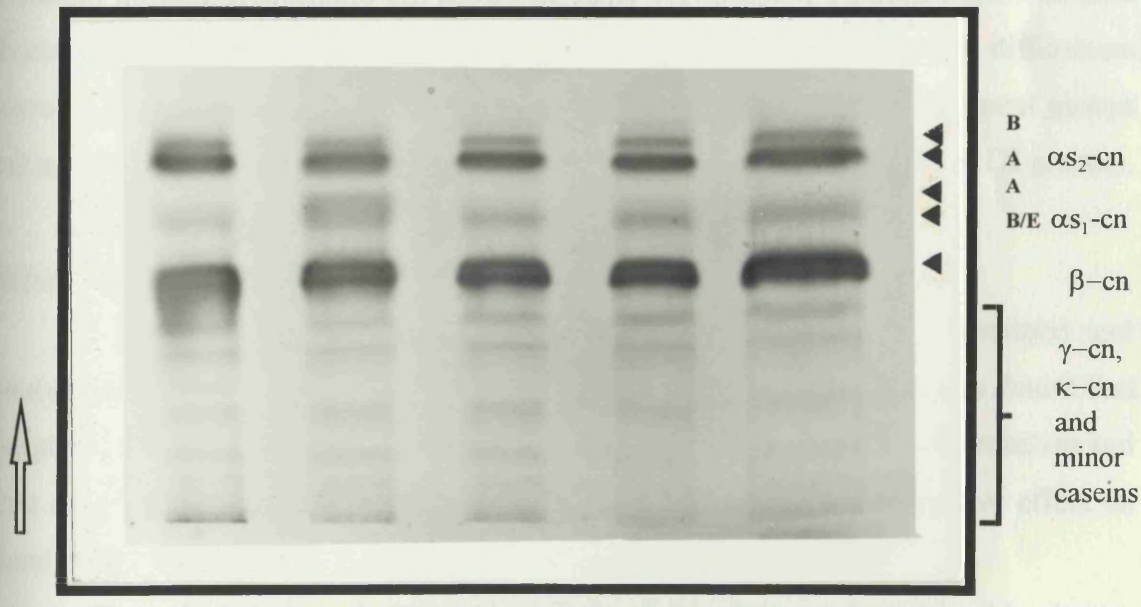


Plate 7.2 Acid PAGE separation of whole casein samples collected from each goat in lactation week 14 of Experiment 1.

5.2 Experiment 2

FPLC

Anion- and cation-exchange FPLC of whole casein samples was conducted and the results and profiles obtained were similar to those seen in experiment 1. The former method provided the best separation of γ-cn and was used to calculate the relative amount of γ-cn present. Cation-exchange profiles confirmed the observations of experiment 1 and again γ-cn and casein breakdown products increased as lactation progressed (results not shown).

PAGE separation of caseins

The samples collected from a control gland milked twice-daily throughout lactation were separated by alkaline PAGE (Plate 7.3). A marked decrease in α_{s2}-cn was seen as lactation progressed while α_{s1}- and β-caseins appeared to remain relatively steady, again confirming the observations made in experiment 1.

Relative amounts of γ-casein

γ-Casein percentages for each treatment group and for glands subjected to different milking frequencies are shown in Table 7.1. Student's paired t-test was used to compare contralateral glands within treatment groups but no significant differences were found ($p \geq 0.47$). Unpaired t-tests were conducted to compare treatment groups but again no significant differences were found ($p \geq 0.84$ in 2X, $p \geq 0.68$ in 1X glands).

Relationship between γ-cn, milk yield and lactation persistency

Lactation persistency over the experimental period has been described and analysed in detail in Chapters 5 (4.2) & 6 (4.3) but, in summary, it was found that perphenazine and oxytocin treatments were not improving persistency in themselves and that once-daily milking in later lactation might be having small but positive effect on persistency regardless of treatment.

The calculations and statistical analysis of this data conducted in this chapter was derived from average daily milk yields obtained on the weeks that samples were collected and γ-cn prepared. Regression lines were fitted to individual lactation curves and the results meaned (Table 7.2). No significant differences were found between contralateral glands within groups ($p \geq 0.42$, paired t-test) or between groups ($p \geq 0.12$, unpaired t-test) and an overall correlation of -0.840 ± 0.028 between yield and stage

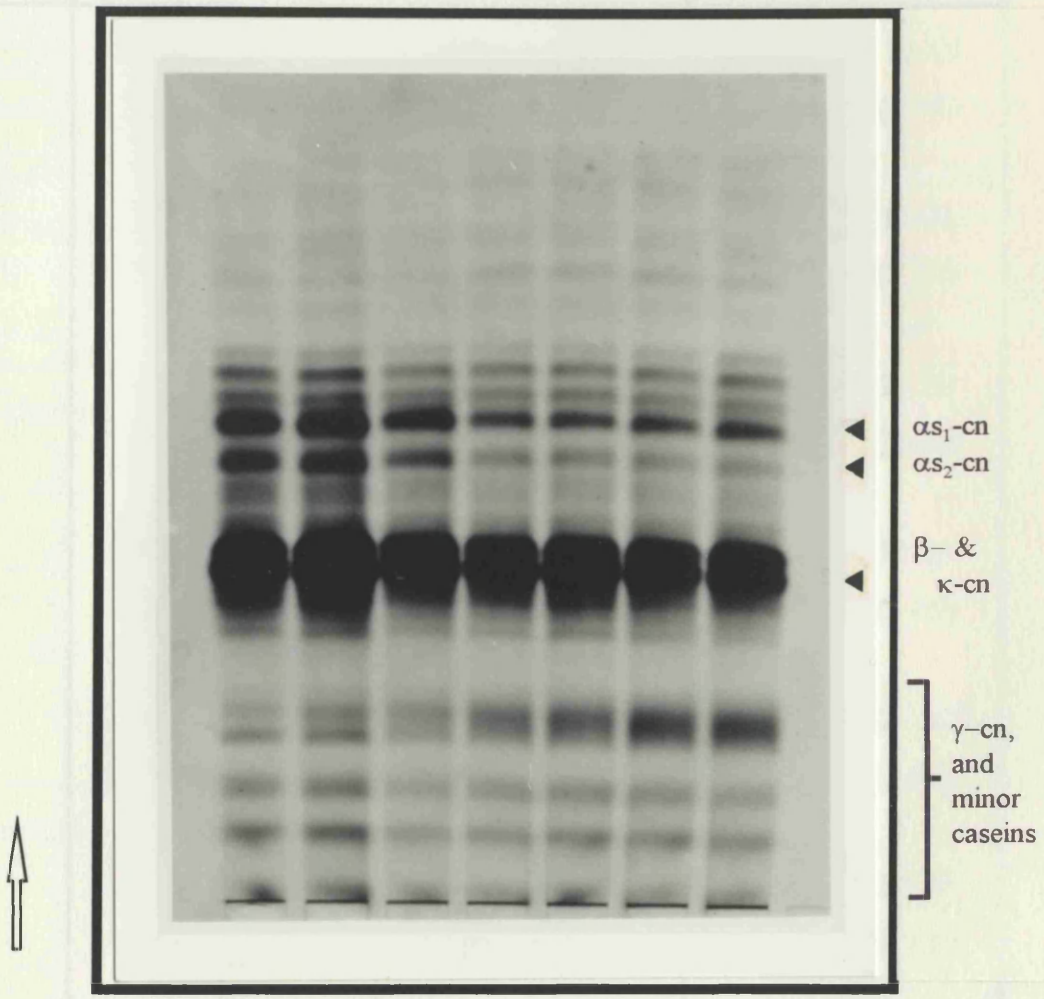


Plate 7.3 Alkaline PAGE separation of whole casein samples collected through lactation from a control animal in Experiment 2.

Group/ Lactation week	Control (% γ-cn)		Oxytocin (% γ-cn)		Perphenazine (% γ-cn)	
	2X	*1X*	*2X*	*1X*	*2X*	*1X*
12	11.43 (1.73)	11.02 (1.33)	9.12 (0.74)	9.35 (0.86)	10.80 (1.49)	11.15 (1.36)
16	17.95 (2.00)	18.55 (2.48)	19.22 (1.43)	19.98 (0.96)	13.98 (1.16)	17.02 (2.24)
20	27.85 (3.92)	32.62 (3.90)	27.55 (3.50)	28.50 (4.13)	23.22 (3.56)	24.70 (3.40)
25	28.22 (2.60)	27.50 (3.98)	28.50 (2.32)	27.58 (3.06)	24.52 (2.33)	27.35 (5.45)
28	29.68 (3.87)	29.90 (3.57)	28.68 (3.86)	28.20 (1.61)	25.15 (1.54)	21.02 (1.84)
32	35.72 (4.98)	36.20 (3.07)	29.48 (3.29)	32.32 (2.94)	38.15 (5.74)	32.22 (1.33)
37	29.40 (5.94)	27.73 (5.05)	34.90 (1.98)	34.18 (1.54)	37.02 (8.91)	36.40 (3.19)

Table 7.1 The effect of treatment on γ-casein percentage (%) in milk samples, determined by anion-exchange chromatography in experiment 2. Values given are means with SEM in brackets, n=4 for each measurement.

Glands/ Group	2X glands m	'1X' glands m
Control	-62.0 -40.9 -11.3 -34.5	-55.9 -48.3 -15.2 -40.8
mean	-32.7 r^2 73.4, p=0.031	-40.0 r^2 78.8, p=0.020
Oxytocin	-17.8 -44.6 -36.4 -16.9	-21.8 -37.5 -36.9 -11.8
mean	-28.9 r^2 59.0, p=0.096	-27.0 r^2 62.2, p=0.086
Perphenazine	-46.3 -50.9 -42.1 -23.5	-50.8 -40.5 -55.6 -27.4
mean	-40.7 r^2 80.1, p=0.009	-43.6 r^2 80.6, p=0.012

Table 7.2 Summary of regression lines fitted to individual milk yield profiles for each animal's glands in experiment 2 where **m** is the change in average daily yield, ml/week. The analysis was on lactation weeks 12-37; 1X milking commenced in week 26 until the end of the study. r^2 and p indicate the accuracy and probability of the best line being fitted to the data.

of lactation was calculated.

Regression analysis of γ-cn levels was also performed (Table 7.3) but again no significant differences were found either between glands ($p \geq 0.14$) or groups ($p \geq 0.32$). An overall correlation between γ-cn and stage of lactation of 0.772 ± 0.031 was calculated.

An overall comparison of the relationship between γ-cn and milk yield was also made (Figure 7.8). Correlations between these parameters were calculated for each individual gland but no significant differences were found between glands (paired t-test, $p \geq 0.26$) or groups (unpaired t-test, $p \geq 0.24$) and the pooled correlation (both glands, all groups) between γ-cn and milk yield was 0.869 ± 0.046.

Glands/ Group	2X glands m (γ-cn)	'1X' glands m (γ-cn)
Control	0.859	0.873
	0.878	0.739
	0.636	0.383
	0.925	1.130
mean	0.825	0.781
	r^2 64.1, p=0.088	r^2 53.8, p=0.161
Oxytocin	0.827	0.721
	0.859	0.803
	0.800	0.969
	0.965	0.935
mean	0.863	0.857
	r^2 65.8, p=0.030	r^2 67.4, p=0.033
Perphenazine	1.400	0.868
	0.648	0.460
	1.120	0.830
	1.300	0.925
mean	1.117	0.771
	r^2 62.2, p=0.046	r^2 58.2, p=0.054

Table 7.3 Summary of regression lines fitted to individual γ-casein results for each animal's glands in experiment 2. **m** is the change in γ-casein, percent per week. The analysis was on lactation weeks 12-37; 1X milking was applied in weeks 26 to 37. r^2 and p indicate the accuracy and probability of the best line being fitted to the data.

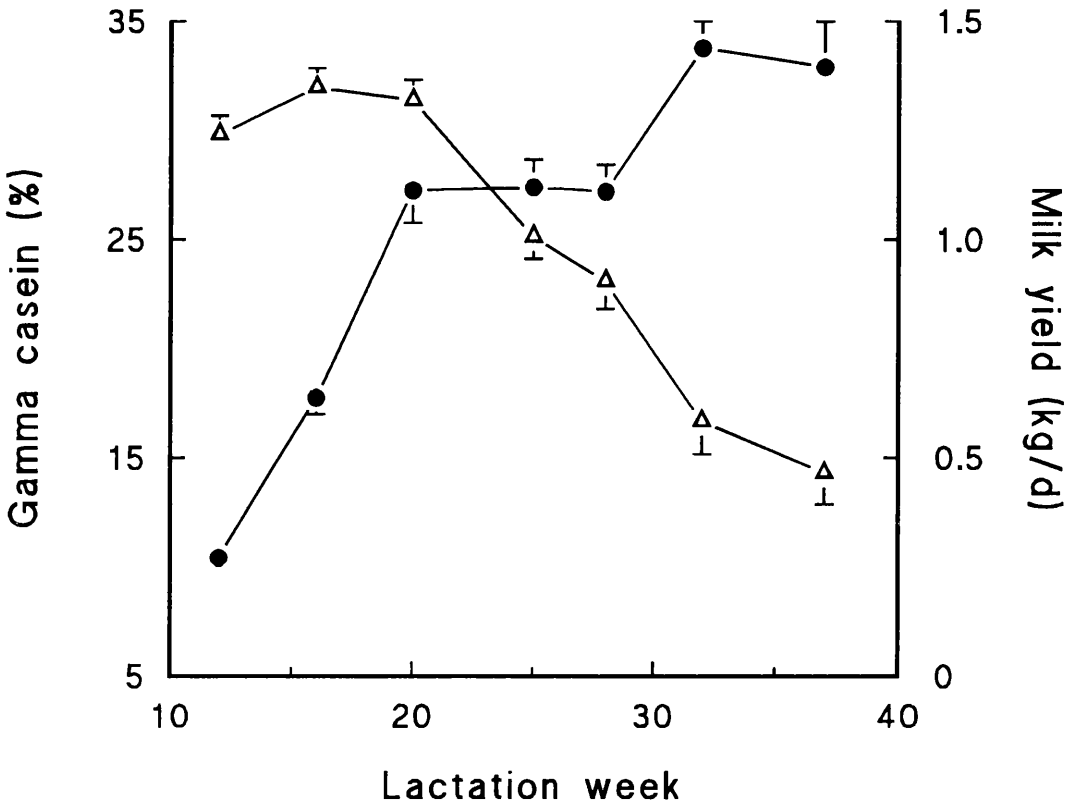


Figure 7.8 Changes in the relative amount of γ-casein (●) and in milk yields (Δ) over lactation in Experiment 2.
Values plotted are the meaned results from control, oxytocin and perphenazine groups and both 2X and 1X glands. SEMs are illustrated and n=24 for each measurement.

6. DISCUSSION

Remodelling of the mouse mammary gland through proteolysis and activation of the plasmin system has been described by Ossowski *et al* (1979). Their experiments removed pups either at parturition or on lactation day 5 to cause a rapid cessation of lactation. This in turn caused a large but transient increase in plasminogen activator (PA) concentrations, mammary weights decreased and protein and DNA contents also dropped. If exogenous oxytocin and prolactin were administered after pups were removed a reduced response in PA activation was seen, illustrating PA's responsiveness to changes in the hormonal environment. This suggests that it might therefore provide a marker for involution in the mammary gland.

Unfortunately different species respond to different lactogenic signals. In rodents the cessation of suckling-induced hormone release is the primary signal for involution while in ruminants the increase in intramammary pressure as less milk is removed is more important (Fleet & Peaker, 1978). Rodents lose large numbers of secretory epithelial cells during involution, in direct contrast to ruminants which appear to have minimal loss of cells and still maintain some secretory capacity even when the gland is fully involuted (Sordillo & Nickerson, 1988; Oliver & Sordillo, 1989). In cows extensive cytoskeletal reorganisation has been observed, organelles involved in secretory functions are retained, although histologically altered, and secretion can continue at a low rate (Holst *et al*, 1987).

However, in many of these studies the stimulus for involution is acute, an immediate cessation of milk removal, which does not mimic the chronic process of slow and gradual involution after peak lactation. Little evidence has been produced for the specific role of the plasmin system in ruminant lactation; PA content in bovine mammary tissue does increase within a few days of the cessation of milking in late lactation (Politis *et al*, 1992b) and plasmin and plasminogen-derived activity both increase in late lactation (Korycka-Dahl *et al*, 1983). However, this could be due to the increased permeability of blood vessels in the mammary gland allowing them access to the milk via leaky tight junctions. This is seen during mastitic infection of glands when the epithelium becomes ruptured, and increased plasmin and plasminogen concentrations are associated with increasing milk somatic cell counts (Politis *et al*, 1989a). Determination of the ratio of plasmin to plasminogen, in combination with determination of sodium, potassium and serum albumin concentrations in milk, could indicate whether the increase was as a result of leakage from the blood or due

specifically to increased activation of the plasmin system.

The results obtained in experiment 1 showed that the proportions of the caseins changed as lactation progressed and milk yields declined. The observations supported the model of α_{s2}- and β-cn being most susceptible to proteolytic attack with γ-cns and proteose-peptones being produced as breakdown products. Formation of the latter was most probably by the action of plasmin on β-cn. Increased γ-cn was seen using all separation methods, but the increase detected by alkaline PAGE was not as marked as expected. Gel separations conducted to specifically resolve γ-cn were loaded with a double concentration of whole casein in an attempt to improve detection. It is possible that this contributed to obscure some changes, or that FPLC methods overestimated the size of the fraction by including other products which were not γ-cns but eluted in a similar position. The relative amount of β-cn decreased by approximately 4% over the lactation which appears quite a small drop, but it was the most abundant casein present, constituting almost half of the total casein, and hence a drop of a few percent was quite large in real terms.

The second experiment attempted to elucidate whether or not changes in lactation persistency, and hence involution, had an effect on casein composition, most especially γ-cn. There were very small differences between the treatment groups and the general trends of changes in the relative amounts of the caseins confirmed the observations of experiment 1. So why were no changes detected? The treatments applied were not as successful in maintaining or improving persistency as had been hoped, indeed perphenazine treatment had a negative effect on milk yield. Oxytocin treatment was more successful but the alteration in persistency was small. In the light of this it was perhaps not surprising that no changes in γ-cn were detected between the groups.

If an effective treatment which improved persistency could have been tested then there would have been the potential to find differences between test and control groups. Growth hormone has delayed mammary regression in goats (Knight *et al*, 1990) and when cows were treated with it there was a reduction in PA concentrations (Politis *et al*, 1990). It would have been interesting to give an additional group growth hormone treatment and to have determined the effect on lactation persistency and milk casein content over a lactation.

It is, however, also possible that the acute activation of the plasmin system when milking is stopped prematurely (eg by pup removal) reflects the rapidity of

change in the mammary gland, in the usual situation there would be a more gradual weaning of the young and therefore the changes during slow involution may not activate the system to the same, or to an easily measurable, extent. It may also be the case that the changes in casein composition, although statistically correlated with lactation and the plasmin system, may not be directly related to each other. However, there is good evidence, already covered in the introduction, which shows that plasmin proteolytic action does indeed have an effect on caseins.

Experiment 1 produced an unexpected result in that one member of the group had a markedly different 'casein profile' in both relative amounts and concentrations of the various casein components. This animal, goat 41, had high levels of α_{s1}-cn and had higher individual casein production than the other members of the group, believed to be homozygous α_{s1}-Cn^{E}. It was deduced from a variety of gel separations that this individual had the genotype α_{s1}-Cn^{AB}. The α_{s1}-Cn gene locus exhibits a high degree of polymorphism and the relative amount of α_{s1}-cn is positively correlated with the total concentration of casein in milk (Remeuf & Lenoir, 1986). Goats with α_{s1}-Cn^{A}, α_{s1}-Cn^{B} and α_{s1}-Cn^{C} alleles generally yield higher levels of α_{s1}-cn than those with α_{s1}-Cn^{D}, α_{s1}-Cn^{E} or α_{s1}-Cn^{F} alleles (Grosclaude *et al*, 1987; Brignon *et al*, 1990). Therefore production of α_{s1}-cn (both relative amount and concentrations) will vary depending on the genotype of the animal (Martin & Grosclaude, 1993). Other differences in milk have been seen between groups of goats expressing high or low levels of α_{s1}-cn. The former produced milk with significantly lower pH and mean micellar size distribution, and had higher total solids, fat, nitrogen and calcium contents (Pirisi *et al*, 1994). It has been observed that for an individual the relative amounts of the secreted caseins did not vary with the total concentration of casein in the milk, although casein proportion could be changed by variations in whey proteins, specifically α-lactalbumin (Kroeker *et al*, 1985). Making cheese from milk containing high α_{s1}-cn produces firmer curds, improves cheese quality (higher solids, fat and casein contents) and yield. However it increases coagulation times and alters textures compared to cheese produced from low α_{s1}-cn milk (Ambrosoli *et al*, 1988; Pirisi *et al*, 1994). Selective breeding for particular genetic polymorphisms leading to increased casein could improve cheese yields (Marziali & Ng-Kwai-Hang, 1986), however it would probably alter the processing characteristics of the milk and would not necessarily be advantageous.

κ-Casein increased with stage of lactation and increasing para-κ-cn formation

was also detectable by PAGE. It is possible that this short peptide would elute in the fraction arbitrarily defined as γ-cn, possibly contributing to the slight difference between PAGE and FPLC estimations of γ-cn. If this were the case it could be assumed that the true γ-cn levels were slightly lower, and κ-cn (and its derived products) slightly higher than assumed from FPLC determinations. Micellar size has been negatively correlated with κ-cn (Davies & Law, 1983); the micellar radius decreased by approximately one-third when the relative amount of κ-cn increased by 225% (Dalgleish *et al*, 1989). In the first experiment, κ-cn increased between peak and late lactation by an estimated 137%, so some decrease in micellar size would be predicted. Micellar diameters were not determined in this work and this aspect could be investigated in future studies of lactational changes in milk casein composition. Making cheese with milk containing smaller micelles could potentially alter the kinetics of aggregation and change the firmness of the curd produced (Dalgleish, 1992).

CHAPTER 8

SUMMARY AND CONCLUSIONS

This thesis has examined some of the factors which might affect lactation persistency in the goat. We have defined persistency as the rate of decline in milk yield after peak lactation and quantified it by linear regression analysis on average daily gland milk yields. However, other authors have measured persistency by, for example, looking at total milk production over a lactation, or by counting the number of days that the animals lactated (Broster & Broster, 1984). So the definition of persistency is itself variable, and comparisons between studies should be made with care. The decline in yield after peak lactation was studied as changes in milk production during this period reflect the activity and differentiative state of the secretory cell population and amount of cell loss from the mammary gland which occurs during involution (Wilde *et al*, 1986). The overall aim of this work was to determine some of the factors that affect lactation persistency and attempt to slow the reduction in milk yield by the application of various treatments which might maintain mammary function.

The mathematical measurement of persistency can be problematic as fluctuations in yield due to, for example, a change in the milking regime, or nutritional constraints on the animal, can alter the gradient of the regression line (m) and reduce the accuracy of its fit (r^2). Periods during which there were large fluctuations in yield were generally excluded from regression calculations, seen particularly when there were alterations in the treatment regime. So the analysis was conducted on periods during which yield curves were relatively 'steady'. An example of this is in Chapter 5, experiment 2, when the fit of linear regression lines was improved by 42% by excluding the first three weeks of the treatment phase. It may be that a more complex mathematical model for persistency needs to be constructed but comparisons between animals may become more difficult while regression analysis provides a straightforward measure of the rate of decline in yield, which is easily comparable between glands and animals.

The goat may not have been the best model to measure alterations in persistency. Firstly they typically produce about 3 l of milk a day while cows regularly yield 30 l a day (Amos *et al*, 1985), so a 10% improvement in the goat would be equivalent to 300ml milk, while the cow would produce an extra 3 litres, so measurements of milk yield in the goat need to be more accurate as the changes in absolute yield will be much smaller. Also goats proved to be very persistent with a

median reduction in yield of approximately 60 ml/week, so perhaps there was less room for improvement in this species. However mature cows show yield reductions in the order of 600ml/week (Amos *et al*, 1985) which is a similar proportional drop to that seen in goats, suggesting that manipulation of persistency will not necessarily be any easier in cows. Goats are easy to manage in husbandry terms, the division of their udders into two halves is ideal for the application of unilateral treatments, and there are economic advantages to studying them. There is also good evidence that goats have the potential for increased milk production. In one study, treatments were applied from lactation week 19 to week 41, increasing milking frequency to 3X resulted in a 6% increase in yield, bST administration gave a 18% enhancement, and 36% was achieved when these treatments were given together (Knight *et al*, 1990). This indicated that the gland had the capacity for higher milk secretion when appropriately stimulated.

A promising avenue for manipulation of lactation is to enhance mammary growth during late gestation. Here, this was achieved by giving exogenous growth hormone and stimulating compensatory growth by hemimastectomy. The latter increased secretory cell numbers and maintained the overall differentiative state of the gland but animals were studied for a maximum of 30 days *post partum*, before peak lactation was reached. Consequently the long-term effect on lactation persistency could not be determined, and this provides an area for further study. Indications were that the proportion of BrdU labelled cells were still well above control levels in final samples (lactation week 4), suggesting that cell numbers were being maintained. However it is not know what effect the labelling has on the subsequent longevity of the cell- it may affect further multiplication, make it more resistant to degradative processes such as apoptosis, so the usefulness of further measurements remains to be confirmed. Growth hormone also provided a stimulus for mammary growth but the long-term effects of its administration during gestation on lactation persistency remain to be discovered. The results from this work showed increased mammary size in response to treatment which is encouraging for longer term improvements in yield.

Another aim of this work was to elucidate the role of prolactin in established lactation by preventing the seasonal drop in prolactin concentrations (Hart, 1975a). We were able to significantly increase circulating prolactin concentrations by infusing perphenazine, even in lactating goats which had high basal prolactin levels. However long-term administration by oral dosing and injection was not successful in elevating prolactin, predominantly due to the intolerance of perphenazine given by these routes.

If recombinant prolactin could be given regularly by, for example, subcutaneous slow release injection or osmotic minipump, then quite possibly the fall in prolactin could be prevented and the effects of this on milk production could be studied. It may be that only low levels of prolactin are required to maintain lactation once it is established, for it to be able to, for example, induce and stabilise casein mRNA and extend its half life (Teyssot & Houdebine, 1980), thereby facilitating milk secretion. However as a consistent elevation of prolactin in ruminants has not been achieved over a lactation, its role remains open to debate, and this could provide an area for further study.

Oxytocin was given to goats immediately before each milking to stimulate milk ejection and ensure emptying of the mammary glands, particularly the alveolar areas. It was hypothesised that this would improve persistency as there would be a reduction in the local inhibition of milk secretion. However oxytocin treatments were only partly successful in improving persistency, and the changes that were achieved (in Expt 1) were numerically small and not statistically significant. This may be, in part, due to the administration route- due to its short half life oxytocin was given by intramuscular injection immediately before milking and some animals became sensitised to the repeated injections. Unfortunately there are few alternative administration routes for oxytocin as experimental conditions must be clearly defined with milking commencing shortly after oxytocin is given. If not, the full effect of milk ejection may be missed due to its short half-life, so giving oxytocin by slow release or continuous infusion is not possible. One option might be giving by nasal spray, used in lactating women (Winter & Robinson, 1964) but this has never been attempted in goats. The results obtained in the experiments described in this thesis may also reflect biological variation between animals in the response to treatment; cows with poorer persistency showed more response to oxytocin (Sprain *et al*, 1954). In the first experiment, oxytocin treatment was associated with maintenance of mammary tissue mass, suggesting a beneficial effect on the mammary gland, perhaps contributing to the preservation of secretory cell function. By lactation week 37, three oxytocin animals were producing measurable amounts of milk compared to one control animal, again suggesting a positive effect on lactation. However these results were not substantiated in the follow up experiment conducted the following year. Oxytocin might be preventing or reducing involution in the gland and, when administered at physiological levels, may contribute to maintaining tight junction integrity, all of which would help to reduce the rate of decline in milk yields. It may also be that oxytocin alone is not enough to alter persistency but is a contributing factor in the 'well being' of secretory cells. Perhaps the best long-term

option for oxytocin is selective administration- giving it to animals with poorer lactation persistency, established from records of previous lactations, and subsequently studying its effects.

The interaction between oxytocin and 1X milking was not expected- it was expected that 1X milking would reduce lactation persistency, but this did not happen. It could be argued that the drop in yield on the switch to 1X milking affected the linear regression analysis of yields, but good fits for the lines were obtained, and the difference in yield between glands reduced as lactation progressed, indicating a consistent effect. If the result had been a quirk of the statistics then one would expect the difference between glands to remain relatively steady but this was not seen. The effect was local and it may well reflect an alteration in the storage characteristics in the udder. This was studied more closely in the second experiment and during the period of oxytocin+1X milking there was an increase in both cisternal volume and percentage. This could partly alleviate the negative effect of FIL on milk secretion (Wilde *et al*, 1995) and consequently maintain or even improve persistency. Differences in storage characteristics between 1X and 2X glands were not found but this may reflect the period of milk accumulation not being sufficiently long enough to detect changes in the partitioning of milk in the udder, which would probably be most marked between 8 and 16 hours after milking.

Application of treatments in early lactation proved to be an effective means of manipulating milk yields, and some carry over effects were seen. Suckling proved to be a potent stimulus for increasing alveolar capacity, but this was not matched by the volume of milk being stored cisternally and there were no apparent carry over effects. This may be due to the frequent milk removal by kids not allowing cisternal storage to increase as much as it would have if it had been regularly filling up with milk. Consequently when switched to regular 2X milking, these glands were subject to increased local inhibition of milk secretion by FIL as a greater proportion of their milk was stored in alveolar areas, and the positive effect of increased mammary growth on yield was subsequently lost. The negative effect of reduced cistern size compared to animals with larger cisterns has already been seen in goats and cows (Peaker & Blatchford, 1988; Dewhurst & Knight, 1994), supporting this hypothesis.

Mammary growth also appeared to have been stimulated by 3X milking as a significant effect of treatment on yield was seen during the post-treatment period in a mixed parity group. This corresponded to a significantly increased alveolar volume

compared to contralateral 1X glands after 5 weeks of treatment. Measurements of cell proliferation and secretory enzyme activities were not made in this study, but previous work has shown that even over short periods (9 days) increased milking frequency increases the activity of the secretory cells, while 13 weeks of 3X milking improved the metabolic efficiency of milk production (Wilde *et al*, 1987b).

The apparent lack of response of multiparous animals to 3X milking may reflect a reduction in the potential of the gland to respond to growth stimuli, but there is also the question of their tolerance for 1X milking. This may be due to changes in the storage of milk in the mammary gland associated with increasing parity. The cisternal capacity of multiparous goats, already large, was increased much more by 1X milking than that of primiparous goats. Longer periods of milk accumulation could be tolerated by milk moving into the large cistern which would not increase the inhibitory effects on the gland, and secretion could continue as long as milk was overspilling into these areas. Multiparous goats also exhibited poorer lactation persistency, and in this regard the explanation may involve factors other than storage characteristics, since the observed ability to overcome FIL activity would also be expected to increase persistency.

There was an overall trend for improved persistency in 1X glands, seen in most experiments, regardless of the treatments being applied, and it appeared that regular 24 hour periods of milk accumulation resulted in more milk moving into cisternal areas. This was not confirmed by measurements of alveolar and cisternal volumes made 5 hours after milking, but this interval was probably too short to detect long-term changes, and measurements made at longer intervals, and at various points in the post-treatment period, would have been more appropriate. The effect was, however, visually clear (Plate 6.3) and the area of the interaction between milk secretion and the storage of milk in the mammary gland is one worthy of further investigation.

The measurement of persistency was generally determined by regression analysis of milk yields but another aspect was considered in this thesis, namely the effect that alterations in lactation persistency might have on caseins (milk proteins). The most abundant casein in goat milk is β-casein which can be proteolytically degraded by plasmin to produce γ-casein fragments (Eigel,, 1977b). The activation of plasmin is positively correlated with stage of lactation (Politis *et al*, 1989a) but is this a relationship between plasmin and the time after parturition, or a reflection of the process of involution occurring in the gland? Unfortunately the treatments applied

(perphenazine to elevate prolactin and oxytocin to improve milk ejection) did not significantly alter persistency, so the question could not be answered in this study. We did find a significant correlation between milk yield and γ-casein percentage in declining lactation, so γ-casein does appear to be a good marker for the process of involution. However as milk yields themselves reflect the extent of mammary function, the measurement of milk yield itself would be a more direct and straightforward measure of mammary function.

In conclusion there are a variety of factors which influence lactation persistency in the goat. The role of prolactin remains to be established and oxytocin treatment may prove useful in improving persistency some animals, particularly those with poor milk ejection. The mammary gland clearly retains the potential for growth both *pre-partum* and in early lactation on the application of appropriate stimuli and alterations in milking frequency and the mode of milk removal can also stimulate mammary growth during the treatment period. Treatments may also alter milk storage characteristics, but responses can be influenced by parity. In particular the tolerance for once-daily milking and relationship with lactation persistency is an area worthy of continued investigation.

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ADDENTUM:

Finidori, J. & Kelly, PA. (1995). Cytokine receptor signalling through two novel families of transducer molecules: Janus kinases, and signal transducers and activators of transcription, *J. Endocrinol.*, **147**, 11-23.