MANIPULATION OF LACTATION PERSISTENCY TO ACHIEVE EXTENDED LACTATION IN DAIRY COWS

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Abstract

This thesis investigated management practices that might be used to manipulate lactation persistency to achieve extended lactation in dairy cows. Factors under investigation were milking frequency, nutrition and calving season. Increased milking frequency resulted in an increased milk yield by as much as 50% and significantly improved lactation persistency. Nutrition and calving season increased milk yield but only had short term effects on lactation persistency. This suggests that while milking frequency has a continuing stimulatory effect on the mammary cell population, nutrition and calving season might have an initial stimulatory effect to adapt to a higher level of milk production but no long lasting effects. The most consistent and significant predictor of lactation persistency was peak milk yield, which was negatively correlated with lactation persistency (-0.68).

Fertility parameters and endocrine profiles were also investigated during extended lactation. No evidence was found to suggest that re-breeding at a later stage of lactation was any easier or worse than during early lactation. Extended lactation did not compromise future reproductive success. It is inevitable that good reproductive management will remain an essential part of any extended lactation strategy.

A persistent lactation was positively correlated with changes in GH (0.14) and negatively with changes in insulin (-0.29), suggesting that nutrient partitioning is important in maintaining lactation persistency. This effect might be indirect since nutrient partitioning affects milk yield.

The last part of the study determined whether the same treatment used to manipulate lactation persistency also affected milk protein quality. Both frequent milking and supplementary feeding increased casein number, indicative of good processing quality. In combination these were able to completely maintain casein number through lactation. The mechanism underlying the effect of frequent milking on milk protein quality was shown to be bi-factorial, reduced storage time of milk within the udder together with decreased involution (and thus better integrity of mammary tight junctions) both contributing to reduced casein proteolysis by components of the plasmin system.

This thesis has shown that it is possible to manipulate lactation persistency to extend lactation in dairy cows but careful management is required.

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ABBREVIATIONS

3x	thrice daily milking
2x	twice daily milking
Anova	analysis of variance
Ancova	analysis of covariance
BST	bovine somatotropin
FIL	feed back inhibitor of lactation
GH	growth hormone
IGF-1	insulin like growth factor –1
IGFBP	insulin like growth factor binding protein
IgG	immunoglobulins
K	potassium
kg	kilogram
LF	lactoferrin
MSe	mean square error
Na	sodium
n.s	non significant
REML	residual maximum likelihood
RIA	radioimmunoassay
SA	serum albumin
SCC	somatic cell count
SE	standard error
SED	standard error of deviation

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CHAPTER ONE

REVIEW OF LITERATURE

1.1. Introduction *Why consider extending lactation?*

The typical lactation curve reaches a peak at around 8-10 weeks *post-partum* and thereafter steadily declines for the remainder of lactation (Wood, 1967). The length of a cow's lactation cycle is usually 12 months and, so far, maximum peak milk yield and minimum calving interval have been considered as being the main criteria for achieving the best profitability (Dijkhuizen *et al.*, 1985; Strandberg & Oltenacu, 1989). However, there are a number of reasons why this might not be the most appropriate system any longer.

Genetic selection and better feeding management have greatly improved dairy cow productivity. However, this has not been achieved by altering the shape of the lactation curve but only by the magnitude of milk production. As yield has increased so have concerns about cow health and welfare. Most health problems such as metabolic diseases, mastitis, lameness and the failure to rebreed occur around peak lactation and approximately 60 % of all veterinary costs are incurred during the first 45 days of lactation (Grohn *et al.*, 1986; Erb *et al.*, 1984). This extreme risk period is undoubtedly related to the level of production and in particular to the metabolic imbalance which this imposes (Domecq *et al.*, 1997). It is unlikely to be solely a consequence of genetic selection (Sorensen *et al.*, 1998). In a comparison of high and average genetic merit cows similar incremental responses to known galactopoietic stimuli were observed, indicating that cows of high genetic merit are not operating closer to their metabolic limit for milk production than the average genetic merit cow and are not, therefore, at greater risk of metabolic collapse. The increased incidence in metabolic diseases has also led to an increase in

culling rate with consequent increases in replacement rate and cost. Analyses have indicated annual culling rates of 24% in the UK, the main reason for culling being reproduction/ infertility failure (36.5%), mastitis (10.1%) and lameness (5.6%) (Kossaibati & Esslemont., 1995). In Denmark, annual culling rates are as high as 40-50%. Furthermore, yearly average national milk production is about 2000 litre higher in Denmark than in the UK. The production life for a dairy cow in the UK is, therefore, between 3 and 4 lactation and only between 2 and 3 lactation in Denmark. Knight (1998) illustrated this point very dramatically by considering the fact that a cow that first calves at two and a quarter years and achieves twelve month lactation cycles will have been productive for only 35 months out of her 68 month life. The welfare implications of a short life are arguable but the economic impact is not. Economic modelling shows benefits from keeping a cow longer in the herd (Scott, 1994).

With increasing milk production, yield at drying off prior to calving can be considerable. Whether this causes any stress or pain in form of udder problems to the cow is unknown, although it does seems like a waste of production capacity.

Interest in extending lactation has increased over the last few years. Increasing the calving interval will inevitably reduce the number of periods where cows are exposed to extreme risk for health problems. Considering a 3 years period cows on 12 months lactation cycles will be exposed to 3 periods of extreme risk while cows on extended lactation cycles of 18 months will only be exposed to two such periods. This may potentially increase productive life. In an American study comparing twelve and eighteen month lactation cycles no difference was found in disease incidences during the first two years of their study. This is

not surprising since two years of study includes two extreme risk periods for both lactations (Van Amburgh *et al.*, 1997).

Twelve month lactation cycles are usually regarded as the most profitable length of the calving interval (Strandberg & Oltenacu, 1989; Dijkhuizen *et al.*, 1985). The economical modelling is based on milk yield, feeding, housing and does not take health and treatment cost into consideration. In Sweden reproductive parameters for cows with a 12, 15 or 18 month lactation cycle have been compared. In the 15 and 18 month groups fewer inseminations per pregnancy were needed but no difference was found in conception rate at 1'st insemination and percentage of cows finally pregnant between the three calving intervals. Furthermore, fewer cases of anoestrus and cystic ovaries in the extended lactation groups were evident (Ratnayake *et al.*, 1998). In an American study (Van Amburgh *et al.*, 1997) economic analysis taking replacement cost, health cost and so on into account was performed on 12 and 18 month lactations. Extended lactation was found to increase the profitability per cow by 75%. However, one thing to bear in mind concerning this work is the use of BST, which in this particular study showed positive effects on lactation persistency. Assuming that lactation persistency does not change with extended lactation 12 month lactation cycles will always produce higher annual milk yields as was calculated by Knight (1998). However, if lactation persistency increases insemination is profitable longer into the lactation (Dekkers *et al.*, 1998). An increase in lactation persistency by 0.142 (calculated as the ratio of yield from day 201 to day 305 of lactation and yield in the first 100 days of lactation) showed that a calving interval of 14 month (maximum calving interval investigated) was more profitable than a calving interval of 11 months. Although feed costs were reduced per lactation with an increase in persistency the effect on milk return was greater than the effect on feed cost. Therefore,

persistency is important in determining the appropriate calving interval. If one could find ways of improving lactation persistency there is no doubt that extended lactation would be truly successful.

Different people have defined and measured lactation persistency in different ways (see McFadden, 1997 for discussion). In this thesis, however, lactation persistency will be defined as the rate of decline in milk yield after peak lactation and it will be measured by regression analysis.

Another issue, which becomes rather important when considering extended lactation, is milk quality. It is well known that the processing quality of milk decreases as lactation progress although total crude protein increases (Politis *et al.*, 1989; Baldi *et al.*, 1996). In New Zealand and Ireland where a seasonal breeding system of dairy cows is in use, milk quality can decrease to such an extent during late lactation that it is unsuitable for cheese manufacture. If extending lactation will result in an overall poorer quality of milk due to the longer period spent in late lactation is unknown. However, if one considers the current milk-paying scheme where the payment for milk is based on the protein and fat content of the delivered milk late lactation milk is valuable, but one needs to take into consideration that this system might eventually change so that farmers will be paid on the actual processing qualities of the milk.

1.2. Ways of manipulating lactation persistency

There is little knowledge of the control of lactation persistency. From mammary perspective milk production is a simple function of the number and activity of the individual secretory cells. In the goat, the period of increasing milk yield from parturition until peak lactation is characterised only by modest amounts of cell proliferation but a very

substantial increase in secretory activity on a per cell basis (Knight & Peaker, 1984; Wilde *et al.*, 1986; Fowler *et al.*, 1990). In contrast, the decline in milk yield after peak lactation is entirely due to a decline in number of secretory cells, activity per cell stays unchanged. The decline in amount of tissue is equivalent to the rate of decline in milk production (Wilde & Knight, 1989). This change in cell number results from an imbalance between cell proliferation and cell death (apoptosis). Mammary apoptosis is controlled by systemic and local factors (Travers *et al.*, 1996). To improve lactation persistency it becomes clear that one needs to be able to prevent or compensate for loss of mammary secretory cells.

Stimulation of milk yield using BST increases lactation length mainly by pushing the yield curve upwards (Bauman, 1992) although recent studies have also shown an increase in lactation persistency (Van Amburgh *et al.*, 1997). Oxytocin injected at every milking can alter the shape of the lactation curve and reduce the post-peak lactation decline (Nostrand *et al.*, 1991). This is an indirect consequence of more efficient milk removal (Knight, 1994) rather than a direct effect of the hormone. In any case endocrine manipulation is not a desirable approach. Use of BST is not permitted in European countries in contrast to America, therefore, other ways of increasing lactation persistency have to be found.

Increased milking frequency also stimulates yield, through more frequent removal of a secretion inhibiting protein present in milk, the feedback inhibitor of lactation, FIL (Wilde *et al.*, 1997a). Long term increased milking frequency has been shown to increase lactation persistency in some studies (Pearson *et al.*, 1979; Amos *et al.*, 1983) but not in others (Allen *et al.*, 1986; Gisi *et al.*, 1986). Lactation persistency might be improved while the treatment is applied, however, once frequent milking is stopped the rate of decline in milk

yield returns to values as for twice daily milking although total yield remains higher (Pearson *et al.*, 1979).

The short term effect of frequent milking on secretion rate has been shown to be a direct consequence of more frequent removal of FIL (Wilde *et al.*, 1997a). In the long term mammary development changes (Knight *et al.*, 1990b; Knight *et al.*, 1992). In cows (Hillerton *et al.*, 1990) a tendency for an increase in cell proliferation and the activity of key enzymes involved in milk synthesis was evident after four weeks of 4 times daily milking.

Nutrition and the partitioning of nutrients is important for sustaining secretion on a day to day basis, but whether is has effects on mammary development is unknown. The partitioning of nutrients towards the mammary gland decreases as lactation progress as the ratio of GH to insulin decreases. But what dictates this change? Is it led by the mammary gland or by the body tissues? A recent study by Friggens *et al.* (1995) suggests that the amount of feed given during lactation does affect mammary development, because of the linear response observed in milk yield to increments in the amount of feed given, milk yield did not reach a plateau within the 4 week treatment period.

It is very evident that season of year has an effect on milk yield (Wood, 1972), although how much of the spring flush is nutritional and how much is due to an increase in daylength which can increase milk yield is uncertain (Tucker, 1985). The nutritional effect on milk yield when the winter ration is substituted with fresh grass will depend on the difference in the nutritional value of the two rations. Summer grazing is certainly at the Hannah associated with an increase in milk production as fresh spring grass is of better nutritional value than grass silage which is the main forage used in the winter ration in

most of the UK and at the Hannah Research Institute. However, at the Centre for Dairy Research in Reading where maize silage is the main forage in the winter ration grazing results in decreases in milk yield. An increase in daylength can not, therefore, override the nutritional changes associated with turn out to grass.

The overall aim of this thesis was to investigate the effect of season, nutrition and milking frequency on lactation persistency and milk quality during extended lactation in dairy cows. We were aiming for an eighteen-month lactation. Anything less would be close to what already occurs often as individual cows fail to rebreed without a delay, and eighteen month has the advantage of supporting autumn and spring calving groups. Since the importance of mammary development was recognised from the outset, a mouse model was developed which will allow us to study the interrelationship between mammary cell proliferation and apoptosis. The remainder of this chapter will review and discuss factors that could affect lactation persistency and in particular discuss the effect that milking frequency, nutrition and calving season could have on lactation persistency.

1.3. Mammary cell proliferation and apoptosis during lactation

Milk yield is determined by epithelial cell number and by secretory activity per cell. A successful lactation in the dairy cow requires therefore that the mammary gland produce a large number of potential milk secreting cells. Yet, despite many years of genetic selection and technological advances lactation remains by its nature a transient process programmed to terminate even as it is initiated.

The majority of mammary growth occurs during the latter part of gestation (Delouis *et al.*, 1980). The degree to which mammary growth is completed at parturition varies between species. In most species an increase in cell number continues into early lactation, although

the extent of lactational mammary growth varies. In rodents, cell proliferation accounts for as much as 75% of the increase in daily milk yield up to peak lactation. The remaining increase in yield is due to an increase in cellular activity (Knight & Peaker, 1984). In goats and cows it was initially believed that mammary cell proliferation ceased at parturition (Anderson *et al.*, 1981). It is now recognised that in the goat, increasing milk yield up to peak lactation is due to an increase in cell number together with an increase in the cellular activity of individual cells (Knight & Peaker, 1984; Wilde *et al.*, 1986; Fowler *et al.*, 1990). Little is known about mammary proliferation during lactation in the cow however lactating bovine mammary epithelial cells are able to divide, as a response to increased milking frequency (Hillerton *et al.*, 1990) and BST at least in mid lactation (Capuco & Byatt, 1998). In goats and probably cows the decrease in milk production after peak lactation results primarily from a decrease in the number of secreting cells, rather than a change in the metabolic capacity of those cells which remain (Knight & Peaker, 1984; Wilde *et al.*, 1986). This is in contrast to the rat where it is a decrease in the cellular activity that is responsible for the decrease in milk production (Knight *et al.*, 1984). In the mouse it is a combination of a decrease in cell number and activity which accounts for the decrease in milk yield (Knight & Peaker., 1982). Mammary cell loss is also observed during tissue involution after cessation of milk removal (Wilde *et al.*, 1997b). In cows a non-lactating period is required for maximum production in the subsequent lactation. Without a dry period, milk production may be reduced by 20% (Swanson, 1965). It is now known that these decreases in cell number in the ruminants (Molenaar *et al.*, 1996; Wilde *et al.*, 1997b) but also in rodents occur by apoptosis (programmed cell death) (Quarrie *et al.*, 1995; Quarrie *et al.*, 1996).

Cell death by apoptosis differs fundamentally from necrotic cell death. Apoptosis is a response to physiological stimuli while necrotic cell death is a result of tissue injury, such as mastitis. Apoptosis involves a series of ultrastructural changes including the compaction of nuclear chromatin and condensation of cell cytoplasm and is associated with the fragmentation of genomic DNA into oligonucleosomal fragments of 180-200 base pair lengths. Contrary to necrosis, cell death by apoptosis takes place in the absence of an immune response. Apoptotic bodies are taken up and degraded by adjacent cells or are engulfed by neighbouring macrophages (Kroemer *et al.*, 1995).

Mammary involution in the dairy cow differs from that in rodents. While the mammary glands of rodents regress to a state resembling that of a virgin animal (Strange *et al.*, 1992) remodelling of the bovine mammary gland proceeds with only partial loss of the mammary population (Holst *et al.*, 1987; Wilde *et al.*, 1997b) and de-differentiation of the remainder (Wilde *et al.*, 1997b; Li *et al.*, 1999). This raises the question of whether de-differentiated epithelial cells are capable of re-differentiating and secreting milk in the subsequent lactation, i.e. are cells carried-over from one lactation to another. Using magnetic resonance imaging (Fowler *et al.*, 1990) illustrated that the udder of the goat does not revert back to its virgin state after the first lactation. The udder commenced the next lactation larger than at the start of the first cycle. This suggests that some cells were carried-over to the subsequent lactation. Li *et al.* (1999) provided evidence that alveolar cells can de-differentiate to ductal epithelial cells. In bovine mammary glands unmilked for 11 days milk yield recovered 28% of pre-treatment yield 3 days after lactation was reinitiated (Noble & Hurley, 1997). Taking into account that after 7 days of cessation of milking the abundance of α_{s1}-casein and α-lactalbumin mRNA in mammary tissue decreased by 99 and 85%, respectively (Wilde *et al.*, 1997b), the reinitiation of milk

secretion, therefore, indicates a potential for re-induction of gene expression in surviving cells. In a comparison of animals which were allowed a dry period and animals which had no dry period no difference was found one week before parturition in the number of mammary epithelial cells between non-lactating and lactating animals. However the rate of [^{3}H]thymidine was 80% greater in mammary tissue from non-lactating cows, indicative of increased cell proliferation (Capuco & Akers, 1999). Because the total number of mammary cells was the same in lactating and non-lactating cows this suggests a higher rate of cell renewal in the non-lactating mammary glands. Thus the dry period might be important for replacement of "old" mammary epithelial cells prior to the next lactation. But do old or re-differentiated cells have a shorter life or reduced secretory capacity than newly proliferated cells? If so could this explain why the heifer lactation is much more persistent than the mature cow lactation? Perhaps the heifer mammary cell population undergoes less apoptosis as lactation progresses due to the entirely new generation of cells. Although another issue to bear in mind is that heifers are still growing and so most probably is their mammary glands. More research is needed to determine the extent of cell renewal and carry-over of cells from one lactation to another and how this affects milk production and lactation persistency.

1.4. Endocrine control of apoptosis and cell proliferation

It is the extent of cell proliferation and apoptosis in the lactating mammary gland that determines milk yield and lactation persistency and to improve the productivity and in particular lactation persistency it is then crucially important that we understand the processes that regulate mammary cell number.

The control of apoptosis has primarily been studied in rodents. Mammary apoptosis is under endocrine and local control. Removal of the suckling induced release of galactopoietic hormones by simply removing the litter triggers apoptosis (Quarrie *et al.*, 1996). In mice administration of prolactin after litter removal reduced apoptosis (Sheffield & Kotolski, 1992). In lactating rats prolactin depletion using bromocriptine decreased milk yield and rapidly induced apoptosis (Travers *et al.*, 1996). Reduction of GH secretion also reduced milk yield and induced apoptosis, however, the effect was smaller than the one observed with bromocriptine treatment. Depletion of both circulating prolactin and GH almost completely stopped milk secretion and stimulated apoptosis to an even greater extent. When prolactin secretion was replaced milk secretion completely recovered and apoptosis was reduced. However when only GH was replaced milk secretion was only partially restored, as was apoptosis. By dual hormone replacement apoptosis was completely abolished (Travers *et al.*, 1996). These studies clearly demonstrate interplay between these to hormones in their ability to protect cells from apoptosis.

In ruminants prolactin is without doubt required for initiation of lactation (Delouis *et al.*, 1980). However, whether it plays any significant role for milk yield once lactation is established is open to debate. Following hypophysectomy in lactating goats milk yield decreased rapidly. Administration of glucocorticoid, thyroid hormones and GH increased milk yield to about 28% of pre hypophysectomy. When prolactin was added, milk yield was completely restored. After milk yield was re-established and prolactin was withdrawn milk yield was not affected (Cowie *et al.*, 1964). Treatment with bromocriptine in lactating cows has shown little or no effect on maintaining milk production (Karg *et al.*, 1972; Hart, 1973) however in goats bromocriptine treatment reduced milk yield by about 20% (Knight *et al.*, 1990a; Forsyth *et al.*, 1995). Plasma levels of prolactin are higher during lactation as

are the number of receptors in mammary tissue (Collier *et al.*, 1984) and prolactin release in response to milking is positively correlated with milk yield (Collier *et al.*, 1984). The mammary gland of both rodents and ruminants is capable of synthesising its own prolactin (Leprovst *et al.*, 1994), so maybe the mammary gland is simply upregulating it's own production when circulating levels are low. Growth hormone on the other hand has been shown to be strongly galactopoietic in ruminants.

Administration of recombinantly derived GH stimulates milk production by 20% or more (Bauman & Eppard, 1985). In some studies GH has shown increased lactation persistency, while in others no effect on lactation persistency has been found. If cows are given an inadequate amount of feed the magnitude of response to GH will decrease according to the extent of the inadequacy (Bauman, 1992). Short-term treatment with BST is associated with an increase in the repartitioning of nutrients towards the mammary gland, at the expense of other body tissues (Bauman, 1992). The initial increase in milk yield is, therefore, attributed to body tissue mobilisation and thereafter feed intake increases (Bauman & Eppard, 1985). However due to the failure of intravascular or abomasal infusion of nutrients to increase milk yield to the same extent (Peel *et al.*, 1981) it appears likely that GH is able to stimulate milk synthesis through an additional mechanism.

Although blood flow to the udder and thereby nutrient supply increases with GH treatment this effect seems to be a response rather than a cause of the increase in milk production (Davis & Collier, 1985).

In pre pubertal heifers mammary parenchyma volume increased with administration of GH (Sejrsen *et al.*, 1986; Sandles & Peel, 1987). In pregnant goats GH treatment pre partum

showed no effects on either DNA synthesis or subsequent milk yield (Lee & Forsyth, 1988). However GH given to goats in late pregnancy increased both mammary cell number and milk yield, but the same treatment starting at parturition only increased milk yield (Knight *et al.*, 1994). Cellular differentiative responses to short term administration of GH during lactation in goats (Knight *et al.*, 1990a) and cows (Knight *et al.*, 1992) showed only a small increase in cellular activity, and in the longer term no difference between GH treated and control animals was found. However treatment with GH in the goat commencing in week 19 of lactation and continued for 22 weeks maintained the mammary cell population completely while a decrease of 23% was seen in control goats (Knight *et al.*, 1990a) which suggests that long term treatment with GH prevents or reduces glandular involution. Later in lactation GH treatment to lactating cows increased mammary cell proliferation from 0.5% to 1.6% (Capuco & Akers, 1998). GH administration to lactating animals increases milk yield only for as long as the treatment is applied (Bauman, 1992), which implies that the developmental changes are not sufficient in themselves to sustain the enhanced yield, but must be combined with the nutrient repartitioning action of GH. Although GH not always improves lactation persistency there can be no doubt that GH is important in sustaining a viable cell population in lactation but GH is also very important in sustaining nutrient partitioning to the mammary gland.

There are still many questions to be answered about the mechanisms by which GH acts on the mammary gland. The evidence indicates that exogenous GH does not exert its effects through a direct action on mammary tissue. No direct effect of GH on milk synthesis in bovine mammary tissue has been demonstrated in vitro (Gertler *et al.*, 1983) and no significant binding of GH by lactating mammary tissue has been detected (Akers, 1985). The lack of a direct effect of GH may be due to the absence of GH receptors although

mRNA for the GH receptor has been described in bovine mammary tissue (Hauser *et al.*, 1990). In other tissues GH acts via IGFs and there is accumulating evidence that this is also the case in mammary tissue. The type I receptor has been found in mammary tissue (Dehoff *et al.*, 1988) and IGF-I is a potent mitogen for the developing and lactating mammary cells (Baumrucker & Stemberger, 1989; Shamay *et al.*, 1987).

Administration of GH increases systemic levels of both GH and IGF-I. However systemic infusion of IGF-I, to mimic the effect of GH, had no effect on milk production (Davis *et al.*, 1989), so if the action of GH on mammary development and milk yield is mediated through IGF-I it is certainly not through increased circulating levels of IGF-I. IGF-I does not circulate in its free form, but bound to binding proteins. Several binding proteins have been identified (Jones & Clemmons, 1995) which raises the possibility of GH/IGF-I acting through one or more of these binding proteins.

IGFBP-5 is synthesised within the mammary gland and the synthesis of this protein is increased during involution (Tonner *et al.*, 1995). Furthermore changes in the production of this binding protein are highly correlated with apoptosis, which was demonstrated in the GH and prolactin deficient rats (Tonner *et al.*, 1995) described above (Travers *et al.*, 1996). These studies led Flint & Knight, (1997) to hypothesise a possible mechanistic explanation for the interactive effect of GH and prolactin on cell survival. GH stimulates IGF-1 production possibly from the mesenchyme and prolactin ensures IGF-1 action by suppressing the synthesis of IGFBP-5 from the epithelial cells (Flint & Knight, 1997). GH receptors have been identified on the stromal mesenchymal cells and binding of GH to this receptor has been shown to induce local production of IGF-I (Ruan *et al.*, 1992), hence the local production of IGF-I. Furthermore administration of GH into the rat mammary gland

can stimulate milk yield in that gland without affecting the contra lateral gland (Flint & Gardner, 1994), suggesting local induction of IGF-1 synthesis. Although the changes in IGFBP-5 production is correlated with apoptosis this does not demonstrate a causal relationship. A transgenic mouse over expressing IGFBP-5 in the mammary gland has therefore been constructed and preliminary result shows a reduction in mammary development in these animals (D. Flint, personal communication).

IGF-BP3 has also been suggested to be important in modulating the mitogenic activity of IGF-1 in mammary tissue at least in undifferentiated mammary epithelial cells (Weber *et al.*, 1999). Like IGF-BP5, IGF-BP3 is synthesised by mammary epithelial cells. Weber *et al.* (1999) demonstrated that addition of IGF-BP3 to primary undifferentiated mammary epithelial cells inhibited DNA synthesis by 26%. Supplementing the media with IGF-1, serum or mammary extract from pre pubertal heifers all increased DNA synthesis, mammary extract being the most potent stimulator. Adding IGF-BP3 inhibited approximately one third of the DNA synthesis stimulated by serum or extracts, presumably by binding IGF-1 and thereby reducing it's access to it's receptor on the mammary epithelial cells. The difference in the potential of serum and mammary extract to stimulate DNA synthesis has been attributed to differences in the IGFBP profile, IGF-BP2 being relatively more abundant in serum compared to mammary tissue extracts (Weber *et al.*, 1999). The same group has recently found that DNA synthesis induced by serum is negatively correlated with IGFBP-2 and positively correlated with IGF-BP3 and IGF-1 while DNA synthesis induced by mammary extract is negatively correlated with IGF-BP3 and no correlation exist between BP-2 and IGF-1 (Purup *et al.*, 2000). This has led the group to speculate that circulating levels of IGFBP-3 might stimulate IGF-1 activity while

IGFBP-3 in mammary tissue might inhibit IGF-1 activity. Interestingly IGF-BP3 is increased in serum from cows treated with BST.

1.5. Local control of mammary apoptosis and cell proliferation

Milk stasis in itself is a potent inducer of apoptosis in rodent mammary tissue (Quarrie *et al.*, 1995), demonstrated by sealing the teats on one body half of lactating mice, and allowing half of the litter to be suckled on the remainder. Apoptosis was induced in the sealed glands, showing that cell death is stimulated by an intramammary mechanism sensitive to milk accumulation (Quarrie *et al.*, 1996). Unilateral milk stasis has the same effect in lactating goats (Quarrie *et al.*, 1994; Li *et al.*, 1999). Any endocrine influences on apoptosis might therefore be modulated locally within each mammary gland by a mechanism sensitive to milk stasis.

1.5.1. Milking frequency

When milking frequency is increased milk yield increases as described above. However, different milking frequencies applied to individual glands within the same animal only increase milk secretion in the gland that receives the additional milking and is limited to the daily period in which the extra milking is applied (Henderson *et al.*, 1983). The mechanism involved is therefore not related to endocrine function but rather to actual milk removal (Henderson *et al.*, 1983).

The acute response has been found to be due to more frequent removal of a milk protein (FIL) which limits milk secretion by a negative feedback mechanism on the secretory cells (Wilde *et al.*, 1997a). In the longer term increased milking frequency causes a small increase in the activity of key enzymes involved in milk synthesis and cell proliferation. In

goats mammary epithelial cell differentiation was stimulated after 10 days of unilateral thrice daily milking before peak lactation (Wilde *et al.*, 1987). In declining lactation the same treatment had no significant effect after 3 weeks of treatment, but after 22 weeks of treatment a significant difference between twice and thrice milked glands was found in both cell differentiation and proliferation (Knight *et al.*, 1990b). In cows 4x daily milking for 4 weeks showed a tendency for an increase in differentiation and proliferation of mammary epithelial cells (Hillerton *et al.*, 1990). Not only does increased milking frequency increase cell proliferation and differentiation but also it is likely to suppress apoptosis. Goats were milked thrice in one gland and once in the other gland starting during lactation week 20. After 4 weeks of treatment the thrice daily milked glands had a reduced amount of apoptosis compared to the once daily milked glands, however, after 10 weeks of treatment no difference was found between the two glands. This suggests that after an initial accelerated loss of cells as an adaptation to once daily milking, the two glands may then lose cells at a similar rate (Li *et al.*, 1999). However, this effect could be due to another phenomena namely compensatory growth. It has been shown in cows that when milking is stopped in one or more mammary glands the remaining milked glands are capable of compensating for the loss of milk production in the unmilked glands (Hamann & Reichmuth, 1990). When once daily milking reverts to twice daily, milk yield recovers completely although activities of key enzymes do not recover to the same extent. However, the enzyme activities and metabolic fluxes measured are indicators of synthetic potential rather than precise measures of synthetic rates. Furthermore, cell proliferation was not measured in this study so it is possible that the gland responded to the increase in milking frequency by increasing cell proliferation, although it is unlikely that this would happen so quickly. This does raise some interesting questions about whether or not the mammary

gland has "spare" capacity, so the gland is normally not using its full potential. This will be discussed later.

It becomes clear that mammary development is under local control, but what mechanisms are involved? Stimulation by galactopoitic hormones is clearly not responsible since this would have resulted in an effect on the whole udder. That said, the consequence of more or less frequent milking is an alteration in the number of receptors for prolactin on the surface of the mammary secretory cell (McKinnon *et al.*, 1988) and in the binding of IGF-I by mammary tissue (Bennett, 1993). Both prolactin and IGF-1 are cell survival factors for the mammary gland so it is possible but not known whether this local modulation of the glands sensitivity to circulating hormone is involved in the changes in mammary development.

The IGFBP's are another potential regulator of mammary cell growth and survival, due to accumulation of these proteins within the gland during milk stasis. Because the mammary gland produces a secretion rich in biologically active substances and then stores that secretion, a variety of possible candidate as regulators of mammary growth and death are available. TGF-β1 is one such. Its secretion is up-regulated during involution and under conditions which stimulate apoptosis *in vitro* (Atwood *et al.*, 1995). Changes in concentration of milk borne factors certainly do provide a mean of regulating mammary apoptosis within each gland. Alternatively local induction of apoptosis could be due to physical distension of the mammary cells due to milk accumulation. In the goat increased intramammary pressure to levels which normally occur following cessation of milking had no effect on milk secretion (estimated from gland volume), mammary blood flow and substrate uptake until 24h after last milking (Peaker, 1980). The rate of milk secretion,

stage of lactation and the storage capacity of the gland will all influence the time scale for when intramammary pressure will affect milk secretion.

The mammary tight junctions which by joining adjacent secretory epithelial cells form a barrier preventing interdiffusion of milk constituent and plasma proteins have been suggested to be possible modulators of pressure-induced inhibition of milk secretion and apoptosis (Davis *et al.*, 1999). An increase in tight junction permeability is accompanied by a decrease in milk secretion. When milking frequency is reduced from twice daily to once daily milk secretion decreases and the mammary tight junction opens up (Stelwagen *et al.*, 1994b). In cows milk secretion and the mammary tight junctions did not became disrupted until 18h after the last milking (Stelwagen *et al.*, 1997). However in goats where mammary pressure was increased immediately after the morning milking by experimental infusion of a sucrose solution the mammary epithelium did not become leaky until the pressure was well above physiological pressures (Peaker, 1980). Disruption of the mammary epithelium is, therefore, not necessarily due to increased mammary pressure. Other factors are likely to be involved in maintaining the integrity of the mammary epithelium. It could be that some factors even the same factors as involved in controlling apoptosis could affect permeability or maybe inactive secretory cells or individual cells that undergo apoptosis fail to maintain the structure of the junctional complexes connecting neighbouring cells. In this case the mammary epithelium should become leakier as lactation progresses which is indeed the case (Baldi *et al.*, 1996).

It is possible that intramammary pressure contributes to the reduction in milk secretion and increase in mammary apoptosis during once daily milking. However, it is unlikely that the increase observed in milk secretion during trice daily milking compared to twice daily

milking is due to a reduction in intramammary pressure simply because of the time scale involved in these changes. Accumulation of local factors and changes in the gland sensitivity to hormones is much more likely to play a role under these circumstances.

Apoptosis could be co-ordinated at an alveolar level through localised milk stasis. Molenaar *et al.*, 1992 found that the pattern of gene protein expression was different between adjacent alveoli in sheep and bovine mammary tissue. The expression patterns of α-S1-casein were similar to those of α-lactalbumin. Lactoferrin, a putative marker of involution, was only expressed in cells which did not express α-lactalbumin. In another experiment Li *et al.* (1999) showed that whereas thrice daily milking maintains a uniform lactating histological appearance once daily milking results in regression of alveolar cells in selected alveoli. These observations do not appear to be due to a disruption in the integrity of the basement membrane, in fact in the ruminant there is no evidence that the basement membrane is involved in apoptosis (Holst *et al.*, 1987), in contrast to the rodent where the basement membrane is important for cell survival (Lund *et al.*, 1996). In mammary organ cultures it has been observed that myoepithelial cells do not respond uniformly to oxytocin stimulation a variation which might be due to the distribution of the oxytocin receptor on the myoepithelial cells (Moore *et al.*, 1987). This could allow only myoepithelial cells in secreting alveoli to undergo contraction thus other cells would accumulate milk.

1.6. Mammary tight junctions and their importance in determining milk quality

Tight junctions are the "gasket" which join neighbouring secretory epithelial cells. They form a barrier, preventing interdiffusion of milk constituents and plasma proteins.

Although total crude protein and casein increase in milk as lactation progresses the processing qualities of milk decrease (Klei *et al.*, 1997; Auldist *et al.*, 1995). Milk contains four different caseins α, β, κ and γ –casein. γ is the breakdown product of α and β casein and is unwanted in milk because it decreases the processing qualities of the milk. During cheese making γ-casein is lost in the whey fraction, which ultimately leads to loss of cheese yield. α and β casein are hydrolysed by the protease plasmin, while κ-casein seems to be resistant to proteolysis. Currently there is no evidence to support a local production of plasmin and its inactive pre-cursor plasminogen in the mammary gland. Some plasmin and all plasminogen must therefore be derived from blood. Evidence for paracellular transfer has been provided. Stelwagen *et al.* (1994b) found positive correlations between the changes in milk plasminogen and plasmin activity when milking frequency is switched from twice to once daily and changes in the concentrations of BSA in milk and lactose in plasma, all indicative of impairment of the mammary tight junctions. No data are available to support or refute a transcellular route. As lactation progress the levels of plasmin and plasminogen increases in milk (Politis *et al.*, 1989; Baldi *et al.*, 1996), this is partly due to accelerated conversion of plasminogen to plasmin but also to an increased permeability of the mammary epithelium (Baldi *et al.*, 1996). An increase in the permeability of the mammary epithelium does not only lead to increased hydrolysis of α- and β-casein but also to an increase in plasma proteins such as BSA in milk and a decrease in milk lactose (Stelwagen *et al.*, 1994b).

1.6.1. Effect of milking frequency on milk quality

Effects of milking frequency on milk composition have primarily been studied in relation to a reduction in milking frequency from twice daily to once daily. Once-daily milking decreases milk yield by approximately 13% in short term trials but losses of 35-50% have

been indicated in full lactation studies (Davis *et al.*, 1999). During once daily milking total protein increases. Of the serum proteins BSA (Stelwagen *et al.*, 1994b) and IgG increase (Davis *et al.*, 1999) as do proteolytic enzymes. The conversion of plasminogen to plasmin is also increased (Stelwagen *et al.*, 1994b), thus an increase in the proteolysis of α-, β-casein to γ-casein would be expected. A small increase in the concentration of casein has been reported, thus as milk volume is lower, casein becomes more concentrated in milk. However the casein/whey protein ratio is reduced by about 10% (Davis *et al.*, 1999). The concentration of lactose, which is only synthesised within the mammary gland, increases in blood and decreases in milk. Sodium increases while the concentration of potassium decreases (Stelwagen *et al.*, 1994b). These changes are typical of those observed with increased permeability of mammary tight junctions (Stelwagen *et al.*, 1994b)

It is quiet clear that once daily milking has a negative effect on the processing qualities of milk and that this effect in part is due to an increase in the permeability of the mammary epithelium. Another factor to take into consideration is the time that milk is stored within the udder. Longer storage time would allow greater conversion of plasminogen to plasmin. The ratio of plasminogen to plasmin is often used as an indicator of conversion of plasminogen to plasmin. This ratio is independent of milk volume (Politis *et al.*, 1989). This ratio decreases with once daily milking indicating an increase in the conversion of plasminogen to plasmin (Stelwagen *et al.*, 1994b).

Studies investigating the effect of more frequent milking on milk composition have primarily focused on fat percentage (Poole, 1982; Amos *et al.*, 1983), this is not really surprising since fat until fairly recently was the most valuable milk component, but 3x milking has no significant effect on milk fat percentage (Gisi *et al.*, 1986; Poole, 1982).

One report (Klei *et al.*, 1997) considers the long-term effect of thrice daily milking on milk proteins. Crude protein percentage was significantly decreased in milk from 3x milked udders compared to 2x milked and with casein protein percentage being unchanged this resulted in a significant increase in casein as a percentage of crude protein (casein number). The authors of this paper argue that the difference, which they observed in casein number, was entirely due to the shorter storage time of milk with plasmin in the udder, hence lesser time for proteolysis when milking frequency, is increased. This hypothesis ignores any possible contributions from developmental changes that might have occurred within the udder caused by the frequent milking. Research is needed to identify the status of the mammary tight junctions during thrice daily milking combined with more detailed information on protein composition.

1.7. Effects of nutrition and nutrient partitioning on milk yield

Although milk yield is a function of the number and activity of mammary secretory cells, the ability of the animal to supply these cells with nutrients once lactation is established is a major determinant of milk production. However, the effect of different feeding levels on lactation persistency has received relatively little attention, since lactation persistency has very much been regarded as a genetic trait (Broster & Broster, 1984). Broster & Broster (1984) found that the response to supplementary feeding was proportional to current yield rather than time post calving and once the response was established lactation persistency was similar in control cows. In the study of Smith *et al.* (1978) cows were fed a high energy ration, medium energy ration or a low energy ration for an entire lactation. Dry matter intake was similar for all groups. Peak milk yield was 3 kg higher for cows consuming the high energy ration compared to the medium and low energy diets, and although weight loss was greater on the medium and low energy diets, mobilisation of

energy stores was not sufficient to meet milk yield potential. Lactation persistency after peak lactation was similar for the low and high energy levels, while persistency was significantly improved on the medium energy ration until week 20 of lactation. At this stage milk yield was similar on the high and medium energy levels and lactation persistency did not differ thereafter. It is difficult from this type of experiment to interpret whether mammary development is affected by moderate under feeding in early lactation. In the following discussion it will become evident that the mammary gland is not milking at it's full potential. It is possible that moderately underfed animals have lost some of their mammary capacity and are more fully utilising what capacity they do have compared to well fed animals. However, severe underfeeding during early lactation does affect subsequent yield and lactation persistency (Schmidt, 1971), indicating that in this situation mammary development is suppressed.

In lactating rats deleterious effects of undernutrition and of restricted protein supply and positive effects of increased energy supply on cell proliferation have been identified (Grimble & Mansary, 1981). In the ruminant we are not aware of any published data investigating the effect of nutrition on developmental changes in the mammary gland during lactation. In a study performed at the Danish agricultural research centre we looked at the effect of diet energy concentration of a total mixed ration on mammary development during the first 8 weeks of lactation (Sorensen *et al.*, 2000). Milk yield was 22% higher in cows fed a high energy diet compared to cows on a low energy diet. This increase was associated with increased proliferation of mammary cells as measured by the proportion of cells staining positive for proliferating cell nuclear antigen, while no difference was found in the proportion of cells undergoing apoptosis. The activity of key enzymes involved in milk synthesis was also higher in mammary tissue from cows on the high energy diet. This

study indicates that diet energy levels are important in mammary development at least during early lactation. However, more studies are necessary to identify the short and long term effects of feeding levels on developmental changes in the mammary gland. The mechanisms involved are not known. Chronic undernutrition has been shown to decrease milk yield but increase circulating levels of GH and decrease IGFBP3. GH treatment to underfed animals fails to stimulate the mammary use of nutrients and milk yield, however, the direct effects of GH on liver and glucose metabolism is still evident (Bauman, 1999).

1.7.1. *Partitioning of nutrients*

Nutrient supply to the mammary gland is under hormonal regulation. GH (also known as BST) and insulin are the most important factors in regulating the partitioning of nutrients between different tissues, including the mammary gland.

Administration of BST increases gluconeogenesis in the liver and reduces glucose oxidation by body tissues resulting in an increased availability of glucose for milk synthesis. Lipid metabolism is also affected by BST treatment. Fat metabolism is stimulated while lipogenesis is inhibited (Bauman, 1999). Insulin, on the other hand, is the hormone promoting lipogenesis, inhibiting lypolysis, stimulating protein synthesis in adipose and stimulating glucose uptake and protein synthesis in muscle. BST treatment has been shown to decrease insulin's ability to stimulate lipogenesis but does not affect any of the others (Bauman, 1999).

Initiation of lactation induces marked changes in the partitioning of nutrients between mammary and non-mammary tissues. These changes are associated with an increase in circulating levels of GH and a decrease in insulin. Hypoinsulinaemia and insulin resistance

of adipocytes in early lactation are also common features (Vernon & Pond, 1997). These changes all support nutrient supply to the mammary tissue and away from body tissue. However, as lactation progress nutrient partitioning to the mammary gland decreases, which is reflected in the decrease of the GH to insulin ratio. Exactly what regulates these changes in nutrient partitioning between mammary gland and body tissues is unknown.

The priority in the metabolism of nutrient towards mammary tissue during early lactation is so great that mobilisation of body stores in high yielding cows is energetically equivalent to about one third of the total milk production (Beever *et al.*, 1998). As lactation progresses milk production decreases and cows start to replenish body stores, as food intake is no longer a limiting factor to milk production (Chillard, 1992).

The cow has sometimes been envisioned as a metabolic appendage to the udder rather than vice versa (Bauman & Currie., 1980). We do not believe this to be true. During early lactation we investigated whether the metabolic restriction point determining maximum milk yield output was determined at the level of the mammary gland itself or elsewhere within the body during early lactation in both high and average genetic merit cows (Sorensen & Knight, 1999). Maximum milk yield was achieved by 5 day treatment periods of 4x milking, 4x milking and BST and finally 4x milking BST and thyroxine. The total increment in milk yield was 9.02±0.60 kg/d in the average genetic merit and 8.17±2.3 in the high genetic merit cows. To determine the metabolic restriction point milking frequency was then reduced in half of the udder to 2x daily. A decrease in milking frequency reduced milk yield by 2.6±0.61 and 2.1±0.50kg/d in high and average genetic merit cows, respectively. However, the udder halves which continued on 4x daily milking increased milk production by 1.8±0.56 and 2.5±0.17kg/d for the two groups. This

compensatory increase shows that it is not the capacity of the mammary gland but rather the cow's capacity to supply substrate that ultimately limits milk production.

There are other examples in the literature indicating that the mammary gland has spare capacity. In late lactation once daily milking reduces milk production, induces apoptosis and reduces activity of key enzymes involved in milk synthesis (Wilde & Knight, 1990; Li *et al.*, 1999). When twice daily milking is resumed milk yield recovers completely although activities of key enzymes do not recover to the same extent (Wilde & Knight, 1990). Although the enzyme activities and metabolic fluxes measured are indicators of synthetic potential rather than actual synthetic rates these data indicate that the gland has a potential which is not used. Administration of BST increases milk yield immediately, due to an increased amount of nutrient reaching the mammary gland (Bauman, 1992). It is unlikely that a developmental response would happen that quickly, again indicating that the mammary gland is not utilising it's full potential. Long term treatment with BST increases milk production and induces changes in mammary development (Knight *et al.*, 1990b), however, once treatment is stopped milk yield drops to control values (Bauman, 1992). Although the mammary gland has a capacity for higher milk production it is not able to utilise it without the changes that occur in nutrient partitioning with BST treatment.

None of these experiments indicate why the capacity of the mammary gland is not fully utilised. A possible explanation could be a limitation in the nutrients available for milk synthesis, either through inadequate food intake or insufficient nutrient processing as for example the formation of glucose in the liver. Food intake seems to be an unlikely explanation for animals in late lactation. Supply or uptake of nutrient by the mammary gland could also be the explanation. However, one thing is clear, the capacity of the

mammary gland is not fully utilised during lactation. The metabolic restriction point determining milk yield must therefore be determined at the level of the whole animal.

1.8. Effect of nutrition on milk composition

It is well established that increased energy intake increases total crude protein in milk (Bartsch *et al.*, 1979; Sutton & Morant, 1989). Whether it also changes the proportions of the individual proteins in milk is unclear. Yousef *et al.* (1969) found an increase in α_{s1}-casein and β-lactoglobulin while serum albumin decreased with increased concentrate feeding while Bartsch *et al.* (1979) found no difference in protein composition as concentrate levels was increased. When increasing concentrate levels the proportion of propionate in the rumen in relation to other volatile fatty acids increases. The principal precursor for gluconeogenesis in the liver is propionate and one hypothesis is that the increased amount of glucose increases lactose synthesis and since lactose is the main osmolar component of milk this then increases milk volume. The increase in milk protein could be explained by a greater amount of amino acid being available for protein synthesis in the mammary gland as propionate becomes the main substrate for glucose production (Sutton & Morant., 1989). However, infusion of glucose, propionate or casein into the abomasum does not increase milk protein content.

Energy balance is a major player in milk protein content. Positive energy balance is usually associated with high protein content in milk. This led to the belief that insulin might be important in determining protein content of milk. However intravenous infusion of insulin did not increase milk protein content (D. Chamberlain, personal communication). Griinari *et al.* (1997) postulated that this was due to failure in keeping the glucose levels high during insulin and aminoacid infusion, and demonstrated that when glucose levels were

maintained milk protein content did increase. However, to maintain glucose levels 3kg of glucose had to be infused. It is highly unlikely that an animal will ever reach this level of glucose in the blood.

The biological mechanism responsible for the increase in milk protein content remains unexplained. However, a delicate balance between forage and concentrate is important. Propionate also stimulates insulin secretion and insulin favours body tissue deposition at the expense of milk yield and milk fat (Sutton & Morant, 1989).

1.9. Effect of season on milk yield

Seasonal variation in milk yield does occur. In a comparison of dairy cows kept indoors all year round fed on grass silage and concentrate with animals which were allowed out on grass during the summer month, Wood (1972) found no seasonal variation in milk yield in the indoor group. However in animals which were allowed out on grass there was a tendency for milk production to increase when grass quality was good. This could be due to an increase in photoperiod which has been shown to increase milk yield (Tucker, 1985). However, it could also be associated with a change in diet or a combination of both. Fresh grass is of better nutritional value than grass silage and an increase in milk production would therefore be expected. On the other hand a winter ration with maize silage as the main forage results in decreases in milk yield when cows are turned out to grass (R. Phipps, personal communication). Dietary changes, therefore, override the photoperiodic effects on milk yield.

In an experiment where cows were exposed to photoperiods of 16h of light and 8h of dark compared to cows subjected to natural photoperiod (9-12h/day of light) milk yield was 10% higher during the first 100 days of lactation. When treatments were reversed between

the 2 groups milk yield dropped in the previous treated cows while milk yield increased in the now supplemented cows. Furthermore, lactation persistency was significantly improved in the now treated animals (Tucker, 1985). However, when cows were exposed to the same light regime as in the above experiment during early or late lactation no difference was found in lactation persistency between treated and controls although milk yield was increased.

The physiological explanation for the galactopoietic effect of photoperiod on milk yield is unknown. Although food intake is increased during extended photoperiod this effect seems to be a response rather than a cause of the increase in milk production (Tucker, 1985). Concentration of insulin, thyroxine, GH and glucocorticoids are not affected by photoperiod, however, prolactin secretion is altered (Peters & Tucker, 1978). Plasma concentrations of prolactin are highest during summer and lowest during winter and the concentration is increased by extended photoperiods. Whether or not prolactin is involved in photoperiod induced increments of milk yield remains uncertain. As previously discussed there is no clear evidence that ruminant milk yield is responsive to prolactin. A recent study investigated the effect of photoperiod on the circulating levels of IGF-1 and IGFBP-2 and 3 (Dahl *et al.*, 1997). Circulating levels of IGF-1 were significantly increased during extended photoperiod while no change was found in either IGFBP-2 or IGFBP-3 or GH. An increase in circulating levels of IGF-1 does not increase milk production (Davis *et al.*, 1989) and it is therefore unlikely that the increase in milk yield with an increase in photoperiod is due to an increase in circulating levels of IGF-1.

Season of year has an effect on milk yield, although how much of the spring flush is nutritional and how much is due to day length which can influences the plasma levels of hormones such as prolactin and IGF-1 remains to be resolved.

1.10. Aims of the thesis

The aim of this thesis was to investigate the biological control of lactation persistency with the objective of achieving extended lactation in dairy cows. Factors investigated were milking frequency, nutrition and calving season. Milk yield, the change in milk yield over time (lactation persistency), milk composition and milk processing quality were the major variables determined. Additional data on fertility parameters and endocrine profiles are also presented and the mechanism underlying the effects of frequent milking on milk quality was determined. All the work presented was undertaken in dairy cows undergoing extended lactation cycles of a nominal eighteen-month duration. Studies of mammary cell turnover during lactation rescue in mice, which were undertaken during the course of the cow work, are not presented but published abstracts of this work are included as annexes.

Chapter two

Materials and methods

2.1. Introduction

This chapter describes the methods used in the experiments shown in this thesis. The overall design of the extended lactation experiment which is used in chapter 3, 4, 5, 6 and 7 will also be presented in this chapter although details about timing of sampling collection is described in the individual chapters. All chemical used, unless otherwise stated, were from Sigma Chemical Company Ltd, Poole, UK.

2.2. Experimental design

Twenty-four cows were used in a factorial design to study the effects of milking frequency, calving season and nutrition on lactation persistency during extended lactation. Twelve of the cows calved during spring 1996 (24/3-1996 to 23/5-1996) and the other twelve calved during autumn 1996 (14/10-1996 to 10/12-1996). Parity ranged from 1-3 in the spring calving group and 1-5 in the autumn calving group.

All cows were fed a grass silage-based total mixed ration (winter) or grazed pasture (summer) supplemented with in parlour concentrate containing 18% protein. When pasture quality declined the animals were buffer fed with sugar beet pulp. To asses the effect of nutrition on lactation persistency half of the spring calvers and half of the autumn calvers were managed on a high nutritional input system while the remaining cows were fed to milk production (conventional input). The cows on the high nutritional input received an additional 3 kg/d of in parlour concentrate.

The effects of milking frequency are mediated locally within individual mammary glands and a half udder approach was therefore adopted to study the effect of milking frequency

on lactation persistency. Half of the udder, diagonally-opposed udder quarters, of every cow was milked thrice daily (milking interval 8:8:8) and the other half twice daily (milking interval 8:16). All treatments were initiated during lactation week 9.5±0.64 (standardised lactation week 9), so around peak lactation.

Rebreeding commenced around lactation week 32, all cows in heat being served, animals which had not been observed in heat before standardised lactation week 36 were synchronised using standard regimes as advised by veterinarian.

Following second calving all the cows in the spring calving group except one cow which did not conceive until 70 weeks into her lactation were managed for another eighteen-month lactation. Each individual cow received the exact same treatment as during her first extended lactation. Calving dates spread from 16/9-1997 to 27/1-1998 and animals were therefore not blocked but submitted to treatment individually as they reached lactation week 9.

In the autumn calving group all cows except for four cows that for different reasons were culled (see chapter 3 for further details) were submitted to a conventional 12 month lactation. The nutritional treatment was not applied during this lactation and all cows were fed as for the conventional fed animals during the extended lactation. Due to the spread in calving dates from 1/5-1998 to 22/11-1998 the frequency treatment was initiated for individual animals at lactation week 9±0.0 on a half udder basis as for the previous lactation. For all of the lactations studied we were aiming for a 60 day dry period.

2.3. Blood sampling

Blood samples were collected by tail vein puncture into heparinised vacutainer tubes (Becton Dickinson, Vacutainer Systems Europe, Meylan-Cedix, France). Shortly after the collection samples were centrifuged (1800g, 15min, 4°C) and plasma gathered and stored at -20°C until further analysis.

2.4. Hormone determination

Double-antibody radioimmunoassay (RIA) was used to determine plasma concentrations of prolactin, GH and insulin as described by Vernon *et al.* (1981).

2.4.1. Growth hormone RIA

Growth hormone standard (10μg/ml) was prepared from ovine GH (AFP-9220A, donated by the National Institute of Health, Bethesda, Maryland, USA (NIH)). Standard curves were then prepared by serial dilution with RIA buffer (0.05M sodium phosphate, 0.15M sodium chloride, 0.02% sodium azide, pH 7.4, .5% w/v bovine serum albumin) at concentrations ranging from 0.31-40ng/ml. Antiserum to ovine GH (AFP-CO123080) was provided by NIH and diluted in RIA buffer for a dilution of 1:15000. Before addition of tracer samples or standard were incubated with first antibody for a minimum of 7 hours. To get an average total binding of 30% a tracer activity of around 10.000 cpm ^{125}I-0GH was added. After an overnight incubation the second antibody was added. The second antibody was prepared by adding an equal amount of polyethylene glycol (16% PEG, BDH, Thornliebank, Glasgow, UK) to RIA buffer then adding 0.83% v/v anti-rabbit (donkey) precipitating serum and 0.03% v/v normal rabbit serum both donated by the Scottish Antibody Production Unit (SAPU), Carluke, Lanarkshire, UK. After 2-4 hours of incubation at room temperature samples were centrifuged (3000g, 30min, room

temperature) and the resulting supernatant discharges. Samples were counted on a gamma counter (Packard, Meriden, USA). All samples from each individual cow were analysed within one assay and intra-assay coefficient of variation was 14.2% while inter-assay coefficient of variation was 15.5%.

2.4.2. Prolactin RIA

The same procedure as for growth hormone was followed. The standard curve (3.12-200ng/ml) was prepared by ovine prolactin (AFP-9221A), antiserum to ovine prolactin was used in a 1:60-80,000 (donated by NIH) and the tracer ^{125}I-oprolactin had an activity around 30,000 cpm. Intra-assay coefficient of variation was 12.3% and inter-assay coefficient of variation was 15.3%.

2.4.3. Insulin RIA

The standard curve (0.08-5.0ng/ml) was prepared by using bovine insulin (Sigma, I-550). Anti porcine insulin (donated by SAPU) was used in a 1:20-40,000 dilution and tracer ^{125}I-binsulin with an activity around 10,000 cpm was used. The second antibody consisted of equal amounts of RIA buffer and 16% PEG, pH 7.4, 0.50% v/v anti-guinea pig serum and 0.03% v/v normal guinea pig serum (gift from SAPU). The assay was performed as for prolactin and GH. Intra-assay coefficient of variation was 10.6% and inter-assay coefficient of variation was 12.2%.

2.4.4. Insulin-like growth factor I RIA

The method described by Flint & Gardner (1989) was used to determine IGF-1 concentration in the plasma. Before assaying samples for IGF-1 concentration IGF-1 was

separated from its binding proteins by acid ethanol extraction. Samples and standards were incubated for 30 min at room temperature with 4 volumes of 2N HCL and ethanol (1:7 v/v). Tubes were centrifuged (3000g, 10min) and a known amount of supernatant was removed and diluted with an equal amount of neutralising buffer (4% w/v TRIS: RIA buffer). The standard curve (10-2500ng/ml) was prepared using recombinant human IGF-I (Bachem, Saffren Walden, Essex, UK). The first antibody (polyclonal rabbit anti rhIGF-I (donated by NIDDK) was used at a 1:2000 dilution. The tracer added ^{125}I-IGF-1 contained around 20,000cpm. The second antibody consisted of equals amounts of RIA buffer and 16% PEG and 6% v/v anti-rabbit IgG precipitating serum and 0.4% v/v normal rabbit serum (SAPU). The assay was performed as for GH, prolactin and insulin. Intra-assay coefficient of variation was 11.2% and inter-assay coefficient of variation was 15.3%.

2.4.5. Progesterone RIA

Plasma concentrations of progesterone were determined as described by Corrie *et al.* (1981). The standard curve (7.8-2000 pg/ml) was prepared by using progesterone available from Sigma (P-0130). Anti rabbit progesterone was used in a 1:16.000 dilution and tracer ^{125}I-progesterone with an activity around 12.000-15.000 cpm was used. Label and first antibody were added to sample or standard and incubated for 3h at RT before addition of second antibody. The second antibody consisted of donkey anti-rabbit IgG precipitating serum (SAPU) diluted 1:35 in 0.1% phosphate gelatin buffer containing 0.15M NaCl and 0.1% thimerosal and 1:300 normal rabbit serum, pH 7.4. The reaction tubes were then incubated overnight at 4°C, washed in phosphate gelatin buffer before centrifugation at 3000g 4°C for 30 min. and the supernatant was discharged before samples were counted on a gamma counter. Intra-assay coefficient of variation was 9.3%

Chapter three

Lactation characteristics during extended lactation

3.1. Introduction

In this chapter the results on fertility parameters and general production characteristics such as lactation length and days dry during extended lactation are discussed.

Involuntary annual culling rate in the UK is around 24% with the biggest culling reason being reproductive problems, which account for as much as 36.5% of the culling that occurs (Kossaibati & Esslemont, 1995). Fertility problems have often been associated with high milk yield and in particular with the metabolic imbalance that is observed with high production levels. Domecq *et al.* (1997) found in a herd of 720 cows that cows that lost 0.4 point in body condition score during the first month of lactation were 1.17 times less likely to conceive at first insemination than were cows that did not lose body condition score during the first month of lactation. The current recommended calving interval of around 12 month requires cows to be rebred at around 50-60 days post partum which is the time in the lactation cycle where cows are exposed to the highest degree of negative energy balance. During early lactation milk production has a very high priority in the metabolism of the cow and in high yielding dairy cows mobilisation of body reserves has been shown to be energetically equivalent to about one third of the total milk produced (Beever *et al.*, 1998). It is likely that milk production takes priority during early lactation at the expense of reproductive function.

One way of solving this problem could be to extend lactation. Extending lactation to 18 month would involve rebreeding at around 9 month of lactation, by which time, cows

would presumably be back in positive energy balance and reproductive function might be better than during early lactation. For extended lactation to be feasible rebreeding at around 9 month into lactation must be, at worst, no more difficult than at 2-3 month of lactation.

3.2. Material and methods

3.2.1. Experimental design

The design of the experiment is given in chapter 2.

Health and reproductive observations were recorded using the Daisy system. With the exception of one cow that was treated successfully for endometritis shortly after calving no parturient abnormalities were apparent in the spring calving group. The autumn calving cows were all bought from other dairy farms. None of these cows calved at the Hannah and their reproductive history is unknown.

Signs of oestrus were detected visually by experienced dairymen and animals were allowed to be served at the first oestrus observed after 264±6day *post partum* during the first extended lactation. During the second extended lactation rebreeding commenced at the first oestrus observed after 240day *post partum*. During the conventional lactation cycle animals were synchronised according to standard synchronising regimes as advised by a veterinarian if not observed in heat around 60 days *post partum*. A veterinarian examined cows that did not show any sign of oestrus within a month after rebreeding was allowed. As advised by the veterinarian animals were then synchronised using standard regimes. Pregnancy was diagnosed by ultrasonography approximately 5 weeks following insemination.

3.2.2. Blood sampling for progesterone analysis

To determine ovarian function during early lactation and at the actual time of rebreeding blood samples were collected three times weekly for one month and analysed for progesterone. In early lactation blood sampling commenced around 55day *post partum*. No significant difference existed between the different groups for day of first sampling which had an overall mean of 58±3.4d. The blood sampling was repeated approximately 28 weeks later at 247±3.0d in the spring calvers and at 272±2.2d in the autumn calvers which was significantly later ($P<0.001$). Blood samples for progesterone analysis were only collected during the first extended lactation. Progesterone was measured by radioimmunoassay as described in chapter 2.

3.2.3. Statistical analysis

To evaluate the effect of nutrition, calving season and lactation number on fertility parameter analysis of variance (anova) was used. Differences in the calculated parameters between two consecutive lactations were compared within cow, while the seasonal comparison was between cows. All data was analysed using Genstat (Genstat 5, 1997). All values are reported as mean±s.e unless otherwise stated.

3.3. Results

3.3.1. *Fertility parameters*

Reproductive data comparing spring and autumn calvers on either high or conventional nutritional input during extended lactation are shown in table 3.1. The seasonal difference that was observed in days to first oestrus is almost certainly a recording error. Progesterone sampling during early lactation indicated that all but 2 cows were cycling between 60 and 80d *post partum*. No further analysis of heat observations was conducted, therefore. Days

to first service differed between spring and autumn calvers however when the actual days from when rebreeding was allowed was taken into account the seasonal difference was no longer evident. In the spring calving group all 12 cows conceived while only 10 out of the 12 autumn calving cows conceived. Although the number of inseminations per cow was higher in the autumn calving group than the spring calving this difference was not statistically significant. Nutrition had no effect on the number of inseminations per cow. Insemination per pregnancy was much higher in the autumn calving group on high nutrition simply due to the 2 animals, which failed to conceive in this group. Conception rate to first insemination, overall conception rate and the interval from first service to conception was highest in the spring calvers on high nutritional input. In the conventional input group no cows held to the first insemination in the spring calving group, however overall conception rate was better than in any of the autumn calving groups. A significant effect of nutrition was found on the interval from first service to conception, which was much lower in the high nutritional input groups compared to the conventional input groups. In the autumn calving group on high nutritional input this interval is based on only 4 animals since 2 failed to conceive. No significant difference was found in veterinary interventions between any of the groups. The two cows which failed to conceive were examined by a veterinarian 7 and 8 times respectively which explains the high number of veterinary interventions per cow in the autumn calving group on high nutritional input. The target calving interval was significantly different for the autumn and spring calving group. The actual calving interval was longer than the target interval for all 4 treatment groups and was not affected by either season or nutritional input.

Milk yield at the start of rebreeding was not affected by calving season but by nutritional input. The high nutritional input cows were producing more milk at this stage compared to

the cows on conventional input. No correlation was evident between conception interval and milk yield.

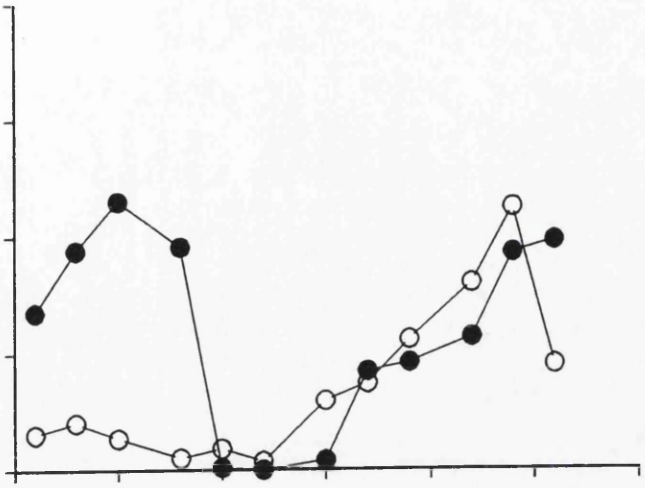
Body condition at the start of the rebreeding period was 2.67±0.31 and 3.17±0.51 in the high and conventional nutritional input groups respectively in the spring calving group. In the autumn calving group body condition was 2.08±0.24 and 1.92±0.27 for the high and conventional input groups, respectively. Within each group and overall, body condition and conception interval were not correlated.

Examples of blood progesterone profiles determined at peak lactation and in late lactation (just prior to or during rebreeding) are shown for 4 cows in figure 3.1. In the majority of cows regular cycles of around 21d duration were detected both during early and late lactation. Only 2 of the cows were not cycling during the early lactation period (figure 3.1d) and 2 others had presumably just started or appeared not to have regular cycles (figure 3.1b). Visual examination of peak height and area under curve of the profiles for all animals indicated no differences between early and late lactation, high or low nutritional input and spring or autumn calvers.

Table 3.1. Comparison of selected fertility parameters between spring and autumn calving cows on either high or conventional nutritional input. Nutritional treatment was conventional input (conv), cows fed to milk production, or high nutritional input (high), cows supplemented with 3 kg of in parlour concentrate. The nutritional treatment was initiated at lactation week 9.5±0.64. Data were analysed by anova.

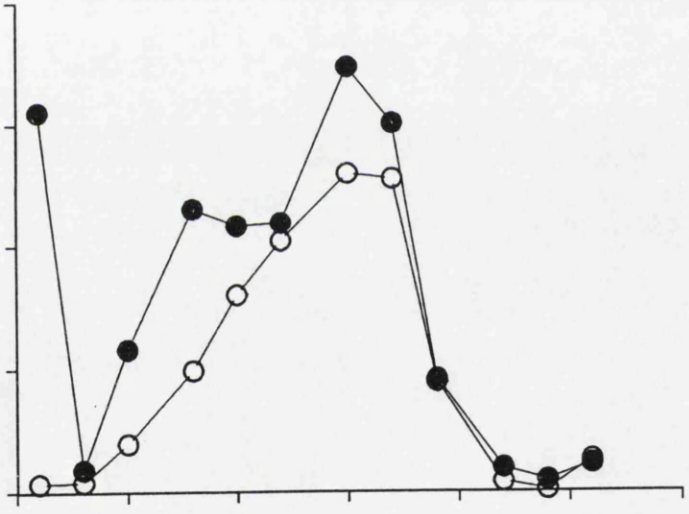
	Spring		Autumn		Nutrition	Season	N *S
Nutritional treatment	High	Conv	High	Conv			
N	6	6	6	6			
Days to 1'st oestrus	103.3± 23.4	89.6± 19.6	208.2± 25.8	144.7± 25.9	n.s	0.003	n.s
Days to 1'st service	273.5± 8.84	280.3± 14.5	328.0± 20.7	289.8± 4.98	n.s	0.029	n.s
Yield at start of breeding	18.6± 0.7	14.4± 1.0	16.5± 1.3	15.0± 1.1	0.013	n.s	n.s
No. conceiving	6/6	6/6	4/6	6/6			
Insemination/cow	1.7± 0.3	2.2± 0.2	2.5± 0.6	2.7± 0.5	n.s	n.s	n.s
Insemination/pregnancy	1.7	2.2	3.8	2.7			
Conception rate to 1'st insemination (%)	50	0	16.7	16.7			
Conception rate overall (%)	60.0	46.0	26.7	37.5			
1'st service to conception (days)	25.2± 12.4	63.8± 23.5	29.3± 11.5	74.5± 23.1	0.05	n.s	n.s
Veterinary intervention /cow	0.33± 0.21	0.83± 0.65	2.83± 1.51	1.0± 0.52	n.s	n.s	n.s
Target calving interval (days)	540± 9.9	543± 8.7	588± 9.4	581± 7.6	n.s	<0.001	n.s
Actual calving interval (days)	588± 17.7	621± 34.7	623± 24.6	648± 25.0	n.s	n.s	n.s
Days lost	43.3± 18.7	81.0± 27.7	35.0± 30.1	58.7± 26.9	n.s	n.s	n.s

a



o-early lactation
●-late lactation

b



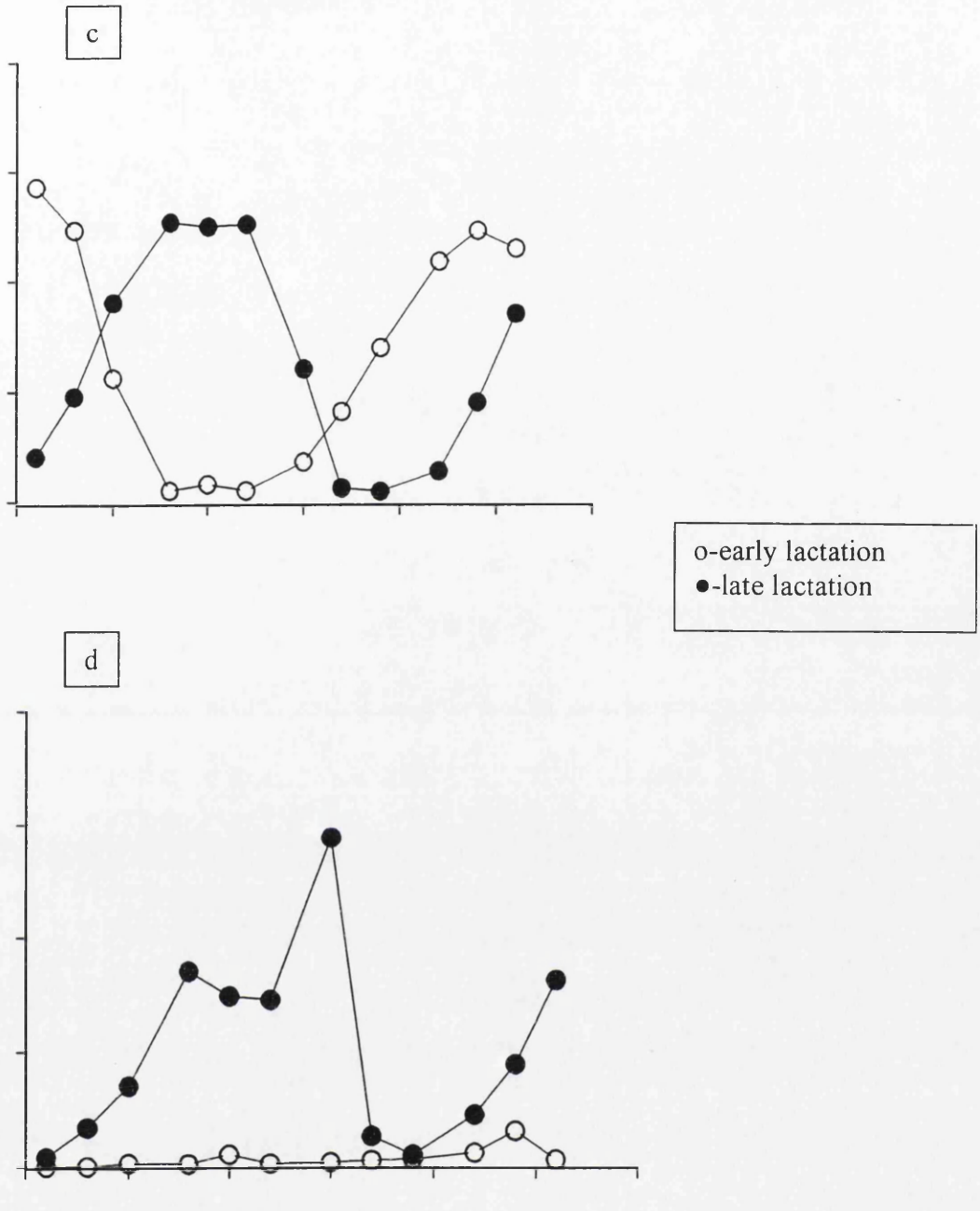


Figure 3.1. Examples of progesterone profiles for extended lactation cows. Axes are unlabelled for clarity; ordinate is 0 to 20ng/ml progesterone and abscissa is 0-30d for each. Open symbol are early lactation sampling period, closed symbols are late lactation.

To compare fertility parameters for 2 consecutive extended lactations fertility parameters were calculated and compared for the original spring calvers, which completed two extended lactations. Results are shown in table 3.2.

Although all the spring calving cows (see table 3.1) conceived during their first extended lactation one cow did not conceive until 70 weeks into her lactation. This cow was not submitted to a second extended lactation simply because her calving date was much later than the rest of the group. For statistical analysis data from this one cow were excluded from both lactations and therefore only 5 cows were present in the conventional nutritional input group.

Days to first service were significantly lower during the second extended lactation compared to the first lactation. When the actual target for calving interval was used as a covariate in the anova this difference still existed. All cows conceived during the first lactation but during the second lactation one cow in the high nutritional input group failed to conceive. Inseminations per cow did not significantly differ between any of the groups. However this was strongly influenced by one cow which during her second lactation had 7 inseminations and still failed to conceive. During the first lactation this particular cow had one insemination to which she conceived. Conception rate to first insemination was higher during the second lactation compared to the first lactation. Overall only 3 cows out of 11 did not conceive at first insemination during the second lactation while in the first lactation only 3 out of 11 cows conceived at their first insemination. None of the cows in the conventional input group held to first insemination during their first extended lactation however all of them held to their second insemination. Conception rate overall was not much different between the nutritional groups and number of extended lactations. Nutrition

had no effect on the interval from first service to conception however this interval was significant lower during the second lactation. No difference was found in the number of veterinary interventions per cow between the two lactations and nutritional input. The target calving interval was held constant and actual calving interval was significantly closer to the target during the second lactation compared to the first lactation. Nutrition had no effect.

Fertility parameters for autumn calvers undergoing an extended lactation followed by a 12 month lactation cycle are shown in table 3.3. As described earlier two animals failed two conceive during their first extended lactation. These animals were culled and obviously did not proceed into a conventional lactation. Another two animals were culled, one due to a leg injury and the other because of lameness. Therefore only 8 animals were submitted to the following 12 month lactation. All animals were fed as for the conventional input cows on extended lactation.

Number of days to first oestrus was not calculated for any of the animals. During the conventional lactation cycle animals were synchronised according to standard synchronising regimes as advised by a veterinarian if not observed in heat around 60 days of lactation. 4 out of 8 animals had been observed in oestrus at this stage, the other 4 cows were synchronised. Calculating days to first oestrus therefore made no sense. As a direct consequence of the experimental protocol days to first service was significantly different between the two groups which also meant that milk yield was considerably lower for the extended lactation cows at the start of rebreeding. Only 7 out of the 8 cows conceived during the following 12 month lactation (2'nd conventional). Inseminations per cow was only 1.4±0.3 in the 12 month lactation cycle compared to 2.4±0.4 in the extended lactation

cycle and conception rate to first service was as high as 75% during the 12 month cycle compared to only 25% in the extended lactation cycle. Due to the failure of one cow to conceive overall conception rate did not differ between the two groups. However days from first service to conception was significantly lower on 12 month cycles compared to extended cycles. Actual calving interval was 50±24.6 days longer than the target calving interval in the extended lactation group and 10±13.7 days shorter in cows on 12 month lactation cycles. Milk yield at the beginning of the rebreeding period was inversely correlated with conception interval (-0.90, $P<0.001$), however within each group milk yield and conception interval were not correlated.

Table 3.2. Comparison of selected fertility parameters between cows undergoing two consecutive extended lactations on either high or conventional nutritional input. Treatment and analysis details as for figure 3.1. The nutritional treatment was initiated at lactation week 9.3±0.49.

	1'st Extended lactation		2'nd Extended lactation		P Nutrition	P lactation	P Nutrition *lactation
Nutritional treatment	High input	Conventional input	High input	Conventional input			
N	6	5	6	5			
Days to 1'st oestrus	103.3± 23.4	96.6± 22.5	31.2± 64.7	109.6± 25.9	n.s	n.s	n.s
Days to 1'st service	273.5± 8.8	274.6± 16.4	237.3± 8.4	247.6± 14.1	n.s	0.016	n.s
Yield at start of breeding	18.6± 0.7	14.8± 1.2	26.6± 1.4	25.6± 1.0	0.04	<0.001	n.s
No. conceiving	6/6	5/5	5/6	5/5			
Insemination/cow	1.7± 0.3	2.0± 0.0	2.2± 1.0	1.4± 0.4	n.s	n.s	n.s
Insemination/pregnancy	1.7	2	2.6	1.4			
Conception rate to 1'st ins.(%)	50	0	67	80			
Conception rate overall (%)	60	50	38	71			
1'st service to conception (d)	25.2± 12.4	41.2± 7.8	8.4± 8.4	8.2± 8.2	n.s	0.029	n.s
Veterinary intervention /cow	0.33±	0.20±	0.50±	0.40±	n.s	n.s	n.s
Target calving interval	540± 9.9	540± 9.4	540± 0.0	540± 0.0	n.s	n.s	n.s
Actual calving interval	587± 17.7	593± 24.9	526± 18.4	536± 18.5	n.s	0.009	n.s
Days lost	47.7± 19.1	54.2± 18.2	-14.4± 18.4	-3.6± 18.5	n.s	0.005	n.s

Table 3.3. Comparison of selected fertility parameters between cows undergoing an extended lactation following a conventional 12 month lactation.

	1'st extended	2'nd conventional	P-value
N	8	8	
Days to 1'st service	296.3± 7.1	71.1± 12.0	<0.001
My at start of breeding	15.8± 1.3	34.8± 1.7	<0.001
No. conceiving	8/8	7/8	
Insemination/cow	2.4±0.4	1.4±0.3	0.03
Insemination/pregnancy	2.4	1.6	
Conception rate to 1'st insemination (%)	25	75	
Conception rate overall	42	55	
1'st service to conception	53.1± 18.3	5.6± 5.6	0.04
Veterinary intervention /cow	1.0± 0.42	0.63± 0.26	n.s
Target calving interval	579.6± 7.3	365± 0.0	<0.001
Actual calving interval	631± 21	355± 14	<0.001
Days lost	51± 25.1	-10± 13.7	0.04

3.3.2 Lactation length and days dry

Lactation length and number of days dry for the spring and autumn calving groups are shown in table 3.4. Lactation length includes all animals, even the ones who failed to conceive. Therefore number of days dry added to lactation length does not equal calving interval. Lactation length was significantly longer in the autumn calving group compared to the spring calving group. Lactation length for the two animals that failed to conceive was 571 and 590 days. The difference in lactation length was not only due to the autumn calving group having a slightly longer calving interval, but also a tendency for a shorter dry period than the spring calvers especially the animals on conventional input. Even when the cow who failed to conceive until 70 weeks into her lactation who had a dry period of 213days was excluded from the analysis the average days dry for the spring calvers on conventional input was still 109± 21.8. The number of days spent dry was also affected by nutrition so that the high nutritional input groups had less number of days dry than the conventional nutritional input groups. We were aiming for 60d dry period. Only one group, the autumn calvers on high nutritional input, full filled this criterion. All the other treatment groups had animals that were dried off due to a low milk yield before their actual due date. Table 3.5 shows number of animals out of total number of pregnant animals for each of the described lactations and treatment groups, which failed to complete their lactation due to low milk production.

Table 3.4. Effect of calving season, consecutive extended lactations and an extended lactation followed by a conventional 12 month lactation on lactation length (days) and days dry. Treatment and analysis details as for figure 3.1. The nutritional treatment was initiated at lactation week 9.5±0.64.

Season	Spring		Autumn		P-value		
Nutrition	High	Conventional	High	Conventional	Nutrition	Season	Nutrition * season
Lactation length	515±16	495±19	571±17	572±30	n.s	<0.01	n.s
Days dry	72±8	126±25	58±4	76±14	<0.05	0.07	n.s
	1'st extended lactation		2'nd extended lactation		Nutrition	Lactation	Nutrition * lactation
Lactation length	516±15.5	484±19.3	481±17.8	469±21.0	n.s	n.s	n.s
Days dry	72±7.5	109±21.8	57±2.2	68±6.5	0.08	<0.05	
	1'st extended lactation		2'nd conventional lactation				
Lactation length	559±23.7		303±15.5		<0.001		
Days dry	72±10.5		61±6.1		n.s		

Table 3.5. Number of animals out of total number of pregnant animals that failed to complete their lactation due to low milk yield. Average milk yield for animals dried off before due (incomplete) and animals, which completed their lactation, is indicated in brackets (incomplete/complete).

	High nutritional input	Conventional nutritional input
Spring	2/6 (2.9±1.0/8.4±1.5)	5/6 (2.5±0.31/14.3)
Autumn	0/4 (6.1±1.5)	2/6 (1.8±0.3/8.6±1.5)
1'st extended lactation (spring)	2/6 (2.9±1.0/8.41±1.5)	4/5 (2.3±0.3/14.3)
2'nd extended lactation	0/5 (8.6±0.93)	2/5 (2.5±0.6/5.8±1.4)
1'st extended lactation (autumn)	2/8 (1.8±0.3/8.0±1.4)	
Conventional lactation	0/8 (12±1.4)	

Calving interval (not significant) and lactation length ($P<0.015$) were longer in the autumn calving group than the spring calving group. However only 2 cows out of a total of 10 were dried off before their actual due date while in the spring calving group 7 out of 12 cows failed to complete their lactation. The majority of these cows were in the group on conventional nutritional input.

The length of the second extended lactation was no different from the length of the first extended lactation although calving interval was significantly longer during the first lactation. Number of days dry was therefore longer during the first compared to the second lactation (see table 3.4). During the second lactation 2 of the cows in the conventional input group failed to complete their lactation but none in the high nutritional input group failed (see table 3.5). These 2 cows were cows that also failed to complete their first

extended lactation. During the first lactation calving interval was 516 and 670 and in the second lactation calving interval was 580 and 492 for these two animals.

Lactation length was obviously significantly different between animals who had an extended lactation and thereafter a conventional 12 month lactation. However there was no difference in number of days dry between the 2 groups (table 3.3) although their were 2 cows (137 and 84 days dry) present in the extended lactation group which failed to complete their extended lactation (table 3.5).

3.4. Discussion

The chapter considers selected fertility parameters during a voluntarily increased calving interval of 18 month (extended lactation) and whether these were affected by feeding levels and calving season. To examine longer term consequences, fertility parameters were compared between animals that completed two consecutive extended lactations and animals which completed one extended lactation and thereafter a conventional 12 month lactation. The calculated fertility measures to assess reproductive efficiency were based on the recommendation of Fetrow *et al.* (1990) and Esslemont (1992).

The main objective of the experiment was to study the biological control of lactation persistency. Included in this chapter are measures of lactation and dry period length. It was evident from this study that not all animals were able to maintain an extended lactation. Some animals were basically drying themselves off more than 60d before their due date. This problem was significantly more pronounced in the conventional fed animals. Lactation and dry period length will be discussed further in chapter 4.

First service was allowed at the first oestrus observed 247±3.0 d post partum in the spring calving group and 272±2.2 days in the autumn calving group. The actual number of days to first service was 276±8.2 in the spring calving group and 309±11.7 in the autumn calving group, so slightly higher for both groups than planned. Progesterone samples which were collected at the same time as rebreeding was initiated indicated that all animals were cycling, however, in the spring calving group only half of the cows were recorded as showing heat during this period. The sampling period commenced in December and was stopped in January. When comparing heat observation and progesterone profiles it was evident that all animals which were in season over the Christmas holiday were the ones not observed in heat. However, in the autumn calving group again only 5 out of the 12 animals were observed in heat although progesterone profiles again showed that all animals were cycling. Days to first oestrus and number of heats were not analysed in this experiment since it was quite clear that the observations of heat were not done properly. In the UK heat detection has been around 55% for the last 25 years while in New Zealand heat detection rates are as high as 80-90% (Kossaibati & Esslemont, 1995). Obviously the UK farmer has not yet seen the necessity to change. Percentage of cows expressing normal heat signs increases up to the 4th ovulation post-calving and thereafter remains stable up to at least the 8th ovulation (Ratnayake *et al.*, 1998). It might be difficult to spot heat signs during the earlier stages of lactation, however, it should become easier as lactation progresses. Number of days to first service was significantly higher in the autumn compared to the spring calvers, however, when the actual number of days from when rebreeding was allowed was taken into account there was no difference between the two groups. During the second extended lactation days to first service was significantly lower than during the first lactation. This suggests that heat detection was more efficient during the second extended lactation.

Conception rate to first insemination ranged between 0 and 50% between the four treatment groups during the first extended lactation. Kossaibati & Esslemont (1995) studied fertility achievements in 90 dairy herds (total number of animals 13,680) in the UK. Average annual milk production in the herds was 6000 l/cow and all farms were aiming for 12 month lactation cycles. Conception rate to first insemination ranged from 25 to 72% with an average rate of 47.3%. During 18 month lactation cycles Ratnayake *et al.* (1998) found a conception rate to first insemination of 52.4%. In our study the conception rate to first insemination was, therefore, very poor. Overall conception rate to first insemination was 20.8% which was even lower than the lowest rate achieved in conventional dairy farms (Kossaibati & Esslemont, 1995). In our own comparison between animals completing an 18 month lactation cycle and thereafter a 12 month lactation cycle conception rate to first insemination was 25% during the extended lactation but 75% during the conventional lactation. It is unlikely that this differences reflects any significant differences in fertility between 12 and 18 month lactation cycles since conception rate to first insemination increased from 25% during the first extended lactation to 73.5% during the second extended lactation.

Overall conception rate in this study was closer to the 46% observed on commercial farms during 12 month lactation cycles and in most scenarios smaller than those 62.5% observed by Ratnayake *et al.* (1998) during 18 month lactation cycles. Overall conception rate is directly depend upon number of inseminations used per animal. At commercial farms 2.1 insemination per cow was needed (Kossaibati & Esslemont, 1995). During 18 month lactation cycles only 1.6 inseminations was used which was significantly lower than the between 2.2 and 3.3 inseminations per cow that was used in the 12 month control groups (Ratnayake *et al.*, 1998). Overall conception rate during our 12 month lactation cycle was

55% and number of inseminations per animal was only 1.4±0.3, however, in these animals previous extended lactation overall conception rate was 42% and number of inseminations used per animal was 2.4±0.4. This might not be due to animals being more fertile during the early part of lactation compared to the later part because during the second extended lactation overall conception rate was 71% and number of inseminations per animal was 1.4 in the conventional nutritional input group. Although in the supplemented animals overall conception rate was only 38% during the second compared to 60% during the first extended lactation; but this was simply because of one cow, which was served 7 times and still failed to conceive. This also explains the high number of inseminations per cow/ pregnancy in this group. During her first extended lactation this animal conceived after first service. Under normal farm management decisions this animal would have been abandoned long before the 7th insemination.

During the first extended lactation overall conception rates were particularly low in the autumn calving group (high: 26.7 and conventional: 37.5). In the high nutritional input group this was due both the fact that 2 cows failed to conceive and that one of these was served 5 times. Of 7 prebreeding examinations made in the autumn calving group for reasons of non-cyclicity 4 animals were diagnosed with ovarian cysts on at least one occasion. Two of the cows were the ones that failed to conceive. The other two animals, which were part of the conventionally fed group, did eventually after 4 inseminations each conceive. In the spring calving group 2 animals were diagnosed anoestrus. During the 12 month lactation cycles one cow was diagnosed anoestrus.

As a result of the greater difficulties in rebreeding animals during the first extended lactation, days from first service to conception was significantly higher during the first

compared to the second extended lactation. Furthermore, this resulted in a significantly longer calving interval during the first extended lactation. The same was the case when comparing cows completing an extended and a conventional lactation. Nutrition showed significant effect on the number of first days to conception during the first extended lactation. Taking into account that there was only 4 animals present in the high nutritional input group of the autumn calvers and that this effect was not found when comparing 2 consecutive extended lactations it is doubtful whether there is any biological significance to this observation. Nutrition had no significant effect on any of the other parameters. Season had no effect on any of the fertility parameters except were it was a direct consequence of the experimental protocol.

It is evident from this study that late rebreeding is not "easy". This was particularly evident during the first extended lactations. During the second extended lactation and indeed the conventional 12 months lactation rebreeding seemed to be more efficient. It is not possible from this study to make any conclusion as to the cause of this. It is important to recognise that relatively few cows were studied in this experiment and poor fertility in one individual could have a disproportionate effect on group values. It is also important to bear in mind that the experiment was not specifically designed to investigate fertility during extended lactation, but rather the persistency of lactation. Only one other study that we are aware of has looked at fertility during lactations as long as 18 month with rebreeding starting around day 240 of lactation (Ratnayake *et al.*, 1998). In this study there was a tendency for inseminations per animal to be lower during extended lactation cycles compared to 12 month cycles (1.6 versus 2.2-3.3) although conception rate to first insemination did not differ. Treatment for anoestrus occurred more often in the conventional compared to extended group of animals. The number of animals studied in this experiment was small

(25). Two other studies have looked at extended calving intervals but not as long as 18 month. Scheider *et al.* (1981) compared rebreeding at 50 days with 80 days of lactation. However only number of inseminations per conception is represented in the paper, which was 1.96 and 1.50 respectively. It is impossible to make any proper conclusions on this one statement. In the other study (Schindler *et al.*, 1991) fertility parameters were compared when rebreeding was commenced 35-59, 60-90 and 120-150 days *post partum*. Conception rate to first insemination was 65.4% in the late bred animals compared to 35.7% in early bred animals. It is clear that more studies with a larger number of animals are needed to clarify the effect of extended calving intervals on reproduction. We found no evidence that an extended lactation compromises future reproductive success (either extended or conventional lactation) indeed quite the reverse. However, on the basis of this experiment and the experiment of Ratnayake *et al.* (1998) it is inevitable that good reproductive management will remain an essential part of an extended lactation system.

Chapter four

The effect of milking frequency, nutrition and calving season on lactation persistency during extended lactation in dairy cows

4.1. Introduction

In the previous chapter I concluded that rebreeding at around 9 month *post partum* was no easier than rebreeding 2-3 month *post partum*, however, the cause of this was not identified. Secondly, it became evident that not all animals were capable of lactating for as long as 18 months but were drying themselves off before 60 days *pre partum*. In this chapter I will describe and discuss the most important aspect of the extended lactation project, namely, how milking frequency, nutritional input and calving season affects lactation persistency.

It is well known that an increase in milking frequency increases milk production. However the effect on lactation persistency is by no means consistent (Pearson *et al.*, 1979; Amos *et al.*, 1983; Allen *et al.*, 1986). The immediate response in milk yield is proposed to be due to more frequent removal of a milk protein (FIL) which exerts a negative inhibitory effect on milk secretion (Wilde *et al.*, 1997a). If sustained for more than a few weeks, increased milking frequency stimulates the activity of key enzymes involved in milk synthesis, increases cell proliferation and decreases apoptosis (Hillerton *et al.*, 1990; Li *et al.*, 1999). From a mammary perspective milk yield is a function of the number and activity of milk secretory cells and the rate of decline in milk yield after peak lactation is entirely due to a decrease in the number of mammary secretory cells (Fowler *et al.*, 1990). On the basis of the documented effect of increased milking frequency on mammary development, increased milking frequency therefore has the potential to increase lactation persistency.

The effect of different feeding levels on lactation persistency is not well established (Broster & Broster, 1984). Broster & Broster (1984) found that the response to supplementary feeding was proportional to current yield rather than time after calving and, once the response was established, lactation persistency was similar to that of control cows. However, severe underfeeding during early lactation does affect subsequent yield and lactation persistency (Schmidt, 1971). Likewise season of year has been shown to affect milk yield but how it affects lactation persistency is relatively unexplored (Wood, 1972).

4.2. Material and methods

4.2.1. Experimental design

The overall design of the experiment is described in chapter 2.

4.2.2. Milking

Cows were milked in an autotandem milking parlour especially modified for routine half udder milking. Half udder milk yield was recorded at each milking to a precision of 100g.

4.2.3. Measurement of body weight and body condition score

Body weight and body condition score were measured weekly in the spring and autumn calvers, the spring calvers consecutive extended lactation and the autumn calvers consecutive conventional lactation. However, due to the instalment of an automatic weighing bridge body weight data are missing from standardised lactation week 51-56 in the spring calvers and standardised lactation week 57-66 in the autumn calving group. Due to technical problems with the weighing bridge body weights are also missing from the first 20 weeks during the second extended lactation.

Body condition score was assessed by a scoring system developed by the East of Scotland College of Agriculture (1976). It is based on measurements of the loin area between the hipbone and the last rib as well as around the tail head, which are considered important areas in determining the mobilisation of the fat tissue. Body condition score was determined on a scale from 1 to 5, where 1 is extremely thin cows and 5 excessively fat cows. The same two people carried out the assessment throughout the experiment.

4.2.4. Statistical analysis

To evaluate the effect on total milk production of milking frequency, nutritional input and calving season and to compare the first and second extended lactations analysis of covariance (ancova) was used. To adjust for any pre-existing differences in milk yield between udder-halves and individual cows pre-treatment milk yield was used as the covariate. To examine the effect of treatment on lactation persistency a best fit linear regression analysis was performed on weekly averaged milk yields for each individual cow from standardised lactation week 9 and onwards (Minitab Release 11, Minitab Inc, State College, PA 16801, USA). Other curve-fits were investigated using Genstat 5 Release 4.1, Lawes Agricultural Trust, Rothamsted Experimental Station and Minitab Release 11, Minitab Inc, State College, PA 16801, USA. The best fit was obtained with a ninth grade polynomial. Third and fourth grade polynomials did not produce a consistently better fit than best fit linear regression analysis. We therefore decided to use best fit linear regression analysis to describe our lactation curves. Slopes from the regression analysis were analysed using ancova. A significant negative relationship existed between peak milk yield and rate of decline in milk production and peak yield was therefore used as a covariate. All data were analysed in Genstat 5 Release 4.1, Lawes Agricultural Trust, Rothamsted Experimental Station. The effect of milking frequency on lactation persistency

was compared within cow as were differences between the first and second extended lactation. Nutritional input and calving season were compared between cows. The effect of pregnancy on lactation persistency was analysed by residual maximum likelihood analysis (REML) due to an unbalanced design. This is explained further in section 4.3.3. All values are reported as mean±s.e. unless otherwise stated.

4.3. Results

4.3.1. Milk production

Table 4.1 shows milk production data adjusted for pre-treatment yield from 3x milked and 2x milked udder-halves of spring and autumn calvers on either a high or a conventional nutritional input system. Values are milk production during the first 25 weeks of treatment (lactation week 9.5±0.64 to lactation week 33.5±0.64) immediately before rebreeding commenced, as well as from start of treatment to drying off and from lactation week 1 to drying off. As described in chapter 3 lactation length was 515±16 days in the spring calving group on high nutritional input and 495±19 days on conventional input. In the autumn calvers lactation length was significantly longer. The high input group lactated for 571±17 days while the conventional group lactated for 572±30 days.

Thrice daily milking increased milk production. During the first 25 weeks of treatment milk yield from 3x udder-halves was 541.60±89.62 kg higher than in 2x milked udder-halves. Furthermore, milk production was significantly greater in cows fed on a high nutritional input system compared to a conventional input system. Calving season also affected milk production, so that milk production was significantly higher in the autumn calvers compared to the spring calvers. The effect of the individual treatments was independent since no interaction was found between any of the treatments.

Overall there was no significant effect of nutrition on milk production. However, due to differences in lactation length the average daily milk yield per day throughout the entire lactation was calculated for each individual animal. The conventional input group produced 9.0±0.40 kg on a daily basis (half udder yields) throughout their entire lactation while the high input group produced 9.8±0.46 kg/d ($P<0.065$, ancova using pre treatment yield as a covariate). Therefore, high nutritional input did result in an overall greater milk production. Milk production was significantly higher from thrice daily milked than twice daily milked udder-halves. Overall, 3x milked udder-halves produced 1545±143kg more milk than 2x milked udder-halves, a percentage difference of 50.35±5.93%. Furthermore, total amount of milk from the autumn calving group was higher than from the spring calving group. However, this was due to a difference in lactation length between the two seasonal groups because when calculating daily milk production for individual animals throughout their entire lactation there was no difference in milk yield from the two seasonal groups. On a half udder basis the spring calving group were producing 9.22±0.42 kg/d through the entire lactation, while the autumn were yielding an average of 9.52±0.46kg/d.

Milk yield from two consecutive extended lactations for the spring calving groups is shown in table 4.2. Again, pre-treatment milk yield was used to adjust for any pre-existing differences in milk yield from udder-halves allocated to either 2x or 3x daily milking.

During the first 25 weeks of treatment and all through experiments the thrice daily milked udder-halves were yielding more milk than the twice daily milked. Overall, the difference amounted to 1306±178kg, an increase of 42.68±6.94%. Milk yield was significantly greater during the second lactation (3x: 699±274 kg (19.13±5.77%), 2x: 987±260kg

(40.55±9.11%)) although lactation length did not differ between the two lactations. Half-udder average daily milk yield was 11.7±0.4kg/d during the second lactation but only 9.4±0.4kg/d during the first extended lactation. High nutritional input also increased milk yield. This was not simply due to a slightly longer lactation length in the animals on high nutritional input since average daily milk yield was 11.0±0.45 in the high nutritional input group and only 10.0±0.51kg/d in the conventionally fed animals ($p<0.01$, ancova using pre-treatment yield as a covariate). No interaction was found between any of the treatments. The greatest amount of milk was therefore produced from thrice daily milked udder-halves of cows on high nutritional input.

On a whole cow basis (2x + 3x) milk production was 10228±366kg during the 1'st lactation and 11541±272kg during the 2'nd lactation. The average calving interval was 559±16 and number of days dry was 64.8±5. Therefore, calculated 365 days yield as an average over a 3 year period on a whole cow basis was 7544 kg/year. This calculation was performed as shown below:

Total milk yield during the two lactations / ((2 x CI – days dry)/365)

Udder-halves milked thrice daily in the high nutritional input group produced on average 6093 ±152kg/lactation. Assuming that both udder-halves would have produced equal amounts of milk if the whole udder had been milked 3x daily, total milk production would have been 12187±304 kg/lactation which equals 8434kg of milk in 365 days. These figures are illustrated in figure 4.1 together with 365 days yield for our autumn cows during their consecutive 12 months lactation cycle as well as for the Hannah herd in general.

The animals which went on to a 12 month lactation cycle after an 18 month cycle produced 4459±329 kg milk from thrice daily milked udder-halves throughout their lactation while

production from the 2x milked udder-halves was 3596±307 kg ($P<0.01$). There was no significant difference in milk yield between the two udder-halves before the treatment was applied. Calving interval for this group was 355±14 days and number of days dry was 61±6. Assuming that milk yield and calving interval would stay the same through 3 lactations 365 day yield on a whole udder basis was, therefore, 9366kg/lactation. The Hannah herd is managed conventionally. Cows are milked two times daily and fed to milk production. Cows are fed a grass silage-based total mixed ration (winter) or grazed pasture (summer) supplemented with in parlour concentrate containing 18% crude protein. Milk production for the Hannah herd over the last three years was 18, 595 kg per animal, the calving interval was 430 days and the dry period 60 days. Therefore, 365 days yield was 5518 kg.

Compared to the Hannah herd average the extended lactation cows produced 36.7% more milk and this increased to 53% when assuming both udder-halves would produce equal amounts of milk if milked 3x daily. However, when comparing the extended lactation cycles with the experimental 12 months cycle milk production was 19.5% lower from cows on extended lactation.

Table 4.1. The effect of milking frequency (thrice daily, 3x versus twice daily, 2x), nutrition (high input versus conventional input) and calving season (spring versus winter) on milk production. Values are half-udder milk production (kg) during the first 25 weeks of treatment (Lac week 9 to 33), from treatment week one until drying off (Lac week 9 to 77±1.7) and finally from lactation week one until drying off. Data were analysed by ancova using pre-treatment yield as a covariate. Values are adjusted for pre-treatment yield, except when comparing milk production between groups from lactation week 1 to drying off. No interaction was found between any of the treatments.

Group	Spring calvers				Autumn/winter calvers							
Nutrition	High input		Conventional input		High input		Conventional input		P-value			
Frequency	2x	3x	2x	3x	2x	3x	2x	3x	SED	Frequency	Nutrition	Season
Lac 9-33	1828	2306	1614	2102	2047	2520	1841	2433	113.7	<0.001	0.01	<0.01
Lac 9-77	3424	4881	2657	3898	3809	5457	3636	5252	553.1	<0.001	n.s	<0.05
Lac 1-77	4259	5969	3563	4861	5322	6218	4612	5959	381.2			

Table 4.2. The effect of milking frequency (thrice daily, 3x versus twice daily, 2x), nutrition (high input versus conventional input) and number of extended lactations (1'st extended versus 2'nd extended) on milk production. Values are half-udder milk production (kg) during the first 25 weeks of treatment (Lac week 9 to 33), from treatment week one until drying off (Lac week 9 to 70±1.3) and finally from lactation week one until drying off. Data were analysed by ancova using pre-treatment yield as a covariate. Values are adjusted for pre-treatment yield, except when comparing milk production between groups from lactation week 1 to drying off. No interaction was found between any of the treatments.

Group	1'st extended lactation				2'nd extended lactation							
Nutrition	High input		Conventional input		High input		Conventional input		P-value			
Frequency	2x	3x	2x	3x	2x	3x	2x	3x	SED	Frequency	Nutrition	Lactation
Lac 9-33	1857	2362	1677	2179	2446	2816	2231	2637	106.9	<0.001	<0.01	<0.001
Lac 9-70	3318	4850	2652	3939	4456	5474	3800	5157	341.5	<0.001	<0.01	<0.001
Lac 1-70	4259	5969	3563	4885	5322	6218	4612	5959	391.5			

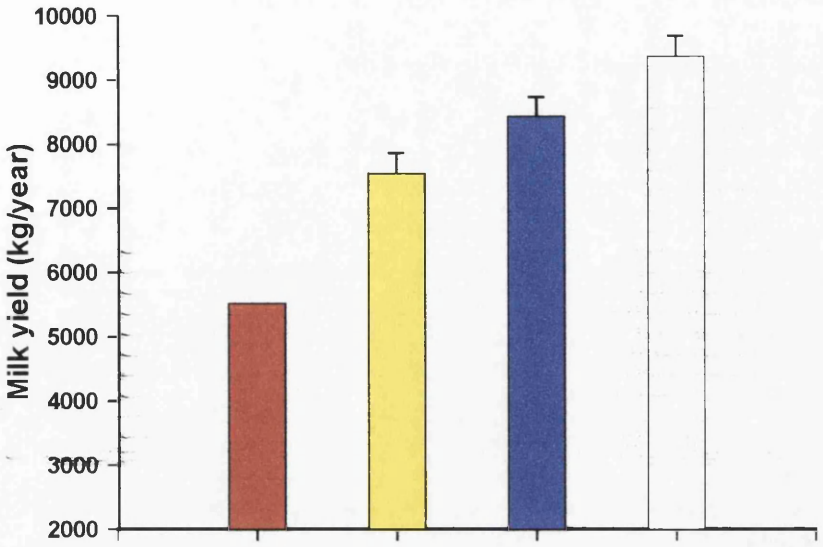


Figure 4.1. Calculated milk production figures annualised to 365d yield. Red bar: Average of the Hannah herd excluding cows on this experiment. Yellow bar: Actual production of cows completing two consecutive extended lactations on high input. Blue bar: Theoretical production of these same cows had the whole udder been milked trice daily. White bar: Extrapolated from actual production of cows during a conventional twelve month lactation following an extended lactation.

4.3.2. Effect of nutrition, milking frequency and season on lactation persistency before rebreeding

Rebreeding commenced around lactation week 32. To determine the effect of nutrition, calving season and milking frequency on lactation persistency a best fit linear regression analysis on weekly averaged milk yields from individual cows was performed using data for weeks 9-33. To test if fitted and actual values differed between treatment anova of mean square error (MSe) for individual animals, which is the estimated standard deviation about the regression line was performed. An interaction between calving season and milking frequency was evident. In the autumn calving cows MSe was higher in thrice daily milked udder-halves (1.4±0.20 vs. 0.85±0.15) and although MSe also was higher (0.59±0.11 vs 0.48±0.08) in thrice daily milked udder-halves in the spring calving cows this difference was bigger in the autumn calvers ($p<0.01$). Lactation curves showing the weekly average milk yields and the fitted regression line for the autumn and spring calving groups are in figures 4.2 and 4.3. Figure 4.4 shows fitted regression lines for each treatment adjusted to a common start-point for ease of visual comparison. A significant negative relationship was found between milk yield at peak lactation and rate of decline in milk yield (correlation –0.543, $P<0.001$). Peak milk yield was therefore, used as a covariate in the statistical analysis. Body condition score at the start of treatment had no effect on rate of decline in milk yield (correlation -0.213, n.s).

Lactation was much more persistent in autumn than spring calvers (table 4.3 and figure 4.4). Increased milking frequency improved lactation persistency, an effect that was significantly greater in the autumn calving group. High nutritional input also improved lactation persistency. No interactions were found between level of nutrition and milking frequency and level of nutrition and calving season.

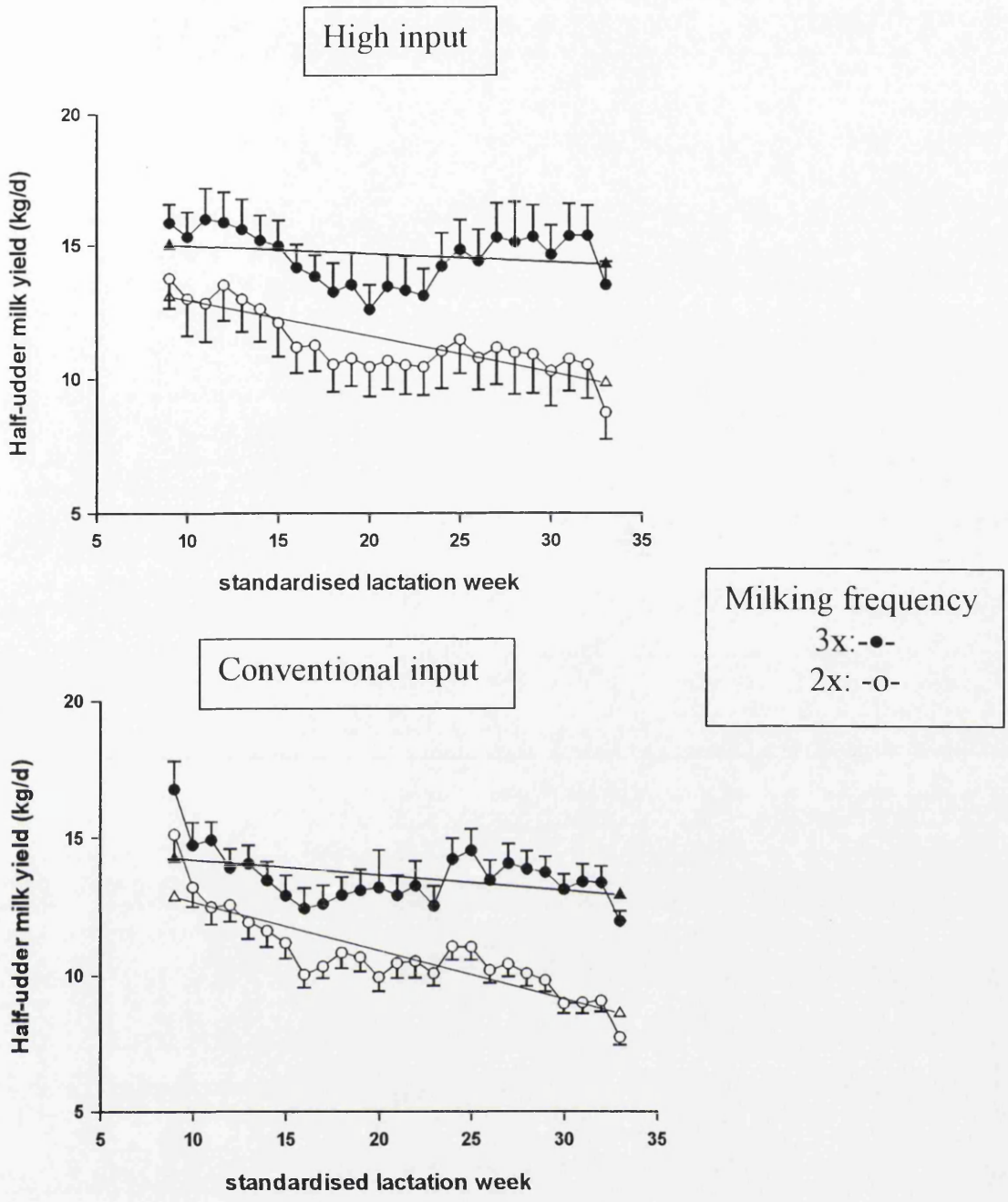


Figure 4.2. Effect of milking frequency and nutrition on milk yield in autumn calving cows during lactation week 9 to 33. Data are weekly averaged half-udder milk yields (kg/d). Solid symbols: milked thrice daily (3x). Open symbols milked twice daily (2x). Cows were fed conventionally according to milk yield (n=6, bottom panel) or received an additional 3kg/d 18% supplement (n=6, top panel). The lactation curve was described by best fit linear regression analysis on individual cow data; these fits are shown for comparison of fitted and actual values.

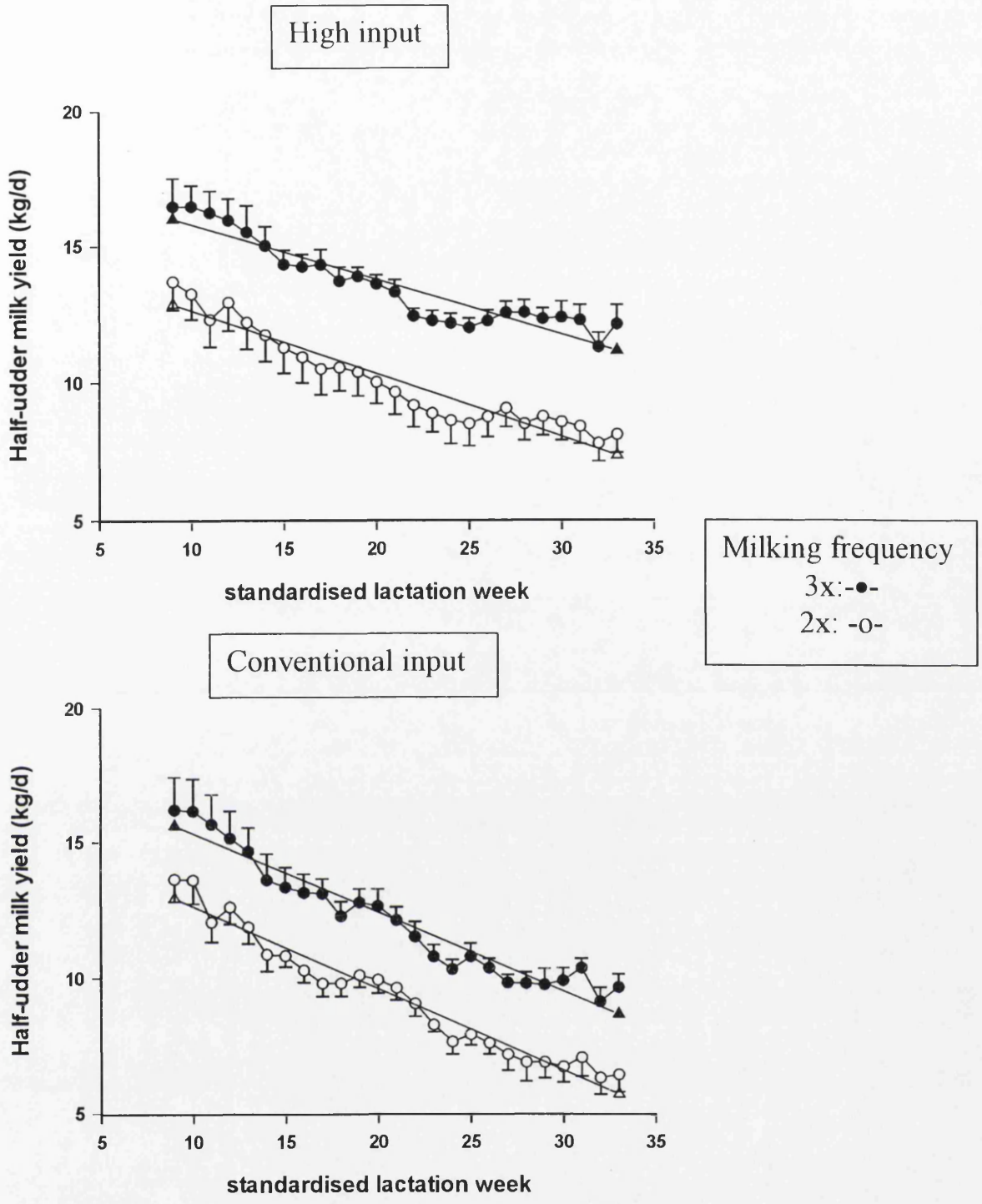


Figure 4.3. Effect of milking frequency and nutrition on milk yield in spring calving cows during lactation week 9 to 33. Treatment and analysis details as for figure 4.2.

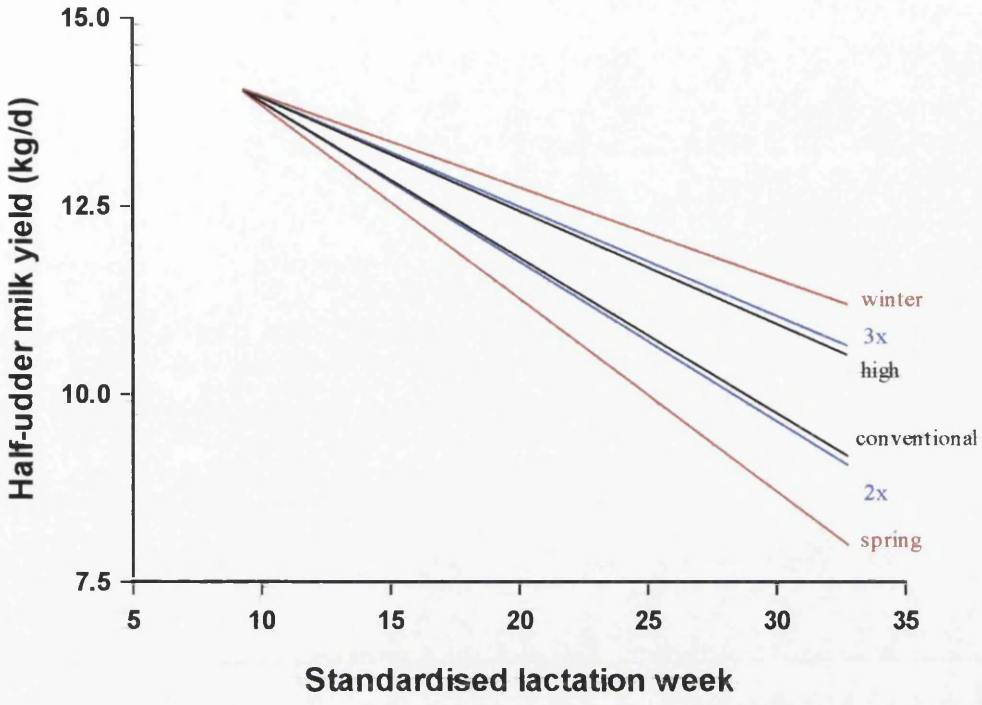


Figure 4.4. Effect of season (–), milking frequency (–) and nutrition (–) on lactation persistency from lactation week 9 to lactation week 33, immediately before rebreeding was commenced. Regression lines were fitted by best linear regression analysis. Data are adjusted to a common start point. Treatment was initiated during lactation week 9.6±0.64.

Table 4.3. Effect of milking frequency (thrice daily versus twice daily), nutrition (high versus conventional input) and calving season on lactation persistency during lactation week 9 to 33. The lactation curve was described by best fit linear regression analysis on weekly averaged milk yields and then analysed by ancova using peak yield as a covariate. Values are adjusted for peak yield. Means for interactions are only shown when significant.

Treatment	Slope 9-33	SED	P-value	N
Calving season				
Autumn	-0.1054	0.028	<0.001	24
Spring	-0.2503			
Frequency				
Thrice	-0.1457	0.015	<0.001	24
Twice	-0.2100			
Nutrition				
High	-0.1474	0.028	<0.05	24
Conventional	-0.2083			
Calving season*frequency				
Spring 2x	-0.2601	0.032	<0.01	12
Spring 3x	-0.2405			
Autumn 2x	-0.1599			
Autumn 3x	-0.0509			

Average weekly milk yield data and the fitted regression lines from lactation week 9 through to lactation week 33 for the spring calving group's second extended lactation are shown in figure 4.5. The standard deviation around the regression line was significantly

greater during the second compared to the first lactation (MSe: 1.2±0.18 vs 0.56±0.07, $P<0.05$). Again a significant negative relationship between peak milk yield and rate of decline in milk yield was evident (correlation –0.54, $p<0.001$). In the analysis of variance peak milk yield was therefore, used as a covariate. Once again, a positive effect of milking frequency was evident, but in this lactation nutritional supplementation had a negative influence on lactation persistency. This is best visualised in figure 4.6, where it is also apparent that persistency was better in the second extended lactation irrespective of other treatment. Statistical analysis of the data is in table 4.4, which summarises persistency slopes for the different treatments. Lactation persistency was significantly greater during the second lactation, thrice daily milking significantly increased lactation persistency and the interaction between nutrition and lactation number was not significant.

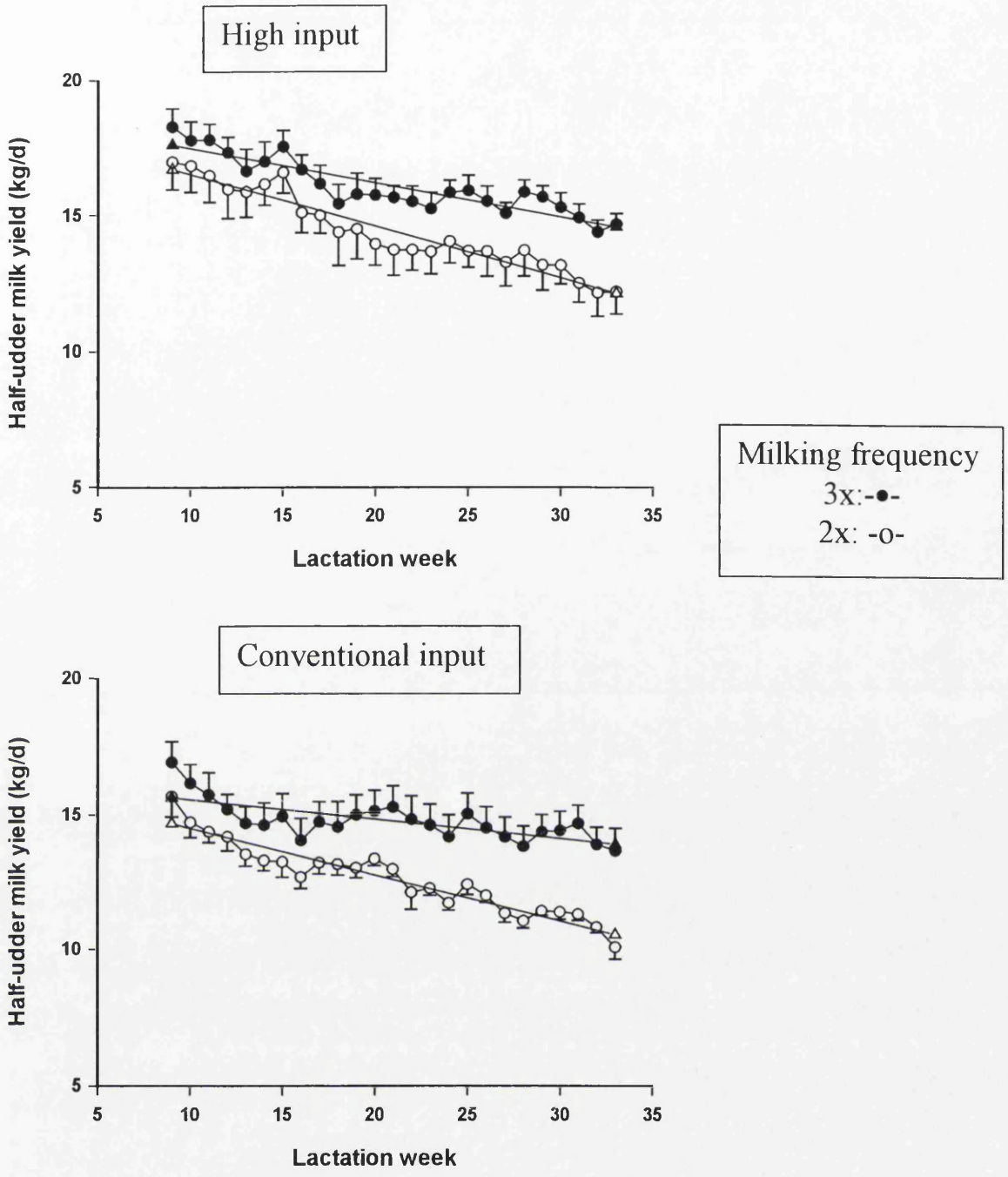
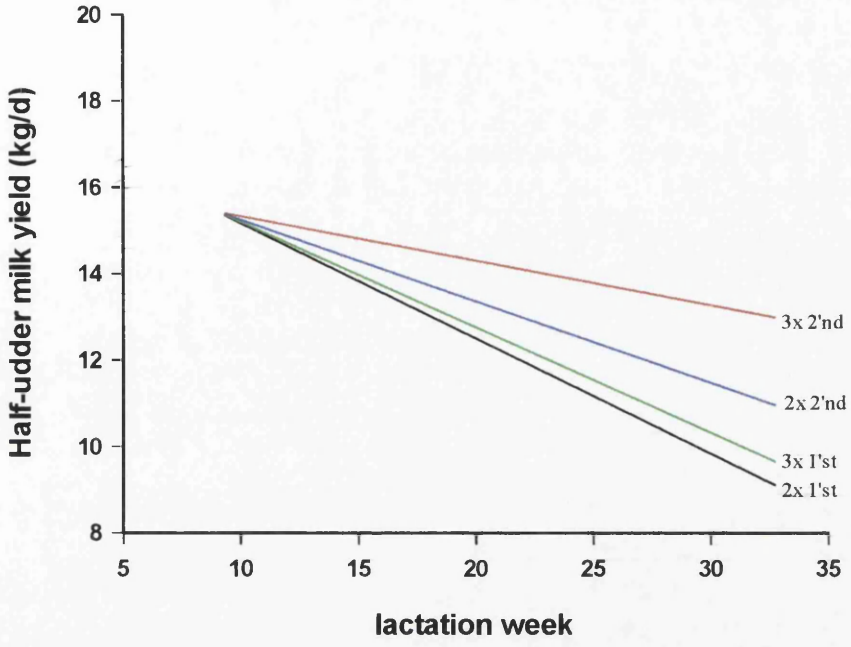


Figure 4.5. Effect of milking frequency and nutrition on milk yield during the spring calvers second extended lactation from lactation week 9 to 33. Treatment and analysis details as for figure 4.2.



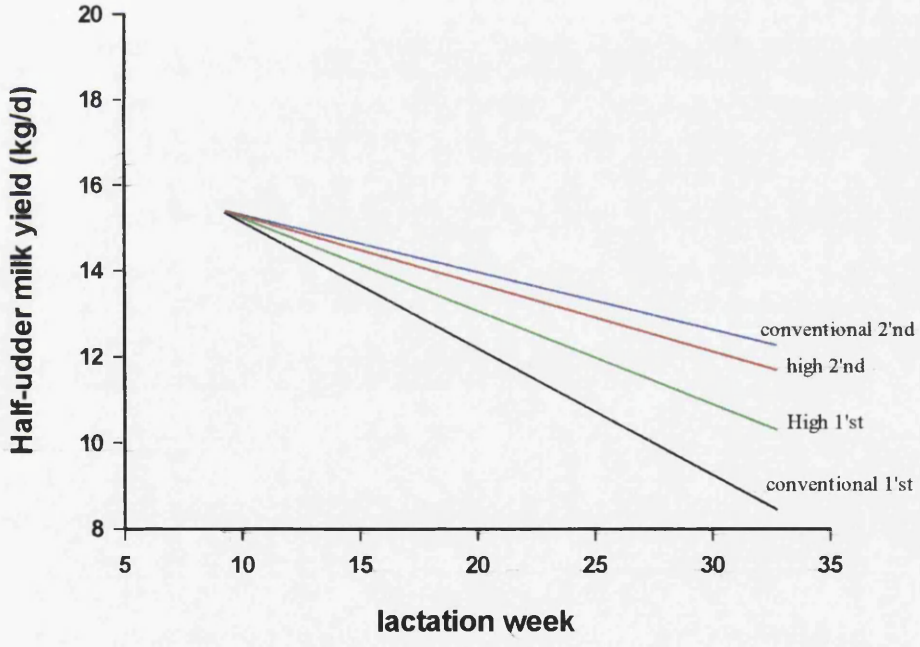


Figure 4.6. Effect of milking frequency (top graph) and nutrition (bottom graph) during two consecutive extended lactations on lactation persistency. Regression lines were fitted by best linear regression analysis. Data are adjusted to a common start point. Treatment was initiated during lactation week 9.3±0.49.

Table 4.4. Lactation persistency during the first and second extended lactations, effect of milking frequency and nutrition during lactation week 9 to 33. Treatment and analysis details as for table 4.3. Each individual animal received the same treatment during her first and second lactation.

Treatment	Slope	SED	P-value	N
1'st extended lactation / 2'nd extended lactation				
1'st extended	-0.2768	0.032	0.001	22
2'nd extended	-0.1220			
Frequency				
Thrice	-0.1755	0.016	<0.01	22
Twice	-0.2232			
Nutrition				
High	-0.1896	0.026	n.s	22
Conventional	-0.2112			
Lactation number * nutrition				
1'st high	-0.2473	0.043	n.s	11
1'st conventional	-0.3121			
2'nd high	-0.1318			
2'nd conventional	-0.1102			

4.3.3. *Effect of pregnancy on milk yield and lactation persistency*

To determine the effect of pregnancy on milk yield and lactation persistency milk yield data were restandardised according to week of pregnancy. The pregnancy period was then divided into three stages. Stage one was the first 10 weeks of pregnancy, stage 2 the second 10 weeks of pregnancy while stage 3 was the last 7 weeks. Pregnant cows that were dried off less than 27 week after conception were excluded from the analysis (details below). Regression lines were fitted to milk yield curves for individual animals within each stage and for a period of 10 weeks prior to conception. Different stages were compared by REML.

In the autumn calving group 2 cows failed to conceive, one cow was culled due to a leg injury and one cow failed to complete 27 weeks of her pregnancy before she dried herself off. This left 4 cows in the high nutritional input group and 4 in the conventional input group. In the spring calving group 2 animals dried themselves off before they reached the 27th week of their pregnancy, both of these animals were part of the conventional feeding system, so only 4 animals were present in this group. During the 2'nd extended lactation one cow in the high nutritional input group failed to conceive, which left 5 animals in each of the two nutritional groups.

Milk yield curves 10 weeks prior to conception throughout to 27 weeks of pregnancy for autumn and the spring calvers 1'st and 2'nd lactation on either high or conventional nutrition are shown in figures 4.7-4.9. Stage of pregnancy showed a clear effect on rate of decline in milk yield, which is analysed statistically in table 4.5. There was no difference in rate of decline between the 10 weeks prior to pregnancy and stage 1 or 2 of pregnancy. However during stage 3 yield started to decline rapidly. With stage 3 excluded from the

analysis there was no significant effect of pregnancy but with stage 3 included there was a highly significant effect. Direct comparison of stage 2 and 3 by paired t-test confirmed this ($P<0.001$).

During all stages yield was more persistent in thrice daily milked udder-halves, except for stage 3 where rate of decline was greater in 3x as shown in table 4.6. Stage 3 was therefore excluded from the data set and analysed separately. No effect was found of level of nutrition or calving season. However rate of decline in yield was significantly greater during the 2'nd extended lactation compared to the 1'st extended lactation.

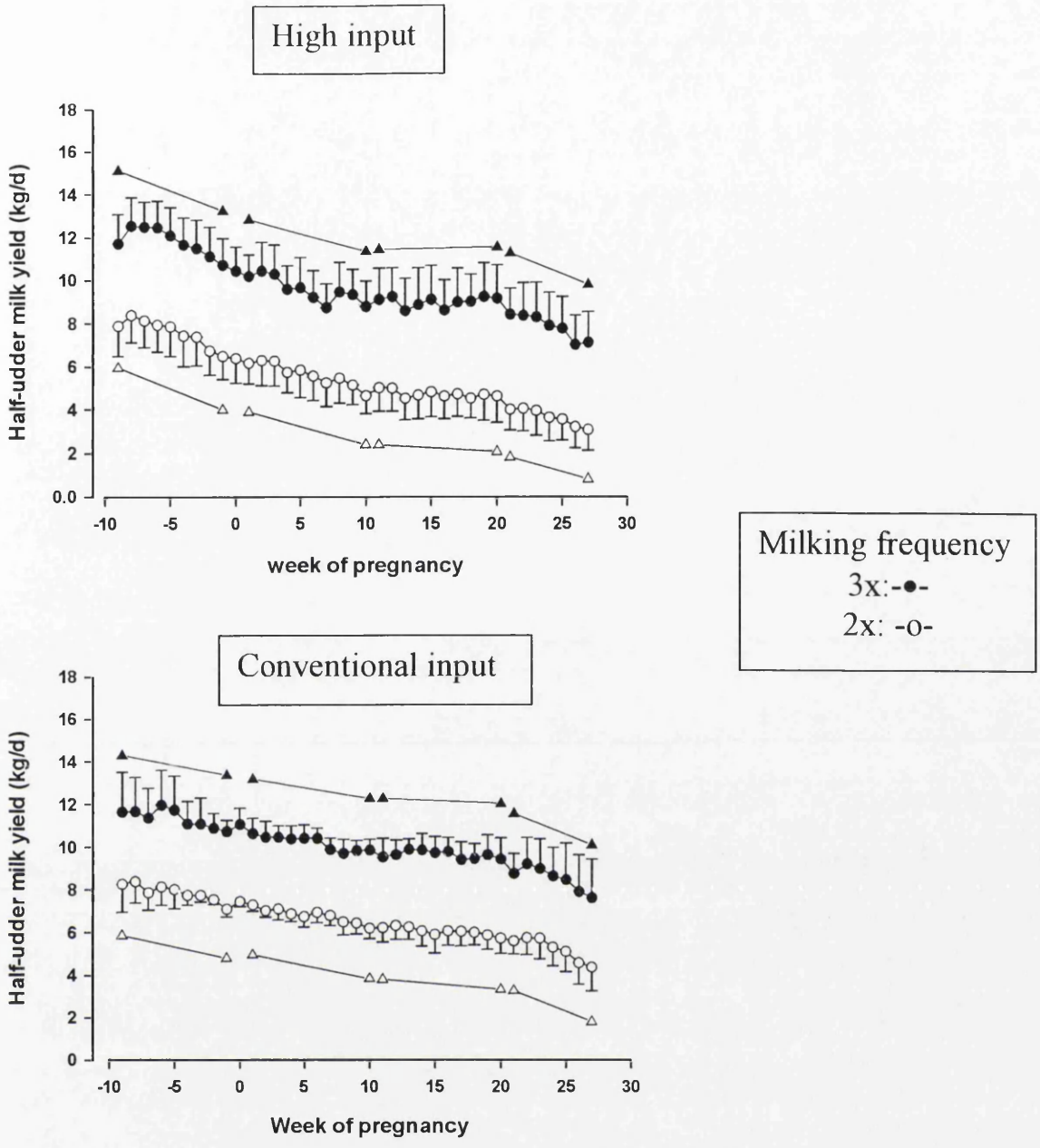


Figure 4.7. Effect of recurring pregnancy on lactation persistency in autumn calving cows. Data are weekly averaged half-udder milk yields (kg/d). Solid symbols: milked thrice daily (3x). Open symbols: milked twice daily (2x). Cows were fed conventionally according to milk yield (n=6, bottom panel) or received an additional 3kg/d 18% protein supplement (n=6, top panel). Best fit linear regression analysis was performed on individual cow data over four periods; these fits are shown displaced vertically for clarity.

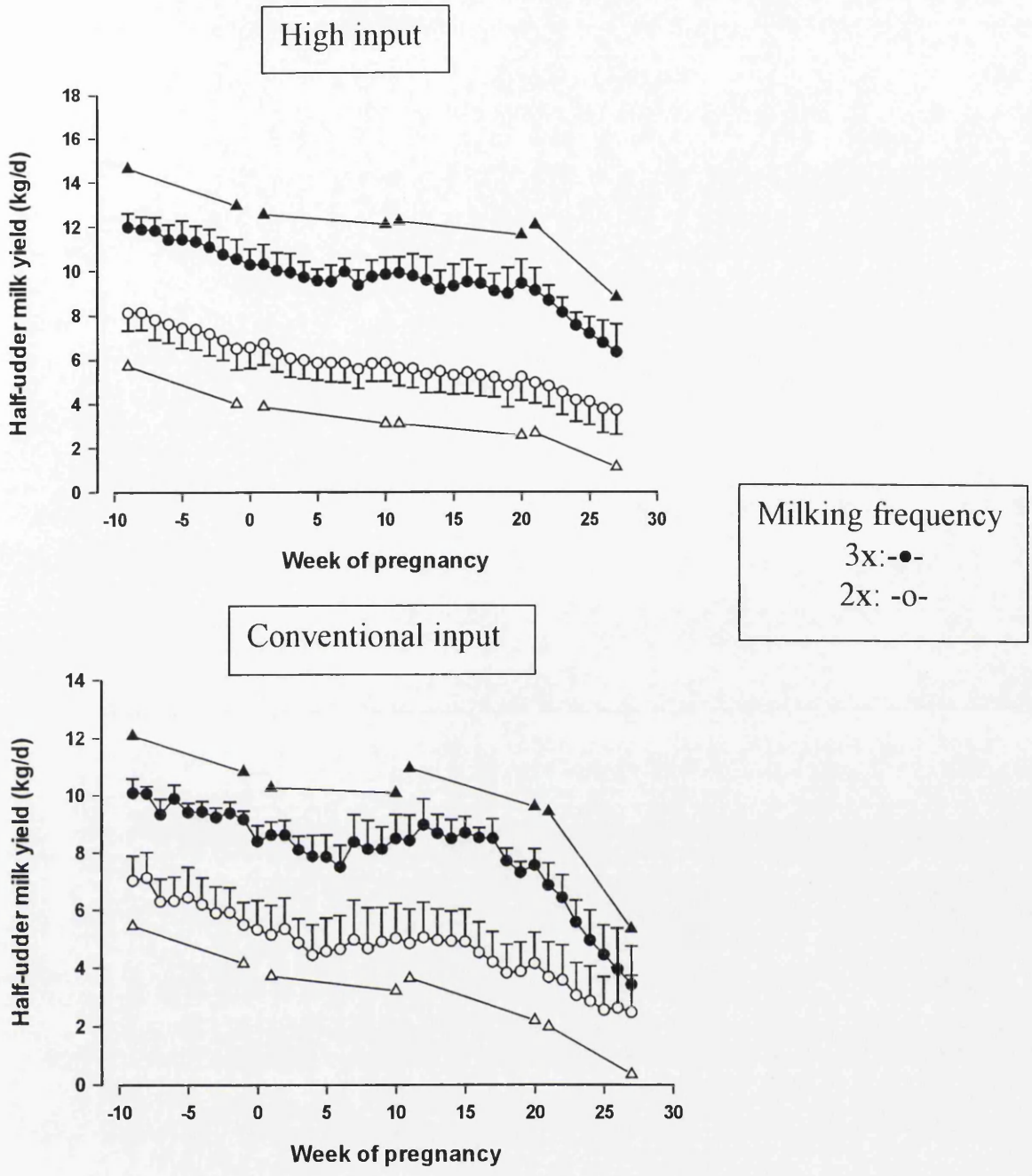


Figure 4.8. Effect of recurring pregnancy on lactation persistency in spring calving cows. Treatment and analysis details as for figure 4.7.

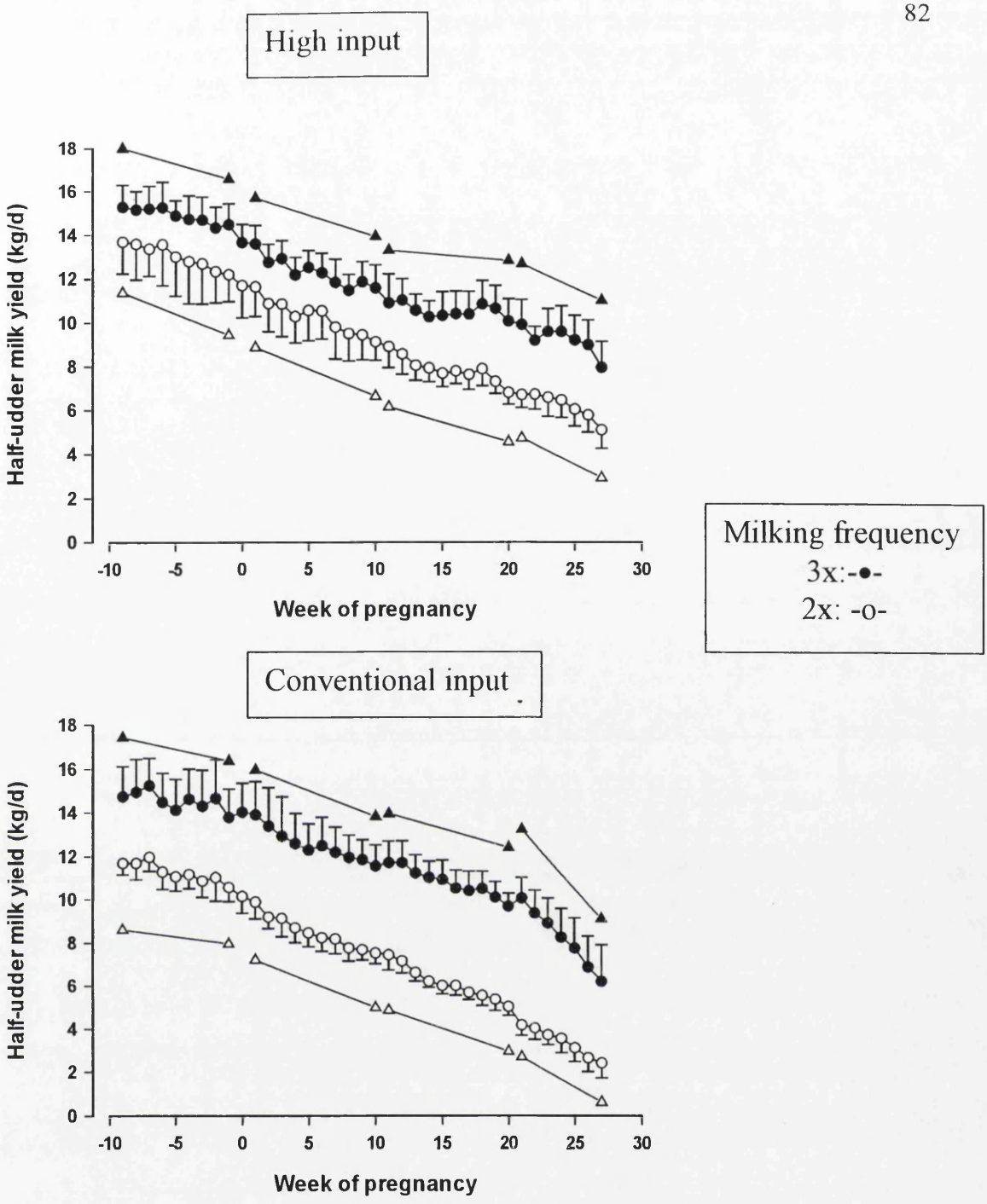


Figure 4.9. Effect of recurring pregnancy on lactation persistency in the spring calvers second extended lactation. Treatment and analysis details as for figure 4.7.

Table 4.5. The effect of recurring pregnancy on lactation persistency. Milk yield data was standardised around conception and the pregnancy period was divided into three stages. Stage one was the first 10 weeks of pregnancy, stage 2 the second 10 weeks and stage 3 final 7 weeks. Regression lines were fitted to milk yield curves for individual animals at each stage and 10 weeks prior to pregnancy. Separate analyses were done with and without stage 3. Stage 3 was excluded from all analysis when analysing the effect of other treatments on lactation persistency. Treatment details are as described in table 4.3. Data were analysed by REML.

Treatment	Slope	SED	P-value	N
Stage of pregnancy				
Pre	-0.1649	0.041	n.s	58
Stage 1	-0.1408			
Stage 2	-0.1038			
Stage 3	-0.3215	0.043	P<0.001	
Frequency				
Twice daily	-0.1513	0.015	<0.05	87
Thrice daily	-0.1217			87
Nutrition				
High	-0.1352	0.043	n.s	84
Conventional	-0.1378			90
Calving season				
Spring	-0.1101	0.051	n.s	60
Autumn	-0.1222			48
1'st extended lactation /2'nd extended lactation				
1'st extended	-0.1116	0.043	<0.05	54
2'nd extended	-0.1887			54

Table 4.6 shows persistency, absolute decrease and percentage decrease in milk yield from pregnancy week 21 to 27 (stage 3). Milk yield decreased from 8.91±0.42 to 5.88±0.56 in

thrice daily milked udder-halves and from 5.39±0.44 kg to 3.46±0.41 in twice daily milked udder-halves. Absolute decrease was higher in 3x milked udder-halves compared to 2x milked udder-halves (3x: 3.04±0.50 2x: 1.93±0.27). However no difference was found in the percentage decrease between 3x and 2x milked udder-halves (116±33.2%, 114±22.9, respectively). This decrease in milk yield happened irrespective of nutritional input, calving season or number of extended lactations.

Table 4.6. Rate of decline, absolute decrease and percentage decrease in milk yield from thrice daily (3x) and twice daily (2x) milked udder-halves from pregnancy week 20 to pregnancy week 27. Data were analysed by anova.

Frequency	3x	2x	P-value
Slope	-0.4085±0.08	-0.2403±0.03	<0.001
Absolute decrease	3.04±0.50	1.93±0.27	0.06
Percentage decrease	116±33.2	114±22.9	n.s

Stage of lactation at the time of conception ranged from 32 to 63 weeks. To determine whether this had any effect on the rate of decline in milk yield during pregnancy, conception week was divided into 4 sections. Section one was from conception week 32 to 40 (n=10 cows), section two included cows which conceived between lactation week 41 and 49 (n= 14 cows), section 3 was from conception week 50 to 58 (n=3 cows) and finally section 4 included cows which conceived during lactation week 59 to 63 (n= 2 cows). Rate of decline in milk yield decreased with increased conception week (slope: –0.2468, -0.1801, -0.1270 and –0.0671 for section 1, 2, 3, and 4 respectively), independent of stage of pregnancy. Furthermore a significant negative correlation was found between milk yield and week of lactation (-0.430).

4.3.4. Effects on persistency throughout the entire lactation

A negative effect of pregnancy on milk yield was apparent from pregnancy week 20 onwards. This last stage of pregnancy was excluded when best fit linear regression lines were fitted to weekly averaged milk yields from individual cows, which was, therefore, from lactation week 9 through to pregnancy week 20. The standard deviation around the regression line was significantly higher in thrice compared to twice daily milked udder-halves (MSe: 1.4±0.15 vs. 1.1±0.15, $p<0.01$). A significant negative relationship between peak yield and rate of decline in milk yield still existed (correlation –0.68, $p<0.001$) and peak yield was, therefore, used as a covariate in the anova. No significant correlation was found between body condition score at the start of treatment and rate of decline in yield (correlation 0.124, n.s).

Weekly averaged milk yield data for the autumn and spring calving groups are shown in figures 4.10 and 4.11 and regression slopes in figure 4.12. Slopes adjusted for peak yield are analysed in table 4.7.

Spring grazing commenced in calendar week 19, 1997, which corresponds to standardised lactation week 23 in the autumn calvers and week 49 in the spring calving group. In figures 4.10 and 4.11 it can be seen that turn out was associated with a rise in milk yield. During the spring calvers first grazing period no apparent rise in milk yield was detectable since this was very early in lactation (week 2) and before treatment began. After 9 weeks on grass milk yield began to decline rapidly and although buffer feeding with sugar beet pulp was begun 4 weeks after yield started to decline absolute yield did not recover. Although lactation was more persistent in the autumn calvers before rebreeding there was no difference overall in persistency between the two seasonal groups (figure 4.12 and table

4.7). There was still a trend for cows on high input to have a more persistent lactation although this effect was not consistent between spring and autumn calving cows. In the spring calving cows lactation was more persistent in the high input group (-0.1428±0.02 vs –0.1681±0.02) while in the autumn calvers it was animals on conventional input which had the most persistent lactation (-0.1495±0.02 vs –0.1556±0.02). Once again lactation was more persistent in the thrice daily milked udder-halves. This effect was present in both groups and there was no interaction between milking frequency and calving season in contrast to before rebreeding where the effect of thrice daily milking was much greater in the autumn calving cows (table 4.8)

Table 4.7. Lactation persistency between week 9 and pregnancy week 20: effect of milking frequency, nutrition and calving season. Treatment and analysis details as for table 4.3.

Treatment	Slope 9-p20	SED	P-value	N
Calving season				
Spring	-0.1554	0.017	n.s	24
Autumn	-0.1525			
Frequency				
Thrice	-0.1468	0.007	0.07	24
Twice	-0.1611			
Nutrition				
High	-0.1492	0.017	n.s	24
Conventional	-0.1588			

Table 4.8 compares statistical analysis of slopes between lactation week 9-33 with slopes through the entire lactation in twice and thrice daily milked udder-halves. In the spring calving cows the persistency slopes for twice and thrice milked udder-halves were steeper before rebreeding than overall. In the autumn calving cows lactation persistency was better before rebreeding in thrice milked udder-halves while there was no difference between twice milked udder-halves resulting in a smaller difference in persistency between twice and thrice milked udder-halves overall.

Table 4.8. Effect of milking frequency on lactation persistency during lactation week 9-33 and lactation week 9 to pregnancy week 20 in spring and autumn calving cows. Treatment and analysis details as for table 4.2. P-value test for difference in persistency between period within frequency (paired t-test).

	3X		2X		SED
Period	Spring	Autumn	Spring	Autumn	
Lac 9-33	-0.2405	-0.0509	-0.2606	-0.1598	0.032
Lac 9-p20	-0.1479	-0.1457	-0.1629	-0.1593	0.019
P-value	<0.001	<0.01	0.001	n.s	

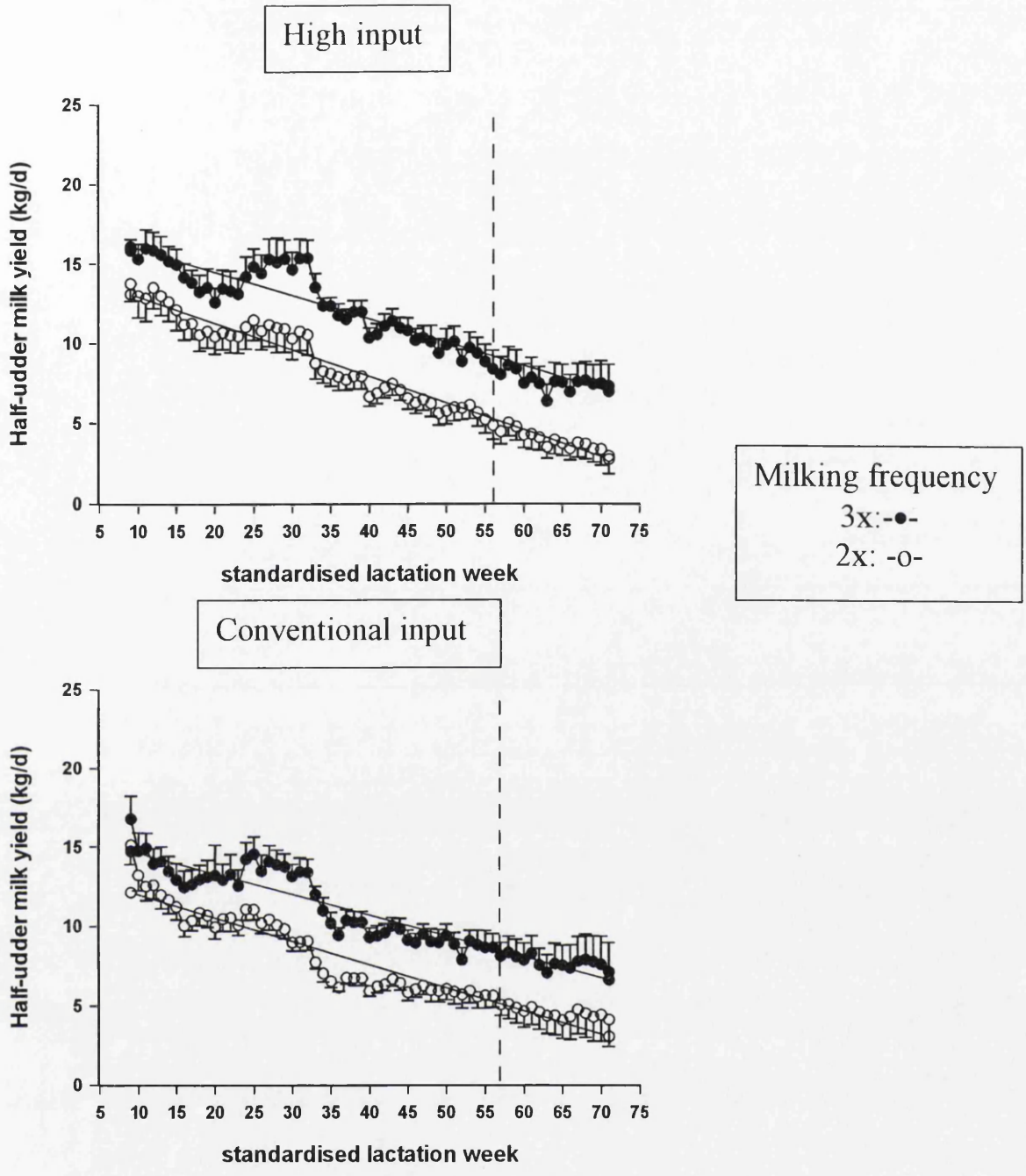


Figure 4.10. Effect of milking frequency and nutrition on milk yield in autumn calving cows during lactation week 9 to pregnancy week 20. Treatment and analysis details as for figure 4.2. Dotted vertical line indicates the start of pregnancy week 20 for the first cow. Data after the dotted line is a combination of actual and extrapolated milk yields, the latter being for cows after pregnancy week 20.

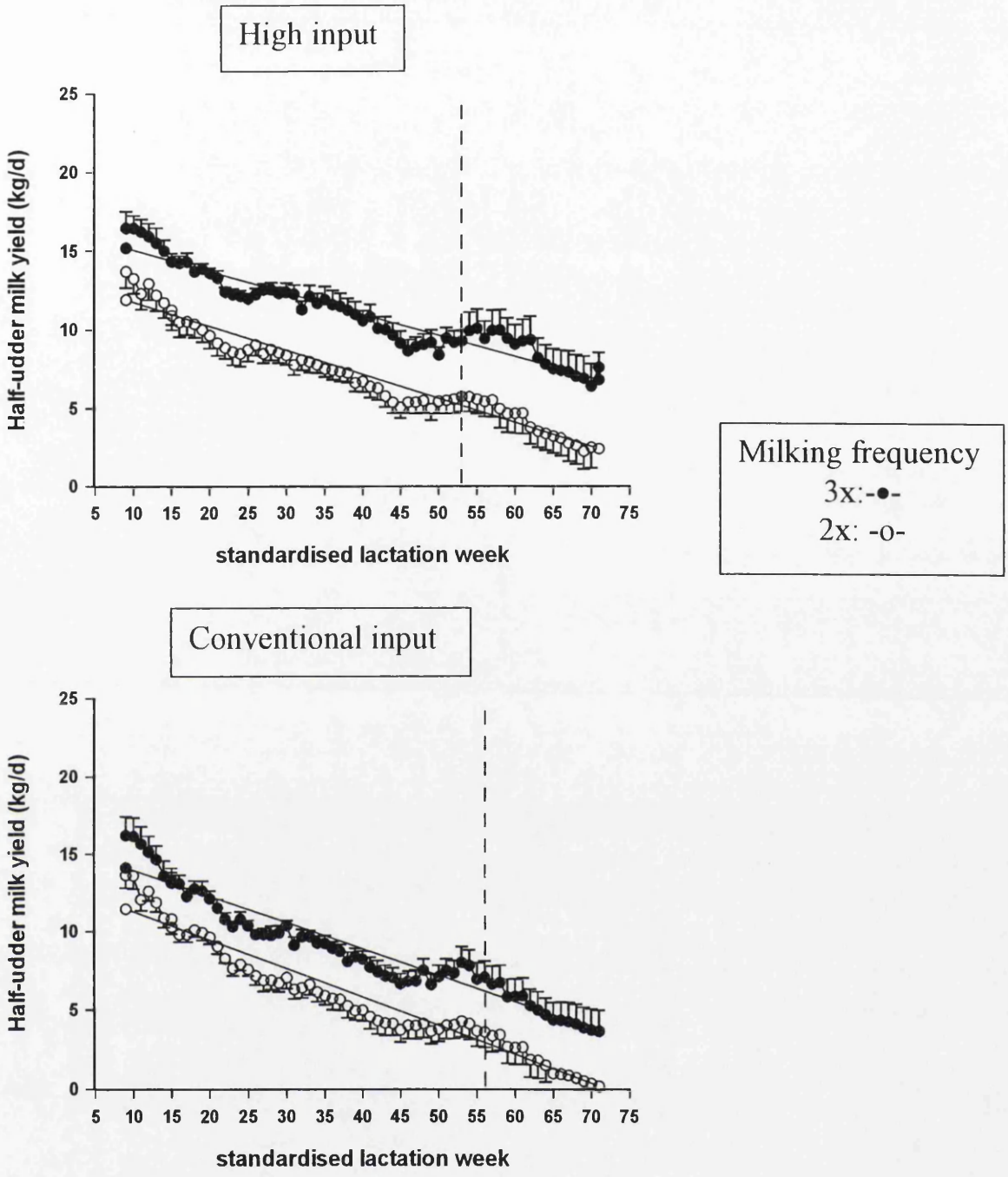


Figure 4.11. Effect of milking frequency and nutrition on milk yield in spring calving cows during lactation week 9 to pregnancy week 20. Treatment and analysis details as for figure 4.2. Dotted vertical line indicates the start of pregnancy week 20 for the first cow. Data after the dotted line is a combination of actual and extrapolated milk yields, the latter being for cows after pregnancy week 20.

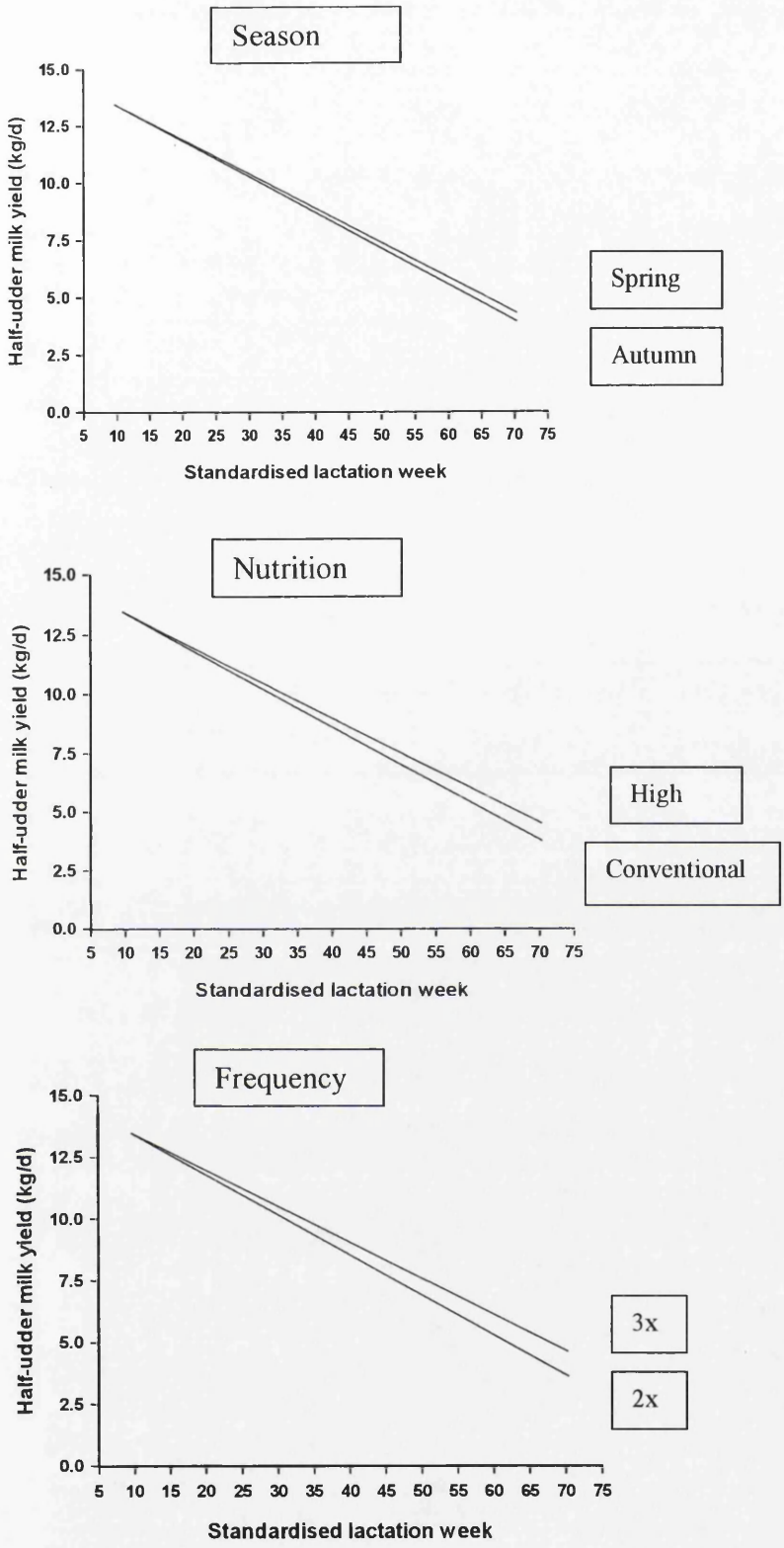


Table 4.12. Effect of calving season, nutrition and milking frequency on lactation persistency during extended lactation. Regression lines were fitted by best fit linear regression analysis. Data are adjusted to a common start point.

Weekly averaged milk yields and slopes for the second extended lactation are shown in figures 4.13 and 4.14. Statistical analysis of slopes adjusted for peak yields are in table 4.9. No difference was found in the standard deviation around the regression line between any of the treatments. Before rebreeding lactation was significantly more persistent in the second lactation, however, overall lactation was more persistent during the first lactation although the difference was not significant. Nutrition had no effect on lactation persistency as before rebreeding the high nutritional input group had a more persistent lactation in the first lactation (-0.1532± 0.02vs –0.1764±0.03) while in the second lactation it was the conventional input group which had the most persistent lactation (-0.1699±0.02 vs – 0.1941±0.02) although these differences were not significant. Increased milking frequency once again produced a significantly more persistent lactation.

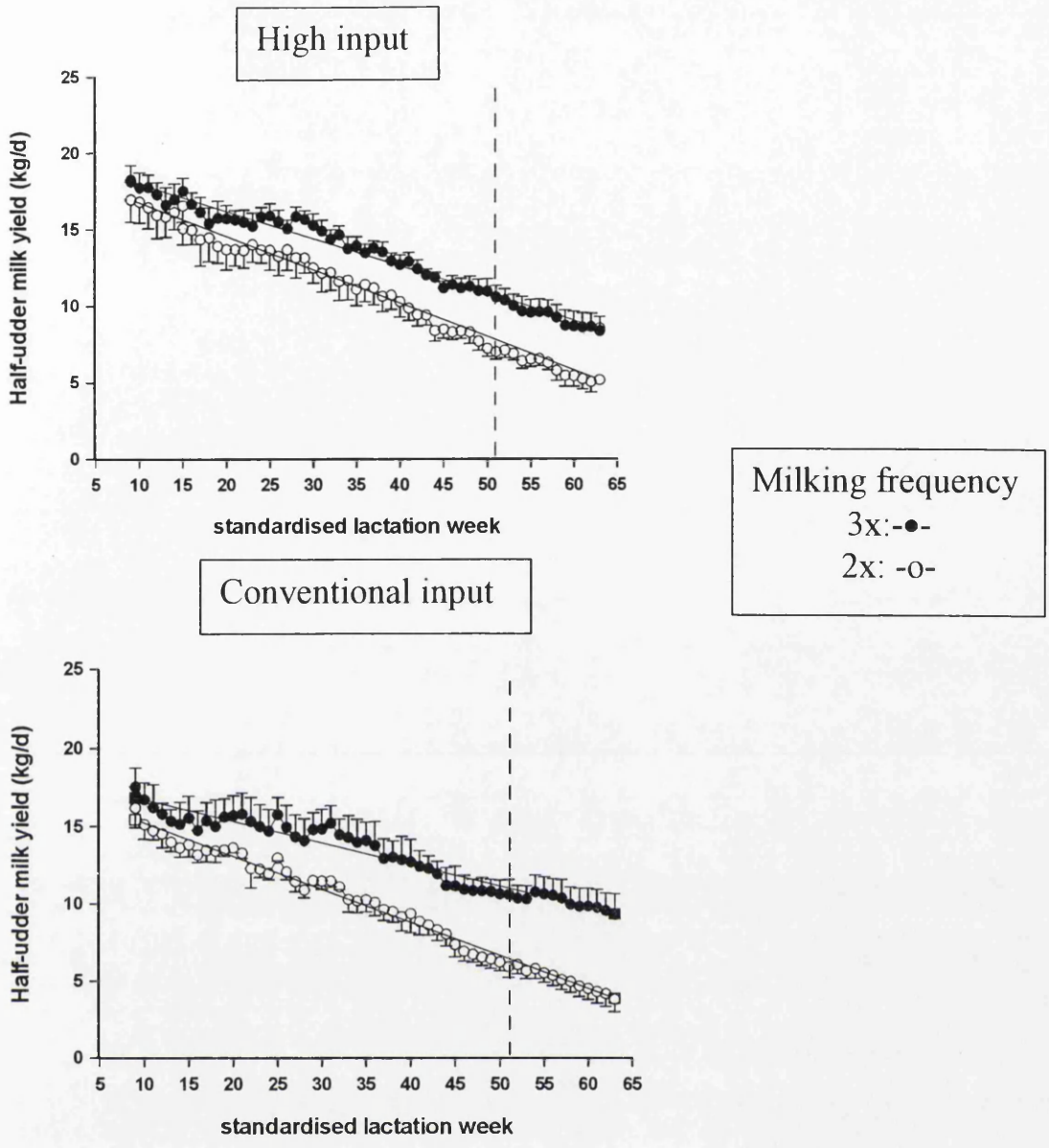


Figure 4.13. Effect of milking frequency and nutrition on milk yield during the spring calvers second extended lactation during lactation week 9 to pregnancy week 20. Treatment and analysis details as for figure 4.2. Dotted vertical line indicates the start of pregnancy week 20 for the first cow. Data after the dotted line is a combination of actual and extrapolated milk yields, the latter being for cows after pregnancy week 20.

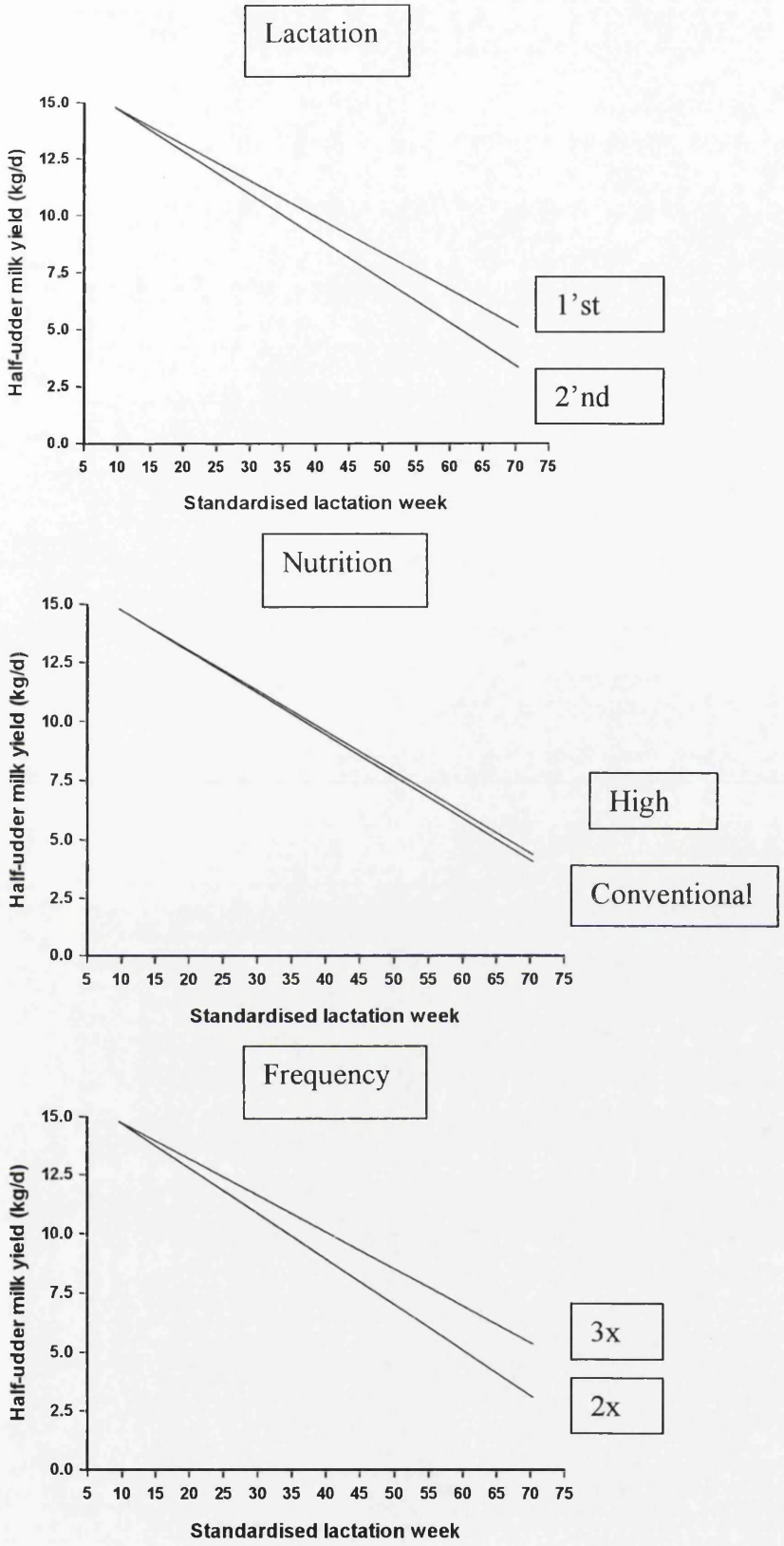


Figure 4.14. The effect of number of extended lactations, nutrition and milking frequency on lactation persistency. Regression lines were fitted by best fit linear regression analysis. Data are adjusted to a common start point.

Table 4.9. Lactation persistency during the first and second extended lactations, effect of milking frequency and nutrition between week 9 and pregnancy week 20. Treatment and analysis details as for table 4.3. Each individual animal received the same treatment during her first and second lactation.

Treatment	Slope 9-p20	SED	P-value	N
1'st extended lactation / 2'nd extended lactation				
1'st extended	-0.1637	0.018	n.s	22
2'nd extended	-0.1831			
Frequency				
Thrice	-0.1579	0.012	0.01	22
Twice	-0.1889			
Nutrition				
High	-0.1736	0.023	n.s	22
Conventional	-0.1732			

Comparisons of slopes before rebreeding and through out the entire lactation between thrice and twice daily milked udder-halves is shown in table 4.10. Lactation persistency was better overall in both twice and thrice daily milked udder-halves than before rebreeding during the first lactation. During the second lactation, however, lactation was more persistent before rebreeding than overall irrespective of milking frequency.

Table 4.10. Effect of milking frequency on lactation persistency during lactation week 9-33 and lactation week 9 to pregnancy week 20 during the first and second extended lactation. Treatment and analysis details as for table 4.2. P-value test for difference in persistency between period within frequency (paired t-test).

	3X		2X		SED
Period	1'st	2'nd	1'st	2'nd	
Lac 9-33	-0.2654	-0.0856	-0.2881	-0.1584	0.035
Lac 9-p20	-0.1550	-0.1608	-0.1725	-0.2054	0.021
P-Value	<0.001	0.03	<0.01	n.s	

Another way of analysing within-cow effects is to consider the absolute difference in milk yield between 3x and 2x milked udder-halves. This is shown from the start of treatment through to pregnancy week 20 in figure 4.15 for autumn, spring and the spring calvers second extended lactation. The absolute differences in yield increased as lactation progressed in all 3 calving groups. When the first ten weeks of treatment (lactation week 9-19) was compared with the last ten weeks of treatment (pregnancy week 11-20) absolute differences between 2x and 3x increased from 2.1±0.47 to 3.5±0.29 kg milk per day ($p=0.02$, paired t-test).

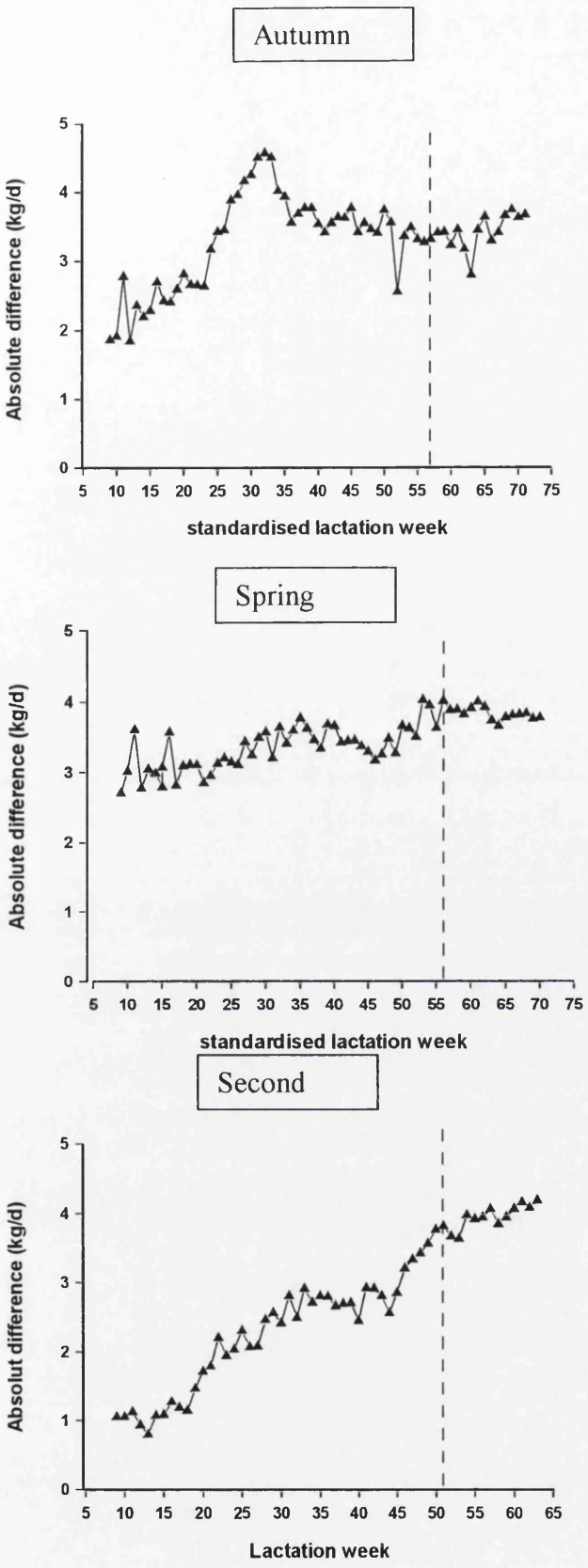


Figure 4.15. Absolute difference in milk yield between thrice and twice daily milked udder halves in autumn, spring and the spring calvers 2'nd lactation. Dotted vertical line indicates the start of pregnancy 20 for the first cow. Values thereafter are estimated and actual values

4.3.5 Predictors of lactation persistency

Figure 4.16 compares lactation persistency between the whole of the first and second extended lactations. Regression analysis showed no significant linear relationship between lactation persistency in the thrice daily milked udder-halves between the two lactations (correlation 0.43, n.s). This was not an effect of the frequent milking treatment being more or less effective because neither was any relationship found between twice daily milked udder-halves between two lactations (0.50, n.s). There were 3 heifers present which all showed better persistency during the first lactation in both 3x (–0.0608±0.005 vs. –0.1444±0.049) and 2x (–0.0758±0.01 vs. -0.1890±0.033). Two of the cows also demonstrated better persistency in the first lactation one showed the opposite but 5 had similar persistency in the two lactations. When heifers were excluded from the analysis there was still no significant linear relationship between persistency during the first and second extended lactation (correlation 3x: 0.50, 2x:0.61, n.s). Figure 4.17 compares lactation persistency between the first and second lactation in twice and thrice daily milked udder-halves before rebreeding. No significant linear relationship was found between the two lactations irrespective of milking frequency (3x:-0.31; 2x:-0.06). All of the twice daily milked udder-halves of the heifers showed less persistency during their first lactation, while 2 of the thrice daily milked udder-halves had a similar persistency. All cows showed better persistency in their second lactation in both twice and thrice daily milked udder-halves.

The relationship between peak milk yield and lactation persistency is shown in figure 4.18. Peak milk yield was a good predicte of lactation persistency in both thrice and twice daily milked udder-halves. The overall correlation in thrice daily milked udder-halves was –0.66 ($p<0.001$) and –0.78 in twice daily milked udder-halves ($p<0.001$). Correlations also

existed for the early lactation period although these were stronger in twice than thrice daily milked udder-halves (2x:0.63, $p<0.001$; 3x:-0.30, $p=0.08$).

Lactation length was positively correlated with lactation persistency as shown in figure 4.19. A longer lactation was associated with a more persistent lactation, however, the relationship was stronger in twice daily milked (correlation 0.53, $p<0.001$) than in 3x milked udder-halves (correlation 0.33, $p=0.05$).

A negative relationship was evident between the changes in body weight and body condition score both before rebreeding and during the entire lactation and lactation persistency as shown in table 4.11, however, none of these correlations were significant.

Table 4.11. Correlation coefficients between the change in body weight and lactation persistency during lactation week 9 to 33 and lactation week 9 to pregnancy week 20.

	Slope 2x	Slope 3x
Change in body weight 9-33	-0.094	-0.124
Change in body condition score 9-33	-0.266	-0.130
Change in body weight 9-p20	-0.263	-0.183
Change in body condition score 9-p20	-0.324	-0.311

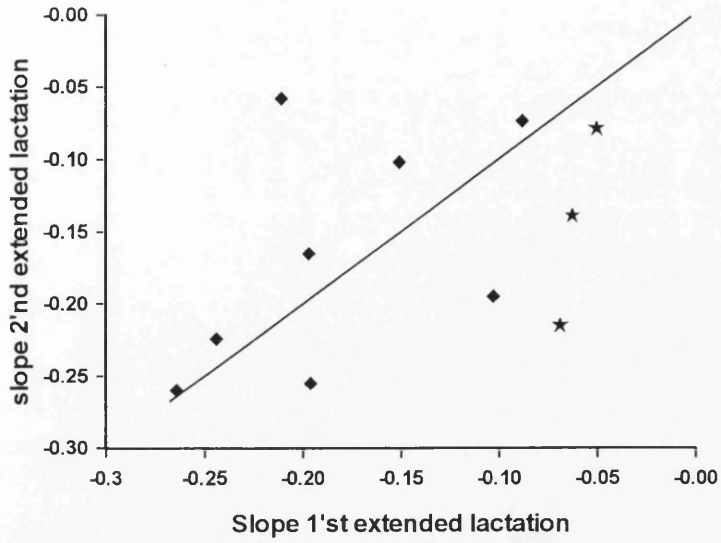


Figure 4.16. Comparison of lactation persistency during lactation week 9 to pregnancy week 20 between animals that completed two consecutive extended lactations. Diamonds are cows while stars are heifers.

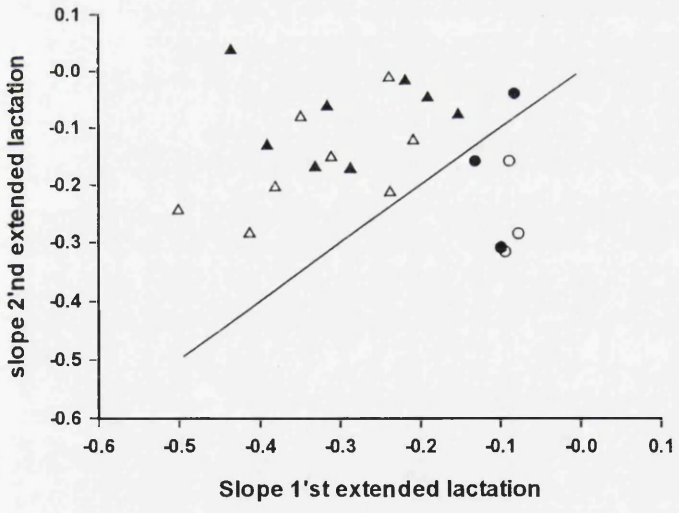


Figure 4.17. Comparison of lactation persistency in twice and thrice daily milked udder halves during lactation week 9 to 33 between animals that completed two consecutive extended lactations. Open squares are cows milked twice daily, closed squares are cows milked thrice daily. Open circles are heifers milked twice daily closed circles are heifers milked thrice daily.

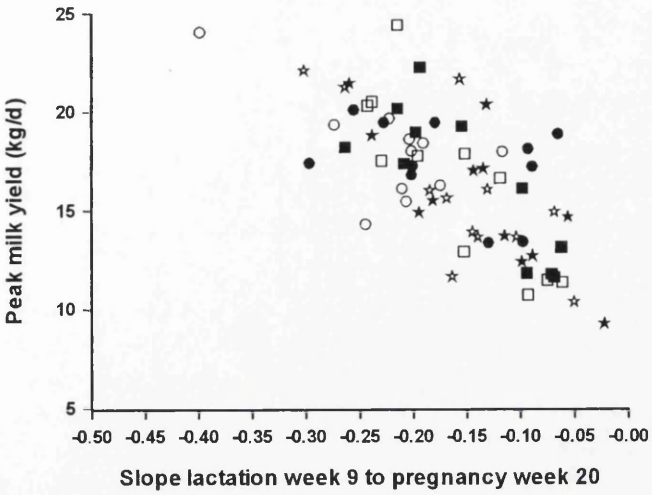


Figure 4.18. Comparison between peak milk yield and rate of decline in milk yield from lactation week 9 to pregnancy week 20. Squares are spring calvers milked thrice (closed) or twice (open) daily. Circles are cows completing a second extended lactation and milked thrice (closed) or twice daily (open). Stars are autumn calvers milked either thrice (closed) or twice daily (open).

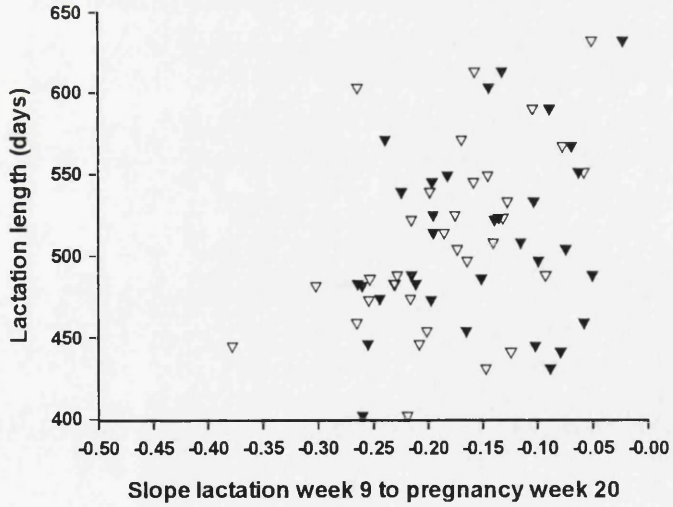


Figure 4.19. Comparison between lactation length and lactation persistency in thrice (closed triangles) and twice daily milked udder halves (open triangles) though the entire lactation.

4.3.6. Body weights and body condition score

Body weights and body condition scores for the spring calvers throughout lactation are shown in figure 4.20 and 4.21, respectively and for the autumn calvers in figure 4.22 and 4.23. Body weight increased in both the autumn and spring calvers as lactation progressed. Body weight and body condition score together with the change in body weight and condition score between standardised lactation week 9 and 33 and 9 and pregnancy week 20 are analysed in tables 4.12 and 4.13 respectively. Body condition score immediately before drying off is also shown. At standardised lactation week 9 no difference was found in body weight between the different treatment groups although body condition score was significantly higher in the spring calving group than the autumn calving group. During the first 25 weeks of treatment (lactation week 9 to 33) body weight increased more in the spring calvers (48.5kg±8.4) than in the autumn calvers (14.8kg±10.8, $P<0.03$). However, no difference was found in the increase in body condition score between the two seasonal groups. Body condition score increased by 0.4±0.2 points in the autumn calving group and by 0.17±0.17 points in the spring calving group. Nutritional input had no significant effect on changes in bodyweight or body condition score. Both tended to be greater in high input in spring calvers but lower in autumn calvers.

At pregnancy week 20 body weight had increased by 106.8kg±9.3 in the spring calving group and 145.3kg±15.9 ($p<0.001$, paired t-test) in the autumn calving group, while body condition score increased by 1.79±0.20 and 1.54±0.26 points ($P<0.001$), respectively. Indeed the trends were reversed compared to early lactation. Nutrition had no significant effect on the increase in body weight. Body condition score showed an interaction between season and nutrition. In the autumn calvers body condition score increased by 2.08±0.36 in the high nutritional input group and 1.5±0.31 in the conventional input group, while in the

spring calving group it was the conventional input group that gained the most points in body condition score (conventional 1.92±0.42 points/ high 1.17±0.26 points). From pregnancy week 20 to drying off body condition increased by a further 0.23±0.13 points in the spring calvers and 0.21±0.16 in the autumn calvers (increase $P<0.05$). However, at the time of drying off both seasonal groups had gained similar amount of body condition and nutrition did not have any effect on this increase.

A positive correlation between the change in body weight and body condition score was evident between standardised lactation week 9 and pregnancy week 20 (0.643, $P<0.01$). However, neither nutrition nor calving season had any effect on this relationship. During the first 25 weeks this correlation was influenced by season. Only in the autumn calving group was there any significant correlation between the change in body weight with the change in body condition score (0.674, $P<0.05$).

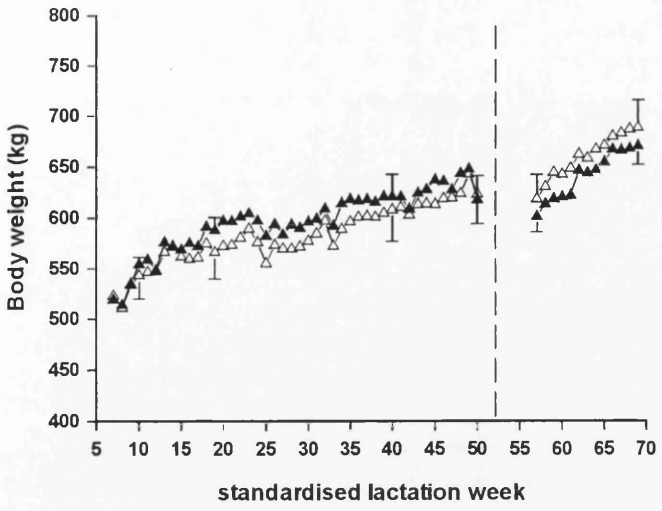


Figure 4.20. Average weekly body weights (kg) from lactation week 7 to lactation week 69 for spring calving cows. Closed triangles are the animals fed on high nutritional input while the open triangles are conventional fed animals. For clarity only selected representative standard errors are shown. Dotted vertical line indicates the start of pregnancy week 20.

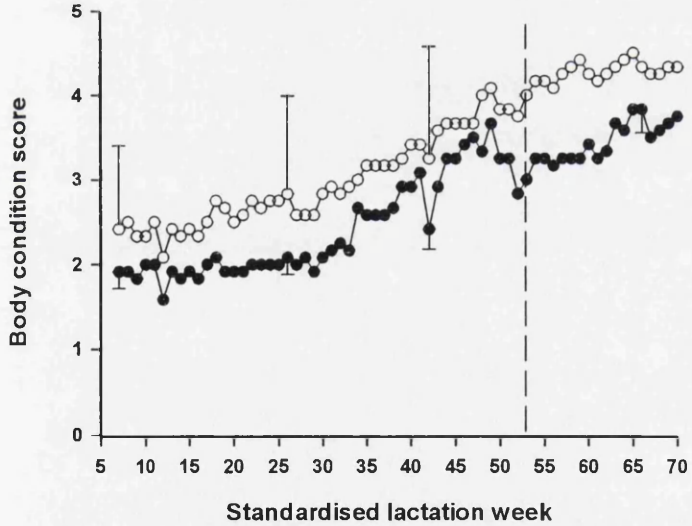


Figure 4.21. Average weekly body condition scores from lactation week 7 to lactation week 69 for spring calving cows. Closed circles are the animals fed on high nutritional input while the open circles are conventional fed animals. For clarity only selected representative standard errors are shown. Dotted vertical line indicates the start of pregnancy week 20.

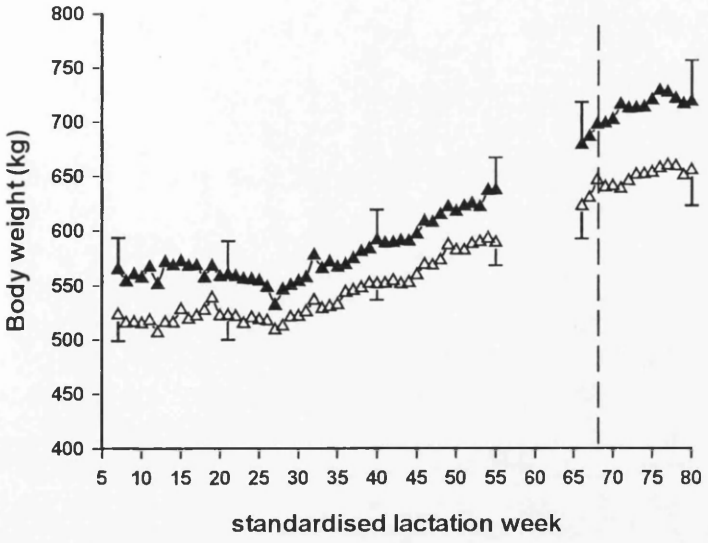


Figure 4.22. Average weekly body weights (kg) from lactation week 7 to lactation week 80 for autumn calving cows. Closed triangles are the animals fed on high nutritional input while the open triangles are conventional fed animals. For clarity only selected representative standard errors are shown. Dotted vertical line indicates the start of pregnancy week 20.

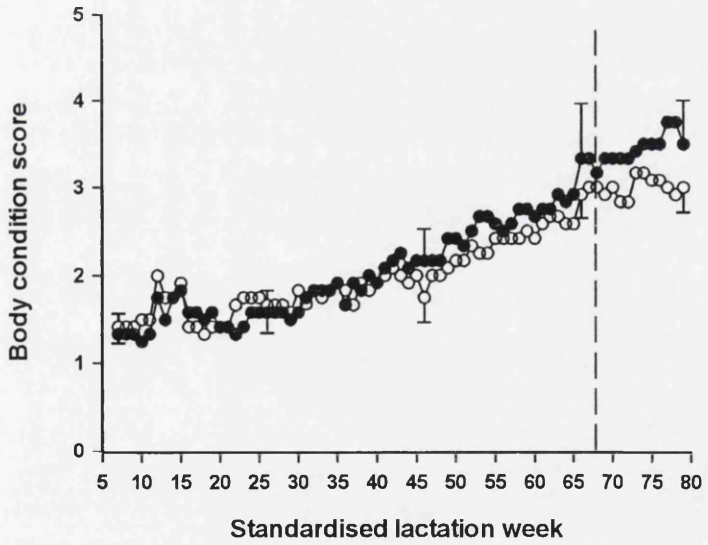


Figure 4.23. Average weekly body condition score from lactation week 7 to lactation week 80 for autumn calving cows. Closed circles are the animals fed on high nutritional input while the open circles are conventional fed animals. For clarity only selected representative standard errors are shown. Dotted vertical line indicates the start of pregnancy week 20.

Table 4.12. Effect of nutrition (high versus conventional input) and calving season (spring versus autumn/winter) on body weight gain. Values are body weight and body weight changes between lactation week 9 to 33 and lactation week 9 to pregnancy week 20 (P20). Data was analysed by anova.

	Spring		Autumn		P-Value		
	High input	Conventional input	High input	Conventional input	Nutrition	Season	Nutrition* Season
Lac 9	545±7.9	560±29.4	559±30.0	515±25.4	n.s	n.s	n.s
Lac 33	598±19.1	604±34.3	571±29.6	532±12.1	n.s	0.07	n.s
Increase 9-33	53±14.5	44±9.4	13±14.5	17±17.3	n.s	<0.05	n.s
P20	647±23.2	672±26.1	713±36	652±15.9	n.s	n.s	n.s
Increase 9-P20	102±18.5	112±5.5	154±26.6	136±19.4	n.s	0.06	n.s

Table 4.13. Effect of nutrition (high versus conventional input) and calving season (spring versus autumn) on body condition score. Values are body condition score and changes in body condition score changes between lactation week 9 to 33, lactation week 9 to pregnancy week 20 (P20) and lactation week 9 to drying off (dry). Data was analysed by anova.

	Spring		Autumn		P-value		
	High input	Conventional input	High input	Conventional input	Nutrition	Season	Nutrition* Season
Lac 9	2.1±0.21	2.3±0.42	1.3±0.10	1.5±0.15	n.s	<0.01	n.s
Lac 33	2.0±0.22	2.8±0.56	1.8±0.26	1.8±0.26	n.s	n.s	n.s
Increase 9-33	-0.1±0.26	0.5±0.16	0.5±0.20	0.3±0.24	n.s	n.s	n.s
P20	3.3±0.28	4.3±0.40	3.4±0.45	3.0±0.28	n.s	n.s	n.s
Increase 9-P20	1.2±0.26	1.9±0.42	2.1±0.36	1.5±0.31	n.s	n.s	0.06
Dry	3.7±0.32	4.3±0.40	3.8±0.49	3.0±0.40	n.s	n.s	n.s
Increase 9-dry	1.6±0.34	2.0±0.44	2.5±0.40	1.5±0.33	n.s	n.s	n.s

Body weights and body condition scores for the high and conventional input groups throughout the second extended lactation are shown in figure 4.24 and 4.25, respectively. Instead of comparing changes in body weight between lactation week 9, 33 and pregnancy week 20 as in previous comparison between spring and autumn calvers a comparison of changes between lactation week 20, 33 and pregnancy week 20 was performed. This was due to missing data during the first 20 weeks of lactation. Statistical analysis of the data is shown in table 4.14. Body condition score which also includes comparison between lactation week 9 and 33; lactation week 9 to pregnancy week 20 and lactation week 9 to immediately before drying off is shown in table 4.15.

In lactation week 9 bodyweight was significantly higher during the second compared to the first lactation. No difference was found in body condition between any of the treatment groups. During the first extended lactation body weight increased by 10.1±12.6 kg in the conventional input group and 1.1±9.5kg in the high input group between week 20 and 33 of lactation. Similar weight gains were observed in these animals during their second lactation (conventional 6.5±10.6 kg / high 25.7±8.3). Nutritional input did not influence the change in body weight gain. An interaction between nutrition and calving season however was evident in body condition score. During the first lactation more body condition point were gained in the conventional fed group (conventional 0.30±0.18 / high 0.04±0.14 points) while in the second lactation it was the other way around (high 0.38±0.06 points / conventional -0.05±0.17). This effect was not apparent when comparing the change in body condition score from lactation week 9 to 33. Body weight gain between lactation week 20 to pregnancy week 20 was 63±9.6 kg during the first lactation and 120±9.2 kg during the second lactation, an increase which was significant ($P<0.001$, paired t-test) but greater during the second lactation. However, the bigger increase in body weight

was not associated with a bigger increase in body condition score. The increase in body condition score was 1.7±0.32 during the first and 0.91±0.22 during the second lactation. Nutritional input had no effect on body weight gain or body condition gain during this period.

Body condition score increased significantly ($P<0.01$) by a further 0.25±0.14 points during the first lactation and 0.43±0.19 points during the second lactation between pregnancy week 20 and drying off. Overall there was no difference in the amount of condition score that had been gained throughout the lactation.

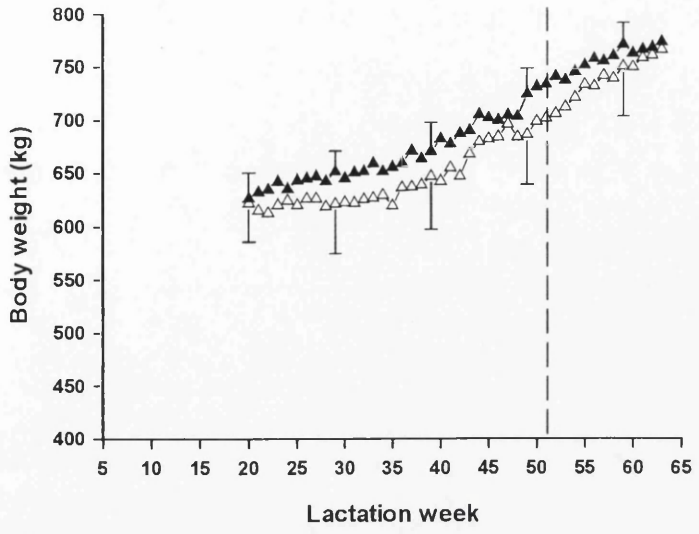


Figure 4.24. Average weekly body weights (kg) from lactation week 20 to 63 for animals completing their second extended lactation. Closed triangles are the animals fed on high nutritional input while the open triangles are conventional fed animals. For clarity only selected representative standard errors are shown. Dotted vertical line indicates the start of pregnancy week 20.

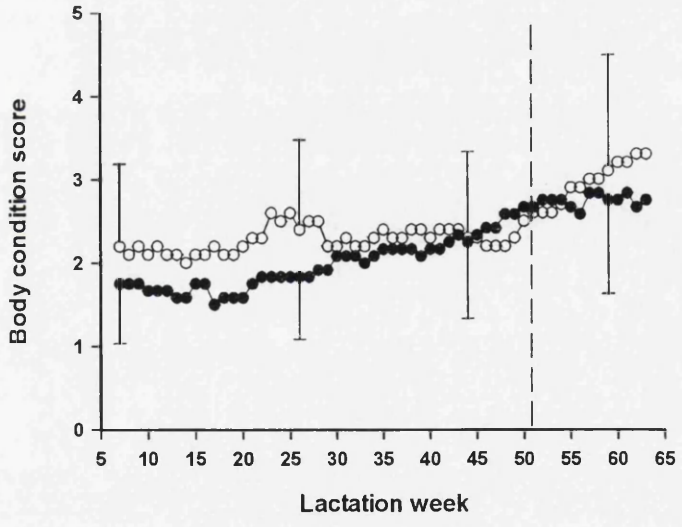


Figure 4.25. Average weekly body condition score from lactation week 7 to 63 for animals completing their second extended lactation. Closed circles are the animals fed on high nutritional input while the open circles are conventional fed animals. For clarity only selected representative standard errors are shown. Dotted vertical line indicates the start of pregnancy week 20.

Table 4.14. Effect of nutrition (high versus conventional input) and number of extended lactations (1'st extended lactation versus 2'nd extended lactation) on body weight gain. Values are body weight and body weight changes between lactation week 20 (Lac 20) and 33 (Lac 33) and lactation week 20, lactation week 9 to pregnancy week 20 (P20), lactation week 20 to pregnancy week 20, and pregnancy week 20 (P20). Data was analysed by anova comparing changes within cow between lactations.

	1'st extended lactation		2'nd Extended lactation		P-value		
	High input	Conventional input	High input	Conventional input	Nutrition	Lactation	Nutrition* Lactation
Lac 20	597±13.3	573±23	630±23	619±40.7	n.s	<0.01	n.s
Lac 33	598±19.1	583±32.7	655±23.3	625±45.2	n.s	<0.01	n.s
Increase 20-33	1±9.5	10±12.6	26±8.3	7±10.6	n.s	n.s	n.s
P20	647±23.2	651±19.2	754±14.3	733±53.7	n.s	<0.01	n.s
Increase 20-P20	50±15.0	78±7.7	124±12.9	115±14.2	n.s	<0.01	n.s

Table 4.15. Effect of nutrition (high versus conventional input) and number of extended lactations (1'st extended lactation versus 2'nd extended lactation) on body condition gain. Values are body condition and changes in body condition score between lactation week 9 (Lac 9) to 20 (Lac 20), lactation week 20 to lactation week 33 (Lac 33), lactation week 20 and pregnancy week 20 (P20), lactation week 9 and pregnancy week 20 and lactation week 9 to drying off (Dry). Data was analysed by anova comparing changes within cow between lactations.

	1'st extended lactation		2'nd Extended lactation		P-value		
	High input	Conventional input	High input	Conventional input	Nutrition	Lactation	Nutrition*Lactation
Lac 9	2.1±0.21	2.0±0.32	1.7±0.16	2.2±0.37	n.s	n.s	n.s
Lac 20	2.0±0.19	2.1±0.32	1.7±0.22	2.3±0.34	n.s	n.s	n.s
Lac 33	2.0±0.22	2.4±0.42	2.0±0.21	2.2±0.26	n.s	n.s	n.s
Increase 9-33	-0.1±0.26	0.4±0.15	0.3±0.08	0.1±0.26	n.s	n.s	n.s
Increase 20-33	0.0±0.14	0.3±0.18	0.4±0.06	-0.1±0.17	n.s	n.s	0.05
P20	3.3±0.28	4.1±0.46	2.8±0.38	2.9±0.44	n.s	0.04	n.s
Increase 9-P20	1.2±0.26	2.1±0.46	1.1±0.33	0.7±0.24	n.s	n.s	n.s
Increase 20-P20	1.3±0.40	2.0±0.50	1.2±0.34	0.6±0.20	n.s	n.s	n.s
Dry	3.7±0.32	4.2±0.46	3.1±0.35	3.5±0.45	n.s	n.s	n.s
Increase 9-dry	1.6±0.34	2.2±0.49	1.4±0.28	1.3±0.26	n.s	n.s	n.s

For cows undergoing an extended lactation and thereafter a conventional 12 month lactation body weight and body condition score is shown in figure 4.26 and 4.27. Table 4.16 and 4.17 is showing body weights and body condition score at week 9 of lactation, pregnancy week 20 and immediately before drying off.

Body weight increased significantly by 131±15.2 kg ($p<0.001$, paired t-test) from lactation week 8.9±1.1 to lactation week 67.4±2.9 (pregnancy week 20) in the extended lactation group. In the conventional 12 months lactation cycle group body weight had only increased by 21±15.2 kg ($p>0.05$, paired t-test) at pregnancy week 20, which was 29.9±2.0 weeks into their lactation. From pregnancy week 20 until the time of drying off body weight increased by a further 38±14.3 kg ($p<0.05$) in the extended lactation group and by 39±13.1 ($p<0.05$) in the conventional group. There was no difference in body weight gain between the two lactations during this period. Overall, the extended lactation cows therefore gained 169±20.5 kg from lactation week 9 to drying off while the conventional managed cows only gained 60±16.0 kg ($p<0.001$). As with body weight body condition score also increased significantly more in the extended lactation group up to pregnancy week 20 and thereafter body condition score increased with the same rate. Overall, the increase in body condition score was significantly greater during the extended lactation compared to the conventional lactation (2.1±0.33 points versus 0.47±0.20 points, $p<0.001$).

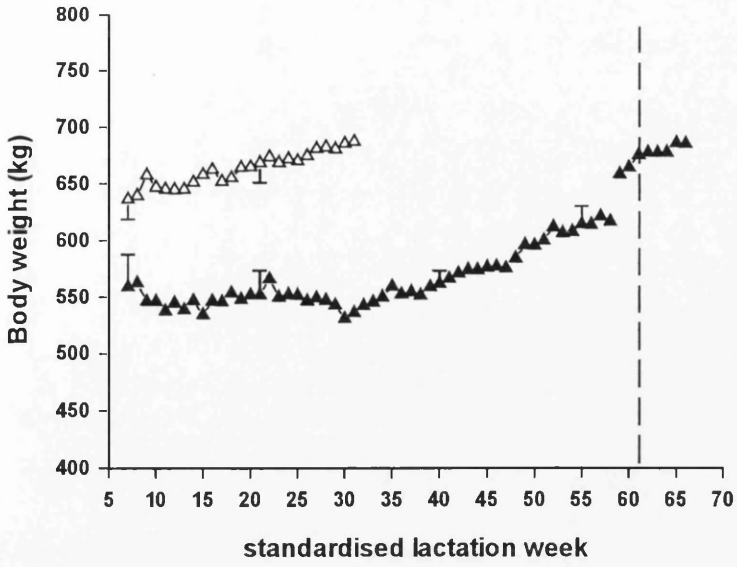


Figure 4.26. Average weekly body weights (kg) from lactation week 7 to lactation 66 during extended lactation (closed triangles) and lactation week 7 to 31 in these animals consecutive conventional lactation (open triangles). For clarity only selected representative standard errors are shown. Dotted vertical line indicates the start of pregnancy week 20.

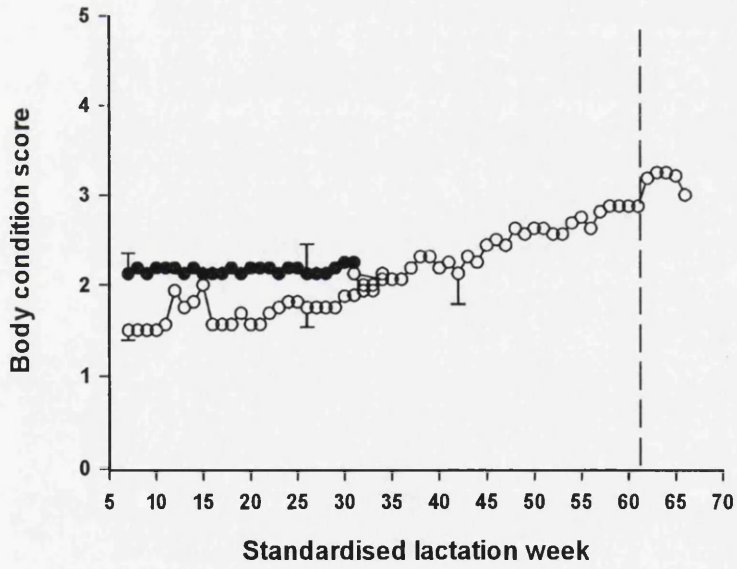


Figure 4.27. Average weekly body condition score from lactation week 7 to lactation 66 during extended lactation (closed circles) and lactation week 7 to 31 in these animals consecutive conventional lactation (open circles). For clarity only selected representative standard errors are shown. Dotted vertical line indicates the start of pregnancy week 20.

Table 4.16. Effect of extended lactation and a conventional lactation on body weight and body weight changes. Values are body weight and body weight changes between lactation week 9 (Lac 9) and pregnancy week 20 (P20) and lactation week 9 and drying off (Dry). Pregnancy week 20 was lactation week 67.4±2.9 for the extended lactation group and 29.9±2.0 for the conventional lactation. Data was analysed by anova comparing changes within cow between lactations.

	1'st extended lactation	2'nd conventional lactation	P-value
9	542±22.8	652±18.6	<0.01
P20	672±14.6	674±19.0	n.s
Increase 9-P20	131±15.2	21±15.2	0.001
Dry	711±21.3	712±18.4	n.s
Increase 9-dry	169±20.5	60±16	0.01

Table 4.17. Effect of extended lactation and a conventional lactation on body condition score and body condition changes. Values are body condition score and changes in body condition score between lactation week 9 (Lac 9) and pregnancy week 20 (P20) and lactation week 9 and drying off (Dry). Pregnancy week 20 was lactation week 67.4±2.9 for the extended lactation group and 29.9±2.0 for the conventional lactation. Data was analysed by anova comparing changes within cow between lactations.

	1'st extended lactation	2'nd conventional lactation	P-value
9	1.5±0.09	2.2±0.27	0.05
P20	3.2±0.28	2.4±0.34	0.001
Increase 9-P20	1.7±0.30	0.2±0.23	<0.01
Dry	3.6±0.34	2.7±0.33	<0.01
Increase 9-dry	2.1±0.33	0.5±0.20	<0.01

4.4. Discussion

The aim of this study was to determine the effect of milking frequency, calving season and nutrition on milk production and especially lactation persistency during extended lactation. Any possible relationship between body weight and body condition score with lactation persistency was also investigated.

The response to thrice daily milking was as great as 50±5.9% during the first extended lactation. During the following lactation the response to thrice daily milking was 24.6±6.05% in the extended lactation group and 37.2±13.2% during the conventional lactation This was slightly lower compared to the response in the previous lactation (extended 42.6±9.09%, conventional 47.4±7.4%) although this difference was not significant. The response to thrice daily milking was therefore high compared to those previously reported. Other studies where thrice daily milking was practised for an entire lactation has reported increases ranging from 6 to 25% (DePeters *et al.*, 1985; Amos *et al.*, 1983). One study has investigated thrice daily milking during a prolonged calving interval on 18 month. In this study thrice daily milking increased milk yield by 10-15% compared to twice daily milked animals (Ratnayke *et al.*, 1999).

It is well established that the response to frequent milking is a local one operating only within the glands to which the treatment is applied (Hillerton *et al.*, 1990). To reduce variation of between animal origin we, therefore, used a half udder design to study the effect of milking frequency. One could argue that a greater response to frequent milking would be observed under such circumstances. Morag, (1973) showed that the increase in milk yield when milking thrice daily is not affected by milking frequency of the other udder half. We have obtained a similar result. When we milked dairy cows four times a

day and after a week reduced milking frequency to twice daily in half of the udder there was a decrease in milk yield from this half but no change in production from the continuing four times daily milked udder-halves (unpublished observation). However a different result was obtained when animals were treated with the combined galactopoietic stimuli of four times daily milking, BST and thyroxine prior to and during the reduction in milking frequency in half of the udder. Under these conditions a compensatory increase equivalent to the decrease in milk yield from the two times daily milked udder-halves was observed in the udder-halves that continued on four times daily milking (Sorensen & Knight, 1999). The hormonal treatment administered in this experiment is known to increase the amount of nutrients directed towards milk production (Bauman, 1992; Davis *et al.*, 1988). A compensatory increase in milk yield when using a half udder design is, therefore, possible although only when nutrient repartitioning is stimulated at the same time. Hence it is likely, that had the whole udder of these animals been milked thrice daily, the response would have been just as great per gram of tissue as it was on a half udder basis.

Another factor that can affect the degree of response observed to frequent milking is cisternal storage space. Cows with small cisternal storage space have a bigger response to an increase in milking frequency than cows with large cisternal capacity (Dewhurst & Knight, 1994). The immediate effect of an increase in milking frequency on milk yield is believed to be due to more frequent removal of a specific autocrine inhibitory protein (FIL, feed back inhibitor of lactation). FIL is secreted by the alveolar cells and acts upon the alveolar cells to inhibit milk secretion (Wilde *et al.*, 1997a) so once the milk has been moved away from the alveolar secretory cells and begins to accumulate in the cistern, FIL effectively becomes inactive (Dewhurst & Knight, 1993). Therefore cows with large

cistern are more tolerant of infrequent milking, while those with small cistern (and therefore with relative more milk stored in alveolar areas) respond best to increases in milking frequency, supposedly due to more frequent removal of FIL.(Dewhurst & Knight, 1994).

Increased milking frequency did not only increase milk yield but it also significantly and consistently improved lactation persistency, which suggests that the mammary cell population was better maintained. In the longer term increased milking frequency has been shown to increase cell proliferation (Wilde *et al.*, 1987) and reduce apoptosis (Li *et al.*, 1999) and it is therefore not unlikely that lactation persistency was increased due to better maintains of the mammary cell population when milking thrice daily. During once daily milking Li *et al.* (1999) found that after an initial accelerated loss of cells in the mammary gland cell loss returned to a similar rate as for thrice daily milked glands, suggesting that lactation persistency did not differ between once and thrice daily milked glands. However thrice daily milking must have had a continuing effect on cell turnover in our experiment otherwise lactation persistency would not have been improved throughout the entire lactation.

Calving season was the treatment which had the largest effect on persistency before rebreeding. This was likely to be related to turn out to grass which because of the higher nutritional value of fresh spring grass than the winter ration fed in this experiment led to increases in milk production. The spring calving cows grazed before peak milk yield a period which was not included in the persistency analysis and in late lactation. During the first grazing period there was no visible effect on milk yield. However, it is likely that the effect was masked by the rise that occurs in milk yield at this stage of lactation anyway.

Furthermore as a result of summer grazing peak milk yield might have been higher than could have been achieved on the winter ration. The autumn calving cows grazed from lactation week 23 as a result milk yield increased and thereby prevented the decline in milk yield which was beginning to show at that stage (figure 4.2). This is also likely to explain why persistency was greater before rebreeding in the spring calving cows second extended lactation. These animals re-calved during autumn/winter time and would therefore have had the benefit of spring grass at a stage of lactation where milk yield is decreasing. From the figures illustrated in this thesis it is not possible for the reader to make any conclusion on the effects of grazing on milk yield and lactation persistency in this lactation, because animals were submitted to treatment as they reach lactation week 9. However when graphs were drawn for each individual animals again a clear effect of fresh early spring grass was evident (results not shown). Another obvious difference between the two consecutive lactations is an increase in parity. However, it is unlikely that the increased persistency during the second lactation was due to an increase in parity since lactation persistency usually decreases with an increase in parity (Auran, 1974). However, parity might be partly responsible for the observation that milk production was higher during the second compared to the first extended lactation., since an increase in milk yield with an increase in parity is a common phenomena (Auran, 1974). An increase in food quality at a very important stage of lactation (beginning of the decline after peak yield) might not be the only factor responsible for the increase in milk yield associated with turn out to grass. An increase in photoperiod has been shown to increase milk yield (Tucker, 1985). The physiological explanation for this is unknown. Plasma levels of prolactin increases as does IGF-1 but there is no effects on plasma levels of GH, glucocorticoid, thyroxin or insulin (Peters & Tucker, 1978). So far there is no clear evidence that ruminant milk yield is responsive to prolactin. Furthermore, there is no evidence to suggest that milk yield is

responsive to plasma levels of IGF-1 (Davis *et al.*, 1989). It was not possible in this experiment to distinguish the effect due to photoperiod from the ones due to the increase in food quality on milk yield at turnout to grass. However in dairy systems where the winter diet is of better nutritional value to the animal than fresh spring grass a decrease in milk yield is associated with turn out to grass (Phipps, R. personal communication). It is therefore quiet clear that an increase in day length can not override the dietary changes associated with turnout to grass.

The effects of calving season on lactation persistency were not evident through the entire lactation. Nine weeks after summer grazing was started a sharp decline in milk yields occurred which was probably due to a decrease in grass quality and grass shortage. Buffer feeding with sugar beet pulp was initiated 4 weeks after the decrease started but this was too late since absolute yield did never recover. One explanation could be that because the nutritional value of sugar beet pulp is not as high as fresh spring grass the mammary gland is simply having fewer nutrient available for milk synthesis, however, it is also possible that within the four weeks before buffer feeding was administered the mammary gland had regressed i.e. had lost some of its potential for milk synthesis. Research into the effect of nutrition on mammary cell function in ruminants has been limited. We recently found that cows fed a low diet energy concentration during early lactation had a lower activity of key enzymes involved in milk synthesis and less proliferation of mammary epithelial cells compared to tissue from animals on a high energy diet. However, the energy concentrations studied in this experiment represented extremes. The high concentrate diet consisted of 75% concentrate and only 25% whole crop barley silage, while the low concentrate diet consisted of 25% concentrate and 75% whole crop barley silage (Sorensen *et al.*, 2000). Severe under feeding has detrimental effects on milk yield and lactation

persistency (Smith *et al.*, 1978) suggesting that under feeding does affect mammary function. Supplementary feeding on the other hand has not been shown to affect lactation persistency once the response in milk yield is established (Broster & Broster, 1984). The effect of supplementary feeding in this experiment was likewise very inconsistent and did not exist in the longer term. A 3 weeks period is usually regarded as the time it takes for a nutritional response to exert its full effect on milk yield. It is not unlikely that cell proliferation could take place within this period however it must be a short term burst of proliferation since if it was a continuing process one would have expected to see long term effect on lactation persistency. This situation would presumably only be relevant if the potential of the mammary gland was fully utilised. The metabolic activity of the mammary epithelial cells do not change as lactation progress and an increase in nutrients available for milk synthesise under circumstances where the potential for milk yield was not fully utilised would therefore only result in short term increases in lactation persistency. Further research is required to determine the effect of nutrition on mammary function.

The decrease in grass quality and grass shortage also had consequence for the spring calvers. In the previous chapter we showed that in the spring calving group there was a higher proportion of animal in the conventionally fed groups than the high input group not completing their lactation, but drying them self off before 6 weeks prior to calving. This was clearly affected by the decrease in grass quality and/or shortage of grass. At this stage the conventional fed animals were not receiving any concentrate in the parlour, while the supplemented animals were still receiving 3 kg of concentrate, which might have helped them cope better. Buffer feeding was not successful in repairing the loss in commitment to lactation caused by the loss of a good quality diet.

During the spring calving cows second extended lactation buffer feeding with sugar beet pulp was initiated before grass shortage and grass quality became a problem to milk yield and as a result the decrease in milk yield was not as drastic but more gradual than observed in the autumn calvers. Overall, this did not result in a better persistency during the second compared to the first extended lactation but vice versa. This suggests that the effect of nutrition on lactation persistency is short term. The question that arises is would a continuing positive effect on lactation persistency have existed if the animals were allowed fresh spring grass or a diet of higher quality than the one fed in this experiment all year round? We don't know. However it would require a continuing effect on cell turnover in the mammary gland and at the moment it is not clear whether this is the case.

It is well established that the milk yield of the cow responds to changes in energy supply. Thus an increase in concentrate input will result in an increase in milk yield (Broster & Thomas, 1981) as was observed in this experiment. However the response in milk yield to increased concentrate input declines as lactation progresses, presumably due to the progressive rise in energy being partitioned into body tissues (Broster & Thomas, 1981) as a result of the endocrine changes that occur as lactation progresses (Schams *et al.*, 1991). The mammary gland regresses as lactation progresses and the change in nutrient partitioning could therefore simply be due to fewer nutrients needed for milk synthesis. This is very unlikely because administration of GH increases milk production in the first instance simply by diverting more nutrient towards the mammary gland (Bauman, 1992) and a ready available capacity to process these nutrients must therefore be present. The body tissues must, therefore, take precedence over the mammary gland for nutrients as lactation progresses. How this mechanism is regulated is unknown. There was no evidence in this experiment to suggest a higher increase in body weight or body condition score with

supplementary feeding indicating that the extra concentrate was converted into milk. An increase in concentrate input is, therefore, not necessarily associated with tissue deposition. However, it is important to keep in mind that besides from monitoring in parlour concentrate, food intake was not measured in this experiment. Increases in body weight and body condition score were negatively correlated with lactation persistency. Although not significant, this indicates that nutrient partitioning between mammary and non-mammary tissues is an important factor in maintaining lactation persistency.

Other factors such as peak milk yield and pregnancy affected lactation persistency. Peak milk yield and lactation persistency were negatively correlated, which has also been observed by Auran (1974) and Danell (1981). Furthermore, Auran (1974) found a negative relationship between parity and lactation persistency with the main effect being seen between first lactation compared to subsequent lactations. Due to the low number of first lactation animals and the spread of animals in subsequent parities no attempt was made to statistically analyse the effect of parity within lactations. There was no significant difference in lactation persistency between animals completing two consecutive lactations suggesting that parity had no effect on lactation persistency. Furthermore, the lack of any significant correlation in persistency within cow between the two lactations could not be explained by a significant difference in milk yield in any of the two udder-halves. In the thrice daily milked udder-halves peak yield was 0.01±1.25 kg/d higher during the second lactation and in the twice daily milked udder-halves peak yield was 0.29±1.23kg/d higher during the second lactation (paired t-test, heifers excluded). However, factors such as management and calving season could mask the effect of parity.

Lactation persistency declines with age within lactation number and it was presumed that this was related to peak yield. However, the relationship is not straightforward since age accounted for around 40% of variation in milk yield in early lactation but less than 2% in the last 3 months (Auran, 1974). No attempt has been made to hypothesise or investigate why peak yield affects lactation persistency. Evidence is accumulating that de-differentiated mammary epithelial cells can re-differentiate and secrete milk in the subsequent lactation, in other words, cells can be carried over from one lactation to another (Fowler *et al.*, 1990; Li *et al.*, 1999). The question is whether the re-differentiated cells have a shorter life or maybe a reduced secretory capacity than newly proliferated cells. If the answer to the question was yes this could explain why the heifer lactation is more persistent than a mature cow lactation because the heifer mammary cell population simply undergoes less apoptosis as lactation progress due to the entire new generation of cells. However, heifers are still growing and so most probably are their mammary glands, which might also contribute to their more persistent lactation. However, it could also explain why peak yield is negatively correlated with lactation persistency. Peak yield will depend on the number of cells carried over, however, the life expectancy of these cells is lower and lactation persistency will decrease as these cells die. In reality it is likely to be more complicated than this. Peak yield is also influenced by food intake and the animals ability for body tissue mobilisation (Garnsworthy, 1988). Animals which are over conditioned at calving (body condition score >4.0), may be at risk for lower milk yield and increased health problems since higher body condition scores at calving is generally associated with lower food intake and a greater loss of body condition during early lactation. In order to achieve high peak milk yields farmers are advised to calve their cows at body condition scores around 3-3.5. In Garnsworthy (1988) a summary of 11 studies looking at the effect of body condition score at calving is presented. Within a range of 2 to 4 in body condition

score at calving only small insignificant effects were found on milk yield. Assuming that body condition score did not change between drying off and calving in all of our studied lactations body condition score was higher than recommended in the spring calving group and in the autumn calvers on high nutritional input. In the second extended lactation body condition score at drying off was between 3 and 3.5. Because body condition score was not measured at calving and during early lactation no conclusion can be made as to whether or not body condition at calving influenced milk yield. Furthermore whether animals calving at higher condition score lost more body condition in this experiment remains unknown, however, milk yield was certainly not affected since milk production both during the first 25 weeks of treatment and overall was significantly higher during the second lactation. Extended lactation cows did however gain more body condition during lactation than did the animals on conventional lactation. This is not unexpected since the extended lactation cows spent a longer time at more moderate production levels.

Recurring pregnancy has a negative effect on milk yield. This study confirmed the inhibitory effect apparent from pregnancy week 20 as found previously (Coulon *et al.*, 1995). In our study the inhibitory effect was dependent on stage of lactation; cows which conceived earlier had poorer persistency although due to the small number of animals and the distribution of animals into stage of lactation at conception week this conclusion must be taken with caution, although the observation agrees with those of Auran (1974) but not Coulon *et al.* (1995).

The absolute decrease in yield during the last third of gestation was higher in thrice than twice daily milked udder-halves, as was persistency. However, the percentage decrease was equal irrespective of milking frequency. Given that the total amount of secretory tissue

was likely to be higher in thrice daily milked udder-halves this suggests that an equal amount of tissue returns to a non secretory state in the two udder-halves. In high producing cows the absolute decrease but not proportional decrease in milk yield is higher than in cows producing a moderate amount of milk (Coulon *et al.*, 1995). High yielding cows certainly do have a higher amount of secretory tissue (Sorensen *et al.*, 1998) once again suggesting that an equal amount of tissue returns to a non secretory state.

The inhibitory effect of pregnancy on milk yield is unlikely to be due to competition between nutrients used for lactation and foetal growth, because not until the last 2 month of pregnancy when the animal is usually dry does foetal demand for specific mammary used nutrients become of any significance (Bauman & Currie, 1980). The inhibitory effect of pregnancy has, therefore, been linked to hormonal changes and in particular the secretion of oestrogen from the placenta. Athie *et al.* (1996) showed that administration of estrogen at the last milking before drying off increased the involution rate of mammary tissue. Oestrogen production does not only increase from the developing foetus but also locally within the mammary gland (Maule Walker *et al.*, 1983) at the latter stages of pregnancy. The significance of this increase in local oestrogen production within the mammary gland on mammary tissue involution remains to be investigated. Due to the influence of pregnancy on lactation persistency from lactation week 20 and onwards we decided to use pregnancy week 20 as our cut-off point when analysing the effect of milking frequency, nutrition, calving season and consecutive lactations on lactation persistency.

Wassell *et al.* (1998) estimated that for extended lactations to be just as profitable as the recommended 12 month calving interval, target yield in 18 months for a 6000l herd should

be 8500kg, while an 8000l herd should aim for 11500kg milk. The model is based on the assumption that all animals completed four lactations, health cost are equal for 12 and 18 month lactations and that milk price and concentrate costs are constant throughout the year. Finally, the model assumed that extended lactation was achieved by pushing the lactation curve upwards without altering lactation persistency. The Hannah herd is a 6000l herd and all except one of our extended lactation groups produced 8500kg in 18 month. The exception was the twice daily milked udder-halves on the conventional fed animals. The calculations are making the assumption that the response to thrice daily milking on a half udder basis equals that on a whole udder basis had the whole udder been milked thrice daily. However, it needs to be taken into consideration that thrice compared to twice daily milking adds an extra cost. On the other hand, money is expected to be saved on the health account. Annual yields calculated for animals that completed two consecutive extended lactations were all higher than those in the Hannah herd. However, none of the treatment groups, even when assuming that the response in milk yield to thrice daily milking on a half udder basis would equal the response on a total udder basis, reached as high an annual milk production as the animals who proceeded into a conventional lactation after their extended lactation. To make any unreasonable conclusion on the economics of our extended lactation proper calculations and assumptions need to be made. No attempt will be made to do so in this thesis but our data have been sent to the Scottish Agricultural College for full economic analysis.

In summary increased milking frequency consistently improved lactation persistency. Nutrition and calving season showed no effect on lactation persistency overall, however, based on the changes in milk yield observed with summer grazing it is clear that a combination of long-term support for cell survival (milking frequency) with continuous

day to day nutritional support for secretion will result in the best persistency being achieved. All year round housing would be a way of achieving nutritional consistencies because nutrition can be more closely controlled. However, the general trend of public opinion is against continuous housing. It appears on the basis of these experiments that careful management will be required especially during summer grazing.

Chapter five

Relationship between endocrine factors and lactation persistency

5.1 Introduction

There are two ways in which the endocrine system could affect lactation persistency. One is through effects on the dynamic of the mammary epithelial cell population the other through nutrient partitioning, although this might only be a short term effect as discussed in chapter 4.

Nutrient partitioning between mammary and non-mammary tissues depends largely upon the balance between hormones such as GH and insulin, which are involved in regulation of metabolic processes. The role of insulin in the control of metabolism is essentially anabolic. Thus the hormone is associated with processes diverting energy away from milk synthesis and toward body tissue (Vernon, 1980). As yet no direct effect of insulin on mammary epithelial cells has been claimed although the receptor is present in the mammary gland. GH on the other hand is positively correlated with milk secretion. Levels of GH are high in early lactation and fall during lactation as milk yield is dropping (Convey, 1973). Milk yield can be increased by GH (BST) administration by as much as 40% (Bauman, 1992). In the majority of trials BST pushes the lactation curve upwards and does not alter the shape of the curve (Bauman, 1992). However BST treatment has been shown to increase mammary cell proliferation (Capuco & Byatt, 1998) and positive effects of BST on lactation persistency have sometimes been found (Van Amburgh *et al.*, 1997). Therefore, GH is not only important in sustaining nutrient partitioning to the mammary gland but also in sustaining a viable mammary cell population.

Prolactin is important for establishing lactation but has never been given much credit for any role in maintenance of lactation (Cowie *et al.*, 1964). This is primarily due to the lack of effects on milk production when circulating prolactin levels are reduced by administration of bromocriptine (Hart, 1973). However, this issue is further complicated by the fact that the mammary gland is capable of producing it's own prolactin.

The aim of this study was to investigate possible relationships between plasma levels of GH, insulin, IGF-1 and prolactin and lactation persistency. Since milking frequency was studied in a within animal design, it was not possible to examine the effect of milking frequency on endocrine factors. It must be stressed that it was not the aim of this experiment to identify mechanisms by which hormones affected lactation persistency but rather to investigate trends between endocrine factors and lactation persistency.

5.2 Material and methods

5.2.1 Experimental design

The overall design of the experiment was described in chapter 2.

5.2.2. Blood sampling

Blood samples were collected on a weekly basis at 9 o'clock, approximately 2-3 hours after the morning milking. This sampling regime was maintained throughout the experiment. Processing of samples and determination of the plasma concentration of insulin, GH, IGF-1 and prolactin is described in chapter 2. Although samples were obtained from the spring calving group, the winter calving group and the spring calving groups second extended lactation plasma concentrations were only measured in the spring and winter calving group.

5.2.2 Statistical analysis

Differences in absolute plasma levels of the measured hormones as a result of feeding level or calving season were evaluated by calculating the mean value for each individual animal between lactation week 9 to 33, lactation week 34 to pregnancy week 20 and pregnancy week 9 to pregnancy week 20. The obtained mean values were then analysed by anova.

Changes in plasma levels as lactation progressed were analysed by fitting best fit linear regression lines from lactation week 9 to 33, and lactation week 34 to pregnancy week 20 and these were then analysed by anova.

The reason for choosing the above time points was based on the previous chapter where lactation persistency was investigated between lactation week 9 to 33 and 9 to pregnancy week 20. Possible relationships between lactation persistency and changes in the measured endocrine factors were investigated by linear regression analysis using the individual regression lines for lactation persistency in both twice and thrice daily milked udder halves and endocrine changes.

To evaluate if prolactin increased with an increase in daylight and decreased with a decrease in daylight best fit linear regression analysis was performed from mid June to October (decrease in daylight) which was a different year for the two groups and March to June within the same year for the two groups. To investigate whether plasma levels of prolactin were related to daylight or stage of lactation the mean plasma concentration of prolactin for each individual animal was calculated for January where daylight is short and June where daylight is at it longest. In the spring calvers January corresponded to lactation week 29-32 (mid lactation) and in the autumn calvers January corresponded with lactation

week 9-11 (early lactation) and lactation week 60-63 (late lactation). June on the other hand corresponded with lactation week 9-10 (early lactation) in the spring calvers and lactation week 59-62 (late lactation). In the autumn calvers June was in mid lactation (lactation week 29-32). All lactational stages (early, mid and late lactation) were therefore represented in January and in June. The data were then analysed by anova. All values are reported as mean±s.e. unless otherwise stated.

5.3. Results

Plasma GH concentrations for the spring and autumn calving groups on either high or conventional nutritional input are shown in figure 5.1. Table 5.1 shows the mean plasma concentration of GH between lactation week 9 to 33, lactation week 34 to pregnancy week 20 and lactation week 9 to pregnancy week 20. At all times plasma levels of GH were significantly higher in autumn than spring calvers but there was no effect of nutrition. Changes in the concentration of GH as lactation progressed are illustrated in figure 5.2, whilst table 5.2 summarises the statistical analysis. Nutrition had no effect on changes in GH levels as lactation progressed and figure 5.2 does, therefore, only illustrate the two seasonal groups. During the early part of lactation (lactation week 9-33) GH concentration increased in the autumn calving group and decreased slightly in the spring calving group. However from lactation week 34 to pregnancy week 20 GH levels decreased in the autumn while only small changes occurred in the spring calving group. Overall plasma levels of GH dropped to the same extent as lactation progressed in the 2 seasonal groups.

GH concentrations were positively correlated with total milk yield (0.38±0.06) and with milk yield from both twice (0.38±0.06) and thrice daily milked udder halves (0.37±0.06). There was no significant linear relationship between lactation persistency either during

lactation week 9-33 or lactation week 9 to pregnancy week 20 and average plasma levels of GH during this same period. However there were tendencies for changes in GH to have a significant linear relationship with lactation persistency during the first part of the lactation as shown in figure 5.3. Correlation between lactation persistency and changes in growth hormone levels were 0.48 ($P< 0.05$) in thrice daily milked udder halves and 0.36 ($P=0.08$) in twice daily milked udder halves. However, overall (lactation week 9 to pregnancy week 20) no linear relationship was found between lactation persistency in thrice daily milked udder halves and changes in plasma levels of GH (correlation 3x: 0.14, n.s) although a significant relationship still existed between lactation persistency in twice daily milked udder halves and changes in plasma levels of GH (correlation: 0.43, $P<0.05$).

Plasma concentrations of IGF-1 of spring and autumn calvers are shown in figure 5.4 and table 5.1 shows mean plasma concentration of IGF-1 at the previous mentioned periods. Neither nutrition nor season affected plasma concentrations of IGF-1. IGF-1 concentrations increased from lactation week 9 to 33 in the autumn calving group while a slight decrease was found in the spring calvers a difference which was significant as shown in table 5.2 and figure 5.5. During the latter part of lactation there was no effect of season on IGF-1 although a small increase was evident in both groups. Overall (lactation week 9 to pregnancy week 20) however the IGF-1 pattern differed between the spring and autumn calvers. While IGF-1 increased as lactation progressed in the autumn calvers a small decrease was found in the spring calvers. Nutrition had no effect on IGF-1 at any point.

No significant correlation was found between changes in plasma IGF-1 concentration and lactation persistency.

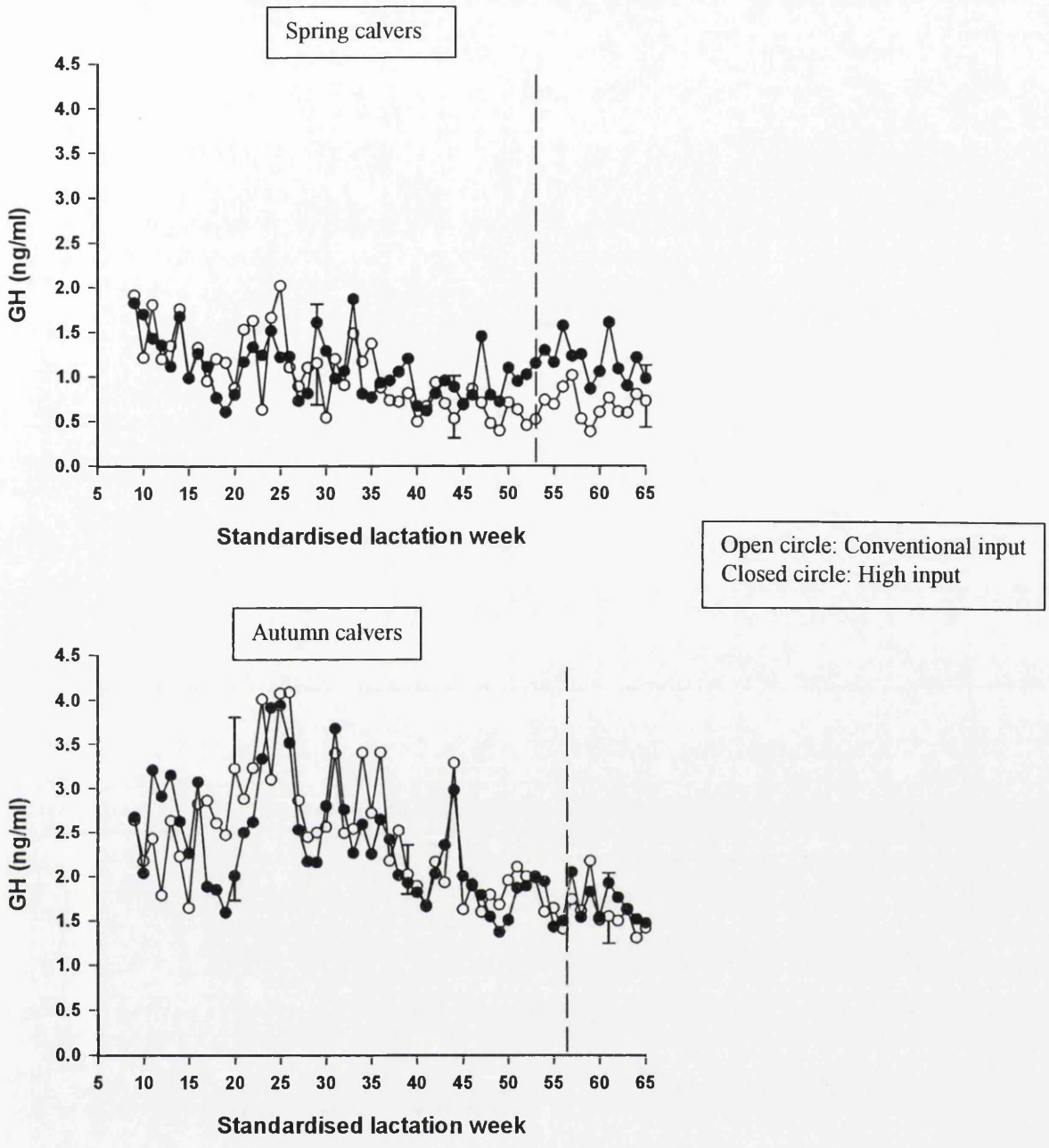


Figure 5.1. Plasma concentration (ng/ml) of GH from lactation week 9 throughout to lactation week 65. Closed circles are cows on high nutritional input and open circles are conventional fed animals. The spring calvers are in the top graph and the autumn calvers in the bottom graph. Dotted vertical line indicates the start of pregnancy week 20 for the first cow.

Table 5.1. The effect of nutrition (high versus conventional) and calving season (spring versus autumn) on plasma concentrations of GH, IGF-1 and insulin. Values are mean±s.e. over lactation week 9 to 33 (Lac 9-33), lactation week 33 to pregnancy week 20 (Lac 33-p20) and lactation week 9 to pregnancy week 20 (Lac 9-p20). Data was analysed by anova.

	Spring		Autumn		Nutrition	Season	Nutrition*Season
	High	Conventional	High	Conventional			
	GH (ng/ml)						
Lac 9-33	1.3±0.18	1.3±0.18	2.0±0.16	2.1±0.29	n.s	<0.01	n.s
Lac 33-p20	1.0±0.16	0.74±0.05	1.9±0.16	1.9±0.14	n.s	<0.001	n.s
Lac 9-p20	1.1±0.16	1.0±0.10	2.8±0.33	2.6±0.16	n.s	<0.001	n.s
	IGF-1 (ng/ml)						
Lac 9-33	157±28	179±31.4	146±14	175±26	n.s	n.s	n.s
Lac 33-p20	145±21.1	165±17.9	172±9.3	196±14.9	n.s	n.s	n.s
Lac 9-p20	162±10.4	186±18.2	162±10.4	186±18.2	n.s	n.s	n.s
	Insulin (ng/ml)						
Lac 9-33	0.92±0.20	0.67±0.07	0.50±0.06	0.50±0.11	n.s	<0.05	n.s
Lac 33-p20	0.85±0.19	0.88±0.15	0.99±0.24	0.87±0.18	n.s	n.s	n.s
Lac 9-p20	0.88±0.19	0.79±0.11	0.81±0.17	0.69±0.13	n.s	n.s	n.s

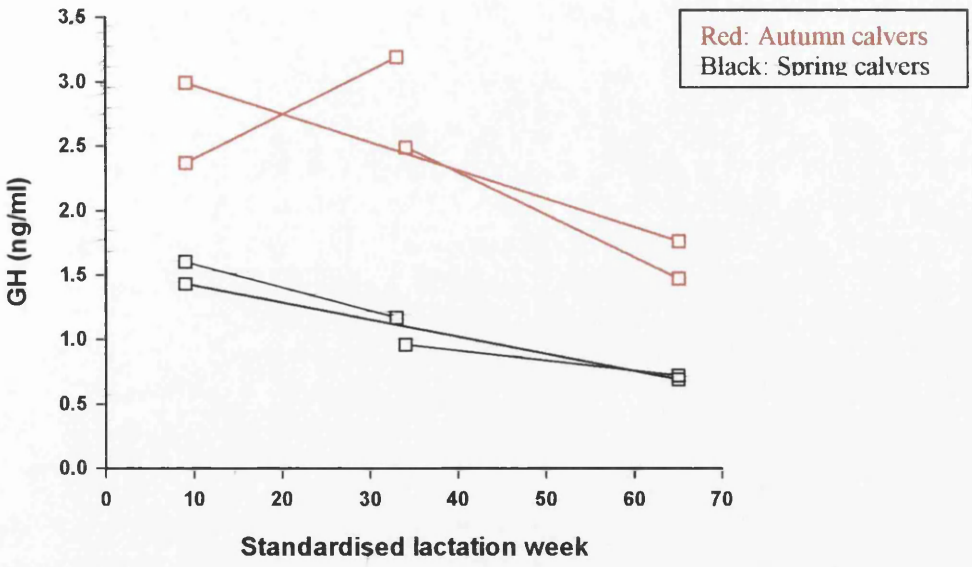


Figure 5.2. Changes in the plasma concentration of GH (ng/ml) from lactation week 9 to 33, lactation week 34 to 65 and from lactation week 9 throughout to lactation week 65 in spring (black line) and autumn (red line) calving cows. Changes were described by fitting best fit linear regression lines to weekly samples.

Table 5.2. Effect of nutrition (high versus conventional) and calving season (spring versus autumn) on changes in plasma levels of GH, IGF-1 and insulin from lactation week 9 to 33 (Lac 9-33), lactation week 33 to pregnancy week 20 (Lac week 33-P20) and lactation week 9 to pregnancy week 20 (Lac 9-P20). The changes in plasma levels of the measured hormones was described by best fit linear regression analysis on weekly samples and then analysed by anova.

	Spring		Autumn		Nutrition	Season	Nutrition *season
	High	Conventional	High	Conventional			
	GH						
Lac 9-33	-0.020±0.013	-0.019±0.015	0.039±0.012	0.030±0.023	n.s	<0.01	n.s
Lac 33-P20	0.005±0.010	-0.02±0.009	-0.035±0.004	-0.031±0.009	n.s	<0.01	n.s
Lac 9-P20	-0.016±0.011	-0.019±0.005	-0.025±0.004	-0.024±0.005	n.s	n.s	n.s
	IGF-1						
Lac 9-33	-0.14±0.37	-0.59±0.90	1.54±0.45	2.37±0.42	n.s	0.001	n.s
Lac 33-P20	0.05±0.43	0.42±0.30	0.36±0.28	0.03±0.45	n.s	n.s	n.s
Lac 9-P20	-0.28±0.22	-0.37±0.45	0.76±0.23	0.73±0.53	n.s	0.01	n.s
	Insulin						
Lac 9-33	0.003±0.004	0.006±0.004	-0.005±0.002	0.002±0.002	n.s	n.s	n.s
Lac33-P20	-0.014±0.005	0.005±0.003	0.023±0.013	0.012±0.004	n.s	<0.01	0.06
Lac 9-P20	-0.004±0.002	0.006±0.003	0.009±0.002	0.015±0.007	n.s	0.01	n.s

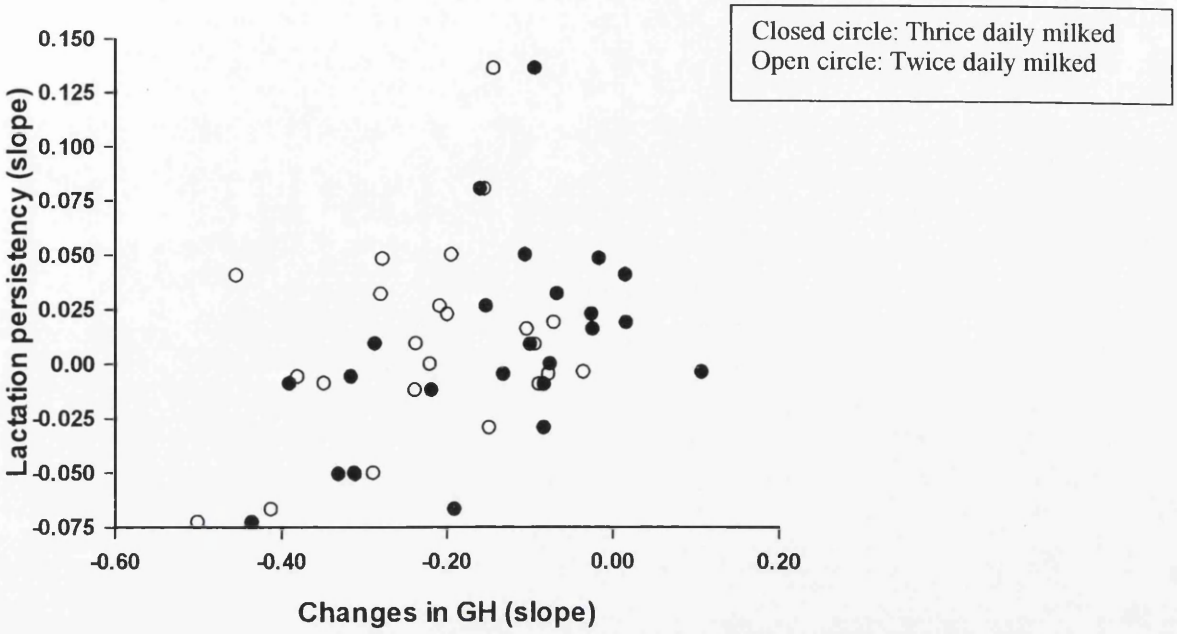


Figure 5.3. Relationship between lactation persistency and changes in GH levels from lactation week 9 to 33. Open circles are lactation persistency in twice daily milked udder halves while closed circles are lactation persistency in thrice daily milked udder halves.

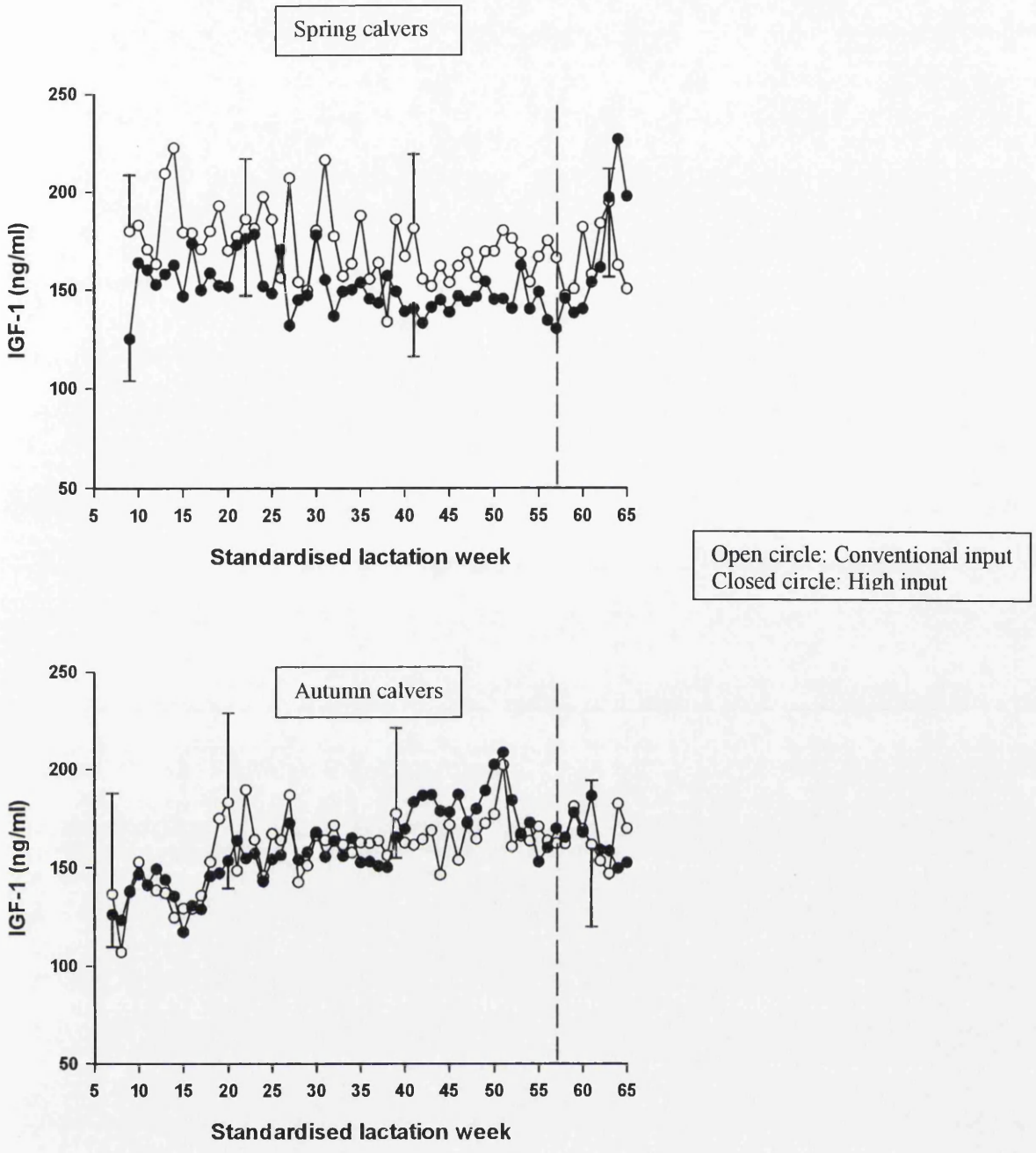


Figure 5.4. Plasma concentration of IGF-1 (ng/ml) from lactation week 9 throughout to lactation week 65. Closed circles are cows on high nutritional input and open circles are conventional fed animals. The spring calvers are in the top graph and the autumn calvers in the bottom graph. Dotted vertical line indicates the start of pregnancy week 20 for the first cow.

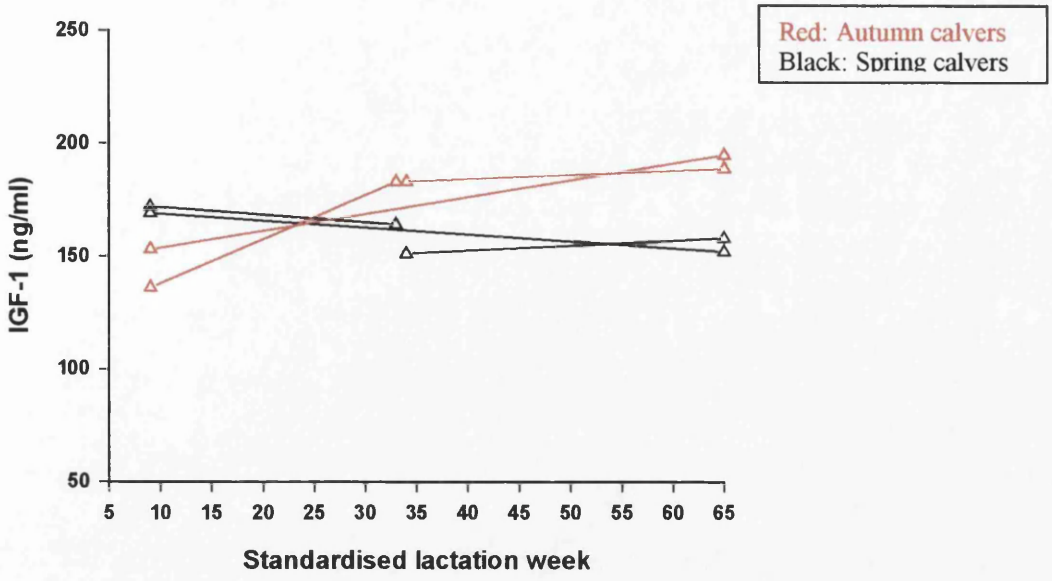


Figure 5.5. Changes in the plasma concentration of IGF-1 (ng/ml) from lactation week 9 to 33, lactation week 34 to 65 and from lactation week 9 throughout to lactation week 65 in spring (black line) and autumn (red line) calving cows. Changes were described by fitting best fit linear regression lines to weekly samples.

Plasma insulin levels for the 4 treatment groups are shown in figure 5.6 and mean concentrations in lactation week 9 to 33, lactation week 34 to pregnancy week 20 and lactation week 9 to pregnancy week 20 are shown in table 5.1. Nutrition had no effect on insulin levels at any stage Season on the other hand did affect insulin levels but only during the early part of lactation where insulin levels were significantly lower in autumn than spring calvers. During the latter part of lactation insulin levels did not differ between the 2 seasonal groups and neither was there any difference in insulin overall. Figure 5.7 and table 5.2 shows changes in insulin levels as lactation progress. Nutrition had no effect on changes in insulin between lactation week 9 to 33 or overall, however, a significant interaction was evident between nutrition and calving season between lactation week 34 and pregnancy week 20. In the autumn calvers insulin increased more in the high input group while in the spring calvers it was vice versa. Overall insulin increased in the autumn calvers and was more or less stable in the spring calvers as lactation progressed.

Lactation persistency was negatively correlated with the changes in insulin as shown in table 5.3. A significant correlation was only found in thrice daily milked udder halves.

Table 5.3. Correlation between lactation persistency in twice and thrice daily milked udder halves and changes in insulin levels during lactation week 9 to 33 and lactation week 9 to pregnancy week 20. Correlation coefficients with * indicates a significant relationship, P<0.05.

	Insulin lac week 9-33	Insulin lac week 9-p20
Thrice daily milked	-0.41*	-0.37*
Twice daily milked	-0.29	-0.20

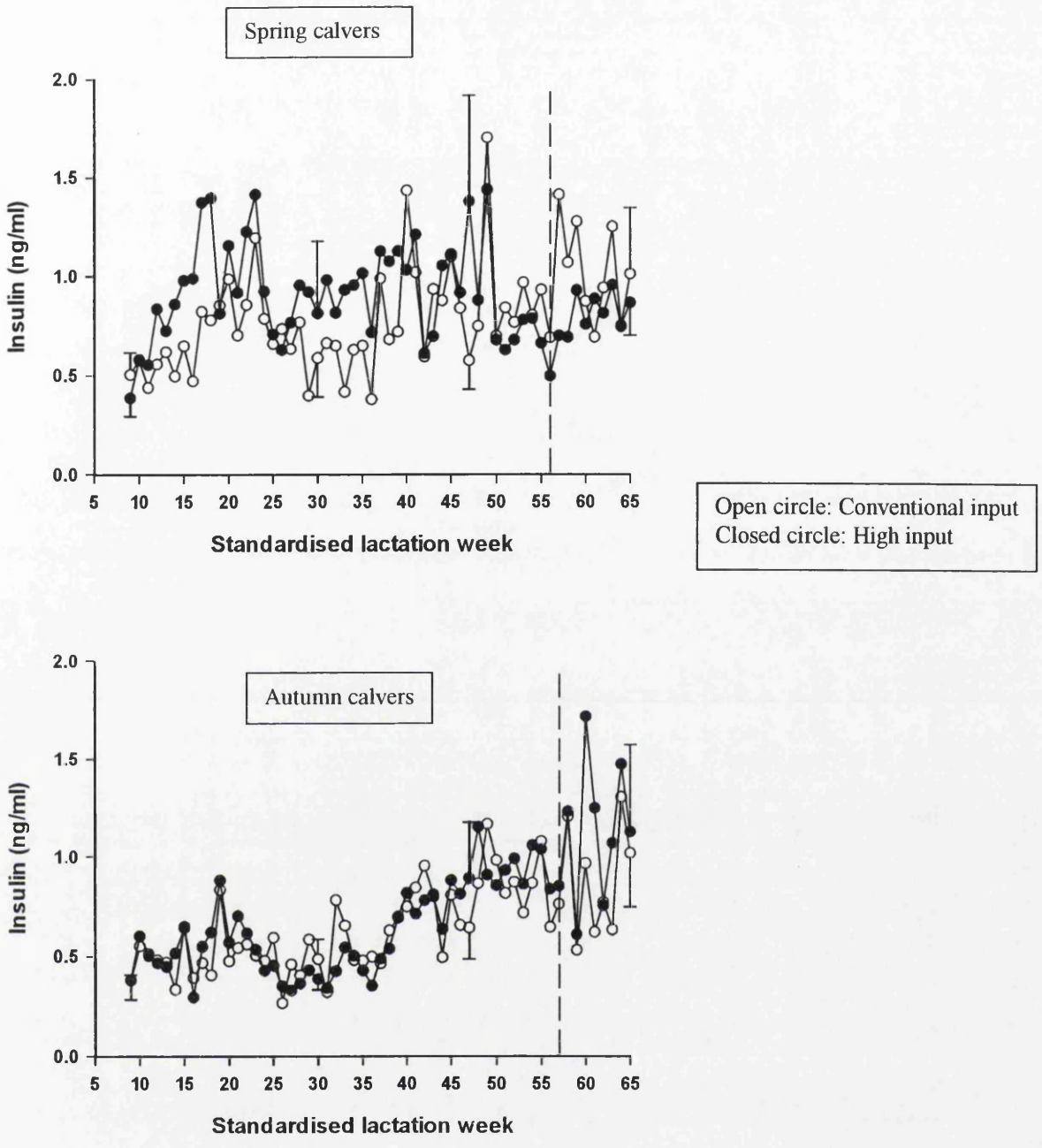


Figure 5.6.. Plasma concentration of insulin (ng/ml) from lactation week 9 throughout to lactation week 65. Closed circles are cows on high nutritional input and open circles are conventional fed animals. The spring calvers are in the top graph and the autumn calvers in the bottom graph. Dotted vertical line indicates the start of pregnancy week 20 for the first cow.

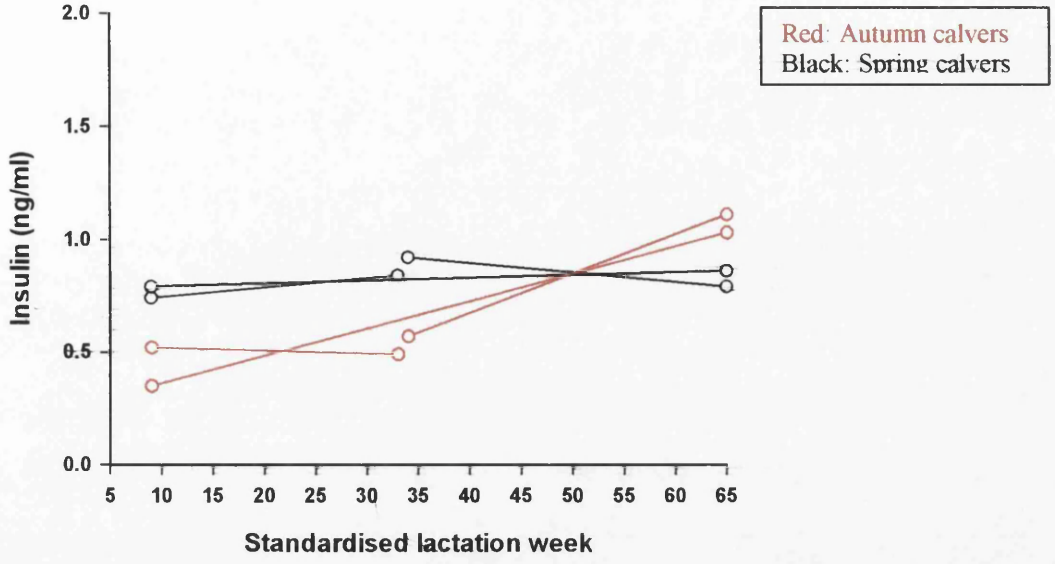


Figure 5.7. Changes in the plasma concentration of insulin (ng/ml) from lactation week 9 to 33, lactation week 34 to 65 and from lactation week 9 throughout to lactation week 65 in spring (black line) and autumn (red line) calving cows. Changes were described by fitting best fit linear regression lines to weekly samples.

The correlation between GH and insulin was –0.29±0.08. Prolactin plasma levels are shown in figure 5.8 for spring and autumn calvers on either high or conventional nutritional input. Plasma levels of prolactin increased from March to the middle of July as day length increased and decreased from July to September as day length decreased as shown in table 5.4. The increase in prolactin with an increase in day length, although it did occur in the autumn calving group was more pronounced in the spring calvers. Plasma levels of prolactin decreased when day length decreased. Nutrition had no effect on the increase in prolactin, however, an interaction between season and nutrition was evident when day length became shorter. Prolactin levels dropped from June to a minimum around September the beginning of October and thereafter prolactin levels started to increase again reaching a peak around December and thereafter decreased (figure 5.8). The increase from September to November was 2.1±0.72 ($p<0.05$) in the autumn calvers and 4.5±0.90 ($p<0.001$) in spring calvers. A significant effect of season was found on prolactin levels, which were higher in June than in January as shown in table 5.5. Stage of lactation also had an effect on prolactin levels, since the concentration of prolactin was lowest in mid lactation and highest in early and late lactation. No interaction was found between stage of lactation and season. No attempt was made to correlate lactation persistency and changes in plasma levels of prolactin due to the strong effects of photoperiod.

Table 5.4. Effect of nutrition (high versus conventional) and calving season (spring versus autumn) on prolactin levels with increasing day length and decreasing day length. The changes in prolactin was described by best fit linear regression analysis on weekly samples and then analysed by anova.

	Spring		Autumn		P-Value		
	High	Conventional	High	Conventional	Nutrition	Season	Nutrition * Season
Increase	March-July 1997 Lac 46-65		March-July 1997 Lac 16-35				
	0.458± 0.058	0.503± 0.063	0.221± 0.071	0.134± 0.065	n.s	<0.001	n.s
Decrease	June-Sep 1996 Lac 9-22		July-Sep 1997 Lac 33-44				
	-0.424± 0.066	-0.470± 0.078	-0.675± 0.0504	-0.352± 0.128	n.s	n.s	0.05

Table 5.5. The effect of season and stage of lactation (early 9-11, mid 29-32, late 59-62) on plasma concentrations of prolactin (ng/ml). All values are mean±s.e. for 6 cows. Within a column (effect of stage of lactation) and row (seasonal effect) values which do not share a common superscript are significantly different, P<0.05.

	June	January
Early	$9.5\pm0.66_a$	$7.8\pm0.88_b$
Mid	$8.7\pm0.54_a$	$5.3\pm0.53_b$
Late	$10.8\pm0.79_a$	$5.9\pm0.73_b$

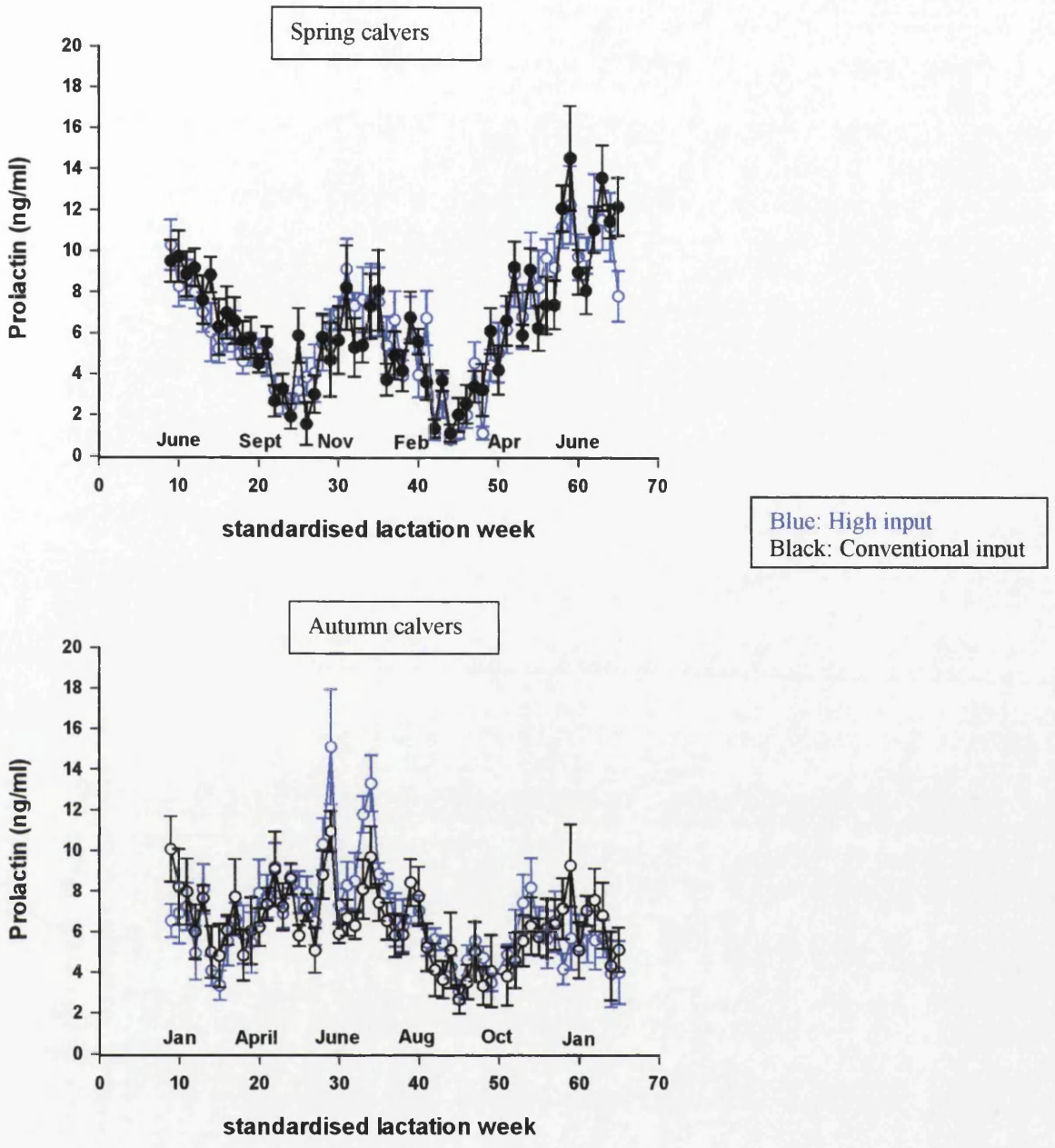


Figure 5.8. Plasma concentration of prolactin (ng/ml) from lactation week 9 throughout to lactation week 65. Blue lines are cows on high nutritional input and black lines are animals fed conventional. The spring calving animals are in the top graph and the autumn calvers in the bottom graph. Time of year together with lactation week is shown along the X-axis were lactation week 10 corresponds to calendar week 26 1996 in the spring calvers and calendar week 4 1997 in the autumn calvers.

5.4. Discussion

The overall aim of this part of the study was to investigate the relationship between lactation persistency and endocrine hormones such as insulin, GH and IGF-1. It is important to keep in mind that when relating hormone concentrations to metabolic events this does not account for possible changes in blood flow to a tissue or in alterations in numbers of hormone receptors in a target tissue. In the previous chapter we found that milk yield and lactation persistency were significantly and consistently improved by increased milking frequency using a half udder design, suggesting that the rate of milk secretion is under local and not endocrine control. With frequent milking the number of prolactin and IGF-1 receptors on mammary epithelial cells increases (Bennett, 1993). Although it is unknown what role this might play in the milk yield and developmental response to frequent milking it illustrates the difficulties in relating plasma hormone concentration to metabolic events.

Mammary apoptosis is under endocrine control as well as local. The involvement of the endocrine system in mammary apoptosis has been demonstrated in rodents where GH and prolactin deficiency increased apoptosis within 48h. However, when prolactin was replaced the induction of apoptosis was completely prevented. GH replacement on the other hand was only partially effective (Travers *et al.*, 1996). In ruminants plasma levels of GH are high during early lactation and decrease as lactation progress (Schams *et al.*, 1991), as was found in our experiment. Furthermore, administration of GH increases milk yield (Bauman, 1992) and in late lactation mammary cell proliferation has been observed with administration of GH (Capuco & Byatt, 1998).

GH could be an important factor in maintaining lactation persistency due to its positive effects on mammary cell proliferation. Bauman (1992) found that administration of GH improved lactation persistency but only for about ten weeks, thereafter, although total milk production remained higher persistency returned to values as for control animals. However, a more recent study showed positive effects of GH on lactation persistency throughout the entire lactation (Van Amburgh *et al.*, 1997). Despite the numerous reports of the mammogenic, galactopoitic and lactogenic effect of GH, the mechanism by which GH exerts its influence on these processes is unclear, due to the failure of detecting the GH receptors in the mammary gland (Akers, 1985). However, a very recent study has localised the receptor in ductal and alveolar epithelia and stromal cells in the mammary gland (Sinowatz *et al.*, 2000). This opens up the possibility that the effects of GH on the mammary gland after all might be through a direct mechanism. However, in vitro studies have shown no direct effect of GH on milk synthesis in bovine mammary tissue (Gertler *et al.*, 1983). The thought that the effects of GH on the mammary gland are indirect is therefore still valid. It is well known and accepted that an increase in nutrient partitioning is partly responsible for the increase in milk production observed with administration of GH. However how GH stimulates mammary growth (Capuco & Byatt, 1998) and serves as an anti apoptotic factor at least in rodents (Travers *et al.*, 1996) has been under a lot of debate. IGF-1 is a potent mitogen for the mammary gland (Baumrucker & Stemberger, 1989) and GH stimulates the secretion of this hormone so it has been suggested that GH exerts its actions on mammary epithelial cells through IGF-1. It is unlikely that IGF-1 mediates these effects through circulating levels of IGF-1 since administration of IGF-1 does not increase milk yield (Davis *et al.*, 1989) and circulating levels of IGF-1 actually increase as lactation progresses and is highest during the dry period (Schams *et al.*, 1991). Although the liver is the main source of IGF-1 in blood, IGF-1 is produced locally in a

number of other tissues including the mammary gland (Ruan *et al.*, 1992). IGF-1 is recognised as a cell survival factor in a number of tissues including the mammary gland (Ruan *et al.*, 1992) although the fact that it reaches its highest concentration during the dry period when apoptosis is greatest does not immediately suggest such a role in the dairy cow. However, specific binding proteins exists in serum which modulate the action of IGF-1 and its clearance from serum (Jones & Clemmons, 1995). At least one of these binding proteins, IGF-BP5, is synthesised in the mammary gland and its secretion is upregulated during involution (Flint & Knight, 1997). These observations led Flint & Knight (1997) to hypothesise that the presence of IGFBP-5 prevents IGF-1 from binding to its receptor and exerting its cell survival function. In rodents at least the local production of IGFBP-5 appears to be negatively regulated by prolactin, since the post lactational appearance of IGFBP-5 was found to decrease when prolactin was administered (Tonner *et al.*, 1995). So in the combined presence of GH and prolactin IGF-1 production is increased, IGFBP-5 production is down regulated and apoptosis prevented. These studies do not demonstrate a causal role for IGFBP-5 in involution, however preliminary results using a transgenic mouse overexpressing IGFBP-5 in the mammary gland has show a depression of mammary development just as would be predicted by the model (D. J. Flint, personal communication).

The absolute concentration of GH showed no significant linear relationship with lactation persistency. Although changes in GH was positively correlated with lactation persistency at least in twice daily milked udder-halves. In thrice daily milked udder-halves the correlation was weaker and in the long term not even significant. As a reminder, hormone action is determined not only by plasma hormone concentration but also by receptor number. An increase in milking frequency has been shown to increase the number of IGF-

1 receptors on the mammary epithelial cells (Bennett 1993). If thrice daily milking indeed increased the number of IGF-1 receptors on mammary epithelial cells and GH exerts its effect on mammary cell turnover through IGF-1 this could explain the lack of a significant relation between the change in plasma levels of GH and persistency in thrice daily milked udder-halves. However, according to the cell survival model described above an increase in the binding of IGF-1 to its receptor requires the absence of IGFBP-5, which appeared to be absent in the presence of prolactin. Contrary to rodents the role of prolactin in ruminant lactation is not clear. Unlike in rodents bromocriptine treatment does not have any significant effect on milk yield (Karg *et al.,* 1972). Plasma levels of prolactin are higher during early lactation, however, prolactin levels in plasma do not correlate with milk yield and stage of lactation (Collier *et al.,* 1984). A significant effect of stage of lactation on prolactin levels was found in this experiment. Plasma levels of prolactin were high in early and late lactation and lower in mid lactation. This might not be a true effect but rather a consequence of the way in which these data were analysed. The mid lactation prolactin value was from the autumn calvers in the month of June where prolactin for some unknown reason dropped. In the same month the decrease was also observed in the spring calvers (late lactation) but the decrease was not as drastic as in the autumn calvers (figure 5.8). This might explain why prolactin levels were slightly lower in mid lactation.

Circulating prolactin is affected by photoperiod, the concentration increases with an increase in day length and decreases with decreasing day length (Tucker, 1985). A photoperiodic effect on prolactin was also demonstrated in this experiment. The shortest hours of daylight is in December and it was interesting to note that prolactin had dropped to it's minimum level already in October when the animals went from grass to housed management. Thereafter a transient increase in prolactin was evident before it decreased to

a minimum level in March. The biological significance of these observations is unknown. However, it is clear that circulating levels of prolactin did not affect milk yield in either this or other experiment. Although it is important to recognise that in experiments where bromocriptine treatment has been used to lower prolactin this does not completely eleminate pituitary release of prolactin. Furthermore the mammary gland might upregulate it's own synthesis of prolactin when circulating levels are low (Leprovst *et al.*, 1994). The number of prolactin receptors in the mammary gland increases in lactation. Furthermore, thrice daily milking increases the number of prolactin receptors in the goat mammary gland while milk stasis in rodents causes a rapid decrease in the number of receptors. Therefore, it might be the number of prolactin receptors and not circulating levels of prolactin which are the important factor in regulating mammary cell survival. If thrice daily milking increased the number of prolactin receptors in the mammary gland in this experiment the concentration of IGFBP-5 would presumably be lower allowing IGF-1 to bind to its receptor and exerts its cell surviving effect which would then improve lactation persistency.

Nutrient partitioning is under endocrine control. An increase in nutrient partitioning increases milk yield (Bauman & Currie, 1980) and therefore indirectly affects lactation persistency. Insulin has no direct effect on mammary tissue although its receptor is present in mammary tissue (Vernon, 1980). However insulin plays a major role in nutrient partitioning between mammary and non-mammary tissues. GH is also involved in nutrient partitioning diverting nutrient towards milk production (Bauman, 1992). IGF-1 is negatively correlated with milk yield, which is believed to reflect the energy status of the animal. Interpretation of these data is difficult. IGF-1 is not only regulated by GH but also factors such as energy balance, energy intake and insulin (Gluckman *et al.*, 1987). Interpretation of plasma levels of IGF-1 are therefore difficult. It is well known that the

plasma concentration of GH decreases while concentrations of IGF-1 and insulin increases as lactation progress (Schams *et al.*, 1991) resulting in a redistribution of nutrient between mammary and non-mammary tissues where it is the mammary gland that loses priority for nutrient. However what is determining this redistribution of nutrients in the first instance is not known as discussed in chapter 4. The energy status was different between the two seasonal groups before rebreeding so that energy was more efficiently directed towards milk production in the autumn calvers. This was reflected in the higher milk yield, higher absolute levels of plasma GH, lower insulin and lower body condition score. While it might be the absolute levels of these hormones that is important to determine the level of milk yield it follows that it is the deviation in this level that affects lactation persistency. Before rebreeding nutrients were more efficiently used for milk synthesis in the autumn calving cows. Milk yield was higher and lactation persistency was better. GH increased in autumn calvers while it decreased slightly in the spring calvers. Insulin was more or less constant in both groups. Although there was no difference in body condition score gain between the two seasonal groups body weight increased more in the spring calving group. Overall the proportion of nutrient diverted into body tissues did not differ between the two seasonal groups, because there was no difference in the changes in GH, insulin, body weight or body condition score. Neither was there any difference in lactation persistency between the two groups.

Lactation persistency was negatively correlated with changes in insulin but positively with GH. In chapter 4 it was evident that increases in body weight and body condition score were negatively correlated with lactation persistency. This indicates that nutrient partitioning between mammary and non-mammary tissues is an important factor in maintaining lactation persistency. This is not surprising because an animals ability to divert

nutrient towards milk production is an important determinant of milk yield and milk yield obviously affects lactation persistency. However, the important question to ask is whether an increase in utilisation of nutrients by the mammary gland is having any direct effect on lactation persistency.

Chapter six

The effect of milking frequency and nutrition on milk composition and processing quality during extended lactation

6.1. Introduction

Improving lactation persistency is not the only important issue when considering extended lactation. During a normal lactation the processing qualities of the milk will usually decrease as lactation progresses. This would be a most undesirable attribute in extended lactation. In this chapter I will, therefore, determine what effects milking frequency and nutrition have on the gross composition of milk during extended lactation, paying particular attention to its processing properties.

Protein is the most valuable component in milk. In August 2000 Scottish Milk paid 2.8 pence per percentage of milk crude protein compared to only 1.6 pence per percentage of milk fat. Typically, the concentration of total protein in milk increases as lactation progresses, however, the proportion of casein to non-casein (commonly referred to as casein number) decreases. Casein number is a good indicator of milk quality and a decrease in casein number is associated with a decrease in milk quality since this typically reflects an increase in serum proteins and casein breakdown products, which are undesirable milk processing characteristics (Feagan, 1979). Individual protein components do not as yet directly affect the payment for milk and late lactation milk is valuable for the farmer although it might not be valuable for the processor. However, in the UK and many other countries current payment schemes bear a penalty (or premium) related to the bulk tank somatic cell count (SCC). Less than or equal to 150,000 cells/ml is rewarded by 0.20 pence/l milk, 151-250,000 cells/ml 0.10 pence/l, 251-350,000 cells/ml 0.00 pence/l and

over 350,000counts/ml is penalised with 0.50 pence/l by Scottish Milk. SCC can be used as an indirect measure of milk quality since the activity of plasmin which is the main proteolytic enzyme in milk, degrading α and β casein to γ casein, increases with an increase in SCC, although this effect does not become significant before SCC reaches a level above 290,000 cells/ml (Politis *et al.*, 1989).

6.2. Materials and methods

6.2.1 Experimental design

The overall design of the experiment is described in chapter 2. Gross composition of milk was studied in the spring calving group and in these animals consecutive extended lactation. All values are reported as mean±s.e. unless otherwise stated.

6.2.2. Collection of milk samples

During the spring calvers first extended lactation milk samples were collected from individual cows every second week in a milking parlour equipped to collect milk separately from half udders (diagonally opposed glands). Milk samples were proportionally bulked in each of the two or three milkings in one day according to milk yield and analysed for percentage fat, lactose and SCC by Livestock Services (UK) Ltd, Paisley, PA3 1TJ, according to standard procedures. Once every month samples were bulked according to treatment (3x high nutrition, 2x high nutrition, 3x conventional nutrition and 2x conventional nutrition) and analysed for crude protein and casein protein.

During the 2'nd extended lactation milk samples were collected from individual cows once a month and analysed for percentage fat, lactose, crude protein, casein protein and SCC as before.

6.2.3 Protein analysis

Protein analysis was performed by the Food Quality Group of the Hannah Research Institute. Total casein was prepared by acid precipitation (Brown *et al.*, 1995). Total nitrogen and casein nitrogen was determined by standard micro-Kjeldahl analysis on skimmed milk and extracted casein, respectively, and non-casein nitrogen determined by difference. All results are expressed on a protein basis by multiplying by 6.38.

6.2.3. Statistical analysis

All data were analysed by anova using Genstat 5 Release 4.1, Lawes Agricultural Trust, Rothamsted Experimental Station comparing stage of lactation and milking frequency within cow while nutrition was compared between cows. No attempt was made to compare concentrations of milk components between lactations.

6.3. Results

Data for crude protein, casein protein and casein number during the first lactation are shown in figure 6.1 while the second lactation data are shown in figure 6.2. For clarity all curves were smoothed using Minitab Release 11, Minitab Inc, State College, PA 16801, USA. For analysis of stage of lactation on milk composition the lactation period was divided into four periods. During the first lactation the 1'st period was from standardised lactation week 10-22, 2'nd period included lactation week 24-32, 3'rd period was lactation week 34-50 and 4'th period started at lactation week 52 and finished at lactation week 60. During the second lactation the lactation period was divided as during the first lactation except for the first and last period which included lactation week 12-22 and 52-56, respectively. The overall means and statistical analysis of the treatment effects on milk

protein composition are shown in table 6.1 and table 6.2 for the first and the second lactation respectively.

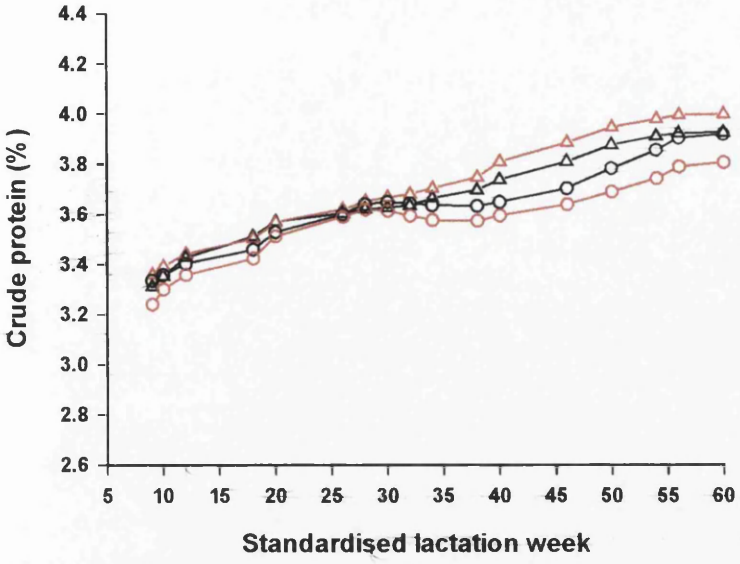
As lactation progressed crude protein increased in all treatment groups and during both lactations, as did casein protein. During the 1'st lactation percentage crude protein increased from 3.44±0.019 in lactation week 12 to 3.83±0.43 in lactation week 60 and casein protein increased from 2.78±0.027 to 3.00±0.059. In the 2'nd lactation percentage crude protein and casein protein in milk was 2.98±0.053 and 2.39±0.051, respectively in lactation week 12 and had increased to 3.94±0.073 and 3.04±0.067 in lactation week 52 respectively. Neither of the treatments had any effect on crude protein during the second lactation, however, during the first lactation high nutritional input increased crude protein and there was also a tendency (P=0.08) for an interaction between nutrition and milking frequency. In the conventional fed group thrice daily milked udder-halves produced milk with higher crude protein while twice daily milked udder-halves had higher crude protein in the high nutritional input group.

Casein on the other hand was higher in the high nutritional input groups during both lactations. In both lactations there was a tendency for milking frequency to interact with nutrition (1'st and 2'nd lactation, P=0.08). Thrice daily milking increased casein in the conventional fed groups while in the high input groups there was a slight increase during the first and a slight decrease during the second lactation. Although both crude protein and casein increased as lactation progressed overall casein numbers decreased. During the 1'st lactation casein number was 80.68±0.43 in lactation week 12 and had decreased to 78.59±1.02 in lactation week 60. In the 2'nd lactation casein number was 79.85±0.50 in lactation week 12 and had decreased to 77.28±0.69 in lactation week 52. However, high

nutritional input and milking frequency both increased casein numbers although an interaction between the two was apparent during the second lactation as thrice daily milking increased casein numbers in milk from conventional fed animals and only small changes were evident in milk from animals on high nutritional input (figure 6.1 and 6.2).

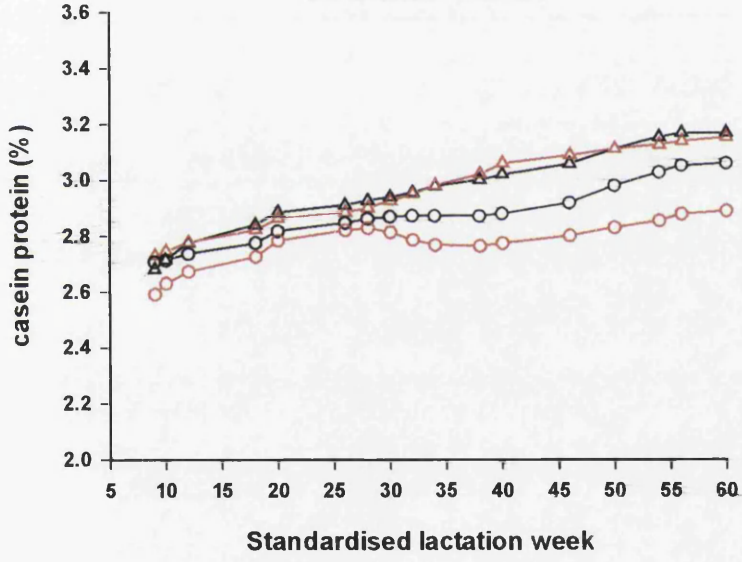
During the first lactation casein numbers were completely maintained at their early lactation value in milk from thrice daily milked udder-halves of high nutritional fed animals (see figure 6.1), whilst in the twice daily milked udder-halves of these animal casein numbers decreased by 1.78 from lactation week 12 to 60. During the second lactation (lactation week 8 to 52) casein numbers dropped by 1.69±1.32 in thrice daily milked and 2.71±1.31 in twice daily milked in milk from high nutritional fed animals. In the conventional input group casein numbers decreased by 2.01±1.00 in thrice daily milked and 3.87±1.12 in twice daily milked udder-halves.

Crude protein



Δ-High input
O-Conventional input
Black-3x
Red-2x

Casein protein



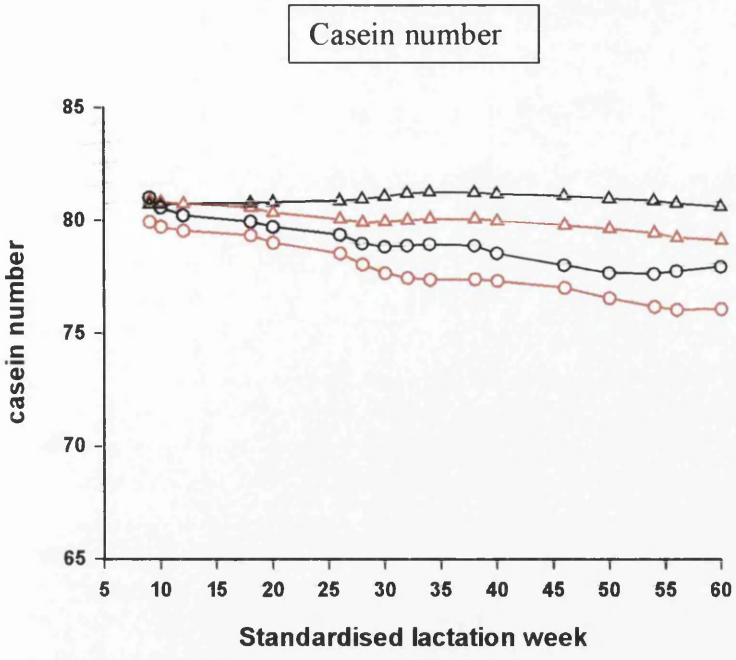
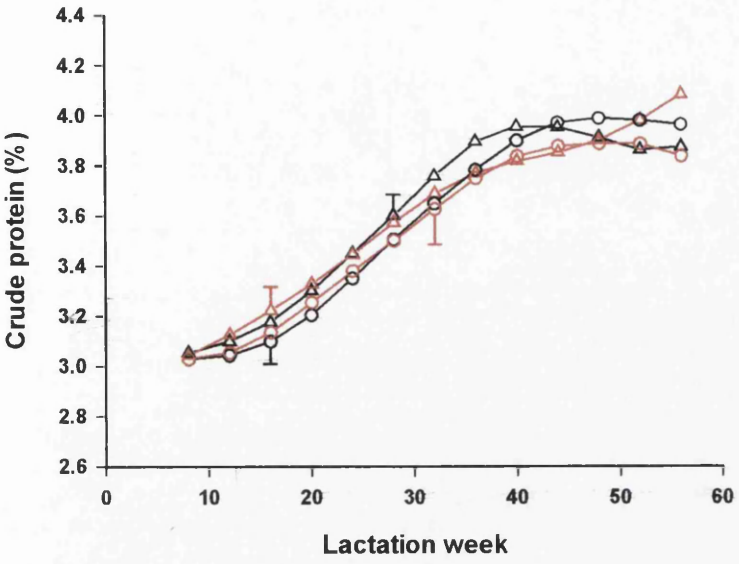
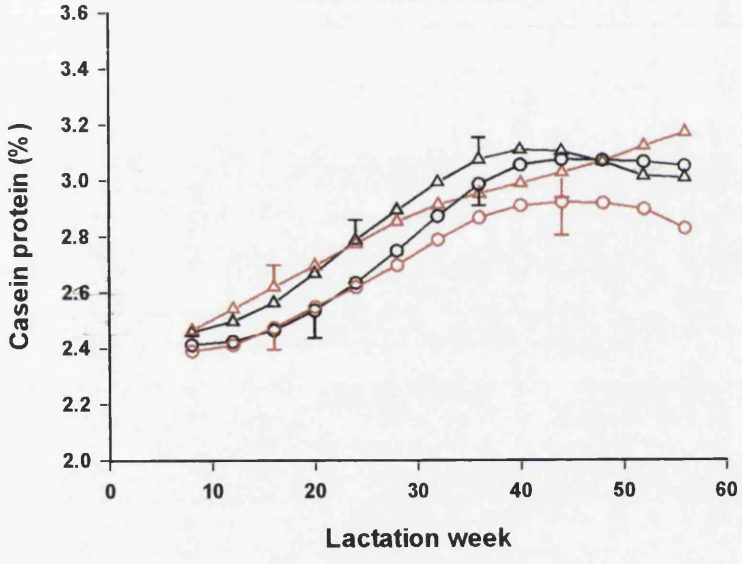


Figure 6.1. The effect of milking frequency and nutrition on crude protein, casein protein and casein number in milk. Triangle are cows on high nutritional input whilst circle is conventional input. Black is thrice and red is twice daily milked udder-halves.

Crude protein



Casein protein



Δ−High input
O- Conventional input
Black-3x
Red-2x

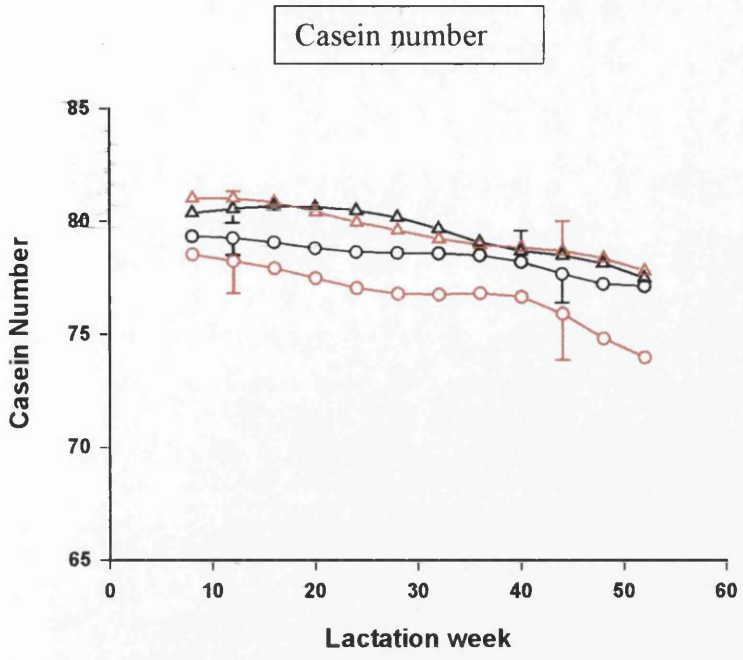


Figure 6.2. The effect of milking frequency and nutrition on crude protein, casein protein and casein numbers in milk during a second extended lactation. Triangles are cows on high nutritional input whilst circles are conventional input. Black is thrice daily and red is twice daily milked udder-halves. For clarity only representative s.e are shown.

Table 6.1. Effect of milking frequency (thrice versus twice daily), nutrition (high versus conventional) and stage of lactation (1'st quarter lactation week 10-20, 2'nd quarter lactation week 26-32, 3'rd quarter lactation week 34-50 and 4'th quarter lactation week 54-60) on milk protein composition. Data was analysed by anova.

Treatment	Protein %	Casein %	Casein number
Frequency			
Thrice	3.68	2.94	79.82
Twice	3.66	2.87	78.53
SED	0.02	0.01	0.17
P-value	n.s	<0.01	<0.001
Nutrition			
High	3.70	2.97	80.16
Conventional	3.64	2.85	78.19
SED	0.02	0.01	0.15
P-value	0.01	<0.001	<0.001
Stage of lactation			
1'st quarter	3.43	2.75	80.20
2'nd quarter	3.65	2.89	79.11
3'rd quarter	3.70	2.94	79.36
4'th quarter	3.89	3.04	78.04
SED	0.02	0.02	0.25
P-value	<0.001	<0.001	<0.001

Table 6.2. Effect of milking frequency (thrice versus twice daily), nutrition (high versus conventional) and stage of lactation (1'st quarter lactation week 12-20, 2'nd quarter lactation week 24-32, 3'rd quarter lactation week 36-48 and 4'th quarter lactation week 52-56) on milk protein composition during a consecutive extended lactation. Data was analysed by anova.

Treatment	Protein %	Casein %	Casein number
Frequency			
Thrice	3.56	2.79	78.26
Twice	3.65	2.83	77.53
SED	0.04	0.04	0.30
P-value	n.s	n.s	<0.001
Nutrition			
High	3.63	2.87	78.94
Conventional	3.58	2.75	76.86
SED	0.04	0.03	0.30
P-value	n.s	0.02	<0.001
Stage of lactation			
1'st quarter	3.11	2.49	79.82
2'nd quarter	3.64	2.88	78.93
3'rd quarter	3.90	3.02	77.23
4'th quarter	3.76	2.84	75.60
SED	0.04	0.04	0.36
P-value	<0.001	<0.001	<0.001

Percentage fat in milk increased as lactation progressed in all four treatments groups as shown in table 6.3 for the first lactation and table 6.4 for the second lactation. Nutrition had no effect on fat percentage, however, there was a suggestion of a higher fat percentage in 3x milked udder-halves compared to 2x milked udder-halves in the second lactation.

Milk lactose content is shown in figure 6.3 from the four treatment groups during the two lactations. Lactose decreased in all treatment groups as lactation progressed as shown in table 6.3 and 6.4, however, the decrease was much greater in twice than thrice daily milked udder-halves. This difference became more apparent as lactation progressed (frequency * stage of lactation, $P<0.01$). Again the effect of milking frequency was greater in milk from the conventional fed animals during the second lactation. Furthermore, lactose was significantly correlated with milk yield (2'nd 0.783, $p<0.001$, figure 6.4), while no linear relationship existed between lactose and lactation persistency (0.226, $p<0.31$) as shown in figure 6.5.

SCC increased during both of the studied lactations as shown in table 6.3 and 6.4. In lactation week 12 SCC was 40.5±7.5 and 56.5±13.7 in the first and second lactation, respectively and steadily increased to 265.5±46.4 in lactation week 60 and 200.1±31.1 in lactation week 52. Level of nutrition had no effect on SCC but increased milking frequency significantly reduced SCC.

Table 6.3. Effect of milking frequency (thrice versus twice daily), nutrition (high versus conventional) and stage of lactation (1'st quarter lactation week 10-22, 2'nd quarter lactation week 24-32, 3'rd quarter lactation week 34-52 and 4'th quarter lactation week 54-60) on milk fat, lactose and somatic cell counts (SCC). Data was analysed by anova.

Treatment	Fat %	Lactose %	SCC*10^3
Frequency			
Thrice	4.18	4.82	121
Twice	4.24	4.55	252
SED	0.04	0.02	22
P-value	n.s	P<0.001	<0.001
Nutrition			
High	4.24	4.76	151
Conventional	4.19	4.60	222
SED	0.04	0.02	22
P-value	n.s	n.s	0.03
Stage of lactation			
1'st quarter	3.69	4.94	64
2'nd quarter	4.28	4.71	124
3'rd quarter	4.34	4.60	238
4'th quarter	4.54	4.47	320
SED	0.07	0.03	41
P-value	<0.001	<0.001	<0.001

Table 6.4. Effect of milking frequency (thrice versus twice daily), nutrition (high versus conventional) and stage of lactation (1'st quarter lactation week 12-20, 2'nd quarter lactation week 24-32, 3'rd quarter lactation week 36-48 and 4'th quarter lactation week 52-56) on milk fat, lactose and somatic cell counts (SCC) during a consecutive extended lactation. Data was analysed by anova.

Treatment	Fat %	Lactose %	SCC*10^3
Frequency			
Thrice	4.28	4.89	148
Twice	4.01	4.69	316
SED	0.06	0.02	35
P-value	<0.01	P<0.001	0.001
Nutrition			
High	4.09	4.80	171
Conventional	4.20	4.78	293
SED	0.06	0.02	35
P-value	n.s	n.s	0.02
Stage of lactation			
1'st quarter	3.81	5.16	116
2'nd quarter	3.86	4.97	189
3'rd quarter	4.27	4.70	153
4'th quarter	4.64	4.32	470
SED	0.09	0.03	50
P-value	<0.001	<0.001	<0.001

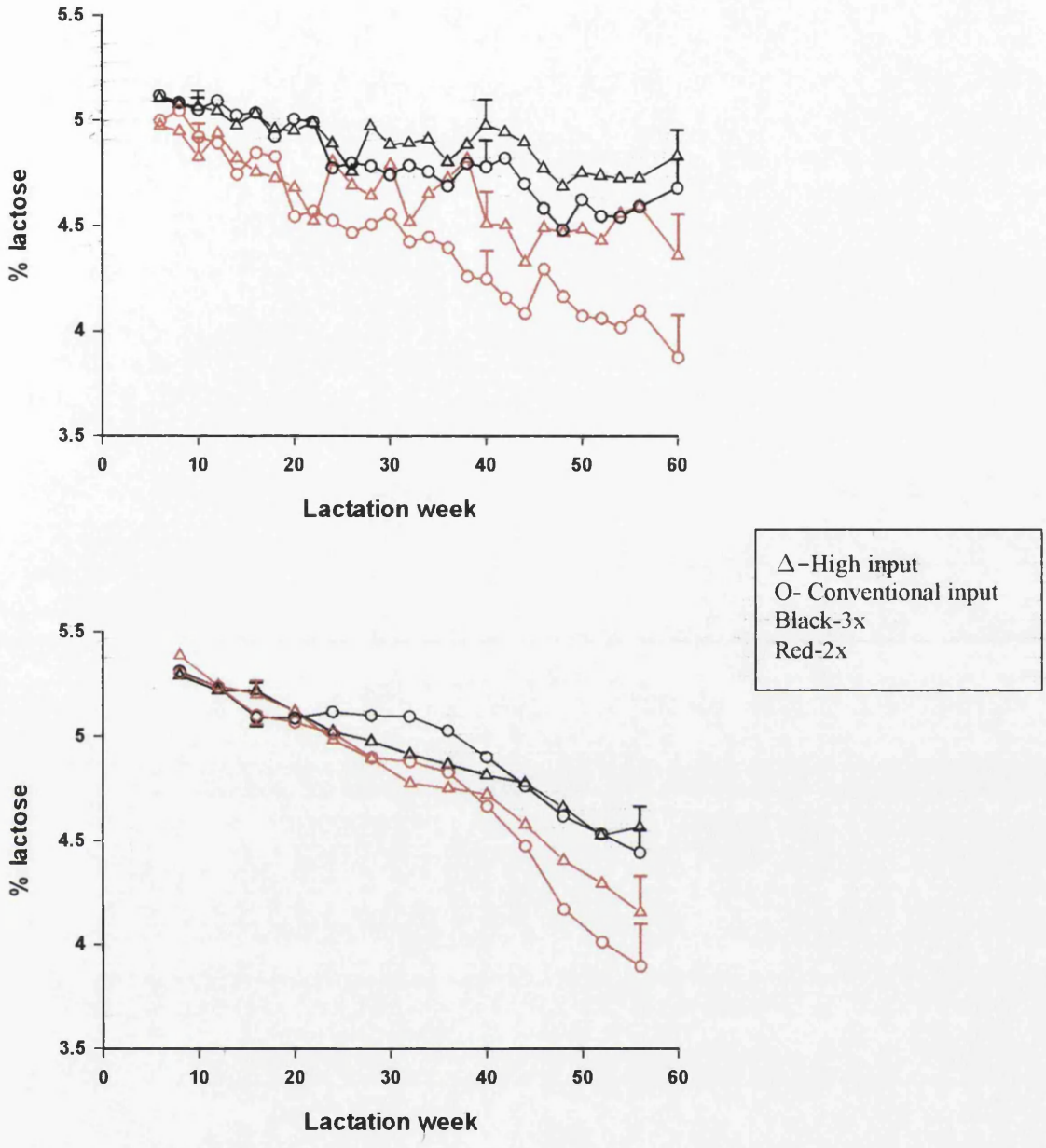


Figure 6.3. The effect of milking frequency and nutrition on lactose content in milk during two consecutive extended lactations (top 1'st lactation bottom 2'nd lactation). Triangles are cows on high nutritional input while circles are conventional fed. Black is thrice daily milked and red is twice daily milked udder-halves. For clarity only representative s.e. are shown.

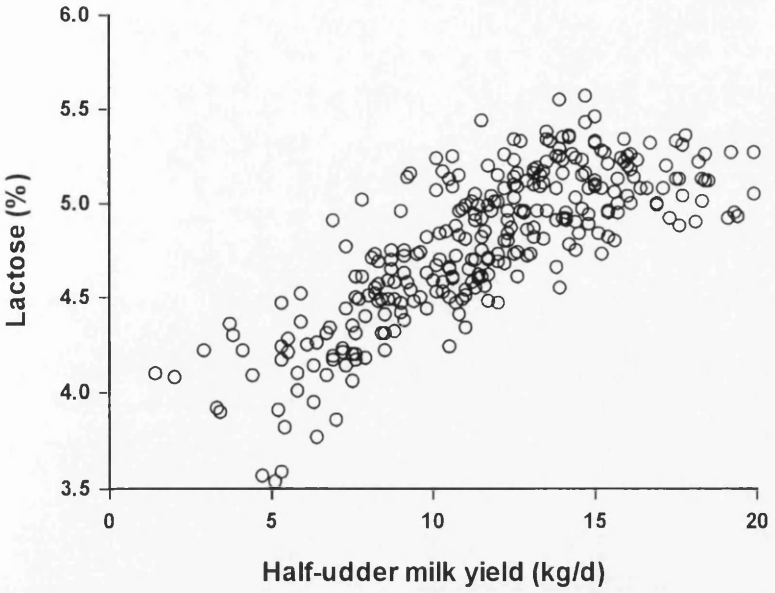


Figure 6.4. Relationship between milk content of lactose and half udder milk yield.

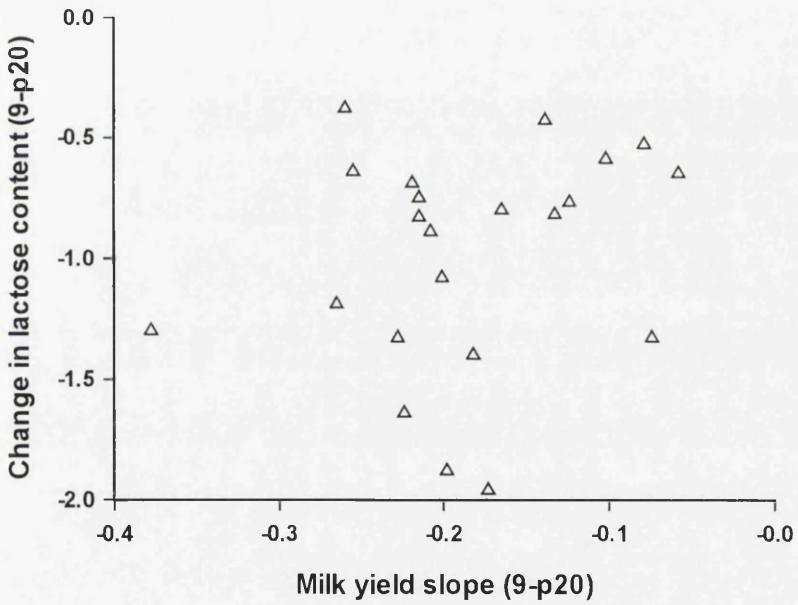


Figure 6.5. Relationship between milk content of lactose and lactation persistency from lactation week 9 through to pregnancy week 20.

6.4. Discussion

The objective of this study was to investigate the effect of increased milking frequency and level of nutrition on gross composition of milk during extended lactation.

It is well known that milk fat, crude protein and casein increase as lactation progresses (Klei *et al.*, 1997; Auldist *et al.*, 1995) and our data confirm this. The increase in milk fat, crude protein and casein has been suggested to be due to improved energy balance as lactation progresses and, therefore, more nutrients available for *de novo* synthesis within the mammary gland (Auldist *et al.*, 1995).

In agreement with Coulon & Perochon (1998) the increase in fat and protein was not related to milk production as no significant relationship was found between milk yield and the concentration of fat and protein in milk. Although protein and fat content was not related to milk production the content of lactose was. Lactose serves as the dominant factor controlling secretion of water into milk and hence is largely responsible for regulating milk volume. Lactose concentration does therefore not vary in freshly secreted milk and a significantly positive relationship was evident between milk yield and lactose. However, lactose decreased as lactation progressed. This may be attributed to the leakage of lactose into blood as the mammary epithelium becomes leaky due to breakdown of the mammary tight junction between neighbouring cells as lactation progress (Baldi *et al.*, 1996). In the mammary gland the tight junctions form a barrier between the blood and interstitial fluid thus preventing serum components from entering blood and *vice versa* (Peaker, 1977). Leaky tight junctions of course have other consequences, since they increase the amount of serum derived constituents in milk. In this study both crude protein and SCC increased as lactation progressed.

Although fat, crude protein and casein increased as lactation progressed the processing qualities of milk decreased. Casein is one of the most important determinants for the processing properties of milk (Dalgleish, 1992) and casein number is a good indicator of the changes in processing qualities of milk. The casein number gives the ratio of casein to non-casein protein. Increased casein number could result from increased casein secretion, from a reduction in non-casein protein or from a combination of these two. Non-casein protein comprises secreted whey proteins, serum proteins transferred directly into milk, either through specific transport mechanisms (eg hormones) or through paracellular leakage and a third category, namely casein breakdown products, although some of the breakdown products might be retained in the casein fraction.

The mammary cell secretes four different caseins α_{s1}, α_{s2}, β and κ-caseins. α and β-casein can be broken down to γ-casein. γ-casein is unwanted in milk because it decreases the processing qualities of milk. Since most of the γ –casein is removed when measuring casein in milk an increase in proteolysis of α and β-casein would result in a decrease in casein protein and therefore a decrease in casein numbers. During the course of a lactation the proportion of γ -casein to α and β-casein increases, an effect which has been attributed to an increasing conversion of the proteolytically inactive protein plasminogen to its active plasmin as lactation progresses but also an increased leakage of these proteins into milk via leaky tight junctions (Baldi *et al.*, 1996; Politis *et al.*, 1989).

Pregnancy affects fat and protein content from around pregnancy week 20, a pattern which mainly depends on stage of lactation (Coulon & Perochon, 1998). In this study, it was not

possible to evaluate the effect of pregnancy on milk constituents, due to the limited numbers of samples obtained (monthly samples).

It is well known that feeding of different energy levels influences milk components. A diet high in energy, which is usually achieved by higher concentrate levels generally increases protein content and reduces fat content while the content of lactose remains unchanged (Thomas & Martin, 1988). In this study, the difference in feeding between the high and conventional input group was 3 kg of in parlour concentrate. There was no significant effect of the extra concentrate on the fat or lactose percentage. It is generally recognised that an increase in the ratio of concentrate to forage depresses milk fat content, however, it is not until the diet contains approximately 55-60 percentage concentrates that extra concentrate begins to shown any significant effect on milk fat (Thomas & Martin, 1988). Bearing in mind that total food intake was not measured in this experiment, the additional in parlour fed concentrate was not enough to change milk fat content. However increased milking frequency significantly increased fat content in one of the lactations, which is not in agreement with other studies Gisi *et al.* (1986) and (Poole, 1982) where thrice daily milking had no or if any a small insignificant depressing effect on milk fat content. Whether this is a significant biological effect or due to sample and analysis problems is uncertain.

In animals underfed, the ratio of lactose to other osmotic constituents in milk is reduced. However, lactose is normally unaffected by dietary changes when animals are fed at or above recommended levels, suggesting that dietary changes do not affect the mammary tight junctions. Indeed Lacy-Hulbert *et al.* (1999) found no alterations in permeability of

the mammary gland when late lactating cows were fed restricted for 26 days. In agreement we found no significant effect of nutrition on lactose content.

The effect of feeding higher concentrate levels on crude proteins was not consistent during the two studied lactations. During the first lactation, crude protein was higher in animals fed extra concentrate, however, this effect was not apparent during the second lactation. Casein content on the other hand was higher in milk from high input animals during both lactations and as a result casein number was also higher during both lactations.

Information on the effect of dietary energy on protein synthesis in the mammary gland compared with serum derived proteins is limited. Grant *et al.* (1980) observed no difference in the proportions of individual caseins from changes in the ratio of forage to concentrate suggesting that proteolysis of caseins does not change. However Yousef *et al.* (1969) reported that, with high concentrate diets, the proportion of total casein as α- and β-casein increased, whereas γ-casein decreased. The underlying mechanism responsible for an increase in milk protein with increased concentrate levels is not well understood. However, it is unlikely to be a local effect residing within the mammary gland since no effect has been found on the integrity of the epithelium during food restriction (Lacy-Hulbert *et al.*, 1999). The insignificant changes observed in lactose in this and other studies (Thomas & Martin, 1988) tend to support this. A systemic effect is likely and is has been suggest that insulin might play a role in this scenario (Griinari *et al.*, 1997).

Intensive studies have been done looking at the effect of once daily milking on milk composition (Davis *et al.*, 1999), however, only one study has looked at the effect of thrice daily milking in detail on milk protein (Klei *et al.*, 1997). Our result confirmed the

observations by Klei *et al.* (1997) that thrice daily milked mammary glands produced milk with higher casein numbers than twice daily milked control glands. During the second lactation an interaction between milking frequency and nutrition was apparent so that the effect of increased milking frequency on casein number was much greater in animals fed conventionally. However, this is not to say that mammary casein synthesis was not increased. Increased milking frequency increases milk production, so to maintain milk protein concentration at approximately the same level mammary synthesis of casein protein must also increase, however, to obtain no change in casein numbers crude protein concentration increased to the same extent.

There was a slight non–significant increase during the first and a slight decrease during the second lactation in crude protein as a result of thrice daily milking. These observations agree with other studies where changes in crude protein observed with thrice daily milking have been small (Henderson *et al.*, 1983; Hillerton *et al.*, 1990).

Casein showed a small but significant increase during the first lactation and a small non-significant decrease during the second lactation as a result of thrice daily milking. Others (Klei *et al.*, 1997) found a small and generally non-significant reduction in casein concentration. Although small, the changes in crude and casein protein are sufficient to increase casein numbers.

Milk content of lactose was significantly elevated by increased milking frequency an effect which increased as lactation progress. This suggests that the integrity of the mammary epithelium was significantly improved. As for casein numbers an interaction between milking frequency and nutrition existed during the second lactation which could maybe

explain why increased milking frequency was less effective in increasing casein numbers in the high nutritional input group during the second lactation.

It has been suggested by Klei *et al.* (1997) that the increase in casein numbers with an increase in milking frequency is due to storage time. Shorter storage time of milk in the udder would mean less chance of proteolysis. We do not agree, and the mechanisms involved in improving milk protein quality as a result of an increase in milking frequency will be the subject of the next chapter.

In conclusion, both high nutritional input and increased milking frequency increased milk protein quality. Casein number decreased as lactation progressed however in combination these two treatments were able in the first lactation to completely maintain casein numbers and in the second lactation maintain casein numbers at a higher level than any of the other treatments. Increased milking frequency also kept lactose levels higher and SCC lower, which probably suggests that the integrity of the mammary tight junctions is better maintained with increased milking frequency.

Chapter seven

The effect of milking frequency on epithelial integrity and milk protein composition

7.1. Introduction

In the previous chapter it was shown that milking frequency does influence milk protein quality. In this chapter the mechanism involved in this phenomenon will be investigated.

The lactation cycle of the dairy cow is characterized by increasing milk production during early lactation followed by a progressive decline until drying off. This decline is a consequence of gradual involution of the gland (loss of secretory cells) through a process of apoptosis (Wilde *et al.*, 1997b). Concomitant with the decline in milk yield are changes in the protein composition of the milk, resulting in a gradual deterioration of processing properties as lactation advances. Total protein content increases, but α–casein and β-casein are progressively degraded by proteolytic enzymes such that the proportion of casein to non-casein protein decreases (Brown *et al.*, 1995). Proteolysis is largely achieved through activation of the serum protease, plasmin. Milk plasmin is derived from blood and is present in milk in the active and the inactive plasminogen form (Korycka-Dahl *et al.*, 1983). The activity of both plasmin and plasminogen in milk increase as lactation progresses, due to increased activation of plasminogen to plasmin and also to increased leakage of these enzymes from serum into milk (Stelwagen *et al.*, 1994b) as tight junctions between neighbouring epithelial cells gradually lose their integrity. Milking more frequently causes an immediate increase in milk yield with only small changes in milk protein (Chapter 6, Hillerton *et al.*, 1990). In the longer term, there is evidence that involution is suppressed by more frequent milking (Wilde *et al.*, 1987) and accelerated by inefficient milking (Wilde & Knight, 1989). A recent study (Klei *et al.*, 1997)

milked thrice daily. The authors argued that this difference was entirely due to the shorter storage time in the mammary gland, hence, lesser time for proteolysis. However, this ignores any possible contribution of developmental changes in the gland caused by the frequent milking.

The aims of the work reported in this chapter were to examine the effect of short and long term changes in milking frequency on proteolysis of milk proteins, using a two day reversal of milking frequency to differentiate between effects due to storage and those due to better maintenance of epithelial integrity.

7.2. Materials and methods

7.2.1. Experimental design

The reader is referred to chapter 2 for detailed information on cow management. 6 animals from the spring calving group were used in the first experiment described here whilst 11 cows from the winter calving group were used for the second experiment reported in this chapter.

7.2.2. Milking and collection of milk samples

Milking was routinely performed in a milking parlour equipped to collect milk separately from half udders (diagonally opposed glands) and to weigh the milk to a precision of 100 g. From parturition until lactation week 9 all cows were milked 2 times daily. Thereafter milking frequency was increased, in one half of the udder only, to 3 times daily at equal 8 h intervals (Frequent) whilst the other half continued on 2 times daily milking (Control; 8:16 h intervals).

In the first experiment monthly milk samples collected from 6 of the spring calving cows as described in chapter 6 was used to study the cross-lactational changes in epithelial integrity.

Concentrations of sodium and potassium in milk were used as a measure of mammary tight junction integrity.

In a second experiment 11 of the winter calving cows were used to study the effect of a brief reversal of milking frequency. Short-term experimental milkings commenced in week 52 ± 2.9 and comprised, for each udder half, a series of eight 2 d periods of either thrice or twice daily milking according to a statistically randomised design. There were thus 4 treatments; Control (twice daily long term, twice daily short term), Increased (twice daily long term, thrice daily short term), Frequent (thrice daily long term, thrice daily short term), and Decreased (thrice daily long term, twice daily short term) with each treatment represented in four of the 2 d periods. Milk samples were collected at each milking and proportionally bulked according to milk yield each day. All day 2 samples within each period were analysed for total crude protein and for casein protein, as well as for epithelial integrity as before. Samples from 2 cows were selected for detailed analysis of protein fractions and proteolysis.

7.2.3. Casein analysis

Crude protein and casein protein were determined as described in chapter 6 and again the analysis was done by our Food Quality Group as was the detailed analysis of protein composition. Individual caseins were determined by ion-exchange FPLC (Davies & Law, 1987). Immunoglobulins (IgG), serum albumin and lactoferrin in combination (SA/Lf), β-lactoglobulin and α-lactalbumin were measured by gel permeation FPLC as described by Law *et al.* (1993).

7.2.4. Milk proteinase activities

Milk samples were defatted by centrifugation (20 min at 3000 rpm). Skim milk was centrifuged at 100,000 g for 1 h at 4^0 C to obtain the supernatant and the pellet (casein). The pellet was

reconstituted in a 50mM Trisbuffer containing 110mM NaCL and ε-amino-n-caproic acid (EACA, Sigma Chemical Co., Poole BH15 1TD, UK). To allow plasmin and plasminogen to dissociate from the casein micelles to the buffer this mixture was incubated for 2h. and centrifuged for 1h at 4^0 C at 100,000 g (Stelwagen *et al.*, 1994b).

To measure the total activity of plasmin and plasminogen 10µl of the supernatant was mixed with 125µl of 50mM Trisbuffer (pH 7.4) containing 110mM NaCl and 2.5mM EACA. To allow conversion of plasminogen to plasmin 150 plough units of urokinase was added (Sigma Chemical Co., Poole BH15 1TD, UK). 6mM H-D-Valyl-L-leucyl-L-lysine-p-nitroanilide di hydrochloride (Sigma Chemical Co., Poole BH15 1TD, UK) which when cleaved by plasmin forms p-nitroanilide was then added and the mixture incubated for 1h at 37^0 C to allow the conversion of plasminogen to plasmin to proceed. The formation of p-nitroanilide during cleavage of the substrate by plasmin was determined by measuring the absorbance at 405nm every hour for 6hours. The rate of p-nitroanilide formation was determined from the linear part of the absorbance versus time and the result expressed as units/ml where one unit of activity of plasmin or plasminogen was defined as the amount of the enzyme that produced a change in absorbance at 405 nm of 0.001 in 1 min at 37^0 C.

Plasmin activity was measured in the same reaction measure as for the total activity of plasmin and plasminogen however without the addition of urokinase.

7.2.5. Ionic composition

Concentrations of sodium and potassium measured by flame photometry were used to assess tight junction permeability

7.2.6. Statistical analysis

All data were obtained using a half-udder experimental design, allowing comparisons to be made within-cow. For the first experiment analysis was by analysis of variance testing for effects of milking frequency (Control, twice daily milked vs Frequent, thrice daily milked) and stage of lactation. For the second experiment milk yield, crude protein, casein protein and epithelial integrity data were analysed by analysis of variance (Minitab Release 11, Minitab Inc, State College, PA 16801, USA) testing for effects of long term milking frequency, short term milking frequency and the interaction between the two as well as for treatment (Control, Increased, Frequent, Decreased). Additional factors in the model were cow and preceding short term milking frequency, to take account of possible carry-over effects from period to period. Since there were no significant effects of preceding treatment a further analysis was run using paired *t*- tests to compare the overall treatment means, ie the mean, for each cow, of the four periods in which each treatment was represented.

Analysis of individual caseins was done on a series of eight samples, four from each of two cows. Differences between treatments were assessed by analysis of variance using the mixed procedure model for repeated measures (SAS, 1992). The model comprised cow, treatment period, long term milking frequency, short term milking frequency and interactions. Additional analysis of variance (Genstat 5 Release 4.1, Lawes Agricultural Trust, Rothamsted Experimental Station) tested independently for differences between repeated measures and for differences between treatments within period. This confirmed the results from the repeated measures analysis, only the former is reported.

7.3. Results

Results of the first experiment are in Fig 7.11 and Table 7.11. Milk sodium concentration increased during the course of lactation and that of potassium decreased (Fig 7.1). Effects were significant (Table 7.1). Compared to Control, Frequent udder-halves had lower sodium concentration throughout lactation and higher potassium concentration for most of lactation. These differences became greater as lactation advanced. The sodium:potassium ratio is inversely correlated with mammary epithelial integrity. This ratio increased through the course of lactation, and was always higher in Control (Fig 7.1). Before week 40 the rate of change was similar in Control and Frequent, but thereafter there was an accelerated increase in Control only (slopes of 0.034±0.009 and 0.015±0.002, $P<0.001$, paired *t* test on log transformed data). Effects of lactation stage and milking frequency were significant (Table 7.1).

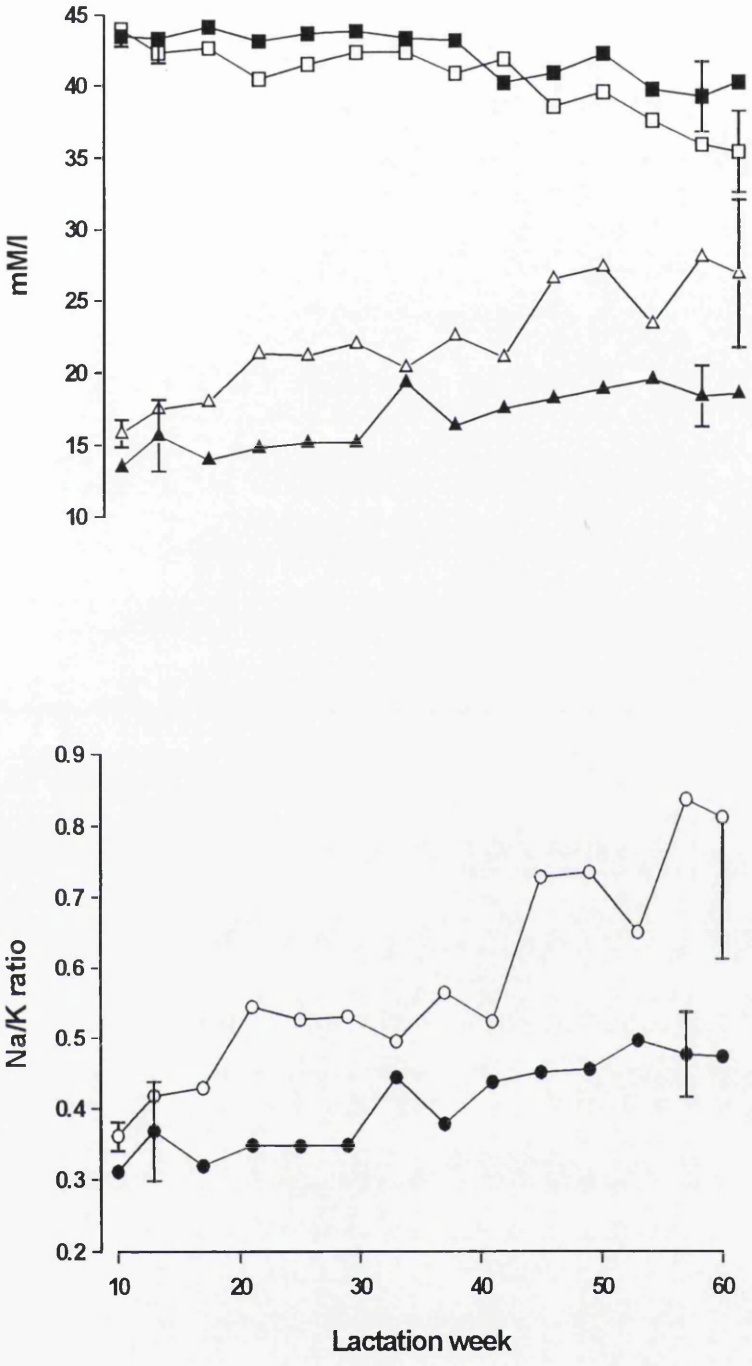


Figure 7.1. Concentrations of sodium (triangles) and potassium (squares) and the ratio of sodium to potassium (circles) in milk from half-udders milked twice daily (open symbols) or thrice daily (closed symbols) from week 9 of lactation onwards. Values are means for 6 cows with representative s.e.

Table 7.1. Milk ionic composition at the start and end of a 60 week lactation for milk from half-udders milked twice-daily (Control) or thrice-daily (Frequent). All values are means ±s.e. for 6 cows. Anova tests for effects of milking frequency (weeks 10 to 60), stage of lactation and their interaction.

	Treatment	Na	K	Na:K
Start	Control	17.11±1.75	43.00±1.41	0.40±0.05
	Frequent	14.39±1.52	43.67±1.67	0.33±0.04
End	Control	26.06±4.68	36.22±2.55	0.77±0.18
	Frequent	18.78±1.89	39.67±2.23	0.48±0.05
Analysis of variance				
Frequency	P<0.001	P<0.001	P<0.001	
Stage	P<0.001	P<0.001	P<0.001	
Interaction	n.s	n.s	n.s	

Immediately before the short-term experimental milkings in the second experiment milk yield was significantly lower in Control udder halves than in Frequent (6.03 ± 0.16 vs. 9.71 ± 0.16 kg/d; $P < 0.001$, paired *t*- test). Short term thrice-daily milking increased yield by 13% (Control 5.9 vs Increased 6.7 kg/d, P<0.001), whereas short term twice-daily milking decreased yield by 10% (Frequent 9.8 vs Decreased 8.9 kg/d, P<0.01). Ionic composition data for the second experiment are in Table 7.2. Values for Control and Frequent were similar to the equivalent end of lactation values reported in the first experiment. Sodium concentration was reduced by short term frequent milking (Increased) to a value intermediate between Control and Frequent, whilst potassium was increased to an intermediate value. A short term reduction in frequency (Decreased), on the other hand, decreased sodium and increased potassium, again to

intermediate values. As a result, the Na:K ratio was highest in Control, lowest in Frequent and intermediate in both Increased and Decreased. Effects of long- and short-term milking frequencies were all significant but there was no interaction between the two.

Values for total protein, casein protein and casein number (casein as a fraction of total protein) are shown in Table 7.2. Analysis of variance revealed significant effects of long-term milking frequency on all three parameters, but short-term frequency only affected casein number. It should be remembered that short term twice daily milking is present in both Control and Decreased treatments, similarly short term thrice daily milking is present in Frequent and Increased treatments. The interaction between long and short term frequencies was significant for casein and casein number, indicating that milking twice followed by thrice produced a different response to milking thrice followed by twice. The four treatments, which represent the various combinations of long and short term frequencies, produced highly significant differences. There were no significant carry-over effects from period to period within the series of short term altered milkings, hence paired *t* test analysis could be done on the overall means to examine these treatment effects. Casein number was significantly higher in Frequent than in Control; similar but smaller differences in total protein and casein protein were not significant. A short-term increase in milking frequency (Increased) reduced total protein (non-significantly) but had no effect on casein protein, resulting in a significant increase by 1.28 ± 0.32 in casein number. This increase was significantly correlated with initial casein number ($r = -0.7$; $P < 0.05$). Total protein was elevated by a short-term decrease in milking frequency (Decreased), leading to a reduction in casein number. No apparent correlation existed between this decrease and the initial casein number ($r = 0.1$; $P > 0.7$). Casein number was lowest in Control, highest in Frequent and intermediate in both Increased and Decreased; the recovery or loss of casein number due to short term changes in milking frequency was, therefore, incomplete.

Individual total protein, casein protein and casein number values for the two cows selected for detailed analysis reflected the group changes (results not shown) and both cows demonstrated the same pattern of changes of all of the milk constituents measured. Individual casein values are in Table 7.3. Frequent udder halves had significantly higher proportions of α- and β-casein, but lower γ-casein. These changes in proportion were a consequence of increased concentrations of α- and β-casein coupled with decreased γ-casein concentration.

Short term changes produced intermediate values, α- and β-casein elevated in Increased and reduced in Decreased, with opposite changes in γ-casein. As with casein number, reversal of milking frequency only partly eliminated differences between the two udder halves (Table 7.3). Although κ-casein is resistant to proteolysis differences were evident between Frequent and Control, with Increased and Decreased being intermediate between these.

Changes in whey proteins and serum proteins were less consistent than the caseins, although clear patterns were still present (Table 7.3). β-lactoglobulin, which is the dominant whey protein in bovine milk, was significantly higher in Frequent than in Control and once again the Increased and Decreased samples had intermediate values. α-lactalbumin, on the other hand, was not affected by milking frequency in either the long or the short term. Values for IgG and for SA/Lf were the reverse of β-lactoglobulin, ie low in Frequent, high in Controls and intermediate for others (Table 7.3).

Plasmin and plasminogen concentrations are in Table 7.3. Both were high in Control and low in Frequent, but the ratio of plasminogen to plasmin was highest in Frequent and lowest in Control. As with other measures the losses or gains caused by the short term change of frequency were incomplete.

Table 7.2. Milk ionic composition, protein composition and casein number (casein as a proportion of total protein) for late lactation milk from half udders milked long-term twice daily (Control), long-term thrice daily (Frequent) or for the same halves after short (2 d) reversal of milking frequency (Increased, Decreased respectively). All values are mean ± s.e. for 11 cows. Within a column, values which do not share a common superscript are significantly different, $P < 0.05$ or greater, paired *t* test. Analysis of variance tests for effects of long-term and short-term milking frequency, their interaction, carry-over between individual periods within the short-term milking frequency schedule and the four treatments.

	Na (mM/l)	K (mM/l)	Na:K	Total proteing/l	Casein protein g/l	Casein number
Control	29.70±3.67^{a}	38.41±1.43^{a}	0.82±0.14^{a}	37.8±1.3^{a,b}	28.8±1.4^{a,b}	75.7±1.3^{a}
Increased	26.00±2.98^{b}	39.45±1.21^{b}	0.69±1.21^{b}	37.3±1.1^{a}	28.7±1.2^{a}	77.0±1.1^{b,c}
Frequent	21.14±2.06^{c}	41.63±1.20^{c}	0.52±0.07^{c}	38.1±1.1^{b}	29.5±1.2^{b,c}	77.4±0.9^{c}
Decreased	22.33±2.32^{d}	41.06±1.18^{d}	0.56±0.08^{d}	39.0±1.2^{c}	29.9±1.1^{c}	76.6±0.9^{a,b}
Analysis of variance						
Frequency, long	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P=0.001
Frequency, short	P=0.01	P<0.05	P<0.05	n.s.	n.s.	P<0.001
Long x short	n.s.	n.s.	n.s.	n.s.	P<0.05	P<0.01
Carry-over	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Treatment	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001

Table 7.3. Individual milk proteins, plasmin and plasminogen values in milk from half udders milked twice daily (Control), thrice daily (Frequent) or for the same halves after 2 d of reversed milking frequency (Increased, Decreased respectively). Individual proteins are expressed as a percent of total protein. Plasmin and plasminogen are arbitrary units. All values are mean ± s.e. for 8 observations from 2 cows. β - LG: β - lactoglobulin. α - LA: α - lactalbumin. SA: serum albumin. LF: lactoferrin. Rows which do not share a common superscript are significantly different, $P < 0.05$, analysis of variance.

	Control	Increased	Frequent	Decreased
α - casein	39.94±0.63 ^{a}	42.19±0.53 ^{b}	44.26±0.18 ^{c}	43.44±0.33 ^{b}
β - casein	25.83±1.39 ^{a}	29.18±0.69 ^{b}	32.82±0.44 ^{c}	30.28±0.62 ^{b}
κ - casein	14.98±0.67 ^{bd}	13.33±0.20 ^{b}	12.16±0.13 ^{a}	13.00±0.29 ^{bc}
γ - casein	18.37±1.56 ^{a}	14.97±0.72 ^{b}	9.95±0.52 ^{c}	12.24±0.51 ^{b}
β - LG	58.52±0.47 ^{a}	59.41±0.38 ^{a}	62.09±0.36 ^{b}	60.54±1.01 ^{ab}
α - LA	12.88±0.31 ^{a}	12.88±0.20 ^{a}	12.86±0.33 ^{a}	12.90±0.46 ^{a}
SA + LF	17.53±0.66 ^{a}	17.07±0.57 ^{a}	15.81±0.78 ^{b}	16.44±0.80 ^{ab}
IgG	11.06±0.55 ^{a}	10.66±0.47 ^{a}	9.22±0.32 ^{b}	10.14±0.37 ^{b}
Plasmin (P)	12.65±0.71 ^{a}	8.19±0.52 ^{b}	5.93±0.42 ^{c}	7.59±0.50 ^{b}
Plasminogen (p)	42.69±1.81 ^{a}	40.73±1.40 ^{a}	35.43±0.57 ^{b}	36.24±0.87 ^{ab}
Sum, P+p	55.34±2.49 ^{a}	48.92±1.67 ^{b}	41.36±0.88 ^{c}	43.83±1.22 ^{c}
p/P ratio	3.40±0.08 ^{a}	5.10±0.31 ^{b}	6.16±0.39 ^{c}	4.89±0.26 ^{bc}

7.4. Discussion

The primary objective of this study was to examine the long-term and short-term effects of milking frequency on the secretion of milk proteins and their degradation by plasmin during late lactation. The results confirm the result obtained in chapter 6 that individual mammary glands milked thrice daily for the preceding 10 months produce milk with a higher proportion of casein to non-casein protein (higher casein number) than twice daily milked control glands. This extends recent observations made in groups of cows (Klei *et al.*, 1997) to show that the mechanism(s) reside within the mammary gland.

Increased casein number could result from increased casein secretion, from a reduction in non-casein protein or from a combination of these two. Non-casein protein comprises secreted whey proteins, serum proteins transferred directly into milk, either through specific transport mechanisms (eg hormones) or through paracellular leakage and a third category, namely casein breakdown products. Thrice daily milking has generally been found to decrease milk protein concentration by a small amount (Campos *et al.*, 1994), the exception being controlled experiments using within-animal designs where individual glands milked more often have either had the same (Henderson *et al.*, 1983) or slightly increased protein content (Hillerton *et al.*, 1990). This suggests that the more commonly seen depression is related to systemic factors (probably nutritional) rather than the gland's capacity to synthesize protein. The same can now be said for caseins themselves; comparison of groups of cows indicated a small and generally non-significant reduction in casein concentration (Klei *et al.*, 1997), whereas our results demonstrate a similarly small increase. Increased milking frequency results in enhanced milk yield, so to maintain milk protein concentration at approximately the same level mammary synthesis of casein protein must also increase. Increased casein secretion in the face of an unchanged secretion of whey proteins and influx of non-mammary protein would produce the

observed increase in casein number. However, concentrations of β-lactoglobulin and α-lactalbumin were unchanged or increased by more frequent milking (Table 7.3), indicating that secretion of whey proteins at least kept pace with milk volume. This leaves influx of non-mammary proteins by paracellular leakage, which is a passive process whereby serum proteins flow into milk down a concentration gradient. Unless there is a change in the permeability of the epithelium, the increased volume of milk of an unchanged protein concentration would cause a commensurate increase in the influx of serum proteins. The explanation for the changes in casein number must, therefore, be more complex.

Milk is synthesised and secreted more or less continuously but removed by milking or suckling only intermittently, and for the time that it is stored within the udder it is potentially subject to modification.The most prevalent and well characterized modification is proteolytic degradation by the serum proteinase, plasmin, whereby α- and β-caseins are broken down to γ-casein (Politis, 1996). Logically, the longer that milk is stored in the gland the greater the chance of degradation, and it has been argued that the observed difference between thrice and twice daily milking is entirely due to storage time (Klei *et al.*, 1997). Plasminogen is the inactive precursor of plasmin and is converted to the active form by the action of plasminogen activators. The ratio of plasminogen to plasmin in milk is inversely related to proteolytic capacity, so the significantly lower ratio of plasminogen to plasmin in twice daily milked udder halves supports the storage hypothesis. If storage time were the only factor influencing the proteolysis of casein, however, we would have expected to see a full recovery or loss of casein number and of the individual caseins when storage times were reversed by short term treatment. This was not the case. Although content of α- and β-casein increased and γ-casein decreased when milking frequency was increased, significant differences remained.

The integrity of the mammary epithelium can influence proteolytic activity in milk. In the

mammary gland tight junctions form a barrier between the blood and interstitial fluid (basolateral side) and milk in the alveolar lumen (apical side) thus preventing serum components from entering into milk and *vice versa* (Peaker, 1997). In most species, including the cow, tight junctions are 'leaky' pre-partum, achieve patency in the first few days of lactation and then gradually lose integrity as lactation progresses. This was evident in the data from the first experiment. We used milk concentrations of sodium and potassium to assess tight junction permeability. Sodium's concentration in milk is normally lower than in plasma due to the presence of sodium pumps only on the basolateral aspect of the secretory cell. An increase in the sodium to potassium ratio is thus indicative of leaky tight junctions, allowing partial equilibration between plasma and milk.

There were clear differences in tight junction integrity between udder halves milked twice and thrice daily. Some of this was a short-term response, most probably related to storage time and udder distension. Stelwagen *et al.* (1994a) assessed tight junction integrity associated with once daily milking in dairy cows. They found that leakiness developed after approximately 17 h of milk accumulation, and this was associated with a reduction in milk secretion and an increase in protease activity (plasminogen and plasmin) in milk. However, the first experiment also clearly demonstrated long-term developmental differences whereby junctional integrity deteriorated more quickly in Control udder halves than in Frequent, particularly during late lactation (after week 41). In the previous chapter we found a decrease in lactose concentration as lactation progressed and this effect was much more pronounced in twice daily compared with thrice daily milked udder halves. Furthermore in agreement with the ratio of sodium to potassium this effect became greater as lactation progressed. Lactose is the principal osmolar component of milk and its concentration dictates milk volume, hence lactose concentration does not vary in freshly secreted milk. The decreased concentration in stored milk represents

a net flux of lactose from milk back into serum, and is thus further evidence of leaky tight junctions in glands milked twice-daily throughout lactation.

There is no evidence that plasminogen or plasmin is produced by mammary tissue, or that either is transferred into milk by a transcellular route. It seems highly likely, therefore, that paracellular transfer accounts for most of the plasminogen and some of the plasmin in milk (Stelwagen *et al.*, 1994b). Our ionic composition data show that tight junctions do gain and lose patency when milking frequency is temporarily increased or reduced, respectively. One effect of increased milking frequency is to reduce the time available for plasminogen to be converted to plasmin, so one would anticipate an increase in its concentration. On the contrary, plasminogen concentration decreased, which suggests that influx had been reduced, which is exactly what one would predict if uptake was passive through tight junctions which had become 'tighter'. This change in integrity also explains why the sum of plasmin plus plasminogen was decreased by short-term thrice-daily milking and increased by short-term twice-daily milking (Table 7.3).

Another possible route of entry of blood components into milk is via 'holes' in the mammary epithelium caused by apoptosis or necrosis during involution and mastitis (Nguyen & Neville, 1998), although there is no documented histological evidence of such holes, and data from other epithelia indicates that complete integrity is restored within a few minutes after cell death (Hudspeth, 1975). Mastitis is another cause of leaky tight junctions, but milk SCC was less than 250,000/ml throughout this study, so mastitis related increase in protease activity is unlikely.

Increased milking frequency sustained for long periods increases mammary cell proliferation and differentiation and reduces mammary apoptosis (Quarrie *et al.*, 1994). If involution had

indeed been suppressed by thrice daily milking, one would anticipate a reduced concentration of plasma proteins (BSA, IgG) and an elevated concentration of mammary derived proteins (caseins, α-lactalbumin, β-lactoglobulin). This is exactly what we observed. Lactoferrin is a mammary derived protein whose production is inversely related to lactational state, ie low during lactation, high in involution (Hurley, 1989). We were not able to distinguish serum albumin from lactoferrin owing to the small difference in molecular mass between the two (66267 versus 83000-87000), but we should have expected both to be lower in thrice daily milked glands and the results support this. All of the evidence points to the fact that involution was indeed suppressed by the thrice daily milking.

The short term changes induced by increased or decreased milking frequency are also consistent with a tight-junction related mechanism. Switching from twice to thrice daily milking caused tight junctions to become less leaky, evident as a reduced Na:K ratio. Total protein decreased as less plasma derived protein was transferred into milk and casein did not change. Reciprocal changes were observed when milking frequency was reduced from thrice daily to twice daily. Results for SA/Lf, IgG and plasmin/plasminogen are all consistent with this mechanism. A second element of consistency is the degree of response, which in all cases was small (often non-significant) and failed to totally reverse the long term effects. In other words, the loss of epithelial integrity caused by long term twice daily milking was only partly reversible by switching to thrice daily milking for two days. It may be that a more complete restoration would have occurred had the treatment been maintained for longer, but this was not tested.

Notwithstanding this, the net effect of a short term change in milking frequency was to move casein number in the appropriate direction to a point intermediate between the long term twice and thrice daily milked values. On the evidence of the changes in γ-casein this resulted at least

in part from decreased proteolysis, for which there are two possible explanations. First is the different storage time, second is the partial restoration/loss of epithelial integrity which would have altered the amount of activated plasmin available. We cannot ascribe relative importance to the two mechanisms from our results, although it may well be that storage time is most important in the short-term.

Chapter eight

Summary and conclusions

This thesis investigated factors, which potentially could manipulate lactation persistency to extend lactation from the conventional 12 month to 18 month lactation in dairy cows. Milk quality is an important issue to take into consideration during an extended lactation. The processing qualities of milk decreases as lactation progresses and it is not known whether this results in an overall poorer quality of milk due to the longer period spent in late lactation. This thesis therefore also examines whether the same factors that potentially can manipulate lactation persistency also improve milk protein quality. The arguments for extending lactation are numerous and involves a decrease in health problems, reduced veterinary bills, increased longevity, reduced replacement cost and so on. Furthermore, since fertility problems are often associated with high milk yield and negative energy balance we anticipated that reproductive success would be greater during extended lactation because cows would presumably be back in positive energy balance by the time of rebreeding. However, this was not the case, when we compared selected fertility parameters for our 18 month lactation with our 12 month lactation and with UK standards (Kossaibati & Esslemont, 1995) we found no evidence that rebreeding later into the lactation was easier or worse than during early lactation. Extended lactation did not compromise future reproductive success whether the following lactation was an extended or a conventional lactation rather the reverse. Body condition score at the start of the rebreeding period was not correlated with conception interval, which suggests that energy balance was not the reason for reproductive failure. It has been suggested that it is the magnitude and severity of loss in body condition score in early lactation that affects reproductive performance (Domecq *et al.*, 1997). Body condition score was not measured during the early part of

lactation in this experiment, so no conclusion can be drawn as to whether or not a correlation existed between loss in body condition score during early lactation and reproductive performance. Ovarian cysts could be another reason for failure to breed and the incidence of these were high. It was not within the remit of this study to look at the possible causes of infertility, however, the problems encountered may be different than those associated with peak lactation.

For extended lactation to be truly successful and at least as economical as a conventional lactation, lactation persistency needs to improve. Lactation persistency was in this thesis defined as the rate of decline in milk yield after peak lactation. R^2 is often used as an indicator to describe the accuracy of the fitted model to the actual data. In our spring calvers R^2 was 51.1±2.6% for a second grade polynomial, 51.3±2.6%for a third grade polynomial and 51.2±2.6% for a fourth grade polynomial grade, none of which produced a significantly better fit than a simple best fit linear regression analysis (R^2=47.6±2.7%). However R^2 does not actually describe the variation between actual and fitted data but rather how much of the variation in milk yield can be explained by lactation week, therefore, in our spring calving group about 50% of the variation in milk yield could be explained by lactation week. When lactation persistency is best, little variation in milk yield is explained by lactation week and R^2 is low. However this is not to say that the fitted model varies from the actual values. MSe describes the variation around the regression line and this measure is therefore a better indicator for the accuracy between fitted and actual data. MSe was 6.7 for the spring calving animals when fitting 2'nd, 3'rd or 4'th grade polynomials to their lactation curve while MSe for linear regression fit was 7.2, a difference that was not significant. Lactation persistency in this thesis was therefore quantified by best fit linear regression analysis performed on weekly averaged milk yields for individual cows.

It is important to distinguish between what affects milk yield and what affects lactation persistency. Since at any stage of lactation milk yield can be increased abruptly by frequent milking and BST treatment. It would appear that cell number is never immediately limiting to milk yield. On the other hand cell number does limit lactation persistency, since it is a gradual loss of cells which accounts for the decline in milk yield after peak lactation. Furthermore, to improve lactation persistency a continuing effect on cell number is required. Short term increases in lactation persistency might be an indirect effect of a stimulation of milk yield. During once daily milking an initial increase in apoptosis is observed but cell turnover then returns to levels as for thrice daily milking. Although lactation persistency was not measured in this experiment one would presume that persistency would be similar in once and thrice daily milked mammary glands. In order to determine the biological control of lactation persistency it is therefore important that we distinguish between long and short term effects on yield, since short term effects are probably a way for the gland to adapt to a certain level of production and do not affect lactation persistency.

A promising treatment for increasing persistency is to increase milking frequency, which has been shown to not only increase mammary cell proliferation (Wilde *et al.*, 1987) but also to reduce apoptosis (Li *et al.*, 1999). We therefore increased milking frequency from twice to thrice daily at peak lactation and continued this treatment throughout the entire lactation. Other treatments under investigation were nutritional input and calving season. Thrice daily milking proved to be the most effective and consistent treatment to increase milk production but also to improve lactation persistency. This suggests that the mammary cell population was better maintained by thrice daily milking. Evidence to suggest that involution was indeed suppressed by frequent milking was gathered in Chapter 7 where we found that plasma proteins (BSA, IgG) were reduced while mammary derived proteins

(caseins, α-lactalbumin, β-lactoglobulin) were higher in milk from long term thrice daily compared to twice daily milked udder-halves. Furthermore, this effect was not completely reversible with a two day change over in milking frequency. It may be that a more complete restoration would have occurred had the treatment been sustained for longer but this was not tested. However differences in milk lactose together with differences in sodium and potassium suggested long term developmental differences in the mammary epithelium between twice and thrice daily milked udder halves. This also had consequences for milk protein quality. One report which investigated the effect of thrice daily milking on casein numbers argued that the increase they observed in casein number with an increase in milking frequency was due to a difference in storage time of milk within the udder and hence more/less time for proteolysis. We demonstrated that although storage time is an important factor in determining proteolysis in milk it is certainly not the only mechanism involved. The mammary tight junctions also have a role to play. It is well established that supplementary feeding increases casein number in milk as we found in this experiment. The mechanism is unknown but it is believed to involve changes in rumen fermentation. Nevertheless a combination of frequent milking and supplementary feeding was able to completely maintain casein number through lactation.

While there can be no doubt that servere underfeeding affects mammary function in both ruminants (Sorensen *et al.*, 2000; Smith, 1978). and rodents (Grimble, 1987) we are not aware of any studies investigating the effect of supplementary feeding on mammary cell turnover. It is well known and accepted that milk yield increases with supplementary feeding, but whether that is simply due to a greater nutrient uptake without any cellular changes in the gland remains to be investigated. Supplementary feeding has not been shown to affect lactation persistency once a response is established. However, none of these earlier

studies were especially set up to measure lactation persistency (Broster & Broster, 1984). It is apparent from the current data that supplementary feeding increased milk yield but the effect on lactation persistency was inconsistent. This suggests that supplementary feeding does not significantly affect cell turnover in the mammary gland at least in the long term. It might be that nutrition is of more importance for absolute milk yield and in that respect also lactation length rather than lactation persistency per se. There is a need for further investigation of the effect of supplementary feeding on mammary function under circumstances where animals are not underfed.

Seasonal variation in milk yield is a common phenomenon, which was also observed in this experiment. We concluded that this effect was due to a change in diet from the winter ration, which had a lower nutritional value than fresh spring grass. However, some of the effect could also be due to photoperiod. Lactation persistency was also affected by calving season, however, this effect was only apparent in the first half of lactation. Like supplementary feeding this would suggest that seasonal changes in nutrition and photoperiod do not have a continuing effect on cell turnover in the mammary gland if any at all. However, it could also be that the late start of buffer feeding provoked an apoptotic response in the mammary gland, although when buffer feeding was initiated earlier (in the second extended lactation) the seasonal effects on lactation persistency were still short term. The buffer feed used in this experiment was of lower nutritional value than fresh spring grass, which again could have changed cell turnover in the mammary gland. It is not possible to conclude from this experiment whether nutrition/calving season has any biological control on lactation persistency. A controlled experiment determining the effect of a high quality diet such as maize silage for example throughout lactation on lactation

persistency combined with measurement of mammary turnover could possibly answer this question.

Recurring pregnancy has a negative effect on milk yield from around pregnancy week 20 and onwards (Coulon & Perochon, 1998). This is believed to be due to secretion of oestrogen from placenta (Bauman & Currie, 1980). A negative effect of pregnancy on lactation persistency was also evident from pregnancy week 20 in this experiment Both persistency and absolute decrease from pregnancy week 20 to 27 was greater in thrice daily milked udder halves, however, the percentage decrease was similar irrespective of milking frequency. If increased milking frequency had indeed increased the amount of secretory tissue this suggests that an equal amount of tissue returned to a non secretory state. Peak yield was negatively correlated with lactation persistency. This observation is not novel (Danell, 1981), however, no research has been conducted to establish the cause of this. We believe that the extent of cell renewal and carry-over of cells from one lactation to another could be one factor involved. If so, how does one encourage cells to undergo apoptosis during the dry period so that as many new cells as possible proliferate? If the same factors controlling apoptosis in rodents are functional in ruminants, administration of IGFBP-5 into the mammary gland should encourage apoptosis, however, whether this would result in a corresponding increase in proliferation is an open question.

This thesis has shown that it is possible to manipulate lactation persistency to extend lactation in dairy cows but careful management is required. Increased milking frequency consistently improved lactation persistency while supplementary feeding and calving season only had temporary effects on lactation persistency. However, both had positive effects on milk yield and therefore have influences on lactation length. Milk protein quality was

improved by frequent milking and supplementary feeding, in combination these were able to completely maintain casein number throughout lactation. Furthermore it is also apparent from this work that more research is needed to clarify control mechanisms for mammary cell turnover because we know very little about what controls lactation persistency. This would enable us to develop new strategies for improving lactation persistency. In the meantime, the advent of robotic milking systems which have the capability to milk frequently without increased labour offer the potential to achieve extended lactation. If this is to happen attention will have to focus on maintaining cow attendance throughout the lactation, because it is apparent that persistency will only be improved if frequent milking is maintained continuously.

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