The effect of protein levels in dairy cow diets on body reserves throughout lactation

End of Project Report to AgriSearch
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STRUCTURE OF REPORT

This report begins with an Executive Summary which provides the background to the research, details of the work undertaken, key findings, and practical implications.

The main body of the report comprises a series of four scientific papers (Chapters 1-4), with each paper examining a specific aspect of the work. Three of these papers have already been published in scientific journals, while the fourth will soon be submitted for publication. Papers contained within chapters 1-4 are as follows:

Chapter 1:
Law, R.A., F.J. Young, D.C. Patterson, D.J. Kilpatrick, A.R.G. Wylie and C.S. Mayne. 2009a. Effect of dietary protein content on animal production and blood metabolites of dairy cows during lactation. *Journal of Dairy Science* 92: 1001-1012.

This chapter examines the effects of dietary crude (CP) protein content on milk production, energy status and blood metabolite concentrations in high yielding dairy cows.

Chapter 2:
Yan, T. 2009. Effects of dietary crude protein concentration on energy and nitrogen utilisation efficiencies and environmental footprint in lactating dairy cows. (For submission).

This chapter examines the effects of dietary CP content on methane emissions and the efficiency of energy and nitrogen utilisation by high yielding dairy cows.

Chapter 3:
Law, R.A., F.J. Young, D.C. Patterson, D.J. Kilpatrick, A.R.G. Wylie and C.S. Mayne. 2009b. Effect of dietary protein content on the fertility of dairy cows during early and mid lactation. *Journal of Dairy Science* 92: 2737-2746.

This chapter examines the effects of dietary CP content, and energy status on the reproductive performance of the high yielding dairy cow.

Chapter 4:

Law, R.A., F.J. Young, D.C. Patterson, D.J. Kilpatrick, A.R.G. Wylie and C.S. Mayne. 2009c. Effect of dietary protein content on oestrous behaviour of dairy cows during early and mid lactation. *Journal of Dairy Science* 92: 1013-1022.

This chapter examines the effects of dietary CP content on the expression of oestrous behaviour by high yielding dairy cows. Furthermore, it examines the use of a low labour input oestrous detection system.

The report finishes with a summary of key presentations and publications which have arisen from this work.

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EXECUTIVE SUMMARY

- Dairy cows require protein for milk production, maintenance, growth, pregnancy and immune function. Protein supply is a key driver of food intake and milk production.

- The protein requirements of a dairy cow are met directly from dietary protein, rumen microbial crude protein (CP), and from the mobilisation of body tissue reserves. Rumen bacteria require a source of nitrogen to grow. If there is inadequate nitrogen in the diet, microbial growth and subsequent digestion of food will be impaired.

- In early and mid lactation, high yielding cows are unable to consume sufficient forage to meet their energy (and sometimes protein) requirements. Consequently, it is normal practice to offer concentrate supplements. The CP content of the concentrate is normally adjusted to achieve a total dietary CP content of 18% (dry matter basis). An 18% CP diet is normally assumed to be optimum to ensure an adequate supply of metabolisable protein, and to achieve an economically optimum milk output response.

- Protein tends to be the most expensive component of the diet, and as such, overfeeding protein will increase feed costs and reduce margins. Furthermore, as dietary protein is used inefficiently by dairy cows, with approximately 70% of protein consumed excreted in faeces and urine, feeding diets containing excessive dietary CP concentrations will result in nitrogen loss to the environment.

- Nitrogen loss to waterways, mainly via leaching, contributes to aquatic eutrophication. Nitrogen lost to the atmosphere as ammonia via volatilisation, and as nitrous oxide (green house gas) via denitrification, contributes to terrestrial eutrophication, and global warming respectively. European Union (EU) and UK Government legislation seeks to control nitrogen losses via each of these routes, for example, through the Nitrates Directive and the Water Framework Directive (nitrate leaching), the EC National Emission Ceilings Directive (ammonia), and the UK Climate Change Bill.

- The EU Nitrates Directive has already had a significant impact on Northern Ireland (NI) dairy farmers, with the whole of Northern Ireland having been designated a Nitrates Vulnerable Zone (NVZ). A key feature of the Nitrates Directive is a limit on manure nitrogen applications of 170 kg organic nitrogen per hectare. However, the Nitrates Directive does not set manure N excretions for different classes of livestock, leaving each country to establish their own values. Within NI, AFBI research has demonstrated an excretion value of 91 kg organic nitrogen per year for a dairy cow, thus allowing a stocking rate of 1.86 cows per hectare (170/91). However, this value of 91 kg per cow per annum is likely to be revised in subsequent reviews of the NI Nitrates Directive action plan. A move towards lower protein diets may be looked on favourably by the EU Commission.

- As high yielding dairy cows are unable to consume sufficient food to support their energy requirements in early lactation, cows tend to experience severe and prolonged periods of negative energy balance. Severe negative energy balance is known to have a detrimental effect on milk production and subsequent reproductive performance. Milk yield is a key driver of negative energy balance. As dairy cows have a limited supply of nitrogen within the body that can be used for milk production, reducing dietary protein content is likely to suppress milk production, and as such, improve energy balance. Therefore, lowering the dietary protein content could be an important strategy to reduce the extent of negative energy balance experienced in early lactation, and this may have a positive effect on reproductive performance. However, this will only be acceptable if it can be achieved without a significant reduction in milk output and without having a detrimental effect on cow health.

- To address this issue, a study was undertaken to examine the effect of reducing dietary CP content on:
    a. Animal performance (energy status and production)
    b. Nitrogen utilisation and methane emissions
    c. Reproductive performance
    d. Oestrous expression

- Experimental Design: the experiments involved the allocation of ninety autumn calving Holstein Friesian dairy cows (45 primiparous and 45 multiparous) to diets containing one of three dietary CP concentrations (173, 144, or 114 g CP/kg DM), from calving until day 150 of lactation. From day 151 onwards, half of the animals in each treatment were allocated an alternative dietary CP concentration (Figure 1), whilst the remaining animals were maintained on their original diets. All diets contained 55% concentrate, 27% grass silage, 18% maize silage (DM basis).

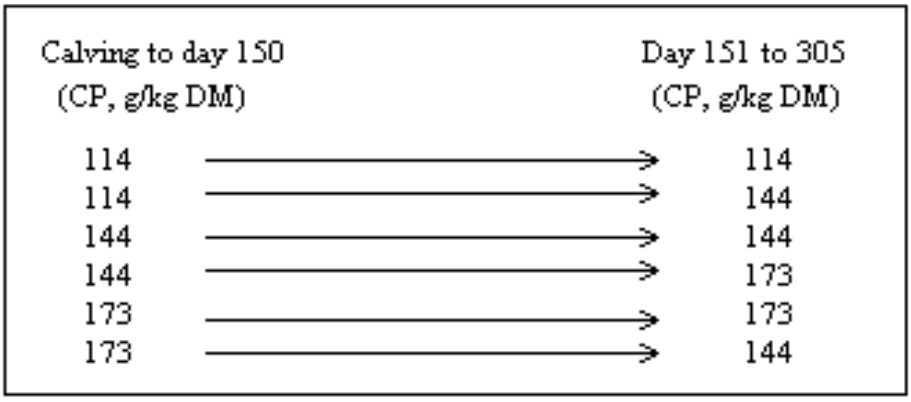

Figure 1: Diet allocation at day 151 of lactation.

- Within this study, a subset of animals (4 cows and heifers from each treatment) was transferred to a metabolism unit on three occasions throughout the lactation (days 80, 160, and 240) to examine the effects of dietary CP concentration on energy and N utilisation efficiency, and on methane production.

- Milk production in early to mid lactation: during the first 150 days post-calving, an increase in dietary CP concentration resulted in an increase in dry matter intake and milk yield (Table 1). Animals offered 114 g CP/kg DM produced milk with a higher milk fat concentration than animals offered 144 or 173 g CP/kg DM. There was no effect of diet on concentrations of milk protein and lactose.

Table 1: Effects of dietary crude protein concentration on dry matter intake, milk yield and milk composition (fat, protein and lactose) during the first 150 days of lactation.

	Dietary CP concentration (g/kg DM)				
	114	144	173	SED	Significance
Dry matter intake (kg/d)	16.5	18.0	18.6	0.35	***
Milk yield (kg/d)	25.4	31.8	35.4	1.14	***
Milk composition (%)					
Fat	4.20	3.83	3.81	0.140	*
Protein	3.14	3.23	3.24	0.061	NS
Lactose	4.82	4.81	4.78	0.046	NS

- Milk production in mid to late lactation: during mid to late lactation (day 151 to 305), decreasing the dietary CP concentration from 173 to 144 g/kg DM had no significant effect on milk yield or dry matter intake (Table 2). This highlights that the efficiency of use of dietary nitrogen can be improved by feeding diets with lower protein concentrations (144 g CP/kg DM) without detrimental effects on production.

Table 2: Effect of altering dietary crude protein concentration at day 151 of lactation on dry matter intake, milk yield, and milk composition between days 151-305 of lactation.

	Dietary CP concentration (g/kg DM)							
Day 1 – 150	114	144	173	114	144	173	SED	Significance
Day 151 - 305	114	144	173	144	173	144		
Dry matter intake (kg/d)	16.8	17.8	19.3	18.0	19.7	18.7	0.45	***
Milk yield (kg/d)	23.0	28.8	29.8	26.3	30.7	29.8	1.07	***
Milk composition (%)								
Fat	4.23	3.83	3.81	4.27	3.98	3.74	0.099	***
Protein	3.32	3.35	3.37	3.26	3.37	3.35	0.060	NS
Lactose	4.84	4.80	4.71	4.89	4.86	4.80	0.053	*

- Body energy status: increasing dietary protein concentration in early lactation resulted in an increase in ME intake (as a result of higher DM intakes). However, the increase in ME intake was not sufficient to supply the extra ME requirement associated with the higher milk output. Therefore, animals receiving 173 g CP/kg DM experienced more severe negative energy balance than animals receiving 144 and 114 g CP/kg DM (Table 3).

Table 3: Effect of dietary protein on energy status during days 1-150 of lactation.

	Dietary CP concentration (g/kg DM)			SED	Significance
Day 1 to 150	114	144	173		
Live weight (kg)	532	548	544	4.18	***
Condition score	2.38	2.41	2.37	0.03	NS
ME intake (MJ/d)	204	223	231	4.54	***
ME requirement (MJ/d)	191	222	242	6.34	***
Energy balance (MJ/d)	12.8	0.24	-11.1	6.20	**
Cumulative EB (MJ)	414	-537	-1801	520	***

- Nitrogen utilisation: increasing dietary CP concentration significantly increased N intake and N output in manure and milk. However, increasing dietary CP concentration significantly reduced milk N output as a proportion of N intake. Furthermore, manure N output associated with one kg milk production increased with increasing dietary CP concentration (Table 4). Decreasing dietary CP concentration improved the efficiency of nitrogen utilisation for milk production.

Table 4: Effects of dietary CP concentration on nitrogen utilisation efficiency

	Dietary CP concentration (g/kg DM)				
	114	144	173	SED	Significance
Nitrogen intake and output (g/d)					
N intake	322	445	562	10.5	***
Manure (faeces + urine) N	227	300	380	5.3	***
Milk N	100	132	144	4.4	***
Nitrogen utilisation efficiency					
Manure N/N intake (g/g)	0.71	0.67	0.68	0.007	***
Manure N/milk yield (g/kg)	11.4	12.0	14.1	0.53	***
Milk N/N intake (g/g)	0.31	0.30	0.26	0.007	***
Methane emission					
CH_4/DM intake (l/kg)	31.1	28.2	28.2	0.83	*
CH_4/milk yield (l/kg)	25.7	20.9	20.4	1.13	**

- Methane emissions: increasing dietary CP content from 114 to 144 g/kg DM resulted in a significant decrease in methane emissions, as a proportion of DM intake ($P < 0.05$) and milk yield ($P < 0.01$). However, there is no benefit in terms of reduced methane emissions, as a proportion of feed intake or milk yield, when dietary CP concentration is over 144 g/kg DM (Table 4).

- Reproductive performance: dietary CP concentration had no significant effect on any of the reproductive parameters assessed. However, animals receiving 114 g CP/kg DM tended to have a higher 100 day in-calf rate compared to animals receiving 144 and 173 g CP/kg DM (Table 5). Within a six month breeding period, 100% of cows became pregnant when allocated a diet containing 114 g CP/kg DM during the first 150 days of lactation, compared to 92.9% and 86.7% of cows receiving diets containing 144 and 173 g CP/kg DM respectively.

Table 5: Effects of dietary crude protein concentration on fertility performance

	Dietary CP content (g/kg DM)			SED	Significance
	114	144	173		
Pregnancy to 1st service (%)	35	30	28	8.6	NS
Pregnancy to 1st and 2nd service (%)	55	63	52	9.2	NS
100d in-calf rate (%)	83	67	62	8.2	NS
Overall pregnancy rate after 6 months (%)	100	93	87	5.4	NS

- Energy effects on reproductive performance: cows with a larger cumulative negative energy balance were less likely to conceive. Additionally, it was found that a larger daily negative energy balance and a larger cumulative negative energy balance were associated with a longer interval to progesterone rise after ovulation. A delay in progesterone rise post ovulation and/or low progesterone concentrations during a cycle are associated with smaller embryos and will reduce the likelihood of conception.

- Oestrous expression: replacing cows that are culled due to reproductive failure is extremely costly (estimated at £18,000/100 cow herd/year). Although reproductive efficiency is influenced by many factors, heat detection is a key management issue which influences reproductive success and one which farmers have a high degree of control over. In addition to investigating the effect of dietary CP concentration on oestrous behaviour and subsequent fertility, the current study evaluated a more focussed approach to heat detection.

- Low input oestrous detection method: cows were observed for 30 minutes, twice daily (9.00 am and 9.00 pm), for the expression of heat. Nine oestrous behaviours were used from a scoring system developed in Holland to identify cows in heat (Table 6). Each behaviour is allocated a given number of points. Once an observation period was complete the total number of points for each cow was calculated. If the total was greater than or equal to 50 points during a single or consecutive (an aggregate score for a particular cycle) observation period(s), then the animal was deemed to be in heat. Using

this low labour input heat detection system, a 65% heat detection rate was achieved. If additional heats were recorded during cow collection and milking, this percentage could be increased to 75%.

Table 6: Scoring scale for observed symptoms of oestrus (Van Eerdenberg *et al.,* 1996)

Symptoms of oestrus	Score
Mucous vaginal discharge	3
Cajoling	3
Restlessness	5
Sniffing the vagina of other cow	10
Chin resting	15
Mounted but not standing	10
Mounting (or attempt) other cows	35
Mounting head-side of other cow	45
Standing heat	100

- Behavioural indicators of oestrus: within this experiment a total of 238 oestrous cycles with a score of 50 points or above were observed. However, dietary protein concentration had no effect on the expression of oestrous behaviour. Despite being deemed the most reliable sign of oestrus, only 51.7% of cycles (238) were characterised as standing immobile on mounting. The most frequent behavioural activity displayed was chin resting (89.5% of cycles) and the most reliable behaviour was standing immobile on mounting (when expressed 96.4% of cows were in oestrus). The most dependable (function of reliability and frequency displayed) sign of oestrus was mounting or attempting to mount another cow (Table 7). This behaviour was expressed in 83% of cycles, and when expressed, 89% of animals were in oestrus.

- Sexually active group: an increase in the size of the sexually active group (animals in oestrus at the same time; up to five) significantly increased the expression of mounting or attempting to mount another cow, the number of cycles in which 'standing immobile on

being mounted' was observed, the total oestrous score, and the proportion of cyclic animals that were diagnosed as being in oestrus. The size of the sexually active group can be increased with a compact calving pattern and/or large groups of cows at a similar stage of lactation.

Table 7: The dependability of oestrous behavioural activities as a means of detecting animals in oestrus.

Oestrous behaviour	Reliability[1]	Percentage[2] expression	Dependability[3]
Mucous vaginal discharge	75.7	8.8	667
Cajoling	86.9	53.4	4640
Restlessness	78.9	81.5	6430
Sniffing the vagina of other cow	75.8	86.6	6564
Chin resting	80.5	89.5	7205
Mounted but not standing	78.3	19.7	1543
Mounting (or attempt) other cows	88.5	83.2	7363
Mounting head side of other cow	95.0	22.3	2119
Standing immobile on being mounted	96.4	51.7	4984

[1] Percentage of animals that expressed this behaviour and were in oestrus based on progesterone profile
[2] Percentage of oestrous cycles in which behaviour was expressed
[3] Function; reliability * percentage expression

Chapter 1: Effect of Dietary Protein Content on Animal Production and Blood Metabolites of Dairy Cows during Lactation

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INTERPRETIVE SUMMARY

Ninety autumn calving dairy animals, upon parturition, were allocated diets containing one of three dietary CP concentrations. On day 151 of lactation, half the animals on each treatment were allocated an alternative dietary CP concentration. Results indicate that from 1 to 150 days in milk, an increase in dietary CP significantly increased milk, fat and protein yield, but decreased energy balance. From days 151 to 305 of lactation, reducing the dietary CP concentration from 173 to 144 g CP/kg DM had no detrimental effect on milk, fat and protein yields but improved the nitrogen efficiency.

Abstract

Ninety autumn calving Holstein dairy cows (45 primiparous and 45 multiparous (mean parity, 3.1)) were allocated to one of three dietary CP concentrations; 173, 144, or 114 g CP/kg DM, from calving until day 150 of lactation. On day 151, half of the animals in each treatment were allocated an alternative dietary protein concentration. Half of the animals receiving 114 g CP/kg DM went onto 144 g CP/kg DM, half of the animals receiving 144 g CP/kg DM went onto 173 g CP/kg DM and half of the animals receiving 173 g CP/kg DM went onto 144 g CP/kg DM, with the remaining animals staying on their original treatment. This resulted in six treatments in the mid to late lactation period: 114/114; 144/144; 173/173; 114/144; 144/173; and 173/144 g CP/kg DM. An increase in dietary CP concentration significantly increased milk, fat and protein yield in early lactation (days 1 to 150). Dry matter intake was also increased with increased dietary protein concentration, however, this was not significant between 144 and 173 g CP/kg DM. Increased dietary CP significantly increased plasma urea, albumin and total protein concentrations but had no significant effect on NEFA, leptin or IGF-1 concentrations. Decreasing the dietary CP concentration in mid-late lactation (days 151 to 305) from 173 to 144 g/kg DM had no significant effect on milk yield, dry matter intake, or milk fat and protein yield, compared to animals that remained on 173 g CP/kg DM throughout lactation. Increasing dietary CP concentration from 144 to 173 g/kg DM significantly increased dry matter intake, compared to animals that remained on the 144 g CP/kg DM throughout lactation. There were no significant dietary treatment effects on live weight or body condition score change throughout the experiment. Results of this study indicate that high protein diets (up to 173 g CP/kg DM) improved food intake and animal performance in early lactation (up to day 150), but thereafter, protein concentration can be reduced to 144g CP/kg DM with no detrimental effects on animal performance.

Keywords: dietary protein, milk production, dry matter intake, energy balance,

Introduction

The ever increasing requirement to maximise the economical efficiency of animal production has driven forward the intensification of farming systems. The exponential increase in the genetic potential for milk production of the dairy cow has resulted in an increase in dietary CP concentration of diets, to ensure a sufficient supply of metabolisable protein to achieve maximal

milk production. At present, for high yielding dairy cows a CP level of approximately 180 g/kg of dry matter (DM) is commonly used in commercial diets (Kung, 2000). However, previous reports have suggested that increasing dietary protein concentration above 167 g/kg DM has no benefit in terms of yield of milk or milk components (Broderick, 2003). High dietary CP levels are positively associated with the degradation of protein in the rumen (increased ammonia concentrations) and have been shown to decrease the efficiency of nitrogen utilisation for milk production (Broderick, 2003; Hristov *et al*., 2004). From an environmental perspective, 0.65 to 0.75 of nitrogen consumed is excreted via urine and faeces (Yan *et al*., 2006; Chase, 1994). Yan *et al*. (2006) showed that nitrogen excretion in manure was highly correlated with dietary nitrogen intake, and hence a key mitigation strategy to reduce manure nitrogen output is to reduce dietary nitrogen concentrations. Furthermore, previous studies have shown that an over-supply of rumen degradable protein (relative to fermentable metabolisable energy supply) will increase the diffusion of ammonia from the rumen to the portal blood supply and subsequently increase urea production (detoxification by-product) in the liver (Roseler, 1994). In addition, ureagenesis (conversion of absorbed ammonia into urea in the liver) is less efficient in cows with fatty liver (Strang *et al*., 1998) which will reduce the detoxification of ammonia in the blood. The toxicity of ammonia has been reported to inhibit vital processes such as gluconogenesis and the functionality of the tricarboxylic acid cycle (Rodriguez *et al*., 1997). Therefore, the balance between rumen degradable protein and fermentable carbohydrate is important in achieving an optimal ammonia concentration in the rumen.

The objective of this study was to investigate the effects of a range of dietary protein concentrations (120, 150 and 180 g/kg DM) on animal production, energy metabolism, blood parameters and nitrogen efficiency during early lactation (1 to 150 d), and the subsequent effects of altering dietary protein concentration at day 151 of lactation.

Materials and Methods

Animals and Housing

Ninety Holstein Friesian dairy cows (45 primiparous and 45 multiparous (mean parity 3.1)) were used in the experiment. Calving commenced on 29 August and ended on 23 December. Following calving, animals were housed as a single unit in free stalls with concrete flooring. The cubicle to cow ratio was ≥ 1:1 at all times, meeting the recommendations set by FAWC (1997). Cubicles had a bed measurement of 2.20 m long and 1.25 m wide, were fitted with rubber mats and bedded with sawdust thrice weekly. Concrete passageway floors were scraped a minimum of four times daily, using an automated system. Cows were milked twice daily at 05.30 and 16.30 hours, with cows travelling about 35 m to the milking parlour. Lights were left on in the buildings at all times.

Experimental Design, Diets and Feeding

The experiment involved allocating 90 freshly calved Holstein Friesian dairy animals to three dietary treatments which differed in overall CP levels, as formulated, in the complete diet (DM basis); 180, 150, and 120 g/kg DM. At day 151 of lactation, half of the animals in each treatment were allocated (balanced for parity, milk yield, calving date and live weight) an alternative dietary CP concentration, whilst the remaining animals were maintained on their original diets. Half of the animals receiving 120 g CP/kg DM went onto 150 g CP/kg DM, half of the animals receiving 150 g CP/kg DM went onto 180 g CP/kg DM and half of the animals receiving 180 g CP/kg DM went onto 150 g CP/kg DM. This resulted in six treatments (1 to 151/151 to 305 days in milk (DIM)): 120/120; 150/150; 180/180; 120/150; 150/180; and 180/150. Primiparous animals were assigned in a balanced manner to treatments based on heifer rearing regime, calving date and live weight. Multiparous animals were assigned to treatments according to parity, previous lactation milk yield, calving date and live weight. The diet was presented as a total mixed ration (TMR) and animals were fed between 10.00 and 11.00 h daily using a diet feeder. Animals had free access to water at all times. The concentrate to forage ratio (DM basis) was 0.55:0.45 for all diets. The forage component of the diet consisted of 0.60 grass silage and 0.40 maize silage (DM basis). Samples of grass and maize silage were taken weekly and analysed using near infrared reflectance spectroscopy (Park *et al*., 1998), and twice weekly for measurement of nitrogen and ammonia nitrogen using methods outlined by Steen

(1989). Two concentrates were formulated to contain 229 and 117 g CP/kg DM in order to achieve target protein levels in the overall diets of 180 and 120 g CP/kg DM respectively. Diets containing 150 g CP/kg DM were produced by complementing the forage component with equal amounts of concentrates containing 117 and 229 g CP/kg DM. The energy and protein concentrations of individual ingredients were based on published values (AFRC, 1993) which were used in the initial formulation of the concentrate proportion of the diet as presented in Table 1.1. Concentrate samples were taken weekly during the experiment and analysed for DM, ash, ADF, NDF and nitrogen as described by Cushnahan and Gordon (1995). The TMR diets were offered *ad libitum* using feed boxes which were placed on a computer recorded load cell system, with controlled access to the boxes using an electronic identification system. This enabled dry matter intakes of individual cows to be recorded continuously via automatic feeding gates (Calan gate feeder), from which a daily intake was calculated and then averaged on a weekly basis.

Measurements

Milk yields were recorded daily at each milking throughout the experiment. Milk composition (fat, protein, lactose and somatic cell count) was determined on a weekly basis from one consecutive am and pm milking. Separate analysis was completed for am and pm samples and milk composition was calculated on the basis of recorded am and pm milk yields. Milk composition was determined using an infrared milk analyzer (IRMA).

Live weight and body condition score (scale: 0-5; Edmonson *et al*., 1989) were recorded on a weekly basis and throughout lactation.

Animals were blood sampled once weekly, between 0930 and 1130, from the coccygeal vein using uncoated, heparin-coated and fluoride oxalate coated tubes (BD, Oxford, UK) from calving until day 100 of lactation, and then fortnightly thereafter. Plasma was recovered by centrifugation for analysis of glucose (fluoride oxalate tubes), total protein, albumin, globulin, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA) and urea (heparinised tubes) respectively by clinical analyser (Olympus UK Ltd, Middlesex, UK). Non-esterified fatty acid concentrations were determined using a standard kit (Wako Chemicals GmbH, Neuss, Germany). Uncoated tubes provided serum for leptin and IGF-1 radio-immunoassays (RIA). Plasma and

serum samples were stored at –20°C until analysed. Radio-immunoassays were balanced for dietary treatments and parity and control samples were included in each assay.

Table 1.1: Ingredient formulation of concentrates (g/kg DM) and energy and protein concentration (as formulated)

Constituent	Low protein (g/kg DM)	High protein (g/kg DM)
Barley (milled)	240	140
Wheat (milled)	240	140
Unmolassed sugar beat pulp	163	94
Citrus pulp	163	94
Maize gluten feed	30	100
Distillers grain (maize)	80	100
Soya bean meal (Hi-Pro)	0	165
Rape meal	0	100
Megalac[1]	22	14
Trace minerals and vitamins	3	3
Salt	4	4
Dicalcium phosphate	12	0
Limestone ($CaCO_3$)	15	15
Magnesium oxide	6	6
Molasses	24	24
ME (MJ/kg DM)	13.1	13.1
CP (g/kg DM)	116.6	228.6
ERDP (g/kg DM)	78.5	134.8
DUP (g/kg DM)	28.9	73.5
Starch (g/kg DM)	310.6	214.9

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Serum leptin concentrations were determined in samples taken from all animals in each of weeks 2, 4, 6, 8, 10, 12, 16 and 20. The primary anti-leptin antibody (OL-3) was raised in guinea pigs using recombinant ovine leptin kindly donated by Professor A. Gertler (The Hebrew University of Jerusalem) and was included at a final dilution of 1:160,000 in each assay. Primary antiserum (100 µl) and plasma (100 µl) were incubated overnight (20 hr) in a refrigerator after which was

added 100 µl (12000-15000 cpm) of ^{125}I-iodinated ovine leptin, prepared using iodogen (Perbio Science, Northumberland, UK). Tubes were then held for a further 24 hr in a refrigerator before addition of 100 µl of a cellulose-immobilised second antibody suspension (Sac-Cel anti-guinea pig IgG; IDS Ltd., Washington Tyne on Wear, UK). Tubes were then left at room temperature for 20 min before addition of 1 ml of deionised water and centrifugation at 1900 x g for 20 min. Following centrifugation, supernatant was removed and radioactivity in each pellet was counted using a Cobra ll gamma counter (Packard Canberra, Reading, UK).

Serum IGF-1 concentrations were determined in samples taken, in weeks 2, 6, 10, 16 and 20, from 4 multiparous and 4 primiparous animals, randomly selected from each of the three dietary treatments. Insulin-like growth factor-1 binding proteins were removed by acid-ethanol cryo-precipitation (Wylie *et al*., 1997) before analysis for free IGF-1. The primary antibody (AFP 4892898) was rabbit anti-human somatomedin C (a gift from Dr A Parlow of the US National Hormone & Pituitary Program, Torrance, CA), used at a final dilution of 1:400,000. Human recombinant IGF-1 purchased from NIBSC (National Institute for Biological Standards and Control, Potters Bar, Herts., UK) was used as the standard while the label was ^{125}I-human recombinant IGF-1 (Amersham International, Bucks., UK) included at 12000 to 15000 cpm per tube. Bound IGF-1 label was recovered by addition of a second antibody (goat anti-rabbit IgG, Sigma Chemical Co, Poole, UK) followed by precipitation with polyethylene glycol succinate (6% w/v solution). After centrifugation and removal of supernatant, pellets were counted using a Cobra ll gamma counter. Blood samples were not analysed for leptin and IGF-1 from 151 to 305 DIM.

Calculation of Energy Balance

The average daily energy balance for each animal was calculated each week of lactation using the equations described by Thomas (2004) [Energy balance = metabolisable energy (ME) intake – ME requirement (-10 + (ME_{preg} + $ME_{maintmilk}$ * $Lwt^{0.75}$) + ((0.0013* Lwt)/K_m)); $K_{m,}$ efficiency of energy use for maintenance (0.35*ME/GE + 0.503)]. Daily milk yield, daily dry matter intake (DMI), weekly milk composition, weekly live weight and feed composition data were used in the calculations. ME contents of grass and maize silage were obtained on a weekly basis using near infrared reflectance spectroscopy (Park *et al*., 1998) and ME contents of the concentrate were as

formulated. Missing values were estimated from the week previous to and the week following missing data. Less than 2% of the data were missing.

Statistical Analysis

A repeated measures approach using the Residual Maximum Likelihood (REML) procedure available in GenStat (Payne *et al*. 1993) was used to analyse the data set. The model fitted fixed effects for parity, dietary protein treatment and stage of lactation (weeks from calving) for each parameter. The model included all two-level interactions between these variables. Additional orthogonal contrasts were calculated for linear and quadratic effects of treatments from 1 to 150 DIM. There was no significant effect of dietary treatment on condition score change or live weight over the first three weeks of lactation. Therefore, the deviation from the mean of these two variables, in multi- and primiparous animals during this period, was used as a covariate. Due to the design of this experiment, data were analysed in three parts: 1 to 150 DIM, 151 to 305 DIM, and 1 to 305 DIM. An additional covariate was included in the analysis of data from 151 to 305 DIM; the difference between the mean of the variable in question from 135 to 150 DIM for each animal, and the overall mean of the same variable from animals within the same treatment from 135 to 150 DIM.

Results

Table 1.2 illustrates the composition (as fed) of diets targeted to contain 120, 150 and 180 g CP/kg DM. Actual values were 114, 144 and 173 g CP/kg DM respectively. All diets were isoenergetic. The effective rumen degradable protein (eRDP, estimated using Feed into Milk (Thomas, 2004)) to digestible undegradable protein (DUP) ratios for diets containing 114, 144 and 173 g CP/kg DM were 2.17, 1.96 and 1.79 respectively (Feedbyte (Feed into Milk, 2004)).

Production Responses to Dietary Protein in Early and Mid Lactation (1 to 150 DIM)

Dietary protein concentration effects on DMI, ME intake, milk yield, milk constituents (fat, protein and lactose), constituent yields (fat and protein) and milk energy output are presented in Table 1.3. Increased dietary protein concentration significantly ($P < 0.001$) increased: milk yield; milk protein concentration; total milk fat yield; total milk protein yield; and milk energy output. Increasing dietary protein concentration from 114 to 144/173 g/kg DM significantly

increased DMI ($P < 0.001$) and decreased milk fat concentration ($P < 0.05$). There was no significant ($P > 0.05$) difference in DMI or milk fat concentration between animals receiving 144 and 173 g CP/kg DM. However, orthogonal contrasts for linear and quadratic effects suggest that the relationship between dietary protein concentration and DMI, and milk fat concentrations is linear ($P < 0.001$ and $P < 0.05$ respectively).

During the first 150 days of lactation, an increase in dietary protein content significantly ($P < 0.001$) increased the average daily ME requirement (Table 1.4). Increasing dietary protein concentration from 114 to 144/173 g/kg DM significantly increased ME intake and live weight. There was no significant difference in ME intake or live weight of animals between 144 and 173 g CP/kg DM. Orthogonal contrasts for linear and quadratic effects suggest that the relationship between dietary protein concentration and live weight is curvilinear (significant ($P < 0.01$) linear and quadratic effects). Increasing dietary protein concentration from 114 to 173 g/kg DM significantly ($P < 0.001$) decreased daily energy balance. However, there was no significant difference in daily energy balance between animals on 144 g CP /kg DM and those on 114 and 173 g CP/kg DM. Increasing dietary protein concentration from 114/144 to 173 g/kg DM significantly decreased cumulative energy balance. There was no significant effect of dietary protein concentration on the change in live weight or body condition score over the first 150 DIM. There was a significant ($P < 0.05$) effect of dietary protein concentration on the interval to energy nadir (cumulative energy balance at lowest point). Dietary protein concentrations of 114, 144 and 173 g/kg DM produced intervals to energy nadir of 7.8, 10.8 and 14.8 weeks (SED, 2.61) respectively.

An increase in the dietary protein concentration significantly ($P < 0.001$) reduced the efficiency of nitrogen use for milk production (Table 1.5). From 1 to 150 DIM animals receiving 114 g CP/kg DM had a nitrogen efficiency of 0.423 in comparison to 0.350 in animals receiving 173 g CP/kg DM.

Table 1.2: Composition of TMR as fed, indicating dry matter, CP and metabolisable energy contents.

Target CP conc. (g/kg DM)[2]	180			150			120		
	Conc.[1]	Grass silage	Maize silage	Conc.[1]	Grass silage	Maize silage	Conc.[1]	Grass silage	Maize silage
Proportion in diet	0.55	0.27	0.18	0.55	0.27	0.18	0.55	0.27	0.18
Dry matter content (g/kg)	870	266	343	870	266	343	870	266	343
CP (g/kg DM)[2]	217	147	78.6	163.5	147	78.6	110	147	78.6
ME (MJ/kg DM)[2]	13.1	11.9	10.8	13.1	11.9	10.8	13.1	11.9	10.8
Total ME (MJ/kg DM)[2]		12.4			12.4			12.4	
Total CP (g/kg DM)[2]		173			144			114	
ERDP (g/kg DM) [2]		112.4			94.5			75.5	
DUP (g/kg DM) [2]		62.6			48.2			34.7	

[1] Conc., concentrate
[2] CP, crude protein; conc., concentration; ME, metabolisable energy; ERDP, effective rumen degradable protein; DUP, digestible undegradable protein

Table 1.3: Effects of dietary protein concentration on dry matter intake, milk yield, milk constituents (fat, protein and lactose) and constituent yields (fat and protein) of animals in early and mid lactation (1 to 150 d)

	Dietary CP concentration (g/kg DM)[2]			Significance[1]		Lin. and Quad. Contrast[3]	
	114	144	173	SED	P-value	Lin.	Quad
Dry matter intake (kg/d)	16.5[b]	18.0[a]	18.6[a]	0.35	***	***	NS
Milk yield (kg/d)	25.4[c]	31.8[b]	35.4[a]	1.14	***	***	NS
Milk constituents (g/kg)							
Fat	42.0[a]	38.3[b]	38.1[b]	1.40	*	*	NS
Protein	31.4	32.3	32.4	0.61	NS	NS	NS
Lactose	48.2	48.1	47.8	0.46	NS	NS	NS
Milk constituent yield (kg/d)							
Fat	1.07[c]	1.21[b]	1.35[a]	0.058	***	***	NS
Protein	0.80[c]	1.03[b]	1.15[a]	0.031	***	***	*
Fat + protein	2.15[c]	2.41[b]	2.70[a]	0.115	***	***	NS
Milk energy output (MJ/d)	80.4[c]	96.2[b]	107.2[a]	3.52	***	***	NS

[1] SED, standard error of the difference; NS, P > 0.05; *, P < 0.05; ***, P < 0.001
[2] CP, crude protein; DM, dry matter
[3] Lin., linear; Quad., quadratic

Table 1.4: Effects of dietary protein concentration on live weight, body condition score and body energy status of animals in early and mid lactation (1 to 150 d)

	Dietary CP concentration (g/kg DM)[2]			Significance[3]		Lin. and Quad. Contrast[4]	
	114	144	173	SED	P-value	Lin.	Quad
Live weight (kg)	531.5[b]	548.3[a]	544.0[a]	4.18	***	**	**
Liveweight gain (kg/d)	0.16	0.27	0.24	0.193	NS	NS	NS
BCS[1]	2.38	2.41	2.37	0.030	NS	NS	NS
BCS change (units/week)[1]	-0.004	0.006	0.001	0.007	NS	NS	NS
ME requirement (MJ/d)[1]	191.3[c]	222.3[b]	242.3[a]	6.34	***	***	NS
ME intake (MJ/d)[1]	204.2[b]	222.8[a]	231.0[a]	4.54	***	***	NS
Daily energy status (MJ/d)	12.78[a]	0.539[ab]	-11.05[b]	6.20	**	***	NS
Cumulative energy status (MJ)	414[a]	-537[a]	-1,801[b]	520	***	***	NS

[1] BCS, body condition score; ME, metabolisable energy
[2] CP, crude protein; DM, dry matter
[3] SED, standard error of the difference; ns, P > 0.05; **, P < 0.01; ***, P < 0.001
[4] Lin., linear; Quad., quadratic

Table 1.5: Effect of altering dietary protein concentration at day 151 of lactation on the efficiency of nitrogen use for milk production from day 1 to 150 and 151 to 305.

Day 1 to 150	Dietary CP concentration (g/kg DM)[2]			Significance[1]		Lin. and Quad. Contrast[3]	
CP (day 1-150) [2]	114	144	173	SED	P-value	Lin.	Quad
Dietary nitrogen intake (kg)	0.300^{c}	0.414^{b}	0.515^{a}	0.0059	***	***	NS
Milk nitrogen output (kg)	0.126^{c}	0.160^{b}	0.179^{a}	0.0020	***	***	***
Efficiency of nitrogen use for milk production	0.423^{a}	0.391^{b}	0.350^{c}	0.0048	***	***	NS

Day 151 to 305								
CP (day 1-150)[2]	114	144	173	114	144	173	SED	P-value
CP (day 151-305)[2]	114	144	173	144	173	144		
Dietary nitrogen intake (kg)	0.307^{e}	0.411^{d}	0.535^{b}	0.414^{d}	0.545^{a}	0.433^{c}	0.0043	***
Milk nitrogen output (kg)	0.120^{d}	0.151^{b}	0.161^{a}	0.135^{c}	0.163^{a}	0.155^{b}	0.0025	***
Efficiency of nitrogen use for milk production	0.390^{a}	0.368^{b}	0.300^{d}	0.326^{c}	0.299^{d}	0.360^{b}	0.0049	***

[1] SED, standard error of the difference; ***, P < 0.001
[2] CP, crude protein
[3] Lin., linear; Quad., quadratic

Blood Responses to Dietary Protein in Early and Mid Lactation (1 to 150 DIM)

The effects of dietary protein concentration on blood parameters are presented in Table 1.6. An increase in dietary protein concentration significantly increased plasma urea ($P < 0.001$), total protein ($P < 0.001$) and albumin ($P < 0.001$) concentrations, and decreased plasma BHB ($P < 0.001$) concentrations. An orthogonal contrast for linear and quadratic effects identified a curvilinear relationship between urea and dietary CP concentration (significant linear ($P < 0.001$) and quadratic trends ($P < 0.01$)). There was no significant ($P > 0.05$) effect of dietary protein concentration on serum IGF-1, leptin, plasma glucose, globulin or NEFA concentrations.

Significant effects of parity were also realised for blood parameters. Primiparous animals had significantly lower albumin (32.3 vs. 33.2; $P < 0.05$), total protein (76.4 vs. 78.7; $P < 0.01$) and urea concentrations (2.70 vs. 2.95; $P < 0.01$) than multiparous animals.

Dietary Protein Adjustment at Day 151 of Lactation

The effects of adjusting dietary protein concentration at day 151 of lactation on production parameters are presented in Tables 1.7 and 1.8. From day 151 to 305 of lactation, increasing dietary protein concentration from 114 to 144 g/kg DM significantly increased: DMI ($P < 0.001$); ME intake ($P < 0.001$); milk yield ($P < 0.001$); milk fat yield ($P < 0.001$); milk protein yield ($P < 0.001$); and fat plus protein yield ($P < 0.001$). Increasing dietary protein concentration from 144 to 173 g/kg DM significantly increased: DMI ($P < 0.001$); ME intake ($P < 0.001$); milk fat yield ($P < 0.001$); milk protein yield ($P < 0.001$); and fat plus protein yield ($P < 0.001$). There was no significant effect ($P > 0.05$) of increasing dietary protein concentration from 144 to 173 g/kg DM on milk yield during the second half of lactation. Decreasing dietary protein concentration from 173 to 144 g/kg DM had no significant effect ($P > 0.05$) on: DMI, ME intake, milk yield, milk fat yield, milk protein yield or fat plus protein yield. There was no significant effect ($P > 0.05$) of dietary protein change on the concentration of milk fat, protein or lactose.

Table 1.6: Effects of dietary protein concentration on blood constituents of animals in early and mid lactation (1 to 150 d)

	Dietary CP concentration (g/kg DM)[1]			Significance[2]		Lin. and Quad. Contrast[4]	
Blood parameter	114	144	173	SED	P-value	Lin.	Quad
Urea (mmol/l)	1.56[c]	2.59[b]	4.32[a]	0.130	***	***	**
Total protein (g/l)	75.5[c]	77.0[b]	80.2[a]	1.01	***	***	NS
Albumin (g/l)	31.2[b]	33.3[a]	33.7[a]	0.53	***	***	NS
Globulin (g/l)	44.3	43.7	46.5	1.31	NS	NS	NS
NEFA (meq/l)[1]	0.386	0.368	0.402	0.029	NS	NS	NS
BHB (mmol/l)[1]	0.558[a]	0.499[ab]	0.476[b]	0.031	*	**	NS
Glucose (mmol/l)	3.33	3.32	3.34	0.044	NS	NS	NS
Leptin (ng/ml)[3]	1.97	2.04	2.00	0.352	NS	NS	NS
IGF-1 (ng/ml)[1,3]	103	103	99	6.5	NS	NS	NS

[1] NEFA, non-esterified fatty acid; BHB, β-hydroxybutyrate; IGF-1, insulin-like growth factor-1; CP, crude protein; DM, dry matter
[2] NS, $P > 0.05$; *, $P < 0.05$, ***, $P < 0.001$
[3] IGF-1 samples (n=24, five time points), Leptin samples (n=72, eight time points), and all other parameters (n=90, 16 time points)
[4] Lin., linear; Quad., quadratic

Table 1.7: Effect of altering dietary protein concentration at day 151 of lactation on; dry matter intake, milk yield, milk constituents (fat, protein and lactose) milk constituent yields (fat and protein) and milk energy output of animals from day 151 to 305

	Dietary CP concentration (g/kg DM)[2]						Significance[1]	
Day 1-150	114	144	173	114	144	173	SED	P-value
Day 151-305	114	144	173	144	173	144		
Dry matter intake (kg/d)	16.8^{d}	17.8^{c}	19.3^{ab}	18.0^{bc}	19.7^{a}	18.7^{b}	0.45	***
Milk yield (kg/d)	23.0^{c}	28.8^{a}	29.8^{a}	26.3^{b}	30.7^{a}	29.8^{a}	1.07	***
Milk constituents (g/kg)								
Fat	42.3^{a}	38.3^{bc}	38.1^{bc}	42.7^{a}	39.8^{b}	37.4^{c}	0.99	***
Protein	33.2	33.5	33.7	32.6	33.7	33.5	0.60	NS
Lactose	48.4^{a}	48.0^{ab}	47.1^{b}	48.9^{a}	48.6^{a}	48.0^{ab}	0.53	*
Milk constituent yield (kg/d)								
Fat	0.97^{c}	1.10^{b}	1.13^{ab}	1.11^{b}	1.21^{a}	1.13^{ab}	0.046	***
Protein	0.76^{d}	0.94^{b}	1.03^{a}	0.86^{c}	1.04^{a}	0.99^{ab}	0.036	***
Fat + protein	1.93^{c}	2.20^{b}	2.25^{b}	2.21^{b}	2.43^{a}	2.27^{ab}	0.092	***
Milk energy output (MJ/d)	72.2^{c}	87.8^{ab}	89.5^{a}	81.7^{b}	94.8^{a}	89.1^{a}	3.57	***

[1] SED, standard error of the difference; NS, P > 0.05, *, P < 0.05 ***, P < 0.001
[2] CP, crude protein; DM, dry matter

Data presented in Table 1.8 illustrates that increasing dietary protein concentration from 114 to 144 g/kg DM at day 151 of lactation significantly increased ME requirement ($P < 0.001$) and significantly decreased cumulative energy balance ($P < 0.001$), compared to animals that remain on 114 g CP/kg DM throughout lactation. There was no significant effect ($P > 0.05$) of increasing dietary protein concentration from 114 to 144 g/kg DM on live weight or daily energy balance. An increase in the dietary protein concentration from 144 to 173 g/kg DM significantly increased live weight ($P < 0.01$), ME requirement ($P < 0.001$) and daily energy balance ($P < 0.01$). There was no significant effect ($P > 0.05$) of altering the protein concentration of the diet from 144 to 173 g/kg DM on cumulative energy balance. Additionally, there was no significant effect ($P > 0.05$) of decreasing dietary protein concentration from 173 to 144 g/kg DM on live weight, ME requirement, daily energy balance or cumulative energy balance. Dietary protein concentration had no significant effect on liveweight change, body condition score or body condition score change.

Data presented in Table 1.5 indicates that the efficiency of nitrogen use for milk production was significantly ($P < 0.001$) higher in animals that were changed from 173 to 144 g CP/kg DM at day 151 of lactation in comparison to those that remained on 173 g CP/kg DM throughout lactation.

The effects of changing dietary protein concentration at day 151 of lactation on blood parameters are presented in Table 1.9. Increasing the dietary CP concentration from 114 to 144 g/kg DM at day 151 of lactation significantly increased the concentrations of urea ($P < 0.001$) and total protein ($P < 0.01$) from 151 to 305 DIM. Increasing the dietary concentration from 144 to 173 g/kg DM at day 151 of lactation significantly ($P < 0.001$) increased the concentration of urea. Decreasing the dietary protein concentration from 173 to 144 g/kg DM at day 151 of lactation significantly ($P < 0.001$) decreased the concentration of urea.

Table 1.8: Effect of altering dietary protein concentration at day 151 of lactation on liveweight, body condition score and body energy status of animals from day 151 to 305

	Dietary CP concentration (g/kg DM)[3]						Significance[1]	
Day 1-150	114	144	173	114	144	173	SED	P-value
Day 151-305	114	144	173	144	173	144		
Live weight (kg)	573^{b}	579^{b}	569^{b}	578^{b}	593^{a}	570^{b}	6.41	**
Liveweight gain (kg/d)	0.47	0.48	0.30	0.49	0.46	0.43	0.078	NS
BCS[2]	2.66	2.60	2.53	2.57	2.65	2.53	0.078	NS
BCS change (units/week)[2]	0.012	0.017	0.006	0.011	0.014	0.008	0.007	NS
ME requirement (MJ/d)[2]	179^{c}	205^{ab}	203^{b}	197^{b}	217^{a}	204^{b}	6.21	***
ME intake (MJ/d)[2]	209^{d}	218^{bc}	232^{b}	221^{c}	244^{a}	226^{bc}	5.79	***
Daily energy status (MJ/d)	31.2^{a}	13.2^{b}	29.3^{a}	20.1^{ab}	28.2^{a}	21.5^{ab}	5.76	**
Cumulative energy status (MJ)	4,218^{a}	987^{cd}	716^{d}	3,202^{b}	1790^{c}	27^{d}	482	***

[1] SED, standard error of the difference; NS, P > 0.05 **, P < 0.01 ***, P < 0.001
[2] BCS, body condition score; ME, metabolisable energy
[3] CP, crude protein; DM, dry matter

Table 1.9: Effect of altering dietary protein concentration at day 151 of lactation on blood constituents of animals from day 151 to 305

	Dietary CP concentration (g/kg DM)[2]						Significance[1]	
Day 1-150	114	144	173	114	144	173	SED	P-value
Day 151-305	114	144	173	144	173	144		
Urea (mmol/l)	2.06^{d}	3.29^{c}	5.43^{a}	3.43^{c}	5.13^{a}	4.16^{b}	0.303	***
Total protein (g/l)	77.2^{c}	80.2^{b}	82.6^{a}	80.4^{b}	79.0^{bc}	81.1^{ab}	1.45	**
Albumin (g/l)	33.3	34.2	34.2	34.4	34.9	34.5	0.60	NS
Globulin (g/l)	43.9	45.8	48.3	46.0	44.3	46.6	1.68	NS
NEFA (meq/l)[3]	0.27	0.29	0.30	0.32	0.24	0.39	0.045	NS
BHB (mmol/l)[3]	0.55	0.51	0.47	0.53	0.53	0.51	0.50	NS
Glucose (mmol/l)	3.31	3.20	3.32	3.30	3.26	3.23	0.078	NS

[1] SED, standard error of the difference; NS, P > 0.05 **, P < 0.01 ***, P < 0.001
[2] CP, crude protein; DM, dry matter
[3] NEFA, non-esterified fatty acids; BHB, β-hydroxybutyrate

Production Responses from 1 to 305 DIM

When evaluated over a full 305 day lactation, there were no significant ($P > 0.05$) differences in: DMI; milk yield; milk fat concentration; milk fat yield; milk protein yield; milk fat plus protein yield; milk energy output; daily energy balance; or cumulative energy balance in animals receiving 173 g CP/kg DM throughout lactation and those receiving 173 g CP/kg DM from 1 to 150 DIM and 144 g CP/kg DM from 151 to 305 DIM (Table 1.10). Animals receiving 144 g CP/kg DM throughout lactation had significantly lower ($P < 0.001$) DMIs than animals receiving 144 g CP/kg DM from 1 to 150 DIM and 173 g CP/kg DM from 151 to 305 DIM (Table 1.10). However, there was no significant difference in: milk yield; milk fat concentration; milk fat yield; milk protein yield; milk fat plus protein yield; milk energy output; daily energy balance; or cumulative energy balance between these two groups. Animals receiving 114 g CP/kg DM throughout lactation had a significantly ($P < 0.001$) higher daily energy balance and lower: milk yields; milk fat yields; milk protein yields; milk fat plus protein yield; and milk energy outputs than animals receiving 114 g CP/kg DM from 1 to 150 DIM and 144 g CP/kg DM from 151 to 305 DIM (Table 1.10). There was no significant difference in DMI, milk fat concentration or cumulative energy balance between these two groups.

Table 1.10: Effect of altering dietary protein concentration at day 151 of lactation on; dry matter intake, milk yield, milk constituents (fat, protein and lactose), milk constituent yields (fat and protein), milk energy output, and daily and cumulative energy balance of animals from day 1 to 305

	Dietary CP concentration (g/kg DM)[2]						Significance[1]	
Day 1-150	114	144	173	114	144	173	SED	P-value
Day 151-305	114	144	173	144	173	144		
Dry matter intake (kg/d)	16.8^{d}	17.8^{bc}	18.6^{ab}	17.5^{cd}	18.9^{a}	18.9^{a}	0.45	***
Milk yield (kg/d)	23.0^{d}	29.8^{bc}	33.0^{a}	26.9^{c}	30.0^{b}	30.7^{ab}	1.49	***
Milk constituents (g/kg)								
Fat	42.5^{a}	39.3^{ab}	36.6^{b}	42.0^{a}	38.6^{b}	40.4^{ab}	1.93	*
Protein	32.6	33.3	32.9	32.2	32.9	33.7	0.75	NS
Lactose	48.0	47.9	47.2	48.9	48.4	48.2	0.61	NS
Milk constituent yield (kg/d)								
Fat	0.97^{b}	1.17^{a}	1.20^{a}	1.13^{a}	1.12^{a}	1.23^{a}	0.067	**
Protein	0.74^{d}	0.99^{b}	1.08^{a}	0.86^{c}	0.98^{b}	1.03^{ab}	0.044	***
Fat + protein	1.94^{b}	2.33^{a}	2.40^{a}	2.27^{a}	2.24^{a}	2.46^{a}	0.135	**
Milk energy output (MJ/d)	73.8^{c}	93.7^{ab}	98.8^{a}	86.0^{b}	91.7^{ab}	97.8^{a}	4.37	***
Daily energy balance (MJ/day)	27.4^{a}	4.40^{b}	4.65^{b}	12.1^{b}	18.0^{ab}	11.3^{b}	7.21	*
Cumulative energy balance (MJ)	2870^{a}	-465^{bc}	-1531^{c}	1067^{ab}	1788^{ab}	200^{bc}	1145	**

[1] SED, standard error of the difference; NS, P > 0.05, *, P < 0.05 ***, P < 0.001
[2] CP, crude protein; DM, dry matter

Discussion

Dietary Protein Effects on Production Parameters

Increasing dietary CP concentrations (1-150 DIM) from 114 to 144, and from 144 to 173 g/kg DM realised a daily milk yield responses of 6.4 and 3.6 kg/day, respectively. Despite these differences suggesting a curvilinear relationship, results from an orthogonal contrast for linear and quadratic effects state that only a linear relationship exists (Table 1.3). These results are in contrast to previous authors who have demonstrated a quadratic relationship between milk output and dietary protein intake. Olmos Colmenero and Broderick (2006) reported an increase in milk yield, with increasing dietary CP concentration up to 165 g/kg DM, after which point a decline in milk yield was noted. They also noted a similar pattern of increase in milk protein and fat yields. Furthermore, Cunningham *et al*. (1996) and Leonardi *et al*. (2003) observed no improvement in milk yield when dietary CP increased from 165 to 185 g/kg DM and from 161 to 189 g/kg DM respectively. In the present study, Figure 1.1 demonstrates a tendency towards a greater milk yield response when increasing dietary protein concentration from 114 to 144 g/kg DM than from 144 to 173 g/kg DM, especially in the early to mid lactation period. Figure 1.1 also indicates that post day 151 of lactation, there is a major decline in the efficiency of nitrogen utilisation for milk production in animals allocated 173 g CP/kg DM throughout lactation (1 to 305 DIM), compared to those allocated a reduced dietary CP concentration (173 to 144 g/kg DM) at day 151 of lactation. Animals receiving 173 g CP/kg DM prior to day 151 of lactation showed no detrimental effects on production when allocated 144 g CP/kg DM at day 151 of lactation. These conclusions are verified in Table 1.5, which illustrates that the nitrogen efficiency significantly increases when animals are reduced from 173 to 144 g CP/kg DM at day 151 of lactation.

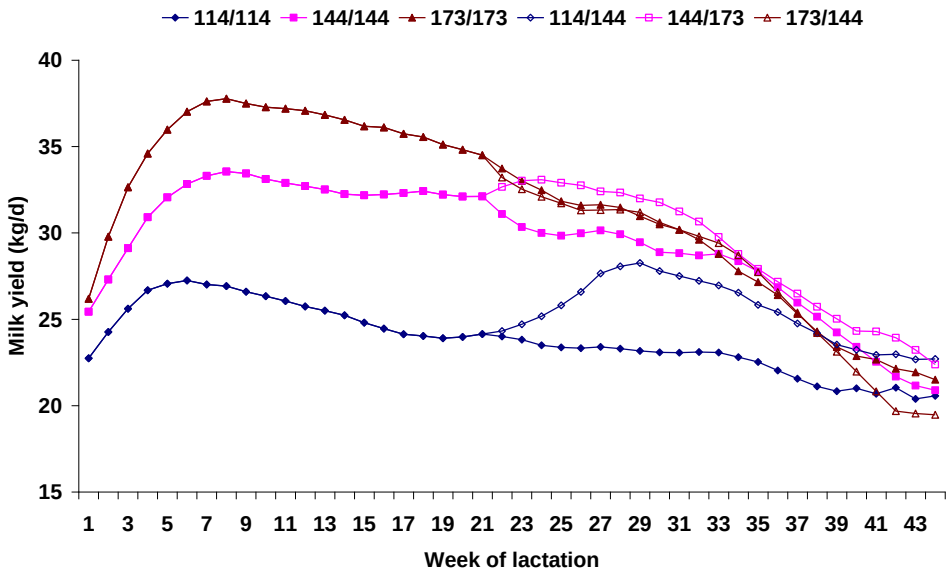

Figure 1.1: Effect of dietary protein concentration on milk production. (legend: dietary protein concentration, 1 to 150 DIM/151 to 305 DIM)

Animals receiving 114 g CP/kg DM had significantly higher milk fat concentrations in comparison to animals on 144/173 g CP/kg DM during the first 150 days of lactation. However, orthogonal contrast for linear and quadratic effects identified a linear relationship between these two variables. These results are in contrast to those reported by Leonardi *et al*. (2003) (161 to 189 g CP/kg DM) and M'Hamed *et al*. (2001) (141 to 170 g CP/kg DM) who found that milk fat concentration increased in response to dietary CP. Additionally, Lundquist *et al*. (1986) found no effect of altering dietary protein concentration on milk fat content. The latter authors had similar protein levels to those used in the current experiment (125, 155 and 180 g CP/kg DM). Total milk fat and protein yields increased with increasing dietary protein concentrations, which is in partial agreement with Broderick (2003). Broderick (2003) stated that yields of fat and protein increased with increases in the concentration of dietary CP from 151 to 167 g/kg DM but showed no further increase when dietary CP concentrations were increased to 184 g/kg DM. In the current experiment a linear response was realised between milk fat yield and the concentration of dietary CP. When considering total milk protein yield, Leonardi *et al*. (2003) found that it was unaffected by an increase in dietary CP content (161 to 189 g/kg DM), with milk protein concentration decreasing. However, in the present study each increment in dietary CP produced a significant increase in milk protein yield; this relationship was curvilinear.

Broderick (2003) reported a linear increase in DMI with increases in dietary CP concentration from 152 to 167 and 183 g/kg DM. A linear relationship was also identified in the current study, however, there was no significant difference in DMI (1 to 150 DIM) between animals receiving 144 and those receiving 173 g CP/kg DM. If fermentable energy supply is not a limiting factor, then increasing the amount of eRDP should realise an increase in microbial protein synthesis and generate increased dietary intakes as a consequence of increased microbial activity in the rumen. In the present study, the eRDP concentration of diets containing 114, 144 and 173 g CP/kg DM were predicted to be 75.5, 94.5 and 112.5 g/kg DM respectively. However, dietary intake responses were not in proportion to eRDP increments which would suggest that either the supply of fermentable energy was limiting, or that bacterial growth was approaching a maximum. In agreement with this, Cunningham *et al*. (1996) found no significant difference in DMI when cows were offered 165 and 185 g CP/kg DM. M'Hamed *et al*. (2001) concluded that increasing the protein content of the diet enhances DMI, milk yield and body weight gain, but that the responses vary greatly according to the type and level of protein supplementation.

Increasing dietary protein concentration from 114 to 173 g/kg DM significantly reduced the daily and cumulative energy balance. Increasing dietary protein concentration from 114 to 173 g/kg DM produced a 13.1 percent increase in ME intake but a 26.7 percent increase in ME requirement, which accounts for the decrease in daily energy balance. Additionally, an increase in dietary protein concentration from 114 to 173 g/kg DM significantly increased the interval to energy nadir. Animals that display a more negative energy balance will take longer to reach an energetic equilibrium (zero energy balance). Results presented in Table 1.4 indicate that the observed variation in cumulative energy balance between animals receiving 114 and 173 g CP/kg DM is not reflected in body condition score change or live weight. A difference in cumulative energy balance of 2215 MJ (414-(-1801)) would equate to a difference in body condition score of 1.25 units (1770 MJ/ unit body condition score loss; AFRC, 1993), and a difference in live weight of 95 kg (23.4 MJ/kg body live weight loss (AFRC, 1990)). The actual difference in body condition score and live weight was 0.01 units and 12.5 kg (higher live weight in animals with more negative energy balance) respectively. These discrepancies may be partially a result of some basic assumptions used in the energy calculations. These assumptions include:

1. The maintenance requirement of all animals is a constant value which is relative to metabolic body weight, irrespective of the level of production.
2. The net efficiency of energy utilisation for lactation (k_l), despite being partially scaled to level of intake (Thomas, 2004), does not go below 0.59.

Dietary Protein Effects on Blood Parameters (1 to 150 DIM)

Plasma urea and total protein concentrations were significantly elevated by an increase in dietary protein content. Increased plasma urea concentrations indicate increased ammonia detoxification in the liver, whilst an increase in total blood protein concentrations indicate intestinal absorption of 'protein', which will be evident at higher dietary protein contents (where fermentable carbohydrate is not limiting). Increased ammonia concentrations in the blood may be caused by an over supply of eRDP in the rumen (Kenny *et al*., 2001). The curvilinear relationship identified between urea and dietary CP concentration would support this suggestion. There was no significant effect of dietary protein content on plasma NEFA, glucose or globulin concentrations which is in agreement with results reported by M'Hamed *et al*. (2001). The latter authors showed no significant effects of increasing dietary protein concentration from 141 to 165 g CP/kg DM on concentrations of these blood parameters. Additionally, there were no significant effects of altering the dietary protein content on serum leptin or IGF-1 concentrations. Adipose tissue is the biggest contributor to plasma leptin in ruminants (Chilliard *et al*., 2001) and is positively correlated with body condition (Kokkonen *et al*., 2005). As there were no significant effects of dietary CP concentration on body condition score, or body condition score change in the current study (Tables 4 and 8), no effects on leptin would be expected. Similarly, Armstrong *et al*. (2001) found no effect of dietary protein on plasma IGF-1 concentrations.

Dietary Protein Effects on Production Parameters (151 to 305 DIM)

Data presented in Figure 1.1 indicates that reducing the dietary protein concentration at day 151 of lactation, from 173 to 144 g/kg DM had no detrimental effect on milk yield compared to animals that remained on 173 g CP/kg DM (Table 1.7). This would imply that reducing the dietary protein concentration of the diet post 150 DIM is a key mitigation strategy to improve the efficiency of nitrogen use for milk production. Animals receiving 114 g CP/kg DM from day 1 to 150 of lactation displayed significant increases in milk yield when allocated 144 g CP/kg DM during the second part of lactation (151 to 305 DIM). There was also a trend (though not

significant, P > 0.05) for an increase in milk yield of 1.9 kg per day (days 151 to 305) in animals changed from 144 to 173 g CP/kg DM at day 151, compared to those that remained on 144 g CP/kg DM.

Increasing protein concentrations from 114 to 144, and 144 to 173 g CP/kg DM at day 151 of lactation produced significant (Table 1.7; Figure 1.2) increases in DMI of 7.1 and 10.6% respectively (Figure 1.2). Reducing protein concentration from 173 to 144 g CP/kg DM tended (P > 0.05) to decrease DMI (Table 1.7). Therefore, increasing dietary protein concentration from 114 to 144 g/kg DM produced a 7.1 percent increase in ME intake, but a 14.3 percent increase in milk yield, which resulted in a decrease in cumulative energy balance (Figure 1.3). Increasing dietary protein concentration from 144 to 173 g/kg DM produced a 10.6 percent increase in ME intake but a 6.5 percent increase in milk yield resulting in a significantly higher energy balance.

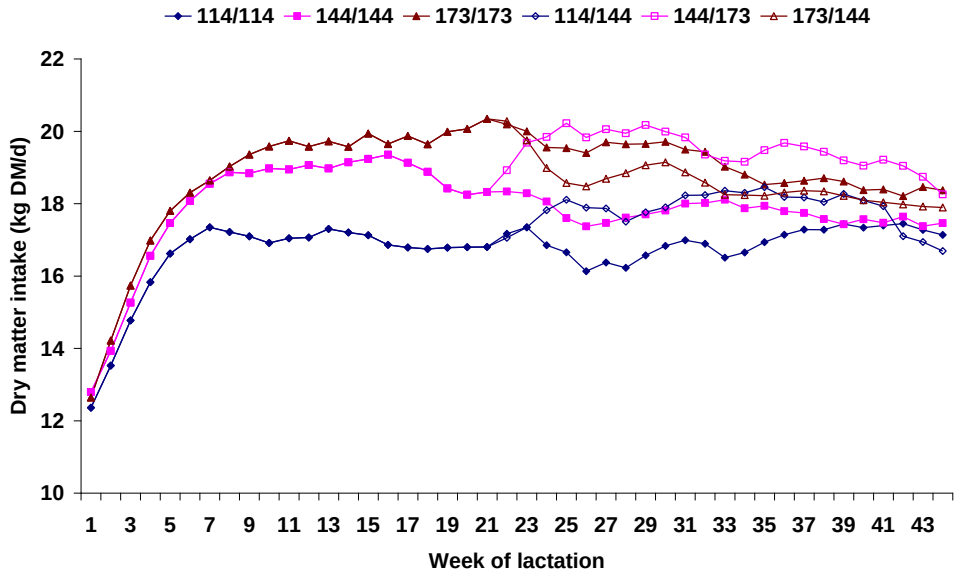

Figure 1.2: Effect of changing dietary protein concentration on dry matter intake. (legend: dietary protein concentration, 1 to 150 DIM/151 to 305 DIM)

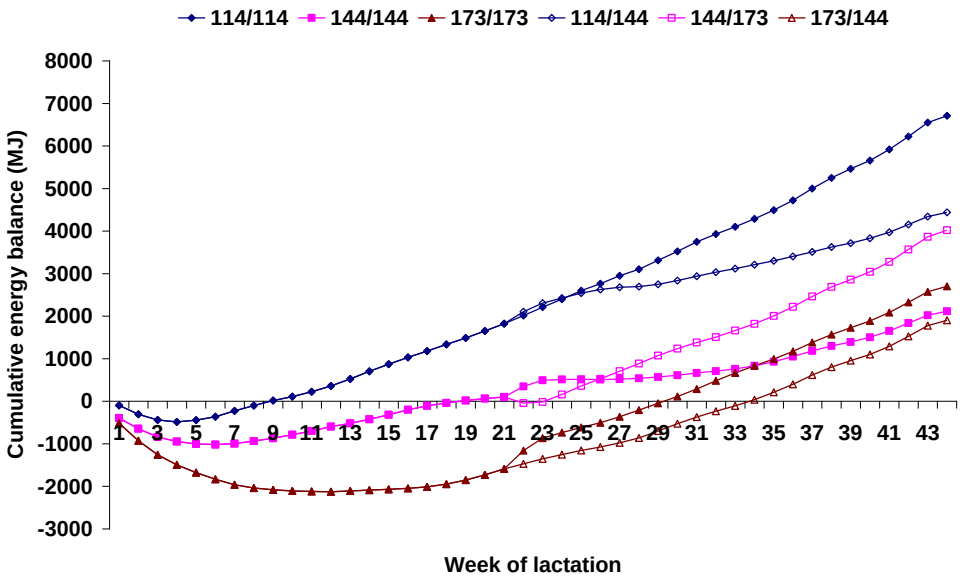

Figure 1.3: Effect of changing dietary protein concentration on cumulative energy balance (legend: dietary protein concentration, 1 to 150 DIM/151 to 305 DIM)

Conclusions

The present study assessed the effects of altering dietary protein concentration on production parameters of lactating dairy cows. The results indicate that in early lactation (day 1 to 150) an increase in dietary protein content up to 173 g/kg DM has beneficial effects on milk yield and DMI. However, in late lactation (151 to 305 DIM), more efficient use of dietary nitrogen can be achieved by feeding diets with lower dietary protein concentrations (between 144 and 173 g CP/kg DM) without realising detrimental effects on production. Improving efficiency and economic sustainability of dairy cow production systems, as well as reducing the environmental impact of intensive dairy farming is critical and these results indicate that lowering dietary protein concentration in mid/late lactation improves the efficiency of use of dietary nitrogen with no detrimental effects on animal performance.

Acknowledgements

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Chapter 2: Effects of dietary crude protein concentration on energy and nitrogen utilisation efficiencies in lactating dairy cows, and on the environmental footprint of milk production systems.

Dr T Yan

Abstract

The Holstein-Friesian dairy cows (twelve first and 12 multi-lactation) used in the present study, were selected from a continuous design study (from week 1 to 44 of lactation) to examine the effects of dietary CP concentration on energy and nutrient utilisation efficiency, and the environmental footprint of dairy systems. The CP concentrations evaluated were 180, 150 and 120 g/kg DM in mixed diets containing 550 g/kg DM of concentrates and 450 g/kg DM of forage (600 g/kg DM grass silage and 400 g/kg DM maize silage). During early (70 to 90 days), mid (150 to 170 days) and late (230 to 250 days) lactation, the same 4 cows and 4 heifers from each treatment were transferred to metabolism units for measurements of energy metabolism and N utilisation, with methane emission and heat production measured in calorimeter chambers. There was no significant difference in live weight or fat, protein or lactose concentration in milk between the 3 diets, but increasing dietary CP concentration significantly increased DM intake and milk yield ($P < 0.001$). Increasing dietary CP concentration significantly increased GE intake, energy outputs in faeces, urine and milk, and heat production ($P < 0.001$). Dietary CP concentration had no significant effect on methane energy output, energy balance, energy partitioning between milk and body tissue, or the efficiency of ME use for lactation. Similarly, increasing dietary CP concentration significantly increased N intake, N outputs in faeces, urine and milk, and N balance ($P < 0.001$). Increasing dietary N concentration resulted in more N being partitioned into milk ($P < 0.001$) and less into body tissue ($P < 0.01$). These data were also used to evaluate the effects of dietary CP concentration on the environment footprint of dairy systems. Increasing dietary CP concentration significantly reduced manure N output as a proportion of N intake, methane emission as a proportion of DM intake, gross energy intake and

milk yield ($P < 0.05$ or less), while significantly increasing manure N output as a proportion of milk yield ($P < 0.001$). When individual treatments were examined, it was found that the effects on manure N output per kg of milk yield were mainly derived from diets with a CP content of 180 g/kg DM, compared to a diet with a CP content of 150 g/kg DM. Decreasing CP concentration further (to 120 g/kg DM) had no significant effect on this variable. However, the opposite effect was observed in relation to methane emission as per kg of milk yield with the effects derived mainly from the diets with CP concentration of 150 vs. 120 g/kg DM. These results demonstrated that a decrease in dietary CP concentration from 180 to 120 g/kg DM resulted in lowered milk production, but had no effect on energy utilisation efficiency. In terms of reducing the environmental footprint, a dietary CP concentration of 150 g/kg DM has the potential to reduce manure N output and methane emissions, whilst avoiding the very substantial increase in milk yield observed with very low protein diets.

Introduction

Nitrogen (N) is of significant environmental concern at present because of losses of ammonia to the air and nitrate contamination of surface water and groundwater (Tamminga, 1992; Van Horn *et al*., 1994). Every year large amounts of N are brought onto dairy farms and much of this N is lost rather than being incorporated into milk, animal tissue and crops that are sold off the farm (Korevaar, 1992; Klausner, 1993). The overall efficiency of utilisation of dietary N in European dairy farming was estimated to be less than 20% and in many situations still decreasing (Bruchem *et al*., 1991). A statistical modelling study using data from studies undertaken AFBI Hillsborough for the last 30 years found a higher efficiency of N utilisation for milk production and body gain (28%) (Yan *et al*., 2006). Consequently, dairy production contributes to the loss of ammonia N and nitrous oxides in air, and to nitrates in soil and ground water (Tamminga, 1992). In 1991 the European Union introduced the Nitrates Directive (European Community, 1991), which aims to prevent the pollution of groundwater and surface water by nitrates arising from agricultural sources. The Directive stipulates mandatory measures that must be included in an action program, one of which involves a limit on the amount of livestock manure (faeces and urine) which may be applied to land each year. This is set at 170 kg organic N (manure N) per hectare. This limit has very significant implications for stocking rates on livestock farms. Therefore there is an increasing interest in developing approaches to mitigate manure N output in

animal production. Many dietary and animal factors can influence manure N output in lactating dairy cows (Yan and Mayne, 2007a). Dietary N concentration and N intake are the two most important factors, due to the strong positive relationship between N intake and manure N output in lactating dairy cows (Yan *et al*., 2006). However, reducing dietary N concentration below the requirements of rumen microbial growth and for milk production would reduce production efficiency and energy utilisation efficiency (Agnew and Yan, 2000). Moreover, reducing dietary CP concentration can increase methane emission as a proportion of feed intake and milk yield (Yan and Mayne, 2007b). Methane emissions from dairy cows is another significant environmental concern at present as methane is an important greenhouse gas and is responsible for global warming. Methane emission from cattle takes account of 9% of total greenhouse gases emission in Northern Ireland. However, there is little information available in the literature on effects of dietary CP concentration on energy and N utilisation efficiency and the subsequent environmental footprint (manure N and methane emissions). The objective of the present study was to evaluate differences in energy and N utilisation efficiency, manure N loss and methane emissions, of lactating dairy cows offered diets with different levels of CP.

Materials and Methods

Experimental Design, Animal and Diets

The present study was part of a large scale project to evaluate the effect of dietary CP concentration on animal performance, nutrient utilisation efficiency and fertility in Holstein dairy cows. Full details of experimental design, animal, diets and management used in the present study were reported in Chapter 1.

In this study, 45 first and 45 multi-lactation Holstein dairy cows were used in a three treatment design study from day 1 to 305 of lactation. Three diets were used which contained different levels of CP (180, 150 and 120 g/kg DM) in mixed diets of 450 g/kg DM of forage (60% grass silage and 40% maize silage in DM basis) and 550 g/kg DM of concentrates. At day 151 of lactation, half of animals in each treatment were allocated to an alternative CP concentration diet, while half of animals remained on their original diet. Two concentrate supplements (high vs. low CP concentration) were used and consisted of different proportions (g/kg DM) of barley (140 vs. 240), wheat (140 vs. 240), unmolassed sugar beet pulp (94 vs. 163), citrus pulp (94 vs.

163), maize gluten feed (100 vs. 30), distillers maize grain (100 vs. 80), soybean meal (165 vs. 0), rape meal (100 vs. 0), tallow (14 vs. 22), molasses (24 vs. 24), and minerals/vitamin (29 vs. 38). The high and low CP concentrates contained the same level of ME (13.1 MJ/kg DM), but differed in CP (229 vs. 117 g/kg DM), effective rumen degradable CP (ERDP, 79 vs. 135 g/kg DM), digestible undegradable CP (DUP, 74 vs. 29 g/kg DM) and starch (311 vs. 215 g/kg DM) content. These variables were calculated using the UK Feed into Milk programme (Feed into Milk, 2004). These two concentrate supplements were used to generate high, medium and low CP diets. The animals were fed *ad libitum* between 10.00 and 11.00 h daily using a diet feeder. All cows were loose housed in cubicle accommodation with free access to water, and milked twice daily starting at 05.00 and 16.30 h.

Digestibility Study and Calorimetric Measurements

During early (70 to 90 days), mid (150 to 170 days) and late (230 to 250 days) lactation, the same 4 cows and 4 heifers from each treatment were selected from continuous feeding regimes (no change in diets throughout the study). Selection criteria were cows which had similar parity (multi-lactation cows only) and live weight, BCS and milk yield (measured at week 10 of lactation) as the average values of the treatment. These animals were transferred to metabolism units and housed in individual stalls for 8 days, with measurements of feed intake and outputs of faeces and urine made during the final 6 days. Immediately following completion of digestibility measurements, all cows were transferred to indirect open-circuit respiration calorimeter chambers for 3 days with gaseous exchange (oxygen, carbon dioxide and methane) measured during the final 2 days. The calorimetric chambers used to measure gaseous exchange in the present study were as described by Gordon *et al*. (1995) and Yan *et al*. (2000).

Measurements

Throughout the study, from calving to 305 days of lactation, total food intake was recorded daily. The grass silage and maize silages were sampled daily for oven DM determination and the dried sample bulked over the week for determination of ADF, NDF and ash concentrations. Fresh silage samples were also taken twice weekly during the production study and daily during the digestibility and chamber measurements, for determination of volatile corrected DM, pH, CP, ammonia-N, GE, lactic acid, volatile fatty acids, ethanol and propanol concentrations. Samples of concentrates were taken weekly during the production study and daily during the digestibility

and chamber measurements, and the weekly bulked sample analysed for oven DM, GE, CP, ADF, NDF and ash concentrations. Faeces and urine outputs with cows in the digestibility measurements were recorded and sampled daily as a proportion (5%) of total excretion of faeces (by weight) and urine (by volume). The six-day samples of faeces and urine were separately mixed and a representative sample taken for analysis as follows: faeces samples were analysed for oven DM, N, GE, ADF, NDF and ash concentrations and urine samples were analysed for GE and N concentrations. The methods adopted for analysis of silage, concentrate, faeces and urine samples were as described by Mayne and Gordon (1984). Crude protein concentration was determined as Kjeldahl N x 6.25. Silage pH was determined using a Corning 113 pH-meter, and ammonia was assayed by bringing the alkalinity of the aqueous extract to pH 10 and using an Orion 95-12-00 ammonia sensing gas electrode. Volatile fatty acids, lactic acid, ethanol and propanol concentrations in silage samples were analysed by gas liquid chromatography using a Perkin-Elmer gas chromatograph having a column packed with 80/120 Carbopack B-DA/4% Carbowax 20m.

Milk yields were recorded daily and milk samples were taken during the morning and afternoon milking (samples analysed separately), once weekly during the production study. During the digestibility and chamber measurements, milk samples were taken during the morning and afternoon milking daily. The weekly milk samples were combined to produce one composite sample. The composite sample for each cow was analysed by the methods of Ling (1963) for fat and lactose concentrations using a Milkoscan Model 605 (Foss Electric, DK-3400, Hillerød, Denmark), and protein concentration as Kjeldahl N x 6.38.

Live weight and body condition score (BSC) of the cattle were determined weekly. Body condition of each cow was determined using the method described by (Edmonson *et al.,* 1989), with 5 categories from 1 (very thin) to 5 (very fat).

Statistical Analysis

The effects of dietary CP concentration on animal performance, energy and N utilisation efficiency and methane and manure N emissions were analysed using one way (dietary CP concentration) analysis of variance with experimental period as block. The statistical program

used in the present study was Genstat 6.1, sixth edition (Lawes Agricultural Trust, Rothamsted, England, UK).

Results and Discussion

The 3 diets were designed to contain CP at 120, 150 and 180 g/kg DM, respectively. The diets were formulated using feedbyte based on tabulated values for concentrate ingredients and actual nutritive values for grass silage and maize silage measured at the start of the present study. The measured CP values, averaged for the 4 periods, were 122, 151 and 180 g/kg DM, for treatments 120, 150, and 180 g CP/kg DM respectively. The actual CP values were the same or very close to the calculated data. All 3 diets were designed to have the same ME concentration, however, actual ME concentrations were 11.2, 11.6 and 12.0 MJ/kg DM with diets containing CP of 120, 150 and 180 g/kg DM, respectively. The differences in ME concentrations are most likely derived from the associative effects.

Effects on Milk Production

Effects of dietary CP concentration on feed intake and milk yield of the 24 cows used in these nutrient utilisation studies are presented in Table 2.1. As expected, increasing dietary CP concentration significantly increased DM intake ($P < 0.001$), and consequently milk yield increased with increasing CP concentration ($P < 0.001$). These results are similar to those from the main feeding study of the present project (Chapter 1). The effect on milk production in the present study was mainly derived from the difference between low and medium CP diets, with a mean increase in milk yield of 5.5 kg/d. The increase in milk yield was much smaller (2.2 kg/d) from medium to high CP diets. However, the quadratic response to dietary CP concentration in the present study are in contrast to previous authors who have demonstrated a much greater quadratic relationship between milk output and dietary protein intake. Olmos Colmenero and Broderick (2006) reported an increase in milk yield, with increasing dietary CP concentration up to 165 g/kg of DM, after which point a decline in milk yield was noted. They also noted a similar pattern of increase in milk protein and fat yields. Furthermore, Cunningham *et al*. (1996) and Leonardi and Armentano (2003) observed no improvement in milk yield when dietary CP increased from 165 to 185 g/kg of DM and from 161 to 189 g/kg of DM, respectively.

Table 2.1. Animal data and milk production (mean of 4 recording periods)

	Dietary CP concentration (g/kg DM)				
	120	150	180	s.e.	Sig
Live weight and feed intake					
Live weight (kg)	554	536	536	13.4	
Body condition score	2.52	2.43	2.44	0.054	
DM intake (kg/d)	16.5	18.4	19.5	0.43	***
Milk yield and composition					
Yield (kg/d)	20.6	26.1	28.3	0.94	***
Fat (g/kg)	41.9	40.4	39.7	1.35	
Protein (g/kg)	34.0	35.3	35.9	0.92	
Lactose (g/kg)	47.2	45.9	46.3	0.85	
Energy (MJ/kg)	3.14	3.09	3.08	0.056	

The present study found no significant effects of dietary CP concentration on live weight, body condition score, or concentration of fat, protein, lactose or energy in milk. Lundquist *et al.* (1986) also found no effect of altering dietary protein concentration (125, 155, and 180 g of CP/kg of DM) on milk fat concentration. However, in the main feeding study a low CP concentration diet produced a significantly higher milk fat concentration than the medium and high CP diets (Chapter 1). Leonardi and Armentano (2003) and M'Hamed *et al.* (2001) also found that milk fat concentration increased in response to dietary CP.

Effects on Energy Utilisation Efficiency

The effects of dietary CP concentration on energy metabolism are presented in Table 2.2. Increasing dietary CP concentration significantly increased GE intake ($P < 0.001$), this effect being largely driven by DM intake, with GE concentrations being 18.3, 18.5 and 18.6 MJ/kg DM for low, medium to high CP diets, respectively. As expected, the lower GE intake with low CP diet resulted in a lower energy outputs from faeces ($P < 0.05$), urine ($P < 0.001$), heat production ($P < 0.001$) and milk ($P < 0.001$). The effects on energy outputs in urine, heat production and milk were equally derived from the differences between low and medium CP diets and between

medium and high CP diets. Methane energy output and energy balance were also increased with increasing dietary CP concentration, but the difference did not reach the significance.

Table 2.2. Effects of dietary CP concentration on energy utilisation efficiency

	Dietary CP concentration (g/kg DM)				
	120	150	180	s.e.	Sig
Energy intake and output (MJ/d)					
GE intake (MJ/d)	301	340	363	7.8	***
Faecal energy	85	94	94	2.6	*
Urine energy	12	13	14	0.3	***
CG_4 energy	20	20	22	0.7	
Heat production	125	132	143	3.1	***
Milk energy	64	80	86	2.4	***
Energy balance	-5	1	4	3.0	
Energy utilisation efficiency					
ME/GE	0.611	0.625	0.643	0.0041	***
HP/ME intake	0.682	0.624	0.617	0.0114	***
Milk energy/ME intake	0.354	0.376	0.367	0.0085	
Energy balance/ME intake	-0.036	0.000	0.016	0.0153	
k_1	0.551	0.579	0.563	0.0130	

Increasing dietary CP concentration significantly increased energy metabolisability ($P < 0.001$), and consequently increased ME concentration in diets (11.2, 11.6 and 12.0 MJ/kg DM for low, medium and high CP diets, respectively). This effect was likely to have arisen from an associative effect between concentrate type and forge, the three diets having been formulated to have the same ME concentration. However, heat production as a proportion of ME intake was higher with the low CP diet ($P < 0.001$). When examining the individual treatment effects, it was found that effect of dietary protein level on heat production as a proportion of ME intake was mainly derived from the difference between low and medium CP diets, the difference between medium and high CP diets being non significant. This effect may be due to the fact that cows offered low CP diet had a lower ME intake and consequently spent a higher proportion of ME

intake for maintenance, compared to those offered medium and high CP diets. This suggestion is based on the fact that there was no difference in ME requirement for maintenance between the three groups of cows when scaled to metabolic live weight ($MJ/kg^{0.75}$), as derived from relationships between ME intake and milk energy output adjusted to zero energy balance (Figure 2.1 and Eq. [1a] to [1b] in Table 2.5). The estimated ME requirement for maintenance for all three groups of cows was the same (0.71 $MJ/kg^{0.75}$).

However, dietary CP had no effect on energy partitioning between milk (milk energy output as a proportion of ME intake) and body tissue (energy balance as a proportion of ME intake). There was no significant difference in the efficiency of ME use for lactation (k_l) between the three diets, when using the ME requirements for maintenance of Agnew *et al*. (2003). Agnew and Yan (2000), in an extensive review of energy metabolism data published in the scientific literature, found that there were no significant effects of either dietary or animal factors, including milk yield, on k_l.

Effects on Nitrogen Utilisation Efficiency

Effects of dietary CP concentration on N utilisation efficiency are presented in Table 2.3. The present study demonstrated that dietary CP concentration had significant effects on N intake and outputs. Nitrogen intake increased significantly with increasing dietary CP concentration ($P < 0.001$), with this effect derived from a higher dietary N concentration and from a higher DM intake (reported previously). Accordingly, N outputs in faeces and urine increased with increasing dietary CP content ($P < 0.001$). Similarly, N incorporated into milk and body tissue increased with increasing dietary CP concentration ($P < 0.001$).

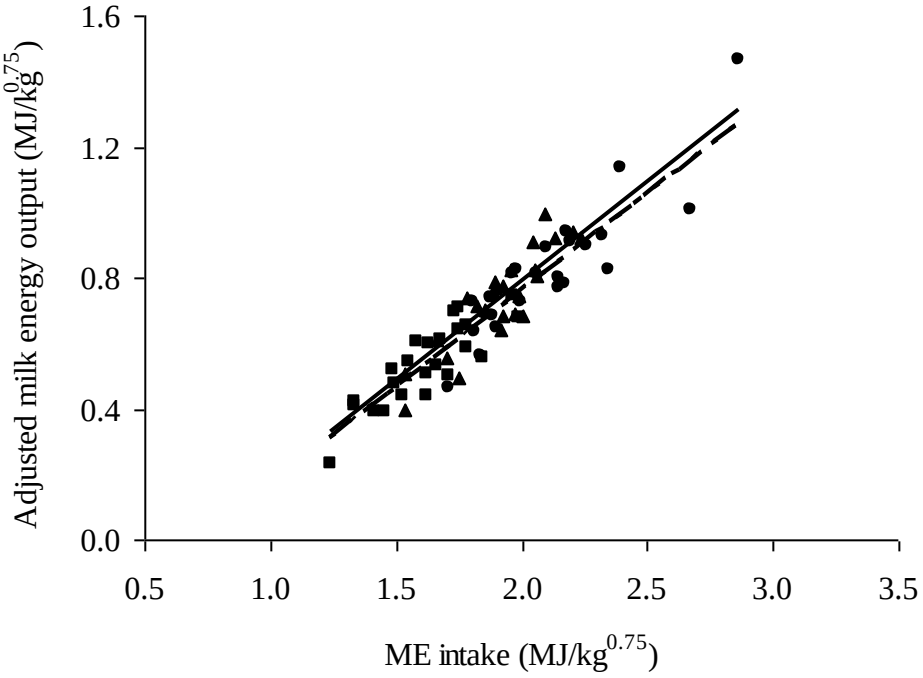

Figure 2.1. Effects of dietary CP concentration on the relationships between ME intake and milk energy output, adjusted to zero energy balance (Low CP: square and short broken line; Medium CP: triangle and solid line; High CP: circle and long broken line)

Dietary CP concentration had significant effects on N utilisation efficiency, when expressed as N output as a proportion of N intake. For example, increasing dietary CP concentration significantly decreased faecal N output as a proportion of N intake ($P < 0.001$), while urine N output as a proportion of N intake was significantly higher with high compared to the low CP diets ($P < 0.001$). The higher N digestibility with the high CP diet is unlikely to reflect a higher N utilisation efficiency. As the supply of degradable CP from the high CP diet is likely to exceed the requirement of microbial activity in the rumen, the excess ammonia from the over-supplied degradable CP will be absorbed through the rumen wall into blood, synthesised into urea in the liver, and excreted from urine. This may explain why there was a higher ratio of urine N output, as a proportion of N intake, with high compared to the low CP diets. Milk N output and N balance were also affected by dietary CP concentrations. Increasing dietary CP concentration significantly decreased milk N output as a proportion of N intake ($P < 0.001$), while N balance as a proportion of N intake increased with increasing CP level ($P < 0.01$).

Table 2.3. Effects of dietary CP concentration on nitrogen utilisation efficiency

	Dietary CP concentration (g/kg DM)				
	120	150	180	s.e.	Sig
Nitrogen intake and output (g/d)					
N intake	322	445	562	10.5	***
Faecal N	135	162	173	5.1	***
Urine N	92	138	207	5.4	***
Milk N	100	132	144	4.4	***
N balance	-5	13	38	1.8	***
Nitrogen utilisation efficiency					
Faecal N/N intake	0.425	0.363	0.308	0.0097	***
Urine N/N intake	0.282	0.311	0.368	0.0082	***
Milk N/N intake	0.310	0.297	0.256	0.0068	***
N balance/N intake	-0.017	0.0303	0.068	0.0043	**

Environmental Impact

The effects of dietary CP concentration on manure N output and methane emissions are presented in Table 2.4. Manure N output as a proportion of N intake was significantly higher with the low, compared to the medium and high CP diets ($P < 0.001$). However, manure N output as a proportion of DM intake or milk yield significantly increased with increasing dietary CP concentration ($P < 0.001$). These results are similar to that reported by Yan *et al*. (2006) in a modelling review of N digestibility data of dairy cows undertaken at AFBI Hillsborough for the last 30 years. In the present study, the effect of dietary CP concentration on manure N output per kg of milk yield was a quadratic function, not a linear relationship. This variable was similar between diets containing 120 and 150 g/kg DM of CP, and was significantly higher when dietary CP level increased to 180 g/kg DM. Therefore, these results indicate that an overall dietary CP concentration of 150 g/kg DM may be appropriate in order to reduce manure N output, whilst minimising the decrease in milk yield normally observed with very low protein diets.

Increasing dietary CP concentration significantly decreased methane emission as a proportion of DM intake ($P < 0.05$) and milk yield ($P < 0.01$), and methane energy output as a proportion of

GE intake ($P < 0.01$). However, when examining individual treatment effects, the effect of dietary CP concentration on proportional methane emission was mainly derived from diets containing between 120 and 150 g CP/kg DM, there were little or no differences in proportional methane emissions between diets containing between 150 and 180 g CP/kg DM. These results indicate that there is no benefit in terms of methane emission as a proportion of feed intake or milk yield when dietary CP concentration is over 150 g/kg DM.

The relationships between milk yield and manure N output, as a proportion of milk yield (Eq. [2a] to [2c]) and methane emission as a proportion of milk yield (Eq. [3a] to [3b]) for the 3 diets are presented in Table 2.5 and Figure 2.2 (A) and (B). With a common constant for each set of equations, increasing milk yield would reduce manure N output or methane emission as a proportion of milk yield for all 3 diets, although the reduction rates were lower with each increase in dietary CP concentration.

Table 2.4. Effects of dietary CP concentration on manure nitrogen excretion and methane emission

	Dietary CP concentration (g/kg DM)				
	120	150	180	s.e.	Sig
Manure N excretion					
Manure N/N intake (g/g)	0.71	0.67	0.68	0.007	***
Manure N/DM intake (g/kg)	13.8	16.3	19.5	0.19	***
Manure N/milk yield (g/kg)	11.4	12.0	14.1	0.53	***
Methane emission					
CH_4/DM intake (l/kg)	31.1	28.2	28.2	0.83	*
CH_4/milk yield (l/kg)	25.7	20.9	20.4	1.13	**
CH_4-E/GE intake	0.07	0.06	0.06	0.002	**

Table 2.5. Effects of dietary CP concentration on the linear relationships

	Equations	R^2	Eq. No
Low CP	$E_{l(0)}$ = 0.59 MEI - 0.42	0.73	[1a]
Medium CP	$E_{l(0)}$ = 0.60 MEI - 0.42	0.77	[1b]
High CP	$E_{l(0)}$ = 0.59 MEI - 0.42	0.80	[1c]
Low CP	Manure N/MY = -0.253 MY + 18	0.29	[2a]
Medium CP	Manure N/MY = -0.205 MY + 18	0.46	[2b]
High CP	Manure N/MY = -0.183 MY + 18	0.42	[2c]
Low CP	CH_4/MY = -0.908 MY + 44	0.43	[3a]
Medium CP	CH_4/MY = -0.892 MY + 44	0.68	[3b]
High CP	CH_4/MY = -0.827 MY + 44	0.59	[3c]

$E_{l(0)}$ = milk energy output adjusted to zero energy balance (MJ/kg$^{0.75}$); MEI = ME intake (MJ/kg$^{0.75}$); MY = milk yield (kg/d); Unit for CH_4 emission is l/d and for manure N (g/d)

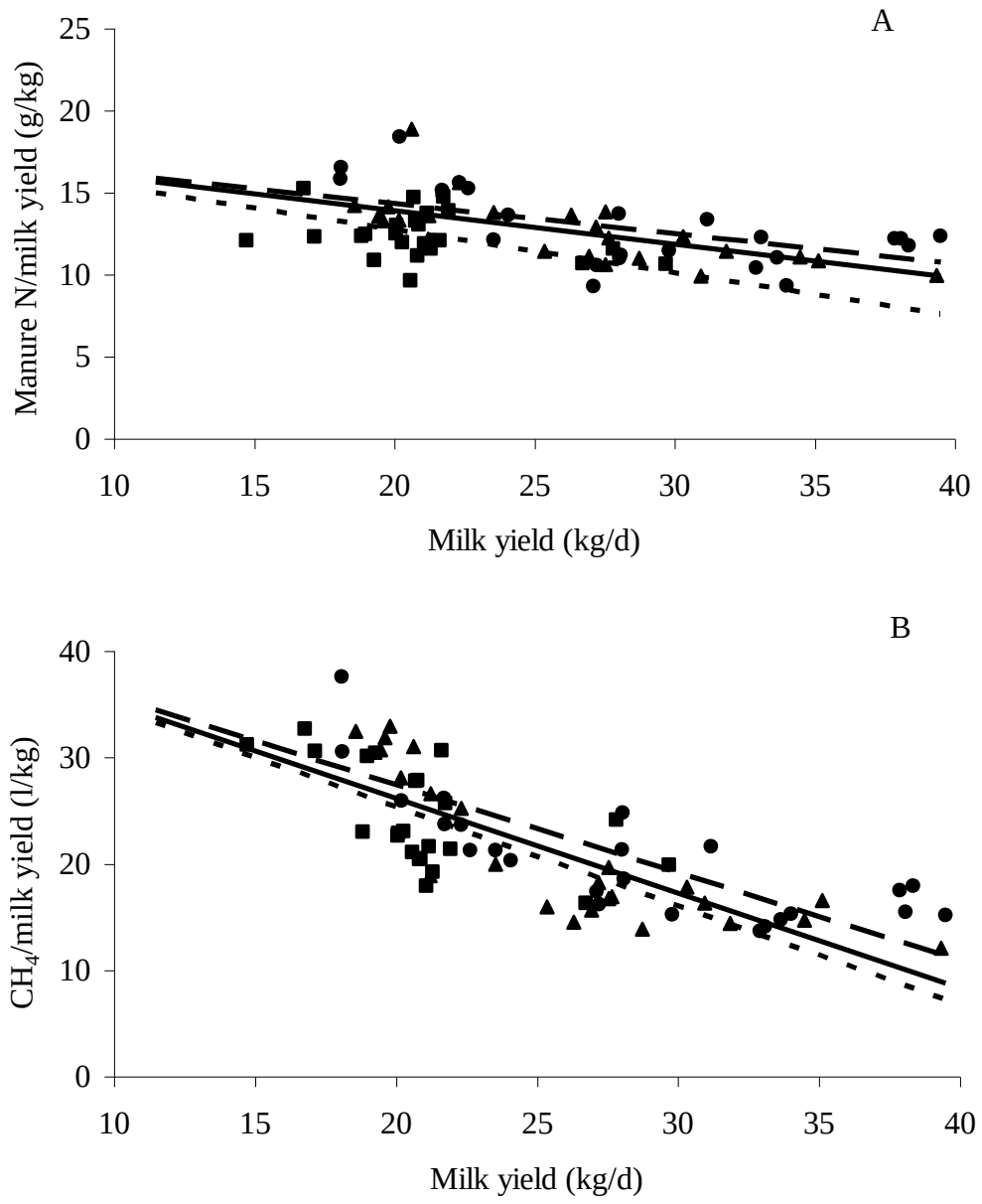

Figure 2.2: Effects of dietary CP concentration on the relationships between milk yield and manure N output as a proportion of milk yield (A) and methane emission as a proportion of milk yield (B) (Low CP: square and short broken line; Medium CP: triangle and solid line; High CP: circle and long broken line.

Conclusions

Reducing dietary CP concentration from 180 to 120 g/kg DM significantly reduced feed intake and energy metabolisability. These effects resulted in a lower ME intake and protein supply for milk production, with cows offered low CP diet. Consequently, milk yield declined with each decrease in dietary CP concentration. However, dietary CP concentration had no effect on energy partitioning between milk and body tissue, or on the efficiency of ME use for lactation, although the high CP diet generated a lower milk N output as a proportion of N intake and a higher N balance as a proportion of N intake. Increasing dietary CP concentration increased manure N output as per kg milk yield, but decreased methane emission per kg milk yield. In order to achieve a balance between the loss of milk, and yet achieve environmental benefits, a dietary CP concentration of 150 g/kg DM may be appropriate. At this level of dietary protein, manure N output and methane emissions will be reduced, while the loss of milk yield normally observed with very low protein diets will be minimised.

Chapter 3: Effect of Dietary Protein Content on the Fertility of Dairy Cows during Early and Mid Lactation

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INTERPRETIVE SUMMARY

The present study focuses on the effects of reducing the dietary protein concentration to improve energy balance and fertility in high yielding Holstein dairy cows. No direct effects of altering dietary protein concentration on reproductive performance were yielded, however, reducing dietary protein concentration tended to improve pregnancy to first service rates and 100 day in-calf rates. A reduced dietary protein concentration improved cow energy status and a more positive average cumulative energy balance was associated with higher conception. The importance of progesterone in maintaining pregnancy was also emphasised.

Abstract

Ninety autumn calving Holstein dairy cows (45 primiparous and 45 multiparous (mean parity, 3.1)) were allocated to one of three treatments; 173, 144, or 114 g CP/kg DM, from calving until day 150 of lactation. On day 151 of lactation, half the animals receiving 114 g CP/kg DM went onto 144 g CP/kg DM, half of the animals receiving 144 g CP/kg DM went onto 173 g CP/kg DM and half of the animals receiving 173 g CP/kg DM went onto 144 g CP/kg DM, with the remaining animals staying on their original treatment. This resulted in six treatments in mid to late lactation period: 114/114; 144/144; 173/173; 114/144; 144/173; and 173/144 g CP/kg DM. Overall, 95.3% of cows intended for breeding conceived during a 6-m breeding period. The average pregnancy rates to first service and first plus second service were 30.9% (SED, 0.05) and 56.7% (SED, 0.05) respectively. The average 100 d in-calf rate, from the start of the breeding period, was 70.5% and at least one abnormal progesterone profile was observed in 62% of animals. An increase in dietary protein content decreased the requirement for treatment of metritis. There was no effect of dietary protein content on any of the reproductive or progesterone measures e.g. days to conception, calving interval, 100 day in-calf rate (from commencement of breeding), days to onset of luteal activity, average luteal phase, average inter ovulatory interval, average inter luteal interval etc. An increase in dietary protein content decreased the average daily energy balance (ADEB). A more positive energy balance was associated with an increased requirement for the treatment of metritis in the current study. Cumulative energy balance was positively associated with conception. There was no effect of the concentration of plasma urea on any of the reproductive variables; however, the concentration of serum leptin was favourably associated with the time to progesterone rise above 3 ng/ml which has been deemed essential for embryo survival. Additionally, the average peak concentration of progesterone and the duration of the average luteal phase were favourably associated with the interval from calving to conception. The latter relationships emphasise the importance of progesterone in achieving and maintaining pregnancy.

Keywords: Reproduction, energy balance, progesterone, conception.

Introduction

Post partum nutrition plays a significant role in the onset of ovarian cyclicity, the expression of normal oestrous cycles and conception rates (Robinson *et al.*, 2006). However, the importance of protein nutrition on the reproductive performance of the modern high yielding dairy cow is ambiguous (Laven and Drew, 1999). There are numerous studies reporting direct negative effects (Larson *et al.*, 1997), indirect or associative effects (Barton *et al.*, 1996), or no effects (Kenny *et al.*, 2001) of elevated dietary protein content on reproductive performance. For example, work by Jordan and Swanson (1979) showed that an increase in dietary CP content increased the interval from calving to first ovulation and reduced pregnancy rate. However, other studies have failed to support this observation (Laven and Drew, 1999). Westwood *et al.* (1998) also reported confounding evidence on the effect of dietary protein concentration on conception rates. However, Westwood *et al.* (1998) concluded that a stronger relationship existed between dietary CP concentration and conception rates in older animals, as opposed to primiparous animals. Work by Butler (2001) illustrated that elevated blood urea concentration coupled with suboptimal early luteal progesterone concentrations are detrimental to embryo survival. Interestingly, Orihuela (2000) indicated that a severe protein deficiency will interfere with reproductive processes, as well as an excessively high protein concentration in the diet. The detrimental effects of protein deficiency were previously emphasised by Westwood *et al.* (1998) who stated that low CP diets result in sub-optimal microbial protein synthesis in the rumen and are associated with reproductive failure.

The current study was undertaken to investigate possible reasons for the inconsistencies in the effects of dietary protein concentration on reproductive performance presented in the literature. The specific aim of the present study was to examine the response of high yielding Holstein cows to low protein diets, in terms of effects on energy balance and fertility. The effects of three levels of total dietary CP on a range of reproductive variables are reported.

Materials and Methods

Animals and Housing

Ninety Holstein dairy cows (45 primiparous and 45 multiparous (mean parity 3.1)) were used in the study. Calving commenced on 29th August and ended on 23rd December. Following calving, animals were housed as a group in free stalls (cubicles) with concrete flooring. The cubicle to cow ratio was ≥ 1:1 at all times, meeting the recommendations set by FAWC (1997). Cubicles had a bed measurement of 2.20 m long and 1.25 m wide, were fitted with rubber mats and bedded with sawdust thrice weekly. Concrete passageway floors were scraped a minimum of four times daily, using an automated system. Cows were milked twice daily through a 50-bail rotary parlour at 05:30 and 16:30, with cows travelling about 35 m to the milking parlour. Lights were left on at all times. Animals were inseminated from a minimum of 42 d post-calving, on the first observed standing heat following the commencement of the breeding period (23rd November).

Experimental Design, Diets and Feeding

The experiment involved allocating 90 freshly calved Holstein Friesian dairy animals to three dietary treatments which differed in overall CP levels, as formulated, in the complete diet (DM basis); 180, 150, and 120 g/kg DM. At day 151 of lactation, half of the animals in each treatment were allocated (balanced for parity, milk yield, calving date and liveweight) an alternative dietary CP concentration, whilst the remaining animals were maintained on their original diets. Half of the animals receiving 120 g CP/kg DM went onto 150 g CP/kg DM, half of the animals receiving 150 g CP/kg DM went onto 180 g CP/kg DM and half of the animals receiving 180 g CP/kg DM went onto 150 g CP/kg DM. This resulted in six treatments (1 to 151/151 to 305 d in milk (DIM)): 120/120; 150/150; 180/180; 120/150; 150/180; and 180/150. Primiparous animals were assigned in a balanced manner to treatments based on heifer rearing regimen, calving date and live weight. Multiparous animals were assigned to treatments according to parity, previous lactation milk yield, calving date and live weight. The diet was presented as a total mixed ration (TMR) and animals had free access to water at all times and were fed between 10:00 and 11:00 daily using a diet feeder. The concentrate to forage ratio (DM basis) was 0.55:0.45 for all diets. The forage component of the diet consisted of 0.60 grass silage and 0.40 maize silage (DM basis). Samples of grass and maize silage were taken weekly

and analysed using near infrared reflectance spectroscopy (Park *et al.*, 1998), and twice weekly for measurement of nitrogen and ammonia nitrogen using methods outlined by Steen (1989). Two concentrates were formulated to contain 229 and 117 g CP/kg DM in order to achieve target protein levels in the overall diets of 180 and 120 g CP/ kg DM respectively. Diets containing 150 g CP/kg DM were produced by complementing the forage component with equal amounts of concentrates containing 117 and 229 g CP/kg DM. The energy and protein concentrations of individual ingredients were based on published values (AFRC, 1993) which were used in the initial formulation of the concentrate proportion of the diet as presented in Table 1.1. Concentrate samples were taken weekly during the experiment and analysed for DM, ash, ADF, NDF and nitrogen as described by Cushnahan and Gordon (1995). The TMR diets were offered *ad libitum* using feed boxes which were placed on a computer recorded load cell system, with controlled access to the boxes using an electronic identification system. This enabled dry matter intakes of individual cows to be recorded continuously via automatic feeding gates (Calan gate feeder), from which a daily intake was calculated and then averaged on a weekly basis.

Following approval from an ethical review committee, the experiment was conducted in a manner that avoided unnecessary discomfort to the animals by the use of proper management and sampling techniques.

Measurements

Milk yields were recorded daily at each milking throughout the experiment. Milk composition (fat, protein, lactose and somatic cell count) was determined on a weekly basis from one consecutive AM and pm milking. Separate analysis was completed for am and pm samples and milk composition was calculated on the basis of recorded am and pm milk yields. Milk composition was determined using an infrared milk analyser (IRMA). Live weight and body condition score (scale: 0-5; Edmonson *et al.*, 1989) were recorded on a weekly basis and throughout lactation. Locomotion scores were carried out fortnightly using the method described by Manson and Leaver (1988). Blood samples were collected weekly between 0930 and 1130 h from the coccygeal vein using uncoated, heparin-coated, and fluoride oxalate coated tubes (BD, Oxford, UK) from calving until day 100 of lactation, and then fortnightly thereafter. Plasma was recovered by centrifugation for analysis of glucose, (fluoride oxalate tubes) total protein, albumin, globulin, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA) and urea

(heparinized tubes) respectively, by clinical analyser (Olympus UK Ltd, Middlesex, UK). NEFA concentrations were determined using a standard kit (Wako Chemicals GmbH, Neuss, Germany). Uncoated tubes provided serum for leptin radio-immunoassays (RIA). Plasma and serum samples were stored at –20°C until analysed. RIAs were balanced for dietary treatments and parity and control samples were included in each assay.

Milk samples were collected twice weekly for use in progesterone analyses (Tuesday and Friday; am milk samples) until pregnancy confirmation at day 30 post insemination. Pregnancy was reconfirmed at day 60 post insemination and in the event of embryo loss, milk sampling for progesterone recommenced. Each sample had preservative added (lactab Mark III, Thompson and Cooper Ltd, Runcorn, UK) and was stored at 4°C until analysis (within 4 weeks). Milk progesterone concentrations were determined using an enzyme-linked immuno-sorbent assay (ELISA) kit (Ridgeway Science Ltd, Lydney, UK) based on the method of Sauer *et al*. (1986).

Reproductive Performance

All fertility events were recorded until 6 m after the commencement of breeding. Insemination details included: date, hour, sire, and inseminator. On average, cows were inseminated 12 h after visual observation of oestrus. Pregnancy was diagnosed via an ultrasound scan carried out by a veterinary surgeon at day 30 and day 60 post insemination. Late embryo loss (> 60 d post insemination) was defined by the return of an animal to oestrus that had previously been diagnosed as pregnant at day 60 post insemination. Conception was defined by parturition, marking the commencement of the subsequent lactation.

Stockpersons examined animals for metritis weekly. Metritis was classified as the presence of uterine discharge from the vagina 21 d or more after calving. Animals displaying uterine discharge were presented for veterinary inspection and following confirmation of metritis, animals were treated with Pevidine (iodine-based solution (60 ml, 1:5 dilution), administered through a stainless steel catheter into the uterine body) and/or pessaries (Utocyl (combination of antibiotics), 6 pessaries administered into the uterus for the prevention of post partum metritis, leucorrhoea, endometritis and pyometra). Additional interventions products include: CIDR/PRID (intravaginal progesterone-releasing devices, used for the treatment of anoestrus or follicular ovarian cysts); Estrumate (prostaglandin analog; cloprostenol, which causes luteolysis

followed by return to oestrus); and Fertagyl (synthetic equivalent of gonadotrophin releasing hormone; Gonadorelin, which optimises time of ovulation and improves conception rate).

Progesterone Measure Definitions

Royal *et al*. (2000) and McCoy *et al*. (2006) provide full descriptions defining ovarian activity and abnormal progesterone profiles respectively. These are summarised below.

Onset of luteal activity (OLA) was defined by the first of at least two consecutive progesterone concentrations ≥ 3 ng/ml in composite milk.

The Luteal phase (LP) of an individual oestrous cycle was defined as the time between the first elevated progesterone concentration measuring ≥ 3 ng/ml and the final consecutive milk progesterone concentration measuring ≥ 3 ng/ml.

Inter-ovulatory interval (IOI) was defined as the time between the first progesterone rise of one cycle to the first progesterone rise of the next cycle.

Inter-luteal interval (ILI) was defined as the time between the demise of one corpus luteum and the rise of the next. It is the interval from the first progesterone concentration < 3 ng/ml at the end of one cycle until the last consecutive progesterone concentration < 3 ng/ml in composite milk at the start of the next cycle.

Abnormal Progesterone Patterns

Delayed ovulation type I (DOVI) was defined as progesterone concentrations < 3 ng/ml in composite milk for ≥ 45 d (prolonged anovulation).

Delayed ovulation type II (DOVII) was defined as progesterone concentrations < 3 ng/ml in composite milk for ≥ 12 d after the onset of luteal activity (prolonged inter-luteal interval).

Persistent corpus luteum type I (PCLI) was defined as progesterone concentrations ≥ 3 ng/ml for ≥ 19 d on the first luteal phase (delayed luteolysis of the corpus luteum during the first oestrous cycle).

Persistent corpus luteum type II (PCLII) was defined as progesterone concentrations ≥ 3 ng/ml for ≥ 19 d on subsequent luteal phases (delayed luteolysis of the corpus luteum during subsequent oestrous cycles).

Calculation of Energy Balance

The average daily energy balance for each animal was calculated each week of lactation using the equations described by Thomas (2004) [Energy balance = ME intake – ME requirement (-10 + (ME_{preg} + $ME_{maintmilk}$ * $Lwt^{0.75}$) + ((0.0013*Lwt)/K_m))]. Missing values were estimated from the week previous to and the week following missing data. Less than 2% of the data were missing.

Statistical Analyses

A repeated measures approach using the Residual Maximum Likelihood (REML) procedure available in GenStat (Payne *et al*. 1993) was used to analyse the data set. The model fitted fixed effects for parity, dietary protein concentration and stage of lactation (weeks from calving) for each dependent variable. The model included all two-level interactions between those independent variables. There was no significant effect of dietary protein concentration on condition score change over the first three weeks of lactation and the deviation from the mean condition score of multi- and primiparous animals during this period was used as a covariate in this experiment.

Multiple linear regression analysis was performed for several independent variables on all the reproductive variables. Independent variables used in the multiple linear regression analysis were: milk yield, milk energy, daily energy balance, cumulative energy balance, average daily energy balance in the first three weeks of lactation, average daily energy balance in the first six weeks of lactation and weeks to energy nadir. A Spearman's correlation matrix was also performed on all continuous variables.

A logistic regression model was used to analyse the following data; pregnancy rate to first service, pregnancy rate to first and second service, 100 d in-calf rate, intervention, delayed

ovulation types I and II, persistent corpus luteum types I and II and whether or not there was more than one abnormal progesterone profile observed.

Results

For the purpose of the analysis, data post day 150 of lactation are not presented because only 16 cycles, from a total of 412 cycles across all animals, occurred on a changed diet (half the animals changed diet at 150 d post-calving).

The composition (as fed) of diets targeted to contain 120, 150 and 180 g CP/kg DM were 114, 144 and 173 g CP/kg DM respectively. All diets were isoenergetic.

Effect of Protein Concentration on Energy Measures (0-150 d)

Dietary protein concentration effects on production measures (milk yield, dry matter intake, ME requirement, ME intake, daily energy balance and cumulative energy balance) are presented in Table 3.1. A full description of the effects of dietary protein on production measures is given in Chapter 1. Increased dietary protein concentration significantly ($P < 0.001$) increased milk yield, dry matter intake, and ME requirement. Increasing dietary protein concentration from 114 to 144 or 173 g/kg DM significantly ($P < 0.001$) increased ME intake. Increasing dietary CP concentration from 114 or 144 to 173 significantly decreased daily energy balance and cumulative energy balance.

Table 3.1 Effect of dietary protein concentration in early and mid lactation on animal performance and energy balance (1 to 140 d)

	Dietary CP concentration (g/kg DM)[3]			Significance[4]	
	114	144	173	SED	P-value
Milk yield (kg/d)	25.4[c]	31.8[b]	35.4[a]	1.14	***
Dry matter intake (kg/d)	16.5[b]	18.0[a]	18.6[a]	0.35	***
ME requirement (MJ/d)[1,2]	191.3[c]	222.3[b]	242.3[a]	6.34	***
ME intake (MJ/d)[1]	204.2[b]	222.8[a]	231.0[a]	4.54	***
Daily energy status (MJ/d)[2]	12.78[a]	0.539[ab]	-11.05[b]	6.20	**
Cumulative energy status (MJ)[2]	414[a]	-537[a]	-1,801[b]	520	***

[1] ME, metabolisable energy
[2] ME requirement, Daily energy balance, and Cumulative energy balance were calculated using the equations described by Thomas (2004)
[3] CP, crude protein; DM, dry matter
[4] SED, standard error of the difference; **, P < 0.01; ***, P < 0.001

Effect of Protein Treatment on Reproductive Variables

An increase in the dietary protein concentration significantly (P < 0.05) decreased the proportion of animals diagnosed as having metritis (Table 3.2). Following veterinary inspection all animals with metritis were treated with Pevidine (iodine-based solution) and/or pessaries. An increase in dietary protein concentration significantly (P < 0.05) decreased the requirement to administer Pevidine and/or pessaries for the treatment of metritis. There were no significant (P > 0.05) effects of dietary protein content on the incidence of any other fertility intervention treatments.

Table 3.2 Effect of dietary protein concentration on the incidence of metritis and the proportion of cows requiring intervention with a reproductive treatment, within each protein treatment

Reproductive ailment[††]/treatment[†]	Protein treatment (g CP/kg DM)			Significance
	114	144	173	
Metritis[††]	0.45	0.25	0.13	*
Pevidine[†]	0.31	0.19	0.06	*
Pessaries[†]	0.34	0.11	0.04	*
CIDR/PRID[†]	0.29	0.22	0.40	NS
Estrumate[†]	0.31	0.31	0.42	NS
Fertagyl[†]	0.58	0.41	0.55	NS

[1] CIDR (Animal Reproductive Technologies Ltd, UK); PRID (CEVA Animal Health Ltd, UK); Pevidine, iodine based solution (1%); Pessaries (Utocyl) (Novartis Animal Health UK Ltd, UK); Estrumate (Schering-Plough Animal Health, UK); and Fertagyl (Janssen Animal Health, UK). Hormonal treatments CIDR and PRID are intravaginal progesterone-releasing devices; Estrumate is a prostaglandin analog, cloprostenol; and Fertagyl is a synthetic GnRH. Utocyl pessaries, containing a combination of antibiotics, and the iodine-based Pevidine were used in treating metritis.

[2] CP, crude protein; DM, dry matter; NS, $P > 0.05$; *, $P < 0.05$

The effects of protein treatment on selected reproductive variables are presented in Table 3.3. No statistically significant ($P > 0.05$) effects of dietary protein concentration on any of the parameters listed in Table 3.3 were found. However, there was a tendency for animals on the 114 g CP/kg DM to have a higher 100 d in-calf rate (82.7%) compared to those on 144 (66.7%) and 173 g CP/kg DM (62.1%). The 100 d in-calf rate was defined as an animal becoming pregnant during the 100 d period after the commencement of breeding (23rd November). This variable indicates the proportion of animals returning to the autumn calving regimen in the subsequent year. There also appears to be some evidence of a beneficial effect of 114 g CP/kg DM on overall conception rate, with 100% of cows becoming pregnant (144 g CP/kg DM, 92.9%; 173 g CP/kg DM, 86.7%). The overall interval to the onset of luteal activity and the pregnancy rate to first service rate in the current study were 32.2 d and 30.6% respectively.

Table 3.3 Predicted means for effects of protein concentration on fertility measures

Treatment	CP concentration (g/kg DM)			Significance		Overall
	173	144	114	SED	P-Value	
OLA[2] (d)	30.9	33.2	32.4	4.6	NS	32.2
Pregnancy to 1st Service (%)	27.6	29.7	34.5	8.6	NS	30.6
Pregnancy to 1st and 2nd Service (%)	51.7	63.0	55.2	9.2	NS	56.6
Services per conception	2.69	2.32	2.44	0.38	NS	2.48
100 d in-calf rate[3] (%)	62.1	66.7	82.7	8.2	NS	70.5
Cumulative pregnancy rate[4] (%)	86.7	92.9	100.0	5.4	NS	93.2
Calving Interval (d)	398	399	398	13.7	NS	398.3

1 There was no significant effect of protein concentration on any of the above fertility measures; SED, standard error of the difference; NS P > 0.05
2 OLA, onset of luteal activity; CP, crude protein
3 100 d in-calf rate was taken from the commencement of breeding (23rd November 2005)
4 Cumulative pregnancy rate was calculated over a 6-m breeding period

Effect of Energy Status on Reproductive Variables

Several individual energy measures were significantly associated with the incidence of metritis (average daily energy balance in first 21 d ($P < 0.01$); average daily energy balance over 40 wk ($P < 0.05$); average cumulative energy balance over 40 wk ($P < 0.05$)). In all cases a more positive energy balance elicited an increased requirement for treatment. Following a Spearman's correlation matrix analysis it was found that the average cumulative energy balance was positively associated with conception ($P < 0.01$; r-value, 0.351), i.e. a more positive average cumulative energy balance was associated with a higher conception rate. Additionally, it was found that a more negative daily ($P < 0.01$; r-value, -0.294) and cumulative ($P < 0.05$; r-value, -0.261) energy balance was associated with a longer interval to progesterone rise (above 3 ng/ml) after ovulation.

Effect of Parity on Reproductive Variables

Parity had a significant influence on the average inter ovulatory interval ($P < 0.01$), the average inter luteal interval ($P < 0.01$), the average peak progesterone concentration ($P < 0.05$), the time to progesterone rise ($P < 0.01$) and occurrence of more than one abnormal progesterone profile

(P < 0.05). Primiparous animals had a shorter average inter ovulatory interval (23.5 vs. 26.9 d; SED, 1.19), average inter luteal interval (8.2 vs. 11.2 d; SED, 0.97) and time to progesterone rise (5.8 vs. 6.6 d; SED, 0.27) than multiparous animals. Primiparous animals had a higher average peak progesterone concentration (34.1 vs. 30.3 ng/ml; SED, 1.70) than multiparous animals and a lower percentage of primiparous animals displayed more than one abnormal profile than multiparous animals (19.2 vs. 39.9 %; SED, 6.64).

Effect of Blood Measures on Reproductive Variables

There was no statistically significant (P > 0.05) relationship between plasma urea and any of the fertility measures. Using a Spearman's correlation matrix and scatter graphs to verify relationships (Table 3.4), it was observed that: leptin concentration was negatively associated with time to progesterone rise (P < 0.05); globulin concentration was negatively associated with peak progesterone concentration (P < 0.01); glucose concentration was positively associated with peak progesterone concentration (P < 0.05); glucose concentration was negatively associated with duration of the inter luteal interval (P < 0.01); and total protein concentration was negatively correlated with peak concentration of progesterone (P < 0.001).

Table 3.4 Effect of blood constituents on reproductive variables

Blood constituent	Reproductive variable	r-value[2]	Significance[3]
Leptin (ng/ml)	Time to progesterone rise[1]	-0.207	*
Glucose (mmol/l)	Peak progesterone concentration	0.144	*
Globulin (g/l)	Peak progesterone concentration	-0.163	**
Glucose (mmol/l)	Duration of inter luteal interval	-0.174	**
Total protein (g/l)	Peak progesterone concentration	-0.211	***

1 Days to progesterone rise above 3ng/ml
2 Correlation coefficient
3 *, P < 0.05; **, P < 0.01; ***, P < 0.001

Correlations between Reproductive Variables

Correlations between reproductive variables are presented in Table 3.5. In the present study, a shorter inter-ovulatory interval was associated with a longer interval from calving to conception ($P < 0.01$). The occurrence of a persistent corpus luteum was associated with a longer calving interval ($P < 0.001$) and with a lower 100 d in-calf rate ($P < 0.01$). Progesterone concentration during the first 7 d post breeding was positively related to conception ($P < 0.001$). An increase in the average peak concentration of progesterone significantly decreased the calving interval ($P < 0.001$). The interval to the onset of luteal activity was unfavourably associated with peak progesterone concentration in the first cycle ($P < 0.01$). The requirement for intervention was associated with a decrease in the 100 d in-calf rate ($P < 0.001$), and an increased calving interval ($P < 0.001$).

Table 3.5 Correlations between reproductive variables

Reproductive variable[1]	Reproductive variable[1]	r-value[2]	Significance[2]
Persistent corpus luteum type II	100d in calf rate	-0.317	**
Intervention required	100d in calf rate	-0.583	***
Av. inter ovulatory interval	Days to concep. from calving	0.354	**
Peak progesterone of first cycle	Days to OLA	-0.342	**
Intervention required	Calving Interval	0.732	***
Persistent corpus luteum type II	Calving Interval	0.442	***
Av. peak conc. of progesterone	Calving Interval	-0.402	***
Progesterone conc. during first 7 d post breeding	Conception	0.332	***

[1] Av., Average; conc., concentration; OLA, Onset of luteal activity; concept., conception
[2] Correlation coefficient; **, $P < 0.01$; ***, $P < .001$

Progesterone Measures and Abnormal Hormonal Patterns

There was no significant effect of dietary protein concentration on any of the progesterone measures. The average interval to the onset of luteal activity was 32.2 d (inter quartile range (IQR), 19.2 to 40.0), the average luteal phase was 19.1 d (IQR, 10.9 to 24.6), the average inter luteal interval was 9.2 d (IQR, 6.6 to 10.6), the average inter ovulatory interval was 24.8 d (IQR, 21.0 to 28.0) and the average number of cycles per cow was 4.5 (IQR, 3.0 to 6.0). There was no

effect of dietary protein concentration on the incidence of any abnormal hormonal patterns following logistic regression analysis. In the current study, 62% of the animals displayed one or more abnormal progesterone profiles. A total of 81% of these animals received an intervention treatment during the breeding period. Delayed ovulation occurred in 20.7% of animals on their first cycle (DOV I) and at least one prolonged inter-luteal interval (delayed ovulation in subsequent cycles) occurred in 27.6% of animals (DOV II). Delayed luteolysis during the first cycle was observed in 19.5% of animals (PCL I) and at least one delayed luteolysis in subsequent cycles occurred in 20.7% of animals (PCL II). Overall, 41 % of animals displayed at least one delayed ovulation (DOV I and DOV II) and 40% displayed at least one persistent corpus luteum (PCL I and PCL II). When assessing the incidence of abnormal progesterone profiles in terms of cycles, it was found that 30.3% of all cycles had prolonged luteal phases (either PCL I or PCL II), 11.7% of all cycles had a prolonged inter-luteal interval (DOV II) and 44.9% of all cycles had an inter-ovulatory interval greater than or equal to 24 d.

Discussion

Effects of Dietary Protein concentration on Reproductive Variables

Altering dietary protein concentration had no direct influence on conventional reproductive variables (Table 3.3). The present study had a relatively low pregnancy to first service rate (30.6%) in comparison to values reported by Mayne *et al*. (2002) and Royal *et al*. (2000); 37.1 % and 39.7 respectively. Interestingly, Canfield *et al*. (1990), stated that animals on a high protein diet, with excess RDP, had a significantly lower (31%) conception to first service rates than animals on a low protein diet (48%), that met the requirements of RDP. The current study failed to illustrate a significant difference in pregnancy to first service rates between dietary protein concentrations but indicated a tendency for animals on a low protein diet to have a higher pregnancy to first service rate. Despite obtaining a low conception rate to first service, the calving interval in the current experiment was shorter than that reported by Mayne *et al*. (2002) (398 vs. 407 d). No significant difference in the 100 d in-calf rate was apparent between dietary protein concentrations, however, the results indicate a tendency for animals receiving 114 g CP/kg DM to have a substantially higher 100 d in-calf rate than animals receiving 144 or 173 g CP/kg DM. Clark *et al*. (1985) indicated that an increase in dietary CP concentration had a tendency to increase the number of services per conception. Results from the current study

partially agree with this. Animals receiving 173 g CP/kg DM had the highest number of services per conception, however, animals receiving 144 g CP/kg DM had fewer services per conception than animals receiving 114g CP/kg DM.

An increase in dietary protein concentration reduced the requirement to administer treatments for metritis. An increase in the requirement for treatment of metritis would suggest a suppression of the animal's ability to deal with an immune challenge. Houdijk *et al*. (2001) stated that competition for metabolisable energy does not produce a breakdown of immunity, but only competition for metabolisable protein. These conclusions are based on the proteinaceous nature of the immune system. In agreement, results of the present study indicate that a higher dietary protein concentration was associated with a more competent immune defence in terms of a reduced incidence of metritis in early lactation. The immune defence mechanisms against bacterial contamination include the attraction of neutrophils to the uterus endometrium and lumen, and the subsequent phagocytosis of bacteria, and the release of antimicrobial proteins (highly proteinaceous). Contrary to these findings, a secondary study carried out by Young *et al*. (unpublished data) on the *in vitro* immune competence of dairy cows receiving 114, 144, and 173 g CP/kg DM indicated that cows receiving 114 g CP/kg DM had a higher immune competence than those on 144 or 173 g CP/kg DM (quantifying interferon gamma production). However, this study was carried out in mid lactation (150 d) at which point the risk of metritis is low and factors affecting immune competence may be different to those in early lactation.

Abnormal Hormonal Patterns and Correlations between Reproductive Variables

In the current experiment, 62% of animals displayed at least one abnormal progesterone profile which is higher than reported in a previous study (McCoy *et al*., 2006; 41%). Overall, 30.3% of oestrous cycles were prolonged due to delayed luteolysis. An extended luteal phase, in the absence of pregnancy will increase the inter-ovulatory interval (IOI). The average IOI in the present study was 24.8 d (44.9% of all cycles above 24). Royal *et al*. (2000) suggested that an IOI with a duration greater than, or equal to, 24 d would be associated with reduced fertility (lower pregnancy rates). This was observed in the present study as the duration of the IOI was negatively associated with the interval from calving to conception.

Post ovulation, the luteal phase is extremely important in maintaining regular reproductive cycles and a high reproductive performance. In the present study, the occurrence of a persistent corpus luteum was associated with a longer calving interval. An extended luteal phase can occur in two situations; 1) when there is early embryo loss following maternal recognition of pregnancy, or 2) when there is delayed luteolysis in the absence of an embryo (pregnancy). A luteal phase greater than 16 d would suggest that maternal recognition of the embryo has occurred. Larson *et al.* (1997) illustrated that non-pregnant animals, with maternal recognition of the embryo (progesterone concentrations greater than 3 ng/ml after day 19 post breeding), had lower progesterone concentrations at day 4.5 post insemination than pregnant animals. In support of this, Wathes *et al.* (2003) reported that a delay in progesterone increase in the early luteal phase (the progesterone concentrations on d 4 and d 5) in high yielding cows will reduce the capability of cows becoming pregnant. In such cases the dam recognises the presence of the embryo but due to a delay in embryo maturity (as a result of a reduced progesterone concentration in the first 7 d post insemination) the embryo is incapable of maintaining pregnancy. In the present study there was a positive correlation between the average progesterone concentration in the first 7 d post breeding and conception, which further strengthens this hypothesis.

Delayed luteolysis, in the absence of pregnancy, can also be caused by a sub-optimal uterine environment which can disrupt normal hormonal and luteolytic mechanisms (Kindahl *et al.*, 1999; Sheldon *et al.*, 2006). In a more favourable uterine environment, prostaglandin $F_{2\alpha}$ acts on the corpus luteum and causes it to regress and cease progesterone production, removing the inhibitory effect on LH release and allowing the next ovulation to occur. Sheldon *et al.* (2006b) stated that in a sub-optimal uterine environment (presence of bacterial pathogens) there is an increase in prostaglandin E_2 production, due to the stimulatory effect of lipopolysaccharides found on the uterine pathogen *E. coli*. Elevated prostaglandin E_2 has a luteotrophic effect which increases progesterone secretion and subsequently prolongs the luteal phase. Prostaglandin E_2 is important in the growth, development and maintenance of a normal corpus luteum (Arosh *et al.*, 2004). Interestingly, Lamming and Darwash (1998) reported that cows that have sub-optimal uterine environments and experience persistent corpora lutea are more at risk of late embryonic loss after becoming pregnant.

The average interval to the onset of luteal activity in the current experiment was 32.2 d, which was an improvement on the previous figures of 36.1 and 37.0 d quoted by McCoy *et al*. (2006) and Opsomer *et al*. (2000) respectively. An extended time to the onset of luteal activity is caused by the inability of the ovulatory follicle to produce adequate amounts of estradiol to allow ovulation. In the present study an increase in the interval to the onset of luteal activity was associated with a decrease in the peak progesterone concentration of the first cycle. This would suggest that factors causing a delay in the onset of luteal activity have subsequent negative effects on the functionality of the corpus luteum, which will reduce the probability of embryo survival and pregnancy. Progesterone is essential for embryo development and a delay in luteinisation, caused by a reduction in the competence of the corpus luteum, will result in a delayed rise in circulating progesterone levels after ovulation, significantly reducing the probability of embryo survival (Wathes *et al*., 2003). Butler (2000) concluded that during a period of negative energy balance the ability of follicles to produce sufficient estradiol for ovulation appears to be dependent on the availability of insulin and IGF-1. Detrimental effects of negative energy balance on IGF-1 and luteal progesterone concentrations have been shown by Pushpakumara *et al*. (2001) and Spicer *et al*. (1990) respectively. In the present study a more negative energy balance was associated with an increased interval from ovulation to a progesterone rise above 3 ng/ml. Starbuck *et al*. (2001) stated that animals with progesterone concentrations below 1 ng/ml on day five after mating had pregnancy rates below 10%.

Effect of Blood Constituents on Reproductive Variables

In the current study, blood urea concentrations did not significantly affect any of the reproductive variables. Interestingly, Butler *et al*. (1996) stated that blood urea concentrations above 19 or 20 mg/dl would result in a 20% decrease in pregnancy rate after insemination. An increase in blood urea nitrogen concentration will decrease the uterine pH, making the uterine environment more hostile and unsuitable for the early developing embryo (Elrod *et al*., 1993). However, in the current experiment only 0.54% of all blood samples had urea concentrations above this threshold (using the conversion: mmol/l divided by 0.357 = mg/dl), which may explain the lack of any effect of urea on fertility measures. Plasma ammonia concentrations, which were not recorded in this experiment, may have better illustrated the toxic effects of elevated protein on reproductive performance. An increase in plasma ammonia concentration occurs when the detoxification capacity of the liver is breached. Ureagenesis (conversion of

absorbed ammonia into urea in the liver) has been reported to be less efficient in animals with fatty liver (Strang *et al.*, 1998), subsequently reducing the capacity to detoxify. This would suggest that negative energy balance can amplify the negative effects of elevated dietary protein on reproductive performance.

In the current study an increase in leptin concentration significantly reduced the interval to progesterone rise above 3 ng/ml after ovulation. Adipose tissue is the biggest contributor to plasma leptin in ruminants (Chilliard *et al.*, 2001) and is positively correlated with body condition (Kokkonen *et al.*, 2005). Leptin concentration in blood is highly sensitive to alterations in energy status, with a more severe negative energy balance being associated with a greater loss of adipose tissue and subsequently large reductions in leptin production (Houseknecht *et al.*, 1998; Wathes *et al.*, 2003). In previous studies conducted by Woods (2005) and Reist *et al.* (2003), a positive correlation between leptin and IGF-1 was observed which could explain the observed relationship between leptin and time to progesterone rise in the current experiment. Unfortunately there was no analysis of IGF-1 against interval to progesterone rise after ovulation in the current study, due to limitations in the number of IGF-1 samples analysed. Wathes *et al.* (2003) suggested that IGF-1 was important in dictating the progesterone output of the corpus luteum. Those authors also stated that increased progesterone secretion from the corpus luteum was related to a large dominant follicle (high estradiol production) that took less time to mature. In support of this, Starbuck *et al.* (2000) reported that a longer interval to progesterone rise after ovulation is associated with poor estradiol secretion during the follicular phase leading up to ovulation.

Conclusions

Much of the previous work on protein nutrition of dairy cattle has focused on increasing the protein concentration of the diet to increase milk yield, whereas the present study focused on the effects of reducing the protein concentration of the diet as a means of improving the energetic balance and fertility of the high yielding Holstein dairy cow. The results from the current study suggest that there is little or no effect of altering dietary protein concentration on the reproductive performance of the contemporary high yielding dairy cow. This is a view shared by Laven *et al.* (2007), who stated that the evidence for negative effects of elevated concentrations

of dietary nitrogen on fertility of dairy cows was not conclusive as many studies have shown little or no effect. Additionally, the high level of abnormal oestrous cycles observed in the current study would suggest that there are more serious underlying physiological problems that may inhibit any potential benefit of altering the dietary CP concentration.

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Chapter 4: Effect of Dietary Protein Content on Oestrous Behaviour of Dairy Cows during Early and Mid-Lactation

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INTERPRETIVE SUMMARY

The ability to detect cows in oestrus plays a vital role in improving pregnancy rates on commercial farms. Lowering the protein concentration of dairy cow diets improved body energy status but had no effect on the display of oestrous behaviour. An increase in the number of animals in oestrus simultaneously increased the intensity of oestrus and the number of occasions in which standing immobile on being mounted was observed. Despite being the most reliable behavioural activity (96.4%) associated with oestrus expression, standing immobile on being mounted was not expressed often enough (51.7% of cycles) to be used exclusively as an indicator of oestrus.

Abstract

One of the main contributing factors to the decline in fertility in contemporary dairy farming is the inability to detect cows in oestrus. In the current study, ninety Holstein dairy cows (45 primiparous and 45 multiparous (mean parity of 3.1)) were allocated to one of three treatments at calving; 173, 144, or 114 g CP/kg DM. Oestrous behaviour was recorded for one 30 minute period every 12 hours from calving until all animals reached 140 days post-partum. Behavioural activities were recorded according to a scoring system developed by Van Eerdenburg *et al*. (1996), with nine key oestrous behavioural activities each allocated a given number of points. If the total score allocated was greater than or equal to 50 points during a single or consecutive observational periods then the animal was deemed to be in oestrus. A total of 238 oestrous cycles scored 50 points or above on the Van Eerdenburg *et al*. (1996) scale in this experiment, with 51.7% of these cycles being characterised as standing immobile on mounting. There were no direct effects of dietary protein content on oestrous behaviour, however, three significant stage of lactation by protein treatment interactions occurred for the behavioural activities; mucous discharge, chin resting and mounting the head side of another cow, but no consistent trends were apparent from the predicted means. There was a significant influence of parity on the frequency of mounting the head side of another cow and total number of behaviour activities displayed per oestrous cycle. In both cases multiparous animals displayed fewer behavioural activities than primiparous animals. An increase in the size of the sexually active group (animals in oestrus at the same time, up to 5) significantly increased the expression of mounting or attempting to mount another cow, the number of cycles in which standing immobile on being mounted was observed, the total oestrous score and the proportion of cyclic animals that were diagnosed as being in oestrus. The most frequent behavioural activity displayed was chin resting (89.5% of cycles) and the most reliable behaviour was standing immobile on mounting (when expressed 96.4% of cows were in oestrus). The most dependable (function of reliability and frequency displayed) sign of oestrus was mounting or attempting to mount another cow. This behaviour was expressed in 83% of cycles, and when expressed, 89% of animals were in oestrus.

Keywords: oestrous behaviour, crude protein, mounting behaviour, oestrous detection

Introduction

Oestrous detection is the most important managerial variable involved in reproductive performance (Van Vliet and Van Eerdenburg, 1996) and is one of the main contributing factors to the ongoing reproductive decline. Van Vliet and Van Eerdenburg (1996) referred to work by Heersche and Nebel (1994) stating that out of 4550 herds in the USA, the mean oestrous detection rate was 38%. Coleman (1993) attributed 90% of low oestrous detection rates to farm staff and 10% to the cow. Accurate oestrous detection is not only important in maintaining short calving intervals but also in defining the time interval to insemination (Lyimo *et al.,* 2000). Inaccurate detection of oestrus may result in insemination of cows not in oestrus or insemination at a time which is not optimal for conception. Van Eerdenberg *et al*. (2002) found that when ovulation occurred after 48 hours post insemination only 15% of the cows conceived, whereas, 52% of cows that ovulated within 24 hours of insemination conceived. Furthermore, Reimers *et al*. (1985) reported that 5.1 % of cows were not in oestrus when inseminated but emphasised that this percentage varied from 0 to 60% among herds. This could be due to early or late insemination or incorrect oestrous detection. Additionally, it has been reported that 5 to 10% of pregnant cows also display some signs of oestrus (Ambrose, 1999). With the introduction of oestrous detection aids, farm staff may not be as vigilant on routine observations as they once were. The maintenance of a good level of knowledge of oestrous symptoms and the observation of cows as frequently as possible, for a sufficient length of time, is critical for farm staff (Van Vliet and Van Eerdenburg, 1996). The duration and intensity of the displayed oestrus is highly variable among individuals and is greatly influenced by the number of cows that are in oestrus simultaneously (Diskin and Sreenan, 2000). Small groups of cows and all-year round calving will greatly reduce the number of simultaneous oestrous cycles.

In the present study the emphasis is on how nutrition, namely protein content of the diet affects the expression of oestrus. There is a common consensus that malnutrition or specific nutrient deficiencies may interfere with and/or inhibit oestrous expression, however it is also considered that as long as cyclic ovarian function is proceeding normally, the expression of oestrus will be unaffected (Allrich, 1993). It is thought that low dietary CP is associated with reduced oestrous behaviour and conception in beef heifers (Robinson, 1996). One possible explanation for this

effect is the suggestion that lower basal serum luteinising hormone concentrations are associated with a lower dietary CP intake, when compared to a higher CP intake (Westwood *et al*., 1998). While luteinising hormone is important in ovarian follicular development post-calving and oestrous behaviour (Ferguson, 1996; Westwood *et al*., 1998), there are suggestions that high protein diets can be detrimental to oestrous expression (Orihuela, 2000). A higher dietary protein intake will increase milk yield which is not emulated entirely by energy intake, subsequently exacerbating negative energy balance. This will inevitably increase the risk of prolonged anoestrus (Ferguson, 1991) due to poor follicular development, which will reduce estradiol production and subsequent expression of oestrus. Spicer *et al*. (1990) found a positive correlation between energy balance and the frequency of visually observed oestrus in the first post-partum ovulation. Ferguson (1996) also reported work from Harrison *et al*. (1990) suggesting that higher yielding cows will have a lower expression of oestrus. Interestingly, Van Eerdenburg *et al*. (1998) reported that the oestrous period was longer in animals with a more pronounced NEB.

The objective of the present study was to evaluate the effect of level of dietary protein on the oestrous behaviour of high yielding dairy cows and the effectiveness of observing cows twice a day, for a duration of 30 minutes, on oestrous detection using a scoring system developed by Van Eerdenburg *et al*. (1996). It has been stated that the accuracy and efficiency of direct observation, as an oestrous detection technique, is affected by the frequency, duration and timing of the observation periods (Hurnik *et al*., 1975). However with increasing herd sizes and reduced levels of staffing available on farms (Mayne, 2006), the manpower input per cow decreases (Senger, 1994), which increases the need for more practical and focused oestrous detection methods.

Materials and Methods

Animals and Housing

Ninety Holstein dairy cows (45 primiparous and 45 multiparous (mean parity 3.1)) were used in the experiment. Cows entered the trial following parturition (29 August to 23 December). Following calving, animals were housed as a single unit in free stalls with concrete flooring. The cubicle to cow ratio was ≥ 1:1 at all times, meeting the recommendations set by FAWC (1997).

All cubicles had a rubber mat bed, measuring 2.20 m long and 1.25 m wide and were bedded with sawdust thrice weekly. Concrete passageway floors were scraped a minimum of four times daily, using an automated system. Cows were milked twice at 05.30 and 16.30 hours, with cows travelling about 35 m to the milking parlour. Lights were left on at all times.

Experimental Design, Diets and Feeding

The experiment involved allocating 90 freshly calved Holstein Friesian dairy cows to three dietary treatments which differed in overall CP levels, as formulated, in the complete diet (DM basis); 180, 150, and 120 g/kg DM. Primiparous animals were assigned in a balanced manner to treatments based on heifer rearing regime, calving date and live weight. Multiparous animals were assigned to treatments according to parity, previous lactation milk yield, calving date and live weight. The diet was presented as a total mixed ration (TMR) and animals were fed between 10.00 and 11.00h daily using a diet feeder. Animals had free access to water at all times. The concentrate to forage ratio (DM basis) was 0.55:0.45 for all diets. The forage component of the diet consisted of 0.60 grass silage and 0.40 maize silage (DM basis). Samples of grass and maize silage were taken weekly and analysed using near infrared reflectance spectroscopy (Park *et al.*, 1998), and twice weekly for measurement of nitrogen and ammonia nitrogen using methods outlined by Steen (1989). Two concentrates were formulated to contain 229 and 117 g CP/kg DM in order to achieve target protein levels in the overall diets of 180 and 120 g CP/kg DM respectively. Diets containing 150 g CP/kg DM were produced by complementing the forage component with equal amounts of concentrates containing 117 and 229 g CP/kg DM. The energy and protein concentrations of individual ingredients were based on published values (AFRC, 1993) and were used to formulate the concentrate proportion of the diet as presented in Table 1.1. Concentrate samples were taken weekly during the experiment and analysed for DM, ash, ADF, NDF and nitrogen as described by Cushnahan and Gordon (1995). The TMR diets were offered *ad libitum* using feed boxes which were placed on a computer recorded load cell system, with controlled access to the boxes using an electronic identification system. This enabled dry matter intakes of individual cows to be recorded continuously via automatic feeding gates (Calan gate feeder), from which a daily intake was calculated and then averaged on a weekly basis.

Measurements

In all animals, oestrous behaviour was recorded for one 30 minute period every 12 hours from calving until all animals had reached 140 days post-partum. During the observational period a trained observer walked through the herd examining animals for activity. All cows had identification collars and were freeze branded, allowing ease of identification. Behavioural activities were recorded according to a scoring system developed by Van Eerdenburg *et al.* (1996) (Table 4.1), with nine key oestrous behavioural activities; each allocated a given number of points. Once each 30 minute observational period was complete the total number of points was calculated. If the total was greater than or equal to 50 points during a single or consecutive (an aggregate score for a particular cycle) observational periods, then the animal was deemed to be in oestrus (Van Eerdenburg *et al.,* 1996). Technicians were trained to record oestrous behaviour over a three week period.

Milk yields were recorded daily at each milking throughout the experiment. Milk composition (fat, protein, lactose and somatic cell count) was determined on a weekly basis from one consecutive am and pm milking. Separate analysis was completed for am and pm samples and milk composition was calculated on the basis of recorded am and pm milk yields. Milk composition was determined using an infrared milk analyser (IRMA). Milk progesterone samples were collected twice weekly (Tuesday and Friday; am milk samples) until pregnancy confirmation at day 30 post service. Pregnancy was reconfirmed at day 60 post insemination and in the event of embryo loss, milk progesterone samples commenced. Pregnancy was confirmed using an ultrasound scan carried out by a veterinarian. Milk progesterone concentrations were used to confirm ovulation and subsequently the validity of oestrous expression. Milk progesterone concentrations were determined using an enzyme-linked immuno-sorbent assay (ELISA) kit (Ridgeway Science Ltd, Lydney, UK) based on the method of Sauer *et al*. (1986). Sequential milk progesterone samples were plotted for each animal. If an animal was deemed to be in oestrus, and was past the voluntary waiting period (42 days), then she was presented for AI approximately 12 hours after the observational period. In the absence of a continuous (24-hour) observation of oestrous activity it is difficult to determine the exact time of onset of oestrous activity. Reduced conception rates have been reported in cows inseminated > 16 (Stevenson *et al.,* 1984) and > 24 hours (Foote, 1979) after an observed oestrus, therefore, a 12-hour interval from oestrus to AI was implemented. Insemination details were recorded (date, hour, sire and inseminator).

Table 4.1: Scoring scale for observed symptoms of oestrus (Van Eerdenburg *et al*., 1996)

Symptom of oestrus	Score
Mucous vaginal discharge	3
Cajoling	3
Restlessness	5
Sniffing the vagina of other cow	10
Chin resting	15
Mounting but not standing	10
Mounting (or attempt) other cows	35
Mounting head side of other cow	45
Standing immobile on being mounted	100

[1] The symptoms are cumulative, once an observation is observed the allocated points are added to the total

Animals were blood sampled once weekly, between 0930 and 1130, from the coccygeal vein using uncoated, heparin-coated and fluoride oxalate coated tubes (BD, Oxford, UK) from calving until day 100 of lactation, and then fortnightly thereafter. Plasma was recovered by centrifugation for analysis of glucose, (fluoride oxalate tubes) total protein, albumin, globulin, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA) and urea (heparinised tubes) respectively, by clinical analyser (Olympus UK Ltd, Middlesex, UK). NEFA concentrations were determined using a standard kit (Wako Chemicals GmbH, Neuss, Germany). Uncoated tubes provided serum for leptin radio-immunoassays (RIA). Plasma and serum samples were stored at –20°C until analysed. RIAs were balanced for dietary treatments and parity and control samples were included in each assay.

Serum leptin concentrations were determined in samples taken from all animals in each of weeks 2, 4, 6, 8, 10, 12, 16 and 20. The primary anti-leptin antibody (OL-3) was raised in guinea pigs using recombinant ovine leptin kindly donated by Professor A Gertler (The Hebrew University of Jerusalem) and was included at a final dilution of 1:160,000 in each assay. Primary antiserum (100 µl) and plasma (100 µl) were incubated overnight (20 hr) in a refrigerator after which was

added 100 µl (12000-15000 cpm) of ^{125}I-iodinated ovine leptin, prepared using iodogen (Perbio Science, Northumberland, UK). Tubes were then held for a further 24 hr in a refrigerator before addition of 100 µl of a cellulose-immobilised second antibody suspension (Sac-Cel anti-guinea pig IgG; IDS Ltd., Washington, Tyne on Wear, UK). Tubes were then left at room temperature for 20 minutes before addition of 1 ml of de-ionised water and centrifugation at 1900 x g for 20 minutes. Following centrifugation, supernatant was removed and radioactivity in each pellet was counted using a Cobra ll gamma counter (Packard Canberra, Reading, UK).

Live weight and body condition score (scale: 0-5; Edmonson *et al*., 1989) were recorded on a weekly basis throughout lactation. Locomotion scores were carried out every two weeks using the method described by Manson and Leaver (1988).

Calculation of Energy Balance

The average daily energy balance for each animal was calculated each week of lactation using the equations described by Thomas (2004) [Energy balance = ME intake – ME requirement (-10 + (ME_{preg} + $ME_{maintmilk}$ * $Lwt^{0.75}$) + ((0.0013*Lwt)/K_m))]. Daily milk yield, daily DMI, weekly milk composition, weekly live weight and feed composition data were used in the calculations. ME contents of grass and maize silage were obtained on a weekly basis using near infrared reflectance spectroscopy (Park *et al*., 1998) and ME contents of the concentrate were as formulated. Missing values were estimated from the week previous to and the week following missing data. Less than 2% of the data were missing.

Statistical Analysis

A repeated measures approach using the Residual Maximum Likelihood (REML) procedure available in GenStat (Payne *et al*. 1993) was used to analyse the data set. The model fitted fixed effects for parity, treatment, size of sexually active group and stage of lactation (0 to 20 weeks). The model included all two-level interactions between these variables. Two covariates were used in the analysis of the data; the deviation from the mean condition score of multi- and primiparous animals during the first three weeks of lactation and the deviation from the mean of the first two locomotion scores recorded of multi- and primiparous animals. There was no significant effect of treatment on the change in condition score in the first three weeks of

lactation or on the difference between the first two locomotion scores recorded, therefore allowing them to be used as covariates.

Regression analysis was performed for several additional independent variables on all the behavioural activities (response variables). In this analysis the fitted terms included; constant + parity + stage of lactation + an additional variable (X). These additional independent variables included the blood constituents; leptin, urea, globulin, albumin, total protein, BHB, NEFA and glucose.

Results

Production Responses to Dietary Protein in Early and Mid Lactation (1 to 140d)

The composition (as fed) of diets targeted to contain 120, 150 and 180 g CP/kg DM were 114, 144 and 173 g CP/kg DM respectively. All diets were isoenergetic. Dietary protein concentration effects on milk yield, dry matter intake, ME requirement, ME intake, daily energy balance and cumulative energy balance are presented in Table 4.2. Increased dietary protein concentration significantly ($P < 0.001$) increased milk yield, dry matter intake, and ME requirement. Increasing dietary protein concentration from 114 to 144/173 g/kg DM significantly ($P < 0.001$) increased ME intake. Increasing dietary CP concentration from 114/144 to 173 significantly decreased daily energy balance and cumulative energy balance.

Table 4.2: Production responses dietary protein in early and mid lactation (1 to 140d)

	Dietary CP Concentration (g/kg DM)[2]			Significance[3]	
	114	144	173	SED	P-value
Milk yield (kg/d)	25.4[c]	31.8[b]	35.5[a]	1.14	***
Dry matter intake (kg/d)	16.4[c]	18.0[b]	18.5[a]	0.35	***
ME requirement (MJ/d)[1]	191[c]	222[b]	243[a]	6.35	***
ME intake (MJ/d)[1]	203[b]	223[a]	230[a]	4.53	***
Daily energy status (MJ/d)	11.9[a]	0.35[a]	-12.7[b]	6.32	**
Cumulative energy status (MJ/d)	341[a]	-568[a]	-1810[b]	502.2	***

[1] ME, metabolisable energy
[2] CP, crude protein; DM, dry matter

[3] SED, standard error of the difference; **, P < 0.01; ***, P < 0.001

Behavioural Trends

Examination of the progesterone profiles of the group of 90 animals indicated that 367 oestrous cycles occurred during the 140-day period post-calving. Of these 367 cycles, 72 were described as being 'silent'; defined as an animal not being observed in oestrus by either the trained dairy technicians during an observational period, or by the farm staff who carried out four 20 minute observational periods during each 24-hour period (8 am, 12 pm, 4 pm and 9 pm, with additional observations during collection). The highest percentage (47.2) of silent oestrous cycles were displayed in the first cycle and decreased thereafter. Interestingly, in their fourth and fifth oestrous cycles, 4 animals failed to display oestrous activity. In 65% of ovulations, a cumulative oestrous behaviour score of 50 points or more was observed by dairy technicians during the 30 minute observational period carried out at 12-hour intervals. In 7.6% of ovulations, behavioural activity was observed but the cumulative total score was below 50 points, which signifies a non-oestrous state, according to the behavioural scoring system. In a further 7.9% of ovulations, behavioural indicators of oestrus were detected by farm staff on their routine observations but not by dairy technicians during the experimental observational periods. An additional 34 positive oestrous cycles (a score of 50 or above) were recorded in animals considered to be acyclic based on their progesterone profiles, and of these, eight positive oestrous cycles (7 different animals) were recorded in pregnant animals. Furthermore, on 63 occasions, oestrous behavioural activity with a score below 50 occurred in animals that were considered to be acyclic from their progesterone profiles.

The mean number of individual behavioural activities recorded across all cycles was 20.9 per cycle (SED, 0.86), with a maximum and minimum of 72 and 3 respectively. The mean oestrous behaviour score was 421 (SED, 19.1) per cycle, with a maximum and minimum of 1480 and 52 respectively. Oestrous behaviour score had no significant effect (P > 0.05) on conception rate, however, animals that conceived tended to have a higher oestrous behaviour score than animals that failed to conceive (475 vs. 396 respectively; SED, 48.0). The reliability of each behavioural activity as an indicator of oestrus, the percentage expression and the average frequency of each behavioural activity is presented in Table 4.3, based on analysis of progesterone profiles. The most reliable behavioural activity in detecting oestrus was standing immobile on being mounted

(when expressed, 96.4% of animals were in oestrus). The least reliable and the least frequently displayed (per cycle) behavioural activity was mucous vaginal discharge (when expressed, 75.7% of animals were in oestrus; 0.11 occurrences per oestrous cycle). The most frequently displayed behavioural activity was chin resting (5.68 times per oestrous cycle). The behavioural activity expressed in the highest number of cycles was chin resting (89.5%). Standing immobile on being mounted was expressed in 51.7% of the cycles. During the first ovulation, only 23% of animals displaying oestrous behaviour stood immobile on being mounted (11% of all animals). During the second ovulation 52% of animals displaying oestrous behaviour stood immobile on being mounted (36% of all animals). There was no effect of cycle number on the intensity of the oestrous behaviour displayed.

Table 4.3: Analysis of oestrous cycles with a cumulative oestrous behaviour score of 50 points or more (n=238)

Oestrous behaviour	Reliability of behavioural activity[1]	Percentage expression[2]	Average counts per cycle[3]
Mucous vaginal discharge	75.7	8.8	0.11
Cajoling	86.9	53.4	1.44
Restlessness	78.9	81.5	3.22
Sniffing the vagina of other cow	75.8	86.6	5.37
Chin resting	80.5	89.5	5.68
Mounting but not standing	78.3	19.7	0.40
Mounting (or attempt) other cows	88.5	83.2	2.88
Mounting head side of other cow	95.0	22.3	0.41
Standing immobile on being mounted	96.4	51.7	1.38

1 Percentage of animals that expressed this behaviour and were in oestrus based on progesterone profile
2 Percentage of oestrous cycles in which behaviour was expressed
3 Number of times each behavioural activity was expressed in the average cycle. The average cycle included 20.9 individual behavioural activities

Effect of Treatment, Parity and the Size of the Sexually Active Group

The REML variance component analysis indicates that there was no significant ($P > 0.05$) direct effect of dietary protein concentration on any of the oestrous behaviours. However, the predicted means suggest that there is a tendency for reduced dietary protein content to increase oestrous behavioural score. Diets containing 173, 144 and 114 g CP/kg DM had mean oestrous behaviour scores of 328, 357 and 404 (SED, 55.7) respectively. Three significant stage of lactation by protein treatment interactions were found for the behavioural activities; mucous vaginal discharge ($P < 0.01$), chin resting ($P < 0.01$) and mounting the head side of a cow ($P < 0.05$), but no consistent trends from the predicted means were apparent. Significant effects of parity were also realised. Compared to multiparous animals, primiparous animals displayed significantly more 'mounting the head side of another cow' (0.41 vs. 0.16; SED, 0.09) per cycle, and displayed a significantly higher total number of behavioural activities per cycle (22.4 vs. 18.7; SED, 1.75). There was no significant ($P > 0.05$) effect of parity on the number of silent oestrous cycles.

The size of the sexually active group had a significant effect on the expression of mounting or attempting to mount another cow ($P < 0.05$), standing immobile on being mounted ($P < 0.001$) and the total oestrous score obtained ($P < 0.001$) (Table 4.4). There was a positive correlation between the number of animals in a sexually active group (up to five) and the above behavioural parameters. The number of occasions in which a sexually active group size was observed decreased with an increase in the size of the sexually active group. Also, the size of the sexually active group had a significant effect on whether or not animals that were cycling (according to the progesterone profiles) were actually detected during the 30 minute observational period ($P < 0.001$). The larger the sexually active group the higher the proportion of cows that were correctly diagnosed as being in oestrus (0.59, 0.91, 0.91, 0.94, 0.96 and 0.99 for a sexually active group size of 1, 2, 3, 4, 5, and 6 respectively; SED, 0.07). There was no significant ($P > 0.05$) effect of dietary protein concentration or stage of lactation on the size of the sexually active group.

Table 4.4: Effect of the size of the sexually active group on the expression of oestrous behaviours (following a square root transformation)

Behavioural activity[1]	Size of sexually active group[2]						SED[3]	Significance[3]
	1	2	3	4	5	6		
Occurences[4]	49	41	31	24	7	6		
Mounting	1.11	1.27	1.38	1.47	1.70	1.66	0.228	*
Standing	0.19	0.48	0.76	1.04	1.14	0.94	0.212	***
Total	15.7	17.1	18.8	20.1	22.1	20.2	1.735	***

[1] Mounting, mounting or attempting to mount another cow; Standing, standing immobile on being mounted; Total, total oestrous score
[2] Size of sexually active group, number of cows that displayed interactive oestrous behaviour during an observational period.
[3] SED, standard error of the difference; *, $P < 0,05$; ***, $P < 0.001$
[4] Occurrences, the number of occasions a sexually active group of this size was observed.

The Effect of Blood Parameters on Behavioural Activities

Effects of blood parameters on the expression of oestrous behavioural activities are presented in Table 4.5. The total behavioural score was significantly affected by plasma urea ($P < 0.05$;

negative relationship). An increase in serum leptin concentrations significantly increased the expression of cajoling ($P < 0.01$) and the expression of mounting the head side of another cow ($P < 0.01$). An increase in globulin and total protein significantly ($P < 0.05$) decreased the expression of restless behaviour.

Table 4.5: Effect of blood parameters on the expression of oestrous behaviours (regression analysis)

Blood parameter	Oestrous behaviour	Relationship	Significance[1]	r^2
Urea	Total behaviour score	Negative	*	0.02
Leptin	Cajoling	Positive	**	0.04
Leptin	Mounting head side of another cow	Positive	**	0.03
Globulin	Restlessness	Negative	*	0.02
Total protein	Restlessness	Negative	*	0.02

[1] *, $P < 0.05$

Discussion

Behavioural Trends

In the current experiment, an oestrous detection rate of 65% was achieved when cows were observed for two 30 minute periods (12 hour interval) every 24 hours. The farm staff, who have a standard operating procedure for oestrous detection, realised a marginally higher (73%) detection rate by observing the animals during four 20 minute observational periods per 24 hour period, with additional observations during the milking periods. These oestrous detection rates are similar to those reported by Van Eerdenburg *et al*. (1996), who stated that two observational periods of 30 minutes duration per 24 hour period could achieve a 74% detection rate. Van Vliet and Van Eerdenburg (1996) also noted that a decrease in the duration of the observational period from 30 to 20 minutes would decrease the detection rate by 20%.

'Silent' ovulations are another common problem associated with oestrous detection and are thought to be widespread among high yielding dairy cows (Shipka, 2000). The absence of

oestrous behaviour prior to the first ovulation is normally attributed to an excessively high concentration of estradiol at the end of pregnancy, which elicits a refractory state in the hypothalamus (Allrich, 1994). It has been suggested that the release of progesterone during the first post partum luteal phase alleviates this refractory state and allows the cow to express oestrus when the normal rise in concentration of estradiol occurs during the second ovulation (Kyle *et al*., 1992; Allrich, 1994). King *et al*. (1976) and Kyle *et al*. (1992) indicated that 50 to 80% of first ovulations are silent, but that by the third ovulation 100% of cows will exhibit oestrous behaviour. In the current study 19.6% of all cycles and 47.2% of first ovulations were silent. These results are similar to those quoted by Shipka (2000), who stated that 22% of all cycles were silent, with 42.1% of first ovulations being silent. The findings of the present study also suggest that silent oestrus may also occur in later cycles (cycles 4 and 5). Gil *et al*. (1997) stated that 48% of animals displayed at least one silent oestrous cycle before becoming pregnant whereas in the current experiment this was found to be the case in 59% of animals. In the present study a silent oestrous cycle was defined as an animal not being observed in oestrus by either the trained dairy technicians during an observational period, or by the farm staff on their routine observations. However, it must be emphasised that this does not mean that the cow did not display any signs of oestrus. Some cows in oestrus do not arouse the interest of others (Ball and Jackson, 1979) which may limit the external signs of oestrus. Additionally, with the duration of oestrus being reported to be as low as 4 hours (Van Vliet and Van Eerdenburg, 1996), a short oestrous cycle could be missed between observational periods.

Standing immobile on being mounted is recognised as the primary and most reliable sign of oestrus and the best indicator of the cow's pre-ovulatory state (Hafez *et al*., 1969). In this study it was found that 96% of cows standing immobile on being mounted were in oestrus. However, not all animals will stand to be mounted during oestrus (Foote, 1974; Pennington *et al*., 1986; Kerbret and Disenhaus, 2004). Historically, relatively high percentages of standing immobile on being mounted have been quoted in the literature. Trimberger and Fincher (1956) reported that by day 90 post-calving 93% of cows displayed this behaviour. Similarly, Hurnik (1987) reported that over 70% of animals in oestrus stood immobile on being mounted. However, more recently the expression of standing immobile on being mounted has been reported to be as low as 37% (of cycles) when cows are in oestrus (Van Vliet and Van Eerdenburg, 1996). The latter authors observed animals for 30 minutes every two hours for a six week period. In the present study the

animals were observed for one 30 minute period every 12 hours. Standing immobile on being mounted was expressed in 51.7% of cycles, however during the first and second cycle it was only expressed in 11 and 36% of animals respectively. On average, standing to be mounted represented only 6.7% of the total behaviour recorded, which is comparable to the 8% reported by Kerbat and Disenhaus (2004). However, Senger (1994) referred to earlier work stating that standing immobile on being mounted represents less than one percent of the period of oestrus (Senger, 1990) and is thus exceptionally difficult to detect without significant devotion to direct observations. Therefore, the use of secondary behavioural activities in the accurate identification of oestrous expression is vital. The most frequently displayed behavioural activities in this experiment were chin resting, sniffing the vagina of another cow and mounting, or attempting to mount, another cow. Although these behaviours aren't as reliable as standing immobile on being mounted, they occur more often which makes them extremely useful. It is suggested that an overall dependability score would be the best method of assessing how useful a behaviour is in detecting oestrus. This score would be a function of reliability and percentage expression. Table 4.6 illustrates a dependability score for the behavioural activities observed in this experiment. This approach would suggest that standing immobile on being mounted is only the fifth most dependable oestrous behaviour in identifying cows that are in the pre-ovulatory stage of the oestrous cycle. Interestingly, mounting or attempting to mount another cow is characterised as the most dependable sign of oestrus. Mounting or attempting to mount another cow was observed in 83% of oestrous cycles and when expressed, 89% of cows were in oestrus. Despite being highly reliable (when expressed, 96% of cows were in oestrus), standing immobile on being mounted was only expressed in 51.7% of oestrous cycles.

Table 4.6: The dependability of oestrous behavioural activities, as a function of reliability and percentage expression, in identifying cows in a pre-ovulatory state.

Oestrous behaviour	Reliability[1]	Percentage expression[2]	Dependability[3]
Mucous vaginal discharge	75.5	8.8	667
Cajoling	86.9	53.4	4640
Restlessness	78.9	81.5	6430
Sniffing the vagina of other cow	75.8	86.6	6564
Chin resting	80.5	89.5	7205
Mounting but not standing	78.3	19.7	1543
Mounting (or attempt) other cows	88.5	83.2	7363
Mounting head side of other cow	95.0	22.3	2119
Standing immobile on being mounted	96.4	51.7	4984

[1] Percentage of animals that expressed this behaviour and were in oestrus based on progesterone profile
[2] Percentage of oestrous cycles in which behaviour was expressed
[3] Function; reliability * percentage expression

Effects of Treatment, Parity and Size of Sexually Active Group

There was no significant effect of dietary protein concentration of the expression of oestrous behavioural activities. It was hypothesised that animals receiving a high concentration of dietary CP would have increased milk yields and subsequently a more negative energy balance. Potentially, this would reduce estradiol production and subsequent oestrous expression. Lyimo *et al*. (2000) stated that maximum estradiol concentrations correlated with total oestrous score. Despite the dramatic effect of dietary protein concentration on milk output and subsequent energy status, no direct influences of dietary protein concentration were realised in the current study. Harrison *et al*. (1990) reported that higher yielding cows had a lower expression of oestrus; however, milk yield differences were a response to genetic merit as opposed to nutrition.

There was a significant influence of parity on the frequency of mounting head side of another cow and total number of behaviour activities displayed per cycle. In both cases, multiparous animals displayed fewer behavioural activities than primiparous animals. In contrast, Van Vliet and Van Eerdenburg (1996) reported that multiparous animals displayed significantly more

intense cycles than primiparous animals with respective oestrous scores of 578 and 361, and this trend was also reported by Gwazdauskas *et al*. (1980). However, in the present experiment there was no significant difference between multi and primiparous animals for the overall behavioural score (intensity of behaviour). In a review, Orihuela (2000) reported that younger cows display fewer silent oestrous cycles than older cows, however this was not proven in the present study.

In this study, artificial synchronisation of oestrus was not implemented (however, reproductive intervention may have influenced synchronisation patterns), which allowed the natural synchronisation of oestrus, reflected in the formation of sexually active groups. Natural synchronisation will be influenced by group size, calving pattern and group composition (Hurnik, 1987). The findings of previous experiments have shown that maximum expression of oestrous behaviour (average mounts per cow) occurs when 3 cows are in oestrus simultaneously (Hurnik *et al.,* 1975). In the current study behavioural expression was displayed at a maximum when 5 cows were in oestrus simultaneously. However, the number of occasions in which five animals were in oestrus simultaneously was one seventh of that observed when only one animal was in oestrus (7 vs. 49). When only one animal is in oestrus, mounting activity and the duration of oestrus is minimal (Hurnik and King, 1987). Small groups and all year round calving will greatly reduce the number of simultaneous oestrous cycles, subsequently reducing the expression of oestrus. A close calving pattern will increase the probability of natural synchronisation or 'clustering' of cows displaying oestrous behaviour within the herd due to social facilitation and sexual stimulation (Kilgour *et al*., 1977). Hurnik *et al*. (1975) found that the number of mounts increased from 11.2 to 52.6 when the number of cows in oestrus increased from one to three. In the present study the number of mounts per cycle (during the observational periods) increased from 0.11 to 2.4 when the size of the sexually active group increased from 1 to 5 cows. Experimental examples of this social influence have also been reported in a review by Orihuela (2000), demonstrating the effect of synergy in the expression of oestrous behaviour due to the presence of companion cows. Orihuela (2000) stated that natural synchronisation of oestrous behaviour indicates the effect of sensory stimulation within a group and its influence on the activity of neuro-hormonal mechanisms in controlling sexual behaviour. Hurnik (1987) stated that pheromones, i.e. biochemical substances dispersed into an animal's external environment, can induce physiological changes when received by other individuals of the same species.

Conclusions

Low rates of oestrous detection are a major problem on commercial dairy farms due to increases in herd size, reduced staffing levels and effects of housing and feeding (Mayne, 2006). Any adjustment in procedures to improve the success of the detection of oestrus will also potentially improve fertility. The experimental protocol implemented for observation and recording of oestrous behaviour, of two 30 minute periods per 24-hour period, would appear to be relatively successful (65% detection rate) and in conjunction with oestrous detection during collection for milking, could be a viable approach to oestrous detection in modern dairy herds. Silent oestrous expression is a major problem in detecting oestrus in cyclic cows, with 19.6% of oestrous cycles being recorded as 'silent' in the present study. Standing immobile on being mounted is the most reliable indicator of oestrus; however, when used exclusively in oestrous detection, the low frequency of expression makes it less useful. The additional use of secondary behaviours, namely; mounting or attempting to mount another cow; sniffing the vagina of another cow; and chin resting, are crucial in increasing the efficiency of oestrous detection. Using these behaviours proved to be valuable in accurate oestrous detection. The size of the sexually active group had a major effect on the intensity of the oestrous expressed. Therefore, large groups of animals and concentrated calving patterns will increase the likelihood of natural synchronisation of oestrus and improve the probability of accurate oestrous detection. There was no direct effect of dietary protein content on the expression of oestrous behaviour in the current study.

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KEY PRESENTATIONS

Presentations at conferences

- Law, R. A, Young, F. J., Patterson, D. C. and Mayne, C. S. (2007) The effect of dietary protein content on the expression and detection of oestrus in high yielding dairy cows. In: *Proceedings of Fertility in Dairy Cows – bridging the gaps, EAAP satellite meeting, BSAS,* page 11.
- Yan, T., Young, F. J., Patterson, D. C. and Mayne, C. S. (2009) Effects of dietary protein concentration on the efficiency of nitrogen utilisation in lactating dairy cows. In: *Advances in Animal Biosciences, Proceeding of the British Society of Animal Science,* Page 78.

Presentations to farmers/industry meetings

- Presentation to Thompson's technical staff 24th July 2008
- Presentation to Volac technical staff 19th August 2008
- Presentation to Kemin group 8th January 2009
- Presentation to Volac technical staff 23rd April 2009

Presentations to farmers groups visiting Hillsborough

- Chilean farmers group Feb 08
- Alltech-Harbro visit 16th April 2008
- Israel visitors and William Crawford 1st May 2008
- Cooprinsem Chile dairy tour 23rd May 2008
- Gary Waghorn 28th May 2008
- VOLAC 19th August 2008
- Enniskillen dairy farmers 26th August 2008
- Hugh Black plus 6 farmers 10th September 2008
- Corby rock 1st October 2008
- Irish farmers group 4th October 2008
- Tiffany Burdack (Australian student) 10th December 2008

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