

Effect of dietary-induced changes in plasma insulin concentrations during the early post partum period on pregnancy rate in dairy cows

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Abstract

Dietary stimulation of insulin in post partum dairy cows has been found to enhance ovarian follicle development but to impair oocyte developmental competence. The objective of this study was to test the hypothesis that pregnancy rate would be improved by feeding a diet to stimulate higher insulin (H) until cows resumed ovarian cyclic activity after parturition, and then feeding a diet to lower insulin (L) during the mating period. Each diet was fed to 30 post partum dairy cows until their first rise in milk progesterone, when 15 cows in each group were transferred to the other diet (treatments HL and LH) and 15 cows in each group remained on their original diet (treatments HH and LL) until 120 days post partum. Treatments did not affect dry matter intake, milk yield and metabolisable energy balance. Plasma insulin concentration was elevated in cows fed on H compared with cows fed on L. Treatment did not affect days to first progesterone rise, first oestrus or first insemination. At 120 days post partum, 27% of cows on each of treatments HH, LL and LH were pregnant, but 60% of cows on treatment HL were pregnant ($P=0.021$). These findings support the concept that physiological relationships between insulin and the reproductive system vary according to stage of the reproductive cycle, and suggest that pregnancy rate can be enhanced by a two-diet strategy tailored to optimise responses before and after the first post partum ovulation.

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Introduction

The decline in dairy cow fertility over the past 30 years has been attributed partly to unfavourable genetic correlations between milk yield and reproductive traits, and partly to increasing imbalance of nutrients leading to metabolic stress (Pryce *et al.* 2004). Much emphasis has been placed on the effects of negative energy balance in early lactation on the length of the post partum anovulatory period and reduced conception rate (Beam & Butler 1999, Butler 2003). Prolonged periods of negative energy balance are associated with suppression of pulsatile luteinising hormone secretion, reduced ovarian responsiveness to luteinising hormone stimulation and reduced oestradiol secretion by the dominant follicle, all of which can influence ovulation of the dominant follicle (Lucy *et al.* 1991, Butler 2003).

The most common strategy for reducing the degree of negative energy balance in early lactation is to increase dietary energy concentration by increasing the starch or fat components of the ration at the expense of forage components. Such strategies alter metabolic hormones, particularly insulin, which can influence ovarian function (Boland *et al.* 2001, Webb *et al.* 2004, Garnsworthy

et al. 2008a). Gong *et al.* (2002a) demonstrated that feeding a high-starch diet to dairy cows for the first 50 days post partum enhanced circulating insulin concentrations and increased the proportion of cows ovulating before 50 days post partum from 55 to 90% without affecting milk yield or energy balance. Part of the mechanism could involve feedback signalling from ovarian follicles to the hypothalamus (Garnsworthy *et al.* 2008a) because dietary stimulation of plasma insulin in high-yielding dairy cows can increase numbers of small follicles (<5 mm diameter) and diameter of the ovulatory follicle (Garnsworthy *et al.* 2008b).

Although increasing insulin by dietary manipulation can be beneficial for resumption of oestrous cycles post partum, there is evidence that high insulin status might have detrimental effects on oocyte developmental competence, as indicated by rate of blastocyst production following *in vitro* maturation and fertilisation. For example, diets designed to increase plasma insulin concentration had negative effects on blastocyst rate in heifers (Adamiak *et al.* 2005, 2006) and in lactating dairy cows (Fouladi-Nashta *et al.* 2005). Furthermore, diets with a high fat content have beneficial effects on

blastocyst rate in lactating dairy cows (Fouladi-Nashta *et al.* 2007), although they may decrease plasma insulin concentrations (Garnsworthy *et al.* 2008c). In addition, supplementary fatty acids can have beneficial effects on plasma progesterone concentration and uterine secretion of prostaglandin-F_{2α} (Staples *et al.* 1998, Staples & Thatcher 2005). There is a potential conflict, therefore, between insulin-stimulating diets, which have beneficial effects on resumption of oestrous cycles post partum, and insulin-depressing or high-fat diets, which improve oocyte quality and embryo development. This is consistent with observations in sheep and non-lactating cattle, as reviewed by Boland *et al.* (2001), where high levels of feeding increased follicle numbers and size of the dominant follicle but reduced early embryo development. The overall suggestion is that optimum feeding strategies might differ according to the stage of the reproductive cycle and the aim of the current study was to explore this concept.

The main objective of the study was to test the hypothesis that pregnancy rate will be enhanced by feeding a diet that stimulates higher plasma insulin (H; Table 1) until cows resume ovarian cycles and then switching to a diet that lowers plasma insulin (L; Table 1) and increases fatty acid supply during the mating period (Treatment HL). A second hypothesis was that the converse strategy (a low-insulin diet followed by a high-insulin diet; Treatment LH) would reduce pregnancy rate. These strategies were compared with continuous feeding of either a high-insulin diet (Treatment HH) or a low-insulin diet (Treatment LL).

Table 1 Formulation and composition of diets designed to induce high (H) or low (L) plasma insulin.

	Diet	
	H	L
Formulation (g/kg DM)		
Grass silage	404	409
Maize/whole-crop wheat silage	119	120
Sugar beet pulp	114	211
Wheat	209	77
Soyabean meal	76	85
Rapeseed meal	57	61
Fatty acid supplement ^a	11	27
Minerals and vitamins ^b	10	10
	1000	1000
Composition		
DM (g/kg)	556	553
ME (MJ/kg DM)	12.5	12.6
CP (g/kg DM)	179	181
NDF (g/kg DM)	285	293
Starch (g/kg DM)	182	98
Sugars (g/kg DM)	57	67
Fat (g/kg DM)	39	53

^aMegalac, calcium salts of palm fatty acids; Volac International, Royston, UK. ^bBibby HiPhos: ABN Ltd, Peterborough: Calcium, 18%; Phosphorus, 10%; Magnesium, 5%; Salt, 17%; Copper, 2000 mg/kg; Manganese, 5000 mg/kg; Cobalt, 100 mg/kg; Zinc, 6000 mg/kg; Iodine, 500 mg/kg; Selenium, 25 mg/kg; Vitamin A, 400 000 IU/kg; Vitamin D3 80 000 IU/kg; Vitamin E, 1000 mg/kg.

Results

Intake, milk production and energy balance responses

There was no treatment effect on intakes of dry matter (DM; Table 2), neutral-detergent fibre (NDF), metabolisable energy (ME) and crude protein (CP). Cows in group HH consumed 1.6 times as much starch ($P < 0.001$) and 0.75 times as much fat ($P < 0.001$) as cows in group LL (Table 2).

There was no treatment effect on milk yield, milk protein and milk lactose, but milk fat content was greater ($P < 0.001$) for LL than for HH (Table 2). There was no treatment effect on (mean \pm S.E.M.) live weight (660 ± 7.1 kg), live-weight change from 0 to 8 weeks post partum (6 ± 6.7 kg), body condition score (BCS; 2.7 ± 0.07) or BCS change from 0 to 8 weeks post partum (-0.9 ± 0.11 units).

There was no treatment effect on net energy output in milk or ME balance (Table 2). Energy balance was negative after calving, but all groups reached positive energy balance in week 7 or 8 post partum (Fig. 1).

Metabolic hormone responses

Mean plasma insulin was higher ($P = 0.039$) throughout the experiment for HH than for LL (Table 3; Fig. 2). There was a significant ($P = 0.028$) interaction between treatment group and week of lactation; insulin concentrations increased for all groups from week 2 to 7 of lactation and was higher when cows were offered H than when they were offered L (Fig. 2). For groups that involved a diet change, insulin increased (LH) or decreased (HL) to levels similar to those of groups that did not involve a diet change (Fig. 2).

There was no treatment effect on mean plasma glucagon (Table 3), although mean plasma glucagon tended to be higher for HH than for LL ($P = 0.089$).

There was no treatment effect on mean plasma insulin to glucagon ratio, insulin-like growth factor I (IGF1), GH or leptin (Table 3).

Blood metabolite responses

There was no treatment effect on plasma non-esterified fatty acids (NEFA; Table 3). Plasma glucose was lower ($P = 0.015$) for LH compared with HH + LL, but no other contrast was significant. Mean plasma β -hydroxybutyrate (BOHB) was lower ($P = 0.011$) for HH than for LL (Table 3). Mean plasma urea-N was lower ($P = 0.004$) for LL than for HH (Table 3).

Health

There was no difference ($P = 0.973$) between treatment groups in mean vaginal mucus score (mean 1.08, standard error 0.15), and the distribution of scores was

Table 2 Mean feed intake and performance over the first 120 days post partum for groups of cows fed on diets that induced high (H) or low (L) insulin before and after the first rise in progesterone post partum^a.

	Treatment group				SED ^c	Contrasts ^b P		
	HH	HL	LH	LL		c1	c2	c3
Dry matter intake (kg/d)	22.8	23.6	22.2	24.2	0.74	NS	NS	NS
Starch intake (kg/d)	4.1	3.1	3.4	2.6	0.21	NS	NS	<0.001
Fat intake (kg/d)	0.9	1.1	1.0	1.2	0.05	NS	NS	<0.001
Milk yield (kg/d)	44.6	44.1	43.7	44.5	2.49	NS	NS	NS
Butterfat (g/kg)	31.0	35.0	33.7	36.7	2.03	NS	NS	<0.001
Protein (g/kg)	32.4	32.9	33.7	32.7	0.78	NS	NS	NS
Lactose (g/kg)	45.2	45.4	44.9	45.1	0.47	NS	NS	NS
Milk energy output (MJ/d)	126	130	121	131	7.9	NS	NS	NS
ME balance (MJ/d)	1.7	2.0	3.0	1.4	2.49	NS	NS	NS

^aValues for intake and milk production parameters were adjusted by covariance for previous lactation milk yield. ^bOrthogonal contrasts used in ANOVA were: c1 (HL versus HH + LH + LL); c2 (LH versus HH + LL); c3 (HH versus LL). ^cStandard error of the difference for comparing treatment group means.

similar among groups. Forty eight per cent of cows had a score of 0 (clear), 12% a score of 1 (flecks of pus), 22% a score of 2 (mild endometritis) and 18% a score of 3 (endometritis).

There was no difference ($P=0.912$) between treatment groups in the incidence of health problems requiring veterinary intervention. Veterinary treatment for retained placenta or severe uterine infection was given to five cows (8%). Seven cows (11%) were treated for mastitis. Four cows (7%) were treated for lameness.

Ovarian function

Progesterone profiles indicated that 62% of cows showed only normal oestrous cycles and 38% of cows showed abnormal oestrous cycles at some point in the

experiment; the proportion of cows with normal oestrous cycles was not affected by treatment. Proportions of cows showing each type of abnormal cycle were: delayed ovulation type 1 (DOV1), 12%; delayed ovulation type 2 (DOV2), 13%; persistent corpus luteum type 1 (PCL1), 2%; and persistent corpus luteum type 2 (PCL2), 15%.

Cows that had not been inseminated or were not cycling normally by 60 days post partum were scanned to record the state of their ovaries (Table 4). Cows offered diet H at 60 days post partum had a greater ($P=0.037$) total number of follicles and tended to have a greater ($P=0.064$) number of small (< 5 mm) follicles than cows offered Diet L at 60 days post partum. The number of cows with a corpus luteum, as a proportion of cows scanned at 60 days post partum, was greater ($P=0.029$) in cows offered Diet L at that time than in cows offered Diet H.

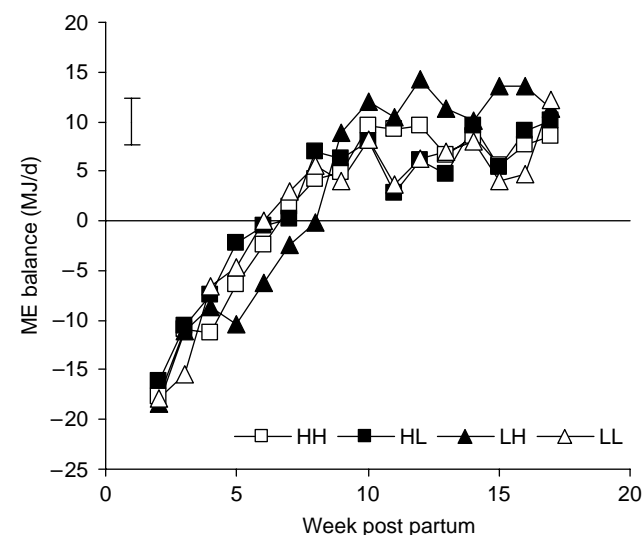


Figure 1 Metabolisable energy (ME) balance for groups of cows ($n=15$) offered diets that induced high insulin (H) or low insulin (L) before and after the first rise in progesterone post partum. Bar shows $2 \times \text{SED}$ for comparing treatment means. Effects of treatment group, NS; group \times week interaction, NS.

Reproductive performance

There was no effect of dietary treatment on days to first progesterone rise, days to first oestrus, days to first insemination or days to conception in cows exhibiting these events (Table 5). All the cows exhibited a progesterone rise during the experimental period and all exhibited oestrus except two cows in group HH. Eight cows were not inseminated during the experimental period, but there was no difference among groups in their distribution.

Compared with cows in the three other groups, for cows in HL (Table 5) pregnancy rate to first insemination tended to be higher ($P=0.054$); pregnancy rate to all inseminations was higher ($P=0.037$); pregnancy rate as a proportion of all cows was higher ($P=0.021$); and pregnancy rate as a proportion of cows served was higher ($P=0.015$). Survival analysis showed that the successful pregnancy curve for treatment HL was significantly different from the combined curve for the

Table 3 Mean plasma hormones and metabolites over the first 120 days post partum for groups of cows fed on diets that induced high (H) or low (L) insulin before and after the first rise in progesterone post partum^a.

	Treatment group				SED ^b	Contrasts ^a P		
	HH	HL	LH	LL		c1	c2	c3
Insulin (ng/ml)	0.49	0.43	0.44	0.40	0.043	NS	NS	0.039
Glucagon (pg/ml)	99.9	93.2	94.5	88.7	6.07	NS	NS	NS
Insulin to glucagon ratio (ng/ng)	5.18	4.98	5.04	4.76	0.63	NS	NS	NS
GH (ng/ml)	4.92	3.98	4.10	4.30	0.677	NS	NS	NS
IGF1 (ng/ml)	134	137	134	113	15.6	NS	NS	NS
Leptin (ng/ml)	1.76	1.88	1.81	1.49	0.324	NS	NS	NS
Glucose (mmol/l)	3.59	3.51	3.39	3.61	0.121	NS	0.015	NS
β -hydroxybutyrate (mmol/l)	0.50	0.63	0.65	0.68	0.067	NS	NS	0.011
Non-esterified fatty acids (mmol/l)	0.33	0.36	0.34	0.44	0.051	NS	NS	NS
Urea-N (mmol/l)	2.82	2.51	2.72	2.33	0.176	NS	NS	0.004

^aOrthogonal contrasts used in ANOVA were: c1 (HL versus HH+LH+LL); c2 (LH versus HH+LL); c3 (HH versus LL). ^bStandard error of the difference for comparing treatment group means.

other three treatments (Log-rank 5.01, $P=0.025$), indicating an increased pregnancy rate for HL across time as well as across groups; Fig. 3 shows that the survival curve for HL started to diverge from the other curves at 78 days post partum. Cows in group HL had significantly shorter calving intervals than those in the other three groups ($P=0.047$).

There was no effect of vaginal mucus score, or veterinary treatment for uterine infection, on proportions of cows with abnormal cycles, days to first progesterone rise, days to insemination or days to conception (data not shown). Cows with a vaginal mucus score of 0 had a significantly higher ($P=0.01$) pregnancy rate (52%) than those with scores of 1 to 3 (19%), but there was no effect of veterinary treatment for uterine infection on pregnancy rate.

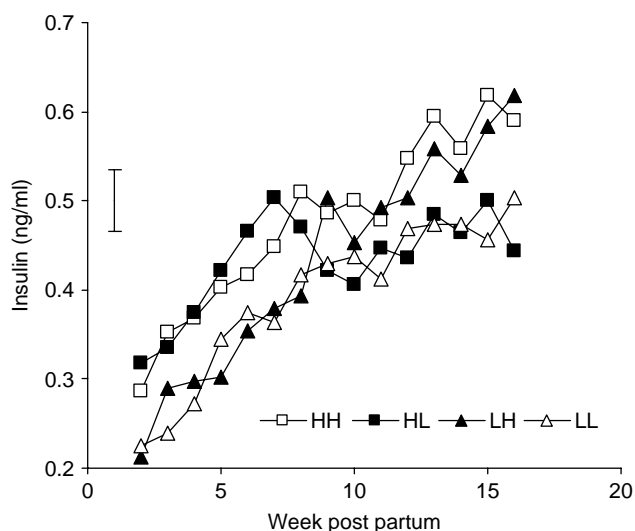


Figure 2 Plasma insulin for groups of cows ($n=15$) offered diets designed to induce high insulin (H) or low insulin (L) before and after the first rise in progesterone post partum. Bar shows $2 \times$ SED for comparing treatment means. Effects of group \times week interaction, $P=0.028$; HH versus LL, $P=0.039$.

Abnormal oestrous cycles affected pregnancy rate at 120 days post partum; for cows exhibiting abnormal oestrous cycles mean pregnancy rate was 13%, which was lower ($P=0.003$) than the mean rate (49%) for cows with normal oestrous cycles. Presence or absence of a corpus luteum at day 60 did not affect pregnancy rate at 120 days post partum ($P=0.596$).

Discussion

The main hypothesis of this experiment was that the best dietary strategy for fertility would be to feed a high-insulin diet (H) until cows resumed ovarian cyclic activity after parturition and then to feed a low-insulin diet (L) during the mating period. The significant improvement in pregnancy rate achieved with treatment HL indicates that this was indeed the best strategy under the conditions of this experiment. As outlined in the introduction, feeding cows diets designed to increase plasma insulin has been found to have beneficial effects on follicular development, leading to earlier resumption of ovarian cyclic activity (Gong *et al.* 2002a), but can have negative effects on oocyte developmental competence (Fouladi-Nashta *et al.* 2005). High insulin also has adverse effects on nuclear maturation of oocytes after follicle culture (Fouladi-Nashta & Campbell 2006). The results of this experiment support the concept that responses to nutrition can vary at different stages of the reproductive cycle.

A major aim when designing this experiment was to induce differences among treatment groups in plasma insulin, without confounding effects of differences in milk production or energy balance. Results for plasma insulin, milk production and energy balance confirm that this aim was achieved. Pregnancy responses can, therefore, be attributed to direct and indirect dietary influences on the reproductive system.

It is important to note that this experiment was performed under carefully controlled research conditions. Clearly, larger studies will be required to test

Table 4 Follicle numbers and proportion of cows with a corpus luteum (CL) at 60 days post partum in cows fed on diets that induced high (H) or low (L) insulin.

	Diet at scanning		SED/CI ^a	P
	H	L		
Number of cows scanned	19	15		
Follicle numbers				
<5 mm	6.1	3.1	1.07	NS
5–10 mm	4.6	4.3	0.65	NS
>10 mm	2.6	2.3	0.37	NS
All follicles	13.3	9.4	1.24	0.037
Proportion of cows with a CL	0.53 (9/19)	0.87 (13/15)	0.03, 0.50	0.029

^aStandard error of the difference for comparing mean follicle numbers, or 95% confidence interval for difference in proportions of cows with a CL.

the concepts under field conditions with greater numbers of cows before they can be applied in commercial practice.

Intake, production and metabolic responses to dietary treatments

As anticipated, there was no significant difference between treatments in the intake of ME or any nutrient except starch and fat. Despite a greater butterfat content of milk from cows fed on diet L, there was no significant effect of diet on net energy output or energy balance. This is important when considering reproduction responses, because energy balance has been shown to affect many aspects of the reproductive system (Butler 2003, Garnsworthy *et al.* 2008a).

For the two treatment groups that remained on the same diets throughout the experiment, insulin was significantly higher when the high-starch diet (HH) was fed to cows, compared with the high-fat diet (LL), in agreement with previous studies (Gong *et al.* 2002a, Garnsworthy *et al.* 2008a, 2008b). As in the study of

Gong *et al.* (2002a), differences in insulin between high-starch and high-fat diets were maintained throughout the experiment, with no difference in milk yield or energy balance. Insulin concentrations changed rapidly in cows that were transferred from one diet to the other, in accordance with the role of insulin in glucose homeostasis (Bauman 2000).

Plasma glucagon tended to be higher for HH than for LL ($P=0.089$). This is contrary to observations of Garnsworthy *et al.* (2008b), where glucagon decreased with increasing dietary starch concentration. In the current experiment, differences in glucagon were observed only in the first 50 days post partum, whereas the study of Garnsworthy *et al.* (2008b) was conducted between 60 and 70 days post partum. The differential response of glucagon to dietary starch is probably due, therefore, to interactions with insulin, which increased throughout the current study in both HH and LL treatment groups.

The lack of dietary effect on plasma GH and IGF1 is in agreement with previous studies into dietary manipulation of metabolic hormones (Garnsworthy *et al.* 2008b,

Table 5 Reproductive performance for groups of cows fed on diets that induced high (H) or low (L) insulin before and after the first rise in progesterone post partum.

	Treatment group				SED/CI ^b	Contrasts ^a P		
	HH	HL	LH	LL		c1	c2	c3
Days post partum for								
First progesterone rise	32.9	30.3	26.9	26.4	5.36	NS	NS	NS
First oestrus	58.2	53.2	54.1	51.2	7.04	NS	NS	NS
First insemination	77.3	68.1	74.4	77.4	7.62	NS	NS	NS
Conception	81.0	83.0	82.2	65.5	12.15	NS	NS	NS
Subsequent parturition	413	404	418	423	8.03	0.047	NS	NS
Numbers of cows (%)								
Showing oestrus	87 (13/15)	100 (15/15)	100 (15/15)	100 (15/15)	-2, 10	NS	NS	NS
Inseminated	73 (11/15)	87 (13/15)	93 (14/15)	93 (14/15)	-20, 20	NS	NS	NS
Pregnancy rate (%)								
To first insemination	9 (1/11)	38 (5/13)	7 (1/14)	21 (3/14)	10, 68	0.054	NS	NS
To all inseminations before 120 days post partum ^c	23 (4/17)	41 (9/22)	15 (4/27)	18 (4/22)	10, 50	0.037	NS	NS
At 120 days post partum (all cows)	27 (4/15)	60 (9/15)	27 (4/15)	27 (4/15)	7, 46	0.021	NS	NS
At 120 days post partum (inseminated cows)	36 (4/11)	69 (9/13)	29 (4/14)	29 (4/14)	5, 44	0.015	NS	NS

^aOrthogonal contrasts used were: c1 (HL versus HH+LH+LL); c2 (LH versus HH+LL); c3 (HH versus LL). ^bStandard error of the difference for comparing treatment group means for days data, or 95% confidence interval of contrast c1 (benefit of treatment HL) for proportions data. ^cNumber of pregnant cows divided by total number of inseminations per group.

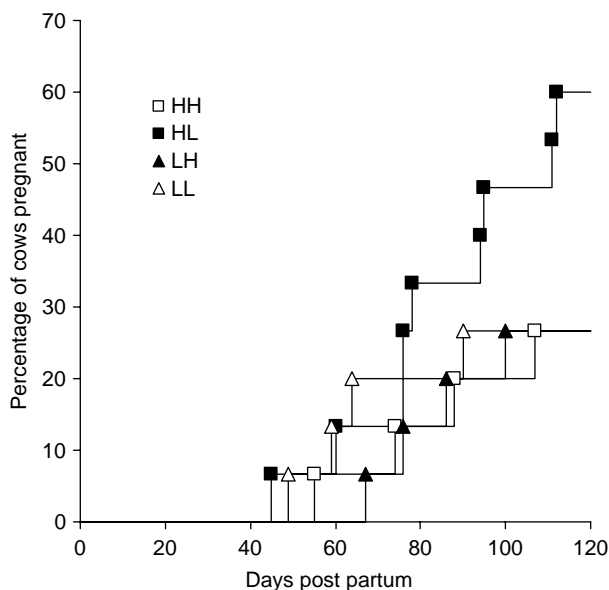


Figure 3 Number of cows pregnant between 0 and 120 days post partum (as a percentage of total cows) for groups of cows ($n=15$) offered diets that induced high insulin (H) or low insulin (L) before and after the first rise in progesterone post partum. The survival curve for HL was higher ($P=0.025$) than curves for the three other treatment groups.

2008c, 2008d) and implies that these hormones are related more to animal factors, such as milk yield, live weight and energy balance, than to diet composition (Lucy 2000). Similarly, leptin is related mainly to animal factors, particularly body fatness, with insulin being involved in regulating leptin responses to nutrition (Armstrong *et al.* 2003).

Of the blood metabolites measured, only glucose, BOHB and urea-N were influenced by dietary treatment. Glucose was lower for LH than for HH + LL, but we have no explanation for this observation because this contrast was far from significance for all measures of intake, milk production, metabolic hormones and other metabolites. Elevated BOHB is associated with NEB (Butler 2003), but there was no effect of diet on energy balance or mobilisation of body fat in this experiment. Differences in BOHB might be due to dietary sugar content, which was however higher for Diet L; sugars encourage production of butyrate in the rumen, which is converted to BOHB in the rumen wall. Plasma urea-N is normally decreased by higher dietary starch concentrations because fermentable starch tends to decrease rumen ammonia concentrations, but the opposite effect was seen here. This might be due to increased gluconeogenesis from amino acids, induced by higher concentrations of glucagon (Garnsworthy *et al.* 2008d).

Reproduction responses

In the study of Gong *et al.* (2002a), a diet that induced higher plasma insulin concentrations in dairy cows increased the proportion of cows that ovulated by

50 days post partum (90 vs 55%). Ovulation was not determined by ultrasound scanning in the current experiment, but 87% of cows fed Diet H before first progesterone rise and 97% of cows fed Diet L showed a rise in progesterone before 50 days post partum, which is considerably greater than the proportions observed by Gong *et al.* (2002a). The most likely explanation for this discrepancy is differences in insulin concentrations between studies. For high- and low-insulin diets respectively, mean insulin concentrations over the first 50 days post partum were 0.32 and 0.21 ng/ml in the study of Gong *et al.* (2002a), and 0.40 and 0.32 ng/ml in the current experiment. This suggests that there is a minimum insulin concentration (between 0.21 and 0.32 ng/ml) necessary for restoration of normal ovarian cycles post partum, which was exceeded for most cows in the current experiment. Gong *et al.* (2002a) noted that all cows exhibited fluctuations in FSH from 3–5 days post partum and follicular waves from 7–10 days post partum, and suggested that ovulation might be limited in low-insulin cows by functional competence of the dominant follicle or inadequate luteinising hormone secretion.

Cows were not subjected to exogenous hormonal intervention to synchronise ovulation in this study. Therefore, cows scanned at 60 days post partum would have been at different stages of the oestrous cycle. Nevertheless, the greater numbers of follicles observed for cows fed on Diet H at 60 days post partum are consistent with findings from other experiments, where high-insulin diets were found to increase follicle numbers in synchronised animals (Gutierrez *et al.* 1997a, Gong *et al.* 2002b, Garnsworthy *et al.* 2008b). Greater numbers of small follicles are associated with lower FSH concentrations, independently of circulating oestradiol, inhibin or IGF1 concentrations (Burns *et al.* 2005). It has been demonstrated that declining FSH and increasing luteinising hormone are associated with the differentiation and maturation of dominant follicles (Webb *et al.* 2004, 2007), thereby increasing the chance of ovulation in response to a luteinising hormone surge (Beam & Butler 1998, 1999). Differentiation and maturation of dominant follicles are controlled by local ovarian growth factors that affect their response to luteinising hormone (see Webb *et al.* 2007). An insulin-stimulated increase in follicle numbers could increase the strength of local signals, thereby leading to better functional competence of dominant follicles. Although, in the current study, there was no apparent difference in timing of the first ovulation post partum, the difference in pregnancy rate between HL and LL suggests that exposure of follicles to high insulin during early development might be beneficial.

The ovulatory follicle develops over a period of ~3 months, but the final processes of selection, dominance and ovulation occur over a period of 20–40 days (Webb *et al.* 2004). Differences in oocyte developmental competence have been detected after dietary treatment

periods of only 14 days in lactating dairy cows (Fouladi-Nashta *et al.* 2005, 2007) and 25 days in heifers (Adamiak *et al.* 2005). Therefore, this supports the concept that both long-term and short-term nutritional milieu might influence both follicle growth and oocyte quality.

As mentioned previously, although diets designed to stimulate plasma insulin secretion can be beneficial for resumption of oestrous cycles post partum, they can also have negative effects on blastocyst rate (Adamiak *et al.* 2005, Fouladi-Nashta *et al.* 2005). This suggests that responses to nutritionally-induced differences in plasma insulin vary at different stages of the reproductive cycle, and at different stages of follicle development, which could explain the dietary effects on pregnancy rate observed in the current study. Evidence from heifers indicates that higher concentrations of circulating insulin and IGF1 stimulate follicular growth and reduce oocyte developmental competence through direct actions on the local binding proteins and receptors of the ovarian IGF system (Lucy 2000, Armstrong *et al.* 2001). However, additional factors that might have contributed to the enhanced pregnancy rate for HL in the current study include the beneficial effects of dietary fatty acids on oocyte developmental competence (Fouladi-Nashta *et al.* 2007) and progesterone secretion (Staples & Thatcher 2005, Garnsworthy *et al.* 2008b). Boland *et al.* (2001) reviewed nutritional effects on reproductive function across species and found consistent evidence that high dietary intake is beneficial for follicle development but reduces developmental capacity of embryos.

Health and reproduction

The overall incidence of abnormal progesterone profiles (38%) is similar to the incidence (44%) observed by Royal *et al.* (2000) in a database of 714 cows. The reduction in pregnancy rate for cows exhibiting abnormal cycles (13 vs 49%) emphasises the need to encourage normal cyclicity.

Based on vaginal mucus score, the incidence of endometritis (40%) was slightly higher than the incidence of 31% reported by Williams *et al.* (2005), although the number requiring veterinary intervention (8%) was considerably lower. The reduction in pregnancy rate for cows with vaginal mucus scores of 1–3 compared with score 0 (19 vs 52%) emphasises the need to reduce the incidence of uterine infections in dairy cows.

Conclusions

The results of this study support the original hypothesis that pregnancy rate will be enhanced by feeding a diet that stimulates higher plasma insulin until cows resume ovarian cycles and then switching to a diet that lowers

plasma insulin and increases fatty acid supply during the mating period. The theory is that feeding a high-insulin diet post partum will benefit follicular development, and then feeding a low-insulin and high-fat diet during the mating period will benefit oocyte developmental competence and possibly improve progesterone status. Based on previous studies, it was anticipated that the high-insulin inducing diet would lead to a greater proportion of cows exhibiting oestrus by 50 days post partum. This was not observed, however, which indicates that there are likely to be benefits from stimulating follicular development even when time of ovulation is not altered. Previous studies further suggested that feeding a low-insulin inducing diet during the mating period would improve developmental competence of oocytes, which was supported by the improved pregnancy rates observed in cows fed on this diet.

Results of this study support the concept that responses to nutritional manipulation of metabolic hormones vary with stage of the reproductive cycle. This significant finding could partly explain why previous attempts to manipulate metabolic hormones through continuous treatment regimes have had limited success at improving pregnancy rates. The results suggest that by applying this novel concept to design a dietary strategy that induces high insulin followed by low insulin, fertility levels can be enhanced without compromising milk production in high-yielding dairy cows. Further research is required to elucidate the exact mechanisms involved, and to confirm the pregnancy rate effect with large numbers of cows under field conditions.

Materials and Methods

Treatments

Two diets were formulated to have equal concentrations of DM, ME and CP, but to differ in starch, fat and NDF concentrations (Table 1). Diet H was expected to induce relatively high plasma insulin concentrations because of its higher starch and lower fat contents; Diet L was expected to induce relatively low insulin concentrations because of its lower starch and higher fat contents. These diets were equivalent to the high and low insulin diets used by both Gong *et al.* (2002a) and Fouladi-Nashta *et al.* (2005).

Sixty high-yielding multiparous Holstein dairy cows were blocked according to calving date and parity, and were allocated at random to four treatment groups. There was no difference between groups (mean \pm S.E.M.) in parity (2.6 ± 1.33) or BCS at calving (3.4 ± 0.04). Cows in group HL were fed on Diet H from calving until the first rise in milk progesterone and were then fed on Diet L until 120 days post partum. Cows in group LH were fed on Diet L and then Diet H. Cows in group HH were fed on Diet H until 120 days post partum, and cows in group LL were fed on Diet L until 120 days post partum. Milk progesterone was measured twice in a week

(either Monday and Thursday or Tuesday and Friday mornings) and a rise in progesterone was defined as above 3 ng/ml for two consecutive samples.

Feeding and milking

Cows were housed as one group throughout the experiment, and were fed individually via electronic feeders (Roughage Intake Control feeders, Fullwood Ltd, Ellesmere, UK) that recorded feed intake automatically. Cows were milked by an automatic (robotic) milking system (AMS; Merlin, Fullwood Ltd.), which they entered voluntarily and were milked on average 2.65 ± 0.09 times per day. In order to encourage cows to use the AMS, 4 kg fresh weight of each cow's daily concentrate allocation was dispensed automatically in the AMS during milking.

Reproductive management

Cows were artificially inseminated at the first or second oestrus following dietary change (or first progesterone rise for HH and LL), provided they had been on their appropriate diet for at least 20 days. Insemination was repeated at any subsequent oestrus until the end of the experiment at 120 days post partum. Oestrus was detected using a combination of behavioural observations, pedometer activity monitoring and milk progesterone profiles. Milk progesterone was monitored daily from 4 days before expected oestrus until signs of oestrus were detected; monitoring then returned to twice in a week until 4 days before the next expected oestrus (21 days later). Progesterone profiles were used subsequently to classify oestrous cycles as normal or abnormal (DOV1, DOV2, PCL1 or PCL2), following the definitions of Lamming & Darwash (1998).

Uterine involution (uterine horns and cervix <40 mm in diameter) was confirmed for every cow by trans-rectal ultrasound scan at 20 days post partum using an Aloka SSD-500 scanner equipped with a 5-MHz linear array transducer (Aloka Co., Ltd., Tokyo, Japan). Also at 20 days post partum, samples of vaginal mucus were collected into 50 ml clear plastic vials to assess endometritis, following the method of Williams *et al.* (2005). Mucus was assessed for colour, proportion and volume of pus, and a character score was assigned as follows: (0) clear or translucent mucus; (1) mucus containing flecks of white or off-white pus; (2) <50 ml exudate containing $\leq 50\%$ white or off-white mucopurulent material; and (3) >50 ml exudate containing purulent material, usually white or yellow, but occasionally sanguineous.

Cows that had not been inseminated, or were not cycling normally, by 60 days post partum were scanned by trans-rectal ultrasound to record numbers of ovarian follicles and corpora lutea. Pregnancy was confirmed by a veterinary surgeon at a routine monthly visit using rectal palpation between 40 and 72 days post-insemination.

Recording, sampling and analysis

Milk yield, feed intake and pedometer activity were recorded daily throughout the experiment. Live weight and BCS (1 = thin

to 5 = fat) were recorded weekly. Milk samples were taken twice in a week (Monday and Thursday mornings) and analyzed for fat, protein and lactose contents by infrared analysis at the National Milk Records Laboratory, Harrogate, UK, using AOAC reference method No. 972.16 (AOAC 1990), and for progesterone by ELISA (Ridgeway Scientific, Alvington, UK). The reliable reading range of the ELISA was from 1.5 to 10.5 ng/ml. Samples reading <1.5 ng/ml were taken as 1.5 ng/ml; samples reading >10.5 ng/ml were diluted to bring the reading within range. If the coefficient of variation (CV) of duplicate sample readings was >15%, the analysis was repeated. The intra- and inter-assay coefficients of variation were <15 and 6.6% respectively.

Feed samples were taken weekly and pooled on a monthly basis for analysis of DM, CP, NDF, starch, sugars, fat and ME, as detailed in Garnsworthy *et al.* (2008b).

Blood samples were taken every Wednesday at 0930 h for measurement of hormones and metabolites. Blood samples were analyzed for the following hormones (in each case, the reference is followed by mean assay sensitivity, intra-assay CV and inter-assay CV): insulin (Adamiak *et al.* 2005; 0.045 ng/ml, 4.0%, 8.4%), GH (Gong *et al.* 1997; 1.2 ng/ml, 3.9%, 11.3%), IGF1 (Gutierrez *et al.* 1997b; 0.11 ng/ml, 3.8%, 12%), glucagon (kit supplied by Linco Research Inc., St Charles, MO, USA; 40.5 pg/ml, 5.3% 6.2%) and leptin (Adamiak *et al.* 2005; 0.2 ng/ml, 3.9%, 12.2%).

Blood samples were analyzed for the following metabolites on a Bayer opera autoanalyzer (Bayer UK Ltd): urea-N (Bayer kit T01 182356), glucose (Bayer kit T01 183356), BOHB (Randox kit Ranbut RB 1008) and NEFA (Waiko kit NEFA-C); intra- and inter-assay coefficients of variation were <5%.

All instances of ill health and veterinary treatments were recorded. The ultimate fate of each cow (subsequent calving or culling) was recorded to allow calculation of calving interval. For cows that were pregnant when culled, subsequent calving date was estimated as date of successful insemination plus 282 days; cows that were not pregnant when culled were omitted from statistical analysis of calving interval.

Statistical analysis

All data were analyzed using Genstat 10th Edition (Lawes Agricultural Trust, Rothamsted, UK). Orthogonal contrasts (Morris 1999) were used to test specific hypotheses. The main hypothesis of the study (that pregnancy rate will be enhanced by treatment HL compared with the other dietary strategies) was tested by contrast c1, which compared treatment HL with the three other treatments (HL versus HH + LH + LL). The second hypothesis (that pregnancy rate will be reduced by treatment LH) was tested by contrast c2, which compared treatment LH with the two continuous treatments (LH versus HH + LL). Finally, contrast c3 compared the two continuous treatments (HH versus LL). Results are presented as treatment group means (with SED) and probability values for each contrast.

Intake, milk production, hormone and metabolite responses were examined by ANOVA. Data from the first week post partum were excluded from the analysis because of variations in the time taken for individual cows to recover from calving

and adapt to the automatic feeding and milking facilities. Week of lactation (2–17) was included in each model, and data were blocked by cow to allow for repeated measures (i.e. the error term for comparing treatment effects was cow nested within diet). For intake and milk production data, previous lactation yield was used as a covariate to allow for any genetic difference among cows. Data consisting of days (e.g. days to first oestrus), which were not normally distributed, were analyzed by generalised linear models with a Poisson error distribution and a log link function. Data consisting of proportions (e.g. pregnancy rate) were analyzed by generalised linear models with a binomial error distribution and a logit link function.

To examine effects of treatments on time from calving to pregnancy, survival analysis was performed. Cows not pregnant at the end of the experiment were censored at 120 days post partum and Kaplan–Meier estimates of the survivor function were compared for each orthogonal contrast using a Log-rank test.

Data for type of ovarian cycle and incidence of health disorders, both of which consisted of counts, were analyzed by Fisher's Exact Test. Vaginal mucus scores were analyzed by ordinal regression using score as a single response factor, treatment group as the model term and a logit link function. Effects of mucus scores and health disorders on reproduction data were examined by generalised linear models with a Poisson error distribution and a log link function for days, and with a binomial error distribution and logit link function for proportions.

Ultrasound scanning data, obtained at 60 days post partum, were analyzed by generalised linear models with a Poisson error distribution and log link function for follicle counts, and a binomial error distribution and logit link function for proportions of cows exhibiting a corpus luteum. Because some cows on treatments HL and LH had not yet changed diets at 60 days post partum, only the diets (H or L) fed at the time of scanning were compared (cows scanned at 60 days post partum had been fed on their respective diets for at least 10 days; mean 33 days, standard error 2.9 days).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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