Nutrition, Metabolism, and Fertility in Dairy Cows: 1. Dietary Energy Source and Ovarian Function

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ABSTRACT

In previous studies, high plasma insulin was associated with earlier resumption of postpartum estrous cycles in dairy cows. The objective of this experiment was to quantify hormonal and ovarian responses to dietary starch and fat contents. Thirty cows were fed on a standard diet from calving until 40 d in milk (DIM) and then 6 cows were allocated to each of 5 isoenergetic diets containing 231, 183, 159, 135, and 87 g of starch and 39, 42, 43, 45, and 48 g of fat/kg of dry matter (DM) for diets 1 to 5, respectively, until 70 DIM. Estrus was synchronized at 60 DIM. Between 60 and 70 DIM, energy intake, milk yield, and energy balance were similar among diet groups. Plasma insulin-to-glucagon ratio increased with increasing dietary starch and decreasing dietary fat concentrations, reaching a break point at 159 g of starch, 43 g of fat/kg of DM (diets 1 to 5: mean 3.86, 3.78, 3.59, 2.98, 2.06 $\pm$ standard error 0.22). Growth hormone, insulin-like growth factor-I, and leptin did not vary among diets. The greatest dietary starch concentration was associated with elevated plasma urea-N (diets 1 to 5: mean 3.69, 3.01, 2.94, 2.95, 2.75, $\pm$ standard error 0.13 mmol/L, respectively) and delayed postovulatory progesterone increase (progesterone at 3 to 5 d postovulation for diets 1 to 5: mean 2.7, 5.9, 4.2, 5.6, 4.3 $\pm$ standard error 0.9 ng/mL, respectively). The number of small (<5 mm) ovarian follicles was positively related to starch intake ($r = 0.381$) and plasma insulin concentration ($r = 0.402$). It is concluded that to maintain adequate insulin-to-glucagon ratio in cows at the start of the breeding period, dietary starch concentration should be above 160 g/kg of DM and dietary fat below 44 g/kg of DM, and this should have a positive effect on ovarian function.

Key words: insulin-to-glucagon ratio, starch, fatty acid, ovarian function

INTRODUCTION

Large increases in milk yield over the past 30 yr were associated with declining fertility of dairy cows all over the world (Butler, 2003). This decline in fertility was attributed partly to unfavorable 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 strong association between negative energy balance (NEB) in early lactation and length of the postpartum anovulatory period (Garnsworthy et al., 2008). Prolonged periods of NEB were associated with suppression of pulsatile LH secretion, reduced ovarian responsiveness to LH stimulation, and reduced estradiol secretion by the dominant follicle, all of which influenced ovulation of the dominant follicle (Butler, 2003). Mobilization of body fat during NEB increased plasma concentrations of NEFA and BHBA, both of which were associated with reduced fertility (Garnsworthy et al., 2008).

Negative energy balance resulted in loss of BCS as the cow mobilized body fat reserves to support milk production. Greater BCS loss was associated with delayed first ovulation postpartum and reduced conception rate (Butler, 2003). The magnitude and duration of BCS loss was directly related to BCS at calving, because dairy cows adjust their DMI in early lactation to move toward a biological target BCS at around 12 wk postpartum (Garnsworthy and Topps, 1982). A recent review (Garnsworthy, 2007) suggested that biological BCS targets were defined by genetics and have reduced over the past 20 yr; therefore, modern dairy cows are more likely to suffer prolonged NEB.

The most common strategy used to reduce the extent of NEB and BCS loss 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 changes in carbohydrate and fat supplies have implications for rumen function, milk composition, nutrient partition, and metabolic hormones.

Changes in metabolic hormones are pertinent for dairy cow fertility, because they interact with repro-

Received January 18, 2008.

Accepted May 19, 2008.

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ductive hormones that control ovarian function (Webb et al., 2004; Garnsworthy et al., 2008). Selection of dairy cows for high milk production was associated with a longer interval from parturition to first ovulation, high plasma concentrations of GH and BHBA, and low plasma concentrations of glucose and insulin (Gutierrez et al., 2006). In an earlier study, we tested the hypothesis that feeding a high-starch diet to increase circulating insulin concentrations for the first 50 DIM could alleviate the delay in the first ovulation postpartum observed in high genetic merit dairy cows (Gong et al., 2002). The high insulin-inducing diet increased the proportion of cows ovulating within 50 d of calving from 55 to 90% and reduced the interval to first ovulation postpartum from 48 to 34 d. In other studies, we found that developmental competence of oocytes was influenced by high versus low dietary concentrations of starch (Fouladi-Nashta et al., 2005) and fat (Fouladi-Nashta et al., 2007) and that responses were related to changes in metabolic hormones. Although these studies provided proof of principles, comparison of extremes does not allow prediction of responses to intermediate diets.

We have conducted a series of experiments to investigate responses to diets designed to alter metabolic hormones, particularly insulin, in high-yielding dairy cows. The overall objective was to determine hormonal responses to diet at a critical stage of the breeding period (40 to 70 DIM) and to relate these to traits of ovarian function.

The specific objective of this experiment was to investigate responses to isoenergetic diets varying in composition from high starch to high fat in a regression approach. The main hypothesis being tested was that plasma insulin concentration would be influenced by dietary concentrations of starch and fat, which would lead to differences in ovarian function.

MATERIALS AND METHODS

Animals, Management, and Diets

Thirty multiparous Holstein dairy cows were group-housed and individually fed through Calan electronic gates from calving until 70 DIM. Diets were fed as TMR, and daily feed allowances were adjusted for each cow to at least 110% of intake, to ensure ad libitum feeding. Daily feed allowances were offered in 2 portions at 0700 and 1300 h each day. Cows were milked twice daily at approximately 0500 and 1530 h.

Cows were fed a standard TMR (diet 3; Table 1) from calving until 40 DIM and were assigned to 6 blocks of 5 cows on the basis of calving date. Blocks were matched for milk yield recorded during the first 20 DIM. From 38

DIM, cows were transferred at random within blocks to 5 treatment TMR over a period of 2 d. Treatment TMR were fed from 40 to 70 DIM.

The TMR (Table 1) were formulated to be isoenergetic and isonitrogenous, supplying ME and MP requirements (Thomas, 2004) for 45 L/d of milk production. All TMR contained the same proportions of grass silage, corn silage, brewers grains, and concentrates. Two concentrates were formulated: one was designed glucogenic (high starch concentration) and the other ketogenic (high fat and fiber concentrations). Five concentrates were produced by mixing these 2 concentrates in the proportions 1:0, 2:1, 1:1, 1:2, and 0:1. These 5 concentrates were mixed with the forage and brewers grains to produce the 5 TMR.

Feed Analysis

Feed intake was recorded daily, and diet samples were taken for analysis at weekly intervals. Forages were analyzed by near-infrared spectroscopy using Forage Analysis Assurance Group equations to predict nutrient contents. All other feeds were analyzed using AOAC (1990) methods. Dry matter, CP, NDF, starch, sugars, and fat (oil acid hydrolysis; OAH) were determined directly. Metabolizable energy was calculated as $(0.14 \times \text{NCGD} + 0.25 \times \text{OAH})$, where NCGD = neutral cellulase plus gamanase digestibility (FSR, 2005). Effective RDP, digestible RUP, and rumen bypass starch were calculated from laboratory-determined CP and starch values, using degradation rates determined by the nylon bag method (Allison and Garnsworthy, 2002) in 4 rumen-fistulated cows.

Estrous Synchronization

Estrous cycles were synchronized by vaginal insertion of a controlled internal drug release (CIDR) progesterone-releasing device (SmithKline Beecham, Tadworth, Surrey, UK) at 50 DIM, with administration of an injection of 2 mL of the PGF_{2α} analog, cloprostenol (Estrumate; Schering-Plough Animal Health, Welwyn, Garden City, UK), when the CIDR was removed at 60 DIM. Cows were allowed to ovulate without further hormonal intervention.

Ovarian activity was monitored by daily transrectal ultrasound scanning from 61 to 70 DIM, using an Aloka SSD-500 scanner equipped with a 5-MHz linear array transducer (Aloka Co. Ltd., Tokyo, Japan). Day of ovulation (3 to 7 d after CIDR removal) was determined as the day on which a large follicle was no longer present and was confirmed by subsequent progesterone increase. Numbers of small (<5 mm) and medium-sized (5 to 10 mm) follicles were recorded at each scan, as

Table 1. Formulation and chemical composition of isoenergetic treatment diets varying from high starch (1) to high fat (5)

Formulation (g per kg of DM)	Diet				
	1	2	3	4	5
Grass ¹ silage	334	334	334	334	334
Corn ² silage	163	163	163	163	163
Brewers grains	55	55	55	55	55
Wheat	249	177	142	106	35
Fatty acid supplement ³	8	13	15	18	23
Molassed sugar beet pulp	37	99	129	159	220
Soybean meal	78	81	83	85	88
Rapeseed meal	66	68	69	70	72
Minerals and vitamins ⁴	6	6	6	6	6
Dicalcium phosphate	4	4	4	4	4
Total	1,000	1,000	1,000	1,000	1,000
Composition ⁵					
DM (g/kg)	445	445	446	446	447
ME (MJ/kg of DM)	12.2	12.2	12.2	12.2	12.2
NDF (g/kg of DM)	301	317	324	332	347
Starch (g/kg of DM)	231	183	159	135	87
Rumen bypass starch (g/kg of DM)	84	66	58	49	32
Sugars (g/kg of DM)	58	72	79	86	100
Fat ⁶ (g/kg of DM)	39	42	43	45	48
CP (g/kg of DM)	181	181	181	181	181
Effective RDP ⁵ (g/kg of DM)	115	114	113	112	111
Digestible RUP ⁷ (g/kg of DM)	54	55	56	56	57

¹Perennial ryegrass (*Lolium perenne*).²Maize (*Zea mays*).³Megalac (calcium salts of palm fatty acids); Volac International, Royston, Herts., UK.⁴Bibby HiPhos; ABN Ltd., Peterborough, UK: calcium, 18%; phosphorus, 10%; magnesium, 5%; salt, 17%; copper, 2,000 mg/kg; manganese, 5,000 mg/kg; cobalt, 100 mg/kg; zinc, 6,000 mg/kg; iodine, 500 mg/kg; selenium, 25 mg/kg; vitamin A, 400,000 IU/kg; vitamin D₃, 80,000 IU/kg; vitamin E, 1,000 mg/kg.⁵Calculated from laboratory analysis of components.⁶Acid hydrolysis method.⁷Calculated from rumen degradability values determined in situ and laboratory analysis of components.

was the diameter of the largest follicle before ovulation and diameter of the developing corpus luteum after ovulation.

Performance Measurements

Milk yield was recorded daily from calving until 70 DIM. Milk samples were collected at 30, 35, and 60 to 70 DIM and analyzed for fat, protein, and lactose contents by infrared analysis at the National Milk Records Laboratory, Harrogate, Yorkshire, United Kingdom, using AOAC reference method no. 972.16 (AOAC, 1990). Body weight and BCS (1 to 5 scale; Mulvanny, 1977) were recorded weekly from 30 to 70 DIM at 0900 h on Monday of each week.

Blood Sampling and Analysis

Blood samples were collected by jugular venipuncture at 0900 h (2 h after morning feeding), on 2 occasions between 30 and 38 DIM for determination of baseline hormones and metabolites. Additional blood

samples were collected daily at 0900 h from 60 to 70 DIM for determination of daily variation in hormones and metabolites.

Blood samples were analyzed for the following hormones (in each case, the reference is followed by mean assay sensitivity, intraassay CV, and interassay CV): insulin (Adamiak et al., 2005; 0.045 ng/mL, 4.0%, 8.4%), growth hormone (GH; Gutierrez et al., 2006; 1.2 ng/mL, 3.9%, 11.3%), IGF-I (Gutierrez et al., 2006; 0.11 ng/mL, 3.8%, 12%), glucagon (Linco Research Inc., St. Charles, MO; 40.5 pg/mL, 5.3%, 6.2%), leptin (Adamiak et al., 2005; 0.2 ng/mL, 3.9%, 12.2%), estradiol (Mann et al., 1995; 0.27 pg/mL, 10%, 9.7%), and progesterone (Mann et al., 1995; 0.3 ng/mL, 5.7%, 9.4%).

Blood samples were analyzed for the following metabolites on a Bayer opera autoanalyzer (Bayer UK Ltd., Newbury, UK): albumin (Bayer kit T01 137702), total protein (Bayer kit T01 130102), globulin (total protein minus albumin), urea-N (Bayer kit T01 182356), glucose (Bayer kit T01 183356), BHBA (Randox kit Ranbut RB 1008), NEFA (Waiko kit NEFA-C), magnesium (Bayer

Table 2. Least squares means over 60 to 70 DIM, adjusted by covariance for values between 30 and 38 DIM, for intakes, milk production and composition, BW, and BCS in cows (n = 6) given diets varying from high starch (1) to high fat (5)

Item	Diet ¹					SED ³	Contrast ²		
	1	2	3	4	5		Diet	Linear	Quadratic
Intake									
DM (kg/d)	23.3	24.5	23.2	24.4	23.8	1.83	0.927	0.846	0.755
Starch (kg/d)	5.4 ^a	4.5 ^b	3.7 ^c	3.3 ^c	2.1 ^d	0.29	<0.001	<0.001	0.694
Fat (kg/d)	1.0 ^a	1.1 ^{ab}	1.1 ^{ab}	1.2 ^b	1.2 ^b	0.09	0.043	0.007	0.841
Sugars (kg/d)	1.3 ^a	1.7 ^b	1.8 ^{bc}	2.0 ^{cd}	2.3 ^d	0.14	<0.001	<0.001	0.796
NDF (kg/d)	7.1	7.8	7.5	8.1	8.2	0.60	0.340	0.058	0.765
ME (MJ/d)	283	296	280	295	287	22.2	0.928	0.861	0.755
Milk production									
Milk yield (kg/d)	43.7	43.7	41.2	43.6	44.1	1.48	0.113	0.870	0.121
Milk fat (g/kg)	36.2 ^a	37.9 ^{ab}	40.4 ^{abc}	43.0 ^c	40.7 ^{abc}	2.25	0.049	0.015	0.182
(g/d)	1,573	1,685	1,675	1,850	1,766	142.1	0.389	0.110	0.513
Milk protein (g/kg)	30.0	29.8	30.9	29.2	28.5	0.82	0.088	0.078	0.157
(g/d)	1,308	1,292	1,268	1,257	1,256	47.6	0.758	0.215	0.733
Milk lactose (g/kg)	47.4	47.3	46.4	47.0	47.3	0.39	0.080	0.560	0.061
(g/d)	2,086	2,074	1,876	2,023	2,081	69.2	0.068	0.736	0.310
Energy balance (MJ/d)	-0.1	4.4	3.1	-6.2	0.7	3.75	0.077	0.491	0.869
Weight and condition									
BW (kg)	611	607	613	612	618	8.1	0.742	0.293	0.485
BW change (kg/d)	-0.01	0.10	0.08	-0.13	0.05	0.144	0.515	0.343	0.286
BCS	2.6	2.4	2.5	2.4	2.5	0.19	0.669	0.794	0.199
BCS change	0.09	-0.15	-0.04	-0.13	0.02	0.180	0.670	0.791	0.195

^{a-d}Means in the same row without a common superscript differ ($P < 0.05$).

¹Diets 1 to 5 contained 231, 183, 159, 135, and 87 g of starch and 39, 42, 43, 45, and 48 g of fat/kg of DM, respectively.

²Significance of diet effect and linear and quadratic effects of dietary starch concentration.

³SED = standard error of the difference between treatment means.

kit T01 287802), and inorganic phosphorus (Bayer kit T01 130302); intra- and interassay CV were <5%.

Statistical Analyses

Metabolizable energy balance was calculated for each cow from BW, milk energy output, and metabolizable energy intake, using UPWin software (AGM Systems, Exeter, UK) and Feed Into Milk equations (Thomas, 2004). Dietary effects on mean performance and blood data during the treatment period (60 to 70 DIM) were examined by analysis of covariance, using Genstat 8 (Lawes Agricultural Trust, Rothamsted, UK), looking at the main effect of dietary treatment group, with observations during the standardization period (30 to 38 DIM) as covariates. When the main effect was significant, differences among treatment means were tested by least significant difference. Linear and quadratic effects of dietary starch concentration were examined. Simple correlation coefficients were determined to examine relationships among variables. The all-subsets regression procedure (Genstat 8, Lawes Agricultural Trust) was used to identify significant multiple relationships among variables, and coefficients for these were determined by multiple linear regression.

Reproductive data, which were not normally distributed, were analyzed by using generalized linear models

with a Poisson error distribution and log link function. For progesterone, estradiol, ovulatory follicle diameter, and corpus luteum diameter, day of ovulation was included as a covariate.

RESULTS

The responses are analyzed and reported as linear and quadratic effects of dietary starch concentration, but they can be interpreted as effects of dietary fat, sugars, or fiber concentrations, all of which changed in a linear fashion across diets.

Intake, Milk Production, and Body Weight

Intakes of DM, NDF, and ME did not vary among treatment groups (Table 2). Starch intake decreased ($P < 0.001$), whereas intakes of sugars and fat increased ($P < 0.001$), with decreasing dietary starch concentration and increasing sugars and fat concentrations.

Milk yield did not vary among treatment groups (Table 2). Milk fat concentration varied among treatment groups ($P < 0.05$) and was linearly related ($P < 0.05$) to dietary starch (negative) and fat (positive) concentrations. Concentrations of milk protein and lactose, and yields of milk fat, protein, and lactose, did not vary among treatment groups. In addition, energy balance,

Table 3. Least squares means over 60 to 70 DIM, adjusted by covariance for values between 30 and 38 DIM, for plasma concentrations of metabolic hormones and metabolites in cows (n = 6) given diets varying from high starch (1) to high fat (5)

Item	Diet ¹					SED ³	Contrasts ²		
	1	2	3	4	5		Diet	Linear	Quadratic
Insulin (ng/mL)	0.40 ^a	0.39 ^a	0.38 ^{ab}	0.34 ^b	0.25 ^c	0.031	<0.001	<0.001	0.019
Glucagon (pg/mL)	102 ^a	108 ^a	108 ^a	115 ^b	119 ^b	5.6	0.037	0.002	0.833
Insulin:glucagon ratio	3.86 ^a	3.78 ^a	3.59 ^a	2.98 ^b	2.06 ^c	0.315	<0.001	<0.001	0.018
IGF-I (ng/mL)	110	71	116	88	102	21.3	0.256	0.921	0.261
Growth hormone (ng/mL)	7.96	7.26	7.86	7.28	7.01	1.474	0.957	0.548	0.989
Leptin (ng/mL)	1.27	1.29	1.43	1.04	1.16	0.174	0.274	0.327	0.609
Albumin (g/L)	34.8	34.4	37.1	36.3	37.4	1.65	0.302	0.082	0.980
Globulin (g/L)	36.3	35.3	36.4	35.1	37.7	1.79	0.616	0.495	0.255
Total protein (g/L)	71	70	73	72	75	3.01	0.446	0.166	0.382
Urea-N (mmol/L)	3.69 ^a	3.01 ^b	2.94 ^b	2.95 ^b	2.75 ^b	0.186	<0.001	<0.001	0.033
BHBA (mmol/L)	0.67	0.70	0.78	1.24	1.15	0.280	0.136	0.027	0.980
NEFA (mmol/L)	0.19 ^a	0.18 ^a	0.19 ^a	0.28 ^b	0.32 ^b	0.039	0.004	<0.001	0.149
Glucose (mmol/L)	3.4	3.6	3.6	3.5	3.4	0.15	0.280	0.672	0.046
Phosphorus (mmol/L)	1.42	1.51	1.55	1.41	1.51	0.120	0.748	0.729	0.759
Magnesium (mmol/L)	1.00	0.90	0.94	0.99	0.97	0.037	0.083	0.769	0.114

^{a-c}Means in the same row without a common superscript differ ($P < 0.05$).

¹Diets 1 to 5 contained 231, 183, 159, 135, and 87 g of starch and 39, 42, 43, 45, and 48 g of fat/kg DM, respectively.

²Significance of diet effect and linear and quadratic effects of dietary starch concentration.

³SED = standard error of the difference between treatment means.

BW, BW change, BCS, and change in BCS did not vary among treatment groups (Table 2).

Metabolic Hormones and Metabolites

Mean plasma insulin and glucagon concentrations, and their ratio, varied ($P < 0.001$) among treatment groups (Table 3), with significant linear ($P < 0.001$) and quadratic ($P < 0.05$) effects of dietary starch concentration. Effects of dietary starch concentration were positive for insulin, negative for glucagon, and positive for the insulin-to-glucagon ratio. For IGF-I, GH, and leptin, there was no difference between treatment groups and no linear or quadratic effect of dietary starch concentration.

There was no difference between treatment groups, and no effect of dietary starch concentration, for plasma concentrations of albumin, globulin, total protein, phosphorus, or magnesium (Table 3). Plasma urea-N concentration was greater for group 1 (greatest starch) than for any other group; therefore, the overall dietary effect was significant ($P < 0.001$) and significant positive linear ($P < 0.001$) and quadratic ($P < 0.05$) effects of dietary starch concentration were observed. There was no difference among treatment groups for BHBA, although there was a linear ($P < 0.05$) increase in BHBA with decreasing dietary starch concentration. Plasma NEFA concentrations were lower ($P < 0.01$) in diets 1 to 3 than in diets 4 and 5, and the negative linear effect of dietary starch concentration was significant ($P < 0.001$). There was no overall difference among

treatment groups for plasma glucose, but diet groups 1 and 5 had lower glucose concentrations than the other groups, and the quadratic effect of dietary starch concentration was significant ($P < 0.05$).

Ovarian Activity

Ovulation was detected by ultrasound scanning in 28 of the 30 cows. For 1 cow in diet group 1 and 1 cow in diet group 3, scanning was not possible due to behavioral problems, and ovulation was confirmed by assessment of estradiol and progesterone concentrations. Cows showed a normal pattern of ovulation with a mean ($\pm$ SE) day of ovulation of 4.3 ± 0.2 (range 3 to 7 d) after CIDR removal.

The maximum number of small (<5 mm) follicles during the preovulatory follicular wave was greater in diet groups 1 and 2 (high starch) than in diet groups 4 and 5 (high fat), and the overall treatment effect was significant ($P < 0.001$; Table 4). Similar effects ($P < 0.01$) on the maximum number of small follicles were seen in the postovulatory follicular wave.

The maximum number of medium-sized (5 to 10 mm) follicles during the postovulatory follicular wave (Table 4) was lower ($P < 0.05$) in diet groups 1 and 2 (high starch and low fat) than in diet group 3; the maximum number of medium-sized follicles for diet groups 4 and 5 (low starch and high fat) was not different from groups 1 to 3. In addition, there was no difference among treatment groups in maximum diameter of the ovulatory follicle (Table 4), but the corpus luteum that

Table 4. Treatment means¹ relative to a synchronized estrus at around 60 DIM for plasma concentrations of estradiol before ovulation and progesterone from d 3 to 5 postovulation, as well as follicle numbers, ovulatory follicle diameter, and corpus luteum (CL) diameter in cows (n = 6) given diets varying from high starch (1) to high fat (5)

Item	Diet ²					SED ³	P-value ⁴
	1	2	3	4	5		
Progesterone (ng/mL)	2.7 ^a	5.9 ^b	4.2 ^{ab}	5.6 ^b	4.3 ^{ab}	1.26	0.043
Estradiol (pg/mL)	1.3	1.4	1.1	1.2	1.3	0.26	0.988
Maximum small (<5 mm) follicles preovulation	13.0 ^a	15.7 ^a	11.8 ^{ab}	8.0 ^b	8.7 ^b	2.03	<0.001
Maximum small (<5 mm) follicles postovulation	14.2 ^{ab}	17.2 ^a	11.8 ^b	9.5 ^c	11.5 ^{bc}	2.14	0.004
Maximum medium-sized (5 to 10 mm) follicles postovulation	2.6 ^a	3.5 ^a	6.0 ^b	4.2 ^{ab}	4.8 ^{ab}	1.23	0.044
Ovulatory follicle diameter (mm)	19	26	24	23	22	2.7	0.109
CL diameter ⁵ (mm)	26 ^a	32 ^b	26 ^a	29 ^{ab}	28 ^{ab}	3.1	0.041

^{a-c}Means in the same row without a common superscript differ ($P < 0.05$).

¹Values are back-transformed from generalized linear models with Poisson error distribution and log link function. For progesterone, estradiol, ovulatory follicle diameter, and CL diameter, day of ovulation was included as a covariate.

²Diets 1 to 5 contained 231, 183, 159, 135, and 87 g of starch and 39, 42, 43, 45, and 48 g of fat/kg of DM, respectively.

³SED = standard error of the difference between treatment means.

⁴P = probability of main treatment effect (diet) on each variable.

⁵On d 5 after ovulation.

developed postovulation was larger for diet group 2 than for diet groups 1 and 3.

After ovulation, cows in diet group 1 (high starch) showed a delayed postovulatory increase in progesterone compared with the other 4 groups (Figure 1). This late progesterone increase resulted in lower mean progesterone concentrations from d 3 to d 5 postovulation for diet group 1 than for diet groups 2 and 4 (Table 4). Plasma concentration of estradiol did not differ among dietary groups on any day of the study, and there was no difference among groups in plasma estradiol measured during the preovulatory phase (d -2 to 0; Table 4).

Relationships Among Variables (Across Individuals)

Plasma insulin was positively correlated with starch intake ($r = 0.56$), energy balance ($r = 0.40$), milk protein content ($r = 0.50$), and plasma glucose concentration ($r = 0.45$). Insulin was negatively correlated with plasma BHBA ($r = -0.39$) and plasma NEFA ($r = -0.58$). Plasma IGF-I was negatively correlated with intakes of DM ($r = -0.43$) and fat ($r = -0.37$), and with milk yield ($r = -0.38$), milk fat concentration ($r = -0.40$), and milk fat yield ($r = -0.53$). Plasma leptin was negatively correlated with intakes of DM ($r = -0.44$) and fat ($r = -0.41$) and with yields of milk ($r = -0.50$), fat ($r = -0.45$), protein ($r = -0.39$), and lactose ($r = -0.48$). Insulin-like growth factor-I was positively correlated with plasma leptin ($r = 0.47$). None of the traits measured were correlated with glucagon or GH.

The maximum number of small (<5 mm) ovarian follicles during the follicular wave preceding ovulation was positively related to insulin, starch intake, and

milk yield and negatively related to milk fat and protein concentrations (Table 5). Apart from milk yield, similar relationships were observed during the first follicular wave postovulation.

The best multiple regression equation for predicting plasma insulin concentration included positive effects of starch intake and plasma glucose and a negative effect of milk yield (Table 6). This equation accounted for 53% of variation in plasma insulin. The best multiple regression equation for predicting plasma IGF-I concentration included positive effects of BCS change and BW and a negative effect of DMI. This equation accounted for 44% of variation in plasma IGF-I. The best regression equation for predicting plasma leptin concentration included only a negative effect of milk yield. This equation accounted for 23% of variation in plasma leptin.

DISCUSSION

The design of the experiment allows examination of responses to isoenergetic diets varying widely in composition across the range found in normal commercial practice. An advantage of providing stepped changes in energy sources from high starch to high fat is that intermediate responses can indicate thresholds. Dry matter intake was not affected by diet; therefore, responses can be attributed to changing nutrients rather than energy intake. At the individual cow level, DMI was correlated with milk yield ($r = 0.76$; $P < 0.001$), indicating that cows were eating to meet their physiological requirements for energy and nutrients.

The main hypothesis was that plasma insulin concentration would be influenced by dietary concentrations

Table 5. Effects¹ of insulin and production traits on numbers of ovarian follicles <5 mm before and after a synchronized ovulation at around 60 DIM

Trait	Preovulation						Postovulation					
	Constant	SE	Slope	SE	RSD ²	P-value	Constant	SE	Slope	SE	RSD	P-value
Insulin (ng/mL)	1.27	0.275	3.301	0.739	2.10	<0.001	1.88	0.251	1.933	0.690	1.86	0.005
Starch intake (kg/d)	1.63	0.206	0.215	0.051	2.12	<0.001	1.98	0.190	0.156	0.048	1.81	0.001
Milk yield (kg/d)	1.45	0.482	0.023	0.011	2.25	0.040						
Milk fat (g/kg)	3.74	0.488	-0.033	0.012	2.22	0.007	3.45	0.459	-0.023	0.012	1.87	0.049
Milk protein (g/kg)	5.59	1.11	-0.107	0.038	2.21	0.004	6.03	1.050	-0.118	0.036	1.80	0.007

¹Values are regression equations from generalized linear models with Poisson error distribution and log link function, fitted to individual cow means. Each equation predicts log of maximum follicle numbers from the relevant trait; for example, preovulation, $\log[\text{follicles}] = 1.45 + (\text{milk yield} \times 0.023)$; so if milk yield = 40 kg/d, predicted maximum small follicles = 10.7.

²Residual standard deviation.

of starch and fat. This hypothesis was supported by the differences in insulin observed among treatments and the relationships between insulin and starch concentration ($r = 0.45$), fat concentration ($r = -0.37$), and starch intake ($r = 0.56$). Insulin plays a central role in metabolism by stimulating utilization of glucose in peripheral tissues such as muscle and adipose tis-

sue and by promoting accumulation of glycogen and lipid reserves. Insulin was increased by diets with high starch content (Reynolds, 2006) and was decreased by diets with high fat content (Choi and Palmquist, 1996), although increased insulin concentrations were found when supplementary fat increased energy intake (Palmquist and Moser, 1981).

Figure 1. Mean plasma concentrations of progesterone in groups ($n = 6$ per group) of lactating Holstein-Friesian cows fed diets varying from high starch (1) to high fat (5). Cycles were synchronized and data then aligned to day of ovulation. Diets 1 to 5 contained 231, 183, 159, 135, and 87 g of starch and 39, 42, 43, 45, and 48 g of fat/kg of DM, respectively. ^aMean progesterone was lower ($P = 0.039$) for diet 1 than for the other diets on d 5.

Table 6. Best regression equations to predict metabolic hormones from intake, milk production, and blood parameters

Item	Constant	Slope 1	Slope 2	Slope 3	RSD ¹	% VAR ²
Insulin (ng/mL)						
Starch intake (kg/d) + milk yield (kg/d) + plasma glucose (mmol/L)	−0.16	0.046	−0.004	0.14	0.054	53.4
IGF-I (ng/mL)						
Condition score change + BW (kg) + DMI (kg/d)	136.1	55.3	0.23	−7.34	27.31	43.5
Growth hormone (ng/mL)						
BW (kg) + DMI (kg/d)	10.7	−0.021	0.396		2.25	10.5
Leptin (ng/mL)						
Milk yield (kg/d)	2.93	−0.039			0.35	22.6

¹Residual standard deviation.²Percentage of variation accounted for by regression equation.

The curvilinear response of insulin to dietary starch concentration suggests that insulin secretion was reduced at low starch concentrations and reached a break point at approximately 160 g of starch/kg of DM and 43 g of fat/kg of DM. The high-merit cows used in our previous study (Gong et al., 2002) had insulin concentrations of 0.21 ng/mL on the low-insulin diet and 0.32 ng/mL on the high-insulin diet over the first 50 DIM; that difference was similar to the difference between equivalent diets (5 and 1) in the current experiment.

Glucagon plays a complementary role to insulin in metabolism; it stimulates mobilization of glycogen and lipid reserves, increases the rate of gluconeogenesis, and promotes a switch from the use of glucose to the use of fatty acids as a source of energy. Diets rich in carbohydrates decrease the concentration of glucagon in the blood, whereas fasting, or feeding low-carbohydrate diets, has the opposite effect. Although glucagon increased linearly with decreasing dietary starch concentration, the difference between diets did not reach significance until dietary starch concentration was below 160 g/kg of DM.

Due to the opposing effects of insulin and glucagon on plasma glucose, insulin-to-glucagon ratio could be more important than absolute levels of each hormone. The ratio started to decrease when starch concentration decreased below 160 g/kg of DM. Low insulin-to-glucagon ratio could indicate compromised gluconeogenesis from propionate and amino acids and may explain some of the reproductive effects seen with low-insulin diets (Gong et al., 2002).

In the current experiment, insulin was positively related to the maximum number of small follicles in follicular waves before and after ovulation, in agreement with the observations of Gutierrez et al. (1997). Greater numbers of small follicles were associated with lower FSH concentrations, independently of circulating estradiol, inhibin, or IGF-I concentrations (Burns et al., 2005). Declining FSH, accompanied by increasing LH pulses, aids the differentiation and maturation of dominant follicles during early lactation, thereby

increasing the chance of ovulation in response to an LH surge (Webb et al., 2004). The increased number of medium-sized follicles for diet 3, compared with diets 1 and 2, suggests that increasing dietary fat concentration stimulated growth of follicles. Importantly, insulin effects in the current experiment were independent of energy balance, whereas in other studies, low insulin accompanied NEB (Butler, 2003).

As with insulin, nutritionally induced changes in systemic IGF-I concentrations have been linked to ovarian activity (Webb et al., 2004). In the current experiment, IGF-I was negatively related to DMI but did not vary among diets and was not related to plasma insulin or GH concentrations. The endocrine role for IGF-I in controlling ovarian activity cannot be viewed in isolation and must be combined with factors affecting its expression in the liver along with nutritionally induced changes in both the serum IGF binding protein profile and IGF binding protein expression in the dominant follicle (Webb et al., 2004). The results of this experiment provide further support for the suggestion that GH does not appear to be directly involved in the physiological mechanisms underlying the nutritional influence on ovarian function in cattle (Webb et al., 2004), although administration of exogenous GH does influence follicular populations (Gong et al., 1991).

Leptin appears to act as a further signal linking nutritional status with reproductive performance (Keisler et al., 1999) and is related to BCS in lactating cows and level of feeding in nonlactating cows (Armstrong et al., 2003). It appears that both insulin and IGF-I are involved in regulating leptin responses to nutrition. In the current study, the lack of variation in leptin among diets is consistent with the lack of variation in BCS and DMI, although the relationship between leptin and DMI ($r = -0.44$) across individual cows is consistent with the role of leptin in regulation of feed intake.

Mean progesterone concentration during the post-ovulatory increase was 40% lower in the greatest starch (lowest fat) group than in the other 4 groups. During this time, progesterone plays a critical role in

supporting uterine function, and numerous studies have linked low progesterone secretion at this time to reduced embryo development and early embryo loss (Mann and Lamming, 2001). Interestingly, cows on diet 2 numerically had the greatest mean progesterone concentration and also the largest mean corpus luteum diameter, both of which were significantly different from diet 1, suggesting that a critical threshold for fatty acid supply had been met. Thus, high dietary starch (>183 g/kg of DM) and low dietary fat (<42 g/kg of DM) concentrations might be detrimental to pregnancy rate by reducing progesterone. Further support for this proposition comes from studies of developmental competence of oocytes. Fouladi-Nashta et al. (2005) found that cows fed a high-starch diet (equivalent to diet 2) produced oocytes that had a significantly lower proportion developing to the blastocyst stage after *in vitro* fertilization than cows fed on a high-fiber diet (equivalent to diet 5). In a later study (Fouladi-Nashta et al., 2007), a high-fat diet produced significantly greater blastocyst yields than a low-fat diet.

Diet composition had no effect on plasma metabolites except BHBA, NEFA, and urea-N. There was a linear increase in BHBA with decreasing dietary starch concentration. Grohn et al. (1983) suggested BHBA levels of <1 mmol/L represented healthy cows, whereas levels of 1 to 3 mmol/L were mildly ketotic cows. Thus, cows on diets 4 and 5 might have been mildly ketotic, but cows on diets 1 to 3 were healthy.

Elevated plasma NEFA concentrations were associated with NEB in early lactation and were linked to reduced reproductive performance (Butler, 2003; Garnsworthy et al., 2008). There was no difference among treatments in energy balance, which was generally positive; therefore, the positive relationship between NEFA and dietary fat concentration suggests that the greater NEFA values seen for diets 4 and 5 resulted from the low insulin-to-glucagon ratios observed in these groups.

The positive relationship between urea-N and dietary starch concentration is unusual; normally high-starch diets improve rumen ammonia capture and decrease plasma urea-N concentrations (Reynolds, 2006). The inverse effect could be due to the use of molassed sugar beet pulp as a fiber source, which reduced plasma urea concentrations (Kenny et al., 2002). The high moisture absorbency of beet pulp, approximately 20× its weight in water, could trap ammonia in solution within the rumen, improving its utilization for microbial protein synthesis. The increases in corpus luteum diameter and progesterone concentration for diet 2 compared with diet 1 could be linked to increased intakes of starch and sugars, or reduced plasma urea-N, the only variables that were significantly different between

these diets. Yet, plasma urea-N concentrations were below the standard range (3.3 to 5 mmol/L) reported by Ward et al. (1995) in all but 9 cows. The implications of low plasma urea-N concentrations are unknown. High plasma ammonia and urea levels were associated with elevated progesterone concentrations, reduced dominant follicle growth, and reduced cleavage rate of oocytes from medium-sized follicles (Sinclair et al., 2000), but whether the relative changes in urea-N found in the current study are important in this respect remains to be tested.

CONCLUSIONS

The results of this experiment demonstrate that insulin and glucagon can be changed by diet in modern high-yielding Holstein dairy cows and provide quantitative data that can be used to determine critical thresholds. The critical threshold for maintaining adequate insulin-to-glucagon ratios in cows at the start of the breeding period appears above 160 g of starch/kg of DM and below 44 g of fat/kg of DM. Although there is less direct evidence from this experiment, associated studies suggest that the greatest starch diet (231 g of starch/kg of DM) could cause problems from low progesterone or impaired developmental competence of oocytes. Further evidence is provided to support the role of insulin as a metabolic signal influencing ovarian function, because nutritionally induced changes in insulin were associated with changes in follicle dynamics.

ACKNOWLEDGMENTS

This study was part of Project LK0646 in the LINK Sustainable Livestock Production program, which was funded by The Scottish Executive Environment and Rural Affairs Department (SEERAD, Edinburgh, UK), ABNA Ltd. (Peterborough, UK), BOCM PAULS Ltd. (Ipswich, UK), and Provimi Ltd. (Sint-Stevens-Woluwe, Belgium). The following people served on the Programme Management Committee and made valuable contributions to the design and interpretation of the experiment: J. Newbold (Provimi), M. Marsden (ABNA), S. Richards (Provimi), A. Henderson (BOCM PAULS), P. Thomas (Artillus Ltd., UK), D. Armstrong (Roslin Institute, Edinburgh, UK), A. Flint (Univ. Nottingham, UK), D. Garwes (Department for Environment, Food and Rural Affairs), and D. Leaver (Royal Agricultural College). We would like to thank the following people for technical assistance: H. Russell (Univ. Nottingham), J. Gong (Roslin Inst.), G. Baxter (Roslin Inst.), M. Mitchell (Univ. Nottingham), N. Armstrong (Univ. Nottingham), D. Scholey (Univ. Nottingham),

M. Hunter (Univ. Nottingham), D. Whitaker (Univ. Edinburgh), and C. Smith (National Milk Records). Statistical advice was provided by J. Craigon (Univ. Nottingham).

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