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Effects of different patterns of feed restriction and insulin treatment during the luteal phase on reproductive, metabolic, and endocrine parameters in cyclic gilts[1,2]

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ABSTRACT: The objectives of the present study were 1) to study potential effects of previous nutritional treatment on developmental competence of early fertilized oocytes in vitro; 2) to study responses to insulin treatment during the period of feed restriction in the late luteal phase which has deleterious effects on subsequent fertility; and 3) to establish the metabolic and endocrine status of gilts during treatment and the subsequent periestrous period. Nineteen trios of littermate gilts were subjected to feed restriction during the first (RH) or second (HR) week of the estrous cycle. A second group of HR gilts received injections of long-acting insulin during their period of feed restriction (HR+I). Intensive sampling was performed in a subgroup of 23 animals on d 15 and 16 of the cycle for analyses of endocrine (gonadotropins and steroid hormones) and metabolic (insulin, IGF-I, leptin, total triiodothyronine [T3], and free T3) variables. Gilts were checked for estrus every 6 h, and time of ovulation was monitored by transcutaneous ultrasonography. Surgeries were performed 12 to 20 h after ovulation, and the early-fertilized oocytes recovered were cultured in vitro under standardized conditions. There was no treatment effect on the developmental competence of fertilized oocytes in vitro; however, ovulation rate was increased in HR+I gilts. No effect of treatment was observed on plasma leptin and IGF-I concentrations on d 15 and 16. However, HR+I gilts had higher ($P < 0.05$) postprandial insulin and lower ($P < 0.05$) postprandial total and free T3 on d 15. Plasma concentrations of LH, FSH, and progesterone on d 15 and 16 and plasma estradiol concentrations on d 16 were not affected by previous nutritional or insulin treatment. In the periestrous period, plasma concentrations of LH, FSH, and estradiol were higher ($P < 0.05$) in RH and HR+I, and the rise in plasma progesterone after the LH surge was lower ($P < 0.05$), than in HR gilts. No effect of treatment was observed on plasma concentrations of metabolic hormones, except on plasma leptin concentrations, which were higher ($P < 0.05$) at the time of the LH surge in RH gilts. These results suggest that feed restriction during the late luteal phase may have deleterious effects on ovarian function in the periestrous period, which may be counteracted by insulin.

Key Words: Gilts, Insulin, Nutrition, Reproduction

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Introduction

Changes in metabolic fuel availability and associated endocrine status are important determinants in the signaling of metabolic status to the reproductive axis (I'Anson et al., 1991; Wade and Schneider, 1992). In particular, the metabolic hormones insulin, IGF-I, leptin, and thyroid hormones could be the link between metabolic state and reproductive function (Monget and Martin, 1997; Mounzih et al., 1997; Viguié et al., 1999).

A previous study in our laboratory (Almeida et al., 2000) used short-term changes in feed intake in cyclic gilts to further study the interactions between nutrition and reproduction. In this model, littermate gilts were fed either at a high plane throughout the estrous cycle

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(**HH**), or feed intake was restricted during the first (**RH**) or second (**HR**) week of the cycle. Although pattern of feeding had no effect on ovulation rate, embryonic survival at d 28 of pregnancy in the HR gilts was significantly lower than in RH or HH gilts, and HR gilts also had lower plasma progesterone concentrations at 48 h after onset of estrus. Moreover, in a subsequent study in our laboratory using the same experimental paradigm (Almeida, 2000), different patterns of feed intake during the previous estrous cycle affected fertilization rate of oocytes recovered immediately after ovulation without any detrimental effect on oocyte developmental competence in vitro.

Based on these results, the objectives of the present study were to continue to use this established cyclic gilt model to 1) extend our observations on potential effects of previous nutritional treatment on developmental competence of early fertilized oocytes in vitro; 2) study responses to insulin treatment during the period of feed restriction in the late luteal phase, which was previously shown to have detrimental effects on subsequent fertility; and 3) establish the metabolic and endocrine status of gilts during treatment and the subsequent periestrous period.

Materials and Methods

Animals

The experiment was conducted at the Swine Research Unit of the University of Alberta, in barns with a totally controlled environment, and the selection, pretreatment, and management of nutritional treatments of gilts were performed as in our previous study (Almeida et al., 2000). All gilts were fed a wheat-barley-soybean grower diet, which was nutritionally balanced in terms of amino acids, vitamins, and minerals to meet NRC (1988) requirements. Nineteen sets of littermate gilts (Pig Improvement Canada Ltd, Camborough × Canabrid terminal line) in their second estrous cycle were initially allocated to one of the following treatments: feed restriction to 2.1× energy requirements for maintenance from d 1 to 7 of the cycle and then fed 2.8× maintenance from d 8 until onset of estrus (RH) or fed 2.8× maintenance from d 1 to 7, restricted to 2.1× maintenance from d 8 to 15, and then refed at 2.8× maintenance from d 16 until onset of estrus, with (**HR+I**) or without (HR) insulin treatment during the period of feed restriction from d 8 to 15. If it was not possible to allocate three littermates to treatments, pairs of gilts were randomly allocated to the main HR and HR+I treatments and matched with a nonlittermate RH gilt according to body weight and age at puberty. However, of a total of 19 groups, only three (one of which was in the subgroup of canulated gilts) were matched with a nonlittermate gilt. Long-acting insulin (Iletin Lente Insulin, Pork, Eli Lilly, Indianapolis, IN) was administered to HR+I as twice-daily injections of 0.4 IU/kg body weight s.c. behind the ear at the time of the morning and afternoon feed using flexible butterfly catheters (21G ¾ needle, Becton Dickinson, Franklin, NJ) without restraint. To avoid severe hypoglycemia, and based on preliminary studies to titrate the hypoglycemic effects of insulin treatment, insulin-treated gilts were given 30 mL of corn syrup in the feed, which corresponded to an increment of about 300 kcal in their daily energy intake. Blood glucose was monitored in all gilts 3 h after the afternoon feed on d 8, 12, and 15 using a glucometer (One Touch II, Lifescan Inc., Milpitas, CA). Body weight and backfat thickness at P_2 (Renco Lean-Meter, Renco Corporation, Minneapolis, MN) were measured in all animals at d 0 (onset of second estrus), d 7, d 15, and at the onset of the third estrus.

Of the 57 gilts initially allocated to treatment, one (HR+I) had polycystic ovaries at slaughter, five (four RH, and one HR+I) had fertilization rates around 10%, and five (two RH, one HR and two HR+I) had embryos with more than two cells at collection. Data from these animals were excluded from the final analysis of treatment effects on oocyte developmental competence, which was therefore based on 13 RH, 18 HR, and 15 HR+I gilts. In a subgroup of 24 gilts designated to receive the jugular catheters, 1 gilt (RH) became sick during the experimental period, and metabolic and endocrine statuses were, therefore, established in 23 animals (7 RH, 8 HR, and 8 HR+I).

All experimental procedures were carried out in accordance with the guidelines of the Canadian Council for Animal Care and under authorization from the Faculty Animal Policy and Welfare Committee of the University of Alberta.

Insemination and Embryo Recovery

Starting at d 18, gilts were checked for estrus every 6 h (0600, 1200, 1800, and 2400) using the back pressure test during periods of fence line contact with mature vasectomized boars. Gilts were artificially inseminated 12 and 24 h after the first observed standing estrus with pooled semen (3×10^9 spermatozoa/dose) from the same group of boars (Alberta Swine Genetics Corporation, Leduc, AB, Canada) specifically designated for this experiment. Time of ovulation was monitored using transcutaneous ultrasonography (Pie Medical Scanner 200, model 41480, Can Medical, Kingston, ON, Canada), using a 5.0- to 7.5-MHz multiple-angle transducer to scan for the presence of preovulatory follicles. Gilts were scanned every 6 h, beginning 24 h after onset of standing estrus, until completion of ovulation. During scanning, follicle diameter was recorded. Time of ovulation was defined as the time of the first scanning when no presumptive ovulatory follicles were seen less in 3 h. In the group of gilts that did not have jugular catheters, 2-mL blood samples were taken by acute venepuncture at ovulation, 12 h after ovulation, 48 h after onset of standing heat, and at surgery.

Surgeries were performed 12 to 20 h after the predicted time of ovulation, under general anesthesia, to recover early-fertilized oocytes. The surgical procedure included laparotomy and exposure of the uterine horns, oviducts, and ovaries. Ovulation rates were recorded and each oviduct was flushed twice using 5 mL of Dulbecco's phosphate buffered saline (DPBS, Sigma Chemical Company, St. Louis, MO), previously warmed at 39°C. Flushings were collected in sterile Falcon dishes (Fisher Scientific, St. Louis, MO) and immediately transported to the laboratory in a Styrofoam box containing a tray and flasks filled with warm water to avoid cooling of the recovered oocytes.

Falcon dishes containing 2 mL of NCSU-23 culture media supplemented with 4 mg/mL BSA (Sigma Chemical Co., catalogue # 8022) were prepared and left in incubators to warm and equilibrate for at least 2 h before surgery. Embryos were immediately transferred into the culture dishes and incubated under standard conditions of 39°C and 5% CO_2. The stage of embryonic development was observed three times a day (0800, 1400, 2200) for 7 d using a dissecting microscope (Wild M3, Wild Heerbrugg, Switzerland). Indications of fertilization (presence of sperm heads in the zona pellucida) and abnormal features (cells dividing unevenly) were observed under an inverted stage, phase-contrast, microscope at 400× magnification (Nikon Corp., Tokyo, Japan).

Blood Sampling

At d 13 of the treatment cycle, an indwelling jugular catheter was surgically implanted, under general anesthesia, via the superficial cephalic vein (Cosgrove et al., 1993), in eight littermate sets per treatment, of which one set had a nonlittermate gilt allocated to the RH treatment. Three-milliliter blood samples were withdrawn at 15-min intervals from 0600 to 1800 on d 15 and 16 of the estrous cycle for analysis of LH. Additional 10-mL samples were collected hourly for analysis of FSH, estradiol, progesterone, insulin, IGF-I, leptin, and free and total triiodothyronine (T3) by RIA. Starting at d 18, 10-mL samples were collected every 6 h (0600, 1200, 1800, and 2400) until surgery for analysis of the same parameters. Blood samples were collected into heparinized tubes and centrifuged at 1,500 × *g* for 15 min, and the plasma was then decanted and stored at –30°C until analysis.

Estimation of Plasma Hormone Concentrations

For RIA analysis, all treatment groups were represented in each assay, and all samples from a gilt were analyzed in the same assay. Assay sensitivity was calculated as Average of the zero binding tube (Bmax) – 2SD(Bmax)/average(Bmax)·100. Plasma progesterone concentrations were determined in duplicate using an established radioimmunoassay (Coat-a-Count Progesterone, Diagnostic Products Corporation, Los Angeles), previously validated for use with porcine plasma without extraction (Mao and Foxcroft, 1998). The sensitivity of the assay, defined as 96.2% of total binding, was 0.1 ng/mL. The intra- and interassay CV were 7.0% and 8.3%, respectively.

Plasma LH and FSH concentrations were determined in duplicate using the homologous double-antibody radioimmunoassays previously described by Cosgrove et al. (1991). For LH, 200 μL of plasma was assayed, and the intra- and interassay CV were 15.7% and 10.4%, respectively. Sensitivity, estimated as 93% of total binding, was 0.03 ng/mL. For FSH, 300 μL of plasma was assayed, the intraassay CV for the single assay used was 9.8%. Sensitivity, defined as 91.4% of total binding, was 12.4 ng/mL.

Plasma insulin and IGF-I concentrations were determined in duplicate using the homologous double-antibody radioimmunoassays previously described by Cosgrove et al. (1992). For insulin, 100 μL of plasma was assayed, and the intra- and interassay CV were 7.0% and 12.8%, respectively. Sensitivity, defined as 94.6% of total binding, was 0.02 ng/mL. For IGF-I, 100 μL of sample was initially extracted and the radio inert recovery efficiency was 111.0%. The intra- and interassay CV were 6.3% and 10.5%, respectively. Sensitivity, defined as 96.0% of total binding, was 8.0 ng/mL.

Plasma leptin concentrations were analyzed using the multispecies double-antibody kit assay (Linco Research, St. Louis, MO) previously validated in our laboratory for use with porcine plasma (Mao et al., 1999). The intra- and interassay CV were 15.0% and 6.3%. Sensitivity, estimated as 114.2% of total binding, was 1.0 ng/mL.

For determination of plasma estradiol-17β concentrations, 1 mL of plasma was extracted by the addition of 5 mL of diethyl ether (VWR Canlab, Mississauga, ON, Canada) and vortexing for eight 1-min pulses. Samples collected on d 15 and 16 of the cycle were only assayed for estradiol if gilts had low or falling levels of progesterone on these days, indicating that luteolysis had occurred and that estrogenic follicles might be developing. Therefore, estradiol analysis was performed on d-16 samples from 14 animals (all balanced for treatment and for littermate groups). Plasma samples obtained at the time of the LH surge and in the previous 24 h were prediluted 2.5-fold with assay buffer (phosphate-buffered saline containing 0.1% gel, pH 7.0), whereas the samples collected on d 16 of the cycle and the other six-hourly samples collected in the periestrous period were not diluted. Average extraction efficiency was 80 ± 0.7% and data were not corrected for recovery. Estradiol concentrations were estimated in a single radioimmunoassay using a double-antibody kit from Diagnostics Products Corp. (Los Angeles, CA), previously validated for use with porcine plasma (Yang et al., 2000b). The intraassay CV for the single assay used was 7.8%. Sensitivity, defined as 97.2% of total binding, was 0.3 pg/mL.

Plasma total T3 concentrations were measured using Diagnostic Products Corporation Coat-a-Count Total T3 kit (Diagnostics Product Corporation, catalogue number TKT31) with the following modifications: 1) removal of the unbound fraction of radiolabeled total T3 was done by aspiration rather than by decanting. The number of tubes aspirated was limited to 5 min per assay (200 tubes with two people aspirating) based on testing for drift in maximal bound tubes. 2) The volume of plasma assayed was 200 μL. 3) Standards were topped up to 200 μL using zero calibrator. 4) An extra standard was made by diluting the lowest standard provided with zero calibrator, providing a range of standards from 10 to 600 pg/100 μL. Parallelism was shown by finding no significant deviation between the slopes of the standard curve and a curve of volume vs binding for a pool of porcine plasma at 200, 100, and 50 μL. Recovery of 100 pg of total T3 from porcine plasma was 90 ± 7.1%. The intra- and interassay CV were 8.2% and 14.5%, respectively. Sensitivity, defined as 91.5% bound, was 11 pg/tube, equivalent to 55 pg/mL of plasma. Data were not corrected for cold recovery.

Free T3 was measured using Diagnostic Products Corporation Coat-a-Count Free T3 kit (Diagnostics Product Corporation, catalogue number TKF31) with the following modifications: 1) removal of the unbound fraction of radiolabeled free T3 was done by aspiration rather than by decanting. The number of tubes aspirated was limited to 10 min per assay (400 tubes with two people aspirating) based on tests for drift in maximal bound tubes. 2) The volume of plasma assayed was 200 μL. 3) Standards were topped up to 200 μL using zero calibrator. 4) An extra standard was made by diluting the lowest standard provided with zero calibrator, providing a range of standards from 25 to 4,700 fg/100 μL. Parallelism was shown by finding no significant deviation between the slopes of the standard curve and a curve of volume vs binding for a pool of porcine plasma at 200, 100, and 50 μL. Recovery of 520 fg of free T3 from porcine plasma was 120.1 ± 7.2%. The intra- and interassay CV were 16.6% and 31.7%, respectively. Sensitivity, calculated at 88.3% bound, was 20 fg/tube, equivalent to 100 fg/mL of plasma. Data were not corrected for cold recovery. Analysis of both free and total T3 during the periestrous period were performed in the samples collected at 0600.

Statistical Analysis

Data were analyzed as a randomized complete block design. Each block consisted of three littermates representing each treatment, with the exception of three blocks in which only gilts allocated to the HR and HR+I treatments were littermates. Treatment effects on ovulation rate; number of embryos recovered; recovery rate; fertilization rate; intervals from last insemination to ovulation, LH peak to ovulation, onset of estrus to LH peak, onset of estrus to estradiol peak, and estradiol peak to LH peak; estrus duration; time of ovulation in relation to estrus onset; feed offered and consumed; lysine and energy intake; body weight and body weight change; backfat and backfat change; blood glucose concentrations; and progesterone concentrations at ovulation, 12 h after ovulation, 48 h after onset of estrus, and at surgery were all analyzed using the GLM procedure of the SAS (SAS Inst. Inc., Cary, NC) and based on 55 gilts (18 RH, 19 HR, and 18 HR+I). The analysis of body weight and body weight change, and backfat and backfat change, during treatment, included the effects of block and treatment in the model, with body weight and backfat at d 0 of the treatment cycle as covariates. After arc sine transformation of the data, evaluation of treatment effects on embryo developmental competence in vitro was also performed by the GLM procedure of SAS (SAS Inst. Inc.). As discussed earlier, this analysis was based on 46 gilts. For both sets of data, the complete model included treatment and block as main effects, gilt was the experimental unit, and gilt within treatment × block interaction was used as the error term. In the event that significant treatment effects were established, multiple comparisons were performed using probability of differences (pdiff) between least square means, adjusted by Tukey-Kramer (SAS Inst. Inc.). For evaluation of treatment effects on endocrine and metabolic parameters, data were analyzed by the repeated measures analysis of variance (SAS Inst. Inc.). Variables that did not represent a normal distribution (progesterone, IGF-I, leptin, FSH) were log-transformed. The complete model included treatment and block as the main effects, day as the repeated measure, gilt as the experimental unit, and gilt within treatment × block interaction was used as the error term. Analysis of plasma insulin, leptin, and free and total T3 concentrations on d 15 and 16 were performed using means from two time periods: preprandial (2 h before the morning feed) and postprandial (1 to 10 h after the morning feed). In the event that significant treatment effects were established, multiple comparisons were performed using probability of differences (pdiff) between least square means, adjusted by Tukey-Kramer (SAS Inst. Inc.).

For the analysis of plasma progesterone in the periestrous period, concentrations at the time of the LH surge (time 0) were used as covariates, and analysis of covariance performed for each of the five subsequent samples, testing for homogeneous (no interaction) and heterogeneous (interaction) slopes. Plasma LH data were characterized initially with the sliding window technique of Shaw and Foxcroft (1985), and maximum, mean, and minimum LH concentrations at d 15 and 16 were used for statistical analysis.

Where appropriate, relationships among measured variables were established using correlation analysis (SAS Inst. Inc.).

Results

Growth Characteristics

Data on feed intake, energy, and lysine consumed are summarized in Table 1. The RH gilts had lower (*P*

Table 1. Daily feed consumed, energy intake, and lysine intake of gilts on treatment relative to metabolic body weight

Item	RH	HR	HR+I
Feed offered, kg			
d 1–7	2.8 ± 0.1	3.7 ± 0.1	3.7 ± 0.1
d 8–15	3.8 ± 0.05	2.8 ± 0.05	2.9 ± 0.05
d 16–estrus	4.0 ± 0.1	3.9 ± 0.1	4.0 ± 0.1
Feed consumed, g/kg $BW^{0.75}$			
d 1–7	61.8 ± 1.8[a]	73.8 ± 1.8[b]	73.6 ± 1.8[b]
d 8–15	84.8 ± 1.2[x]	65.7 ± 1.1[y]	68.7 ± 1.1[y]
d 16–estrus	64.6 ± 3.1	65.4 ± 3.1	69.7 ± 3.3
Energy intake, kcal/kg $BW^{0.75}$			
d 1–7	205.0 ± 6.2[a]	244.5 ± 6.0[b]	244.0 ± 6.0[b]
d 8–15	281.0 ± 3.9[x]	217.7 ± 3.7[y]	227.7 ± 3.7[y]
d 16–estrus	213.8 ± 9.0	225.7 ± 9.5	230.7 ± 9.5
Lysine intake, mg/kg $BW^{0.75}$			
d 1–7	0.61 ± 0.02[a]	0.73 ± 0.02[b]	0.73 ± 0.02[b]
d 8–15	0.84 ± 0.01[x]	0.65 ± 0.01[y]	0.68 ± 0.01[y]
d 16–estrus	0.64 ± 0.03	0.65 ± 0.03	0.69 ± 0.03

[a,b]Least squares means within a row with different superscripts differ ($P < 0.0005$); [x,y]Least squares means within a row with different superscripts differ ($P = 0.0001$). Abbreviations: RH = feed restriction during 1st wk of estrous cycle; HR = feed restriction during 2nd wk of estrous cycle; HR+I = feed restriction during 2nd wk of estrous cycle plus insulin treatment.

= 0.0001) energy and lysine intakes during the period of feed restriction (d 1 to d 7) compared with the HR and HR+I gilts. Similarly, energy and lysine intakes were lower ($P = 0.0001$) in HR and HR+I gilts during the period of feed restriction (d 8 to d 15) compared with their RH counterparts. Following d 15, no differences in energy or lysine intakes were observed among groups ($P > 0.05$).

Data on body weight and body weight change, backfat thickness at P_2, and backfat change of gilts are summarized in Table 2. Feed intakes recorded, relative to changing metabolic body weight, produced expected differences in growth (body weight change). The RH gilts had a lower growth rate ($P = 0.09$) during the period of feed restriction (d 1 to 7) than their HR and HR+I counterparts. Moreover, HR gilts also had a lower growth rate ($P = 0.02$) during this period of feed restriction (d 8 to 15) than RH and HR+I gilts. There was no difference ($P > 0.05$) in growth rate among groups from d 16 until onset of third estrus, but food intake was relatively low during this period. Backfat changes were not different among groups during the experimental period.

Reproductive Characteristics

Reproductive characteristics of gilts (Table 3) showed that ovulation rate was greater ($P = 0.06$) in the insulin-treated group than in the RH group, but was similar to the HR group. Consequently, the number of embryos recovered was higher ($P = 0.056$) in HR+I gilts than in RH gilts, and similar to HR gilts. However, previous nutrition and insulin treatments did not affect embryo recovery rate or fertilization rate. In the embryo data set, recovery rate was higher ($P = 0.06$) in HR+I gilts than in RH gilts, and similar to HR gilts. Previous nutritional regimen did not influence cycle length and duration of estrus among groups. A significant association was established between estrous cycle length and body weight at the onset of third estrus ($r = 0.41$, $P = 0.02$). Treatment affected time of ovulation relative to the onset of estrus, which occurred later ($P = 0.02$) in the HR+I gilts than in their RH and HR counterparts. However, in the data set of the canulated gilts, ovulation occurred earlier ($P = 0.024$) in RH gilts than in their HR and HR+I counterparts. There was no treatment effect on the interval from last insemination to ovulation.

Embryo Development Competence

The development of embryos recovered from each treatment group is summarized in Figure 1. There was a gradual decrease in the percentage of embryos reaching successive stages of early development, but a marked decrease in the percentage of embryos making the transition from morula to blastocyst. No treatment effects were observed on the development of embryos to the 4- to 8-cell and morula stages or on the percentage of blastocysts obtained at 96 and 144 h of culture.

Glucose Measurements

Blood glucose concentrations were lower ($P = 0.0001$) in the HR+I gilts on d 8, 12, and 15 as expected, than

Table 2. Body weight and BW change, and backfat thickness and backfat change of RH, HR, and HR+I gilts at d 0, d 7, d 15, and at estrus (least squares means ± SEM)

Parameter	RH (n = 18)	HR (n = 19)	HR+I (n = 18)
Body weight, kg			
d 0	143.0 ± 3.0	138.5 ± 2.4	136.5 ± 2.7
d 7	144.6 ± 0.9[x]	147.2 ± 0.7[y]	146.8 ± 0.8[y]
d 15	151.0 ± 0.6	151.5 ± 0.6	152.8 ± 0.7
Estrus	153.4 ± 1.6[y]	152.8 ± 1.4[y]	157.0 ± 1.9[x]
Body weight change, kg			
d 1–7	5.0 ± 0.9[x]	7.7 ± 0.7[y]	7.2 ± 0.7[y]
d 8–15	7.3 ± 0.6[a]	5.3 ± 0.5[b]	7.4 ± 0.5[a]
d 16–estrus	1.9 ± 1.2	0.7 ± 1.0	4.0 ± 1.3
Backfat, mm			
d 0	11.6 ± 0.7	11.0 ± 0.5	12.3 ± 0.6
d 7	12.6 ± 0.3	12.7 ± 0.3	12.4 ± 0.3
d 15	13.5 ± 0.4	12.8 ± 0.3	12.7 ± 0.3
Estrus	12.5 ± 0.4	12.8 ± 0.3	12.5 ± 0.4
Backfat change, mm			
d 1–7	0.9 ± 0.4	0.9 ± 0.4	–0.9 ± 0.4
d 8–15	1.0 ± 0.3	0.4 ± 0.3	–.04 ± 0.4
d 16–estrus	0.7 ± 0.3	0.5 ± 0.3	–0.3 ± 0.4

[a,b]Least squares means within rows with different superscripts differ ($P < 0.05$); [x,y]Least squares means within rows with different superscripts differ ($P < 0.1$). Abbreviations: RH = feed restriction during 1st wk of estrous cycle; HR = feed restriction during 2nd wk of estrous cycle; HR+I = feed restriction during 2nd wk of estrous cycle plus insulin treatment.

Table 3. Reproductive characteristics of gilts on treatment (least squares means ± SEM). All gilt data: data set including all gilts allocated to treatment (n = 55); embryo data set: data set in which gilts that had embryos with more than two cells at the time of collection and had fertilization rate around 10% were excluded(n = 46); endocrine data set: data set from cannulated gilts (n = 23)

Parameter	RH	HR	HR+I
All gilt data (n = 55)	18	19	18
Ovulation rate	16.0 ± 1.8[a]	18.0 ± 1.6[ab]	22.0 ± 1.6[b]
Number of embryos recovered	14.0 ± 1.8[a]	16.4 ± 1.5[ab]	20.3 ± 1.8[b]
Recovery rate, %	82.8 ± 3.0	89.4 ± 2.5	92.0 ± 2.8
Fertilization rate, %	95.5 ± 5.0	87.8 ± 4.3	96.2 ± 5.0
Interval last insemination to ovulation, h	12.4 ± 1.8	12.0 ± 1.7	16.4 ± 1.8
Estrous cycle length, d	20.0 ± 0.3	19.8 ± 0.3	20.0 ± 0.3
Estrus duration, h	51.5 ± 2.0	52.0 ± 1.7	53.8 ± 1.7
Ovulation in relation to estrus onset, h	40.7 ± 1.5[c]	41.7 ± 1.4[c]	46.5 ± 1.5[d]
Embryo data set (n = 46)	13	18	15
Ovulation rate	16.0 ± 2.2	17.7 ± 2.1	21.0 ± 2.0
Number of embryos recovered	13.5 ± 2.2[c]	16.3 ± 1.7[cd]	20.9 ± 2.1[d]
Recovery rate, %	82.8 ± 3.1[c]	88.5 ± 2.5[cd]	93.6 ± 3.0[d]
Fertilization rate, %	96.3 ± 6.3	87.3 ± 5.0	97.0 ± 6.0
Estrus duration, h	51.8 ± 2.8	51.7 ± 2.2	56.0 ± 2.5
Ovulation in relation to estrus onset, h	41.0 ± 2.4	42.8 ± 2.0	45.8 ± 2.0
Endocrine data set (n = 23)	7	8	8
Ovulation rate	15.8 ± 2.3	18.8 ± 1.8	19.0 ± 1.6
Number of embryos recovered	13.6 ± 1.4	17.6 ± 1.0	17.5 ± 1.1
Recovery rate, %	81.6 ± 4.3	94.9 ± 3.0	93.5 ± 3.4
Fertilization rate, %	88.3 ± 12.2	82.2 ± 8.8	94.7 ± 9.7
Estrus duration, h	52.0 ± 5.0	52.0 ± 3.0	57.4 ± 2.8
Ovulation in relation to estrus onset, h	35.8 ± 3.0[c]	45.5 ± 1.9[d]	48.3 ± 1.9[d]

[a,b]Least squares means within a row with different superscripts differ ($P \leq 0.06$); [c,d]Least squares means within a row with different superscripts differ ($P \leq 0.05$). Abbreviations: RH = feed restriction during 1st week of estrous cycle; HR = feed restriction during 2nd wk of estrous cycle; HR+I = feed restriction during 2nd wk of estrous cycle plus insulin treatment.

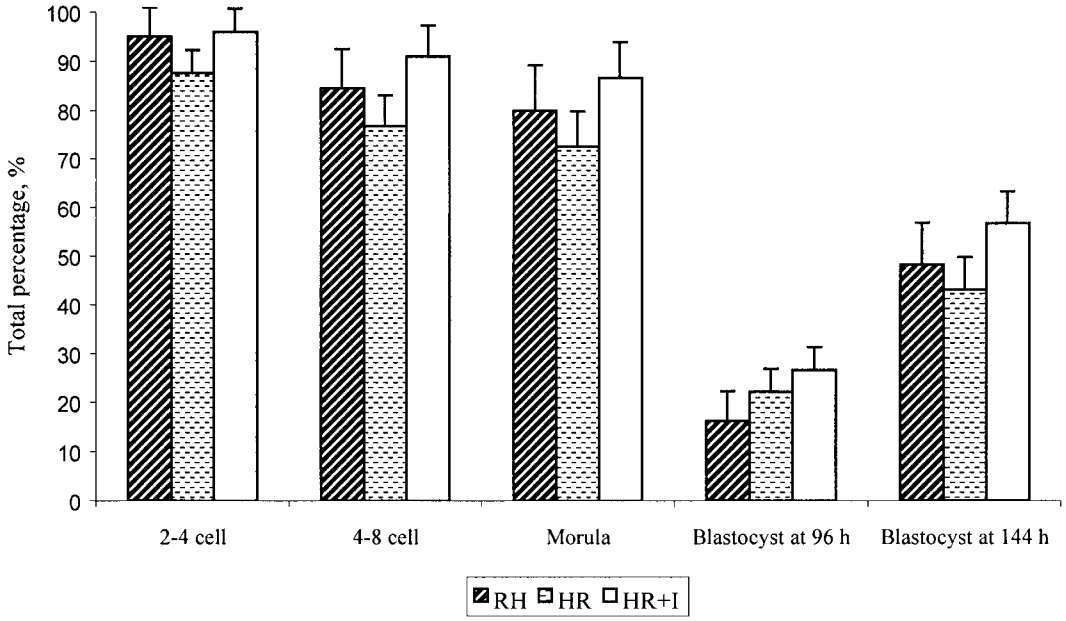

Figure 1. Percentage of fertilized oocytes recovered 12 to 20 h after ovulation reaching successive stages of development during culture in vitro for 144 h.

in RH and HR gilts, which had similar glucose concentrations (Table 4).

Plasma Hormone Concentrations

Plasma insulin concentrations increased after the morning feeding on d 15 ($P = 0.0001$) and d 16 ($P = 0.0001$), and as expected, a time × treatment interaction ($P = 0.009$) on d 15 resulted in higher postprandial insulin concentrations in the HR+I (2.0 ± 0.2 ng/mL) than in the RH (1.2 ± 0.2 ng/mL) and HR (1.0 ± 0.2 ng/mL) groups. Feeding again increased ($P < 0.0001$) insulin concentrations on d 16, but there was no time × treatment interaction. The difference between pre- and postprandial insulin was influenced by the interaction between day and treatment ($P = 0.008$), with greater increase in the HR+I (1.1 ± 0.1 ng/mL) than in the RH (0.4 ± 0.1 ng/mL) and HR (0.3 ± 0.1 ng/mL) gilts on d 15, and no differences on d 16. However, there was no overall effect of interaction between day and treatment in preprandial mean plasma insulin concentrations. Treatment did not affect mean plasma insulin concentrations during the periestrous period.

Plasma IGF-I concentrations did not change after the morning feeding ($P > 0.05$). However, there was a day effect ($P = 0.055$) for mean plasma IGF-I concentrations, which increased from d 15 to d 16. Although much of this effect seemed to relate to changes in HR gilts (97.8 ± 5.0 and 106.3 ± 6.0 ng/mL on d 15 and 16, respectively), no overall day × treatment interaction was observed. Treatment did not affect mean plasma IGF-I concentrations during the periestrous period.

Plasma leptin concentrations increased after the morning feeding on d 15 (1.9 ± 0.4 to 2.6 ± 0.4 ng/mL; $P = 0.0001$) and on d 16 (1.9 ± 0.3 to 2.4 ± 0.3 ng/mL; $P = 0.055$). However, there were no overall effects of day, treatment, or day × treatment interaction on plasma leptin or on the difference between pre- and postprandial leptin. In the periestrous period (Figure 2), a time × treatment interaction was observed ($P = 0.08$); RH gilts had higher ($P = 0.02$) leptin concentrations at the time of the LH surge (time 0) than HR or HR+I gilts, and associations were established between leptin concentrations at the time of the LH surge, and backfat measurements at d 15 ($r = 0.56$, $P = 0.02$) and at the onset of third estrus ($r = 0.53$, $P = 0.03$).

Plasma concentrations of total T3 increased after the morning feeding on d 15 ($P = 0.0001$) and on d 16 ($P = 0.025$), and there was a time × treatment interaction ($P = 0.009$) on d 15, resulting in higher postprandial concentrations in the HR group (634 ± 34 pg/mL) than in HR+I gilts (518 ± 34 pg/mL), with intermediate concentrations in RH gilts (580 ± 38 pg/mL). There was a trend ($P = 0.07$) for a time × treatment interaction on d 16. The difference between pre- and postprandial total T3 was influenced by day ($P = 0.004$) and by treatment ($P = 0.009$), with a greater rise in the HR than in the HR+I gilts, but similar to RH gilts, on d 15 and 16. Similarly, plasma concentrations of free T3 increased after the morning feeding on d 15 (518 ± 73 to 1,006 ± 90 fg/mL; $P = 0.0001$) and on d 16 (2,104 ± 357 to 2,875 ± 109 fg/mL; $P = 0.009$). However, no time × treatment interaction was observed. The difference between pre- and postprandial free T3 was influenced by day ($P = 0.013$) and was smaller on d 16 than on d 15 (257 ± 148 and 488 ± 105 fg/mL, respectively). However, no day × treatment interaction was observed. Overall, preprandial plasma free and total T3 concentrations increased from d 15 to d 16 in all treatment groups ($P = 0.008$ and $P = 0.004$, respectively). However, no overall interaction between day and treatment was observed. There was no effect of treatment on postprandial mean plasma free and total T3 among groups. During the periestrous period, plasma free and total T3 concentrations were not affected by previous nutritional treatments.

There was an increase from d 15 to d 16 in mean (0.31 ± 0.03 to 0.33 ± 0.03 ng/mL; $P = 0.045$) and minimum (0.17 ± 0.02 to 0.22 ± 0.02 ng/mL; $P = 0.001$) LH concentrations; however, no treatment × day interaction was observed ($P > 0.05$). During the periestrous period, a time × treatment interaction ($P = 0.03$) was observed and the area under the LH peak was greater ($P = 0.048$) in the RH gilts than in the HR gilts, but was similar to HR+I gilts (Figure 3).

Plasma FSH concentrations were not affected by day. However, there was a trend ($P = 0.07$) for a day × treatment interaction. During the periestrous period, there was a time × treatment interaction ($P = 0.04$), and, at the time of the LH surge (time 0), FSH concentrations in HR gilts were lower ($P = 0.06$) than in their RH and HR+I counterparts (Figure 3).

Overall, progesterone concentrations decreased ($P = 0.0001$) from d 15 (12.0 ± 1.3 ng/mL) to d 16 (6.6 ± 2.1 ng/mL); however, there was no treatment × day interaction ($P > 0.05$). In the periovulatory period, a treatment × time interaction ($P = 0.049$) was observed (Figure 3). A comparison of slopes fitted to the rising progesterone concentrations showed that previous nutrition and insulin treatment affected the pattern of the progesterone rise after the LH surge ($P = 0.036$), with a slower rise in progesterone in the HR gilts than in their RH and HR+I counterparts. However, progesterone concentrations in RH, HR, and HR + I gilts at

Table 4. Blood glucose concentrations (mg/dL) of gilts on treatment (least squares means ± SEM)

Treatment	d 8	d 12	d 15
n = 55[a]	n = 18	n = 19	n = 18
RH	68.6 ± 3.7[x]	65.2 ± 4.0[x]	64.6 ± 3.8[x]
HR	62.7 ± 2.8[x]	63.5 ± 3.0[x]	63.0 ± 3.0[x]
HR+I	39.0 ± 2.6[y]	38.3 ± 4.0[y]	37.2 ± 2.8[y]

[a]Data set including all gilts allocated to treatment (n = 55); [x,y]Least squares means within a column with different superscripts differ ($P = 0.0001$). Abbreviations: RH = feed restriction during 1st week of estrous cycle; HR = feed restriction during 2nd wk of estrous cycle; HR+I = feed restriction during 2nd wk of estrous cycle plus insulin treatment.

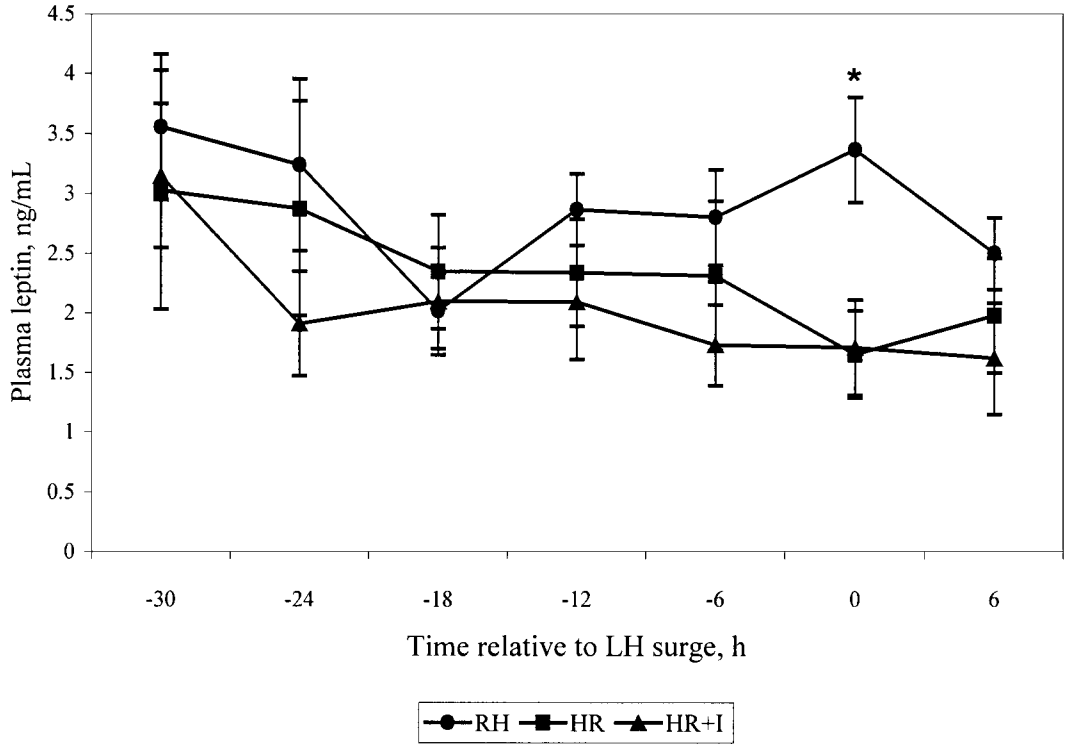

Figure 2. Plasma leptin concentrations during the periestrous period in gilts according to treatment. RH gilts had higher leptin concentrations at the time of the LH surge than HR or HR+I gilts ($P < 0.05$).

ovulation (1.1 ± 0.1, 1.0 ± 0.1, and 1.0 ± 0.1 ng/mL, respectively), 12 h after ovulation (2.6 ± 0.3, 2.0 ± 0.3, and 2.0 ± 0.3 ng/mL, respectively), 48 h after onset of estrus (2.1 ± 0.3, 1.5 ± 0.2, and 1.5 ± 0.2 ng/mL, respectively), and at surgery (2.7 ± 0.3, 2.4 ± 0.3, and 2.0 ± 0.3 ng/mL, respectively) were not different. A significant correlation was established between ovulation rate and progesterone concentrations at surgery (r = 0.45, $P = 0.004$).

There was no effect of previous nutritional treatment on plasma estradiol concentrations at d 16 ($P > 0.05$). However, the area under the estradiol peak during the follicular phase of the cycle in RH and HR+I gilts was greater ($P = 0.01$) than in HR gilts (Figure 3).

The intervals between the estradiol peak and the LH peak (14.9 ± 2.5 h), LH peak and ovulation (29.9 ± 1.9 h), and between the onset of estrus and either the estradiol peak (–0.6 ± 4.3 h) or the LH peak (11.9 ± 3.6 h), were not affected by previous nutritional treatments.

Effect of Litter

A litter effect was observed for body weight at d 0 ($P = 0.0001$), d 7 ($P = 0.0001$), d 15 ($P = 0.0001$), and at the onset of third estrus ($P = 0.025$), for growth rate ($P = 0.02$) during d 1 to 7 and d 8 to 15, and for backfat at d 0 ($P = 0.018$), d 7 ($P = 0.09$), d 15 ($P = 0.001$) and at the onset of third estrus ($P = 0.003$). Associated with these effects, there was a litter × day interaction for blood glucose ($P = 0.052$), postprandial insulin ($P = 0.037$), pre- ($P = 0.024$) and postprandial total T3 ($P = 0.001$), postprandial free T3 ($P = 0.023$), preprandial leptin ($P = 0.044$), mean LH concentrations ($P = 0.03$), minimum LH concentrations ($P = 0.04$), and progesterone ($P = 0.008$).

Estrous cycle length was also affected by litter ($P = 0.025$), and a positive association was observed between cycle length and progesterone concentrations on d 15 (r = 0.86, $P = 0.0001$) and d 16 (r = 0.90, $P = 0.0001$). During the periestrous period a litter effect was also observed for estradiol ($P = 0.001$) and total T3 ($P = 0.06$), and a time × litter interaction was observed for FSH ($P = 0.0001$), progesterone ($P = 0.0001$), IGF-I ($P = 0.0001$), insulin ($P = 0.0004$), and leptin ($P = 0.0004$). There was a trend for a litter effect on estrus duration ($P = 0.08$) and for the percentage of oocytes developing to blastocysts at 96 h ($P = 0.08$), but no litter effect was observed for ovulation rate ($P > 0.05$). A positive correlation was observed between cycle length and body weight at third estrus (r = 0.41, $P = 0.02$), and body weight at d 15 and at estrus were positively associated with age at puberty (r = 0.56, $P = 0.0001$; r = 0.33, $P = 0.06$, respectively). The association between litter effects on cycle length and characteristic endocrine profiles during the periestrous period are shown in Figure 4.

Discussion

The design of the present study was based on an experimental model developed by Almeida et al. (2000). Although the consequences of different periods of feed

restriction on subsequent embryonic survival were not assessed in the present study, we consider that our strict adherence to the same experimental paradigm used previously enabled us to investigate the mechanisms that might mediate the latent effects of feed restriction on subsequent reproductive function. In particular, we sought to establish whether the critical period of feed restriction in the late luteal phase of the cycle has effects on 1) the subsequent developmental competence of fertilized oocytes cultured in vitro and 2) the endocrine status of gilts during and after treatment. Furthermore, we wished to use a modification of this

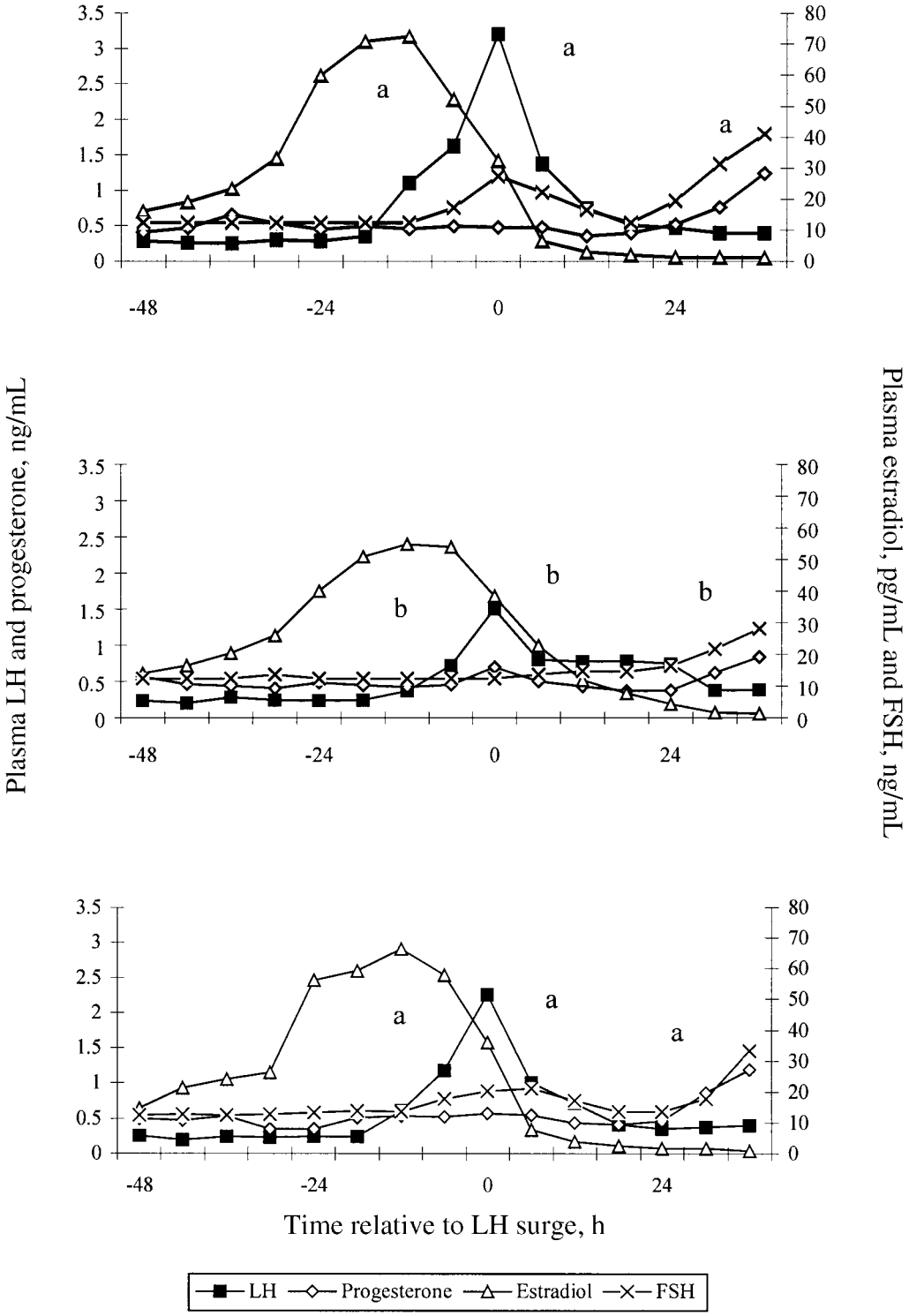

Figure 3. Gonadotropin and steroid profiles of gilts during the periestrous period according to treatment. Estradiol, LH, and FSH surges were lower in HR than in RH or HR+I gilts (a,b values differ $P < 0.05$). The progesterone raise after the LH surge occured more slowly in HR gilts than in RH or HR+I gilts (a,b values differ, $P < 0.05$).

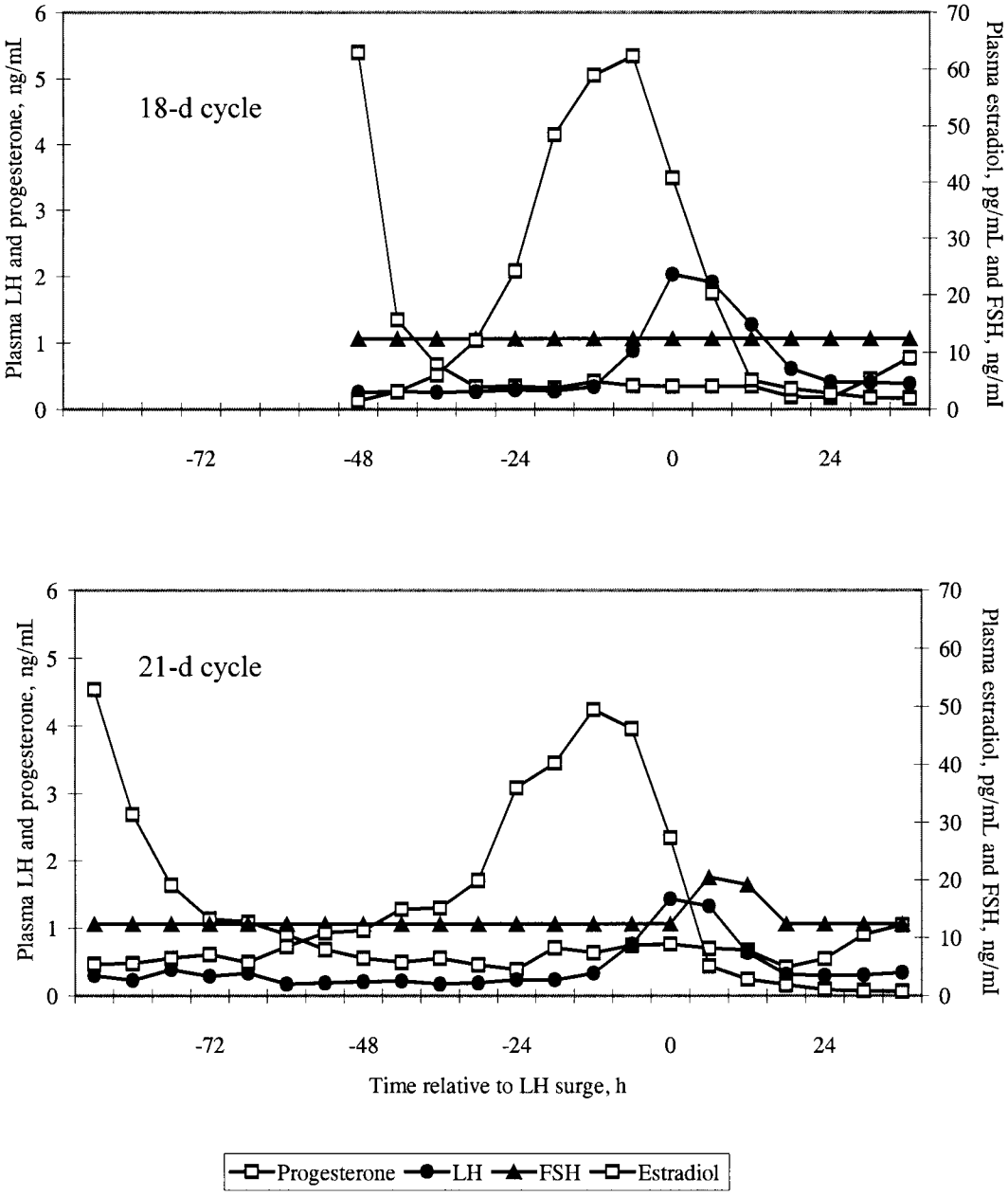

Figure 4. Gonadotropin and steroid hormone profiles during the periestrous period from littermate gilts with an 18-d estrous cycle (n = 3) or a 21-d estrous cycle (n = 2). Luteal regression and a decline in plasma progesterone occurred on d 14 in the 18-d-cycle gilts and on d 16 in the 21-d-cycle gilts.

experimental model to determine whether insulin could counteract the detrimental effects of feed restriction in the later part of the luteal phase of the cycle.

Consistent with this model, the patterns of feed intake imposed during the estrous cycle resulted in differential patterns of body weight change in RH, HR, and HR+I gilts, and differences in plasma progesterone concentrations in the immediate postovulatory period in RH and HR gilts. In terms of other treatment effects on BW change, the ability of insulin treatment to counteract the effects of feed restriction is consistent with several studies in humans, suggesting that hyperinsulinemia leads to an increase in BW (Odeleye et al., 1997; Purnell and Brunzell, 1998; Guven et al., 1999), presumably related to a general increase in glucose utilization.

Our findings on culture of preimplantation embryos are consistent with our preliminary study (Almeida, 2000), in which previous nutritional treatment did not affect embryo developmental competence within 144 h of culture. Furthermore, previous insulin treatment also did not affect developmental competence of fertilized oocytes. A greater developmental competence of oocytes in gilts receiving insulin might have been expected, as insulin influences granulosa cell steroidogenic activity (Channing et al., 1976; Barbieri et al.

1983) and induces LH receptors in porcine granulosa cells in culture (May and Schomberg, 1981) and maturation of porcine oocytes (Tsafriri and Channing, 1975). Studies in ewes have also suggested that the level of priming progesterone, modulated by preovulatory nutrition, influenced embryo survival through direct effects on the developing oocyte (McEvoy et al., 1995a). In subsequent studies, McEvoy et al. (1995b) reported that the provision of supplementary progesterone to ewes on a high plane of feeding during the preovulatory priming phase elevated plasma progesterone and enhanced subsequent ovum development. Because treatment did not affect preovulatory progesterone concentrations in the present study, it may be that a critical mechanism mediating nutritional effects on oocyte maturation was not activated.

In contrast to this apparent lack of an effect on oocyte competence, our data suggest that insulin treatment may have exerted other effects on ovarian function, which became evident in the periestrous period. The observed differences in estradiol concentrations in HR and RH gilts during the follicular phase of the cycle suggest that a period of feed restriction in the late luteal phase critically affects follicular maturation. The greater increase in follicular phase concentrations of plasm estradiol HR+I compared with HR gilts, supports previous evidence that insulin can act at the ovarian level to stimulate steroidogenesis (Channing et al., 1976; Barbieri et al., 1983). The less-robust estradiol surge in the HR compared with HR and HR+I gilts, may be responsible for the less-robust preovulatory LH and FSH surges seen in the HR animals (Figure 3), as the biphasic effects of increasing plasma estradiol, acting at both the hypothalamic and pituitary level, triggers the preovulatory LH surge. In turn, the more rapid rise in progesterone after ovulation in RH and HR+I compared with HR gilts indicated that previous effects of feed restriction and insulin treatment also affected the luteinization process, and hence the production of progesterone in the immediate postovulatory period. However, if difference in progesterone production is one of the mechanisms mediating latent effects of feed restriction on early embryonic survival, our embryo data suggest that this effect was not evident at the early blastocyst stage.

The role of periovulatory progesterone as a possible mediator of nutritionally induced effects on embryonic survival has been studied in ewes and gilts (Ashworth et al., 1989; Ashworth, 1991; Jindal et al., 1997). Ashworth (1991) and Jindal et al. (1997) demonstrated in gilts that progesterone injections in the early stages of pregnancy could counteract a nutritionally induced increase in embryonic loss. This confirms the importance of adequately monitoring the patterns of progesterone secretion in the critical postovulatory period (Foxcroft, 1997) and also raises the additional possibility, previously discussed by Hunter and Wiesak (1990), that differences in follicular maturation before ovulation may be reflected in subtle, but physiologically important, differences in progesterone secretion in early pregnancy.

As previously mentioned, insulin effects on the ovary include decreased follicle atresia, allowing more follicles to enter the preovulatory pool, and ovulation rate to increase (see Cox, 1997 for review). In the present study, exogenous insulin injection during the late luteal phase of the cycle, even in the presence of moderate feed restriction, also increased ovulation rate, consistent with findings in cyclic gilts (Cox et al., 1987) and in lactating sows (Whitley et al., 1998a). In contrast, Kirkwood and Thacker (1991), Quesnel and Prunier (1998), and Whitley et al. (1998b) did not find any effects of insulin on ovulation rate in lactating sows. Of the other reproductive characteristics analyzed, a greater number of embryos recovered in the HR+I than in the RH and HR gilts was likely due to the greater number of ovulations in this group.

From a metabolic perspective, insulin treatment resulted in an increase in plasma postprandial insulin concentrations on d 15 in the HR+I group, as anticipated. Even though supplemental energy was provided to insulin-treated gilts, this elevation of insulin was accompanied by a decrease in glucose concentrations, without any effect on plasma IGF-I or pre- and postprandial leptin. Several previous studies in pigs have reported that feed restriction reduced plasma insulin and IGF-I (Booth et al., 1994; Zak et al., 1997; Quesnel et al., 1998) and postprandial leptin (Mao et al., 1999; Barb, 1999). In the present study, the lack of an effect of feed restriction on plasma concentrations of these metabolic hormones on d 15 in RH and HR gilts is likely due to the modest level of feed restriction used (2.1 vs 2.8× maintenance).

The increase in plasma concentrations of total and free T3 after feeding in the present study is in agreement with the findings of Dauncey et al. (1983) in pigs and Williams et al. (1996) in primates, suggesting that a meal directly stimulates the secretion of T3 from the thyroid gland as well as affecting peripheral conversion of T4 to T3. Insulin injections blunted the postprandial rise in total T3 on d 15, and, because thyroid hormones are regulators of energy homeostasis, low glucose concentrations in HR+I gilts probably acted as a signal of energy deficiency, triggering the fall in plasma T3 concentrations. However, the lack of effects of feed restriction from d 8 to 15 on pre- and postprandial total and free T3 measured on d 15 suggests that thyroid hormones did not play a role in mediating nutritional effects on subsequent reproductive function in the present study; nor did the other metabolic regulators analyzed (IGF-I, insulin, and leptin). Although our data suggest that a critical period of feed restriction in the late luteal phase can have detrimental effects on the endocrine status of gilts in the periestrous period and that these effects likely originate from differences in follicular development prior to recruitment into the follicular phase of the cycle, the identity of the link between feed restiction and ovarian function was not

established. The higher leptin concentrations in RH gilts at the time of the LH surge was likely due to their body fat content during that period, as plasma leptin concentrations in the periestrous period were positively associated with backfat at d 15 and at the onset of third estrus.

In the present study, LH maximum, mean, and minimum concentrations on d 15 and 16 were also not affected by previous nutritional treatments. The increases in mean and minimum LH concentrations on d 16 coincided with a decrease in plasma progesterone concentrations due to luteolysis. Therefore, it is likely that progesterone exerts such a dominant effect on LH secretion that the level of feed restriction applied between d 8 and 15 of the cycle does not further modify the pattern of LH secretion. We conclude that centrally mediated effects on gonadotropin secretion at the time of feed restriction do not mediate effects of feed restriction on subsequent reproductive function.

The effect of litter on many of the variables measured in this study indicates the importance of the use of littermates when designing experiments of this kind, and the ability to adjust for this family effect in the statistical analysis by using a randomized complete block design, fitting litter as a block to account for the differences due to family. An interesting finding of our study was the litter effect on estrous cycle length. Our results showed that cycle length was apparently determined by the length of the luteal phase, as cycle length was highly and positively associated with plasma progesterone concentrations on d 15 and 16. Although the hormonal events of the periestrous period did not vary with litter, data in Figure 4 suggest that the interval between luteolysis and the rise in plasma estradiol was probably shorter in short-cycle gilts. However, incomplete data on the time of the initial fall in plasma progesterone did not allow us to test the relationship statistically across all litters. Notwithstanding the litter effect on estrous cycle length, the positive correlation between BW at third estrus and cycle length suggests that, besides litter, BW also plays a role in determining length of the estrous cycle (heavier gilts have longer cycles). Therefore, controlling body weight of cyclic gilts may be an important factor for achieving improvements in reproductive efficiency. Although in contrast to our previous studies (Almeida, 2000; Almeida et al., 2000), no litter effect was observed for ovulation rate, litter may be an important factor influencing ovulation rate.

In conclusion, our results demonstrate that a moderate level of feed restriction imposed on gilts during the luteal phase of the cycle does not affect plasma concentrations of metabolic hormones (insulin, IGF-I, leptin, total and free T3), gonadotropins (LH, FSH), or steroid hormones (progesterone and estradiol) during this period of feed restriction, but had latent effects on endocrine events in the periestrous period. Effects of feed restriction seemed to be largely counteracted by insulin treatment. Although previous nutritional and insulin treatment did not affect the early developmental competence of fertilized oocytes measured in vitro, treatment effects (RH vs HR) on embryonic survival to d 28 seen previously may be mediated by differences in progesterone status in early pregnancy.

Implications

Because moderate feed restriction during the late luteal phase of the estrous cycle had lasting effects on ovarian function and the endocrine status of gilts in the periovulatory period, this may be an important mechanism mediating previously established effects of feed restriction on subsequent fertility. It seems that insulin treatment can counteract the deleterious effects of feed restriction on reproductive function seen in this study. Our data suggest that previous feed restriction and insulin treatment do not affect the developmental potential of the fertilized oocyte, which may be important in determining the source of oocytes that can be successfully used for in vitro maturation and fertilization systems. The better understanding of the interactions between nutrition and reproduction gained in this study will contribute to enhanced reproductive efficiency of breeding sow herds through the development of optimal nutritional management strategies.

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