BIOLOGY OF REPRODUCTION **73,** 918–926 (2005)
Published online before print 22 June 2005.
DOI 10.1095/biolreprod.105.041483

Impact of Nutrition on Oocyte Quality: Cumulative Effects of Body Composition and Diet Leading to Hyperinsulinemia in Cattle[1]

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ABSTRACT

The present study sought to assess the combined effects of body composition and diet (level of feeding) on the postfertilization developmental potential of oocytes recovered from heifers using ultrasound-guided transvaginal follicular aspiration and to relate oocyte quality to the metabolic status of these animals. By collecting oocytes on repeated occasions spanning several weeks, it was possible to assess the cumulative effects of changes in nutritional status on oocyte quality over this period. Twenty-four heifers of low and moderate body condition were placed on one of two levels of feeding (equivalent to once or twice the maintenance requirements of these animals). Oocytes were recovered at two defined time points within each of three successive estrous cycles and were matured, fertilized, and cultured to the blastocyst stage in vitro. The results show that the effect of feeding level on oocyte quality is dependent on the body condition of the animal, with the high level of feeding being beneficial to oocytes from animals of low body condition but detrimental to oocytes from animals of moderately high body condition. Furthermore, the effects of high levels of feeding on oocyte quality were cumulative, with blastocyst yields for relatively fat heifers on twice the maintenance requirement deteriorating with time relative to yields for relatively thin heifers on the same level of feeding. Finally, a significant proportion of the moderately fat animals on the high level of feeding were hyperinsulinemic, and we show, to our knowledge for the first time in ruminants, that this condition is associated with impaired oocyte quality.

embryo, in vitro fertilization, insulin, leptin, oocyte development

INTRODUCTION

Environmental influences, such as chronic and acute changes in dietary intake and body composition, can have a profound effect on fertility and, specifically, ovarian function in all mammalian species studied, including humans [1, 2]. In ruminants, these changes can occur without significant variation in circulating gonadotroph concentrations but are correlated with changes in circulating concentrations of various metabolites and metabolic hormones, including insulin, insulin-like growth factor 1 (IGF1), and leptin [3]. It is rare for young cattle under good systems of management to encounter periods of food shortage. On the contrary, such animals are more likely to encounter prolonged periods of high-plane feeding, because they are encouraged to attain puberty at appropriately early ages [4]. Our understanding about the effects of nutritional status on oocyte and embryo quality under such circumstances is limited, so an urgent need exists to conduct thoughtfully designed, whole-animal studies that adequately characterize both the dietary treatments and the metabolic status of the animals involved [5]. Those studies that have assessed the effects of plane of nutrition (normally expressed as multiples of the energy requirements for body maintenance) have tended to work with moderately fat animals (animals of moderately high body condition) and have produced a consensus that high planes of nutrition in nulliparous ruminants are detrimental to oocyte quality, with low postfertilization developmental rates [6, 7]. By default, those who choose to use oocytes from abattoir-derived ovaries for in vitro studies are constrained to working with germ cells from a nondescript but potentially diverse population of animals and body conditions, which will tend to increase the variance of experimental results. It is highly probable that the effects of nutrient supply (either in vivo or in vitro through the supplementation of culture media with specific metabolites or growth factors) will be influenced by the body composition of the oocyte donor, but this has never been formally determined.

The primary objective of the present study was to assess the combined effects of body condition and level of feeding on the postfertilization developmental potential of oocytes recovered from heifers using ultrasound-guided transvaginal follicular aspiration (ovum pickup [OPU]). A secondary objective was to relate oocyte quality to the metabolic status of these animals, specifically to plasma concentrations of insulin, IGF1, and leptin. Finally, by collecting oocytes from repeated sessions of OPU (at two defined time points within each of three estrous cycles), it was possible to determine the effects of changes in nutritional status (i.e., the combined effects of level of feeding and changes in body composition and metabolism) over this period (lasting several weeks) on oocyte quality.

MATERIALS AND METHODS

The experiment described in the present paper was reviewed by the Animal Ethics Committee of the Scottish Agricultural College and was conducted under the auspices of, and in accordance with, the requirements of the Home Office Animals (Scientific Procedures) Act 1986.

Animals and Experimental Design

Twenty-four beef × dairy heifers (Scottish Agricultural College) approximately 20 mo of age and with an average initial weight (mean ±

[1]Supported by the Department for Environment, Food, and Rural Affairs. The Scottish Agricultural College receives financial assistance from Scottish Executive for Environment and Rural Affairs Department. S.J.A. was supported by the Perry Foundation and the University of Nottingham.
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Received: 4 March 2005.
First decision: 26 April 2005.
Accepted: 20 June 2005.

ISSN: 0006-3363. http://www.biolreprod.org

TABLE 1. Composition and chemical analysis of feeds.

Concentrate ingredient	g/kg	Chemical analysis[a]	Concentrate	Straw
Barley	180	DM (g/kg)	873.0	846.0
Wheat	400	ME (MJ/kg DM)	13.2	4.6
Maize grain	90	CP (g/kg DM)	163.0	30.0
Sugar beet pulp	90	NDF (g/kg DM)	157.0	832.0
Maize gluten	100	Starch (g/kg DM)	456.0	11.0
Rapeseed meal	70	AHEE (g/kg DM)	43.0	14.0
Molasses	60			
Urea	10			
Total	1000			

[a] DM, dry matter; ME, metabolizable energy; CP, crude protein; NDF, neutral detergent fiber; AHEE, acid hydrolyses ether extract.

SEM) of 432.7 ± 14.6 kg were used. All animals were accommodated in individual pens on slatted floors. Four months before the start of the experiment, the live weight and body condition score (BCS) of each heifer was recorded. Body condition was scored on the six-point scale of Lowman et al. [8], where 0 = lean and 5 = obese. To create homogeneous groups of animals of both low and moderate BCS at the beginning of the experimental period, heifers were allocated to one of several levels of feeding based on Agricultural and Food Research Council [9] values for the energy requirements of cattle. Once this had been achieved, animals were allocated at random within each of the two groups (low and moderate BCS) to one of two levels of feeding, either 500 kJ (maintenance [M]) or 1000 kJ of metabolizable energy per kilogram of metabolic live weight per day (twice maintenance [2M]), resulting in a 2 × 2 factorial design in which the factors were low versus moderate BCS and M versus 2M level of feeding. The diets were offered as two meals (at 0800 and 1600 h) daily. Details of diet composition and chemical analysis are presented in Table 1. The experiment was conducted over three successive estrous cycles and repeated 2 mo later (denoted as periods 1 and 2). To reduce excessive gain or loss of live weight throughout the study, animals were realigned to the same levels of BCS between the end of the first and the beginning of the second periods of study. Furthermore, the levels of feeding (M or 2M) for animals within each BCS group during period 2 were switched from that offered in period 1 so that animals fed at the M level during period 1 were fed at the 2M level during period 2, and vice versa.

Estrous Synchronization, Oocyte Recovery, and Follicular Growth

To establish a reference estrus at the beginning of each period, heifers were synchronized using a 10-day, progesterone-releasing, controlled internal drug-release dispenser (CIDR; SmithKline Beecham, Tadworth, Surrey, UK) containing 1.9 g of progesterone. A prostaglandin $F_{2\alpha}$ analog (Luprositol 15 mg; Prosolvin, Intervet, Cambridge, UK) was administered i.m. 8 days after CIDR insertion. Heifers were predicted to be in estrus 2 days after CIDR removal (Day 0 = day of reference estrus) (Fig. 1).

Animals were subjected to ultrasound-guided transvaginal follicular aspiration (i.e., OPU) as described by Goodhand et al. [10], on Days 5 and 12 of each of the three successive estrous cycles within each of the two periods (Fig. 1). Briefly, at the beginning of each OPU session, heifers were sedated with 28 mg of xylazine hydrochloride (Rompun; Bayer AG, Leverkusen) administered i.m. before epidural anesthesia with 5 ml of lignocaine hydrochloride (Locovetic; Bimeda, UK) between the first and second coccygeal vertebrae. A 5-MHz curvilinear transducer (Aloka SSD-500 V; BCF Technology Ltd., Livingstone, UK) was inserted vaginally, and all visible follicles greater than 2 mm were aspirated using a single-lumen needle (20 gauge) at a vacuum pressure of 70 mm Hg into conical tubes containing 5 ml of warm PBS supplemented with 0.1% (w/v) BSA fraction V, 50 IU/ml of heparin, and antibiotics (50 IU/ml of penicillin, 50 μg/ml of streptomycin sulfate, and 100 μg/ml of neomycin).

Ovarian follicular growth was monitored daily for a 6-day period commencing 3 days after the second OPU session leading up to estrus within each estrous cycle (Fig. 1). Ovarian follicles were visualized by transrectal real-time ultrasonography using a 5-MHz transducer (Aloka SSD-500 V). The total number of visible (≥2 mm) follicles and their diameters were recorded. Each day before the early morning meal during the period leading up to estrus within each cycle, blood samples were collected by jugular venipuncture into lithium-heparin tubes on ice. Additional blood samples were similarly collected on the day before, the day of, and the day after each session of OPU. Plasma harvested from these samples was stored at −20°C until analysis.

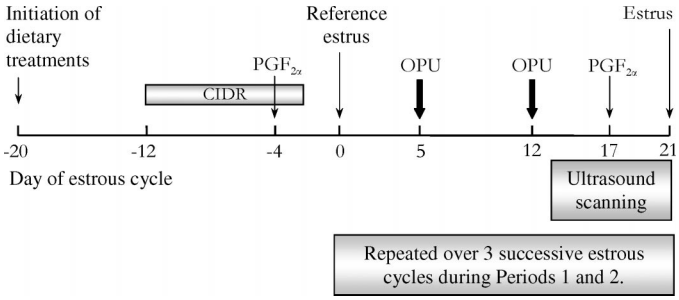

FIG. 1. Experimental procedures and time scales within each period, indicating initiation of dietary treatments (feeding levels), establishment of a reference estrus, time points during each of the three successive estrous cycles within each period when OPU was conducted, and 6-day period of ovarian ultrasound scanning during estrous synchronization (see text for details).

In Vitro Embryo Production

All reagents and media used in the present experiment were obtained from Sigma Chemical Co. (Dorset, UK) unless otherwise stated. Animal identity was retained throughout. Tubes containing aspirants were transported in a polystyrene box (at 37°C) to the laboratory, where the aspirants were filtered through sterile, 70-μm nylon gauze (BD Falcon, BD Biosciences, Oxford, UK) into PBS supplemented with 0.2% (w/v) BSA fraction V and antibiotics (50 IU/ml of penicillin, 50 μg/ml of streptomycin sulfate, and 100 μg/ml of neomycin), and cumulus-oocyte complexes (COCs) were graded according to the number of compact cumulus cell layers and granulation of the oocyte cytoplasm as described previously [10]. Only grade-1 through grade-3 COCs were selected for maturation. Selected COCs were washed twice in maturation medium (bicarbonate-buffered TCM199 with Earle salts supplemented with 0.4% [w/v] BSA fraction V, 10 μg/ml of FSH, 10 μg/ml of LH, and antibiotics [50 IU/ml of penicillin and 50 μg/ml of streptomycin sulfate]) and transferred into 50-μl drops of the same medium with granulosa cell monolayers (≤20 oocytes/drop) overlaid with mineral oil. The granulosa cell monolayers were prepared from a single batch of frozen-thawed granulosa cells used throughout the experiment that were cultured for 4 days before each OPU session, with half the medium being renewed every 48 h. Oocytes were matured for 24 h at 38.8°C in a humidified atmosphere of 5% CO_2 in air.

Frozen semen from a single bull was thawed at 35°C and layered (85 μl) under 1 ml of a modified calcium-free Tyrode albumin lactate pyruvate medium (TALP; capacitation medium; 0.6% [w/v] BSA fraction V, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin sulfate; pH 7.4) in conical tubes for a swimup procedure [11]. The upper 0.7 ml of medium was then collected after incubation for 0.5 h at 38.8°C. The pooled medium containing spermatozoa was centrifuged (300 × *g*) for 10 min, and the supernatant was removed, leaving the pellet in approximately 200 μl of capacitation medium. The pellet was resuspended, and motile spermatozoa were counted and added in a maximum of 4 μl of capacitation media to a 46-μl drop of fertilization media to give a final concentration of 1×10^6 motile spermatozoa/ml. Fertilization medium was a modified TALP [12] supplemented with 0.2 nmol/ml of penicillamine, 0.1 nmol/ml of hypotaurine, 0.02 nmol/ml of epinephrine, 0.6% (w/v) BSA (fatty acid-free), 30 μg/ml of heparin, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin sulfate (pH 7.8). Following maturation, most of the cumulus cells were removed by gentle pipetting, and oocytes were washed twice in Hepes-buffered TALP supplemented with 0.3% (w/v) BSA fraction V, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin sulfate. Matured oocytes were placed in a 46-μl drop of fertilization media. The oocytes and spermatozoa were incubated in 50-μl drops under mineral oil (up to 20 ova/drop) for 22 h at 38.8°C in a humidified atmosphere of 5% CO_2 in air.

Following in vitro insemination, putative zygotes were removed and washed twice in synthetic oviduct fluid medium [13] containing 25 μmol/ml of $NaHCO_3$, 108 μmol/ml of NaCl, 7.2 μmol/ml of KCl, 1.7 μmol/ml of $CaCl_2$, 1.2 μmol/ml of KH_2PO_4, 0.5 μmol/ml of $MgCl_2$, 1.5 μmol/ml of glucose, 3.3 μmol/ml of sodium pyruvate, 33 μmol/ml of sodium lactate, 1 μmol/ml of L-glutamine, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin sulfate supplemented with 0.3% (w/v) BSA fatty acid-free, 1% (v/v) nonessential amino acids, and 2% (v/v) essential amino acids.

Groups of up to 20 putative zygotes were then placed in 20-μl microdrops of the culture medium in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.8°C, and droplets were renewed every 48 h. On Day 2 of development (Day 0 = day of in vitro fertilization), the cleavage rate was recorded, and cleaved zygotes were then cultured in 20-μl microdrops until Day 8. On Day 8, blastocyst yield, blastocyst quality [14], and blastocyst developmental stage were recorded. Day-8 blastocysts were then fixed individually in 1 ml of 3:1 acetic acid:ethanol solution per well in 24-well plates, stained with 10 μg/ml of Hoechst 33342 solution, and immediately visualized by fluorescence microscopy. Total cell number per blastocyst was recorded.

Glucose Tolerance and Insulin Resistance

An assessment of glucose tolerance was conducted during an induced estrus within 3 wk of the end of period 2. Estrus was induced on Day 12 of the estrous cycle following i.m. administration of Luprositol (15 mg) on Day 9. On the day before the glucose tolerance test, flexible catheters (18 gauge) were placed into the jugular vein of each heifer. The glucose tolerance test commenced 5 h after the animal's morning meal with an i.v. infusion of sterile glucose solution (Glucose 40% [w/v]; Arnolds Veterinary Products Ltd., Shrewsbury, UK) at 0.3 g/kg. Three blood samples, collected at 20-min intervals before glucose infusion, established glucose and insulin baselines. Glucose infusion was completed within 5 min, and this was followed by the collection of further blood samples at 5, 10, 20, 30, 40, 60, 80, 100, and 120 min postinfusion. All blood samples were collected into heparinized vacuum tubes and stored on ice until centrifugation (15 min at 600 × g). Plasma was transferred into cuvettes and stored at −20°C until analysis. Plasma samples were analyzed for glucose using a BMD/Hitachi 705 autoanalyzer. The kit for glucose was supplied by Randox Laboratories Ltd. (Glucose GOD PAP; catalog no. GL2623).

Hormone analysis

Plasma insulin concentrations were determined using a ^{125}I-labeled insulin double-antibody RIA based on the method of Starr et al. [15]. The assay was modified to use porcine insulin (I-3505; 24 IU/mg) obtained from Sigma Co. Ltd. (UK) and guinea pig anti-porcine insulin, normal guinea pig serum, and sheep anti-guinea pig immunoglobulin G obtained as a gift from the Scottish Antibody Production Unit, Law Hospital (Carluke, Lanarkshire, Scotland). The [^{125}I]porcine insulin solution was prepared using the chloramine T method [16]. The minimum detection limit of the assay (defined at the 85% effective dose) was 2.6 μIU/ml. The interassay coefficients of variation for low, medium ,and high controls were 8.2%, 9.0%, and 10.0%, respectively, and the corresponding intra-assay coefficients of variation were 6.7%, 7.9%, and 7.1%, respectively.

Plasma IGF1 concentrations were determined using a [^{125}I]IGF1 double-antibody RIA based on the method of Armstrong et al. [17]. Plasma IGF1 was first separated from its binding proteins by acid-gel high-performance liquid chromatography according to the method of Owens et al. [18]. The RIA used recombinant human IGF1 and anti-human IGF1 polyclonal antiserum (rabbit), both obtained from GroPep Ltd. (Adelaide, Australia); normal rabbit serum; and donkey anti-rabbit immunoglobulin G obtained from Diagnostics Scotland (Law Hospital, Carluke, Scotland). The [^{125}I]IGF1 solution was prepared using the chloramine T method [16]. All samples analyzed for IGF1 concentrations were processed in a single assay with a detection limit of 12.5 ng/ml and in which the coefficients of variation for low- and high-quality controls were 11.7% and 8.4%, respectively.

Plasma leptin concentrations were determined in duplicate using the method of Blache et al. [19]. The primary antibody, normal emu serum, and sheep anti-emu serum were both provided by Dr. Blache (University of Western Australia, Perth, Australia). Ovine leptin (supplied by Dr. Keisler, University of Missouri, Columbia, MO) was iodinated in-house as described above. The detection limit for leptin was 0.2 ng/ml, and the interassay coefficients of variation for low, medium, and high controls were 13.0%, 9.7%, and 10.5%, respectively. The corresponding intra-assay coefficients of variation were 12.8%, 9.9%, and 10.3%, respectively.

Data analysis

Changes in animal live weight, body condition, and plasma hormone concentrations during the three successive estrous cycles were analyzed by repeated-measures ANOVA within GenStat 6 [20], in which the main effects of body condition (low vs. moderate), level of feeding (M vs. 2M), period (1 vs. 2), week within period (OPU sessions 1–6), and all interactions were tested. Residual variation was partitioned into between-animal variation, period-within-animal variation, and week-within-period variation. The within-animal degrees of freedom were adjusted using the calculated Greenhouse-Geiser ε to account for nonindependence of measurements between weeks in the same animal. The growth rate of the largest follicle during each of the six estrous cycles was calculated for each animal by regressing follicle diameter against day and then analyzing the regression coefficients by ANOVA. Data represent the predicted means with either appropriate standard error of difference (SED) or SEM.

Oocyte recovery and in vitro embryo production data (i.e., oocytes cleaved and Day-8 blastocyst yields) were analyzed using generalized linear models assuming binomial errors with logit link functions within the same statistical package. Terms fitted to the model were animal, period, body condition, level of feeding, and OPU session within period. Comparisons between means were conducted by analysis of deviance. Data are presented as predicted means from these models. Changes in blastocyst yields during the 7-wk experimental period (i.e., for Weeks 1, 2, 4, 5, 7, and 8 during which OPU was conducted) are presented as the change in logit proportions, with time (t) expressed as weeks.

Glucose tolerance was calculated according to the method described by Opsomer et al. [21] and was determined as fractional glucose turnover rate (k) and the half-life ($T_{½}$) starting at 5 min after the infusion (T_5). The k value represents the clearance of infused glucose and was calculated as

$$k = ((\text{glucose } T_{10} - \text{glucose } T_{40})/(T_{40} - T_{10}))\cdot 100 = \%\text{min}$$

The glucose $T_{½}$ is the time required for the glucose concentration to fall by half. Using this k value, the glucose $T_{½}$ was calculated as

$$T_{½} = (0.693/k)*100 = \text{min}$$

The insulin response following glucose infusion was demonstrated as the insulin peak concentration and ΔMax (insulin peak level − insulin basal level). All parameters of the glucose tolerance test were tested for normality using the Ryan-Joiner test statistic and then analyzed by ANOVA.

RESULTS

Live Weight and Body Condition

Mean live weight and BCS were lower ($P < 0.001$) at the beginning of each of the two experimental periods for heifers allocated to the low compared to those in the moderate BCS groups (Table 2). As anticipated, during the experimental period, animals fed at the M level retained their live weight and BCS. In contrast, for heifers fed at the 2M level, live weight and BCS increased ($P < 0.001$) (Table 2). The pattern and extent of the increases in live weight and BCS were the same for both periods of study.

Metabolic Hormones

Both moderate BCS and the 2M level of feeding increased ($P < 0.001$) plasma insulin concentrations compared to low BCS and the M level of feeding (Table 2). Mean plasma insulin concentrations did not differ between cycles but increased ($P < 0.001$) during the days leading up to estrus in each cycle (mean values of 21.0, 26.9, and 28.4 μIU/ml for the 2 days preceding and the day of estrus, respectively; SED = 1.50).

Mean plasma IGF1 concentrations increased ($P < 0.01$) with successive estrous cycles (230.4, 246.2, and 249.8 ng/ml; SED = 6.34) and were greater ($P < 0.05$) for heifers with moderate compared to low BCS (Table 2). However, mean IGF1 concentrations were unaffected by the level of feeding. As with insulin, plasma concentrations of IGF1 peaked ($P < 0.001$) on the day of estrus (mean values of 227.5, 242.9, and 255.9 ng/ml for the 2 days preceding and the day of estrus, respectively; SED = 4.23). An interaction ($P < 0.01$) was observed between BCS, level of feeding, and day of cycle on plasma IGF1 concentrations, which indicated that the increase in plasma IGF1 concentrations in the days leading up to estrus was greatest in low-BCS heifers fed at the 2M level (207.3, 226.9, and 253.9 ng/ml; SED = 12.68).

TABLE 2. Mean body condition score (BCS), live weight, and plasma concentrations of metabolic hormones during the experimental periods.

	Low BCS (n = 24)[a]		Moderate BCS (n = 24)[a]			*P* value		
Parameter	M	2M	M	2M	SED	BCS	F[b]	BCS × F
Body condition (units)								
Observations/animal	1	1	1	1				
Initial	2.0[c]	2.0[c]	3.7[d]	3.7[d]	0.12	<0.001		
Final	2.0[c]	2.9[d]	3.6[e]	4.1[f]	0.12	<0.001	<0.001	<0.050
Live weight (kg)								
Observations/animal	1	1	1	1				
Initial	394[c]	390[c]	516[d]	517[d]	16.60	<0.001		
Final	401[c]	480[d]	524[e]	605[f]	16.60	<0.001	<0.001	
Metabolic hormones								
Observations/animal	9	9	9	9				
Insulin(μIU/ml)	10.8[c]	22.2[d]	18.0[cd]	48.3[e]	4.70	<0.001	<0.001	<0.010
IGF1 (ng/ml)	224.3[c]	229.4[c]	267.0[d]	247.7[cd]	15.30	<0.050		
Leptin (ng/ml)	1.8[c]	3.2[cd]	4.4[d]	6.7[e]	0.66	<0.001	<0.001	<0.050

[a] n = number of animals.
[b] F = level of feeding.
[c–f] Means within a row with different superscripts are significantly different ($P < 0.05$).

Mean plasma leptin concentrations were greater ($P < 0.001$) for animals of moderate compared to low BCS and for animals fed at the 2M compared to the M level (Table 2). The interaction ($P < 0.05$) between BCS and level of feeding indicated that mean plasma leptin concentrations did not differ significantly between levels of feeding for animals of low BCS but did differ between levels of feeding for animals of moderate BCS. Mean plasma leptin concentrations increased ($P < 0.001$) with time (cycle number) on experiment (3.8, 4.0, and 4.3 ng/ml for cycles 1, 2, and 3, respectively; SED = 0.09), and an interaction ($P < 0.001$) was observed between level of feeding and cycle number, indicating that plasma leptin concentrations increased with time for heifers on the 2M (4.5, 4.9, and 5.4 ng/ml; SED = 0.25) but not for heifers on the M (3.1, 3.0, and 3.2 ng/ml; SED = 0.25) diets. In contrast to insulin and IGF1, plasma leptin concentrations did not vary over the 6-day period leading up to estrus in any of the three cycles averaged over both periods.

Glucose Tolerance Test

Baseline glucose concentrations were greater ($P < 0.01$) for heifers fed at the 2M compared to the M level but did not differ between BCS groups (Table 3). Peak concentrations of glucose following glucose infusion were greater ($P < 0.05$) for heifers of moderate compared to low BCS, and peak concentrations also were greater ($P < 0.06$) for heifers fed at the M compared to the 2M level. Glucose clearance rate (k) was greater ($P < 0.05$), and the half-life ($T_{½}$) was lower ($P < 0.05$), for animals of moderate compared to low BCS.

Before glucose infusion, baseline insulin concentrations were greater ($P < 0.01$) for animals fed at the 2M compared to the M level, and baseline insulin concentrations were greater ($P < 0.06$) for animals in moderate compared to low BCS (Table 3). However, mean treatment differences in peak insulin concentrations and the increase in insulin concentration (ΔMax) following glucose infusion were not significantly different, although they were greater ($P < 0.05$) for heifers in the moderate-BCS/2M group than for those in the low-BCS/M group.

Follicular Growth

The total number of visible follicles per animal averaged 12.8 ± 0.23 and did not differ between treatments (Table 4). However, the number of large (>8 mm) follicles was greater ($P < 0.01$) for animals fed at the 2M compared to the M level. Similarly, the number of medium-sized (4–8 mm) follicles was greater ($P < 0.01$) for animals fed at the 2M compared to the M level. The number of small (<4 mm) follicles averaged 7.6 ± 0.23 and was unaffected by treatments. The growth rate and maximum diameter of the dominant follicle, as monitored over the 6-day period before estrus in each cycle, were greater for heifers fed at the 2M than at the M level.

TABLE 3. Plasma glucose and insulin response following an intravenous infusion of glucose (0.3 g/kg of body weight).

	Low BCS (n = 12)[a]		Moderate BCS (n = 12)[a]			Significance of effect		
Parameter	M	2M	M	2M	SED	BCS	F[b]	BCS × F
Basal glucose[c] (μmol/ml)	3.70[d]	4.00[e]	3.80[de]	4.00[e]	0.14		<0.05	
Peak glucose (μmol/ml)	17.40[d]	16.70[d]	19.10[e]	17.60[d]	0.69	<0.05	<0.06	
k (%/min)	21.20[d]	21.00[d]	26.70[e]	22.10[d]	1.73	<0.05	0.06	<0.10
T½ (min)	3.30[d]	3.30[d]	2.60[d]	3.20[e]	0.23	<0.05	0.07	
Basal insulin[c] (μIU/ml)	20.10[d]	37.90[de]	30.30[d]	55.40[e]	9.71	<0.06	<0.01	
Insulin peak (μIU/ml)	178.30[d]	239.50[de]	237.20[de]	367.30[e]	79.67			
ΔMax (μIU/ml)	158.20[d]	201.60[de]	206.90[de]	311.90[e]	71.56			

[a] n = number of animals.
[b] F = level of feeding.
[c] Basal samples collected 4 h after the morning feed.
[d,e] Means within a row with different superscripts are significantly different ($P < 0.05$).

TABLE 4. Mean follicle number, maximum diameter of the dominant follicle (DF) during the 6-day period prior to ovulation in each cycle, and the growth rate of this DF.

	Low BCS (n = 24)[a]		Moderate BCS (n = 24)[a]			*P* value		
Parameter	M	2M	M	2M	SED	BCS	F[b]	BCS × F
Observations per animal	3	3	3	3				
Follicle number								
Total	13.00	12.70	12.20	13.30	0.980			
Small (<4 mm)	8.30	7.30	7.40	7.30	0.890			
Medium (4 to 8 mm)	3.90	4.40	3.80	4.50	0.500		<0.010	
Large (>8 mm)	0.70[c]	1.10[c]	1.00[c]	1.50[d]	0.280		<0.010	
Dominant follicle								
Maximum diameter (mm)	11.80[c]	12.90[cd]	12.60[cd]	13.80[d]	0.580		<0.001	
Growth rate (mm/day)	1.13[c]	1.39[d]	1.28[c]	1.42[c]	0.126		<0.050	

[a] n = number of animals.
[b] F = level of feeding.
[c,d] Means within a row with different superscripts are significantly different ($P < 0.05$).

Oocyte and Embryo Development In Vitro

The number of follicles observed before OPU averaged 11.9 ± 0.24 per animal and did not vary between treatments. The mean recovery rate (percentage of oocytes recovered per follicle aspirated) throughout the experimental period averaged 53.6% ± 1.16%, and an average of 5.8 ± 0.21 oocytes were recovered per animal. Of these, 5.3 ± 0.19 oocytes per animal were selected for maturation. None of these parameters was affected by the experimental treatments.

Cleavage rates averaged 58.1% ± 1.76%, and these also did not differ significantly between treatments. An interaction ($P < 0.05$) was observed, however, between BCS and level of feeding on mean blastocyst yield (Day-8 blastocysts of oocytes cleaved), indicating that blastocyst yields were lower for the two extreme nutritional combinations (i.e., low-BCS/M vs. moderate-BCS/2M) (Fig. 2a). An interaction ($P < 0.05$) also was observed between BCS, level of feeding, and OPU session, which indicated that as the six sessions of OPU (averaged over both periods) progressed, blastocyst yields decreased for heifers of moderate BCS fed at the 2M level relative to heifers of low BCS fed at the 2M level (Fig. 2b). Total cell number per Day-8 blastocyst did not differ significantly between treatments (mean ± SEM, 105.3 ± 3.85).

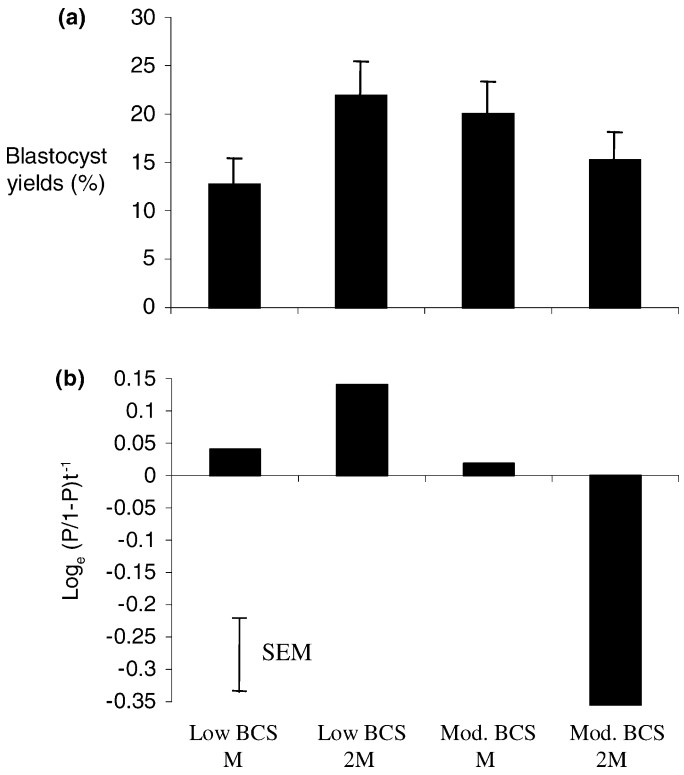

FIG. 2. Postfertilization development in vitro, presented as (**a**) mean (± SEM) blastocyst yields (%) and (**b**) mean (± SEM) change in blastocyst yields (change in logit proportion with time [wk]) over the six sessions of OPU spanning 7 wk.

Metabolic Hormones and Oocyte Quality

Plasma insulin concentrations across treatments were positively correlated with both BCS ($r = 0.63$, $P < 0.01$) and plasma leptin concentrations ($r = 0.71$, $P < 0.001$). Plasma leptin concentrations also were positively correlated ($r = 0.72$, $P < 0.001$) with BCS, as were plasma IGF1 concentrations ($r = 0.42$, $P < 0.05$). As a consequence of these relationships, and because of the positive effects of feeding level on both plasma insulin and leptin concentrations (Table 2), none of these terms (i.e., insulin, IGF1, and leptin) had any additional effect, nor did they reduce the error terms substantially when fitted to multiple-regression models of cleavage rate and blastocyst yield that included animal BCS and feeding level as factors.

To assess the effects of high concentrations of plasma insulin and leptin on oocyte quality, a similar regression approach was initially applied to a restricted data set that included all animals within the moderate-BCS/2M group (n = 12). This analysis indicated no effect of plasma leptin within this subgroup of animals on postfertilization development of oocytes. Similarly, no effect of insulin on cleavage rates was shown. However, a negative relationship was found between plasma insulin concentrations and blastocyst yield (regression coefficient = −0.035 ± 0.012, $P < 0.05$). Furthermore, an interaction ($P < 0.01$) was observed between plasma insulin concentrations and OPU session on blastocyst yield such that relative to the first OPU session, in which the regression coefficient for blastocyst yield against insulin was 0.024 ± 0.016, corresponding coefficients for OPU sessions 2–6 were −0.004 ± 0.028, −0.147 ± 0.070, −0.357 ± 0.165, −0.504 ± 0.117, and −0.710 ± 0.312, respectively. To confirm these observations, a hyperinsulinemic state was formally designated as fasting

TABLE 5. Differences between heifers in the moderate BCS, 2M treatment group with plasma insulin concentrations either within the normal range (normal) or >3 SD above the mean of heifers in the low BCS 2M and moderate BCS M treatment groups (hyperinsulinemic).

Parameter	Normal (≤37.20 μIU/ml)	Hyperinsulinemic (>37.20 μIU/ml)	*P*
No. of animals	4	8	
Metabolic hormones			
Observations per animal	18	18	
Insulin (μIU/ml)	29.00 ± 5.16	58.00 ± 3.65	<0.010
IGF1 (ng/ml)	255.10 ± 7.56	244.10 ± 5.34	
Leptin (ng/ml)	7.10 ± 0.41	6.50 ± 0.28	
Follicular development			
Observations per animal	6	6	
No. of follicles			
Total	16.00 ± 0.93	12.00 ± 0.58	<0.050
Large	1.30 ± 0.22	1.60 ± 0.26	
Medium	6.30 ± 0.59	3.50 ± 0.33	<0.001
Small	8.30 ± 0.50	6.80 ± 0.60	
Maximum diameter of DF (mm)	13.40 ± 0.77	14.00 ± 0.67	
Growth rate of DF (mm/day)	1.30 ± 0.04	1.40 ± 0.10	
Oocyte and early embryo development in vitro			
Observations per animal	12	12	
Oocytes matured (n)[a]	7.10 ± 0.67	4.90 ± 0.50	<0.010
Cleavage rates (%)	65.90 ± 4.17	62.30 ± 4.13	
Blastocysts of cleaved (%)	26.20 ± 4.53	13.40 ± 3.56	<0.050

[a] n = number of oocytes.

plasma insulin concentrations more than three standard deviations above the combined mean of animals in the low-BCS/2M and moderate-BCS/M groups; animals in these groups had moderate concentrations of plasma insulin (Table 2). This formal designation revealed that 8 of the 12 heifers in the moderate-BCS/2M group were hyperinsulinemic. Neither plasma IGF1 nor plasma leptin concentrations differed between the eight hyperinsulinemic and four ''normal'' animals within this treatment group (Table 5). However, the hyperinsulinemic animals had fewer follicles (particularly medium-sized follicles), produced significantly fewer oocytes, and had lower blastocyst yields following in vitro fertilization and embryo culture.

DISCUSSION

The major new findings of the present study are as follows: 1) The effects of level of feeding on oocyte quality are dependent on the initial BCS of the heifer, 2) the effects of high levels of feeding on oocyte quality over time are cumulative, and 3) high levels of feeding to moderately fat heifers can lead to hyperinsulinemia and impaired oocyte quality. In terms of postfertilization development to the blastocyst stage, the high level of feeding (2M) was beneficial for animals of low body condition (BCS 2 on the six-point scale of Lowman et al. [8]) but, importantly, was detrimental for animals in moderately high body condition (BCS 3.5 or more on the same scale). Furthermore, blastocyst yields for relatively thin heifers on the 2M level diverged from that of relatively fat heifers on the same level of feeding over the six sessions of OPU. Until now, the general consensus has been that high planes of nutrition in nonpregnant, nonlactating ruminants are detrimental for oocyte quality, with low postfertilization developmental rates in sheep [22–24] and cattle [6, 7, 25]. A consistent feature of these reports, however, was that animals were in moderately good (preslaughter) body condition and, consequently, were akin to the moderate BCS group of the present study, in which the effects of high levels of feeding also had a detrimental effect on oocyte quality. In contrast, the effects of high levels of feeding in relatively thin heifers from the present study were beneficial. Finally, the present study produced evidence, to our knowledge for the first time in ruminants, to indicate that a significant proportion (8/12) of the moderately fat animals on the high level of feeding were hyperinsulinemic and that these very high plasma insulin concentrations were associated with impaired oocyte quality. Regression analysis further revealed that the negative effects of insulin on blastocyst yields in this population of moderately fat and well-fed animals increased with time on experiment.

Endocrinology

The factorial nature of the present study was purposely designed to create three groups of animals with low (~10 μIU/ml), moderate (~20 μIU/ml), and high (~50 μIU/ml) mean basal plasma insulin concentrations (Table 2), which are representative of the broad range of concentrations encountered in practice, with the lower values representative of those recorded with lactating cattle (2–18 μIU/ml) [26, 27]. The greater fasting (48 μIU/ml) and nonfasting (55 μIU/ml) plasma insulin concentrations associated with the fatter and better-fed animals are indicative of increased peripheral insulin resistance [28]. However, such animals remained capable of adequately metabolizing an i.v. infusion of glucose (0.3 g/kg) by further increasing peripheral circulating concentrations of insulin (Table 3). Glucose infusion has previously led to peak plasma insulin concentrations of approximately 210 μIU/ml in obese, nonlactating sheep [28]; 200 μIU/ml in midlactation dairy cows [21]; and 200 μIU/ml in obese, maiden heifers [29]. These values are broadly in line with those for low-BCS/2M and moderate-BCS/M heifers in the present study. The more extreme insulin values observed for moderate-BCS/2M heifers in the present study (basal, 55 μIU/ml; post-glucose infusion, 367 μIU/ml) are greater than those reported previously for ruminants and more akin to the extreme values reported for women with polycystic ovarian syndrome (PCOS; fasting insulin, 40–52 μIU/ml; peak insulin post-glucose infusion, 400–650 μIU/ml) [30]. Indeed, at the time of the glucose tolerance test, one animal in the present study had basal insulin concentrations of approximately 100 μIU/ml and a peak post-glucose infusion insulin con-

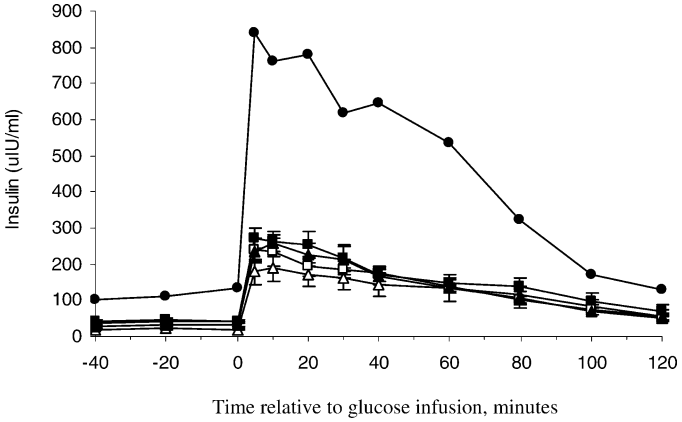

FIG. 3. Response (mean ± SEM) in plasma concentrations of insulin following infusion of 0.3 g/kg of 40% glucose. The extreme hyperinsulinemic animal is indicated (black circle). Treatments are indicated as follows: low BCS, M (white triangle) and 2M (white square); moderate BCS, M (black triangle) and 2M (black square).

centration of approximately 850 μIU/ml (Fig. 3). Closer inspection of the plasma insulin data over the 12 sessions of OPU for this group of animals identified several other single-sample occasions with basal (fasting) insulin concentrations as high as 111 μIU/ml.

Inevitably, the present experimental design also led to the creation of three groups of animals with low (~2 ng/ml), moderate (~4 ng/ml), and high (~7 ng/ml) mean plasma leptin concentrations (Table 2); therefore, the effects of insulin and leptin on follicular growth and oocyte quality cannot be easily separated. As with the other mammalian species examined, plasma leptin concentrations in ruminants are related to body fatness and level of feeding [31]. This latter effect can be attributed, at least in part, to the combined actions of insulin on leptin gene expression [32] and insulin-stimulated glucose uptake by adipocytes [33]. Surprisingly, we detected only a very modest effect of BCS on plasma IGF1 concentrations in this study. We had previously observed, using the same assay, a significant effect of feeding level on plasma concentrations of this growth factor, paralleling that of insulin [7].

Ovarian Folliculogenesis

Working with a heifer genotype similar to that used in the present study, Gong et al. [34] reported a significant increase in the number of small (2–4 mm) follicles, leading to an increase in the number of large (>9 mm) follicles following FSH treatment in heifers fed at the 2M compared to the M level. The population of medium-sized follicles was unaffected by the level of feeding in that study. Although broadly in agreement with the present results (Table 4), the apparent discrepancy with regard to the effects of diet on the number of follicles within each size category between studies may be caused by differences in the duration of exposure to the levels of feeding (3 wk in the study of Gong et al. [34] vs. a means of approximately 6, 9, and 12 wk in the present study). Similarly, Armstrong et al. [35] failed to observe any effect of feeding level (M vs. 2M) on granulosa cell proliferation in vitro, but the BCS of their animals was closer to that of the moderate-BCS group of heifers in the present study.

Body condition and level of feeding influence a number of factors that regulate ovarian follicular development, granulosa cell proliferation, and steroidogenesis. In this regard, the effects of insulin are particularly well documented. Insulin stimulates bovine granulosa [36] and thecal [37] cell proliferation, as well as steroidogenesis in vitro [38, 39]. Similarly, IGF1 stimulates granulosa cell proliferation and steroidogenesis in vitro [40, 41]. In contrast, leptin inhibits gonadotroph and/or insulin-induced steroidogenesis by granulosa and thecal cells in vitro, although it does not seem to be able to affect granulosa cell proliferation at physiological levels [42, 43]. The present study can shed no further light on these mechanisms, but it highlights the need to better understand the potentially interactive effects of BCS and level of feeding on granulosa cell proliferation and physiology.

Postfertilization Development

The highest blastocyst yields in the present study were for the treatment combinations low-BCS/2M and moderate-BCS/M (Fig. 2). These treatment combinations were associated with moderate concentrations of both plasma insulin and leptin (Table 2). It is tempting to speculate that moderate plasma concentrations of these two metabolic hormones are associated with improved postfertilization embryo development, whereas either low or high concentrations are associated with impaired postfertilization embryo development. This conclusion would certainly be consistent with the data of Armstrong et al. [7]. Regression analysis in which these two terms were fitted to a model that included BCS and feeding level as factors, however, indicated that neither plasma insulin nor plasma leptin could account for any substantial variation in postfertilization development that had not already been explained by these factors.

Hyperinsulinemia and Oocyte Quality

To our knowledge, this is the first report of hyperinsulinemia associated with impaired oocyte quality in ruminants. Indeed, hyperinsulinemia is poorly studied in ruminants, and little information is available concerning the effects of elevated plasma insulin concentrations on the postfertilization developmental potential of oocytes in cattle and sheep. Fasting plasma insulin concentrations for the eight hyperinsulinemic animals identified in the present study (58 μIU/ml) were greater than those previously reported for obese cattle and sheep (24–33 μIU/ml) [28, 29] but comparable to the extreme values (40–52 μIU/ml) reported in women with PCOS by Dunaif [30]. Indeed, in human subjects, the median (interquartile range) of serum insulin concentrations are 12 (10–18) and 27 (16–43) μIU/ml for normal and weight-matched anovulatory women with PCOS, respectively [44]. Large (14–20 mm), persistent follicles were observed in four of the eight hyperinsulinemic animals on separate occasions following OPU during the six estrous cycles in the present study (data not shown). Large, persistent follicles yield developmentally less-competent oocytes [45], but because these follicles were not present at the time of follicular aspiration, this mechanism cannot explain the low blastocyst yields following OPU and in vitro fertilization in the present study. Instead, the data suggest that elevated concentrations of insulin in these animals may have had a more direct, negative impact on the follicle-enclosed oocyte, thus impairing postfertilization development. A causal mechanism for insulin is suggested, because the negative association of elevated plasma insulin concentrations with postfertilization development occurred in the absence of differences in circulating IGF1 and leptin (Table 5). This negative association also increased with time on

experiment, suggesting that the cumulative effects of nutritional status reported in Figure 2 may result, at least in part, from the long-term, detrimental effects of high insulin concentrations on oocyte development. This raises the interesting, albeit speculative, idea that nutritional status and/or plasma insulin may exert greater effects on oocytes within preantral follicles. Alternatively, duration of exposure may be important.

Our laboratory has demonstrated previously that high-plane feeding, resulting in elevated plasma concentrations of both insulin and IGF1 in heifers, leads to impaired embryo development following in vitro maturation and fertilization of oocytes recovered from these animals [7]. These observations were associated with reduced steady-state concentrations of mRNA encoding IGF-binding protein-2 and -4 and the insulin receptor locally within small (<4 mm) antral follicles. Evidence suggests that expression of the IGF1 receptor also was reduced but did not reach statistical significance. Although it was hypothesized that the expected increase in the bioavailability of intrafollicular IGF1 and -2 may have been detrimental to the developing oocyte, the precise mechanisms of such an effect are not understood, and it is not known if similar effects were operating in the present study. However, peripheral concentrations of insulin for the eight hyperinsulinemic animals in the present study were significantly greater than those reported earlier. At present, the detrimental effects of hyperinsulinemia on the follicle-enclosed oocyte are not understood. However, Eppig et al. [46] showed that the coincubation of insulin (~126 mIU/ml) and FSH with murine oocyte-granulosa cell complexes derived from preantral follicles reduced the percentage of fertilized oocytes developing into blastocysts, and this observation is broadly in keeping with that reported here.

In conclusion, results from the present study show that the effects of level of feeding on oocyte quality are dependent on the body condition of the animal. High levels of feeding improve postfertilization development for animals in low body condition but reduce postfertilization development for animals in good body condition. Furthermore, the effects of high levels of feeding are cumulative, with blastocyst yields for heifers in moderately good body condition deteriorating over time relative to heifers in low body condition. The present study also shows, to our knowledge for the first time, evidence of impaired oocyte quality associated with hyperinsulinemia in ruminants. The data presented here establish a nutritional framework to enable quantifiable assessments of future dietary effects on oocyte quality in cattle, and they form the basis for further detailed studies into the underlying mechanisms of such effects.

ACKNOWLEDGMENTS

The authors would like to express their gratitude to J.C. Rae and staff for animal supervision and to A. Thain and M. Mitchell for assistance with hormone analyses. The authors gratefully acknowledge the statistical advice given by Dr. Jim Craigon.

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