Interrelationships of growth hormone *Alu*I polymorphism, insulin resistance, milk production and reproductive performance in Holstein-Friesian cows

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ABSTRACT: Healthy multiparous Holstein-Friesian cows (n = 22, parity: 2–4) from a large-scale dairy herd in Hungary were subjected to an intravenous glucose tolerance test 10–15 days after calving. *Alu*I genotype of growth hormone, several plasma metabolites and metabolic hormones were determined, and current and previous lactation yields were recorded. We also used the Revised Quantitative Insulin Sensitivity Check Index (RQUICKI) and its modified version ($RQUICKI_{BHB}$) for the estimation of peripheral insulin sensitivity. The majority of cows (n = 18) was leucine homozygous (LL), four were heterozygous (LV) and there were no valine homozygous (VV) animals in the population. Current average milk production was not different between *Alu*I genotypes, but LV cows tended to have higher 305-day previous lactation yields (P = 0.13). *Alu*I polymorphism was not associated with any of the calculated glucose and leptin parameters of the intravenous glucose tolerance test (P > 0.58). Heterozygous cows were prone to higher basal insulin levels (P = 0.064), longer time to reach half of the maximal and basal insulin concentrations (P = 0.035 and P = 0.054, respectively) and larger insulin area under the curve (P = 0.032). Both RQUICKI and $RQUICKI_{BHB}$ estimated decreased insulin sensitivity in LV compared to LL cows (P = 0.055 and P = 0.044, respectively). Higher plasma NEFA and BHB levels accounted for slower glucose disappearance and lower insulin release and insulin clearance rate (P < 0.05). Average yield was inversely related to glucose area under the curve (P = 0.040) and time to reach baseline concentration (P = 0.005). Plasma cortisol lowered glucose clearance rate (P = 0.040) and prolonged time to reach basal levels (P = 0.006). More weight loss was associated with higher glucose peak and prolonged glucose disappearance time (P = 0.055 and P = 0.024, respectively). All cows became cyclic and showed signs of estrus during the study period. There were no differences between leucine homozygous and heterozygous animals in the onset of ovarian activity and in the time of first observed estrus (P > 0.540). We conclude that Holstein-Friesian cows heterozygous for *Alu*I polymorphism of the growth hormone gene may be more likely to develop insulin resistance during early lactation than leucine homozygous cows. Decreased insulin sensitivity could be part of a homeorhetic adaptation process that supports nutrient partioning for the use of the mammary gland and may allow LV cows to reach higher yields throughout lactation.

Keywords: growth hormone; *Alu*I polymorphism; dairy cow; postpartum; glucose tolerance test; first ovulation

In high-yielding postpartum (PP) dairy cows insulin resistance (IR) develops to help directing nutrients from insulin sensitive tissues such as skeletal muscle and adipose tissue to the lactating mammary gland (Holtenius and Traven, 1990; Bell, 1995). Insulin response to glucose challenge was

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depressed, while glucose clearance rate increased after calving compared to prepartum values possibly due to greater glucose utilization by the udder (Holtenius et al., 2003; Bossaert et al., 2008). However, PP increase in glucose disappearance rate was smaller in cows fed high energy diet before calving due to IR in fat cows (Holtenius et al., 2003). Lactating dairy cows had lower pancreatic insulin output and consequently lower hepatic insulin uptake than non-lactating animals (Lomax et al., 1979). They also showed reduced insulin responsiveness to intravenous (*i.v.*) glucose and propionate challenges and a decrease in hepatic glucose output equal to the rate of glucose infusion.

Several endocrine and metabolic factors are implicated in the initiation of an insulin resistant state during early lactation. Growth hormone (GH) seems to be one of these endocrine signals, as GH concentration is usually increased PP and its metabolic effects are antagonistic to insulin by enhancing lipolysis in adipose tissue and gluconeogenesis in the liver (Bell, 1995; Block et al., 2001; Ingvartsen, 2006). After chronic GH treatment blood glucose was increased (Putnam et al., 1999) and insulin resistance occurred within a few hours in insulin-sensitive tissues (Yokota et al., 1998).

Reports on endocrine profiles of *Alu*I genotypes showed conflicting data in growing calves. Basal and stimulated GH release was higher in leucine homozygous (LL) calves (Sorensen et al., 2002; Katoh et al., 2008), while Grochowska (2001) found increased GH release in valine homozygous (VV) animals and higher basal insulin-like growth factor I (IGF-I) levels in LL calves. Leucine homozygous animals excelled in plasma IGF-I and insulin concentrations and VV calves in leptin and triglyceride levels (Katoh et al., 2008). Contradictory results on milk production have been previously achieved favoring the leucine (Lee et al., 1996; Shariflou et al., 2000; Dybus, 2002) or the valine allele (Sabour et al., 1997; Zwierzchowski et al., 2002; Kovacs et al., 2006). Concerning reproductive traits, Lechniak et al. (1999, 2002) did not find a significant relationship between *Alu*I polymorphism and bulls' sperm characteristics or parameters of *in vitro* fertilization and embryo development.

It is possible that the degree of insulin sensitivity may vary between cows carrying the leucine or the valine allele due to differences in GH release and/or in daily milk yield and the early rise of the lactational curve. We are not aware of any reports on the association of *Alu*I polymorphism and insulin resistance in lactating dairy cows. Knowledge on the relationship of reproductive performance with *Alu*I genotype is also limited. Our objectives were to estimate the degree of insulin resistance in Holstein-Friesian cows through an intravenous glucose tolerance test (ivGTT) in the second week of lactation and to investigate whether milk yield and reproductive performance (primarily the resumption of cyclic ovarian activity PP) may vary between *Alu*I genotypes.

MATERIAL AND METHODS

Animals and management

This study was carried out on winter-calving (January–February, 2002) multiparous Holstein-Friesian cows (n = 32, parity: 2–4) in a large-scale dairy herd in Hungary. Animals were kept in free-stall barns partially covered on all sides and had shavings used for bedding. Cows were dried off at 7th month of pregnancy and kept separately in dry-cow pens. During the close-up period (from 21 days before expected due date onwards) they were regrouped and stayed in preparatory pens. Within one week of expected calving date cows were transferred into maternity units (max. four individuals in each) and remained there until Days 4–5 after parturition. Thereafter they returned to the lactating herd and were kept in groups of 50 to 80 animals according to stage of lactation and yield performance (e.g. to the group of fresh cows first, four weeks later to groups of cows producing ≥ 43 kg or 40 kg milk/day). Cows were milked three times a day (6 am, 2 pm, 10 pm) and were fed a total mixed ration (TMR) that was distributed after each milking. Nutrient, mineral and vitamin requirements were calculated according to the National Research Council (2001) recommendations, however, MJ was used instead of Mcal (1 Mcal = 4.184 MJ). Natural ingredients and nutritive value of daily ration are shown in Table 1 and 2. Drinking water was available *ad libitum*. During the periparturient period niacin and propylene-glycol were also added to TMR in order to support liver function.

Study design

On Day 9-14 PP cows were subjected to a general physical examination, vaginoscopy and palpation

Table 1. Natural ingredients of cows' daily ration in kg (average live weight: 700 kg; 3.5% fat-corrected milk)

	Dry cow I	Dry cow II	Fresh cow	Lactation	
				40 kg/day	43 kg/day
Corn silage	11.5	12.5	15.0	18.5	17.5
Grass hay	6.0	5.0	–	–	–
Alfalfa haylage	2.0	1.0	2.0	2.5	2.5
Alfalfa hay	–	–	3.5	3.0	3.0
Chopped alfalfa	–	–	0.5	2.0	1.5
Concentrate I	1.5	–	–	–	–
Concentrate II	–	2.5	–	–	–
Concentrate III	–	–	5.0	8.2	9.0
Wet sugar beet pulp	–	–	2.0	3.0	3.0
Dry sugar beet pulp	–	–	0.5	–	–
Brewer's grain, wet	–	–	0.5	1.0	1.0
Corn gluten	–	0.6	2.6	1.8	2.4
Water	–	–	1.2	1.2	1.2
Molasses	–	–	0.6	0.6	0.6
Additives	–	0.15*	0.4*	–	0.2*

Dry cow I: first 5 weeks of the dry period; Dry cow II: last 3 weeks of the dry period; Fresh cow: from calving to 30 days PP
Concentrate I: 30% corn, 20% barley, 30% sunflower meal, extr. solvent, 10% corn distiller dried grain, 10% mineral-vitamin premix
Concentrate II: 31.3% corn, 20% full-fat soybean, 20% corn gluten, 10% corn distiller dried grain, 14.7 % mineral-vitamin premix, 4% *Saccharomyces cereovisiae* living culture
Concentrate III: 55.5% corn, 10% full-fat soybean, 10% barley, 5% corn distiller dried grain, 4% sunflower meal, extr. solvent, 13.5% mineral-vitamin premix, 2% *Saccharomyces cereovisiae* living culture
*Additives: propylene-glycol, monensin, niacin, cobalt chloride and *Saccharomyces cereovisiae*

per rectum, and those with clinical signs of metabolic disorders and/or inflammatory diseases (e.g. puerperal metritis, mastitis) were excluded (n = 10). Thereafter, healthy cows were weighed and body condition scores (BCS) were assessed the same day and again on Day 24–28 after calving on a 5-point scale with 0.25 unit increments (Skidmore et al., 1997).

On Day 10–15 PP healthy animals were subjected to the intravenous glucose tolerance test and sampled for several plasma metabolites, metabolic hormones, enzymes and *Alu*I genotype determination. The night before the ivGTT animals were moved to a tie-stall barn with straw bedding. Fasting of cows started two hours before and continued throughout the ivGTT.

Daily milk yields of the current lactation between 4–45 days PP for each cow were obtained from recordings of the automated milking system. Postpartum resumption of cyclic ovarian activity was monitored by individual milk progesterone profiles. Day of first observed estrus, days from calving to conception and number of services per conception within the first 200 days PP were also recorded.

Intravenous glucose tolerance test

One hour after the morning milking a basal (0 min) blood (t_0) was drawn from the jugular vein and immediately thereafter 0.15 g/kg body weight (BW) glucose (as an iv infusion of 40% glucose

Table 2. Nutritive value of cows' daily ration

	Dry cow I	Dry cow II	Fresh cow	Lactation	
				40 kg/day	43 kg/day
As fed weight (kg)	21.0	21.8	33.8	42.8	41.9
Dry matter (kg)	12.1	11.8	18.1	22.9	22.7
NE_M (MJ)	71.7	64.2	–	–	–
NE_G (MJ)	42.3	38.5	–	–	–
NE_L (MJ)	68.6	73.8	133.9	167.9	170.9
Crude protein (g)	1 379	1 655	3 303	4 022	4 064
Ether extract (g)	341	398	990	1 362	1 300
Crude fiber (g)	2 961	2 659	2 811	3 461	3 233
Calcium (g)	72	71	182	235	220
Phosphorus (g)	54.5	72.6	82.9	119.1	119.8
Sodium (g)	23	27	38	53	56
Magnesium (g)	34	29	69	96	101
Vitamin A (IU)	112 500	225 000	147 000	195 000	191 000
Vitamin D (IU)	15 000	45 000	36 750	48 750	47 750
Vitamin E (mg)	600	1 500	708	988	998
RUP (% of crude protein)	–	–	40	38	40
NDF (kg)	6.55	5.40	5.86	7.19	6.99
ADF (kg)	3.78	3.06	3.57	4.39	4.22
NSC (kg)	–	–	7.03	9.22	9.24
MPE (g)	1 013	1 160	1 943	2 432	2 505
MPN (g)	902	1104	2101	2574	2633
MPN-MPE (g)	–11.04	–5.56	15.79	14.28	12.87

Dry cow I: first 5 weeks of the dry period; Dry cow II: last 3 weeks of the dry period; Fresh cow: from calving to 30 days PP
NE_M = net energy maintenance; NE_G = net energy gain; NE_L = net energy lactation; RUP = rumen undegraded (intake) protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; NSC = non-structural carbohydrate; MPE = metabolizable protein – energy; MPN = metabolizable protein – nitrogen; MPN-MPE = rumen N-balance

solution given in < 3 min) was administered into the superficial cranial epigastric (mammary) vein. Placement of a jugular catheter was not permitted by the farm management, so blood samples were further collected by jugular venipuncture at 5, 15, 30, 45, 60, 75, 90, 120, 150 and 180 min following glucose infusion.

Handling of blood samples

Collection tubes containing sodium-fluoride were used for the measurement of plasma glucose concentration. Heparinized samples were taken for the analysis of insulin, leptin, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), total cholesterol (TCh), aspartate-aminotransferase activity (AST), thyroxine (T_4), triiode-thyronine (T_3) and cortisol. All sodium-fluoride and heparinized samples were immediately centrifuged (3 000g for 10 min), plasma was harvested, placed into small vials and kept frozen (–20°C) until laboratory analysis. Blood drawn into K_3EDTA containing tubes (Vacuette®; Greiner Bio-One, Kremsmuenster, Austria) at t_0 was immediately frozen and kept at –20°C until evaluation of *Alu*I genotype. Plasma

glucose, insulin and leptin were assayed at all time points of the ivGTT, while BHB, NEFA, TCh, AST, T_4, T_3 and cortisol were measured only at t_0.

Monitoring commencement of ovarian activity

Milk samples (8 to 10 ml) were taken three times a week before the morning milking from 10 to 15 days PP until confirmation of the first luteal phase but no longer than 120 days PP into plastic tubes containing 7.5 mg potassium-dicromate (Reanal Ltd., Budapest, Hungary) for the detection of luteal activity. Samples were kept refrigerated at +4°C until milk progesterone concentration was determined. All assays were carried out within 21 days of collection.

Laboratory procedures

Growth hormone *Alu*I genotype was determined by a polymerase chain reaction, restriction fragment length polymorphism (PCR-RFLP) method (Lucy et al., 1993). Primers were designed to amplify a 428 bp sequence of the bovine GH gene that included 55 bp of the 4th exon, the entire 4th intron, and 99 bp of the 5th exon (Genbank Accession Number J00008; Woychik et al., 1982; Zhang et al., 1992). Digestion of PCR products with *Alu*I restriction endonuclease was conducted at 37°C overnight. DNA fragments were run in 4% high-resolution agarose gels (Cambrex Biosciences, San Diego CA, USA) stained with ethidium bromide and fragments typical of either leucine (265, 96, 51 and 16 bp) or valine (265, 147 and 16 bp) alleles (Lucy et al., 1993) were visualised under UV light.

All metabolites and metabolic hormones were determined from plasma. Analyses of BHB and NEFA were carried out by enzymatic methods (D-3-Hydroxybutyrate kit, Kat. # RB 1007 and NEFA kit, Kat. # FA 115, from Randox Laboratories Ltd, Ardmore, UK). Intra- and interassay coefficients of variation (CVs) were ≤ 5.7% and ≤ 7.5% for BHB, respectively and ≤ 4.1% and ≤ 9.2% for NEFA, respectively. TCh was assayed by CHOD-PAP reaction (Cholesterol-PAP kit, Kat. # 40121, Diagnosztikum RT, Budapest, Hungary), intra- and interassay CVs were ≤ 1.5% and ≤ 3.8%, respectively. Plasma glucose was determined by GOD-POD reaction (Glucose kit, Kat. # 40841, Diagnosztikum RT, Budapest, Hungary) with intra- and interassay CVs of ≤1.5% and ≤4.3%, respectively. AST activity was determined by the IFCC method in UV range with an AST Kit (Kat. # 7249, Reanal RT, Budapest, Hungary; intra- and interassay CVs ≤ 6.2% and ≤ 7.8%, respectively). Plasma insulin was assayed with a commercial ^{125}I-RIA kit (^{125}I-Insulin RIA CT kit; CIS Bio International Ltd, Gif-Sur-Yvette, France) developed for human and validated (Huszenicza et al., 1998; Nikolic et al., 2003) for bovine and ovine samples (sensitivity: 3.85 pmol/l, intra- and interassay CVs: 5.5– 8.4% and ≤ 8.8%, respectively). T_4 and T_3 were determined by ^{125}I-T_4-Spec and ^{125}I-T_3 coated tube RIA kits (Institute of Isotopes Co., Ltd. Budapest, Hungary) previously developed for animal or human samples and both validated for bovine and ovine plasma (Nikolic et al., 2003). Assay sensitivities were 0.5 nmol/l for T_4 and 0.19 nmol/l for T_3. Intraassay CVs were 6.4–8.1% (T_4) and 6.0–8.3% (T_3) and interassay CVs were ≤ 5.8 % and ≤ 6.5%, respectively. Leptin was determined by a ruminant-specific, homologous, double-antibody, non-equilibrium ^{125}I-RIA method (Delavaud et al., 2002) modified and validated for bovine plasma in our lab (assay sensitivity: 0.032 nmol/l; intra- and interassay CVs: 4.6–10.1% and 5.6–12.2%, respectively; Kulcsar et al., 2006). Plasma cortisol was determined by a direct ^{3}H-RIA method developed for human (Csernus, 1982) and equine (Nagy et al., 1998) samples and validated for bovine plasma without modification (sensitivity: 0.26 nmol/l, intra- and interassay CVs: ≤ 5.6% and ≤ 9.5%, respectively; Nikolic et al., 1998; Janosi et al., 2003).

Individual progesterone profiles were obtained from serial milk samples assayed by a microplate ELISA method (Nagy et al., 1998) modified for whole milk (Taponen et al., 2002; Huszenicza et al., 2005). Intra- and interassay CVs were < 8% and 10%, respectively, and sensitivity ranged from 0.17–0.25 nmol/l. Luteal activity was confirmed when milk progesterone concentrations reached ≥ 1.5 nmol/l for two or more consecutive samples.

Statistical evaluation

Pearson's Chi-square test was applied to prove whether a Hardy-Weinberg genetic equilibrium was fulfilled in the population.

From serial glucose and insulin measurements of the ivGTT the following parameters were calculated and used in the statistical analysis: basal

concentration (conc.t_0), peak concentration, increment (peak-conc.t_0), area under the curve in the first 60 min (AUC_{60}), mean concentration between 75–180 min ($mean_{75-180}$), clearance rate (CR, %/min), half-life ($t_{1/2}$, min), time to reach basal concentration (t_{basal}, min). Area under the curve was calculated using the trapezoidal method and corrected for baseline concentration (Holtenius et al., 2003). All parameters were actual concentrations except for glucose and insulin clearance rates, $t_{1/2}$ and t_{basal}, which were estimated by exponencial curve fitting between t_5 and t_{60} based on the equation of $F(t)=Ae^{-kt}$, where $F(t)$ is the concentration at time t, A is the estimated maximum value, t is the time (min) and k is the regression coefficient (Pires et al., 2007a). Thereafter, calculations of $CR = 100 * k$, $t_{1/2} = 1/k * \log(t_5/2A)$ and $t_{basal} = 1/k * \log(t_0/A)$ were carried out. Glucose half-life was disregarded due to the smaller than two-fold increment from basal concentrations. From serial measurements of plasma leptin concentrations, mean t_{60} and mean t_{180} (average concentration in the first 60 min and 180 min, respectively) were used. Single trait linear models were built with dependant variables of glucose (conc.t_0, peak concentration, increment, AUC_{60}, $mean_{75-180}$, CR, t_{basal}), insulin (conc.t_0, peak concentration, increment, AUC_{60}, $mean_{75-180}$, CR, $t_{1/2}$, t_{basal}) and leptin ($mean_{60}$, $mean_{180}$) parameters. Independent variables were *Alu*I genotype (1 : LL and 2 : LV), parity (2–4), 305-day previous lactation yield (kg), average milk yield (kg) of the current lactation (4–45 days PP), basal BHB and NEFA concentrations, body weight (BW) and BCS 9–14 days PP, BW change and BCS change (from 9–14 days to 24–28 days PP), and in case of glucose and leptin parameters plasma cortisol at t_0 was also included. These models were then subjected to a stepwise variable selection procedure (Venables and Ripley, 2002).

The Revised Quantitative Insulin Sensitivity Check Index (RQUICKI) adapted from human medicine and used in cattle by Holtenius and Holtenius (2007) for the rapid estimation of insulin sensitivity was calculated in this study following the equation of RQUICKI = 1/[log(conc.t_0 glucose) + log(conc.t_0 insulin)+log(conc.t_0 NEFA)]. In general, a higher value of the index means higher insulin sensitivity. Hyperketonemia has been previously reported to interfere with insulin resistance. Depressed pancreatic insulin secretion following ivGTT and lower glucose and insulin clearance rates have been shown by several authors (Hove, 1978; Sakai et al., 1993, 1996; Samanc et al., 1996; Steen et al., 1997). Therefore, we further modified the RQUICKI adding basal plasma concentration of β-hydroxybutyrate into the equation, which then became $RQUICKI_{BHB}$ = 1/[log(conc.t_0 glucose) + log(conc.t_0 insulin) + log(conc.t_0 NEFA) + log(conc.t_0 BHB)]. We expect that the inclusion of BHB will help to quickly, easily and more accurately assess insulin sensitivity.

Student's two-sample *t*-test allowing for unequal variances was used to compare reproductive parameters (time of first ovulation and first observed estrus PP), milk yield, indexes of insulin sensitivity (RQUICKI and $RQUICKI_{BHB}$) and measurements of body condition (BW, BW change, BCS, BCS change) between LL and LV cows.

Pearson's correlation coefficient was used to evaluate the strength of association between basal plasma parameters (BHB, NEFA, AST, T_4, T_3, TCh, cortisol, glucose, insulin and leptin) and also between calculated glucose, insulin and leptin measurements of the ivGTT with each other and with RQUICKI, $RQUICKI_{BHB}$, BHB, NEFA, BCS, BCS change, BW, BW change.

Level of significance was adjusted at $P < 0.05$. Calculations of exponential curve fitting and all statistical evaluations were carried out by Excel (Version 5.0; Microsoft Corporation, Redmond, WA, USA) and R (Version 2.4.1, R Development Core Team, 2006) program packages.

RESULTS

Out of all cows examined on Day 9–14 (n = 32) only 22 animals were included in the study and 10 were excluded for having clinical symptoms of (toxic) puerperal metritis. Among cows that participated in the experiment 18 were LL, four were LV and there were no VV animals in the population. The frequency of the leucine allele was $p_{leucine} = 0.909$ and the valine allele was $q_{valine} = 0.091$. Expected genotype frequencies were LL: n = 18.2, LV: n = 3.6, VV: n = 0.2, so based on Pearson's Chi-square test the pool was in Hardy-Weinberg genetic equilibrium ($\chi^2 = 0.246$, df = 1, $P > 0.05$).

In the beginning of lactation (4–45 days PP) LL and LV cows produced approximately the same amount of milk (36.9 ± 5.8 kg and 38.7 ± 2.3 kg, respectively, P = 0.31) and LV cows tended (P = 0.13) to have higher 305-day previous lactation yields (LV: 11 060.5 ± 1 386.8 kg and LL: 9 647.4 ± 1 145.3 kg; mean ± SD).

In general, following the intravenous glucose infusion, all cows reached peak plasma glucose concentration 5 min later, which returned to baseline level in the 45–75 min. Accordingly, all cows mounted an insulin response to the glucose challenge that reached peak concentration 5 to 15 min later and returned to baseline between 45–75 min. Plasma leptin did not change significantly after glucose infusion. *Alu*I genotype was not associated with any of the calculated glucose parameters of the ivGTT ($P > 0.64$). Heterozygous cows, on the other hand, were prone to higher t_0 and mean$_{75\text{-}180}$ insulin levels ($P = 0.064$ and $P = 0.002$; respectively), longer time to reach half of the maximal and basal insulin concentrations ($P = 0.035$ and $P = 0.054$, respectively) and larger insulin AUC$_{60}$ ($P = 0.032$) compared to leucine homozygous cows. Leptin response to the glucose challenge was not associated with *Alu*I polymorphism ($P > 0.58$). The actual plasma glucose, insulin and leptin responses to ivGTT are shown in Figure 1.

Higher plasma NEFA levels accounted for higher glucose peak and increment, longer time to reach baseline glucose concentration and larger glucose AUC$_{60}$ ($P < 0.05$). Plasma BHB was negatively associated with insulin traits e.g. peak, increment,

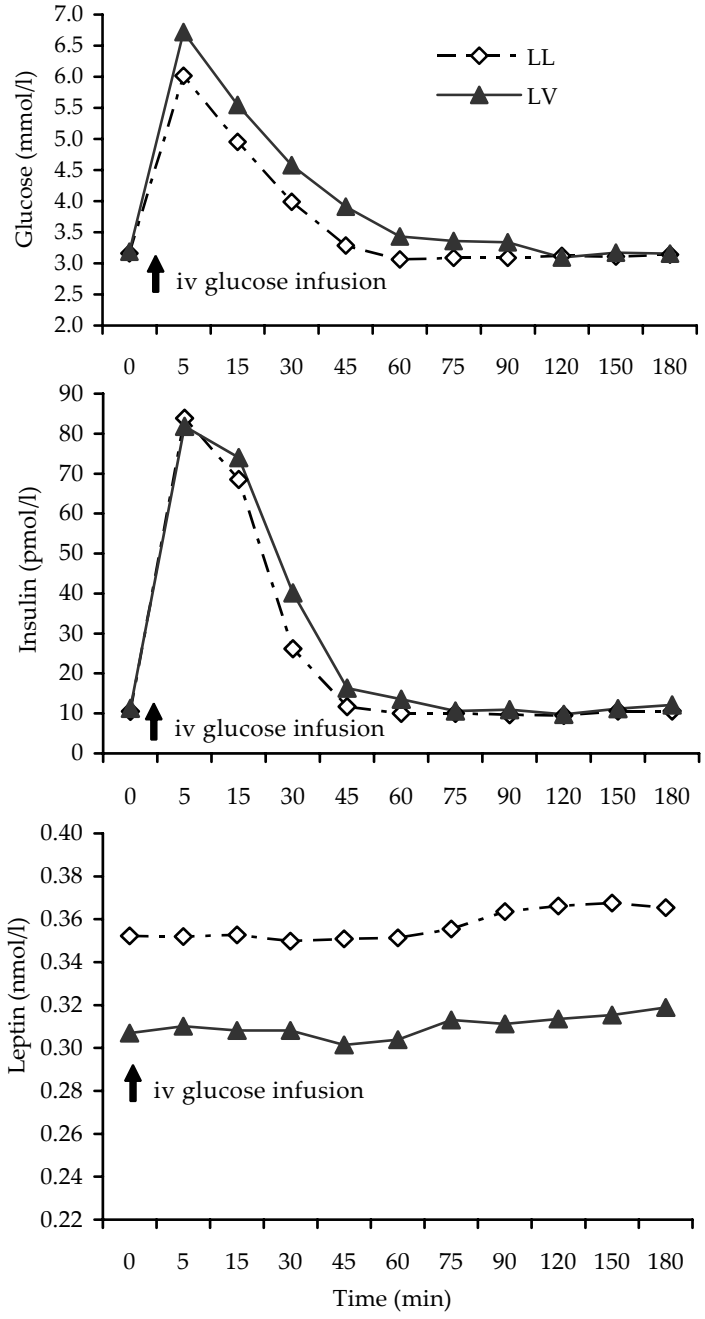

Figure 1. Actual plasma glucose, insulin and leptin responses following ivGTT by GH genotype

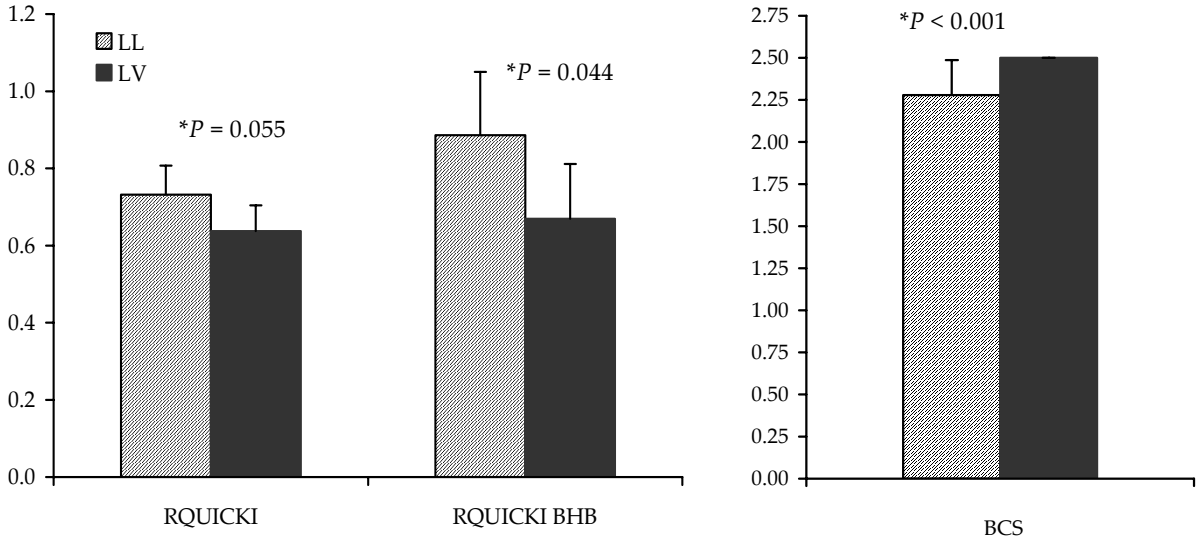

Figure 2. Indexes of insulin sensitivity and BCS by GH genotype groups (mean and SD)

*Student's *t*-test

CR, AUC_{60} ($P < 0.001$) and t_{basal} ($P = 0.045$). Higher average milk yield was related to decreased plasma glucose peak and AUC_{60} ($P = 0.058$ and $P = 0.040$, respectively), a shorter time to reach baseline glucose concentration ($P = 0.005$) and higher leptin levels ($P < 0.018$). Previous lactation yield was inversely related to t_0 and $mean_{75\text{-}180}$ insulin levels ($P = 0.007$ and $P < 0.001$, respectively). Plasma cortisol lowered glucose CR ($P = 0.040$) and prolonged t_{basal} ($P = 0.006$). As parity number increased so did baseline glucose level ($P = 0.025$). Older cows also had higher peak insulin concentrations and insulin increment ($P = 0.042$ and $P = 0.058$, respectively) and tended to have lower leptin levels during ivGTT ($P > 0.068$). Cows that lost more weight PP had higher glucose peak concentration and longer time to reach conc.t_0 ($P = 0.055$ and $P = 0.024$, respectively). Challenged plasma leptin levels were higher in cows with more BW ($P < 0.001$) and/or less BW loss ($P < 0.009$).

Based on RQUICKI and $RQUICKI_{BHB}$, LV cows showed decreased insulin sensitivity on 10–15 days PP and they were also in a slightly better body condition compared to LL animals (Figure 2). There were no differences in BW, BW change and BCS change between *Alu*I genotypes.

All cows became cyclic and showed signs of estrus during the study period. There were no differences between LL and LV animals in the onset of ovarian activity and in the time of first observed estrus (Figure 3). From the LL group 17 cows were inseminated out of which 12 conceived (days open: 119.8 ± 66.2 days PP) and on average needed 1.9 services per conception. Three of the LV cows were served and conceived (days open: 110.7 ± 30.0 days PP) and needed two services per conception.

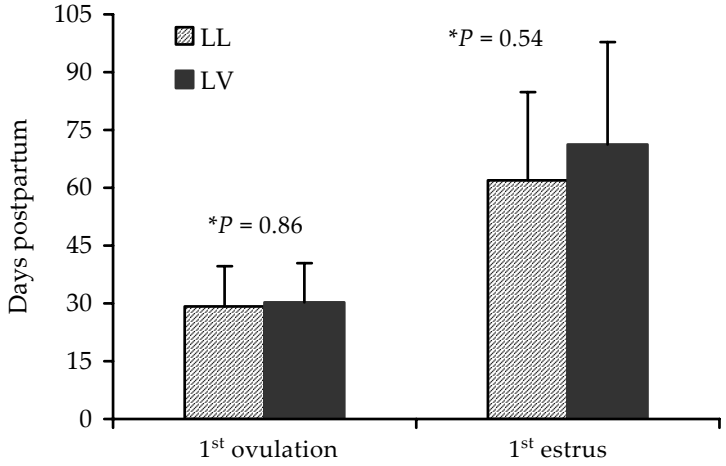

Figure 3. Days from calving to first ovulation and to first observed estrus by GH genotype (mean and SD)

*Student's *t*-test

Table 3. Correlation coefficients between basal plasma parameters (only significant correlations are shown)

	BHB	NEFA	AST	T_4	T_3	TCh
BHB	1	0.529**	0.631**	–0.412*	–0.647***	NS
NEFA		1	0.510*	–0.485*	–0.510*	NS
AST			1	NS	NS	–0.422*
T_4				1	0.779***	NS
T_3					1	NS
TCh						1

NS = not significant; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

BHB = β-hydroxybutyrate, NEFA = non-esterified fatty acids, AST = aspartate-aminotransferase activity, T_4 = thyroxine, T_3 = triiode-thyronine, TCh = total cholesterol

There was a moderate positive correlation between plasma BHB and NEFA and both were positively related to AST, and negatively to T_4 and T_3. Plasma AST had a negative relationship with TCh, while T_4 and T_3 depended closely and positively on each other (Table 3).

Glucose peak, increment, t_{basal} and AUC_{60} were all negatively correlated with both insulin sensitivity indexes and at the same time, positively with BHB and/or NEFA. On the other hand, insulin peak, increment, CR and AUC_{60} were negatively related to BHB and/or NEFA. Insulin increment and clearance rate showed a positive correlation with RQUICKI and $RQUICKI_{BHB}$, while resting (t_0) and $mean_{75\text{-}180}$ concentration of insulin had an inverse relationship with the index of insulin sensitivity (Table 4).

Table 4. Correlation coefficients of estimated glucose and insulin measurements of ivGTT with insulin sensitivity indexes, plasma- and body condition parameters (only significant relationships are shown)

	RQUICKI	$RQUICKI_{BHB}$	BHB	NEFA	BW	BW change
Glucose peak	–0.634**	–0.422*	NS	0.628**	NS	–0.418*
Glucose increment	–0.645***	–0.487*	NS	0.720***	NS	–0.479*
t_{basal} Glucose	–0.605**	–0.558**	0.580**	0.602**	NS	NS
AUC_{60} glucose	–0.663***	–0.565**	0.416*	0.735***	NS	NS
Conc.t_0 insulin	–0.493*	NS	NS	NS	NS	NS
Insulin peak	NS	NS	–0.601**	NS	NS	NS
Insulin increment	NS	0.388§	–0.600**	–0.360§	NS	NS
CR insulin	0.417*	0.473*	–0.635**	–0.383§	NS	NS
$t_{1/2}$ Insulin	NS	NS	NS	0.467*	NS	–0.406§
t_{basal} Insulin	NS	NS	NS	NS	0.427*	NS
AUC_{60} insulin	NS	NS	–0.509*	NS	NS	NS
$Mean_{75\text{-}180}$ insulin	–0.448*	NS	NS	NS	NS	NS

NS = not significant, §$P \leq 0.1$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

Parameters of the intravenous glucose tolerance test: t_{basal} = time to reach basal concentration, AUC_{60} = area under the curve in the first 60 min, conc.t_0 = basal concentration, CR = clearance rate, $t_{1/2}$ = time to reach half of peak concentration, $mean_{75\text{-}180}$ = average concentration between 75–180 min

RQUICKI = Revised Quantitative Insulin Sensitivity Check Index, $RQUICKI_{BHB}$ = Revised Quantitative Insulin Sensitivity Check Index modified with β-hydroxybutyrate, BHB = β-hydroxybutyrate, NEFA = non-esterified fatty acids, BW = body weight

Leptin ($mean_{60}$ and $mean_{180}$ concentration) was not an indicator of insulin sensitivity, but showed a moderate correlation (R = 0.492; P = 0.02) with body weight. Both BCS and BW were negatively related to $RQUICKI_{BHB}$ (R = −0.414, P = 0.05 and R = −0.459, P = 0.03, respectively) and BW also showed a positive correlation with BHB and NEFA (R = 0.490, P = 0.02 and R = 0.427, P = 0.04, respectively). BCS change and BW change were positively related to each other (R = 0.633, P = 0.002).

DISCUSSION

Patterns of plasma glucose and insulin responses to ivGTT in this study were comparable to those described elsewhere (Holtenius et al., 2003; Pires et al., 2007a). Leptin concentrations were not affected by the glucose challenge test. Gabai et al. (2002) and Chagas et al. (2006) could not induce a leptin response by infusing glucose or amino acid into late pregnant or lactating Simmenthal cows and to primiparous Holstein-Friesian cows.

Resting glucose concentrations, glucose peak and glucose disappearance following ivGTT were similar in LV and LL cows despite higher baseline and $mean_{75\text{-}180}$ insulin levels, longer half-life and larger insulin AUC_{60} in LV animals, reflecting a state of IR. Basal and challenged leptin levels were not associated with *Alu*I genotype. Katoh et al. (2008) found higher insulin levels in LL compared to LV Japanese Black calves despite similar glucose concentrations, and the highest plasma leptin levels in VV calves. Valid comparison of our results to this study is not possible due to breed, age and physiological differences between the two populations. In lactating dairy cows IR develops concomittantly with depressed pancreatic insulin secretory capacity (Holtenius and Traven, 1990; Holtenius et al., 2003). In our study, LV cows had larger insulin area under the curve, which might be due to increased insulin secretion and/or decreased insulin metabolism. Glucose disappearance after ivGTT is the sum of glucose utilization by peripheral tissues, absorption from the intestine, hepatic glucose output and excretion through the kidney. Glucose utilization by the mammary gland is significantly increased PP (Holtenius et al., 2003) and the degree of glucose drain from the circulation depends on milk yield. In our study milk production was not different between LL and LV cows. They received the same diet and were both fasted shortly before and during ivGTT, so glucose absorption from the intestine should have been minimal. We have no knowledge about the effect of ivGTT on renal glucose excretion. Although lactating cows have increased hepatic gluconeogenesis (Ingvartsen, 2006), high insulin levels reached during ivGTT should efficiently suppress gluconeogenesis and hepatic glucose output (Brockman and Laarveld, 1986). Therefore, glucose uptake by insulin-dependant peripheral tissues should have been similar in both genotypes as glucose clearance rate was not different. However, LV cows needed more insulin to trigger the same glucose response without provoking hypoglycemia compared to LL cows, which often accompanies IR conditions (Kushibiki et al., 2001). Accordingly, both RQUICKI and $RQUICKI_{BHB}$ were reduced in LV cows further pointing to possibly lower insulin sensitivity. However, due to the small number of LV animas (n = 4) these results should be interpreted with caution.

Both RQUICKI and $RQUICKI_{BHB}$ showed significant negative correlations with many of the glucose parameters of the ivGTT and with basal and $mean_{75\text{-}180}$ insulin concentrations, and were positively related to insulin clearance rate. Therefore, we infer that both indexes may be useful for the rapid estimation of insulin sensitivity in dairy cows.

In our study, NEFA and BHB were negatively associated with peripheral glucose utilization, pancreatic insulin secretion and insulin disappearance during ivGTT. NEFA predominantly compromised glucose response, while high BHB levels primarily accounted for decreased insulin response and clearance rate. NEFA not only impairs insulin actions in several pathways, but could also impair pancreatic insulin output. Bossaert et al. (2008) found that plasma NEFA was negatively related to insulin AUC and peak, but did not influence glucose parameters in PP cows. Pires et al. (2007a,b) showed that elevated triglycerid, and more closely, NEFA levels promoted IR that could be reversed by the administration of nicotinic acid. Several studies reported marked reduction of pancreatic insulin secretory capacity and decreased insulin responsiveness in hyperketonemic cows (Hove, 1978; Sakai et al., 1993, 1996), which is in agreement with our findings. We also showed that plasma cortisol was associated with decreased glucose clearance rate and prolonged time to reach baseline. Cortisol is usually increased in early lactation (Ingvartsen, 2006) and its actions on blood glucose concentration are opposite to that of insulin promoting hyperglycemia, therefore it may impair glucose disappearance.

Cows that lost more weight PP had higher glucose peak and glucose increment, needed longer time to reach basal concentration and had longer insulin half-life, all these changes pointing to a state of insulin resistance. In periparturient dairy cows blood NEFA, and consequently BHB increases as lipolysis advances (Pullen et al., 1989; Rukkwamsuk et al., 1999), so decreased peripheral insulin sensitivity in cows with more weight loss may be due to higher NEFA and BHB levels. Both BCS and BW were negatively related to $RQUICKI_{BHB}$ indicating that cows with higher weight or in better condition probably have diminished insulin sensitivity. Holtenius et al. (2003) previously found that overconditioned periparturient cows were more likely to develop IR and glucose intolerance than their herdmates. Indeed, there was a significant negative linear relationship between BCS and RQUICKI in all animals using the same dataset (Holtenius and Holtenius, 2007).

Milk production in the beginning of lactation was not different between *Alu*I genotypes, but LV cows had a tendency for higher 305-day lactation yields. Several reports have already investigated the association between *Alu*I polymorphism and milk production traits (Lee et al., 1996; Sabour et al., 1997; Shariflou et al., 2000; Dybus, 2002; Zwierzchowski et al., 2002). A recent study carried out in six large-scale Holstein herds in Hungary showed the advantage of LV dams over LL cows in 305-day lactation and test-day milk yields (Kovacs et al., 2006).

There were no differences between LL and LV cows in the commencement of luteal activity and in the time of the first observed estrus. We have only limited information about the involvement of *Alu*I polymorphism in reproductive traits in cattle (Lechniak et al., 1999, 2002), and neither of these reports investigated fertility characteristics of the high-yielding PP dairy cow. Results from our previous experiment conducted in four large-scale dairy herds (Balogh et al., in press) showed no advantage of either the leucine or the valine allele in resuming ovarian activity sooner after calving, which supports findings of our current study even though numbers of participating cows were much smaller here, and that should be taken into consideration when interpreting results.

We conclude that Holstein-Friesian cows heterozygous for *Alu*I polymorphism of the GH gene seem more likely to develop insulin resistance during early lactation than leucine homozygous cows. Decreased insulin sensitivity could be part of a homeorhetic adaptation process that supports nutrient partioning for the use of the mammary gland and may allow heterozygous cows to reach higher yields throughout lactation. *Alu*I genotype does not seem to be involved in the onset of postpartum ovarian activity and in the time of first observed estrus. The Revised Quantitative Insulin Sensitivity Check Index and its modified variant ($RQUICKI_{BHB}$) seem equally able to estimate changes in insulin sensitivity.

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