Title: Insulin resistance: relationship between indices during late gestation in dairy cows and effects on newborn metabolism

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ABSTRACT

Purpose: To investigate the relationship between insulin resistance (IR) indices ["Revised quantitative insulin sensitivity check index" (RQUICKI; RQ), "Revised quantitative insulin sensitivity check index - β-hydroxybutyrate" ($RQUICKI_{BHB}$; RQ_{BHB}), and "Homeostasis model assessment-insulin resistance" (HOMA-IR; HR)], and metabolic parameters in dams during late gestation, and their newborn calves.

Procedures: Blood was sampled twice weekly during the experimental period in 30 dry Holstein cows. In calves, blood sampling and body weight measurements were performed immediately after birth, and in 1-week-old male calves, liver and muscle biopsy samples were obtained for determining metabolic factor mRNA levels.

Findings: RQ and RQ_{BHB} were negatively correlated with insulin, non-esterified fatty acid, BHB and albumin, and positively correlated with leptin levels in blood during late gestation ($P<0.05$). RQ, rather than RQ_{BHB}, reflected metabolism of dams, while stronger positive correlations were present between HR and blood insulin concentrations than other parameters, and calves of dams with high HR had low body weight, and high liver and muscle expression of growth hormone and insulin receptor mRNA ($P<0.05$).

Conclusions: RQ and HR of dams during late gestation could serve as indicators of dam metabolism and predictors of metabolism in newborn calves, respectively.

Key words: *calf, dairy cow, insulin resistance, late gestation, metabolism*

INTRODUCTION

Insulin resistance (IR) is characterized by a deficiency in the effectiveness of insulin (Kahn, 1978), and is evaluated in terms of insulin responsiveness (the response of insulin to glucose) and insulin sensitivity (the responsiveness of tissues to insulin) (Hayirli, 2006). IR is commonly associated with obesity, hyperinsulinemia, a high-fat diet, hyperlipidemia, and malnutrition (Hayirli, 2006). IR is also observed during gestation, and can promote the provision of energy for fetal and placental development (Hayirli, 2006). Moreover, insulin secretion from pancreatic β-cells is enhanced during gestation to maintain glucose homeostasis (Wilcox, 2005; Catalano, 2010; Freemark, 2010). However, severe IR is often observed during late gestation as a consequence of elevated energy requirements during the periods of fetal growth and lactation (Hayirli, 2006). Severe IR induces low insulin sensitivity and β-cell dysfunction, and these can in turn cause hyperglycemia (Cerf, 2013). Such metabolic changes, particularly those associated with glycometabolism, induced by IR during late gestation, may affect subsequent metabolism in the offspring, as the maternal endocrine and metabolic milieu transferred through the placenta during late pregnancy affects the fetal environment (Rasby et al., 1990; Holt, 2002; Micke et al., 2010).

In humans, a poor fetal environment can be a precursor of future obesity, lifestyle-related diseases, and IR development (Gluckman and Hanson, 2004). Maternal hyperglycemia in the gestational period can also induce fetal and neonatal hyperglycemia, hyperinsulinemia, macrosomia, and congenital heart disease, in addition to increased maternal risk at delivery (The HAPO, 2009; Kamana et al., 2015; Helle et al., 2018), and IR in the mother is associated with low infant birth weight (Barker, 1995). In beef cattle, maternal malnutrition during gestation is related to retarded development

of both the placenta and the fetus (Rasby et al., 1990; Micke et al., 2010). Furthermore, in dairy cows, IR in the peripartum period is associated with several diseases, including ketosis (Kerestes et al., 2009). Therefore, IR and energy status during gestation can have subsequent effects on energy status after parturition, occurrence of disease, and fetal growth and health. However, little information is currently available regarding the relationships between IR in dairy cows during late gestation, the metabolic status of dams, and their calves. Moreover, although we showed in a previous study that the IR of dairy cows in late gestation is associated with lower calf body weight (BW) immediately after birth (Kawashima et al., 2016), there is at present no standardized procedure for IR evaluation in cattle, and consequently different studies have tended to adopt different methods.

The methods of IR evaluation commonly performed for cattle include the hyperinsulinemic euglycemic clamp test (HEC), the intravenous glucose tolerance test (IVGTT), and the insulin tolerance test (ITT) (Hayirli, 2006; De Koster et al., 2016; Kawashima et al., 2016). However, these methods are not suitable for cattle in late pregnancy, owing to the necessity to inject large amounts of insulin and/or glucose, and to hold the animals while these tests are being performed, which are potential sources of stress for cows and the developing fetus. In humans, insulin resistance, sensitivity, and secretion ability are determined using the oral glucose tolerance test (OGTT) and hyperinsulinemic euglycemic clamp test (HEC), among others. In addition, surrogate indices for insulin sensitivity, including the revised quantitative insulin sensitivity check index (RQUICKI; RQ), revised quantitative insulin sensitivity check index - β-hydroxybutyrate ($RQUICKI_{BHB}$; RQ_{BHB}) and the homeostasis model of insulin resistance (HOMA-IR; HR) are often used for IR evaluation in humans (Matthews et al.,

1985; Rabasa-Lhoret et al., 2003). These indices are determined based only on blood glucose, non-esterified fatty acid (NEFA), β-hydroxybutyrate (BHB), and insulin concentrations. However, De Koster et al. (2016) concluded that there is no association between the HEC test and any of the surrogate indices for insulin sensitivity such as RQ, HR, QUICKI, and RQ_{BHB}, and these indices reflect energy deficit rather than IR, although Holtenius and Holtenius (2007) showed that the RQ index could be used to identify insulin function in lactating cows. In addition, Balogh et al. (2008) showed that both RQ and RQ_{BHB} may be useful for the estimation of insulin sensitivity based on the results of correlation analyses of these indices, and glucose and insulin parameters of IVGTT. Moreover, Guyot et al. (2017) concluded that RQ, RQ_{BHB}, and HR differed significantly, allowing discrimination between healthy cows and cows with downer syndrome, although RQ_{BHB} does not add information relative to RQ. Therefore, the relationship between the gold standard and more practical methods of measurement for IR, such as HEC, IVGTT and ITT, and surrogate indices, such as RQ, RQ_{BHB}, and HR, have not been fully elucidated in dairy cattle. However, it is common knowledge that female mammals during late gestation usually have IR, and that their nutritional and metabolic status changes during the peripartum period (Bell, 1995; Hayirli, 2006).

Thus, in the present study, we sought to investigate the relationships between surrogate indices of IR, namely the RQ, RQ_{BHB}, and HR indices, in dairy cows during late pregnancy and the metabolic parameters of these cows at this time, and also the BW and metabolic status of their calves after birth, to examine whether IR indices can be used as metabolic indicators for dams during late pregnancy and/or metabolic predicators for their newborn calves.

MATERIALS AND METHODS

Animals, feeding, and management

The experimental procedures performed in the present study complied with the Guide for the Care and Use of Agricultural Animals of Obihiro University (approval number: #27-184, 28-40 and 29-41). The experiment was carried out at the Field Center of Animal Science and Agriculture, Obihiro University of Agriculture and Veterinary Medicine. As study animals, we used 30 Holstein cows with a 273-287 days gestation period, which had calved between January 2016 and December 2017, and their calves. The mean parity, calving interval, dry period, and body condition score (BCS) of the experimental cows at initiation of the study were 2.6 ± 0.1, 406.5 ± 9.2 days, 56.1 ± 6.3 days and 3.57 ± 0.05, respectively. Dry-off treatments were carried out for cows approximately 6 weeks (wk) before the expected calving date; all cows were moved to a paddock for the close-up dry period. Cows with mastitis (n = 2, dry period; 158.5 days), delayed conception (n = 1, dry period; 104 days), or decrease in daily milk production (n = 5, dry period; 57.8 days) were housed in a paddock for the far-off dry period after dry-off treatment earlier than 6 wk before the expected calving date, and were moved to a paddock for the close-up dry period about 1 month before the expected calving date. During the close-up dry period, all cows were fed a limited total mixed ration (TMR; 10.2 kg/day/head) consisting of grass silage, maize silage, and concentrate for dry cows, and were offered feed once daily at 11.30 h. In addition, grass hay, minerals, and water were freely accessible (Table 1). BW during the close-up dry period was 811.5 ± 13.6 kg, and dry matter intake predicted by the NRC (2001) equations was 16.0 ± 0.3 kg/day. All calves were fed good quality colostrum, defined by density higher than 1,044 kg/m^3 (Fleenor and Stott, 1980). Each pack of colostrum from an individual cow was stored at

−20°C, and thawed with warm water before feeding. The calves were separated from the dams immediately after birth, and fed colostrum three times on the first day (2 L every 10 h; 6 L in total). From the second day, 2 L of milk replacer (150 g/L of Calftop EX, National Federation of Dairy Cooperative Associations, Tokyo, Japan; TDN >103%, CP >28%, crude fat >15%) was fed twice a day at 8.00 and 16.30 h.

Sampling

BCS was assessed twice weekly from 5 wk before the expected parturition until calving, by the same operator using a 1 to 5 scale with 0.25 intervals according to Ferguson et al. (1994). Blood samples of dams were obtained at approximately the same time of the day between 07.00 and 08.00 h (approx. 3 h before feeding) via caudal venipuncture twice weekly from 5 wk before the expected parturition until calving. In addition, blood samples were taken from the jugular veins of calves immediately after birth before the first colostrum feeding. A non-heparinized and silicone-coated 9-mL tube (Venoject, Autosep, Gel + Clot. Act., VP-AS109K; Terumo Corporation, Tokyo, Japan) was used for biochemical analyses, and a sterile 9-mL tube containing 200 μL stabilizer solution (0.3 M EDTA-2Na and 1% acetyl salicylic acid, pH 7.4) was used for hormonal analyses. To obtain serum, blood samples were coagulated for 10 min at 38°C in an incubator. All tubes were then centrifuged at 2,328 *g* for 10 min at 4°C, and serum and plasma samples were stored at -30°C until analysis. Newborn calves were cleaned and dried with a towel, and were weighed before the first colostrum feeding.

Tissue collection, RNA extraction, and quantitative real-time reverse transcription PCR

To examine the expression of growth hormone (GH) receptor (GHR), insulin receptor (INSR), and β-actin mRNA, the muscle (n = 11) and liver (n = 8) of male

calves were sampled on a single occasion between 8 and 10 days of age via percutaneous needle biopsy (Bard Monopty Disposable Core Biopsy Instrument., 16G × 9 cm; Bard Peripheral Vascular, Inc., AZ, USA) under local anesthesia (Lidocaine hydrochloride; Xylocaine® Jelly 2%, AstraZeneca, Sweden) after intravenous administration of xylazine (Ceractal®, 2% solution, Bayer, Osaka, Japan) and 5% tranexamic acid (Vasolamin Injection, Meiji Seika Pharma, Tokyo, Japan), and stored at -80°C until analyzed. Total RNA from homogenized tissue samples was extracted using TRIZOL® regent, as described previously (Çolak et al., 2011). Real-time PCR quantification of GHR, INSR, and β-actin gene expression was performed in an iQ5 Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using a commercial kit (QuantiTect™ SYBR® Green PCR; Qiagen GmbH, Hilden, Germany) as described previously (Çolak et al., 2011). The primers used for GH, INSR, and β-actin amplification were those described previously (Çolak et al. 2011, Table 2). GH and INSR gene expression values were normalized using β-actin as the internal standard.

Measurement of hormones and metabolites

Maternal plasma insulin and leptin concentrations were determined once weekly from 4 to 1 wk before calving, and plasma insulin, GH and total insulin-like growth factor 1 (IGF-1) concentrations in calves were determined after birth. Plasma insulin and leptin concentrations were determined using enzyme-linked immunosorbent assay (ELISA) kits (Bovine Insulin ELISA 10–1201-01; Mercodia, Uppsala, Sweden, and Bovine Leptin ELISA CSB-E06771b; Cusabio, Houston, USA, respectively). The mean intra-assay and inter-assay coefficients of variation (CVs) were 4.8% and 13.5% for insulin, and 3.4% and 6.9% for leptin, respectively. GH and IGF-1 concentrations were determined using enzyme immunoassays based on the biotin–streptavidin amplification

technique after protein extraction using acid ethanol, as described previously (Kawashima et al., 2007). The mean intra-assay and inter-assay CVs were 7.7% and 7.9% for GH, and 6.5% and 8.1% for IGF-1, respectively. For all serum samples, the serum concentrations of glucose, NEFA, BHB, total cholesterol (T-Cho), aspartate aminotransferase (AST), and albumin (Alb) were measured using a clinical chemistry automated analyzer (TBA120FR; Toshiba Medical Systems Co., Ltd., Tochigi, Japan). In addition, the serum concentrations of total protein (TP) in male calves between 8 and 10 days of age were measured using a clinical chemistry automated analyzer (TBA120FR), in order to determine the presence or absence of failure of passive transfer (FPT) (Shinmori. et al., 2013).

IR evaluation indices

IR was evaluated using RQ, RQ_{BHB}, and HR as insulin sensitivity indices. Although these indices are typically calculated based on basal blood concentration at fasting (overnight; approximately 10 h) in humans (Rabasa-Lhoret et al., 2003), it is impractical to fast cows during late gestation. Therefore, we regarded blood concentration before feeding (3 h before limited TMR feeding) as the basal level for calculation of RQ, RQ_{BHB}, and HR.

RQ was calculated based on the concentrations of glucose and NEFA in serum and insulin in plasma using the following equation (Holtenius and Holtenius, 2007): RQ = 1 / [log (glucose; mg/dL) + log (insulin; μIU/mL) + log (NEFA; mmol/L)].

RQ_{BHB} was calculated based on the concentrations of glucose, NEFA, and BHB in serum and insulin in plasma using the following equation (Balogh et al., 2008): RQ = 1 / [log (glucose; mg/dL) + log (insulin; μIU/mL) + log (NEFA; mmol/L) + log (BHB; mmol/L)].

HR was calculated based on the concentrations of serum glucose and plasma insulin using the following equation (Yang et al., 2016): HR = insulin (μIU/mL) × glucose (mmol/L)/22.5.

Statistical analysis

Plasma GH and IGF-1 concentrations in one calf were not analyzed owing to insufficient samples. Prior to data analysis, the weekly data for BCS and serum metabolite concentrations were averaged. The relationship between weekly IR indices and parameters of dams in the same week of prepartum and the parameters of their calves after birth or at 1 wk of age were analyzed using Pearson correlation or Spearman rank correlation analysis after statistical testing for normality using the Shapiro–Wilk test (SigmaPlot® 13; Systat Software, Inc., San Jose, CA, USA.). Moreover, to compare the blood parameters of dams during the prepartum period, the data were analyzed using a one-way repeated-measures analysis of variance (SigmaPlot®). Results are reported as the mean ± standard error of the mean (SEM). *P* values < 0.05 were considered to indicate a statistically significant difference.

RESULTS and DISCUSSION

IR indices, blood hormone and metabolite concentrations during late gestation

Concentrations of insulin in plasma and glucose, NEFA, and BHB in serum, which were used for calculation of the IR indices, and values of RQ, RQ_{BHB}, and HR are shown in Table 3. Values of RQ, RQ_{BHB}, and HR in the present study were higher than those previously reported using clinically healthy dry cows at 3 wk before the expected calving date (De Koster et al., 2016), and clinically healthy cows during early lactation (Holtenius and Holtenius, 2007; Cincović et al., 2017; Guyot et al., 2017). This

might be because the cows used in the present study had higher plasma insulin and serum glucose concentrations, and lower serum NEFA and BHB concentrations than those examined in previous studies (Holtenius and Holtenius, 2007; De Koster et al., 2016; Cincović et al., 2017; Guyot et al., 2017). Thus, the energy status of dams in the present study might be higher than that of the cows in previous studies. Figure 1 shows the average IR indices, blood hormone, and metabolite concentrations of the cows during the last 4 wk of gestation. Values of RQ and RQ_{BHB} at 1 wk prepartum were lower than those at 2 wk prepartum ($P < 0.05$). Low RQ and RQ_{BHB} values indicate low insulin sensitivity (Holtenius and Holtenius, 2007; Balogh et al., 2008). Therefore, the dams in the present study may have low insulin sensitivity 1 wk before the calving day. In contrast, HR values decreased gradually as the calving day approached; higher HR values indicate low insulin sensitivity (Matthews et al., 1985). Cincović et al. (2017) demonstrated a negative relationship between HR and IGF-1, and a positive relationship between HR and BHB in cows during early lactation. Moreover, Guyot et al. (2017) showed that downer cows had higher HR than healthy cows during early lactation. HR could thus be used as an indicator of IR during early lactation but not during late gestation in dairy cows.

We found that BCS and plasma leptin concentrations remained unchanged, whereas plasma insulin and serum glucose, T-Cho, Alb concentrations, and AST activity decreased and serum NEFA and BHB concentrations increased as the calving day approached. BCS, serum glucose, NEFA, and T-Cho concentrations, and serum AST activities were within the standard values for the dry period reported by Kida (2002b). In addition, the changes in plasma concentrations of insulin and leptin, and the serum concentrations of BHB (Zhang et al. 2015) and Alb (Kawashima et al. 2016)

during late gestation were within approximately the same ranges as reported previously. Therefore, the herd examined in the present study appeared to have normal levels of the selected metabolites.

Relationship between IR evaluation indices and the metabolic parameters of dams during late gestation

Figure 2 shows the linear regression between IR indices and the metabolic parameters used to calculate these indices during the last 4 wk of gestation. RQ and RQ_{BHB} values were negatively correlated with serum concentrations of glucose (RQ and RQ_{BHB}; $P < 0.01$), NEFA (RQ and RQ_{BHB}; $P < 0.01$), and BHB (RQ and RQ_{BHB}; $P < 0.01$), and plasma insulin concentrations (RQ and RQ_{BHB}; $P < 0.01$). In contrast, HR values were positively correlated with serum glucose ($P < 0.01$) and BHB ($P < 0.01$) concentrations and plasma insulin concentrations ($P < 0.01$), and negatively correlated with serum NEFA concentrations ($P < 0.01$). Therefore, positive or negative correlations with all parameters were shown not only for RQ_{BHB}, which was calculated based on all parameters, but also for RQ and HR. Table 4 shows the relationships between the value of each IR index during each prepartum week and the parameters measured in dams. We found that for each week, RQ and RQ_{BHB} values were negatively correlated with plasma insulin concentrations ($P < 0.01$). In addition, a negative correlation was noted between RQ values and serum NEFA concentrations in each week (-4 wk; $P < 0.1$, -3 and -2 wk; $P < 0.05$, -1 wk; $P < 0.01$), and BHB concentrations except at 3 wk prepartum (-4 and 1 wk; $P < 0.05$, -2 wk; $P < 0.1$), whereas there was a negative correlation between RQ_{BHB} values and serum NEFA concentrations at only 1 wk prepartum ($P < 0.01$) and BHB concentrations in each wk (-3 wk; $P < 0.1$, -4, -2 and

-1 wk; $P < 0.01$). However, there was no correlation between serum glucose concentrations and RQ and RQ_{BHB} values in each week except at 1 wk prepartum (RQ; $P < 0.01$, RQ_{BHB}; $P < 0.05$). Additionally, RQ and RQ_{BHB} values were negatively correlated with BCS at 1 wk prepartum (RQ and RQ_{BHB}; $P < 0.05$) and serum AST activities at 4 wk prepartum (RQ and RQ_{BHB}; $P < 0.05$), and positively correlated with plasma leptin concentrations at 2 wk (RQ; $P < 0.05$, RQ_{BHB}; $P < 0.1$) and 1 wk prepartum (RQ; $P < 0.01$, RQ_{BHB}; $P < 0.05$). Furthermore, there was a negative correlation between RQ and RQ_{BHB} values and serum Alb concentrations in each wk except at 2 wk prepartum (RQ: -4 wk; $P < 0.1$, -3 wk; $P < 0.05$ and -1 wk; $P < 0.01$, RQ_{BHB}: -4 wk; $P < 0.1$, -3 wk; $P < 0.01$ and -1 wk; $P < 0.05$), and RQ and RQ_{BHB} values were positively correlated with serum T-Cho concentrations at 2 wk (RQ and RQ_{BHB}; $P < 0.1$) and 1 wk (RQ; $P < 0.1$) prepartum. HR values were positively correlated with plasma insulin concentrations in each week ($P < 0.01$), whereas there was no correlation between serum glucose concentrations and HR values in each week except at 1 wk prepartum ($P < 0.01$). In addition, HR values were positively correlated with serum BHB concentrations in each week (-3 wk; $P < 0.1$, -4 and -1 wk; $P < 0.05$, and -2 wk; $P < 0.01$), and were negatively correlated with serum NEFA concentrations in each week except 1 wk prepartum (-4, -3 and -2 wk; $P < 0.05$). In contrast, there was a positive correlation between HR values and BCS at 3 wk and 1 wk prepartum ($P < 0.05$), and serum Alb concentrations at 3 wk and 1 wk prepartum ($P < 0.01$). For concentrations of leptin in plasma and T-Cho in serum, and serum AST activities, there was no correlation with HR values.

Insulin is secreted from the pancreas in response to an increase in blood glucose concentrations; it regulates glucose homeostasis by stimulating glucose uptake into

insulin-responsive target tissues, such as adipocytes and skeletal muscle (Bergman, 2007; Diamanti-Kandarakis and Dunaif, 2012), and a reduction in the insulin sensitivity of insulin-responsive target tissues is characterized by increasing levels of circulating insulin (Diamanti-Kandarakis and Dunaif, 2012). In addition, in dairy cows, circulating insulin concentrations are influenced by the energy level of feed and energy balance (Armstrong et al. 2003; Holtenius et al. 2003). Moreover, insulin suppresses lipolysis by impairing hormone sensitive-lipase activity and reduces the mobilization of body fat from adipose tissue (Brockman, 1978; Hayirli, 2006). Accordingly, an increase in blood insulin levels causes a decrease in blood NEFA levels (Brockman, 1978; Hayirli, 2006). However, under conditions of energy deficiency, serum NEFA levels increase due to increased induction of adipose tissue mobilization (Grummer, 1993; Grummer, 1995; Puppel and Kuczynska, 2016), and severe energy deficiency can result in disordered lipid metabolism in the liver and increase blood BHB concentrations (Puppel and Kuczynska, 2016; Grummer, 1993; Grummer, 1995). De Koster et al. (2016) showed that blood NEFA concentrations reflect energy status rather than IR, although an association did exist between blood NEFA concentrations and IR in over-conditioned cows; they concluded that RQ, RQ_{BHB}, and HR are not associated with insulin sensitivity as determined by the HEC test in dairy cows at the end of the dry period (De Koster et al., 2016). We concur with the results of this previous study in that, based on our findings in the present study, we consider IR indices to have applicability as indicators of energy status rather than insulin sensitivity in dams during late gestation. Moreover, in the present study, RQ and RQ_{BHB} were negatively correlated with metabolic parameters, and HR values were positively correlated with concentrations of BHB in serum and insulin in plasma. However, serum NEFA concentrations were

negatively correlated with HR values. Both low RQ and RQ_{BHB} values and high HR values indicate low insulin sensitivity (Matthews et al., 1985; Holtenius and Holtenius, 2007; Balogh et al., 2008). Cincović et al. (2017) demonstrated a positive relationship between HR and BHB, as also indicated by our data, but did not present the results of the correlation with NEFA. Thus, it seems that our observation of the negative correlation between serum NEFA concentrations and HR was not consistent with insulin sensitivity determined based on HR values. As previously mentioned, high blood insulin levels causes a decrease in blood NEFA levels in order to suppress lipolysis by impairing hormone sensitive-lipase activity and reduce the mobilization of body fat from adipose tissue (Brockman, 1978; Hayirli, 2006). In the present study, HR values had a stronger positive correlation with plasma insulin concentrations than with other metabolic parameters. Therefore, the high plasma insulin concentrations of dams with high HR values might have caused the low serum NEFA concentrations observed in the present study. In contrast, blood glucose concentrations in dry cows hardly decrease even if energy status is low owing to differences in blood metabolic hormone levels (Kawashima et al., 2007), since insulin is enhanced during gestation to maintain glucose homeostasis (Wilcox, 2005; Catalano, 2010; Freemark, 2010). Thus, we consider that low IR indices at 1 wk prepartum affected the result of correlations with serum glucose concentrations during the last 4 wk gestation in the present study.

Low blood albumin concentrations reflect the progression of fatty liver, and high concentrations indicate hemoconcentration due to dehydration (Kida, 2002a; Puppel and Kuczynska, 2016). Additionally, leptin is secreted under conditions of adiposity, and low plasma leptin levels are indicative of energy deficiency or fasting (Friedman and Halaas, 1998; Ahima and Flier, 2000; Block et al., 2001). In the present study, cows

with low RQ and RQ_{BHB} values had high serum Alb concentrations and low plasma leptin concentrations. Therefore, we speculate that cows with low RQ and RQ_{BHB} values have reduced drinking water intake combined with low feed intake, based on the results of high serum Alb; they then develop energy deficiency owing to low plasma leptin concentrations. However, the results of this study were insufficient to uncover why HR and leptin concentrations were not related. In addition, we did not measure the amount of feed and drinking water intake; thus, further investigations are needed to measure not only metabolic parameters in blood but also feed and drinking water intake to clarify the relationship between IR indices and these parameters. In addition, except at 4 wk before calving, we detected no correlation between RQ and RQ_{BHB} values and serum AST activity, and there was no correlation between RQ and RQ_{BHB} values and BCS apart from 1 wk before calving. Higher AST activities in blood indicate hepatic dysfunction (Puppel and Kuczynska, 2016); although the reason for the high AST activity observed in blood at 4 wk prepartum is unclear based on the results of this study, we consider that low RQ and RQ_{BHB} values at 1 wk prepartum and a lack of change in BCS during late gestation or the high serum AST activity at 4 wk prepartum affected the results of these correlations in the present study. In contrast, HR values were positively correlated with BCS at 3 wk and 1 wk prepartum in the present study. As previously mentioned, higher insulin concentrations in blood reflect higher energy status in dairy cows (Armstrong et al. 2003; Holtenuis et al. 2003). The strong positive correlations between HR values and plasma insulin concentrations lead us to consider that cows with high HR showed high BCS in this study.

Cincović et al. (2017) showed that RQ might be the most appropriate predictor of metabolic status in dairy cows during early lactation, and Guyot et al. (2017) concluded

based on studies using dairy cows that RQ_{BHB} does not add information relative to RQ. As RQ values showed correlations with a greater number of metabolic parameters than did RQ_{BHB} and HR in the present study, we consider that RQ values could be used as an index for the metabolic status of dairy cows during late gestation.

Relationship between IR evaluation indices in dams and metabolic parameters of their newborn calves

Table 5 presents the relationships between the values of each IR index determined for each prepartum week in the dams and the parameters measured in their newborn calves. Serum TP concentrations in male calves between 8 and 10 days of age was 5.8 g/dL (5.1 - 6.5 g/dL). Shinmori et al. (2013) showed that a cut-off point of serum TP concentration in FPT Holstein calves at 7 days of age was 4.2 g/dL. Thus, we consider that there was no FPT calves in the present study.

There were negative correlations between serum T-Cho concentrations of newborn calves and RQ ($P < 0.05$) and RQ_{BHB} ($P < 0.1$) values of dams at 3 wk prepartum, and serum AST activity of newborn calves and RQ and RQ_{BHB} values of dams at 2 wk (RQ; $P < 0.1$, RQ_{BHB}; $P < 0.05$) and 1 wk (RQ; $P < 0.05$, RQ_{BHB}; $P < 0.01$) prepartum. In addition, plasma GH concentrations of newborn calves were positively correlated with RQ values of dams at 1 wk prepartum ($P < 0.1$), and RQ_{BHB} values at 2 wk ($P < 0.05$) and 1 wk ($P < 0.05$) prepartum. Moreover, RQ and RQ_{BHB} values of dams were highly negatively correlated with GHR mRNA expression in the muscle (RQ at -1 wk; $P < 0.01$, RQ_{BHB} at -3 wk; $P < 0.05$, RQ_{BHB} at -1 wk; $P < 0.01$) and liver (RQ and RQ_{BHB} at -2 wk; $P < 0.1$, RQ and RQ_{BHB} at -1 wk; $P < 0.05$) of calves at 1 wk of age ($P < 0.05$). Furthermore, there was a negative correlation between RQ

and RQ_{BHB} values of dams at 1 wk prepartum and INSR mRNA in the muscle (RQ and RQ_{BHB}; $P < 0.1$) and liver (RQ_{BHB}; $P < 0.1$) of male calves at 1 wk of age. There were also negative correlations between the HR values of dams and BW in newborn calves (-4 wk; $P < 0.05$, -3 and -1 wk; $P < 0.1$). Moreover, we detected highly positive correlations between the expression of GHR and INSR mRNA in the muscle and liver of male calves at 1 wk of age and the HR values of dams (GHR in the muscle: -3 and -1 wk; $P < 0.01$, GHR in the liver: -4 and -3 wk; $P < 0.01$, -2 and -1 wk; $P < 0.1$, INSR in the muscle: -4 and -1 wk; $P < 0.05$, -3 wk; $P < 0.01$, INSR in the liver: -4 wk; $P < 0.05$). Other parameters of newborn calves showed no significant correlations with the values of IR indices in dams.

In humans, sheep, and cows, malnutrition in mothers during middle and late gestation delays placental development and fetal growth and results in low offspring BW (Rasby et al., 1990; Redmer et al., 2004; Belkacemi et al., 2010; Micke et al., 2010), whereas malnutrition in mothers during early pregnancy promotes placental development in order to enhance fetal growth (Redmer et al., 2004; Belkacemi et al., 2010). Moreover, in humans, a poor fetal nutritional environment may be a predisposing factor for future obesity, lifestyle-related diseases, and development of IR (Gluckman and Hanson, 2004). In the present study, dams with low RQ and RQ_{BHB} and high HR values, indicating low insulin sensitivity (Matthews et al., 1985; Holtenius and Holtenius, 2007; Balogh et al., 2008), had low energy status, as also reported in previous studies (Cincović et al., 2017; Guyot et al., 2017). However, HR values, rather than RQ and RQ_{BHB} values, had a strong correlation with metabolic parameters of newborn calves, although we considered that RQ values could be used as an index of metabolic status for dairy cows during late gestation. In the present study, the positive

correlations between HR values and plasma insulin concentrations were stronger than those with other metabolic parameters. Thus, we considered that HR values strongly associated with blood insulin concentrations in dairy cows during late gestation. Blood insulin concentrations are influenced by the energy level of feed and energy balance in dairy cows (Armstrong et al. 2003; Holtenuis et al. 2003). However, in mice, it has similarly been found that the offspring of mothers with hyperinsulinemia have low BW after birth (Kahraman et al., 2014). We found that the calves of dams with high HR values had low BW after birth despite having high expression of GHR and INSR mRNAs in the muscle and liver tissues, which are relatively easy to grow. Hence, we considered that dams with high HR values might have hyperinsulinemia. Moreover, gene expression of GHR and INSR in the muscle and liver tissues was strongly correlated with HR values of dams at 4 wk and 3 wk prepartum. Fetal BW increased by more than 1.5-fold in the last month of gestation in Holstein cattle (Krog et al., 2018). Therefore, we consider that the HR values of dams at 4 wk and 3 wk prepartum rather than at 2 wk and 1 wk prepartum were related to BW and gene expression of metabolic hormones in the muscle and liver. GH induces IGF-1 production via binding to GHR in the liver, and then IGF-1 affects the muscle, liver, and other targets through its receptor (Renaville et al., 2002). Moreover, there is a positive correlation between GHR and IGF-1 mRNA in the liver and muscle, respectively (Liu et al., 1999). The longissimus muscle in the fetuses of beef cattle that received restricted feeding during mid to late gestation had a higher mRNA level of IGF-1, its receptor, and INSR (Paradis et al., 2017). In addition, Micke et al. (2011) showed that calves born to malnourished mothers showed high expression of IGF-1 receptor mRNA in beef cattle, indicating that growth and the induction of obesity in these calves is easier than those in calves born to

mothers with stable nutrient status under the same feeding system. Moreover, in humans, a poor fetal nutritional environment may be a predisposing factor for future obesity, lifestyle-related diseases, and development of IR (Gluckman and Hanson, 2004). Furthermore, we speculate that it is conceivable that the calves of dams with high HR values induced by hyperinsulinemia, which are exposed to a nutrient-limited prenatal environment, will develop severe IR or obesity in the future. Therefore, follow-up studies are required for to assess growth, metabolic status, and production after the 1st calving of the calves of dams with high HR in the future.

In conclusion, in dairy cows during late gestation, RQ, RQ_{BHB}, and HR values, used for the evaluation of IR in humans, were found to reflect their metabolic status. In particular, RQ values calculated based on insulin, glucose, and NEFA concentrations in blood could be used as an index reflecting the metabolic status of dairy cows during late gestation rather than using RQ_{BHB} and HR values, and low RQ values reflect a low energy status, increased body fat mobilization, and disordered lipid metabolism of dairy cows during late gestation. However, HR values of dams during late gestation, especially 4 to 3 wk prepartum, were found to reflect the metabolic status of their newborn calves. High HR values of dams during the late gestation were correlated with low BW and high gene expression of metabolic parameters, as dams with high HR values might have hyperinsulinemia. Overall, RQ and HR indices in dams during late gestation could represent a useful indicator of energy and metabolic status of dams and their newborn calves, respectively.

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Figure Legends

Figure 1.

Insulin resistance indices, serum metabolite concentrations and activities of enzymes, plasma metabolic hormones, and body condition score (BCS) of prepartum cows during late gestation (n = 30). The data for plasma insulin and leptin concentrations are weekly values, whereas those for other parameters are averages of weekly values. Values are presented as the mean ± SEM. Means with different superscripts (a, b, c) are significantly different ($P < 0.05$). ns, not significant. RQ: Revised quantitative insulin sensitivity check index; RQ_{BHB}: Revised quantitative insulin sensitivity check index - β-hydroxybutyrate; HR: Homeostasis model assessment-insulin resistance; Alb: albumin; AST: aspartate aminotransferase; BHB: β-hydroxybutyrate; NEFA: nonesterified fatty acid; T-Cho: total cholesterol.

Figure 2.

Linear regression between IR indices and serum glucose, NEFA and BHB and plasma insulin concentrations of dams (n = 30) during the last 4 weeks of gestation. r; coefficient of correlation. RQ: Revised quantitative insulin sensitivity check index; RQ_{BHB}: Revised quantitative insulin sensitivity check index - β-hydroxybutyrate; HR: Homeostasis model assessment-insulin resistance; BHB: β-hydroxybutyrate; NEFA: nonesterified fatty acid.

Table 1. Ingredients and nutrient composition of diets during the close-up dry period

Item	Close-up dry period	
Ingredient composition, kg DM/d		
Corn silage	3.0	
Grass silage	4.4	
Concentrate for dry cows[(a)]	2.6	
Orchard hay	*ad libitum*	
Vitamins, minerals	0.2	
Nutrient composition		
ME, Mcal/d (% Red)	28.5	(116)
MP, g/d (% Red)	1080.6	(104)
NFC, % DM	32.0	
peNDF, % DM	36.2	
CP, % DM	13.3	

a) Farm aid 18 (nutrient composition on a DM basis: 18.0% CP, 2.0% crude fat, 10.0% crude fiber, 10.0% crude ash, 0.5% Ca, and 0.4% P; 74.0% TDN, including corn, rapeseed cake, soybean oil cake, corn gluten feed and corn distiller by-products, bran; Snow Brand Seed Co. Ltd., Hokkaido, Japan).

DM, Dry matter; ME, Metabolizable energy; MP, Metabolizable protein; NFC, Non-fibrous carbohydrates; peNDF, Physically effective Neutral detergent fiber; CP, crude protein.

Table 2. Sequence of primers used for real time PCR (Çolak et al. 2011).

Gene	Primer sequence	Size (bp)
GHR	For: 5'–CAC ACC AGC TTT CCT TGT CA-3'	177
	Rev: 5'–GCA GAG ACG ACC ACT TTT GT-3'	
INSR	For: 5'–GCT GCT GCC TGG GAA TTA-3'	213
	Rev: 5'–CCA TCT GGC TGC CTC TTT-3'	
β-Actin	For: 5'–CCA AGG CCA ACC GTG AGA AGA T-3'	256
	Rev: 5'–CCA CGT TCC GTG AGG ATC TTC A-3'	

For, Forward; Rev, Reverse.

GHR, growth hormone receptor; INSR, insulin receptor.

1

Table 3. Plasma insulin and serum glucose, NEFA and BHB concentration, and values of RQ, RQ_{BHB} and HR indices of dams in the last 4 weeks of gestation.

	Values	SD	Minimum	Maximum
Insulin, μIU/mL	13.4 ± 0.7	8.1	2.9	43.3
Glucose, mg/dL	69.2 ± 0.4	4.0	56.0	83.6
(mmol/L)	(3.85 ± 0.02)	0.22	(3.11)	(4.64)
NEFA, mmol/L	0.14 ± 0.01	0.13	0.04	0.82
BHB, mmol/L	0.42 ± 0.01	0.11	0.20	0.69
RQ	0.53 ± 0.01	0.07	0.36	0.69
RQ_{BHB}	0.68 ± 0.01	0.15	0.41	1.33
HR	2.26 ± 0.12	1.35	0.48	7.37

Values; mean ± SEM.

RQ, Revised quantitative insulin sensitivity check index; RQ_{BHB}, Revised quantitative insulin sensitivity check index - β-hydroxybutyrate; HR, Homeostasis model assessment-insulin resistance; BHB, β-hydroxybutyrate; NEFA, nonesterified fatty acid.

1

Table 4. Coefficient of correlation (r) between values of IR indices and the metabolic parameters of dams in the last 4 weeks of gestation.

	RQ (n = 30)				RQ_{BHB} (n = 30)				HR (n = 30)			
	-4 wk	-3 wk	-2 wk	-1 wk	-4 wk	-3 wk	-2 wk	-1 wk	-4 wk	-3 wk	-2 wk	-1 wk
BCS	0.095	-0.093	-0.175	-0.393*	0.072	-0.085	-0.085	-0.398*	0.261	0.438*	0.075	0.446*
Glucose	-0.208	-0.248	-0.275	-0.471**	-0.227	-0.223	-0.275	-0.450*	0.279	0.102	0.118	0.494**
Insulin	-0.693**	-0.496**	-0.565**	-0.603**	-0.704**	-0.582**	-0.631**	-0.635**	0.995**	0.988**	0.980**	0.995**
NEFA	-0.318†	-0.391*	-0.387*	-0.525**	-0.243	-0.243	-0.224	-0.483**	-0.388*	-0.463*	-0.371*	-0.257
BHB	-0.449*	0.111	-0.342†	-0.365*	-0.656**	-0.343†	-0.688**	-0.635**	0.461*	0.337†	0.597**	0.435*
Leptin	-0.176	0.149	0.394*	0.572**	-0.107	0.011	0.328†	0.472*	0.129	0.162	-0.076	-0.295
T-Cho	0.185	0.207	0.361†	0.307†	0.277	0.186	0.353†	0.209	-0.066	0.098	-0.125	-0.176
AST	-0.420*	-0.013	-0.084	-0.169	-0.425*	-0.019	-0.162	-0.168	0.189	-0.074	0.117	0.014
Alb	-0.318†	-0.459*	-0.351	-0.499**	-0.322†	-0.566**	-0.295	-0.433*	0.100	0.628**	0.212	0.585**

† $P < 0.1$; * $P < 0.05$; ** $P < 0.01$.

RQ, Revised quantitative insulin sensitivity check index; RQ_{BHB}, Revised quantitative insulin sensitivity check index - β-hydroxybutyrate; HR, Homeostasis model assessment-insulin resistance; Alb, albumin; AST, aspartate aminotransferase; BCS, body condition score; BHB, β-hydroxybutyrate; NEFA, nonesterified fatty acid; T-Cho, total cholesterol.

1

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Table 5. Mean values of metabolic parameters of newborn calves and coefficient of correlation (r) between IR indices of dams in the last 4 weeks of gestation.

	Values	RQ (n = 30)				RQ_{BHB} (n = 30)				HR (n = 30)			
	(min-max)	-4 wk	-3 wk	-2 wk	-1 wk	-4 wk	-3 wk	-2 wk	-1 wk	-4 wk	-3 wk	-2 wk	-1 wk
BW, kg	47.3 ± 1.0 (38.0 - 60.0)	0.185	0.012	-0.208	-0.057	0.193	-0.025	-0.196	-0.027	-0.419*	-0.329†	-0.170	-0.319†
Glucose, mg/dL	95.8 ± 7.3 (38.0 – 239.9)	0.150	-0.109	-0.074	-0.038	0.199	0.019	-0.111	-0.004	0.121	0.041	0.103	-0.048
Insulin, μIU/mL	14.1 ± 2.7 (2.3 – 69.7)	-0.139	0.131	-0.147	-0.197	-0.135	0.214	-0.087	-0.206	0.138	0.044	0.005	0.262
NEFA, mmol/L	0.79 ± 0.08 (0.13 – 1.63)	0.116	0.000	0.251	-0.039	0.001	0.099	0.108	-0.019	0.062	0.236	0.179	0.033
BHB, mmol/L	0.04 ± 0.00 (0.03 – 0.07)	0.152	-0.248	-0.121	-0.112	0.092	-0.245	-0.190	-0.159	-0.029	0.171	0.011	-0.176
T-Cho, mg/dL	20.3 ± 1.1 (11.0 – 35.6)	-0.061	-0.418*	-0.202	-0.166	0.036	-0.324†	-0.120	-0.142	-0.297	-0.053	-0.226	-0.069
AST, IU/L	32.4 ± 2.6 (17.0 - 70.0)	-0.075	-0.223	-0.355†	-0.464*	-0.159	-0.220	-0.407*	-0.537**	-0.056	0.106	0.193	0.213
Alb, g/dL	3.0 ± 0.0 (2.7 – 3.3)	-0.150	-0.038	-0.102	0.003	-0.066	0.003	-0.088	-0.051	-0.088	-0.055	-0.301	-0.142
GH[(a)], ng/mL	15.3 ± 1.6 (5.1 - 46.4)	0.251	0.047	0.290	0.358†	0.290	-0.051	0.425*	0.399*	-0.168	-0.070	-0.218	-0.115
IGF-1[(a)], ng/mL	70.6 ± 5.5 (35.0 - 162.6)	0.122	0.259	0.220	0.163	0.187	0.080	0.303	0.135	-0.013	-0.050	-0.192	0.125
Muscle-GHR[(b)]	$1\times10^{11} \pm 6\times10^{10}$ (9×10^{-2} - 6×10^{11})	-0.164	-0.509	-0.309	-0.773**	-0.355	-0.609*	-0.491	-0.736**	0.464	0.900**	0.418	0.727**
Muscle-INSR[(b)]	$3\times10^{-8} \pm 1\times10^{-8}$ (2×10^{-9} - 1×10^{-7})	-0.245	-0.327	-0.091	-0.545†	-0.373	-0.455	-0.327	-0.591†	0.609*	0.855**	0.436	0.664*
Liver-GHR[(c)]	$8\times10^{24} \pm 7\times10^{24}$ (8×10^{-1} - 5×10^{25})	-0.310	-0.429	-0.619†	-0.714*	-0.286	-0.429	-0.667†	-0.786*	0.857**	0.833**	0.619†	0.619†
Liver-INSR[(c)]	$5\times10^{-7} \pm 1\times10^{-7}$ (2×10^{-8} - 9×10^{-7})	-0.152	0.233	-0.170	-0.462	-0.143	-0.224	-0.382	-0.694†	0.690*	0.548	0.190	0.476

Mean ± SEM., † $P < 0.1$; * $P < 0.05$; ** $P < 0.01$., (a) n = 29. (b) n = 11. (c) n = 8.

RQ, Revised quantitative insulin sensitivity check index; RQ_{BHB}, Revised quantitative insulin sensitivity check index - β-hydroxybutyrate; HR, Homeostasis model assessment-insulin resistance; Alb, albumin; AST, aspartate aminotransferase; BHB, β-hydroxybutyrate; BW, body weight; GH, growth hormone; GHR, growth hormone receptor; IGF-1, insulin-like growth factor 1; INSR, insulin receptor; NEFA, nonesterified fatty acid; T-Cho, total cholesterol.

Figure 1

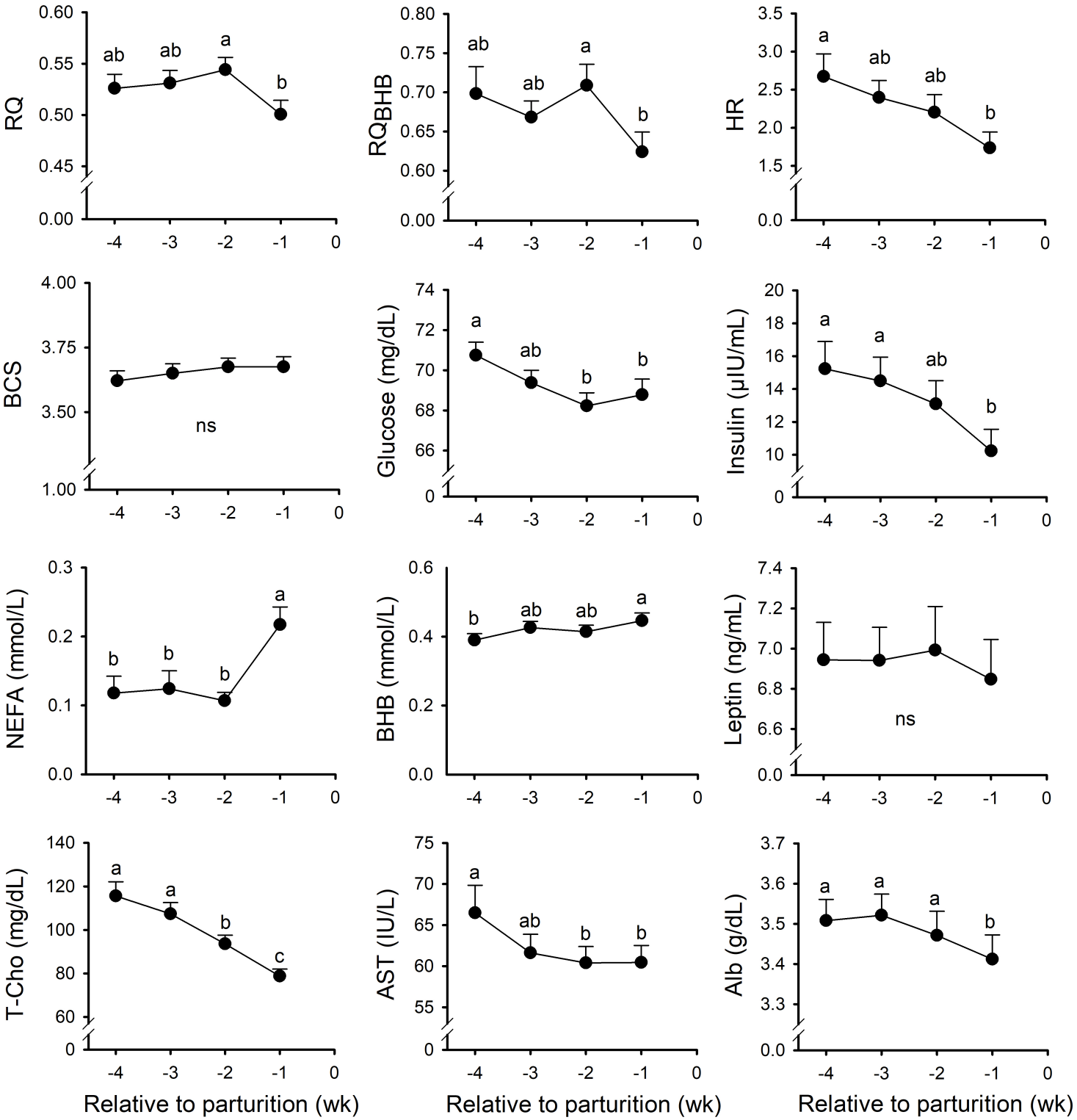
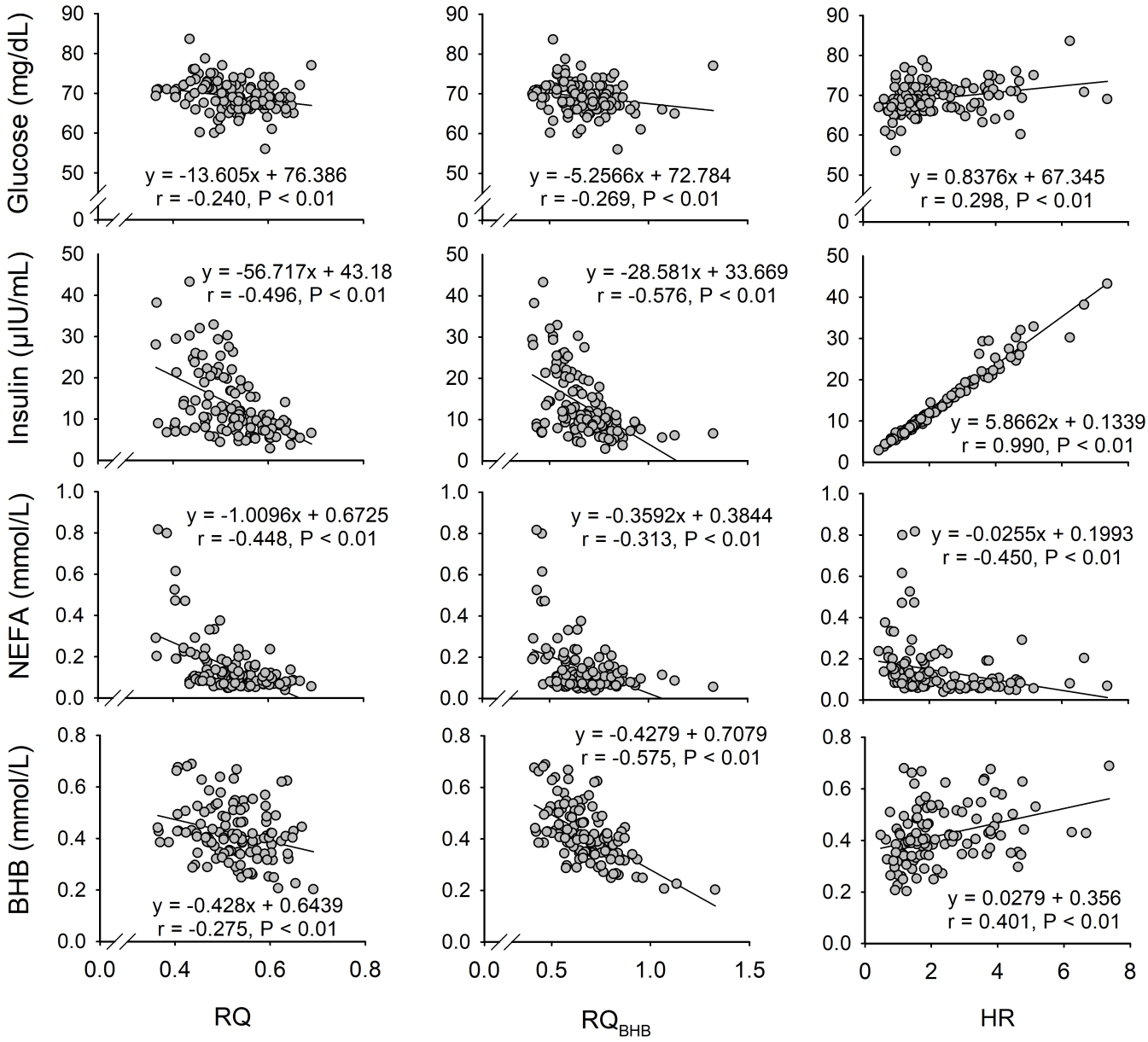

RQ
RQBHB
HR
BCS
Glucose (mg/dL)
Insulin (μIU/mL)
NEFA (mmol/L)
BHB (mmol/L)
Leptin (ng/mL)
T-Cho (mg/dL)
AST (IU/L)
Alb (g/dL)
ns
Relative to parturition (wk)
Relative to parturition (wk)
Relative to parturition (wk)

Figure2


Glucose (mg/dL)
y = -13.605x + 76.386
r = -0.240, P < 0.01
y = -5.2566x + 72.784
r = -0.269, P < 0.01
y = 0.8376x + 67.345
r = 0.298, P < 0.01
Insulin (μIU/mL)
y = -56.717x + 43.18
r = -0.496, P < 0.01
y = -28.581x + 33.669
r = -0.576, P < 0.01
y = 5.8662x + 0.1339
r = 0.990, P < 0.01
NEFA (mmol/L)
y = -1.0096x + 0.6725
r = -0.448, P < 0.01
y = -0.3592x + 0.3844
r = -0.313, P < 0.01
y = -0.0255x + 0.1993
r = -0.450, P < 0.01
BHB (mmol/L)
y = -0.428x + 0.6439
r = -0.275, P < 0.01
y = -0.4279 + 0.7079
r = -0.575, P < 0.01
y = 0.0279 + 0.356
r = 0.401, P < 0.01
RQ
RQBHB
HR