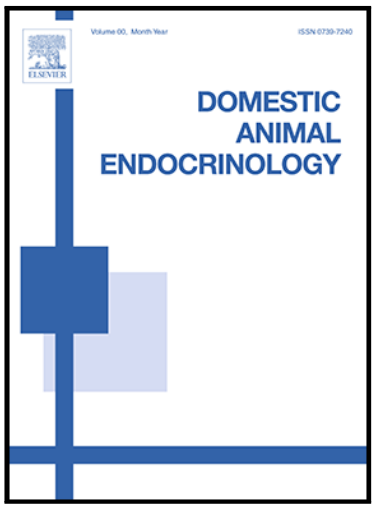

Effect of precalving body condition score on insulin signaling and hepatic inflammatory state in grazing dairy cattle

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Effect of precalving body condition score on insulin signaling and hepatic inflammatory state in grazing dairy cattle

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Highlights

- Hepatic insulin resistance could be an important factor during the transition of dairy cows.
- The hosphatidylinositol-3-kinase signaling pathway is altered in cows with high body condition score.
- Both high body condition score and pro-inflammatory state could be associated with hepatic insulin resistance.

Abstract

During postpartum, high-production dairy cows show a temporary period of insulin resistance, during which glucose uptake by peripheral tissues is reduced to prioritize milk production. However, this can further increase their negative energy balance by compromising liver function, especially in cows with excessive body condition score (BCS) and a pro-inflammatory state. Based on this, the aim of this study was to evaluate the hepatic expression of proteins of the insulin signaling pathway (PI3K) and of the cytokines TNFα, IL-6 and NF-κB, as well as the plasma concentrations of non-esterified fatty acids (NEFA), beta-hydroxybutyrate, glucose, triglycerides (TAG), insulin and insulin-like growth factor-1, insulin sensitivity indexes, and the hepatic content of TAG during the transition period in cows with different BCS. Sixteen Holstein cows were selected fourteen days before the expecting calving date and classified into two groups: low BCS (LBCS) ≤ 3.25 (n = 9) and high BCS (HBCS) ≥ 3.5 (n = 7). Blood and liver samples were obtained 14 (±3) days before the expected calving date and 4 (±3), 14 (±3) and 28 (±3) days after calving. The concentration of NEFA was higher in the HBCS group than in the LBCS group. Glucose concentration showed an interaction effect, with a greater concentration on day 28 in HBCS. Insulin concentration showed no changes. While the pAkt/total Akt ratio was lower in the HBCS group, the TNFα protein expression was higher only on day 4 postcalving in the HBCS group. In agreement with these results, the insulin sensitivity indexes RQUICKI and $RQUICKI_{BHBA}$ were lower in the HCBS group. The results suggest an insulin resistance and a pro-inflammatory state in the liver of cows with HBCS.

1. Introduction

In dairy cows, high milk production sets a scenario in which glucose needs to be redirected to the mammary gland for lactose synthesis. This leads to a systemic decrease in insulin levels and greater concentrations of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA) [1]. After binding to its receptor, insulin leads to the activation of different signaling pathways, including the phosphatidylinositol-3-kinase (PI3K) pathway, which is responsible for most of the metabolic actions of insulin [2]. Like in other mammals, a temporary period of insulin resistance, which aims to reduce the glucose uptake by peripheral tissues to prioritize milk production, has been well documented in the postpartum of high-production dairy cows [3,4]. However, this physiological pathway can further increase the negative energy balance (NEB) of dairy cows, compromising health and reproduction. Insulin resistance may also lead to the development of hepatic lipidosis, a disease with systemic consequences, and compromise liver function during the transition period, especially gluconeogenesis, which, in ruminants, is relevant for glucose supply to the mammary gland for milk synthesis [5].

Several studies have shown that cows with high body condition score (BCS) at parturition may have several problems [5,6–9]. The peripartum of dairy cows has been described as a pro-inflammatory period characterized by frequent infectious diseases and related to high BCS and fat mobilization [11,12], similar to that described in humans with type II diabetes [13]. In this sense, pro-inflammatory interleukins such as tumor necrosis factor (TNFα) and interleukin 6 (IL-6) have been indicated as important elements in the development of this inflammatory state [14,15]. In addition, nuclear factor kappa B (NF-κB) has been proposed as an element that could connect the pro-inflammatory state with insulin resistance. NF-κB is sequestered in the cytoplasm

bound to inhibitor kappaB (IκB) proteins. After the stimulation with different activators that also mediate the innate and adaptive immune response, such as pro-inflammatory cytokines (TNFα and IL-1), the IκB kinase (IKK) complex is activated and leads to the degradation of IκB. This triggers the translocation of NF-κB to the nucleus and the upregulation of target genes that encode inflammatory mediators such as TNF-α, IL-1β, and IL-6. Moreover, the activation of the IKK complex could affect the insulin signaling pathway through the phosphorylation of the serine residues of insulin receptor substrate 1 (IRS1) [16].

Considering the close relationship of the insulin action with inflammatory elements and an excessive BCS and its importance during the transition period of dairy cows, we hypothesized that an alteration in the hepatic insulin signaling pathway along with the development of a local pro-inflammatory state could be associated with a high BCS. Therefore, we aimed to study the hepatic expression of relevant proteins from the PI3K signaling pathway, TNFα, IL-6, and NF-κB, along with some plasma parameters, insulin sensitivity indexes, and the hepatic content of triglycerides (TAG), during the transition period of cows with different BCS from a commercial dairy farm.

2. Materials and methods

2.1. Ethics

The present study was approved by the Ethics Committee of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Santa Fe, Argentina (Protocol number 158/2013), and all the procedures were conducted according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, 2010).

2.2. Animals

Sixteen Holstein cows were selected from a commercial grazing dairy farm in Hipatia (31°07'S, 61°03'W), Santa Fe, Argentina, close to entering their second to fourth lactation and with an estimated mean body weight of 650 kg. Fourteen days before the expecting calving date, the animals were classified into two groups: cows with low BCS (LBCS) ≤ 3.25 (n = 9) and cows with high BCS (HBCS) ≥ 3.5 (n = 7) based on a 1 to 5 scale with 0.25 intervals, as proposed by Edmonson et al. [17]. Only healthy animals without retained placenta, clinical milk fever, mastitis, metritis, clinical ketosis, displaced abomasum, lameness and/or clinical gastrointestinal disorder were included in the study. After parturition, cows were milked twice a day and milk production was recorded monthly up to 120 days in milk with milk meters (Waikato Milking Systems, Hamilton, New Zealand).

The animals were fed as previously described in Angeli et al. [18] (Table 1). Postcalving animals received a diet based on alfalfa and ryegrass, whereas the remaining animals received a mixed diet. The consumption of alfalfa and ryegrass was *ad libitum* and the amount consumed was estimated as the difference between pregrazing and postgrazing mass per m^2 and multiplied by the area allocated/cow.

2.3. Blood and liver sampling

Blood and liver samples were obtained 14 (±3) days before the expected calving date and on days 4 (±3), 14 (±3) and 28 (±3) after calving. Blood samples from precalving cows were taken from the jugular vein between 1400 h and 1900 h, whereas blood samples from postcalving cows were taken at the same time after milking. The blood was collected in tubes with EDTA (#601702, Eurotubo, Deltalab, Spain), cooled at 5 °C and then centrifuged for 10 min at 2,000 x g to obtain the plasma and then stored

at – 20 °C until analyzed. The concentration of BHBA was measured in whole blood by using reactive strips.

The liver was sampled via puncture biopsy as previously described [19]. Briefly, under local anesthesia by infiltration of 15 mL of lidocaine hydrochloride 2% (OVER, Santa Fe, Argentina), a skin incision was made with a scalpel in the right 11th intercostal space at the level of the greater trochanter. A percutaneous true-cut biopsy needle (273 mm long), described by Buckley et al. [20], and modified *ad hoc* (without the external collar), was inserted into the muscular and subcutaneous tissues. Approximately 500 mg of liver tissue was collected in a tube that was immediately snap-frozen in liquid nitrogen and subsequently stored at – 80 °C. The stab incision was closed with nonabsorbable suture. The health status was monitored by a veterinary specialist, who checked for clinical signs of disease. The suture was removed 10-14 days after surgery.

2.4. Plasma metabolites and hormone measurements

The concentration of BHBA was measured in whole blood by using reactive strips (FreeStyle Optium Xceed, Abbott Diabetes Care Ltd., Oxon, UK). Plasma concentrations of NEFA, glucose and TAG were determined enzymatically with commercial kits (NEFA: #FA115, RANDOX Laboratories LTD, UK; glucose and TAG: #1400101 and #1780107, respectively, WIENER Lab, Rosario, Argentina), by using a spectrometer (ultra-fast UV/Vis spectrometer SPECTROstar Nano, BMG LABTECH GmbH, Ortenberg, Germany). The intra-assay coefficients of variation (CVs) were 5 % for NEFA, 10 % for glucose and 3 % for TAG. Plasma insulin concentration was measured by radioimmunoassay (RIA) by using an anti-bovine insulin antibody (#I6136, Sigma, St. Louis, MO, USA) and a human insulin standard

provided by Laboratorios Beta (Buenos Aires, Argentina), as previously described [21,22]. The minimum detectable concentration of insulin was 0.05 ng/mL. The intra- and inter-assay CVs for insulin were 8 % and 11 %, respectively. The concentration of IGF-1 was determined using an antibody (#UB2-495, Hormone Distribution Program of the NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases, Rockville, MD, USA) and a recombinant human IGF-1 (rhIGF1, Chiron Corp., Emeryville, CA, USA) was used as radioligand and unlabeled ligand [23]. The minimum detectable concentration of IGF-1 was 0.4 ng/mL. The intra- and inter-assay CVs for IGF-1 were 6 % and 9 %, respectively.

2.5. Insulin sensitivity indexes

The different insulin sensitivity indexes were calculated according to Guyot et al. [24]. Briefly, the homeostasis model assessment index (HOMA-IR) was determined by the following formula: glucose (mmol/L) x insulin (μU/mL); the quantitative insulin sensitivity check index (QUICKI) as follows: 1 / [log (glucose) + log (insulin)]; the revised quantitative insulin sensitivity check index (RQUICKI) as follows: 1 / [log (glucose) + log (insulin) + log (NEFA)]; and the RQUICKI modified with beta-hydroxybutyrate ($RQUICKI_{BHBA}$) as follows: 1 / [log (glucose) + log (insulin) + log (NEFA) + log (BHBA)]. In these formulas, glucose is expressed as mg/dL, insulin as μU/mL, NEFA as mmol/L and BHBA as mmol/L.

2.6. Liver TAG content analysis

Total lipids were extracted from liver tissue homogenates by a mixture of chloroform and methanol (2:1 v/v) [25] and the liver TAG content was determined

enzymatically using a commercial kit (#1780107, WIENER Lab, Rosario, Argentina) [19].

2.7. Protein expression

2.7.1. Preparation of protein lysates

Approximately 50 mg of frozen liver sample was homogenized with an homogenizer (#3737002, IKA T 10 Basic ULTRA-TURRAX^{TM}, Germany) in an ice bath for 2 min, as previously described [19]. The lysis buffer consisted of 0.1 % w/v SDS, 50 mmol/L sodium fluoride, 1 % v/v IGEPAL CA630 (octylphenyl-polyethylene glycol), 0.5 % w/v sodium desoxycholate, 1 mmol/L EDTA (all from Sigma-Aldrich Corp.), 0.1 mol/L PBS, and a protease and phosphatase inhibitor cocktail, according to the manufacturer´s specifications (#A32961, Pierce Protease Inhibitor Tablet, Thermo Fisher Scientific Company, Finland). Then, tissue lysates were centrifuged at 12,000 x *g* for 20 min and the supernatant was separated and collected at -80 °C. Protein concentrations were quantified using the Lowry method with the Bio-Rad Protein Assay Kit (#5000116, Bio-Rad Laboratories, Hercules, CA, USA) [26].

For NF-κB protein determination, liver tissue samples were used for the subcellular fractionation as described by Dimauro et al. [27] with some modifications. Briefly, liver tissue (100 mg) was homogenized using a potter-elvehjem homogenizer with 400 µL of cold cytoplasmic protein extraction buffer containing: 10 mM Tris-HCl pH 7, 10 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.025% Triton X-100 and a protease inhibitor cocktail, according to the manufacturer´s specifications (#A32961, Pierce Protease Inhibitor Tablets, Thermo Fisher Scientific Company, Finland). After 30 min on ice, the homogenate was centrifuged at 800 x *g* for 15 min at 4 °C. The resulting supernatant was centrifuged again for 5 min at 12,000 x *g*

to obtain a new supernatant containing the cytoplasmic fraction. The initial pellet was washed twice with the cytoplasmic protein extraction buffer, centrifuging for 15 min at 4 °C at 500 x *g* and 1,000 x *g*, respectively. The final pellet was resuspended in a cold buffer for nuclear protein extraction containing: 10 mM Tris-HCl pH 7, 10 mM Tris-HCl pH 8, 500 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.025% Triton X-100, 4% glycerol and protease inhibitor cocktails, according to the manufacturer´s specifications (#15634189, Pierce Protease Inhibitor Tablets, Thermo Fisher Scientific Company, Finland). After allowing to stand on ice for 30 min and sonicating, the final pellet was centrifuged at 12,000 x *g* for 20 min at 4 °C. The resulting supernatant contained the nuclear fraction.

2.7.2. Western blot analysis

Denaturing electrophoresis in discontinuous polyacrylamide gels (SDS-PAGE) was performed with a pore size for the resolution gel in relation to an acrylamide concentration according to the molecular weight of the proteins to determine: 10% for insulin receptor (IR), PI3K, total Akt (also known as protein kinase B) and phosphorylated Akt (pAkt), and 12% for nuclear NF-kB (nNF-κB), cytoplasmic NF-kB (cNF-κB), IL-6 and TNFα. In the case of IRS1, due to its weight and the internal control used (β-actin), a 7-15% gradient gel was used. Electrophoresis was performed and resolved proteins were transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). The membranes were blocked for 1 h with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBST, Sigma–Aldrich Corp.) at room temperature and then incubated overnight at 4 °C with the specific primary antibodies (Table 2). Then, membranes were washed with TBST and incubated for 1.5 h at room temperature with the corresponding secondary peroxidase-conjugated

antibody (Table 2). Finally, membranes were washed with TBST and the specific reactions were evidenced using a chemiluminescence detection kit (ECL Prime Western Blotting System, GE Healthcare) on hyperfilm-ECL film (GE Healthcare). PCNA and beta-actin were used as internal controls (Table 2). Preliminary studies showed linearity of the Western blot assay from 20 to 80 µg of protein and at increasing exposure times. The intensity of the bands was quantified using the IMAGE PRO-PLUS 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA) and a standard sample from a pool of six animals was blotted on each membrane to adjust band values from different membranes. The specificity of the antibodies used has been proven by the suppliers as well as by other researchers [28–34]. In addition, as in previous studies [19,35], we established the homology between the target peptide of each antibody and the homologous bovine protein by using the Basic Local Alignment Search Tool (BLAST) software (http://www.ncbi.nlm.nih.gov/BLAST) to determine the peptide locations and confirm antigen specificity.

2.8. Statistical analysis

The data were analyzed with the statistical software package SPSS 22.0 for WINDOWS (SPSS Inc., Chicago, IL, USA) and a repeated-measures analysis was performed using the Generalized Linear Model (GENLIN) approach. The distribution of data was tested using the Kolmogorov-Smirnov test. The variables BCS, NEFA, BHBA, liver TAG content, IR, IRS1, Akt, pAkt/Akt, nNF-κB and cNF-κB were positively skewed adjusting to a gamma distribution, whereas the other variables showed a normal distribution. For the variables with gamma distribution, we used the module GENLIN with a logarithmic link function, whereas for the variables with normal distribution, we used the module GENLIN with identity link function. Repeated measures were assessed

using the first-order autoregressive covariance structure. Selection of this covariance structure was based on the lowest Akaike information criterion between first-order autoregressive, compound symmetry and unstructured structures. The model consisted of BCS, time (T), and BCS × T as fixed effects. When the BCS × T interaction was significant, differences between treatments were tested for significance with the estimated marginal measures of the GENLIN approach. A value of $P < 0.05$ was considered significant. The results are expressed as mean ± SEM.

3. Results

3.1. Milk production, plasma parameters and hepatic TAG content

Milk production at 120 days in milk was higher in the HBCS group (3847 ± 162) than in the LBCS group (3358 ± 143; $P = 0.02$). No differences in the lactation number were observed between groups (HBCS = 3.00 ± 0.15; LBCS = 2.78 ± 0.13; $P = 0.25$). NEFA concentration was higher in the HBCS group ($P < 0.05$), reaching a maximum on day 4 ($P < 0.05$) and remaining high during the rest of the study. No BCS x T interaction effect was observed ($P > 0.05$). The concentration pattern of BHBA was similar to that of NEFA, although the peak was reached on day 14 postcalving, remaining high on day 28 ($P < 0.05$), without differences between groups or interaction effect ($P > 0.05$), but with a tendency to be higher in the HBCS group ($P = 0.09$). Glucose concentrations were lower on days 4 and 14 than on day 28 ($P < 0.05$). Particularly, on day 28, a BCS x T interaction effect was observed, where the HBCS group had a higher glucose concentration ($P < 0.05$). Plasma TAG showed the same distribution over time as glucose ($P < 0.05$), but without differences between groups or BCS x T interaction effect. Insulin concentration decreased on day 4 and remained low during the postcalving period ($P < 0.05$), without differences between groups or

interaction effect (P > 0.05). Finally, the hepatic TAG content increased in the postcalving period (P < 0.05), but without differences between groups or BCS x T interaction effect (P > 0.05) (Table 3).

3.2. Insulin sensitivity indexes

The HOMA index showed no differences between groups, variations in time or interaction effects (P > 0.05). The QUICKI index showed variations in time, decreasing between 14 days precalving and 14 days postcalving (P < 0.05), but no differences between groups or interaction effect (P > 0.05). Finally, the RQUICKI and $RQUICKI_{BHBA}$ indexes were lower in the HCBS group and showed variations in time, being lower at postcalving than at precalving (P < 0.05) (Table 4).

3.3. Liver protein expression of IR, IRS1, PI3K, Akt, pAkt, TNFα, IL-6 and NF-κB

The protein expression of the insulin signaling intermediates IR, IRS1 and PI3K showed no variations in time, differences between groups, or BCS x T interaction effect (P > 0.05). While Akt showed variations in time, decreasing in the postcalving period and then increasing until day 28 postcalving (P < 0.05), pAkt only showed a tendency in the BCS x T interaction (P = 0.11). The pAkt/total Akt ratio showed a greater relationship in the cows of the LBCS group (P < 0.05) (Figure 1).

The hepatic protein expression of IL-6 showed no variations in time and no effect of the BCS or BCS x T interaction (P > 0.05). In contrast, TNFα showed a BCS x T interaction effect, where protein expression was higher on day 4 postcalving in the cows of the HBCS group (P < 0.05). Finally, the protein expression of NF-κB showed no differences between groups or interaction effect, both in the nucleus and in the cytoplasm (P > 0.05) (Figure 2).

4. Discussion

It is well known that problems during the transition period in dairy cows have a negative impact on their health and milk production [36]. In this sense, the BCS is a very important parameter to control the incidence of postcalving pathologies. Cows with high BCS are predisposed to metabolic and infectious diseases [37]. Recently, some authors have proposed that cows with a BCS between 3.0 and 3.5 at calving show no evident productive differences, and those with a BCS above that value show an increased risk of suffering postpartum diseases, thus recommending a BCS between 3.00 and 3.25 at calving [38]. In this study, before calving, the cows were divided into two groups: cows with LBCS (≤ 3.25) and cows with HBCS (≥ 3.5). Cows with a HBCS showed greater milk production. Results of previous studies about the effect of BCS on milk production are inconsistent, possibly because the relationship between the BCS at partum and subsequent milk production is not linear, and the benefit decreases as the BCS increases until a point where milk production begins to decrease [37].

In the present study, we evaluated parameters that characterize the NEB as NEFA and BHBA. We found that while NEFA concentration was higher, the BHBA concentration tended to be higher in the cows of the HBCS group. Previous studies have shown that cows with high BCS tend to exhibit more lipomobilization and more BCS loss during postpartum [39]. This effect would be related to a lower DMI in cows with a HBCS, which would increase the NEB. In addition, the higher milk production increases the requirements in HBCS cows, contributing to the higher NEB. In addition, we observed that, on days 14 and 28 postcalving, cows with HBCS had BHBA concentrations above the recommended values [40]. The concentrations of NEFA in the HBCS cows were also higher than the recommended values throughout the postcalving

period. The high concentrations of NEFA and BHBA in the cows of the HBCS group on day 28 indicate that these animals continue in NEB and support the idea that they could continue to lose weight, being able to lose more than 1 point of BCS. In this regard, cows with high BCS at calving and high NEFA and BHBA concentrations have a higher risk of suffering metabolic disorders and infectious diseases, compromising milk production and reproductive performance [9,41]. It is important to highlight that, in our study, only cows without clinical evidence of any disease were considered for the analyses.

Another metabolite related to the NEB is glucose, whose concentrations decrease at postcalving as a result of the demand by the mammary gland for the synthesis of lactose. In this sense, previous studies have shown that cows with metabolic disorders or lower reproductive performance show lower glucose concentrations [42,43]. In contrast, in our study, despite having parameters that indicated higher NEB and higher milk production, cows with HBCS showed a higher concentration of glucose on day 28 postcalving. Cows with excessive BCS have been associated with type II ketosis, high or normal glucose concentrations and a postpartum insulin resistance state [44]. In our study, cows from the HBCS group presented subclinical ketosis values (> 1.2 mmol/L). Type II ketosis has also been associated with insulin concentrations higher than those of healthy cows [44]. However, in the present study, we found no differences in insulin concentrations. The metabolic state previously described has also been related to fatty liver [45]. In our study, no differences in this regard were observed between the groups; however, on day 28 postcalving, cows from the HBCS group had a mild fatty liver (2% liver TAG on wet weight basis), while cows from the LBCS group had a normal liver (< 1% liver TAG on wet weight basis), according to the classification of Bobe et al. [7].

The importance of insulin resistance in cattle and its relationship with calving and obesity is not completely clear. Previous studies have shown that cows with higher BCS or higher concentrations of NEFA or BHBA show lower response to reduce the systemic glucose concentration after treatment with commercial insulin [3,46]. Moreover, Pires et al. [47] reported that cows with high NEFA concentrations require a greater secretion of insulin to achieve glycemic control after intravenous glucose infusion. In contrast, in a glucose tolerance test, a negative relationship of high BCS, NEFA and BHBA with insulin concentration has been observed, which could suggest not only an insulin resistance state but also a lower response of the beta cells of the pancreas [5,46]. Also, Bossaert et al. [48] showed a negative effect of the high chronic NEFA concentrations on the insulin secretory capacity of the pancreas. In our study, we did not perform glucose tolerance or insulin resistance tests, but observed a higher glucose concentration, which could not be explained by a difference in insulin concentration. In this sense, Jaakson et al. [49] described an increase in glucose concentration with higher insulin concentrations in HBCS cows associated with the decrease in the glucose transporter 4 (GLUT4) in adipose tissue. In the present study, we also calculated insulin sensitivity indexes, which are widely used in human medicine to predict insulin sensitivity with a single blood sample after an overnight fast and predict an insulin resistance state. Regardless of the difficulty to achieve a fasting state in ruminants, some researchers have used these indexes to predict insulin resistance in dairy cows, with good results comparing with glucose tolerance tests [5]. In our study, we found differences between groups for RQUICKI and $RQUICKI_{BHBA}$, where cows with HBCS showed lower indexes, indicating a possible lower insulin sensitivity [50]. Similarly, a previous study found lower RQUICKI and $RQUICKI_{BHBA}$ values in downer

dairy cows, but only at early postcalving, indicating a possible relation with metabolic disturbances [24].

In the metabolic context described in this study, we also evaluated the insulin signaling pathway in the liver, a central organ in the metabolism of transition dairy cows. We found that the protein expression of pAkt tended to be greater in the cows of the LBCS group, and that the difference became more evident when we analyzed the relationship between pAkt and total Akt. The PI3K pathway consists of successive phosphorylations of different proteins, among which one of the most important ones is that of Akt, which is responsible for the activation of different pathways within the cell. We found no differences in other elements of the insulin signaling. Liu et al. [51] reported a decrease in the IR gene expression in the liver, but comparing healthy cows with cows whose concentration of BHBA and hepatic TAG content were higher than in our study. Moreover, when analyzing the protein expression of other downstream intermediaries of the PI3K pathway, in our study, the lower activation of Akt in cows with HBCS could be explained by a difference in insulin concentration and was in concordance with the lower values of insulin indexes described. Therefore, this could be indicating that cows with high BCS have a lower response to insulin in liver tissue.

In humans, type II diabetes and insulin resistance have been associated with a pro-inflammatory state [16] and there is much evidence of the relationship between obesity and higher plasma NEFA concentrations and a chronic inflammatory condition, the latter of which is characterized by the abnormal production of pro-inflammatory cytokines such as IL-6 and TNFα. In cattle, several researchers have described the transition period as a pro-inflammatory period and have studied this state through the analysis of the plasma variations in acute phase proteins, which are synthesized mainly in the liver. Their results have shown a relationship between these proteins and both the

productive and reproductive performance of dairy cows [18,52,53]. In this study, we found an increase in the protein expression of TNFα in the liver of cows with HBCS, possibly related to a greater amount of NEFA and their pro-inflammatory action.

The relationship between the pro-inflammatory process and the insulin resistance state could be indicated by the phosphorylation of the serine residues of IRS1 by TNFα and IL-6 through NF-κB activation [16]. In contrast to the normal phosphorylation of tyrosine residues, the phosphorylation of serine residues has an inhibitory effect on the insulin signal. In this sense, Pantelić et al. [54] demonstrated a lower phosphorylation of serine residues of IRS1 in cows supplemented with chromium during the transition period, and the consequent higher activation in the insulin signaling pathway. Despite this background, in our study, we did not find a greater expression of NF-κB in the nucleus, which represents the active state of this transcription factor. Shi et al. [55] found increases in the activation of NF-κB in the liver, but only in cows with higher levels of hepatic lipidosis than those found in our study. It is important to note that the effect through NF-κB is not the only mechanism through which TNFα can influence the insulin signaling pathway. An action by means of mitogen-activated protein kinases on the phosphorylation of IRS1 has also been described, but this was not analyzed in this study [16].

In recent years, some authors have discussed the possible similarities of type II diabetes and metabolic syndrome in humans with the peripartum of dairy cows [5], but have not reached conclusive results. In this context, we here evaluated cows with different BCS, and found greater lipomobilization in cows with HBCS and greater NEFA and BHBA concentrations. We also found a decrease in the insulin signal in the liver of cows with HBCS along with an increase in glucose concentration, but without changes in insulin concentration and with alterations in insulin sensitivity indexes.

Finally, related to the pro-inflammatory state described in conditions of greater lipomobilization, we found greater local hepatic expression of TNFα, which could be related to the decrease in the insulin signal. However, further studies are necessary to determine the magnitude and importance of insulin resistance in cattle during the peripartum. This knowledge could be important to improve the adaptation of postpartum dairy cows and in turn improve their productivity and the efficiency of dairy systems.

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References

[1] Sundrum A. Metabolic Disorders in the Transition Period Indicate that the Dairy Cows' Ability to Adapt is Overstressed. Anim an Open Access J from MDPI

2015;5:978–1020. https://doi.org/10.3390/ani5040395.

[2] Schultze SM, Hemmings BA, Niessen M, Tschopp O. PI3K/AKT, MAPK and AMPK signalling: protein kinases in glucose homeostasis. Expert Rev Mol Med 2012;14:e1. https://doi.org/10.1017/S1462399411002109.

[3] De Koster J, Hostens M, Van Eetvelde M, Hermans K, Moerman S, Bogaert H, et al. Insulin response of the glucose and fatty acid metabolism in dry dairy cows across a range of body condition scores. J Dairy Sci 2015;98:4580–92. https://doi.org/10.3168/jds.2015-9341.

[4] Abuelo A, Hernández J, Benedito JL, Castillo C. Association of oxidative status and insulin sensitivity in periparturient dairy cattle: an observational study. J Anim Physiol Anim Nutr (Berl) 2016;100:279–86. https://doi.org/10.1111/jpn.12365.

[5] De Koster JD, Opsomer G. Insulin resistance in dairy cows. Vet Clin North Am - Food Anim Pract 2013;29:299–322. https://doi.org/10.1016/j.cvfa.2013.04.002.

[6] Stockdale CR. Body condition at calving and the performance of dairy cows in early lactation under Australian conditions: a review. Aust J Exp Agric 2001;41:823. https://doi.org/10.1071/EA01023.

[7] Bobe G, Young JW, Beitz DC. Invited Review: Pathology, Etiology, Prevention, and Treatment of Fatty Liver in Dairy Cows. J Dairy Sci 2004;87:3105–24. https://doi.org/10.3168/jds.S0022-0302(04)73446-3.

[8] Roche JR, Meier S, Heiser A, Mitchell MD, Walker CG, Crookenden MA, et al. Effects of precalving body condition score and prepartum feeding level on production, reproduction, and health parameters in pasture-based transition dairy cows. J Dairy Sci 2015;98:7164–82. https://doi.org/10.3168/jds.2014-9269.

[9] Roche JR, Kay JK, Friggens NC, Loor JJ, Berry DP. Assessing and managing

body condition score for the prevention of metabolic disease in dairy cows. Vet Clin North Am - Food Anim Pract 2013;29:323–36. https://doi.org/10.1016/j.cvfa.2013.03.003.

[10] LeBlanc S. Monitoring metabolic health of dairy cattle in the transition period. J Reprod Dev 2010;56 Suppl:S29-35. https://doi.org/10.1262/jrd.1056S29.

[11] Bionaz M, Trevisi E, Calamari L, Librandi F, Ferrari A, Bertoni G. Plasma Paraoxonase, Health, Inflammatory Conditions, and Liver Function in Transition Dairy Cows. J Dairy Sci 2007;90:1740–50. https://doi.org/10.3168/jds.2006-445.

[12] Bradford BJ, Yuan K, Farney JK, Mamedova LK, Carpenter AJ. Invited review: Inflammation during the transition to lactation: New adventures with an old flame. J Dairy Sci 2015;98:6631–50. https://doi.org/10.3168/jds.2015-9683.

[13] Bertoni G, Trevisi E. Use of the liver activity index and other metabolic variables in the assessment of metabolic health in dairy herds. Vet Clin North Am - Food Anim Pract 2013;29:413–31. https://doi.org/10.1016/j.cvfa.2013.04.004.

[14] Kushibiki S. Tumor necrosis factor-α-induced inflammatory responses in cattle. Anim Sci J 2011;82:504–11. https://doi.org/10.1111/j.1740-0929.2011.00931.x.

[15] Trevisi E, Amadori M, Cogrossi S, Razzuoli E, Bertoni G. Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows. Res Vet Sci 2012;93:695–704. https://doi.org/10.1016/j.rvsc.2011.11.008.

[16] Chen L, Chen R, Wang H, Liang F. Mechanisms Linking Inflammation to Insulin Resistance. Int J Endocrinol 2015;2015:508409. https://doi.org/10.1155/2015/508409.

[17] Edmonson AJ, Lean IJ, Weaver LD, Farver T, Webster G. A Body Condition Scoring Chart for Holstein Dairy Cows. J Dairy Sci 1989;72:68–78. https://doi.org/10.3168/jds.S0022-0302(89)79081-0.

[18] Angeli E, Rodríguez FM, Rey F, Santiago G, Matiller V, Ortega HH, et al. Liver fatty acid metabolism associations with reproductive performance of dairy cattle. Anim Reprod Sci 2019;208:106104. https://doi.org/10.1016/j.anireprosci.2019.06.016.

[19] Angeli E, Trionfini V, Gareis NC, Matiller V, Huber E, Rey F, et al. Protein and gene expression of relevant enzymes and nuclear receptor of hepatic lipid metabolism in grazing dairy cattle during the transition period. Res Vet Sci 2019;123:223–31. https://doi.org/10.1016/j.rvsc.2019.01.020.

[20] Buckley WT, Eigendorf GK, Dorward WJ. A liver biopsy instrument for large animals. Can J Anim Sci 1986;66:1137–40. https://doi.org/10.4141/cjas86-125.

[21] Becú-Villalobos D, García-Tornadú I, Shroeder G, Salado EE, Gagliostro G, Delavaud C, et al. Effect of fat supplementation on leptin, insulin-like growth factor I, growth hormone, and insulin in cattle. Can J Vet Res 2007;71:218–25.

[22] Hein GJ, Panzani CG, Rodríguez FM, Salvetti NR, Díaz PU, Gareis NC, et al. Impaired insulin signaling pathway in ovarian follicles of cows with cystic ovarian disease. Anim Reprod Sci 2015;156:64–74. https://doi.org/10.1016/j.anireprosci.2015.02.010.

[23] Giuliodori MJ, Delavaud C, Chilliard Y, Becú-Villalobos D, Lacau-Mengido I, De la Sota RL. High NEFA concentrations around parturition are associated with delayed ovulations in grazing dairy cows. Livest Sci 2011;141:123–8. https://doi.org/10.1016/j.livsci.2011.05.007.

[24] Guyot H, Detilleux J, Lebreton P, Garnier C, Bonvoisin M, Rollin F, et al. Comparison of various indices of energy metabolism in recumbent and healthy dairy cows. PLoS One 2017;12:1–11. https://doi.org/10.1371/journal.pone.0169716.

[25] Folch J, Lees M, Stanley GHS. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497–509. https://doi.org/10.1007/s10858-011-9570-9.

[26] Hein GJ, Bernasconi AM, Montanaro MA, Pellon-Maison M, Finarelli G, Chicco A, et al. Nuclear receptors and hepatic lipidogenic enzyme response to a dyslipidemic sucrose-rich diet and its reversal by fish oil n-3 polyunsaturated fatty acids. AJP Endocrinol Metab 2010;298:E429–39. https://doi.org/10.1152/ajpendo.00513.2009.

[27] Dimauro I, Pearson T, Caporossi D, Jackson MJ. A simple protocol for the subcellular fractionation of skeletal muscle cells and tissue. BMC Res Notes 2012;5:513. https://doi.org/10.1186/1756-0500-5-513.

[28] Zachut M, Honig H, Striem S, Zick Y, Boura-Halfon S, Moallem U. Periparturient dairy cows do not exhibit hepatic insulin resistance, yet adipose-specific insulin resistance occurs in cows prone to high weight loss. J Dairy Sci 2013;96:5656–69. https://doi.org/10.3168/jds.2012-6142.

[29] Montgomery SR, Mamedova LK, Zachut M, Kra G, Häussler S, Vaughn M, et al. Effects of sodium salicylate on glucose kinetics and insulin signaling in postpartum dairy cows. J Dairy Sci 2019;102:1617–29. https://doi.org/10.3168/jds.2018-15312.

[30] Kinoshita A, Kenéz Á, Locher L, Meyer U, Dänicke S, Rehage J, et al. Insulin Signaling in Liver and Adipose Tissues in Periparturient Dairy Cows Supplemented with Dietary Nicotinic Acid. PLoS One 2016;11:e0147028. https://doi.org/10.1371/journal.pone.0147028.

[31] Gareis NC, Huber E, Hein GJ, Rodríguez FM, Salvetti NR, Angeli E, et al. Impaired insulin signaling pathways affect ovarian steroidogenesis in cows with

COD. Anim Reprod Sci 2018;192:298–312. https://doi.org/10.1016/j.anireprosci.2018.03.031.

[32] Yao X, EI-Samahy MA, Fan L, Zheng L, Jin Y, Pang J, et al. In vitro influence of selenium on the proliferation of and steroidogenesis in goat luteinized granulosa cells. Theriogenology 2018;114:70–80. https://doi.org/10.1016/j.theriogenology.2018.03.014.

[33] Baravalle C, Dallard BE, Cadoche MC, Pereyra EAL, Neder VE, Ortega HH, et al. Proinflammatory cytokines and CD14 expression in mammary tissue of cows following intramammary inoculation of Panax ginseng at drying off. Vet Immunol Immunopathol 2011;144:52–60. https://doi.org/10.1016/j.vetimm.2011.07.003.

[34] Baravalle C, Silvestrini P, Cadoche MC, Beccaria C, Andreotti CS, Renna MS, et al. Intramammary infusion of Panax ginseng extract in bovine mammary gland at cessation of milking induces changes in the expression of toll-like receptors, MyD88 and NF-kB during early involution. Res Vet Sci 2015;100:52–60. https://doi.org/10.1016/j.rvsc.2015.03.025.

[35] Stassi AF, Gareis NC, Marelli BE, Matiller V, Leiva CJM, Rey F, et al. Follicular structures of cows with cystic ovarian disease present altered expression of cytokines. Zygote 2019;27:285–98. https://doi.org/10.1017/S0967199419000285.

[36] Drackley JK. Biology of Dairy Cows During the Transition Period: the Final Frontier? J Dairy Sci 1999;82:2259–73. https://doi.org/10.3168/jds.S0022-0302(99)75474-3.

[37] Roche JR, Friggens NC, Kay JK, Fisher MW, Stafford KJ, Berry DP. Invited review: Body condition score and its association with dairy cow productivity, health, and welfare. J Dairy Sci 2009;92:5769–801.

https://doi.org/10.3168/jds.2009-2431.

[38] Roche JR, Lee JM, Macdonald KA, Berry DP. Relationships among body condition score, body weight, and milk production variables in pasture-based dairy cows. J Dairy Sci 2007;90:3802–15. https://doi.org/10.3168/jds.2006-740.

[39] Friggens NC, Ingvartsen KL, Emmans GC. Prediction of Body Lipid Change in Pregnancy and Lactation. J Dairy Sci 2004;87:988–1000. https://doi.org/10.3168/jds.S0022-0302(04)73244-0.

[40] Ospina PA, McArt JA, Overton TR, Stokol T, Nydam D V. Using nonesterified fatty acids and ??-hydroxybutyrate concentrations during the transition period for herd-level monitoring of increased risk of disease and decreased reproductive and milking performance. Vet Clin North Am - Food Anim Pract 2013;29:387–412. https://doi.org/10.1016/j.cvfa.2013.04.003.

[41] Barletta RV, Maturana Filho M, Carvalho PD, Del Valle TA, Netto AS, Rennó FP, et al. Association of changes among body condition score during the transition period with NEFA and BHBA concentrations, milk production, fertility, and health of Holstein cows. Theriogenology 2017;104:30–6. https://doi.org/10.1016/j.theriogenology.2017.07.030.

[42] Bicalho MLS, Marques EC, Gilbert RO, Bicalho RC. The association of plasma glucose, BHBA, and NEFA with postpartum uterine diseases, fertility, and milk production of Holstein dairy cows. Theriogenology 2017;88:270–82. https://doi.org/10.1016/j.theriogenology.2016.09.036.

[43] Garverick HA, Harris MN, Vogel-Bluel R, Sampson JD, Bader J, Lamberson WR, et al. Concentrations of nonesterified fatty acids and glucose in blood of periparturient dairy cows are indicative of pregnancy success at first insemination. J Dairy Sci 2013;96:181–8. https://doi.org/10.3168/jds.2012-5619.

[44] Mair B, Drillich M, Klein-Jöbstl D, Kanz P, Borchardt S, Meyer L, et al. Glucose concentration in capillary blood of dairy cows obtained by a minimally invasive lancet technique and determined with three different hand-held devices. BMC Vet Res 2016;12:34. https://doi.org/10.1186/s12917-016-0662-3.

[45] Herdt TH. Ruminant adaptation to negative energy balance. Influences on the etiology of ketosis and fatty liver. Vet Clin North Am Food Anim Pract 2000;16:215–30. https://doi.org/10.1016/S0749-0720(15)30102-X.

[46] Kerestes M, Faigl V, Kulcsár M, Balogh O, Földi J, Fébel H, et al. Periparturient insulin secretion and whole-body insulin responsiveness in dairy cows showing various forms of ketone pattern with or without puerperal metritis. Domest Anim Endocrinol 2009;37:250–61. https://doi.org/10.1016/j.domaniend.2009.07.003.

[47] Pires JAA, Pescara JB, Grummer RR. Reduction of Plasma NEFA Concentration by Nicotinic Acid Enhances the Response to Insulin in Feed-Restricted Holstein Cows. J Dairy Sci 2007;90:4635–42. https://doi.org/10.3168/jds.2007-0146.

[48] Bossaert P, Leroy JLMR, De Vliegher S, Opsomer G. Interrelations Between Glucose-Induced Insulin Response, Metabolic Indicators, and Time of First Ovulation in High-Yielding Dairy Cows. J Dairy Sci 2008;91:3363–71. https://doi.org/10.3168/jds.2008-0994.

[49] Jaakson H, Karis P, Ling K, Ilves-Luht A, Samarütel J, Henno M, et al. Adipose tissue insulin receptor and glucose transporter 4 expression, and blood glucose and insulin responses during glucose tolerance tests in transition Holstein cows with different body condition. J Dairy Sci 2018;101:752–66. https://doi.org/10.3168/jds.2017-12877.

[50] Holtenius P, Holtenius K. A model to estimate insulin sensitivity in dairy cows. Acta Vet Scand 2007;49:3–5. https://doi.org/10.1186/1751-0147-49-29.

[51] Liu GW, Zhang ZG, Wang JG, Wang Z, Xu C, Zhu XL. Insulin Receptor Gene Expression in Normal and Diseased Bovine Liver. J Comp Pathol 2010;143:258–61. https://doi.org/10.1016/j.jcpa.2010.04.004.

[52] Bertoni G, Trevisi E, Han X, Bionaz M. Effects of Inflammatory Conditions on Liver Activity in Puerperium Period and Consequences for Performance in Dairy Cows. J Dairy Sci 2008;91:3300–10. https://doi.org/10.3168/jds.2008-0995.

[53] Zhou Z, Trevisi E, Luchini DN, Loor JJ. Differences in liver functionality indexes in peripartal dairy cows fed rumen-protected methionine or choline are associated with performance, oxidative stress status, and plasma amino acid profiles. J Dairy Sci 2017;100:6720–32. https://doi.org/10.3168/jds.2016-12299.

[54] Pantelić M, Jovanović LJ, Prodanović R, Vujanac I, Đurić M, Ćulafić T, et al. The impact of the chromium supplementation on insulin signalling pathway in different tissues and milk yield in dairy cows. J Anim Physiol Anim Nutr (Berl) 2018;102:41–55. https://doi.org/10.1111/jpn.12655.

[55] Shi X, Li D, Deng Q, Li Y, Sun G, Yuan X, et al. NEFAs activate the oxidative stress-mediated NF-κB signaling pathway to induce inflammatory response in calf hepatocytes. J Steroid Biochem Mol Biol 2015;145:103–12. https://doi.org/10.1016/j.jsbmb.2014.10.014.

Legends to figures

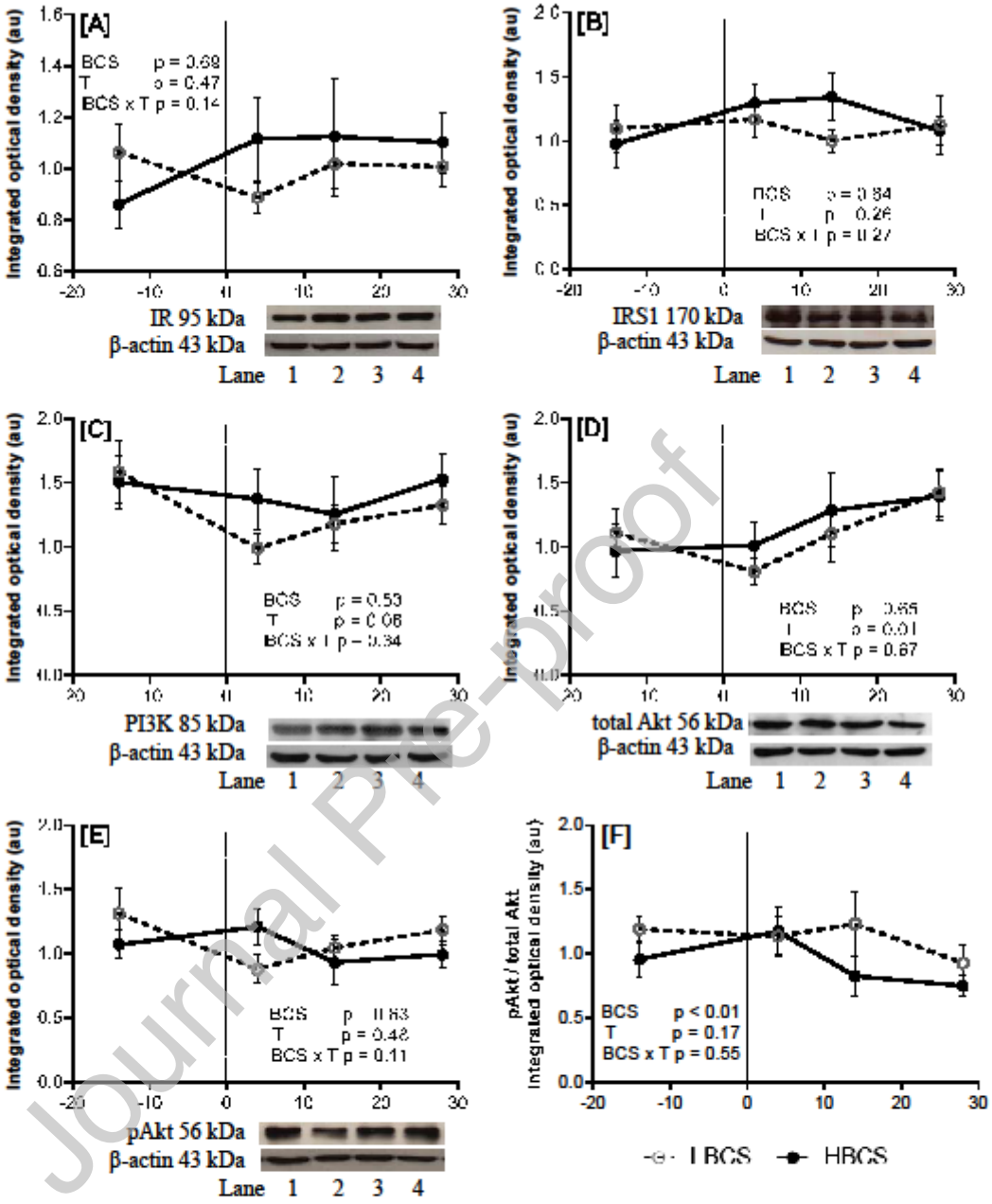

Figure 1. Protein expression of: hepatic insulin receptor (IR, panel A), insulin receptor substrate 1 (IRS1; panel B), phosphatidylinositol-3-kinase (PI3K, panel C), protein

kinase B (total Akt; panel D), phosphorylated Akt (pAkt; panel E) and pAkt/Akt rate (panel F). Animals were grouped as cows with high body condition score (HBCS; n = 7) or low body condition score (LBCS; n = 9) at 14 days precalving, and at 4, 14 and 28 days postcalving. Top of each panel: Integrated optical density (au: arbitrary units) determined by Western blot analysis. Values are expressed as mean ± SEM. The statistical effects of BCS, time (T) and BCS x T are indicated ($P < 0.05$). Bottom of each panel: Representative immunoblots of each determination. The molecular weight is shown on the right. Lane 1, day 14 precalving; lanes 2 to 4, days 4, 14 and 28 postcalving.

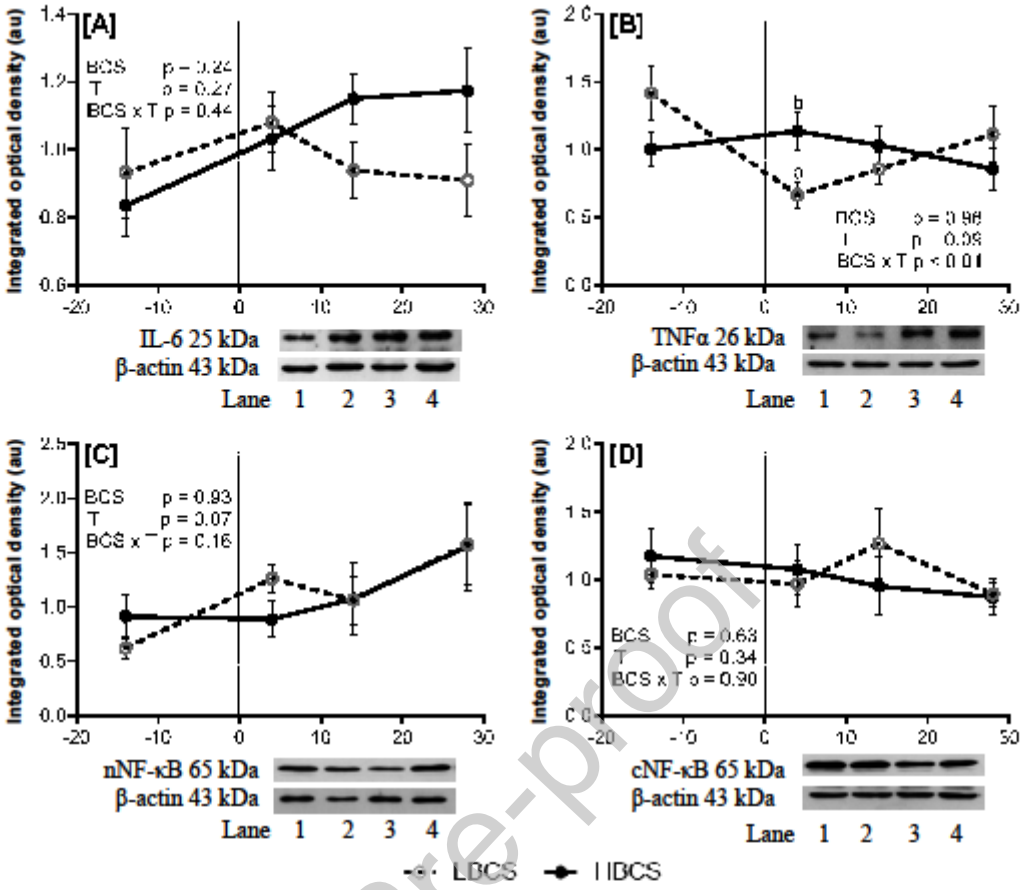

Figure 2. Protein expression of hepatic interleukin 6 (IL-6; panel A); tumor necrosis factor (TNFα; panel B), nuclear factor kappa B in the nucleus (nNF-κB; panel C) and nuclear factor kappa B in the cytoplasm (cNF-κB; panel D). Animals were grouped as

cows with high body condition score (HBCS; n = 7) or low body condition score (LBCS; n = 9) at 14 days precalving, and at 4, 14 and 28 days postcalving. Top of each panel: Integrated optical density (au: arbitrary units) determined by Western blot analysis. Values are expressed as mean ± SEM. The statistical effects of BCS, time (T) and BCS x T are indicated. [a-b] BCS × T among BCS at a given week ($P < 0.05$). Bottom of each panel: Representative immunoblots of each determination. The molecular weight is shown on the right. Lane 1, day 14 precalving; lanes 2 to 4, days 4, 14 and 28 postcalving.

Table 1. Ingredients and chemical composition of the experimental diet for precalving and postcalving herds

	Precalving	Postcalving
Ingredients (% DM)		
Corn silage	24.07	17.10
Cracked corn grain	21.65	17.10
Sunflower meal		21.38
Wheat straw		25.65
Grass hay		17.10
Alfalfa	9.63	
Ryegrass	24.07	
Soybean silage	9.63	
Soybean meal	4.81	
Wheat grain	4.81	
Mineral and vitamin pack[1]	1.33	1.67
DMI (kg/d)	11.9	14.9
Chemical		
CP (% DM)	13.3	16.5
NDF (% DM)	49.5	36.3
ADF (% DM)	32.0	20.7
NFC (% DM)	32.6	40.6
NEl (Mcal/kg)	1.40	1.63

[1] The prepartum pack contained Mg (15%), Cr (1%), Zn (1600 mg/kg), Mn (650 mg/kg), Cu (750 mg/kg), I (25 mg/kg), Se (2 mg/kg), Co (19 mg/kg), Vit A (350000 IU/kg), Vit D (60000 IU/kg), Vit E (6500 IU/kg), rumen-protected choline (25%), and rumen-protected methionine (8%), whereas the postpartum pack contained Ca (24%), Mg (8%), Na (15%), Cl (10%), monensin 4000 mg/kg, Zn (3400 mg/kg), Mn (1000 mg/kg), Cu (800 mg/kg), I (46 mg/kg), Se (25 mg/kg), Co (8 mg/kg), Vit A (300000 IU/kg), Vit D (80000 IU/kg) and Vit E (1000 IU/kg).

%DM: dry matter basis; ADF: acid detergent fiber; NFC: non-fiber carbohydrate; DMI: dry matter intake.

Table 2. Antibodies and conditions used for Western blot assays

Antibodies	*Type*	*Suppliers*	*Dilution*	*Total Protein/lane*	*References*
Primary Antibodies					
IR	Rabbit Polyclonal C-19: sc-711	Santa Cruz Biotechnology, Inc., CA, USA	1/500	40 µg	[28]
IRS1	Rabbit Polyclonal C-20: sc-559	Santa Cruz Biotechnology, Inc., CA, USA	1/1000	40 µg	[29]
PI3K	Rabbit Polyclonal Z-8: sc-423,	Santa Cruz Biotechnology, Inc., CA, USA	1/500	40 µg	[30]
Akt	Rabbit Polyclonal ab18785	Abcam, Cambridge, UK	1/600	40 µg	[31]
pAkt	Mouse monoclonal B-5: sc-271966	Santa Cruz Biotechnology, Inc., CA, USA	1/250	40 µg	[32]
IL-6	Mouse monoclonal 77830, MAB686	Novus Biologicals, LLC, USA	1/500	40 µg	[33]
TNFα	Rabbit Polyclonal AHP852Z	Bio-Rad Laboratories Inc. CA, USA	1/1500	20 µg	[33]
NF-κB p65	Rabbit Polyclonal C-20: sc-372	Santa Cruz Biotechnology, Inc., CA, USA	1/3000	40 µg	[34]
β–actin	Mouse monoclonal JLA20	DSHB, Iowa City, IA, USA	1/1500		
PCNA	Mouse monoclonal PC10: sc-56	Santa Cruz Biotechnology, Inc., CA, USA	1/250		
Secondary Antibodies					
Mouse anti-rabbit IgG	Monoclonal sc-2357	Santa Cruz Biotechnology. Inc. CA, USA	1/10000		
Mouse IgGκ binding protein	Binding protein: sc-516102	Santa Cruz Biotechnology. Inc. CA, USA	1/10000		

Table 3. BCS, plasma concentrations of NEFA, BHBA, glucose, TAG, insulin, IGF1 and liver TAG content of dairy cattle

		Precalving day	*Postcalving days*					
		-14	**4**	**14**	**28**	**BCS**	**T**	**BCS x T**
BCS	LBCS	3.00 ± 0.08	2.83 ± 0.11	2.58 ± 0.10	2.66 ± 0.09	< 0.01	< 0.01	0.25
	HBCS	3.71 ± 0.09	3.46 ± 0.14	3.14 ± 0.11	3.00 ± 0.07			
NEFA	LBCS	0.61 ± 0.09	1.32 ± 0.16	1.09 ± 0.16	0.94 ± 0.17	0.05	< 0.01	0.16
(mmol/L)	HBCS	0.71 ± 0.09	1.30 ± 0.17	1.42 ± 0.23	1.67 ± 0.24			
BHBA	LBCS	0.31 ± 0.06	0.70 ± 0.14	0.94 ± 0.19	0.88 ± 0.18	0.09	< 0.01	0.16
(mmol/L)	HBCS	0.32 ± 0.07	1.07 ± 0.22	1.64 ± 0.34	1.40 ± 0.29			
Glucose	LBCS	77.65 ± 8.41	78.11 ± 4.46	72.85 ± 4.75	83.70 ± 4.85[a]	0.57	< 0.01	< 0.01
(mg/dL)	HBCS	79.17 ± 6.41	73.45 ± 4.12	74.16 ± 4.18	96.16 ± 4.21[b]			
TAG	LBCS	42.40 ± 4.22	33.04 ± 1.72	42.04 ± 5.34	48.20 ± 3.86	0.35	< 0.01	0.82
(mg/dL)	HBCS	34.71 ± 5.07	29.82 ± 5.54	36.58 ± 4.79	48.68 ± 5.44			
Insulin	LBCS	0.79 ± 0.06	0.65 ± 0.05	0.74 ± 0.08	0.69 ± 0.04	0.42	< 0.01	0.41
(ng/mL)	HBCS	0.76 ± 0.02	0.69 ± 0.04	0.61 ± 0.04	0.65 ± 0.03			
IGF-1	LBCS	165.39 ± 18.87	121.74 ± 18.94	248.50 ± 31.68	187.57 ± 21.56	0.12	< 0.01	0.18
(ng/mL)	HBCS	100.37 ± 20.94	96.19 ± 19.20	235.05 ± 34.53	196.94 ± 20.45			
Liver TAG	LBCS	5.38 ± 0.82	17.36 ± 3.14	15.45 ± 3.70	9.60 ± 1.88	0.28	<0.01	0.22
(mg/g wt)	HBCS	6.50 ± 1.21	16.05 ± 4.50	18.01 ± 2.26	15.83 ± 3.41			

Animals were grouped as cows with high body condition score (HBCS; n = 7) or low body condition score (LBCS; n = 9) at 14 days precalving and at 4, 14 and 28 days postcalving. Values are expressed as mean ± SEM. The statistical effects of BCS, time (T) and BCS x T are indicated. [a-b]BCS × T among BCS at a given week ($P \leq 0.05$). BCS: body condition score; NEFA: non-esterified fatty acids; BHBA: beta-hydroxybutyric acid; TAG: triglycerides; Liver TAG (mg/g wt): TAG content (mg/g wet tissue).

Table 4. Insulin sensitivity indexes of dairy cattle

		Precalving	*Postcalving days*					
		-14	**4**	**14**	**28**	**BCS**	**T**	**BCS x T**
HOMA	LBCS	0.148 ± 0.015	0.122 ± 0.014	0.140 ± 0.018	0.142 ± 0.013	0.80	0.09	0.20
	HBCS	0.167 ± 0.013	0.153 ± 0.022	0.102 ± 0.013	0.117 ± 0.013			
QUICKI	LBCS	0.578 ± 0.025	0.606 ± 0.017	0.590 ± 0.022	0.576 ± 0.014	0.90	0.05	0.19
	HBCS	0.550 ± 0.012	0.571 ± 0.019	0.632 ± 0.021	0.607 ± 0.017			
RQUICKI	LBCS	0.350 ± 0.011	0.319 ± 0.005	0.324 ± 0.007	0.334 ± 0.012	0.02	< 0.01	0.34
	HBCS	0.330 ± 0.005	0.310 ± 0.007	0.323 ± 0.007	0.309 ± 0.007			
$RQUICKI_{BHBA}$	LBCS	0.459 ± 0.025	0.338 ± 0.017	0.339 ± 0.016	0.349 ± 0.018	0.01	< 0.01	0.34
	HBCS	0.396 ± 0.016	0.331 ± 0.016	0.312 ± 0.013	0.309 ± 0.018			

Animals were grouped as cows with high body condition score (HBCS; n = 7) or low body condition score (LBCS; n = 9) at 14 days precalving and at 4, 14 and 28 days postcalving. Values are expressed as mean ± SEM. The statistical effects of BCS, T and BCS x T are indicated ($P \leq 0.05$). BCS: body condition score; HOMA: homeostasis model assessment index. QUICKI: quantitative insulin sensitivity check index. RQUICKI: revised quantitative insulin sensitivity check index. $RQUICKI_{BHBA}$: revised quantitative insulin sensitivity check index modified with beta-hydroxybutyrate

CRediT roles

E. Angeli: Conceptualization, Investigation, Project administration, Writing - review & editing. **D. Barcarolo:** Conceptualization, Investigation, Project administration, Writing - review & editing. **L. Durante:** Data curation, Formal analysis, Writing - review & editing. **S. Gonzalo:** Methodology, Writing -review & editing. **V. Matiller:** Methodology, Conceptualization, Writing - review & editing. **F. Rey:** Methodology, Conceptualization, Writing - review & editing. **H.H. Ortega:** Methodology, Conceptualization, Resources, review & editing. **G.J. Hein:** Methodology, Conceptualization, Project administration, Resources, Writing -review & editing - original draft.