Body condition alters glutathione and nuclear factor erythroid 2-like 2 (NFE2L2)–related antioxidant network abundance in subcutaneous adipose tissue of periparturient Holstein cows

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ABSTRACT

Dairy cows with high body condition score (BCS) in late prepartum are more susceptible to oxidative stress (OS). Nuclear factor erythroid 2-like 2 (NFE2L2) is a major antioxidant transcription factor. We investigated the effect of precalving BCS on blood biomarkers associated with OS, inflammation, and liver function, along with mRNA and protein abundance of targets related to NFE2L2 and glutathione (GSH) metabolism in s.c. adipose tissue (SAT) of periparturient dairy cows. Twenty-two multiparous Holstein cows were retrospectively classified into a high BCS (HBCS; $n = 11$, $BCS \geq 3.5$) or normal BCS (NBCS; $n = 11$, $BCS \leq 3.17$) on d 28 before parturition. Cows were fed a corn silage- and wheat straw-based total mixed ration during late prepartum, and a corn silage- and alfalfa hay-based total mixed ration postpartum. Blood samples obtained at -10 , 7 , 15 , and 30 d relative to parturition were used for analyses of biomarkers associated with inflammation, including albumin, ceruloplasmin, haptoglobin, and myeloperoxidase, as well as OS, including ferric reducing ability of plasma (FRAP), reactive oxygen species (ROS), and β -carotene. Adipose biopsies harvested at -15 , 7 , and 30 d relative to parturition were analyzed for mRNA (real-time quantitative PCR) and protein abundance (Western blotting) of targets associated with the antioxidant transcription regula-

tor nuclear factor, NFE2L2, and GSH metabolism pathway. In addition, concentrations of GSH, ROS and malondialdehyde were measured. High BCS cows had lower prepartum dry matter intake expressed as a percentage of body weight along with greater BCS loss between -4 and 4 wk relative to parturition. Plasma concentrations of ROS and FRAP increased after parturition regardless of treatment. Compared with NBCS, HBCS cows had greater concentrations of FRAP at d 7 postpartum, which coincided with peak values in those cows. In addition, NBCS cows experienced a marked decrease in plasma ROS after d 7 postpartum, while HBCS cows maintained a constant concentration by d 30 postpartum. Overall, ROS concentrations in SAT were greater in HBCS cows. However, overall mRNA abundance of *NFE2L2* was lower and cullin 3 (*CUL3*), a negative regulator of NFE2L2, was greater in HBCS cows. Although HBCS cows had greater overall total protein abundance of NFE2L2 in SAT, ratio of phosphorylated NFE2L2 to total NFE2L2 was lower, suggesting a decrease in the activity of this antioxidant system. Overall, mRNA abundance of the GSH metabolism-related genes glutathione reductase (*GSR*), glutathione peroxidase 1 (*GPX1*), and transaldolase 1 (*TALDO1*), along with protein abundance of glutathione S-transferase mu 1 (*GSTM1*), were greater in HBCS cows. Data suggest that HBCS cows might experience greater systemic OS after parturition, while increased abundance of mRNA and protein components of the GSH metabolism pathway in SAT might help alleviate tissue oxidant status. Data underscored the importance of antioxidant mechanisms at the tissue level. Thus, targeting these pathways in SAT during the peripar-

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turient period via nutrition might help control tissue remodeling while allowing optimal performance.

Key words: body condition score, oxidative stress, NFE2L2, adipose

INTRODUCTION

Body condition score is used to evaluate the degree of apparent adiposity in dairy cows (Roche et al., 2013). A high body condition score (BCS $\geq$ 3.5) at calving is negatively associated with early-lactation DMI and milk yield, and is positively related to the incidence of periparturient metabolic disorders (Roche et al., 2009). For instance, cows calving at a high BCS are more likely to experience fatty liver, subclinical ketosis, and chronic oxidative stress (OS) during the transition period (Reid et al., 1986; Bernabucci et al., 2005; Loor et al., 2013a). Despite extensive research on the use of BCS as a management tool and its association with important physiological aspects such as lipid metabolism, insulin resistance and inflammation (De Koster et al., 2015; Depreester et al., 2018; Newman et al., 2019), molecular mechanisms of OS associated with BCS in adipose tissue are not well-known.

Nuclear factor erythroid 2-like 2 (NFE2L2), considered a master antioxidant transcription factor, plays a critical role against OS damage via regulating a wide range of antioxidant response-dependent genes in mammals (Ma, 2013). Changes in transcription of *NFE2L2* in the liver during the transition period are thought to play a role in regulating tissue antioxidant response (Gessner et al., 2013). More recent in vitro and in vivo data indicate that activation of NFE2L2 (and its target genes) could serve as a mechanism to maintain oxidant status in the mammary gland (Han et al., 2018a,b; Ma et al., 2018). Greater protein abundance of targets associated with the NFE2L2 pathway, coupled with elevated plasma malondialdehyde, was reported in subcutaneous adipose tissue (SAT) in cows calving during the summer compared with winter, suggesting this pathway also might be important in coping with OS in SAT (Zachut et al., 2017). Indeed, an essential role of the NFE2L2 pathway in the antioxidant response in bovine adipose tissue was underscored by a recent in vitro study demonstrating that mild OS led to greater abundance of NFE2L2 at both transcription and translation levels, while severe OS resulted in lower abundance (Sun et al., 2019).

Glutathione (GSH) is a well-known antioxidant in cells, and contributes to eliminating H₂O₂ within the cytosol, hence, preventing oxidative damage and regulating the thiol-redox status in tissues (Aquilano et al., 2014). A previous study from our group revealed that enhanced post-ruminal supply of Met, the source of

thiol-groups, led to alleviated OS, along with greater mRNA abundance of glutamate-cysteine ligase modifier subunit, glutathione reductase (*GSR*), and glutathione peroxidase 1 (*GPX1*). The greater activity of various GSH-related antioxidant enzymes in periparturial dairy cow SAT underscored the importance of GSH metabolism and its responsiveness to changes in physiologic state (Batistel et al., 2017; Liang et al., 2019).

Our general hypothesis was that normal prepartal BCS leads to the activation of the NFE2L2 pathways, ensuing greater GSH synthesis in SAT. The main objective of this study was to investigate changes in mRNA and protein abundance of major components related to the NFE2L2 and GSH pathways in SAT, along with plasma and tissue biomarkers of OS in periparturial cows calving at a high or low BCS.

MATERIALS AND METHODS

Experiment Design

All procedures were conducted under protocols approved by the University of Illinois Institutional Animal Care and Use Committee (Urbana; protocol #17168). Body condition score was monitored weekly by 3 individuals from -4 wk to 4 wk relative to expected parturition date, and mean values were used for classifying cows in the current study. Twenty-two clinically healthy multiparous Holstein cows were retrospectively classified into 2 groups: high BCS (**HBCS**; 3.75 $\pm$ 0.25, 3.5–4.0; mean $\pm$ SD; n = 11) and normal BCS (**NBCS**; 3.07 $\pm$ 0.07, 3.0–3.17; mean $\pm$ SD; n = 11), at d 28 before parturition based on a 5-point scale (Edmonson et al., 1989). The average (mean $\pm$ SD) BW at -4 wk relative to parturition was 896 $\pm$ 51 kg and 786 $\pm$ 48 kg in HBCS and NBCS, respectively. The average for parity (mean $\pm$ SD) was 3.5 $\pm$ 1.6 in HBCS cows and 3.0 $\pm$ 1.1 for NBCS. Cows were fed a corn silage- and wheat straw-based TMR during late prepartum period and a corn silage- and alfalfa hay-based TMR after parturition (Table 1). Cows were fed once daily (0600 h) with ad libitum access to the diet. Dry cows were housed in a freestall barn with an individual Calan gate feeding system (American Calan, Northwood, NH). After calving, cows were housed in a tie-stall barn and milked 3 times daily at approximately 0600, 1400, and 2200 h. Milk production and feed refusals were recorded daily for each cow. Diets were formulated to meet predicted requirements for dairy cows according to NRC (2001).

Feed Sample Collection

Individual ingredients and TMR samples were collected once a week to determine the DM and used

to adjust the DM of the TMR accordingly. Weekly samples of ingredients and TMR were frozen at -20°C and pooled monthly for nutrient composition analysis, as described previously by Batistel et al. (2017). The ingredients and nutrient compositions of the diets are reported in Table 1.

Blood Collection and Analyses

Blood was obtained from the coccygeal vein before morning feeding at $-10 (\pm 1 \text{ d})$, 7, 15, and 30 d relative to parturition. Samples were collected in Vacutainer tubes containing lithium heparin (BD Vacutainer, Becton, Dickinson and Co., Franklin Lakes, NJ) and were immediately placed on ice. Plasma was harvested by centrifugation at $2,000 \times g$ for 15 min at 4°C , and aliquots stored at -80°C until further analysis. Activities of aspartate aminotransferase (**AST**), γ -glutamyl transpeptidase (**GGT**), alkaline phosphatase, myeloperoxidase and paraoxonase, and concentrations of albumin, total bilirubin, total plasma reactive oxygen species (**ROS**), ferric reducing ability of plasma (**FRAP**), haptoglobin, ceruloplasmin, nitric oxide and nitric oxide metabolites, β -carotene, retinol, and tocopherol were analyzed as described by Lopreiato et al. (2019).

Adipose Tissue Biopsies

Cows in HBCS and NBCS averaged $28 \pm 3 \text{ d}$ in the close-up dry period. All (11/group) were free of clinical disorders and had the full set of biopsies. Tissue was harvested from the tail head (alternating between the right and left tail head region) at $-15 (\pm 2 \text{ d})$, 7, and 30 d relative to parturition according to previous procedures from our laboratory (Ji et al., 2012). Upon collection, adipose tissue was immediately placed in screw-capped microcentrifuge tubes, snap-frozen in liquid nitrogen, and preserved at -80°C until further analysis. Health was monitored for 7 d after surgery, and surgical clips were removed after 7 d post-biopsy. No antibiotics were administered post-biopsy.

RNA Isolation, cDNA Synthesis and Quantitative PCR

Total RNA isolation was performed as described in our previous study (Liang et al., 2019). Briefly, total RNA was isolated from 200 mg of adipose tissue using the miRNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The RNA samples were digested with DNaseI and quantification was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The quality

of RNA samples was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The quantitative PCR (**qPCR**) was performed as described by Osorio et al. (2014). The internal controls for adipose tissue were *RPS9*, *GAPDH* and *ACTB*. These internal control genes were previously confirmed as suitable for adipose tissue gene expression analysis (Vailati-Riboni et al., 2015, 2016, 2017). Gene symbols and names, qPCR performance, and primer informa-

Table 1. Ingredient and nutrient composition of diets fed to Holstein cows with prepartum high (≥ 3.5) or normal (≤ 3.17) BCS during the close-up (-28 d to calving) dry period and early lactation (calving to 30 d)

Item	Close-up	Lactation
Ingredient, % of DM		
Corn silage	37.45	41.18
Ground shelled corn	11.10	23.40
Wheat straw	21.80	2.30
Canola meal	11.66	3.20
Soybean meal	6.30	13.00
Alfalfa hay	—	8.60
Soychlor ¹	3.37	—
Corn gluten	2.80	2.50
ProvAAL2 AADvantage ²	0.47	0.72
Biotin ³	0.10	0.08
Rumensin ⁴	0.19	0.02
Calcium sulfate	0.53	0.12
Magnesium oxide	0.10	0.12
Ca	0.66	1.00
P	0.33	0.35
Salt	0.10	0.25
Na	0.12	0.45
Cl	0.78	0.68
Mg	0.45	0.38
K	1.36	1.45
S	0.33	0.20
Nutrient composition		
CP, % of DM	14.50	17.00
NDF, % of DM	43.30	21.50
ADF, % of DM	33.80	16.76
aNDFom, ⁵ % of DM	49.21	27.01
NFC, % of DM	28.22	46.83
NE _L , Mcal/kg of DM	1.37	1.65
NE _L allowable milk, kg/d	—	25.85
MP allowable milk, kg/d	—	28.66
RDP, % of DM	8.45	11.00
RUP, % of DM	6.05	6.00
RDP required, g/d	1,165	1,873
RDP supplied, g/d	1,152	1,995
RDP balance, g/d	-18	122
RUP required, g/d	158	1,510
RUP supplied, g/d	821	1,088
RUP balance, g/d	662	-421
MP required, g/d	821	2,404
MP supplied, g/d	1,360	2,041
MP balance, g/d	539	-362

¹Anionic supplement; West Central Soy (Ralston, IA).

²Rumen-protected methionine; Perdue AgriBusiness (Salisbury, MD).

³ADM Animal Nutrition (Quincy, IL).

⁴Monensin ionophore; Elanco Animal Health (Greenfield, IN).

⁵aNDFom = neutral detergent fiber after amylase treatment and ashing.

tion are reported in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-17813>).

Western Blot Analysis

Total protein was extracted from 100 mg of adipose tissue using a tissue protein extraction reagent (catalog no. 78510; Thermo Fisher Scientific) containing a Halt protease and phosphatase inhibitor cocktail (100×, catalog no. 78442; Thermo Fisher Scientific). The concentration of total protein was determined using a Pierce bicinchoninic acid protein assay kit (catalog no. 23227; Thermo Fisher Scientific). Details of Western blot were reported in a previous study from our group (Liang et al., 2019). Briefly, protein samples were denatured by heating at 95°C for 5 min before loading 10 µL of protein into each lane of a 4–20% SDS-PAGE gel (catalog no. 4561096; Bio-Rad, Hercules, CA). Reactions were run for 10 min at 180 V, and then for 45 to 60 min at 110 V. Then the protein sample was transferred to the membrane in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (catalog no. 170-3940, Bio-Rad). Membranes were blocked in 1× Tris-buffered saline (TBST) containing 5% nonfat milk for 2 h at room temperature. The membranes were incubated in TBST containing primary antibodies to glutathione S-transferase mu 1 (GSTM1), kelch-like ECH associated protein, extracellular signal-regulated protein kinases 1 and 2, phospho-extracellular signal-regulated protein kinases 1 and 2(Thr202/Tyr204), NFE2L2 and phospho-NFE2L2(Ser40) (catalog # and dilution ratio are included in Supplemental Table S2, <https://doi.org/10.3168/jds.2019-17813>) overnight at 4°C. The membranes were then washed with 1× TBST and incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (catalog no. 7074S; Cell Signaling Technology, dilution 1:1,000). Subsequently, the membranes were washed with 1× TBST and then incubated with ECL reagent (catalog no. 170-5060; Bio-Rad) before image acquisition. β-Actin (catalog no. 4967S; Cell Signaling Technology, Danvers, MA) was used as the internal control. Images were acquired using the ChemiDOC MP Imaging System (Bio-Rad). The intensities of the bands were measured with Image-Pro Plus 6.0 software. Specific target protein band density values were normalized to β-actin density values. Representative blots are included in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2019-17813>).

Biomarker Analysis in Subcutaneous Adipose Tissue

As in our previous study (Liang et al., 2019), the following OS biomarkers in SAT were determined using

Figure 1. Change in BCS and BCS loss between –4 and 4 wk relative to parturition in Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥3.5) or normal BCS (NBCS, BCS ≤3.17). Data are LSM ± pooled SEM, n = 11 cows per group. ^{a,b}Means groups differ ($P \leq 0.05$).

commercial kits according to manufacturer's instructions: ROS (catalog no. STA-347, Cell Biolabs, San Diego, CA), malondialdehyde (catalog no. 10009055; Cayman Chemical), and GSH (catalog no. NWK-GSH01; Northwest Life Science Specialties, Vancouver, WA). Adipose tissue total protein concentration was measured using the Pierce BCA assay kit (catalog no. 23227; Thermo Scientific).

Statistical Analysis

The data were analyzed using the MIXED procedure of SAS v.9.4 (SAS Institute Inc., Cary, NC) according to the following model with repeated measures:

$$Y_{jil} = \mu + M_j + T_l + MT_{jl} + e_{jil},$$

where Y_{jil} = dependent, continuous variable, μ = overall mean, M_j = fixed effect of BCS (j = HBCS vs. NBCS), T_l = fixed effect of day (for blood biomarkers, –10, 7, 15, and 30 d; for qPCR, Western blot, and OS biomarker in SAT analysis, –15, 7, and 30 d), MT_{jl} = interaction between BCS and Day, and e_{jil} = residual error. Cow, nested within BCS, was the random effect. The Kenward-Roger statement was used for computing the denominator degrees of freedom. The covariance structure of the repeated measurements was spatial power [SP(POW)]. When the interaction was significant, least squares means separation between and within time points was performed using the PDIF statement with Tukey adjustment. Normality of the

Figure 2. Prepartum and postpartum DMI, DMI as % of BW, and milk yield of Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥ 3.5) or normal BCS (NBCS, BCS ≤ 3.17) -30 to 30 d relative to parturition. Data are LSM $\pm$ pooled SEM, $n = 11$ cows per group.

Table 2. Least squares means ($n = 11$) $\pm$ pooled SEM for plasma biomarkers of inflammation and oxidative stress in Holstein cows with prepartum high or normal BCS

Item ¹	Group ²		SEM	P-value		
	HBCS	NBCS		BCS	Day	BCS × Day
Inflammation						
Albumin, g/L	36.4	35.3	0.59	0.22	0.40	0.42
Ceruloplasmin, μmol/L	3.21	3.11	0.14	0.59	<0.01	0.10
Haptoglobin, g/L	0.35	0.35	0.03	0.92	<0.01	0.68
Myeloperoxidase, U/L	526	518	15.2	0.71	0.01	0.63
Oxidative stress						
FRAP, μmol/L	123	120	3.99	0.66	<0.01	0.02
ROS, H ₂ O ₂ /100 mL	16.0	15.2	0.50	0.28	<0.01	0.06
NO, μmol/L	26.3	26.4	0.33	0.74	<0.01	0.55
Nitrite, μmol/L	3.86	3.57	0.21	0.31	<0.01	0.26
Nitrate, μmol/L	21.9	22.5	0.35	0.23	<0.01	0.92
β-Carotene, mg/100 mL	0.17	0.23	0.02	0.08	<0.01	0.29
Retinol, μg/mL	24.9	26.5	2.01	0.58	<0.01	0.64
Tocopherol, μg/mL	2.85	3.18	0.18	0.21	<0.01	0.96
Liver function						
Alkaline phosphatase, U/L	43.9	52.8	4.42	0.13	0.22	0.04
AST, U/L	102	103	5.85	0.96	<0.01	0.20
GGT, U/L	23.5	20.6	1.58	0.18	<0.01	0.26
Paraoxonase, U/L	74.1	69.9	4.06	0.47	<0.01	0.44
Bilirubin, μmol/L	5.03	4.06	0.49	0.13	<0.01	0.22

¹FRAP = ferric-reducing ability of plasma; ROS = reactive oxygen species; AST = aspartate aminotransferase; GGT = γ -glutamyl transpeptidase.

²HBCS = high BCS (≥ 3.5); NBCS = normal BCS (≤ 3.17).

residuals was checked with normal probability and box plots, and homogeneity of variances was checked with plots of residuals versus predicted values. Outliers were removed when the absolute value of studentized residual was >2 . Significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS AND DISCUSSION

Body Condition and Animal Performance

High BCS cows had greater BCS compared with NBCS cows from -4 to 4 wk relative to calving date ($P < 0.01$; Figure 1). Additionally, HBCS cows had greater BCS loss than NBCS cows ($P < 0.05$; Figure 1). Both prepartum and postpartum DMI did not differ between HBCS and NBCS cows ($P = 0.77$ and $P = 0.89$; Figure 2A and C), which is in line with Alharthi et al. (2018) and Pires et al. (2013). However, when expressed as percent of BW, NBCS cows had greater prepartum DMI ($P = 0.04$; Figure 2B), and tended to have greater postpartum DMI ($P = 0.09$; Figure 2D). Feed intake and milk yield might play a role in regulating BCS when cows are fed and managed under the same conditions (Rocco and McNamara, 2013). Due to the lack of difference in actual amounts of DMI and milk yield ($P = 0.77$; Figure 2E), DMI as % of BW seems to be a more reasonable indicator of BCS effect on performance.

Blood Parameters Associated with Inflammation and Oxidative Stress

Compared with the prepartum, plasma ceruloplasmin and haptoglobin concentrations increased after parturition in both HBCS and NBCS cows (Day, $P < 0.01$; Table 2, Figure 3A and B). However, ceruloplasmin tended to decrease from 7 to 30 d postpartum in NBCS cows, whereas HBCS cows showed the opposite trend (BCS $\times$ Day, $P = 0.10$; Figure 3A). Overall, plasma myeloperoxidase activity increased between -10 and 15 d around parturition, followed by a sudden decrease at 30 d after parturition, irrespective of BCS (Day, $P = 0.01$; Figure 3C). Regardless of BCS, AST activity and bilirubin concentration (indicators of liver function) were greater after parturition and reached a peak at d 7 (Day, $P < 0.01$; Figure 4B and C). Similarly, GGT increased after parturition, regardless of BCS (Day, $P < 0.01$; Figure 4A). Additional results are given in Supplemental Figure S2 (<https://doi.org/10.3168/jds.2019-17813>).

Acute-phase proteins (APP), a critical part of the acute-phase response, include positive APP (i.e., increase during inflammation), such as haptoglobin, ceruloplasmin, and serum amyloid-A, and negative APP (i.e., decrease during inflammation), such as albumin, apolipoproteins, retinol-binding protein, and paraoxonase (Trevisi et al., 2013; Loores et al., 2013b). Through its antimicrobial activity, myeloperoxidase is a critical

Figure 3. Plasma biomarkers of inflammation in Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥ 3.5) or normal BCS (NBCS, BCS ≤ 3.17). (A) Ceruloplasmin; (B) haptoglobin; and (C) myeloperoxidase. Data are LSM $\pm$ pooled SEM, $n = 11$ cows per group.

Figure 4. Plasma biomarkers of liver function in Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥ 3.5) or normal BCS (NBCS, BCS ≤ 3.17). (A) γ -Glutamyl transpeptidase (GGT); (B) aspartate aminotransferase (AST); and (C) bilirubin. Data are LSM $\pm$ pooled SEM, $n = 11$ cows per group.

Figure 5. Plasma biomarkers of oxidative stress in Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥ 3.5) or normal BCS (NBCS, BCS ≤ 3.17) (A) Ferric-reducing ability of plasma (FRAP); (B) reactive oxygen species (ROS); and (C) β -carotene. Data are LSM $\pm$ pooled SEM, $n = 11$ cows per group. Means with different letters (a, b) differ (BCS $\times$ Day, $P \leq 0.05$).

enzyme in regulating innate immunity (Depreester et al., 2017). Changes in the various APP along with markers of liver function are commonly used to study inflammation status of periparturient cows (Bionaz et al., 2007; Bertoni et al., 2008; Graugnard et al., 2013). Increased ceruloplasmin concentration is associated with inflammation (Ceron et al., 2005); thus, its sharp increase after parturition in HBCS and NBCS cows was suggestive of a greater chronic inflammatory response postpartum (Bionaz et al., 2007; Batistel et al., 2018). However, the subsequent decrease in ceruloplasmin in NBCS cows suggested they experienced a shorter inflammatory period (Figure 3A). Although the greater inflammatory status during transition is one adaptive mechanism for dairy cows to cope with acute metabolic changes, a prolonged inflammatory response exacerbates the induction of metabolic disorders (Bertoni et al., 2008). Thus, as reported previously (Treacher et al., 1986; Roche et al., 2009), a prolonged inflammatory response in HBCS cows might contribute to greater susceptibility to metabolic disorders.

The lower concentration of plasma ROS prepartum and increase postpartum (Figure 5B) were in agreement with previous studies (Bernabucci et al., 2005; Batistel et al., 2018). The change in ROS between pre- and postpartum might have been due to the well-known increases in metabolic rate (Reynolds et al., 2003), along with potential direct effects of free fatty acids and BHB on circulating immune cells (Lacetera et al., 2005) or the liver (Sun et al., 2019). Overproduction of ROS results in OS and an ensuing inflammatory response, both of which increase the incidence of metabolic disorders (Bertoni et al., 2008). Thus, we speculate that the relative stability of plasma ROS concentration in HBCS cows after d 7 postpartum (unlike the lower plasma ROS level in NBCS cows) denoted a more prolonged inflammatory state, which agrees with some of the plasma biomarkers analyzed.

The cellular antioxidant β -carotene is mainly stored in the adipose tissue (Tourniaire et al., 2009), and is the major dietary precursor of vitamin A in dairy cattle, as well as a precursor for the synthesis of retinoic acid, a metabolite of vitamin A (LeBlanc et al., 2004; Frey and Vogel, 2011). Supplementation with β -carotene contributes to reduced risk of mastitis and retained placenta, a response associated with its antioxidant properties (Spears and Weiss, 2008). In humans, obesity is associated with lower β -carotene concentrations in adipocytes (Östh et al., 2014). Although we are unaware if adipose tissue mobilization contributes to the circulating β -carotene level during the transition period, the fact that all-*trans* retinoic acid supplementation inhibited inflammation in bovine adipocytes challenged with lipopolysaccharide suggests

a potentially important indirect effect of this vitamin (Xu et al., 2019). We speculate that maintaining higher concentrations of β -carotene in the circulation, either through supplementation or optimizing DMI, might directly or indirectly contribute to antioxidant status in SAT during the transition period.

Insulin supplementation in culture medium increased β -carotene content in bovine adipose explants, whereas epinephrine decreased it, suggesting that hormones related to lipid metabolism influence β -carotene mobilization from adipose tissue (Arias et al., 2009). However, BCS does not necessarily affect plasma insulin concentrations (e.g., Alharthi et al., 2018). Dairy cows experience increased lipolysis during the transition period, especially after parturition (Lor et al., 2013b). Intense lipolysis is linked to OS and uncontrolled inflammatory responses (Sordillo and Raphael, 2013). In the current study, the postpartal decrease in plasma concentration of β -carotene, regardless of BCS, was consistent with previous results (Osorio et al., 2014; Batistel et al., 2018). These responses suggest that increased lipolysis, along with OS and enhanced inflammatory response, might contribute to lower levels of circulating β -carotene. Thus, without differences in DMI, we speculate that HBCS cows are likely to utilize more circulating β -carotene due to their greater BCS loss. Taken together, the greater overall plasma β -carotene concentrations in NBCS cows might contribute to their reduced inflammatory response.

Similar to concentrations of ROS, FRAP increased after parturition regardless of BCS, and we detected a BCS $\times$ Day effect ($P < 0.01$) due to a greater response in plasma FRAP on d 7 postpartum in HBCS cows, followed by a decrease until 30 d postpartum (Figure 5A). These results are consistent with plasma ROS and

β -carotene data, and support the view that HBCS cows might have experienced greater OS status, especially after parturition (Bernabucci et al., 2005). The effect of lipolysis rate in bovine adipose tissue on β -carotene metabolism and utilization merits further study.

Oxidative Stress Biomarkers in Adipose Tissue

Main effects of BCS, Day, and their interaction on OS biomarkers are reported in Table 3, Figure 6, Figure 7, and Figure 8. High BCS cows had lower overall abundance of *NFE2L2* ($P = 0.03$; Table 3). In rodents, *NFE2L2* plays a critical role in the liver in regulating OS via increasing mRNA abundance of key antioxidant enzymes (Ma, 2013). In dairy cows, mRNA abundance of *NFE2L2* was first reported in the liver during the periparturient period (Lor, 2010) and recent studies revealed that *NFE2L2* is also expressed in the mammary gland and SAT (Zachut et al., 2017; Han et al., 2018b; Liang et al., 2019). In vitro, enhanced activity of *NFE2L2* and its target heme oxygenase-1 contributed partly to controlling oxidant status in bovine mammary epithelial cells (Ma et al., 2019).

Overall, the concentration of ROS in SAT was greater in HBCS than NBCS cows ($P < 0.01$; Figure 6A). Free radicals are essential for normal cellular metabolism, but overproduction without sufficient antioxidant capacity often results in DNA and protein damage and apoptosis (Valko et al., 2007). Reactive oxygen species can activate *NFE2L2* to protect cells from OS damage (Ray et al., 2012). A recent in vitro study reported that an increase in H_2O_2 concentration from 0 to 100 μM upregulated mRNA abundance of *NFE2L2* in bovine adipocytes; however, mRNA abundance of *NFE2L2* decreased when the concentration of H_2O_2 reached 200

Table 3. Least squares means ($n = 11$) $\pm$ pooled SEM for mRNA abundance of genes related to the *NFE2L2* pathway and glutathione metabolism in Holstein cows with prepartum high or normal BCS

Pathway and gene ¹	HBCS ²	NBCS ²	% Difference ²	SEM	P-value		
					BCS	Day	BCS × Day
NFE2L2 pathway							
<i>NFE2L2</i>	0.90	1.05	−13.5	0.04	0.03	<0.01	0.09
<i>KEAP1</i>	1.22	1.05	16.3	0.08	0.11	0.05	0.49
<i>CUL3</i>	1.54	1.25	22.7	0.10	0.03	<0.01	0.01
Glutathione metabolism							
<i>ME1</i>	0.84	0.75	12.3	0.09	0.45	<0.01	0.32
<i>TALDO1</i>	1.31	1.08	22.0	0.08	0.04	<0.01	0.27
<i>GSR</i>	0.20	0.14	41.1	0.01	<0.01	0.33	0.10
<i>GCLM</i>	0.51	0.51	−0.58	0.04	0.96	0.01	0.51
<i>GPX1</i>	0.79	0.64	22.4	0.05	0.02	0.05	0.23
<i>GCLC</i>	0.86	0.79	9.66	0.05	0.24	0.08	0.23

¹*NFE2L2* = nuclear factor, erythroid 2 like 2; *KEAP1* = kelch-like ECH-associated protein 1; *CUL3* = cullin 3; *ME1* = malic enzyme 1; *TALDO1* = transaldolase 1; *GSR* = glutathione reductase; *GCLM* = glutamate-cysteine ligase modifier subunit; *GPX1* = glutathione peroxidase 1; *GCLC* = glutamate-cysteine ligase catalytic subunit.

²HBCS = high BCS (≥ 3.5); NBCS = normal BCS (≤ 3.17); difference in mRNA abundance = (HBCS − NBCS)/NBCS $\times$ 100.

Figure 6. Concentrations in subcutaneous adipose tissue of Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥ 3.5) or normal BCS (NBCS, BCS ≤ 3.17). (A) Reactive oxygen species (ROS); (B) glutathione (GSH); and (C) malondialdehyde (MDA). Data are LSM $\pm$ pooled SEM, $n = 11$ cows per group.

μM (Sun et al., 2019). We speculate that the lower overall abundance of *NFE2L2* in HBCS cows (Table 3), coupled with greater ROS in SAT, was suggestive of diminished capacity of the tissue to mount an antioxidant response.

Studies in rodents have demonstrated that NFE2L2 function is not only important in regulating OS, but also for adipose development and insulin sensitivity (Schneider and Chan, 2013; Seo and Lee, 2013). The latter is particularly important because periparturient cows experience insulin resistance, especially early postpartum (Holtenius et al., 2003), while recent studies demonstrated that adipose tissue (AT) insulin resistance, especially in over-conditioned cows, develops prepartum (Jaakson et al., 2018). Over-conditioned cows have larger adipocytes in both SAT and omental AT; furthermore, larger adipocytes are more sensitive to lipolytic signals (De Koster et al., 2016). A recent study revealed that BCS loss is positively associated with macrophage infiltration in SAT during early lactation (De Koster et al., 2018). Macrophage infiltration leads to overproduction of ROS and inflammatory cytokines in human and rodent AT (Surmi and Hasty, 2010). In the present study, greater ROS concentration in SAT along with greater BCS loss in HBCS cows led us to speculate that macrophage infiltration might play a role in controlling oxidant status in cows calving at HBCS. The link between NFE2L2 and macrophage infiltration in regulating insulin resistance and adipocyte differentiation as it relates to calving BCS merits further study.

The decrease in plasma ROS after 7 and 15 d postpartum in NBCS and HBCS cows, respectively (Figure 5B), is noteworthy because ROS concentration in SAT was relatively steady from -15 d prepartum to 30 d postpartum, regardless of BCS (Figure 6A). These data suggest that SAT might take a longer time to recover from OS. Compared with NBCS, HBCS cows had greater overall abundance of cullin 3 (*CUL3*; $P = 0.03$; Table 3). Both KEAP1 and *CUL3* are inhibitors of NFE2L2 (Suzuki and Yamamoto, 2017); hence, greater abundance of *CUL3* explains at least in part the lower abundance of *NFE2L2* in HBCS cows.

In contrast to mRNA abundance, greater overall protein abundance of NFE2L2 and lower phosphorylated (p)-NFE2L2:NFE2L2 ratio was observed in HBCS cows ($P < 0.01$ and $P < 0.01$; Figure 7A and C). The difference between mRNA and protein abundance of NFE2L2 suggests that the activity of NFE2L2 is not regulated at the transcription level. In the present study, there was a BCS $\times$ Day effect for p-NFE2L2 ($P < 0.01$) due to a decrease in abundance of p-NFE2L2 in HBCS cows, and an increase in NBCS cows from 7 to 30 d after parturition (Figure 7B). These data provide

additional support for the idea that OS status increases with time in HBCS cows during the transition period.

Glutathione Metabolism

Main effects of BCS, Day, and their interaction related to GSH metabolism are reported in Table 3 and Figure 8. The greater overall abundance of genes associated with GSH metabolism including *GXP1*, *GSR* and transaldolase 1 (*TALDO1*) in HBCS cows ($P = 0.02$; $P < 0.01$; $P = 0.04$; Table 3) was surprising in part because those cows had lower abundance of *NFE2L2* ($P = 0.03$; Table 3). Cows in HBCS also had greater

overall protein abundance of GSTM1 ($P = 0.03$; Figure 8A). Despite these differences at the transcription and translation levels of GSH metabolism components, there was no difference in tissue GSH concentration ($P > 0.05$; Figure 6B).

Glutathione is a crucial antioxidant in mammalian cells (Aquilano et al., 2014), and the GSH metabolism pathway is one target regulated by NFE2L2 (Harvey et al., 2009). Although GSH metabolism is closely regulated by OS status in nonruminants (Dickinson and Forman, 2002), other factors such as nuclear factor- κ B (NF- κ B) activity (Buelna-Chontal and Zazueta, 2013) and availability of substrates such as Cys, Gly, and

Figure 7. Protein abundance (relative to β -actin) of the nuclear factor, erythroid 2 like 2 (NFE2L2; inactive, panel A), phosphorylated (p)-NFE2L2 (active, panel B), ratio of p-NFE2L2/NFE2L2 (panel C), NFE2L2 repressor kelch-like ECH associated protein (KEAP1; panel D) in subcutaneous adipose tissue of Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥ 3.5) or normal BCS (NBCS, BCS ≤ 3.17). Data are LSM, $n = 11$ cows per group, $\pm$ pooled SEM. Means with different letters (a, b) differ (BCS $\times$ Day, $P \leq 0.05$).

Figure 8. Protein abundance (relative to β -actin) of the glutathione S-transferase mu 1 (GSTM1; panel A), extracellular signal-regulated protein kinases 1 and 2 (ERK1/2; inactive, panel B), phosphorylated (p)-ERK1/2 (active, panel C), and ratio of p-ERK1/ERK1/2 (panel D) in subcutaneous adipose tissue of Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥ 3.5) or normal BCS (NBCS, BCS ≤ 3.17). Data are LSM $\pm$ pooled SEM, $n = 11$ cows per group. Means with different letters (a, b) differ (BCS $\times$ Day, $P \leq 0.05$).

Ser affect the pathway (Wu et al., 2004; Lu, 2009). It could be that differences in the rate of mobilization of body protein and differences in DMI to satisfy energy needs determine the availability of AA and other intermediates of the GSH pathway in SAT (Pires et al., 2013; Batistel et al., 2018; Liang et al., 2019). If true, lower availability of AA would lead to decreased mRNA and protein abundance of targets in the GSH metabolism pathway. This idea is partly supported by data from cows fed rumen-protected Met in which a greater DMI was associated with greater mRNA abundance of

GCLM, *GSR*, and *GPX1* in SAT (Liang et al., 2019). Due to the lack of difference in DMI, we speculate that in the present study GSH metabolism was partly regulated by protein mobilization.

Glutathione peroxidases play a crucial role in scavenging and inactivating hydrogen and lipid peroxides in mammalian cells (Cohen and Hochstein, 1963; Drevet, 2006), as well as controlling the inflammatory response (Bozinovski et al., 2012). Thus, it is commonly accepted that greater GPX activity is a positive indicator of health. However, a study in mice demonstrated that

overexpression of *GPX* promotes inflammation in lung (Bozinovski et al., 2012). Additionally, decreased GPX activity in mouse adipocytes led to the accumulation of GSH and reduced insulin sensitivity (Kobayashi et al., 2009). Thus, the difference in gene expression of *GPX1* between HBCS and NBCS cows might be associated with inflammatory response and insulin resistance in SAT. Although there are no available data in bovine demonstrating a direct link between GSTM1 and oxidative stress in adipose tissue, dairy cows calving in summer exhibited signs of OS, along with lower s.c. abundance of GSTM1 (Zachut et al., 2017). In human lymphocytes, the absence of *GSTM1* did not lead to abnormal susceptibility to an oxidant challenge in vitro (Onaran et al., 2001). We speculate that increased mRNA and protein abundance of targets associated with GSH metabolism in SAT were adaptive responses in HBCS cows to counteract the negative effect caused by increased ROS concentration. Overall, these data seem to underscore the need for further studies to better understand the mechanistic role of GSH metabolism in bovine adipose tissue.

CONCLUSIONS

Although both HBCS and NBCS cows experience OS and inflammation during the periparturient period, these events are likely more pronounced in cows with HBCS, e.g., they had greater overall plasma β -carotene and ROS concentrations in SAT, especially after parturition. Activation of NFE2L2 in SAT might partly explain the reduced inflammatory response in dairy cows with NBCS. The role of GSH metabolism in bovine adipose tissue merits further study.

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