# Interpretive Summary

**Body condition alters glutathione and nuclear factor erythroid 2-like 2 (NFE2L2)-related antioxidant network abundance in subcutaneous adipose tissue of periparturient Holstein cows.** ***By Liang et al.*** We explored periparturient s.c. adipose tissue (SAT) antioxidant mechanisms in cows with high and low body condition score (BCS) in late-prepartum. Although overall activation of the antioxidant transcription regulator NFE2L2 was lower and reactive oxygen species concentrations were greater in SAT from high BCS cows, the greater protein abundance of glutathione S-transferase mu 1 associated with glutathione (an antioxidant) metabolism in those cows underscored the importance of antioxidant mechanisms at the tissue level.

**RUNNING TITLE: ADIPOSE ANTIOXIDANT NETWORKS AND BODY CONDITION SCORE**

# 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 pre-calving 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 ≥ 3.5) or low BCS (LBCS; n = 11, BCS ≤ 3.17) on d 28 before parturition. Cows were fed a corn silage- and wheat straw-based total mixed ration (TMR) during late-prepartum and a corn silage- and alfalfa hay-based TMR 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, and oxidative stress 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 (RT-PCR) and protein abundance (Western blotting) of targets associated with the antioxidant transcription regulator nuclear factor, erythroid 2 like 2 (NFE2L2) and GSH metabolism pathway. In addition, concentrations of GSH, ROS and malondialdehyde (MDA) were measured. HBCS cows had lower prepartum dry matter intake (DMI) expressed as a percentage of body weight (BW) along with greater BCS loss between -4 to 4 wk relative to parturition. Plasma concentrations of ROS and FRAP increased after parturition regardless of treatment. Compared with LBCS, HBCS cows had greater concentrations of FRAP at d 7 postpartum, which coincided with peak values in those cows. In addition, LBCS 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 (p)-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 periparturient period via nutrition might help control tissue remodeling while allowing optimal performance.

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

## INTRODUCTION

Body condition is used to evaluate the degree of apparent adiposity in dairy cows (Roche et al., 2013). High body condition (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 (**HBCS**) 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, Schulz et al., 2014). 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 oxidative stress 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 were suggested to play a role in regulating tissue antioxidant response (Gessner et al., 2013). More recent *in vitro* and *in vivo* data indicated 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, Han et al., 2018b, Ma et al., 2018). Greater protein abundance of targets associated with the NFE2L2 pathway coupled with elevated plasma malondialdehyde (**MDA**) was reported in s.c. adipose tissue (**SAT**) in cows calving during the summer compared with winter, suggesting this pathway also might be important in coping with oxidative stress 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 $H_2O_2$ 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 oxidative stress along with greater mRNA abundance of glutamate-cysteine ligase modifier subunit (***GCLM***), glutathione reductase (***GSR***), and glutathione peroxidase 1 (***GPX1***). The greater activity of various GSH-related antioxidant enzymes in peripartal 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 low 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 peripartal 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). BCS was monitored weekly by three 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: HBCS (3.75 ± 0.25, 3.5 to 4.0; mean ± SD; n = 11) and LBCS (3.07 ± 0.07, 3.0 to 3.17; mean ± SD; n = 11), at d 28 before parturition based on a 5-point scale (Edmonson et al., 1989). The average (mean ± SD) BW at -4 wk relative to parturition was 896 ± 51 kg and 786 ± 48 kg in HBCS and LBCS, respectively. The average for parity (mean ± SD) was 3.5 ± 1.6 in HBCS cows and 3.0 ± 1.1 for LBCS. 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 free-stall barn with an individual Calan gate feeding system (American Calan, Northwood, NH, USA). 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 (Batistel et al., 2017). The ingredient and nutrient compositions of the diets fed are reported in Table 1.

**Blood Collection and Analyses**

Blood was obtained from the coccygeal vein before morning feeding on d -10 (± 1 d), 7, 15, and 30 relative to parturition. Samples were collected into 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 × *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 (**PON**), 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 LBCS averaged 28 ± 3 d in the close up dry period. All (i.e., 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 (± 2 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 exactly 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 was performed as described previously (Osorio et al., 2014). The internal controls for adipose tissue were ribosomal protein S9 (***RPS9***), ***GAPDH*** and actin beta (***ACTB***). These internal control genes were previously confirmed as suitable for adipose tissue gene expression analysis (Vailati-Riboni et al., 2015, Vailati-Riboni et al., 2016, Vailati-Riboni et al., 2017). Gene symbols and names, quantitative PCR performance, and primer information are reported in Supplemental Table S1.

**Western Blot Analysis**

Total protein was extracted from 100 mg adipose tissue using a tissue protein extraction reagent (catalog no. 78510; Thermo Fisher Scientific) containing Halt protease and phosphatase inhibitor cocktail (100x, catalog no. 78442; Thermo Fisher Scientific). The concentration of total protein was determined using the Pierce BCA 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 protein into each lane of a 4-20% SDS-PAGE gel (catalog no. 4561096; Bio-Rad). 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 then blocked in 1× Tris-buffered saline (1×TBST) containing 5% nonfat milk for 2 h at room temperature. The membranes were then incubated in TBST containing primary antibodies to glutathione S-transferase mu 1 (**GSTM1**), Kelch-like ECH associated protein 1 (**KEAP1**), extracellular signal-regulated protein kinases 1 and 2 (**ERK1/2**), phospho-ERK1/2(Thr202/Tyr204), NFE2L2 and phospho-NFE2L2(Ser40) (catalog # and dilution ratio are included in Supplemental Table S2) overnight at 4 °C. The membranes were then washed with 1x TBST and incubated with anti-rabbit HRP-conjugated

184 secondary antibodies (catalog no. 7074S; Cell Signaling Technology, dilution 1:1000).
185 Subsequently, the membranes were washed with 1× TBST and then incubated with ECL reagent
186 (catalog no. 170-5060; Bio-Rad) prior to image acquisition. Actin beta (catalog no. 4967S; Cell
187 Signaling Technology) was used as the internal control. Images were acquired using the
188 ChemiDOC MP Imaging System (Bio-Rad). The intensities of the bands were measured with
189 Image-Pro Plus 6.0 software. Specific target protein band density values were normalized to β-
190 actin density values. Representative blots are included in Supplemental Figure S2.

191 **Biomarker Analysis in Subcutaneous Adipose Tissue**

192 As in our previous study (Liang et al., 2019), the following OS biomarkers in SAT were
193 determined using commercial kits according to manufacturer's instructions: ROS (catalog no.
194 STA-347, Cell Biolabs, San Diego, CA), malondialdehyde (**MDA**; catalog no. 10009055;
195 Cayman Chemical), and GSH (catalog no. NWK-GSH01; Northwest Life Science Specialties,
196 Vancouver, WA). Adipose tissue total protein concentration was measured using the Pierce BCA
197 assay kit (catalog no. 23227; Thermo Scientific).

198 **Statistical Analysis**

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

201 $$Y_{jl} = \mu + M_j + T_l + MT_{jl} + e_{jl},$$

202 where $Y_{jl}$ = dependent, continuous variable, $\mu$ = overall mean, $M_j$ = fixed effect of BCS (j =
203 HBCS vs. LBCS), $T_l$ = fixed effect of Day (for blood biomarkers, -10, 7, 15, and 30 d; for qPCR,
204 western blot, and oxidative stress biomarker in SAT analysis, -15, 7, and 30 d), $MT_{jl}$ =
205 interaction between BCS and Day, and $e_{jl}$ = residual error. Cow, nested within BCS, was the

206 random effect. The Kenward-Roger statement was used for computing the denominator degrees
207 of freedom. The covariance structure of the repeated measurements was spatial power
208 [SP(POW)]. When the interaction was significant, least squares means separation between and
209 within time points was performed using the PDIFF statement with Tukey adjustment. Normality
210 of the residuals was checked with normal probability and box plots, and homogeneity of
211 variances was checked with plots of residuals versus predicted values. Outliers were removed
212 when the absolute value of studentized residual was greater than 2. Significance was declared at
213 $P \leq 0.05$ and tendencies at $P \leq 0.10$.

214 **RESULTS AND DISCUSSION**

215 **Body Condition and Animal performance**

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

226 **Blood Parameters Associated with Inflammation and Oxidative Stress**

227 Compared with the prepartum, plasma ceruloplasmin and haptoglobin concentrations
228 increased after parturition in both HBCS and LBCS cows (Day, $P < 0.01$; Table 2, Figure 3A
229 and B). However, ceruloplasmin tended to decrease from 7 to 30 d postpartum in LBCS cows,
230 while HBCS cows had an opposite trend (BCS×Day, $P = 0.10$; Figure 3A). Overall, plasma
231 myeloperoxidase activity increased between -10 and 15 d around parturition followed by a
232 sudden decrease at 30 d after parturition irrespective of BCS (Day, $P = 0.01$; Figure 3C).
233 Regardless of BCS, AST activity and bilirubin concentration (indicators of liver function) were
234 greater after parturition and reached a peak at d 7 (Day, $P < 0.01$; Figure 5B and C). Similarly,
235 GGT increased after parturition regardless of BCS (Day, $P < 0.01$; Figure 5A).

236 Acute-phase proteins (**APP**), a critical part of the acute-phase response, include positive
237 APP (i.e. increase during inflammation) such as haptoglobin, ceruloplasmin, and serum amyloid-
238 A and negative APP (i.e. decrease during inflammation) such as albumin, apolipoproteins,
239 retinol-binding protein, and also PON (Ceciliani et al., 2012, Trevisi et al., 2013, Tothova et al.,
240 2014). Through its antimicrobial activity, myeloperoxidase is a critical enzyme in regulating
241 innate immunity (Depreester et al., 2017). Changes in the various APP along with markers of
242 liver function are commonly used to study inflammation status of periparturient cows (Bionaz et
243 al., 2007, Bertoni et al., 2008, Graugnard et al., 2013). Increased ceruloplasmin concentration is
244 associated with inflammation (Cerón et al., 2005), thus, its sharp increase after parturition in
245 HBCS and LBCS cows was suggestive of a greater chronic inflammatory response postpartum
246 (Bionaz et al., 2007, Batistel et al., 2018). However, the subsequent decrease in ceruloplasmin in
247 LBCS cows suggested they experienced a shorter inflammatory period (Figure 3A). Although
248 the greater inflammatory status during transition is one adaptive mechanism for dairy cows to
249 cope with acute metabolic changes that occur, a prolonged inflammatory response exacerbates

250 the induction of metabolic disorders (Bradford et al., 2015). Thus, as reported previously
251 (Treacher et al., 1986, Roche et al., 2009), a prolonged inflammatory response in HBCS cows
252 might contribute to greater susceptibility to metabolic disorders.

253 The lower concentration of plasma ROS prepartum and the increase postpartum (Figure 4
254 B) were in agreement with previous studies (Bernabucci et al., 2005, Batistel et al., 2018). The
255 change in ROS between pre and postpartum might have been due to the well-known increases in
256 metabolic rate (Reynolds et al., 2003) along with potential direct effects of free fatty acids (**FFA**)
257 and β-hydroxybutyrate (**BHB**) on circulating immune cells (Lacetera et al., 2005) or the liver
258 (Sun et al., 2019). Overproduction of ROS results in OS and an ensuing inflammatory response
259 both of which increase the incidence of metabolic disorders (Abuelo et al., 2015). Thus, we
260 speculate that the relative stability of plasma ROS concentration in HBCS cows after d 7
261 postpartum (unlike the lower plasma ROS level in LBCS cows) denoted a more prolonged
262 inflammatory state, which agrees with some of the plasma biomarkers analyzed.

263 β-carotene, an important cellular antioxidant, is mainly stored in the adipose tissue
264 (Tourniaire et al., 2009), and not only is the major dietary precursor of vitamin A in dairy cattle
265 but is also a precursor for the synthesis of retinoic acid, a metabolite of vitamin A (LeBlanc et
266 al., 2004, Frey and Vogel, 2011). β-carotene supplementation contributes to reduced risk of
267 mastitis and retained placenta, a response associated with its antioxidant properties (Spears and
268 Weiss, 2008). In humans, obesity is associated with lower β-carotene concentrations in
269 adipocytes (Östh et al., 2014). Although we are unaware if adipose tissue mobilization
270 contributes to the circulating β-carotene level during the transition period, the fact that all-trans
271 retinoic acid supplementation inhibited inflammation in bovine adipocytes challenged with
272 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 while epinephrine decreased it, which suggested that hormones related to lipid metabolism influence β-carotene mobilization from adipose tissue (Arias, 2009). However, BCS does not necessarily impact plasma insulin concentrations (e.g. Alharthi et al., 2018). It is well-recognized that dairy cows experience increased lipolysis during the transition period especially after parturition (Contreras et al., 2018). Intense lipolysis is linked to oxidative stress 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 oxidative stress 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 LBCS cows might contribute to their reduced inflammatory response.

Similar to concentrations of ROS, FRAP increased after parturition regardless of BCS, and there was a BCS×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 4A). 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 (Abuelo et al., 2013,

295 Bernabucci et al., 2005). Whether the lipolysis rate in bovine adipose tissue affects β-carotene
296 metabolism and utilization merits further study.

297 **Oxidative Stress Biomarkers in Adipose Tissue**

298 Main effects of BCS, Day, and their interaction on oxidative stress biomarkers are
299 reported in Table 3, Figure 6, Figure 7, and Figure 8. HBCS cows had lower overall abundance
300 of *NFE2L2* ($P = 0.03$; Table 3). In rodents, *NFE2L2* plays a critical role in liver in regulating OS
301 via increasing mRNA abundance of key antioxidant enzymes (Ma, 2013). In dairy cows, mRNA
302 abundance of *NFE2L2* was first reported in liver during the periparturient period (Loor, 2010)
303 and recent studies revealed that *NFE2L2* is also expressed in mammary gland and SAT (Zachut
304 et al., 2017, Han et al., 2018b, Liang et al., 2019). *In vitro*, enhanced activity of NFE2L2 and its
305 target heme oxygenase-1 (**HMOX1**) contributed partly to controlling oxidant status in bovine
306 mammary epithelial cells (**BMEC**) (Ma et al., 2019).

307 Overall, the concentration of ROS in SAT was greater in HBCS than LBCS cows ($P <$
308 0.01; Figure 6A). Free radicals are essential for normal cellular metabolism, but overproduction
309 without sufficient antioxidant capacity often results in DNA and protein damage and apoptosis
310 (Valko et al., 2007). Reactive oxygen species can activate *NFE2L2* to protect cells from OS
311 damage (Ray et al., 2012). A recent in vitro study reported that an increase in $H_2O_2$ concentration
312 from 0 to 100 μM upregulated mRNA abundance of *NFE2L2* in bovine adipocytes; however,
313 mRNA abundance of *NFE2L2* decreased when the concentration of $H_2O_2$ reached 200 μM (Sun
314 et al., 2019). We speculate that the lower overall abundance of *NFE2L2* in HBCS cows (Table 3)
315 coupled with greater ROS in SAT were suggestive of diminished capacity of the tissue to mount
316 an antioxidant response.

317 Studies in rodents have demonstrated that NFE2L2 function is not only important in
318 regulating OS, but also for adipose development and insulin sensitivity (Schneider and Chan,
319 2013, Seo and Lee, 2013). The latter is particularly important because periparturient cows
320 experience insulin resistance especially early postpartum (Bell and Bauman, 1997, Holtenius et
321 al., 2003, De Koster et al., 2018a), while recent studies demonstrated that AT insulin resistance,
322 especially in over-conditioned cows, develops prepartum (Jaakson et al., 2018). Over-
323 conditioned cows have larger adipocytes in both SAT and omental AT; furthermore, larger
324 adipocytes are more sensitive to lipolytic signals (De Koster et al., 2016). A recent study
325 revealed that BCS loss is positively associated with macrophage infiltration in SAT during early-
326 lactation (De Koster et al., 2018b). The fact that macrophage infiltration leads to overproduction
327 of ROS and inflammatory cytokines in human and rodent AT (Surmi and Hasty, 2010). In the
328 present study, greater ROS concentration in SAT along with greater BCS loss in HBCS cows led
329 us to speculate that macrophage infiltration might play a role in controlling oxidant status in
330 cows calving at HBCS. The link between NFE2L2 and macrophage infiltration in regulating
331 insulin resistance and adipocyte differentiation as it relates to calving BCS merits further study.

332 The decrease in plasma ROS after 7 and 15 d postpartum in LBCS and HBCS cows,
333 respectively (Figure 4B), is noteworthy because ROS concentration in SAT was relatively steady
334 from -15 d prepartum to 30 d postpartum regardless of BCS (Figure 6A). Thus, these data
335 suggest that SAT might take a longer time to recover from OS. Compared with LBCS, HBCS
336 cows had greater overall abundance of cullin 3 (***CUL3***; $P = 0.03$; Table 3). Both KEAP1 and
337 CUL3 are inhibitors of NFE2L2 (Suzuki and Yamamoto, 2017), hence, greater abundance of
338 *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 p-NFE2L2/NFE2L2 ratio was observed in HBCS cows ($P < 0.01$ and $P < 0.01$; Figure 7A and Figure 7C). 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×Day effect for p-NFE2L2 ($P < 0.01$) due to a decrease in abundance in HBCS cows and an increase in LBCS cows from 7 to 30 d after parturition (Figure 7 B). 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 non-ruminants (Dickinson and Forman, 2002), other factors such as NF-kB activity (Buelna-Chontal and Zazueta, 2013) and availability of substrates such as Cys, Gly, and Ser impact the pathway (Wu et al., 2004, Lu, 2009). It could be possible that differences in the rate of mobilization of body protein and differences in DMI to

362 satisfy energy needs is one determinant of the availability of AA and other intermediates of the
363 GSH pathway in SAT (Pires et al., 2013, Batistel et al., 2018, Liang et al., 2019). If that is true,
364 lower availability of AA would lead to decreased mRNA and protein abundance of targets in the
365 GSH metabolism pathway. This idea is partly supported by data from cows fed rumen-protected
366 Met in which a greater DMI was associated with greater mRNA abundance of *GCLM*, *GSR*, and
367 *GPX1* in SAT (Liang et al., 2019). Due to the lack of difference in DMI, we speculate that in the
368 present study GSH metabolism was partly regulated by protein mobilization.

369 Glutathione peroxidases play a crucial role in scavenging and inactivating hydrogen and
370 lipid peroxides in mammalian cells (Cohen and Hochstein, 1963, Drevet, 2006), and also in
371 controlling the inflammatory response (Bozinovski et al., 2012). Thus, it is commonly accepted
372 that greater GPX activity is a positive indicator of health. However, a study in mice
373 demonstrated that overexpression of *GPX* promotes inflammation in lung (Bozinovski et al.,
374 2012). Additionally, decreased GPX activity in mouse adipocytes led to the accumulation of
375 GSH and reduced insulin sensitivity (Kobayashi et al., 2009). Thus, the difference in gene
376 expression of *GPX1* between HBCS and LBCS cows might be associated with inflammatory
377 response and insulin resistance in SAT. Although there are no available data in bovine
378 demonstrating a direct link between GSTM1 and oxidative stress in adipose tissue, dairy cows
379 calving in summer exhibited signs of oxidative stress along with lower s.c. abundance of GSTM1
380 (Zachut et al., 2017). In human lymphocytes, the absence of *GSTM1* did not lead to abnormal
381 susceptibility to an oxidant challenge *in vitro* (Onaran et al., 2001). We speculate that increased
382 mRNA and protein abundance of targets associated with GSH metabolism in SAT were adaptive
383 responses in HBCS cows in order 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 LBCS 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 LBCS. The role of GSH metabolism in bovine adipose tissue merits further study.

## ACKNOWLEDGMENTS

Y. Liang is a recipient of a doctoral fellowship from China Scholarship Council (CSC, Beijing, China). A. S. Alharthi received a fellowship from King Saud University to perform his PhD studies at the University of Illinois (Urbana). A. A. Elolimy was recipient of a fellowship from Higher Education Ministry, Egypt to perform his Ph.D. studies at the University of Illinois (Urbana). We thank Perdue AgriBusiness (Salisbury, MD) for the donation of ProvAAL2 AADvantage, and Phibro Animal Helath Corp. (Teaneck, NJ) for the donation of Animate® during the course of the experiment.

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**Table 1**. Ingredient and nutrient composition of diets fed to Holsten cows with prepartum high (HBCS, BCS ≥ 3.5) or low body condition score (LBCS, BCS ≤ 3.17) 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, % 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/ | 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 |

[1]West Central Soy.

[2]Perdue AgriBusiness (Salisbury, MD).

[3]ADM Animal Nutrition (Quincy, IL).

[4]Rumensin, Elanco Animal Health (Greenfield, IN).

**Table 2.** Least square means (n = 11) ± pooled SEMs for plasma biomarkers of inflammation and oxidative stress in Holstein cows with prepartum high (HBCS, BCS ≥ 3.5) or low body condition score (LBCS, BCS ≤ 3.17).

| | Group | | | *P*-value | | |
|---|---|---|---|---|---|---|
| Item | HBCS | LBCS | SEM | 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[1], μmol/L | 123 | 120 | 3.99 | 0.66 | <0.01 | 0.02 |
| ROS[2], $H_2O_2$/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 |
| $NO2^-$, μmol/L | 3.86 | 3.57 | 0.21 | 0.31 | <0.01 | 0.26 |
| $NO3^-$, 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[3] ,U/L | 102 | 103 | 5.85 | 0.96 | <0.01 | 0.20 |
| GGT[4],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 |

[1] FRAP= Ferric-reducing ability of plasma.

[2] ROS= Reactive oxygen species.

[3] AST = Aspartate aminotransferase.

[4] GGT = γ-glutamyl transpeptidase.

**Table 3.** Least square means (n = 11) ± pooled SEMs for mRNA abundance related to NFE2L2 pathway and glutathione metabolism in Holstein cows with prepartum high (HBCS, BCS ≥ 3.5) or low body condition score (LBCS, BCS ≤ 3.17).

| | | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|
| Gene[1] | HBCS | LBCS | % Difference[2] | SEM | BCS | Day | BCS×Day |
| NFE2L2 pathway | | | | | | | |
| *NFE2L2* | 0.90 | 1.05 | -13.5 | 0.04 | 0.03 | <0.01 | 0.09 |
| *KEAP1* | 1.22 | 1.05 | 16.3 | 0.08 | 0.11 | 0.05 | 0.49 |
| *CUL3* | 1.54 | 1.25 | 22.7 | 0.10 | 0.03 | <0.01 | 0.01 |
| Glutathione metabolism | | | | | | | |
| *ME1* | 0.84 | 0.75 | 12.3 | 0.09 | 0.45 | <0.01 | 0.32 |
| *TALDO1* | 1.31 | 1.08 | 22.0 | 0.08 | 0.04 | <0.01 | 0.27 |
| *GSR* | 0.20 | 0.14 | 41.1 | 0.01 | <0.01 | 0.33 | 0.10 |
| *GCLM* | 0.51 | 0.51 | -0.58 | 0.04 | 0.96 | 0.01 | 0.51 |
| *GPX1* | 0.79 | 0.64 | 22.4 | 0.05 | 0.02 | 0.05 | 0.23 |
| *GCLC* | 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 protein1; *CUL3*=Cullin3; *ME1*= enzyme 1; *TALDO1*= Transaldolase 1; *GSR* =Glutathione reductase; *GCLM* =Glutamate-cysteine ligase modifier s *GPX1*=Glutathione peroxidase 1; *GCLC* =Glutamate-cysteine ligase catalytic subunit.

[2]Difference in mRNA abundance = (HBCS – LBCS)/LBCS × 100.

**Figure 1.**

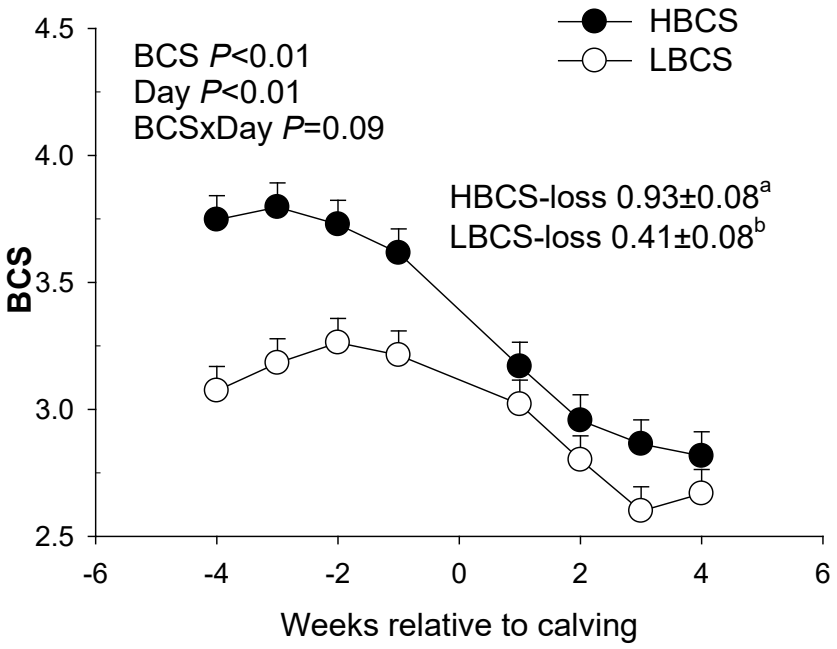
HBCS
LBCS
BCS P<0.01
Day P<0.01
BCSxDay P=0.09
HBCS-loss 0.93±0.08a
LBCS-loss 0.41±0.08b
BCS
4.5
4.0
3.5
3.0
2.5
-6
-4
-2
0
2
4
6
Weeks relative to calving

**Figure 2.**

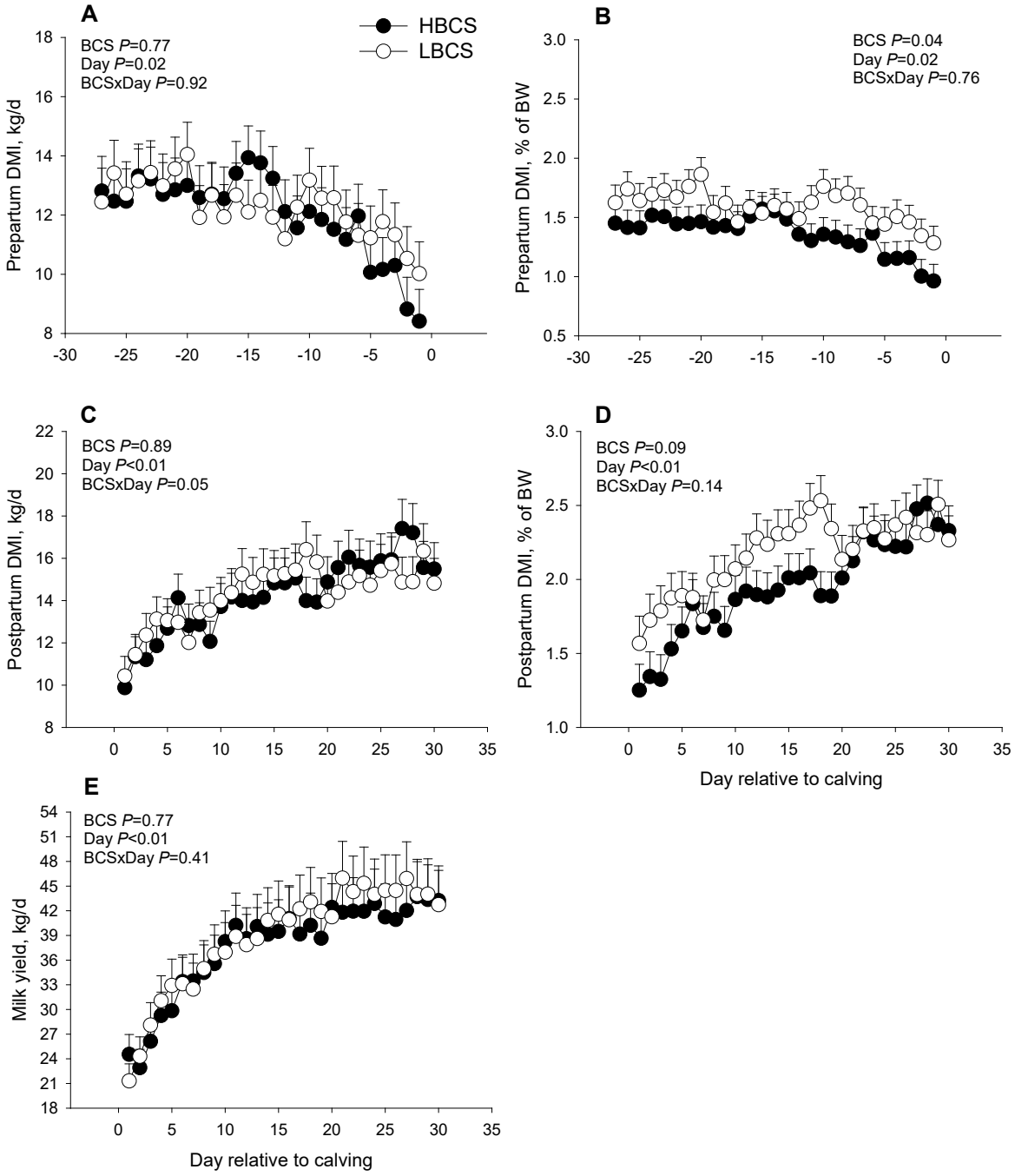
A
HBCS
LBCS
BCS P=0.77
Day P=0.02
BCSxDay P=0.92
Prepartum DMI, kg/d
B
BCS P=0.04
Day P=0.02
BCSxDay P=0.76
Prepartum DMI, % of BW
C
BCS P=0.89
Day P<0.01
BCSxDay P=0.05
Postpartum DMI, kg/d
D
BCS P=0.09
Day P<0.01
BCSxDay P=0.14
Postpartum DMI, % of BW
Day relative to calving
E
BCS P=0.77
Day P<0.01
BCSxDay P=0.41
Milk yield, kg/d
Day relative to calving

**Figure 3.**

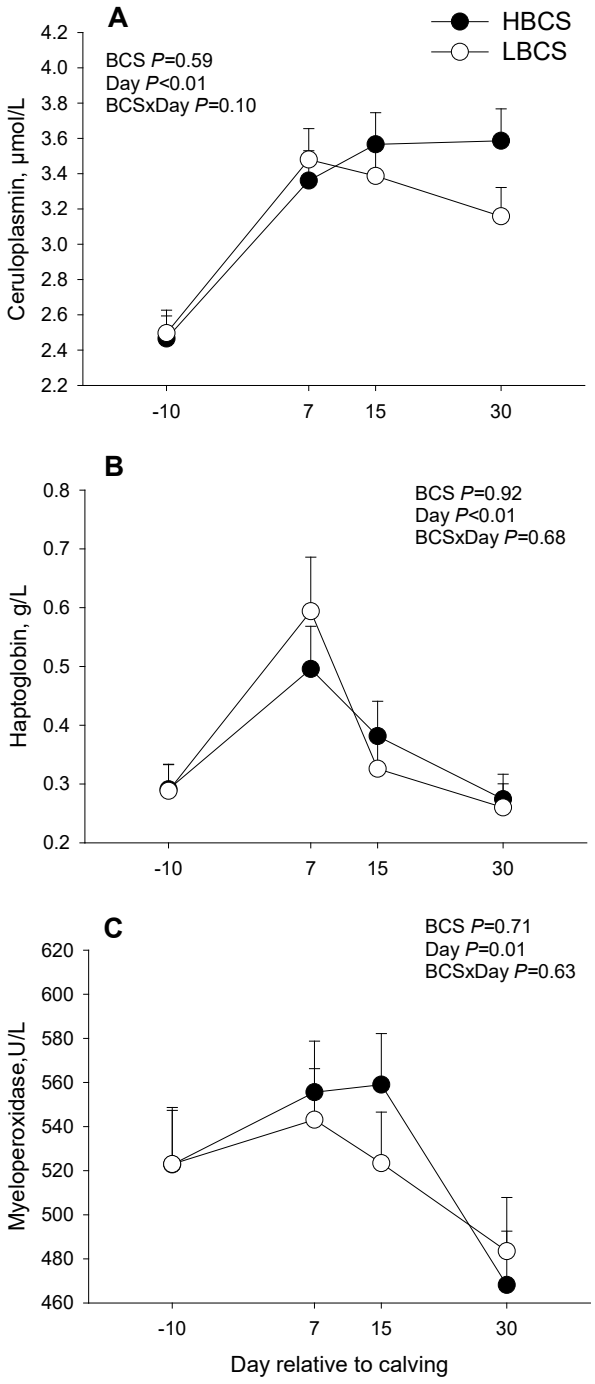
A
HBCS
LBCS
BCS P=0.59
Day P<0.01
BCSxDay P=0.10
Ceruloplasmin, µmol/L
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
-10
7
15
30
B
BCS P=0.92
Day P<0.01
BCSxDay P=0.68
Haptoglobin, g/L
0.8
0.7
0.6
0.5
0.4
0.3
0.2
-10
7
15
30
C
BCS P=0.71
Day P=0.01
BCSxDay P=0.63
Myeloperoxidase,U/L
620
600
580
560
540
520
500
480
460
-10
7
15
30
Day relative to calving

**Figure 4.**

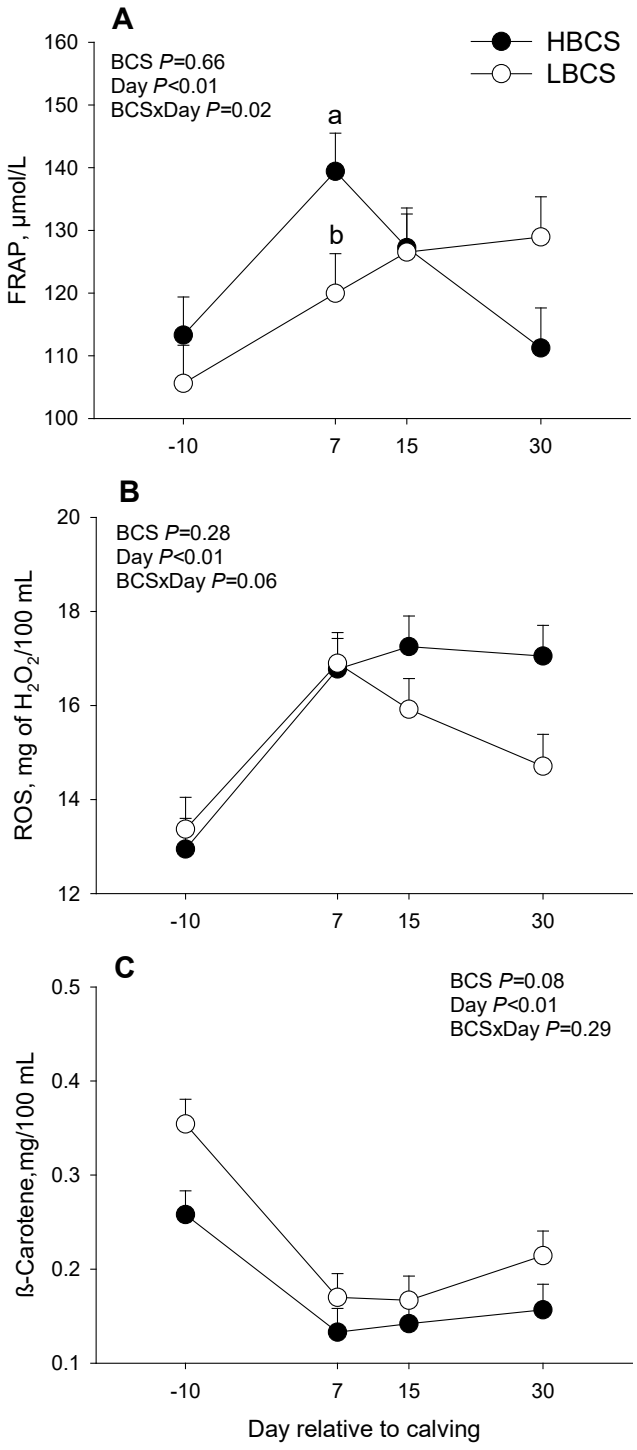
A
HBCS
LBCS
BCS P=0.66
Day P<0.01
BCSxDay P=0.02
a
b
FRAP, µmol/L
160
150
140
130
120
110
100
-10
7
15
30
B
BCS P=0.28
Day P<0.01
BCSxDay P=0.06
ROS, mg of H2O2/100 mL
20
18
16
14
12
C
BCS P=0.08
Day P<0.01
BCSxDay P=0.29
ß-Carotene,mg/100 mL
0.5
0.4
0.3
0.2
0.1
Day relative to calving

**Figure 5.**

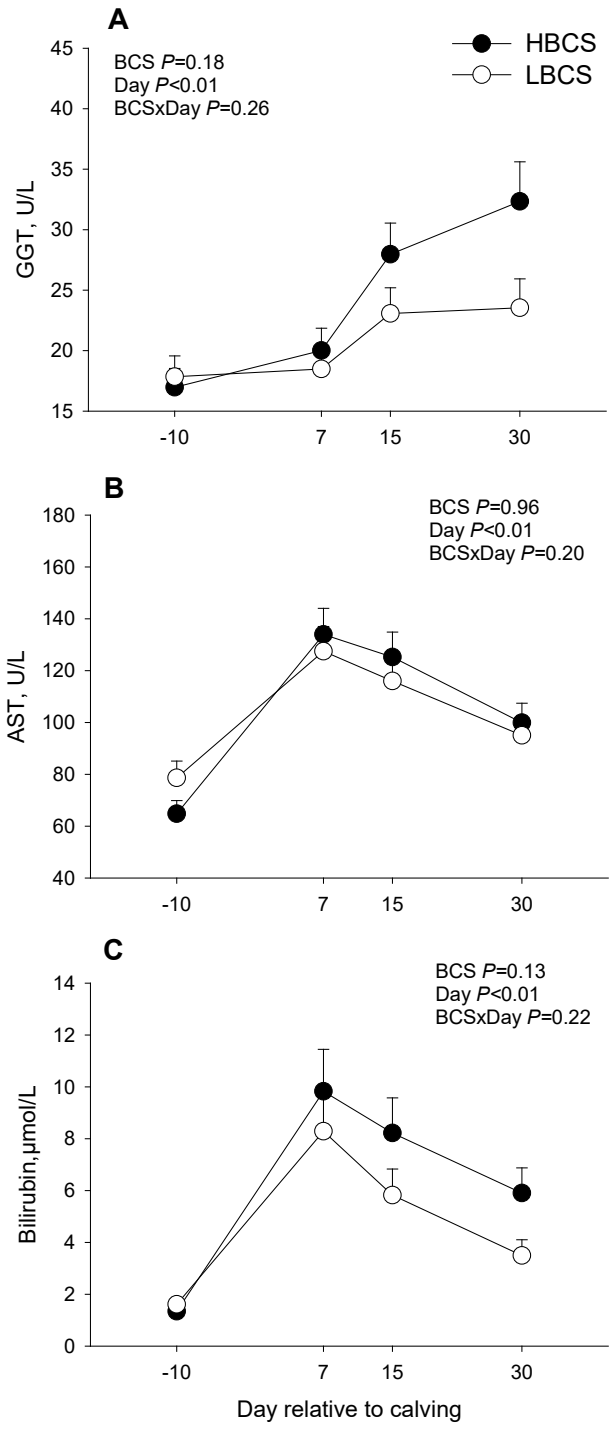
A
HBCS
LBCS
BCS P=0.18
Day P<0.01
BCSxDay P=0.26
GGT, U/L
45
40
35
30
25
20
15
-10
7
15
30
B
BCS P=0.96
Day P<0.01
BCSxDay P=0.20
AST, U/L
180
160
140
120
100
80
60
40
-10
7
15
30
C
BCS P=0.13
Day P<0.01
BCSxDay P=0.22
Bilirubin,µmol/L
14
12
10
8
6
4
2
0
-10
7
15
30
Day relative to calving

**Figure 6.**

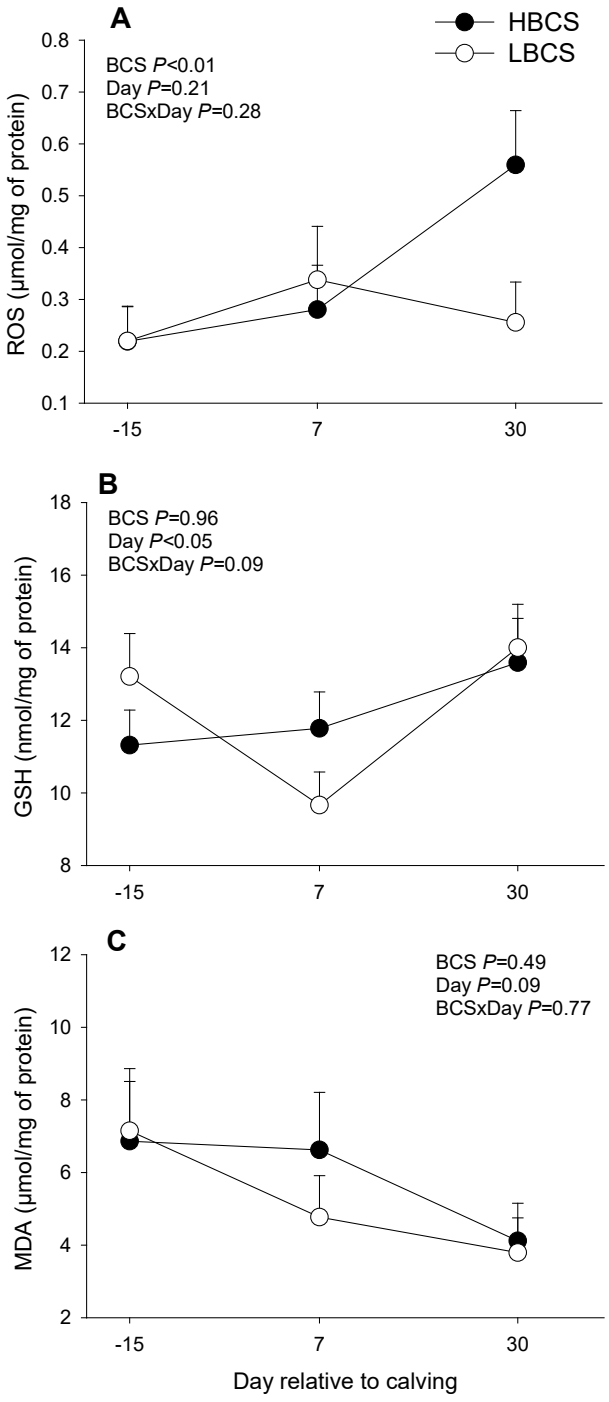
A
HBCS
LBCS
BCS P<0.01
Day P=0.21
BCSxDay P=0.28
ROS (µmol/mg of protein)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
-15
7
30
B
BCS P=0.96
Day P<0.05
BCSxDay P=0.09
GSH (nmol/mg of protein)
18
16
14
12
10
8
-15
7
30
C
BCS P=0.49
Day P=0.09
BCSxDay P=0.77
MDA (µmol/mg of protein)
12
10
8
6
4
2
-15
7
30
Day relative to calving

**Figure 7.**

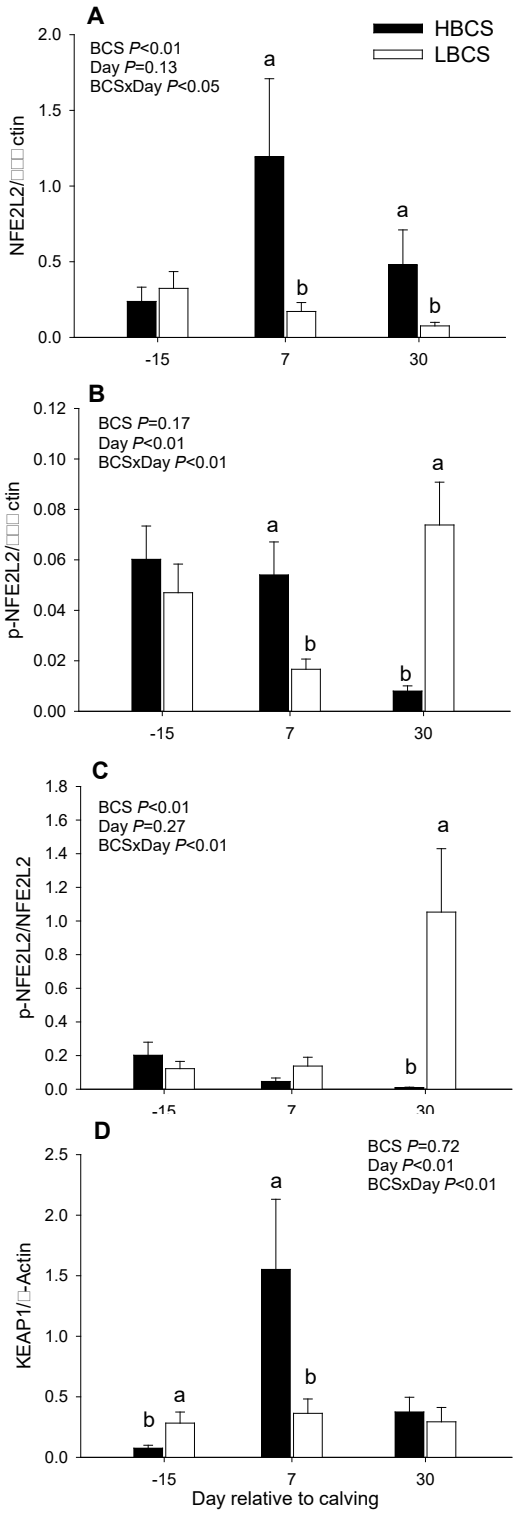
A
HBCS
LBCS
BCS P<0.01
Day P=0.13
BCSxDay P<0.05
NFE2L2/□□□ctin
B
BCS P=0.17
Day P<0.01
BCSxDay P<0.01
p-NFE2L2/□□□ctin
C
BCS P<0.01
Day P=0.27
BCSxDay P<0.01
p-NFE2L2/NFE2L2
D
BCS P=0.72
Day P<0.01
BCSxDay P<0.01
KEAP1/□-Actin
Day relative to calving

**Figure 8.**

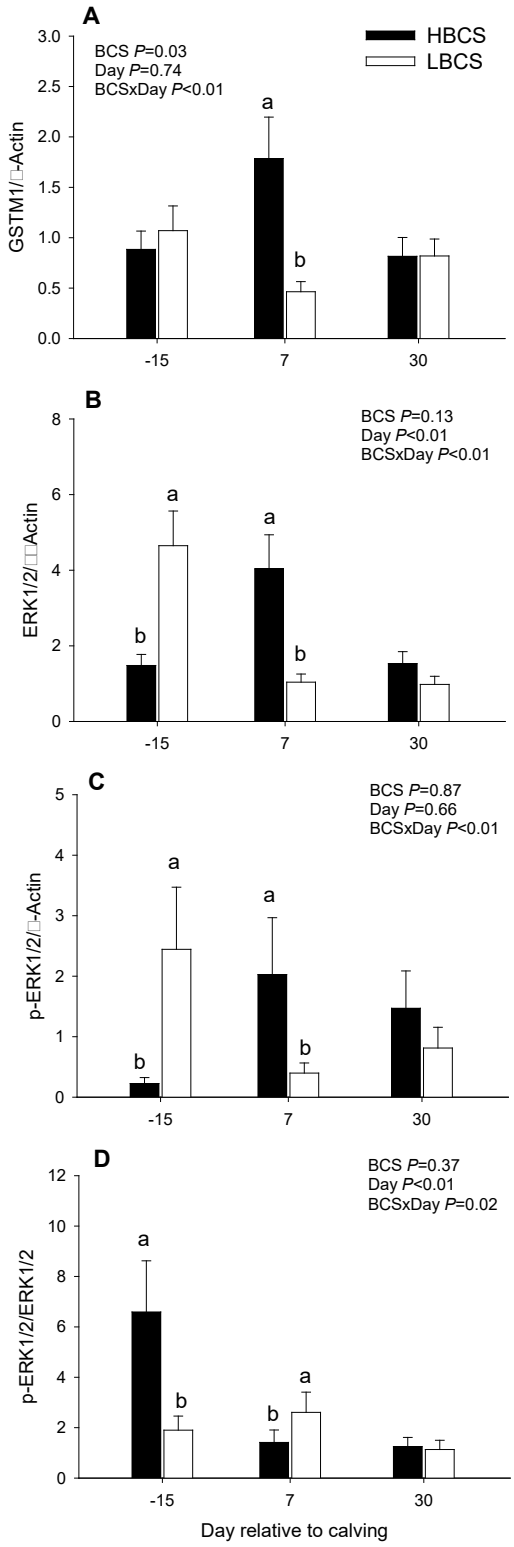
A
HBCS
LBCS
BCS P=0.03
Day P=0.74
BCSxDay P<0.01
GSTM1/□-Actin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
-15
7
30
B
BCS P=0.13
Day P<0.01
BCSxDay P<0.01
ERK1/2/□□Actin
8
6
4
2
0
a
a
b
b
-15
7
30
C
BCS P=0.87
Day P=0.66
BCSxDay P<0.01
p-ERK1/2/□-Actin
5
4
3
2
1
0
a
a
b
b
-15
7
30
D
BCS P=0.37
Day P<0.01
BCSxDay P=0.02
p-ERK1/2/ERK1/2
12
10
8
6
4
2
0
a
b
b
a
-15
7
30
Day relative to calving

**Figure Legend**s

**Figure 1.** Change in body condition score (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 low body condition score (LBCS, BCS ≤ 3.17). Data are LS means, n = 11 cows per group, ± pooled SEMs. [ab]Means groups differ ($P \leq 0.05$).

**Figure 2.** Prepartum and postpartum DMI, DMI as % of body weight and milk yield of Holstein cows with prepartum (28d before expected parturition) high (HBCS, BCS ≥3.5) or low body condition score (LBCS, BCS≤ 3.17) through -30 to 30 d relative to parturition. Data are LS means, n = 11 cows per group, ± pooled SEMs.

**Figure 3.** Plasma biomarkers of inflammation in Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥ 3.5) or low body condition score (LBCS, BCS ≤ 3.17) (panel A= Ceruloplasmin; panel B= Haptoglobin; panel C= Myeloperoxidase). Data are LS means, n = 11 cows per group, ± pooled SEMs. [ab]Means differ (BCS × Day, $P \leq 0.05$).

**Figure 4.** Plasma biomarkers of oxidative stress in Holstein cows with prepartum (28d before expected parturition) high (HBCS, BCS ≥ 3.5) or low body condition score (LBCS, BCS ≤ 3.17) (panel A= FRAP; panel B= ROS; panel C= β-Carotene). FRAP= Ferric-reducing ability of plasma; ROS= Reactive oxygen species. Data are LS means, n = 11 cows per group, ± pooled SEMs. [ab]Means differ (BCS × Day, $P \leq 0.05$).

**Figure 5.** Plasma biomarkers of liver function in Holstein cows with prepartum (28d before expected parturition) high (HBCS, BCS ≥ 3.5) or low body condition score (LBCS, BCS ≤ 3.17) (panel A= GGT; panel B= AST; panel C= Bilirubin). AST = Aspartate aminotransferase; GGT = γ-glutamyl transpeptidase. Data are LS means, n = 11 cows per group, ± pooled SEMs. [ab]Means differ (BCS × Day, $P \leq 0.05$).

**Figure 6.** Concentrations of reactive oxygen species (ROS) (panel A), glutathione (GSH) ( panel B), and malondialdehyde (MDA) (panel C) in SAT of Holstein cows with prepartum (28d before expected parturition) high (HBCS, BCS ≥ 3.5) or low body condition score (LBCS, BCS ≤ 3.17). Data are LS means, n = 11 cows per group, ± pooled SEMs. [ab]Means differ (BCS × Day, $P \leq 0.05$).

**Figure 7.** Protein abundance (relative to β-actin) of the NFE2L2 (inactive, panel A), p-NFE2L2 (active, panel B), ratio of p-NFE2L2/NFE2L2 (panel C), NFE2L2 repressor KEAP1 (panel D) in SAT of Holstein cows with prepartum (28d before expected parturition) high (HBCS, BCS ≥ 3.5) or low body condition score (LBCS, BCS ≤ 3.17). NFE2L2=nuclear factor, erythroid 2 like 2; KEAP1= kelch like ECH associated protein. Data are LS means, n = 11 cows per group, ± pooled SEMs. [ab]Means differ (BCS × Day, $P \leq 0.05$).

**Figure 8.** Protein abundance (relative to β-actin) of the GSTM1 (panel A), ERK1/2 (inactive, panel B), p-ERK1/2 (active, panel C), ratio of p-ERK1/ERK1/2 (panel D) in SAT of Holstein cows with prepartum (28d before expected parturition) high (HBCS, BCS ≥ 3.5) or low body condition score (LBCS, BCS ≤ 3.17). GSTM1= glutathione S-transferase mu 1; ERK1/2= extracellular signal-regulated protein kinases 1 and 2. Data are LS means, n = 11 cows per group, ± pooled SEMs. [ab]Means differ (BCS × Day, $P \leq 0.05$).