ENDOCANNABINOID AND LIPID METABOLISM GENES NETWORK ANALYSIS IN ADIPOSE AND LIVER TISSUE OF DAIRY COWS DURING THE TRANSITION PERIOD

BY

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THESIS

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ABSTRACT

During the transition period dairy cows are exposed to enormous metabolic changes. These changes could affect the overall health and production. Two experiments were conducted on transition cows to evaluate the 1) effects of body condition score (BCS) on the endocannabinoid system and lipid metabolism gene expression in adipose tissue; and 2) effects of rumen-protected methionine on the endocannabinoid system in liver tissue. In the first study, cows were retrospectively classified according to their BCS at -3 wk from parturition into two groups: HiBCS (BCS ≥ 3.75) or LoBCS (BCS ≤ 3.25). Adipose tissue at -10, 7, and 20 d around parturition was used to examine mRNA expression via qPCR of endocannabinoid receptors (*CNR1, CNR2*), enzymes that synthesize endocannabinoid (*NAPEPLD*), enzymes that degrade endocannabinoids (*FAAH, NAAA, MGLL*), and the hormone precursor proopiomelanocortin (*POMC*). We also examined mRNA expression via qPCR of genes associated with lipolysis (*LIPE*, *ABDH5*, *ATGL*), fatty acid oxidation (*CPT1A*, *CPT2*, *ACADVL*, *ACOX1*), oxidative stress (*SOD1*, *SOD2*), and genes that are involved in inflammation (*TLR9*, *TLR4*, *NFE2L2*). Expression of *CNR2* and *NAPEPLD* was greater at 7 d in LoBCS due to lower expression at the same time in HiBCS. The expression of *FAAH* was upregulated at d 7 and 20 in LoBCS than HiBCS cows. Expression of *MGLL* was overall greater across time in LoBCS than HiBCS, LoBCS had a tendency for greater overall expression of *POMC* across time. Regarding the genes associated with lipolysis, LoBCS compared with HiBCS cows had overall greater expression of *ABDH5, LIPE* and *ATGL*, indicating a greater state of basal lipolysis over time. Among genes related with fatty acid oxidation the expression of *CPT1A* and *ACADVL* was greater in HiBCS than LoBCS due to greater expression at -10 and 7 d. For the mitochondrial enzyme *SOD2*, important for

clearing reactive-oxygen species that cause cellular stress and inflammation, we observed an interaction of BCS × day due to higher expression at d 7 in LoBCS than HiBCS. There was an overall BCS effect on the expression of *SOD1* due to greater expression in LoBCS compared with HiBCS.

In the second experiment, cows were fed experimental diets consisting of a basal control diet (CON) or rumen-protected methionine-supplemented (MET) during the transition period (-21 through 30 days in milk). The liver was biopsied at -10, 7, 20 and 30 days relative to parturition. Gene expression was determined through qPCR for endocannabinoid receptors (*CNR1, CNR2*), enzymes that synthesize endocannabinoid (*NAPEPLD*), enzymes that degrade endocannabinoid (*FAAH, NAAA, MGLL*), and the hormone precursor proopiomelanocortin (*POMC)*. A significant interaction of treatment × day was observed for the endocannabinoid receptor *CNR2* associated with lower expression in MET compared with control cows on d -10. There was an overall greater expression of *FAAH*, *MGLL*, *NAAA* and the EC-synthesizing enzyme *NAPEPLD* in MET compared with control cows. Cows supplemented with MET had greater in vitro blood neutrophil phagocytosis, neutrophil oxidative burst and monocyte oxidative burst.

Results from experiment 1 indicate that expression of the endocannabinoid system and lipid metabolism genes in adipose tissue may be associated with BCS. A potential linkage between those pathways and risk of disorders postpartum remains to be determined. Results from experiment 2 suggest that the alterations in the hepatic EC signaling network in response to MET might be involved in the positive effect on performance and liver function.

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AEA	anandamide
ATGL	adipose triglyceride lipase
BCS	body condition score
CPT	carnitine palmitoyl transferase
CON	control diet
CNS	central nervous system
d	day(s)
DIM	days in milk
DMI	dry matter intake
EC	endocannabinoids
ECS	endocannabinoid system
FA	fatty acid
FAA	fatty acid amide
HSL/LIPE	hormone sensitive lipase
kg	kilogram(s)
LCFA	long chain fatty acids
Mcal	megacalorie(s)
ME	metabolizable energy
MET	methionine- supplemented
NEB	negative energy balance
NRC	National Research Council

NEFA	nonesterified fatty acids
OEA	oleoylethanolamine
PKA	protein kinase A
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
SAM	S-adenosylmethionine
TMR	total mixed ration
TG	triglycerides/triacylglycerides
VLDL	very low density lipoproteins

INTRODUCTION

During the transition period in dairy cows, major metabolic changes occur, which negatively impact the immune function, milk production and feed intake (Drackley, 1999). To meet their energy requirements, close to parturition in, a huge decrease in dry matter intake (DMI) exposes cows to negative energy balance (NEB) (Bauman and Currie, 1980). The NEB leads to the extensive mobilization of fatty acids stored in adipose tissue, and could affect liver and immune function further exacerbating the stress on the animal. If severe, the state of NEB will increase the chance of metabolic and infectious disorders. Feeding management during transition period could have an impact on cow health, milk production and postpartum DMI (Dann et al., 2006).

Several researches using model organisms have investigated the importance of endocannabinoid system (ECS) in regulating lipid metabolism, food intake, and immune system. The endocannabinoid system is well-studied in non-ruminants and reported to control energy balance, feed intake, and other metabolic processes. The ECS includes endocannabinoids, cannabinoid receptors, and enzymes for synthesis and degradation of endocannabinoid (Maccarrone et al., 2010). Data from a previous study investigated the role of ECS in bovine liver in response to prepartal plane of nutrition and uncovered a potential link between this system and lipid metabolism (Khan et al., 2012).

Body condition score (BCS) helps to evaluate the nutritional status of the animal, and could have an enormous effect on DMI and energy balance postcalving (Hayirli et al., 2002). The effect of BCS on milk production has been observed in different studies (Garnsworthy and Topps, 1982; Bourchier et al., 1987). An ideal BCS score at calving could help cows have a

successful transition period and maximize the performance during postpartum period (Akbar et al., 2015). Also, a relationship between BCS and oxidative stress has been observed in dairy cows (Bernabucci et al., 2005a) demonstrating that cows with higher BCS at calving and cows with higher change in BCS from prepartum to postpartum are more sensitive to oxidative stress.

Methionine is a limiting amino acid for milk synthesis in dairy cows (Schwab et al., 1992), hence, the supplementation of rumen-protected methionine during transition period has improved milk yield, milk protein and postpartum immune function (Schmidt et al., 1999; Osorio et al., 2013). The relationship between ECS and methionine deficiency on liver development and function was investigated in a recent study using zebra fish (a model organism) (Liu et al., 2016), and results revealed that methionine supplementation can prevent many metabolic deficiencies associated with EC signaling.

The objectives of these studies were 1) to evaluate the effect of BCS on endocannabinoid system and lipid metabolism in bovine adipose tissue during the transition period, and 2) the effects of rumen-protected methionine supplementation on the endocannabinoid system in bovine liver during the transition period.

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CHAPTER I

LITERATURE REVIEW

Transition period

Changing from a non-lactating to a lactating state is known as the "transition period" in dairy cows, and this stage is the most critical time in the life cycle. The transition period typically starts from 3 weeks prepartum until 3 weeks postpartum (Grummer, 1995). During this period the animal is susceptible to different metabolic disorders, which could negatively affect immune function, milk production and feed intake (Drackley, 1999). A successful transition period is imperative for ensuring an optimal lactation in dairy cows.

During the transition period, the nutrient requirements increase to maintain fetal growth and milk synthesis (Grummer, 1995). The dry matter intake (DMI) decreases close to calving (Bertics et al., 1992). The decrease in DMI starts 3 weeks before calving (Drackley, 1999). The reduction in DMI prepartum and increase in nutrient requirements affect metabolism, specifically lipid metabolism (Grummer, 1995). This change in lipid metabolism in tissues like liver and adipose occurs to cover the gap between DMI and nutrient requirements. Due to the variation between DMI and nutrient requirements, the NEB during the prepartum period will affect the overall health and reproductive system. Several researchers have investigated the effects of level of dietary energy during the dry period in the context of a successful transition in dairy cattle (Douglas et al., 2006, Loor et al., 2006, Janovick and Drackley, 2010).

When the time of calving approaches, dairy cows experience a change in their behavior, feed intake pattern and their physiology (Huzzey et al., 2005). All these alterations in the body

cause stress for the animal which will negatively affect the immune system and increase the chances of disease during this time. Feeding management during this period could have a positive effect on cow health, milk production and postpartum DMI (Dann et al., 2006).

Importance of adipose tissue

There are two types of adipose tissue in mammals; brown adipose tissue and white adipose tissue. The primary function of brown adipose tissue is to regulates heat generation (Cannon and Nedergaard, 2004), whereas white adipose tissue plays a major role in energy homeostasis by serving as a storage of lipids when nutrient supply is increased and releasing the fatty acids when the nutrient supply is below the body requirements (Trayhurn and Beattie, 2001). During early pregnancy, the storage of lipid in adipose tissue increases, while in late pregnancy and early lactation this lipid store will rapidly be mobilized due to hormonal changes and the sudden increase in mammary gland requirements (McNamara, 1989).

Fat deposition is determined by the balance between lipolysis and lipogenesis. During feeding of lower energy diets in dairy cows, they tend to increase lipolysis and decrease lipogenesis by altering in some hormones such as insulin and leptin (Roche et al., 2009). Increasing lipid mobilization after calving leads to an increase in the hepatic uptake of nonesterified fatty acids (NEFA) causing TG accumulation, which could cause different metabolic disorders such as fatty liver.

During energy restriction lipolysis within adipose tissue, which involves TG breakdown into free fatty acid (FFA) and glycerol, provides FFA as a source of energy to tissues like the liver. Hormone sensitive lipase (*HSL*) and monoacylglycerol lipase (*MGLL*) are responsible for hydrolyzing the ester bonds of tissue triglyceride. Some of the glycerol is used to re-synthesize

triglyceride through the process of "glyceroneogenesis" which leads to production of glycerol-3-phosphate from substrates like lactate or amino acids feeding into the TCA cycle (Nye et al., 2008). A portion of the FFA released can be re-esterified, exported into the blood, or oxidized for energy within the adipose. The use of glycerol to re-synthesize triglyceride is called esterification, a process distinct from lipogenesis, which involves synthesis of fatty acids de novo (e.g. 16:0). Both lipolysis and re-esterification are persistently occurring in adipocytes (Herdt, 2000). Some of the glycerol that is released from the adipose tissue is used by the liver to provide glucose (via gluconeogenesis) for the synthesis of lactose in the mammary gland (Hanigan et al., 2002).

The nutritional state of the animal is very important in terms of fatty acid utilization, i.e. when the animal has adequate dietary energy from carbohydrates and lipid they will convert some of the carbohydrate to fatty acid and use it for esterification; however, when the animal does not receive enough dietary energy from the feed, adipose tissue lipolysis provides fatty acids to other tissues to oxidize them for energy. Adipose tissue itself can also oxidize fatty acids during periods of dietary energy-insufficiency. In non-ruminants, fatty acid synthesis takes place in liver and adipose tissue, but in the ruminant the adipose and mammary gland (during lactation) are the most important tissues synthesizing fatty acids de novo. Long chain fatty acids can be obtained from the diet or from lipogenesis using acetyl-coenzyme A (acetyl-CoA). Acetyl CoA is used by the first enzyme of fatty acid synthesis, acetyl-CoA carboxylase-alpha (ACACA) (Wakil and Abu-Elheiga, 2009). Fatty acid synthesis requires acetyl-CoA, malonyl-CoA (synthesized from ACAC), and other enzymes and transcription factors that control NADPH synthesis and fatty acid synthesis prior to esterification into triglyceride.

Lipolysis has been investigated in dairy cattle by many researchers (Shirley et al., 1973; Yang and Baldwin, 1973), and they were able to determine alterations in enzymes that control lipid metabolism. The key hormones involved in lipolysis regulation include catecholamines, insulin, growth hormone, and thyroid hormones with catecholamines considered as the essential hormone that stimulates lipolysis by binding to the β-adrenergic receptor followed by activation of G- protein coupled receptors (Sekar and Chow, 2014).

During early lactation the lipolytic pathway increases to meet the energy requirements of the mammary gland. Increases in the rate of lipolysis in adipose tissue depends on the production ability (i.e. genetic merit of the cow) and dietary energy level (McNamara, 1991). Hormone-stimulated lipolysis is regulated by *HSL* which catalyzes the release of fatty acids and glycerol from TG. *HSL* is activated via phosphorylation by protein kinase A (PKA). The activity of *HSL* peaked at 60 d after calving and decline at late gestation (Smith and McNamara, 1990).

Adipose triglyceride lipase (*ATGL*) is responsible for basal lipolysis, the activation of *ATGL* requires the activator protein abhydrolase domain containing 5 (*ABDH5*) (Duncan et al., 2007). The activation of G-protein coupled receptor leads to the production of cAMP which then activates protein kinase A leading to phosphorylation of *HSL* (Kraemer and Shen, 2002). In monogastric animals AMP-activated protein kinase (AMPK) is involved in the regulation of lipolytic processes (Gauthier et al., 2008). In a recent study (Locher et al., 2012), it was determined that lipolysis during early lactation is associated with an increase in phosphorylation of AMPK in bovine adipose tissue.

Importance of liver

The liver plays a major role in terms of NEB and lipid mobilization through transition period. There is an effect of NEB in dairy cow liver during early and late lactation periods (Gross et al., 2013). Increasing lipid mobilization results in a large amount of NEFA released from the adipose tissue (Imhasly et al., 2015). The oxidation of long chain fatty acids within adipose tissue and liver takes place in mitochondria and peroxisomes (Drackley et al., 2001). The liver removes a large amount of NEFA and use them to synthesize ketone bodies or to synthesize triglyceride. Ketone bodies released from the liver can be used as an energy source by peripheral tissues during periods of NEB (i.e. when glucose availability decreases). The oxidation of fatty acids produces Acetyl CoA and ketone bodies, excessive production of ketone bodies could negatively affect the health and production of cows (Herdt, 2000).

Increases in the concentration of NEFA in the blood and the inability of the liver to completely oxidize them often leads to synthesis of a large amount of triglycerides within liver. Very low density lipoproteins are essential for the removal of triglyceride from the liver into the blood (Brickner et al., 2009). Most of the TG exported by the liver will be used by different tissues as an energy source, and some will go to the mammary gland to synthesize milk fat (Drackley et al., 2006). When the rate of triglyceride-fatty acids that is released from adipose lipid mobilization is higher than what is exported by the liver the accumulated TG in the liver can get above the threshold that is considered adequate, and cause fatty liver. This is an important metabolic disorder that is associated with many different diseases and can decrease milk yield (Grummer, 1993; Drackley, 1999).

In non-ruminants, insulin and glucagon signaling within liver play important roles during NEB. It is well-established that increased insulin causes a reduction in carnitine palmitoyltransferase I (*CPT1A*) activity in liver, which can lead to decreased NEFA transport

into mitochondria causing a decrease in ketogenesis (McGarry and Foster, 1980). On the other hand, the increase of insulin promotes esterification and increases the synthesis of triglyceride in adipose tissue (Keller et al., 1988). Glucagon works to stimulate gluconeogenesis, promote the activation of *CPT1A* and stimulate the entry of NEFA into mitochondria to produce Acetyl-CoA for synthesis of ketone bodies (Kerner and Hoppel, 2000). The transport of monocarboxylic acids like ketone bodies and lactate plays a major role in body weight and fat deposition (Pierre and Pellerin, 2005).

Body condition score

In the United States, dairy farmers utilize a five-point scale to evaluate body condition score; whereas a score 1 indicates very thin cows, a score of 5 denotes fat or "overconditioned" cows (Wildman et al., 1982). Body condition scores help to evaluate the amount of fat deposition in the body, and give a good indication to the nutritional status of the animal. Body condition score plays an important role in animal health, with a high body condition score during the prepartum period often being related to different postpartum metabolic disorders in dairy cows (Cameron et al., 1998; Buckley et al., 2003). Also, high body condition is associated with high NEFA concentration in the blood (Rukkwamsuk et al., 1998). Cows with high body condition score prepartum often lose more body weight and condition postpartum than thin cows (Treacher et al., 1986).

Body condition score at calving could affect DMI postcalving. There is evidence to show that DMI postpartum decreases with increased BCS at calving (Hayirli et al., 2002). Furthermore, an increase in fat depots precalving leads to inhibition of feed intake postcalving (Garnsworthy and Topps, 1982). Busato et al. (2002) determined the relationship among

metabolic and endocrine changes with pre-calving and post-calving BCS loss. They observed that cows with prepartum BCS > 3.25, which also lost more than 0.75 BCS points in the first 2 months of lactation, were more susceptible to subclinical ketosis than cows with prepartum BCS < 3.25 and lost less than 0.75 BCS point postpartum.

Body condition score at calving and the loss of BCS after calving often affect milk yield, but the evidence of the effect of BCS on milk yield is conflicting. Some studies reported a negative relationship between BCS and milk yield, particularly due to the difference in DMI between fat cows and thin cows, this is because cows with high BCS prepartum consume less DMI postpartum than cows with low BCS (Garnsworthy and Topps, 1982; Bourchier et al., 1987). Other data show that cows that had BCS at calving 2.5 produced more milk than cows that had BCS 4 at a 5- point scale (Treacher et al., 1986). Other research reported no significant effect of BCS at calving on milk yield. Some studies reported a positive effect of BCS on milk yield (Waltner et al., 1993; Stockdale, 2004).

Oxidative stress

Increase metabolic rate during the transition period leads to an increase in the requirement of oxygen for metabolism in organs such as the liver. As such, there is an increase in the production of reactive oxygen species (ROS). The disequilibrium between ROS production and antioxidant defenses could expose dairy cows to increased oxidative stress. Oxidative stress plays a major role in the pathogenesis of different diseases in dairy cattle (Miller et al., 1993; Castillo et al., 2005). Oxidative stress results in damage of unsaturated lipids with a subsequent change of cell membrane and other cellular chemical properties (Toyokuni, 1999) leading to an overall change of tissue metabolic function.

Negative energy balance could be a primary reason for the development of oxidative stress during the transition period (Pedernera et al., 2010). In humans and mice, there is a relationship between fat deposition and systemic oxidative stress where the production of ROS increases in white adipose tissue in obese mice as a result of increased expression of NADPH oxidase and reduced expression of antioxidant enzymes (Furukawa et al., 2004). In dairy cows, some data showed that cows with high BCS during the prepartum period and with a bigger loss of BCS postpartum were more susceptible to oxidative stress (Bernabucci et al., 2005a).

Superoxide dismutase (SOD) is one of the most important defense mechanisms involved in the control of ROS concentrations (Martindale and Holbrook, 2002), and specifically the removal of superoxide. There are different types of SOD enzymes found in mammalian including *SOD1* and *SOD2*. Superoxide dismutase (*SOD1*) was the first enzyme characterized and it is found in intracellular cytoplasmic spaces (Zelko et al., 2002). In contrast, SOD2 is primarily located in the mitochondria and because this organelle is the primary site for the production of ROS, mitochondrial *SOD2* plays an important role in maintaining cellular ROS balance (Drose and Brandt, 2012). For example, the inhibition of *SOD2* led to accumulation of ROS which caused an increase in the intracellular oxidative stress (Hu et al., 2005).

Methionine

Methionine is one of the most-limiting amino acids for milk synthesis in dairy cows (Schwab et al., 1992). During the transition period, the decrease in DMI and the increase in nutrient demands by the mammary gland are the key causes that increase the methionine requirements and the need for supplementation (Grummer, 1995). In addition to production outcomes, the increase in the availability of methionine could positively affect S-

adenosylmethionine (SAM), which is the primary biological methyl donor (Finkelstein, 1990), concentration at the tissue level. To further complicate the issue, the rumen microbes decrease the dietary availability of methionine which makes the supplementation of rumen-protected methionine necessary. Several researchers demonstrated a benefit of rumen-protected methionine supplementation in terms of milk yield (Schmidt et al., 1999) and milk fat content (Socha et al., 2005). Other more recent studies observed a positive effect of rumen-protected methionine during the peripartal period on alleviating oxidative stress (Osorio et al., 2014).

Methionine is also involved in very low-density lipoprotein (VLDL) synthesis, and plays an important role as a lipotropic agent which could help clear lipid from the liver (Auboiron et al., 1995). Some studies have observed lower liver TG with rumen-protected choline (Zom et al., 2011), whereas other studies reported no effect of methionine supplementation on TG level (Piepenbrink et al., 2004; Osorio et al., 2013).

Endocannabinoid System

Endocannabinoids (ECs) are fatty acid amides that in non-ruminants have the ability to control feed intake, energy balance, and have anti-inflammatory properties (Guindon and Beaulieu, 2006; Maccarrone et al., 2010). The endocannabinoid system (ECS) includes at least two receptors (*CNR1* and *CNR2*) that were originally discovered as the target of the Cannabis sativa activate molecule Δ9-tetrahydrocannabinol (Pertwee et al., 2010). Cannabinoid receptors are G-protein coupled receptors, and in non-ruminants are expressed in central nervous system (CNS) and peripheral tissues that include liver, adipose tissue and skeletal muscle (Matias and Di Marzo, 2007; Khan et al., 2012).

The ECS also include cannabinoid receptor agonists called endocannabinoids the most studied of which are anandamide [N-arachidonoylethanolamine (AEA)] and 2-arachidonoyl glycerol (2-AG) (Devane et al., 1992). These EC are synthesized on demand (Di Marzo et al., 2005). The endocannabinoid system also includes enzymes to regulate the EC level such as N-acyl-phosphatidylethanolamines (NAPE)-hydrolyzing phospholipase D (NAPE-PLD) the main enzyme to synthesize AEA, palmitoylethanolamine (PEA), and oleoylethanolamine (OEA) and also diacylglycerol lipase that leads to production of 2-AG (Bisogno et al., 2003). There are different enzymes involved in the degradation of AEA and 2AG of which the most important are fatty acid amide (*FAAH*) (Fezza et al., 2008) and monoacylglycerol lipase (*MGLL*) (Dinh et al., 2002b).

In non-ruminants, endocannabinoids have the ability to control appetite, food intake and energy balance (Randall, 2007; Pacher et al., 2008; Pagano et al., 2008). For example, low doses of anandamide can increase food intake by actions at the brain level of *CNR1* (Hao et al., 2000), and administration of 2-AG also increases food intake (Kirkham et al., 2002). Some data showed that *CNR1* knockout mice had lower rates of feed intake compared with wild-type mice (Jelsing et al., 2008). However, the level of AEA and 2-AG is affected by nutrient status such that fasting increase the level of cannabinoids especially 2AG; feeding decreased the 2AG level (Hanus et al., 2003).

In non-ruminants, endocannabinoids can regulate lipid and glucose metabolism by binding to peroxisome proliferator-activated receptors (Bensinger and Tontonoz, 2008) and they have the ability to enhance adipogenesis by promoting lipoprotein lipase (*LPL*) and fatty acid synthase activity in the adipose tissue. The *LPL* activity increased during the activation of *CNR1* (Cota et al., 2003). On the other hand, the endocannabinoids are able to inhibit lipolysis and fatty

acid oxidation through preventing adenylyl cyclase and AMPK activity (Maccarrone et al., 2010).

In liver tissue, the relationship between ECS and various liver diseases has been investigated in several studies with non-ruminants, and they demonstrated that the normal liver contains a low level of endocannabinoids (Siegmund et al., 2006; Teixeira-Clerc et al., 2006). Several hepatic disorder such as fatty liver and fibrosis cause upregulation of endocannabinoid levels in the liver (Teixeira-Clerc et al., 2006). The increase level of AEA during the early stages of fatty liver was caused by a decrease in the degradation of AEA by *FAAH* (Siegmund et al., 2006). The expression of endocannabinoid receptors *CNR1* and *CNR2* is very low in normal liver, and is upregulated in various liver diseases, thus, providing more evidence that the activity of the endocannabinoid system is low under normal conditions (Siegmund and Schwabe, 2008).

As discussed above, BCS and feeding management during the transition period play an important role in overall animal health and production. The role of the endocannabinoid system in regulating feed intake and immune system in non-ruminant has been well studied. As far as we know, only one study has been done in bovine related to the endocannabinoid system and demonstrated that ECS may be associated with lipid metabolism which could be affected by energy balance (Khan et al., 2012). Investigating the effect of BCS and feeding management during the transition period on the endocannabinoid system could increase our understanding of this system in the context of metabolism and immune function.

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CHAPTER II

ENDOCANNABINOID AND LIPID METABOLISM GENE NETWORK EXPRESSION IN ADIPOSE TISSUE OF PERIPARTAL COWS WITH HIGH OR LOW BODY CONDITION SCORE

ABSTRACT

Our previous research has revealed a strong inflammatory response within adipose tissue during the transition into lactation. Whether this localized effect is a result of oxidative stress induced by lipolysis and fatty acid oxidation or via the production of endocannabinoids remains to be determined. The objective of this study was to investigate the expression of genes composing the endocannabinoid signaling system and lipid metabolism in adipose tissue during the transition period in dairy cows. Twenty multiparous Holstein cows were retrospectively divided by body condition score (BCS) into two groups (10 cows/group): BCS ≤ 3.25 (LoBCS) and BCS ≥ 3.75 (HiBCS). Adipose tissue was biopsied at d -10, 7 and 20 relatives to parturition. Tissue RNA was used to evaluate 28 target genes via quantitative real time RT-PCR. Among the endocannabinoid-related genes, a BCS × day was observed for *NAPEPLD*, *CNR2* and *FAAH*. Expression of *NAPEPLD* and *CNR2* was greater at d 7 in LoBCS than HiBCS cows, while *FAAH* was upregulated at d 7 and 20 LoBCS than HiBCS cows. Expression of monoglyceride lipase (*MGLL*), which inactivates 2-rachidonoylglycerol, was overall greater across time in LoBCS than HiBCS. Regarding the genes related with lipid metabolism, a BCS × day was observed for the mitochondrial enzyme *SOD2*, important for clearing reactive-oxygen species that cause cellular stress and inflammation, because of greater expression at d 7 in LoBCS than HiBCS. The expression of *SOD1* was greater in LoBCS vs. HiBCS. A tendency for a BCS × day

was observed for *LIPE* due to greater expression at d 7 and 20 in LoBCS than HiBCS. Among genes associated with lipolysis, LoBCS compared with HiBCS cows had overall greater expression of *ABDH5* and *ATGL*, indicating a greater state of basal lipolysis over time. Among genes related with fatty acid oxidation and transporter *CPT1A* and *ACADVL* was greater in HiBCS than LoBCS due to greater expression at -10 and 7 d. Among the genes related to glyceroneogenesis, higher expression of *PCK1* in LoBCS than HiBCS indicates a greater rate of FA recycling in LoBCS. Overall, data indicated that cows with BCS 3.25 or lower before calving experienced greater alterations in lipid metabolism and endocannabinoid signaling whereas, cows with BCS 3.75 or higher showed higher expression of genes related to inflammation like *TLR4* and *TLR9*. A potential linkage between those pathways and risk of disorders postpartum remains to be determined.

INTRODUCTION

The endocannabinoid system (ECS) contains cannabinoid receptors, endogenous cannabinoid agonists known as endocannabinoid, and the enzymes involved in endocannabinoid synthesis and degradation (Vahatalo et al., 2015). Endocannabinoid receptors *CNR1* and *CNR2* are G- protein coupled receptors. They are mainly expressed in central nervous system, and in immune cells but also in peripheral tissues (Mechoulam and Parker, 2013). The most important endogenous cannabinoids are N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). Endocannabinoid-like molecules like N-palmitoylethanolamide (PEA) and N-oleoylethanolamide (OEA) are endogenous fatty acyl amides, but they do not have the ability to activate cannabinoid receptors (Maccarrone et al., 2010). Anandamide and 2-AG are synthesized by multiple biosynthetic pathways. N-acyl-phosphatidylethanolamines (NAPE)- hydrolyzing phospholipase D (*NAPE-PLD*) is the first enzyme in the pathway to synthesize AEA, PEA, and OEA (Petrosino et al., 2009). The degradation of endocannabinoids occurs through fatty acid amide hydrolase (*FAAH*), N-acylethanolamine acid amidase (*NAAA*), and monoglyceride lipase (*MGLL*) (Matias and Di Marzo, 2006).

The discovery of the role of the endocannabinoid system in modulating energy balance and controlling feed intake led to demonstration the effect of this system in adipose tissue. Several studies indicate a direct effect of endocannabinoid in adipose tissue (Cota et al., 2003; Hildebrandt et al., 2003). The level of AEA and 2-AG is highly affected by nutrient status; fasting can increase levels of 2-AG and AEA in the part of the brain involved in regulation of food intake, whereas feeding reduces these levels (Soria-Gomez et al., 2014). N-oleoylethanolamide can stimulate a satiety signal and inhibit food intake via activation of peroxisome proliferator-activator receptor α (PPARα) (Gomez-Boronat et al., 2016). Data from rodents reported the effect of endocannabinoid

in the regulation of fat metabolism, where the activation of *CNR1* in mice increased the expression of lipogenic and fatty acid synthesis genes (Osei-Hyiaman et al., 2005). In addition, EC prevents adenylyl cyclase and AMPK activity which will lead to inhibition of lipolysis and fatty acid oxidation (Maccarrone et al., 2010).

During the transition from a non-lactating to a lactating stage, dairy cows are susceptible to metabolic disorders and immunologic challenges. Dry matter intake (DMI) decreases in the prepartum and rapidly declines when calving date is approaching (Robinson and Garrett, 1999). Due to the variation between DMI and nutrient requirements, most dairy cows will experience negative energy balance (NEB) (Drackley et al., 2001). Negative energy balance affects metabolism in different tissues, and particularly adipose tissue (Grummer, 1995). Adipose tissue plays an important role in the maintenance of metabolic homeostasis during the transition period (McNamara, 1991). During late pregnancy and early lactation, the adipose tissue starts to break down the triglyceride to generate fatty acids (FAs) and glycerol in a process known as lipolysis. The main purpose of lipolysis is providing energy to other organs among the body. Lipolysis and lipogenesis in adipose tissue are regulated by different hormones around parturition (Sumner-Thomson et al., 2011). Non-esterified fatty acids (NEFA) concentration in blood is a good indicator of adipose tissue mobilization (Bell, 1995). Understanding the function of adipose tissue and lipid metabolism might help to decrease the occurrence of metabolic disorders.

After parturition in dairy cows, the mobilization of fat stored in adipose tissue leads to the loss in body condition score. Roche et al. (2013) showed the relationship between body condition score (BCS) and cow metabolic profiles. Body condition score at calving and early lactation are related to the occurrence of several metabolic disorders like ketosis and fatty liver (Drackley, 1999; Gillund et al., 2001). It was reported that cows with high body condition score at calving lose more

body weight and body condition than cows with low BCS (Treacher et al., 1986; Bernabucci et al., 2005a). There is a relationship observed between obesity and oxidative stress in humans (Ozata et al., 2002; Morrow, 2003). Bernabucci et al. (2005) reported a connection between BCS, lipid mobilization and the imbalance in oxidative status in transition cows.

The postnatal effect in non-ruminants of the EC system in pathogenesis of obesity and their role in controlling food intake, body weight and metabolic processes is relatively well-known (Rossi et al., 2016). Endocannabinoid receptors in adipose tissue prevent lipolysis and fatty acid oxidation and increase the expression of lipogenic and fatty acid synthesis genes (Osei-Hyiaman et al., 2005). Dry matter intake postpartum decreases in cows with high BCS prior to calving (Hayirli et al., 2002) and also the increase in fat depots precalving leads to inhibition of feed intake postcalving. (Garnsworthy and Topps, 1982). A previous study investigated the role of EC system in bovine liver in response to prepartal plane of nutrition (Khan et al., 2012), and demonstrated that the expression of ECS and POMC in bovine liver was effected by prepartal plane of nutrition. This response could associate with lipid metabolism and energy balance by controlling hepatic intracellular signals.

The primary objective of this study was to evaluate the effect of body condition score befor calving on the endocannabinoid signaling system and lipid metabolism genes in adipose tissue during the transition period in dairy cows retrospectively grouped into a high or low BCS at calving.

MATERIALS AND METHODS

Animals and diets

All the procedures for this study were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Illinois (Protocol #13023). Twenty Holstein cows were selected according to the body condition score (3.75 ±0.12) or higher (n = 10) and cows have (3.25±0.15) or lower (n = 10). All cows received the same diet; from -50 to -23 d before expected calving they received a far-off diet (1.40 Mcal/kg of DM, 10.2% RDP, and 4.1% RUP), from -21d to expected calving they received a close-up diet (1.52 Mcal/kg of DM, 9.1% RDP, and 5.4% RUP), and from calving until 30 DIM they received a lactation diet (1.71 Mcal/kg of DM, 9.7% RDP, and 7.5% RUP) (Table 2.1). Diets were fed as a total mixed ration (TMR) once daily (0630 h). Dry cows were housed in a free-stall barn with an individual Calan (American Calan, Northwood, NH, USA) gate feeding system. Cows had access to sand-bedded free stalls until 3 d before expected calving date, when they were moved to an individual maternity pen bedded with straw until the calved. After calving, cows were housed in a tie-stall barn and were fed a common lactation diet once daily in the morning and milked 3 times daily at approximately 6:00, 14:00, and 22:00.

Adipose tissue

Subcutaneous adipose tissue biopsies were collected from the tail-head region at -10, 7 and 20 days relative to parturition. The samples were immediately frozen in liquid nitrogen and transferred to –80 °C freezer for future analysis.

RNA extraction, PCR, and design and evaluation of primers

RNA extraction

The frozen tissues were used to extract the RNA using protocols established in our laboratory (Loor et al., 2007). Briefly, adipose tissue samples were weighed (~0.2-0.4 g) and immediately placed 1.2 ml of ice-cold Qiazol reagent (Qiagen 75842; Qiagen Inc., Valencia, CA) for homogenization. After homogenization, genomic DNA was removed from RNA with DNase using RNeasy Mini Kit columns (Qiagen, Hilden, Germany). The Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA) was used to measure the concentration of RNA, while The quality of RNA was evaluated using the Agilent Bioanalyzer system (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA).

qPCR analysis

The cDNA was synthesized with 100 ng RNA. The RNA was mixed with the Master Mix-1(MM1) containing 9 μL DNase/RNase free water and l μL random primers (Roche® Cat. No. 11 034 731 001, Roche Diagnostics GmbH, Mannheim, Germany), then incubated at 65°C for 5 min and kept in ice for 3 min. The reaction was perform using Eppendorf Mastercycler®. 9 μl of Master Mix-2 (MM2) consists of 1.625 μL DNase/RNase free water, 4 μL 5X First-Strand Buffer, 1 μL Oligo dT18, 2 μL 10 mM dNTP mix (10 mM; Cat. No. 18427-088; Invitrogen), 0.25 μL of Revert aid (200 U/μL; Cat. No. EP 0441; Fermentas), and 0.125 μL of RNase inhibitor (20 U/μL; Cat. No. EO 0382; Fermentas). Samples then were incubated (MM1+RNA and MM2) at the following temperature program: 25°C for 5 min, 42°C for 60 min and 70°C for 5 min. An aliquot of undiluted cDNA from all samples was pooled to make samples of standard curve by diluting with DNase/RNase free water, then the cDNA was diluted 1:4 with DNase/RNase free water.

Quantitative PCR was performed using 4 μL diluted cDNA combined with 6 μL of a mixture contain 5 μL 1 × SYBR Green master mix (Applied Biosystems, CA, USA), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems, CA, USA). An ABI prism 7900 HT SDS instrument was used at the following program: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. (Gene symbol, ID and accession number are shown in table2.2)

The final data were normalized using the geometric mean of three internal control genes (ICG): UXT, GAPDH and RPS9. The relative mRNA abundance was calculated as previously reported (Bionaz and Loor, 2008) using the median ΔCt (ΔCt = Ct of the gene – geometrical mean Ct of internal control genes) corrected by efficiency (E), where % relative mRNA abundance = [$1/E^{\Delta Ct}$] / ∑[$1/E^{\Delta C}$] all measures genes × 100 . The PCR efficiency was calculated for each gene using the standard curve method] $E=10^{(-1/slope)}$] .

Statistical analysis

After the data had been normalized with the geometric mean of the internal control genes, the quantitative PCR data were log2 transformed before statistical analysis to obtain a normal distribution. Statistical analysis was performed with SAS (SAS Institute, Inc., Cary, NC, USA). Normalized, log2-transformed data, were subjected to ANOVA and analyzed using repeated measures ANOVA with PROC MIXED. The statistical model included time (day; −10, 7 and 20 d relative to parturition), BCS (HiBCS and LoBCS), and their interactions (BCS × time) as fixed effects. Data were considered significant at $P \leq 0.05$ and tendencies at $P \leq 0.15$.

RESULTS

Dry matter intake and milk production

Prepartum, we observed a tendency for an effect of BCS on DMI (BCS $P = 0.15$) (Figure 2.1) due to more feed intake in LoBCS than HiBCS. There was also a tendency in the first days (BCS × Time $P = 15$) after calving where LoBCS score tended to eat more than HiBCS (Figure 2.1). We did not observe any effect of BCS on milk yield between the two groups (Figure 2.2).

Genes involved in the endocannabinoid system

The expression of genes involved in the ECS system is shown in Figure 2.3. There was an interaction of BCS x time ($P \leq 0.05$) on the mRNA expression of EC receptor *CNR2* due to a greater expression at 7 d in LoBCS than HiBCS cows. Similar to *CNR2*, the expression of *NAPEPLD* the first enzyme responsible for the synthesis of AEA and OEA increased during postpartum, which resulted in higher expression (BCS × time $P \leq 0.05$) at 7 d in cows with LoBCS. Fatty acid amide hydrolase (*FAAH*) was upregulated at d 7 and 20 in Lo BCS compared with HiBCS cows. Expression of monoglyceride lipase (*MGLL*), which inactivates 2-arachidonoylglycerol, was overall greater ($P \leq 0.05$) across time in LoBCS than HiBCS. In addition, LoBCS compared with HiBCS cows had a tendency ($P = 0.06$) for greater overall expression of POMC across time.

Genes involved in lipolysis

Regarding to the genes involved in lipolysis (Figure 2.4), the expressions of *ATGL* which catalyzes the first step in triglyceride hydrolysis and *LIPE* were affected by BCS ($P \leq 0.05$), with *ATGL* having overall greater expression in LoBCS than in HiBCS cows. There was a strong

tendency in the expression of *LIPE* (BCS × time $P = 0.07$) due to greater expression at d 7 and 20 in LoBCS than HiBCS. An overall greater expression of *ABDH5* ($P = 0.04$) was detected in LoBCS cows.

Fatty acid oxidation and transport genes

There was an interaction (BCS × time $P \leq 0.05$) in the expression of *CPT1A*, which is the key enzyme in fatty acid oxidation due to its increase at -10 and 7 d in HiBCS compared with LoBCS (Figure 2.5). There was no BCS or BCS × time effect for the expression of *CPT2*. However, HiBCS experienced a gradual increase (time $P = 0.05$) between -10 and 20 d. Acyl-CoA dehydrogenase, very long chain (*ACADVL*) is another enzyme that plays a key role in the oxidation of long chain fatty acids. The expression of *ACADVL* was higher in HiBCS than LoBCS across time ($P \leq 0.05$). There was a significant interaction ($P \leq 0.05$) due to higher expression at -10 and 7 d in HiBCS than LoBCS. Similar to *ACADVL* there was a BCS ($P \leq 0.05$) effect for the expression of *ACOX1* due to higher expression in HiBCS during prepartum and postpartum.

The gene *ADIPOQ* was affected by BCS due to greater expression in LoBCS than HiBCS ($P \leq 0.05$). The expression of solute carrier family 16 member 1(*SLC16A1*) which is involved in short chain fatty acid transport (Hadjiagapiou et al., 2000) was affected by BCS ($P \leq 0.05$) due to the higher expression in LoBCS than HiBCS (Figure 2.6). We observed a BCS effect ($P \leq 0.05$) for the expression of *FABP4* due to high expression of LoBCS at day 7 comparing to -10 and 20d.

Aquaporin 7 (*AQP7*) allows movement of water and a glycerol across cell membranes and highly expressed in adipose tissue; *AQP7* plays important role in glycerol transport from adipose tissue (Rodriguez et al., 2006). The expression of *AQP7* was not affected by BCS or BCS × time, but there was a time effect ($P \leq 0.05$) due to a decrease the expression in both groups between -10

and 20 d. The expression of *PCK1* (Figure 2.7), which play important in glyceroneogenesis, was higher in LoBCS ($P \leq 0.05$) due to higher expression at -10 and a sharp decrease at postpartum period (Figure 2.7). Glycerol kinase (*GK*) was not affected by BCS; there was a time effect ($P \leq 0.05$) observed due to an increase in expression at 7 and 20 d compared with -10 d in both HiBCS and LoBCS (Figure 2.7).

Inflammation and oxidative stress

The expression of *NFE2L2*, which is involved in inflammation, was not affected by BCS, time or their interaction. We detected a BCS effect ($P \leq 0.05$) for the expression of *SOD1* due to higher expression of LoBCS at -10 and 7 d. There was an interaction (BCS × Tim $P \leq 0.05$) observed for the mitochondrial enzyme *SOD2* because of greater expression at d 7 in LoBCS than HiBCS (Figure 2.8). Although Toll-Like Receptor 4 (*TLR4*) was not affected by BCS or time, we observed an interaction resulting in higher expression at -10 d in HiBCS. We detected a BCS effect for the expression of *TLR9* ($P \leq 0.05$) because of an increase in the expression at 20 d in HiBCS compared with LoBCS.

DISCUSSION

Although cannabinoid receptor 1 (*CNR1*) was not expressed in adipose tissue, we found a significant difference for *CNR2* (BCS × Time, $P \leq 0.05$) because of higher expression in LoBCS cows at day 7. Those data may indicate that the adipose tissue in bovine is a minor site of endocannabinoid signaling. As far as we know, only one study related to the endocannabinoid system has been performed in ruminants, and the data suggested the synthesis of endocannabinoids in ruminant liver is minor (Khan et al., 2012). Data from rodents indicate that the lack of *CNR2* in adipose tissue causes an increase in body weight and food intake (Agudo et al., 2010). The activity

of endocannabinoid signaling during consumption of a high-fat diet seems to increase the expression of *CNR2* in adipose tissue specially when there is an inflammatory stimulus occurring (Deveaux et al., 2009).

Anandamide and 2AG are synthesized on demand through multiple pathways, N Acylphosphatidylethanolamine phospholipase D (*NAPEPLD*) is the main enzyme involved in the synthesis of endocannabinoids, especially AEA (Fonseca et al., 2013). The endogenous cannabinoid AEA can increase food intake in mice by activating *CNRs* (Maccarrone et al., 2010). Body condition score at calving negatively affects DMI during the postpartum period, with 3.5 BCS or higher at calving being associated with lower DMI after calving (Roche et al., 2009). Our data suggest that increased expression of *NAPEPLD* after calving regardless of BCS, the first enzyme involved in AEA synthesis, could be associated with changes in feed intake through greater production and export of AEA. Future research of the plasma profiles of endocannabinoids during the transition period could enhance our understanding of their role in the peripheral control of signals that may induce satiety through CNRs.

The degradation of AEA and 2AG mainly occurs through fatty acid amide hydrolase (*FAAH*) and monoacylglycerol lipase (*MGLL*), respectively (Alswat, 2013). Monoacylglycerol lipase also plays an important role in the conversion of monoacylglycerides to free fatty acids and glycerol. The greater expression of *MGLL* in LoBCS indicated a higher degradation of 2AG as in rodents (Maccarrone et al., 2010), and seems to agree with the greater expression of *NAPEPLD*. Data from rodents showed that deficiency of monoglycerode lipase increased the level of 2AG and impaired lipolysis (Taschler et al., 2011). The expression of *ATGL* and *LIPE* was higher in LoBCS which is opposite to what would be expected, i.e. cows with HiBCS would be expected to have accumulated more fat depots and potentially have greater lipolytic activity partly through the

action of *ATGL* and *LIPE* (Ji et al., 2012). Data from dairy cows revealed a greater expression of *MGLL* prepartum in adipose tissue of overfed versus control cows (Ji et al., 2012), and also postpartum in liver of cows overfed energy prepartum compared with cows fed a diet to closely meet energy requirements during the dry period. Those overfed cows also had higher concentration of TAG in liver after calving (Khan et al., 2012).

The N-acylethanolamine-hydrolyzing acid amidase (*NAAA*) enzyme is involved in the degradation of endocannabinoids, and its expression increased gradually in both groups indicating more degradation of EC during the postpartum. Such response could indicate that *NAAA* may play a role in reducing the amount of EC that could elicit a biologic response within the adipose tissue, e.g. production of inflammatory molecules. Proopiomelanocortin (*POMC*) plays an important role in body weight and appetite regulation in non-ruminants (Zemel and Shi, 2000). In addition, *POMC* neurons are important in coordinating some activities of leptin, during negative energy balance, the level of leptin and insulin in the blood decrease and these two hormones are involved in several peripheral functions (Varela and Horvath, 2012). Additional studies to investigate the mechanisms of *POMC* in bovine adipose tissue will help to understand its potential role in regulating food intake and body weight in dairy cows.

Around calving, adipose tissue becomes active by increasing the mobilization of body fat reserves to provide energy to other tissues. Adipose triglyceride lipase (*ATGL*) is upregulated during fasting and induces lipolysis because it is the rate-limiting enzyme of lipolysis (Duncan et al., 2007). The degradation of triacylglycerols is regulated by *ATGL* and *LIPE,* the two key enzymes in basal and stimulated lipolysis (Morak et al., 2012). The complete activation of *ATGL* requires binding of the protein *ABHD5* which is the activator of *ATGL* (Ji et al., 2012). Hormone sensitive lipase (*LIPE*) is able to hydrolyze triacylglycerols, with the activation of *LIPE* occurring

via cyclic AMP (Kraemer and Shen, 2002). Thus, the increase in the expression of *ATGL*, *LIPE* and *ABDH5* (Figure 2.4) in LoBCS compared with HiBCS indicated a greater state of basal and stimulated lipolysis over time.

Adipose tissue sensitivity to insulin and insulin concentration affect the degree of lipolysis and lipogenesis (De Koster and Opsomer, 2013). Data from rats and humans demonstrated that late-pregnancy is an insulin resistant state (Sevillano et al., 2007). The mRNA expression of *CPT1A*, *ACADVL* and *ACOX1* (genes involved in FA oxidation) (Figure 2.5) was higher in HiBCS cows, indicating an increase in the use of FA as energy within adipose tissue. Some of the fatty acids produced through lipolysis can be transported into the blood and taken up by liver or muscle cells to be oxidized for generation of energy (Serra et al., 2013). The greater expression of *CPT1A* in HiBCS at -10 and 7 day relative to parturition indicated a higher level of fatty acid utilization by the mitochondria for β-oxidation, hence, underscoring the robust capacity of adipose for utilization of fatty acids. Our data indicated that LoBCS cows likely were mobilizing more fat than HiBCS but at the same time they did not seem to have the ability to increase the utilization of these FA through oxidation.

Data from non-ruminants indicated that obese subjects have a lower concentration of adiponectin in the circulation (Arita et al., 2012), which could partly explain the high level of *ADIPOQ* in the LoBCS cows. Solute carrier family 16 member 1 (*SLC16A1*) is responsible for the transport of short chain monocarboxylates such as pyruvate, lactate and volatile fatty acids. (Wang and Morris, 2007). Higher expression of *SLC16A1* in LoBCS at -10 d indicated more transport of short chain fatty acids. Treatment with SCFA in non-ruminants can reduce gain in body weight (Canfora et al., 2015).

Aquaglyceroporin aquaporin-7 (*AQP7*) is highly expressed in non-ruminant adipose tissue, because *AQP7* facilitated the efflux of glycerol that is released from adipose tissue during lipolysis. A deficiency in *AQP7* is related to TG accumulation in adipose tissue (Lebeck, 2014). There was no BCS effect on the expression of *AQP7*, but there was a sharp decrease between prepartum (Figure 2.6) to postpartum. Data from rodents showed that *AQP7* knockout mice had a lower plasma concentration of glycerol under fasting and fed conditions than wild-type mice, but they had a normal plasma concentration of FFA (Maeda et al., 2004).

Fatty acid binding protein 4 (*FABP4*) is involved in the intracellular transport of fatty acids primarily during adipogenesis. There is some evidence for a relationship between *FABP4* and some diseases in humans such as type 2 diabetes and insulin resistance (Terra et al., 2011). However, there was no BCS effect on the expression of *FABP4*. Among the genes related to glyceroneogenesis (i.e. de novo synthesis of glycerol-3-phosphate for TG production) the expression of *PCK1* was higher in LoBCS than HiBCS. Higher expression of *PCK1* in LoBCS could be a response to utilize more FA released as a result of greater *ATGL* and *LIPE* in those cows. A high rate of FA recycling indicates that lipolysis releases more FA than what may be required to generate energy (Nye et al., 2008). It is possible that recycling of the excess amount of FA that were hydrolyzed after calving in LoBCS is partly regulated by hormonal signals (e.g. epinephrine) as a way to maintain TG stores in those cows. However, comparing -3 wk vs. 3 wk relative to parturition reveals that HiBCS lost more BCS than LoBCS which supports the role of *PCK1* in maintaining TG stores (Figure 2.9).

Excessive lipid mobilization in the adipose tissue leads to a high concentration of FFA in the blood and is associated with the incidence of metabolic disorders (Ospina et al., 2010). Hence, during a state of high lipolytic activity of adipose tissue as in the transition period, dairy cows are

more susceptible to inflammation and oxidative stress. Oxidative stress, resulting in the increase in reactive oxygen species (ROS) production, can cause alterations of cell membranes and changes in cellular function (Bernabucci et al., 2005b, Nordberg and Arner, 2001). The enzyme superoxide dismutase (SOD) is one antioxidant mechanism that catalyzes the reduction of ROS, thus, plays an important role in maintenance of proper antioxidant capacity in tissues (Sordillo and Aitken, 2009). It was previously reported that cows with high BCS before calving and with more BCS losses had lower *SOD* and higher ROS in the circulation (Bernabucci et al., 2005a). Furthermore, several studies in humans linked obesity with higher oxidant and lower antioxidant concentrations (Keaney et al., 2003; Fernandez-Sanchez et al., 2011).

The expression of *SOD1*, one of at least three isotypes of SOD, was higher in LoBCS than HiBCS cows; expression of *SOD2* was also higher at day 7 in LoBCS cows (Figure 2.8). Because a previous study detected that the inhibition of *SOD2* expression caused accumulation of ROS (Hu et al., 2005), the response in LoBCS cows could have helped the adipose tissue to maintain a proper antioxidant status. From a mechanistic standpoint, the greater *ATGL* and *LIPE* in LoBCS cows could have generated more FA and greater concentrations of ROS that led to the upregulation of *SOD2*.

Toll-like receptors are essential in the defense mechanism against microbes and activate the innate immune response during inflammation (Medzhitov, 2001). Higher expression of *TLR9* in HiBCS compared with LoBCS and the higher expression of *TLR4* at day -10 in the same group of cows indicated a higher state of inflammation in this group of cows. Toll-like receptor 4 binds to bacterial lipopolysaccharide which is the main component of all Gram-negative bacteria (Shi et al., 2006). Also, *TLR4* could be activated via saturated fatty acids (Contreras and Sordillo, 2011), and such response could be a reason for the increase in expression of *TLR4* at -10 d in HiBCS. A

recent study in mice demonstrated that obesity is associated with the release of cell-free DNA (cfDNA) which could stimulate the resident macrophages via the *TLR9* pathway (Nishimoto et al., 2016). Further research could help demonstrate if the same linkage exists in dairy cow adipose tissue.

CONCLUSIONS

The endocannabinoid system in non-ruminants is reported to regulate immune function, control feed intake, and energy balance. Differences in the mRNA expression of components of this system between the BCS groups underscore the potential link with lipid metabolism in transition cow adipose tissue. The greater expression of genes associated with lipolysis in LoBCS indicated a greater state of basal lipolysis in this group, and at the same time those cows had higher expression of *PCK1*, which indicated more re- esterification of FA to maintain TG stores. The greater expression of genes involved in FA oxidation in the HiBCS indicated a higher use of FA as energy substrates within adipose tissue. The greater expression of *TLR* in HiBCS cows and the lower expression of *SOD* in the same group could be associated with higher loss of BCS postpartum, which could render cows more susceptible to health problems.

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TABLES AND FIGURES

Table 2.1. Ingredients and chemical composition of experimental diets.

	Diet		
Ingredient (% of DM)	Far-off	Close-up	Lactation
Alfalfa silage	12.00	8.34	5.07
Alfalfa hay	-	4.29	2.98
Corn silage	33.00	36.40	33.41
Wheat straw	36.00	15.63	2.98
Cottonseed	-	-	3.58
Wet brewers grains	-	4.29	9.09
Ground shelled corn	4.00	12.86	23.87
Soy hulls	2.00	4.29	4.18
Soybean meal, 48% CP	7.92	2.57	2.39
Expeller soybean meal[1]	-	2.57	5.97
Soychlor[2]	0.15	3.86	-
Blood meal, 85% CP	1.00	-	-
ProVAAl AADvantage[3]	-	0.86	1.50
Urea	0.45	0.30	0.18
Rumen-inert fat[4]	-	-	1.02
Limestone	1.30	1.29	1.31
Salt	0.32	0.30	0.30
Dicalcium phosphate	0.12	0.18	0.30
Magnesium oxide	0.21	0.08	0.12
Magnesium sulfate	0.91	0.99	-
Sodium bicarbonate	-	-	0.79
Potassium carbonate	-	-	0.30
Calcium sulfate	-	-	0.12
Mineral vitamin mix[5]	0.20	0.17	0.18
Vitamin A[6]	0.015	-	-
Vitamin D[7]	0.025	-	-
Vitamin E[8]	0.38	0.39	-
Biotin	-	0.35	0.35

[1]SoyPLUS (West Central Soy, Ralston, IA)
[2]By West Central Soy
[3]Perdue AgSolutions LLC (Ansonia, OH)
[4]Energy Booster 100 (Milk Specialties Global, Eden Prairie, MN)
[5]Contained a minimum of 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5000 mg of Cu/kg, 250 mg of I/kg, 40 mg of Co/kg, 150 mg of Se/kg, 2200 kIU of vitamin A/kg, 660 kIU of vitamin D3/kg, and 7,700 IU of vitamin E/kg.
[6]Contained 30,000 kIU/kg
[7]Contained 5,009 kIU/kg
[8]Contained 44,000 kIU/kg

Table 2.2. Gene ID, GenBank accession number, sequence and amplicon size of primers used in adipose tissue

Gene ID	Accession #	Gene	Primers	Primers (5'-3')	bp[1]
539769	NM_001192303.1	*CNR2*	F.791	TCTTCGCCGGCATCATCTAC	110
			R.900	CATCCGGGCTATTCCAGACA	
541291	NM_001015680.1	*NAPEPLD*	F.400	AGAGATCACAGCAGCGTTCCAT	95
			R.494	ACTCCAGCTTCTTCAGGGTCATC	
540007	NM_001099102.1	*FAAH*	F.1332	TTCCTGCCAAGCAACATACCT	105
			R.1436	CACGAAATCACCTTTGAAGTTCTG	
515375	NM_001100369.1	*NAAA*	F.223	CAGCACTACGACCGGGACTT	110
			R.322	CCGGGACGACTTTTCTGATC	
505290	XM_581556.5	*MGLL*	F.2	GCAACCAGCTGCTCAACAC	137
			R.138	AGCGTCTTGTCCTGGCTCTT	
281416	NM_174151.1	*POMC*	F.855	CTTGTCACGCTGTTCAAAAACG	101
			R.951	GTCAACTTTCCGCGGAGAGA	
535588	NM_001076063.1	*ABDH5*	F. 1141	CTGCAGATGATGTGGGAAAGC	100
			R.1240	GACTGCCTGGTTCTCGTGTCA	
286879	EF140760.1	*LIPE*	F.1674	TCAGTGTCCAAGACAGAGCCAAT	106
			R.1779	CATGCAGCTTCAGGCTTTTG	
504502	XM_005204534	*CPT2*	F. 149	ATCATTTCTTTCACTCTGCAGAACA	98
			R.203	GGAGGCCTGAGGGAGTCATT	
281495	XM_005201085.1	*SOD1*	F. 256	GGCTGTACCAGTGCAGGTCC	101
			F.356	GCTGTCACATTGCCCAGGT	
508493	NM_001046005.1	*ATGL*	F.675	CACCAGCATCCAGTTCAACCT	102
			R.866	CTGTAGCCCTGTTTGCACATCT	
281759	NM_174314	*FABP4*	F.403	TGGTGCTGGAATGTGTCATGA	101
			R502	TGGAGTTCGATGCAAACGTC	
281496	NM_201527.2	*SOD2*	F.620	TGTGGGAGCATGCTTATTACCTT	95
			R.714	TGCAGTTACATTCTCCCAGTTGA	

[1]Amplicon size in base pair

Table 2.2. (Continued) Gene ID, GenBank accession number, sequence and amplicon size of primers used in adipose tissue

Gene ID	Accession #	Gene	Primers[1]	Primers (5'-3')	bp[1]
506812	XM_005227376	*CPT1A*	F. 141	TCGCGATGGACTTGCTGTATA	100
			R. 240	CGGTCCAGTTTGCGTCTGTA	
615498	NM_001076378	*AQP7*	F.880	ACTGGCATCCTTGTTGTC	104
			R.983	GCGAGGAAGGTGAAGAAG	
282130	NM_174494.2	*ACADVL*	F. 1140	TGCTGCTAACCGTACCCAGTTT	106
			R. 1245	CGCCATGGACTCAGTCAGTCACAT	
513996	NM_001035289.2	*ACOX1*	F.180	ACCCAGACTTCCAGCATGAGA	100
			R.279	TTCCTCATCTTCTGCACCATGA	
282865	BC140488	*ADIPOQ*	F. 214	GATCCAGGTCTTGTTGGTCCTAA	131
			R. 344	GAGCGGTATACATAGGCACTTTCTC	
281536	NM_174198.6	*TLR4*	F.555	TAGTTAAAGCTCAGGTCCAGCATCT	110
			R.664	TAGTTAAAGCTCAGGTCCAGCATCT	
282602	NM_183081	TLR9	F.52	GCCAAGCATCCTTCCCTG	115
			R.166	GCACCAGGAGAGAAAGGG	
282855	NM_174737	PCK1	F. 601	AAGATTGGCATCGAGCTGACA	120
			R. 720	GTGGAGGCACTTGACGAACTC	
82617541	NM_001037319.1	SLC16A1	F. 1701	CCTGTGGGACTGAAGGGTAAAT	110
			R.1813	ATGATTCCCACAGAAATGTCCAGTAT	
		NFE2L2		GGGAATATCAGGAACAAGTGATTGA	
				AGCAGATGATTTGTACTTCGATGACT	
505987	NM_001075236	GK	F. 1193	AAAGCTCCGAGGAAATTGAAAAAC	90
			R. 1282	GTGCATACAGCCCCGAAAAT	

[1]Amplicon size in base pair (bp)

Table 2.3. PCR product sequences of the primers after BLAST with NCBI.

Gene	Sequence
CNR2	CGAAGTTCCTCTTCGCCGGCATCATCTACATGGAAGGCCCATCA GGCATTCCGGAGCCAGCTTGGCTGAGCACCGGGACAGACACCTG TCTGA
NAPEPLD	GCATAGCGTTGATGAGACACCTCCGGGGTGCCTTAGGCCATATT TTATTGATGACCCTGAAGAAGCCTGGAGTACAAT
FAAH	GCGTGCACGGGCAGGGGTGCTGTTCAGTGACGGTGGCACGACCT TCCTACAGAACTTCAAAGGTGATTTCGTGA
NAAA	CTCGTCCAATCATCGGAGAATTATGTCCCGCAGTGGGTCCTTGCA TTGATCAGAAAAGTCGTCCCGGGAC
MGLL	GCTGGAGGCGCTGCCAGCTGAGCTGCCCTTCCTGCTGCTGCAGG GCTCTGCCGACCGCCTCTGTAACAGCAGGGGCGCCTACCTGCTC ATGGAGTCAGCCAAGAGCCAGGACAAGACGCTAAT
POMC	CCTAACGCCACAGAGAGGCCAGTGTGAGGGCGCAGCGGGCAGG GGGGCCTCCTCTCCGCGGAAAAGTTTGAACAAA
ABHD5	CGTCCACCTCTCGCGTATTACCGCTCTGGGACGCAGAGTAAGGG AATCTGACACGAGAACCAGGCAGTTAA
LIPE	AGCAGCCCTGACCCGGCCGGAGGGCTCACTGGGAACCGACTCCC TCAAAAGCCTGAAGCTGCATGAA
CPT2	GCATAGCGTTGATGAGACACCTCCGGGGTGCCTTAGGCCATATT TTATTGATGACCCTGAAGAAGCCTGGAGTACAAT
SOD1	GCGTGCACGGGCAGGGGTGCTGTTCAGTGACGGTGGCACGACCT TCCTACAGAACTTCAAAGGTGATTTCGTGA
SOD2	GCATGTTTGGCCGATTATCTGAGGCCATTTTGGAATGTGATCAAC TGGGAGAATGTAACTGCAATAC
ATGL	GCCTCGCCTTCAGGCCTGTTCCGCCCGAGCCCTGGTNCTTCGAGA GATGTGCAAACAGGGCTACAGAACCC
FABP4	CCCGAGTTATGAGAGAGCGTAGCCAAGGGATATTGAAATGGATG ACGTTTGCATCGAACCTCCAAA
SOD2	GCATGTTTGGCCGATTATCTGAGGCCATTTTGGAATGTGATCAAC TGGGAGAATGTAACTGCAATAC
CPT1A	GGACTATGAAGGTAAACCAGGCCCGGGACGCCCTTCGTACAGGC CTCTCGCTCCAGCTGGCTCATTACAAGGGACCA
AQP7	ATTGTGACTGGCATCCTTGTTGTCATCATCGGAATATCCCTGGGC ATGAACTCAGGATATGCCATCAACCCATCCCGGGACCT
ACADVL	GAAACTAACTTTGTGGCGTATCCAGGAGAAGCGTGCCCGGAAGT GCTATGCTGCAGTATGTGACTGAGAATCCATGGCGA
ACOX1	ATCCTCGTATCCGCGTTCAGGGTGCGTTTAAGAAGAGTGCCATC ATGGTGCAGAAGATGAGGAAATCCCC
ADIPOQ	GGCAGTGGTAACTGGATCACTGGGATCGAGGTCCCCGAGGCTTT CCAGGAACCCCAGGCAGAAAGGGAGAACCTGGAGAAAGTGCCT ATGTATACACGCTCTATG

Table 2.3. (Continued) PCR product sequences of the primers after BLAST with NCBI.

Gene	Sequence
TLR4	GCATCCCTCACCGTTATGGTCAGGTGAATTCCTGGGATAAGGCCAGG CTTCCTCTTGTTGGTTACTTCAGCCAGAAA
TLR9	GGCACGGGAAGTGGGCGCCAAGCATCCTTCCCTGCAGCTGCCTCCC AACCTGCCCGCCAGACCCTCTGGAGAAGCCGCATTCCCTGTCATGGG CCCCTACTGTGCCCCGCACCCCCTTTCTCTCCTGGTGC
PCK1	GCCATGTGTACAGCAGTCGCATCATGACGAGGATGGGCACCAGCGT CCTGGAAGCGCTGGGGGGACGGCGAGTTCGTCAAGTGCCTCCACAAA
SLC16A1	GCACTCGTCACCAATATTCTATGTGGCCTGGGTGATCCTACCAGGTG GGTGCCTCAGGTGCAAATACCTGGACATTTCTGTGGGAATCATATGA A
GK	TACTTCTTATGGCTGCTATTTCGTCCCAGCATTTTCGGGGGCTGTATG CACAAA
TLR4	GCATCCCTCACCGTTATGGTCAGGTGAATTCCTGGGATAAGGCCAGG CTTCCTCTTGTTGGTTACTTCAGCCAGAAA
TLR9	GGCACGGGAAGTGGGCGCCAAGCATCCTTCCCTGCAGCTGCCTCCC AACCTGCCCGCCAGACCCTCTGGAGAAGCCGCATTCCCTGTCATGGG CCCCTACTGTGCCCCGCACCCCCTTTCTCTCCTGGTGC

Table 2.4. qPCR performance of genes measured in adipose tissue.

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
CNR2	27.99	6.68	-3.22	0.99	2.05
NAPEPLD	23.88	2.52	-3.11	0.99	2.10
FAAH	23.57	2.21	-2.94	0.99	2.19
NAAA	25.08	3.57	-3.08	0.99	2.11
MGLL	21.94	0.35	-3.85	0.98	1.82
POMC	22.84	1.41	-3.13	0.99	2.09
ABDH5	25.48	3.84	-3.19	0.99	2.06
LIPE	17.87	-3.61	-3.14	0.99	2.08
CPT2	23.89	2.24	-3.20	0.99	2.05
SOD1	21.50	-0.06	-3.66	0.97	1.88
ATGL	19.30	-2.38	-3.42	0.97	1.96
FABP4	15.12	-6.45	-3.13	0.98	2.09
SOD2	23.17	1.84	-3.56	0.97	1.91
CPT1A	23.61	1.77	-3.11	0.99	2.10
AQP7	23.20	1.56	-3.67	0.99	1.87
ACADVL	22.74	1.13	-3.00	0.99	2.15
ACOX1	22.22	0.66	-3.06	0.97	2.12
ADIPOQ	18.70	-2.65	-3.33	0.99	2.00
TLR4	29.83	8.67	-2.50	0.99	2.51
TLR9	28.46	7.07	-3.02	0.99	2.15
PCK1	27.05	5.30	-3.23	0.99	2.04
SLC16A1	23.73	1.92	-3.19	0.99	2.06
NFE2L2	19.96	-1.47	-3.04	0.98	2.13
GK	26.56	5.10	-3.46	0.99	1.94
RPS9	20.08	--	-3.51	0.97	1.93
UXT	24.23	--	-3.16	0.99	2.07
GAPDH	20.35	--	-3.05	0.98	2.13

[1]The median is calculated considering all time points and all cows.

[2] The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct of 3 internal control genes] for each time point and each cow.

[3] Slope of the standard curve.

[4] R^2 stands for the coefficient of determination of the standard curve.

[5] Efficiency is calculated as [$10^{(-1/slope)}$].

Table 2.5. Least squares mean of gene expression data in transition cows with high (HiBCS) or low (LoBCS) body condition score at -10, 7 and 20 days relative to parturition.

Gene	BCS		SEM[1]	HiBCS			SEM[1]	LoBCS			SEM[1]	*P* value		
	Hi BCS	Lo BCS		-10	7	20		-10	7	20		BCS	day	BCS×day
CNR2	1.44	1.80	0.03	1.23	1.06*	2.05	0.51	0.54	2.91*	1.95	0.53	0.43	0.04	0.01
NAPEPLD	1.11	1.31	0.08	1.03	1.11*	1.21	0.11	0.94	1.48*	1.51	0.11	0.09	<0.01	0.02
NAAA	1.41	1.36	0.08	0.86	1.66	1.72	0.11	0.81	1.61	1.67	0.1	0.64	<0.01	0.99
FAAH	1.28	1.45	0.11	0.97	1.52	1.36	0.14	0.8	1.84	1.71	0.13	0.35	<0.01	0.01
MGLL	1.01[B]	1.78[A]	0.14	1.25	1.02	0.76	0.22	2.15	1.66	1.52	0.21	<0.01	0.05	0.24
POMC	2.47	2.98	0.19	2.87	2.67	1.87	0.29	3.28	3.28	2.39	0.29	0.13	<0.01	0.88
ABDH5	0.90[B]	1.17[A]	0.14	1.03	0.77	0.92	0.26	1.16	1.13	1.23	0.28	0.04	0.86	0.91
LIPE	0.99[B]	1.38[A]	0.14	1.06	1.13	0.75	0.25	1.09	1.86	1.16	0.27	<0.01	<0.01	0.08
ATGL	0.78[B]	1.11[A]	0.10	1.15	0.62	0.58	0.19	1.47	1.08	0.78	0.20	0.02	<0.01	0.25
CPTIA	0.97[A]	0.68[B]	0.06	0.56*	1.21*	1.14	0.11	0.22*	0.86*	0.96	0.11	<0.01	<0.01	<0.01
CPT2	0.96	0.94	0.12	0.88	0.96	1.04	0.12	1.09	0.90	0.82	0.21	0.42	0.05	0.19
ACADVL	0.84[A]	0.51[B]	0.04	0.93*	0.89*	0.69	0.08	0.53*	0.46*	0.54	0.08	<0.01	0.02	<0.01
ACOX1	0.74[A]	0.25[B]	0.04	0.78	0.78	0.67	0.08	0.22	0.28	0.24	0.08	<0.01	0.18	0.59

[A-B] indicate to values with statistical difference ($P \leq 0.05$) among treatments, * Indicate significant difference between groups at the same time points.

[1]Greatest SEM

Table 2.5. (Continued) Least squares means of gene expression data in transition cows with high (HiBCS) or low (LoBCS) body condition score at -10, 7 and 20 days relative to parturition.

Gene	BCS		SEM[1]	HiBCS			SEM[1]	LoBCS			SEM[1]	*P* value		
	Hi BCS	Lo BCS		-10	7	20		-10	7	20		BCS	day	BCS×day
SLC16A	0.99[B]	1.33[A]	0.10	2.06	0.74	0.38	0.20	2.68	0.74	0.56	0.20	<0.01	<0.01	0.68
AQP7	0.38	0.33	0.03	0.70	0.24	0.19	0.05	0.56	0.27	0.18	0.05	0.90	<0.01	0.33
FABP4	1.16	1.36	0.16	0.89	1.30	1.29	0.29	0.86	1.95	1.26	0.30	0.39	0.04	0.44
PCK1	1.13[B]	2.30[A]	0.15	2.79	0.31	0.28	0.31	5.61	0.72	0.57	0.33	<0.01	<0.01	0.63
ADIPOQ	0.89[B]	1.29[A]	0.09	1.19	0.91*	0.57*	0.16	1.29	1.65	0.94	0.16	<0.01	<0.01	0.12
GK	0.69	0.52	0.08	0.24	0.88	0.94	0.15	0.20	0.63	0.71	0.13	0.20	<.001	0.96
NFE2L2	0.76	0.72	0.06	0.73	0.73	0.80	0.10	0.61	0.79	0.77	0.10	0.30	0.13	0.17
SOD2	1.07	1.33	0.25	0.88	0.84*	1.48	0.41	1.09	1.89*	1.01	0.43	0.08	0.19	0.04
SOD1	0.78[B]	0.85[A]	0.10	0.78	0.66	0.88	0.17	0.96	0.86	0.73	0.18	<0.01	<0.01	0.88
TLR4	1.96	0.98	0.23	3.79*	0.57	1.52	0.51	0.59*	1.11	1.25	0.49	0.17	0.20	<0.01
TLR9	1.62[A]	0.75[B]	0.29	0.99	0.85	3.03	0.55	0.32	0.92	1.01	0.50	<0.01	<0.01	0.06

[A-B] indicate to values with statistical difference ($P \leq 0.05$) among treatments, * Indicate significant difference between groups at the same time points.

[1]Greatest SEM

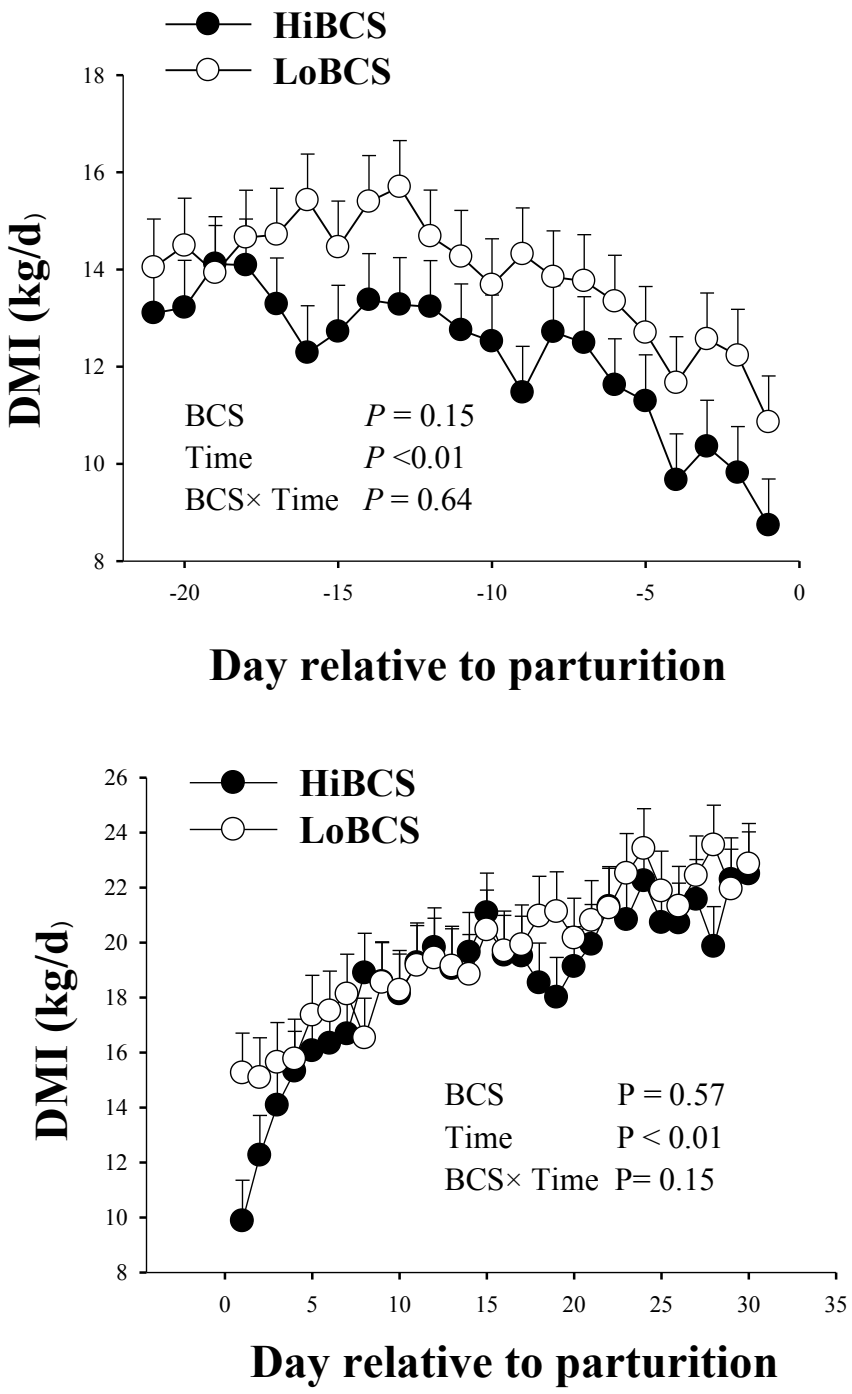

Figure 2.1. Daily dry matter intake (kg/d) between two groups of cows HiBCS and LoBCS during prepartum and postpartum period.

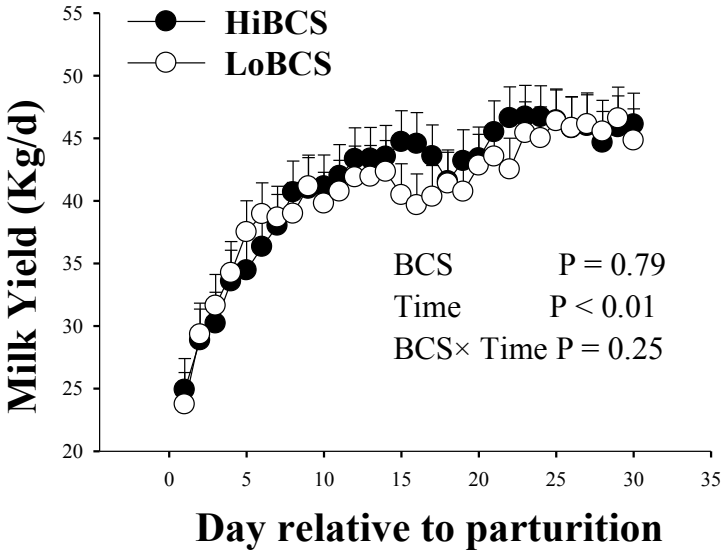

Figure 2.2. Daily milk yield (kg/d) between two groups of cows HiBCS and LoBCS.

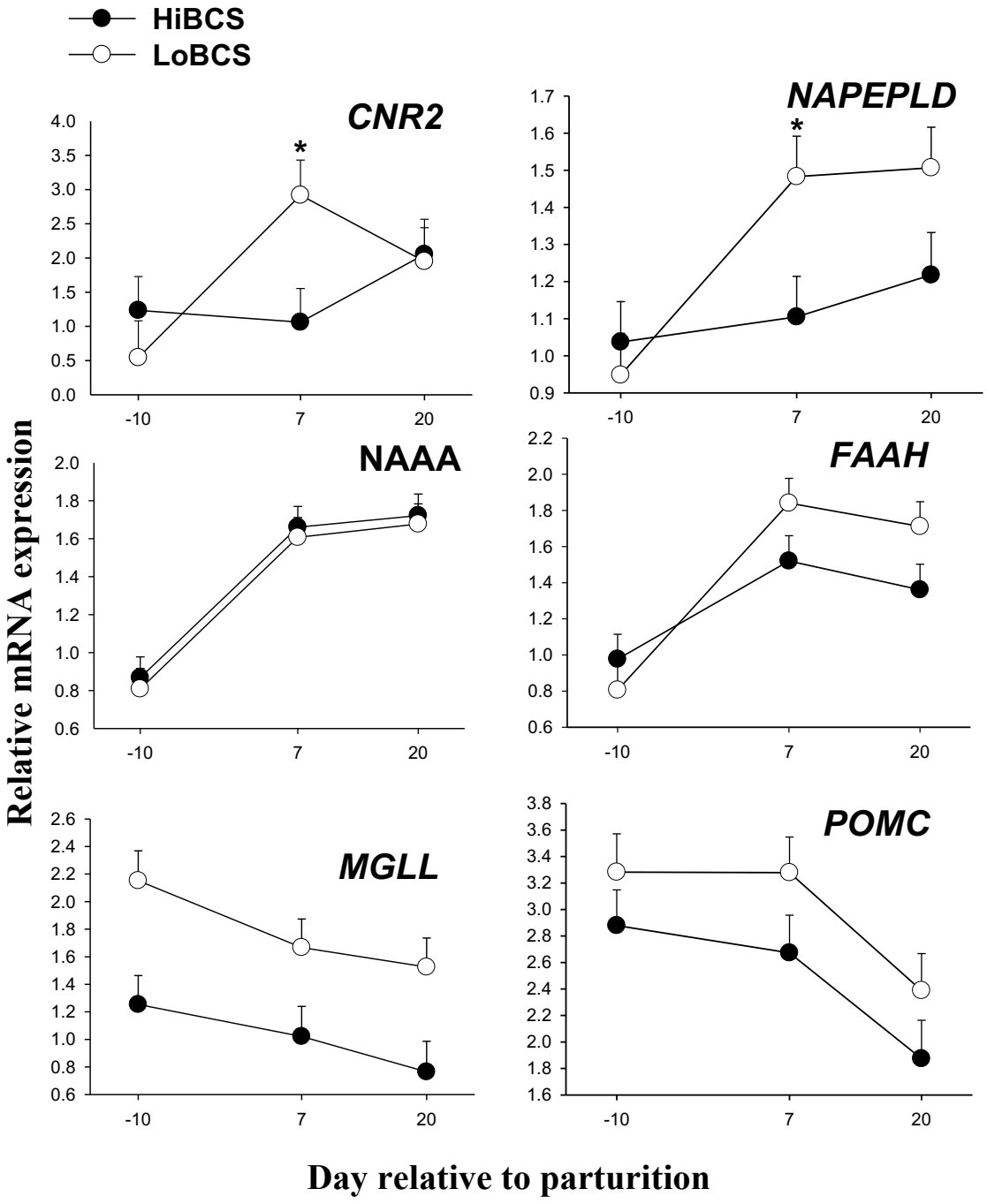

Figure 2.3. mRNA expression (least squares mean ± SEM) of genes involved in the endocannabinoid system in cows with HiBCS or LoBCS at calving * indicates an interaction effect (BCS × time $P \leq 0.05$) at the specific time point.

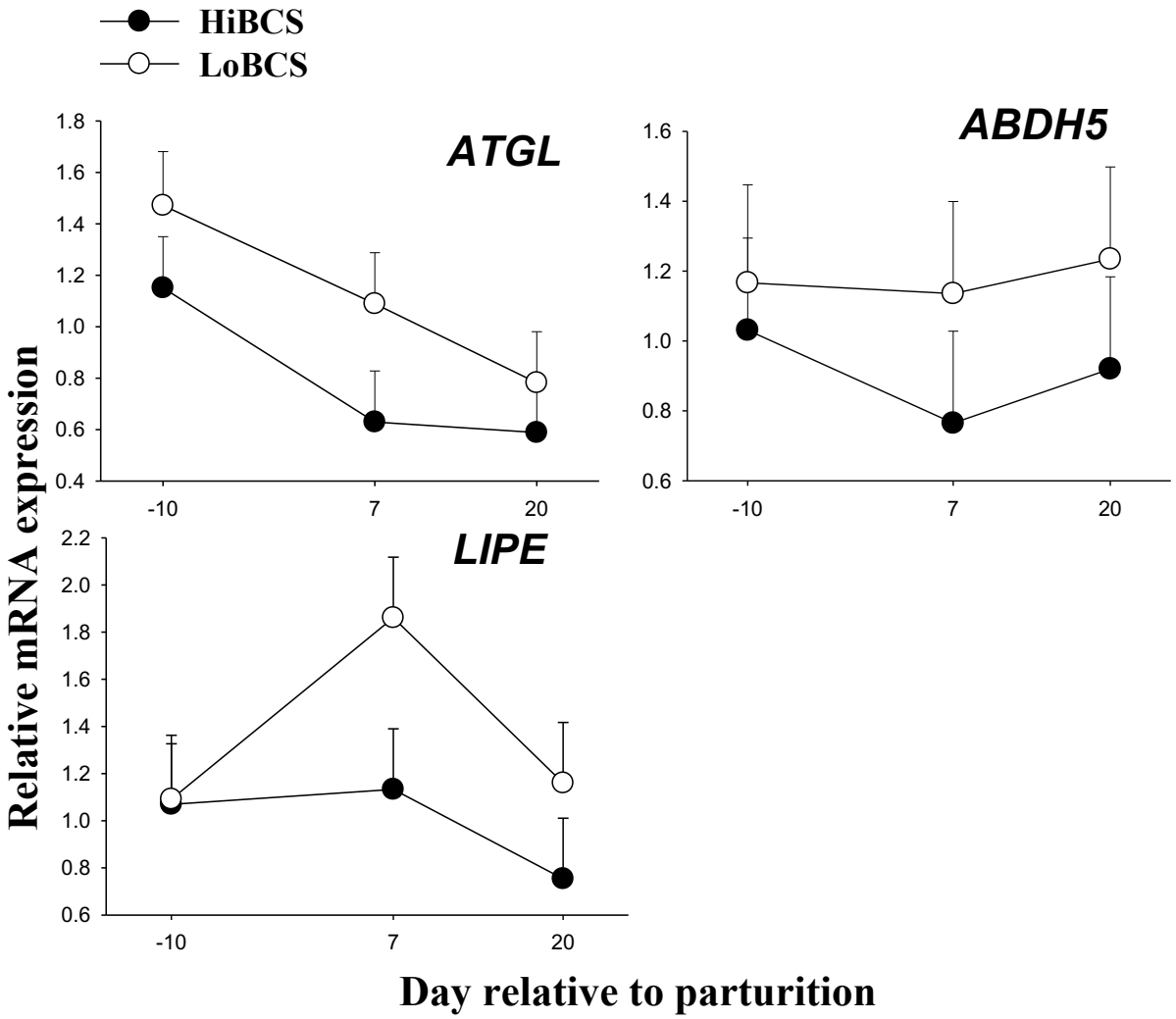

Figure 2.4. mRNA expression (least squares mean ± SEM) of genes involved in lipolysis in cows with HiBCS or LoBCS at calving * indicates an interaction effect (BCS × time $P \leq 0.05$) at the specific time point.

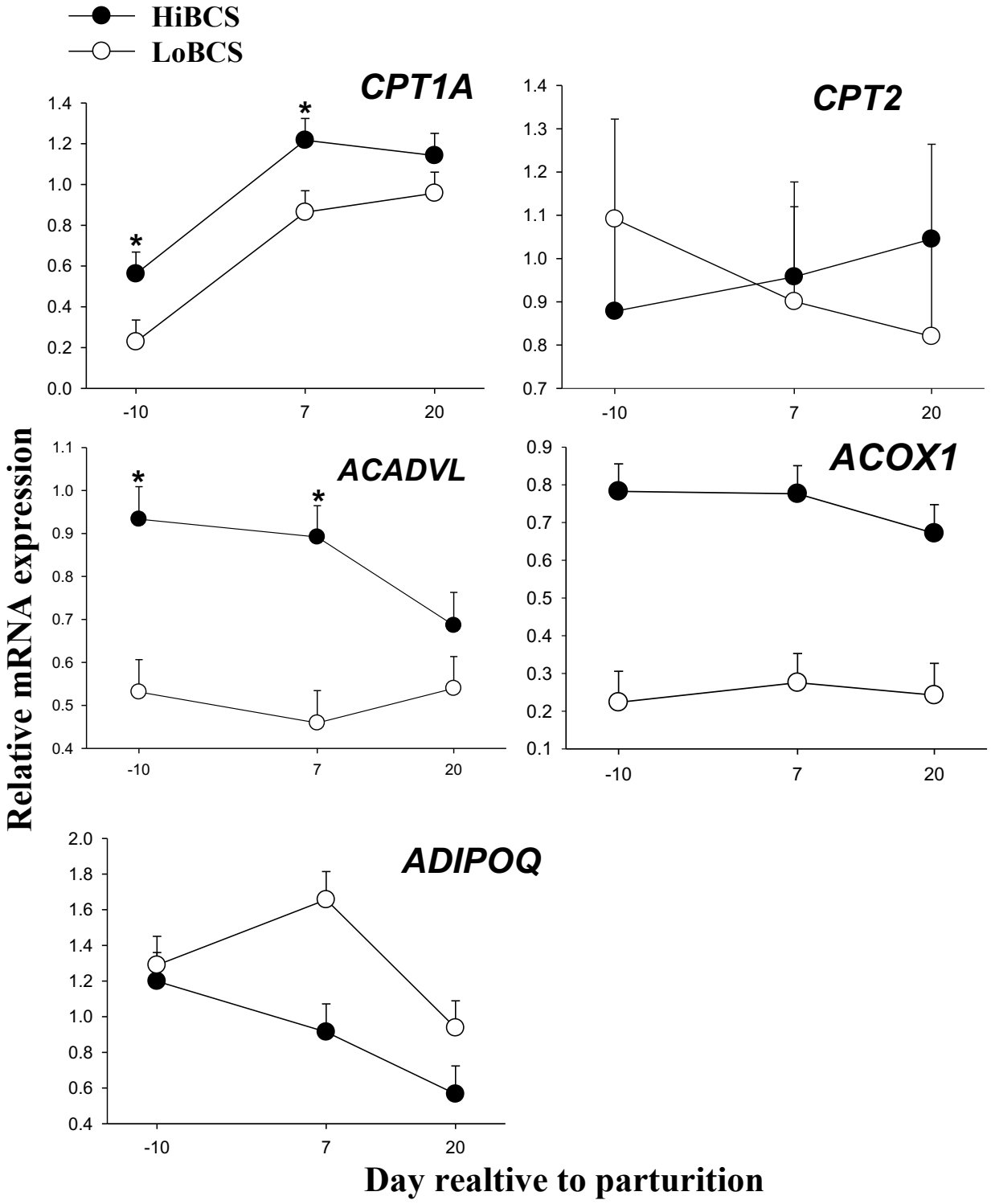

Figure 2.5. mRNA expression (least squares mean ± SEM) of genes involved in fatty acid oxidation in cows with HiBCS or LoBCS at calving.* indicates an interaction effect (BCS × time $P \leq 0.05$) at the specific time point.

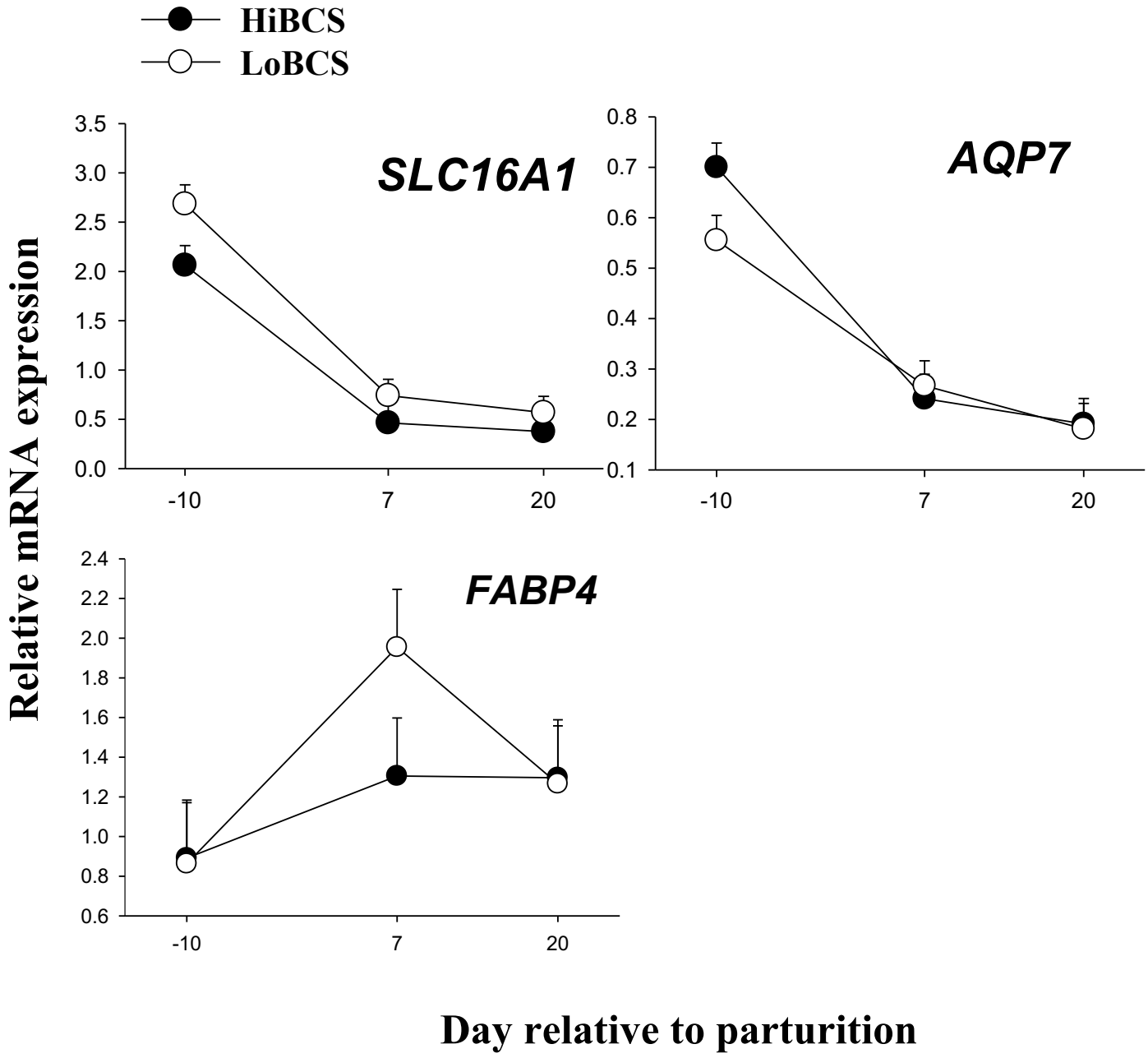

Figure 2.6. mRNA expression (least squares mean ± SEM) of genes involved in fatty acid transporter in cows with HiBCS or LoBCS at calving * indicates an interaction effect (BCS × time $P \leq 0.05$) at the specific time point.

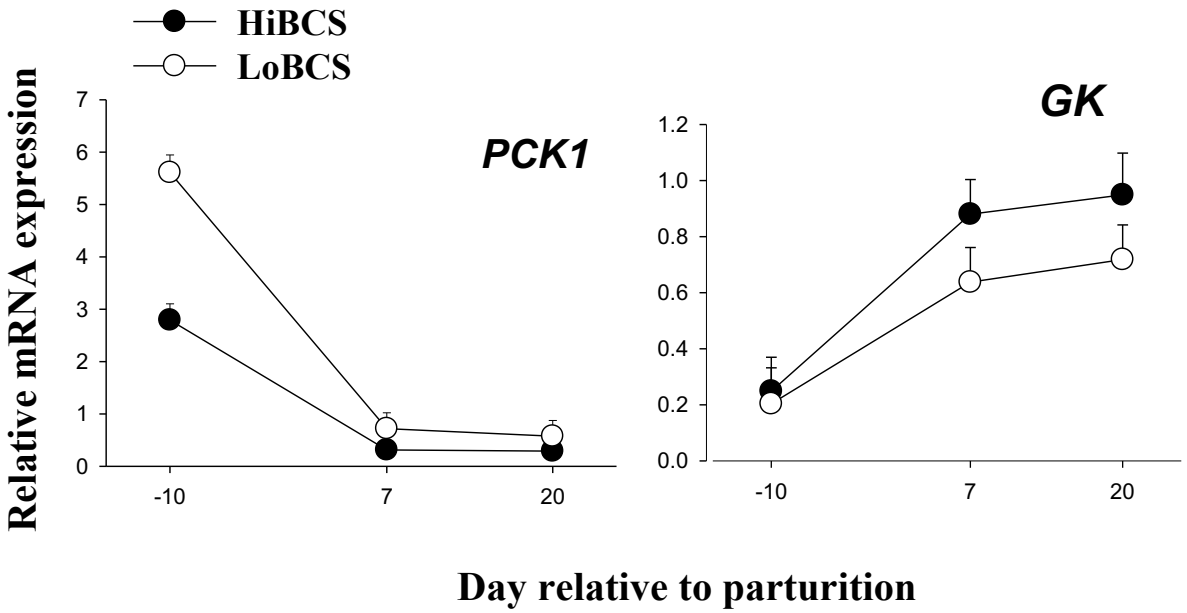

Figure2.7.mRNA expression (least squares means ± SEM) of genes involved in glyceroneogenesis in cows with HiBCS or LoBCS at calvinr* indicates an interaction effect (BCS × time $P \leq 0.05$) at the specific time point.

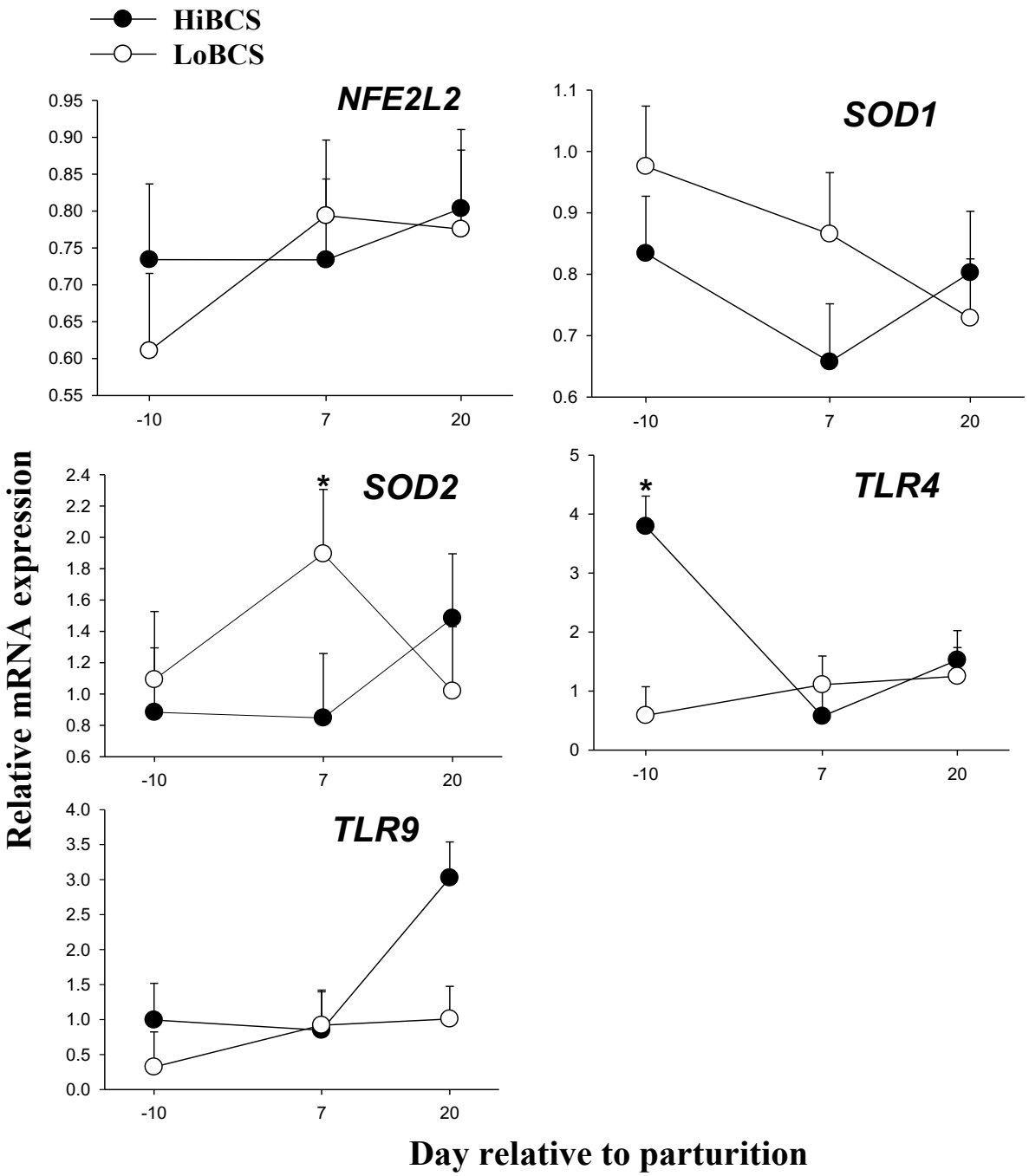

Figure 2.8. mRNA expression (least squares means ± SEM) of genes involved in inflammation and oxidative stress in cows with HiBCS or LoBCS at calving. * indicates an interaction effect (BCS × time $P \leq 0.05$) at the specific time point.

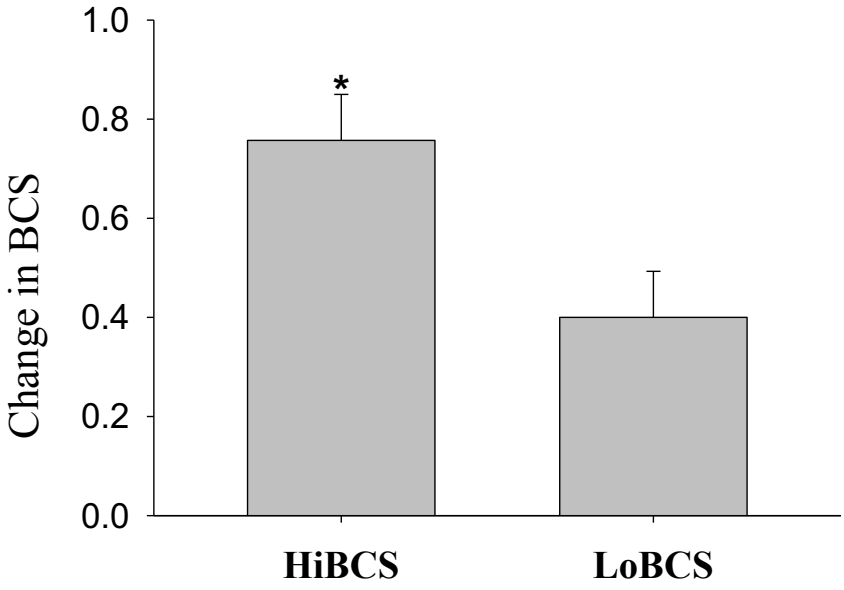

Figure 2.9. Change in BCS between -3 and 3 weeks relative to parturition of HiBCS and LoBCS. * indicates a significant difference between the groups ($P \leq 0.05$).

CHAPTER III

ENDOCANNABINOID NETWORK GENE EXPRESSION IN PERIPARTAL BOVINE LIVER IN RESPONSE TO RUMEN- PROTECTED METHIONINE SUPLEMENTATION

ABSTRACT

Results from our previous work revealed a beneficial effect of rumen-protected Met (MET) supplementation during the transition period on postpartal immune function, inflammation, and cow performance. Endocannabinoids (EC; 2-Arachidonoylglycerol, oleoylethanolamide, and anandamide) are produced upon stimulation of EC receptors expressed in central nervous system and peripheral tissues. These compounds have orexigenic, anorexigenic or pro- and anti-inflammatory properties. Because cows supplemented with rumen-protected methionine (RPM) from -21 through 30 days in milk had a better immune and liver function response postpartum, we sought to examine changes in the expression of EC-related genes in liver. Twenty-two multiparous Holstein cows were fed experimental treatments consisting of a basal control diet (**CON**; n = 11) and CON plus Met (Smartamine M, Adisseo NA) (**MET**; n = 11). All cows received the same far-off diet from -50 to -22 d before expected calving, close-up diet from -21 d to expected calving, and lactation diet from calving through 30 days in milk (DIM). MET supplementation was adjusted daily from -21 d to 30 DIM at a rate of 0.08% (DM basis) of diet DM. The liver was biopsied at -10, 7, 20 and 30 days relative to parturition. RNA was extracted and gene expression was determined via real-time RT-PCR for endocannabinoid receptors (*CNR1, CNR2*), enzymes that synthesize endocannabinoid (*NAPEPLD*), enzymes that degrade endocannabinoid (*FAAH, NAAA, MGLL*), and the hormone precursor proopiomelanocortin

(*POMC)*. A significant difference for a treatment × day effect was observed for the EC receptor *CNR2*, the interaction for *CRN2* was associated with lower expression in MET compared with control cows on d -10. There was an overall greater expression of *FAAH*, *MGLL*, *NAAA* and *NAPEPLD* in MET compared with control cows. Overall, results indicate that alterations in the hepatic EC signaling network in response to MET might be involved in the positive effect on performance and liver function. Additional studies to investigate the mechanism of action of MET on the hepatic endocannabinoid system appear warranted.

INTRODUCTION

Interest in the endocannabinoid system (ECS) in the context of animal physiology has receive increased attention in recent years. The ECS consists of endocannabinoid receptors CNRs (*CNR1* and *CNR2*) which are G - protein coupled receptors that are highly expressed in the central nervous system (CNS), but are also found in peripheral tissues (Rajaraman et al., 2016). The CNR endogenous ligands Anandamide [N -arachidonoylethanolamine (AEA)] and 2-arachidonoyl glycerol (2-AG) have been the most studied (Mechoulam and Parker, 2013). In addition to the endogenous ligand, there are endocannabinoid-like compounds including the anti-inflammatory compound N- plamitoylethanolamaide (PEA) and the appetite suppressor N-olylethanlomaide (OEA). Both have the same structural characteristics of endocannabinoids but do not activate CNRs (Kleberg et al., 2014). The last components of the ECS system are the enzymes involved in the degradation and synthesis of endocannabinoids. The primary enzyme involved in endocannabinoid synthesis is N- acylphosphatidylethanolamine phospholipase D (*NAPEPLD*). Other enzymes involved in the degradation of endocannabinoids are N-acylethanolamine acid amide (*NAAA*), fatty acid amide hydrolase (*FAAH*) and monoglyceride lipase (*MGLL*) (Bari et al., 2011).

Endocannabinoids are synthesized on demand and there is no evidence for endocannabinoid storage (Di Marzo, 2008). The biosynthesis of endocannabinoids occurs through multiple pathways, anandamide synthesis via N-arachidonoyl phosphatidylethanolamine (NAPE), followed by the hydrolysis of NAPE by a phospholipase D (NAPEPLD) (El Manira and Kyriakatos, 2010). The biosynthesis of 2-AG can occur through the hydrolysis of arachidonic acid (Sugiura et al., 2002). Fatty acid amide hydrolase (*FAAH*) is the primary enzyme for the degradation of AEA and OEA. The enzyme MGLL mainly degrades 2-AG, but

also can degrade AEA (Maccarrone et al., 2010). The enzyme NAAA can hydrolyze both AEA and 2-AG (Muccioli, 2010).

The transition period in dairy cows is considered the most important in their life cycle in terms of health and productivity (Drackley, 1999). During this period, energy requirements increase to supply the mammary gland and the fetus (late pregnancy), often causing adjustments in metabolism to restrict the variance between nutrient and energy demand. Furthermore, in addition to negative energy balance, cows also are in negative protein and essential amino acid balance, especially Methionine a methyl donor with important roles beyond protein synthesis.

Methyl donors are essential for many biological processes such as DNA methylation, via the production of S-adenosylmethionine (SAM), as an epigenetic modification involved in the regulation of gene expression in different tissues. In addition to DNA methylation, SAM is also involved in transsulfuration and polyamine biosynthesis (Lu and Mato, 2012; Osorio et al., 2016). Besides the role of ECS in the control of appetite, food intake and energy balance the ECS also is involved in the regulation of immune function (Maccarrone et al., 2010).

Results from previous work (Osorio et al., 2013) revealed a beneficial effect of rumen-protected Met supplementation during the transition period on postpartum immune function, inflammation, and cow performance. Furthermore, the study of Khan et al. (2012) comparing cows overfed energy or fed to requirements during the entire dry period provided evidence of alterations in expression of genes associated with the EC during the transition period. Therefore, the main objective of this study was to determine changes in the genes associated with the EC network and the hormone precursor proopiomelanocortin (*POMC*) during the transition period in cows supplemented with rumen- protected methionine (Zhou et al., 2015).

MATERIALS AND METHODS

Animals and diets

All the procedures for this study were approved by the institutional Animal care and use committee (IACUC) of the University of Illinois (protocol 13023). Twenty-two multiparous Holstein cows were blocked according to parity, previous lactation milk yield, and expected day of calving (Zhou et al., 2015). The cows were fed experimental treatments consisting of basal control (CON) diet (n = 11) and control plus Smartamine M (MET; n = 11) at a rate of 0.08% of DM. All cows received the same far-off diet (1.40 Mcal/ kg of DM, 4.1 % RUP, AND 10.2% RDP) from -50 until -21 d before expected calving, the same close-up diet (1.52 Mcal/kg of DM, 9.1% RDP, and 5.4% RUP) from -21d to expected calving, and the same lactation diet right after calving until 30 DIM (1.71 Mcal/kg of DM, 7.5% RUP and, 9.7% RDP). Methionine was top-dressed from -21 to 30 days in milk one time a day at the AM feeding using around 50 g of ground corn as a carrier of MET. During dry period cows were housed in a ventilated enclosed barn, cows were fed individually once daily at 0630 h using an individual gate system (American Calan Inc., Northwood, NH). Cows had access to sand-bedded free stalls until 3 d before expected parturition, before calving cows moved to individual maternity pens bedded with straw until parturition. After calving, cows were housed in a tie-stall barn and were fed a common lactation diet once daily in the AM (Table 2.1) cows were milked 3 times a day at 6:00, 14:00 and 22:00. The feed was adjusted daily to reach to 10% refusals.

Blood sampling

Blood was sampled from the coccygeal vein on days 1, 4, 7, 14, and 28 relatives to calving. Samples collected into evacuated serum tubes (BD vacationer, BD and CO., Franklin Lakes, NJ) after collocation tubes with lithium heparin were placed on ice until analysis.

Innate immune function assay

Phagocytosis capacity and oxidative burst activity of peripheral monocytes and neutrophils was determined upon challenge with enteropathognic bacteria (*Escherichia coli*0118:H8). Forty µL 100 µM dihydrohodamine 123 (Sigma- Aldrich, St Louis, MO) with 200 µL of blood, and 40 µL of propidium iodine labeled bacteria (10^9 cfu/mL) for 10 min at 38.5 °C. Ice could MilliQ water was used to lysed red blood cells and the cells were re-suspended in PBS solution. Monocytes marked with APC anti- CD14 antibody (cat. NO. 301808; Biolegend, San Diego, CA). Neutrophils were stained with CH138A primary anti- bovine granulocyte monoclonal antibody (Cat. No. BOV2067, Washington State University, WA) and PE-labeled secondary antibody (Cat. No.1020-095, Southern Biotech, AL). Finally, cells re-suspended in PBS solution for flow cytometry analyses (LSR II; Becton Dickinson Dickinson, San Jose, CA).

Liver tissue biopsy

Liver was sampled via puncture biopsy from cows under local anesthesia before morning feeding on days -10, 7, 20 and 30 days relative to parturition. Liver samples was immediately frozen in liquid nitrogen and transferred to - 80 °C freezer for future analysis.

RNA extraction

RNA was extracted from the tissue using protocols established in our laboratory (Loor et al. 2007). Briefly, liver sample was weighed (~0.3-0.5 g) and immediately placed 1.2 ml of ice-cold Qiazol reagent (Qiagen 75842; Qiagen Inc., Valencia, CA) for homogenization. After homogenization, DNase was used to remove any genomic DNA from RNA using RNeasy Mini Kit columns (Qiagen, Hilden, Germany). The concentration was measured using the Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA), and Agilent Bioanalyzer system (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). Used to measure the quality of RNA.

qPCR analysis

The cDNA was synthesized with 100 ng RNA. The RNA was mixed with the Master Mix-1(MM1) containing 9 µL DNase/RNase free water and l µL random primers (Roche® Cat. No. 11 034 731 001, Roche Diagnostics GmbH, Mannheim, Germany), then incubated at 65°C for 5 min and kept in ice for 3 min the reaction was perform using Eppendorf Mastercycler®. 9 µl of Master Mix-2 (MM2) consists of 1.625 µL DNase/RNase free water, 4 µL 5X First-Strand Buffer, 1 µL Oligo dT18, 2 µL 10 mM dNTP mix (10 mM; Cat. No. 18427-088; Invitrogen), 0.25 µL of Revert aid (200 U/µL; Cat. No. EP 0441; Fermentas), and 0.125 µL of RNase inhibitor (20 U/µL; Cat. No. EO 0382; Fermentas). Then incubate (MM1+RNA and MM2) at the following temperature program: 25°C for 5 min, 42°C for 60 min and 70°C for 5 min. An aliquot of undiluted cDNA from all samples was pooled to make samples of standard curve by diluting with DNase/RNase free water, then the cDNA was diluted 1:4 with DNase/RNase free water.

Quantitative PCR was performed using 4 µL diluted cDNA combined with 6 µL of a mixture contain 5 µL 1 × SYBR Green master mix (Applied Biosystems, CA, USA), 0.4 µL each

of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems, CA, USA). Using ABI prism 7900 HT SDS instrument at the following program: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems, CA, USA) used to calculated data. The final data were normalized using the geometric mean of three internal control genes (ICG): UXT, GAPDH and RPS9. The relative mRNA abundance was calculated as previously reported (Bionaz and Loor, 2008) using the median ΔCt (ΔCt = Ct of the gene – geometrical mean Ct of internal control genes) corrected by efficiency (E), where % relative mRNA abundance = $[\ 1/E^{\Delta Ct}\ ] / \sum[1/E^{\Delta C}]$ all measures genes × 100 . The PCR efficiency was calculated for each gene using the standard curve method] $E=10^{(-1/slope)}$] .

Statistical analysis

After the data have been normalized with the geometric mean of the internal control genes, the quantitative PCR data were log2 transformed before statistical analysis to obtain a normal distribution. Statistical analysis was performed with SAS (SAS Institute, Inc., Cary, NC, USA). Normalized data, log2-transformed data, were subjected to ANOVA and analyzed using repeated measures ANOVA with PROC MIXED. The statistical model included Trt (CON and MET), day (Day; −10, 7, 20 and 30 relatives to parturition), and interaction (Trt × Day). Data were considered significant at a $P \leq 0.05$ and tendencies at $P \leq 0.15$.

RESULTS

The relative mRNA abundance of *CNR2* was 2.56% of total mRNA transcripts measured (Figure 3.1). There was a significant difference (Trt × day $P \leq 0.05$) for an effect on the expression of *CNR2* due to higher expression in the CON group at d -10 compared with MET in

which the expression of *CNR2* at -10 and 7 d remained fairly stable. This was followed by a dramatic decrease in expression between d 7 and 20 relative to parturition. The relative mRNA abundance of *FAAH* and *MGLL*, encoding enzymes responsible for the degradation of endocannabinoid-like compounds, was greater than *CNR2* and *NAPELD* which indicated a higher degree of degradation than synthesis of endocannabinoids in liver during transition.

We detected that relative mRNA abundance of *MGLL* accounted for ~59% of total genes measured, which made it the most abundant gene among those measured. Furthermore, we observed a tendency in the expression of *MGLL* (Trt × day $P \leq 0.15$) in cows supplemented with MET than CON. This result was associated with greater expression in MET compared with control cows on d 7. We observed an overall treatment effect for the expression of *MGLL* (Trt $P \leq 0.05$) due to higher expression in cows supplemented with MET than CON.

The relative mRNA abundance of *FAAH* accounted for ~28% of total genes measured in this study, making it the second most abundant gene *FAAH* (Fig 3.1). Although the expression pattern of *FAAH* did not have a significant day or Trt × day, cows supplemented with MET had an overall greater expression of *FAAH* (Trt $P \leq 0.05$, Table 3.5). The expression of *NAAA*, another enzyme that degrades endocannabinoids (Maccarrone et al., 2010), was greater in MET cows than CON (Trt $P \leq 0.05$). Concerning the interaction effects, there was a tendency (Trt × day $P \leq 0.15$) between the two groups of cows at -10 and 7 d with higher level of expression in MET than CON cows (Fig. 3.3). However, there was no interaction for the expression of *NAPEPLD*, but its overall expression was greater in MET-fed cows (Trt $P \leq 0.05$). In terms of time effects, there was a significant difference ($P < 0.01$) between time points in MET-fed cows with expression being at its highest level at -10 d and significantly decreasing at d 20. For CON cows, the expression of this gene experienced a sharp decrease between -10 and 20 d.

There was an irregular pattern of *POMC* expression in MET- and CON-fed cows, even though, there was a tendency (Trt × day $P \leq 0.15$) in term of interactions due to greater expression of *POMC* in MET compared with CON cows at d 7. Furthermore, there was a significant difference between -10 and 7 d owing to the effect of time in MET supplemented cows, the expression of *POMC* increased between -10 and 7 d reaching a peak at d 20 (Fig. 3.3). In terms of time effect in the CON cows, the expression of *POMC* significantly increased from 7 to 20 d relative to parturition.

Blood Phagocytosis and Oxidative burst

A greater increase in neutrophil phagocytosis capability was detected in cows supplemented with MET ($P \leq 0.05$, Table 2.6). Cows supplemented with MET had greater blood neutrophil oxidative burst ($P \leq 0.05$, Table 3.6). Similarly, monocyte oxidative burst was greater in MET than control cows ($P \leq 0.05$, Table 3.6). We observed a tendency ($P = 0.12$, Table 3.6) in monocyte phagocytosis in cows fed MET compared with CON.

Dry Matter intake and milk yield

There was a tendency ($P = 0.11$) for an effect on prepartum DMI due to greater consumption of DMI in cows supplemented with MET than CON. For DMI during postpartum we also observed a tendency ($P = 0.08$) in MET compared with CON cows. Cows supplemented with methionine had a tendency ($P = 0.09$) for greater milk yield compared with CON (Table 3.7).

DISCUSSION

The cannabinoid receptor 1 (*CNR1*) was not expressed in our samples; we also found that the relative mRNA abundance of genes that degrade endocannabinoids was higher than *CNR2*

and *NAPELD*. The relative mRNA abundance of *NAPEPLD* was 1.60 % which represented the lowest value among the genes measured. Those data are similar to a previous study where it was suggested that the degradation of endocannabinoids in peripartal cow liver is a more prevalent process than their synthesis (Khan et al., 2012). Endocannabinoids are not stored in the body, they are produced on demand, and they activate CNRs (Costa, 2016). Data from non-ruminants showed that endocannabinoid signaling can regulate methionine metabolism via sterol regulatory element binding transcription factors (Srebfs) that are downstream of EC signaling in the liver (Liu et al., 2016).

A previous study showed that the endocannabinoid system was up-regulated during fatty liver disease, and also that *CNR2* plays important roles during inflammation (Alswat, 2013). Other data suggested that *CNR1* signaling may promote inflammation (Mukhopadhyay et al., 2010). In our study, the down-regulation of *CNR2* prepartum in cows supplemented with MET may indicate a better immune status. Data from a previous study showed that Met supplementation during the peripartal period might affect lipid metabolism in the liver (Osorio et al., 2013). Another study observed lower liver TAG concentration in cows fed rumen-protected choline (Zom et al., 2011). Lower expression of *CNR2* at -10 d ($P = 0.05$) in MET-supplemented cows could be related to more removal of TAG from the liver. Data from mouse indicated that CNR1 signalling indirectly mediated metabolic steatosis (Mallat and Lotersztajn, 2008). Another study indicated that *CNR2*, not *CNR1*, in bovine liver may be the receptor involved in this signal (Khan et al., 2012).

Fatty acid amide hydrolase (*FAAH*) has the ability to degrade orexigenic (e.g., AEA) and anorexigenic (e.g., OEA) endocannabinoids (Tourino et al., 2010). The endocannabinoid AEA can increase food intake (Maccarrone et al., 2010) and OEA is able to increase adipose lipolysis

and induce satiety (Gomez-Boronat et al., 2016). The endogenous OEA also has analgesic properties by binding to peroxisome proliferator-activated receptor- α (PPAR- α) (Suardiaz et al., 2007). Under normal conditions, the liver in non-ruminant contains a small concentration of the endocannabinoids OEA, AEA, and 2-AG due to the high expression of *FAAH* (Cravatt et al., 2004). The expression of *FAAH* was higher ($P \leq 0.05$) in MET supplemented cows than CON cows suggesting that MET supplementation could have increased the degree of liver tissue OEA degradation. Regarding to a potential link between OEA degradation and feed intake, cows supplemented with MET consumed ($P = 0.11$) 14.03 kg/d whereas CON consumed 12.76 kg/d during the last 21 d prepartum. A greater increase in neutrophil phagocytosis capability, neutrophil oxidative and monocyte oxidative burst in cows supplemented with MET ($P \leq 0.05$) than CON cows indicated a better immune system in MET supplemented cows.

The N-acylethanolamine acid amidase (*NAAA*) is another enzyme involved in the degradation of AEA and PEA (Maccarrone et al., 2010), and it seems to have higher selectivity for PEA than AEA in vitro (Ueda et al., 2013). The endogenous PEA can act as an anti-inflammatory and analgesic compound (Tai et al., 2012). Data from mice showed that the level of *NAAA* in the liver was the lowest among other organs such as lung and spleen (Tai et al., 2012). In the same study, they measure the level of *NAAA* in $FAAH^{-/-}$ mice and there was no significant difference in *NAAA* level between wild-type and $FAAH^{-/-}$ (Tai et al., 2012). However, in our study, the relative mRNA abundance of *NAAA* was ~5.63% of total genes measured which represented the lowest abundance among the genes involved in the degradation of endocannabinoids.

Monoglyceride Lipase (*MGLL*) is an enzyme that inactivates 2-arachidonoylglycerol and converters monoglycerides to fatty acids and glycerol (Guindon and Hohmann, 2009). Data from

previous work indicated that *MGLL* in bovine liver correlated with serum NEFA, which suggested that *MGLL* could control lipidosis (Khan et al., 2012). In non- ruminants, *MGLL* plays a more important role than *FAAH* in the degradation of endocannabinoids (Dinh et al., 2002a). Mice treated with an *MGLL* inhibitor had a higher level of 2-AG in the brain and decreased the level of arachidonic acid (AA) (Nomura et al., 2011). The relative mRNA abundance of *MGLL* accounted for ~59% of total genes measured. The expression of *MGLL* was greater (Trt $P \leq 0.05$) in cows fed MET than CON. A previous study demonstrated the importance of lipotropic agents in the prevention of liver lipid accumulation (Cooke et al., 2007), hence, an increase in the expression of *MGLL* in the MET group could be one such response to a lipotropic agent (i.e. methionine) to clear lipid accumulation from the liver.

Proopiomelanocortin (*POMC*) controls many biological processes in the body. The *POMC* gene produces many different peptides such as melanocyte-stimulating hormones (MSHs), corticotrophin (ACTH) and β-endorphin (Millington, 2007). The deficiency of *POMC* in mice was characterized by early onset of obesity (Challis et al., 2004). Data from rodents showed a relationship between *POMC* neurons and energy balance (De Jonghe et al., 2011). The production rate of endocannabinoids from POMC neurons is similar to other neurons with a lower ability to degrade cannabinoids (Hentges et al., 2005). However, our data did not reveal any treatment effect for the expression of *POMC.* The interaction between treatment and time had a tendency (Trt × day $P \leq 0.15$) due to the greater expression of *POMC* in MET supplementation cows at d 7 than CON cows. Additional research to investigate the mechanisms of *POMC* and endocannabinoids in the bovine liver may help to clarify the importance of these compounds as it relates to rumen-protected methionine, the immune system, and metabolism in dairy cows.

CONCLUSIONS

Results of the present study revealed a relationship between MET supplementation and several components of the endocannabinoid signaling system and proopiomelanocortin in bovine liver. These results raise the possibility that the ECS and POMC in bovine liver may be involved in the positive effect of rumen-protected methionine on hepatic metabolism and the innate immune system and overall cow performance.

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TABLES AND FIGURES

Table 3.1. Ingredients and chemical composition of experimental diets.

	Diet		
Ingredient (% of DM)	Far-off	Close-up	Lactation
Alfalfa silage	12.00	8.34	5.07
Alfalfa hay	-	4.29	2.98
Corn silage	33.00	36.40	33.41
Wheat straw	36.00	15.63	2.98
Cottonseed	-	-	3.58
Wet brewers grains	-	4.29	9.09
Ground shelled corn	4.00	12.86	23.87
Soy hulls	2.00	4.29	4.18
Soybean meal, 48% CP	7.92	2.57	2.39
Expeller soybean meal[1]	-	2.57	5.97
Soychlor[2]	0.15	3.86	-
Blood meal, 85% CP	1.00	-	-
ProVAAl AADvantage[3]	-	0.86	1.50
Urea	0.45	0.30	0.18
Rumen-inert fat[4]	-	-	1.02
Limestone	1.30	1.29	1.31
Salt	0.32	0.30	0.30
Dicalcium phosphate	0.12	0.18	0.30
Magnesium oxide	0.21	0.08	0.12
Magnesium sulfate	0.91	0.99	-
Sodium bicarbonate	-	-	0.79
Potassium carbonate	-	-	0.30
Calcium sulfate	-	-	0.12
Mineral vitamin mix[5]	0.20	0.17	0.18
Vitamin A[6]	0.015	-	-
Vitamin D[7]	0.025	-	-
Vitamin E[8]	0.38	0.39	-
Biotin	-	0.35	0.35

[1]SoyPLUS (West Central Soy, Ralston, IA)
[2]By West Central Soy
[3]Perdue AgSolutions LLC (Ansonia, OH)
[4]Energy Booster 100 (Milk Specialties Global, Eden Prairie, MN)
[5]Contained a minimum of 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5000 mg of Cu/kg, 250 mg of I/kg, 40 mg of Co/kg, 150 mg of Se/kg, 2200 kIU of vitamin A/kg, 660 kIU of vitamin D3/kg, and 7,700 IU of vitamin E/kg.
[6]Contained 30,000 kIU/kg
[7]Contained 5,009 kIU/kg
[8]Contained 44,000 kIU/kg

Table 3.2. Gene ID, GenBank accession number, sequence and amplicon size of primers used in bovine liver.

Gene ID	Accession #	Symbol	Primers	Primers (5'-3')	bp[1]
281087	U77348.1	*CNR1*	F.68 R.172	AACCCCAGCCAGCAGCTT GAGGCTGGAATGGAGGATGA	105
539769	NM_001192303.1	*CNR2*	F.791 R.900	TCTTCGCCGGCATCATCTAC CATCCGGGCTATTCCAGACA	110
540007	NM_001099102.1	*FAAH*	F.1332 R.1436	TTCCTGCCAAGCAACATACC T	105
505290	XM_581556.5	*MGLL*	F.2 R.138	GCAACCAGCTGCTCAACAC AGCGTCTTGTCCTGGCTCTT	137
541291	NM_001015680.1	*NAPEPLD*	F.400 R.494	AGAGATCACAGCAGCGTTCC AT	95
515375	NM_001100369.1	*NAAA*	F.223 R.322	CAGCACTACGACCGGGACTT CCGGGACGACTTTTCTGATC	100
281416	NM_174151.1	*POMC*	F.855 R.951	CTTGTCACGCTGTTCAAAAA CG	97

[1] Amplicon size in base pair (bp).

Table 3.3. PCR product sequences of the primers after BLAST with NCBI

Gene	Sequence
CNR1	GTTTTCGCCAGCTTGCCACGCTGTGCACTTTAAGCGCTTGTTTGGCACCTTCACGGTCCTGGAACCTGCTGGTGCTGTGTCATCCTCCATTCCAGC
CNR2	CGAAGTTCCTCTTCGCCGGCATCATCTACATGGAAGGCCCATCAGGCATTCCGGAGCCAGCTTGGCTGAGCACCGGGACAGACACCTGTCTGGA
FAAH	GCGTGCACGGGCAGGGGTGCTGTTCAGTGACGGTGGCACGACCTTCCTACAGAACTTCAAAGGTGATTTCGTGA
MGLL	GCTGGAGGCGCTGCCAGCTGAGCTGCCCTTCCTGCTGCTGCAGGGCTCTGCCGACCGCCTCTGTAACAGCAGGGGCGCCTACCTGCTCATGGAGTCAGCCAAGAGCCAGGACAAGACGCTAAT
NAPEPLD	GCATAGCGTTGATGAGACACCTCCGGGGTGCCTTAGGCCATATTTTATTGATGACCCTGAAGAAGCCTGGAGTACAAT
NAAA	CTCGTCCAATCATCGGAGAATTATGTCCCGCAGTGGGTCCTTGCATTGATCAGAAAAGTCGTCCCGGGAC
POMC	CCTAACGCCACAGAGAGGCCAGTGTGAGGGCGCAGCGGGCAGGGGGGCCTCCTCTCCGCGGAAAAGTTTGAACAAA

Table 3.4. qPCR performance of genes measured in bovine liver.

Gene	Median	Median	Slope[3]	(R^2)[4]	Efficiency[5]
CNR2	26.85	5.46	-3.68	0.94	1.87
FAAH	22.91	1.54	-3.51	0.96	1.93
MGLL	21.74	0.37	-3.19	0.96	2.06
NAAA	25.08	3.73	-3.27	0.99	2.02
POMC	26.34	4.85	- 3.16	0.99	2.07
NAPEPLD	26.99	5.57	-3.30	0.98	2.01
UXT	24.37	--	-3.39	0.97	1.97
GAPDH	20.01	--	-3.18	0.99	2.07
RPS9	20.09	--	-3.23	0.99	2.04

[1]The median is calculated considering all time points and all cows.

[2] The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct of 3 internal control genes] for each time point and each cow.

[3] Slope of the standard curve.

[4] R^2 stands for the coefficient of determination of the standard curve.

[5] Efficiency is calculated as [$10^{(-1/slope)}$].

Table 3.5. Least squares means of gene expression data in transition cows supplemented with rumen-protected methionine (MET) or control cows (CON).

Gene	Tretment		Control				SEM[1]	Methionine				SEM[1]	*P* value		
	CON	Met	-10	7	20	30		-10	7	20	30		Trt	Day	Trt×day
CNR2	1.04	0.89	1.65*	1.08	0.60	0.84	0.15	1.09*	1.12	0.57	0.82	0.14	0.30	<0.01	0.05
FAAH	0.79[B]	0.93[A]	0.76	0.67	0.87	0.84	0.11	0.95	1.03	0.95	0.80	0.11	0.04	0.91	0.23
MGLL	0.58[B]	0.78[A]	0.41	0.63	0.73	0.57	0.13	0.32	1.17	0.85	0.77	0.12	0.04	<0.01	0.08
NAAA	0.94[B]	1.33[A]	1.42	0.85	0.64	0.87	0.18	1.89	1.72	0.65	1.09	0.17	<0.01	<0.01	0.08
NAPEPLD	0.87[B]	1.07[A]	1.16	0.91	0.55	0.85	0.15	1.47	1.26	0.55	1.01	0.14	0.03	<0.01	0.60
POMC	1.21	1.16	1.09	0.90	1.68	1.18	0.19	0.79	1.46	1.58	0.84	0.19	0.71	<0.01	0.07

[A-B] Mean values with statistical differences ($P \leq 0.05$) between treatments.

* indicates an interaction effect (Trt × day $P \leq 0.05$) at the specific time point.

[1]Greatest SEM.

Table 3.6. The effects of methionine supplementation on blood neutrophil, monocyte phagocytosis, and oxidative burst.

Parameter	Tretment		Control					SEM[2]	Methionine					SEM[2]	*P* value		
	CON	MET	1	4	7	14	28		1	4	7	14	28		T	D	T×d
P_mono[1]	40.5	46.7	39.6	38.1	38.9	40.4	45.5	2.9	48.0	45.0	46.5	47.0	47.0	3.6	0.12	0.26	0.55
P_neutro	50.9[B]	62.2[A]	45.8	49.7	46.2	54.6	58.2	4.1	60.9	62.7	59.0	65.1	63.3	5.1	<0.01	0.19	0.81
O_Mono	17.4[B]	28.1[A]	18.0	19.2	16.4	20.0	13.3	4.7	32.3	22.5	35.2	21.5	29.0	5.7	<0.01	0.71	0.28
O_Neutro	42.7[B]	58.2[A]	41.4	42.1	38.8	44.4	47.0	6.9	57.6	55.6	56.0	56.1	65.9	8.5	0.03	0.69	0.98

[1]P_mono = monocytes phagocytosis; P_neutro = neutrophils phagocytosis; O_mono = monocytes oxidative burst;

O_neutro = neutrophils oxidative burst.

[2]Greatest SEM.

[A-B] Mean values with statistical differences ($P \leq 0.05$) between treatments.

Table 3.7. Diet effects on DMI and milk yield during prepartum and postpartum periods.

Parameter	Treatment		SEM[1]	P- value		
	control	methionine		Treatment	day	T × day
Prepartum						
DMI (kg/d)	12.76	14.03	0.56	0.11	<0.01	0.96
Postpartum						
DMI (kg/d)	17.21	19.33	0.84	0.08	<0.01	0.49
Milk yield (kg/d)	40.99	45.12	1.74	0.09	<0.01	0.71

[1]Greatest SEM.

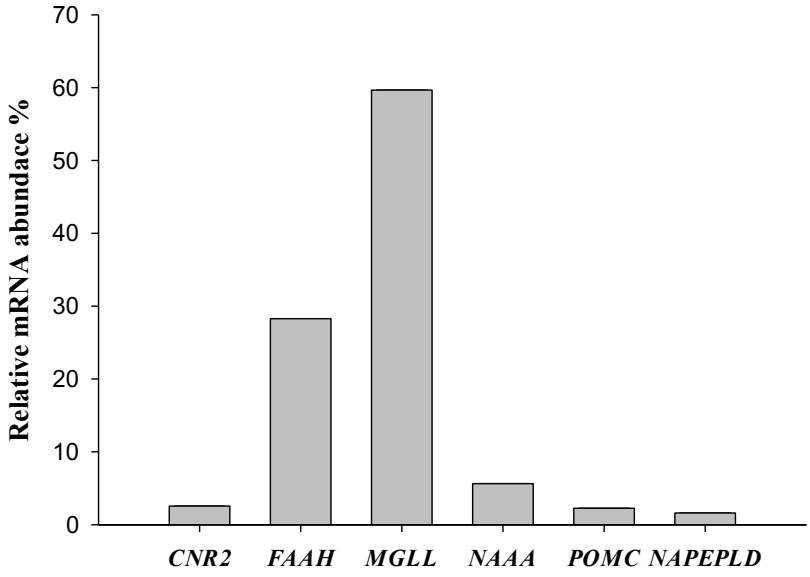

Figure 3.1. mRNA abundance of ECS genes in bovine liver in response to rumen-protected Methionine (MET) or control (CON) diet

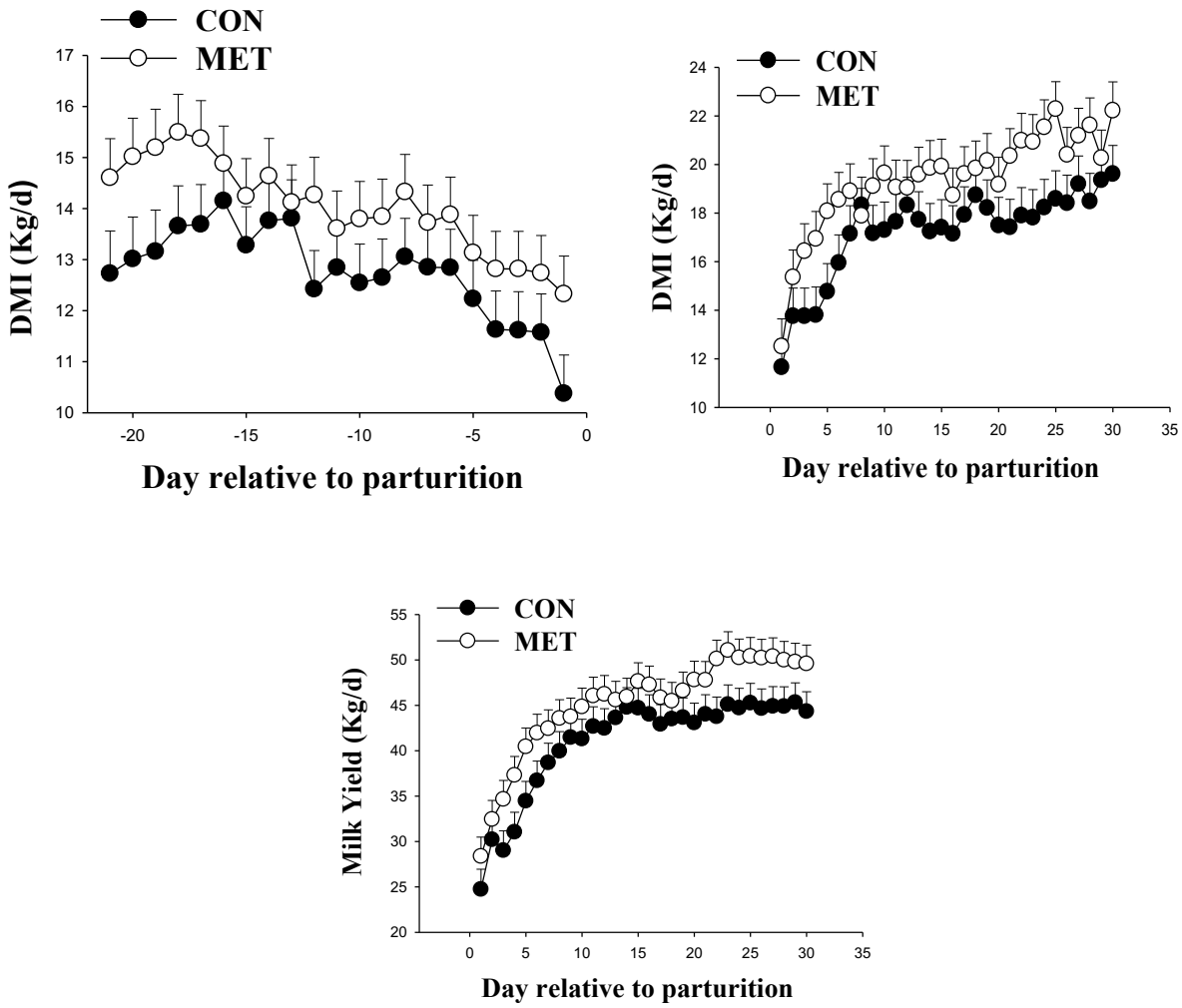

Figure 3.2. Dry matter intake (kg/d) during prerartum and postpartum periods and milk production in response to rumen-protected methionine supplementation (MET) or control (CON) diet.

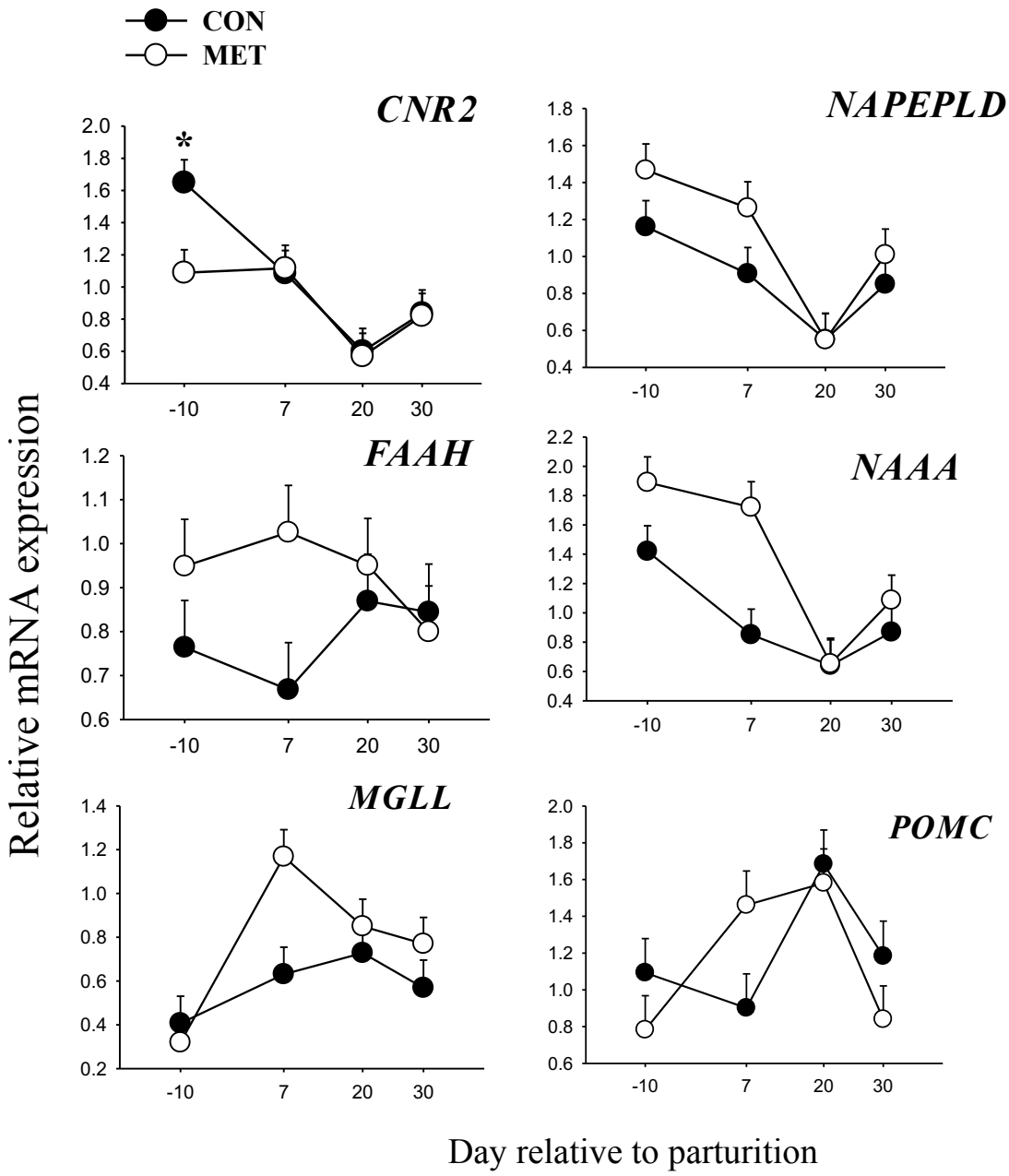

Figure 3.3. mRNA expression of endocannabinoid genes in response to methionine supplementation (MET) or control diet (CON). * indicates an interaction effect (Trt × day $P \leq 0.05$) at the specific time point.