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The Effect of Lactation and Energy Status on Gene Expression in the Main Reproductive Tissues of Lactating Dairy Cattle

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THE EFFECT OF LACTATION AND ENERGY STATUS ON GENE EXPRESSION IN THE MAIN REPRODUCTIVE TISSUES OF LACTATING DAIRY CATTLE

by

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ABSTRACT

The Effect of Lactation and Energy Status on Gene Expression in the Main Reproductive Tissues of Lactating Dairy Cattle

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In recent decades, fertility in dairy cows has declined as cows have been highly selected for milk production. Dairy cows typically experience a state of negative energy balance (NEB) during the first few months of lactation that is associated with hormonal and metabolic fluctuations due to excessive body fat mobilization. However, the significant increase in milk production has augmented the side effects associated with NEB which makes it the major challenge for the modern high-yielding dairy cows during early lactation. Fertility is a multifactorial trait that can be impacted by several factors, through multiple pathways during the cow's reproductive cycle. NEB has an adverse effect on the main reproductive tissues including the oocytes, early embryos, and endometrium. NEB is associated with low glucose and elevated levels of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA) in the circulation. NEB can increase oxidative stress in the intraovarian follicular environment which affects the molecular composition of the oocyte, especially, during final growth and maturation

when the oocyte is very responsive to its surrounding environment. Altering oocyte molecular composition and dysregulation of key genes involved in oocyte developmental competence result in the generation of an embryo with poor quality. Early embryo quality is critical for a successful pregnancy, and most of embryo losses have been shown to occur within the first two weeks of pregnancy during which time the embryo is highly responsive to its surrounding uterine environment. High levels of BHBA and NEFA are detrimental to oocyte and embryo quality and were found to mediate the suppression of immune cells, which predispose the cow to various diseases including uterine infection. NEB promotes a pro-inflammatory uterine environment that is inappropriately suited for supporting early embryo development and negatively affects the overall fertility in dairy cows. We hope this work will help in understanding how lactation and energy status impact the molecular constituents of the main reproductive tissues. Identifying pathways and the key genes impacted by NEB will help in developing profound strategies to improve fertility in dairy cows.

(254 pages)

PUBLIC ABSTRACT

The Effect of Lactation and Energy Status on Gene Expression in the Main Reproductive Tissues of Lactating Dairy Cattle

Sameer Alhojaily

Modern high-yielding dairy cows are currently producing far more milk than their ancestors due to a prolonged and intensive genetic selection for milk production trait accompanied by the revolutionary improvement in technology, management, and nutrition. On the other hand, a noticeable decline in fertility and reproductive performance was undeniably consistent with the increase in milk yield. This decline in fertility and reproductive performance are recognized worldwide and well documented in several studies. Dairy cows typically experience a period of energy deficit during the first few months of lactation due to the rapid increase in milk production and limited feed intake. This shortage of energy requirements results in loss of body fat which is associated with the disturbance of the normal levels of certain hormones and metabolites. The significant increase in milk yield has increased the severity and duration of the energy deficit which has an adverse effect on the main reproductive cells and tissues that profoundly contribute to fertility. These include the egg from the ovary, the early embryo, and the internal lining of the uterus. Fertilization of a healthy egg results in the development of an embryo with an excellent quality that can survive through the multiple stages of gestation, especially during the first two weeks of gestation when many embryos die. The embryos in the early stages are the most susceptible to the disturbance in their environment. Energy deficit was shown to negatively impact the egg and embryo

quality and make the uterus lining suboptimal to support early embryo development. Understanding the mechanisms by which energy deficit influences the main reproductive tissues will help in developing profound strategies to improve fertility in dairy cows.

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Hours, days, and years are the units we use them to measure the time in our lives. However, it is not simply a matter of time counting, it's a matter of how much we progress and learn through our lives. I count my life by the progress I achieve and the knowledge I acquire. Pursuing a PhD degree was a great learning experience that is underlying many prominent changes and a major step accounting for many steps in my life. I am taking this opportunity to express my profound gratitude to everyone who walked me through these steps, especially my major professor, Dr. S. Clay Isom, for his exemplary guidance, monitoring, and constant encouragement to complete the PhD program. The helpful tips and sincere guidance given by him time to time shall help me walk my first steps in the journey of science and research on which I am about to embark. I want to thank my committee members, Dr. Allen Young, Dr. John Stevens, Dr. Irina Polejaeva, and Dr. Heloisa Rutigliano for their great support and continues help through the entire process. Also, I would like to express my gratitude and sincere appreciation to Dr. Rusty Stott and Sarah Pierce for their help in collecting all of our samples and to the staff and everyone at the USU dairy farm for their help in the management and transportation of the animals. Moreover, I want to thank my lovely wife and sweet daughters for being in my life and for inspiring me to succeed and make the best use of my time. At the end, I want to thank my home country, Saudi Arabia, for giving me this scholarship with the generous support to continue my education.

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CHAPTER 1

REVIEW OF LITERATURE

Introduction

The dairy industry has evolved fundamentally worldwide in many aspects due to the enormous increase in knowledge and usage of technology in dairy nutrition, health, genetics, and management. This allowed expansion of dairy production and increased herd size and milk yield up to 4-fold between 1944 and 2007 (Capper et al., 2009). Producing large amounts of milk efficiently to supply the unceasing and growing demand for milk and dairy products is a necessity especially with the growing populations. Producing milk efficiently means the intensification of the dairy industry in such a way to increase milk yield per cow, optimizing feed efficiency, and lowering management costs. The significant improvement in the efficiency of the dairy industry has allowed farms now to produce more milk with fewer cows which is critical nowadays with the limited land for agriculture, rapid urbanization, and increased concerns of the greenhouse gas emissions (von Keyserlingk et al., 2013). At the same time, it raises concerns of animal welfare since it puts enormous stress on the animals at the cost of their health (von Keyserlingk et al., 2013).

The primary objective of dairy farms is to produce a sufficient amount of milk suitable for human consumption. However, milk production is strongly associated with reproductive performance and fertility. Several studies conclude that efficient reproductive performance improves profitability and increased net returns (Butler, 2003; Giordano et al., 2011; Cabrera, 2012; Kalantari and Cabrera, 2012; Galvao et al., 2013).

The ability of the cow to become pregnant is the initiator of a new lactation cycle. Fertility is the ability to conceive and maintain pregnancy by producing an ovum capable of being fertilized and having a uterus capable of supporting the pregnancy to term (Pryce et al., 2004).

The increased demand for milk production and modernization of the dairy industry has created many new challenges that affect fertility and caused a decline in reproductive efficiency in modern dairy cows. There is no single factor identified that causes decline in fertility, but it seems to be a combination of a variety of physiological and management factors since fertility is a multifactorial trait that can be impacted from several aspects. However, the biggest challenge for dairy cows is that they are inseminated and expected to be pregnant while they are at the peak of their milk production. This overlap between gestation and lactation amplifies the physiological stress on dairy cows and shorten the time to restore energy and rest before the next lactation.

In the last two decades, the decline in fertility and impaired reproductive efficiency of dairy cattle gained attention, and it is well documented in several studies worldwide (Macmillan et al., 1996; Butler, 1998; Roche et al., 2000; Royal et al., 2000; Lucy, 2001; Lopez-Gatius et al., 2003; Leroy et al.; 2008b). Conception rate at observed estrus declined in dairy cows from 55% in 1960 (Casida, 1961) to approximately 35% four decades later (Schmitt et al., 1996; Pursley et al., 1997a; Pursley et al., 1997b; Dransfield et al., 1998; Pursley et al.; 1998). The number of services per conception in Holstein and Jersey cows increased from 1.62 to 2.91 between 1972 to 1996 based on data obtained from the Dairy Records Management System (DRMS) from 561 herds

from ten different states (Washburn et al., 2002). Until the beginning of this century, the reported conception rate in dairy cows was 39% while the conception rate of dairy heifers with similar genetic merit was 64% and has not been affected by the genetic selection for milk production (Pryce et al., 2004). This decline in conception rate in lactating cows suggests the indirect adverse effect of milk production that may heavily contribute to the reduced reproductive performance. Recent incorporation of reproductive traits in addition to milk production traits to modern genetic selection indices has reversed the decline. However, it did not improve it to the level it used to be (Lucy, 2019).

There is obviously an opposed association between milk production and reproduction in dairy cattle (Dematawewa and Berger, 1998; Hansen, 2000), but there is no major evidence suggesting a direct adverse effect of increased milk production on fertility when compared with other factors as management, season, and diseases (Grohn and Rajala-Schultz, 2000). Milk production is not necessarily to be the causative effect of the decline in fertility. Nevertheless, it can be a consequence of milk production. One of the main consequences of milk production is nutrient partitioning toward milk production and excessive mobilization of fat from adipose tissue when the cows can not meet the energetic demand solely from feed intake, which puts them in a state of negative energy balance (NEB). Bauman and Currie suggested a clear distinction between high milk production and NEB, as most cows undergo a normal process of weight loss, and decreased body condition score (BCS) that occur during early lactation (Bauman and Currie, 1980). However, the severity of NEB is the key when the increased milk production exceeds the cow's ability to maintain the ideal balance between basic physiological functions and lactation (Leroy et al., 2008b; Esposito et al., 2014).

However, cows vary in response to NEB, and several factors including BCS, weight, genetics, milk yield, and days in milk (DIM) influence the amount and the composition of fat they can mobilize as (Tamminga et al., 1997).

Until the early 2000s, dairy genetic selection programs were mainly focused on increasing milk yield per cow with less consideration to reproductive performance and fertility traits (Berry et al., 2014). Genetic selection and improved nutrition have increased milk yield per cow, but pregnancy rates after insemination have been declined by 0.45 – 1 % every year (Butler, 2003; Royal et al., 2016). Selection for high milk production is associated with excessive ability to mobilize fat from adipose tissue during early lactation to provide energy for the increasing milk yield.

Mobilization of fat during early lactation is a normal physiological process due to the rapid increase in milk production with limited feed intake capacity, which results in dramatic fluctuations in circulating levels of a variety of metabolites, and most cows can manage this metabolic change. However, when this metabolic change exceeds cow's capacity to maintain basic physiological functions or when the metabolic load defined as "the total energy burden imposed by the synthesis and secretion of milk" become too great, it causes a sort of metabolic stress (Crowe et al., 2018; Knight et al., 1999). Based on the Knight et al. (1999) definition of metabolic stress it is "the amount of metabolic load that cannot be sustained by this mobilization, leading to the down-regulation of some energetic processes, including those that maintain general health". This suggests that excessive fat mobilization is the crucial element influencing overall health and reproductive performance in dairy cows.

Fat mobilization is not initiated by milk production; it actually starts pre-calving and adds to the stress during calving which in turn, aggravates the process (Sheehy et al., 2017). Even though NEB and loss in body weight start before calving, cows do not reach maximum feed intake to compensate for the lost body reserves and increased energy demand for milk production until 42 to 56 DIM on average (Tamminga et al., 1997). The highest amount of body fat reserve mobilization due to NEB takes place in the first week after parturition, and it may take several weeks to recover (Tamminga et al., 1997). The duration and intensity of NEB vary according to genetic, milk production, dry matter intake, pre-calving body BCS, nutrition, and management (Grummer, 1995).

The increased milk yield in modern dairy cows intensifies the impact of NEB, which places the dairy cows under a considerable amount of metabolic stress in early lactation. It is important to understand the direct and indirect effect of NEB due to increased milk yield on fertility, especially during early lactation when the cows reach the peak of milk production and at the same time are expected to be bred and conceive. Understanding pathways in which NEB and lactation contribute to decreased fertility in modern dairy cows may help to develop strategies to improve reproductive performance and alleviate the impact of NEB on fertility. Several traits and tissues contribute to the overall fertility and can be impaired by multiple mechanisms. Nevertheless, a regular estrous cycle, normal ovarian function, ovulation of a competent oocyte, development of a quality embryo, and optimal uterine environment to support early development and embryo attachment are the key elements for a successful pregnancy which is the ultimate objective.

Hormonal Regulation of the Estrous Cycle and Resumption of Postpartum Ovarian Cyclicity

Reproductive performance in dairy cows is evaluated by their ability to conceive and maintain pregnancy. Normal ovarian cyclicity and showing sufficient estrous signs are essential for the cow to be inseminated. Cows are polyestrous which means they regularly go through estrus and ovulate several times during the year. The average duration of the estrous cycle in dairy cows is 21 days, and they exhibit estrous behavior for 6 to 24 hours (Senger, 2012). The cows estrous cycle is regulated by the hypothalamic-pituitary-gonadal (HPG) axis which includes gonadotrophin-releasing hormone (GnRH) from the hypothalamus, follicle-stimulating hormone (FSH) from the anterior pituitary, luteinizing hormone (LH) from the anterior pituitary, progesterone (P4) from the corpus luteum (CL) in the ovaries, estradiol (E2) from ovarian follicles, prostaglandin F2α (PGF2α) from the uterus, and activin and inhibin from the ovaries (Senger, 2012).

Reproductive hormones regulate the estrous cycle by positive and negative feedback mechanisms (Forde et al., 2011a). Gonadotrophin-releasing hormone regulates the estrous cycle by controlling the release of gonadotropic hormones, LH and FSH from the anterior pituitary. The pulsatile secretion of GnRH from the tonic center in the hypothalamus maintains the basal level of GnRH during the estrous cycle, and positive feedback of estrogen when it reaches a threshold level stimulates the pre-ovulatory surge of GnRH from the surge center. Follicle-stimulating hormone induces recruitment of gonadotropin sensitive follicles that begin to produce E2 and inhibin which has a negative feedback effect on the anterior pituitary to inhibit FSH release. The dominant

follicle continues to grow and produce E2 that eventually has a positive feedback effect on the surge center in the hypothalamus, resulting in a pre-ovulatory surge of GnRH and a corresponding sharp increase in LH that leads to ovulation (Senger, 2012). Ovulation usually occurs within 17 to 20 hours after LH surge and approximately around 26 hours after the onset of estrus (Mondal et al., 2006).

At the site of ovulation, luteal tissue begins to grow from the collapsed ovulated follicle where granulosa and theca cells luteinize and form the CL. During the luteal phase, the CL becomes the dominant ovarian structure and synthesizes and secretes copious amounts of P4 (Forde et al., 2011a). PGF2α from the endometrium causes luteolysis of the CL and accordingly removes the negative feedback effect of P4 on the GnRH neurons and allow a new estrous cycle to begin with a new follicular phase. In the case of pregnancy, CL must be maintained to continue producing P4 which is essential for the maintenance of pregnancy. Therefore, maternal recognition of pregnancy must occur before luteolysis of the CL. Maternal recognition of pregnancy is initiated by the developing free-floating blastocyst that produces trophoblast protein-1 known as bovine Interferon- tau (bIFN- τ). Bovine IFN- τ inhibit the synthesis of oxytocin receptors in the endometrium that mediate the release of PGF2α (Senger, 2012).

Follicles develop in wave-like patterns during estrous cycles, and cattle typically have 2 or 3 follicular waves before ovulation. Follicular waves occur even in prepubertal heifers and postpartum cows without ovulation. Follicular waves occur during pregnancy and continue regularly with 7 to 10-day intervals. However, in late pregnancy and especially in the last 22 days, the high level of progestogen from the CL and placenta inhibits recruitment and growth of new follicles by suppressing FSH (Crowe, 2008).

Resumption of follicular waves occurs immediately at the time of parturition when P4 and estrogen concentrations sharply decline to the basal level, thus removing the negative feedback effect on GnRH and allowing the increase in FSH level within 3 to 5 days postpartum (Crowe, 2008). Resumption of follicular waves in cattle typically generates a dominant follicle within 7-10 days after calving. However, the fate of this dominant follicle depends on its ability to produce enough E2 to stimulate the LH surge and induce ovulation. The amount of E2 depends on the follicle size, and generally, the first postpartum dominant follicle is small and does not secrete sufficient amount of E2 to induce ovulation and instead, undergoes atresia (Crowe, 2008).

First postpartum ovulation usually occurs without display of estrous behavior and is generally followed by a short cycle of one follicular wave (Kyle et al., 1992). The fact that high expression of oxytocin receptors in the endometrial tissue after parturition induces premature PGF2α production and premature luteolysis of the CL around day 8-10 of the cycle (Zollers et al., 1993). The estrous signs are exhibited normally with the second postpartum ovulation after the short cycle, and followed by normal estrous cycle length (Crowe, 2008).

The ovaries of cycling lactating cows and heifers normally can have up to four follicular waves before ovulation during the estrous cycles. However, lactating dairy cows more often have two follicular waves (Sartori et al., 2004). The level of pulsatile release of LH determines the duration of the dominant follicle in the cycling animals. Progesterone suppresses LH, and lactating dairy cows generally have a lower P4 level when compared with non-lactating cycling heifers, which allows a slight increase in pulse frequency of LH resulting in increased persistence of the dominant follicle instead of

undergoing atresia (Sartori et al., 2004). The duration of the dominant follicle determines the number of follicular waves. Austin et al. (1999) compared the effect of different duration of the dominant ovulatory follicle in heifers and observed higher conception rate in animals with shorter durations of the dominant ovulatory follicles (Austin et al., 1999). This may explain the difference in conception rate between heifers and lactating cows, which tend to have a higher metabolic clearance rate of progesterone, increased duration of the dominant follicle, and fewer follicular waves during the estrous cycle.

Folliculogenesis and Oocyte Development

The growth and development of the ovarian follicles and oocytes can be tracked to early fetal development when primordial germs cells migrate to the genital ridge and form clusters or nests of germ cells. The formed nest contains a number of germ cells called oogonia at this stage, surrounded by somatic cells that later differentiate to become granulosa cells. Oogonia undergo mitosis with incomplete cytokinesis to form a nest of multi-nucleated germ cell interconnected by cytoplasmic bridges (Aerts and Bols, 2010a). After going through a number of mitotic divisions, oogonia enter the first meiotic division and immediately arrest in the diplotene stage of prophase I and become the primary oocytes. Simultaneously, primordial follicles are formed by a single layer of flat granulosa cell precursors containing individual primary oocyte and form the initial follicle pool (Aerts and Bols, 2010a). Approximately at day 90 of gestation in cattle, developed primordial follicles start to occur (Yang and Fortune, 2008).

The majority of primordial germ cells fail to develop into primary oocytes at this stage and undergo apoptosis during multiple rounds of mitotic divisions. In cattle, apoptosis reduces the initial estimated number of primordial germ cells from 2,100,000 to

130,000 at birth (Aerts and Bols, 2010a). Similarly, a large number of formed primordial follicles undergo atresia while the remaining follicles become dormant until the animal starts cycling. The remaining follicle pools establish the ovarian reserve for oocytes and determine the reproductive lifespan of the female (Tingen et al., 2009). These primordial follicles are activated for development at puberty in a wave-like pattern during each ovarian cycle while the rest remain quiescent. With each wave, a number of primordial follicles are triggered to develop by the effect of gonadotropin hormones to enter the growing pool of follicles and become the primary, secondary and tertiary follicles (Tingen et al., 2009).

The transition of primordial follicles is marked by histological and physiological changes. In the primary follicle, granulosa cells continue to grow and isolate the follicle from the ovarian stromal tissue and form a single layer of cuboidal epithelium. The secondary follicles have more follicular cells, follicular fluid starts to accumulate in the intracellular spaces to form an antrum, and a thick layer of glycoprotein layer coats the oocyte to form the zona pellucida. The tertiary follicle is characterized by the accumulation of fluid between the granulosa cells and formation of a large antral cavity, multiple layers of granulosa cells, theca interna, a basal lamina, and a theca externa. The granulosa cells that directly surround oocyte are called cumulus cells, and with the oocyte form the cumulus oocyte complex (COC) (Rimon-Dahari et al., 2016).

Mechanisms associated with primordial follicle activation to enter the growing pool of follicles remain to be elucidated. However, it seems the oocyte plays a central role in determining the capacity of the primordial follicle to be activated through the PTEN-PI3K pathway. Knockout of Pten, a suppressor of PI3K, in mice oocytes results in

activation of all primordial follicles (Reddy et al., 2008). Other factors produced by the oocyte as growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (*BMP15*) are also important for primordial follicle growth and activation (Aerts and Bols, 2010a).

The oocyte communicates with the surrounding follicular cells, granulosa cells, through the gap junctions between them, and helps to regulate the proliferation and differentiation of the granulosa cells. Anti-Mullerian hormone (AMH) produced by the granulosa cells regulates the number of primordial follicles to be activated and maintain the balance between dormant primordial follicles and recruited follicles. AMH has a suppressive effect on primordial follicle activation and the deletion of AMH in mice resulted in faster depletion of the primordial follicles, as a result of increased follicular recruitment (Aerts and Bols, 2010a). Once the primordial follicle is activated and developed to the antral follicle stage, it becomes responsive to and dependent on gonadotropins FSH and LH for further development. Follicle-stimulating hormone stimulates proliferation of granulosa cells, production of E2 and expression of LH receptor. Luteinizing hormone is essential for oocyte maturation and ovulation (Rimon-Dahari et al., 2016).

Development of the antral follicle can be divided into three main phases: recruitment, selection, and dominance. Recruitment depends on the high level of FSH in the circulation, and typically 5-10 follicles in the cow are recruited but the number can be up to 24 follicles (Aerts and Bols, 2010b). The recruited follicles undergo a process of selection where typically in monovulatory animals one follicle is selected to develop into a dominant follicle while the remaining undergo atresia. The dominant follicle produces

more estrogen than other follicles and induces more negative feedback effect on FSH release in the circulation. The dominant follicle has more granulosa cells that express anti-apoptotic factors to enable survival in the absence of FSH and expresses LH receptors (Rimon-Dahari et al., 2016). High levels of E2 from the dominant follicle trigger the preovulatory LH surge which stimulates the oocyte to resume meiosis and induces ovulation. After the release of the COC, the cells from the ruptured follicular undergo luteinization and form corpus luteum and start to produce P4 instead of E2.

Oocyte Resumption of Meiosis

During folliculogenesis, oocytes grow in size while remain arrested in the prophase of the first meiotic division at the germinal vesicle (GV) stage. For the oocyte, being quiescent and arrested at a specific phase of cell division does not silence transcription and other intracellular activities. Actually, the oocyte is transcriptionally active and accumulates mRNA, synthesizes protein and organelles, stores lipid droplets and energy, and communicates with surrounding granulosa cells through gap junctions. These intracellular activities are essential for acquiring competence for maturation, fertilization, and early embryonic development (Sen and Caiazza, 2013).

Oocytes remain meiotically arrested due to the high level of intracellular cyclic adenosine monophosphate (cAMP), which inactivates maturation promoting factor (MPF). Active MPF stimulates resumption of meiosis, allowing the oocyte to proceed to the anaphase, and complete the first meiotic division. The high level of cAMP is maintained by the intrinsic production of cAMP due to constitutive activation of G-protein coupled receptors, and extrinsic continuous supply of cAMP and cyclic guanosine monophosphate (cGMP) from granulosa cells through gap junctions. cGMP inactivates

phosphodiesterase 3A (PDE3A) which hydrolyzes cAMP (Sen and Caiazza, 2013). The preovulatory LH surge initiates oocyte resumption of meiosis by three main mechanisms: First, causing closure of gap junctions between cumulus and oocytes, thus preventing further diffusion of cAMP and cGMP; Second, inactivation of the G-protein coupled receptor pathway, that is responsible for cAMP synthesis; and Third, restoration of PDE3A activity, which further decreases cAMP levels (Sen and Caiazza, 2013). Accordingly, the signaling of LH initiates essential events that promote meiotic maturation and conclude the prophase I arrest.

Meiotic maturation is characterized by the meiotic spindle formation and extrusion of the first polar body (Sen and Caiazza, 2013). This concludes the first meiotic division and oocytes immediately proceed to the second meiotic division where they remain arrested at the metaphase II stage until fertilization. High level of MPF is the key factor for maintaining the metaphase II arrest. Typically, MPF is targeted by the anaphase-promoting complex (APC) for degradation and allows second meiosis to finish. However, the active cytostatic factor (CSF) inhibits the destructive effect of APC and thus sustain a high level of MPF (Sen and Caiazza, 2013).

Oocyte Developmental Competence

The oocyte obtains the developmental competence throughout the course of oogenesis within the ovarian follicle. Oocyte quality and developmental competence are assessed based on the oocyte's ability to resume meiosis, cleave after fertilization, develop to the blastocyst stage, establish a pregnancy, and develop to term in good health (Sirard et al., 2006; Moussa et al., 2015). During oogenesis, the oocyte is dynamically responsive to and regulated by maternal physiological and cellular factors through the

ovarian intra-follicular environment which profoundly contributes to oocyte quality and the subsequent developmental competence of the oocyte (Hsueh et al., 2015). Poor oocyte quality has been the chief culprit and the main interpretation of early embryonic loss in dairy cattle (Sirard et al., 2006). About 90% of ovulated oocytes are successfully fertilized and start developing; however, only 55% survive to term. Most of the embryonic loss occurs within the first two weeks of gestation and accounts for 70-80% of total embryonic loss (Moussa et al., 2015).

Accumulation of maternal RNA transcripts during oogenesis and abundance of key transcriptional factors are vital for obtaining oocyte developmental competence. For example, the production of sufficient cyclin B is critical for the acquisition of meiotic competence, and its mRNA translation is controlled by cytoplasmic polyadenylation in the oocytes (Tay et al., 2000). The combination of the regulatory subunit cyclin B and the catalytic subunit cyclin-dependent protein kinase 1 (Cdk1) forms MPF that promotes maturation. A highly abundant JY-1 transcript was identified and found to regulate cumulus expansion and control early embryogenesis in cattle (Bettegowda et al., 2007). Follistatin mRNA transcripts found to be overrepresented in good quality oocytes and play a functional role in the control of early embryogenesis in bovine (Lee et al., 2009). KPNA7, a member of the importin-α family is predominantly expressed in mature oocytes and plays a functional role in the transportation of nuclear proteins essential for early embryonic development (Tejomurtula et al., 2009). Growth differentiation factor-9 (GDF9) and bone morphogenetic protein 15 (BMP-15) are important oocyte-derived growth factors. Both have critical roles in controlling folliculogenesis, recruitment of the primordial follicle, steroidogenesis of granulosa cells, cumulus cells expansion, oocyte

maturation, and ovulation (Moussa et al., 2015). The oocyte-specific factor in the germline (FIGLA) is essential during early oogenesis for primordial follicle formation and regulates the expression of zona pellucida genes *Zp1, Zp2,* and *Zp3* (Soyal et al., 2000). The nucleotide-binding oligomerization domain/ leucine rich repeat/ pyrin domain containing (NLRP) is a family of proteins which are maternal factors that accumulate during oogenesis and are essential to support the early embryonic development. In mouse, in vitro knockdown of some Nlrp2 gene resulted in the early embryonic developmental arrest, while overexpression lead to normal development. However, the overexpression of Nlrp2 was associated with increased apoptosis in blastomeres (Peng et al., 2012).

The oocyte synthesizes and accumulates cytoplasmic organelles during the course of oogenesis to acquire developmental competence. One of the vital organelles that are required for the oocytes to be competent is the mitochondria. Building up a large number of maternal mitochondria during oocyte development provides ATP for oocyte maturation and fertilization and early stages of embryonic development up until implantation (Jansen, 2000). In bovine, *in vitro* studies showed that variation in oocyte mtDNA with different ATP levels at GV and MII stages, affect the oocyte developmental capacity after fertilization (Tamassia et al., 2004). Several maternal and environmental factors can influence oocyte mitochondrial activity, including nutrition, which can alter mitochondrial activity causing an increase in reactive oxygen species (ROS) in oocytes and zygotes (Igosheva et al., 2010). Similarly, in somatic cells, high fatty acid supplementation increases mitochondrial activity and subsequently increased ROS production (Iossa et al., 2002). The elevated level of ROS in oocyte increases oxidative

stress and; possibly responsible for the reduced oocyte developmental competence (Van Hoeck et al., 2014).

Maternal nutrition can have an immediate effect on the oocyte intra-follicular environment and the entire reproductive system through different metabolic and endocrine signaling pathways. Oocytes during development and maturation are very responsive to their surrounding environment, and any metabolic and hormonal fluctuation in the follicular environment can ultimately have an impact on the molecular constituents and functions of the oocytes. Trace elements such as zinc, copper, and manganese are essential for numerous physiological and molecular processes. Zinc and copper are vital during oocyte maturation for the DNA integrity of the cumulus cells, and in an *in vitro* experiments, they are found to protect the bovine COCs from apoptosis and improve subsequent embryo development (Anchordoquy et al., 2014; Rosa et al., 2016). The active form of vitamin A regulates gene expression and required for oocyte cytoplasmic maturation and acquisition of developmental competence (Ikeda et al., 2005). Vitamins E and C have a potent antioxidant activity that supports the cell membranes and prevents apoptosis. Supplementation of embryo culture media with vitamin E and C improved zygotes development to the expanded blastocyst stage (Olson and Seidel, 2000). Supplementation of the maternal diet with long chain fatty acids also was found to improve fertility in high producing cows and improved embryo quality (Santos et al., 2008). Cleavage rate was increased after *in vitro* fertilization of oocytes retrieved from dairy cows fed a diet high in α-linolenic acid (Zachut et al., 2010).

Oocyte Activation by Fertilization

The oocyte remains arrested at MII until fertilization, which activates the oocyte and allows re-entrance into the cell cycle by triggering the calcium oscillation signaling cascade that activates enzymes involved in CSF degradation. Accordingly, MPF levels decline, allowing resumption of meiosis and extrusion of the second polar body. Initiation of the calcium signaling cascade begins with a single sperm when it binds to the zona pellucida and starts the acrosome reaction. Sperm releases hyaluronidase and acrosin enzymes to dissolve the zona pellucida and allow the sperm actin filament to come in contact with the oocyte cell membrane. This process results in the depolarization of the oocyte membrane and causes calcium influx (Kashir et al., 2012). Membrane depolarization prevents other sperms from binding to the zona pellucida and blocks polyspermy (Ducibella, 1996). The sperm then fuses with the oocyte cell membrane and releases the paternal DNA material and PLC zeta in the cytoplasm. PLC zeta is a sperm-specific enzyme that cleaves phosphatidylinositol 4,5-bisphosphate (PIP2), a minor phospholipid component of the cell membrane of the oocyte, to release inositol trisphosphate (IP3) and diacylglycerol (DAG). Inside the oocyte, IP3 then binds to its receptors on the endoplasmic reticulum to release intracellular calcium and initiate calcium oscillation which occurs in waves starting from the penetrating site (Kashir et al., 2012). There is another model of how sperm activates the oocyte via phosphoinositide signaling pathway. Dr. Oko and colleagues (2007) proposed a specific protein of the sperm head, named sperm post-acrosomal WW binding protein (PAWP), get released into the oocyte and propagate calcium signaling cascade in the fertilized oocyte (Wu et al., 2007; Anifandis et al., 2016).

High levels of calcium cause exocytosis of cortical granules that are located below the plasma membrane. They contain proteases and peroxidase enzymes that clip perivitelline proteins, harden the zona pellucida, and attract water into the perivitelline space causing it to expand (Ducibella, 1996; Gupta et al., 2012). This process increases the size of the perivitelline space and provides a further block to polyspermy. Ovistacin, another enzyme released from the cortical granules, hydrolyzes the sperm-binding protein on the zone pellucida and hardens the zona pellucida; thus no longer sperm can bind (Gupta et al., 2012; Stocker et al., 2014).

Intracellular calcium acts as the second messenger during the process of oocyte activation. The increase in calcium concentrations regulates several signaling pathways and activates enzymes involved in the process of oocyte activation. The downstream targets for calcium are calmodulin, calpain, and calcineurin (Miao and Williams, 2012). Calmodulin is a calcium binding protein that regulates calmodulin-dependent protein kinase II (CaMKII) which inhibits CSF. Calpain regulates some proteins essential for cell cycle such as microtubules, that are important for the resumption of meiosis and separation of chromatids. Calcineurin acts as a transcription regulator and plays an important role in zygote genome activation. Also, calcium and DAG promote plasma membrane translocation and activation of protein kinase C (PKC) which has multiple downstream effects including remodeling of oocyte microtubule protein, regulation of transcription and expulsion of the second polar body (Miao and Williams, 2012).

Embryonic Genome Activation

After fertilization, maternal oocyte and paternal sperm fuse to establish the totipotent one cell zygote. The haploid genomes of the oocyte and sperm each have own

characteristics and specific pattern of epigenetic marks that have been established during gametogenesis and gonadal development. Before fertilization, the sperm DNA is packaged with protamines that replace histone proteins during sperm maturation. This process helps to condense and package the paternal chromosome in the sperm head. The sperm provides the paternal contribution of DNA for the male pronucleus which is necessary for oocyte activation by fertilization (Jukam et al., 2017).

Before ovulation, the oocyte undergoes nuclear maturation which is a nuclear modification during the resumption of meiosis characterized by having a haploid chromosome, formation of the 1st polar body, and the DNA become packaged with hyperacetylated maternal histone proteins (Gu et al., 2010; Rybska et al., 2018). Male and female pronuclei remain physically separate, and the DNA of both oocyte and sperm undergo global demethylation, and paternal DNA get repackaged with hyperacetylated maternal histones. Ten-eleven Translocation (TET) enzyme complex actively mediates complete DNA demethylation of the paternal genome before the first zygote division and DNA replication. Active demethylation of the paternal genome is mediated by the oxidation process that converts the 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (Hackett and Surani, 2013). In contrast, maternal DNA demethylation is a passive process, and it depends on DNA replication to erase methylation marks with each replication with no reestablishment or maintenance, and it takes several replications to be totally erased. Imprinted genes in both male and female DNA resists the DNA demethylation process and keep the same pattern of the origin for monoallelic gene expression (Hackett and Surani, 2013).

After global DNA demethylation of both paternal and maternal DNA, de novo methylation patterns associated with subsequent cell differentiation during development are catalyzed by the DNA methyltransferases (*DNMTs*) 3a and 3b (Hackett and Surani, 2013). In the beginning, the zygote is totally dependent on maternally stored components and transcripts until the embryonic genome is activated. The quality and abundance of RNA transcripts stored in the oocyte during meiotic and cytoplasmic maturation determine the oocyte competence and dictate the principal events essential for embryonic genome activation (Memili and First, 2000). The expression of selected transcripts from oocytes at different maturation stages and derived from follicles of different sizes was found to be more abundant in oocytes obtained from 2 to 8 mm follicles compared with <2-mm follicles, indicating the importance of the follicle size or stage on oocyte quality and RNA transcript abundance (Racedo et al., 2008).

Activation of the embryonic genome occurs in waves, and there is a minor wave with some micro RNAs involved in inactivation of the maternal transcripts, and a major wave when the embryonic genome has the total control of the subsequent events. The timing of the main zygote genome activation is different from one species to another, and in cattle, it occurs at the 8 to 16 cell stage. The transition from maternal to embryonic control of development requires degradation of the maternal RNA transcripts to be replaced with the embryonic transcripts in a very organized and regulated pattern (Memili and First, 2000).

Early Embryonic Development and Implantation

Oocyte fertilization takes place in the oviduct, and the new zygote undergoes mitotic divisions and increases cell numbers, and by the 16-cell stage, the developing

embryo enters the uterus. Around day 4 post-fertilization, it develops to a morula with tight junctions. When the cells of the morula start to polarize and pump sodium into the intracellular space, water follows osmotically, and fluid accumulates to form a cavity known as the blastocoele. At this stage it is called a blastocyst with an inner cell mass which will develop into the embryo. The peripheral cells become the trophectoderm which develops into the chorion, the fetal contribution to the placenta. The blastocyst continues to grow and accumulate more fluid in the blastocoel which increases the pressure and with the help of proteolytic enzymes from the trophoblastic cells it causes the zona pellucida to rupture. This process is known as hatching from the zona pellucida as the blastocyst squeezes out and become free-floating embryo within the uterine lumen (Senger, 2012).

After hatching, which typically occurs around on day 9 - 10 post-fertilization, the blastocyst continues to grow and elongate and change from a spherical shape to filamentous around day 16 - 17 and increases in size more than 1000-fold (Degrelle et al., 2005; Lonergan et al., 2016a). The trophectoderm of the elongated conceptus attaches to the endometrial epithelium to start the implantation process approximately at day 19 (Lonergan et al., 2016a). The chorion, originated from the embryonic trophectoderm, develop tiny villi known as chorionic villi which protrude toward the uterine wall and penetrate and interdigitate with the endometrial caruncles. The chorionic villi become the fetal cotyledon, and in conjunction with the maternal caruncle, they both form the placentomes which develop at restricted areas over the endometrium. The placentomes are essential for fetal-maternal interaction as they provide nutrients to the fetus and mediate gaseous and metabolic waste exchanges. In bovine, the placentomes from

convex structures and there are between 70 to 120 distributed across the endometrium. They grow in size during gestation and may measure up to 6 cm (Senger, 2012).

Embryo Quality and the Maternal Effect

The quality of the preimplantation embryo is mainly influenced by the oocyte developmental competence and the surrounding uterine environment. Oocyte quality determines the intrinsic developmental competence of the embryo since the oocyte's maternal RNA transcripts control and regulate early intracellular biological processes for the developing embryo until the complete activation of the embryonic genome (Lonergan et al., 2001). During oogenesis and maturation, the oocyte synthesizes organelles and stores energy to support early embryonic events. The reproductive tract, including the oviduct and uterus, provides a suitable environment for a healthy embryo development through maternal recognition of pregnancy and implantation and contributes extrinsic factors for the embryo quality (Lonergan et al., 2001).

Early embryonic loss is believed to be the major cause of reduced fertility in dairy cows, and up to 40% of the embryo losses happen within the first two weeks of gestation before implantation (Leroy et al., 2008a). This indicates how important the early embryonic quality during this critical time of gestation. At early stages of embryo development and before hatching from the zone pellucida, mostly at the blastocyst stage, embryo quality can be graded and classified based on the morphology and integrity of the embryos. The morphological criteria consider the general appearance of the embryo and follow a numerical coding system for the quality based on the stage of development. The quality is determined by the number and the symmetry of blastomeres, trophectoderm

appearance, differentiation of the inner cell mass, and blastocoel expansion (Racowsky et al., 2010; Rocha et al., 2016).

Morphological classification is widely used in the assisted reproductive technology and basic research and serve as a noninvasive technique to evaluate the quality of the *in vitro* produced embryos and to predict the outcomes for embryo transfer. However, this classification is inapplicable for in vivo developing embryos, but it can help to understand how the embryo grow and respond to different culture media that mimic the uterine environment under certain physiological conditions (Rocha et al., 2016).

The growing embryo is highly sensitive to fluctuations in its surrounding environment and a suboptimal uterine environment due to maternal health, nutrition, metabolism, endocrine, or stress ultimately has an impact on the embryo quality and survival. Maternal metabolic disorders are associated with fluctuations in certain metabolites and hormones Lonergan et al., 2016a). Elevated concentration of non-esterified fatty acids (NEFA) in the circulation due to excessive fat mobilization during NEB alter the uterine environment and impact the ability of the reproductive tract to support normal embryo development. Exposing bovine oviductal epithelial cells to high NEFA negatively affected the cell number and decreased sperm binding affinity (Jordaens et al., 2015). The ability of the embryo to the reach blastocyst stage was impaired when bovine embryos were exposed to elevated NEFA concentrations during *in vitro* development (Van Hoeck et al., 2014). High NEFA due to NEB is typically associated with elevated β-hydroxybutyrate (BHBA) and low glucose in the circulation which is a good indication of (sub)clinical ketosis. Cleavage rate at 48 h after fertilization

and the number of blastocysts at day eight were reduced after *in vitro* maturation of oocytes in media with elevated BHBA and low glucose to mimic the (sub)clinical ketosis (Leroy et al., 2006).

In addition to the NEB, lactation, in general, seems to affect the embryo quality of dairy cows and it was demonstrated that the quality of embryos from lactating dairy cows was inferior to those from non-lactating dairy cows or heifers (Sartori et al., 2002). Embryos from lactating dairy cows appeared to be much darker and contained up to 50% more intracellular lipids when compared to embryos from heifers or non-lactating beef cows at day seven following AI (Leroy et al., 2005a).

Progesterone is the main hormone required for normal embryo development and maintenance of pregnancy. High-yielding dairy cows demonstrate delayed rise of P4 after ovulation and inadequate P4 concentrations during the luteal phase which may contribute to the decreased conception rates in lactating dairy cows (Leroy et al., 2008a). Even though lactating dairy cows have larger CL when compared with that of non-lactating heifers, the maximal P4 concentration is lower (Sartori et al., 2002). Lower P4 can be explained by the impaired luteal capacity to synthesize and secrete a sufficient amount of P4 or due to a higher rate of steroid metabolization including in the liver (Sangsritavong et al., 2002). However, the association between embryo quality and maternal P4 concentration does not imply a direct effect of P4 on embryo survival.

Progesterone induces changes in the uterine environment to make it suitable for embryo survival and stimulate the production of histotroph which provides nutrients for the developing embryo (Lonergan et al., 2016b). Histotroph is produced by the uterine gland and is vital for embryo development. In sheep experiments, uterine gland knockout

resulted in failure of embryos to develop beyond the blastocyst stage (Gray et al., 2001). Accordingly, the effect of P4 on embryo quality is mediated mainly through enhancing the endometrial environment. Embryo development to blastocyst stage and elongation does not require P4 as its supplementation to the *in vitro* embryo culture medium had no effect on embryo survival after transfer to synchronized recipients (Clemente et al., 2009; Larson et al., 2011).

Uterine Function and Role in Fertility

The uterus is the central organ in the female reproductive tract and a healthy, functional, and receptive uterus is indispensable to establish and maintain pregnancy. It provides nutrients and an optimal environment for the embryo to grow. Anatomically, the uterus is made up of two uterine horns and a body in bovine. Uterine horns extend to the oviducts where fertilization take place, and the uterine body is the site for implantation, placentation, and fetal development (Senger, 2012). Histologically, the uterus consists of three different layers; endometrium, myometrium, and perimetrium. The perimetrium is the serosal layer and forms the external surface of the uterus and is contiguous with the peritoneum. The perimetrium mostly consists of connective tissues that provide strength and support to the uterus. The myometrium is the middle layer of the uterus and mostly consists of smooth muscle and is responsible for uterine contraction and tonicity. The degree of myometrial tone varies from being very hard and easily distinguished and palpated when estrogen is high during estrus to a very soft and flaccid when P4 is high, and estrogen is low (Senger, 2012). The endometrium is the luminal epithelial layer and contains the uterine glands that originate from the mucosal layer of the uterus (Senger, 2012).

The endometrium is constantly subjected to dynamic histological and functional changes through the different stage of the estrous cycle and during pregnancy (Espejel M, 2017). The major hormones promoting the changes in the endometrium are estrogens and progesterone, depending on the phase of the estrous cycle (Ohtani et al., 1993). Estrogen produced from the growing follicles during the follicular phase of the cycle promotes endometrial tissue proliferation and increased uterus thickness and vascularization. This stage is known as the proliferative stage of the endometrium (Ohtani et al., 1993). After ovulation, the CL become the dominant ovarian structure and produces P4 in high amounts which promotes endometrial glandular secretory activity and prepares the endometrium for embryo implantation and the establishment of pregnancy. This stage is known as the secretory stage (Ohtani et al., 1993). In case of no fertilization or no maternal recognition of pregnancy, endometrial tissue undergoes hypotrophy, and the luteolysis of the CL ends the secretory stage and allow a new cycle to begin (Espejel M, 2017).

The uterus is the main source of PGF2α, and its release is mediated by oxytocin receptors which are highly expressed in the endometrium and myometrium. During pregnancy, the CL is maintained by the downregulation of oxytocin receptor expression in the endometrium and accordingly, blocking the release of PGF2α (Silvia et al., 1991). This process is initiated by the release of IFN- τ from the elongated conceptus preimplantation (Senger, 2012). The main sources of oxytocin are the pituitary gland and the ovaries, and the oxytocin reaches the uterus through blood circulation. While endometrial oxytocin receptors mediate PGF2α release, oxytocin receptors in the

myometrium induce uterine contraction and play an essential role during calving and postpartum uterine contraction (Fuchs, 1987).

Proper communication between the conceptus and maternal endometrium is crucial for the establishment and maintenance of pregnancy. The conceptus is responsible for initiating this cross-talk and improper communication results in early embryonic loss due to unprepared uterine receptivity (Lonergan and Forde, 2014). Sufficient amount of IFN- τ secretion is maintained for a period over a week or so, but it can induce an immediate effect on the endometrium as the conceptus can prevent luteolysis within a day after transfer to a recipient cow (Forde et al., 2011b). Prevention of luteolysis pauses the temporal changes that endometrium typically undergoes during the estrous cycle and is the main step toward maternal recognition of pregnancy. Progesterone remains high during pregnancy and functions to prevent the resumption of ovarian cyclicity and estrus.

Uterine Health and its Effect on Fertility

During pregnancy, the uterus increases in size and the vasculature and myometrial muscle stretch to the limit to accommodate the growing fetus. After calving and expulsion of the placenta, endometrial tissue undergoes a regenerative mechanism known as uterine involution during which blood flow is reduced, the vasculature is decreased, and smooth muscle starts to contract. Proper uterine involution is important to eliminate residual mucus, tissue debris, and bacterial contamination and to resume non-pregnant uterine function.

Delayed uterine involution increases the risk of bacterial infection and metritis (Sheldon and Dobson, 2004). Metabolic disorders such as NEB, ketosis, and hypocalcemia weaken the reproductive tract and uterine smooth muscle contraction

resulting in delayed uterine involution and increase the risk of bacterial infection and metritis (Whiteford and Sheldon, 2005; Esposito et al., 2014). The incidence of metritis was increased in cows with high BHBA and NEFA concentrations due to the NEB when compared with healthy cows (Giuliodori et al., 2013). Negative energy balance and BCS during the transition period of dairy cows are good predictors of peripartum diseases including metritis and demonstrate the association between metabolism and reproductive health (Ruprechter et al., 2018).

Metritis is one of the major causes of decreasing reproductive performance and contributes heavily to the declining fertility in dairy cows by decreasing uterine receptivity and conception rate resulting in prolonged calving interval (Salasel et al., 2010; Walsh et al., 2011; Giuliodori et al., 2013). Several factors contribute to postpartum metritis such as retained fetal membrane, dystocia, and stillbirth and all of them can be linked to the metabolic disorders and NEB (Esposito et al., 2014). Numerous studies demonstrated that dairy cows with NEB are more likely to develop uterine diseases (Bromfield et al., 2015).

Treatment of clinical metritis and endometritis is straightforward, but it requires veterinary intervention which adds extra expenses and lower profitability. Besides, the consequences of uterine infection and inflammation persist beyond the resolution of the clinical symptoms. Even with successful treatment, conception rate is 20% lower in the affected animals when compared with healthy ones, and 3% of the affected animals became infertile after the treatment and culled from the herd (Sheldon et al., 2009). The pathogenic bacteria invade the uterus and cause an inflammatory reaction. Infiltration of

the inflammatory cells leaves histological lesions in the endometrium that negatively impair uterine receptivity and perturbs the survival of the new embryo.

Bacterial lipopolysaccharides (LPS) are the major components of the outer membrane of all Gram-negative bacteria and are the common pathogen in metritis. Bacterial LPS was found to disturb endocrine signaling pathway (Peter et al., 1989). Disturbance of the endocrine system affects hormones that regulate the estrous cycle which negatively affect reproductive function and impair fertility. For example, experimental intrauterine infusion of *Escherichia coli* endotoxin in heifers resulted in decreased GnRH secretion from the hypothalamus and reduced LH pulse (Peter et al., 1989). Similarly, intravenous administration of LPS in lactating dairy cows resulted in delayed LH surge and ovulation which definitely decreases the chances of successful fertilization (Lavon et al., 2008). The negative effect of postpartum uterine infection on cows' reproductive performance was reported in multiple studies and manifested as reduced E2 in the circulation, persistent CL, delayed ovulation, slower follicular growth, and increased incidence of anovulation (Sheldon et al., 2002; Bromfield et al., 2015). The healthy endometrium is the main source of luteolytic PGF2α which is responsible for CL regression to end the luteal phase and allowing a new cycle to begin. However, in the case of uterine infection, the bacterial LPS induces endometrium to switch from PGF2α to the inflammatory modulator and luteotropic prostaglandin E2 (PGE2) which results in the extension of the luteal phase (Herath et al., 2009a).

The inflammatory reaction is a normal physiological response to infection and the endometrium responds to infectious pathogens by up-regulating the expression of proinflammatory genes. The expression of the proinflammatory genes (*IL-1A, IL-1B, IL-*

6, and prostaglandin E synthase (*PTGES*)) was found to be upregulated in endometrial biopsy samples collected multiple times postpartum from a group of infertile cows suffering from chronic endometritis when compared with endometrial samples from healthy animals (Herath et al., 2009b). The release of the proinflammatory cytokines recruits neutrophils and macrophages to the inflammatory site. Recruitment of the inflammatory cells and mediators release antimicrobial factors to combat pathogens and resolve the uterine infection. However, this response to infection may alter the uterine environment and make it less suitable for the preimplantation embryo which may explain infertility in cows with high expression of proinflammatory genes (Herath et al., 2009b; Bromfield et al., 2015).

Bacterial contamination is inevitable during and after calving, and successful uterine involution and healthy immune response are crucial to eliminate and fight pathogens and prevent infection (Földi et al., 2006). At parturition, dystocia and retained placenta increase the incidence of uterine contamination and metritis (Tenhagen et al., 2007). Metritis takes time to recover from, and it delays the resumption of normal uterine and ovarian function, which negatively affects conception rate and increases calving interval (Giuliodori et al., 2013; Struve et al., 2013).

The postpartum period is a critical time for proper activation of the immune system that endured a prolonged period of immune suppression during gestation. Uterine immune suppression is required during pregnancy to protect the allogeneic conceptus from the rejection by the maternal immune responses (Esposito et al., 2014). This localized immune suppression in the uterus is induced by P4 which acts as natural immunosuppressive and demonstrate one of the fundamental roles of P4 in maintaining

pregnancy (Hansen, 2013). However, immediate postpartum activation of the uterine immune response is essential after being suppressed for a long period. Negative energy balance and metabolic disorders impair and suppress the immune function and predispose cows to periparturient diseases (Esposito et al., 2014).

Cows suffering from metabolic disorders during early lactation are more susceptible to infection (Hansen, 2013). Fatty liver due to excessive postpartum fat mobilization was associated with the reduced expression of function-associated surface molecules on blood neutrophils (Zerbe et al., 2000). Peripartum evaluation of peripheral blood neutrophil function was significantly impaired in cows with higher NEFA and BHBA and was associated with an increased incidence of uterine infection (Hammon et al., 2006). Periparturient metabolic disorders such as milk fever and ketosis increase the incidence of endometritis (Roche, 2006). The improper interaction between metabolism and the immune system during the transition period intensely contribute to infertility. Therefore, an accurately formulated ration and a balanced diet are necessary during this critical period.

Transition Period and Energy Metabolism

Dairy cows experience dynamic physiological and metabolic changes with complex hormonal regulation during their reproductive cycle when transitioning from non-lactating to lactating, and from pregnant to non-pregnant. The transition period begins during late pregnancy when the fetus reaches maximum growth, and extends to early lactation when milk production reaches the peak. Furthermore, the lactation curve and gestation overlap in dairy cows. Dairy cows are managed to maintain milk production during gestation up to 45 days prepartum, and expected to come in heat, be

breed and conceive within three months postpartum to ensure profitability (De Vries, 2006). The transition period is a critical time and generally extends from 6 to 8 weeks between late pregnancy and early lactation (Roche et al., 2017).

Major events that occur during the transition period are drying from milk production, final growth and development of the fetus, calving, initiation of milk production, rapid increase in milk production, and resumption of ovarian cyclicity. All of these consequent events within a relatively short period of time induce stress on the cow resulting in increased incidence of metabolic and health issues accompanied with severe energetic and nutrient demand (Ingvartsen, 2006; Salasel et al., 2010). In recent decades, intensive genetic selection for milk production resulted in an increase in the incidence of metabolic disorders due to more extreme physiological adaptation to meet the nutritional and energetic demand for milk production.

Cows can not meet the enormous energy requirements of early lactation solely from nutrition due to limited dry matter intake. The deficit in energy requirements is compensated from body fat reserves (Grummer et al., 2004). Going trough a period of energy deficit makes transition period the most critical time during lactation for dairy cows, accompanied with a significant shift in nutrient partitioning toward the mammary gland to support milk production (Bauman and Currie, 1980), which increases the requirement for glucose, amino acids, and fatty acids (Bell, 1995).

Negative energy balance and low BCS negatively affect reproductive performance and aggravate the consequences of dystocia and retained placenta (Berry et al., 2007; Roche et al., 2009). Cows normally lose body weight and BCS during the transition period due to increased energetic demand for milk production accompanied by

stress from calving. Therefore, it is recommended to manage the nutrition during dry and transition period to keep BCS between 2.75 and 3.0 on a 5-point scale at calving and not to lose more than 0.5 at the time of breeding (Crowe, 2008). A well-balanced ration during the dry period and early lactation can minimize the negative effect of NEB and reduce the incidence of metabolic disorders (Thatcher et al., 2010).

Gluconeogenesis and glycogenolysis increase in the liver to provide glucose to the mammary for lactose synthesis while consumption of glucose in the peripheral tissues decreases resulting in reduced insulin concentrations in the plasma (Bauman and Currie, 1980). Low insulin and glucose in the circulation and peripheral tissues promote lipolysis and mobilization of fat from adipose and muscle tissue to be used as a source of energy (Hammon et al., 2009).

Low insulin concentration does not affect or prevent the mammary gland from utilizing glucose, giving the priority for milk production as part of glucose partitioning process toward the mammary gland (van Knegsel et al., 2007). Dairy cows and ruminants, in general, rely on gluconeogenesis, which mostly takes place in the liver to provide glucose since most of the dietary carbohydrates are fermented in the rumen. Ruminal fermentation produces volatile fatty acids (acetic, propionic and butyric acids) which are absorbed by the ruminal epithelium and carried to the liver by circulation where most of the propionic acid serves as a substrate for gluconeogenesis (Aschenbach et al., 2010). Amino acids mostly go toward milk protein, but they also can be used for gluconeogenesis in case of reduced feed intake. Dietary amino acid deficiency results in the breakdown of skeletal muscle and mobilization of amino acids (Bell, 1995).

Body fat reserves are stored as triacylglycerols (TAG) in adipose tissue and hydrolyzed to NEFA via lipolysis to release glycerol into the circulation which serves as substrates for gluconeogenesis. The elevated level of NEFA in the circulation is an indication of increased energetic demand and excessive fat mobilization (van Knegsel et al., 2007). Through β-oxidation, NEFA is activated by coenzyme A, acyl-CoA, to acetyl-CoA which enters the Krebs cycle to generate energy (Zammit, 1990). Excessive acetyl-CoA that does not enter the Krebs cycle due to limited bioavailability of oxaloacetate enters the ketogenic pathway. Through ketogenesis, acetyl-CoA is converted into acetoacetate, and some get further dehydrogenated to BHBA that get released into the circulation and can be used as a source of energy in the peripheral tissues (Drackley et al., 2001). These metabolic changes are normal physiology during the transition period within a limit, but failure to adjust results in a number of metabolic disorders as ketosis and fatty liver. The consequences of these metabolic disorders can directly or indirectly impair reproductive performance in dairy cows resulting in poor fertility (Butler, 2000).

Ketosis is defined as a high level of ketone bodies in the circulation that exceeds the capacity of peripheral tissues to utilize for energy. Ketosis incidence is high in fresh cows when hepatic ketogenesis is induced by massive body fat mobilization when carbohydrate supply is insufficient to provide acetyl-CoA for energy. High level of ketone bodies in the circulation can result in clinical or subclinical ketosis which is defined by the threshold at ≥ 1.2 mmol/L BHBA (Duffield et al., 1998). Ketosis is often associated with fatty liver and increases the incidence of the other metabolic disorders as hypocalcemia and left abomasal displacement and also suppress immune function and increase susceptibility to mastitis and metritis which also contribute to poor fertility

(Roche, 2006). Immunosuppression during the transition period delays recovery and weakens the disease resistance due to low concentration and response of immune cells, making the animal vulnerable to infection (Esposito et al., 2014).

Strategies to Improve Fertility

Fertility is a multifactorial trait and can be affected by several factors through multiple pathways at different time points through the cow's reproductive cycle. The incidence of these factors varies depending on the time during lactation and reproductive cycle. Developing strategies to improve the declining fertility in dairy cows requires a thorough understanding of the causes of infertility. Even though multiple factors can interfere with fertility at different levels, most of them can be tracked to the basic principles of dairy management: balanced nutrition, a well-planned and strategic breeding program, and maintenance of overall health for the optimal performance. However, this review will focus on strategies related to the declining fertility in modern high-yielding dairy cows.

Improve Nutrition to Minimize the Metabolic Stress. Since the main culprit in declining reproductive performance in high-yielding dairy cows is the increased metabolic stress, many nutritional strategies have been developed to minimize the metabolic stress and support the overall health during the transition period (Knight et al., 1999). Formulation of a well-balanced ration to support the cows during the transition period in coping with NEB become one of the main objectives of the nutritionists, in addition to increase the milk yield (Garnsworthy et al., 2008).

The reproductive system is controlled by and strongly associated with the endocrine system. Several hormones involved in milk production and reproduction are

involved in metabolism as well, and are influenced directly or indirectly by nutrition and circulating metabolites such as insulin, growth hormone, and insulin-like growth factor I (Gong et al., 2002). Improvement of the composition of the glucogenic diets can support ovarian restoration of postpartum fertility in dairy cows. Insulin mediates postpartum resumption of ovarian cyclicity, and a ration high in glucogenic nutrients is recommended during the postpartum period to boost insulin in order to support the normal resumption of ovarian cyclicity (Gong et al., 2002). An insufficient glucogenic diet causes a decline in body weight and BCS and is associated with anovulation in dairy cattle which results in reduced conception at the first postpartum insemination (Santos et al., 2016). Negative energy balance causes severe catabolism of adipose tissue and suboptimal concentrations of essential metabolites which further compromises fertility and oocyte competence (Leroy et al., 2008b). Even though some cows under severe NEB can develop follicles until the preovulatory stage, many of these dominant follicles lose their dominance and regress (Gumen et al., 2003). Also, elevated concentration of insulin in the circulation induced by the glucogenic diet during early lactation is found to shorten the time for the first postpartum ovulation (Gong et al., 2002).

Protein is one of the components of dairy cow rations that is critical for high milk yield, however elevated peripheral urea levels as a result of protein degradation has negative impact on reproductive performance as it significantly compromises the oocyte developmental capacity and negatively impacts early embryonic development (Sinclair et al., 2000; Crowe et al., 2018). However, the conclusive association between a high level of circulating urea and reduced fertility in dairy cows is still debatable (Rodney et al., 2018).

Fatty acid supplementation is one of the strategies to improve fertility in dairy cows. Fatty acids are energy dense nutrients and very efficient at supporting the increasing energy demand for milk production. They also contain essential substrates for steroidal hormones involved in reproduction and play an essential role in inflammatory response and regulation. Omega-6 fatty acids have a pro-inflammatory effect, while omega-3 fatty acids have an anti-inflammatory effect. Diet supplementation with omega-3 fatty acids during the transition period increased pregnancy per AI and reduced pregnancy loss (Silvestre et al., 2011). However, the impact of fat supplementation on oocyte quality and embryo survival is a matter of debate as it may alter the compositions of the follicular fluid which has a direct effect on the growing and maturing oocyte and subsequent early embryonic development (Leroy et al., 2005b; Leroy et al., 2008b; Leroy et al., 2008c).

Vitamins and minerals are essential for cows' overall health and optimal reproduction efficiency. There is a variety of commercially available vitamin and mineral additives and incorporating them in the ration is vital (Hurley and Doane, 1989). For example, supplementing the diet with vitamin E and selenium significantly reduced the incidence of retained placenta, stillbirth, and pregnancy loss (Pontes et al., 2015).

***Optimizing Breeding Programs*.** Well managed and planned breeding programs are essential in dairy farms to ensure profitability and can be optimized to improve reproduction. Synchronized ovulation and timed artificial insemination involve the use of reproductive hormones which may help in solving some infertility issues. Synchronization programs benefit fertility not only by managing the estrous cycle, but also helps to recover cows suffering from uterine diseases. Administration of PGF2α

combined with intra-uterine antimicrobial therapy has been shown to improve the fertility of cows with endometritis (Galvao et al., 2009). Prostaglandin F2α helps to eliminate microbial contamination and reduce the incidence of uterine infections, especially in cows with a responsive CL (Yu et al., 2016). Furthermore, the application of synchronization protocols involving the use of CIDR, controlled internal drug releasing, improved the pregnancy rate per AI at 60 d after first AI and reduced pregnancy loss (Chebel et al., 2006). Also, using GnRH to induce ovulation during synchronization treatments reduces the length of follicle dominance and improves embryo quality (Cerri et al., 2009).

Introduction of Fertility Traits in Genetic Selection. Incorporation of traits associated with reproductive performance in genetic selection is one of the main strategies to improve reproduction in dairy cows. Cows were mostly selected for high milk production trait until the early 2000s before the including of fertility trait as part of the genetic selection program (Crowe et al., 2018). Several traits contribute to overall fertility, and proper evaluation and examination need to be done to each trait and their associated phenotypes before selection. Unlike milk yield traits, fertility traits are more diverse and including them requires incorporation of heritable and measurable phenotypes. Some of the traits associated with reproductive performance are routinely measured such as the calving interval, first postpartum heat, the intensity of estrus signs, and services per conception (Berry et al., 2014). There are several detailed traits associated with fertility, and it is not practical to measure them routinely such as reproductive tract examination, regular hormonal profiling, luteal activity, endometrial cytometry, and multiple ovulations, (Royal et al., 2002; Berry et al., 2012; Carthy et al.,

2014). There are more detailed traits that can be selected, but the main issue remains the heritability and practicality to measure and incorporate them. The two major limitations of the genetic selection of the reproductive traits are the low heritability estimate, and the fact that it takes time to evaluate the outcomes of the selection (Crowe et al., 2018).

Some measurable traits are indirectly associated with fertility and can be used as heritable predictor traits. Such traits can help in shortening the time to evaluate the genetic selection as the BCS phenotype which is genetically correlated with fertility and routinely measure (Berry et al., 2003). Several external factors can affect the accuracy of the estimation of the heritability and the correlation with the predictor phenotypes as the environment, management, population, and the statistical model used to estimate the correlation (Berry et al., 2014; Carthy et al., 2014).

Advancements in genomic technologies and new findings in genetic research in the recent years, has helped in increasing the accuracy of finding predictor traits, shorten the evaluation time of genetic selection, and generated large databases of sequencing data, genotyping and associated phenotypes (Capitan et al., 2014). Determination of genomic regions associated with reproduction using single-nucleotide polymorphism (SNP's) genotyping has accelerated the development of genomic selection with accurate estimation of the genetic merit based on the genotype and the associated genetic markers (van der Werf, 2013). Tracking the causes of infertility to the genomic and molecular level will help to find profound strategies to rescue the declining fertility and accelerate the improvement process.

Impact of the Project

In recent decades, fertility in lactating dairy cows has declined as cows have been highly selected for milk production. Since then, the low conception rate in dairy cows has been strongly correlated with high milk production. However, the conception rate among the non-lactating dairy heifers of the same breed and genetics has not declined. Lactating dairy cows normally go through a state of negative energy balance during the transition period due to the rapid increase in milk production and limited feed intake capacity. Negative energy balance is associated with significant fluctuation in the metabolic and hormonal profile that has a potential adverse effect on reproductive performance. Modern high-yielding dairy cows require more energy to meet the demand for the high milk yield which intensifies the severity and duration of the negative energy balance beyond their ability to adapt. Modern high-yielding dairy cows are under a considerable amount of metabolic stress during early lactation that interacts with their reproductive performance and significantly contributes to infertility.

Fertility is a multifactorial trait and can be affected by a network of factors including genetics, environment, nutrition, and management at different levels which makes it hard to identify the primary cause. However, in our study, we want to focus on the effect of high milk production and the energy status on the molecular components of the primary tissues/cells involved in fertility that are determinants of their quality during early lactation. The primary tissues/cells that constitute the essential foundations of healthy fertility are a competent oocyte, good quality embryo, and optimal uterine environment. The overall hypothesis we tested for this project is that lactation and negative energy balance can alter the expression of key genes involved in oocyte

developmental competence, embryo quality, and endometrial receptivity to the embryo. To examine this hypothesis, we analyzed the relative changes in gene expression between individual MII oocytes, individual in vivo developed embryos, and endometrial biopsy samples obtained from non-lactating dairy heifers and lactating dairy cows at different times during lactation. We compared the relative mRNA abundance of selected genes from a variety of functional categories such as metabolism, inflammation, apoptosis, oxidative stress, heat shock proteins, and epigenetics.

Analysis of the differentially expressed genes will help us to understand how lactation and energy status interact with fertility at the molecular level and identify which pathway is the most affected. Identification of the affected pathways will explain the association between lactation, metabolism and reproduction and aid in finding profound solutions for the declining fertility and alleviate the adverse effect of the negative energy balance and high milk yield. It is essential now for reproductive biologists, nutritionists, and geneticists to obtain a more in-depth understanding, at the molecular level, of the underlying reproductive biology that contributes to infertility of dairy cows in order to develop effective strategies to improve it.

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CHAPTER 2

ALTERED GENE EXPRESSION IN OOCYTES OF LACTATING DAIRY COWS DUE TO NEGATIVE ENERGY BALANCE

ABSTRACT

In recent decades, fertility in dairy cows has declined as cows have been highly selected for milk production. Dairy cows often experience a state of negative energy balance (NEB) during the first few months of lactation that is typically associated with metabolic fluctuation and excessive body fat mobilization. However, the increase in milk yield in modern dairy cows has augmented the side effects of NEB and increased oxidative stress which can have a profound impact on the intraovarian follicular environment. Perturbed follicular condition indeed affect the molecular composition and developmental capacity of the oocyte during final growth and maturation when the oocyte is highly responsive to its surrounding environment. This study aimed to identify how the stage of lactation and/or energy status impact gene expression in oocytes. In vivo matured oocytes were collected by ovum pick-up (OPU) from the preovulatory follicles once from each of eight Holstein heifers, and three times from each of thirteen lactating Holstein cows at 47, 75, and 130 days in milk (DIM). RNA was extracted from every individual oocyte for relative transcript abundances for each of 64 genes from a variety of functional categories. Gene expression was evaluated by qPCR. Out of the 64 genes tested, *DNMT1, DNMT3B, GLUD1, GSR, HSP90A1,* and *SUV39H1* were found to be significantly impacted (adj. $P < 0.05$) by NEB that was determined by the level of beta-hydroxybutyrate (BHBA) within lactating cows. None of the genes were found to be

differentially expressed based on stage of lactation. Follicular fluid and corresponding serum samples were collected for metabolomic analysis. Partial correlation ($r = 0.74$) was observed between follicular fluid and serum metabolites. In the serum samples, the concentration of three predominant fatty acids was found to be significantly impacted by stage of lactation and BHBA was significantly lower in heifers' serum. We conclude that metabolic and oxidative stress induced by NEB may impact oocyte quality by altering the molecular constituents related to redox homeostasis and DNA methylation.

INTRODUCTION

The mammalian oocyte comprises the initial foundation of embryonic development. It is not only responsible for the maternal genetic contribution to the zygote, but it also provides nutrients, energy, organelles, and plenty of proteins and RNA transcripts to control and regulate critical early embryonic intracellular biological processes until the complete activation of the embryonic genome. The release of a competent oocyte for fertilization is a pivotal step in the entire reproductive cycle and a substantial element for a successful pregnancy. The oocyte acquires the developmental competence within the ovarian follicle during several stop-and-go periods during the long course of oogenesis that began with the first meiotic division during embryonic development (Sen and Caiazza, 2013). Oocyte developmental competence is defined as the oocyte's ability to resume meiosis after a long period of quiescence, cleave after fertilization, develop to the blastocyst stage, establish a pregnancy, and develop to term in a healthy animal (Sirard et al., 2006; Moussa et al., 2015). The acquisition of developmental competence is associated with a complex and tightly orchestrated process

of follicular, cytoplasmic, and nuclear changes that enable the oocyte to proceed to the next step successfully (Mermillod et al., 1999; Moussa et al., 2015).

During oogenesis and follicular development, the oocyte is responsive to maternal physiological and health status. The oocyte dynamically communicates with its surrounding environment through the ovarian intra-follicular fluid and cumulus cells (Leroy et al., 2011; Hsueh et al., 2015; Moussa et al., 2015). The oocyte is very sensitive to any perturbation, and any adverse follicular conditions, especially during oocyte final growth and maturation, can profoundly impact oocyte developmental competence and, subsequently, the embryo quality and survival (Leroy et al., 2008b; Leroy et al., 2011).

More than two decades ago, Britt (1992) proposed the potential effect of follicular conditions on oocyte quality of high-yielding dairy cows. His hypothesis drew on the association between oocyte developmental competence and the putative adverse follicular conditions during the metabolically challenging period of negative energy balance (NEB). He concluded that such adverse follicular conditions are more likely to result in an oocyte with inferior quality that is incompetent to support early embryonic development (Britt, 1992). Dairy cows often experience a state of NEB during the first few months of lactation due to the rapid increase in milk production and limited feed intake capacity. The deficit in energy supply is compensated for by increasing the rate of fat mobilization from adipose tissue (Contreras et al., 2017). Going through a period of energy deficit is part of a normal transition period in dairy cows (Roche et al., 2017). However, in high-yielding dairy cows excessive fat mobilization results in drastic fluctuations in concentrations of the hormones and metabolites that may exceed the cows' ability to adapt and put them under a considerable amount of metabolic stress. This

metabolic and hormonal dysregulation is reflected in the ovarian follicular fluid which constitutes the micro-environment for the growing and maturating oocyte (Dumesic et al., 2015).

Negative energy balance is associated with an elevated concentration of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA) and low glucose in the circulation (Jorritsma et al., 2003; Chapinal et al., 2012). In addition, the incidence of metabolic disorders increases during the period of NEB that was reported in several studies to be detrimental to the reproductive performance (Jorritsma et al., 2003; Walsh et al., 2011; Opsomer, 2015; Santos et al., 2016). High levels of NEFA and BHBA have an adverse effect on fertility, and replicating their levels in an in vitro maturation system was found to be toxic to the maturing oocytes, resulting in reduced developmental competence (Leroy et al., 2005; Leroy et al., 2008a; Van Hoeck et al., 2013; Van Hoeck et al., 2014).

In recent decades, the intensive genetic selection for milk production traits has augmented the undesirable side effects of NEB and increased the severity and duration of the metabolic disorders (Opsomer, 2015). Such issue resulted in an internationally recognized association between poor fertility and high milk yield in lactating dairy cows. The decrease in conception rate in high-yielding dairy cows is well documented in several studies worldwide (Macmillan et al., 1996; Butler, 1998; Roche et al., 2000; Royal et al., 2000; Lucy, 2001; Lopez-Gatius et al., 2003). The reduced conception rate is believed to be due to the early embryonic loss which is identified as the major cause of reduced fertility in lactating dairy cows (Walsh et al., 2011). Most embryonic losses occur within the first two weeks of gestation, accounting for 70-80% of total embryonic

loss (Moussa et al., 2015). The conception rate in dairy heifers of similar genetic merit has not been affected by the genetic selection for milk production, which suggests an indirect adverse effect of lactation on fertility (Pryce et al., 2004). Lactation-induced metabolic changes were found in a number of studies to alter the steroidogenic activity and transcriptomic profile of the follicular cells in lactating cows when compared to non-lactating heifers (Bender et al., 2010; Walsh et al., 2012a; Walsh et al., 2012b).

Poor oocyte quality has been hypothesized to be a chief culprit and the main interpretation of early embryonic loss and reduced fertility in high-yielding lactating dairy cows (Sirard et al., 2006; Leroy et al., 2008b; Keefe et al., 2015; Santos et al., 2016) as about 90% of oocytes are successfully fertilized and start developing, but only 55% of them survive to term and most die within the first two weeks (Moussa et al., 2015). We hypothesized that the consequences of the metabolic and physiological changes induced by lactation and/or NEB adversely impact oocyte quality by altering the expression of critical genes. In order to test our hypothesis, we evaluated the relative expression levels of selected genes from a variety of functional categories including metabolism, apoptosis, redox, heat shock proteins, epigenetic, imprinting, pluripotency, and oocyte-specific in individual preovulatory (MII stage) cumulus-denuded oocytes using quantitative qPCR analysis. We compared the expression level of the selected genes in individual oocytes aspirated from a group of lactating dairy cows at multiple timepoints during early lactation, and one time from a group of non-lactating dairy heifers. Also, the BHBA concentration, body condition score (BCS), and milk production were reported in the lactating cows before oocyte collection to estimate the energy status of each animal.

We also, collected follicular fluid and corresponding serum samples from another group of heifers and lactating cows following the same experimental design used to collect oocytes. A non-targeted metabolomic analysis was conducted to assess the impact of NEB and stage of lactation on metabolite profiles in follicular fluid and serum that may explain the alteration in gene expression in oocytes. The analysis of the differential gene expression along with the metabolomic analysis may help in identifying key genes and pathways connecting energy balance and oocyte developmental competence in the high-yielding dairy cows.

MATERIALS AND METHODS

Animal Management and Experimental Groups

This experiment was conducted at the Utah State University Caine Dairy Research and Teaching facility and all procedures were carried out under license in accordance with guidelines of the Utah State University Animal Care and Use Committee (IACUC#2438). Thirteen multiparous Holstein-Friesian dairy cows from different parities, second to the seventh lactation, were randomly selected and used in the experiment. Cows were selected a few weeks prior to expected calving, and BCS using a 5-point scale with 0.25-point increments and body weights were taken within the first week post-partum then every other week until wk 19, around 130 days in milk (DIM). Cows were housed in a tie-stall barn during the experiment which was conducted between October and May. All cows were fed a total mixed ration (TMR) twice daily with ad libitum access to feed and water. Cows were milked twice daily, and the milk yield was recorded daily and averaged weekly.

Oocyte samples were collected one time from a group of eight Holstein heifers, average age 14 months, that were randomly selected and kept in a free-stall barn. Oocytes from heifers were collected to be used as samples from non-lactating animals, apparently not experiencing NEB as lactating cows. Oocytes from lactating cows were collected three times from each animal at 47 ± 4.7, 75 ± 4.7, and 130 ± 4.9 DIM. Dairy cows, on average, returned to positive energy balance around 80 DIM (Coffey et al., 2002), and by collecting the samples at 130 DIM we assumed these samples were collected from animals during positive energy balance, unless a serious health issue or a significant drop in body weight was reported.

BHBA and Glucose Measurement

Blood samples were collected from the coccygeal vein two to three times each week post-calving from wk 1 to wk 19 after the evening milking using plain vacutainer blood collection tubes and needles. Blood samples from heifers were collected one time before sample collection. Fresh whole blood was used to measure BHBA and glucose immediately after collection using a digital hand-held device (Precision Xtra, Abbott Diabetes Care, Abingdon, UK). The multiple glucose and BHBA measurements from each cow were averaged by week. The weekly BHBA concentration for each animal was categorized as high (BHBA >12 mg/dL), which is the common cutoff point for subclinical ketosis (Iwersen et al., 2009), and BHBA concentration < 12 mg/dL was further divided into medium (BHBA 8 – 12 mg/dL) and low (BHBA < 8 mg/dL).

Estrous Synchronization and Superovulation

All lactating cows and heifers used in the study were synchronized and superovulated prior to oocyte aspiration following the 7-day CO-Synch + CIDR protocol (Whittier et al., 2013). In brief, a Controlled Internal Drug Release (CIDR) device (Eazi-Breed CIDR®, Pfizer-Pharmacia Animal Health; Kalamazoo, MI) was placed intravaginally for seven days. A dose of 2 ml (100 mcg) gonadotropin-releasing hormone (GnRH) was given two days after placing the CIDR. Then two days later, 17 ml (20 mg/ml) of follicle stimulating hormone (FSH; Folltropin, Bioniche Animal Health, Belleville, ON, Canada) was given in eight decreasing doses 12 hrs. apart for 4 days am. and pm. On the last day of FSH treatment, two doses of 10 and 5 ml Lutalyse ® (5 mg/ml dinoprost tromethamine injection; Pfizer Animal Health, Kalamazoo, MI, USA) were given am. and pm. respectively. The CIDR was removed with the last Lutalyse injection.

Ovum Pick-Up

Oocytes were aspirated from the preovulatory follicles using transvaginal ultrasound-guided ovum pick-up (OPU) technique 24 hrs. before the expected ovulation. Briefly, the cows were restrained in a chute, and the perineal area was thoroughly washed by water and dried. Caudal epidural anesthesia (6 ml of lidocaine hydrochloride 2%) was administered and the OPU procedure was conducted transvaginally by inserting an ultrasound scanner with stainless steel guided 12G x 60 cm needle attached to a vacuum system that connected to a filter dish size 50 µm Millipore (MAI Animal Health, Elmwood, WI) to recover the aspirated cumulus–oocyte complexes (COCs). The needle, tubing, and filter were filled with 20-25 ml of complete flush media (AgTech Inc., Manhattan, KS) supplemented with 50 IU/ml heparin, and incubated at 37°C before

follicle aspiration to avoid blood clotting and clogging of the vacuum system. All follicles ≈14 mm in diameter or greater were aspirated at a vacuum pressure of 80 mmHg. The filter dish with the recovered COCs and follicular fluid was transferred immediately to an on-site lab in the veterinary clinic. All the oocyte retrieval procedures were performed by the same veterinarian in the same clinic and under similar conditions.

Oocyte Processing

In the lab, the filter dish containing the follicular fluid and COCs was rinsed several times with the same complete flush medium used for follicle aspiration to remove tissue debris and blood cells. Under a stereomicroscope, COCs were identified, picked, and transferred to a new small petri dish containing fresh media on a warm stage at 39°C. After transferring all the recovered COCs to the dish, they were transferred to a 1.5 ml tube containing 0.5 ml of hyaluronidase diluted to 10mg/ml in HEPES-Buffered Tyrode's Lactate solution (Lai et al., 2003) and vortexed for 10-15 min at 1900 rpm for cumulus cell removal. Cumulus denuded oocytes were washed 4 times in fresh media and transferred individually into labeled 0.6 ml microcentrifuge tube, snap frozen in liquid nitrogen, and stored at -80°C for future use. Every individual oocyte has a unique ID number indicating the animal number and collection time.

RNA Isolation and Reverse Transcription

Total RNA was isolated from every single cumulus-denuded oocyte using commercial RNA extraction kit (Quick-RNA™ Micro Kit from Zymo Research). Following the recommended protocol in the instruction manual, 100 µl of RNA lysis buffer was added directly into the tube containing the single oocyte and vortexed briefly.

The protocol was exactly followed with an exception at the final elution of the RNA which was performed twice with 10 µl DNase/RNase free water for a final elution volume of 20 µl, and stored at -80°C until further use. Reverse transcription was performed using the GoScript Reverse Transcription kit from Promega (Madison, WI). Ten µl of single oocyte RNA elution was mixed with 1 µl oligo-dT and 1 µl random primers and incubated at 70°C for 5 min using the thermocycler. Four µl 5x reaction buffer, 2 µl MgCl2 (final concentration of 4.8 mM), 1 µl nucleotide mix, and 1 µl of reverse transcriptase (all were supplied with the kit) were added to RNA mix to make a total of 20 µl reaction and incubated at 25°C for 5 min for initial annealing then at 42°C for 60 min. The synthesized cDNA from each oocyte sample was stored at -20°C until further use. A reference cDNA sample was synthesized using the same reverse transcription protocol from a mixture of RNA isolated from different bovine tissues including endometrial tissue, ovarian tissue, pooled blastocysts, and pooled COCs. This sample was prepared to be used as a positive control and a calibrator sample for qPCR.

Fluidigm qPCR Analysis

The BioMark system was used for the qPCR analysis in this experiment which is an innovative and unique platform from the Fluidigm Corporation (South San Francisco, CA) which allows analyzing expression of up to 96 genes of 96 samples. The primers used were designed based on NCBI's reference sequence for Bos taurus through the Fluidigm Corporation's DeltaGene assay design service, and Table 2-1 provides the definition of each gene's symbol used in the qPCR analysis (for primer sequences, see Supplementary Table 2-1). In this experiment, 96 by 96 microfluidic chip of nanoliter-scale was used to test the expression of 64 genes from different functional categories

including housekeeping, apoptosis, redox, heat shock protein, metabolism, epigenetic modifiers, imprinted, pluripotency, and oocyte-specific (Table 2-2). Before qPCR analysis, cDNA samples of each single oocyte were enriched by specific target amplification (STA). One µl of each primer pair (forward and reverse) was mixed into a tube to be used for the STA reaction. For each of 1.25 µl of cDNA, 1.25 µl primer mix and 2.5 µl of TaqMan PreAmp Master Mix (Applied Biosystems; Foster City, CA) was added for STA reaction to make a total of 5 µl reaction. Using the thermocycler, the STA amplification reaction was initially activated by incubation at 95°C for 10 min then followed by 14 cycles of 95°C for 15 seconds and 60°C for 4 min. Exonuclease I treatment was recommended to digest any unincorporated primers and was performed by adding 0.2 µl Exonuclease I Reaction Buffer, 0.4 µl Exonuclease I enzyme (ExoI; New England Biolabs; Ipswich, MA), and 1.4 µl nuclease-free water to each STA reaction to make a total of 7 µl and incubated at 37°C for 30 min. After digestion of unincorporated primers, the reaction was incubated at 80°C for 15 min to inactivate the Exonuclease I enzyme followed by 5- fold dilution by adding 18 µl nuclease-free water to each reaction to make a total of 25 µl of Exonuclease I-treated STA oocytes/embryo cDNA. For Fluidigm qPCR cycling, 2 µl of the diluted and Exonuclease I-treated STA oocytes/embryo cDNA was mixed with 2.5 µl of the 2x TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) 0.25 µl of Sample Loading Reagent (Fluidigm), and 0.25 µl 20x EvaGreen DNA Binding Dye (Biotium, Hayward, CA, USA). On the other hand, 2.5 µl of each primer pair (forward and reverse 20 µM) was mixed with 0.25 µl nuclease-free water and 2.5 µl Assay Loading Reagent (Fluidigm). A 96.96 Dynamic Array Integrated Fluidic Circuits (IFC) was inserted in the IFC Controller

HX for priming using the script run Prime (136x) script before loading the samples and primers. After priming, 5 µl from each primer mix and sample mix were loaded into the designated well in the IFC using the multichannel pipette and returned to the IFC Controller HX for loading by running the Load Mix (136) script. After the Load Mix script has finished, IFC was ejected from the Controller and inserted in the BioMark thermal cycler (Fluidigm) for qPCR. The recommended protocol GE 96x96 Standard v1 was used with initial enzyme activation for 5 min then 35 cycles of 95°C for 15 sec and 60°C for 60 sec and at the end 3 min final extension.

Analysis of qPCR Data and Statistics

Raw qPCR data were analyzed using the comparative cycle threshold (Ct) method using the Fluidigm Real-Time PCR Analysis Software. The software determined the raw Ct value for each reaction and then normalized it to the average Ct of four selected housekeeping genes (*EIF4A1, GAPDH, TAF11*, and *YWHAZ*) to determine the delta Ct (ΔCt) which is the difference between Ct value for each gene tested and Ct of the housekeeping genes. Then, the delta delta Ct (ΔΔCt) value was determined for each reaction by calculating the difference between the ΔCt of each sample and the ΔCt of a selected calibrator sample (cDNA derived from RNA extracted from pooled embryos and COCs, and ovarian and endometrial tissue); described above.

ΔΔCt values were used for the statistical analysis using SAS (SAS, Institute Inc., Cary, NC) to determine the significant difference between samples. A Generalized Linear Mixed Model (GLMM) was fit to allow the gene expression to be dependent upon the type of animal (lactating or non-lactating) and the time of sample oocyte collection which was nested within lactation while accounting for the effect of each animal and BHBA

measurement at the time of collection. Further, we analyzed data based on the effect of BHBA level only at the time of sample oocyte collection while accounting for the effect of each animal and DIM. A significant difference between samples was reported at FDR adjusted *P*-value < 0.05 for multiple comparisons. Further, relative fold change for the significantly expressed genes was calculated by the equation $2^{-(\Delta\Delta Ct)}$ to make the final determination of the significant difference, and the cut-off was set at a fold-change equal to one, which is about 100% increase. The increase in fold change was calculated by determining the difference in fold change between two significantly different ΔΔCt values and divided on the fold change of the largest ΔΔCt value. For example, the value of (ΔΔCt X = 1.388) , and the value of (ΔΔCt Y = -1.141) the equation used is $\frac{2-\hat{}(\Delta\Delta Ct\ Y)-\ 2\hat{}-(\Delta\Delta Ct\ X)}{2-\hat{}(\Delta\Delta Ct\ X)} = \frac{2.207 - 0.382}{0.382}$ = 4.77 which means 477% increase in Y relative to X. Log2 of fold change was calculated to determine the relative expression level between samples and was used to create the figures.

Follicular Fluid and Serum sample Collection and Processing

Five multiparous Holstein-Friesian dairy cows and five non-lactating heifers were randomly assigned to be used in this experiment. Follicular fluid was collected from the lactating cows three times at 41, 71, and 131 DIM on average and one time from a group of heifers. The estrous cycle of all animals was synchronized before aspiration of the follicular fluid as described above excluding the FSH treatment for superovulation. Preovulatory follicles were aspirated from each animal using the transvaginal ultrasound-guided stainless needle in a procedure similar to the OPU technique except, no flush medium or heparin were used. Pure follicular fluid was allowed to drain from the follicles

into a 50 ml tube. Between 500 to 1000 µl of the recovered follicular fluid was transferred into a 1.5 ml centrifuge tube and centrifuged for 5 min at 10,000 x g to allow the tissue debris and blood cells to sediment out, and 200 µl of the clear follicular fluid was aliquoted into a new tube, snap frozen in liquid nitrogen, and stored at -80 °C until analysis.

Corresponding blood samples were drawn from the coccygeal vein from each animal before aspiration of the follicular fluid. From the whole blood, BHBA and glucose were immediately measured using the digital hand-held device (Precision Xtra). Whole blood was allowed to clot for 30-60 minutes at room temperature, then placed on ice. After complete clot, serum was isolated by centrifugation for 15 minutes at 2,000 x g, aliquoted, and stored at -80 °C until future analysis.

Metabolomic Analysis of Follicular Fluid and Serum Samples

In a 1.5 ml centrifuge tube, 4 µl of internal standard (1000 ppm of ribitol) was added to 50 µl of each serum and follicular fluid sample. Then, 1 ml of cooled extraction buffer (a mixture of acetonitrile, isopropanol, and water) was added to the sample and briefly vortexed for 10 sec. The mixture was placed on ice and put in the shaker for 5 min. After shaking, the samples were centrifuged for 2 minutes at 10,000 x g. Into clean 1.5 ml centrifuged tube, 450 µl of the supernatant was aliquoted and allowed to evaporate by centrifugation for about 2 hours for complete dryness. The dried aliquot was resuspended in 450 µl nitrogen-degassed acetonitrile/water (50/50) at room temperature. After 20 min centrifugation (10,000 x g) of the resuspended samples, the supernatant was transferred to a fresh 1.5 ml polypropylene tube. The supernatant was evaporated again to complete dryness in the Speed vacuum concentrators. Derivatization was performed by

adding 30 µl of MeOX (20 mg/mL Methoxyamine hydrochloride) solution to each dried sample and shaken at maximum speed for 1.5 hours at 30°C. Finally, the resuspended contents were transferred to glass vials with micro-inserts inserted and capped immediately.

Metabolome analysis was performed by gas chromatography-mass spectrometry (GC-MS). In brief, one µl of the resuspended sample after derivatization was injected into glass liners with one pre-injection wash and two post-injection washes with ethyl acetate. The flow of helium column carrier gas was 1 ml/min, and the initial oven temperature was 60 °C for 60 seconds. In the oven, the ramp rate was 10 °C/min with 10 minutes until the final temperature of 325 °C. Transfer line temperature was 290 °C for the mass spectrometer, and ion source temperature was 230°C and quadrupole temperature was 150°C.

The raw GC-MS data were normalized to the added internal standard (ribitol) and applied to AMDIS program, and CSV files with peak intensity were generated from http://spectconnect.mit.edu. The peak intensity values were uploaded to https://www.metaboanalyst.ca for the statistical analysis and to determine the correlation between follicular and serum samples. The correlation was based on the peak intensity values of 133 different metabolites that were detected in more than 75% of the samples. AMDIS program was used to identify the significant compounds which were reported at FDR adjusted *P*-value < 0.05 for multiple comparisons (Tukey's test).

RESULTS

The mean body weight for the lactating cows used in the study was 725.74 ± 13.60 kg within the first week postpartum. By the time of the 1st OPU at 47 ± 4.7 DIM,

they lost about 2.7% from the postpartum weight. They continued to lose weight until they reached the lowest point of 703.97 kg around 60 DIM then they gradually started to gain weight until they reached 736.18 kg by the time of the 3rd OPU procedure (Figure 2-1A). The mean of BCS values was 3.12 ± 0.3 within the first week after calving and dropped to 2.71 ± 0.45 after 45 DIM. Milk yield was recorded daily and averaged for each week. The mean milk yield was 27.85 ± 4.23 kg/day within the first week, and rapidly increased to reach the peak of 42.88 ± 5.53 kg/day, by 45 DIM (Figure 2-1B). Table 2-3 shows the mean of DIM, BHBA, glucose, milk yield, body weight, and BCS at the time of oocyte collection for the lactating dairy cows. BHBA and glucose were measured two to three times per week starting the first week after calving until wk 19. Figure 2-2 demonstrates the changes in BHBA and glucose during early lactation from wk 1 to wk 19 for the lactating cows used in the study.

All the cows and heifers used in the study were synchronized and treated with FSH for superovulation before OPU procedure (Figure 2-3). Oocytes were collected for the same animals three times, but the number of oocytes used in the final statistical analysis and animals represented for each time point was variable. From the 1st OPU, twenty-seven oocytes from ten animals were used. From the 2nd OPU, thirty-six oocytes from eleven animals were used. From the 3rd OPU, thirty-one oocytes from nine animals were used. From the heifers' group, eighteen oocytes from eight animals were used. The effect of the animal (the source of the oocyte) was accounted for in the statistical model.

In the analysis of the differential gene expression between individual oocytes, we performed the analysis in three different ways. First, we compared the oocytes collected from non-lactating heifers with the oocytes collected from lactating cows at each of the

three different times. Second, we compared between oocytes collected from lactating cows at the three different times during lactation. Third, we compared between oocytes based on the BHBA level as low , medium, or high of the lactating cows at the time of OPU while accounting for the fixed effect of DIM. The number of oocytes was variable between different BHBA groups; twenty-three in the low BHBA group, forty-three in the medium BHBA group, and twenty-eight in the high BHBA group.

In the comparison between lactating cows and heifers, statistical analysis did not show any genes to be significantly expressed at adjusted *P*-value < 0.05 (see Supplementary Figures 2-1 through 2-8). However, the fold change of three genes was noticeably higher in the oocytes from non-lactating heifers in comparison with those from lactating cows. These genes are; glutaredoxin (*GLRX*) was 4.78-fold higher, the imprinted maternally expressed transcript of *H19* was 20.57-fold higher, and necdin homolog (*NDN*) was 12.66-fold higher. After careful analysis of these genes, we noticed a wide range of standard error due to low expression in some reactions which prevented them from being statistically different (see supplementary Figures 2-4 and 2-8).

In the comparison between individual oocytes within the group of lactating cows, no genes were found to be differentially expressed due to progression in DIM. However, six genes were found to be significantly affected by the BHBA level (see Table 2-4 and Figure 2-4). Three of them were from the epigenetic category: DNA (cytosine-5-)-methyltransferase 1 (*DNMT1*) was 2-fold higher in the medium BHBA group compared with low (adj. *P* = 0.02). DNA (cytosine-5-)-methyltransferase 3 beta (*DNMT3B*) was 1.54-fold higher in the medium BHBA group relative to low BHBA group (adj. *P* = 0.04). Suppressor of variegation 3-9 homolog 1 (*SUV39H1*) was 1.21-fold higher in high

BHBA group relative to low BHBA group (adj. $P = 0.04$). From the metabolism category, glutamate dehydrogenase 1 (*GLUD1*) was 1.22-fold higher in high BHBA group in compare with medium BHBA group (adj. $P = 0.04$). From the redox regulation category, glutathione reductase (*GSR*) was 1.19-fold higher in medium BHBA group compared with the low BHBA group (adj. $P = 0.04$). From the heat shock protein category, heat shock protein 90kDa alpha class A member 1(*HSP90AA1*) was one-fold higher in high BHBA group in compare with medium BHBA group. For more details about all genes tested that were grouped by BHBA level see Supplementary Figures 2-9 through 2-16.

In the metabolomic analysis Table 2-5 and Figure 2-5 show the correlation between follicular fluid and serum sample. The average correlation coefficient across all follicular fluid and matching serum samples from the same animal at the same time was 0.74 ± 0.15. In the serum samples, the NEFA profile of the three predominant fatty acids (palmitic acid, stearic acid, and oleic acid) found to be significantly affected by the stage of lactation as their concentration was at the highest in serum samples collected around 41 DIM (Oleic acid adj. $P = 0.01$, palmitic acid adj. $P = 0.03$, and stearic acid adj. $P = 0.03$). The concentration of serum beta-hydroxybutyrate was, in general, higher in lactating cows compared to non-lactating heifers (adj. $P = 0.03$) (Figure 2-6). Even though there was a correlation between serum and follicular fluid samples ($r = 0.74$), none of the metabolites detected by GC-MS in all samples was found to be significantly different in follicular fluid from lactating cows due to changes in DIM or between lactating cows and heifers.

DISCUSSION

It is well documented that modern high-yielding dairy cows have a noticeable low conception rate and weak reproductive performance relative to their ancestors more than 30 years ago (Lucy, 2001; Weigel, 2006; Leroy et al., 2008c). Fertility is a multifactorial trait, and several factors can contribute to low reproductive performance such as uterine infection, silent estrous signs, incompetent oocytes and low-quality embryos (Walsh et al., 2011). Since major challenges facing modern dairy cows are the increased energetic demands and severe metabolic changes in a relatively short time, it is reasonable to expect their effects to be reflected, to a certain degree, on the intraovarian follicular environment which in turn may influence the oocyte quality and subsequent embryo survival. Therefore, the objective of the current study was to investigate the effect of lactation and energy status on the molecular constitution of the oocyte. This study used the powerful BioMark™ platform for single-cell gene expression analysis from Fluidigm which allowed us to evaluate the expression of sixty-four genes from a variety of functional categories by qPCR in every single oocyte. This way allowed us to account for variation between individual oocytes even when they were aspirated from the same animal. Also, the oocytes were collected from the same lactating cow multiple times during lactation at specific timepoint which allowed us to account for the maternal effect and use the same animal as control (i.e., compare oocytes from the same animal during NEB period and then when they shift to positive energy balance). Unlike lactating cows, dairy heifers are not facing the same metabolic challenge induced by lactation and NEB. Thus, they are a good counterpart to compare with as they are supposed to be in positive energy balance, gaining weight, and have relatively stable metabolic profile compared to

lactating cows (Bender et al., 2010). Age and other physiological differences between cows and heifers were taken into consideration in our model, so there was a clear distinction between lactating and non-lactating animals when the statistical analysis was performed.

The fertility of dairy heifers is not declining like the fertility of lactating dairy cows (Pryce et al., 2004), which supports the concept that this phenomenon is mostly due to the increase in milk yield which promotes excessive fat mobilization (Leroy et al., 2008a). Elevation of BHBA and NEFA in the circulation of lactating dairy as a result of the excessive fat mobilization during the period of NEB was proposed in several studies to be detrimental to the oocyte developmental competence and embryo quality (Leroy et al., 2008c; Van Hoeck et al., 2013; O'Doherty et al., 2014). Changes in the circulation are reflected in the follicular fluid as it is derived from the blood through thecal capillaries which make the intrafollicular environment the major link between systemic metabolism and oocyte quality (Leroy et al., 2011).

Ovulation of a competent oocyte is the first step toward a successful pregnancy. It is generally accepted that oocyte quality profoundly influences embryo developmental competence and survival (Keefe et al., 2015). Early embryonic loss is believed to be the major cause of reduced fertility in dairy cows (Walsh et al., 2011). Up to 90% of ovulated oocytes are successfully fertilized, but only 55% survive to term, and 70 – 80% of the lost embryos die within the first two weeks of gestation (Moussa et al., 2015). It is very important to identify the pathways by which NEB and associated metabolic fluctuations interfere with reproductive performance through oocyte and the resulting embryo at the molecular level in order to substantially improve fertility. Van Hoeck et al. (2013)

incubated bovine oocytes in media mimicking elevated NEFA conditions in high-yielding dairy cows as they had been determined in a previous study by Leroy et al. (2005b). They used a group of NEFA-exposed oocytes for in vitro embryo production and evaluated the expression of genes involved in energy metabolism and mitochondrial function. They found altered expression of genes involved in REDOX regulation in NEFA-exposed oocytes, their cumulus cells, and the generated blastocysts. Interestingly, they found that the embryos developed from NEFA-exposed oocytes had higher expression of genes related to fatty acid synthesis which suggested increased lipogenesis in a mechanism leading to sequestering fatty acids as triacylglycerols in lipid droplets. Intracellular accumulation of lipid droplets was found to be indicative of lower embryos quality as they failed to reach the blastocyst stage (Leroy et al., 2005a). Van Hoeck et al. (2013) concluded that perturbations in the environment where the oocyte completes final maturation may persist and have a substantial consequence on the developing embryo as it alters the molecular constituents related to metabolic functions and REDOX maintenance.

In the experiment described herein, we conducted non-targeted metabolomic analysis for follicular fluid and corresponding serum samples from lactating cows and non-lactating heifers. Significant differences were observed in the three predominant NEFA (palmitic acid, oleic acid, and stearic acid) in the serum samples depending on the time during lactation. Serum samples collected from lactating cows around 41 DIM had a higher concentration of NEFA compared with those collected from the other days or heifers. These results were anticipated as dairy cows typically experience the highest rate of fat mobilization around this time in lactation. Beta-hydroxybutyrate was significantly

lower in heifers' serum, and that was normal as they are gaining weight rather than mobilizing fat. However, the same significant difference was not observed in the follicular samples even though there was a general overall correlation between the metabolite in the serum and follicular samples detected by GC-MS. The insignificant variation in NEFA in the follicular fluid suggests a degree of follicular homeostasis that needs to be maintained regardless of the constant metabolic fluctuation in the serum or indicate partial reflection of serum metabolic profile. Bender et al. (2010) compared the metabolic differences between follicular fluid and serum obtained from lactating cows and heifers using GC–MS. The analysis of metabolomic data of their experiment revealed that follicular fluid had a unique fatty acid composition in comparison with serum. Interestingly, the concentration of these fatty acids was higher in the follicular fluid than serum (Bender et al., 2010), unlike our results which display a moderately lower concentration of the detected fatty acids in follicular fluid compared to serum. Nevertheless, our results are in agreement with an earlier study by Leroy et al. (2005b) where they showed that NEFA concentrations in dairy cows during NEB are partly reflected in the follicular fluid and remain 40% lower than serum. Even though the metabolic fluctuation in the serum is not highly reflected in the follicular fluid, indeed a prolonged period of NEB and elevated NEFA, to some degree, is likely to have an impact on the intrafollicular metabolic and chemical compositions, and adversely influence the maturing oocyte that is actively communicating with its surrounding.

Based on our gene expression findings, it seems NEB, not lactation or the stage of lactation, is driving the alteration in the gene expression of the selected RNA transcripts. The state of energy balance was determined by the level of BHBA in the circulation prior

to the OPU procedure. The level of BHBA in the circulation is commonly used as an indicator of the energy status in dairy cows as it positively correlates with the amount of adipose tissue that is being mobilized for energy (Barletta et al., 2017). Even though the change in the concentration of the circulating BHBA follows a typical pattern in dairy cows and correlates closely with DIM, our results do not suggest an immediate effect of the stage of lactation on the gene expression in oocytes. This can be explained by the individual variation in terms of fat mobilization and BHBA level among animals in response to NEB as it is highly associated with subcutaneous adipose tissue (Barletta et al., 2017). In the current experiment, it was noticeable that the cows with higher BCS consistently tend to have higher BHBA level through multiple weekly measurements of BHBA concentration for nineteen weeks. The result also implies that the intensity of NEB, rather than duration, is what may drive the alteration of gene expression in oocytes.

The relative expression level of six genes out of sixty-four selected for qPCR quantification was significantly impacted by the BHBA level in the circulation. Interestingly, three of them were from the epigenetic category (*DNMT1, DNMT3B,* and *SUV39H1*). Messenger-RNA transcripts of *DNMT1* and *DNMT3B* were found to be more highly expressed in oocytes collected from lactating cows with elevated BHBA concentration which is a positive indication of high-fat mobilization rate. Fat mobilization is associated with increased oxidation of NEFA in the liver leading to excessive production of reactive oxygen species (ROS) which raise the level of oxidative stress in the body (Morris et al., 2009). The relative overexpression of the epigenetic transcripts mentioned above in the same oocytes could be linked to the oxidative stress (Menezo et al., 2016). The significant increase in the expression of these genes could be

an indication of ongoing methylation process in oocytes that apparently recovered from preovulatory follicles and supposedly had completed their growth phase and have already acquired their DNA methylation (O'Doherty et al., 2014). Oxidative stress was demonstrated to weaken the affinity between DNA and DNMTase leading to disrupted and poor methylation, which could result in over-expression of DNMTase activity (Menezo et al., 2016). In an experiment examining the dynamic changes of DNA epigenetic marks in bovine oocytes, the expression of SUV39H1 and SUV39H2 has been found to have lower mRNA in mature oocytes, that theoretically reached epigenetic stability, relative to those at germinal vesicle stage (Zhang et al., 2016). In our experiment, SUV39H1 had higher expression in oocytes collected from lactating cows with high BHBA concentration which may suggests ongoing epigenetic activity in these oocytes.

Oxidative stress has a direct negative impact on male and female gametes (Menezo et al., 2016). Increased intrafollicular oxidative stress was found to be deleterious to the oocyte quality in several mammalian species including human (Prasad et al., 2016). Oxidative stress and excessive generation of ROS disrupts redox signaling status and alter intracellular redox potential (Jones, 2006). Our analysis of relative gene expression shows an increased expression of *GSR* in oocytes retrieved from lactating cows with elevated BHBA. *GSR* encodes glutathione-disulfide reductase, an enzyme critical to resist oxidative stress and maintain redox homeostasis (Couto et al., 2016). The relative overexpression of *GSR* in these oocytes can be an indication of a disrupted redox state and oxidative stress.

One of the genes involved in metabolism, *GLUD1*, was found to be higher in oocytes from high BHBA group. *GLUD1* encodes glutamate dehydrogenase, an enzyme that catalyzes the oxidative deamination of glutamate to alpha-ketoglutarate and ammonia (Plaitakis and Zaganas, 2001) and is involved in various cellular processes including redox homeostasis and metabolism of ammonia (Plaitakis et al., 2017). Overexpression of *GLUD1* could be an indication of increased demand for glutamate dehydrogenase due to the altered redox state or reflection of increased utilization of body protein and amino acids for energy during the period of NEB. Further, the expression of *HSP90AA1* was relatively higher in the oocytes from the high BHBA group. Heat shock protein 90α (Hsp90α) is encoded by the *HSP90AA1* gene, and is one of the heat shock proteins that function as chaperonins and are evolutionary conserved and normally expressed in cells, but overexpressed in response to cellular stress caused by various stimuli (Zuehlke et al., 2015). Up-regulation of *HSP90AA1* in these oocytes might be due to increased cellular stress caused by altered redox state.

In conclusion, the result shows BHBA-induced alteration in relative expression level of six genes in oocytes of lactating dairy cows. These genes are selected from different functional categories including epigenetics, metabolism, heat shock, and redox regulation. Thorough analysis of the qPCR data of these genes by grouping the oocytes by DIM and BHBA level indicates that the intensity of NEB, rather than duration, is what may impact oocyte quality. Our findings suggest an interference with epigenetic stability in oocytes of lactating dairy cows during NEB. This process might be mediated by the increased oxidative stress due to excessive fat mobilization and high rate of β-oxidation

of fatty acids. However, further research is required to obtain a deeper understanding of how NEB interferes with oocyte quality.

TABLES AND FIGURES

Table 2-1. Definition of gene names used in qPCR analysis of individual oocytes

Gene symbol	Gene name
ACACA	Acetyl-CoA carboxylase alpha
ACSL1	Acyl-CoA synthetase long-chain family member 1
ACSL3	Acyl-CoA synthetase long-chain family member 3
ACTB	Beta Actin
ASH2L	Ash2 (absent, small, or homeotic)-like
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BMP15	Bone morphogenetic protein 15
CAT	Catalase
CCNB1	Cyclin B1
CDK2	Cyclin dependent kinase 2
DNMT1	DNA (cytosine-5-)-methyltransferase 1
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
EHMT2	Euchromatic histone-lysine N-methyltransferase 2
EIF4A1	Eukaryotic translation initiation factor 4A1
EZH2	Enhancer of zeste homolog 2
FABP3	Fatty acid binding protein 3
FADD	Fas associated via death domain
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLRX	Glutaredoxin
GLRX3	Glutaredoxin 3
GLUD1	Glutamate dehydrogenase 1
GNAS	GNAS complex locus
GPX1	Glutathione peroxidase 1
GRB10	Growth factor receptor-bound protein 10
GSR	Glutathione reductase
H19	H19, imprinted maternally expressed transcript
HDAC1	Histone deacetylase 1
HDAC2	Histone deacetylase 2
HSF1	Heat shock transcription factor 1
HSP10	Heat shock protein family E (Hsp10) member 1
HSP110	Heat shock protein family H (Hsp110) member 1
HSP60	Heat shock protein family D (Hsp60) member 1 (HSPD1)
HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1
HSP90AB1	Heat shock protein 90 alpha family class B member 1
HSPA1A	Heat shock 70kDa protein 1A

HSPA2	Heat shock protein family A (Hsp70) member 2
IGF1R	Insulin like growth factor 1 receptor
IGF2R	Insulin-like growth factor 2 receptor
KLF4	Kruppel-like factor 4
LIN28A	Lin-28 homolog A
MAPK3	Mitogen-activated protein kinase 3
MAPK8	Mitogen-activated protein kinase 8
MDM2	Mdm2, p53 E3 ubiquitin protein ligase homolog
NDN	Necdin homolog
PEG10	Paternally expressed 10
POU5F1	POU class 5 homeobox 1
PPARG	Peroxisome proliferator activated receptor gamma
SCD	Stearoyl-CoA desaturase (delta-9-desaturase)
SCO2	SCO cytochrome oxidase deficient homolog 2
SLC2A1	Solute carrier family 2 member 1
SOD1	Superoxide dismutase 1, soluble
SOD2	Superoxide dismutase 2, mitochondrial
SOX2	SRY (sex determining region Y)-box 2
SUV39H1	Suppressor of variegation 3-9 homolog 1
TAF11	TATA-box binding protein associated factor 11
TXN	Thioredoxin
TXNRD1	Thioredoxin reductase 1
UBE3A	Ubiquitin protein ligase E3A
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
ZAR1	Zygote arrest 1
ZP2	Zona pellucida glycoprotein 2

Table 2-2. Functional category of genes tested by qPCR in individual oocytes

Functional categories	Number of genes tested	Name of genes tested
Apoptotic Regulators	6	*BAX, BCL2, FADD, MAPK3, MAPK8, MDM2*
Epigenetic Modifiers	8	*DNMT1, DNMT3A, DNMT3B, EHMT2, EZH2, HDAC1, HDAC2, SUV39H1*
Heat shock Proteins	8	*HSF1, HSP10, HSP110, HSP60, HSP90AA1, HSP90AB1, HSPA1A, HSPA2*
Housekeeping	5	*ACTB, EIF4A1, GAPDH, TAF11, YWHAZ*
Imprinting	7	*ASH2L, GNAS, GRB10, H19, NDN, PEG10, UBE3A*
Metabolism	10	*ACACA, ACSL1, ACSL3, FABP3, G6PD, GLUD1, IGF1R, IGF2R, PPARG, SLC2A1*
Oocyte Specific	6	*BMP15, CCNB1, CDK2, SCD, ZAR1, ZP2*
Pluripotency Factors	4	*KLF4, LIN28A, POU5F1, SOX2*
Redox Regulation	10	*CAT, GLRX, GLRX3, GPX1, GSR, SCO2, SOD1, SOD2, TXN, TXNRD1*

Table 2-3. Oocyte collection time during lactation with BHBA, glucose, milk yield, and body weight

OPU	DIM	BHBA (mg/dL)	Glucose (mg/dL)	Milk yield (kg/day)	Body weight (kg)	BCS (1-5 scale)
Initial	7 ± 1.30	7.41 ± 0.88	49 ± 2.9	27.66 ± 1.27	725.74 ± 13.20	3.0 ± 0.11
1st Collection	47 ± 1.30	10.54 ± 2.04	52.00 ± 1.90	42.38 ± 1.37	706.83 ± 17.59	2.71± 0.12
2nd Collection	75 ± 1.30	13.23 ± 2.08	51.85 ± 2.08	42.61 ± 1.14	704.53 ± 13.67	2.60 ± 0.09
3rd Collection	130 ± 1.35	9.23 ± 0.87	57.08 ± 1.70	36.80 ± 1.30	736.38 ± 13.24	2.96 ± 0.10

Ovum pick-up (OPU) was performed three times during lactation in a group of lactating dairy cows (n = 13) at specific days in milk (DIM) with the average of beta-hydroxybutyrate (BHBA) concentration, glucose concentration, milk yield, body weight, and body condition score (BCS) for the initial week and the week prior to OPU procedure. Data are presented as means ± SEM.

Table 2-4. List of genes tested by qPCR in individual oocytes and found to be differentially expressed based on BHBA level

Gene name	Lactating cows						
	BHBA level	Avg. Relative Expression Level	S.E.M	BHBA level	Avg. Relative Expression Level	S.E.M	Adj. *P*-value
DNMT1	Low	4.41	0.36	Medium	5.99	0.23	0.02
DNMT3B	Low	9.34	0.33	Medium	10.69	0.21	0.04
GLUD1	Medium	1.26	0.23	High	2.42	0.26	0.04
GSR	Low	3.49	0.29	Medium	4.62	0.19	0.04
HSP90AA1	Medium	1.04	0.19	High	2.05	0.22	0.04
SUV39H1	Low	6.59	0.34	High	7.93	0.26	0.04

Oocytes were collected from lactating cows (n = 13) three times during early lactation and before each collection, weekly averaged concentration of BHBA for each animal was categorized as high (BHBA >12 mg/dL), medium (BHBA 8 – 12 mg/dL) and low (BHBA < 8 mg/dL). * Data are the means ± S.E.M

Table 2-5. Correlation coefficient between serum and corresponding follicular fluid sample

Sample #	Correlations (r) per sample	Correlations (r) per group	
1A	0.80		
2A	0.52		
3A	0.46	**(A)** 41 DIM	0.59 ± 0.18
1B	0.89		
2B	0.86		
3B	0.68		
4B	0.89		
5B	0.80	**(B)** 71 DIM	0.82 ± 0.09
1C	0.52		
2C	0.67		
3C	0.58		
4C	0.82		
5C	0.87	**(C)** 131 DIM	0.69 ± 0.15
6H	0.80		
6H	0.89		
7H	0.90		
8H	0.82		
10H	0.60	**(H)** Heifers	0.80 ± 0.12
Average all samples	**0.74 ± 0.15**		

* In the sample ID, the number denotes the animal number and the letter denotes the stage of lactation in lactating cows which includes (A) 41 days in milk (DIM), (B) 71 DIM, and (C) 131 DIM, or (H) non-lactating heifers.

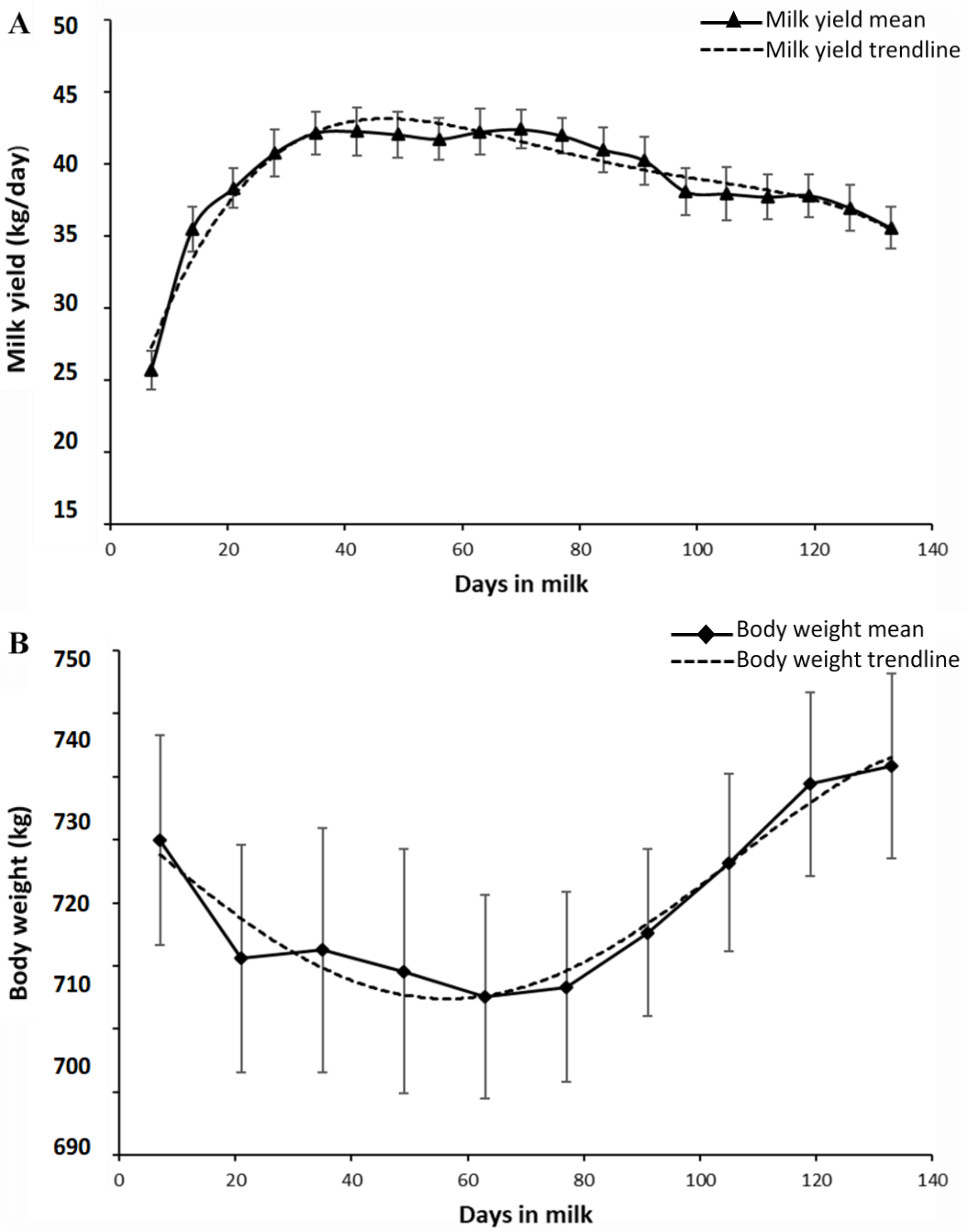

Figure 2-1. Lactation curve and changes in body weight for lactating cows used in the study to collect oocytes. (A) shows milk yield and (B) shows changes in body during the first 130 days in milk for lactating cows (n =13) during the trial. Data are the mean ± S.E.M.

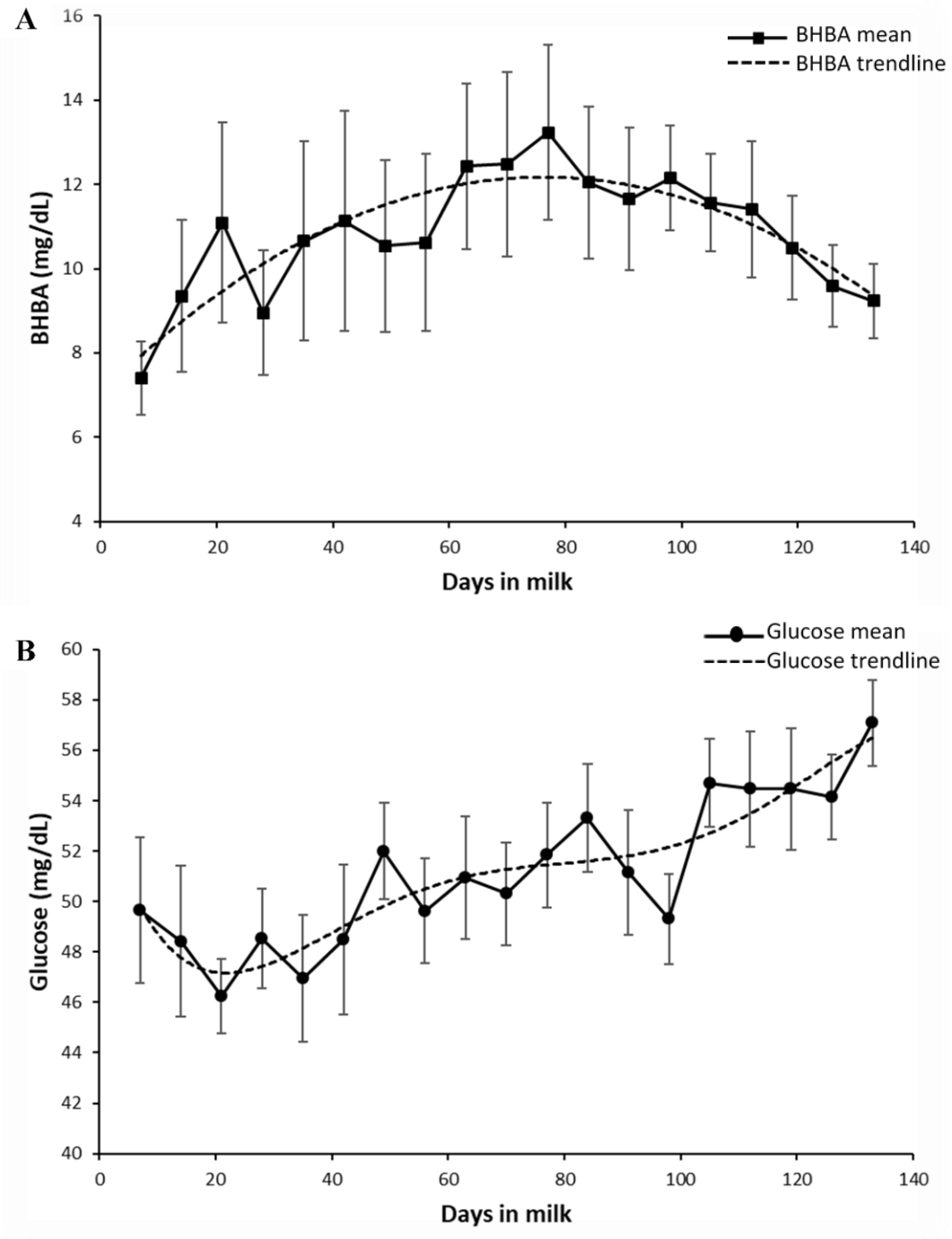

Figure 2-2. Blood concentrations of beta-hydroxybutyrate (BHBA) and glucose of lactating cows used to collect oocytes. (A) shows weekly averaged BHBA concentration (mg/dL) and (B) shows weekly averaged glucose concentration (mg/dL) during the first 130 days in milk for lactating cows (n = 13) during the trial. Data are the mean ± S.E.M.

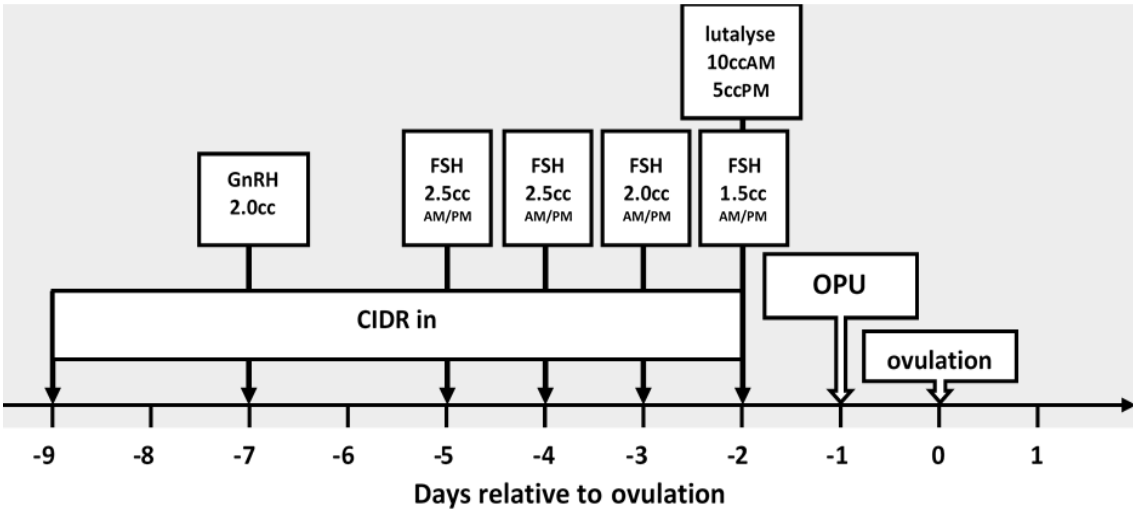

Figure 2-3. Protocol used for estrus synchronization, superovulation, and ovum pick-up (OPU)

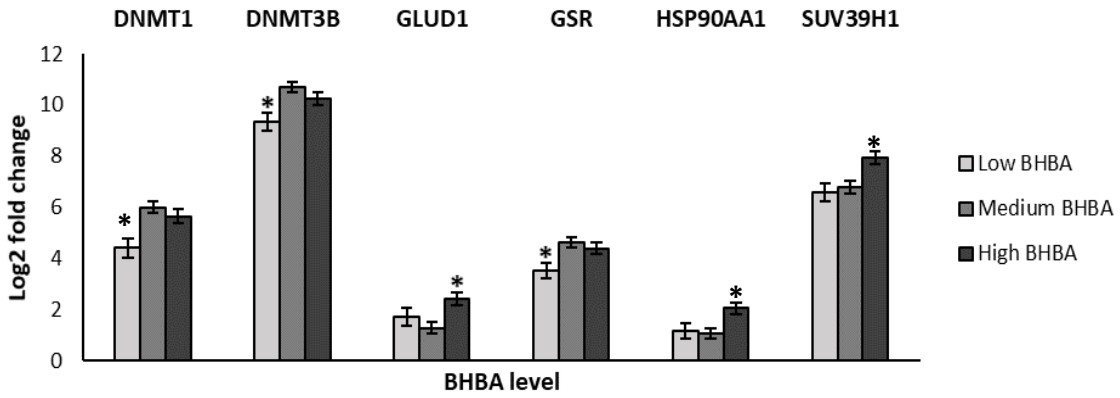

Figure 2-4. Genes that show significantly altered expression in individual oocytes collected from lactating cows grouped by beta-hydroxybutyrate (BHBA) level. Oocytes were collected from lactating cows (n = 13) three times during early lactation and before each collection, weekly averaged concentration of BHBA for each animal was categorized as high (BHBA >12 mg/dL), medium (BHBA 8 – 12 mg/dL) and low (BHBA < 8 mg/dL). The present figure shows relative expression of six genes normalized to five housekeeping genes and a calibrator sample in individual oocytes grouped by BHBA level of each animal prior to each oocyte collection. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.* indicate significant difference at adj. $P < 0.05$.

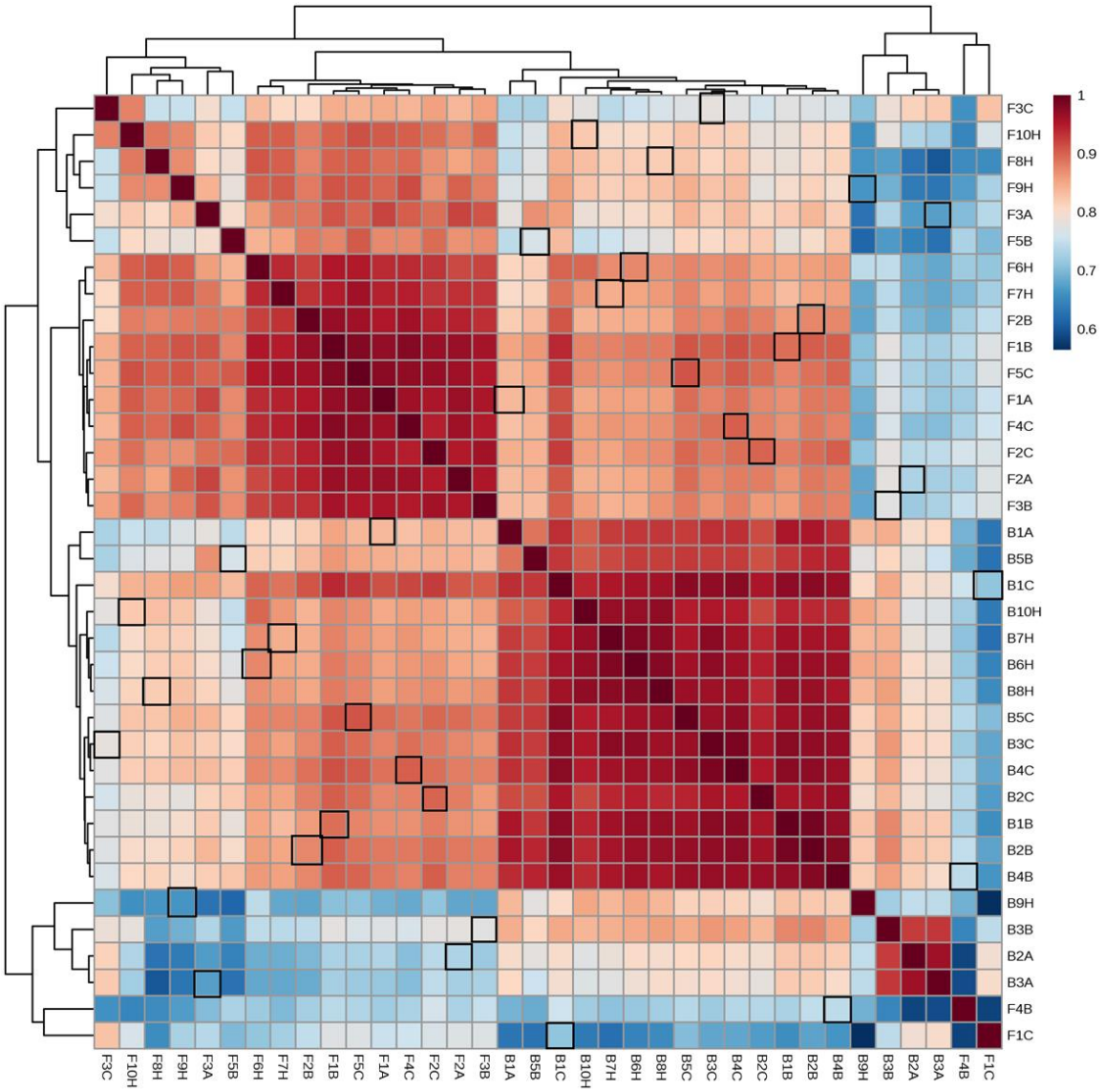

Figure 2-5. Heatmap visualization of the correlation matrix between follicular fluid and serum samples. Follicular fluid and serum samples were collected from lactating cows (n = 5) three times during lactation and one time from a group of heifers (n = 5). The hierarchical clustering is the result of Pearson's correlations between all samples. Bold square indicates a correlation between a serum sample and a follicular fluid sample collected from the same animal at the same time. The first letter in the sample ID denotes blood (B) or follicular fluid (F), the middle number denotes the animal number, and the last letter denotes the time of sample collection which was at (A) = 41 days in milk (DIM), (B) = 71 DIM, and (C) = 131 DIM or (H) = non-lactating heifer. Dark red indicates strong positive correlation (r = 1) and dark blue indicates weak positive correlation (r = 0.6).

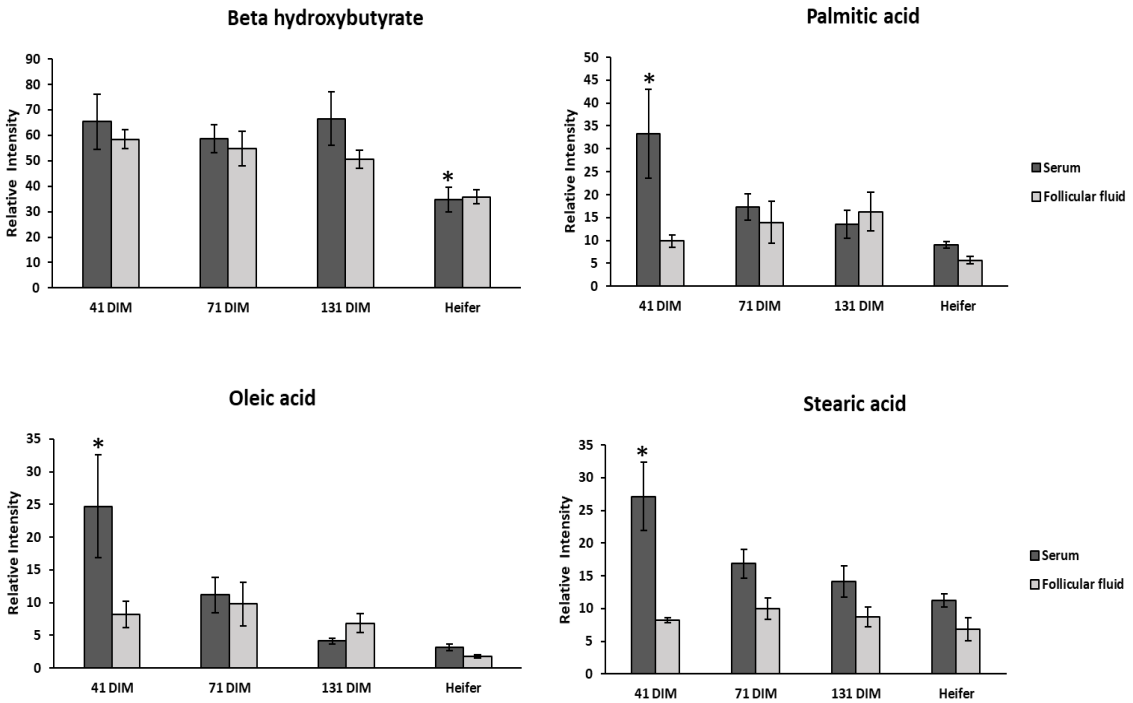

Figure 2-6. Relative peak intensity of beta-hydroxybutyrate (BHBA), palmitic acid, oleic acid, and stearic acid in serum and follicular fluid samples. The present figure shows pairwise comparison between samples collected from lactating cows (n = 5) three times during early lactation at 41, 71, 131 days in milk (DIM) and one time from a group of non-lactating heifers (n =5). * indicate significant difference within the same sample type (serum or follicular fluid) at adj. $P < 0.05$. Data are the mean ± S.E.M.

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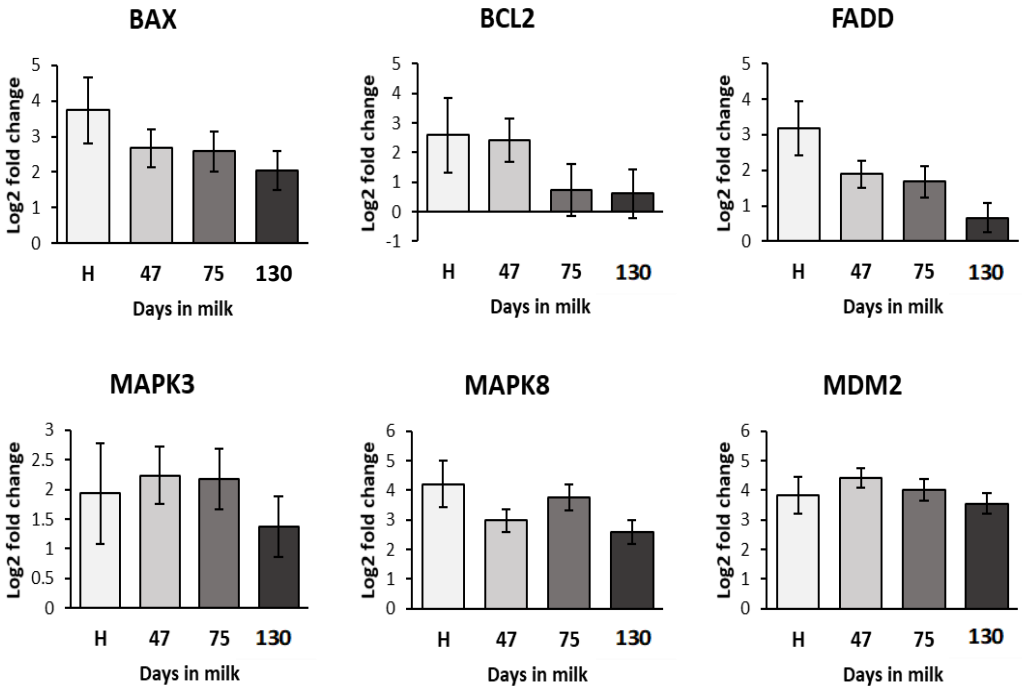
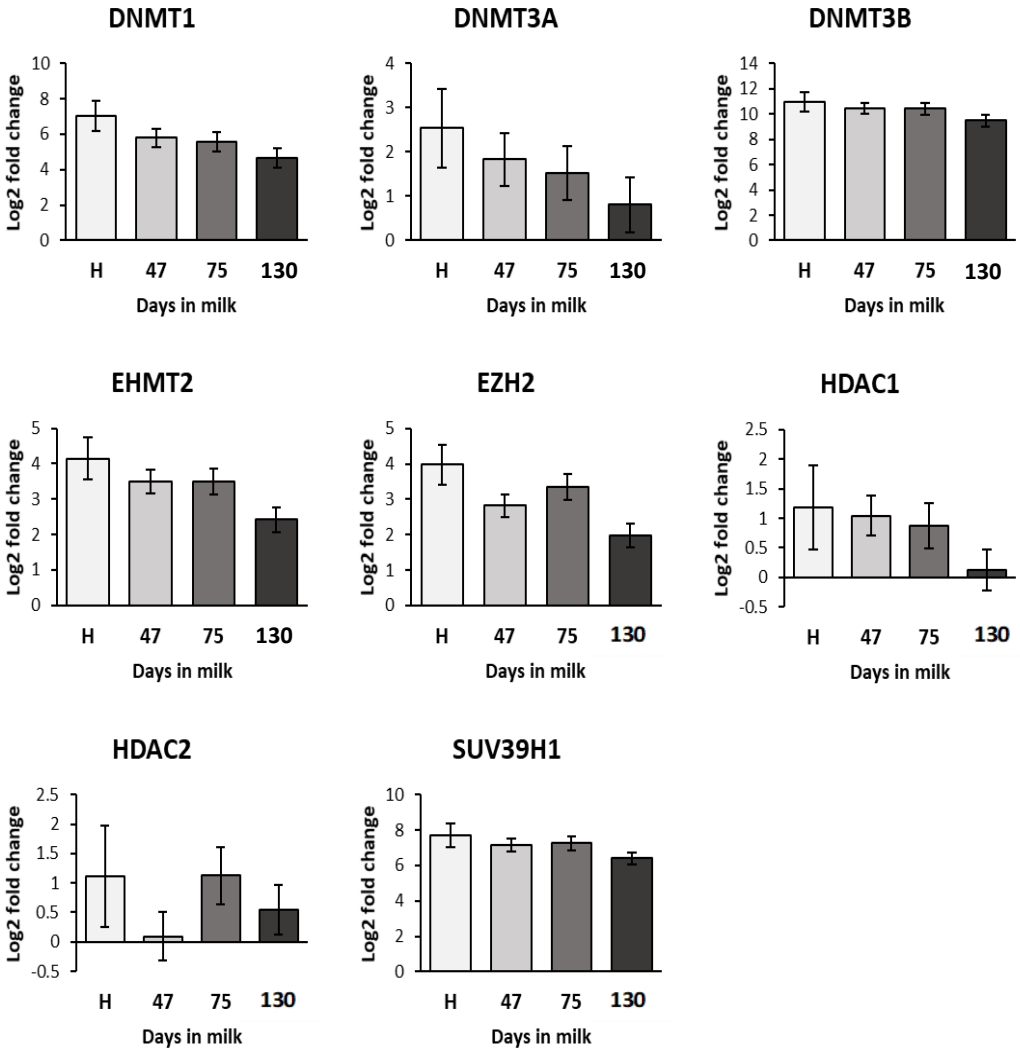
Supplementary Table 2-1. Forward and reverse primer sequences for genes used in qPCR analysis in individual oocytes

Gene	5'-forward primer-3'	3'-reverse primer-5'
ACACA	GCTAACTCAACTCAGCAAGACC	GGATGGCAAATGGGAAGCAA
ACSL1	AAGGTTTCCAGAGGGGCTTAC	TCATAGGGTTGGTCTGGTTTCC
ACSL3	TGGCGGAAAGGATTCCAGAA	AGACAGACAAGCTCAGCACTTA
ACTB	GCCAACCGTGAGAAGATGAC	CCTGGATGGCCACGTACA
ASH2L	TGCCATCATAGCGGGAACAC	ACGTTAGGTTGGCCAAAGCA
BAX	CGGGTTGTCGCCCTTTTCTA	GCCCATGATGGTCCTGATCAA
BCL2	ATGTGTGTGGAGAGCGTCAA	GGTTCAGGTACTCGGTCATCC
BMP15	TGGACACCCTAGGGAAAACC	GAGGAAAGTCCAGGGTCTGTA
CAT	CATCCAGGCTCTTTTGGACAA	TGAGACCCATGCTGCACATA
CCNB1	TTTAGTCTGGGTCGCCCTCTA	GGCCAGAGTATGTAGCTCAACA
CDK2	TTTTGGGGTCCCTGTTCGTAC	TGCAGCCCAGAAGGATTTCC
DNMT1	AGAGACGTCGAGTTACATCCA	GTGTTCCTGGTCTTACTCTTCC
DNMT3A	CCATGTACCGCAAGGCTATCTA	GCTGTCATGGCACATTGGAA
DNMT3B	GCCAAAGCTCTTCCGAGAAA	GGGTGGAGGTACTGCTGTTA
EHMT2	GACGTCCACCATGAACATTGAC	CTGGAGCAGTCATCCACACA
EIF4A1	CAGAAGCTCAACAGCAACAC	CTTCTTGGTCACCTCAAGCA
EZH2	GAGCCATCCAGACTGGTGAA	TTCGATGCCCACGTACTTCA
FABP3	ACCACAGCAGATGACAGGAA	GACAAGTTTGCCGCCATCC
FADD	TCGAGCAGCGACCTGAC	GCTCCAGCTTCCTCTTGCTA
G6PD	GTCTGGTGGCCATGGAGAA	CTGCACCTCTGAGATACACTTCA
GAPDH	GGGTCTTCACTACCATGGAGAA	GTTCACGCCCATCACAAACA
GLRX	CTCGGGTCTTCATCGGTCAA	GTCAACAGTTCCCCTCTCTCA
GLRX3	TAAGTGTGGCTTCAGCAGAC	TCATCCTCCAATATATCAAATGTCTCA
GLUD1	CCGACAACTCCAGAAGCTGATA	TGTCACTCCTCCAGCATTCA
GNAS	CCCGGGCCAAGTACTTCA	GGTGAAGTGAGGGTAGCAGTA
GPX1	CGGGACTACACCCAGATGAA	CTTCAGGCAATTCAGGATCTCC
GRB10	TTGCTGGCAGGAAGCAGTA	GCCTCGTTCCTGACTCTGTTA
GSR	AGTTGGGGATGTGTGTGGAA	GTTTTCGGCCAGCAGCAA
H19	CTTGGAACACGGACTTCTTCAA	AGGGTGTGTAGTGGTTCCAA
HDAC1	ATGTCCGAGTACAGCAAGCA	CAGAACTCAAACAGGCCATCAAA
HDAC2	AAGGAGGCGGCAAGAAGAAA	TGGGATGACCCTGTCCGTAATA
HSF1	GCAGCTCCTCGAGAACATCA	ACACTGTCCTGGCGAATCTTTA
HSP10	AGCTGTTGGATCAGGCTCTAA	GCCTCCATATTCTGGGAGAAGAA
HSP110	TTCTGATCCTCAAGGAGTTCCA	CCATCCTTCTGTGCTGAAACA
HSP60	CTTTTAGCCGATGCTGTAGCC	GGACTTCCCCAACTCTGTTCA
HSP90AA1	CATGGATAACTGCGAGGAGCTA	AGATCCTCAGAATCCACCACAC
HSP90AB1	GGCAGAAATTGCCCAACTCA	AGGGTCTGTCAGGCTCTCATA
HSPA1A	CTGATCAAGCGCAACTCCA	TTGTCCGAGTAGGTGGTGAA
HSPA2	ACGGCATCTTCGAGGTGAA	CCATGCGGTTATCGAAGTCC
IGF1R	GAACCTGCGCCAGATCCTA	GCTGCAAGTTCTGGTTGTCA

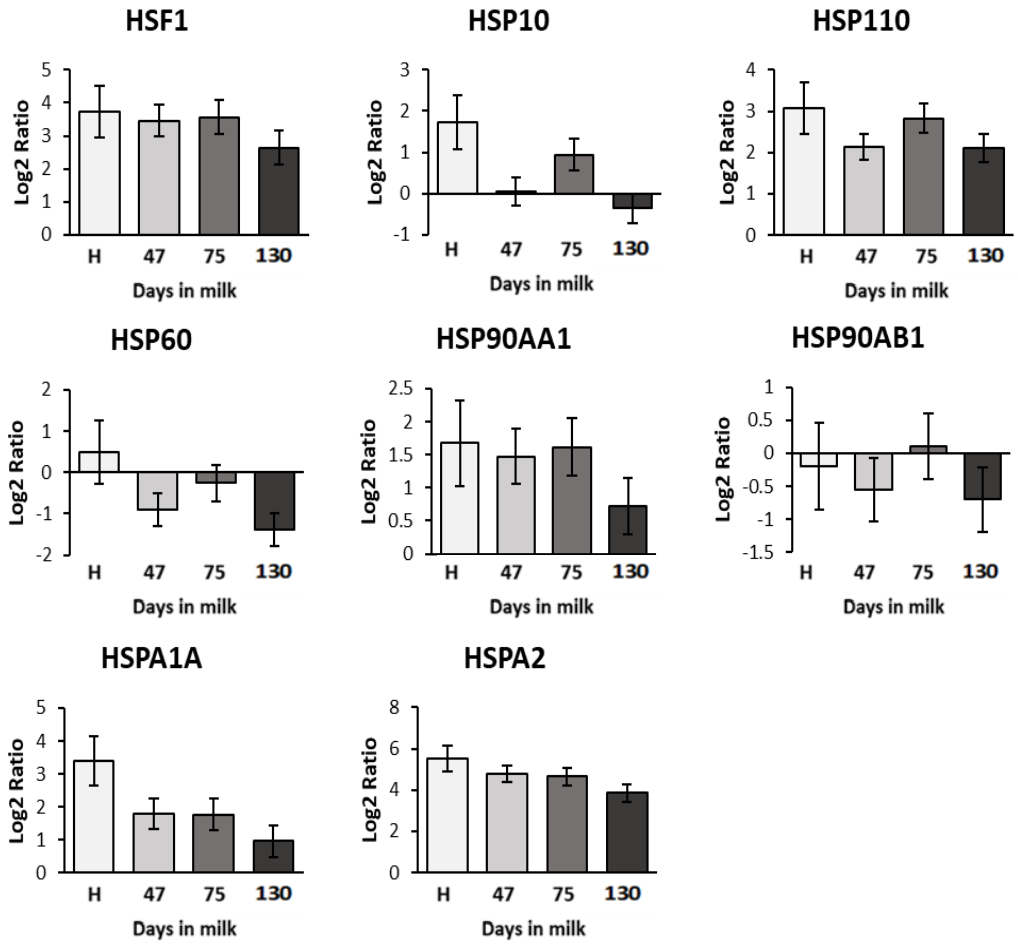
IGF2R	GGCAGATTCCACTCAAGTCA	AGATCAAGGTGAGGTCTCCA
KLF4	GCGGCAAAACCTACACGAA	CCATCCCAGTCACAGTGGTAA
LIN28A	GCGGCCCAAAGGGAAGAATA	CACTCCTTGGCATGATGGTCTA
MAPK3	CGTCATTGGCATCCGAGACA	TGTCTCCATCAGGTCCTGTAC
MAPK8	GCAGTTAGATGAAAGGGAACACA	GGTTCTCTCCTCCAAGTCCATA
MDM2	CAGATTCCAGCTTCGGAACAA	CTGTGCACCAACAGACTTCA
NDN	ATGTGGTACGTGCTGGTCAA	AACTGCCGATGACATCCTTCA
PEG10	GTGGGACCCCGTCTTTCC	GCGCGTAGTAGCTTCACTCC
POU5F1	AGAAGCTGGAGCCGAACC	CTGCTTTAGGAGCTTGGCAAA
PPARG	CTTGCTGTGGGGATGTCTCA	ATCTCCGCTAACAGCTTCTCC
SCD	ATACCGCCCTTATGACAAGACC	GGTGTGGTGGTAGTTGTGGAA
SCO2	GGCGCGAAGCAAGTAGAAG	CAGCAGCAGCATGGACCT
SLC2A1	GCTGTGCAGTGCTCATGAC	ATGGCCACAATGCTCAGGTA
SOD1	CGGTGTTGCCATCGTGGATA	TCCACCTCTGCCCAAGTCA
SOD2	GCCTACGTGAACAACCTCAAC	CTGCAGAGCTATCTGAGCTGTA
SOX2	CCCAAGAGAACCCTAAGATGCA	CCGTCTCGGACAAAAGTTTCC
SUV39H1	TCGAGTACCTGTGCGATTACA	GGGTACCCACGCCATTTTAC
TAF11	AGAGAAGAAGCAGAAAGTGGATGAA	GGTTCAGCTGCTCCTCAGAA
TXN	CGTGGTGTTCCTTGAAGTAGATG	CTGGAAGGTTGGCATGCATT
TXNRD1	CTGAGGAGAAAGCTGTGGAGAA	CGTCCATTCCAATGGCCAAAA
UBE3A	TGACGACATTGAAGCTAGCC	ACTGGTGGTAGTAGCGTTCTA
YWHAZ	AACAGCAGATGGCTCGAGAA	GAAGCGTTGGGGATCAAGAAC
ZAR1	ACCCTTACCGAGTGGAGGATA	TCCACATGGCGAAGTTTCAC
ZP2	AGAACCAGAAGATGAGCATCCA	AGCTTCAGAGGCACCATGTA



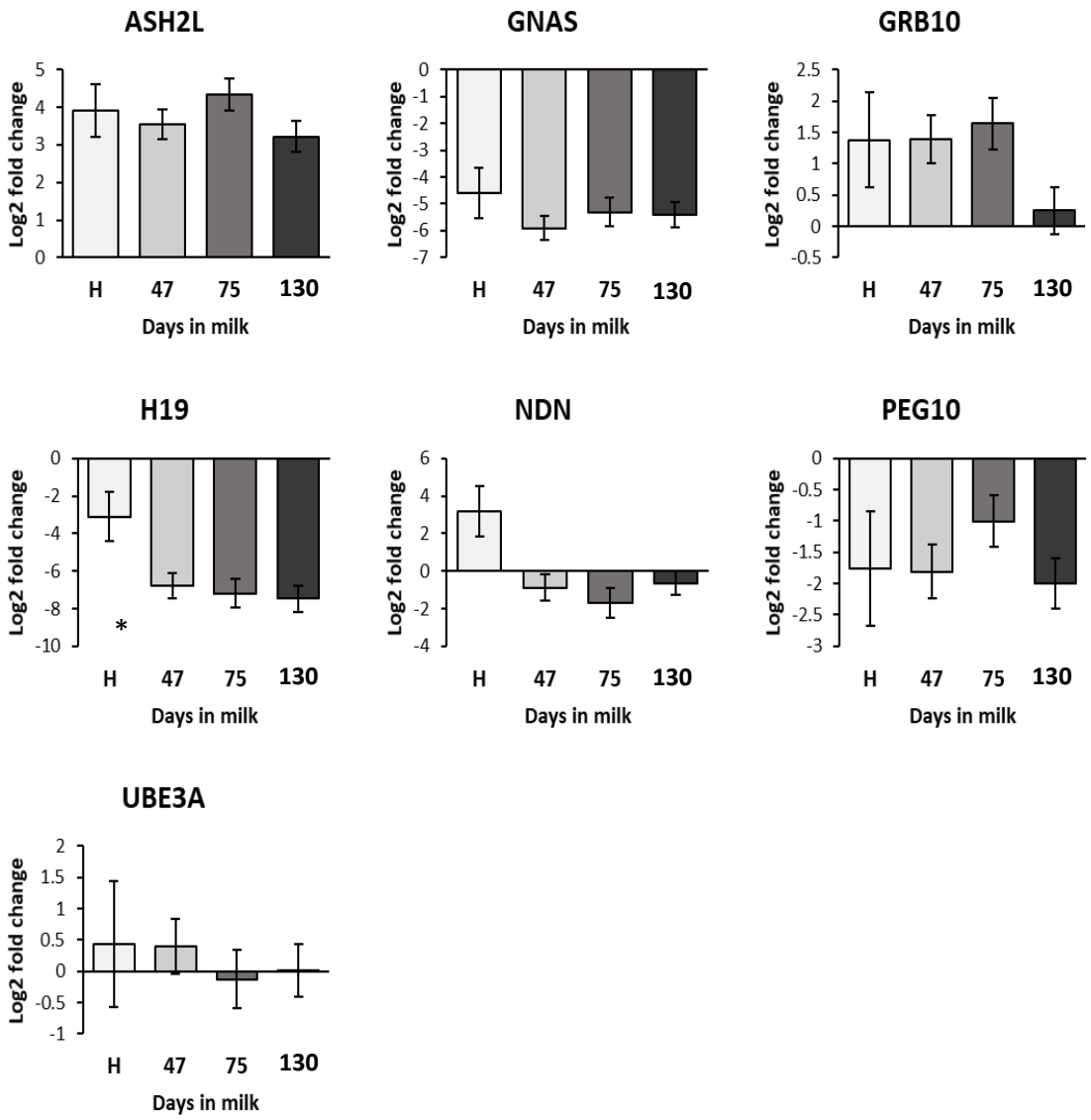
Supplementary Figure 2-1. Relative expression of genes involved in apoptotic regulation tested by qPCR in individual oocytes grouped by DIM. Oocytes were collected one time from heifers (H) and three times from lactating cows during lactation**.** None of the six genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



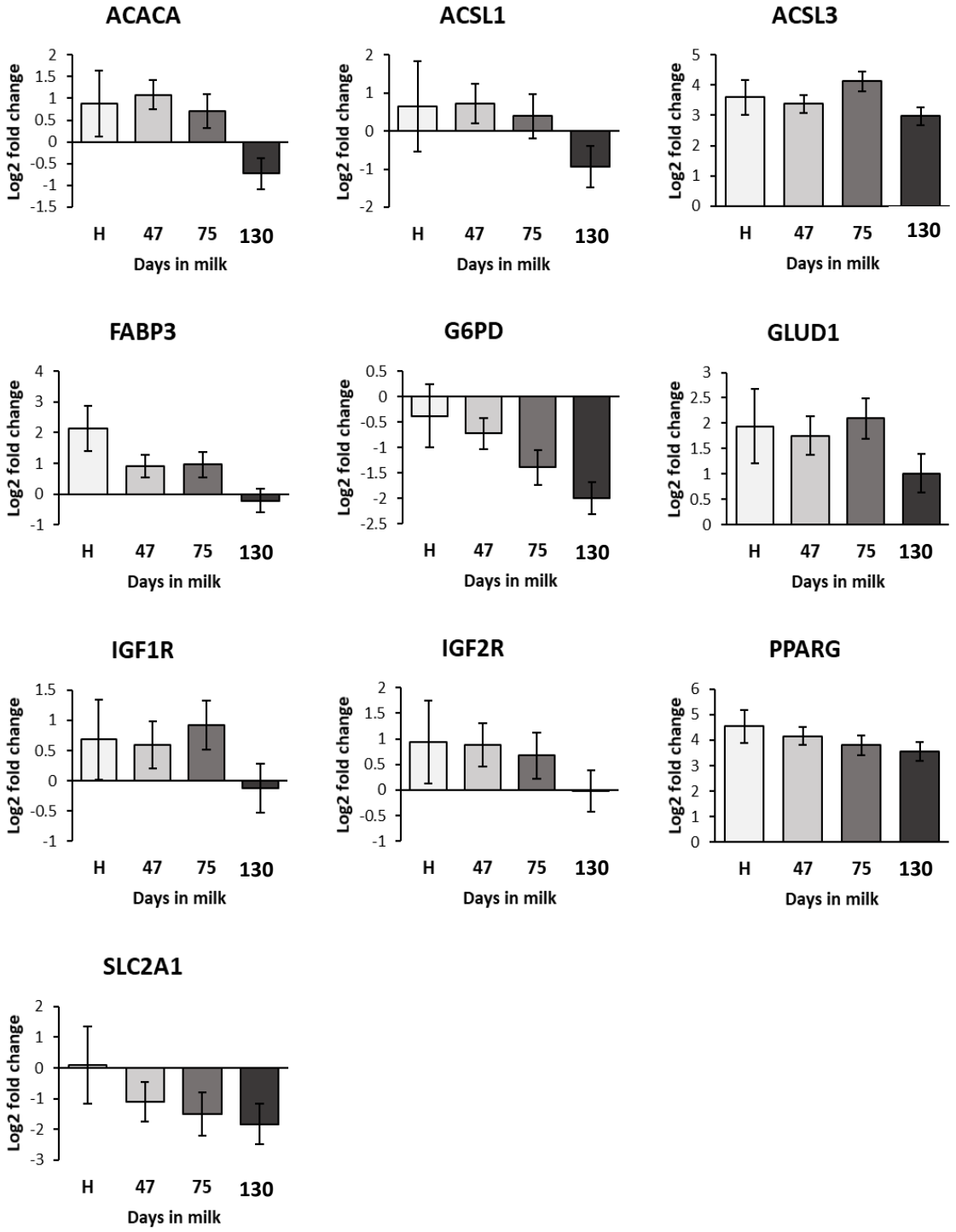
Supplementary Figure 2-2. Relative expression of genes in involved epigenetic regulation tested by qPCR in individual oocytes grouped by DIM. Oocytes were collected one time from heifers (H) and three times from lactating cows during lactation**.** None of the eight genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



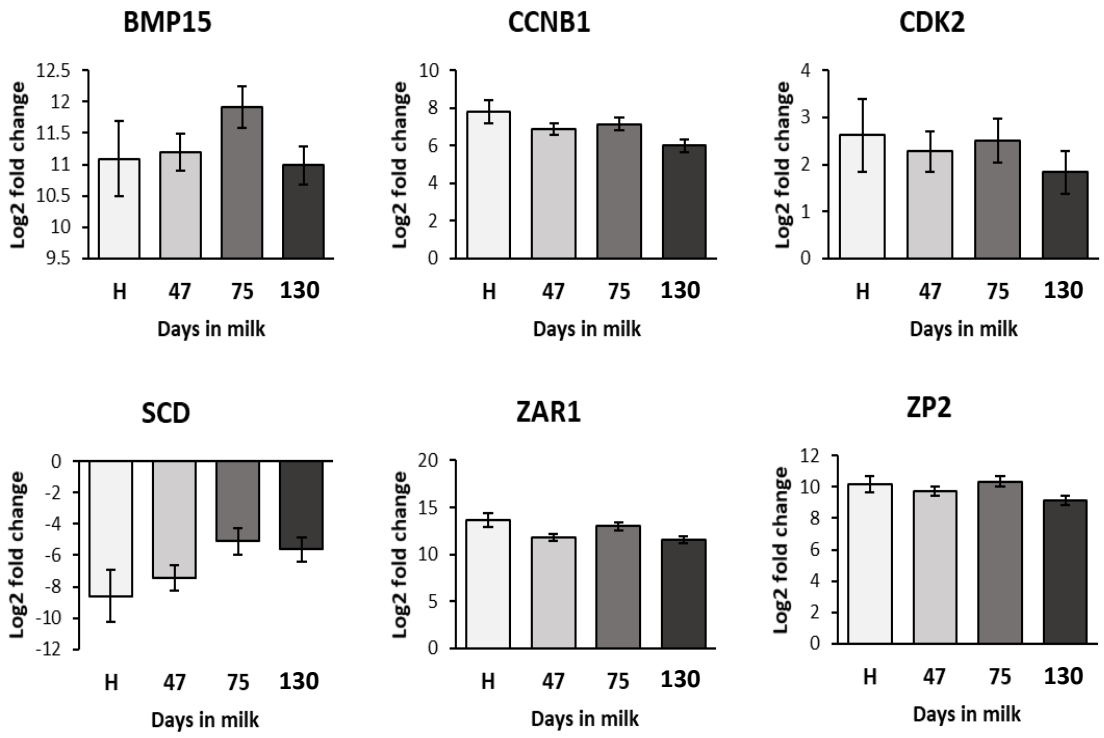
Supplementary Figure 2-3. Relative expression of genes involved in heat shock protein regulation tested by qPCR in individual oocytes grouped by DIM. Oocytes were collected one time from heifers (H) and three times from lactating cows during lactation. None of the eight genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



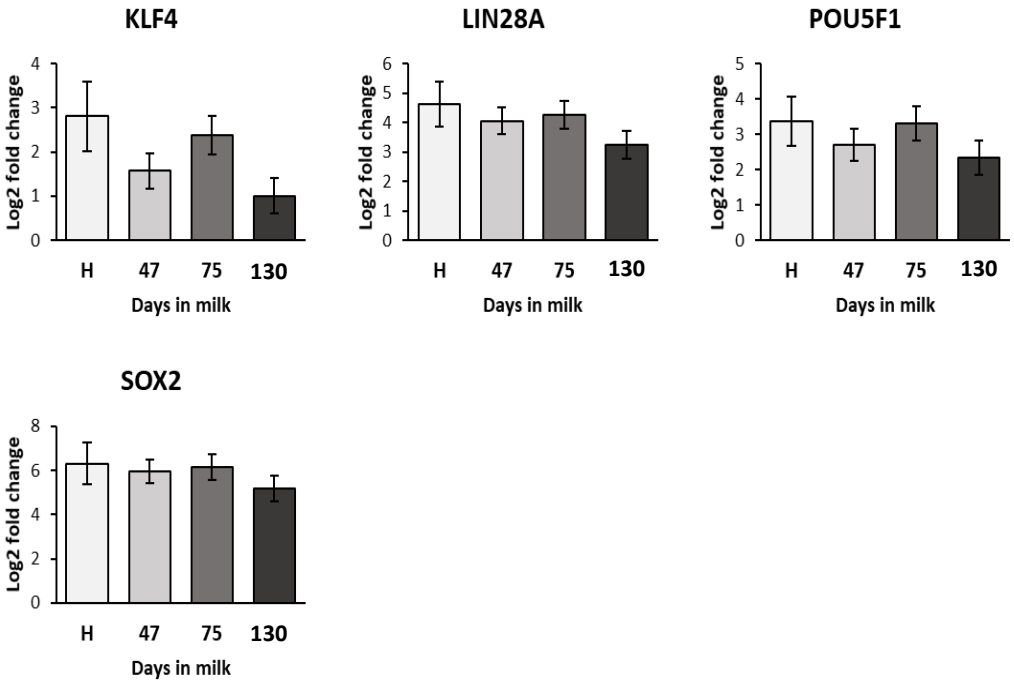
Supplementary Figure 2-4. Relative expression of genes from imprinting category tested by qPCR in individual oocytes grouped by DIM. Oocytes were collected one time from heifers (H) and three times from lactating cows during lactation. None of the seven genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



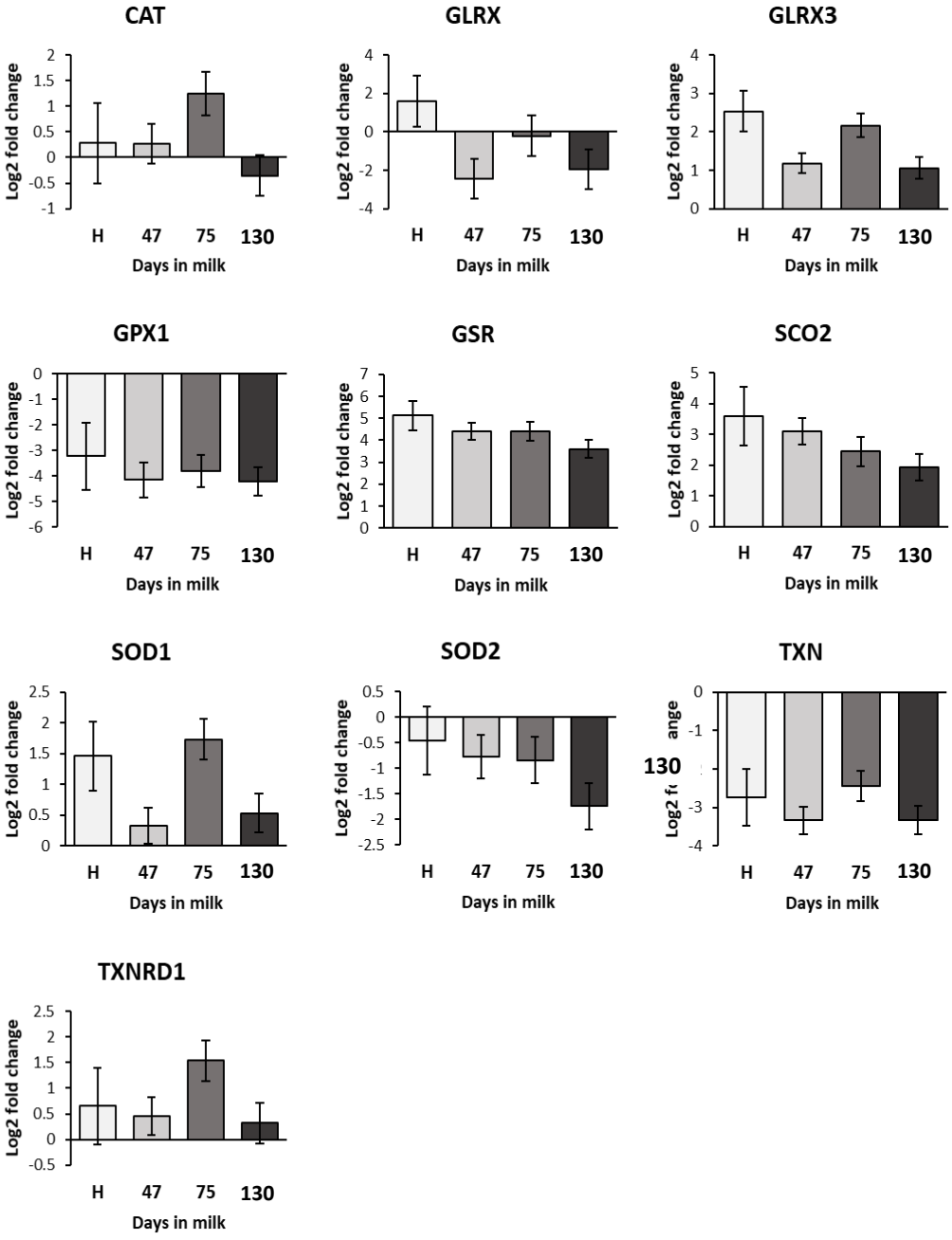
Supplementary Figure 2-5. Relative expression of genes involved in metabolism tested by qPCR in individual oocytes grouped by DIM. Oocytes were collected one time from heifers (H) and three times from lactating cows during lactation. None of the ten genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



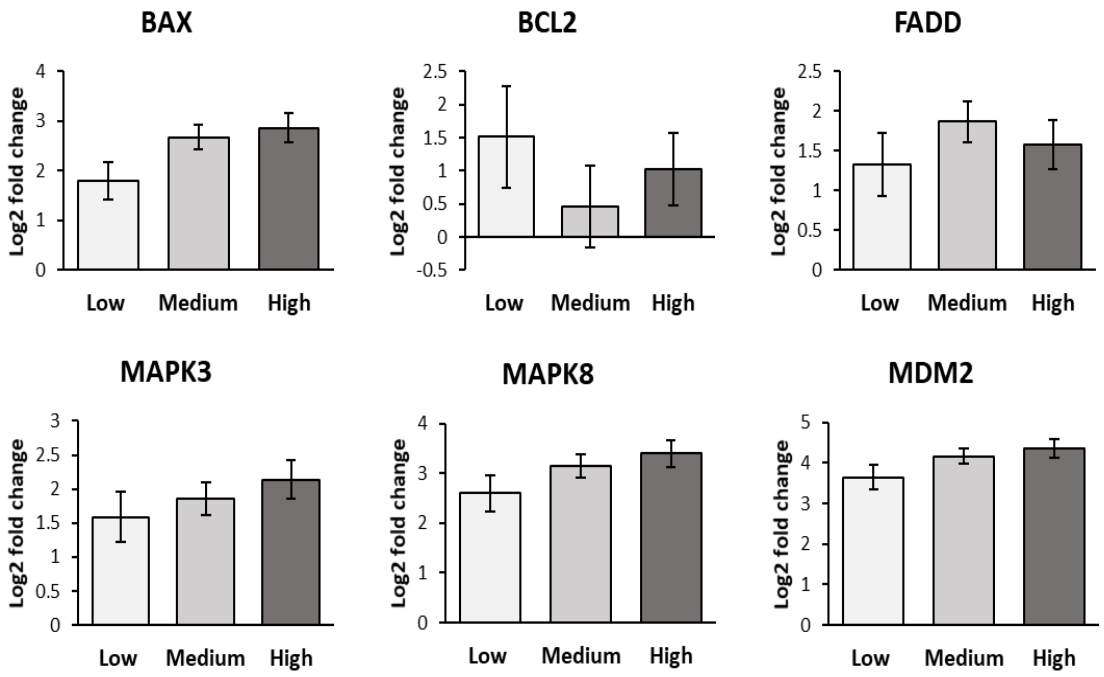
Supplementary Figure 2-6. Relative expression of genes from oocyte specific category tested by qPCR in individual oocytes grouped by DIM. Oocytes were collected one time from heifers (H) and three times from lactating cows during lactation. None of the six genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



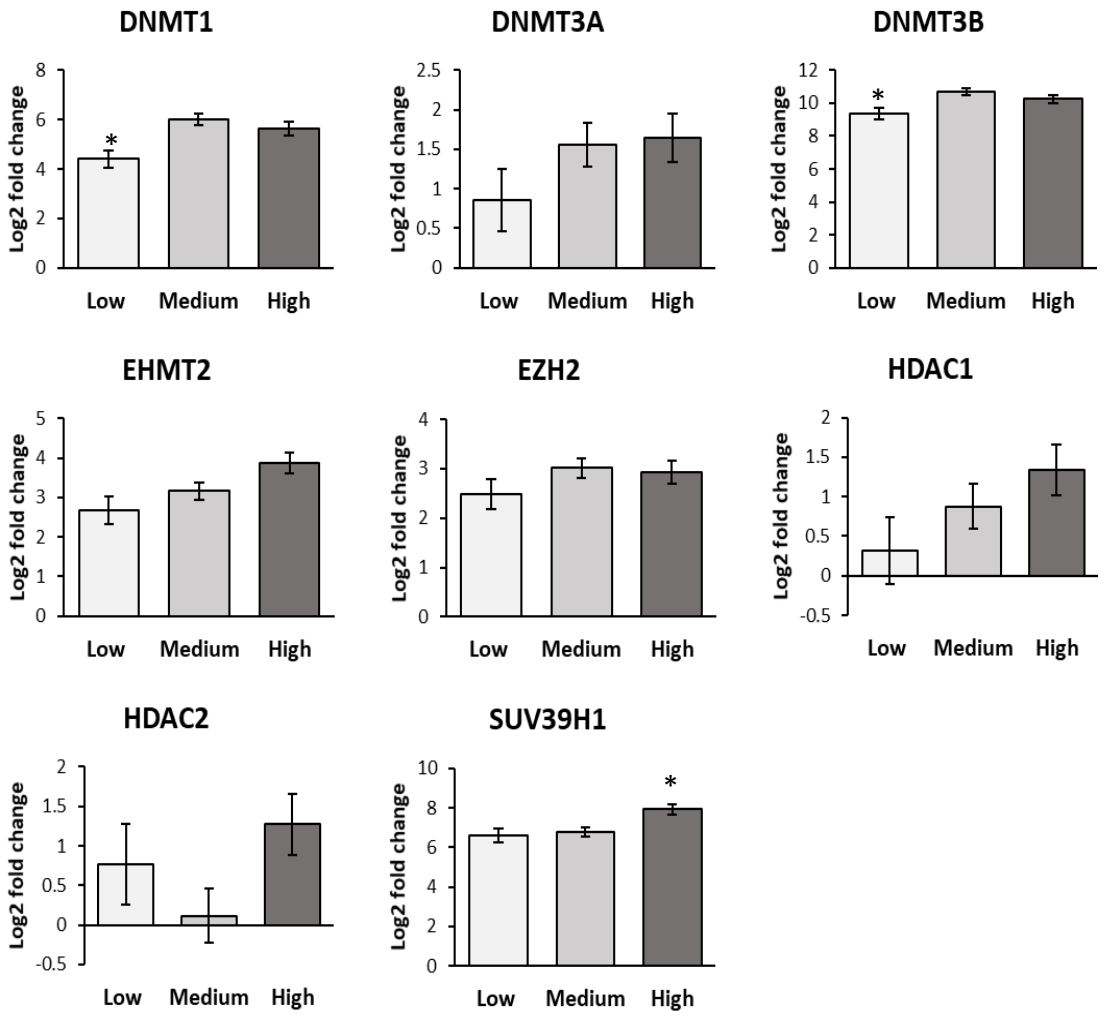
Supplementary Figure 2-7. Relative expression of genes from pluripotency factor category tested by qPCR in individual oocytes grouped by DIM. Oocytes were collected one time from heifers (H) and three times from lactating cows during lactation. None of the four genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



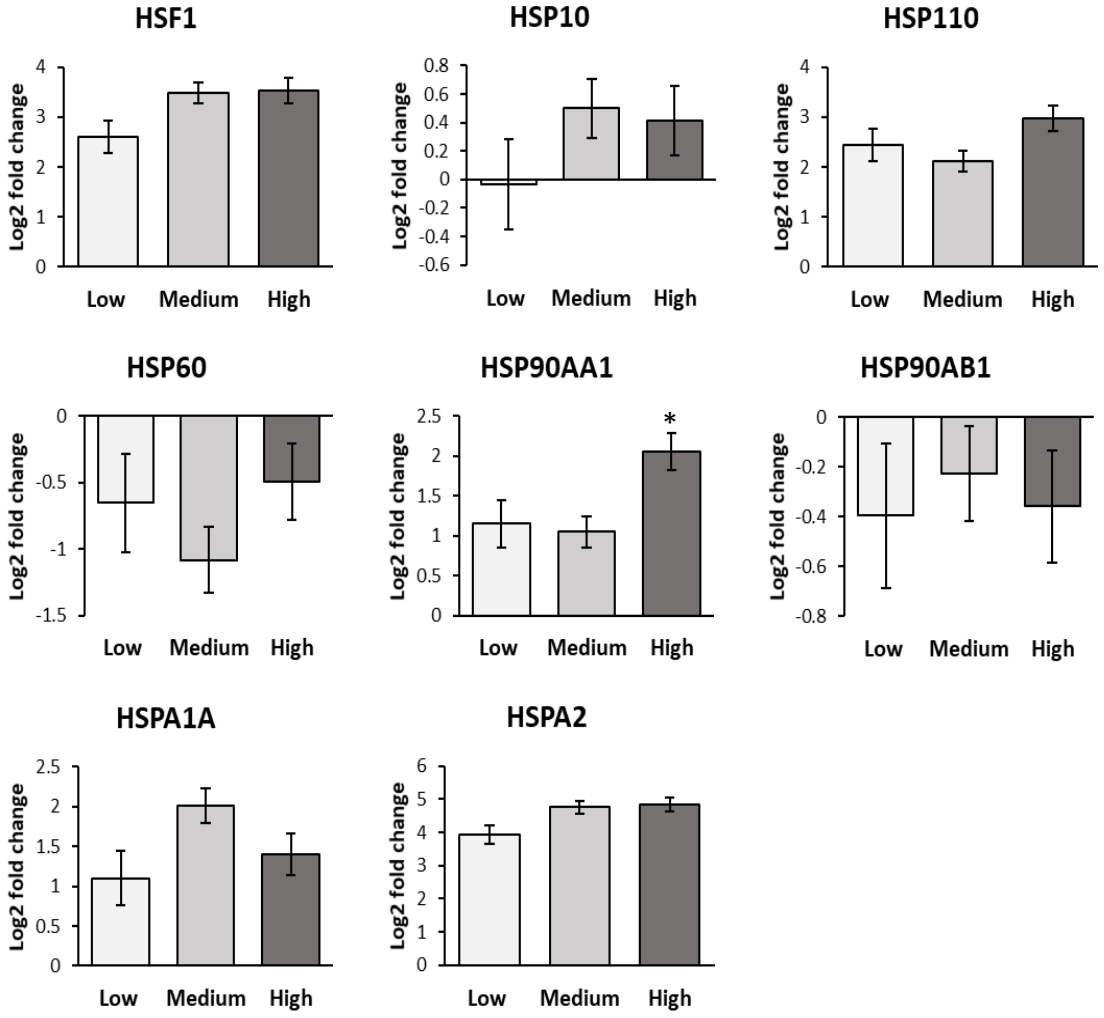
Supplementary Figure 2-8. Relative expression of genes involved redox regulation tested by qPCR in individual oocytes grouped by DIM. Oocytes were collected one time from heifers (H) and three times from lactating cows during lactation. None of the ten genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



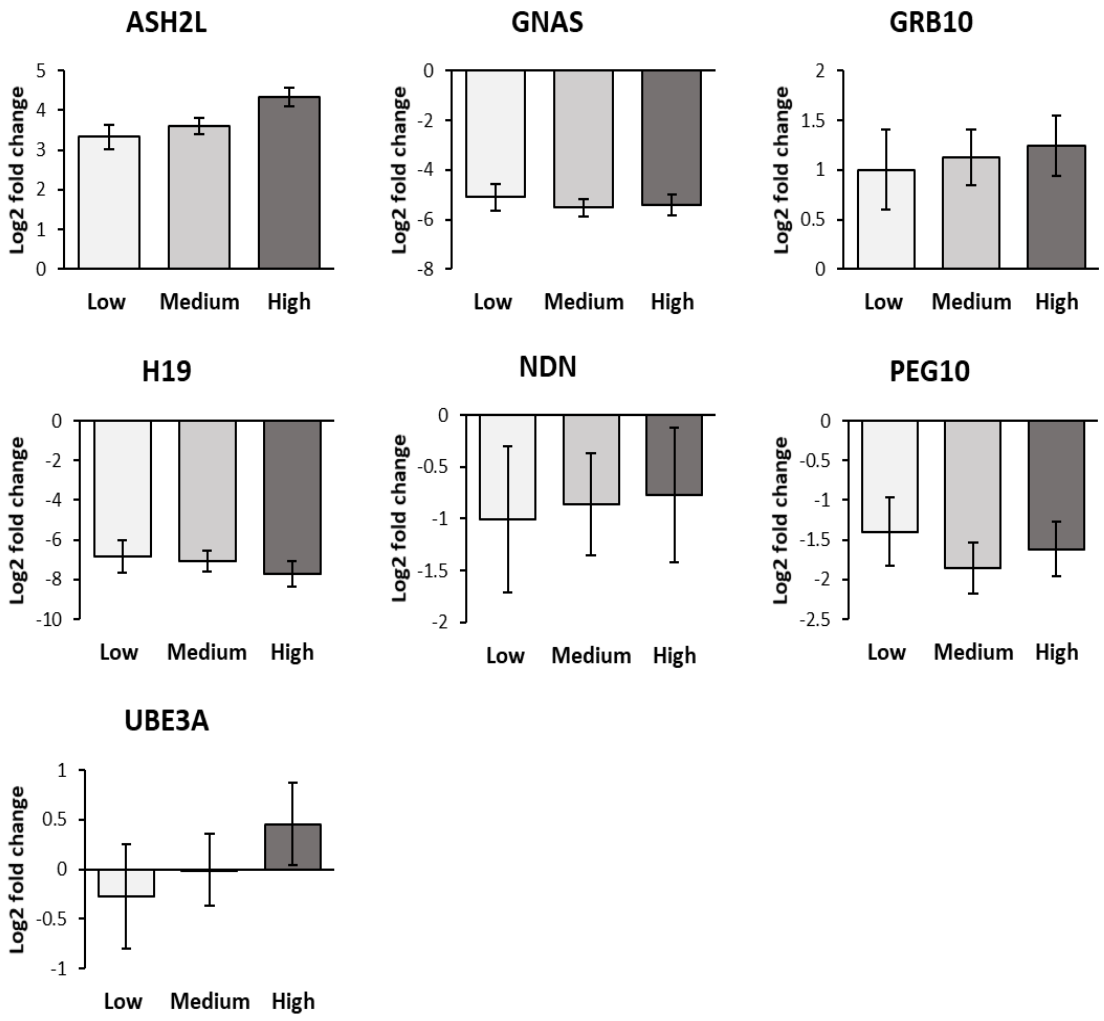
Supplementary Figure 2-9. Relative expression of genes involved in apoptotic regulation tested by qPCR in individual oocytes from lactating cows grouped by BHBA level. None of the six genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



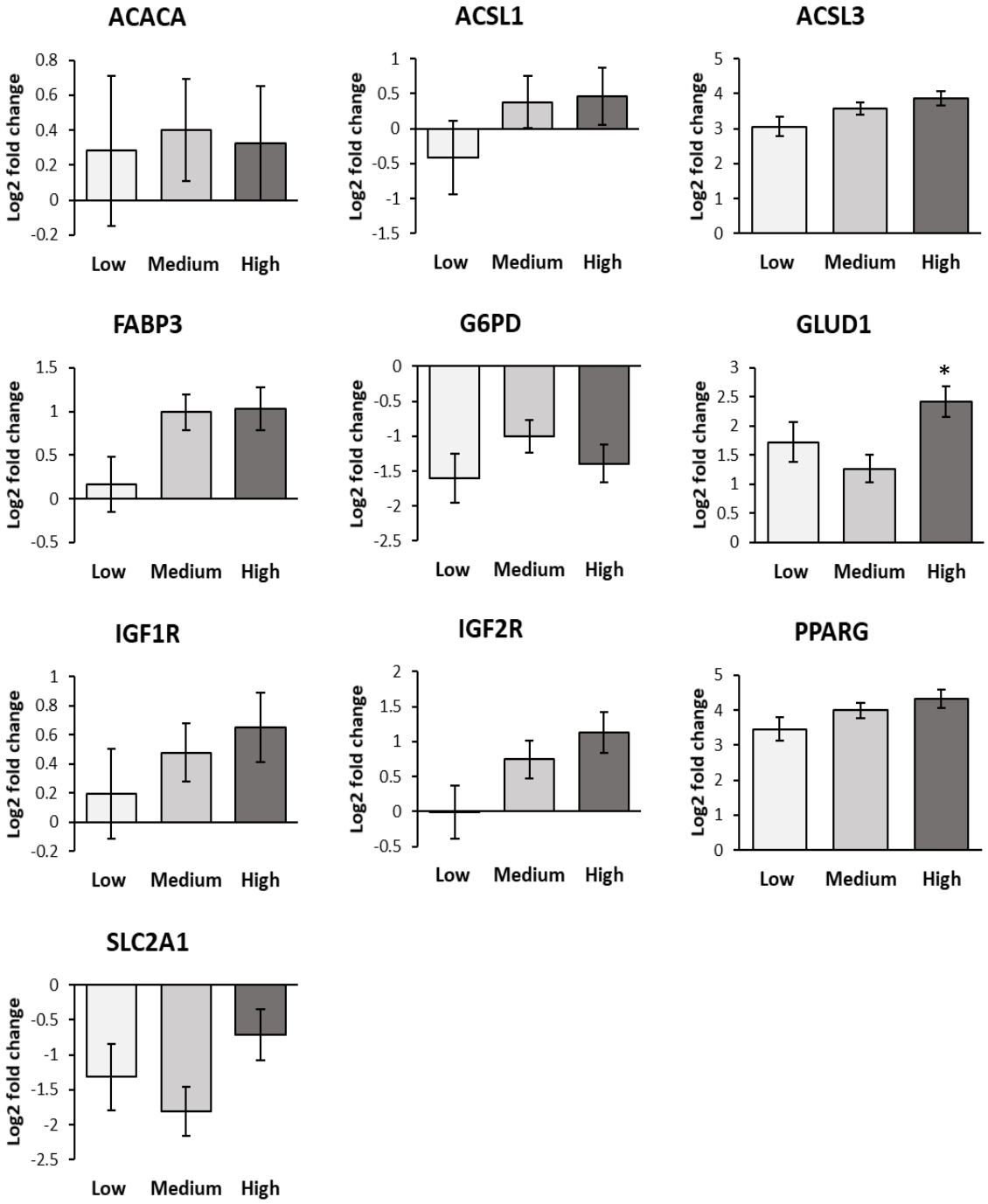
Supplementary Figure 2-10. Relative expression of genes in involved epigenetic regulation tested by qPCR in individual oocytes from lactating cows grouped by BHBA level. Three of the seven genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



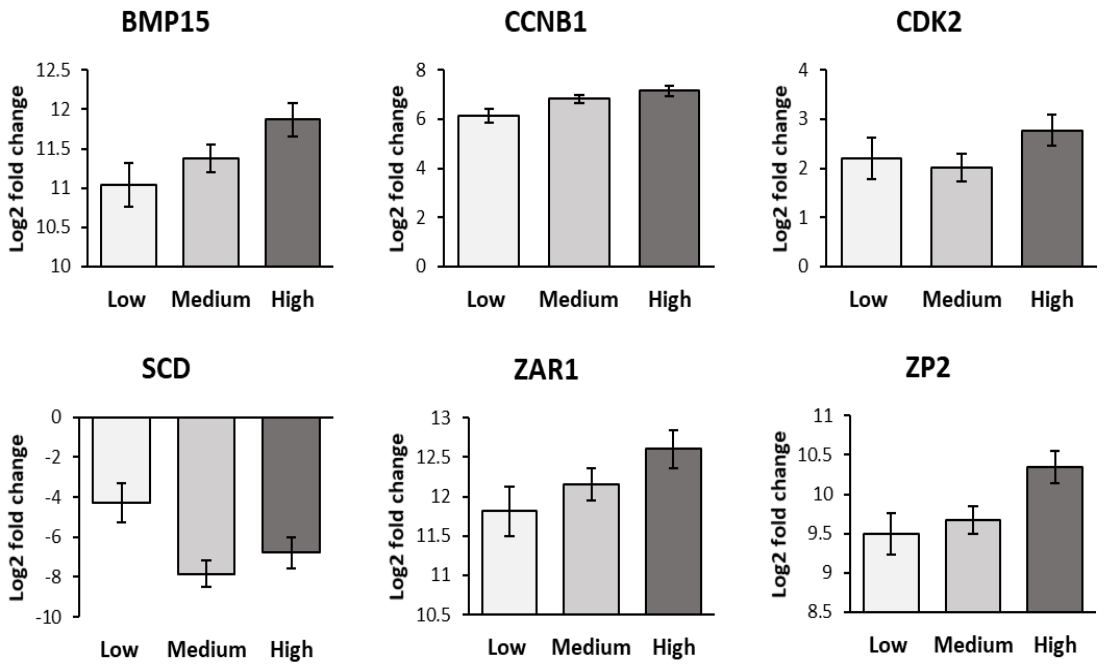
Supplementary Figure 2-11. Relative expression of genes from heat shock protein category tested by qPCR in individual oocytes from lactating cows grouped by BHBA level. One of the eight genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



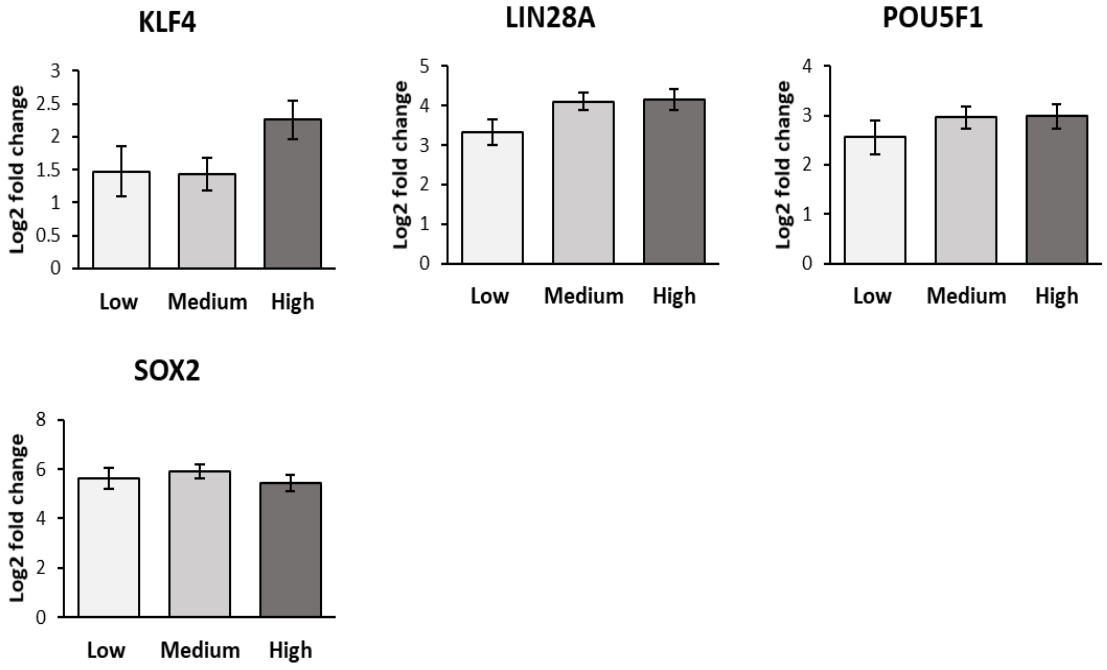
Supplementary Figure 2-12. Relative expression of genes from imprinting category tested by qPCR in individual oocytes from lactating cows grouped by BHBA level. None of the seven genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



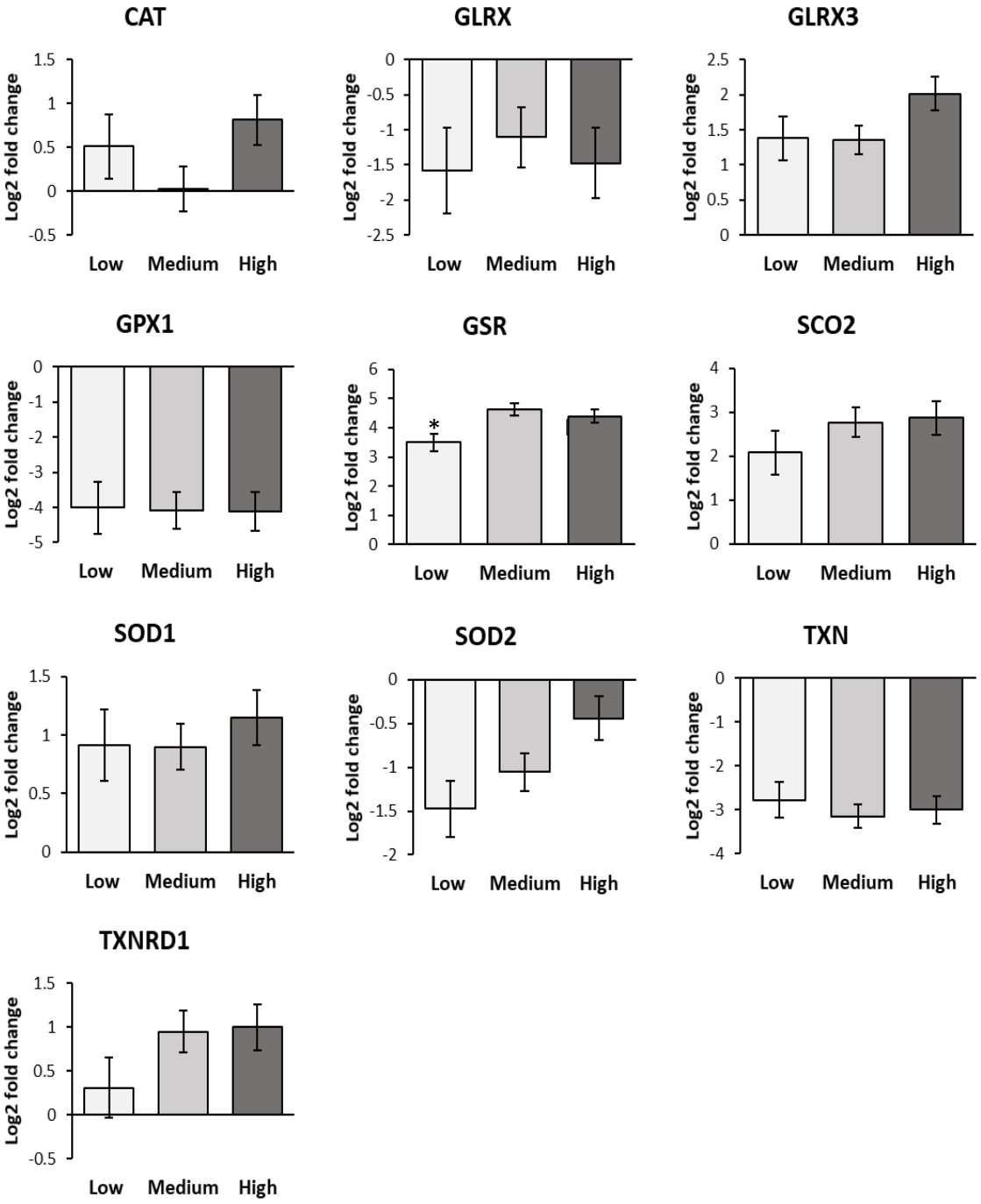
Supplementary Figure 2-13. Relative expression of genes involved in metabolism tested by qPCR in individual oocytes from lactating cows grouped by BHBA level. One of the ten genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



Supplementary Figure 2-14. Relative expression of genes from oocyte specific category tested by qPCR in individual oocytes from lactating cows grouped by BHBA level. None of the six genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



Supplementary Figure 2-15. Relative expression of genes from pluripotency factor category tested by qPCR in individual oocytes from lactating cows grouped by BHBA level. None of the four genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



Supplementary Figure 2-16. Relative expression of genes involved redox regulation tested by qPCR in individual oocytes from lactating cows grouped by BHBA level. One of the ten genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.

CHAPTER 3

COMPARISON OF EXPRESSION OF SELECTED GENES IN INDIVIDUAL IN VIVO PRODUCED PRE-IMPLANTATION EMBRYOS OF LACTATING DAIRY COWS AND NON-LACTATING HEIFERS

ABSTRACT

While pregnancy rates in lactating high-yielding dairy cows have declined drastically in recent decades, non-lactating dairy heifers have remained consistently high pregnancy rate. Unlike non-lactating heifers, lactating dairy cows go through a period of negative energy balance during early lactation which results in drastic changes in circulating metabolites and hormones. Most of the embryo loss has been shown to occur within the first two weeks of pregnancy, before implantation, and during which time the embryos are highly responsive to their surrounding environment. The aim of this study was to identify how lactation and negative energy balance (NEB) impact the relative mRNA abundance of selected genes from a variety of functional categories in individual pre-implantation embryos of lactating dairy cows (n = 20) and non-lactating heifers (n = 5). Embryos were flushed on day seven after superovulation and timed-artificial insemination (TAI) once from heifers and three times from lactating cows at 54, 82, and 137 days in milk (DIM). Energy status was determined for each animal before embryo flushing by measuring the concentration of circulating beta-hydroxybutyrate (BHBA). RNA was extracted from each embryo with a total of 22 embryos from heifer and 18, 22, and 21 embryos from lactating cows at 54, 82, and 137 DIM respectively. Relative transcript abundances for each of 63 genes selected was evaluated by qPCR. None of the

genes tested were found to be differentially expressed (adj. $P < 0.05$) between lactating cows and heifers or due to DIM and BHBA level in lactating cows. Further studies are needed for more accurate transcriptomic assessment of bovine embryos at later stages. Such studies may help in providing a better understanding of the effect of lactation and NEB on early embryonic development.

INTRODUCTION

Declining fertility in high-yielding dairy cows during early lactation is one of the major challenges in dairy management (Macmillan et al., 1996; Lucy, 2001; Leroy et al., 2008b). Conception rates at observed estrus declined in dairy cows from 55.3% in 1961 (Mares et al., 1961) to approximately 38% a few decades later (Pursley et al., 1997). While pregnancy rates in lactating high-yielding dairy cows have declined drastically in recent years, non-lactating dairy heifers of the same genetic merit have had persistently high pregnancy rates (Casida, 1961; Mares et al., 1961; Pursley et al., 1997). These observations have drawn attention toward the adverse effect of lactation and high milk yield on fertility and urged evaluation of the consequences of prolonged and extensive genetic selection for milk yield over the last decades (Macmillan et al., 1996; Hansen, 2000; Lucy, 2001).

Early embryonic loss is the primary reason of pregnancy loss in dairy cows (Diskin and Morris, 2008). About 90% of the ovulated oocytes are successfully fertilized and start developing; however, only 55% survive to term. Most of the embryonic loss occurs within the first two weeks of gestation, accounting for 70-80% of total embryonic mortality (Moussa et al., 2015). Embryonic loss mostly occurs before implantation,

during which time the embryo is highly responsive to its surrounding uterine environment. Uterine health and proper maternal-embryo interactions are critical to successful development (Maillo et al., 2016). As the developing embryo produces Interferon- tau (bIFN- τ) to initiate the maternal recognition of pregnancy, the uterus provides the optimal environment for growth and development and accepts implantation (Lonergan and Forde, 2014).

The quality of the preimplantation embryo is mainly influenced by two main factors: the oocyte developmental competence which determines the intrinsic embryo quality, and the uterine environment which represents the extrinsic factors that contribute to the embryo survival (Gray et al., 2001; Sirard et al., 2006). These two fundamental factors are primarily influenced by maternal health and physiological status. Unlike non-lactating heifers, lactation and rapid increase in milk production cause drastic changes in the circulating metabolites and hormones in the lactating dairy cows that have been found to be associated with poor reproductive performance (Jorritsma et al., 2003; Garnsworthy et al., 2008; Chapinal et al., 2012; Roche et al., 2017).

The oocyte developmental competence and quality contribute profoundly to the potential viability of the developing embryo (Keefe et al., 2015). Through the course of oogenesis, the oocyte accumulates RNA transcripts, organelles, and key transactional factors that are determinants of the developmental competence (Sirard et al., 2006). During follicular development and maturation, the oocyte is very responsive to the ovarian intra-follicular environment which is considered the major link between maternal metabolism and oocyte quality (Leroy et al., 2011). Poor oocyte quality has been

suspected as the chief culprit of early embryonic loss in lactating dairy cattle (Sirard et al., 2006).

The main physiological consequences of lactation are the nutrient partitioning toward milk production and negative energy balance (NEB) resulting in fat mobilization from adipose tissue when females can not meet the energetic demand solely from feed intake (Opsomer, 2015). Negative energy balance is associated with metabolic and hormonal dysregulation, and typically, elevated concentration of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA), and low glucose in the circulation (Jorritsma et al., 2003; Chapinal et al., 2012). Besides, NEB is associated with the increased incidence of the metabolic disorders that interact with the reproductive performance which makes it one of the primary causes of infertility in dairy cows (Jorritsma et al., 2003; Walsh et al., 2011; Opsomer, 2015; Santos et al., 2016). High levels of NEFA and BHBA have adverse effects on fertility and replicating their levels in the in vitro maturation model and embryo culture systems was found to be toxic to the developing embryo and oocytes resulting in reduced quality and developmental competence respectively (Leroy et al., 2005b; Leroy et al., 2008a; Van Hoeck et al., 2013; Van Hoeck et al., 2014).

As the early embryonic loss is the primary cause of reduced fertility in lactating dairy cows, we hypothesized that the consequences of the metabolic and physiological changes induced by lactation and/or NEB would adversely impact embryo quality by altering the expression of critical genes involved in viability leading to early embryonic loss. In order to test our hypothesis, we evaluated the relative expression levels of selected genes from a variety of functional categories such as metabolism, apoptosis,

redox, heat shock proteins, and epigenetic function in individual pre-implantation embryos using quantitative qPCR analysis. We compared the expression levels of the selected genes between individual embryos obtained multiple times during early lactation from a group of lactating dairy cows and one time from a group of non-lactating dairy heifers. Also, the energy status, body condition score (BCS), and milk production were identified in the lactating cows before embryo collection to evaluate the effect of NEB. This analysis of the differential gene expression between the individual embryos obtained from animals with different lactation and metabolic status will provide information that will help in understanding the molecular pathways leading to the reduced embryo viability in the high-yielding dairy cows.

MATERIALS AND METHODS

Animal Management and Experimental Groups

This experiment was conducted at the Utah State University Caine Dairy Research and Teaching facility and all procedures were carried out under license in accordance with guidelines of the Utah State University Animal Care and Use Committee (IACUC#2438). Twenty-five multiparous Holstein-Friesian dairy cows from different parity, second to the seventh lactation, were randomly selected and used in this experiment. Cows were identified a few weeks prior to expected calving, and initial BCS using a 5-point scale with 0.25-point increments and body weight were taken by within the first week post-partum then every other week until the wk 19, around 130 days in milk (DIM). Cows were housed in a tie-stall barn during the experiment which was conducted between October and May. All cows were fed a total mixed ration (TMR)

twice daily with ad libitum access to feed and water. Cows were milked twice daily, and the milk yield was recorded daily and averaged weekly.

Embryos were collected one time from a group of five Holstein heifers, average age 14 months, that were randomly selected and kept in a free-stall barn. Embryos from heifers' group were collected to be used as samples from non-lactating animals that apparently not experiencing NEB as lactating cows. Embryos from lactating cows were collected three times from each animal at 54 ± 4.2, 82 ± 4.2, and 137 ± 4.4 DIM. Dairy cows on average return to positive energy balance around 80 DIM (Coffey et al., 2002), and by collecting the embryos for a 3rd time at 137 DIM we assumed these embryos are being collected from animals during positive energy balance unless a serious health issue or a significant drop in body weight was reported.

BHBA and Glucose Measurement

Blood samples were collected two to three times every week post-calving from wk 1 to wk 19 after the evening milking from the coccygeal vein using plain vacutainer blood collection tubes and needles. Blood samples from heifers were collected one time before embryo flushing. Fresh whole blood was used to measure BHBA and glucose immediately after drawing using the digital hand-held device (Precision Xtra, Abbott Diabetes Care, Abingdon, UK). The multiple glucose and BHBA measurements from each cow were averaged by week. The weekly BHBA concentration for each animal was categorized as high (BHBA >12 mg/dL), which is the common cutoff point for subclinical ketosis (Iwersen et al., 2009), and BHBA concentration < 12 mg/dL was further divided into medium (BHBA 8 – 12 mg/dL) and low (BHBA < 8 mg/dL).

Superovulation, Estrus synchronization, and Timed-Artificial Insemination (TAI)

All animals used in the study were synchronized and superovulated prior to TAI following the 7-day CO-Synch + CIDR protocol (Whittier et al., 2013). In brief, a Controlled Internal Drug Release (CIDR) device (Eazi-Breed CIDR®, Pfizer-Pharmacia Animal Health; Kalamazoo, MI) was placed intravaginally for seven days. A dose of 2 ml (100 mcg) gonadotropin-releasing hormone (GnRH) was given two days after placing the CIDR then two days later, 17 ml (20 mg/ml) of follicle stimulating hormone (FSH) (Folltropin; Bioniche Animal Health, Belleville, ON, Canada) was given in eight decreasing doses, 12 hrs. apart for four days (am. and pm.). In the last day of FHS treatment, two doses of 10 and 5 ml of Lutalyse ® (5 mg/ml dinoprost tromethamine injection; Pfizer Animal Health, Kalamazoo, MI, USA) was given am. and pm. respectively. The CIDR was removed with last Lutalyse injection and 40 hrs. later cows were given a dose of 2 ml GnRH to induce ovulation, and were artificially inseminated (AI) three times with 12 hrs. interval. AI was performed by the same technician and semen used in the study to breed the cows and heifers was collected from the same bull from one collection of known fertility.

Embryo Collection and Processing

Embryo flushing was performed non-surgically on day 7 of the cycle. Donor cows were restrained in the chute and given caudal epidural anesthesia (6 ml lidocaine hydrochloride 2%). Through manipulation per rectum, ovaries were manually palpated to assess the ovarian response to the superovulation treatment by estimating the number of palpable corpora lutea (CLs) in each ovary. The perineal area was thoroughly washed by water and dried and a two-way Foley catheter (AgTech Inc., Manhattan, KS) was placed

over a metal stylet and inserted in the vagina as the other arm was placed in the rectum to guide the catheter through the cervix until it reached the uterine body and directed into each horn separately. Once the uterine horns were reached, the stylet inside the catheter was withdrawn, and the balloon of the Foley catheter was inflated with 7 ml of flush media. Each uterine horn was flushed three times with commercially available complete flush media (AgTech Inc., Manhattan, KS) by introducing increasing volume each time from 50 to 100 ml of media that was allowed to drain through an embryo filter dish of filter size 50 µm Millipore (MAI Animal Health, Elmwood, WI). After the flush was completed, the balloon of the Foley catheter was deflated, and all media in the catheter was thoroughly drained to flow into the embryo filter dish and immediately transferred to the vet clinic lab. Under a stereomicroscope, embryos were identified and only viable embryos of excellent, good, or fair quality between morula and blastocyst stage were picked while embryos of poor quality and degenerate, or unfertilized oocytes were discarded. Viable embryos were washed three times with fresh flush media and placed individually into 0.6 ml labeled microcentrifuge tube and snap frozen in liquid nitrogen and stored at -80°C for future use.

RNA Isolation and Reverse Transcription

Total RNA was isolated from individual embryos using commercial RNA extraction kit (Quick-RNA™ Micro Kit from Zymo Research). Following the recommended protocol in the instruction manual, 100 µl of RNA lysis buffer was added directly into the tube the containing embryo and vortexed briefly. The protocol was exactly followed except the final elution of the RNA was performed twice with 10 µl DNase/RNase free water for a final elution volume of 20 µl and stored at -80°C until

further use. Reverse transcription was performed using the GoScript Reverse Transcription kit from Promega (Madison, WI). Ten µl of individual embryo RNA elution was mixed with 1 µl oligo-dT and 1 µl random primers and incubated at 70°C for 5 min using the thermocycler. Four µl of the 5x reaction buffer, 2 µl of MgCl2 (final concentrations of 4.8 mM), 1 µl nucleotide mix, and 1 µl of reverse transcriptase (all were supplied with the kit) were added to RNA mix to make a total of 20 µl reaction and incubated at 25°C for 5 min for initial annealing then at 42°C for 60 min. The synthesized cDNA from each embryo sample was stored at -20°C until further use. A reference cDNA sample was synthesized using the same reverse transcription protocol from a mixture of RNA isolated from different bovine tissues as endometrial tissue, ovarian tissue, blastocysts, and cumulus-oocyte complexes (COCs). This sample was prepared to be used as a positive control and a calibrator sample for qPCR.

Fluidigm qPCR Analysis

The BioMark system was used for the qPCR analysis in this experiment which is an innovative and unique platform from the Fluidigm Corporation (South San Francisco, CA), which allows for expression analysis of up to 96 genes in each of up to 96 samples. The primers used for these genes were designed based on NCBI's reference sequence for Bos taurus through the Fluidigm Corporation's DeltaGene assay design service, and Table 3-1 provides the definition of the genes' symbols and names used in the qPCR analysis (for primer sequences, see Supplementary Table 3-1). In this experiment, a 96 by 96 microfluidic chip of nanoliter-scale was used to test the expression of 63 genes from different functional categories: housekeeping, apoptosis, redox, heat shock protein, metabolism, epigenetic modifiers, imprinted genes, pluripotency, and trophoblast specific

(Table 3-2). Before qPCR analysis, cDNA samples of each embryo were enriched by specific target amplification (STA). One µl of each primer pair (forward and reverse) was mixed into a tube to be used for the STA reaction. For each of 1.25 µl of cDNA, 1.25 µl primer mix and 2.5 µl of TaqMan PreAmp Master Mix (Applied Biosystems; Foster City, CA) was added for STA reaction to make a total of 5 µl reaction. Using the thermocycler, the STA amplification reaction was initially activated by incubation at 95°C for 10 min then followed by 14 cycles of 95°C for 15 seconds and 60°C for 4 min. Exonuclease I treatment was recommended to digest any unincorporated primers and was performed by adding 0.2 µl Exonuclease I Reaction Buffer, 0.4 µl Exonuclease I enzyme (ExoI; New England Biolabs; Ipswich, MA), and 1.4 µl nuclease-free water to each STA reaction to make a total of 7 µl and incubated at 37°C for 30 min. After digestion of unincorporated primers, the reaction was incubated at 80°C for 15 min to inactivate the Exonuclease I enzyme followed by 5-fold dilution by adding 18 µl nuclease-free water to each reaction to make a total of 25 µl of Exonuclease I-treated STA embryo cDNA. For Fluidigm qPCR cycling, 2 µl of the diluted and Exonuclease I-treated STA embryo cDNA was mixed with 2.5 µl of the 2x TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), 0.25 µl of Sample Loading Reagent (Fluidigm), and 0.25 µl 20x EvaGreen DNA Binding Dye (Biotium, Hayward, CA, USA).

On the other hand, 2.5 µl of each primer pair (forward and reverse 20 µM) was mixed with 0.25 µl nuclease-free water and 2.5 µl Assay Loading Reagent (Fluidigm). A 96.96 Dynamic Array Integrated Fluidic Circuits (IFC) was inserted in the IFC Controller HX for priming using the Prime (136x) script before loading the samples and primers. After priming, 5 µl from each primer mix and sample mix were loaded into the

designated well in the IFC using the multichannel pipette and returned to the IFC Controller HX for loading by running the Load Mix (136) script. After the Load Mix script was finished, the IFC was ejected from the Controller and inserted in the BioMark thermal cycler (Fluidigm) for qPCR. The recommended protocol GE 96x96 Standard v1 was used with initial enzyme activation for 5 min then 35 cycles of 95°C for 15 sec and 60°C for 60 sec and at the end 3 min final extension.

Analysis of qPCR Data and Statistics

Raw qPCR data were analyzed using the comparative cycle threshold (Ct) method using the Fluidigm Real-Time PCR Analysis Software. The software determined the raw Ct value and then normalized it to the average Ct of four selected housekeeping genes (*EIF4A1, GAPDH, TAF11,* and *YWHAZ*) to determine the delta Ct (ΔCt) which is the difference between Ct value for each gene tested and Ct of the housekeeping genes. Then, the delta delta Ct (ΔΔCt) value was determined for each reaction by calculating the difference between the ΔCt of each sample and the ΔCt of a selected calibrator sample (cDNA derived from pooled embryos and cumulus-oocyte complexes, and ovarian and endometrial tissue); described above.

ΔΔCt values were used for the statistical analysis using SAS (SAS, Institute Inc., Cary, NC) to determine the significant difference between samples. A Generalized Linear Mixed Model (GLMM) was fit to allow the gene expression to be dependent upon the type of cow (lactating or non-lactating) and the time of embryo flushing which was nested within lactation while accounting for the effect of each animal and BHBA measurement at the time of collection. Further, we analyzed data based on the effect of BHBA level only at the time of embryo flushing while accounting for the effect of each

animal and time of sample collection. The significant difference between samples was reported at FDR adjusted *P*-value < 0.05. Further, relative fold change for the significantly expressed genes was calculated by the equation $2^{-(\Delta\Delta Ct)}$ to make the final determination of the significant difference, and the cut-off was set at a fold-change equal to one, which is about 100% increase. The increase in fold change was calculated by determining the difference in fold change between two significantly different ΔΔCt values and divided on the fold change of the largest ΔΔCt value. For example, the value of (ΔΔCt X = 1.388) , and the value of (ΔΔCt Y = -1.141) the equation used is $\frac{2-\hat{}(\Delta\Delta Ct\ Y) - 2\hat{}-(\Delta\Delta Ct\ X)}{2-\hat{}(\Delta\Delta Ct\ X)} = \frac{2.207 - 0.382}{0.382}$ = 4.77 which means 477% increase in Y relative to X. Log2 of fold change was calculated to determine the relative expression level between samples and was used to create the figures.

RESULTS

Twenty-five multiparous lactating dairy cows were used in this experiment, and the milk yield was recorded daily and averaged weekly starting wk 1 until wk 19. Figure 3-1 shows the lactation curve for the cows used in this study and the changes in body weight. The initial average milk yield for all cows during the first week was about 29.30 ± 4.3 kg/day and then started to increase rapidly until reached the peak 44.03 ± 5.5 kg/day around 45 DIM (Figure 3-1A). Average of the initial body weight was 724.70 ± 53 kg and was measured within the first week after calving and then once every two weeks for the rest of the trial. Body weight started to decline as milk production increased, until it reached the lowest point of 704.05 ± 53.9 kg by 63 DIM then started to increase gradually to reach 733.20 ± 47.74 kg by 133 DIM (Figure 3-1B). Blood

concentrations of BHBA and glucose for the first week after calving were 6.43 ± 4.53 and 53.60 ± 2.14 mg/dL respectively. Embryo flushing was performed three times during lactation at 54 ± 6.8, 82 ± 6.9, and 137 ± 6.3 DIM and Table 3-3 provides a summary for milk yield, body weight, BCS, and concentrations of BHBA and glucose for each week before embryo collection. Figure 3-2 shows the changes in the blood concentrations of BHBA and glucose during the of embryo flushing. The average blood concentrations of BHBA and glucose for the non-lactating heifers before embryo flushing were 3.12 and 64 mg/dL, respectively.

Figure 3-3 demonstrates the percentage of cows identified with low, medium, and high BHBA levels at the time of embryo flushing. At the time for the first embryo flushing, about 17% of the lactating cows had high BHBA level, 34% had medium, and 47% low. By the time of the second embryo flushing, the percentage of cows with high BHBA increased to 39% while the percentage of cows with low BHBA dropped to 26% and about 34% had medium BHBA. By the time of the last embryo collection, most of the cows, about 60%, had low BHBA level and only 21% had high BHBA level.

All twenty-five cows used in this study were superovulated and synchronized before the TAI (Figure 3-4). However, the response to the superovulation and the number of embryos recovered and used in the qPCR analysis were variable. Table 3-4 shows the average numbers of palpable corpora lutea, recovered embryos, and viable embryos yield at each embryo collection, which were used as parameters to evaluate ovarian response to superovulation treatment in corresponding to DIM. Further, we evaluated the response to superovulation based on the concentration of the circulating BHBA for each animal at the time of embryo collection (Table 3-5).

Some cows randomly did not respond appropriately to the superovulation treatment through the three times they have been stimulated, but the 2nd embryo collection, around 82 DIM, generally had the lowest response and number of recovered and viable embryos. In the evaluation of superovulation based on BHBA concentration, it appeared that the cows with high BHBA tend to have fewer CLs, and yielded the lowest number of viable embryos that were used for qPCR (see Figure 3-5 and Table 3-5). Five cows were excluded from the study and from further analysis because they did not respond properly to superovulation treatment and no embryos were recovered from them after they have been flushed three times. The total number of embryos included in the qPCR analysis for this study was 18 from the first embryo collection, 22 from the second collection, 21 from the third collection, and 22 from the heifers' group.

In the analysis of the differential gene expression between the individual embryos, the analysis was performed in three different ways. First, we compared between the embryos obtained from non-lactating heifers and lactating cows to test the effect of lactation and being multiparous vs nulliparous. Second, we compared between embryos obtained from lactating cows at different times during lactation to test the effect of DIM and changes in energy balance as the milk yield changes over time. Third, we compared between the embryos obtained from lactating cows with different BHBA levels while accounting for the fixed effect of DIM to test the effect of NEB.

Using the Fluidigm qPCR analysis, we tested 63 genes from different functional categories and Table 3-2 provides information about their functional group, and the number of genes tested from each group. After the statistical analysis of the qPCR data, none of the genes tested were found to be differentially expressed at adj. $P < 0.05$ and

Supplementary Figures 3-1 through 3-16 demonstrate relative expression of all gene that were tested and grouped by their functional category. However, in the comparison between individual embryos from heifers and lactating cows, four genes demonstrated more than one-fold increase in embryos from lactating cows at adj. $P = 0.17$ (Table 3-6). From the epigenetic regulation category, Enhancer of zeste homolog 2 (*EZH2*) was 1.95-fold higher in embryos from lactating cows. From the pluripotency category, Kruppel-like factor 4 (*KLF4*) was 1.69-fold higher in embryos from lactating cows. From the redox regulation category, Glutaredoxin 3 (*GLRX3*) and Thioredoxin (*TXN*) were 1.69-fold and 1.14-fold higher in embryos from lactating cows respectively (Figure 3-6).

DISCUSSION

The purpose of the present study was to evaluate the effect of lactation and/or NEB on the relative expression level of selected genes in individual in vivo produced pre-implantation embryos. We tested genes from a variety of functional categories that are involved in early embryonic development and survival. Also, we estimated the energy status of all animals used in the study by determining the blood concentrations of BHBA. Typically, dairy cows experience a state of NEB during early lactation as milk production increase rapidly with limited feed intake. The peak of milk production varies depending on management, nutrition, genetics, and breed. However, Holstein dairy cows typically reach the maximum milk yield between 6 to 8 weeks in lactation, and after that milk production slowly decreases until the end of lactation (Aschenbach et al., 2010; Strucken et al., 2015). During early lactation, they are at the highest demand of energy requirement that they cannot meet exclusively from their diet due to physiological limitations, and instead rely on bodily energy resources to compensate (Allen, 1996). The cows we used

in this study were no exception, and they follow the typical pattern of lactation curve where the milk production peaked around 45 DIM (six weeks), and they lost about 3% of their initial body weight that was taken within the first week after calving and dropped 0.25 point in BCS. These results imply that these cows were at energy deficit and went through a state of NEB.

Negative energy balance has been demonstrated in numerous studies to have a detrimental effect on fertility, interact with normal body physiology, and impact overall health (Dekkers et al., 1998; Ingvartsen et al., 2003). NEB promotes excessive fat mobilization from adipose tissue during early lactation and is associated with metabolic and hormonal dysregulation. Typically, NEB is characterized by elevated concentration of NEFA and BHBA and low glucose in the circulation (Jorritsma et al., 2003; Chapinal et al., 2012), which are known to have adverse effects on fertility (Block et al., 2001; Leroy et al., 2005b; Leroy et al., 2008a; Van Hoeck et al., 2013; Van Hoeck et al., 2014). In this study, we collected embryos from lactating cows multiple times during lactation from the same animals when they are at the period of NEB at 54 and 82 DIM and then two months later at 137 DIM when they shifted to the positive energy balance (Coffey et al., 2002) to examine the effect of energy status on embryo gene expression. These cows were supposedly at NEB around the first embryo collection as they reached the peak of milk production and lost body weight. By the second embryo collection, milk production had declined slightly, and the cows had stopped losing weight. However, BHBA levels were highest at the second collection which may indicate a prolonged effect of NEB that requires more time to recover even after the decline of milk production. By the time of the third embryo collection, milk production had declined about 13% from the peak, body

weight increased about 4% from the lowest weight around 8 weeks in lactation, BCS increased 0.25 point, BHBA levels were the lowest measured from each animal, and 60% of the cows had BHBA < 8 mg/dL which is considered as low and normal for cows in positive energy balance (Iwersen et al., 2009). Based on these parameters, these cows were in positive energy balance when we performed the third collection. This experimental design allowed us to use the same animals as the control to compare with our treatment (i.e., compare between embryos from cows when at NEB and then when they moved to positive energy balance).

The decline in milk production and progression in DIM are reliable indicators of the positive energy status as healthy dairy cows with balanced nutrition typically start to shift to the positive energy balance after eight weeks into lactation (Collard et al., 2000; Strucken et al., 2015). The level of BHBA in the circulation is commonly used as an indicator of the energy status in dairy cows as it positively correlates with the amount of adipose tissues that are being mobilized for energy. However, there is a variation in BHBA level in the circulation among individual animals in response to the NEB as it is highly associated with subcutaneous adipose tissue and BCS (Barletta et al., 2017). Over-conditioned cows around calving more often experience abnormal cyclicity and increased incidence of embryo mortality (Cutullic et al., 2011). For this reason, we compared gene expression in embryos obtained from cows with different BHBA levels while accounting for the DIM in our statistical analysis as a fixed effect. This allowed us to differentiate between the effect of progression in lactation and the effect of BHBA at the time of embryo collection. Cutullic et al. (2011) investigated the effects of milk yield and body condition on post-partum fertility in Holstein dairy cows and indicated that their effects

might be different at different stages during lactation. Early embryo survival seemed to be mostly affected by the BCS, while ovarian cyclicity and late embryonic survival seemed to be mainly affected by lactation and milk yield.

Lactation and high milk yield have adverse effects on fertility and were discussed in several studies (Macmillan et al., 1996; Lucy, 2001; Rodriguez-Martinez et al., 2008). High milk yield was shown to delay the onset of the first postpartum ovulation, increase the incidence of prolonged luteal cycles, and shorten the duration of standing heat (Opsomer et al., 2000; Lopez et al., 2004; Petersson et al., 2006). Sartori et al., (2004) compared the ovarian function between lactating cows and non-lactating heifers and reported lower concentrations of circulating steroid hormones in lactating dairy cows even though they have larger ovulatory follicles and CLs; this is perhaps due to the high clearance rate of steroid hormones as a result of high milk production in lactating cows (Sangsritavong et al., 2002). These low hormones low levels may explain the low conception rate and ovulation failure in comparison with non-lactating heifers. We noticed a negative correlation between the number of CL on the day of embryo collection and the concentration of circulating BHBA within the lactating cows used in the present study. The number of CLs was used to evaluate the ovarian response to superovulation treatment. High blood BHBA and NEFA and accumulation of triglycerides in the liver were found to delay the LH surge that is required for ovarian stimulation (Butler et al., 2006). Additionally, low glucose and insulin concentrations due to NEB results in insufficient IGF-I production in the liver leading to inadequate ovarian responsiveness to gonadotropins (Butler et al., 2003).

In the present study, we compared gene expression in individual embryos obtained from lactating cows and non-lactating heifers to investigate the effect of lactation on embryo quality. It is well documented that the conception rate in dairy cows is negatively associated with the increased milk yield while the conception rate of dairy heifers with similar genetic merit has not been affected (Pryce et al., 2004). Leroy et al. (2005a) compared the quality of Day 7 embryos between lactating dairy cows and non-lactating dairy heifers based on morphological appearance and color that is indicative of intracellular lipid accumulation. And in their previous work, they found a correlation between embryo color and lipid content as the darker embryos have significantly more lipids droplets. They concluded that embryos from lactating dairy cows have more lipid droplets and display lower quality compared to those from non-lactating heifers (Leroy et al., 2005a). However, even though determining the embryo quality based on the morphological appearance and color is an appropriate, non-invasive and applicable technique, it is subjective and can include some bias in observations. Embryo grading based on the developmental stage and morphological appearance is a common practice to determine the embryo quality and commonly used to identify the viable and transferable embryos (Cerri et al., 2009; Sartori et al., 2010; Makarevich et al., 2016; Mikkola and Taponen, 2017). In the present study, we included only viable embryos with transferable quality (i.e., embryos with quality comparable to those qualified to be transferred to a recipient cow) in the qPCR analysis to determine the relative expression level of the selected transcripts. We selected 63 candidate genes from a variety of functional groups that are essential for early embryonic development and survival to use their mRNA expression as an objective approach to identify what constitutes embryo quality. It is

essential to understand the molecular constituents of embryo quality and identifying the genes and pathways involved in those processes in order to develop profound strategies to support early embryo survival. Accumulation of cytoplasmic lipid droplets may not necessarily to be the cause of low embryo quality, but rather an indication of other problems.

Right after fertilization, the embryonic genome undergoes extensive epigenetic remodeling to reprogram the parental DNA, achieve a totipotent state, and allow successful activation of the embryonic genome. During the early stages of embryonic cleavage, the blastomeres are transcriptionally silent until the major activation of the embryonic genome around 8- to 16-cell stage in bovine embryos (Graf et al., 2014). Prior to the embryonic genome activation, maternal transcriptional factors and mRNAs, that have been accumulated in the oocyte through the course of oogenesis, regulate the early embryonic events and epigenetic reprogramming (Memili and First, 2000). We tested seven epigenetic modifiers that regulate DNA methylation and histone modification. In the present study, the expression of *EZH2* from the epigenetic modifier category was found to be 1.95-fold higher in embryos from lactating cows in comparison with those collected from heifers. *EZH2* is a histone-lysine N-methyltransferase enzyme that catalyzes histone methylation process associated with transcriptional silencing (Morey and Helin, 2010). It is involved in normal cell differentiation and mesenchymal stem cell lineage determination (Hemming et al., 2014). In the mouse preimplantation embryo, *EZH2* was found to be essential for early development, and Ezh2-deficient embryos demonstrated reduced outgrowth potential (Huang et al., 2014). Higher level of *EZH2* in embryos from cows may suggest a potential variation in the differentiation level of the

early embryonic cells when maintaining an undifferentiated state is critical for the embryonic stem cells at this stage.

From the four pluripotency factors we tested, *KLF4* was found to be 1.69-fold higher in embryos from lactating cows relative to heifers. *KLF4* regulates cell proliferation and controls terminal cellular differentiation (Chen et al., 2003). In embryonic stem cells, *KLF4* plays an essential role in self-renewal and maintenance (Shi and Ai, 2013). *KLF4* is also involved in the inflammatory response as it was shown to mediate proinflammatory signaling in macrophages (Feinberg et al., 2005; Nayak et al., 2013) and inhibits interleukin-1 beta (*IL-1β*). The overexpression of *KLF4* was shown to be induced in LPS treated macrophages to decrease the expression of *IL-1β*, a potent pro-inflammatory cytokine, while the suppression of *KLF4* increased the expression of *IL-1β* (Liu et al., 2012). These findings may explain the increased fold change of *KLF4* in the embryos collected from the lactating cows in the present study as they grow in a relatively more proinflammatory uterine environment compared to those obtained from heifers. Also, in vitro overexpression of *KLF4* was demonstrated during serum starvation (Shields et al., 1996) and cellular arrest in response to DNA damage (Yoon et al., 2003), and that might be linked to increased oxidative stress in lactating dairy cows during NEB which can adversely impact embryo quality (Guérin et al., 2001; Leite et al., 2017).

Excessive fat mobilization and increased oxidation of NEFA in the liver during the period of NEB result in increased production of reactive oxygen species (ROS) which raise the level of oxidative stress in the body (Morris et al., 2009). Excessive generation of ROS disrupts redox signaling status and alters the control of intracellular redox potential causing redox stress. Redox stress is defined as the intracellular imbalance of

pro-oxidants and antioxidants that leads to disturbance of normal metabolism and physiology (Jones, 2006). Maintaining the balance of the redox state is critical for intracellular biological processes and can be either specific in nature, targeting specific signaling pathways, or stressful non-specific in nature causing damage to macromolecules and toxic, leading to cell death or loss of function (Timme-Laragy et al., 2018). During embryonic development, redox signaling plays a critical role in regulating cellular differentiation, proliferation, and apoptosis (Covarrubias et al., 2008; Ufer et al., 2010). During this time, redox states are more susceptible to dysregulation, and the embryos exhibit greater sensitivity to toxicants including those of exogenous origin such as ROS (Timme-Laragy et al., 2018). The level of ROS must be maintained under balance and it is regulated by several antioxidant enzymes such as glutathione peroxidase (*GPX*), superoxide dismutase (*SOD*), catalase (*CAT*), and glutathione reductase (*GSR*). Increased expression of genes involved in antioxidant and redox regulation was demonstrated in vertebrate embryos in response to oxidative stress (Stover et al., 2000; Dennery, 2007). In the present study, we analyzed the expression of eleven genes involved in redox regulation and two of them (*GLRX3* and *TXN*) showed more than one-fold increase in embryos from lactating cows relative to heifers. *GLRX3* and *TXN* genes encode glutaredoxin and thioredoxin respectively, and they are key antioxidants that protect against the oxidative stress through their disulfide reductase activity (Holmgren, 2000; Lu and Holmgren, 2014). Their increased expression in the embryos from lactating cows might be an indication of disturbed redox status as the preimplantation embryo has the capacity to upregulate genes involved in redox homeostasis in response to oxidative stress.

In conclusion, the current study used qPCR of selected transcripts to analyze the impact of lactation, DIM, and NEB on gene expression of preimplantation embryos. No genes were found to be differentially expressed between embryos from lactating cows and heifers. Also, within embryos collected from lactating cows, no genes were found to be impacted by stage of lactation or different BHBA levels. However, four genes from different functional categories demonstrated more than one-fold increase in embryos from lactating cows relative to heifers. Studying the function of these genes may help in understanding the molecular pathways of how lactation impact embryo quality in dairy cows. Embryo quality is primarily driven by two main factors: oocyte developmental competence and uterine environment hosting the embryos. Further research is needed to differentiate between the impact of these two factors on embryos, and to determine which one has the most significant impact on embryo quality and survival.

TABLES AND FIGURES

Table 3-1. Definition of gene names used in the qPCR analysis of individual embryos

Gene symbol	Gene name
ACACA	Acetyl-CoA carboxylase alpha
ACSL1	Acyl-CoA synthetase long-chain family member 1
ACSL3	Acyl-CoA synthetase long-chain family member 3
ASH2L	Ash2 (absent, small, or homeotic)-like
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BID	BH3 interacting domain death agonist
CAT	Catalase
CDX2	Caudal type homeobox 2
DNMT1	DNA (cytosine-5-)-methyltransferase 1
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
EHMT2	Euchromatic histone-lysine N-methyltransferase 2
EIF4A1	Eukaryotic translation initiation factor 4A1
ESRRB	Estrogen related receptor beta
ETS2	ETS proto-oncogene 2, transcription factor
EZH2	Enhancer of zeste homolog 2
FABP3	Fatty acid binding protein 3
FADD	Fas associated via death domain
FAS	Fas (TNF receptor superfamily member 6)
G6PD	Glucose-6-phosphate dehydrogenase
GATA6	GATA binding protein 6
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLRX	Glutaredoxin
GLRX2	Glutaredoxin 2
GLRX3	Glutaredoxin 3
GLUD1	Glutamate dehydrogenase 1
GNAS	GNAS complex locus
GPX1	Glutathione peroxidase 1
GRB10	Growth factor receptor-bound protein 10
GSR	Glutathione reductase
H19	H19, imprinted maternally expressed transcript
HDAC1	Histone deacetylase 1
HDAC2	Histone deacetylase 2
HSF1	Heat shock transcription factor 1
HSP10	Heat shock protein family E (Hsp10) member 1
HSP110	Heat shock protein family H (Hsp110) member 1
HSP60	Heat shock protein family D (Hsp60) member 1 (HSPD1), transcript variant 3

HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1
HSP90AB1	Heat shock protein 90 alpha family class B member 1
HSPA1A	Heat shock 70kDa protein 1A
HSPA2	Heat shock protein family A (Hsp70) member 2
IGF1R	Insulin like growth factor 1 receptor
IGF2R	Insulin-like growth factor 2 receptor
KLF4	Kruppel-like factor 4
LIN28A	Lin-28 homolog A
MAPK3	Mitogen-activated protein kinase 3
MAPK8	Mitogen-activated protein kinase 8
MDM2	Mdm2, p53 E3 ubiquitin protein ligase homolog
NDN	Necdin homolog
P53	Tumor protein 53
POU5F1	POU class 5 homeobox 1
SCO2	SCO cytochrome oxidase deficient homolog 2
SLC2A1	Solute carrier family 2 member 1
SOD1	Superoxide dismutase 1, soluble
SOD2	Superoxide dismutase 2, mitochondrial
SOX2	SRY (sex determining region Y)-box 2
TAF11	TATA-box binding protein associated factor 11
TEAD4	TEA domain transcription factor 4
TXN	Thioredoxin
TXNRD1	Thioredoxin reductase 1
UBE3A	Ubiquitin protein ligase E3A
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

Table 3-2. Functional category of genes tested by qPCR in individual embryos

Functional categories	Number of genes tested	Name of genes tested
Apoptotic Regulators	9	*BAX, BCL2, BID, FADD, FAS, MAPK3, MAPK8, MDM2, P53*
Epigenetic Modifiers	7	*DNMT1, DNMT3A, DNMT3B, EHMT2, EZH2, HDAC1, HDAC2*
Heat shock Proteins	8	*HSF1, HSP10, HSP110, HSP60, HSP90AA1, HSP90AB1, HSPA1A, HSPA2*
Housekeeping	4	*EIF4A1, GAPDH, TAF11, YWHAZ*
Imprinting	6	*ASH2L, GNAS, GRB10, H19, NDN, UBE3A*
Metabolism	9	*ACACA, ACSL1, ACSL3, FABP3, G6PD, GLUD1, IGF1R, IGF2R, SLC2A1*
Pluripotency Factors	4	*KLF4, LIN28A, POU5F1, SOX2*
Redox Regulation	11	*CAT, GLRX, GLRX2, GLRX3, GPX1, GSR, SCO2, SOD1, SOD2, TXN, TXNRD1*
Trophoblast Specific	5	*CDX2, ESRRB, ETS2, GATA6, TEAD4*

Table 3-3. Time of embryo collection from lactating cows with BHBA, glucose, milk yield, and body weight

Embryo collection	DIM	BHBA (mg/dL)	Glucose (mg/dL)	Milk yield (kg/day)	Body weight (kg)	BCS (1-5 scale)
Initial	7 ± 1.36	7.45 ± 1.20	49 ± 2.92	29.30 ± 1.35	724.70 ± 11.11	3.0 ± 0.10
1st collection	54 ± 1.36	9.61 ± 1.68	53.65 ± 2.12	42.87 ± 1.19	706.09 ± 12.95	2.69 ± 0.15
2nd collection	82 ± 1.38	10.67 ± 1.44	50.92 ± 2.43	42.30 ± 1.01	704.32 ± 10.53	2.62 ± 0.10
3rd collection	137 ± 1.26	8.29 ± 0.69	57.08 ± 1.76	38.15 ± 1.25	733.20 ± 9.95	3.0 ± 0.20

The table reports the time for embryo collection during lactation in a group of lactating dairy cows (n = 25) which was performed three times at specific days in milk (DIM) with the average of beta-hydroxybutyrate (BHBA) concentration, glucose concentration, milk yield, body weight, and body condition score (BCS) for the initial week and the week prior to embryo collection. Data are presented as means ± SEM.

Table 3-4. Parameters used to evaluate the response to the superovulation treatment in lactating cows by DIM

Parameter	DIM		
	54	82	137
Number of corpora lutea	9.30 ± 1.23	5.20 ± 0.78	6.47 ± 1.32
Number of recovered embryos	5.04 ± 0.97	2.32 ± 0.53	3.53 ± 0.99
Number of viable embryos	2.39 ± 0.64	1.00 ± 0.43	1.64 ± 0.68

DIM = days in milk. * Data are the mean ± S.E.M.

Table 3-5. Parameters used to evaluate the response to the superovulation treatment in lactating cows by BHBA level

Parameter	BHBA		
	Low	Medium	High
Days in milk	83.44 ± 7.33	65.90 ± 4.85	79.50 ± 8.08
Number of corpus luteum	7.85 ± 1.13	6.55 ± 1.01	6.13 ± 1.27
Number of recovered embryos	4.41 ± 0.91	2.80 ± 0.63	3.25 ± 0.84
Number of viable embryos	1.96 ± 0.56	1.55 ± 0.58	1.19 ± 0.84

Cows were grouped by the average concentration of beta-hydroxybutyrate (BHBA) for the week prior to embryo collection and categorized as high (BHBA >12 mg/dL), medium (BHBA 8 – 12 mg/dL), and low (BHBA < 8 mg/dL).* Data are the mean ± S.E.M.

Table 3-6. List of genes showing more than 100%-fold change increase in expression level in individual embryo obtained lactating cows relative to non-lactating heifers

Gene name	Non-lactating Heifers		Lactating Cows		
	Avg. Relative Expression Level	S.E.M	Avg. Relative Expression Level	S.E.M	Percentage of fold change increase
EZH2	1.72	0.32	3.27	0.14	195%
KLF4	3.95	0.26	5.38	0.12	169%
GLRX3	-0.35	0.27	1.07	0.12	169%
TXN	-1.60	0.23	-0.51	0.10	114%

Average relative expression data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.

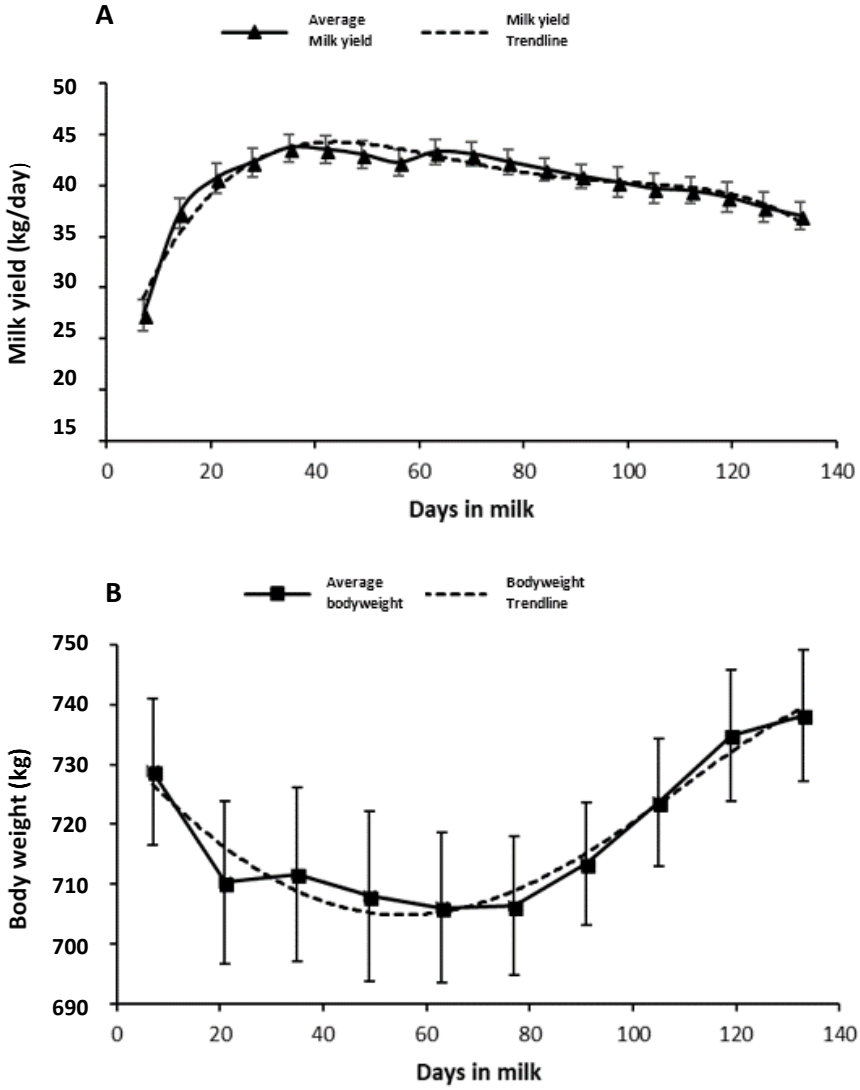


Figure 3-1. Lactation curve and change in body weight for lactating cows used to collect embryos. (A) shows milk yield and (B) shows changes in body weight for lactating cows (n = 25) during the trial for the first 133 days in milk. Data are the mean ± S.E.M.

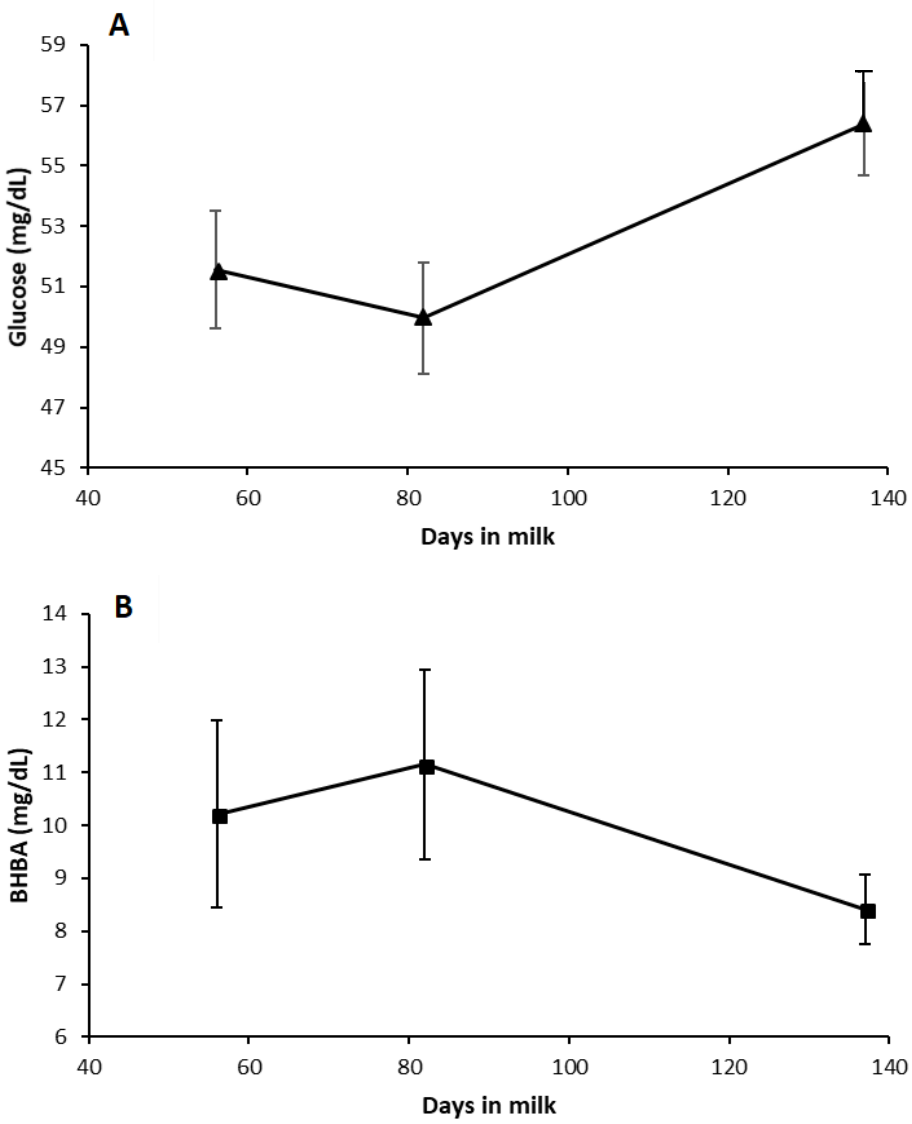


Figure 3-2. Blood concentrations of glucose and beta-hydroxybutyrate (BHBA) of the lactating cows at the time of embryos collection. (A) shows concentration of glucose and (B) shows concentration of BHBA in lactating cows (n = 25) at the time of embryo collection that was performed three times during lactation at 56, 82, and 137 days in milk. BHBA concentrations are averages of three measurements taken within a week prior to embryo collection. Data are the mean ± S.E.M.

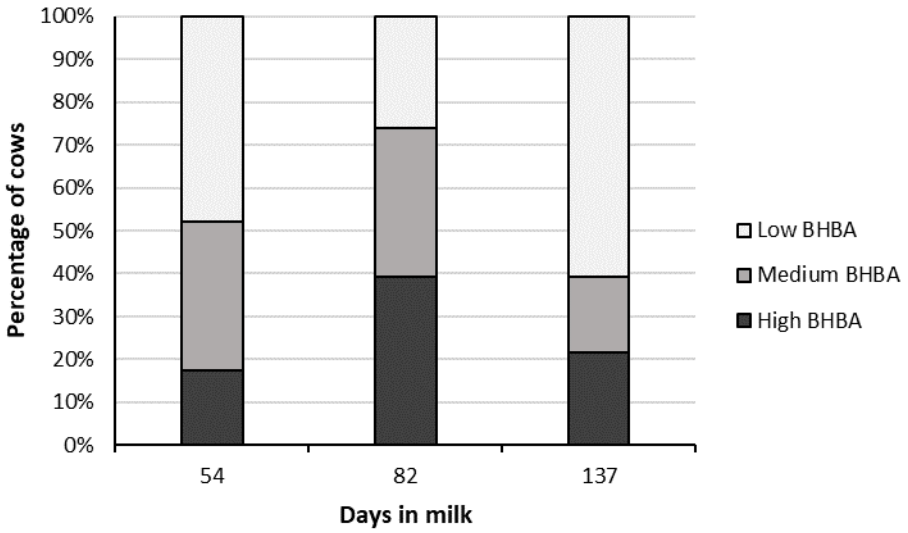


Figure 3-3. Percentage of cows with low, medium, or high beta-hydroxybutyrate (BHBA) level at the time of embryos collection. Weekly averaged BHBA concentration before each embryo collection from lactating cows (n = 25) at 54, 82, and 137 days in milk was categorized as high (BHBA >12 mg/dL), medium (BHBA 8 – 12 mg/dL), and low (BHBA < 8 mg/dL).

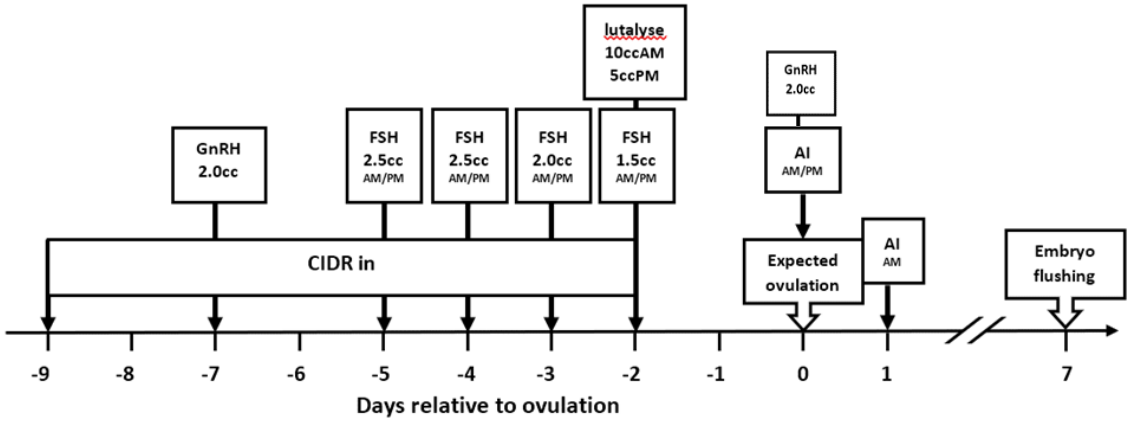


Figure 3-4. Protocol for estrus synchronization, superovulation, and timed-artificial insemination (TAI).

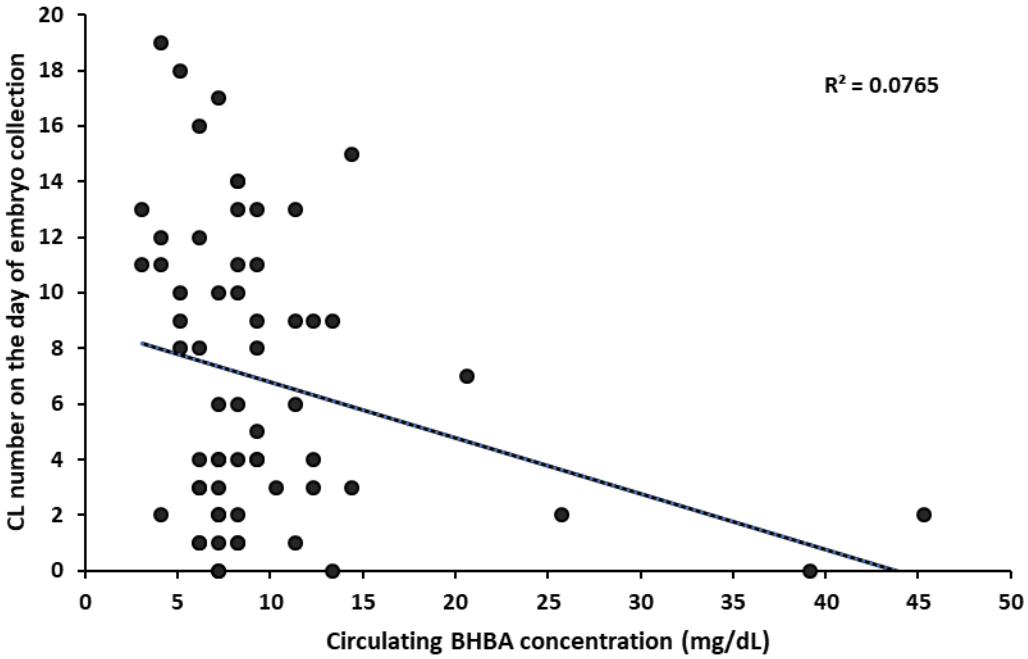


Figure 3-5. Correlation between circulating beta-hydroxybutyrate (BHBA) concentration and the number of CL on the day of embryo collection from lactating cows. Embryos were collected three times from lactating cows (n = 25) and before each embryo collection weekly averaged BHBA concentration was reported and the number of palpable CL was estimated.

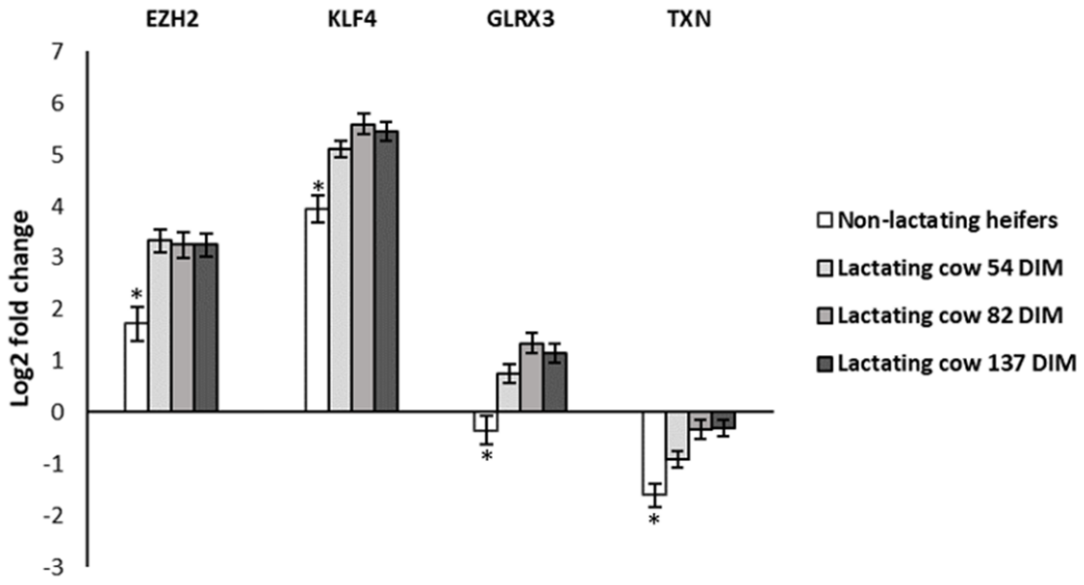


Figure 3-6. Relative expression level of genes that are showing more than 100%-fold increase in embryo from lactating cows relative to heifers. Embryos were collected from lactating cows (n = 25) at three different times during lactation and one time from a group of non-lactating heifers (n = 5). * indicates more than 100%-fold change. Data are the mean + S.E.M

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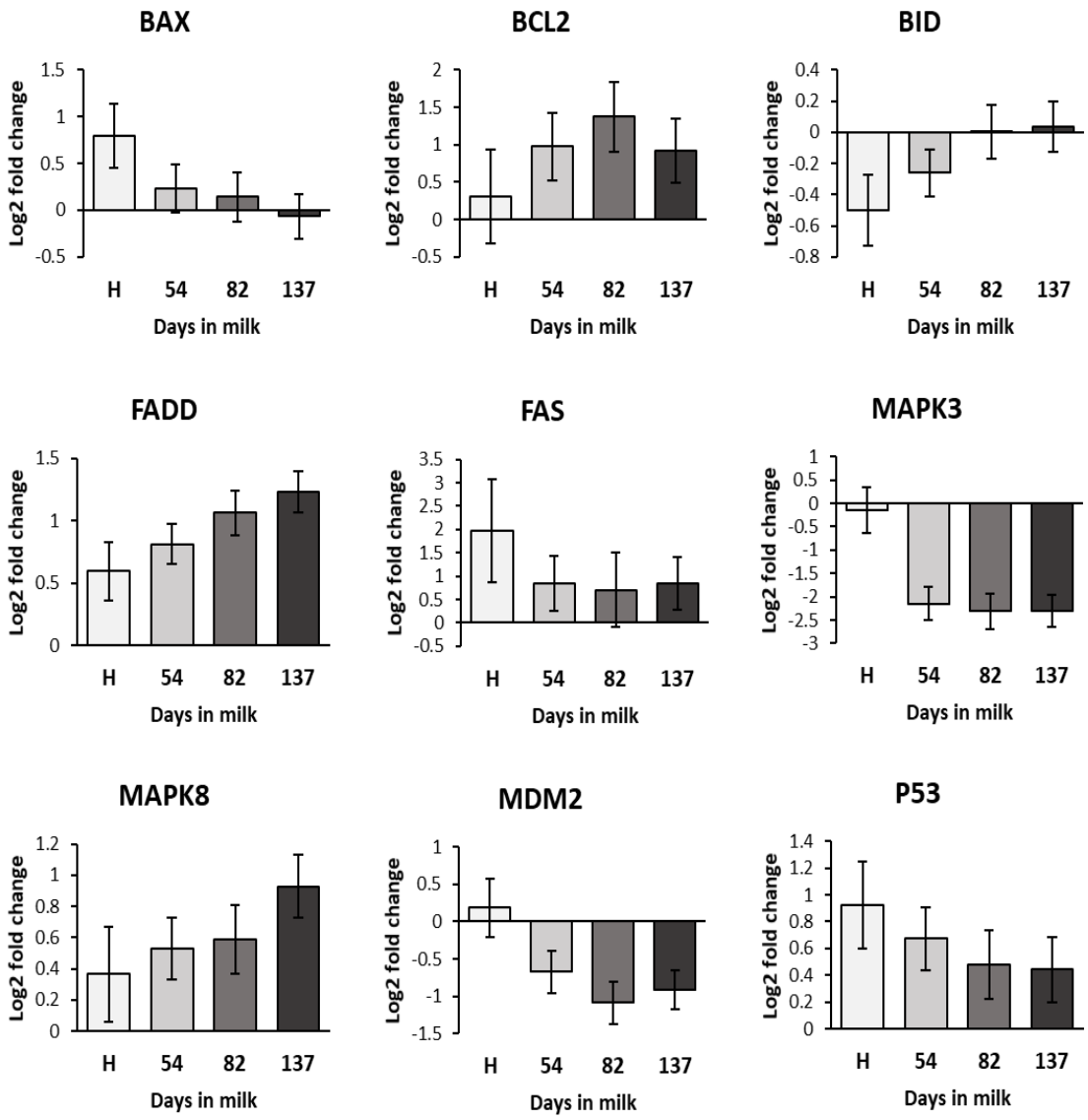
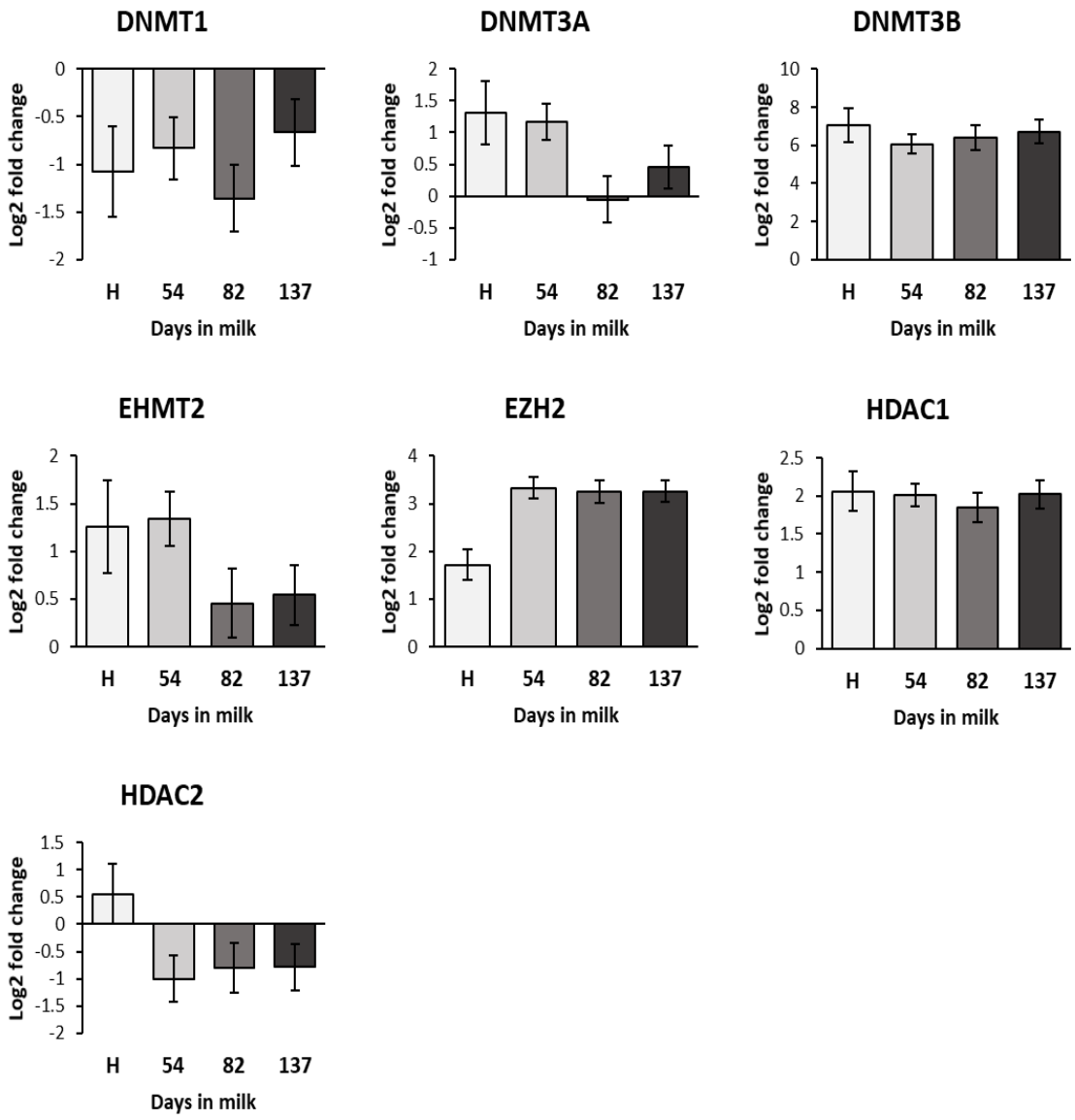
Supplementary Table 3-1. Forward and reverse primer sequences for genes used in qPCR analysis in individual embryos

Gene	5'-forward primer-3'	3'-reverse primer-5'
ACACA	GCTAACTCAACTCAGCAAGACC	GGATGGCAAATGGGAAGCAA
ACSL1	AAGGTTTCCAGAGGGGCTTAC	TCATAGGGTTGGTCTGGTTTCC
ACSL3	TGGCGGAAAGGATTCCAGAA	AGACAGACAAGCTCAGCACTTA
ASH2L	TGCCATCATAGCGGGAACAC	ACGTTAGGTTGGCCAAAGCA
BAX	CGGGTTGTCGCCCTTTTCTA	GCCCATGATGGTCCTGATCAA
BCL2	ATGTGTGTGGAGAGCGTCAA	GGTTCAGGTACTCGGTCATCC
BID	ACGGTGACCTTCATCAACCA	CCGAGTGGTCACTCAGTCC
CAT	CATCCAGGCTCTTTTGGACAA	TGAGACCCATGCTGCACATA
CDX2	TCCGGAGGAAAGCCGAAC	CTTTGCTCTGCGGTTCTGAAA
DNMT1	AGAGACGTCGAGTTACATCCA	GTGTTCCTGGTCTTACTCTTCC
DNMT3A	CCATGTACCGCAAGGCTATCTA	GCTGTCATGGCACATTGGAA
DNMT3B	GCCAAAGCTCTTCCGAGAAA	GGGTGGAGGTACTGCTGTTA
EHMT2	GACGTCCACCATGAACATTGAC	CTGGAGCAGTCATCCACACA
EIF4A1	CAGAAGCTCAACAGCAACAC	CTTCTTGGTCACCTCAAGCA
ESRRB	CAGCCCATACCTGAGCTTACA	CCACCAGCAGGTAGGAGAC
ETS2	GCAGGGCAAACCAGTTATACC	AGAAACTGCCACAGCTGGATA
EZH2	GAGCCATCCAGACTGGTGAA	TTCGATGCCCACGTACTTCA
FABP3	ACCACAGCAGATGACAGGAA	GACAAGTTTGCCGCCATCC
FADD	TCGAGCAGCGACCTGAC	GCTCCAGCTTCCTCTTGCTA
FAS	GAAATGCACACCAACGAGCAA	AGGATCAGGAGGGCCCATAAA
G6PD	GTCTGGTGGCCATGGAGAA	CTGCACCTCTGAGATACACTTCA
GATA6	AGCAAGATGAACGGCCTCA	TGGCGCAGGACAATCCAA
GAPDH	GGGTCTTCACTACCATGGAGAA	GTTCACGCCCATCACAAACA
GLRX	CTCGGGTCTTCATCGGTCAA	GTCAACAGTTCCCCTCTCTCA
GLRX2	CTCTTCACAAAATGACTGGCGAAA	TGTCAGTTGCGCCTCCAATA
GLRX3	TAAGTGTGGCTTCAGCAGAC	TCATCCTCCAATATATCAAATGTCTCA
GLUD1	CCGACAACTCCAGAAGCTGATA	TGTCACTCCTCCAGCATTCA
GNAS	CCCGGGCCAAGTACTTCA	GGTGAAGTGAGGGTAGCAGTA
GPX1	CGGGACTACACCCAGATGAA	CTTCAGGCAATTCAGGATCTCC
GRB10	TTGCTGGCAGGAAGCAGTA	GCCTCGTTCCTGACTCTGTTA
GSR	AGTTGGGGATGTGTGTGGAA	GTTTTCGGCCAGCAGCAA
H19	CTTGGAACACGGACTTCTTCAA	AGGGTGTGTAGTGGTTCCAA
HDAC1	ATGTCCGAGTACAGCAAGCA	CAGAACTCAAACAGGCCATCAAA
HDAC2	AAGGAGGCGGCAAGAAGAAA	TGGGATGACCCTGTCCGTAATA
HSF1	GCAGCTCCTCGAGAACATCA	ACACTGTCCTGGCGAATCTTTA
HSP10	AGCTGTTGGATCAGGCTCTAA	GCCTCCATATTCTGGGAGAAGAA
HSP110	TTCTGATCCTCAAGGAGTTCCA	CCATCCTTCTGTGCTGAAACA
HSP60	CTTTTAGCCGATGCTGTAGCC	GGACTTCCCCAACTCTGTTCA
HSP90AA1	CATGGATAACTGCGAGGAGCTA	AGATCCTCAGAATCCACCACAC
HSP90AB1	GGCAGAAATTGCCCAACTCA	AGGGTCTGTCAGGCTCTCATA

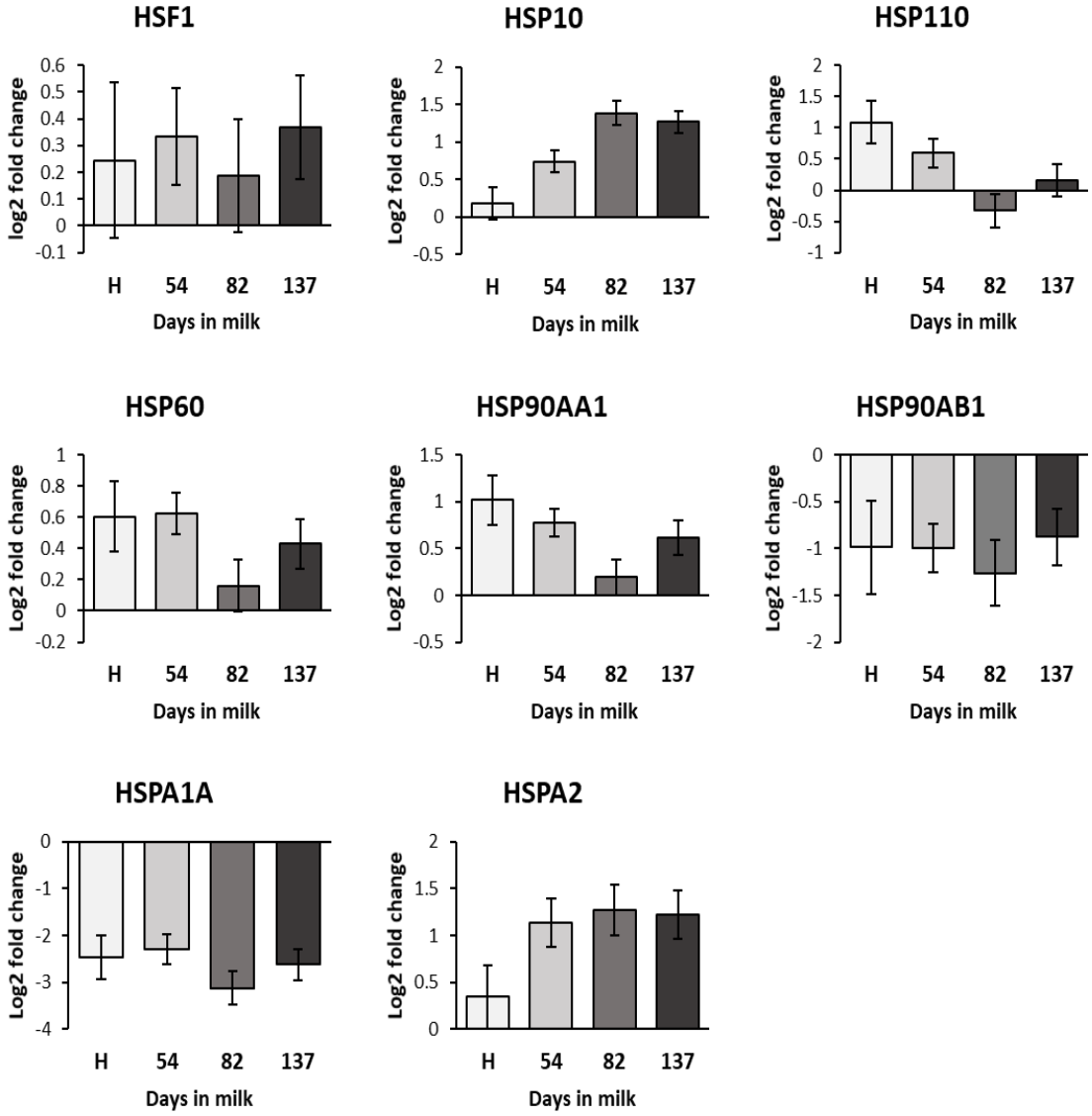
HSPA1A	CTGATCAAGCGCAACTCCA	TTGTCCGAGTAGGTGGTGAA
HSPA2	ACGGCATCTTCGAGGTGAA	CCATGCGGTTATCGAAGTCC
IGF1R	GAACCTGCGCCAGATCCTA	GCTGCAAGTTCTGGTTGTCA
IGF2R	GGCAGATTCCACTCAAGTCA	AGATCAAGGTGAGGTCTCCA
KLF4	GCGGCAAAACCTACACGAA	CCATCCCAGTCACAGTGGTAA
LIN28A	GCGGCCCAAAGGGAAGAATA	CACTCCTTGGCATGATGGTCTA
MAPK3	CGTCATTGGCATCCGAGACA	TGTCTCCATCAGGTCCTGTAC
MAPK8	GCAGTTAGATGAAAGGGAACACA	GGTTCTCTCCTCCAAGTCCATA
MDM2	CAGATTCCAGCTTCGGAACAA	CTGTGCACCAACAGACTTCA
NDN	ATGTGGTACGTGCTGGTCAA	AACTGCCGATGACATCCTTCA
P53	CCCATCCTCACCATCATCACA	GCACAAACACGCACCTCAA
POU5F1	AGAAGCTGGAGCCGAACC	CTGCTTTAGGAGCTTGGCAAA
SCO2	GGCGCGAAGCAAGTAGAAG	CAGCAGCAGCATGGACCT
SLC2A1	GCTGTGCAGTGCTCATGAC	ATGGCCACAATGCTCAGGTA
SOD1	CGGTGTTGCCATCGTGGATA	TCCACCTCTGCCCAAGTCA
SOD2	GCCTACGTGAACAACCTCAAC	CTGCAGAGCTATCTGAGCTGTA
SOX2	CCCAAGAGAACCCTAAGATGCA	CCGTCTCGGACAAAAGTTTCC
TAF11	AGAGAAGAAGCAGAAAGTGGATGAA	GGTTCAGCTGCTCCTCAGAA
TEAD4	ATCATCACCTGCTCCACCAA	CCATTCTCGTAGCGTGCATAC
TXN	CGTGGTGTTCCTTGAAGTAGATG	CTGGAAGGTTGGCATGCATT
TXNRD1	CTGAGGAGAAAGCTGTGGAGAA	CGTCCATTCCAATGGCCAAAA
UBE3A	TGACGACATTGAAGCTAGCC	ACTGGTGGTAGTAGCGTTCTA
YWHAZ	AACAGCAGATGGCTCGAGAA	GAAGCGTTGGGGATCAAGAAC



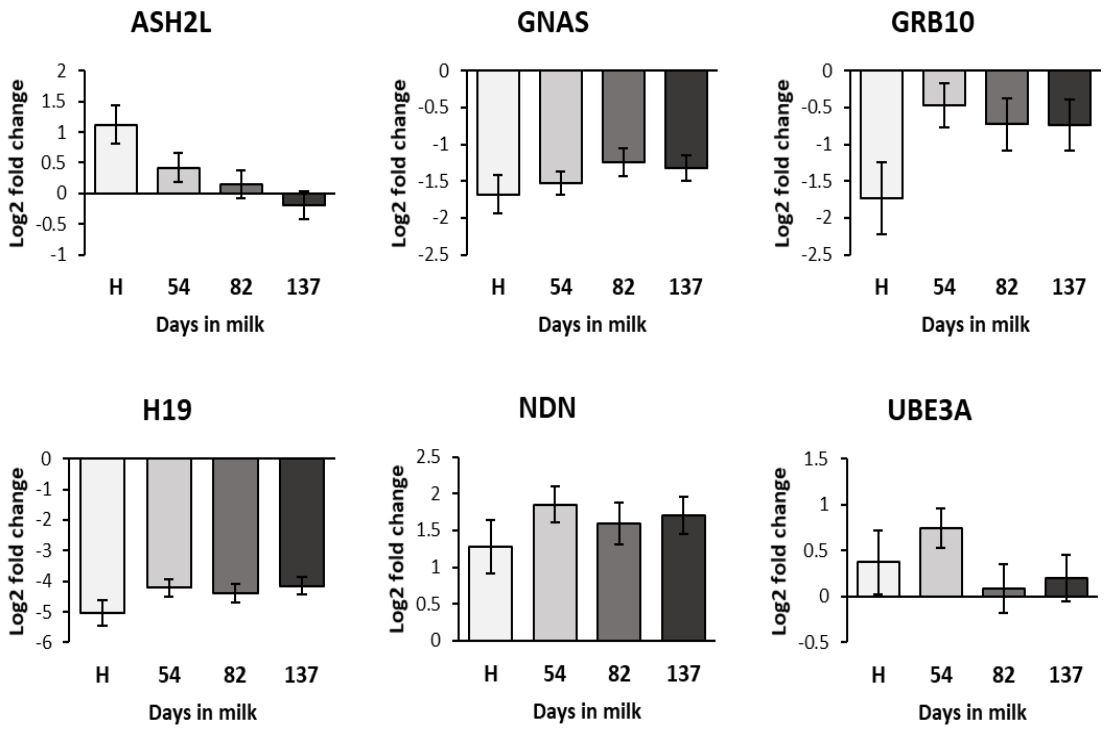
Supplementary Figure 3-1. Relative expression of genes involved in apoptotic regulation tested by qPCR in individual embryos grouped by DIM. Embryos were collected one time from heifers (H) and three times from lactating cows during lactation. Zero out of the nine gene tested were differentially expressed * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



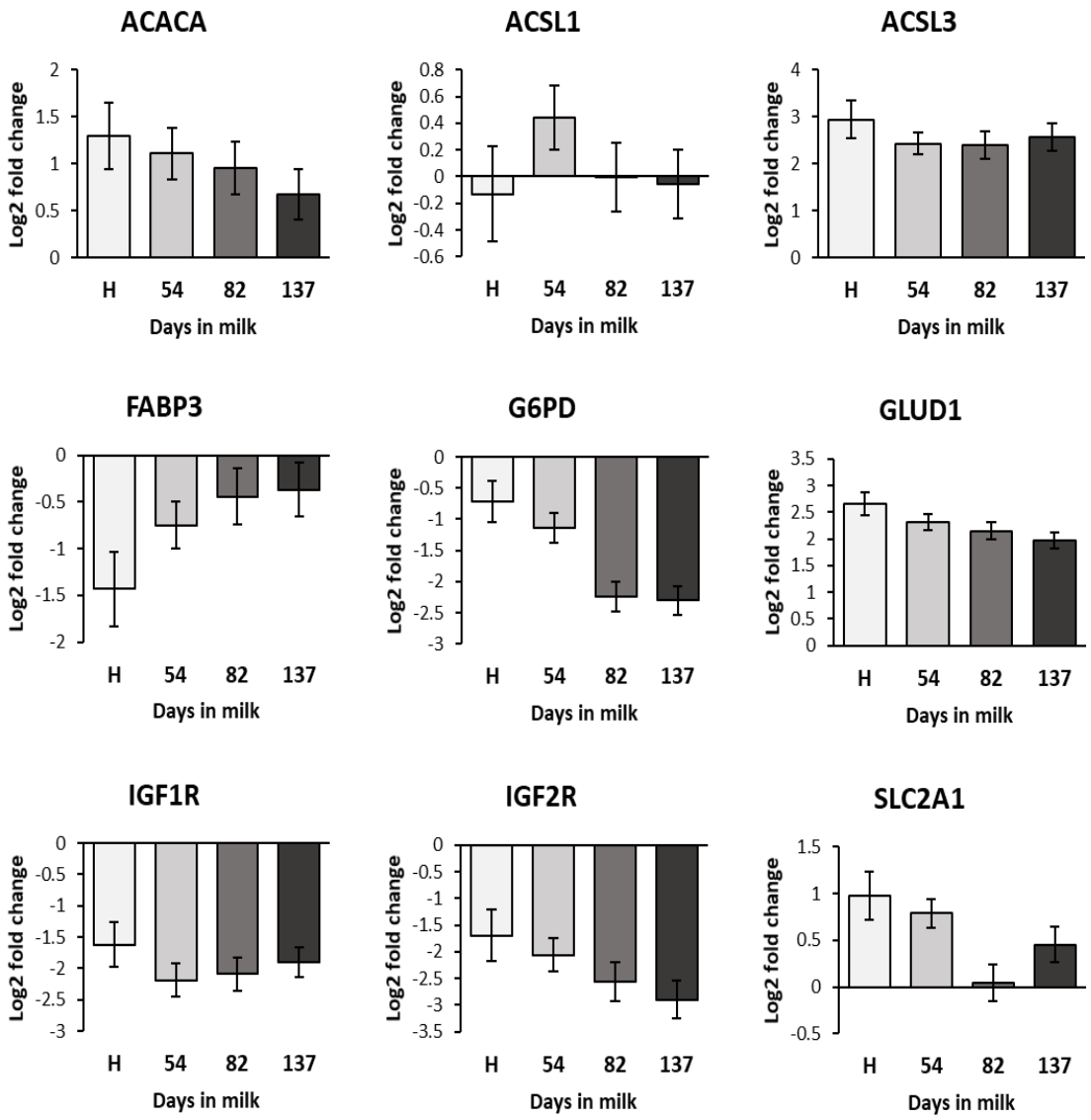
Supplementary Figure 3-2. Relative expression of genes involved in epigenetic regulation tested by qPCR in individual embryos grouped by DIM. Embryos were collected one time from heifers (H) and three times from lactating cows during lactation. Zero out of the seven genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



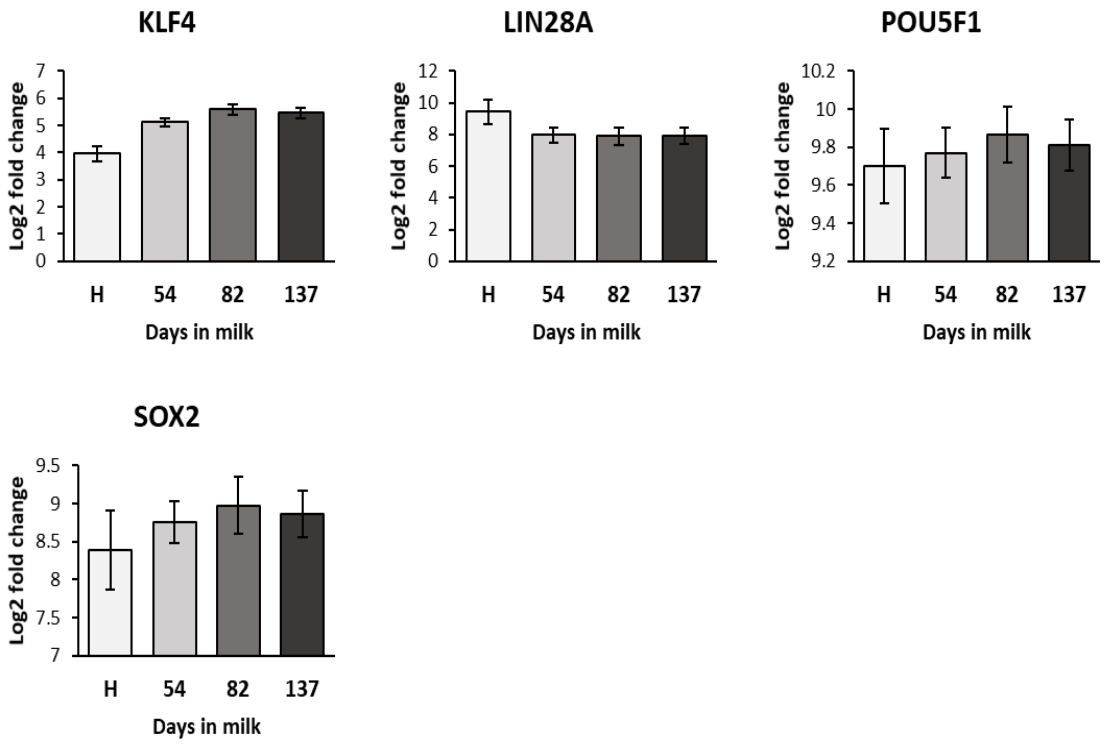
Supplementary Figure 3-3. Relative expression of genes from heat shock proteins category tested by qPCR in individual embryos grouped by DIM. Embryos were collected one time from heifers (H) and three times from lactating cows during lactation. Zero out of the eight genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



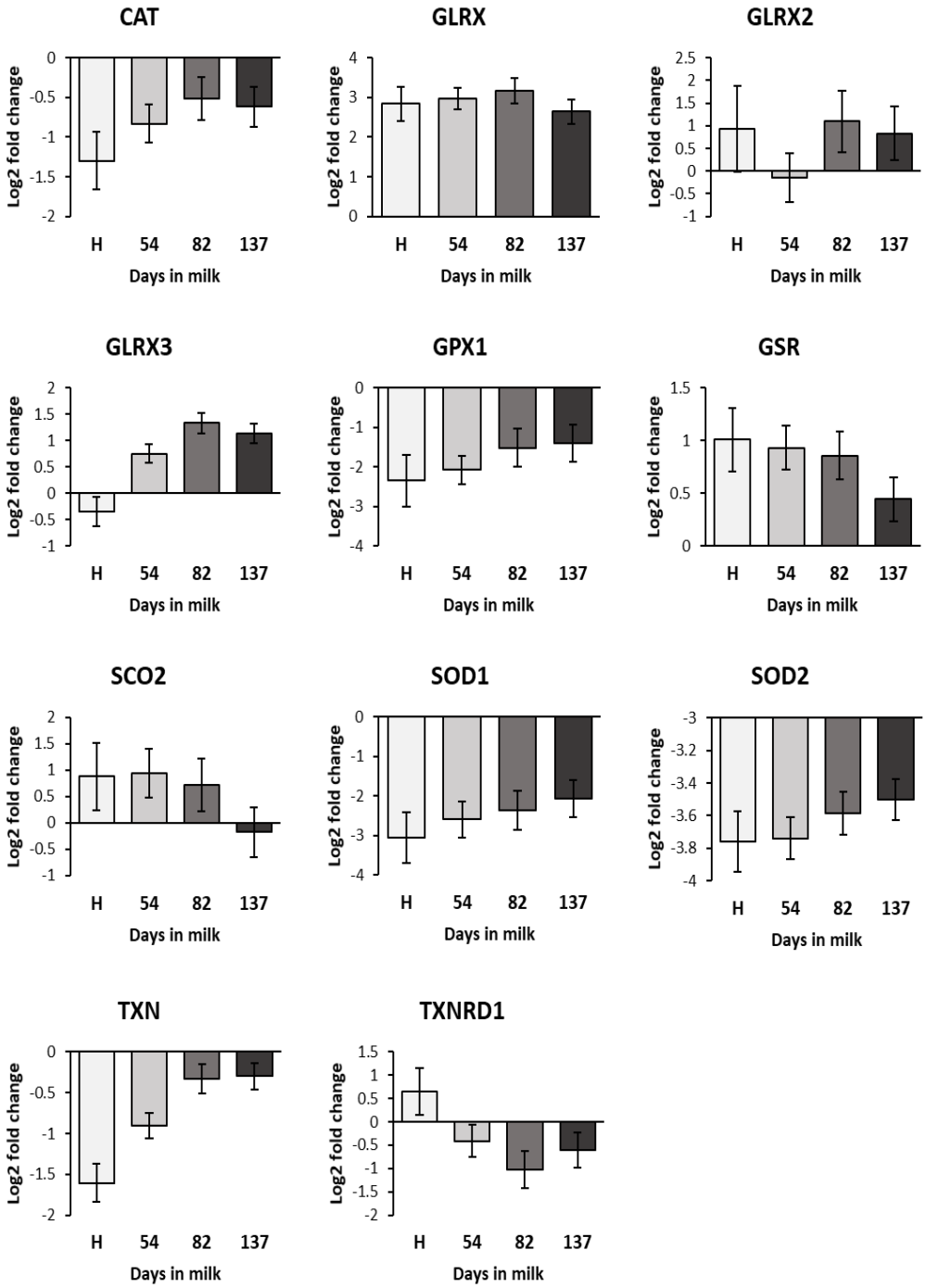
Supplementary Figure 3-4. Relative expression of genes from imprinting genes category tested by qPCR in individual embryos grouped by DIM. Embryos were collected one time from heifers (H) and three times from lactating cows during lactation. Zero out of the six genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



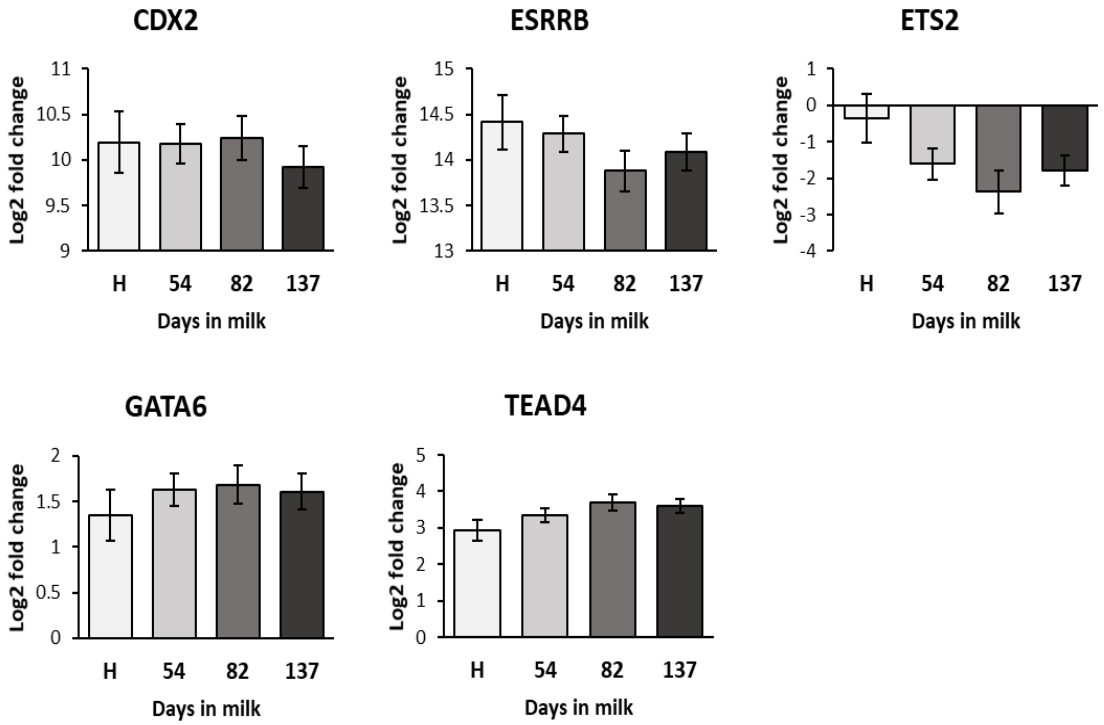
Supplementary Figure 3-5. Relative expression of genes from metabolism category tested by qPCR in individual embryos grouped by DIM. Embryos were collected one time from heifers (H) and three times from lactating cows during lactation. Zero out of the nine genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



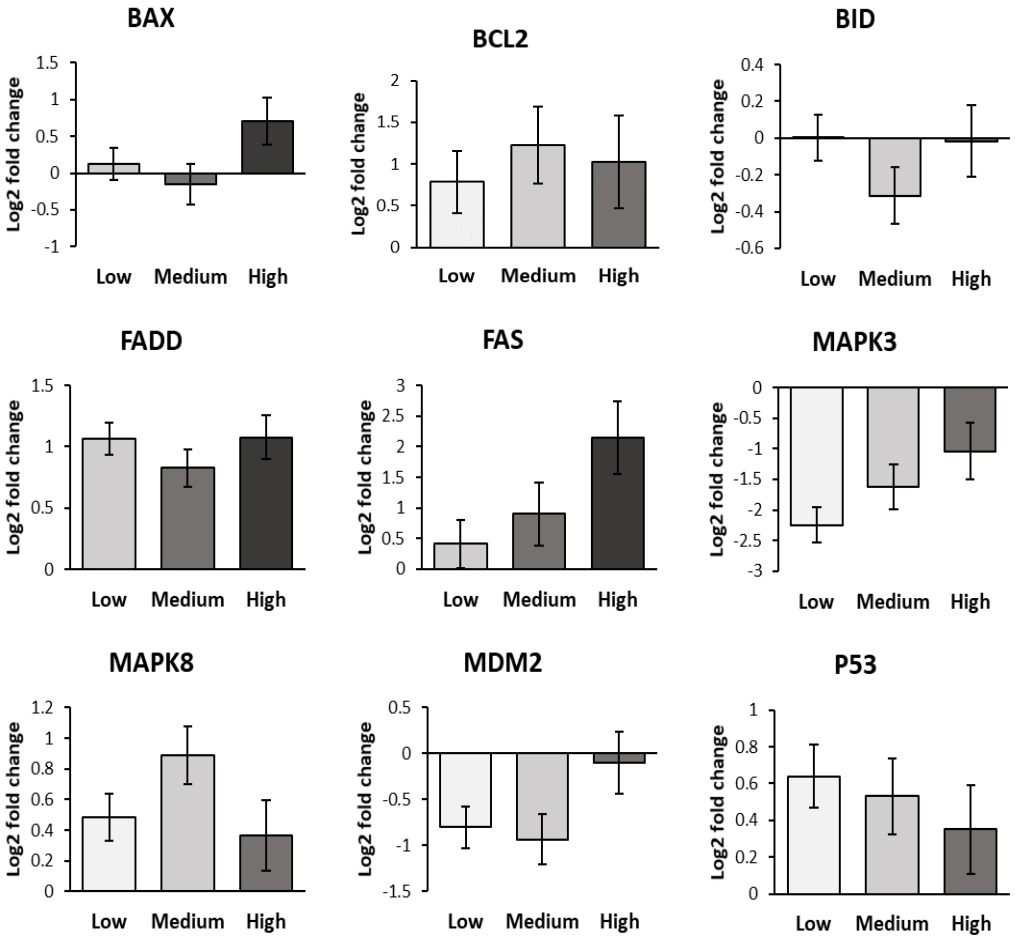
Supplementary Figure 3-6. Relative expression of genes from pluripotency category tested by qPCR in individual embryos grouped by DIM. Embryos were collected one time from heifers (H) and three times from lactating cows during lactation. Zero out of the four genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



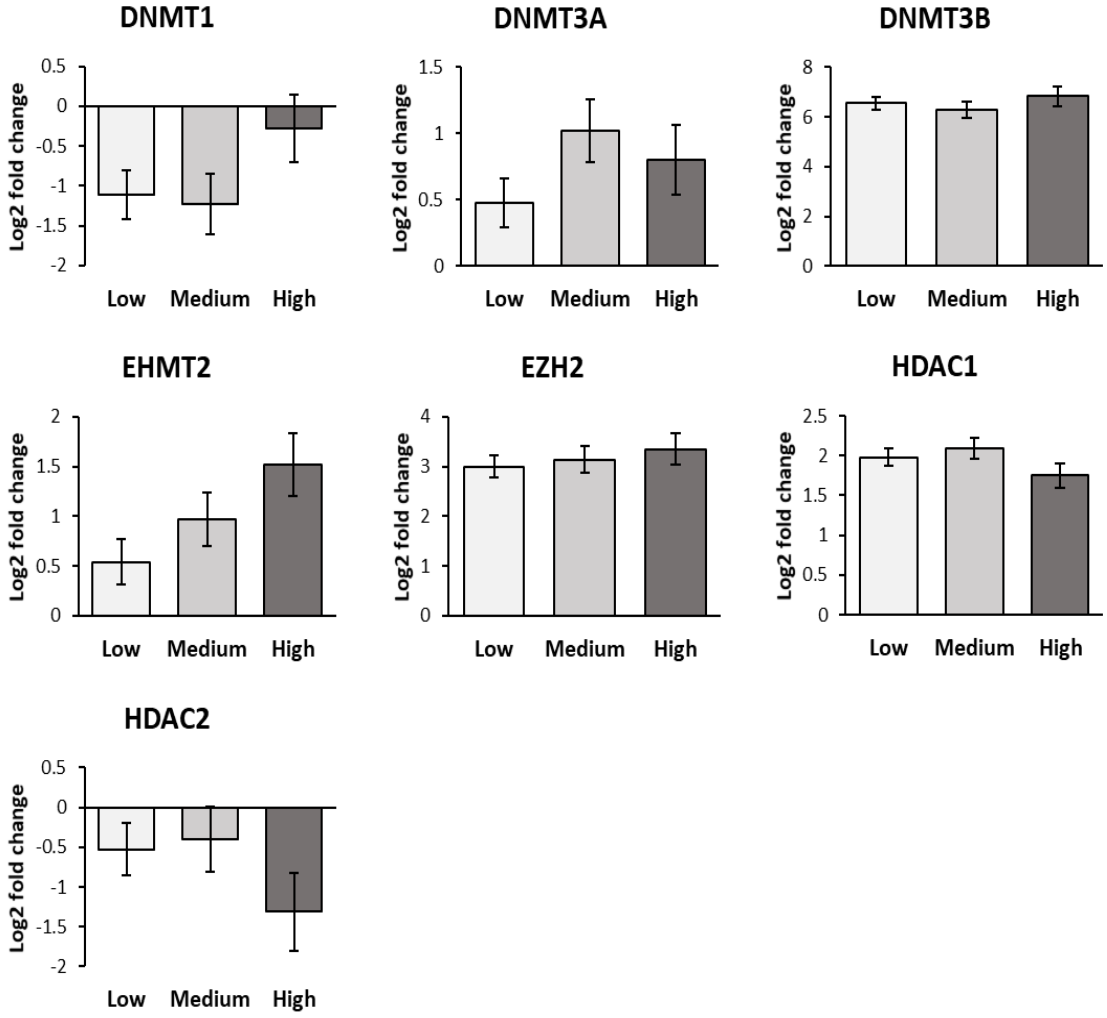
Supplementary Figure 3-7. Relative expression of genes involved in redox regulation tested by qPCR in individual embryos grouped by DIM. Embryos were collected one time from heifers (H) and three times from lactating cows during lactation. Zero out of the eleven genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



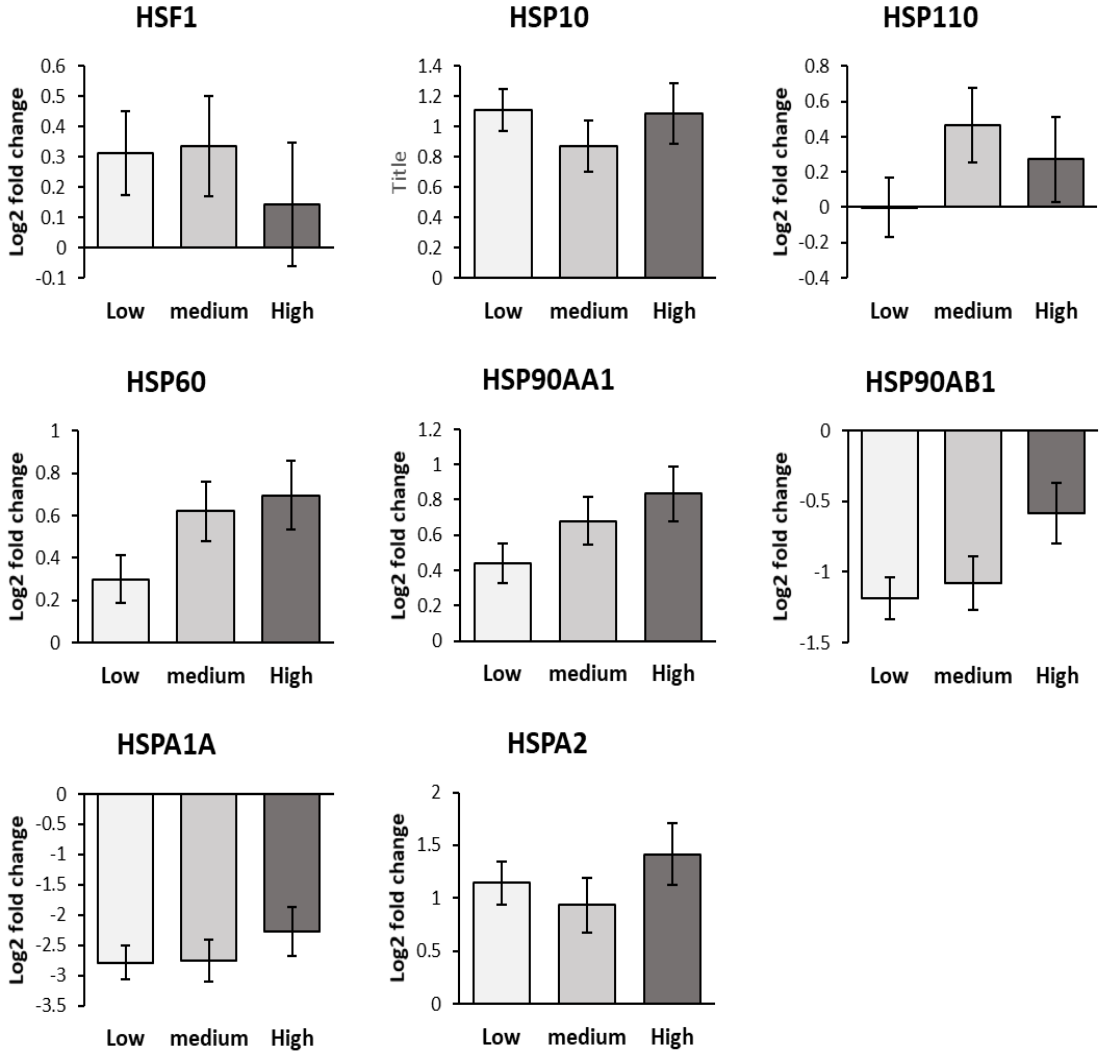
Supplementary Figure 3-8. Relative expression of genes from trophoblast category tested by qPCR in individual embryos grouped by DIM. Embryos were collected one time from heifers (H) and three times from lactating cows during lactation. Zero out of the five genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



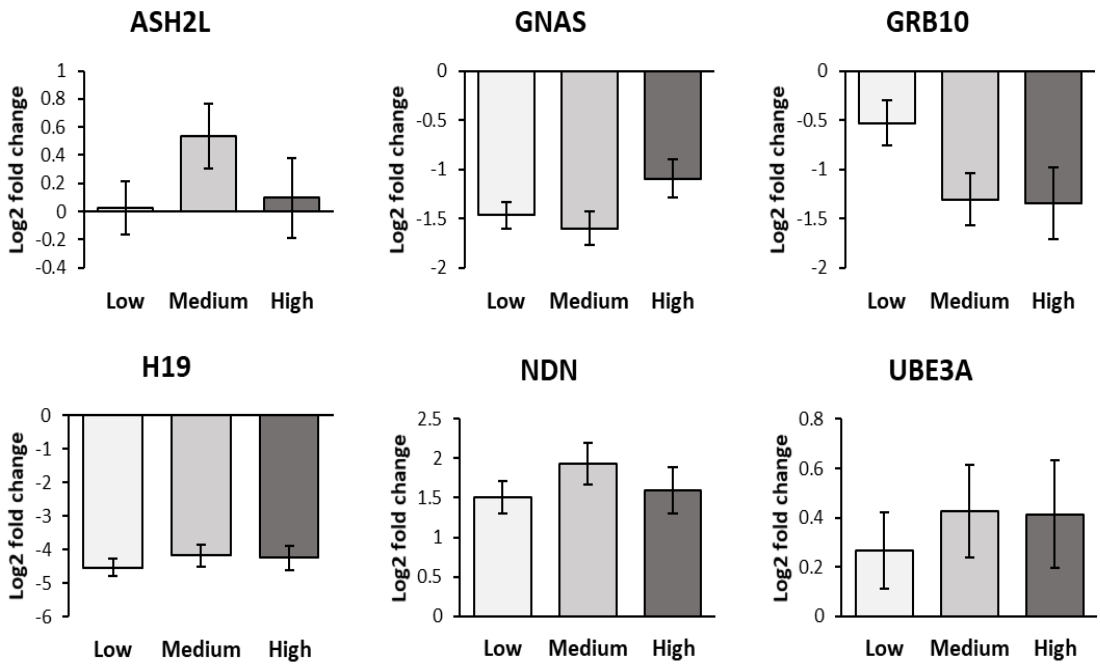
Supplementary Figure 3-9. Relative expression of genes involved in apoptotic regulation tested by qPCR in individual embryos from lactating cows grouped by the BHBA level. Zero out of the nine genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



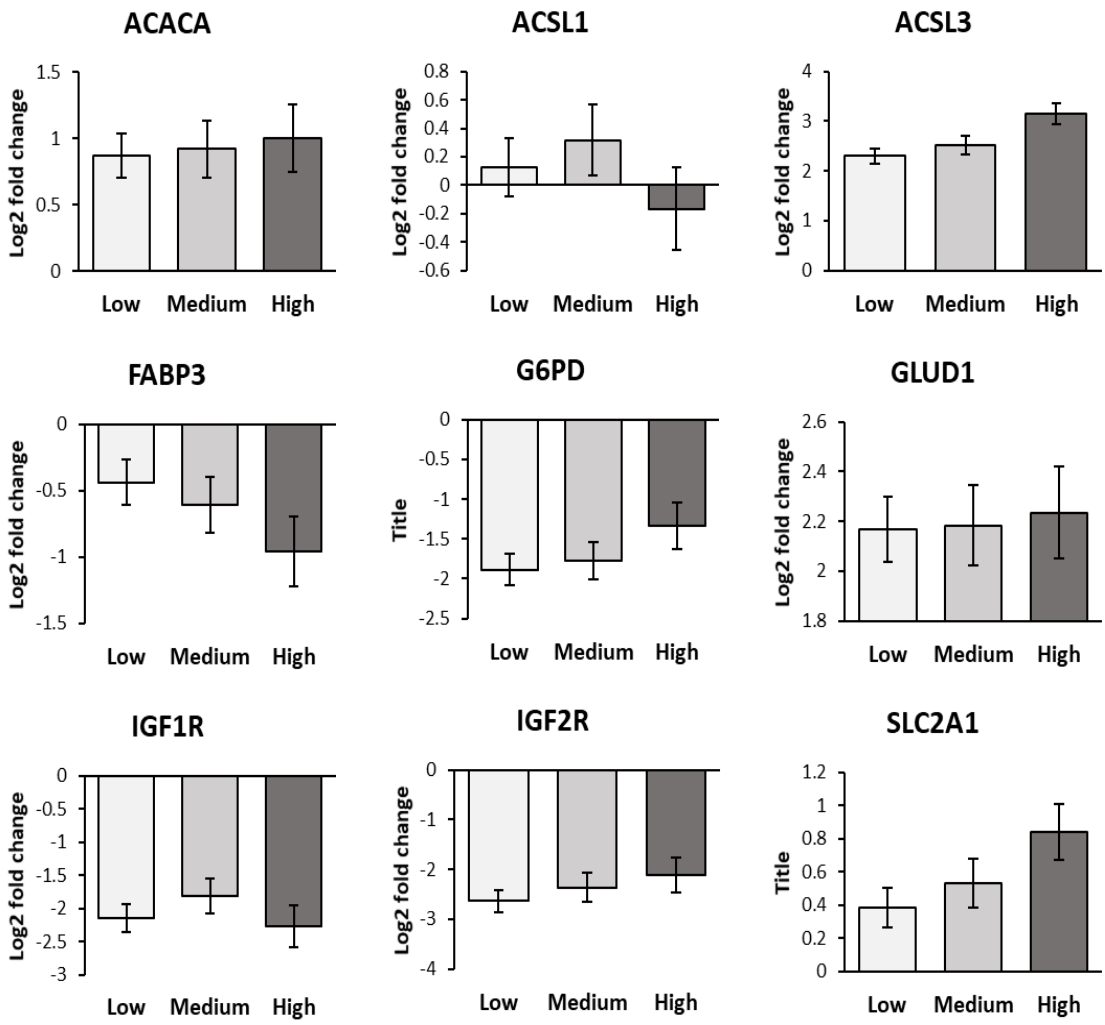
Supplementary Figure 3-10. Relative expression of genes involved in epigenetic regulation tested by qPCR in individual embryos from lactating cows grouped by the BHBA level. Zero out of the seven genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



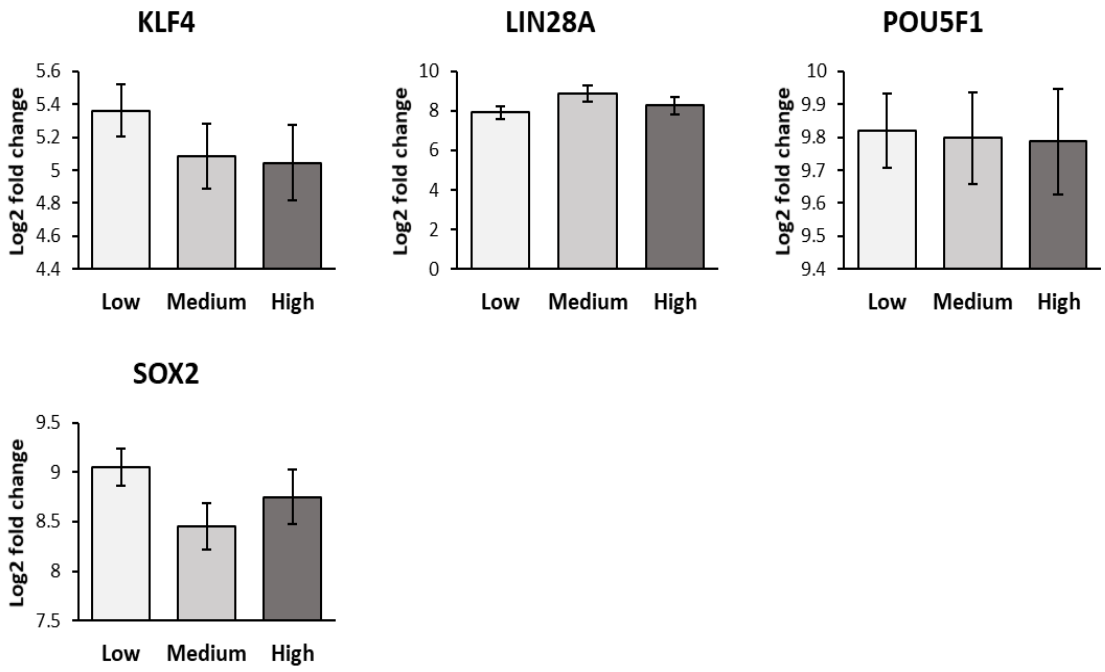
Supplementary Figure 3-11. Relative expression of genes from heat shock proteins category tested by qPCR in individual embryos from lactating cows grouped by the BHBA level. Zero out of the eight genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



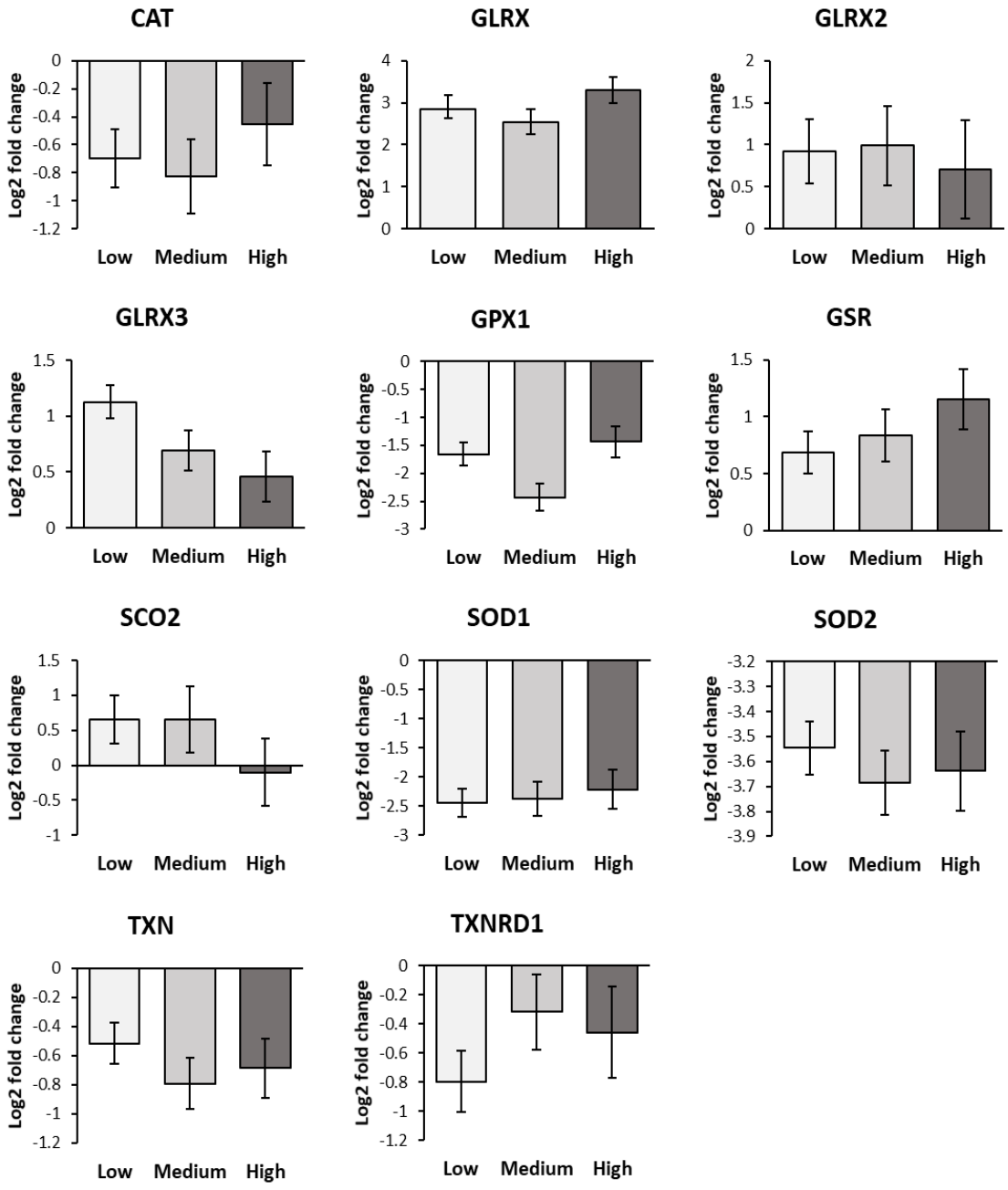
Supplementary Figure 3-12. Relative expression of genes from imprinting genes category tested by qPCR in individual embryos from lactating cows grouped by the BHBA level. Zero out of the six genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



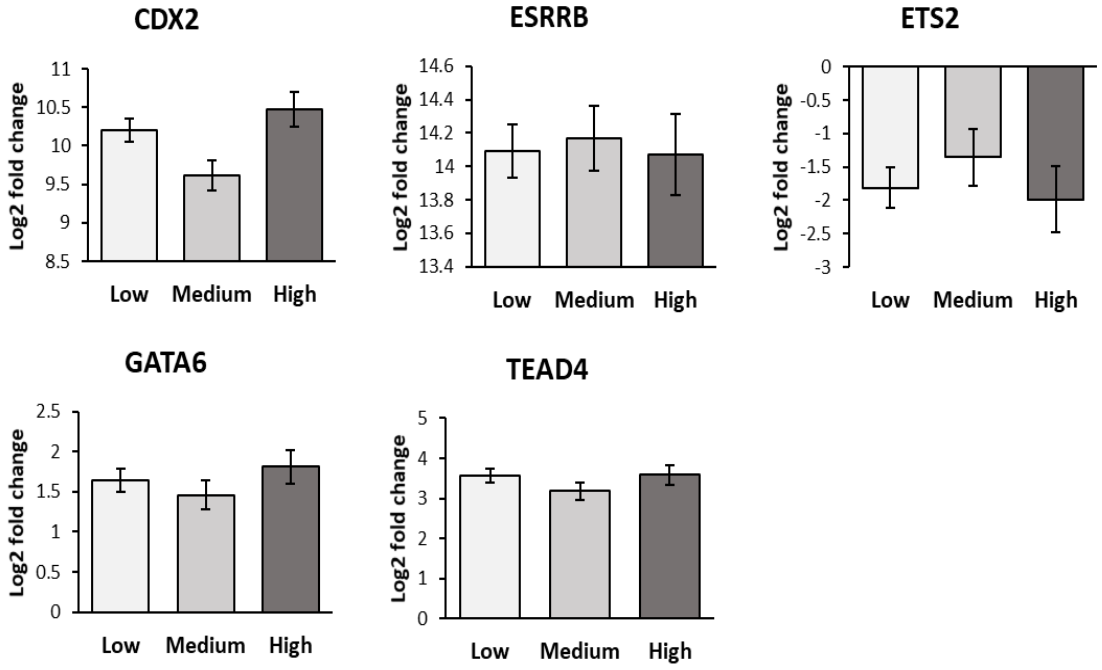
Supplementary Figure 3-13. Relative expression of genes from metabolism category tested by qPCR in individual embryos from lactating cows grouped by the BHBA level. Zero out of the nine genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



Supplementary Figure 3-14. Relative expression of genes from pluripotency category tested by qPCR in individual embryos from lactating cows grouped by the BHBA level. Zero out of the four genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



Supplementary Figure 3-15. Relative expression of genes involved in redox regulation category tested by qPCR in individual embryos from lactating cows grouped by the BHBA level. Zero out of the eleven genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



Supplementary Figure 3-16. Relative expression of genes from trophoblast specific category tested by qPCR in individual embryos from lactating cows grouped by the BHBA level. Zero out of the five genes tested was differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.

CHAPTER 4

EFFECTS OF LACTATION AND NEGATIVE ENERGY BALANCE ON ENDOMETRIAL EXPRESSION OF SELECTED TRANSCRIPTS OF HOLSTEIN DAIRY COWS AT DAY 7 POST-OVULATION

ABSTRACT

Dairy cows often experience a state of negative energy balance (NEB) during the first few months of lactation, resulting in dramatic fluctuations in circulating levels of hormones and metabolites that can have a negative impact on the uterine environment and overall cow fertility. The objective of this study was to identify how the lactation and energy status of dairy cows impact gene expression in the endometrium during early pregnancy. Endometrial biopsies were collected on day 7 post-ovulation from Holstein heifers (n=3) and from lactating Holstein cows (n=22) at 54, 82, 137 days in milk (DIM). beta-hydroxybutyrate (BHBA) concentration was measured to determine the energy status of each cow as low, medium, or high BHBA. RNA was extracted from each endometrial sample for qPCR analysis of 41 genes involved in metabolism, heat shock/chaperone functions, and inflammatory response. Expression of *IL-2, IL2RA, CD28, CTLA4*, and *GATA3* from inflammatory response category was significantly higher (adj. $P < 0.05$) in lactating cows relative to heifers. Expression of *ACACA, G6PD, IGF2R, and SLC16A3* from metabolism category was significantly higher in heifers relative to lactating cows. Within lactating cows, the expression of *IL-10, IL2RA,* and *CD28* was significantly altered by BHBA level. We conclude from these data that lactation and NEB-induced metabolic changes may promote a pro-inflammatory uterine

environment that is inappropriately suited for supporting early embryo development in dairy cows.

INTRODUCTION

The uterus is the central organ in the female reproductive tract, and a healthy and receptive uterus is indispensable to establish and maintain pregnancy. It provides nutrients and optimal environment for the embryo to grow and thrive. Following calving, the uterine tissue undergoes extensive remodeling to regain its pre-pregnancy physiological function and size in a process called uterine involution. Proper and timely postpartum uterine involution is critical to remove cellular debris, and microbial contamination and any delay in this process is likely to increase the incidence of uterine infection and disturb the estrous cycle (Sheldon et al., 2002; Sheldon and Dobson, 2004). Postpartum bacterial contamination is inevitable and successful uterine involution accompanied with healthy immune response are crucial to eliminate and fight pathogens and to prevent infection (Földi et al., 2006).

Typically, the postpartum uterus relies on innate immunity and mucosal barriers for defense against microbial contamination instead of adaptive immunity (King et al., 2003; Sheldon et al., 2006). Inadequate immune response results in uterine disease including metritis, endometritis and subclinical endometritis that are associated with reduced reproductive performance and increased calving interval (LeBlanc et al., 2002; LeBlanc, 2014). Metritis is the inflammation of the uterus and is characterized by systemic signs of sickness. Within the first two weeks postpartum, up to 20% of dairy cows experience metritis, that can turn into purulent vaginal discharge at 4 to 5 weeks after recovering from systemic illness. Endometritis is the local inflammation of the

endometrium and only affects the luminal lining of the uterus with no systemic illness, and up to 50% of cows experience endometritis within 4 to 8 weeks postpartum (LeBlanc, 2014). Subclinical endometritis is the inflammation of the endometrium in the absence of purulent discharge, and for accurate diagnosis, it requires endometrial cytology to be confirmed (Sheldon et al., 2006; Barlund et al., 2008). Subclinical endometritis is diagnosed by measuring the proportion of neutrophils in the endometrial cytology sample and is defined by the presence of >18% neutrophils in the sample by 20–33 DIM or >10% neutrophils at 34–47 DIM (Kasimanickam et al., 2004).

The neutrophil infiltration is part of the innate immune response and recruitment of immune cells and secretion of chemokines and cytokines are essential to clear the bacterial infection and restore homeostasis in the uterus (Sheldon and Roberts, 2010). The inflammatory reaction is a normal physiological response during infection, and the endometrial cells respond to the infectious pathogens by up-regulating the expression of proinflammatory genes (Herath et al., 2009). The immune system responds to the bacterial lipopolysaccharide (LPS) by activating the toll-like receptor-4 (TLR4)-dependent signaling pathway in the endometrial cells which leads to the secretion of proinflammatory cytokines and chemokines such as TNFα, IL-1β, IL-8 and prostaglandin E2 (Sheldon and Roberts, 2010; LeBlanc, 2014). The release of the proinflammatory cytokines recruits neutrophils and macrophages to the inflammatory site that release antimicrobial factors to combat pathogens and resolve the uterine infection. However, this response to infection may alter the uterine environment and make it less suitable for the preimplantation embryo which may explain infertility in cows with high expression of proinflammatory genes (Herath et al., 2009; Bromfield et al., 2015). The inflammatory

response is crucial for the protection of the uterus and to support pregnancy, but the increased intensity or duration of the response may impair rather than enhance fertility. Thus, the immune system must balance between successful protection against infections and inflammatory insults that may interfere with the reproductive capacity.

A poor postpartum innate immune response can result in persistence of the bacterial infection, increased duration and intensity of endometrial inflammatory response, and delay the recovery process leading to increased calving interval or culling. During the transition period, a rapid increase in nutritional and energetic demands with limited feed intake can result in negative energy balance (NEB), that is associated with metabolic and hormonal dysregulations which interact with the reproductive performance (Opsomer, 2015). Negative energy balance has been linked to many reproductive diseases and issues such as retained placenta, dystocia, metritis, subclinical endometritis, and low conception rate which can negatively impact overall fertility (Butler, 2003).

Negative energy balance and metabolic disorders interact with the immune system and suppress the function of immune cells and predispose cows to periparturient diseases and uterine infection (Esposito et al., 2014). Therefore, the incidence of uterine disease and infection is higher in cows during NEB (Sheldon et al., 2006). The increased severity and duration of NEB in high-yielding dairy cows put them under a considerable amount of metabolic stress that goes beyond their physiological capacity. Negative energy balance leads to excessive fat mobilization that is indicated by dysregulation of levels of circulating metabolites such as non-esterified fatty acids (NEFAs) and βhydroxybutyrate (BHBA) in the blood. Elevated levels of BHBA and NEFA, together with a decline in the body condition score (BCS) are indicative of NEB (Barletta et al., 2017). Negative

energy balance and fatty liver can impair peripheral blood neutrophil function and suppress immune function (Zerbe et al., 2000). Neutrophil function was shown to be significantly impaired in peripartum cows with high blood concentration of NEFA due to NEB (Hammon et al., 2006; Ster et al., 2012).

The experiment described here was designed to test the hypothesis that NEB-induced metabolic fluctuations can alter endometrial gene expression, which may result in a suboptimal uterine environment that is unlikely to support early embryonic development and implantation. Suboptimal uterine environment may explain the reduced conception rate in lactating dairy cows when compared with non-lactating dairy heifers of the same genetic merits. To test this hypothesis, we compared the expression level of 41 genes involved in metabolism, heat shock/chaperone functions, and inflammatory response in endometrial tissues. Endometrial tissue samples were collected on day 7 post-ovulation from a group of non-lactating heifers one time and from a group of lactating cows at multiple times during early lactation. This analysis of differential gene expression between these two groups may help in understanding the molecular changes in the endometrium of dairy cows during early lactation.

MATERIALS AND METHODS

Animal Management and Experimental Groups

This experiment was conducted at the Utah State University Caine Dairy Research and Teaching facility and all procedures were carried out under license in accordance with guidelines of the Utah State University Animal Care and Use Committee (IACUC#2438). Twenty-two multiparous Holstein-Friesian dairy cows from different parity, second to the seventh lactation, were randomly selected and used in this

experiment. Cows were identified a few weeks prior to expected calving, and initial BCS using a 5-point scale with 0.25-point increments and body weight were taken within the first week post-partum then every other week until the wk 19, around 130 days in milk (DIM). Cows were housed in a tie-stall barn during the experiment which was conducted between October and May. All cows were fed a total mixed ration (TMR) twice daily with ad libitum access to feed and water. Cows were milked twice daily, and the milk yield was recorded daily and averaged weekly.

Endometrial tissue samples were collected one time from a group of three Holstein heifers, average age 14 months, that were randomly selected and kept in a free-stall barn. Endometrial tissues from heifers' group were collected to be used as samples from non-lactating animals that apparently not experiencing NEB as lactating cows. Endometrial tissue samples from lactating cows were collected three times from each animal at 54 ± 4.2, 82 ± 4.2, and 137 ± 4.4 DIM. Dairy cows on average return to positive energy balance around 80 DIM (Coffey et al., 2002), and by collecting the samples at 137 DIM we assumed these samples are being collected from animals during positive energy balance unless a serious health issue or a significant drop in body weight was reported.

BHBA and Glucose Measurement

Blood samples were collected two to three times every week post-calving from wk 1 to wk 19 after the evening milking from the coccygeal vein using plain vacutainer blood collection tubes and needles. Blood samples from heifers were collected one time before endometrial biopsy collection. Fresh whole blood was used to measure BHBA and glucose immediately after drawing using the digital hand-held device (Precision Xtra, Abbott Diabetes Care, Abingdon, UK). The multiple glucose and BHBA measurements

from each cow were averaged by week. The weekly BHBA concentration for each animal was categorized as high (BHBA >12 mg/dL), which is the common cutoff point for subclinical ketosis (Iwersen et al., 2009), and BHBA concentration < 12 mg/dL was further divided into medium (BHBA 8 – 12 mg/dL) and low (BHBA < 8 mg/dL).

Endometrial Biopsy

Endometrial tissue biopsies were collected from each animal on day 7 post-ovulation. The estrous cycle of all cows used in this study was synchronized following the 7-day CO-Synch + CIDR protocol (Whittier et al., 2013), to allow the collection of endometrial tissue samples at a specific time during lactation. The cows were restrained in a chute and given caudal epidural anesthesia (6 ml lidocaine hydrochloride 2%). The perineal area was thoroughly washed by water and dried. Sterile equine endometrial biopsy instrument (JorVet, Loveland, CO, USA) of cutting area 4mm x 28mm and shaft length 60 cm was inserted transvaginally with sterile lubricating jelly while the other hand was placed in the rectum to guide the instrument through the cervix. Once inside the uterine body the instrument was pushed to reach the uterine wall, and a small endometrial tissue sample between 1 to 2 cm was obtained and placed into a sterile petri dish. The biopsy sample was washed with normal saline and immediately transferred into a 1.5 ml centrifuge tube and snap frozen in liquid nitrogen and stored at -80°C for future use.

Endometrial Tissue Sample Processing, RNA Extraction, and cDNA Synthesis

Total RNA was isolated from the endometrial tissue samples using EZNA total RNA kit (Omega Bio-tek, Norcross, GA, USA). The manufacturer's protocol was followed precisely to extract RNA with an exception at the beginning with the

homogenization process. The collected endometrial sample was allowed to thaw for a minute in a petri dish and with sterile RNase-free scissor and blade was minced and mixed thoroughly. About 30 mg of minced tissue was immediately transferred into 15 ml disposable glass tube with 1ml TRK lysis buffer and placed in a beaker with ice-water and homogenized for 2 min using benchtop homogenizer (Polytron PT 3100; Kinematica AG, Lucerne, Switzerland) at the speed of 8000 rpm. The lysate was transferred into 1.5 ml centrifuge tube and centrifuged for 5 min at ≥ 13,000 x g, and about 300 µl of the cleared supernatant was pipetted into a clean 1.5 ml centrifuge tube. The remaining steps were followed according to the manufacturer's protocol, with the exception of the optional on-column DNase I digestion. The concentration of the extracted RNA was measured with a NanoDrop spectrophotometer and normalized by adding DNase/RNase-free water to reach a final concentration of 90 ng/µl. All RNA samples were treated with DNase Treatment (Invitrogen, Ambion DNA-free kit) following the manufacturer's protocol to remove contaminating DNA and DNase from the samples. The concentration of the DNase treated RNA was measured with a NanoDrop spectrophotometer and normalized by adding DNase/RNase-free water to reach a final concentration of 40 ng/µl. Reverse transcription was performed using the GoScript Reverse Transcription kit (Promega, Madison, WI). Nine µl of DNase treated RNA was spiked with 1 µl of β-galactosidase (β-gal) mRNA of concentration of 5pg/µl (TriLink BioTechnologies, San Diego, CA, USA), to serve as an exogenous reference gene for qPCR and mixed with 1 µl oligo-dT and 1 µl random primers and incubated at 70°C for 5 min using the thermocycler. Four µl of 5x reaction buffer, 2 µl of 4.8 mM MgCl2, 1 µl nucleotide mix, and 1 µl of reverse transcriptase (all were supplied with the kit) were added to RNA mix

to make a total of 20 µl reaction and incubated at 25°C for 5 min for initial annealing then at 42°C for 60 min. The synthesized cDNA from each sample was stored at -20°C until further use. A reference cDNA sample was synthesized using the same reverse transcription protocol from a mixture of RNA isolated from different bovine tissues as endometrial tissue, ovarian tissue, blastocysts, and cumulus–oocyte complexes (COCs). This sample was prepared to be used as a positive control and a calibrator sample for qPCR.

Fluidigm qPCR Analysis

The BioMark system was used for the qPCR analysis in this experiment which is an innovative and unique platform from the Fluidigm Corporation (South San Francisco, CA), which allows for expression analyzsis of up to 96 genes in each of up to 96 samples. The primers used for these genes were designed based on NCBI's reference sequence for Bos taurus through the Fluidigm Corporation's DeltaGene assay design service, and Table 4-1 provides the definition of the genes' symbols and names used in the qPCR analysis (for primer sequences, see Supplementary Table 4-1). In this experiment, a 96 by 96 microfluidic chip of nanoliter-scale was used to test the expression of 41 genes from different functional categories: metabolism, heat shock protein, inflammatory response, and prostaglandin synthesis (Table 4-2). Before qPCR analysis, synthesized cDNA from each sample were enriched by specific target amplification (STA). One µl of each primer pair (forward and reverse) was mixed into a tube to be used for the STA reaction. For each of 1.25 µl of cDNA, 1.25 µl primer mix and 2.5 µl of TaqMan PreAmp Master Mix (Applied Biosystems; Foster City, CA) was added for STA reaction to make a total of 5 µl reaction. Using the thermocycler, the STA amplification reaction was initially

activated by incubation at 95°C for 10 min then followed by 14 cycles of 95°C for 15 seconds and 60°C for 4 min. Exonuclease I treatment was recommended to digest any unincorporated primers and was performed by adding 0.2 µl Exonuclease I Reaction Buffer, 0.4 µl Exonuclease I enzyme (ExoI; New England Biolabs; Ipswich, MA), and 1.4 µl nuclease-free water to each STA reaction to make a total of 7 µl and incubated at 37°C for 30 min. After digestion of unincorporated primers, the reaction was incubated at 80°C for 15 min to inactivate the Exonuclease I enzyme followed by 5- fold dilution by adding 18 µl nuclease-free water to each reaction to make a total of 25 µl of Exonuclease I-treated STA endometrial sample cDNA. For Fluidigm qPCR cycling, 2 µl of the diluted and Exonuclease I-treated STA cDNA was mixed with 2.5 µl of the 2x TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), 0.25 µl of Sample Loading Reagent (Fluidigm), and 0.25 µl 20x EvaGreen DNA Binding Dye (Biotium, Hayward, CA, USA).

On the other hand, 2.5 µl of each primer pair (forward and reverse 20 µM) was mixed with 0.25 µl nuclease-free water and 2.5 µl Assay Loading Reagent (Fluidigm). A 96.96 Dynamic Array Integrated Fluidic Circuits (IFC) was inserted in the IFC Controller HX for priming using the Prime (136x) script before loading the samples and primers. After priming, 5 µl from each primer mix and sample mix were loaded into the designated well in the IFC using the multichannel pipette and returned to the IFC Controller HX for loading by running the Load Mix (136) script. After the Load Mix script was finished, the IFC was ejected from the Controller and inserted in the BioMark thermal cycler (Fluidigm) for qPCR. The recommended protocol GE 96x96 Standard v1

was used with initial enzyme activation for 5 min then 35 cycles of 95°C for 15 sec and 60°C for 60 sec and at the end 3 min final extension.

Analysis of qPCR Data and Statistics

Raw qPCR data were analyzed using the comparative cycle threshold (Ct) method using the Fluidigm Real-Time PCR Analysis Software. The software determined the raw Ct value and then normalized it to the average Ct values of four selected housekeeping genes (*EIF4A1, GAPDH, TAF11,* and *YWHAZ*) to determine the delta Ct (ΔCt) which is the difference between Ct value for each gene tested and Ct of the housekeeping genes. Then, the delta delta Ct (ΔΔCt) value was determined for each reaction by calculating the difference between the ΔCt of each sample and the ΔCt of a selected calibrator sample (cDNA derived from pooled embryos and COCs, and ovarian and endometrial tissue); described above.

ΔΔCt values were used for the statistical analysis using SAS (SAS, Institute Inc., Cary, NC) to determine the significant difference between samples. A Generalized Linear Mixed Model (GLMM) was fit to allow the gene expression to be dependent upon the type of cow (lactating or non-lactating) and the time of endometrial biopsy collection which was nested within lactation while accounting for the effect of each animal and BHBA measurement at the time of collection. Further, we analyzed data based on the effect of BHBA level only at the time of endometrial biopsy collection while accounting for the effect of each animal and time of sample collection. The significant difference between samples was reported at FDR adjusted *P*-value < 0.05. Further, relative fold change for the significantly expressed genes was calculated by the equation $2^{-(\Delta\Delta Ct)}$ to make the final determination of the significant difference, and the cut-off was set at a

fold-change equal to one, which is about 100% increase. The increase in fold change was calculated by determining the difference in fold change between two significantly different ΔΔCt values and divided on the fold change of the largest ΔΔCt value. For example, the value of (ΔΔCt X = 1.388) , and the value of (ΔΔCt Y = -1.141) the equation used is $\frac{2-\hat{}(\Delta\Delta Ct\ Y)-\ 2\hat{}-(\Delta\Delta Ct\ X)}{2-\hat{}(\Delta\Delta Ct\ X)} = \frac{2.207\ -\ 0.382}{0.382}$ = 4.77 which means 477% increase in Y relative to X. Log2 of fold change was calculated to determine the relative expression level between samples and was used to create the figures.

RESULTS

Twenty-two multiparous lactating dairy cows were used in this experiment, and the milk yield was recorded daily and averaged weekly for starting wk 1 until wk 19. Figure 4-1 shows the lactation curve for the cows used in this study and the changes in body weight. These cows followed the typical pattern of lactation curve where the milk yield reached the peak around 45 DIM until 60 DIM then started to decline gradually (Figure 4-1A). Body weight started to decline as the milk production increased until it reached the lowest point by 63 DIM then started to gradually increase when cows shifted to positive energy balance and started to gain more weight as the feed intake increased and milk yield started to decline (Figure 4-1B). Figure 4-2 shows the changes in blood concentrations of BHBA and glucose by the time of the endometrial biopsy. The highest average BHBA level for this group of animals was recorded around the second collection at 82 DIM. On the contrary, the lowest level of glucose was recorded around the second collection, and this result was expected as BHBA and glucose negatively correlate with each other (Chapinal et al., 2012). Table 4-3 provides a summary for milk yield, body

weight, BCS, and concentrations of BHBA and glucose for each week prior to the time of sample collection. Figure 4-3 demonstrates the percentage of cows with low, medium, or high BHBA level at the time of the endometrial biopsy.

In the analysis of the differential gene expression between endometrial samples collected from non-lactating heifers and lactating cows, we found eight genes that were differentially expressed between the two groups. The transcripts of five genes out of seventeen genes tested involved in inflammatory response category showed significantly increased expression in the endometria of the lactating cows in comparison with non-lactating heifers (Table 4-4 and Figure 4-4). Interleukin 2 receptor subunit alpha (*IL2RA*) was 8.74-fold higher (adj. $P = 0.001$). Interleukin 2 (*IL-2*) was 7.18-fold higher (adj. $P = 0.034$). CD28 molecule (*CD28*) was 6.94-fold higher (adj. $P = 0.02$). Cytotoxic T-lymphocyte associated protein 4 (*CTLA4*) was 12.87-fold higher (adj. $P = 0.04$). GATA binding protein 3 (*GATA3*) was 1.80-fold higher (adj. $P = 0.02$). On the other hand, four genes showed significantly increased expression level in the endometrial samples from non-lactating heifers in comparison with those collected from lactating cows (Table 4-5). From the metabolism category, acetyl-CoA carboxylase alpha (*ACACA*) was 1.14-fold higher (adj. $P = 0.02$), glucose-6-phosphate dehydrogenase (*G6PD*) was 2.14-fold higher (adj. $P = 0.001$), and solute carrier family 16 member 3 (*SLC16A3*) was 1.62-fold higher (adj. $P = 0.03$). Insulin-like growth factor 2 receptor (*IGF2R*) was 1.6-fold higher (adj. $P = 0.03$). Interestingly, pentraxin 3 (*PTX3*) from the inflammatory response category was up to 6.37-fold higher in heifers' endometrium, but the adj. *P*-value only tended toward significance with $P = 0.15$ (Figure 4-5).

Within the lactating cows, none of the genes tested were found to be differentially expressed due to DIM. However, three genes from the inflammatory response (*IL2RA, IL-10, and CD28*) showed significantly altered expression when samples grouped by BHBA level (Table 4-6). *IL2RA* was 1.09-fold higher (adj. $P = 0.007$) in samples from lactating cows with low BHBA relative to high BHBA. Interleukin 10 (*IL-10*) was 1.15-fold higher (adj. $P = 0.03$) in samples from lactating cows with low BHBA relative to high BHBA. *CD28* was 1.72-fold higher (adj. $P = 0.01$) in samples from lactating cows with low BHBA relative to high BHBA (Figure 4-6). For more details about the qPCR data of all genes that were tested, supplementary figures 4-1 through 4-8 shows the relative expression of these genes grouped by their functional category.

DISCUSSION

The aim of this study was to evaluate the impact of lactation and NEB on the expression of genes involved in inflammatory response, heat shock proteins, and metabolism in the endometrial tissue of dairy cows throughout early lactation. After calving, the increase in energetic demand and nutritional requirements for milk production accompanied with limited dry matter intake results in severe metabolic changes that interfere with the reproductive performance (Esposito et al., 2014). Typically, dairy cows experience a state of energy deficit during early lactation as their milk production increases rapidly until it reaches the peak between week 6 to 8 in lactation and then it starts to slowly decline until the end of lactation (Aschenbach et al., 2010; Strucken et al., 2015). The shortage in energy requirement is compensated by excessive fat mobilization from body fat reserves (Allen, 1996) and the consequences of NEB have been proposed in numerous studies and shown to have a detrimental effect on

fertility, interact with normal body physiology, and impact overall health (Dekkers et al., 1998; Ingvartsen et al., 2003). NEB is characterized by high concentrations of NEFA and BHBA and low glucose in the circulation (Jorritsma et al., 2003; Chapinal et al., 2012) and has been associated with declined fertility (Block et al., 2001; Leroy et al., 2005; Leroy et al., 2008; Van Hoeck et al., 2013; Van Hoeck et al., 2014).

The liver plays a central role in metabolic homeostasis, and excessive fat mobilization leads to accumulation of lipid in the liver causing metabolic disorders (Gross et al., 2013). Fatty liver syndrome is linked to impaired neutrophil function leading to immune suppression in postpartum dairy cows (Zerbe et al., 2000). Also, neutrophil function was shown to be significantly affected in peripartum cows with high blood concentration of NEFA (Hammon et al., 2006; Ster et al., 2012). Excessive fat mobilization increases oxidation of NEFA in the liver during the period of NEB, resulting in increased production of reactive oxygen species (ROS) which in turn raise the level of oxidative stress in the body (Morris et al., 2009). Excessive generation of ROS disrupts redox signaling status and alters the control of intracellular redox potential causing redox stress that was defined as the intracellular imbalance of pro-oxidants and antioxidants (Jones, 2006) leading to disturbance of normal metabolism and physiology. Moreover, increased production of ROS causes cellular damage through peroxidation of lipid contents in the membrane (Sordillo, 2005). A high amount of polyunsaturated fatty acids in the membrane of immune cells makes them very susceptible to oxidative stress and peroxidation (Spears and Weiss, 2008). Neutrophils are the first line of innate immunity, and the interaction between metabolic disorders and immune function makes the cows very susceptible to various postpartum uterine infections (Esposito et al., 2014). In

addition to the impaired neutrophil function, other metabolic disorders such as hypocalcemia, delay and weaken the process of uterine involution which results in a greater susceptibility to uterine infection (Heppelmann et al., 2015). Weak postpartum immune response to infection impair bacterial clearance process and lead to an extended period of inflammatory response (Földi et al., 2006).

The innate immune system is the initial defense mechanism during postpartum uterine infection. Yet, persistence of infection over time activates the adaptive immune response which becomes important at later stages (Wathes et al., 2009). The ongoing postpartum inflammatory response in the uterus results in the production of inflammatory cytokines. These cytokines including IL-1, IL-6 and TNF stimulate the production of anti-microbial peptides to eliminate pathogens and clear bacterial infection (Herath et al., 2009). During uterine inflammation the mRNA expression of genes encoding pro-inflammatory mediators increases in the endometrium in addition to TLR4 (Herath et al., 2009). The inflammatory response must be maintained under control to avoid excessive immune activation by bacteria which negatively impact fertility. Herath et al. (2009) reported increased expression of *IL1-A* or *IL1-B* to *IL-10* in infertile cows when compared with fertile cows. Interestingly, no significant difference was observed in the expression of innate immune receptors between the two groups of animals. The main difference between fertile and infertile cows appeared to be in the expression of mRNA of inflammatory cytokines. These findings suggest that improper adaptive immune response may have long-term effect on fertility rather than innate immunity.

In the present study, the expression of mRNA of *CD28*, *IL2RA*, and *IL-10*, components of adaptive immunity, was found to be altered in endometrial tissue due to

fluctuation in BHBA level. Cows were categorized into three groups according to their concentration of BHBA in the circulation as low, medium, and high at the time of endometrial biopsy collection. The concentration of BHBA in the circulation is frequently used as an indicator of the energy status in dairy cows as it positively correlates with the amount of adipose tissue being mobilized for energy. The results of this study show a general pattern of downregulation of the genes from the inflammatory response category in cows with high BHBA level. However, only the three genes, mentioned above, were found to be significantly impacted by the BHBA level. Their expression was found to be more than one-fold higher in cows identified as low BHBA. The result suggests that NEB could impact fertility through weakening the immune and inflammatory response in the endometrium leading to impaired bacterial clearance. Also, it may indicate interference of NEB with postpartum uterine adaptive immunity in dairy cows.

Interestingly, our findings are not in agreement with Wathes et al. (2009). They used two groups of dairy cows with mild or severe NEB by varying the feeding and milking regimes and confirmed NEB by changes in the measured metabolites, including BHBA. They used bovine Affymetrix arrays to measure gene expression level and found higher expression of chemokines, cytokines, and several interferon-inducible genes in endometrial samples from cows with severe NEB. They concluded that severe NEB compromises subsequent fertility by preventing cows from producing an effective immune response to the postpartum microbial contamination which may lead to a prolonged uterine recovery period. Even though their and our results are contradictory, both conclusions suggest insufficient immune and inflammatory response. However,

there are some concerns and differences between both experiments that may explain the difference in the results. First, they artificially manipulated the feeding and milking regimes to produce cows with severe NEB while it is commonly known there is a variation among individual animals in response to the NEB as it is highly associated with the amount of subcutaneous adipose tissue and BCS (Barletta et al., 2017). Besides, several factors can influence the amount of fat mobilization as weight, genetics, milk yield, and DIM (Tamminga et al., 1997). Second, they collected the endometrial samples two weeks after calving from only 12 cows before complete uterine involution which takes up to 40 days (Okano and Tomizuka, 1987). This short period may not give enough time for full recovery of the uterine tissue and stable inflammatory state that can be accurately evaluated. In the experiment described herein, three endometrial biopsy samples were collected from 22 after complete uterine involution at 54, 82, and 137 DIM. This design allowed us to examine the effects of DIM, BHBA, and their interaction. We expected to see a difference in gene expression due to DIM, but it seems it mostly driven by the BHBA level and duration of NEB that may impair the immune function and delay the inflammatory response and uterine recovery.

Postpartum uterine bacterial contamination is unavoidable and insufficient innate immune response increases the incidence of uterine infections causing metritis and clinical endometritis (LeBlanc et al., 2002; LeBlanc, 2014). Treatment of clinical metritis and endometritis is straightforward, but it takes time and requires veterinary intervention which adds extra expenses, increases calving interval, and lowers profitability. Besides, the consequences of uterine infection and inflammation persist beyond the resolution of the clinical symptoms. Even with successful treatment, the conception rate is 20% lower

in the affected animals when compared with healthy ones, and 3% of the affected animals become infertile after the treatment and must be culled from the herd (Sheldon et al., 2009). The apparent clinical signs of metritis and endometritis are easily diagnosed, which shortens the time for culling decision or treatment. On the contrary, subclinical endometritis is often under-diagnosed, and the affected cows may remain untreated for a longer period of time until identified later during the breeding season (Van Schyndel et al., 2018).

Subclinical endometritis is a chronic inflammation of the uterus due to improper inflammatory response or persistence of the infection that makes the uterine lumen inappropriate to support early embryonic development and compromises endometrial receptivity for blastocyst (Salilew-Wondim et al., 2016). The diagnosis of subclinical endometritis is confirmed by endometrial cytological findings, and the most common techniques performed in bovine are the cytobrush-method and low-volume uterine flushing (Kasimanickam et al., 2004). The objective of these techniques is to obtain a representative number of endometrial cells from cows without clinical signs of disease and determine the proportion of polymorphonuclear cells (PMN) in the sample evaluated under a microscope. The technique of sampling is straightforward and non-invasive and has no effect on fertility. However, it is unpractical for routine veterinary practice due to the sampling and laboratory process which makes the diagnosis time-consuming. Furthermore, the determined threshold of PMN proportion is questionable as the number of counted cells varies between studies (Wagener et al., 2017a). Also, the proportion of PMN changes by DIM as it is typically higher postpartum and then decreases over time (Prunner et al., 2014). Kasimanickam et al. (2004) determined the PMN threshold in

correlation with DIM and confirmed positive subclinical endometritis if > 18% PMN between 20 and 33 DIM or > 10% PMN between 34 to 47 DIM. Accordingly, PMN count above these thresholds in postpartum cows increase the risk of conception failure after multiple subsequent inseminations making them defined as repeat breeder (Wagener et al., 2017b).

The uterine tissue, especially the endometrial lining, responds to the persisting infection and chronic inflammation by activating or repressing specific biochemical and molecular pathways. Identifying these pathways will help in understanding the molecular changes and biomarkers in the endometrium associated with subclinical endometritis. Several studies have utilized molecular techniques to measure the differential genes expression of endometrial samples obtained from cows diagnosed with clinical or subclinical endometritis (Wagener et al., 2017a). Cows with subclinical endometritis were found to have higher expression of mRNA of pro-inflammatory cytokines and enzymes involved in prostaglandins synthesis in the endometrial samples (Gabler et al., 2009; Fischer et al., 2010). The upregulated cytokine mRNA in the endometrium of cows diagnosed with subclinical endometritis include *IL1A, IL1B, IL-6* and *TNFa* (Ghasemi et al., 2012). However, even healthy cows have higher expression of cytokine mRNA during the third week postpartum then start to decline over time. The postpartum endometrial inflammatory response is an essential part of the uterine involution process to clear bacterial contamination and restore pre-pregnancy endometrial function (Gabler et al., 2010; Chapwanya et al., 2012). The expression of inflammatory factors in the endometrium is mediated by Toll-like receptors when stimulated by the bacterial lipopolysaccharide (LPS) that invade the uterus during calving (Cronin et al., 2012).

Besides the effect of lactation, calving causes major uterine physiological and histological changes that may persist beyond recover and lower the capability of supporting early embryo development than that of nulliparous heifers which may explain the lower conception rates observed in multiparous cows (Rizos et al., 2010).

The endometrium of nulliparous heifers, that did not go through pregnancy and calving, is supposedly not affected by any bacterial invasion that may cause immunological and pathological changes. In the present study, the expression of genes involved in inflammatory response and cytokine synthesis was, in general, lower in the endometrial biopsy samples collected from heifers in comparison with those collected from lactating cows. Five of them (*IL2RA*, *IL-2*, *CD28, CTLA4,* and *GATA3*) were found to be significantly downregulated in heifers. These results may explain the unaffected conception rate in the dairy heifers of the same genetic merits and under the same management (Pryce et al., 2004). Bauersachs et al. (2017) investigated the association of lactation with the altered ability of the endometrium to respond appropriately to the conceptus by examining endometrial gene expression from confirmed pregnant lactating cows, non-lactating cows, and heifers on day 19 of pregnancy. Their results show differences in endometrial gene expression among tested groups of animals. However, the hierarchical cluster analysis of the Differentially Expressed Genes (DEGs) demonstrates more similarity in endometrial gene expression between lactating and non-lactating cows in comparison with heifers. The categories of genes with higher expression in heifers' endometrial samples included defense response, cell migration, and fatty acid metabolic process. In the present experiment, three genes (*ACACA, G6PD,* and *SLC16A3*) from the metabolism group showed significantly increased expression level in

the endometrial samples from heifers. Heifers are in positive energy balance relative to the lactating dairy cows, especially during early lactation. ACACA gene encodes acetyl-CoA carboxylase-α, a rate-limiting enzyme involved in the biosynthesis of monounsaturated fatty acids (Crepaldi et al., 2013), and in the present experiment, it was downregulated in lactating cows relative to heifers. This outcome is not unexpected as 21lactating cows are mobilizing fat rather than synthesizing. *G6PD* encode glucose 6-phosphate dehydrogenase which plays an important role in a ruminant's metabolism as a rate-limiting enzyme in the pentose phosphate pathway (PPP) for carbohydrates processing. The expression of *G6PD* gene found to be enhanced by dietary carbohydrates at both transcriptional and posttranscriptional level (Salati and Amir-Ahmady, 2001). In the present study, the expression of *G6PD* was significantly downregulated in lactating cows as they have low glucose in the circulation and are mostly relying on fat mobilization for energy during the period of NEB. In contrast, overfed cows consuming high energy grain diet demonstrated higher expression of *G6PD* in comparison to the control group (Zhou et al., 2015). There is a positive correlation between *G6PD* expression and energy balance, and as it responds to the changes in energy intake, it was suggested to be used as a biochemical marker of lipogenesis (Laliotis et al., 2012; Triantaphyllopoulos et al., 2014).

SLC16A3 is a member of Solute Carrier Family 16 and also known as monocarboxylate transporter 4 (*MCT4*) (Wang and Morris, 2007). *MCT4* is widely expressed in a variety of tissues that mostly rely on glycolysis as a source of energy and is responsible for lactate uptake from the circulation (Bonen, 2001). In an in vitro study, *MCT4* was found to be up-regulated in activated macrophages and needed for proper

inflammatory response through high glycolytic activity (Tan et al., 2015). In the present study, downregulation of *SLC16A3* in lactating cows relative to heifers might be explained by insufficiently activated macrophages or lower glycolytic activity of endometrial tissue due to lower glucose in the circulation. Interestingly, we found that the expression of *PTX3* to be more than 6-fold higher in the endometrium of heifers than lactating cows (adj. $P = 0.01$). This finding comes in agreement with Bauersachs et al. (2017) experiment which compared differential gene expression of endometrial samples collected from lactating cows, non-lactating cows, and heifers and found significantly higher expression of *PTX3* in heifers' endometria. *PTX3* plays an essential role in implantation as it is highly expressed in the receptive endometrium (Tranguch et al., 2007).

In conclusion, available results suggest that there is a complex interaction between lactation, metabolism, and uterine function in postpartum dairy cows depending on the severity and duration of NEB. High-yielding dairy cows more often experience an intense and longer period of NEB during the first few months of lactation, resulting in dramatic fluctuations in circulating levels of hormones and metabolites that may result in a pro-inflammatory uterine environment that is inappropriately suited for supporting early embryo development and negatively impact overall fertility. Dysregulated inflammatory response might be due to a compromised immune function during the NEB that results in a practical clearance of bacterial contamination or failure to resolve endometrial inflammation. We emphasize on the role of stable endometrial inflammatory state and uterine health to improve fertility and increase conception rate. Also, further investigation

is needed to determine the mechanisms underlying the differences in gene expression between cows with different BHBA levels.

TABLES AND FIGURES

Table 4-1. Definition of gene names used in qPCR analysis of endometrial tissue samples

Gene symbol	Gene name
ACACA	Acetyl-CoA carboxylase alpha
ACADL	Acyl-Coenzyme A dehydrogenase, long-chain
ACSL1	Acyl-CoA synthetase long-chain family member 1
ACSL3	Acyl-CoA synthetase long-chain family member 3
CD28	CD28 molecule
CTLA4	Cytotoxic T-lymphocyte associated protein 4
FOXP3	Forkhead box P3 (FOXP3)
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA3	GATA binding protein 3
GLUD1	Glutamate dehydrogenase 1
HSF1	Heat shock transcription factor 1
HSP10	Heat shock protein family E (Hsp10) member 1
HSP110	Heat shock protein family H (Hsp110) member 1
HSP60	Heat shock protein family D (Hsp60) member 1
HSPA1A	Heat shock 70kDa protein 1A
HSPA2	Heat shock protein family A (Hsp70) member 2
IFNAR1	Interferon alpha and beta receptor subunit 1
IFN-g	Interferon gamma
IGF1	Insulin like growth factor 1
IGF1R	Insulin like growth factor 1 receptor
IGF2R	Insulin-like growth factor 2 receptor
IL-10	Interleukin 10
IL-12B	Interleukin 12B
IL-13	Interleukin 13
IL-18	Interleukin 18
IL-1b	Interleukin 1 beta
IL-2	Interleukin 2
IL-23A	Interleukin 23 subunit alpha
IL2RA	Interleukin 2 receptor subunit alpha
PGR	progesterone receptor
PTGES	prostaglandin E synthase
PTGS2	Prostaglandin-endoperoxide synthase 2
PTX3	Pentraxin 3
SLC16A3	Solute carrier family 16 member 3
SLC2A1	Solute carrier family 2 member 1
TAF11	TATA-box binding protein associated factor 11
T-bet	T-box 21

TGF-b1	Transforming growth factor beta 1
TNFa	Tumor necrosis factor
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

Table 4-2. Functional category of genes tested by qPCR in endometrial tissue samples

Functional categories	Number of genes tested	Name of genes tested
Endometrium specific and prostaglandin synthesis	4	*IFNAR1, PGR, PTGES, PTGS2*
Heat shock Proteins	6	*HSF1, HSP10, HSP110, HSP60, HSPA1A, HSPA2*
Housekeeping	3	*GAPDH, TAF11, YWHAZ*
Inflammatory response and regulation	17	*CD28, CTLA4, FOXP3, GATA3, IFN-g, IL-10, IL-12B, IL-13, IL-18, IL-1b, IL-2, IL-23A, IL2RA, PTX3, T-bet, TGF-b1, TNFa*
Metabolism	11	*ACACA, ACADL, ACSL1, ACSL3, G6PD, GLUD1, IGF1, IGF1R, IGF2R, SLC16A3, SLC2A1*

Table 4-3. Endometrial biopsy collection time during lactation with BHBA, glucose, milk yield, and body weight

Embryo collection	DIM	BHBA (mg/dL)	Glucose (mg/dL)	Milk yield (kg/day)	Body weight (kg)	BCS (1-5 scale)
Initial	7 ± 1.36	7.45 ± 1.20	49 ± 2.92	29.30 ± 1.35	724.70 ± 11.11	3.0 ± 0.10
1st collection	54 ± 1.36	9.61 ± 1.68	53.65 ± 2.12	42.87 ± 1.19	706.09 ± 12.95	2.69 ± 0.15
2nd collection	82 ± 1.38	10.67 ± 1.44	50.92 ± 2.43	42.30 ± 1.01	704.32 ± 10.53	2.62 ± 0.10
3rd collection	137 ± 1.26	8.29 ± 0.69	57.08 ± 1.76	38.15 ± 1.25	733.20 ± 9.95	3.0 ± 0.20

The table reports the time for endometrial biopsy collection during lactation in a group of lactating dairy cows (n = 22) which was performed three times at specific days in milk (DIM) with the average of beta-hydroxybutyrate (BHBA) concentration, glucose concentration, milk yield, body weight, and body condition score (BCS) for the initial week and the week prior to endometrial biopsy collection. Data are presented as means ± SEM.

Table 4-4. List of genes that show significantly increased expression level in endometrial samples from lactating cows relative to non-lactating heifers

Gene name	Non-lactating Heifers		Lactating Cows		
	Avg. Relative Expression Level	S.E.M	Avg. Relative Expression Level	S.E.M	Adj. *P*-value
IL2RA	-4.35	0.51	-1.06	0.11	0.001
IL-2	-3.49	0.96	-0.46	0.17	0.03
CD28	-2.78	0.83	0.20	0.17	0.02
CTLA4	-4.45	1.33	-0.66	0.25	0.04
GATA3	-0.74	0.44	0.74	0.10	0.02

* Relative expression level is Log2 fold change of the least square means of ΔΔCt values ± S.E.M. Significance is at adj. *P*-value < 0.05.

Table 4-5. List of genes that show significantly increased expression level in endometrial samples from non-lactating heifers relative to lactating cows

Gene name	Lactating Cows		Non-lactating Heifers		
	Avg. Relative Expression Level	S.E.M	Avg. Relative Expression Level	S.E.M	Adj. *P*-value
ACACA	-0.42	0.06	0.67	0.31	0.02
G6PD	-0.05	0.06	1.60	0.27	0.001
SLC16A3	0.36	0.11	1.74	0.45	0.03
IGF2R	-0.12	0.06	0.69	0.27	0.03

* Relative expression level is Log2 fold change of the least square means of ΔΔCt values ± S.E.M. Significance is at adj. *P*-value < 0.05.

Table 4-6. List of genes that show significantly altered expression in endometrial samples from lactating cows grouped by BHBA level

Gene name	Lactating cows				
	High BHBA		Low BHBA		
	Avg. Relative Expression Level	S.E.M	Avg. Relative Expression Level	S.E.M	Adj. *P*-value
IL2RA	-1.7840	0.2116	-0.71	0.1559	0.007
IL_10	-0.3078	0.3028	0.79	0.2169	0.03
CD28	-0.6855	0.3272	0.75	0.2442	0.01

* Relative expression level is Log2 fold change of the least square means of ΔΔCt values ± S.E.M. Significance is at adj. *P*-value < 0.05

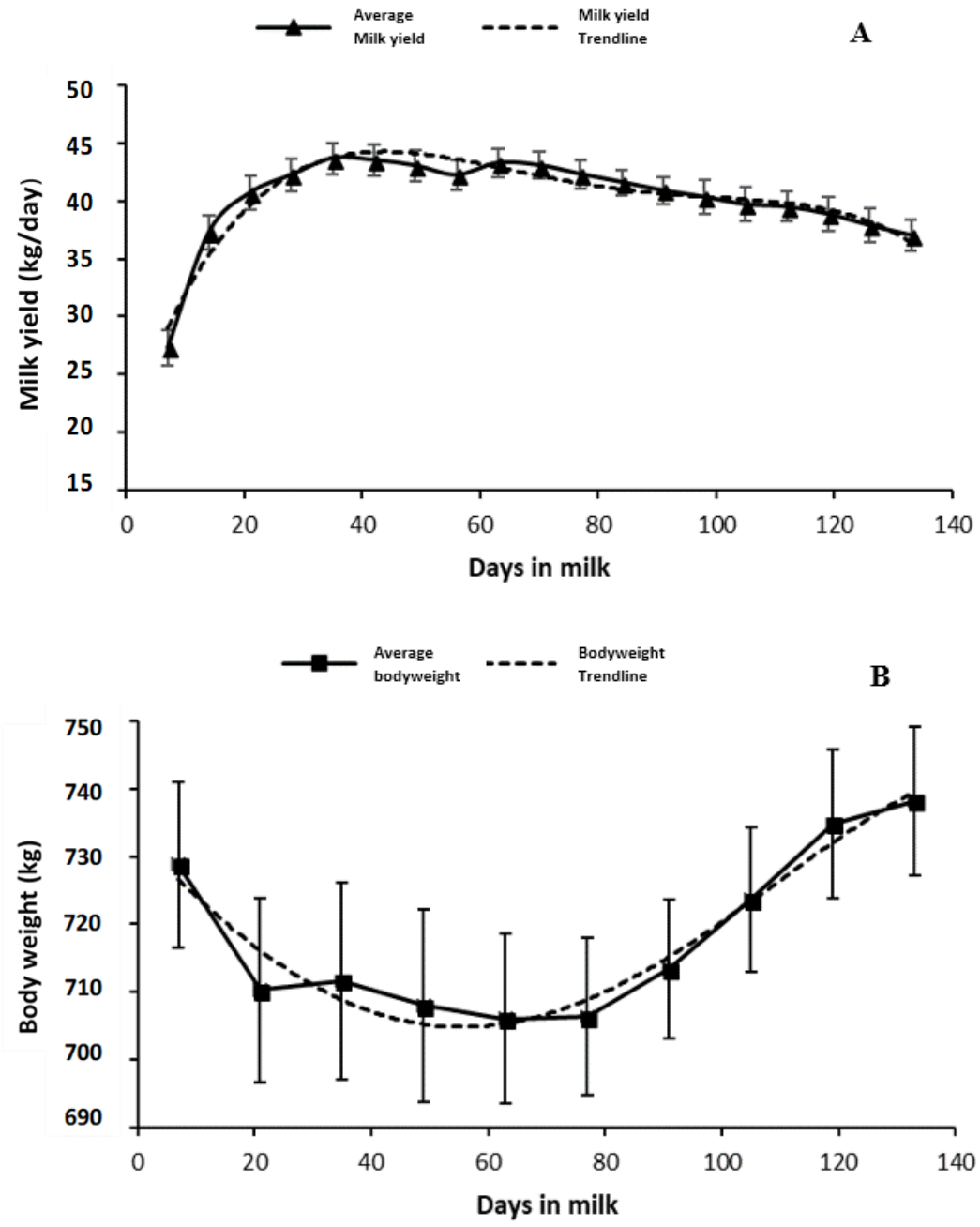


Figure 4-1. Lactation curve and change in body weight for lactating cows used in the study to collect endometrial samples. (A) shows milk yield and (B) shows changes in body weight for lactating cows (n = 22) for the first 133 days in milk during the trial. Data are the mean ± S.E.M.

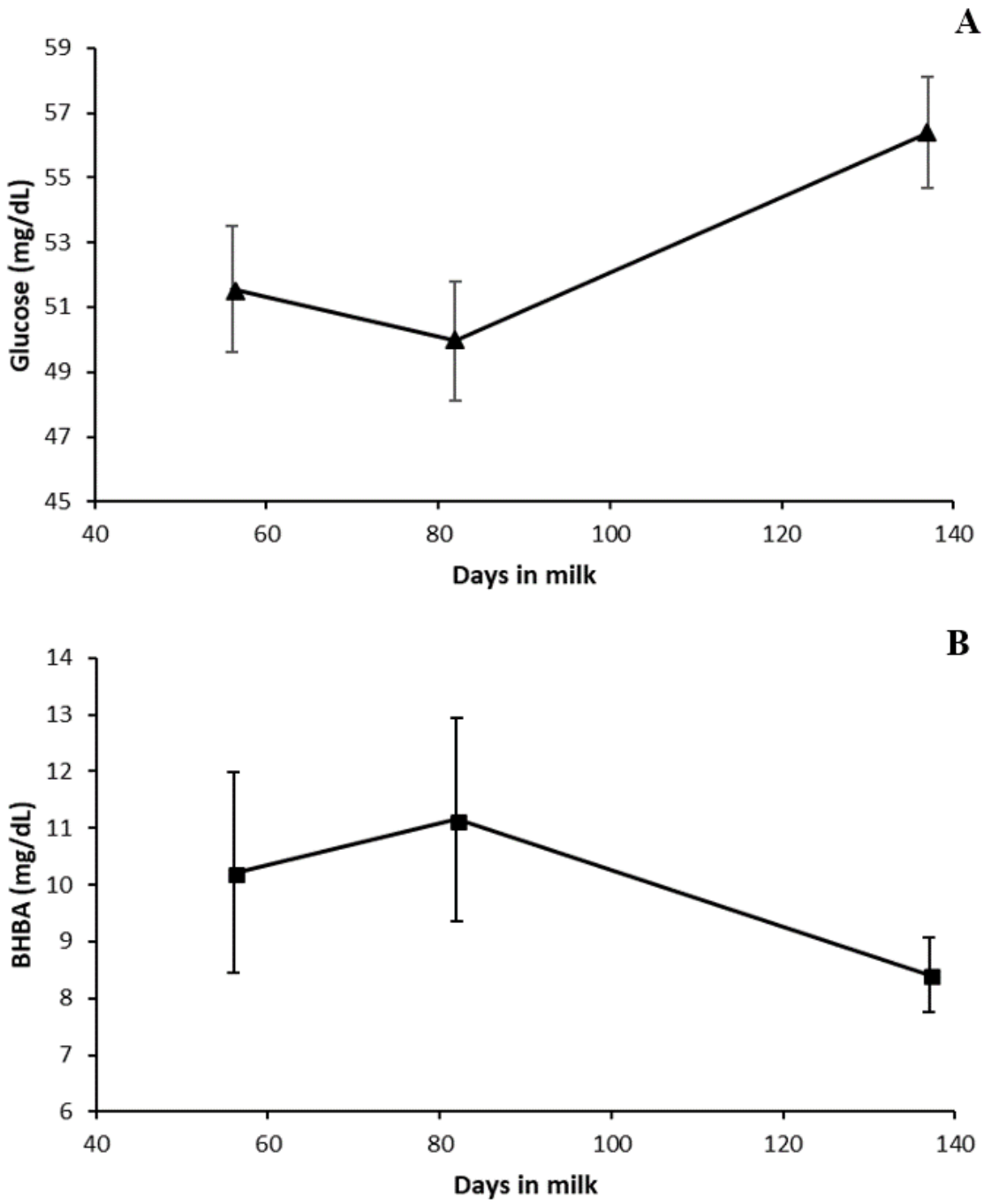


Figure 4-2. Average concentration of glucose and beta-hydroxybutyrate (BHBA) of lactating cows during the week of endometrial sample collection. (A) shows concentration of glucose and (B) shows concentration of BHBA in lactating cows (n = 22) during the week of endometrial sample collection that was performed three times during lactation at 56, 82, and 137 days in milk. Data are the mean ± S.E.M.

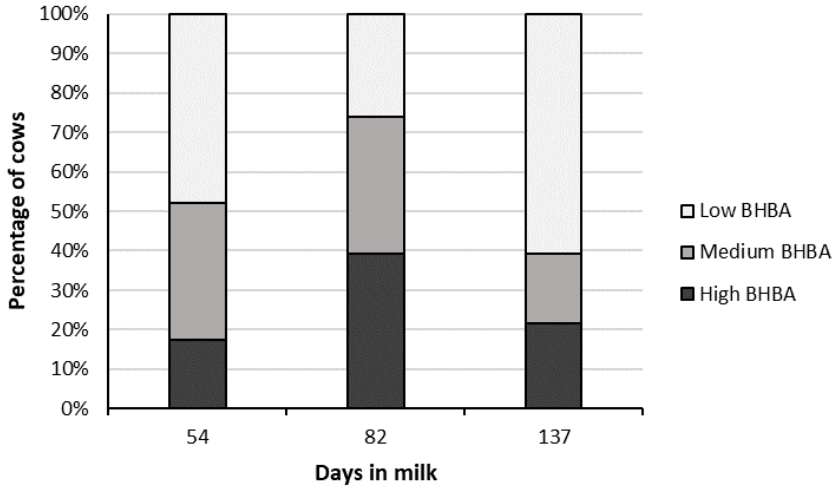


Figure 4-3. Percentage of cows with low, medium, or high BHBA level at the time of endometrial biopsy. Weekly averaged BHBA concentration before each endometrial biopsy collection from lactating cows (n = 22) at 54, 82, and 137 days in milk was categorized as high (BHBA >12 mg/dL), medium (BHBA 8 – 12 mg/dL), and low (BHBA < 8 mg/dL).

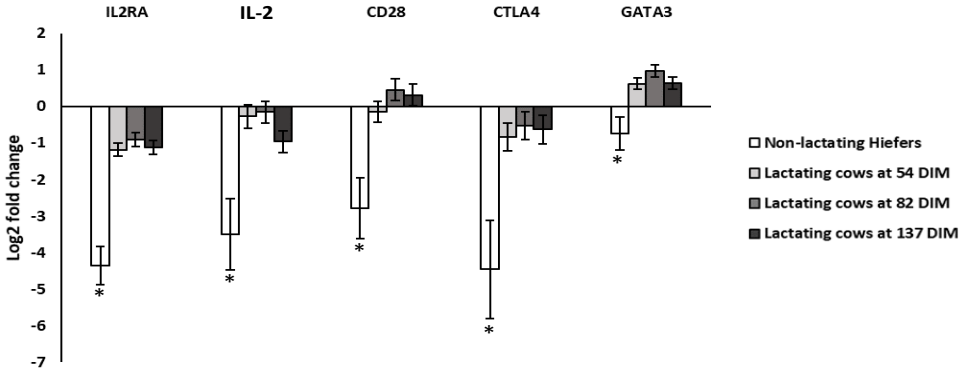


Figure 4-4. Genes that show significantly decreased expression in endometrial samples from heifers relative to lactating cows. Endometrial samples were collected three times from the lactating cows at 54, 82, and 137 days in milk (DIM) and one time from a group of non-lactating heifers. * indicates significant difference at adj. $P < 0.05$. Data are the mean + S.E.M

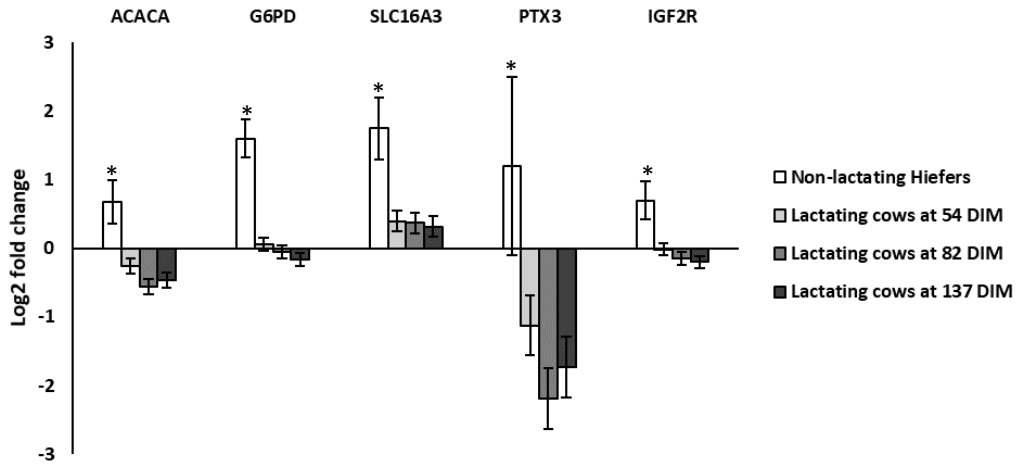


Figure 4-5 Genes that show significantly increased expression level in endometrial samples from heifers relative to lactating cows. Endometrial samples were collected three times from the lactating cows at 54, 82, and 137 days in milk (DIM) and one time from a group of non-lactating heifers. * indicates significant difference at adj. $P < 0.05$. Data are the mean + S.E.M

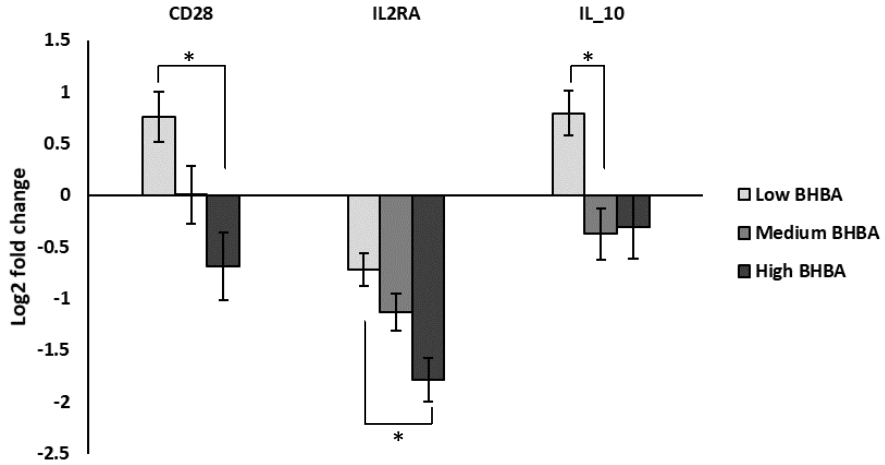


Figure 4-6. Genes that show significantly altered expression in endometrial samples from lactating cows grouped by BHBA level. Endometrial biopsy was collected from lactating cows (n = 22) three times during early lactation and before each collection, weekly averaged concentration of BHBA for each animal was categorized as high (BHBA >12 mg/dL), medium (BHBA 8 – 12 mg/dL) and low (BHBA < 8 mg/dL). Data are the means ± S.E.M.* indicate significant difference at adj. $P < 0.05$.

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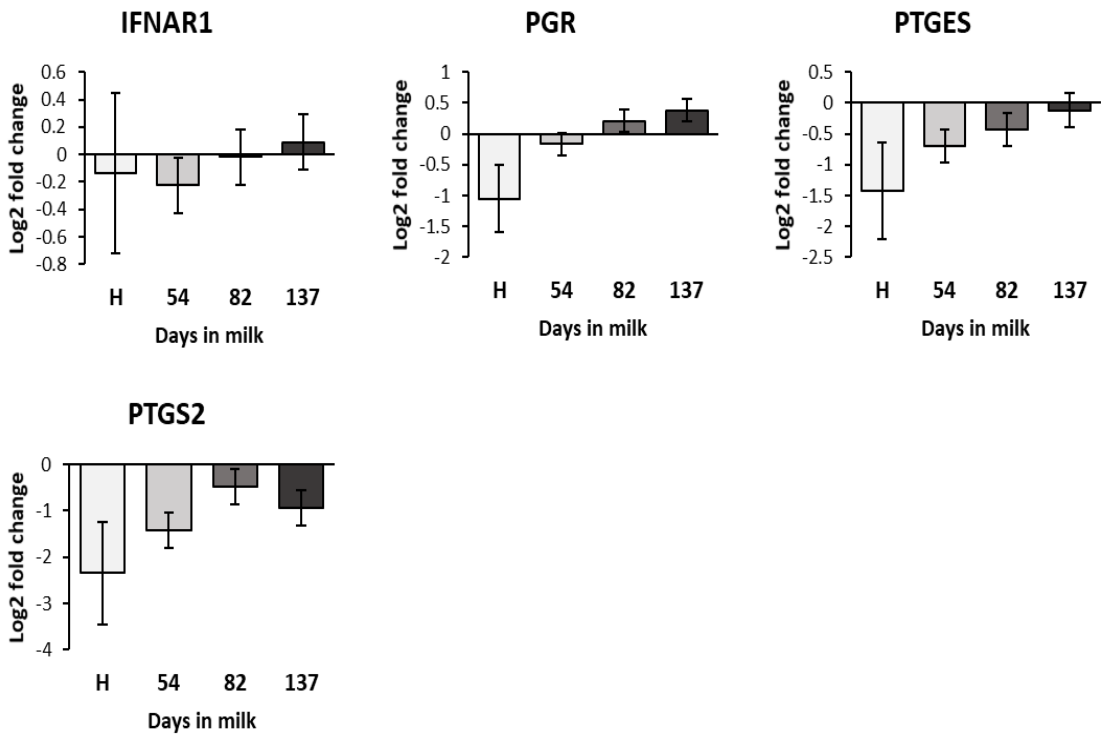
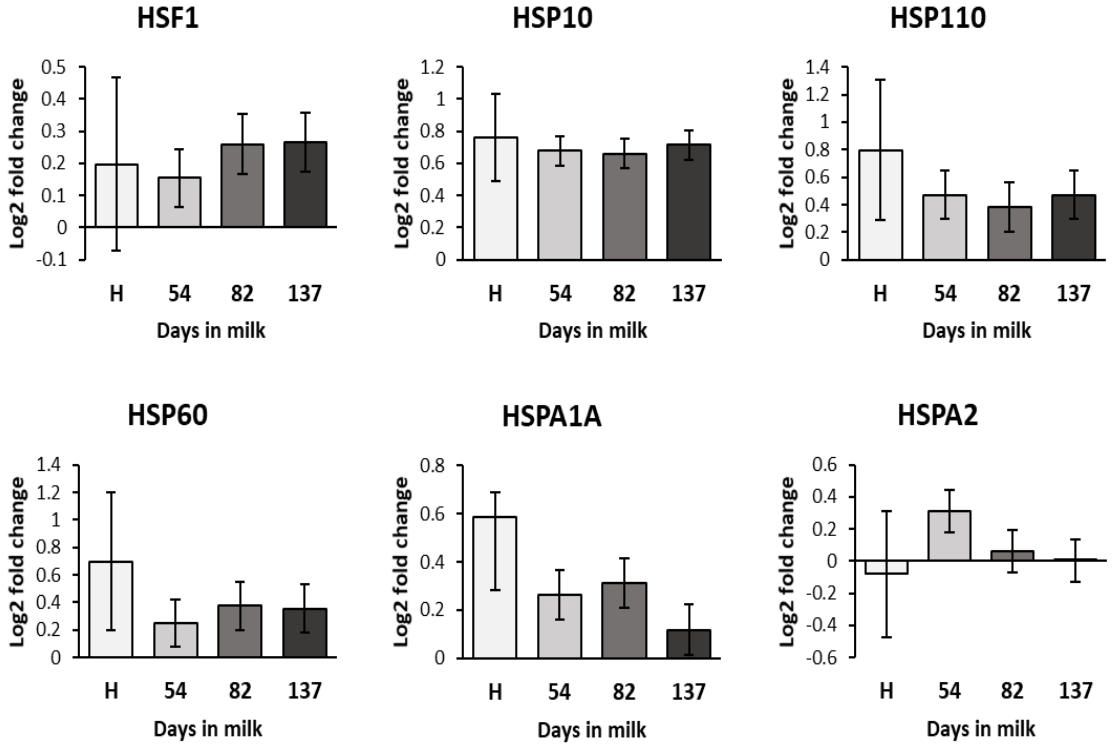
Supplementary Table 4-1. Forward and reverse primer sequences for genes used in qPCR analysis for endometrial tissue

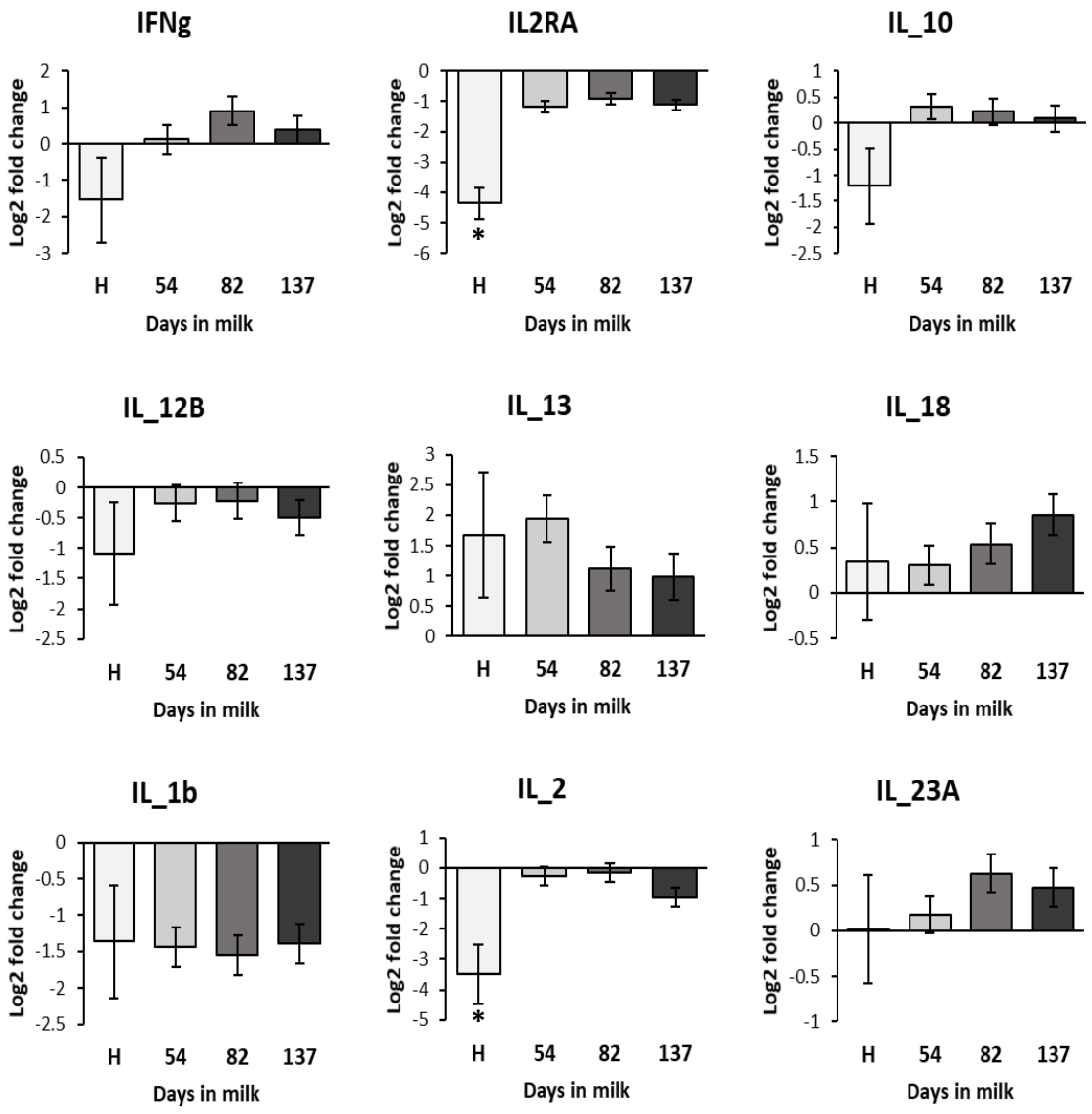
Gene	5'-forward primer-3'	3'-reverse primer-5'
ACACA	GCTAACTCAACTCAGCAAGACC	GGATGGCAAATGGGAAGCAA
ACADL	TACCTCATGCAAGAGCTTCC	AGTCCCTGGTTTCCTCAAAC
ACSL1	AAGGTTTCCAGAGGGGCTTAC	TCATAGGGTTGGTCTGGTTTCC
ACSL3	TGGCGGAAAGGATTCCAGAA	AGACAGACAAGCTCAGCACTTA
CD28	GAGGTTCTTTATCCTCCTCCTTACA	ACTCCGTTTACCACCACCAA
CTLA4	CGGCAATGGAACCCAGATTTAC	CTAACTGCTGCCAGGATCCA
FOXP3	ACGGGGCTCTTCTCTCTCAA	TCCCTGGAAACCCACTCCA
G6PD	GTCTGGTGGCCATGGAGAA	CTGCACCTCTGAGATACACTTCA
GAPDH	GGGTCTTCACTACCATGGAGAA	GTTCACGCCCATCACAAACA
GATA3	AATGCCTGTGGGCTCTACTA	TTCTGGTCTGGATCCCTTCC
GLUD1	CCGACAACTCCAGAAGCTGATA	TGTCACTCCTCCAGCATTCA
HSF1	GCAGCTCCTCGAGAACATCA	ACACTGTCCTGGCGAATCTTTA
HSP10	AGCTGTTGGATCAGGCTCTAA	GCCTCCATATTCTGGGAGAAGAA
HSP110	TTCTGATCCTCAAGGAGTTCCA	CCATCCTTCTGTGCTGAAACA
HSP60	CTTTTAGCCGATGCTGTAGCC	GGACTTCCCCAACTCTGTTCA
HSPA1A	CTGATCAAGCGCAACTCCA	TTGTCCGAGTAGGTGGTGAA
HSPA2	ACGGCATCTTCGAGGTGAA	CCATGCGGTTATCGAAGTCC
IFNAR1	AGCGTCTTCTTGAGATGTGTCA	CCTTAGTGGCTGATCGGAGAAA
IFN-g	CAGCTCTGAGAAACTGGAGGA	TTATGGCTTTGCGCTGGATC
IGF1	GAGTTGGTGGATGCTCTCCA	GACTGCTCGAGCCATACCC
IGF1R	GAACCTGCGCCAGATCCTA	GCTGCAAGTTCTGGTTGTCA
IGF2R	GGCAGATTCCACTCAAGTCA	AGATCAAGGTGAGGTCTCCA
IL-10	CCCTGCGAAAACAAGAGCAA	CTCACTCATGGCTTTGTAGACAC
IL-12B	TTGTGTTCAGGTCCAGGGAAA	GCATCCTTGTGGCATGTGAC
IL-13	TGTGGAGCCTCAACCTGAC	TGGATGACACTGCAGTTGGA
IL-18	TCTACTCTCTCCTGTGAGAACA	TGTCCTGGAACACTTCTTTGAA
IL-1b	TGTGTGCTGAAGGCTCTCC	CCTTGCACAAAGCTCATGCA
IL-2	CTCTTGCACTCGTTGCAAAC	TCCAGCAGCAATGACTTCAC
IL-23A	CTGGAGTGCACACCTACCAA	AGCCATCCTCACACTGGATAC
IL2RA	AGGCTCAAGTGCATACGTGAA	GGAGCTTCCGTGCTCTCC
PGR	CTCAGGACCTGGACGGGAA	CGGCTTCCCCTCCGGAATA
PTGES	GCGCTGCTGGTCATCAAAA	TCCTCGGGGTTGGCAAAA
PTGS2	CTGGAACATGGTCTCACTCA	TGCGACTGGAAGATTCCTAC
PTX3	GCAGTGTCGGCGGAGAA	TGTCCCACTCGGAGTTCTCA
SLC16A3	ACAGCCTGGATCTCCTCCA	GCCAAAGCGATTCACACACA
SLC2A1	GTGGGCATGTGCTTCCAGTA	GAACCAGGAGCACGGTGAA
TAF11	AGAGAAGAAGCAGAAAGTGGATGAA	GGTTCAGCTGCTCCTCAGAA
T-bet	AAGTGGGTGCAATGTGGAAA	GGGGAATCTGGATGGACATAC
TGF-b1	GCCTGCTGAGGCTCAAGTTAA	AGCGCCAGGAATTGTTGCTA
TNFa	CAAGTAACAAGCCGGTAGCC	GGCATTGGCATACGAGTCC
YWHAZ	AACAGCAGATGGCTCGAGAA	GAAGCGTTGGGGATCAAGAAC



Supplementary Figure 4-1. Relative expression of genes expressed in the endometrium and involved in prostaglandin synthesis tested by qPCR in the endometrial samples grouped by DIM. Endometrial samples were collected one time from heifers (H) and three times from lactating cows during lactation. Zero out of four gene tested were differentially expressed. * indicate significantly different at adj. *P*-value < 0.05. Data are Log2 fold change of the LSM ± S.E.M.



Supplementary Figure 4-2. Relative expression of genes from heat shock protein category tested by qPCR in the endometrial samples grouped by DIM. Endometrial samples were collected one time from heifers (H) and three times from lactating cows during lactation. Zero out of six gene tested were differentially expressed. * indicate significantly different at adj. *P*-value < 0.05. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.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IL_10
IL_12B
IL_13
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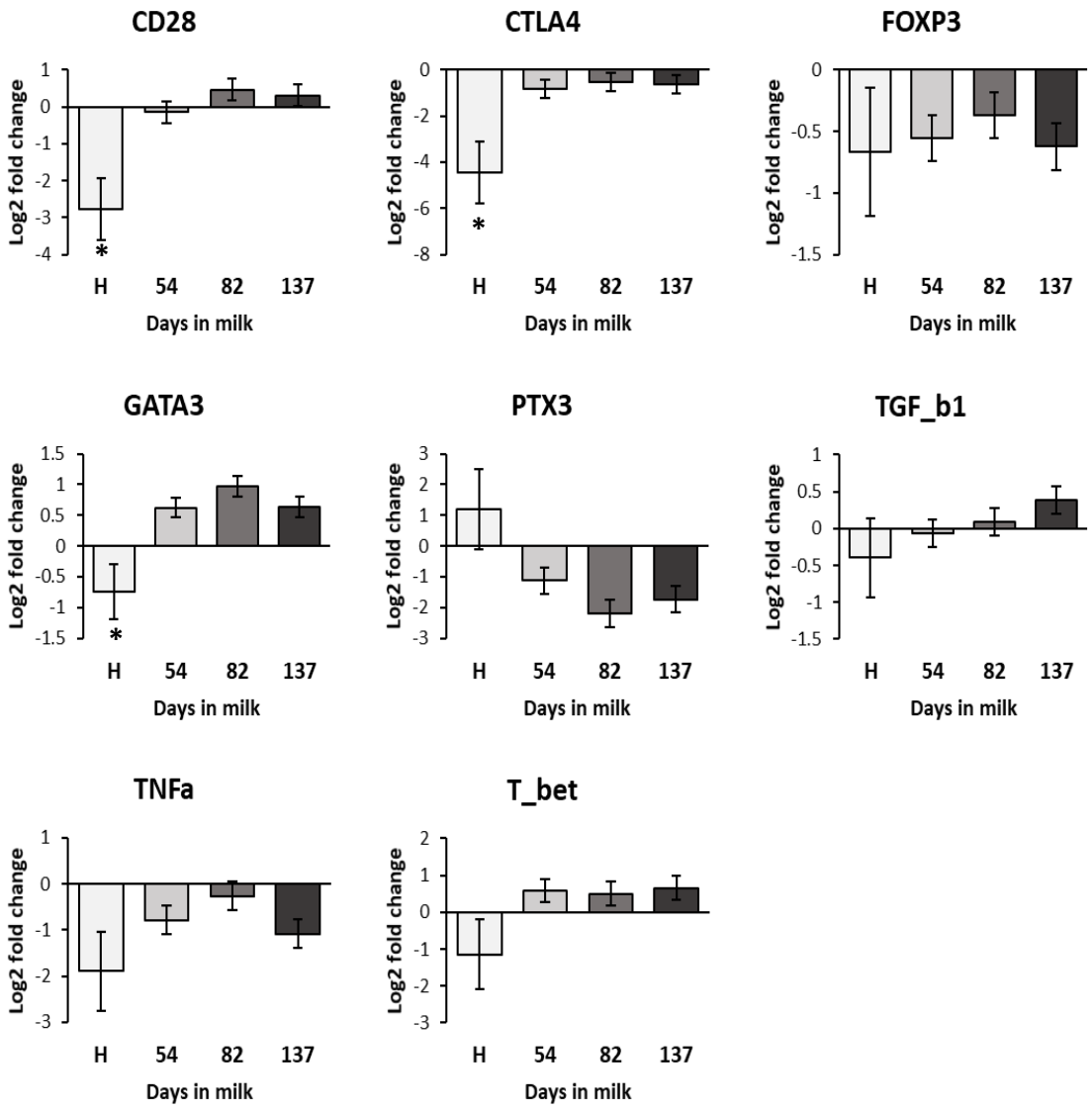
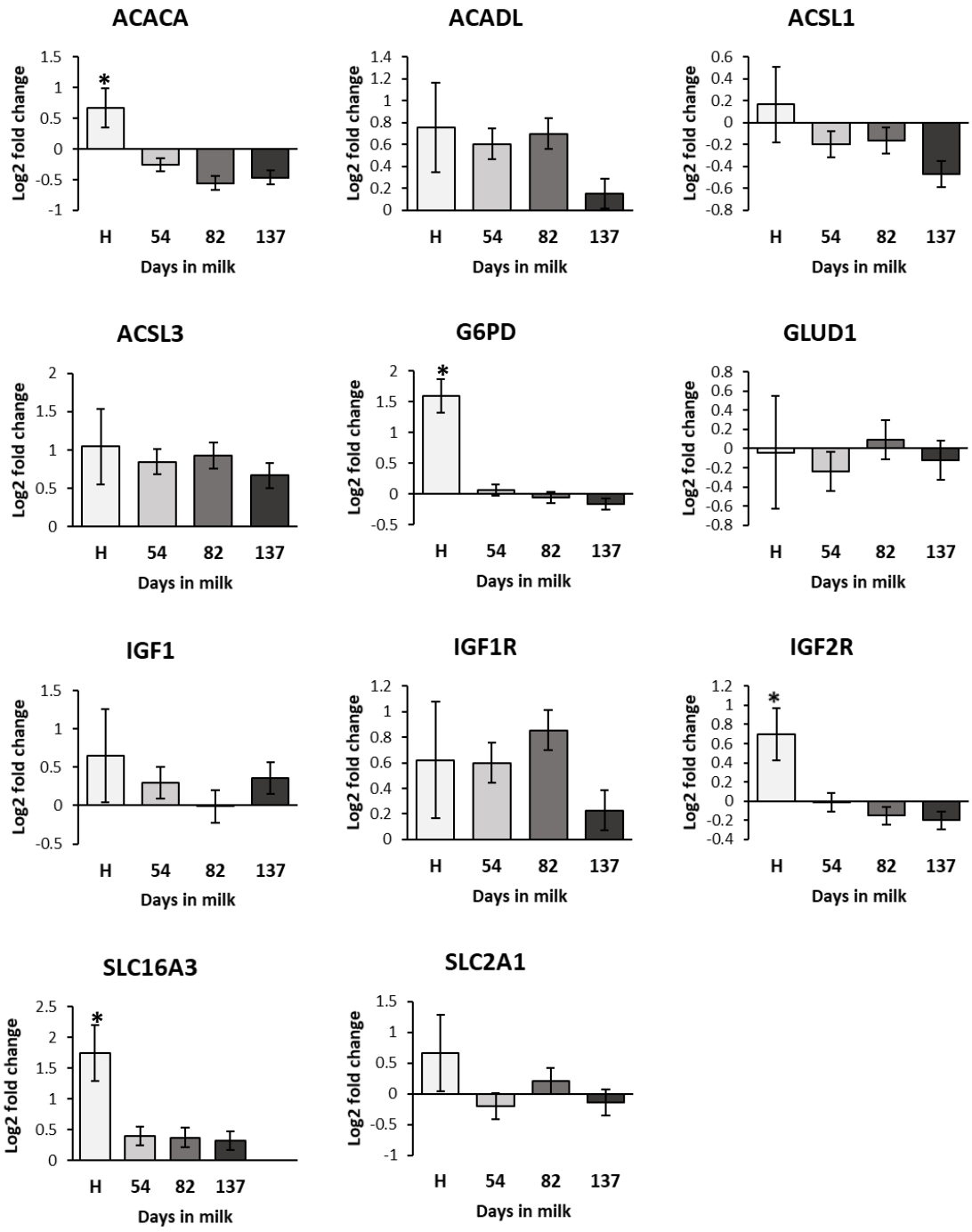
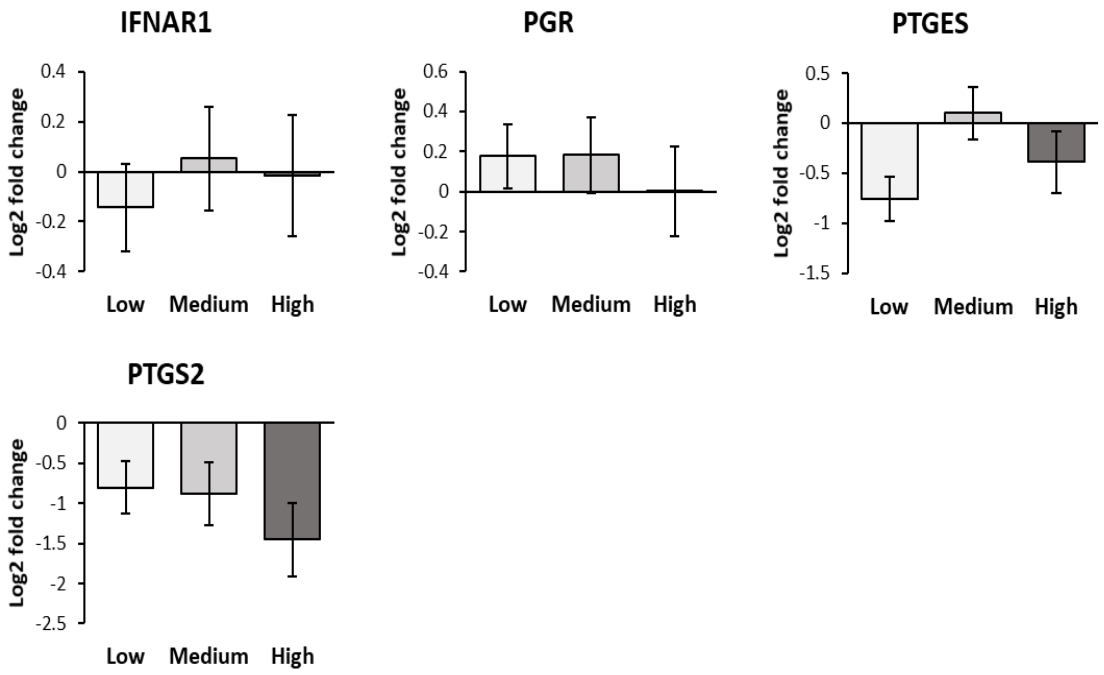
IL_2
IL_23A
Log2 fold change
H
54
82
137
Days in milk
*



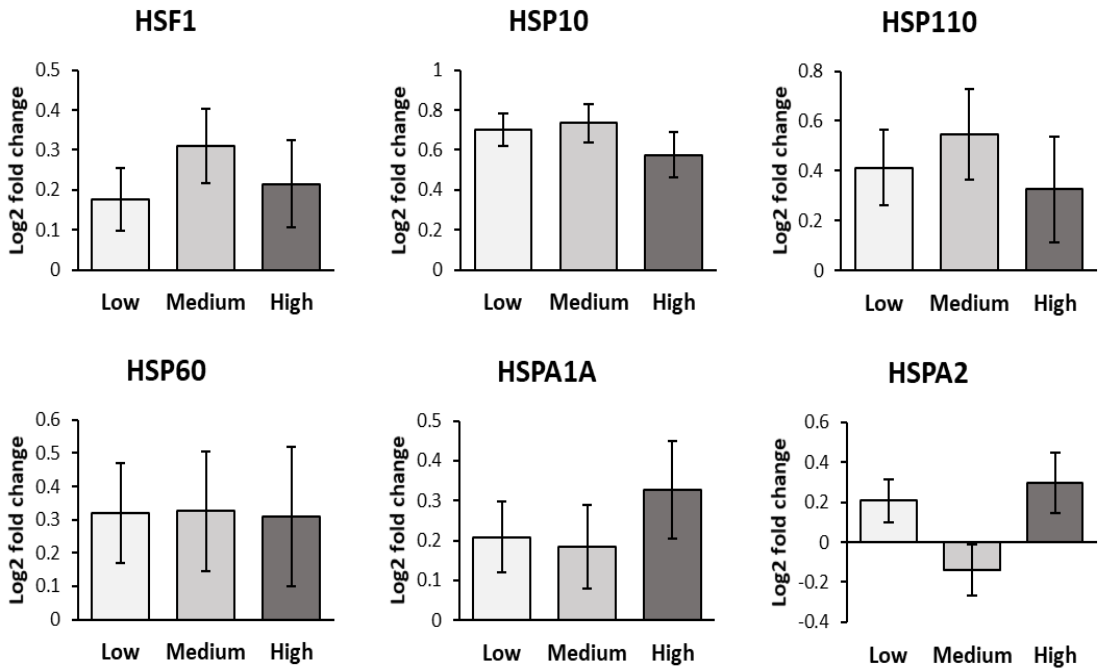
Supplementary Figure 4-3. Relative expression of genes involved in inflammatory response and regulation tested by qPCR in the endometrial samples grouped by DIM. Endometrial samples were collected one time from heifers (H) and three times from lactating cows during lactation. Five out of seventeen gene tested were differentially expressed. * indicate significantly different at adj. *P*-value < 0.05. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



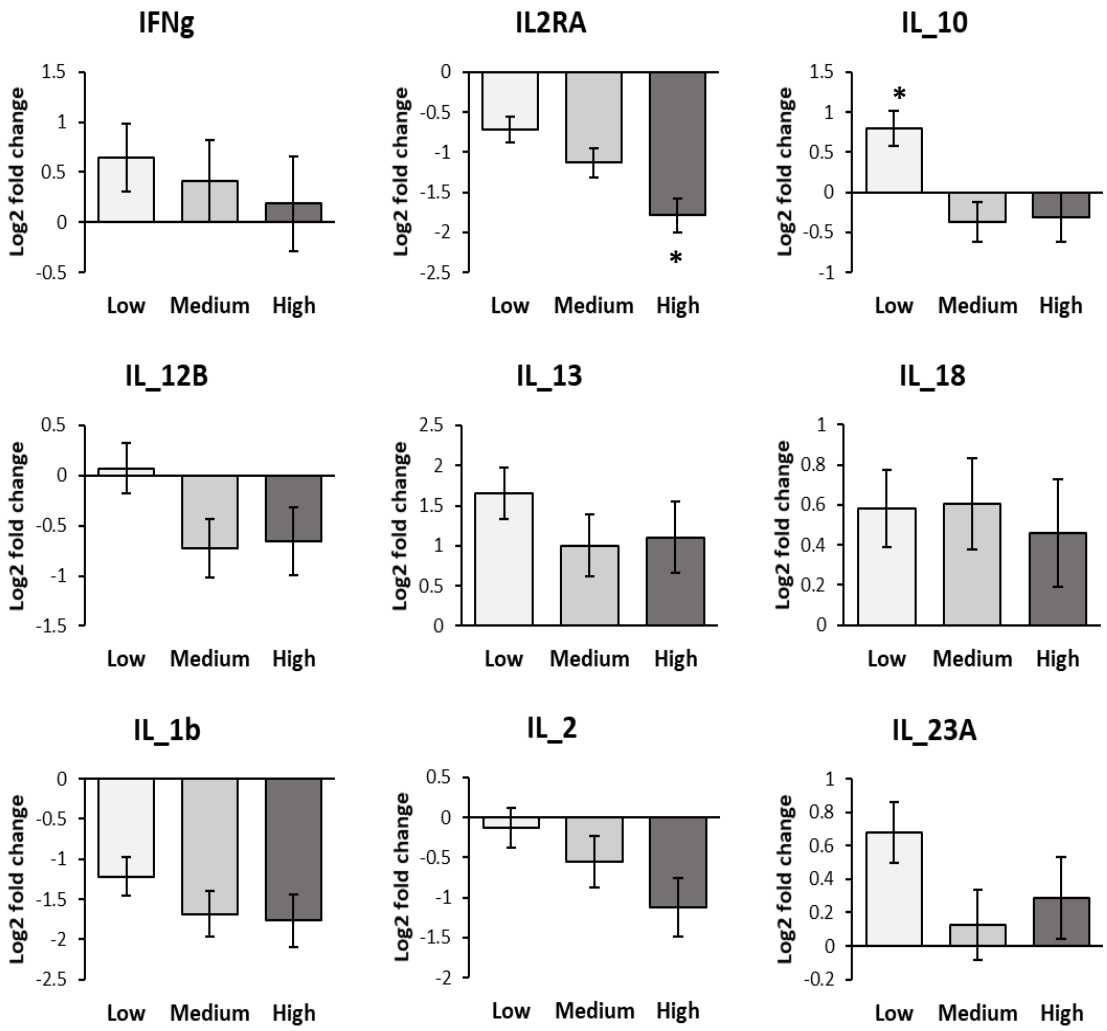
Supplementary Figure 4-4. Relative expression of genes from metabolism category tested by qPCR in the endometrial samples grouped by DIM. Endometrial samples were collected one time from heifers (H) and three times from lactating cows during lactation. Four out of eleven gene tested were differentially expressed. * indicate significantly different at adj. *P*-value < 0.05. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



Supplementary Figure 4-5. Relative expression of genes expressed in the endometrium and involved in prostaglandin synthesis tested by qPCR in endometrial tissue from lactating cows grouped by the BHBA level. Zero out of four genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



Supplementary Figure 4-6. Relative expression of genes from heat shock protein category tested by qPCR in endometrial tissue from lactating cows grouped by the BHBA level. Zero out of six genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.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-1.5
-2
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Low
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IL_10
Log2 fold change
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-1
*
Low
Medium
High
IL_12B
Log2 fold change
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Low
Medium
High
IL_13
Log2 fold change
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2
1.5
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Low
Medium
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IL_2
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-1.5
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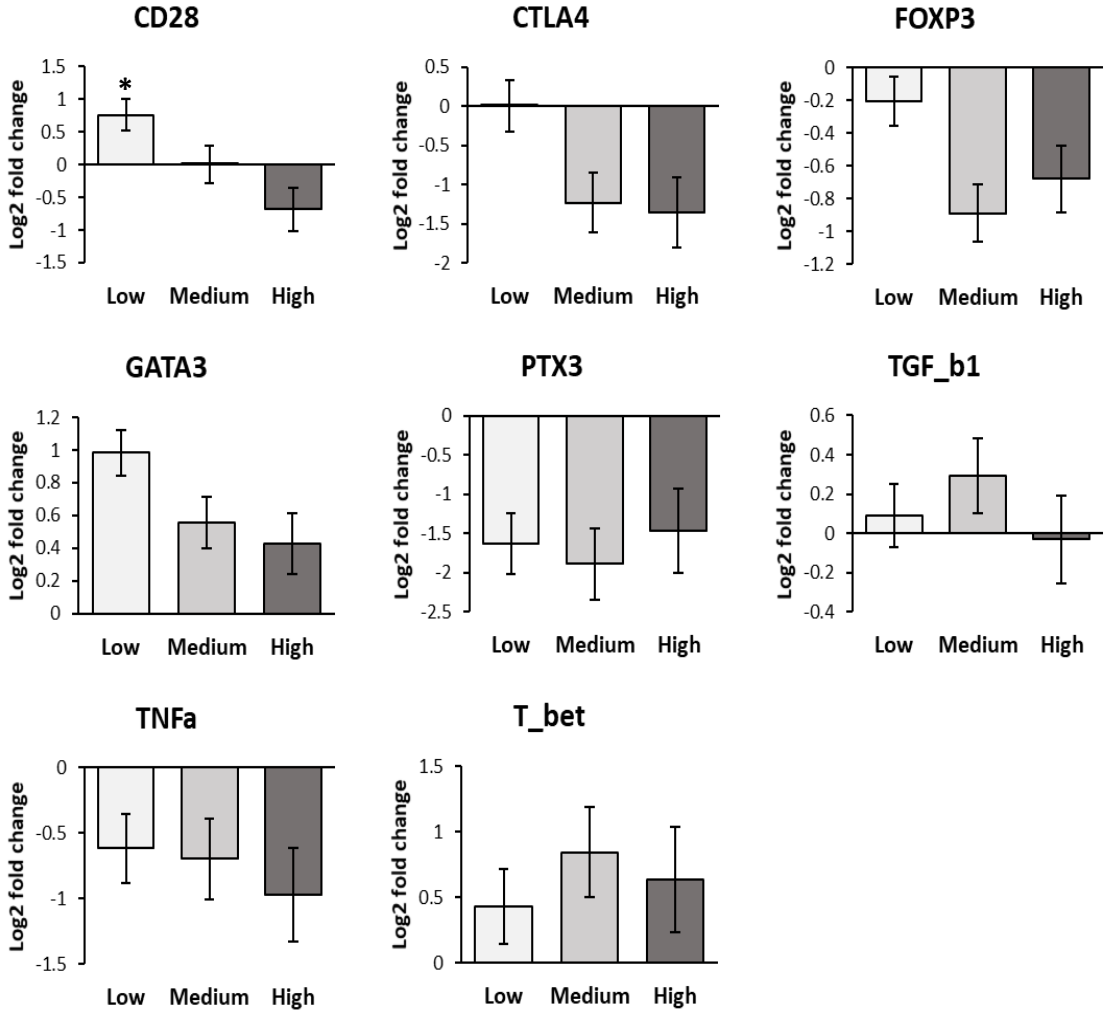
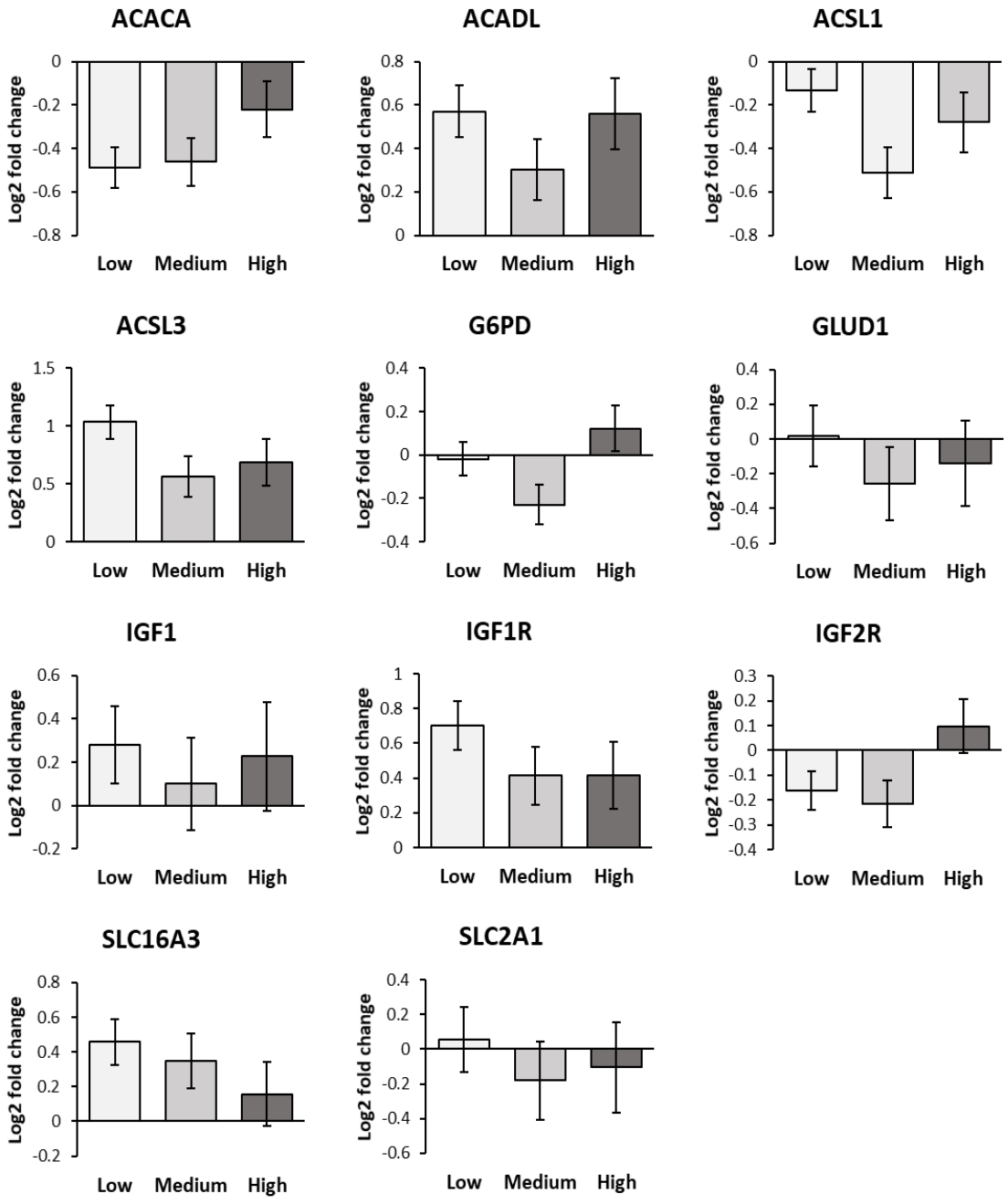
High



Supplementary Figure 4-7. Relative expression of genes from inflammatory response and regulation category tested by qPCR in endometrial tissue from lactating cows grouped by the BHBA level. Three out of seventeen genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.



Supplementary Figure 4-8. Relative expression of genes from metabolism category tested by qPCR in endometrial tissue from lactating cows grouped by the BHBA level. Zero out of eleven genes tested were differentially expressed. * indicate significantly different at adj. $P < 0.05$. Data are Log2 fold change of the LSM of ΔΔCt values ± S.E.M.

CHAPTER 5

SUMMARY

Dairy cows typically go through a period of negative energy balance (NEB) during the first few months in lactation as the abrupt increase in energy demand for rapid milk production immediately after calving can not be met entirely from feed intake. The difference in energy expenditure is compensated by mobilization of body fat as no esterified fatty acids (NEFA) into the circulation and carried to the liver. NEFA are utilized by hepatic tissue for gluconeogenesis in addition to glycogenolysis to provide glucose to supply the peripheral tissues and the mammary for lactose synthesis (Gross et al., 2013). Through β-oxidation, NEFA are converted to acyl-CoA to enter the Krebs cycle to generate energy (Zammit, 1990). Extra acetyl-CoA that does not enter the Krebs cycle enters the ketogenesis pathway and is converted into ketone bodies predominantly β-hydroxybutyrate (BHBA) (Drackley et al., 2001). These metabolic changes are part of normal physiology during the transition period and failure to adjust results in a number of metabolic disorders that may directly or indirectly interfere with the reproductive performance in dairy cattle (Butler, 2000). Animals in general, including dairy cattle, are capable of adapting to fluctuation in a range of physiological conditions to maintain tissue homeostasis and to protect the animal from the side effects of these changes. However, extreme changes that go beyond their physiological capacity to sustain, allow undesirable side effects to be manifested. In the case of modern dairy cows, the superior milk yield potential pushes the cows to their physiological limit, leading to amplification of the associated undesirable side effects (Bauman and Currie, 1980; Roche et al., 2017).

Modern high-yielding dairy cows are currently producing far more milk than their ancestors due to prolonged and intensive genetic selection for milk production traits, accompanied with revolutionary improvements in technology, management, and nutrition (Miglior et al., 2017). On the other hand, a noticeable decline in fertility and reproductive performance has been undeniably consistent with the increase in milk yield. This decline in fertility and reproductive performance is recognized worldwide and internationally well documented in several studies (Macmillan et al., 1996; Butler, 1998; Roche et al., 2000; Royal et al., 2000; Lucy, 2001; Lopez-Gatius et al., 2003; Leroy et al., 2008). Fertility is a multifactorial trait that is technically can be affected by several factors, through multiple pathways, and at different time points through the cow's reproductive cycle (Walsh et al., 2011). The decline in fertility is manifested by poor reproductive performance in a number of essential physiological activities that lead to a successful pregnancy and delivering a healthy newborn calf. These physiological activities include the following: exhibiting normal estrous cycle and ovarian function, showing sufficient expression of estrus with detectable heat signs, ovulation of a competent oocyte that is able to be fertilized and cleave after fertilization, producing embryos of excellent quality, providing optimal uterine environment that supports healthy embryonic development to term, displaying normal calving without complications, completing successful and timely uterine involution, and finally resumption of normal ovarian cyclicity. Even though these are indispensable characteristics of acceptable reproductive performance, the incidence of the related infertility issues and disorders is variable among them (Walsh et al., 2011).

Early embryonic death is one of the major issues leading to infertility and reproductive failure in dairy cattle (Walsh et al., 2011). Approximately 90% of the

spontaneously ovulated oocytes are successfully fertilized and start developing, but only 55% of them survive to term, and 70-80% of the lost embryos die within the first two weeks of gestation before implantation (Moussa et al., 2015). The quality of the preimplantation embryo is mainly influenced by the inherent oocyte developmental competence and the surrounding uterine environment. Oocyte quality determines the intrinsic developmental competence of the embryo since the maternal oocyte's RNA transcripts and organelles control and regulate early intracellular biological processes until the complete activation of the embryonic genome. Concurrently, uterine tissue provides nutrients and optimal environment for the embryo to grow. A healthy, functional, and receptive uterus is a requisite to establish, recognize, and maintain pregnancy (Lonergan and Forde, 2014; Moussa et al., 2015; Maillo et al., 2016).

In the set of the experiments we performed, we focused on embryo quality, its origin (oocyte), and its immediate surrounding environment (endometrium) as the three main tissues that directly contribute to fertility. The main objective of these experiments was to evaluate and understand, at the molecular level, how these three main tissues are being impacted by lactation and NEB as the major challenge facing modern high-yielding dairy cows. The overall hypothesis we tested for this project is that lactation and negative energy balance can alter the expression of key genes involved in oocyte developmental competence, embryo quality, and endometrial receptivity to the embryo. To examine this hypothesis, we analyzed the relative changes in gene expression of the single in vivo matured oocytes, individual in vivo developed preimplantation embryos, and endometrial biopsies obtained from non-lactating dairy heifers and lactating dairy cows at different times during lactation. Analysis of the differentially expressed genes will help to

understand how lactation and energy status interact with fertility at the molecular level and identify which pathway and which tissue type is the most affected.

The level of BHBA concentration in the circulation was used to determine the energy status of each animal before the collection of samples. Energy status changes during lactation as the cows shift from negative to positive energy balance as the feed intake increase, and milk yield reach the peak and start to decline gradually. Typically, by 80 days in milk (DIM) most of the dairy cows should shift to positive energy balance and start gaining weight (Roche et al., 2017). Because of that, we aimed to collect the sample multiple times during lactation before and after the NEB. We collected oocytes using ultrasound-guided transvaginal follicular aspiration technique for ovum pick-up (OPU) at 45, 75, and 130 DIM. A non-surgical uterine flushing technique was performed to recover 7 days preimplantation embryos followed by endometrial biopsy at 54, 82, and 137 DIM. The same protocol for estrus synchronization and induction of superovulation using follicle stimulating hormone (FSH) was applied to all animals before each sample collection at each time. This procedure allowed us to collect multiple samples (oocytes or embryos) at comparable stages of the estrous cycle. The multiple samples recovered from the same animal and at the same time were treated and analyzed individually while accounting for the animal, collection time, and NEB effects in the statistical analysis. We performed RT-qPCR for relative quantification of mRNA abundance of a number of selected genes from a variety of functional categories such as metabolism, inflammation, apoptosis, oxidative stress, heat shock proteins, and epigenetics. The generated delta delta Ct ($\Delta\Delta$Ct) value was determined for each reaction and used for the statistical analysis. The statistical analysis was performed to test the effect of lactation (difference between

non-lactating heifers and lactating cows), the effect of DIM within the lactating cows (difference between first, second, and third sample collection), and the effect of NEB within lactating cows (difference between low, medium, and high BHBA).

In the analysis of relative gene expression levels of 64 selected transcripts in individual oocytes, six genes (*DNMT1, DNMT3B, GLUD1, GSR, HSP90A1*, and *SUV39H1*) were found to be differentially expressed (adj. $P < 0.05$) with at least a one-fold difference. The relative different expression levels of these genes appeared to significantly impacted by the energy status of rather than the stage of lactation. Their expression was higher in lactating cows identified with high BHBA level in the circulation. Overexpression of most of these genes was shown in a number of studies to be related to an altered redox homeostasis and aberrant DNA methylation, which could be linked to the increased oxidative stress in oocytes collected from cows during NEB (Jones, 2006; O'Doherty et al., 2014; Couto et al., 2016; Menezo et al., 2016).

In the analysis of relative gene expression levels of 63 selected transcripts in individual embryos, no genes were found to be differentially expressed between lactating cows and heifers or within lactating cows due to change in DIM or different BHBA levels. However, four genes (*EZH2, KLF4, GLRX3,* and *TXN*) appeared to be differentially expressed with more than one-fold difference between embryos from lactating cows and heifers. The relative difference in fold-change of these genes appeared to be impacted by lactation as it was higher in embryos recovered from lactating cows relative to those recovered from non-lactating heifers. *GLRX3* and *TXN* encode key antioxidants that protect against the oxidative stress through their disulfide reductase activity (Holmgren, 2000; Lu and Holmgren, 2014). Their increased expression in the

embryos from lactating cows could be an indication of ongoing redox activity as the preimplantation embryo has the capacity to upregulate genes involved in redox homeostasis in response to oxidative stress (Stover et al., 2000). *KLF4* is involved in the inflammatory response, mediates proinflammatory signaling in macrophages (Feinberg et al., 2005; Nayak et al., 2013) and inhibits interleukin-1 beta (*IL-1β*). The overexpression of *KLF4* was shown to be induced by pro-inflammatory cytokine (Liu et al., 2012) which may suggest an upregulation in response to the more proinflammatory uterine environment in lactating cows compared to heifers. In the embryonic stem cells, *KLF4* plays an essential role in stem cell self-renewal and maintenance (Shi and Ai, 2013).

In the analysis of relative expression levels of 41 selected transcripts in the endometrial tissue samples, five genes (*IL-2*, *IL2RA*, *CD28*, *CTLA4*, and *GATA3*) from the inflammatory response and regulation were found to be significantly more expressed (adj. $P < 0.05$) with at least a one-fold difference. Their expression was higher in the endometria of lactating cows relative to heifers. The higher expression of these genes is expected even in healthy primiparous and multiparous cows as they typically have higher expression of cytokine mRNA during the third week postpartum then start to decline over time. The postpartum endometrial inflammatory response is essential and part of uterine involution process to clear bacterial contamination and restore pre-pregnancy endometrial function (Gabler et al., 2010; Chapwanya et al., 2012). Unlike primiparous and multiparous cows, nulliparous heifer did not experience pregnancy, and the endometrium was not affected by any bacterial invasion that may cause an inflammatory response. In contrast, the expression of four genes involved in metabolism (*ACACA*, *G6PD*, *IGF2R*, and *SLC16A3*) was significantly higher in heifers in comparison with lactating cows. The

difference in the expression levels of these genes could be due to the difference in energy status and metabolism rate between non-lactating heifers and lactating cows. Only three genes (*IL-10, IL2RA,* and *CD28*) from the inflammation category were significantly affected by energy status within lactating cows. Their expression was found to be more than one-fold higher in cows identified as low BHBA. This finding may suggest that NEB impacts fertility through weakening the immune and inflammatory response in the endometrium leading to impaired bacterial clearance (Wathes et al., 2009).

In conclusion, the results of these experiments demonstrate the complex interaction between lactation, metabolism, and reproductive performance in dairy cattle. The increased severity and duration of NEB are the major challenges facing modern high-yielding dairy cows as it results in dramatic fluctuations in the level of the circulating hormones and metabolites for an extended period. However, the findings of these experiments suggests the severity of NEB due to lactation and high milk yield, rather than duration, what may drive the alteration in gene expression of reproductive tissues. NEB can impact fertility through multiple pathways, and oxidative stress appears to be a common denominator across the main reproductive tissues tested herein. Excessive fat mobilization and oxidation of NEFA to compensate for the energy deficit during the period of NEB raises the level of reactive oxygen species (ROS) leading to oxidative stress (Contreras and Sordillo, 2011; Sordillo and Raphael, 2013). High level of ROS impairs the function of the immune cells, alters redox homeostasis, and interfere with DNA methylation leading to epigenetic errors in female gametes (Menezo et al., 2016). We emphasize on the role of stable endometrial inflammatory state and uterine health to improve fertility and increase conception rate. Also, we encourage further

research in the use of antioxidant supplementation to counteract the adverse effect of oxidative stress dairy cows in order to enhance fertility. We hope this work helps in understanding how lactation and energy status impact the molecular constituents of the main reproductive tissues. Identifying these pathways and the key genes involved will definitely help in developing profound strategies to improve fertility in dairy cows.

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