Metabolic and ovarian functions of lactating dairy cows

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"*Stand up for what you believe in, even if it means standing alone…*”
-Anonymous

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LIST OF ABBREVIATIONS

3β-HSD	3β-hydroxysteroid dehydrogenase
A4	androstenedione
ACTβ	actin β
ADAM17	a disintegrin and metalloprotease-17
ADAMTS1	a disintegrin and metalloproteinase with thrombospondin motif 1
ANPT	angiopoietin
APOB100	apolipoprotein B100
AREG	amphiregulin
AST	aspartate aminotransferase
BCS	body condition score
BHBA	β-hydroxybutyric acid
BSA	bovine serum albumin
CEBPB	CCAT/enhancer-binding protein beta
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CL	corpus luteum
CXCR4	CXC receptor 4
CYP11A1	cholesterol side-chain cleavage enzyme
CYP17A1	cytochrome P450 family 17 subfamily A member 1
CYP19A1	aromatase
CYP7A1	cholesterol 7 alpha-hydroxylase

DNA	deoxyribonucleic acid
DEG	differentially expressed gene
E_2	estradiol
EGR1	early growth response 1
EREG	epiregulin
ERK1/2	extracellular signal-regulated kinase 1 and 2
ET-1	endothelin-1
FBS	fetal bovine serum
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
FRAP	ferric reducing ability of plasma
GnRH	gonadotropin-releasing hormone
GPR109A	niacin receptor 1
GSH	glutathione
IFNγ	interferon gamma
HDL	high-density lipoprotein
IGF1	insulin-like growth factor 1
IGFBP	insulin-like growth factor-binding protein
IL1B	interleukin 1 beta
IL6	interleukin 6
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LH	luteinizing hormone

LHCGR	luteinizing hormone receptor
LPI	lifetime profitability index
LRP8	low-density lipoprotein receptor related protein 8
MAPK 3/1	mitogen-activated protein kinase 3 and 1
MCP-1	chemokine monocyte chemoattractant protein-1
MCT1	monocarboxylate transporter 1
mRNA	messenger ribonucleic acid
NEB	negative energy balance
NEFA	non-esterified fatty acid
NPPC	natriuretic peptide precursor C
NR5A2	nuclear receptor subfamily 5 group A member 2
P_4	progesterone
PAPP-A	pregnancy-associated plasma protein A
PI3K/Akt	phosphatidylinositide 3-kinase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGF2α	prostaglandin F2 α
PGR	progesterone receptor
PPARG	peroxisome proliferator-activated receptor gamma
PTGS2	prostaglandin-endoperoxide synthase 2
RGS2	regulator of G protein signaling 2
RIN	RNA integrity number
RNA	ribonucleic acid

ROS	reactive oxygen species
RPL19	ribosomal protein L19
SCARB1	scavenger receptor class B member 1
SREBP	sterol regulatory element-binding protein
SLC16A1	solute carrier family16 member 1
STAR	steroidogenic acute regulatory protein
STAT3	signal transducer and activator of transcription 3
TACSTD2	tumor-associated calcium signal transducer 2
TBA	total bile acids
TG	triglycerides
TIMP1	tissue inhibitor of matrix metalloproteinases 1
TIMP2	tissue inhibitor of matrix metalloproteinases 2
TNFα	tumor necrosis factor alpha
TNFAIP6	tumor necrosis factor alpha induced protein 6
VEGF	vascular endothelial growth factor
VLDL	very-low-density lipoprotein
VNN2	vanin 2
VWP	voluntary waiting period

ABSTRACT

Reproductive longevity amongst dairy cattle is a hallmark for sustainable dairy production. Nevertheless, subfertility has plagued modern dairy cows. More specifically, failure of cows to physiologically adapt during the transition period can negatively impact subsequent reproductive performance, which is the major cause of involuntary culling in Canadian dairy herds.

Although nulliparous heifers are known to have higher reproductive success than their lactating counterparts, there is lack of research investigating the similarities and differences of the metabolic indicators and the oxidative stress markers between nulliparous heifers, primiparous cows, and multiparous cows. Analysis of plasma metabolites revealed that primiparous and multiparous cows have lower levels of circulating glucose, triglycerides, and very low-density lipoprotein and higher levels of total bile acids, total-cholesterol, high-density lipoprotein, low-density lipoprotein, and the ferric reducing ability of plasma compared to nulliparous heifers in the weeks leading up to planned time of breeding. In general, the metabolic profile was drastically altered in multiparous cows where β-hydroxybutyric acid levels at one week prior to planned time of breeding was 2-fold higher compared to heifers. We expect that these metabolic profiles were reflected to a large extent in the follicular fluid.

Body condition score (BCS) assessment is a management tool to visually appraise lipid mobilization during the transition period. Severe loss of body condition during the transition period until the planned time of breeding has been associated with reduced fertility. Our data showed that cows losing severe body condition had higher levels of circulating non-esterified fatty acids (NEFAs) and γ-glutamyltransferase (GGT) suggesting greater difficulty of liver adaptation to early lactation. These metabolic changes appear to have altered the ovarian follicular microenvironment, since the follicular fluid from the dominant follicle collected at 7 weeks post-partum from cows losing more body condition had lower levels of insulin-like growth factor 1 (IGF1) and estradiol. This is in line with the gene transcription profile of the granulosa cells that was also drastically altered as *CYP19A1, NR5A2, IGF1R,* and *LHCGR* transcript levels were lower in cows losing more body condition. Taken together, failure to adapt to early-lactation due to increased lipid mobilization has long-term consequences hindering the development of a competent dominant follicle.

To further study the underlying mechanisms of infertility in cattle, we chose to investigate the role of extracellular signal-regulated kinase pathway 1 and 2 (ERK1/2) in bovine ovulation.

To study ERK1/2 signaling *in vivo* for the first time in cattle, we administered PD0325901 (ERK1/2 signaling inhibitor) directly into the dominant follicle of cattle followed by an intramuscular injection of gonadotropin-releasing hormone (GnRH) to stimulate a luteinizing hormone (LH) surge. First, we found that inhibition of ERK1/2 prevented ovulation in cattle. Second, we repeated the experiment collecting the ovary by colpotomy 6h post-GnRH. We then studied global transcriptomics in granulosa cells using RNA-seq technology and established a list of 285 LH-ERK1/2 regulated genes in granulosa cells. In this cohort of genes, we established that *EGR1, ADAMTS1, STAT3* and *TNFAIP6* expression was drastically decreased in cows treated with PD0325901 + GnRH, where the opposite is true for *SLC16A1*, which is a monocarboxylate transporter, transporting substances such as β-hydroxybutyrate, across the plasma membrane, and no change in *STAR* expression was observed.

Taken together, this thesis provides an insight into the relationship between metabolism and ovarian function in the modern dairy cow.

RÉSUMÉ

La longévité reproductive chez les vaches laitières est une caractéristique de la production laitière durable. Les problèmes de reproduction sont les raisons principales pour lesquelles les vaches sont involontairement retiré des troupeaux laitiers canadiens.

Bien que l'on sache que les génisses sont plus fertiles que les vaches en lactation, on manque de recherches sur les similitudes et les différences du profil métabolique et de l'état oxydatif entre génisses, vaches primipares et vaches multipares. L'analyse des métabolites plasmatiques a révélé que les vaches primipares et multipares ont des taux plus faibles de glucose, de triglycérides et de lipoproteins de très basse densité (VLDL) et des taux plus élevés d'acides biliaires totaux, de cholestérol total, de lipoprotéines de haute densité, lipoprotéines de densité moyenne (LDL), et de capacité réductrice ferrique du plasma par rapport aux génisses dans les semaines précédant la saillie. Le profil métabolique a été radicalement modifié chez les vaches multipares où les niveaux d'acide hydroxybutyrique à une semaine avant le moment prévu de la saillie étaient 2 fois plus élevés par rapport aux génisses. Nous prédisons que ces profils de métabolites se sont reflétés dans le liquid folliculaire.

L'évaluation de l’état de chair (BCS) est un outil de gestion permettant d'évaluer visuellement la mobilisation des lipides pendant la période de transition. La perte excessive de l’état de chair pendant la période de transition jusqu'au temps prévu de saillie a été associée à une fertilité réduite. Nos données ont montré que les vaches don’t la perte de l’état de chair est excessive ont des taux plus élevés plasmatique d'acides gras non estérifiés et de γ-glutamyltransférase (GGT), ce qui laisse croire à une plus grande difficulté d'adaptation en début de lactation. Ces changements métaboliques semblent avoir altéré l'environnement folliculaire ovarien, puisque le liquid folliculaire provenant du follicule dominant prélevé 7 semaines après le vêlage des vaches perdant plus de BCS présentait des taux plus faibles de facteur de croissance analogue à l’insuline et d'estradiol. Ceci est en ligne avec le profil de transcription génique des cellules de la granulosa; car, les niveaux de transcription de *CYP19A1*, *NR5A2*, *IGF1R*, et *LHCGR* étaient inférieure dans kes vaches perdant plus de BCS. Pris ensemble, l'incapacité à s'adapter au début de la lactation en raison de l'augmentation de la mobilisation des lipides a des conséquences à long terme qui entravent le développement d'un follicule dominant compétent.

Pour étudier d’avance les mécanismes biologiques de l'infertilité chez les bovins, nous avons choisi d'étudier le rôle de signalisation par le kinase: ERK1/2 dans l'ovulation bovine. Pour

examiner la signalisation ERK1/2 in-vivo pour la première fois chez la vache, nous avons administré PD0325901 (inhibiteur de signalisation ERK1/2) directement dans le follicule dominant des bovins suivi d'une injection intramusculaire d'hormone gonadotrophine (GnRH) pour stimuler une hormone lutéinisante (LH). Nous avons constaté que l'inhibition de ERK1/2 empêchait l'ovulation chez les bovins. Nous avons répété l'étude de collecte de l'ovaire par colpotomie 6h après-GnRH. Nous avons ensuite examiné la transcriptomique globale dans les cellules de la granulosa en utilisant la technologie RNA-seq et établi une liste de 285 gènes régulés par LH-ERK1/2 dans les cellules de la granulosa. Dans cette cohorte de gènes, nous avons établi que l'expression de *EGR1, ADAMTS1, STAT3* et *TNFAIP6* est considérablement réduite chez les vaches traitées avec PD0325901 + GnRH, alors que le contraire est vrai pour *SLC16A1*, transporteur de monocarboxylate transportant des substances telles que l'hydroxybutyrate à travers la membrane plasmique, et pas de changement était observer avec l'expression de *STAR*.

Cette thèse fournit un aperçu de la relation entre le métabolisme et la fonction ovarienne chez la vache laitière.

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People are always surprised when I tell them that I am still in school, after all these years. I have spent over 9 years studying at the Macdonald Campus, I think it is safe to say, that it has been a long journey with good times, failed times (so many cell cultures), and most certainly entertaining times.

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CONTRIBUTIONS OF AUTHORS

This thesis was prepared in accordance with the McGill University thesis preparation guidelines in a manuscript-based format. The following thesis was written by Yasmin Schuermann with editorial assistance from Drs. Raj Duggavathi & Vilceu Bordignon.

The first two chapters include the introduction, hypothesis, and objectives, literature review, written by Yasmin Schuermann.

In this thesis, three co-authored manuscripts are presented:

Authors of manuscript 1 (Chapter III)

The third chapter includes the first manuscript entitled "Association between pre-breeding metabolic profiles and reproductive performance in heifers and lactating dairy cows" submitted the to *Theriogenology* (THERIO_2018_485).

Yasmin Schuermann, Gerald Welsford, Evan Nitschmann, Linda Wykes, and Raj Duggavathi

Yasmin Schuermann contributed to ideas and was responsible for the experimental design, conduction of experiments, data collection/analysis and manuscript writing. Dr. Linda Wykes and Evan Nitschmann helped establish the protocols for oxidative stress marker analysis. Assistance in blood sampling was conducted with the help of Gerald Welsford. Finally, Dr. Raj Duggavathi contributed to the experimental design and manuscript assembly.

Authors of manuscript 2 (Chapter IV)

The fourth chapter includes the second manuscript entitled "Body condition loss on the metabolic, hepatic, and ovarian health of dairy cows", which is being prepared for submission to *Reproduction*.

Yasmin Schuermann, Audrey St. Yves, Naomi Dicks, Rodrigo Bohrer, Valerie Higginson,
Rafael G Mondadori, Mariana Priotto de Macedo, Milena Taibi, Arif Mustafa, Bushansingh
Baurhoo, Vilceu Bordignon, and Raj Duggavathi

Yasmin Schuermann contributed to ideas and was responsible for the experimental design, the conduction of experiments, data collection/analysis and manuscript writing. Dr. Naomi Dicks assisted Yasmin Schuermann in performing liver biopsies. Dr. Rodrigo C. Bohrer and Dr. Rafael Mondadori performed ovarian follicular aspirations. Additionally, Mariana Priotto de Macedo assisted Yasmin Schuermann with manipulation of ovarian samples. Audrey St. Yves, Valerie Higginson, and Milena Taibi assisted in blood sampling, while Audrey further assisted with body condition scoring and downstream analysis. Drs. Bushansingh Baurhoo and Arif Mustafa assisted with animal maintenance and gas chromatography. Dr. Raj Duggavathi conceived the study, contributed to the experimental design and edited the manuscript.

Authors of manuscript 3 (Chapter V)

The fifth chapter includes the third a entitled "ERK1/2-dependent gene expression in the bovine ovulating follicle", accepted by *Scientific Reports* (PMID: 30385793).

Yasmin Schuermann, Monique T. Rovani, Bernardo Gasperin, Rogério Ferreira, Juliana Ferst,
Ejimedo Madogwe, Paulo B. Goncalves, Vilceu Bordignon, and Raj Duggavathi

Yasmin Schuermann contributed to ideas, designed experiments, took part in the field work, collected, assembled, analyzed, and interpreted data, prepared figures and wrote the manuscript. Monique T. Rovani took part in the field work and carried out the hormone and metabolic assays. Bernardo Gasperin and Rogério Ferreira contributed to ideas and assisted in the follicular microinjection and colpotomy. Juliana Ferst in field work and data collection. Ejimedo Madogwe assisted in bioinformatics analysis. Paulo B. Goncalves and Vilceu Bordignon contributed to ideas. Raj Duggavathi conceived the study, designed experiments, analyzed data, and edited manuscript.

The sixth chapter was written by Yasmin Schuermann and provides a global discussion and conclusion of the three research articles. The seventh chapter represents an appendix to the third chapter and was written by Yasmin Schuermann and Raj Duggavathi. A short article was written for Progressive Dairyman: Canada to enhance the understanding of cattle in different physiological states.

CONTRIBUTION TO KNOWLEDGE

Chapter III

In the first study, we set out to compare the metabolic profile and the oxidative stress markers in plasma of primiparous cows, multiparous cows, and nulliparous heifers. We showed that the percent pregnancy rates tended to be lower in lactating cows compared to heifers. By superimposing the plasma profiles for all animals in the weeks leading up to the planned time of breeding we reported a 2-fold increase in β-hydroxybutyric acid 1 week prior to expected breeding in multiparous compared to heifers. Furthermore, all lactating cows had lower levels of circulating glucose, triglycerides, and VLDL, and higher levels of total bile acids, total-cholesterol, HDL, and LDL compared to nulliparous heifers. In terms of the antioxidant systems, multiparous and primiparous cows have higher levels of circulating antioxidants established by the ferric reducing ability of plasma assay. The drastically different metabolic profiles observed in the plasma between nulliparous heifers and lactating cows contributes to the differences in reproductive performance as a similar profile is expected to be reflected in the follicular microenvironment.

Chapter IV

In the second study, we examined the impact of body condition loss through the transition period up until the anticipated breeding period on liver function, metabolism, and follicle development. Cows losing more body condition also had a greater BCS at time of parturition. Furthermore, those same cows had higher levels of circulating NEFAs and GGT, linked with lipid mobilization and liver damage, respectively. In addition, greater body condition loss caused an increase in *CYP7A1*, important for cholesterol and bile acid homeostasis, in hepatic tissue. In the follicular microenvironment of the dominant, we found that greater body condition loss was associated with decreased levels of estradiol and IGF1 in the follicular fluid, which was line with decreased expression of *CYP19A1, NR5A2, IGF1R,* and *LHCGR* in granulosa cells of those same follicles. We emphasize that it is important to prevent over-conditioning during the dry period and prevent severe body condition loss in dairy cows as they experience greater difficulty in adapting to early lactation and this can lead to subfertility.

Chapter V

In the third study, we tested the role of ERK1/2 signaling *in vivo* in the ovulatory follicle of cattle. We established that inhibition of ERK1/2 signaling, by intrafollicular injection of PD0325901, during preovulatory development abolished ovulation and thus, the formation of the corpus luteum. Using RNA-sequencing we established a list of LH-ERK1/2 induced genes in granulosa cells including: *EGR1, ADAMTS1, STAT3* and *TNFAIP,* which were validated by qPCR. This list is similar to that reported in the mouse model. However, species differences were observed, whereby LH-stimulation in the presence of the ERK1/2 inhibitor led to an increase in STAR protein abundance in granulosa cells and in theca cell *STAR* gene expression. Interestingly, we also reported enhanced expression of *SLC16A1* in theca and granulosa cells in PD0325901 treated pre-ovulatory follicles, which we speculated may act as a transporter of BHBA. Overall, ERK1/2 signaling in the bovine ovulatory follicle is essential for normal ovulation.

Taken together, compromised fertility in dairy cattle is a major concern for cow longevity and consequently, sustainable dairy production. Results from these studies can contribute to the development of management strategies to minimize the stress of the transition period by adapting the nutritional requirements for cows of particular parities, as well as proper monitoring of the change in body condition scores. Lastly, the imperative role of ERK1/2 signaling in the ovulating follicle will contribute to the overall understanding of possible susceptibility to ovarian dysfunction observed in cattle.

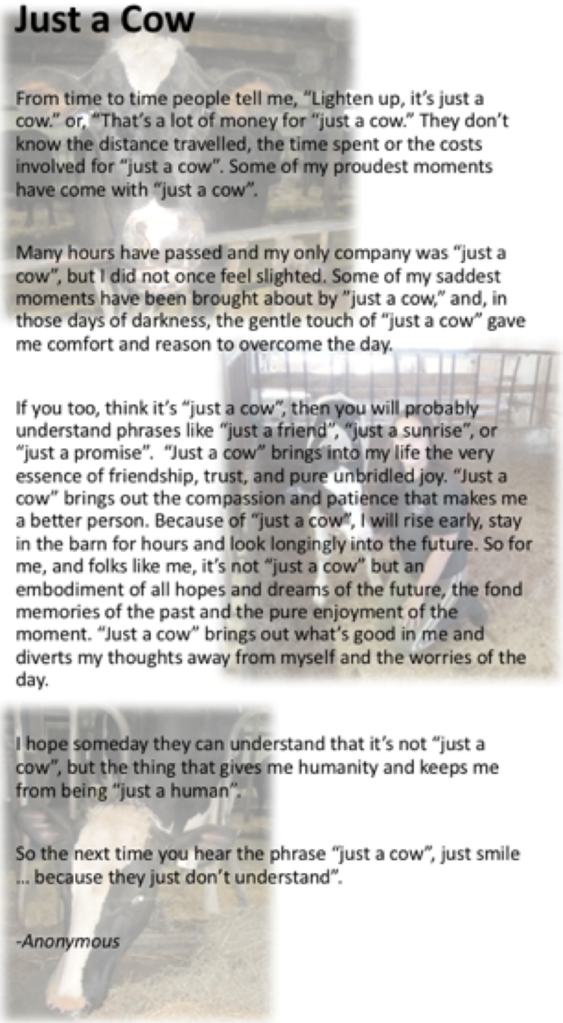
CHAPTER I

RATIONAL

1.1 INTRODUCTION

The cow has become known as a symbol of productivity, fertility, strength, richness, and renewal across religious and cultural beliefs (Alavijeh, 2014). To truly understand the passion and dedication that dairy producers have for their animals take the time and read the anonymously written poem "Just a Cow" (Figure 1.1). This provides a prime example as to why many producers do not view farming as simply a job, but a way of life. Therefore, as researchers in the field of dairy science, we are part of the team helping dairy producers work towards successful and sustainable ventures.

Figure 1.1 Just a Cow - anonymous



Just a Cow

From time to time people tell me, "Lighten up, it's just a cow." or, "That's a lot of money for "just a cow." They don't know the distance travelled, the time spent or the costs involved for "just a cow". Some of my proudest moments have come with "just a cow".

Many hours have passed and my only company was "just a cow", but I did not once feel slighted. Some of my saddest moments have been brought about by "just a cow," and, in those days of darkness, the gentle touch of "just a cow" gave me comfort and reason to overcome the day.

If you too, think it's "just a cow", then you will probably understand phrases like "just a friend", "just a sunrise", or "just a promise". "Just a cow" brings into my life the very essence of friendship, trust, and pure unbridled joy. "Just a cow" brings out the compassion and patience that makes me a better person. Because of "just a cow", I will rise early, stay in the barn for hours and look longingly into the future. So for me, and folks like me, it's not "just a cow" but an embodiment of all hopes and dreams of the future, the fond memories of the past and the pure enjoyment of the moment. "Just a cow" brings out what's good in me and diverts my thoughts away from myself and the worries of the day.

I hope someday they can understand that it's not "just a cow", but the thing that gives me humanity and keeps me from being "just a human".

So the next time you hear the phrase "just a cow", just smile ... because they just don't understand".

-Anonymous

The last few decades have seen major shifts in overall dairy farm technology, production and management in North America. Enhanced feeing strategies as well as genetic improvement by way of artificial insemination with the option of sexed semen, embryo transfer, and genotyping represent some of the advancements that have helped dairy producers quickly improve their herds by increasing milk production (Van Doormaal, 2008, 2010, Osorio et al., 2013). In Canada, the dominating dairy breed is Holstein and on average this breed can produce 10 512 Kg per lactation (305 days) (Centre, 2017). The progress in the field of dairy science has led to breeding world-renowned dairy cows, which include record breakers for highest milk production (365 days): Ever-Green-View My 1326-ET who produced 32 735 Kg (2010), Bur-Wall Buckeye Gigi who produced 33 861 Kg (2016), and Ever-Green-View My Gold-ET who produced 35 144 Kg (2017) (Bullvine, 2017a, Ever-

Green-View, 2018). In the fall of 2017, Selz-Pralle Aftershock 3918 snatched the title as the highest producing cow with a whopping 35 457 Kg (2017)(Boylen, 2018). Although dairy producers in the United States have bred the highest producing cows per lactation, Canada has had its own national treasure by the name of Gillette Emperor Smurf (1996-2015). To this day, Smurf from Embrun, Ontario holds the record for the most milk produced over a lifetime as can be seen in the Guinness Book of World Records. She produced an astounding 216 983 Kg of milk (by 2012), which is the equivalent to approximately 1 million glasses of milk (Duckworh, 2017). Unfortunately, in the last 6 years, the Canadian dairy industry has failed to produce more such record breakers. Moreover, it is very uncommon to see older cows in the herd, which would be able to challenge Smurf. In a typical Quebec herd, only about 40% of cows are in their 3rd or greater lactation (Valacta, 2017). Therefore, improving longevity must be addressed as a key factor in sustainable and profitable dairy farming (Murray, 2013, De Vries, 2017, Delgado, 2018). More specifically, it is essential to address reproductive longevity, as reproduction is one of the most important causes of involuntary culling, whereby in Quebec alone, 15.99% of cows are removed from the herd (Valacta, 2017).

The dairy sector plays a key role in agriculture with the dairy cash receipt of $6.17 billion in 2016 (Centre, 2016). However, decreased reproductive performance leading to reduced longevity of high-yielding cows has become a major issue for the dairy sector (Sewalem et al., 2008). Dairy cattle infertility has been linked to changes in management (Lomander et al., 2013), increased milk production (Lucy, 2001) and nutrition (Lucy et al., 1992a). All of these factors become even more critical during the transition period – defined as 3 weeks pre-partum to 3 weeks post-partum – when cows undergo dramatic metabolic adjustments to accommodate increasing energy demands for milk production (Drackley, 1999, Nydam et al., 2013).

At the onset of lactation, cows experience negative energy balance that can lead to metabolic stress (Sordillo and Raphael, 2013), which has adverse effects on ovarian functions. Metabolic stress in cows is characterized by generalized inflammation (Trevisi et al., 2012), oxidative stress (Fatima et al., 2014), increased β-hydroxybutyric acid (BHBA) (Matoba et al., 2012), decreased glucose and variation in other poorly defined metabolic parameters (Matoba et al., 2012). Metabolic stress has been linked with a decrease in reproductive performance (Huzzey et al., 2015, Shin et al., 2015), as described by anovulation (Walsh et al., 2007), reduced conception rate (Ospina et al., 2010b), increase in the number of days open, and a greater number of services per pregnancy (Bertoni et al., 2008). In a state of negative energy balance and metabolic stress, uterine health is compromised as the cow has a lower tolerance to combat infection and is more vulnerable to decreased fertility(Wathes et al., 2012, Souza et al., 2014). Also, the altered metabolic hormones and metabolites in circulation are reflected in the ovarian follicular fluid, raising the possibility that they may affect follicular health and thus, ovarian functions and fertility (Leroy et al., 2004b, Sanchez et al., 2014). Nonetheless, there may be additional extracellular pathways and metabolic perturbations that may influence ovarian function. From oocyte activation from the ovarian reserve through follicle growth to ovulatory size requires approximately 80 to 100 days, the follicles ovulating at the time of breeding (60 days into lactation) develop during the period of intense metabolic changes of early lactation (Britt, 2008). Characterizing metabolic parameters during the transition period to planned time of breeding is important to better understand the pathogenesis of ovarian dysfunction in lactating dairy cows.

In addition, assessing the body condition score (BCS) of a cow has become a useful tool in visually appraising lipid mobilization that a cow is experiencing during the transition period. A few groups have noted that the severity of body condition loss can lead to the development of health-related disorders, in addition to negatively impacting reproductive performance in cattle (Pryce et al., 2001, Lopez-Gatius et al., 2003, Carvalho et al., 2014a, Shin et al., 2015). Moreover, the hepatic transcriptome can be altered post-calving in response to the body condition (Vailati-Riboni et al., 2016). However, the link between body condition, hepatic, and ovarian function remains unknown.

Lastly, by addressing specific pathways in the ovary, it will be possible to establish links between fertility and metabolic changes. The role of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling pathway has been studied in the mouse model. Abrogation of ERK1/2 signaling in ovarian follicular granulosa cells in mice results in infertility due to complete abolishment of ovulation (Fan et al., 2009, Siddappa et al., 2015). However, few studies have addressed the significance of ERK1/2 in bovine reproduction, where studies address ERK1/2 signaling in cultured granulosa and theca cells (Ryan et al., 2007, Ryan et al., 2008, Sayasith et al., 2013c, Sayasith and Sirois, 2014, Gasperin et al., 2015, da Rosa et al., 2016). With recent studies demonstrating metabolites such as BHBA can signal through ERK1/2 pathway and downstream targets (Fu et al., 2015a, Fu et al., 2015b) it is possible that the altering metabolites may affect ovarian follicular cells directly.

The transition period may alter many facets from circulating metabolites, lipid mobilization, and the hepatic and granulosa cell transcriptomes. Therefore, the overarching goal of this doctoral research was to investigate the link between metabolites, body condition, the liver, and the ovarian environment, in addition to the ERK1/2 signaling pathway in the ovary to better understand mechanisms affecting reproductive longevity.

1.2HYPOTHESES

To address the issues associated with poor reproductive performance in dairy cows with respect to metabolic changes and signaling pathways required for ovulation, we put forth the following hypotheses.

Hypothesis 1: The profiles of metabolic indicators and oxidative stress markers in heifers will favour reproductive success in the period leading up to planned time of breeding, while those profiles in lactating dairy cows will hinder reproductive success. We speculate that the transition period becomes more difficult to overcome by advancing parity. Therefore, the stress caused by transition period will be reflected by drastic metabolic changes requiring more time to stabilize.

Hypothesis 2: Excess loss of body condition through the transition period leading up to the planned time of breeding will impair proper hepatic function, alter metabolite concentrations, all of which would impede development of a competent dominant ovarian follicle.

Hypothesis 3: Signaling pathways required for ovulation remain poorly defined in cattle by means of *in vivo* approach. We speculate that ERK1/2 is indispensable to ovulation in cattle and in its absence, both the gene expression profiles of granulosa and theca cells in the pre-ovulatory follicle will be altered to prevent ovulation.

Overall, the results of the proposed studies will contribute to further our understanding of metabolic basis on ovarian dysfunctions in dairy cows.

1.3 OBJECTIVES

Objective 1: To establish a profile for the metabolic indicators and oxidative stress markers in circulation of dairy heifers and lactating dairy cows leading up to planned time of breeding. (Chapter III)

Objective 2: To study the molecular phenotype of the hepatic tissue and dominant follicle in lactating dairy cow losing moderate vs severe degrees of body condition. (Chapter IV)

Objective 3: To examine the role of extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway in bovine ovulating follicles. (Chapter V)

CHAPTER II
LITERATURE REVIEW

2.1 CANADIAN DAIRY INDUSTRY

Innovation and perseverance perfectly describe the continued success of the Canadian dairy industry on the world stage.

2.1.1 THE CANADIAN DAIRY COW

As of 2017, there are 945 000 milking dairy cows distributed over 10 951 farms shipping milk across Canada – the majority of the farms being located in Ontario and Quebec (Centre, 2018a). The overall size (Figure 2.1A) and barn system (Figure 2.1B) vary greatly across the country. Western-based farms are less in number, but with a greater number of animals housed in free-stall settings, while there is a larger number of small tie-stall farms based in Central Canada (Ontario and Quebec).

The average milk yield per lactation, conformation traits and genetic potential of the Canadian dairy cow are some of the best in the world. Research with respect to nutrition, calving practices (Villettaz Robichaud et al., 2016), cow comfort/welfare (Chapinal et al., 2013, Solano et al., 2016), advancements in technologies for estrous detection (Michaelis et al., 2013, Silper et al., 2015) has helped elucidate some of the key factors affecting performance of the dairy cow.

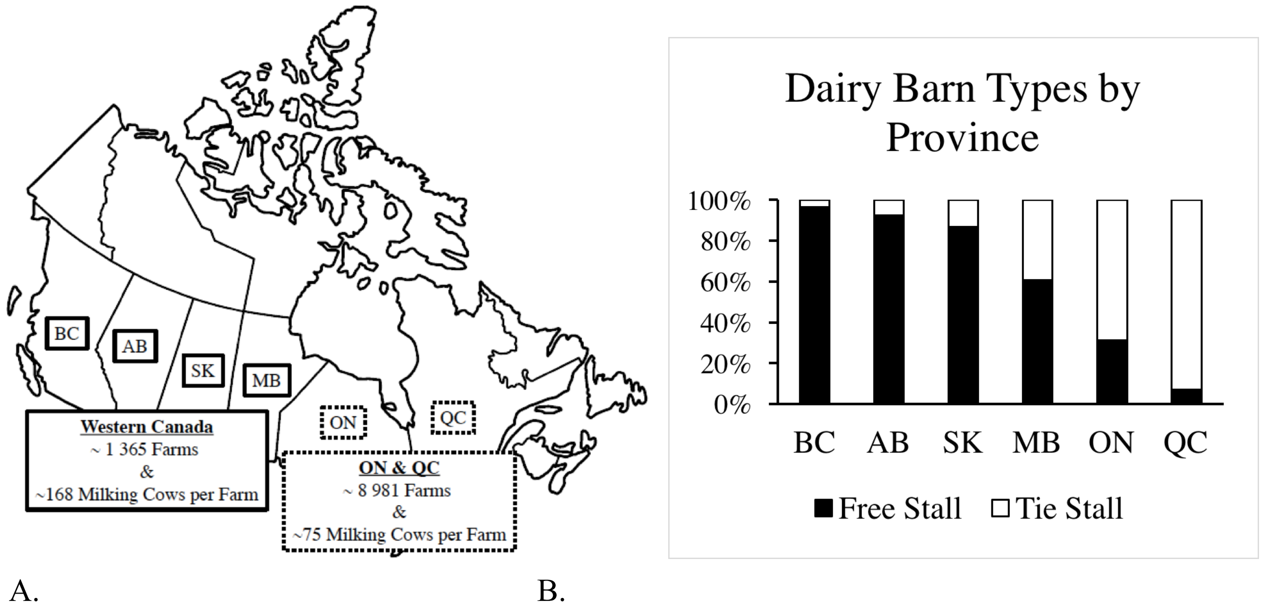
Production performance within a herd will determine whether or not a cow will remain in the herd. The Lifetime Profitability Index (LPI) is an indicator of the value of a specific cow, which is calculated from three components including production traits, durability traits, and health and fertility traits. A producer can use the LPI to make decisions with respect to genetic progress by breeding only superior animals, while culling cows that are not profitable for the business (Network, 2014).

2.1.2 LEAVING TOO SOON

The average Canadian cow becomes profitable to the producer at the onset of her third lactation (Pellerin et al., 2014). The 2017 annual report from Valacta indicated that only about 40% of cows enter their third or greater lactation (Quebec-Atlantic)(Valacta, 2018). However, the Canadian Holstein cow has the potential to live for 9.1 years, which translates to approximately 6 lactations, yet only 2.0% of Canadian dairy cows are culled due to old age (Van Doormal, 2009, Centre, 2018b). Moreover, the cost of raising a replacement heifer in Canada is anywhere from CAN$2500 to CAN$3500, a concerning value for the producers since longevity appears to be a compromised trait (Roy, 2012).

Currently, the top four reasons a cow is subject to involuntary culling from the herd include: reproduction (15.99%), mastitis/ high somatic cell count (11.68%), feet and leg problems (7.14%), and injuries (2.73%)(Valacta, 2017). Unfortunately, from those values we are unable to further establish the proportions of cows leaving due to infertility or sub-fertility in relation to ovarian and/or uterine pathology. Nonetheless, poor reproductive performance is a determining factor resulting in a decline in dairy cattle longevity (Sewalem et al., 2008, Adamczyk et al., 2016). Therefore, understanding the mechanistic basis of infertility in the dairy cow is crucial to achieve sustainable dairy production.

Figure 2.1 A. Distribution of cows and farms B. Distribution of barn types (Free-Stall or Tie-Stall) by Province. Adapted from: Canadian Dairy Information Center (Centre, 2017)



2.2 BOVINE REPRODUCTION

Milk production is dependent on reproductive success, which is a result of interactions among multiple organs. The hypothalamus, the pituitary, and the uterus are dependent on signaling between one another. The ovary is a highly complex organ houses a finite number of oocytes. Follicles are composed of granulosa cells, theca cells, and follicular fluid all of which are part of the support network involved in the release of a fertilizable oocyte (reviewed by (Fortune, 1994, Kanitz, 2003). Moreover, a series of signaling cascades are responsible for the transduction of messages between numerous organs and cells through endocrine, paracrine, and autocrine communication (Richards and Pangas, 2010).

2.2.1 THE HYPOTHALAMUS, PITUITARY, OVARY & UTERUS

Signaling cascades and hormone mediators are responsible for various physiological fluctuations that occur throughout the estrous cycle. Overall, these cascades of events are dependent on the communication between the hypothalamus, pituitary, uterus, and ovary. The onset of drastic changes in communication takes place at puberty, which in cattle ranges from 6 to 8 months of age with animals weighing 200 to 250 Kg (Day et al., 1987, Forde et al., 2011). The period leading up to puberty is characterized by a decrease in the negative feedback of estradiol on gonadotropin-releasing hormone (GnRH) secretions, beginning approximately 50 days prior to puberty, where luteinizing hormone (LH) pulses will grow in amplitude until an eventual LH surge is induced leading to the first ovulation (Gonzalez-Padilla et al., 1975, Day and Anderson, 1998).

The ovulating follicle is grown from a small cohort of antral follicles recruited for growth, where most will become atretic and one will ovulate. There is an increase in pulse amplitude and frequency of the GnRH from the hypothalamus, which in turn regulates and increases follicle-stimulating hormone (FSH) and LH pulses from the anterior pituitary (Schally et al., 1971). This comes as a result of changes in the feedback sensitivity in the ovary to estradiol (E_4) levels. Estradiol concentrations in the follicular fluid increase in the dominant follicle as it grows in size. The dominant follicle also acquires luteinizing hormone receptors on the granulosa and theca cells, allowing it to respond to the LH surge, which in turn orchestrate the onset of genes required for ovulation (revisited later) at which point the follicle will rupture and form the corpus luteum (CL) (Peters and Lamming, 1983, Fortune et al., 2001). This endocrine structure then goes on to produce progesterone (P_4), which will be maintained if the expulsed oocyte becomes fertilized and pregnancy is established. If fertilization does not take place, prostaglandin (PGF2α) produced in the uterus will initiate luteolysis (regression of the CL)(Lamothe et al., 1977). The actions of the uterus by means of secretion of PGF2α is mandatory for the regression process, and thus, a new estrous cycle will succeed (Loeb, 1927, Wiltbank and Casida, 1956, Peters and Lamming, 1983). Therefore, it is evident that communication between the hypothalamus, anterior pituitary, ovary (follicle and CL) and uterus are vital for successful ovulation to occur (Figure 2.2).

Figure 2.2: Communication between the hypothalamus, pituitary, ovary and the uterus (adapted from (Animal Health and Merk, 2018)).

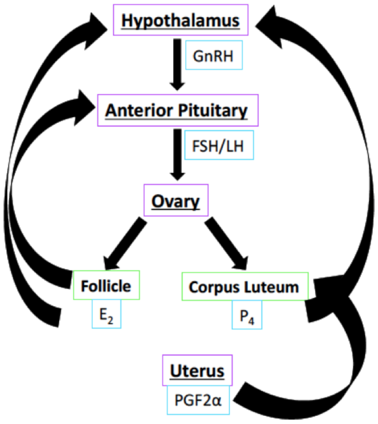
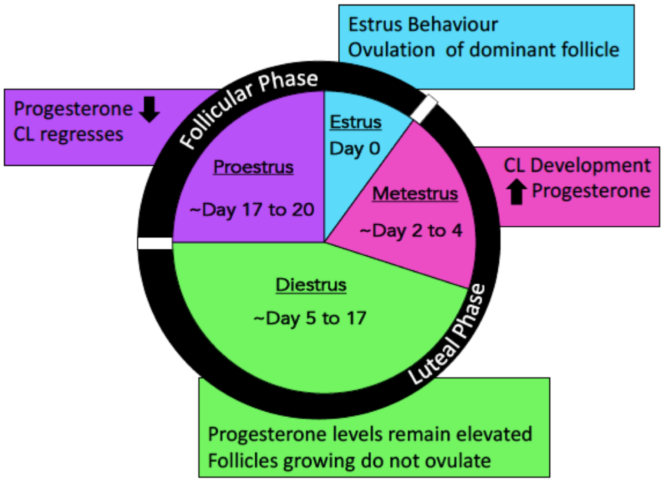


Figure 2.3: Estrous cycle divided into a follicular and luteal phase (adapted from (Wisconsin, 1998)).

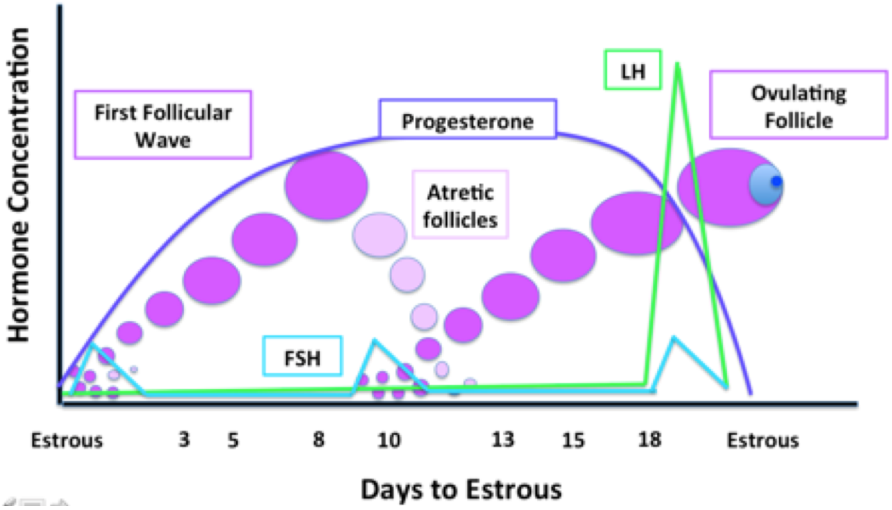


2.2.2 FOLLICLE RECRUITMENT, SELECTION, DEVIATION & DOMINANCE

On average the estrous cycle of cattle is between 18 and 24 days and can be divided into 2 phases: luteal phase (14-18 days) and follicular phase (4-6 days). Estrus can be visualized by behavioural changes in the animal brought on by elevated levels of estradiol. The dominant follicle will grow during proestrus and ovulate at the end of the estrus phase (W, 1961, Hansel and Seifart, 1967, Niswender et al., 2000, Forde et al., 2011) (Figure 2.3).

Follicle growth is divided into a gonadotropin independent stage (preantral follicle growth) and the gonadotropin dependent stage (antral follicle growth). Gonadotropin independent stage requires the actions of growth factors including transforming growth factor β (TGFβ) and insulin-like growth factors (IGFs). Next, the gonadotropin dependent stage requires GnRH, FSH, and LH signaling. Paracrine and autocrine signaling between the oocyte, granulosa cells, and theca cells is required for the growth and development of follicles. Taken together follicle growth requires approximately 3 months from the primordial stage to early antral stage thus, being recruited into the follicular wave. Growth through this gonadotropin independent phase is influenced by nutritional and health status of the cow (Reviewed by (Webb et al., 2004)). A small pool of follicles is recruited into the follicular wave in response to an increase in circulating FSH concentrations, whereby the cohort of follicles grows greater than 5 mm in diameter in response to an FSH peak (Ginther et al., 1996). The general consensus for cattle is that there are 2 to 3 follicular waves per estrous cycle. In each wave, one follicle will be selected for dominance, while the others become subordinate follicles and regress. Although dominance is acquired by one follicle per wave, only the dominant follicle from the last wave will ovulate (Sirois and Fortune, 1988, Ginther et al., 1989) (Figure 2.4).

Figure 2.4: Diagram of a two-wave estrous cycle in cattle (adapted from (Javic, 2003)).



When a new wave emerges, follicles undergo changes in cell morphology, lipid metabolism, cell death, cell proliferation, and immune response (Liu et al., 2009). The term deviation is used to describe the time when there is the greatest change in growth rate, where one follicle continues growth, while the others cease to grow. In the Holstein cow, deviation takes place when the diameter of the largest follicle reaches approximately 8 to 9 mm (Ginther et al., 1996, Ginther et al., 1997, Beg et al., 2001). At the time of deviation, the dominant follicle differs from subordinate follicles with respect to the gene expression program in somatic cells (granulosa and theca cells) and growth factor, hormone, and metabolites in the follicular microenvironment (Beg et al., 2001).

Traditional identifiers of dominance include follicle diameter, indicators of granulosa cell proliferation, acquisition of LH receptors and estradiol production (Beg et al., 2001, Fortune et al., 2001). Follicular growth is also associated with changes in growth factor secretion, which include, but not limited to the production of insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), inhibin, and activin by granulosa cells and epidermal growth factor (EGF) by theca cells (Driancourt, 1991). The rise in estradiol production is initiated by increased sensitivity of the dominant follicle to lower levels of circulating FSH. It has been proposed that the IGF system plays a critical role in estradiol synthesis in concert with gonadotropin stimulation (Spicer and Echternkamp, 1995). The bioavailability of IGFs is dependent on the presence of IGF-binding proteins most notably, IGFBP-2, -4 and -5. The dominant follicle expresses IGFBP-4 proteases, which degrade IGFBP-4, IFGBP-2 and IGFBP-5 and thus, increasing the availability of free IGF (Spicer et al., 2002). The subordinate follicles do not experience these changes, instead there is no increase in free IGF, estradiol, and thus, the growth rate is plateaued with insufficient FSH to sustain viability leading to atresia (Fortune et al., 2001).

Essentially, the synthesis of estradiol is dependent on steroidogenic enzymes. The LH pulse leads to the production of androgens in theca cells. The Cytochrome P450, Family 17, Subfamily A, Polypeptide 1 (CYP17A1) enzyme converts progesterone (P4) in the theca cells to androstenedione (A4). Next, A4 is translocated to granulosa cells, where it is converted to estradiol by the enzymatic activity of aromatase (CYP19A1). The large concentration of estrogen released into the growing pool of follicular fluid followed by entry into circulation initiates a negative feedback to the anterior pituitary, which inhibits FSH release (as reviewed by (Forde et al., 2011, Scaramuzzi et al., 2011)).

Overall, a number of genes have been identified for a role in follicular growth from the onset of wave emergence to the establishment of dominance. These genes are primarily induced by gonadotropin signaling. See Table 2.1.

Table 2.1: Genes involved in growth, atresia, and dominance

Comparison	Cell types	Transcript abundance	References
Largest vs. Second Largest Follicle	**Granulosa & Theca Cells (Merged for analysis)** Sample details: ovaries from pregnant cows collected from abattoir	Largest: ↑ *AMH, PLA2G1B, SCD, & TRB2* (cell growth & survivability), ↑*GPX3* (anti-apoptotic) & ↑*PIGF* (angiogenesis) Second Largest: ↑ *CCL2, SELP,* & SPP1 (immune response), ↑ *GADD45A* (response to DNA damage), & ↑ *PLAUR, TIMP1* (Tissue remodeling)	(Hayashi et al., 2010)
Dominant vs. Subordinate Follicle	**Theca Cells** Sample details: ovariectomy on 2.5-3.5 days of follicular wave	Subordinate: ↑ *FKHRL1* (apoptosis), *SRF* (Cell survival), *CEBPB* (luteinization) & Midnolin (mRNA transport in cells)	(Zielak et al., 2008)
Small VS Large Follicles (Antral)	**Granulosa Cells** Sample details: ovaries from non-pregnant cycling cows collected from abattoir	Small Antral: ↑*KIT* (Follicle survival) & ↑*IHH* & *MEST* (immature phenotype maintenance) →compared to large follicles Large Antral: ↑*IL4R* & *IL6R* (Inflammation Signaling), ↑CITED1 & CITED2 (transcription factors), ↑VCAM1 & TNFAIP6(matrix adhesion), ↑*PLAT, ADAM9, TIMP2, & SERPINA5* → compared to small follicles	(Hatzirodos et al., 2014b)
Small VS Large Follicles (Antral)	**Theca Cells** Sample details: ovaries from non-pregnant cycling cows collected from abattoir	Large Antral:↓ *WNT2B* & ↑*FRZB* (Wnt Signaling), ↑*AOX1* (oxidative stress), ↑*LTBP1* (extracellular matrix proliferation) → compared to Small antral follicles	(Hatzirodos et al., 2014a)
Small VS Large Follicles (Antral)	**Theca & Granulosa Cells** Sample details: ovaries from non-pregnant cycling cows collected from abattoir	Theca: *FBLN5, OGN,* & *RAMP2* (extracellular matrix protein genes) found uniquely in Large and Small Theca Cells, but not Granulosa Cells.	(Hatzirodos et al., 2015)
Growing, Plateau & Atretic Dominant Follicles	**Granulosa Cells** Sample details: ovaries from cows collected from abattoir, with unknown reproductive status	Plateau:↑ *TYRO3* & ↓*JAM2* (differentiation) → compared to Growing Atretic: ↑*VNN1* (oxidative stress) & *ANGPT2* (angiogenesis)→ compared to Plateau Atretic:↓ *BUB1, CCNB1, CKS2, TUBB6,* & *PRC1* (cell proliferation)→ compared to Growing	(Girard et al., 2015a)

2.2.3 THE OVULATORY FOLLICLE

The dominant follicle of the last wave ovulates in response to the pre-ovulatory LH surge, which alters the gene expression pattern of the oocyte and the granulosa and theca cells. They include: 1) resumption of meiosis 2) cumulus cell expansion 3) luteinization which involves a shift from estrogen to progesterone synthesis and 4) follicle rupture and repair (Richards and Pangas, 2010). LH mediates downstream signaling pathways through luteinizing hormone receptor (LHCGR). For example, LH exerts its effect through the second messenger cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway, phosphatidylinositide 3-kinase (PI3K/Akt), and extracellular-regulated kinase 1 and 2 (ERK1/2) pathway in bovine granulosa and theca cells as shown by un vitro experiments (Tajima et al., 2005, Fukuda et al., 2009).

In cattle, ovulation occurs approximately 26-30h post-LH surge. The bovine model offers advantages of collecting pure populations of granulosa cells from an ovulating follicle at precise time points after the LH surge. Therefore, studies have been conducted examining early LH-responsive genes (~6h post-LH) to late LH-responsive genes (~22h post-LH)(Ndiaye et al., 2005, Gilbert et al., 2011). Similar to the mice, the following genes have been identified as LH responsive in cattle (Table 2.2).

Table 2.2: Well-established LH-regulated genes in the bovine pre-ovulatory follicle

Gene	Role	Cell Type	Time of increase	Reference
Early Growth Response 1 (*EGR1*)	Transcription factor regulating downstream genes (Ex: *PTGS2*)	Granulosa & Theca	6h post Hcg	(Sayasith et al., 2006)
Prostaglandin-endoperoxide synthase 2 (*PTGS2*)	Follicle rupture & cumulus cell expansion	Granulosa	21h post-LH	(Sayasith et al., 2006)
Amphiregulin (*AREG*)	Cumulus Cell Expansion	Granulosa	6h post Hcg	(Sayasith et al., 2013a)
Epiregulin (*EREG*)	Cumulus Cell Expansion	Granulosa	6h post Hcg	(Sayasith et al., 2013a)
Progesterone Receptor (*PGR*)	Progesterone Regulation	Granulosa & Theca	4h post-LH & 24 post-LH	(Jo et al., 2002)
Tumor necrosis factor alpha induced protein 6 (*TNFAIP6*)	Cumulus Cell Expansion	Granulosa & Theca	6h to 24h post-Hcg	(Sayasith et al., 2008)
Tissue inhibitor of metalloproteinase 1 (*TIMP1*)	Extracellular matrix remodeling	Whole Follicular Tissue	8h & 48h post-GnRH	(Smith et al., 1996)
Tissue inhibitor of metalloproteinase 2 (*TIMP2*)	Extracellular matrix remodeling	Whole Follicular Tissue	8h & 48h post-GnRH	(Smith et al., 1996)
A disintegrin and metalloproteinase with thrombospondin motif 1 (*ADAMTS1*)	Extracellular matrix remodeling (downstream target of PGR)	Granulosa	6h to 24h post-Hcg	(Sayasith et al., 2013b)
CXC Receptor 4 (*CXCR4*)	Angiogenesis & regulation of other genes required for ovulation.	Granulosa & Theca	6 to 24h post Hcg	(Sayasith and Sirois, 2014)
Regulator of G-Protein Signaling Protein-2 (*RGS2*)	Unclear. Potential link with PGR signaling.	Granulosa	6 to 24h post-Hcg	(Sayasith et al., 2014)

Other recently discovered genes involved in pre-ovulatory development include, Vanin-2 (VNN2) (increased from 12h to 24h post –Hcg) (Sayasith et al., 2013c), involved in inflammatory reactions and a disintegrin and metalloprotease-17 (ADAM17) (increased from 6 to 12h)(Sayasith and Sirois, 2015), involved in extracellular remodeling. Large scale gene expression analysis techniques such as microarrays have allowed for the identification of a greater number of LH-regulated genes in bovine granulosa and theca cells. A recent study by Christenson et al (2013) used microarray to determine changes in gene abundance in granulosa and theca cells at 21h post-GnRH, where out of approximately 11 000 genes, 226 genes in theca cells, 2741 genes in cumulus granulosa cells and 2417 genes in mural granulosa cells were differentially expressed in response to the LH (Christenson et al., 2013).

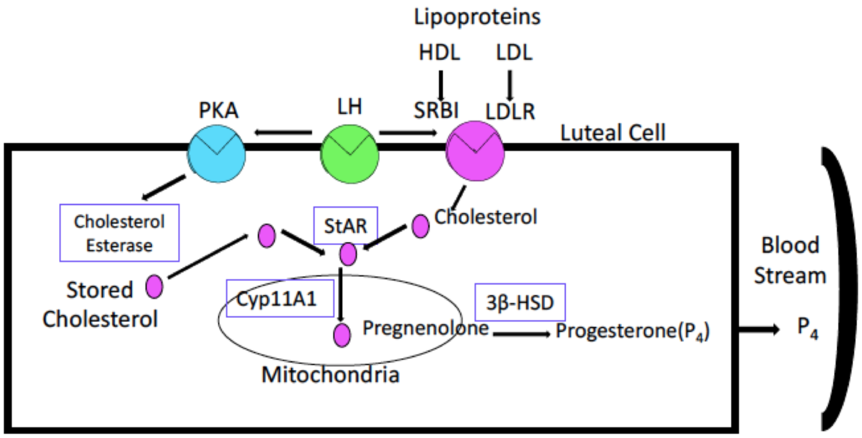
2.2.4 THE CORPUS LUTEUM

The corpus luteum (CL) is a transient, but complex endocrine gland consisting of luteal cells, endothelial cells, fibroblast cells, and immune cells (Niswender et al., 2000, Stocco et al., 2007). The CL is crucial for the maintenance of pregnancy by secreting progesterone (O'Shea et al., 1989). During the estrous cycle, the CL undergoes an initial stage of development and functions through the actions of luteotropic factors followed by regression onset by luteolytic factors (Schams and Berisha, 2004).

Following ovulation, the remnants of the ruptured follicle undergoes tissue remodeling involving angiogenesis, the development of a new vascular network, intermingling of theca cells among granulosa cells and the reprogramming of granulosa and theca cells into progesterone (P4) producing large and small luteal cells, respectively. These changes are a result of the actions of the angiopoietin (ANPT) family and growth factors including, fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF) family and the insulin-like growth factor (IGF) family (Schams and Berisha, 2004).

The small and large luteal cells are predominantly responsible for the production of progesterone during the functional phase of the CL through endocrine regulators, where 80% of the total progesterone production comes from the large luteal cells (Niswender et al., 2000). During CL development, there is an increase in *LHCGR* expression located on the steroidogenic (large and small) luteal cells, with the highest detection during the mid-luteal phase (Yoshioka et al., 2013). The CL synthesizes progesterone from the initial substrate of cholesterol through various cellular processes. Cholesterol is synthesized from the liver and transported to luteal cells through circulation. Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) are the lipoproteins that are used for progesterone synthesis, while ruminant luteal cells predominantly use the latter. However, during periods of decreased lipid metabolism, luteal cells can synthesize cholesterol de novo from acetate [reviewed by (Niswender et al., 2000, Schams and Berisha, 2004)]. Entry of LDL and HDL into the cell occurs through binding to receptors such as LDL receptor (Argov et al., 2004, Argov and Sklan, 2004) and scavenger receptor class B member 1 (SCARB1)(Acton et al., 1996), respectively. Next, transport of cholesterol from the outer to the inner mitochondrial membrane is strictly dependent on the presence of steroidogenic acute regulatory protein (STAR), which is activated through LH stimulation (Rekawiecki et al., 2005). Once inside the mitochondria, the cholesterol side-chain cleavage enzyme (CYP11A1) converts cholesterol to pregnenolone, which is further transported to the smooth endoplasmic reticulum, where it is converted to P4 via the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD). The P4 hormone is subsequently diffused through the luteal cells and enters into circulation until the late stages of the luteal phase when the CL regresses (in the scenario where fertilization does not take place), which is met with a decrease of P4 synthesis and secretion (Figure 2.5) (reviewed in (Niswender et al., 2000)).

Figure 2.5: Steroidogenic activity in luteal cells. Adapted from (Niswender et al., 2000).



Luteolysis is divided into a functional phase, characterized by the decline of P4 initiated by an increase in endometrial PGF2α and a structural phase, designated by cell death (Silvia et al., 1991). A decrease in blood flow through the ruminant CL is caused by the uterine release of PGF2α (Ford and Chenault, 1981), which results in structural luteolysis in the form of vascular remodeling as described earlier through changes in growth factor and angiopoietin expression. Furthermore, members of the immune system are involved in regression of the CL. Cytokines such as tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) are up regulated in the CL and have been shown to stimulate the expression of the chemokine monocyte chemoattractant protein-1, which assists in the overall infiltration of macrophages and lymphocytes into the CL via the capillary network (Cavicchio et al., 2002, Townson et al., 2002, Schams and Berisha, 2004). The presence of PGF2α in the bovine CL (Pate, 1988) is suggested to increase in the presence of vasoactive peptides, localized on large luteal cells, such as endothelin-, known to have inhibitory effects on progesterone (Girsh et al., 1996, Miyamoto et al., 1997). Moreover, changes in steroidogenic activity occur when there is a decrease in expression of *LHCGR* and *CYP11A1* as well as *SCARB1* and *LDLR*, further decreasing the production of progesterone and thus, inducing functional luteolysis (Horihata et al., 2016). Overall, the CL is a complex endocrine gland indispensable for proper establishment of pregnancy through proper signaling and gene expression. A cow's ability to establish and maintain pregnancy is a key factor in her on-farm longevity.

2.2.5 FERTILITY IN NULIIPAROUS HEIFERS VS. LACTATING DAIRY COWS

In dairy cattle, successful ovulation is dependent upon a follicle's acquisition and maintenance of dominance, followed by the ability to of the dominant follicle to respond to the LH surge and ovulate. Granulosa cells, theca cells, and follicular fluid are key players in the release of a fertilizable oocyte (reviewed by (Fortune, 1994, Kanitz, 2003). Although the process of ovulation is the same amongst nulliparous heifers and lactating cows, the former group is considered more fertile than the latter (Kuhn et al., 2006). More specifically, first and second insemination pregnancy rates decrease with age/parity: heifers, 1st parity, 2nd parity, and 3rd/4th parity cows had pregnancy rates of 84.3%, 51.5%, 31.4%, and 19.5%, respectively(Balendran, 2008). Elsewhere, dairy cows entering their first parity have a higher conception rate in response to first breeding at 34% compared to 24% for cows entering their 6th parity and beyond (Norman et al., 2009). These drastic changes between heifers and lactating have been linked to the differences in the follicular microenvironment of the dominant follicle (Bender et al., 2010, Sanchez et al., 2014). It has been shown that there are staggering differences between the metabolic environment of the follicular fluid, where early-lactating cows have lower levels of glucose and IGF1, and higher concentrations of saturated fatty acids, bile acids, cholesterol, and beta-hydroxybutric acid compared to nulliparous heifers (Bender et al., 2010, Sanchez et al., 2014). Together, these hormone and metabolite imbalances are expected to have detrimental effects on oocyte development and overall fertility. To understand the predisposition of lactating cows to sub-fertility in relation to metabolism, it is important to take into consideration the impact of parturition on subsequent reproduction as reviewed in the next section.

2.3 POST-PARTUM FERTILITY IN THE DAIRY COW

The successful breeding of dairy cows resulting in conception is dependent on fertilization of a healthy oocyte and proper implantation. Therefore, optimal management of the dairy cow during the transition period is crucial to increase the likelihood of a successful pregnancy. The following section will address physiological and pathophysiological changes in the cow during the transition period that can affect ovarian health.

2.3.1 NEGATIVE ENERGY BALANCE, METABOLIC STRESS, & ILLNESS

The transition period is defined as three weeks pre-partum to three weeks post-partum, which encompasses the time when a cow is likely to be in suboptimal health and is thus, predisposed to illness, which can further translate to reduced performance exhibited by decreased milk yield and infertility (Grummer, 1995, Drackley, 1999). It is invaluable to understand and meet the specific needs of the animal during the transition period with respect to nutrition (Diskin et al., 2003, Leroy et al., 2008, Cardoso et al., 2013, Drackley and Cardoso, 2014) and cow comfort (Campler et al., 2015, Villettaz Robichaud et al., 2016). Understanding and minimizing the metabolic and oxidative stress that may result from a poor transition period have been key areas of interest in order to address reproductive issues following the voluntary waiting period (60 days in milk) (Leroy et al., 2008, Scaramuzzi et al., 2010, Trevisi et al., 2012, Turk et al., 2013, Folnozic et al., 2015). The concept of metabolic stress has been defined as "the portion of negative energy balance that cannot be sustained by the cow" (Westall et al., 2006).

In Dr. Drackley's famous 1999 review, he elegantly described that at the onset of lactation, the energy demand for milk production is not met with adequate feed intake resulting in a state of negative energy balance (NEB). In early lactation, a cow will mobilize her own adipose tissue and muscle proteins, the latter being limited, as a source of energy. Fat mobilization is characterized by the breakdown of triglycerides (TGs), which enter circulation as non-esterified fatty acids (NEFA) and are transported to the liver in order to generate energy by β-oxidation. The metabolic end product of β-oxidation, acetyl coA can be oxidized to ketone bodies in the form of β-hydroxybutyric acid (BHBA), which can be used by other cells as energy. However, the liver has a limited capacity to metabolize NEFAs and the excess NEFAs are re-esterified into TGs in the liver and stored or exported as very low-density lipoproteins (VLDL). Ultimately, when the capacity to synthesize TGs exceeds the rate of TG removal through VLDL, a cow can suffer from metabolic diseases including ketosis and fatty liver (Drackley, 1999, Adewuyi et al., 2005).

Ketosis and fatty liver are among other post-partum diseases including milk fever, mastitis, metritis, and retained placenta that are key markers of animals experiencing poor adaptation to the onset of lactation(LeBlanc, 2010). Many diseases are initiated prior to calving (example: related to issues with dry period management) and can have negative effects on animal performance into early-lactation (Shin et al., 2015). They lead to increased predisposition to other illnesses due to decreased feed intake and a weakened immune system (Zerbe et al., 2000, Suthar et al., 2013). Moreover, ketosis and fatty liver can lead to delayed and poor reproductive performance (Shin et al., 2015). Table 2.3 provides a typical representation of metabolic markers at different stages of the transition period, persisting into later lactation.

Table 2.3: Typical blood metabolite profile in dairy cows (Matoba et al., 2012)[1] &(Leroy et al., 2004b)[2].

Traditional Markers	**Before Calving**	**At Calving**	**After Calving**		
	Day -7	*Day 0*	*Day 7*	*Day 21*	*Day 50*
NEFA	0.2mmol/L[1]	0.7mmol/L[1]	0.6mmol/L[1]	0.8mmol/L[1]	0.3mmol/L[1]
BHBA	0.5mmol/L[1]	0.5mmol/L[1]	1.0mmol/L[1]	1.0mmol/L[1]	0.5mmol/L[1]
Glucose	3.3mmol/L[1]	4mmol/L[1]	2.8mmol/L[1]	2.9mmol/L[1]	3.2mmol/L[1]
Triglycerides	20mg/dl[2]	10mg/dl[2]	10mg/dl[2]	11mg/dl[2]	8mg/dl[2]

2.3.2 SHE GIVES US HINTS: BODY CONDITION SCORE (BCS)

It has been well-established that the dry matter intake (DMI) typically declines as the cow approaches parturition, where the decrease can easily reach 30 to 35% during the last 3 weeks prior to calving (Marquardt et al., 1977, Grummer, 1995). The body condition score (BCS) is a subjective visual appraisal of body fat reserves based on the pelvic area and the rump. In North America, we use a scale of BCS that has been developed including 1 to 5 units, where a score of 1 is given when the individual is considered emaciated, a score of 5 is given when the individual is considered obese, and 3 is assigned to a lactating cow in optimal condition (Wildman et al., 1982, Edmonson et al., 1989). Producers can monitor cattle weight and establish proper nutrition and or/exercise regimens for the stage of production by monitoring BCS. Guidelines have been put forth for different stages of lactation, whereby at parturition the BCS target is 3.0 to 3.5, which represents a wide range and may be management system specific (Drackley and Cardoso, 2014, Puppel and Kuczynska, 2016). Nonetheless, the most important factor is the change in body condition during the transition period to the planned time of breeding (approximately 60 days in milk). This change should be as minimal as possible (Heinrichs et al., 2016). Excess body condition loss, would suggest severe lipid mobilization and a state of drastic negative energy balance, characterized by elevated levels of blood NEFAs and BHBA (Luttgenau et al., 2016, Barletta et al., 2017). A meta-analysis showed that loss of >1.0 unit of BCS in early lactation should be avoided to prevent a negative impact on subsequent reproductive success (Lopez-Gatius et al., 2003). Nonetheless, most recently, a group in Poland reported that cows experiencing >0.49 unit drop of BCS in early lactation (56 days in milk) had higher levels of serum NEFA and BHBA, with lower concentrations of IGF1, an important component in the reproductive axis (Stefańska et al., 2016). This same group showed evidence that a BCS of >3.5 at time of parturition increased the days open and decreased the conception rate of the first insemination (Stefańska et al., 2016). Therefore, BCS is being implemented as a management tool to improve reproductive success as a negative correlation between BCS loss and fertility has been observed (Pryce et al., 2001, Lopez-Gatius et al., 2003, Santos et al., 2009, Carvalho et al., 2014a, Shin et al., 2015).

2.3.3 BLOOD BIOMARKERS OF METABOLIC DISEASES

NEFA and BHBA are the most commonly used parameters to determine health status of a cow with respect to metabolic stress (Ospina et al., 2010a). During the pre-partum period, NEFA and BHBA levels should not exceed 0.3-0.5 mM and 0.6-0.8 mM, respectively. During the post-partum period, NEFA and BHBA levels should not exceed 0.7-1.0 mM and 1.0 mM, respectively. Elevated levels of both markers suggest issues in physiological adaptation during the transition period often marked by ketosis and fatty liver. Subclinical ketosis is characterized by levels of 1.0 to 1.4 mM BHBA and (higher values would lead to clinical signs of the disease)(Suthar et al., 2013). A cow suffering from subclinical or clinical ketosis in early lactation, is more precisely suffering from type II ketosis. This form of ketosis is experienced by cows who at the onset of lactation are often over-conditioned (high BCS), experience an infiltration of triglycerides into the liver (fatty liver), and high levels of serum NEFAs and of course the trademark ketone, BHBA. To prevent type II ketosis, it is important to feed a ration favouring and maintaining a BCS below 3.5 units during the dry period and minimizing BCS loss at the onset of lactation (Puppel and Kuczynska, 2016)

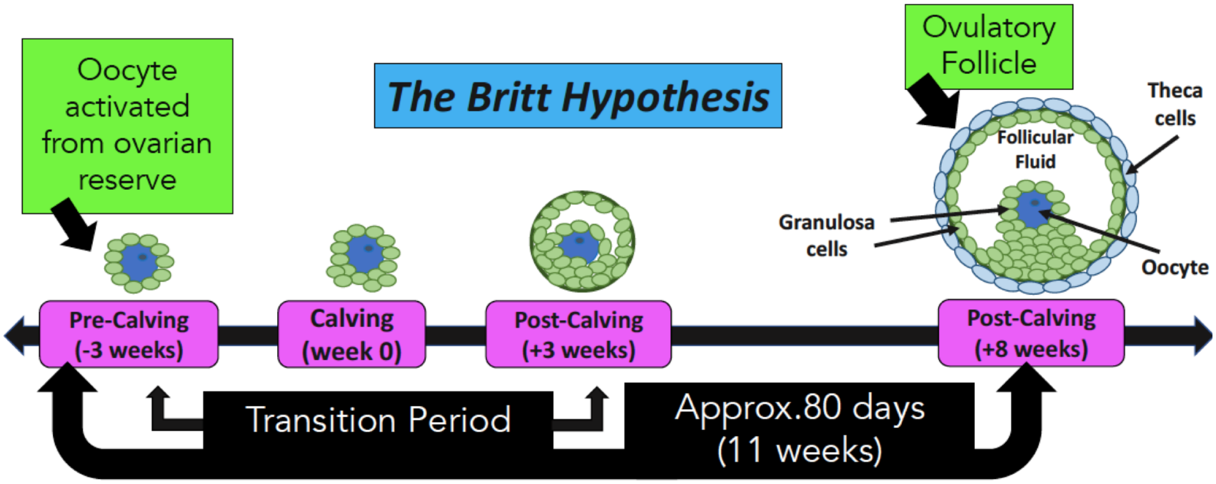
On another note, by separating cows into BHBA positive (+) and BHBA negative (–) groups, Kessel et al, (2008) observed that there was no difference in milk yield, number of services per conception and days open. The trial by Kessel et al, (2008), took place from 2 weeks pre-partum to 14 weeks post-partum and a cow was placed in the BHBA+ if she tested positive for a BHBA level greater than 1 mM, at least once during the sampling trial (Kessel et al., 2008). One positive test greater than 1mM may not have been sufficient to characterize a cow as BHBA+. Elsewhere, large-scale studies have revealed that cows with increased BHBA from 1 week pre-partum to 1 week post-partum experienced decreased pregnancy at first artificial insemination (AI) (Chapinal et al., 2012). Moreover, high levels of BHBA inhibits gluconeogenesis and can replace glucose as an energy source, which may be undesirable in the case of fertility (Zarrin et al., 2017). Moreover, molecular studies have in fact investigated the impact of metabolites including BHBA on somatic cells of the ovary. A recent study compared granulosa cells from the dominant follicle at 60 days post-partum collected from cows experiencing mild BHBA (n=12, 645+/-94 µM) or severe BHBA (n=12, 1102+/-174µM) on day of collection (Girard et al., 2015b). Surprisingly, cow health during the transition period was not monitored and cows from 10 farms with different management and dietary regimens were used (anywhere from 1 to 5 cows per farm). Nonetheless, it was shown that gene expression was altered in granulosa cells 60 days post-partum between the two groups, whereby genes regulated by vitamin A and vitamins D were down-regulated in the severe BHBA group. In addition, genes associated with proliferation (tumor-associated calcium signal transducer 2 (*TACSTD2*)) and fatty acid metabolism (*VNN2*) were down-regulated. The authors suggest a general increase in vitamin A supplementation required for dairy cows (Girard et al., 2015b).

Important to consider it that is common practice in today's North American dairy industry is to provide a cow with a voluntary waiting period (VWP) of 60 days post-partum, suggesting that the planned time of breeding begins at 60 days post-partum (Stangaferro et al., 2018). Therefore, negative energy balance that persists from the transition period into the planned time of breeding may have severe negative impacts on reproductive success with high levels of circulating NEFA and BHBA.

2.3.4 BLOOD-DERIVED METABOLITES ACTION IN THE OVARIAN FOLLICLE

Blood metabolites, such as NEFAs, BHBA, cholesterol, IGF1, triglycerides, bile acids, and glucose are reflected in the ovarian follicular fluid (Leroy et al., 2004b, Bender et al., 2010, Sanchez et al., 2014, Sanchez-Guijo et al., 2016). Therefore, dramatic changes in metabolism witnessed in circulation will be mirrored in the follicular microenvironment and may have a direct effect on follicle development and oocyte health (Jorritsma et al., 2003a, Leroy et al., 2004b, Sanchez et al., 2014). This is based on "The Britt hypothesis" (see figure 2.6) which suggests that the hormones and metabolites in circulation affect the somatic and germ cells of follicles, where it takes approximately 60-80 days for a bovine follicle from the primary stage to mature into an ovulatory follicle (Britt, 1992, Lucy et al., 2014). A VWP of 60 days post-partum would suggest that the ovulatory follicle meant for first attempt at breeding has been impacted by the metabolic and hormone profiles present during the transition period to the planned time of breeding. Therefore, an unfavourable metabolic and hormonal profile traditionally characterized by low concentrations of glucose and IGF1 and high levels of BHBA and NEFAs may hinder the development of a competent oocyte (Sanchez et al., 2014). This factor alone would be proposed to contribute to the high percentage of involuntary culling rates (15.99%) reflected by reproductive issues (Valacta, 2017).

Figure 2.6: Visual representation of " The Britt Hypothesis" (Britt, 1992).



In vitro analysis of bovine granulosa cell cultured with 1 mM of NEFA (oleic, C18:1), a concentration suggested to mimic metabolic stress, revealed a negative effect on cell proliferation, but no effect of progesterone synthesis (Jorritsma et al., 2004). This group also cultured bovine cumulus-oocyte-complexes with 1 mM of NEFAs resulted in reduced fertilization, cleavage and embryonic development (Jorritsma et al., 2004). Elsewhere, Vanholder et al (2005) established that in vitro culture of bovine granulosa cells with NEFAs specifically palmitic (C16:0) and stearic (C18:0) with a lesser extent oleic acid (concentrations ranging from 150µM to 500µM) negatively impacted bovine granulosa cell proliferation, whereby cell survival and proliferation were impaired due to increased apoptosis. Nonetheless, estradiol concentrations increased as the dosage of NEFA's increased, which can be explained by the potential of certain stages of apoptosis still involved in steroidogenesis. Altogether, the group suggested that decreased cell proliferation may explain smaller early post-partum dominant follicles (Vanholder et al., 2005). This same group also evaluated the impact of BHBA on somatic cells, where bovine granulosa and theca cells were subject to varying concentrations of BHBAs (0.5, 1.0 and 1.5 Mm BHBA) in an in vitro setting.

BHBA at all concentrations increased granulosa cell proliferation, since cells will use ketones as a source of energy. However, BHBA led to reduction of progesterone and estradiol in cultured granulosa cells, while no effect was observed in cultured theca cells (Vanholder et al., 2006).

2.3.5 CHOLESTEROL METABOLISM

Lipids are heavily involved in cattle performance during the transition period. Lipoproteins (HDL, LDL, and VLDL) are composed of triglycerides, cholesterol, phospholipids, and apoproteins (Ikonen, 2008). In circulation, lipids are transported with proteins due to their low solubility (Puppione, 1978). In cattle, it has been shown that total cholesterol (TC) concentrations in circulation varies greatly during the periparturient period. Leroy et al, (2004) showed that from 7 days pre-calving to 46 days post-calving, TC decreases until reaching a nadir at calving, followed by a gradual increase post-calving. Moreover, TC rises in both the serum and follicular fluid, where the concentration of TC in follicular fluid is 43-48% that of the serum on days 14 and 46 post-partum (Leroy et al., 2004b).

As previously mentioned, cholesterol plays a significant role in steroidogenesis as it is the precursor of steroid hormones. Lipoproteins are used as transporters of cholesterol to the theca and granulosa cells. Prior to ovulation, theca cells convert cholesterol into androstenedione upon stimulation by LH, which is further converted into estrogen in granulosa cells by the aromatase enzyme. Post-ovulation, small and large luteal cells require cholesterol for estrogen and progesterone production (reviewed by (Christenson and Devoto, 2003, van Montfoort et al., 2014)). HDL and LDL have long been identified as important lipoproteins for the synthesis of progesterone by the CL in the cow (O'Shaughnessy, 1985, O'Shaughnessy et al., 1990). However, species differences have suggested that in cattle, HDL is preferred source of cholesterol for luteal steroidogenesis (Christenson and Devoto, 2003).

Uptake of lipoproteins into steroidogenic cells is dependent on receptors including LDLR, which uptake cholesterol by means of endocytosis. Scavenger receptor class B type 1 (SCARB1), an HDL receptor, does not work by endocytosis, but rather by selective uptake. *SCARB1* expression tended to increase with diameter size of the antral follicle in cows(Argov et al., 2004). Moreover, a 7-fold increase of *SCARB1* is detected in luteinizing cells during the corpus luteum formation in cows; an increase representing enhanced steroidogenic activity (Rajapaksha et al., 1997). Mouse studies have shown that deletion of the *Scarb1* gene leads to female infertility, due to abnormal oocyte development. However, *Scarb1* deficient mice experienced a normal estrous cycle and ovulation with similar levels of circulating progesterone compared to their wildtype counterpart mice(Trigatti et al., 1999). Elsewhere, human studies revealed that cultured granulosa cells with lower SCARB1 protein abundance secreted a lower level of progesterone (Kolmakova et al., 2010). Another group showed that women undergoing in vitro fertilization treatment with lower granulosa cell *SCARB1* expression have lower estradiol levels in addition to lower levels of retrieved and fertilized oocytes (Velasco et al., 2006). In dairy cows, cholesterol metabolism is greatly affected by the transition period (Kessler et al., 2014). Profiling the blood lipoproteins in dairy cattle of different physiological stages would be helpful in providing a window into the follicular microenvironment at the planned time of breeding.

The liver is involved in cholesterol synthesis, VLDL assembly, and cholesterol transport. The rate of synthesis, assembly, and transport has been investigated through specific changes in hepatic mRNA abundance of *HMGCR* and *HMGCS1* (cholesterol synthesis), *MTTP* and *ApoB100* (VLDL assembly and secretion), *ABCA1* and *ABCG1* (cholesterol transport), *SREBF-1* and *SREBF-2* (regulatory genes of cholesterol metabolism) and *ACAT1* (esterification of cholesterol) (Schlegel et al., 2012, Gross et al., 2015). Schlegel et al (2012) has shown that in the cow, hepatic cholesterol levels increase from 3 weeks pre-partum to 1 week post-partum and vice versa for plasma (Schlegel et al., 2012). Moreover, *ACAT1* is up-regulated in the liver during early lactation, it esterifies free cholesterol so that liver can expel cholesterol esters via LDL. Also, *MTTP* and *ApoB100* expressions are increased by one week post-partum and are involved in the assembly and secretion of VLDL (Hussain et al., 2003). While low plasma cholesterol is observed at 1-week post-partum due to increased *LDLR,* important for the transport of cholesterol from blood to cells. Cholesterol synthesis in the liver and uptake from the blood via *LDLR* is controlled by *SREBF-2*. The *SREBF-2* genes are up-regulated from 3 weeks pre-partum to 1 week postpartum (Schlegel et al., 2012). This same group also reported an increase in hepatic cholesterol 7 alpha-hydroxylase (*CYP7A1*) from 3 weeks pre-partum to 1 week post-partum required for the maintenance of bile acid and cholesterol homeostasis (Schlegel et al., 2012). Although cholesterol metabolism in the liver is altered during the transition period, the impact of these changes on early lactation in the period leading to breeding remains ill-defined.

2.3.6 ASSSESING LIVER FUNCTION

Under metabolic stress the dairy cow is prone to changes in the immune response characterized by inflammation and thus, decreased liver activity, which negatively impacts milk production (Bertoni et al., 2008). The degree of inflammation can be assessed using the Liver Functionality Index (LFI). The index takes into consideration acute-phase proteins (APP) originating from the liver and linked with inflammation. APPs are further separated into positive (example: haptoglobulin and ceruloplasmin) or negative (example: cholesterol and albumin) APP, with alterations in the level of both occurring during the transition period. Therefore, high concentrations of positive APPs would suggest an acute immune response, which is accompanied by a decrease of negative APPs, resulting in low liver function (Bertoni et al., 2008, Trevisi et al., 2012). For example, Trevisi et al (2012), evaluated metabolic and inflammatory parameters, from 4 weeks pre-partum to 4 weeks post-partum in high-yielding Holstein cattle, including albumin, interleukin-6, haptoglobulin, ceruloplasmin, total cholesterol, bilirubin, NEFA and glucose comparing high LFI (HL) and low LFI (LF) transition cows. Ultimately, the serum of HL was higher in albumin and cholesterol, but lower in haptoglobulin post-partum. NEFA, ceruloplasmin and bilirubin were lower at different times during the entire sampling period in HL cows compared to LF cows (Trevisi et al., 2012). Similar groupings by Bertoni et al, 2008, suggested that cows in the HL quartile of a herd experienced improved reproductive performance as shown by a decrease in days open (Bertoni et al., 2008).

Albumin and cholesterol, as well as enzymes such as gamma-glutmayl transferase (GGT) are used as serum/plasma markers to assess hepatic health and functions(Bionaz et al., 2007). GGT is an enzyme which is highly sensitive marker, where enhanced GGT has been associated with hepatic diseases including fatty liver (Sevinc, 2001). A recent study, examined the presence of serum GGT and showed that persistently high levels of GGT were found in anovular cows (Kawashima et al., 2016). Levels of GGT ranging around 23-25 U/L, which have been previously characterized by a mild form of fatty liver (Sevinc, 2001).

2.3.7 THE ANTIOXIDANT SYSTEM

Failure of a cow to successfully adapt to the onset of lactation can be characterized by oxidative stress. The balance between production and degradation of reactive oxidative species (ROS) will determine the level of oxidative stress experienced by a cow, where oxidative stress is linked to dysfunctional inflammatory response (Abuelo et al., 2015). Physiologically, non-enzymatic (example: carotenoids, vitamins E and C, and glutathione) and enzymatic (example: glutathione peroxidase and superoxide dismutase) antioxidants systems are in place to prevent accumulation of ROS that can lead to reduced milk yields and poor reproductive performance, as reviewed by (Abuelo et al., 2015).

It has been suggested that cows with a greater BCS at parturition and greater body condition loss in early lactation are predisposed to oxidative stress (Bernabucci et al., 2005). Previous reports have suggested that cows suffering from ketosis will also have a predisposition to oxidative stress, since serum vitamin E levels were significantly decreased in circulation (Li et al., 2016b). Vitamin E supplementation from 14 days pre-calving to 7 days post-calving has led to reproductive improvements: decrease in the number of services and days to conception (Baldi et al., 2000).

The non-enzymatic antioxidants glutathione (GSH) has been linked to placental retention, where the oxidized form GSSG was elevated in animals suffering from retained fetal membranes. In addition, the enzymatic antioxidative defense provided by glutathione peroxidase (GSH-Px) catalyzes the reaction between GSH and hydrogen peroxide forming oxidized GSH (GSSG). This activity is higher in animals that experience retained fetal membranes (Kankofer, 2001a, b). Moreover, GSH-Px activity has been shown to increase around parturition as a potential homeostatic control mechanisms (Bernabucci et al., 2005). A lack of research in dairy cows has investigated the role of glutathione during the transition period.

Elsewhere, the ferric reducing ability of plasma (FRAP) assay is used to detect global antioxidant potential by measuring concentrations of ascorbic acid, uric acid, bilirubin, albumin and vitamin E analogs (Benzie et al, 1996). A higher concentration of FRAP indicates a higher ability and need of the plasma to protect against oxidative stress. Therefore, the level of FRAP is affected by the level of vitamin C, uric acid, bilirubin, total protein and albumin. Few studies have addressed the level of FRAP in dairy cows. An early study in cattle, has shown indifferent FRAP levels before and after calving, it was only lower on the day of calving suggesting a short-term alteration of redox-reactions (Gaal et al., 2006). Later, it was suggested that supplementing Vitamin E during the dry period tended to increase FRAP two weeks pre-calving compared to unsupplemented cows (Bouwstra et al., 2010). Nutritional management of the pre-partum cow is a key aspect in reducing the likelihood of oxidative stress through vitamin supplementation. Both oxidative and metabolic stress resulting from NEB can be harmful in subsequent reproduction and require a deeper understanding on their effect on ovarian health and the overall pathways regulating folliculogenesis.

2.4. MAPK PATHWAY IN THE OVARY

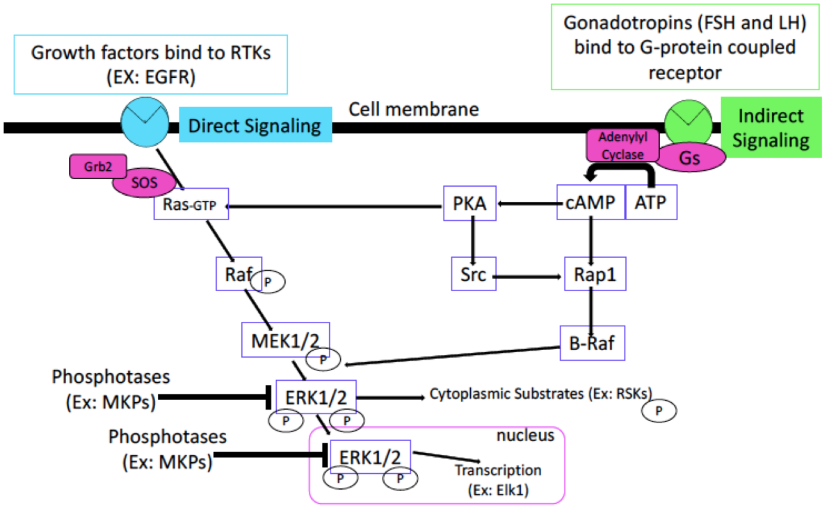
A number of morphological and functional changes take place throughout the life span of the antral/dominant follicle, which ovulates, and eventually forms the corpus luteum. In fact, these alterations are dependent on numerous factors including growth factors and cytokines all of which signal in concert with gonadotropins and their downstream signaling cascades.

The mitogen-activated protein kinase (MAPK) is comprised of three core families: c-Jun amino-terminal kinases or stress-activated protein kinases (JNK/SAPK) 1 to 3, p38MAPK α, β, γ and δ, and lastly, the extracellular regulated kinases (ERKs) 1 to 8. Successful ovulation is dependent on the luteinizing hormone (LH) surge, which initiates the following signaling cascades: (MAPK) phosphatidylinositide 3-kinase (PI3K/Akt) and cAMP/Protein kinase A (PKA) in cattle (Tajima et al., 2005, Fukuda et al., 2009). Theses pathways may interact to regulate one another and mediate downstream functions. The focus of this literature review is on the ERK1/2 pathway.

2.4.1 ERK1/2 PATHWAY

The extracellular signal-regulated kinase 1 and 2 (ERK1/2), also referred to as mitogen-activated protein kinase 3/1, activation is dependent on an initial direct signal from growth factors (via receptor tyrosine kinases (RTKs)) and phorbol esters or indirect signal from G protein-coupled receptors (GPCRs), cytokines, transforming growth factors, osmotic stress or microtubule disorganization (Roux and Blenis, 2004). As ERK 1 and ERK 2 have a high sequence of homology (84% of amino acid identity), they also share substrate specificities and are thus, commonly referred to as ERK1/2 (Busca et al., 2016). Phosphorylated ERK1/2 signal can activate approximately 175 cytosolic/nuclear target substrates (Yoon and Seger, 2006, Roskoski Jr, 2012).

Figure 2.7: ERK1/2 signaling pathway activated directly or indirectly adapted from (Ramos, 2008, Roskoski, 2012)



An example of the signaling pathway is as follows (Figure 2.7): a growth factor binds to RTKs (e.g. epidermal growth factor-epidermal growth factor receptor (EGF-EGFR)), which in turn passes a signal downstream to activate Ras, by recruiting the guanine exchange factor, son of sevenless (SOS), with the assistance of protein growth-factor-receptor-bound-2 (Grb 2). An active Ras (Ras-GTP) interacts with the kinase Raf causing a conformational change in the latter and activating its kinase activity. Subsequently, stimulated Raf phosphorylates MEK1 and MEK2, which leads to the phosphorylation of threonine and tyrosine residues of ERK1/2. Activated ERK1/2 dissociates from the MEK1/2/ERK1/2 complex and is translocated to the nucleus. Once in the nucleus active ERK1/2 phosphorylates various substrates including Elk1 (ELK1), a transcription factor important for proliferation. Additionally, activated ERK1/2 also phosphorylates cytoplasmic substrates. Ultimately, ERK1/2 function in a cell is a result of cell-type-specific substrates. Termination of ERK1/2 signaling generally occurs by dephosphorylation, which is reliant on the dual specificity threonine/tyrosine MAP kinase phosphatases (MKPs)-1,-2,-3, otherwise referred to as DUSPs. Positive and negative feedback loops control the length and intensity of ERK1/2 signaling. MKP3 has been put forth as the phosphatase required to shutdown ERK1/2 signaling in the rat preovulatory follicle (Reizel et al., 2010).

Alternatively, ERK1/2 can be activated via cyclic adenosine monophosphate (cAMP), whereby ligand bound GPCRs (for example LH-LHR) can evoke downstream activation of cAMP followed by a PKA-independent or a PKA-dependent pathway. It has been previously established that the PKA inhibitor H-89 can obstruct gene transcription down-stream the ERK1/2 pathway signifying pathway cross-talk in human, rodent, and bovine theca and granulosa cells (Seger et al., 2001, Tajima et al., 2005, Gerits et al., 2008, Menon et al., 2011, Menon and Menon, 2014).

2.4.2 ERK1/2 INHIBITION TECHNIQUES

Techniques to inhibit the ERK1/2 pathway have been utilized as loss-of-function paradigm to investigate the role of this pathway in normal and cancer cells. Both a pharmacological inhibition and a conditional knockout mouse model have been used to study ERK1/2 signaling in the ovary (Fan et al., 2009, Siddappa et al., 2015). Pharmacological inhibition of the enzymatic activity of MEK1/2 is used to study ERK1/2 signaling. A number of MEK1/2 inhibitors have been developed: CI-I040 (PD0184352)(Rinehart et al., 2004, Lorusso et al., 2005), PD98059 (Dudley et al., 1995), U0126 (Favata et al., 1998), and lastly, PD0325901 (Brown et al., 2007), a follow-up design to CI-I040. PD0325901 is a specific non-ATP competitive inhibitor of MEK1/2, therefore the inhibitor will prevent the ligands from binding to the ERK1/2 receptor. Most importantly, the inhibitors act on both the MEK1 and 2 ligands, which do not differentiate ERK1 from ERK2 (Aouadi et al., 2006). These inhibitors are commonly studied for a potential in cancer therapy due to the role of the ERK1/2 pathway in cellular proliferation and differentiation. A recent publication has revealed the differences in inhibitor targets, where both U0126 and PD98059 affect calcium entry into the cell, suggesting less off-target effects by the PD0325901 inhibitor (Wauson et al., 2013).

2.4.3 ERK1/2 in GRANULOSA & THECA CELLS

The bulk of ERK1/2 signaling in the ovary has been studied in the mouse using both *in vitro* and *in vivo* approaches, while in bovine and other monoovulatory species ERK1/2 signaling in the follicles has been investigated predominantly in cultured granulosa and theca cells (Ryan et al., 2008, Sayasith et al., 2013c, Sayasith and Sirois, 2015). An initial study performed on the Rlhr-4 and Rfshr-17 preovulatory rat granulosa cell lines showed that gonadotropins induce the cAMP/PKA pathway, which activates the down-stream ERK1/2 cascade (Seger et al., 2001). In cultured bovine granulosa cells, treatment with IGF or FSH activated ERK1/2 and AKT pathways (Ryan et al., 2008). *In vitro* detection of active ERK1/2 has been shown in porcine granulosa cells subject to both FSH and LH gonadotropins (Cameron et al., 1996). In rat granulosa cells, FSH has been known to activate ERK1/2 (Das et al., 1996). In the mouse, LH was shown to induce ERK1/2 activity in granulosa cells (Siddappa et al., 2015).

FSH-stimulated bovine granulosa cells treated *in vitro* with PD98059 (inhibitor of ERK1/2 signaling) increased expression of CYP19 (aromatase), therefore suggesting that ERK1/2 negatively regulates aromatase (Silva et al., 2006). In another LH-stimulated cultured bovine theca cells, were subject to pharmacological inhibition (PD98059 or U0126) of the ERK1/2 pathway (Tajima et al., 2005). These authors observed altered steroidogenic functions, whereby under normal LH-stimulated conditions, there was an increase of both progesterone and androstenedione. However, when LH-stimulated theca cells were treated with PD98059, progesterone concentrations were higher, while androstendione significantly decreased. Further analysis revealed that inhibition of the ERK1/2 pathway (U0126) in LH-stimulated theca cells led to a significant increase in STAR protein abundance and a decrease in CYP17 protein abundance (Tajima et al., 2005). Similarly, this group had previously shown similar differences in STAR abundance and progesterone levels in cultured preovulatory rat granulosa cells and human granulosa cells (Tajima et al., 2003).

Immortalized rat granulosa cell lines, subject to ERK1/2 inhibition via the pharmacological inhibitor PD9085, during FSH- and LH-induced steroidogenesis, revealed elevation of cAMP-induced progesterone production and an increase in *Star* expression (Seger et al., 2001). Su et al, 2006 revealed that *in vitro* LH-stimulated mouse granulosa cells treated with the U0126 pharmacological ERK1/2 signaling inhibitor decreased *Star* expression in addition to *cyp11a1*, while similar to bovine studies aromatase activity was increased (Su et al., 2006).

Fan et al, 2009 developed a breakthrough mouse model in which the function of ERK1/2 was specifically studied in the ovary. Mice lacking Erk1/2 in granulosa cells of antral follicles (Erk1/2$^{gc-/-}$) were infertile demonstrating that ERK1/2 is indispensable for ovulation. The CCAAT/Enhancer-binding protein-β (C/ebpβ) transcription factor was identified as a downstream regulator of ERK1/2 signaling. The LH-regulated genes that required ERK1/2 activity in granulosa cells were *Rsk3, Star, Cyp11a1, Ptgs2, Tnfaip6, Ptx3* and *Pgr,* all of which are down-regulated in the knockout mouse model when subject to LH-stimulation (Fan et al., 2009). Likewise, murine pharmacological studies have shown similar results, with additional demonstration of Egr1 as downstream LH-ERK1/2-regulated transcription factor required for follicular rupture (Siddappa et al., 2015).

Previous *in vitro* studies in rat granulosa cells (Tajima et al., 2003), cattle theca cells (Tajima et al., 2005) and human granulosa cells (Tajima et al., 2003) suggest that ERK1/2 negatively regulates STAR. Conversely, the Erk1/2$^{gc-/-}$ mouse model and pharmacological inhibition of ERK1/2 signaling suggest that of ERK1/2 positively regulates Star. Therefore, species difference can provide results requiring further investigation in the bovine model.

2.4.4 REGULATION OF ERK1/2 BY METABOLIC PROCESSES IN THE BODY

Successful reproductive performance relies on combined physiological interactions between various systems including metabolic and hepatic pathways. During a period of stress, such as illness, altered levels of circulating cytokines and hormones may generate unwanted responses at distant sites such as the ovary (Bertoni et al., 2008). In women suffering from polycystic ovary syndrome (PCOS), a 50% decrease in ERK1/2 signaling in theca cells was observed and led to increased androgen production. Furthermore, theca cells treated with the PD98059 inhibitor led to an increase in CYP17 expression, the enzyme responsible for driving steroidogenesis toward androgenesis production in ovarian cells (Nelson-Degrave et al., 2005). Obese patients with PCOS had an increased level of free fatty acids in circulation and ovarian follicular fluid, which suggests abnormal lipid metabolism leading to decreased reproductive efficiency (Niu et al., 2014).

Free fatty acids (FFAs) represent a source of energy. Free fatty acid receptors (FFARs) such as GPR40/FFAR1 have been identified as a receptor for medium and long chain fatty acids. FFAs bind to GPR40 and subsequently, activate the ERK1/2 signaling pathway in human embryonic kidney (HEK293) cell lines and bovine mammary gland (Yonezawa et al., 2008, Seljeset and Siehler, 2012, Miyamoto et al., 2016). To date, the presence and role for free fatty acid receptors have not been addressed in the bovine ovary. Nonetheless, in cows, large quantities of free fatty acids have been suggested to lead to abnormal changes in steroidogenesis and cell proliferation in granulosa cells (Jorritsma et al., 2003b, Jorritsma et al., 2004, Vanholder et al., 2005, Vanholder et al., 2006).

As previously mentioned in dairy cattle, accumulation of non-esterified fatty acids (NEFA) in circulation is a result of immobilizing large quantities of adipose tissue, often linked to the transition period. Subjecting bovine adrenal cells in culture to high levels of NEFAs has illustrated a link between decreased ERK1/2 activity and increased *CYP17A1* and thus, androgen production

(Bellanger et al., 2012). Therefore, there is evidence for a relationship between lipid metabolism, ERK1/2 signaling and steroidogenesis. Another metabolite β-hydroxybutyric acid (BHBA), which rises in cows during the transition period, has been associated with abnormal steroidogenesis in cultured bovine granulosa cells (Vanholder et al., 2006). GPR109A, niacin receptor, has been identified as a receptor for BHBA (Taggart et al., 2005) and to date this receptor has been identified in bovine liver, brain and muscle tissue (Titgemeyer et al., 2011). Recently, BHBA has been shown to signal through its receptor GPR109A and ERK1/2 pathway to regulate growth hormone releasing hormone (GHRH) synthesis in rat the hypothalamus (Fu et al., 2015a). However, BHBA-GPR109A signaling in the bovine ovary remains unexplored.

ERK1/2 signaling may play several roles in the cow with respect to reproductive performance. The mechanisms of action are incompletely understood especially, if interactions involving the ovary, metabolism, and hepatic function are concerned. As a cow undergoes several physiological changes throughout her lifetime, it is imperative to investigate the role of ERK1/2 in the cow and potentially suggest links with metabolic health and subsequent reproductive performance.

2.5 CONCLUDING REMARKS

The dairy cow is a valuable asset to the Canadian economy, yet she is leaving the herd too early due to poor reproductive health. Determining potential metabolic alterations during the transition period and the mechanisms by which those alterations affect ovarian functions during early-lactation will help us understand the pathogenesis of infertility in lactating dairy cows. Data from my proposed studies are expected to contribute towards better understanding sub-fertility in lactating dairy cows.

CHAPTER III

This manuscript has been submitted to the “Theriogenology” for publication (THERIO-2018-485). Elsewhere, a very condensed version of this manuscript was published in the non-peer reviewed journal Progressive Dairyman: Canada journal (October 2017). Please see Chapter VIII: Appendix 8.1: *It’s not easy getting older: Heifers versus lactating dairy cows as they transition*

Association between pre-breeding metabolic profiles and reproductive performance in heifers and lactating dairy cows

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3.1 ABSTRACT

Lactating cows and nulliparous heifers are in distinctive and unique physiological conditions when they are approaching the planned time of breeding, at approximately 60 days in milk and 13-15 months of age, respectively. The oocyte activated from the ovarian reserve requires approximately 80 to 100 days to reach the ovulatory stage, therefore, the metabolic changes occurring in lactating cows could impact the follicular microenvironment. This study aimed to profile the metabolic milieu in heifers (N=14) and lactating cows (N=15) in the weeks leading up to planned time of breeding and relate that to their reproductive performance. All cows were followed for a period of 15 weeks, from 3 weeks pre-calving to 12 weeks post-calving, while heifers were monitored for a period of 4 weeks leading up to the tentative week of breeding (pre-breeding period). For data analysis, we further divided cows into primiparous (N=8) and multiparous (N=7) cows owing to the significant difference in their milk yield. Assessment of reproductive performance showed that primiparous and multiparous cows tended to have lower pregnancy rates compared to heifers ($P<0.1$). Plasma concentrations of β-hydroxybutyric acid were about 2-fold higher in multiparous cows than those of heifers in the week leading up to planned time of breeding ($P<0.05$). Total bile acid levels during the pre-breeding period were higher in all lactating cows compared to heifers ($P<0.05$) and glucose levels were lower in lactating cows ($P<0.05$). Triglyceride concentrations were lowest in multiparous cows than primiparous cows and nulliparous heifers ($P<0.05$). In addition, lactating cows had higher concentrations of total-cholesterol and the high-density lipoprotein and low-density lipoprotein compared to heifers ($P<0.05$). Conversely, concentrations of very low-density lipoprotein were lower in multiparous cows than primiparous cows and nulliparous heifers ($P<0.05$). There were no differences in plasma glutathione levels between the groups, but the ferric reducing ability of plasma was higher in lactating cows compared to heifers ($P<0.05$). These data establish the differences in the profile of the metabolic and oxidative markers during period approaching planned breeding in lactating cows compared to nulliparous heifers. As metabolites in the plasma have been shown to be represented in the ovarian follicular microenvironment, these differences may be responsible for reproductive issues in lactating cows.

3.2 Introduction

Achieving economical sustainability on dairy herds is largely dependent on cow longevity and reproductive success (Delgado, 2018). On North American dairy herds, the average age of milking cows has been reported as 4 years and 3 months, which translates to slightly over 2 lactations (Murray, 2013). Yet, these cows have the potential to live for 9.1 years, which translates to slightly less than 6 lactations (Van Doormal, 2009). Most importantly, these cows only begin contributing to the financial success of the herd by the 3rd lactation (Pellerin et al., 2014). Reduced longevity is a consequence of involuntary culling most typically related to poor reproductive performance, which represents 15.99% of involuntary culling (Valacta, 2017).

The transition period, defined as three weeks pre-calving to three weeks post-calving, comprises a time of enhanced susceptibility to diseases including milk fever, metritis, displaced abomasum, and ketosis (Grummer, 1995, Drackley, 1999). Moreover, 75% of metabolic related diseases occur within the first month after calving (LeBlanc et al., 2006). Cumulative effects of disorders in the transition period can extend for more than 4 months, which can severely compromise reproductive performance (Ribeiro et al., 2016). Excessive lipid mobilization to meet the energy demands of lactation leads to metabolic stress characterized by well-defined increased circulating levels of non-esterified fatty acids (NEFAs) and β-hydroxybutyric acid (BHBA) and decreased levels of glucose (Drackley, 1999, Leroy et al., 2008). Oxidative stress evaluated through the presence of reactive oxidative species (ROS) and antioxidant systems provides an insight to a cow's health status, where high levels of ROS and low levels of antioxidants have been reported in cows experiencing negative energy balance (Bernabucci et al., 2005). Both elevated BHBA and NEFA during the transition period have shown to decrease reproductive performance (Huzzey et al., 2015, Shin et al., 2015), as described by anovulation (Walsh et al., 2007), decreased conception (Ospina et al., 2010b) increased number of days open, and increased number of services per pregnancy (Bertoni et al., 2008).

Fertility is among the most important factors evaluated on a dairy operation. Moreover, fertility of dairy heifers is higher than that of lactating dairy cows, which is attributed to the fact that nulliparous heifers do not experience the stress of the transition period (Kuhn et al., 2006, Wathes et al., 2014). One study in particular, showed that the first and second insemination pregnancy rates were 84.3% and 19.5% for heifers and 3rd/4th parity cows, respectively (Balendran, 2008). Following calving, it is common practice amongst North American dairy herds to allow for a voluntary waiting period of approximately 60 days (8.5 weeks) from calving to breeding (Miller et al., 2007, Stangaferro et al., 2018). During this time, the animal is expected to recover from calving, with respect to resumption of ovarian activity, estrous behavior, and uterine health (Stangaferro et al., 2018). Nonetheless, the process of activation of an oocyte from the ovarian reserve until it becomes of ovulatory capacity requires approximately 80 to 100 days. This time course suggests that the ovulatory follicle ready at breeding developed during the transition period until the end of the voluntary waiting period. The metabolic changes during that time are expected to affect the follicular microenvironment as put forth by the Britt Hypothesis (Britt, 1992, 2008, Lucy et al., 2014).

Although successful pregnancy is multifactorial, the female must produce a developmentally competent oocyte. Both granulosa cells and the follicular fluid in the ovary are part of the microenvironment responsible for the production of a fertilizable oocyte (Britt, 2008, Thatcher, 2017). In cattle, it has been previously shown that the presence of metabolites in circulating blood is reflected in the follicular fluid (Sanchez et al., 2014), where for example cholesterol levels are approximately 55% lower in follicular fluid compared to blood, while the level of BHBA is nearly identical between blood and the follicular fluid (Leroy et al., 2004b). On another note, elevated levels of circulating BHBA have shown to be associated with decreased fertility through changes in the steroidogenic profile (Vanholder et al., 2006) and decreased success of conception (Ospina et al., 2010b). Overall, blood analyses provide a mirror into the follicular microenvironment, which in turn shed light on the changes in metabolic indicators and oxidative stress markers that have the potential to impact follicle development.

Characterizing plasma metabolic parameters and oxidative stress markers of dairy cattle in different physiological stages (nulliparous heifers, primiparous cows, and multiparous cows) in the month leading up to planned time of breeding can open a window into the follicular microenvironment in which the oocyte is developing. Therefore, the aim of this study was to follow lactating dairy cows through the transition period to the subsequent breeding period and breeding aged heifers for a one month sampling period and to superimpose the pre-breeding period of all animals.

3.3 Materials and methods

3.3.1 Ethics statement

All animal procedures were approved by the Animal Care Committee of the Faculty of Agricultural and Environmental Sciences of McGill University. This study was conducted on two Canadian dairy farms with Holstein cattle applying similar herd management practices: 1) the Macdonald Campus Farm from McGill University and 2) a commercial dairy farm on the outskirts of Montreal.

3.3.2 Animals and husbandry

Primiparous (P) cows (N=8), multiparous (M) cows (N=7) and nulliparous heifers (N) (N=14) were used in this study. Cows entering their first lactation (P) calved at 24.65 ± 1.01 months of age, while multiparous cows (M) were of 50.54 ± 7.45 months of age (entering their 3.14 ± 0.55 lactation), and heifers were 13.15 ± 0.17 months of age. We acknowledge that our sample size is small. Animals from both farms, were housed under similar conditions, where all lactating cows were housed in a tie-stall setting and all heifers were kept in a free-stall setting. Cows on both farms were fed a base diet of alfalfa silage, corn silage, and soybean meal at the onset of lactation, while alfalfa silage and dry hay were predominantly fed during the dry period. Heifer rations on both farms were similar, which was lower in energy and higher in fiber when compared to lactating cows. At the onset of lactation, cows from both farms were milked twice daily at approximately 0600h and 1730h, where individual milk yields were recorded at each milking. Reproductive parameters were monitored for subsequent breeding: percent pregnancy rates, calculated based on the number of services, and the calving interval in days (restricted measurement for only the two groups of lactating cows).

3.3.3 Blood collection

The experimental design is shown in Figure 3.8.1. Weekly blood samples were collected from heifers (N=14) for approximately 1 month starting at 4 weeks prior to the week of planned time of breeding, and primiparous (N=8) and multiparous (N=7) cows for fifteen weeks starting from 3 weeks pre-partum to 12 weeks post-partum. In our model, we addressed a typical voluntary waiting period, where the onset of breeding for lactating dairy cows commences at approximately 8 weeks in milk. We superimposed the weekly blood collections from nulliparous heifers onto the 4 weeks prior to planned time of breeding for lactating cows (i.e. 4 weeks to 8 weeks post-calving) as identified by the “pre-breeding period”.

Blood samples were collected from all animals from the coccygeal vein between 1300h and 1500h on the day of sampling. This time corresponded to the period between morning (6h30) and evening (18h30) feedings in order to avoid spikes in metabolic parameters due to feed intake. Blood samples were collected in EDTA treated vacutainers and all samples were cooled down on ice, centrifuged for 10 min at 1500 rpm, and immediately frozen in liquid nitrogen until arrival at the laboratory, where they were stored in a -80°C freezer. Blood samples for glutathione analysis were processed on farm as described below.

3.3.4 Metabolic parameters

We measured the plasma concentration of the following metabolites: β-hydroxybutyric acid (BHBA), glucose, triglycerides, total bile acids, total cholesterol, high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), and very low-density lipoprotein-cholesterol (VLDL-C). All analytes were measured in duplicate.

Due to the fact that BHBA profiles in transition cows are well-established, they were measured by clinical analyzer at the Prairie Diagnostic Services Inc. (Saskatoon, Canada) in samples of select time-points. The time-points for the cows included 3 weeks pre-calving, the week of calving, and weeks 3, 7, and 12 weeks post-calving. In heifers, BHBA was measured in one sample collected at 1 week prior to planned time of breeding, which in our model would be the equivalent to 7 weeks post-partum in lactating cattle.

The other target metabolites were measured in all weekly samples of cows and heifers using colorimetric/enzymatic assays following the manufacturer's protocol unless otherwise specified: 1) Glucose - Glucose kit from Sekisui Diagnostics (220-32, BioPacific Diagnostics Inc, North Vancouver, BC, Canada). The intra- and inter-assay coefficients of variation were 2.59% and 3.10%, respectively. 2) Triglycerides - Triglycerides-SL kit from Sekisui Diagnostics (236-60, BioPacific Diagnostics Inc., North Vancouver, BC, Canada). The intra- and inter-assay coefficients of variation were 1.16% and 3.71%, respectively. 3) Total bile acids - Total Bile Acid kit from Diazyme Laboratories (DZ042A-K, BioPacific Diagnostics Inc., North Vancouver, BC, Canada). The intra- and inter-assay coefficients of variation were 16.77% and 19.76%, respectively. 4) Total Cholesterol -Cholesterol-SL kit from Sekisui Diagnostics (234-60, BioPacific Diagnostics Inc., North Vancouver, BC, Canada). The intra- and inter-assay coefficients of variation were 7.15% and 16.24%, respectively. 5) HDL-C - HDL-C kit from Wako Diagnostics (997-72591 and 993-72691, Wako Life Sciences, Inc., Mountain View, CA, USA), where the second incubation period was increased by 1.5h to assess a stable reaction. The absorbance in individual plasma samples was measured using the FilterMax^{TM} F5 Multi-Mode. The intra- and inter-assay coefficients of variation were 2.47% and 5.43%, respectively.

Concentrations of VLDL-cholesterol (VLDL-C) and LDL-cholesterol (LDL-C) were calculated using the following validated equations:

A) VLDL-C = Triglycerides/5 (Friedewald et al., 1972)
B) LDL-C = Total cholesterol – (HDL-C + VLDL-C) (Friedewald et al., 1972, Kessler et al., 2014)

3.3.5 Assessment of oxidative stress markers

Circulating levels of glutathione (GSH) values and the Ferric Reducing Ability of Plasma (FRAP) assay were used to assess the antioxidant systems in cattle.

GSH being very reactive and unstable required immediate derivatization from blood samples upon collection. First, 500µl of whole blood (from EDTA treated vacutainers) was placed in a 2ml tube with 10µl of 10mM γ-glutamyl-leucine (γGL), which was used as an internal standard, and 200µl of 100mM N-ethylmaleimide (NEM). This mixture was quickly vortexed, then placed on ice for 5-min. Following incubation, 50µl of zinc sulphate ($ZnSO_4 .7H_2O$) was used to lyse the NEM-treated blood and 1ml of ice cold methanol was added to the mixture for protein-precipitation, where the mixture was centrifuged at 800 rpm for 1-2 min. The supernatant was stored in liquid nitrogen, until arrival at the laboratory, where it was transferred to a -80°C freezer. Samples were analyzed by liquid chromatography-tandem mass spectrometry (LC/MS) as previously described (Vassilyadi et al., 2016). In brief, chromatography was performed using the Agilent 1290 UHPLC system (Agilent Technologies) and mass spectrometry was accomplished using the Agilent 6460 Triple Quadrupole (QQQ) system (Agilent Technologies) in positive electrospray mode. MassHunter Qualitative and Quantitative Analysis software (Agilent Technologies) was used to the measure GSH by means of integrating chromatographic peaks, followed by the creation of a standard curve, and the response ratios calculated against γGL.

To measure the total antioxidant activity, we performed the FRAP assay as described previously (Benzie and Strain, 1996, Schlesier et al., 2002), with some modifications for bovine plasma samples. Briefly, we prepared a series of standards of ferrous sulfate ($FeSO_4. 7H_2O$) from 0.1 mM to 1mM. Next, we prepared the FRAP reacting reagent from: 200ml of 300mM Acetate Buffer (at pH 3.6), 20 ml of 10mM TPTZ (2, 4, 6-tripyridyl-s- triazine), 20ml of 20mM Iron (III) chloride hexahydrate ($FeCl_3$. 6H2O), and 24 ml of distilled water. The straw coloured FRAP reagent was subsequently incubated in a water bath at 37°C for 10 minutes. The assay uses antioxidants as reductants in a redox-linked colorimetric method, whereby ferric tripyridyl triazine (Fe III TPTZ) is reduced to its ferrous form. This reaction causes the straw coloured substance to become blue, and thus, absorbance is measured at 593 nm. Thus, by means of a microtiter plate, we added 30 µl H_2O and 10µl standards or samples, followed by 200 µl FRAP-solution, mixed for 10 seconds and the absorbance was measured after approximately 8 minutes. The intra- and inter-assay coefficients of variation were 4.84% and 3.10%, respectively.

3.3.6 Statistical analysis

The data are presented as LS means ± standard errors of the mean (SEM). All data (milk production, reproductive performance, metabolites, and oxidative stress markers) with repeated measures were analyzed using the MIXED model procedure with SAS (version 9.4, SAS Institute Inc., Cary, NC, USA). For BHBA analysis, where the various time points were not equally spaced, an exponential correlation covariance structure SP (POW) was used for repeated measures. To compare the week prior to the onset of breeding between all three groups were compared by analysis of variance. Metabolite and oxidative stress data were analyzed to compare primiparous cows to multiparous cows from 3 weeks pre-calving to 12 weeks post-calving, while analysis of the pre-breeding period was used to compare primiparous cows, multiparous cows and nulliparous heifers. All data was tested for normality using the Shapiro-Wilk test (PROC UNIVARIATE) and if needed were log-transformed. Post-analysis, all data were back transformed for graphical representation. The following model was used:

$$Y_{ijkm} = \mu + G_i + T_j + F_k + GT_{ij} + C_{m(ik)} + e_{ijkm}$$

Where Y_{ijkm} represents the parameter being measured, μ =represents the overall mean G_i is the effect of the i^{th} group (three stages of production: primiparous (P), multiparous (M), and heifers (H)), T_j is the effect of the j^{th} time (in weeks), F_k is the effect of the k^{th} farm (two farms), GT_{ij} is the effect of the interaction between the i^{th} group and the j^{th} time point, $C_{m(ik)}$ is the effect of the m^{th} cow within the i^{th} group and the k^{th} farm and, e_{ijkm} represents the residual error. The random effect was cow nested within stage of production (primiparous (P), multiparous (M), and heifers (H)) and farm, while the fixed effect included the farm, the weekly blood samples, and the interaction between the weekly blood samples and the stage of production. Differences between groups were detected by Scheffés method. The *P*-values of <0.05 were declared significant, while *P*-values of <0.1 tended to be different.

3.4 Results

3.4.1 Production and reproduction

We first compared the milk yield from week 1 post-partum to week 12 post-partum for cows entering their first lactation with those entering the second or greater lactation. As expected, the milk yield during the sampling period of multiparous cows was higher than that of primiparous cows (Figure 3.8.2; $P<0.05$). We then compared the pregnancy rates and calving interval, two principal reproductive management parameters, between heifers and cow groups. While primiparous and multiparous cows had similar pregnancy rates, nulliparous heifers had a tendency for higher pregnancy rates than lactating cows (Table 3.9.1; $P<0.1$). Lactating cows required approximately one additional service per pregnancy compared to nulliparous heifers (about 2.4 services for lactating cows and 1.5 services for nulliparous heifers). There was no difference in calving interval between lactating cows (Table 3.9.1; $P>0.05$). Even though the calving interval was on average 18 days longer for multiparous than primiparous cows.

3.4.2 Blood metabolites

Multiparous cows had higher plasma concentrations of BHBA during the experimental period than primiparous cows (Figure 3.8.3A, $P<0.05$). Because heifers were not monitored for fifteen weeks, we compared BHBA levels during the week prior to the anticipated week of breeding. Multiparous cows had 2-fold higher levels of BHBA than heifers (Figure 3.8.3B, $P<0.05$) with primiparous cows having intermediate levels that were not different from either group of animals (Figure 3.8.3B, $P>0.05$). The plasma glucose levels were lower in lactating cows than heifers during the pre-breeding period (Figure 3.8.4A, $P<0.05$). On average, primiparous and multiparous cows had 29.89% and 40.99% less circulating glucose, respectively, compared to heifers.

There were fluctuations in the total bile acids in the weekly plasma samples throughout the sampling period. Nonetheless, the overall concentrations of total bile acids during the pre-breeding period, were higher in lactating cows compared to heifers (Figure 3.8.4B, $P<0.05$). The plasma concentrations of triglycerides reached a nadir at one week post-calving in both groups of lactating cows. Overall, the triglyceride levels in lactating cows remained lower post-calving compared to pre-calving. Multiparous cows had the lowest concentrations of plasma triglycerides in the pre-breeding period compared to both primiparous cows and nulliparous heifers (Figure 3.8.4C, $P<0.05$).

The total cholesterol in circulation was at its lowest concentration in the weeks around calving for both primiparous and multiparous cows. With the onset of lactation, there was a gradual increase in total cholesterol in both groups of cows reaching a plateau between 6 through 12 weeks in milk. We also observed a significant group and time (weeks) interaction for the lactating cows (Figure 3.8.5A, $P<0.05$). During the pre-breeding period, there were significantly higher concentrations of total cholesterol in both primiparous and multiparous cows compared to heifers. On average, there was 36.36% and 50.65% more total cholesterol in primiparous and multiparous cows than heifers, respectively (Figure 3.8.5A, $P<0.05$). We then analyzed the profiles of cholesterol fractions, HDL-C, LDL-C and VLDL-C. Levels of HDL-C were higher in both primiparous and multiparous cows when compared to heifers during the pre-breeding period (Figure 3.8.5B, $P<0.05$). Levels of plasma HDL-C were 31.82% and 39.05% higher for primiparous and multiparous cows, respectively, compared to heifers. The LDL-C levels were higher in both primiparous and multiparous cows than heifers, respectively, during the pre-breeding period (Figure 3.8.5C, $P<0.05$). Levels of VLDL-C in all groups were very low with a nadir at 1 week post-calving in both primiparous and multiparous cattle. Unlike HDL-C and LDL-C levels, the circulating levels of VLDL-C during the pre-breeding period were lowest in multiparous cows compared to both primiparous cows and nulliparous heifers (Figure 3.8.5D, $P<0.05$).

3.4.3 Biomarkers of oxidative stress markers

Assays for GSH and FRAP were used to evaluate the ability of the cow to counteract reactive oxidative species. The levels of GSH remained stable in lactating cows during the transition period through the voluntary waiting period. Furthermore, there was no difference in the concentration of circulating GSH during the pre-breeding period of both lactating cows and nulliparous heifers (Figure 3.8.6 A, $P>0.05$). However, both primiparous and multiparous cows had higher levels of circulating FRAP in the 12 weeks post-calving compared to the 3 weeks pre-calving. Moreover, during the pre-breeding period, FRAP levels were higher for both groups of lactating cows compared to nulliparous heifers (Figure 3.8.6 B, $P<0.05$).

3.5 Discussion

In this study, we characterized plasma metabolic and oxidative stress indicators of lactating cows from the transition period until 12 weeks post-calving to encompass the pre-breeding period. Furthermore, we collected blood samples from nulliparous heifers during their respective pre-breeding period, which we subsequently superimposed onto the pre-breeding period of lactating cows, separated into primiparous and multiparous cows. The results from this study demonstrated the dramatic changes in profiles of metabolic indicators in plasma of lactating cows and how these profiles are different in heifers during the pre-breeding period. As we (Sanchez et al., 2014) and others (Sartori et al., 2004) have shown that metabolites in plasma are also found in the ovarian follicular fluid, our results from this study provide a reflection of the metabolic profile of follicles growing during the transition period through the voluntary waiting period of approximately 8 weeks in milk.

It has been documented that fertility among dairy heifers is higher than that of their lactating counterparts and also that heifers, unlike lactating cows, do not seem to be as susceptible to environmental factors such as heat stress (Kuhn et al., 2006). Evidence exists that first and second insemination pregnancy rates decrease with age/parity, where heifers, 1st parity, 2nd parity, and 3rd/4th parity cows had pregnancy rates of 84.3%, 51.5%, 31.4%, and 19.5%, respectively (Balendran, 2008). Similarly, we show a tendency for reduced reproductive performance in lactating cows, where their percent pregnancy rates averaged around 46%, while that of nulliparous heifers averaged around 76%. For the average cow, additional services per pregnancy causing lower pregnancy rates adds to a herd's cost of production by increased number of days open, which in turn increased the calving interval and thus, increased the days between the peak in milk production (Laven, 2018). More precisely, prolonging the calving interval for an average cow from the recommended 365 days to 425 days would decrease milk production and efficiency since end of lactation cows require more feed per litre of milk produced (Laven, 2018). Although we did not observe a significant difference between the calving interval of primiparous and multiparous cows with 414.45 ± 26.76 days and 433.39 ± 31.74 days, respectively, they are in both cases longer than economically advantageous. Nonetheless, our data is in line with the average for Quebec Holstein cows (3 521 herds) where lactating cows have a calving interval of 416 days and require approximately 2.4 services to achieve conception (Valacta, 2018).

Overall, the reduced fertility in lactating cows is likely as result of metabolic shifts between heifers and lactating cows. While multiple studies have addressed the direct effects of enhanced NEFA and BHBA on oocyte and granulosa cells (Vanholder et al., 2005, Vanholder et al., 2006), the potential effects of other metabolic changes cannot be ruled out. Thus, we set out to establish additional metabolic indicator and oxidative stress marker differences among heifers and cows of different parities in this study. As expected, here we report higher BHBA levels in multiparous cows compared to primiparous cows throughout the study period. A recent study showed significantly higher levels of BHBA in multiparous cows compared to primiparous cows, with the highest differences at weeks 1 and 4 post-partum (Morales Pineyrua et al., 2018). Furthermore, we show that multiparous cows had twice the concentration of BHBA compared to heifers in the week leading up to the planned time of breeding. The concentrations of BHBA are expected to be nearly identical between blood circulation and the follicular fluid (Leroy et al., 2004b), suggesting that the ovulating follicle in multiparous cows contains twice the amount of BHBA found in the ovulating follicle of heifers. Higher levels of BHBA have been associated with a longer voluntary waiting period (Ospina et al., 2010b) and pregnancy at first artificial insemination (Chapinal et al., 2012). Increased BHBA was associated with altered expression of a number of genes involved in proliferation and fatty acid metabolism in follicular granulosa cells (Girard et al., 2015b). Elsewhere, BHBA treatment reduced estradiol and progesterone synthesis in cultured granulosa cells (Vanholder et al., 2006). These observations demonstrate that BHBA can impact granulosa cells of ovulating follicles. We also found that increased age and number of parities enhance the volume of milk produced, this higher demand is likely met through lipid mobilization observed through higher levels of BHBA. It has been suggested that metabolic indicators and oxidative stress markers are closely intertwined, where higher BHBA levels have been linked with lower levels of antioxidants and higher levels of reactive oxidative species addressed later on in the discussion (Bernabucci et al., 2005). The negative correlation between BHBA levels and reproductive success is increased in multiparous cows, which would suggest a need for individualized nutritional management strategies.

Although we did not observe an effect of parity, there were lower levels of glucose in lactating cows compared to heifers in the 4 weeks leading up to the breeding period, explained by a demand of glucose for milk production. This is in contrast to a recent study that reported lower levels of glucose in multiparous cows compared to primiparous cows (Morales Pineyrua et al., 2018). Levels of glucose are slightly higher in the follicular fluid compared to blood (Leroy et al., 2004b, Sanchez et al., 2014). Cows with ovulatory dysfunction have lower glucose and insulin like-growth 1 (IGF1) levels than normal cows (Kawashima et al., 2007). Pregnancy at first artificial insemination was lower in cows with lower levels of glucose (Green et al., 2012, Garverick et al., 2013).

Bile acid synthesis has been shown to be increased at the onset of lactation in dairy cattle through presence of increased cholesterol as a precursor for bile acid synthesis and upregulation of the expression of the hepatic cholesterol 7 alpha-hydroxylase (CYP7A1) gene (Schlegel et al., 2012). Although we observed a great deal of fluctuations in plasma bile acids, overall values were higher in lactating cows compared to heifers in the pre-breeding period. Bile acids have been shown in follicular fluid of both human and bovine ovaries (Sanchez et al., 2014, Nagy et al., 2015, Sanchez-Guijo et al., 2016). Profiling of bile acid composition found that bovine follicular fluid predominantly contains cholic acid, cheodeoxycholic acid, deoxycholic acid and their glycine and taurine conjugates (Sanchez-Guijo et al., 2016). We previously showed threefold higher concentrations of total bile acids in the follicular fluid of the dominant follicle in early-lactating cows compared to nulliparous heifers (Sanchez et al., 2014). Furthermore, it has been suggested that cultured bovine granulosa cells treated with 5 nM of cholic acid, a concentration expected in the follicular fluid, inhibits estrogen synthesis (Gagnon-Duval et al., 2015). Given that bile acids have recently been shown to act as signals to regulate metabolic functions in humans (Shapiro et al., 2018), these initial observations in cows certainly trigger interest in the effects of bile acids on cow fertility.

Triglyceride levels reached a nadir at one week after parturition for both groups of lactating cows and remained lower in lactating cows compared to heifers, more obvious in multiparous cows. Triglyceride levels are low in bovine follicular fluid (Leroy et al., 2004b) and remain stable irrespective of concentrations found in circulation (Wehrman et al., 1991). However, cows with higher levels of triglyceride accumulation on the liver had a 35% decreased probability of estrous and a 30% decreased chance of pregnancy (Jorritsma et al., 2000). In this study, the lower levels of plasma triglycerides persisted through to the pre-breeding period.

Interestingly, this study showed a gradual increase in the levels of total cholesterol, HDL-C, and LDL-C in cows from the onset of lactation and reaching a plateau at time of anticipated breeding. Levels of the aforementioned cholesterol fractions and cholesterol itself were significantly higher in the lactating cows compared to heifers. There were no differences between primiparous and multiparous cows in total cholesterol and HDL-C, which is in agreement with a previous report (Folnožić, 2016). However, we found a significant effect of parity on LDL-C, which were higher in multiparous than primiparous cows. There is strong correlation between levels of cholesterol in blood and follicular fluid (Leroy et al., 2004b, Sanchez et al., 2014) with higher cholesterol entry into the large follicles compared to small follicles (Leroy et al., 2004a). HDL has long been detected in follicular fluid, while LDL was previously believed to not have been localized in the follicular fluid due to its larger size, making it less likely to cross the blood-follicle barrier, but has recently been located in follicular fluid of cattle (Schneider et al., 2013). Interestingly, higher levels of total cholesterol, HDL, and LDL were found in the follicular fluid of healthy versus atretic follicles (Schneider et al., 2013). Some in vitro studies showed that addition of LDL and HDL increased progesterone synthesis in bovine granulosa cells (O'Shaughnessy, 1985, O'Shaughnessy et al., 1990). However, lactating multiparous cows were shown to have higher BHBA and cholesterol, and lower glucose in circulation than heifers (Walsh et al., 2012). Altered metabolic profiles were associated with reduced estradiol and progesterone concentrations in the dominant follicles. This study also showed that expression of the gene encoding for steroidogenic acute regulatory protein (STAR), a rate limiting enzyme in cholesterol transport, was lower in cows (Walsh et al., 2012). These observations suggest that while cholesterol alone may have positive effects on ovarian steroidogenesis, their effects could be opposite in association with high BHBA and low glucose. However, the low concentrations of circulating C-VLDL in lactating cows, specifically in multiparous cows, during the pre-breeding period would suggest that the process of removing lipids from the liver in the form of C-VLDL is less efficient in lactating cows and may be in relation to a liver which is not functioning at the same capacity as that of nulliparous heifers.

GSH and FRAP are key markers used to evaluate the antioxidant system in plasma and they complement each other as the FRAP assay does not take into consideration the level of reduced glutathione (Hennipman, 2014). The transition period has been described by a depletion of antioxidant status characterized by an imbalance between the reactive oxygen metabolites and elimination by the antioxidant system leading to oxidative stress (Abuelo et al., 2015). It is important to maintain levels of glutathione as it is a natural antioxidant produced in large amounts by the liver. Glutathione is central for preventing fatty liver and mitochondrial injury as shown in mice lacking the Gclc gene required for GSH synthesis(Chen et al., 2007). Many previous studies have measured glutathione peroxidase activity (GSH-Px) as opposed to measuring reduced glutathione (GSH), where the latter is considered a more accurate indicator of the antioxidant capacity of a cell (Bernabucci et al., 2005). It was shown that GSH-Px activity was higher in post-calving than pre-calving cows suggesting oxidative stress (Bernabucci et al., 2005). However, we found no difference in GSH levels at any time of the sampling period, nor between the sampling groups during the pre-breeding period. Our observation of higher FRAP in cows than heifers does suggest that there may be hepatic adjustments in transition cows to alleviate any oxidative stress due to parturition or lactational stress. In line with this, a previous study demonstrated that primiparous cows experienced an increase in FRAP from the pre-calving to post-calving period (Wullepit, 2009).

Taken together, primiparous and multiparous cows, compared to nulliparous heifers, experience greater metabolic imbalance from the transition period through the voluntary waiting period when the processes of oocyte activation, follicle development and ovulation (80-100 days) are taking place. Our observations of slight differences specifically with cholesterol fractions between primiparous and multiparous cows suggest that metabolic changes may contribute to the decline in fertility with every passing parity. We speculate that higher levels of BHBA, cholesterol, bile acids and the lower concentrations of glucose in multiparous cows may hinder the follicular microenvironment and the ability of a follicle to become competently dominant followed by ovulation. Experiments to test this hypothesis are needed. We suggest that individualized nutritional management for dairy cows of different parities may prove beneficial in increasing reproductive longevity and thus their lifetime production. Also, it could be reproductively advantageous to wait for the metabolic process to stabilize in lactating cows by adapting a longer voluntary waiting period that commensurate with milk production levels and parity, but of course the financial implications of increasing the calving interval need to be considered. Nonetheless, further studies are needed to understand how the altered metabolic profiles directly affect reproductive processes during early lactation when the cows are bred.

3.6 Conflict of Interest

None of the authors of the above manuscript has declared any conflict of interest.

3.7Acknowledgments

We would like to thank the Macdonald Campus Farm and Ferme Norline for access to their dairy cattle. In addition, we would like to thank Dr. Luis Agellon for use of his facility, as well as Dr. Roger Cue for assistance with statistical analysis. YS was supported by the Réseau Quebecois en Reproduction (RQR)-CREAT scholarship, Department of Animal Science Graduate Excellence Fellowship, and the Fonds de recherche du Québec – Nature et Technologie (FRQNT) (B2) and GW was supported by the Natural Science and Engineering Research Council- Undergraduate Student Research Awards (NSERC-USRA). Lastly, this study was supported by both FRQNT and NSERC grants (RD).

3.8 Figures

Figure 3.8.1: Experimental design. Holstein dairy cows were subject to weekly blood sampling from three weeks pre-calving until twelve weeks post-calving. Dairy heifers were subject to weekly blood sampling for a total of about one month. We designated an analysis overlap between heifers and cows, where the blood samples from heifers were matched to the period from four to eight weeks post-calving. In both scenarios, animals were nearing their respective planned time of breeding and thus, designated as the pre-breeding period. Animals were divided into three groups: Primiparous cows (N=8), Multiparous cows (N=7), and Nulliparous Heifers (N=14).

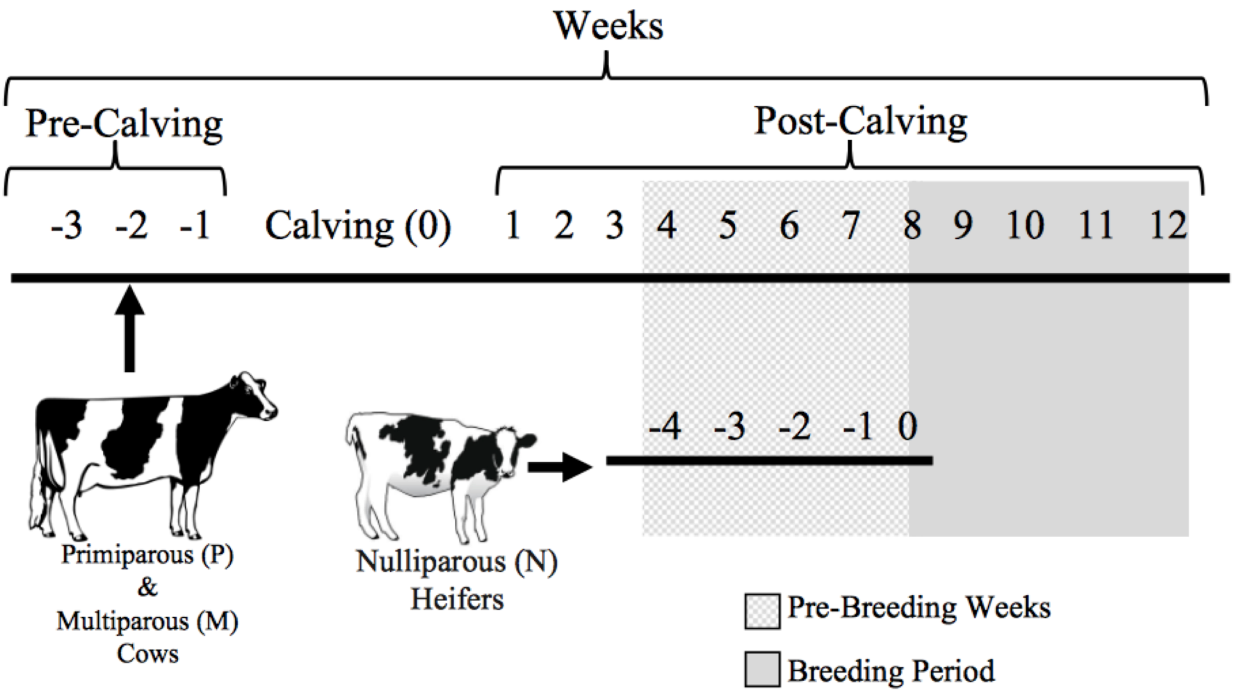


Figure 3.8.2: Milk yield of primiparous and multiparous dairy cows. Milk yields collected from week one to week twelve post-calving from primiparous and multiparous dairy cows. Data are expressed as a LS mean ± S.E.M. An overall contrast between Groups (Primiparous (P) and Multiparous (M)) is reported as significantly different with *P*<0.001.

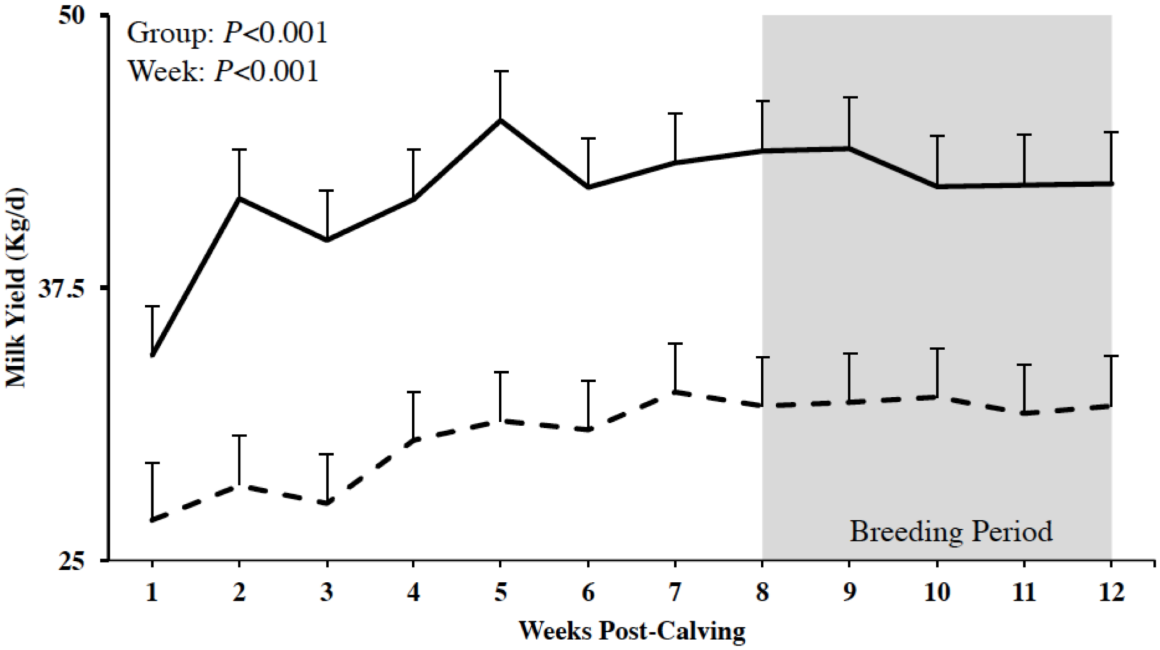


Figure 3.8.3: Plasma levels of β-hydroxybutyric acid (BHBA) in heifers and dairy cattle. Plasma levels of β-hydroxybutyric acid (BHBA) in heifers and dairy cattle. BHBA levels were measured pre-calving, at calving and at three time-points post-calving for lactating dairy cows (A). BHBA levels were compared between heifers, primiparous cows, and multiparous cows at an one week prior to planned time of breeding (B). Data are expressed as a LS mean ± S.E.M. An overall contrast between Groups (Primiparous (P) and Multiparous (M)) is shown (A). Gradual and significant increase in BHBA levels observed in nulliparous heifers compared to multiparous cows as designated by letter superscripts (B), *P*<0.05.

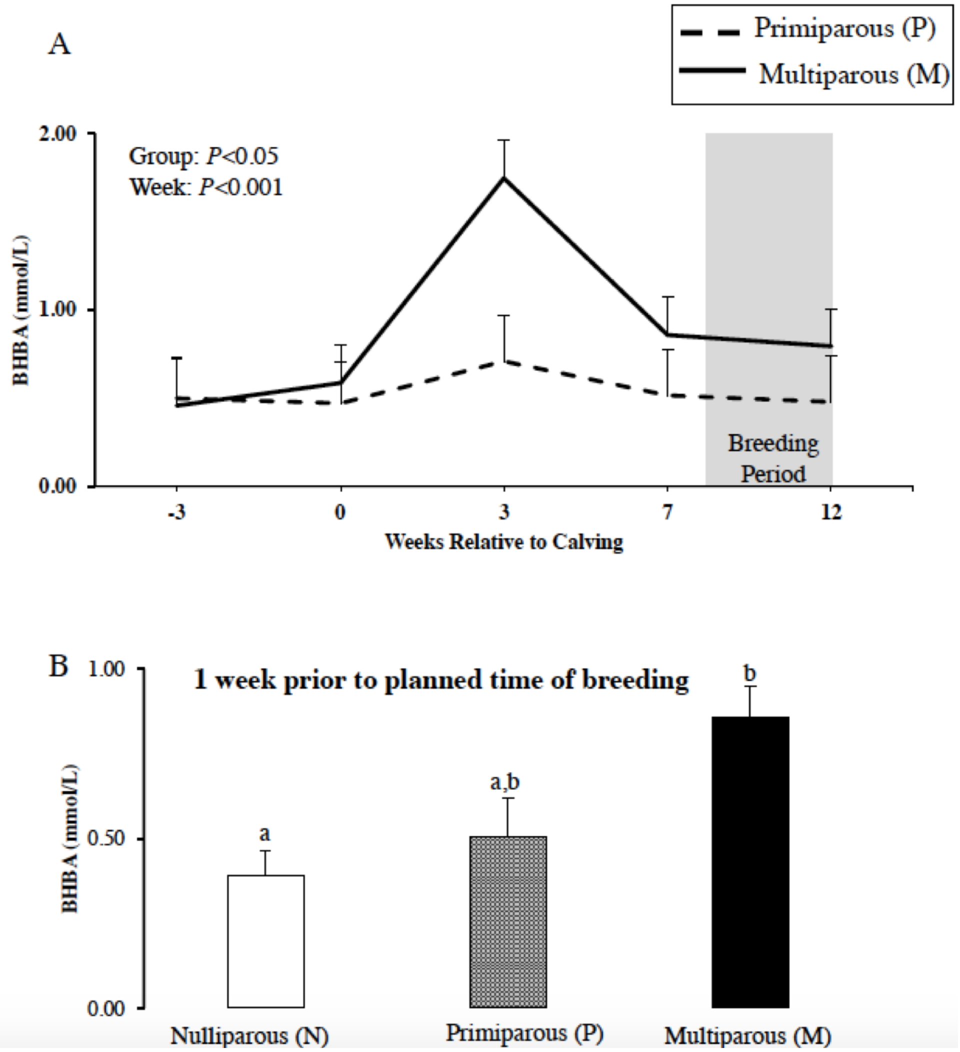


Figure 3.8.4: Profile of plasma metabolites from heifers and dairy cows. Glucose (A), total bile acids (B), and triglycerides (C) were measured weekly using commercially available assays from plasma of dairy cows sampled from three weeks pre-partum to twelve weeks post-partum and for a total of about one month in breeding aged heifers. Data are expressed as a LS mean ± S.E.M. An overall contrast between Groups (Primiparous (P), Multiparous (M), and Heifers (N)) in the pre-breeding period is shown by a significant difference, where *P*<0.05.

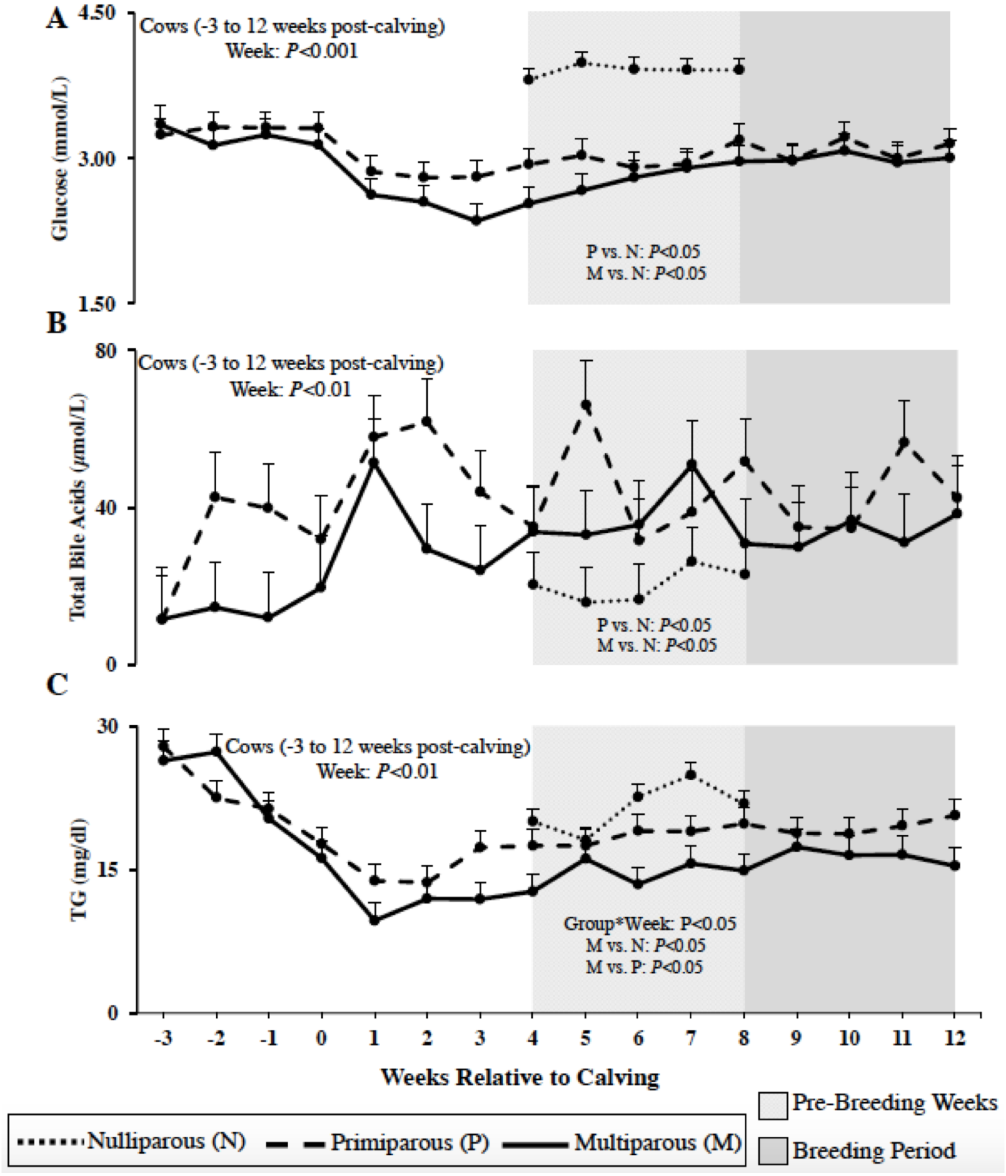


Figure 3.8.5: Representation of the plasma cholesterol profile from dairy cattle of different ages and physiological stages. Total-cholesterol, HDL-Cholesterol, LDL-Cholesterol, and VLDL Cholesterol were assessed weekly using commercially available assays or previously described calculations from plasma of dairy cows sampled from three weeks pre-partum to twelve weeks post-partum and for a total of five weeks in breeding aged heifers. Data are expressed as a LS mean ± S.E.M. An overall contrast between Groups (Primiparous (P), Multiparous (M), and Heifers (N)) in the pre-breeding period is shown by a significant difference, where $P<0.05$.

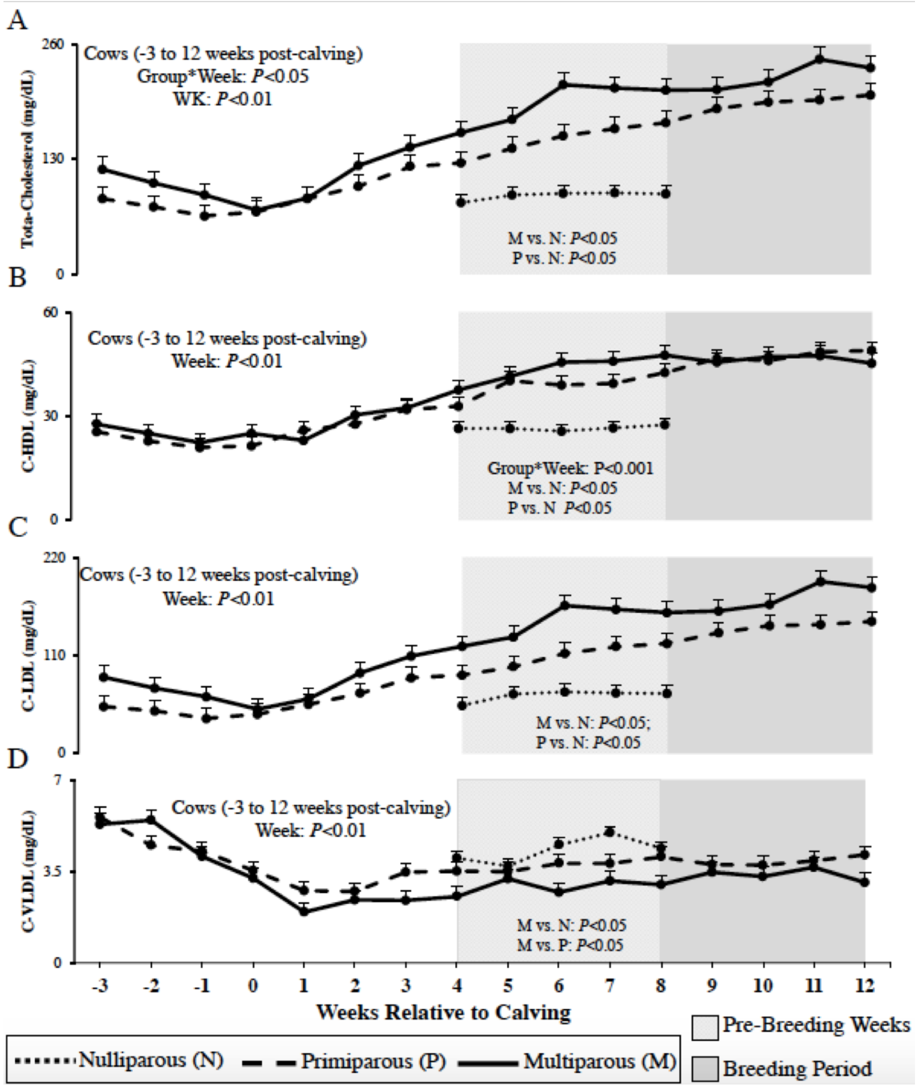
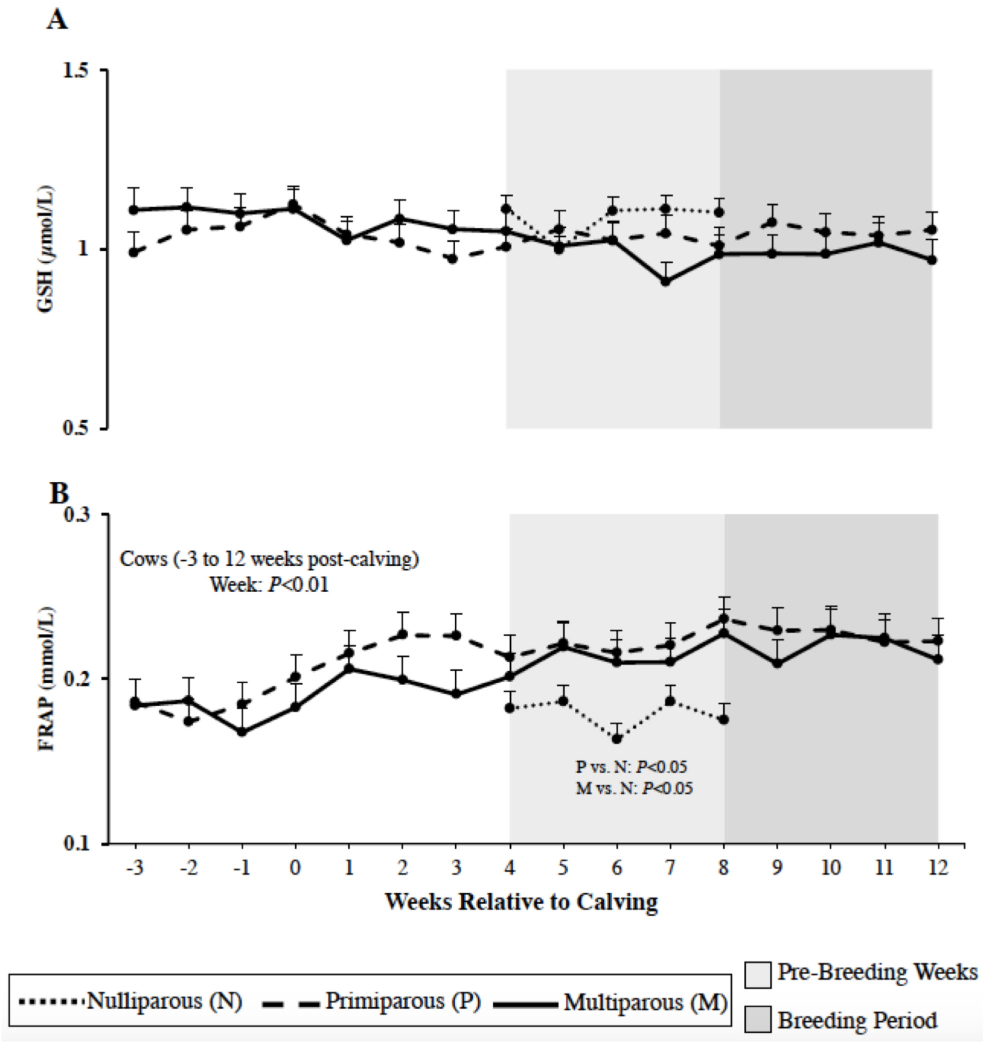
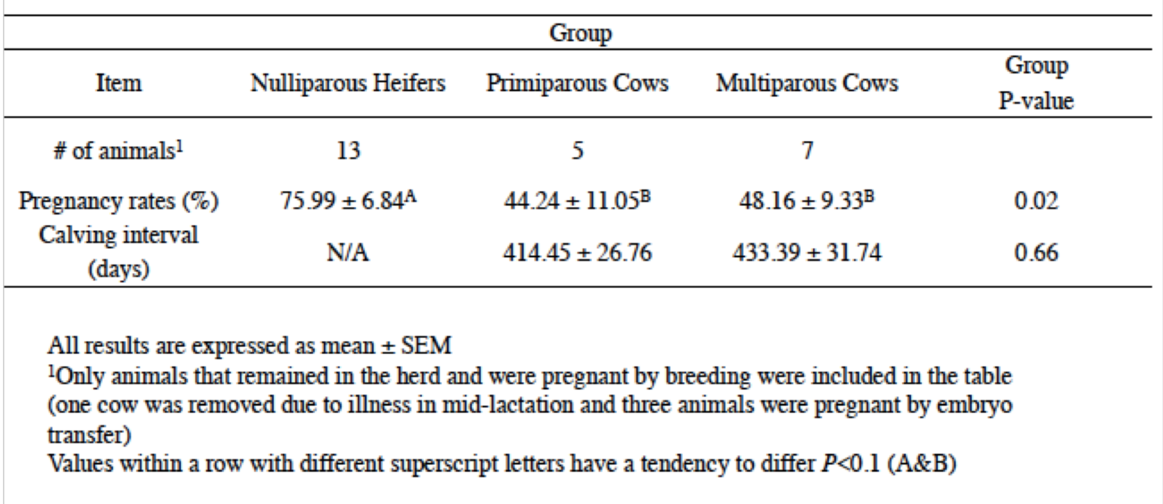


Figure 3.8.6: Oxidative stress markers measured in plasma of dairy heifers and cows. Oxidative stress markers measured in plasma of dairy heifers and cows. Blood samples were specifically processed as described in the material and methods to measure the concentration of glutathione in circulation of dairy heifers and cows using liquid chromatography-tandem mass spectrometry (LC/MS). Plasma samples from cattle were measured for FRAP using an adapted protocol as described in the materials and methods. Data are expressed as a LS mean ± S.E.M. An overall contrast between Groups (Primiparous (P), Multiparous (M), and Heifers (N)) in the pre-breeding period is shown by a significant difference, where *P*<0.05.



3.9 Table

Table 3.9.1: Effect of age and parity on reproductive performance



	Group			
Item	Nulliparous Heifers	Primiparous Cows	Multiparous Cows	Group P-value
# of animals[1]	13	5	7	
Pregnancy rates (%)	75.99 ± 6.84^{A}	44.24 ± 11.05^{B}	48.16 ± 9.33^{B}	0.02
Calving interval (days)	N/A	414.45 ± 26.76	433.39 ± 31.74	0.66

All results are expressed as mean ± SEM

[1] Only animals that remained in the herd and were pregnant by breeding were included in the table (one cow was removed due to illness in mid-lactation and three animals were pregnant by embryo transfer)

Values within a row with different superscript letters have a tendency to differ $P<0.1$ (A&B)

CONNECTING STATEMENT

In the first study, we assessed profiles of metabolic indicators and oxidative stress markers of primiparous cows, multiparous cows, and nulliparous heifers in the weeks leading up to anticipated time of breeding. We established that lactating cows experience lower levels of circulating glucose, triglycerides, and VLDL and higher levels of BHBA, total bile acids, total-cholesterol, HDL, and LDL compared to nulliparous heifers in the timeframe leading up to breeding. Moreover, the drastic changes in BHBA, total cholesterol, LDL, and triglycerides were most pronounced in multiparous cattle in the weeks leading up to breeding. Overall, we, like others before us, have shown that multiparous cows may experience a greater difficulty in adapting to early lactation compared to primiparous cows (Ruprechter et al., 2018).

The transition period is well-defined as a challenging period for cattle as they undergo negative energy balance (Drackley, 1999). Multiparous cattle are more likelu to undergo higher levels of metabolic stress and lipid mobilization to compensate for the energy required for maintenance and milk production than primiparous cattle. Furthermore, the increase in number of parities has been linked to decreased fertility (Balendran, 2008). Therefore, to better understand the impact of the transition period into the breeding period, we aimed to assess the effect of lipid mobilization on liver health, metabolism, and ovarian function in multiparous cattle.

In the following study, we investigated the impact of moderate and severe body condition loss of multiparous dairy cows from the transition period to the weeks leading up to anticipated time of breeding. We performed numerous liver biopsies in the aforementioned timeline as well as collected the dominant follicle in the 7th week of lactation, which is the week nearest to the typical voluntary waiting period of 60 days in milk. Changes in transcript abundance in hepatic tissue and granulosa cells was used to assess overall health and function of the liver and the ovary in response to varying degrees of lipid mobilization. Furthermore, metabolic and hormone profiles were measured in blood samples and the follicular fluid to provide additional information on the underlying factors contributing to changes in early lactation that may influence the molecular basis of fertility.

CHAPTER IV

This manuscript is being prepared for submission to "Reproduction".

Body condition loss on the metabolic, hepatic, and ovarian status of dairy cows

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Short title: Body condition loss and dairy cow fertility

Keywords: Body condition score, dairy cow, metabolites, dominant follicle, IGF1

4.1 Abstract

Monitoring the change in body condition score (BCS) of dairy cattle has become a useful tool to visually appraise lipid mobilization. The objectives of this study were to determine the impact of body condition loss of multiparous cows from 3 weeks pre-partum to 7 weeks post-partum on metabolism, liver function, and ovarian health. Cows were retrospectively grouped based on the units of body condition lost; 1) Moderate (MOD) Loss (<0.75 units) or 2) Severe (SEV) Loss (≥ 0.75 units). SEV cows had significantly higher BCS at time of parturition compared to MOD cows. Furthermore, SEV cows had higher levels of plasma non-esterified fatty acids, a marker of lipid mobilization, and γ –glutamyl transferase, suggesting an increased likelihood of liver disease. Analysis of the hepatic transcriptome revealed that *CYP7A1*, required to maintain homeostasis between cholesterol and bile acids, was highest in SEV cows. The potentially impaired liver function in SEV cows further explained the lower levels of plasma insulin like-growth factor 1 (IGF1) and also, IGF1 follicular fluid at 7 weeks post-partum, which plays an imperative role in fertility. The follicular microenvironment proved to be drastically different between the groups, where SEV cows had significantly lower estradiol concentrations, rationalized by lower granulosa cell transcript abundance of *CYP19A1*, *NR5A2, IGF1R* and *LHCGR*, all integral to the competence of the dominant follicle. A loss of ≥ 0.75 units of BCS during the transition period leading up to the planned start of breeding caused an unfavourable metabolic profile and microenvironment of the dominant follicle.

4.2 Introduction

The transition period, defined as 3 weeks pre-partum to 3 weeks post-partum, represents a physiologically challenging phase in a cow's productive lifetime (Grummer, 1995). With the onset of lactation, a dairy cow requires an abundance of energy to produce high volumes of milk (Grummer, 1995). This energy demand cannot be met simply with dietary intake; therefore, cows enter a state of negative energy balance (NEB) (Wankhade et al., 2017) and depend on lipid mobilization. Cows undergo varying degrees of lipid mobilization and in response release non-esterified fatty acids (NEFAs) from the adipose tissue (Drackley, 1999, Trevisi and Minuti, 2018). NEFAs are transported to the liver and are metabolized to generate glucose as well as an alternate source of energy in the form of ketone bodies such as β-hydroxybutyric acid (BHBA) (Drackley, 1999). However, the liver has limited capacities to metabolize NEFAs and the excess NEFAs are re-esterified into triglycerides (TGs) and exported as very low-density lipoproteins (VLDL). When the capacity to synthesize TGs in the liver exceeds the rate of TG removal through VLDL, the TGs accumulate in the liver leading to fatty liver (Goff and Horst, 1997, Bobe et al., 2004, LeBlanc, 2010).

NEB brings about dramatic alterations in the liver during the transition period and the altered metabolic outcomes have the potential to affect other physiological processes such as immunity and reproduction (LeBlanc, 2012). A number of groups have reported changes in the liver transcriptome associated with gluconeogenesis, fatty acid oxidation, and cholesterol metabolism during the transition period (Schlegel et al., 2012, Kessler et al., 2014, Gross et al., 2015, Ha et al., 2017).

The body condition score (BCS) chart (Edmonson et al., 1989) has been used as a management tool to visually evaluate changes of subcutaneous fat and thus, lipid mobilization (Wildman et al., 1982). The change in BCS during the transition period has been shown to impact the liver transcriptome in dairy cows from pasture-based systems (Akbar et al., 2015, Vailati-Riboni et al., 2016).

Early research has revealed that cows losing more than 1 unit of BCS in the first five weeks of lactation suffer from fatty liver, delayed days to first ovulation and estrus, and increased number of days to first service, with significantly lower first-service conception rates compared to cows losing less than 0.5 units (Butler and Smith, 1989). Others have shown that cows that lost BCS during the transition period as opposed to maintaining or gaining weight had higher levels of circulating NEFAs and BHBA (Carvalho et al., 2014b, Barletta et al., 2017). These same cows were less likely to resume estrous cycling by 50 days in milk and they had longer time to first post-partum ovulation. Lastly, cows losing body condition had lower percent pregnancy/artificial insemination at 32 and 60 days in milk (Barletta et al., 2017). It should be noted that over-conditioned cows at parturition are more likely to lose more BCS during the early post-partum period (Roche et al., 2007). Therefore, it has been suggested that an optimal BCS at time of parturition be 3-3.5, where a BCS loss during early post-partum should not exceed 0.5-1 BCS units (Butler, 2014, Drackley and Cardoso, 2014, Puppel and Kuczynska, 2016). Although it has been shown that excess lipid mobilization during the transition period negatively impacts reproductive performance (Britt, 1992, Lopez-Gatius et al., 2003, Bewley and Schutz, 2008, Carvalho et al., 2014b, Luttgenau et al., 2016, Barletta et al., 2017), the molecular mechanisms by which liver function is altered and competence of the dominant follicle is affected requires further investigation. To our knowledge, no one has yet simultaneously evaluated the impact of BCS loss on the hepatic gene profile and the follicular microenvironment of the dominant follicle from the transition period leading up to the onset of breeding. Therefore, the objective of the present study was to assess the effect of excess body condition loss on the aforementioned tissues from 3 weeks pre-partum to 7 weeks post-partum (week approaching the planned start of breeding).

4.3 Materials and Methods

4.3.1 Ethics statement

This study was conducted on two Canadian dairy farms: 1) the Macdonald Campus Farm from McGill University and 2) a commercial dairy farm located close to McGill University. All animal procedures were approved by the Animal Care Committee of the Faculty of Agricultural and Environmental Sciences of McGill University.

4.3.2 Animals and body condition scoring

Twenty multiparous Holstein dairy cows from two farms (Farm A = 14 cows and Farm B= 6 cows) were included in this study. We acknowledge that the number of animals used in this study is small. Cows from both farms were housed in a tie-stall setting and fed a base diet of alfalfa silage, corn silage, and soybean meal at the onset of lactation, while alfalfa silage and dry hay were predominantly fed during the dry period. All cows were milked twice daily at approximately 0600h and 1730h. Animals were subject to blood sampling, evaluation of the body condition score, liver biopsies, and ovarian follicular aspiration at select time-points relative to parturition from 3 weeks pre-partum to 7 weeks post-partum as shown in Table 4.8.1. This time-frame was selected to include the transition period (Grummer, 1995) to the week approaching the planned start of breeding (i.e week 7 post-partum)(Miller et al., 2007). The traditional voluntary waiting period represents the first 8 weeks post-calving at which point the onset of breeding commences, which was considered in developing the model for our study (Stangaferro et al., 2018). A sample may have been removed from a particular downstream analysis in case of minimum sample quantity and/or poor quality.

Evaluation of the body condition score (BCS) was conducted by 2 independent and trained evaluators using a 5-point scale with 0.25 increments, where 1= under-conditioned to 5= over-conditioned, developed by Edmonson, et al (1989) (Edmonson et al., 1989). Cows were retrospectively grouped by units of body condition score lost throughout the 10-week sampling period in one of two groups. 1) Cows losing less than 0.75 units were considered to be losing a moderate level of body condition: Moderate (MOD) Loss (<0.75 units) (N=11) or 2) Cows losing equal to or greater than 0.75 units were considered to be losing a severe level of condition: Severe (SEV) Loss (≥ 0.75 units) (N=9). A cut off point of 0.75-unit loss was selected as research and management services now suggest that during early post-partum cows should not exceed a loss of more than 0.5 - 1 BCS units, where minimizing overall loss is recommended (Jones, Butler, 2014). Furthermore, a group in Poland has recently used the same cut-off and observed changes in circulating metabolites, however, they used a shorter window of change (2 weeks pre-partum to 2 weeks post-partum) and did not investigate the impact on ovarian health and changes in gene expression in hepatic tissue(Šamanc, 2015). More specifically, some researchers have shown that under optimal conditions, the reproductive health of cows is best when the early post-partum cow is maintaining or gaining weight, while general BCS loss is disadvantageous (Carvalho et al., 2014b, Barletta et al., 2017).

4.3.3 Blood collection and biomarker analysis

Blood samples were collected from individual cows over a 10-week period from 3 weeks pre-partum to 7 weeks post-partum. Samples were obtained via venipuncture of the coccygeal vein at least 30 minutes prior to morning feeding. Both plasma and serum samples were collected from cattle in blood collection tube Monoject 10ml (ethylenediaminetetraacetic (EDTA) K3) and blood collection tube Monoject 10ml (serum), respectively, using 21G vacutainer needles. All samples were cooled down on ice, centrifuged for 10 min at 1500 rpm, and immediately frozen in liquid nitrogen until arrival at the laboratory, where they were stored in a -80°C freezer. Plasma samples were analyzed at the Animal Health Laboratory (University of Guelph, ON, Canada) for β-hydroxybutyric acid (BHBA), non-esterified fatty acids (NEFAs), glucose, γ -glutamyltransferase (GGT) and haptoglobin using a Roche Cobas 6000 c501 automated chemistry analyzer (Roche, Mississauga, Canada). All test reagents were supplied by Roche diagnostics (Indianapolis, IN) except for BHBA and NEFAs which were supplied by Randox Laboratories (Crumlin, UK). Total insulin-like growth factor 1 (IGF1) was measured in serum samples at week 7 post-partum only, was performed by the Vet Biomedical Sciences in Saskatoon, SK using the IMMULITE/IMMULITE 1000 IGF1 enzyme-labeled chemiluminescent immunometric assay (SIEMENS). Samples used for blood analysis: MOD; N=9 and SEV; N=8.

4.3.4 Liver biopsy

The liver was sampled on three mornings: 3 weeks pre-partum, week of parturition, and 7 weeks post-partum using the Tru-Cut type biopsy tool (Care Express Products, Cary, Illinois, USA). This procedure was conducted with the assistance of a trained veterinarian. First, the area of insertion was identified at the 10th intercostal space on the right side of the cow at approximately 2/3 from the top of the cow. Second, the area was thoroughly clipped and disinfected before the cow was administered with 2ml of 2% lidocaine solution (Lidocaine HCL 2%, Bimeda, Cambridge, CA), a local anesthetic. Third, a scalpel blade was used to cut the skin (2cm) for insertion of the 14G, six inch-long Tru-Cut needle. After collection, the area was treated with Blu-Kote (Blu-Kote, Dr. Naylor, Parry Sound, CA) wound spray. The Tru-Cut needle allowed for a collection of approximately 10-12mg of liver tissue as previously described (Herdt, 2013). The procedure was repeated two to three times on each collection day to obtain adequate amounts of hepatic tissue for downstream. The hepatic tissue was immediately frozen in liquid nitrogen until arrival at the laboratory, where it was stored in a -80°C freezer for downstream RNA extraction.

4.3.5 Follicular wave synchronization and granulosa cell and follicular fluid collection

Upon entering the 6th week of lactation, each cow was subjected to a follicular wave synchronization protocol as previously described by our group (Sanchez et al., 2014). In brief, on the first day, the ovaries were examined by transrectal ultrasonography using a 7.5MHz linear-array transducer, where all follicles greater than 5mm were aspirated by transvaginal aspiration. On the same day, cows were administered with 2ml of a synthetic prostaglandin Lutalyse® 5mgl/ml (103843, CDMV, Saint-Hyacinthe, QC, Canada) and an intravaginal progesterone release insert containing progesterone CIDR 1380 Intravaginal Progesterone (108316, CDMV, Saint-Hyacinthe, QC, Canada). This treatment allowed for the emergence of a new follicular wave between 36-48 hours post follicular aspiration. Six days later (in the 7th week of lactation), the CIDR implant was removed and follicular fluid and granulosa cells from the dominant follicle (>10mm) were collected by ultrasound-guided intrafollicular aspiration. In addition, the system was flushed with phosphate buffered saline to remove the remaining granulosa cells. The procedure was conducted with the assistance of a trained veterinarian. All aspirated fluids were centrifuged at 3000 rpm for 7 minutes to collect granulosa cells. Both follicular fluid and granulosa cells were snap frozen in liquid nitrogen and stored at -80°C until further analysis.

4.3.6 Follicle and Follicular fluid analysis

Follicle size was measured by ultrasonography at time of collection. Hormones were measured in the follicular fluid: estradiol (E_2), Progesterone (P_4), & IGF1 as well as total bile acids. E_2 levels in the follicular fluid were measured using the multispecies E_2 ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). Samples were diluted as necessary to fit within the standard curve. . P_4 levels in the follicular fluid were measured using the Progesterone ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). The intra- and inter-assay coefficients of variation for the E_2 3.48% and 14.86%, respectively and for and P_4 assays they were 6.47% and 14.44%, respectively. Total bile acids were measured in the follicular fluid by means of the total Bile Acid kit from Diazyme Laboratories (DZ042A-K, BioPacific Diagnostics Inc., North Vancouver, BC, Canada) following the manufacturer's protocol. The intra- and inter-assay coefficients of variation were 6.56% and 8.97%, respectively. Samples used for the aforementioned analyses: MOD; N=9-11 and SEV; N=6-9.

4.3.7 RNA extraction and Real-Time PCR

Total RNA was purified from hepatic cells using TRIzol® (Thermo Fisher Scientific [Life Technologies, Inc.], Burlington, ON, Canada) and from granulosa cells using the Direct-Zol RNA MiniPrep Isolation kit (R2050, Zymo Research, Cedarlane Laboratories, Burlington, Canada) as per manufacturer's protocol. Samples were quantified by absorbance at 260nm using the DeNovix DS 11 FX+ Spectrophotometer/Fluorometer. Quality assessment of samples for downstream analysis revealed lower number of liver samples from MOD (N=7-8) and SEV (N=5-6) and from granulosa cells MOD (N=5) and SEV (N=4). Complementary DNA was synthesized from 250ng of total RNA using the iScript cDNA Synthesis kit (Bio-Rad, Mississauga, Canada) with the following temperature program: 25°C for 5 min (Priming), 46°C for 20 min (Reverse transcription) and 95°C for 1 min (Reverse transcription inactivation). All qPCR assays were performed using previously described protocols(Schuermann et al., 2018). Sample dilution factors for hepatic tissue and granulosa cells were 1:30 and 1:50, respectively. Relative transcript abundance for each gene of interest was calculated by dividing their respective starting quantity (SQ) values by the mean SQ values of reference genes in hepatic tissue (*ACTB* and *L19*) and granulosa cells (*ACTB, CYCLOPHILIN,* and *L19*). Furthermore, hepatic tissue was also normalized to 3 weeks pre-partum time point. The primer sequences of transcripts measured in this study can be found in Table 4.8.2, where primer design was performed using the NCBI Primer-BLAST. If variants of a gene are present, the primers were designed to include all variants.

4.3.8 Statistical Analysis

All data were analyzed with PROC MIXED of SAS (SAS 9.4 Inst. Inc., Cary, NC, USA). For analyses where the various time points were not equally spaced, an exponential correlation covariance structure SP (POW) was used for repeated measures. Data were checked for normality using Shapiro-Wilk (PROC UNIVARIATE) and if needed were log transformed. Post-analysis, all data were back transformed for graphical presentation. The random effect was cow nested within stage of production (primiparous (P), multiparous (M), and heifers (H)) and farm, while the fixed effect included farm, group, time and the interaction between time and group. Differences between groups were detected by Scheffés method. The data are presented as LS means ± standard errors of the mean (SEM). The P-values of <0.01 and <0.05 were declared as significantly different, while 0.05 < P <0.1 were declared as tendencies for differences.

4.4 Results

4.4.1 Body condition score

Multiparous Holstein dairy cows from two farms were retrospectively grouped based on the change in body condition score (BCS) from 3 weeks pre-partum to 7 weeks post-partum. Cows experiencing a change of less than 0.75 units (0.38 units ± 0.08) of body condition had an initial body condition score of approximately 3.2 at 3 weeks pre-partum and approximately 2.8 at 7 week post-partum. These cows were grouped as Moderate (MOD) Loss. Cows losing equal to or greater than 0.75 units (1.08 units ± 0.10) had an initial body condition score around 3.6 at 3 weeks pre-partum and approximately 2.6 at 7 week postpartum. These cows were grouped as Severe (SEV) Loss. Analysis of BCS profile from week -3 to +7 relative to parturition showed that there was no time effect on the BCS in MOD cows (Figure 4.7.1; *P*>0.05). However, SEV cows have lower BCS at week 7 post-partum compared to 3 weeks before and the week of parturition (Figure 4.7.1; *P*<0.05). Also, SEV had a higher BCS than MOD cows in the week of parturition (Figure 4.7.1; *P*<0.05). Moreover, normalizing the %BCS lost during the sampling period to three weeks pre-partum revealed a decrease in BCS from 100% to 88% in MOD cows and 100% to 70% for SEV cows (Figure 4.7.1).

4.4.2 Plasma metabolites

We evaluated NEFA and BHBA concentrations in the plasma samples collected at 3 weeks pre-partum, the week of parturition, and 1 and 7 weeks post-partum as they provide well-defined markers of lipid mobilization. The SEV cows tended to have higher overall levels of NEFA during the period from 3 weeks pre-partum to 7 weeks post-partum (Figure 4.7.2 A; *P*<0.1). Within each group of cows, there was a significant effect of time where NEFA levels were higher at weeks 0 and 1 relative to parturition compared to the pre-partum period and week 7 post-partum (Figure 4.7.2 A; *P*<0.05). Surprisingly, there was no effect of group on BHBA levels even though there were numerically higher BHBA levels post-partum than pre-partum period (Table 4.8.3; *P*>0.05). The levels of GGT, a marker of liver injury, were higher in SEV than MOD cows during the weeks 1 and 7 post-partum (Figure 4.7.2 A; *P*<0.05). There were no significant differences between MOD and SEV cows with regard to other metabolites: glucose and haptoglobin (Table 4.8.3; *P*>0.05). Furthermore, the total bile acid concentration at 7 weeks post-partum was indifferent between the two study groups (MOD: 41.44 ± 9.69 μM and SEV: 43.26 ± 5.95 μM).

4.4.3 Hepatic gene expression

Genes involved in cholesterol metabolism were investigated in hepatic cells. Hepatic mRNA abundance was normalized to 3 weeks pre-partum. The overall transcript abundance of *CYP7A1* (maintaining cholesterol and bile acid homeostasis) was higher in SEV cows compared to MOD cows (Figure 4.7.2 B; *P*<0.05). However, all other genes investigated for cholesterol transport (*ABCA1*), esterification of cholesterol *(ACAT1),* nuclear receptor/ cholesterol level sensor *(LXRα),* and lipoprotein receptors *(SCARB1* and *LRP8)* mRNA expression neither showed differences between the weeks of sampling nor between the two groups of cows (Table 4.8.4; *P*>0.05).

4.4.4 Follicular development and steroidogenesis

Ultrasonography showed that there was no difference in the size of the dominant follicle of the synchronized follicular wave between MOD and SEV cows at 7 weeks post-partum (Figure 4.7.3 A; *P*>0.05). However, the follicular fluid of the dominant follicles from SEV cows had lower estradiol concentration compared to those from MOD cows (Figure 4.7.3 B; *P*<0.05). There was no difference in follicular fluid progesterone levels in the dominant follicles of the two groups (data not shown).In addition, the total bile acid concentrations in the follicular fluid were not different between MOD & SEV cows groups (MOD: 40.89 ± 2.63 μM and SEV: 44.42 ± 7.04 μM).

4.4.5 IGF1 in circulation and follicular fluid

The serum concentrations of total IGF1 were lower in SEV than MOD cows at 7 weeks post-partum (Figure 4.7.3 C; *P*<0.01). This difference between the two groups of cows was represented in the follicular microenvironment. The follicular fluid of the dominant follicles from SEV cows had lower total IGF1 than those from MOD cows (Figure 4.7.3 C; *P*<0.01).

4.4.6 Granulosa cell mRNA abundance of the dominant follicle

To investigate the molecular basis of reduced estradiol levels in the dominant follicles of SEV cows, we measured gene expression in granulosa cells from MOD and SEV cows at 7 weeks post-partum. Transcript levels of *CYP19A1* were lower in granulosa cells of SEV compared to MOD cows (Figure 4.7.4; $P<0.05$). Furthermore, *NR5A2* and *IGF1R* transcript abundance were also lower in SEV than MOD cows (Figure 4.7.4; $P<0.05$). The luteinizing hormone receptor (*LHCGR*) mRNA abundance tended to be lower in granulosa cells of SEV cows (Figure 4.7.4; $P<0.1$).

4.5 Discussion

This study covered a 10-week period, from 3 weeks pre-partum to 7 weeks post-partum, where MOD and SEV cows lost on average approximately 0.4 and 1.0 BCS, respectively. Some initial studies investigating the impact of BCS loss on reproductive performance were conducted by Dr. Jack Britt. His early retrospective study showed that cows lose the most amount of body condition in the first five weeks post-partum. He showed that a loss of 0.58 in this time-period increased the days from calving to first ovulation and drastically reduced first service conception rates to 25% and overall conception rates to 42% when compared to cows that maintained body weight and had first service conception rates of 62% and overall conception rates of 61% (Britt, 1992). Previous reports suggested that a loss of greater than 1.0 BCS in the first 30 days post-partum increased number of days to first ovulation (Beam and Butler, 1999). A meta-analysis revealed that cows losing more than 1.0 BCS in early-lactation were more susceptible to a longer voluntary waiting period (calving to the onset of breeding), which subsequently increased the likelihood of culling of such cows (Lopez-Gatius et al., 2003). In this study, we investigated metabolic profiles, hormone concentrations and gene expression of hepatic and ovarian tissue and found that a loss of ≥ 0.75 BCS is sufficient to have drastic implications leading to compromised post-partum dominant follicle competence.

SEV cows fell in the category previously identified in other studies as unfavourable levels of loss (Kim and Suh, 2003). Cows losing greater BCS are also the cows that have a higher BCS at parturition and are likely the cows with reduced appetite due to being over-conditioned (Britt, 1992, Rukkwamsuk et al., 1999, Roche et al., 2007, Bewley and Schutz, 2008) . Multiple studies have established that excess BCS prior to parturition is linked with impaired reproductive performance (Buckley et al., 2003, Roche, 2006, Roche et al., 2009, Stefańska et al., 2016). One key characteristic of excess lipid mobilization is circulating NEFAs. We observed a tendency for higher levels of NEFA in SEV cows, with pronounced peaks at both parturition and 1 week post-partum. In line with this, several studies have reported that higher levels of NEFAs were associated with greater body condition loss during early lactation (Akbar et al., 2015, Luttgenau et al., 2016, Barletta et al., 2017). High levels of NEFA during early-lactation in cows, which are over-conditioned at parturition, have negative effect on reproduction with delayed time to first ovulation and decreased conception rates (Rukkwamsuk et al., 1999). Culturing bovine granulosa cells in the presence of palmitic and stearic acid, predominant NEFAs found in the follicular fluid, has been shown to decrease their proliferation, but enhance estradiol production (Vanholder et al., 2005). However, we did not observe any change in follicular fluid NEFA composition or concentrations between our groups of cows at 7 weeks post-partum. We did note reduced *CYP19A1* expression and lower estradiol concentrations in the dominant follicles of SEV cows at 7 weeks post-partum. These observations suggest that NEFAs are less likely to directly dysregulate the dominant follicles as their levels return to normal range by breeding period. Further, these data do not disqualify the potential direct effects of NEFAs on ovarian functions if their concentrations are higher at the planned time of breeding.

We also observed overall higher concentrations of GGT in plasma of SEV cows. GGT is an enzyme which is highly sensitive and provides a helpful marker in assessment of liver function, where enhanced GGT has been associated with hepatic diseases including fatty liver (Sevinc, 2001). A recent study showed that cows maintaining higher levels of GGT throughout early-lactation were found to be anovular (Kawashima et al., 2016). It should be mentioned that we did not diagnose fatty liver disease in cows of this study. Nonetheless, in line with our data, increased GGT and hepatic expression of *CYP7A1* were seen in rats with non-alcoholic fatty liver *(Lionarons et al., 2016)*. Those authors suggested that fatty liver promotes bile salt synthesis by enhancing *CYP7A1* transcription (Lionarons et al., 2016). Previous reports have identified hepatic *CYP7A1* expression induced by lactation in both the mouse(Wooton-Kee et al., 2010) and bovine (Schlegel et al., 2012, Liang et al., 2017). Our results suggest that an increase in hepatic *CYP7A1* expression, may be even more pronounced in cows experiencing a greater body condition loss in early lactation.

To our knowledge, we are the first to investigate the relationship between the change in BCS during the transition period leading up to breeding and hepatic gene expression in cows under a conventional management system. Limited research has addressed the impact of BCS on hepatic gene expression during the transition period(Akbar et al., 2015, Vailati-Riboni et al., 2016). Under grazing-management, a recent study investigated hepatic gene expression, where cows with higher BCS at calving (approximately 3.3 on a 1-5 scale(Roche et al., 2004)/ high for pasture-based system) had a greater mRNA abundance of ketogenesis, hepatokines and fatty acid oxidation. Most interestingly, these cows had lower hepatic expression of genes related to the growth hormone/IGF-1 axis, including growth hormone receptor 1 A *(GHR1A)* and *IGF1 (*involved in IGF1 production) by 5 weeks post-partum(Akbar et al., 2015). IGF1 is a key metabolic hormone involved in ovarian folliculogenesis (Lucy et al., 1992b, Spicer et al., 1994, Beam and Butler, 1999). The liver is responsible for the majority of IGF1 synthesis, although IGF1 can also be produced locally by the ovary (Sjogren et al., 1999, Velazquez et al., 2008). At 7 weeks post-partum, there were lower levels of serum and follicular fluid IGF1 in SEV than MOD cows. This observation adds to our previous data that early-lactating cows have lower levels of IGF1 in both circulation and follicular fluid of the dominant follicle compared to heifers (Sanchez et al., 2014). Given that heifers do not have loss of BCS around breeding period, they are expected to have higher IGF1 and thus, healthier follicles. It has been shown that cows in NEB experience uncoupling of the growth hormone/IGF1 axis, which in turn decreases hepatic IGF1 synthesis (Santos et al., 2012). In mice, the *igf1* knockout model has been shown to be infertile as follicles were unable to grow past the antral stage and ovulate(Baker et al., 1996). In cattle, the marked decrease in IGF1 can have dire consequences on reproductive performance, where lower plasma concentrations of IGF1 in early lactation are linked with decreased follicular competence, estrogen-inactive dominant follicles, anovulation, lower conception rates to first service and longer interval to commencement of luteal activity (Beam and Butler, 1997, 1999, Patton et al., 2007, Castro et al., 2012, Dupont et al., 2014). Circulating IGF1 assists in the induction of *LHCGR* expression in granulosa cells of the dominant follicle (Spicer and Echternkamp, 1995, Beam and Butler, 1999, Rawan et al., 2015). Therefore, lower IGF1 concentrations would explain the tendency of decreased *LHCGR* expression observed in granulosa cells of SEV cows.

The presence of IGF1 is involved in steroidogenesis and proliferation of granulosa cells, where it increases FSH-induced estradiol production in granulosa cells of larger follicles (Spicer et al., 1993). A recent study showed that IGF1 alone increased expression of *CYP19A1*, in cultured granulosa cells, in the absence of FSH (Stocco, 2008, Mani et al., 2010). The increase in I*GF1R* expression is also associated with increase in granulosa cell estradiol production in a growing and differentiating dominant follicle (Spicer et al., 1994, Beam and Butler, 1999). Moreover, mice null of *Igf1r* gene are infertile (Baumgarten et al., 2017). In line with these data, SEV cows had lower *IGF1R* expression, which appears to be at least in part responsible for reduced estradiol synthesis in the dominant follicles of SEV cows.

Even though *Nr5a2* deletion was associated with increased *Cyp19a1* expression in mice (Duggavathi et al., 2008), there is no consensus on the importance of the nuclear receptor for estrogen synthesis in granulosa cells. Knockdown of *Nr5a2* resulted in abrogation of testosterone induced expression of *Cyp19a1* in rat granulosa cells (Saxena et al., 2007, Wu et al., 2011). A recent study demonstrated that overexpression of *NR5A2* in luteinized bovine granulosa cells modestly increased the activity of bovine *CYP19A1* promoter activity, suggesting that *NR5A2* plays a role in *CYP19A1* expression (Sahmi et al., 2014). Taking these data together, our data of lower *NR5A2* expression in SEV granulosa cells may also be, at least in part, responsible for lower *CYP19A1* expression and estradiol production in the dominant follicles of these cows.

In conclusion, although others have looked at more dramatic changes in body condition loss in early lactation (Kim and Suh, 2003), we show that even at 0.75 loss from 3 weeks pre-partum to 7 weeks post-partum, hepatic and metabolic profile in lactating cows are altered. These metabolic perturbations were associated with reduced follicular health in cows that had severe loss of BCS. The take-home message from this study is to minimize a dairy cow's *loss* in BCS from the transition period through the planned time of breeding. On-farm nutrition management strategies need to be implemented to prevent over-conditioning in the pre-partum period (Cardoso et al., 2013, Drackley and Cardoso, 2014) to ease adaptation into early-lactation by properly monitoring energy components in the ration. Proper feed management in the late-dry period are crucial in order to reduce negative energy balance even prior to the onset of lactation(Britt, 1992). In addition, recent research has shown that intramuscular administration of vitamin B12 and folic acid during the transition period may favour granulosa cell function (Gagnon et al., 2015) and feeding of rumen-protected methionine and choline increased dry matter intake, regulated hepatic lipid metabolism and immune function (Sun et al., 2016) and also, increased the survival capacity of preimplantation embryos (Acosta et al., 2016). By preventing drastic body condition loss in early lactation cows will have less fluctuations in blood metabolites and improved liver health, with higher levels of IGF, which positively regulates acquisition and maintenance of the dominant follicle for eventual ovulation

4.6 Acknowledgements

We would like to thank the Macdonald Campus Farm and Ferme Norline for access to their cattle. In addition, we would like to thank Santiago Palacio for training in BCS evaluation, as well as Dr. Roger Cue for assistance with statistical analysis. YS was supported by the Réseau Quebecois en Reproduction (RQR)-CREAT scholarship, Department of Animal Science Graduate Excellence Fellowship, and the Fonds de Recherche du Québec – Nature et Technologie (FRQNT) (B2). Lastly, this study was supported by both FRQNT and NSERC grants obtained by Dr. RD.

4.7 Figures

Figure 4.7.1: Body condition score (BCS). Graph of the change in body condition score from 3 weeks pre-partum to 7 weeks post-partum from retrospectively grouped cows losing Moderate (MOD) or Severe (SEV) units of BCS. All data are expressed as a mean ± S.E.M, where significant differences over time within a group are labelled with superscripts A and B and significant differences between the two groups at a particular time-point are labelled with the superscript Z, *P*<0.05. In addition, the loss of body condition represented as a percentage and normalized to 3 weeks pre-partum.

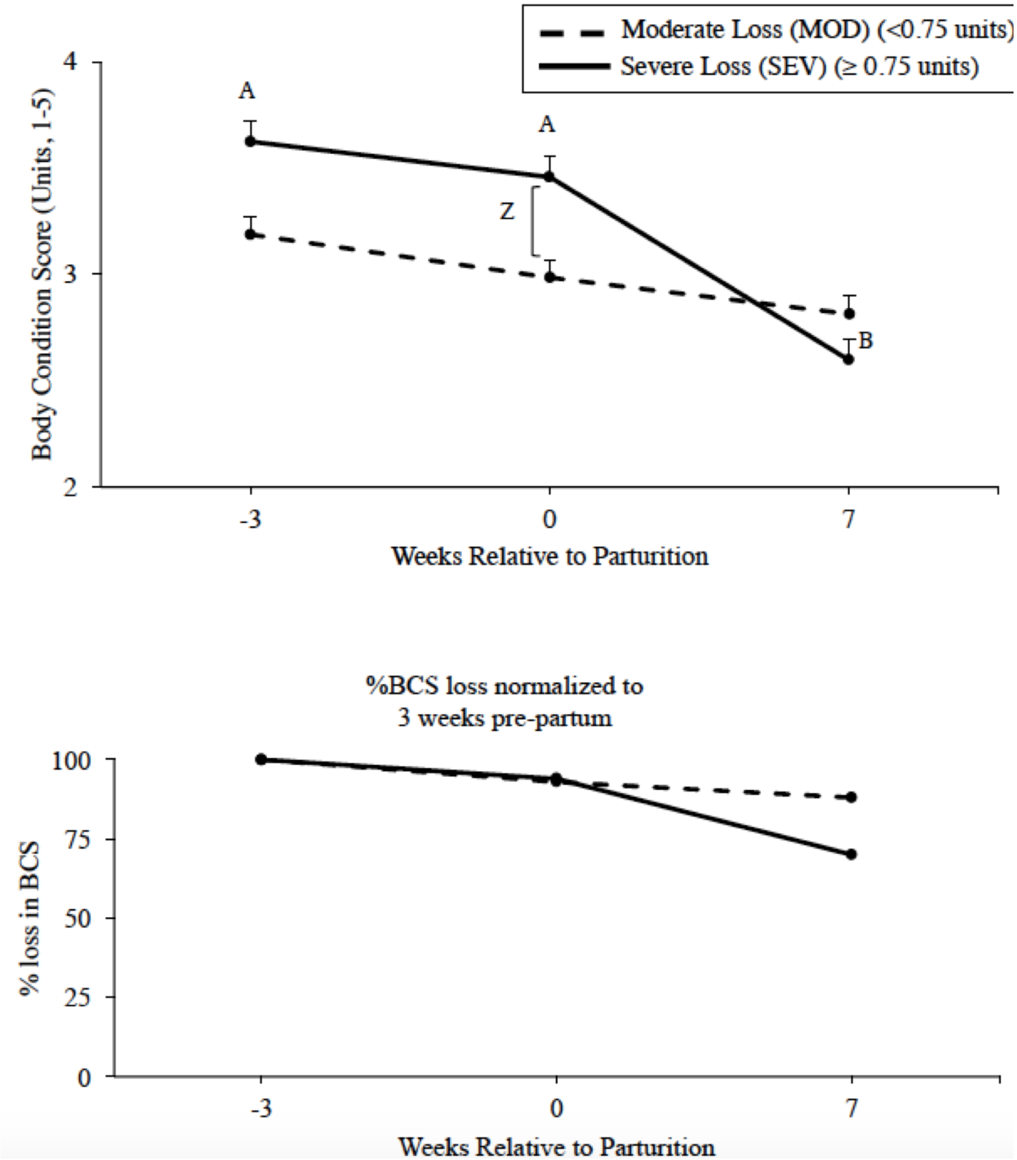


Figure 4.7.2: Metabolic and hepatic profile of cows losing different degrees of BCS units. Plasma concentrations of NEFA and GGT in weeks 3 pre-partum, week of parturition, and weeks 1 and 7 post-partum from cows losing Moderate (MOD) or Severe (SEV) units of body condition score throughout the sampling period. Changes over time within a group are labelled with superscripts A and B, *P*<0.05. Changes between the groups MOD and SEV over the entire sampling period are marked with *P* values, where *P*<0.1 was considered to trend towards a difference, respectively (A). Hepatic cells were collected at 3 weeks pre-partum, week of parturition and 7 weeks post-partum. Transcript abundance of each gene was normalized to week 3 pre-partum and reference genes *ACTB* and *L19*. Changes between the groups MOD and SEV over the entire sampling period are marked with *P* values, where a *P*<0.05 was considered statistically different (B). All data are expressed as a mean ± S.E.M.

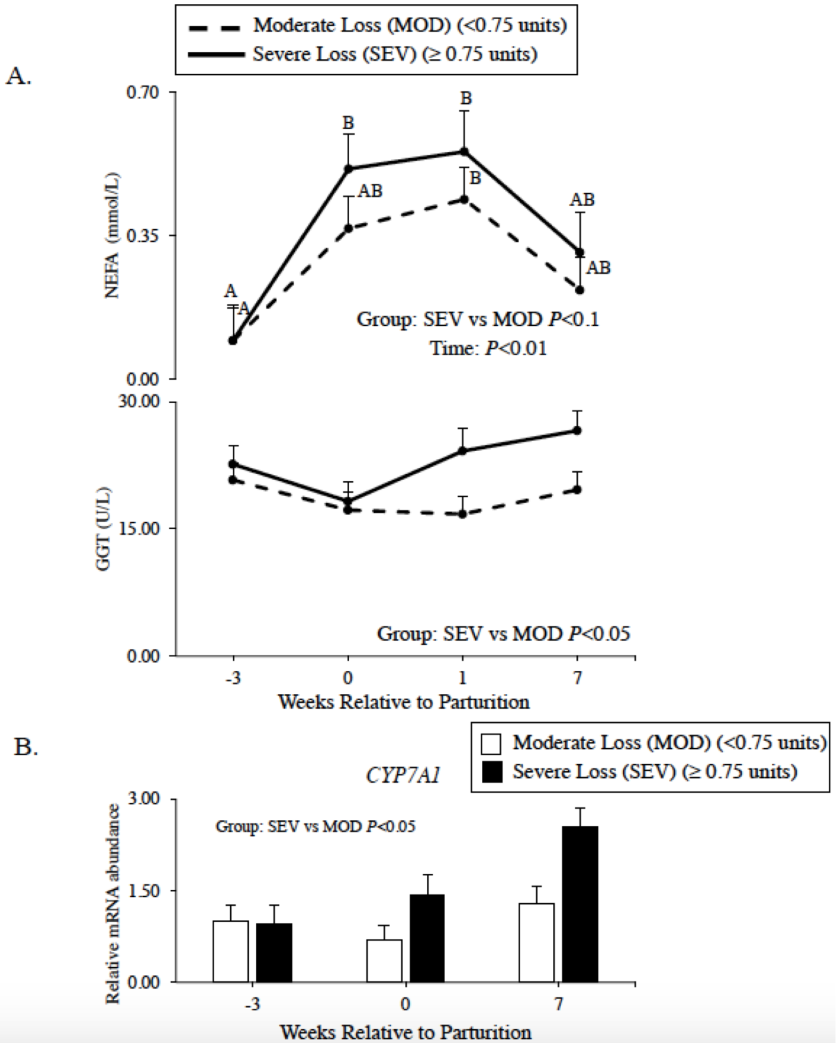


Figure 4.7.3: Follicle size and hormone analysis of the dominant follicle and serum in week 7 post-partum between cows experiencing a Moderate (MOD) or Severe (SEV) degree of body condition loss. The follicle size was measured for all cows at the time of follicular aspiration (A). Estradiol levels measured in the follicular fluid of dominant follicles (B). IGF1 concentrations measured in the follicular fluid and serum at 7 weeks post-partum (C). Significant differences between groups are marked with the superscript * (*P*<0.05) and ** (P<0.01), while tendencies for differences are marked with the superscript # (*P*<0.1). All data are expressed as a mean ± S.E.M.

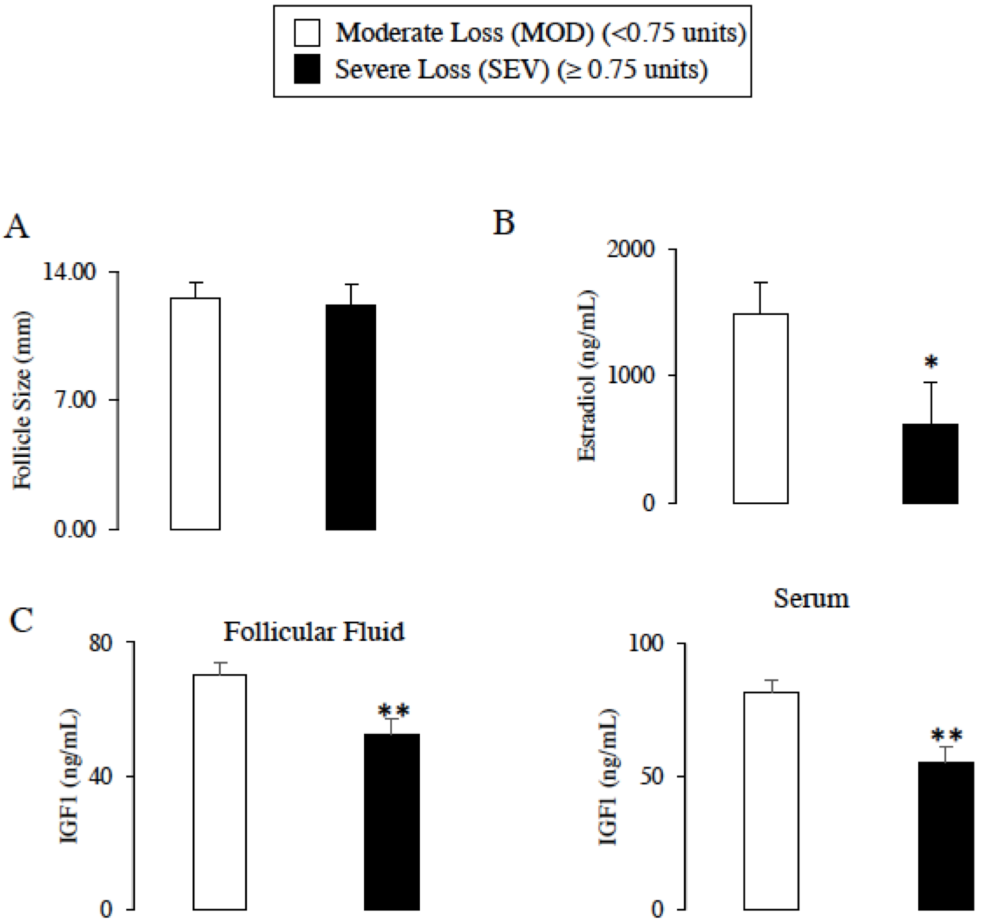
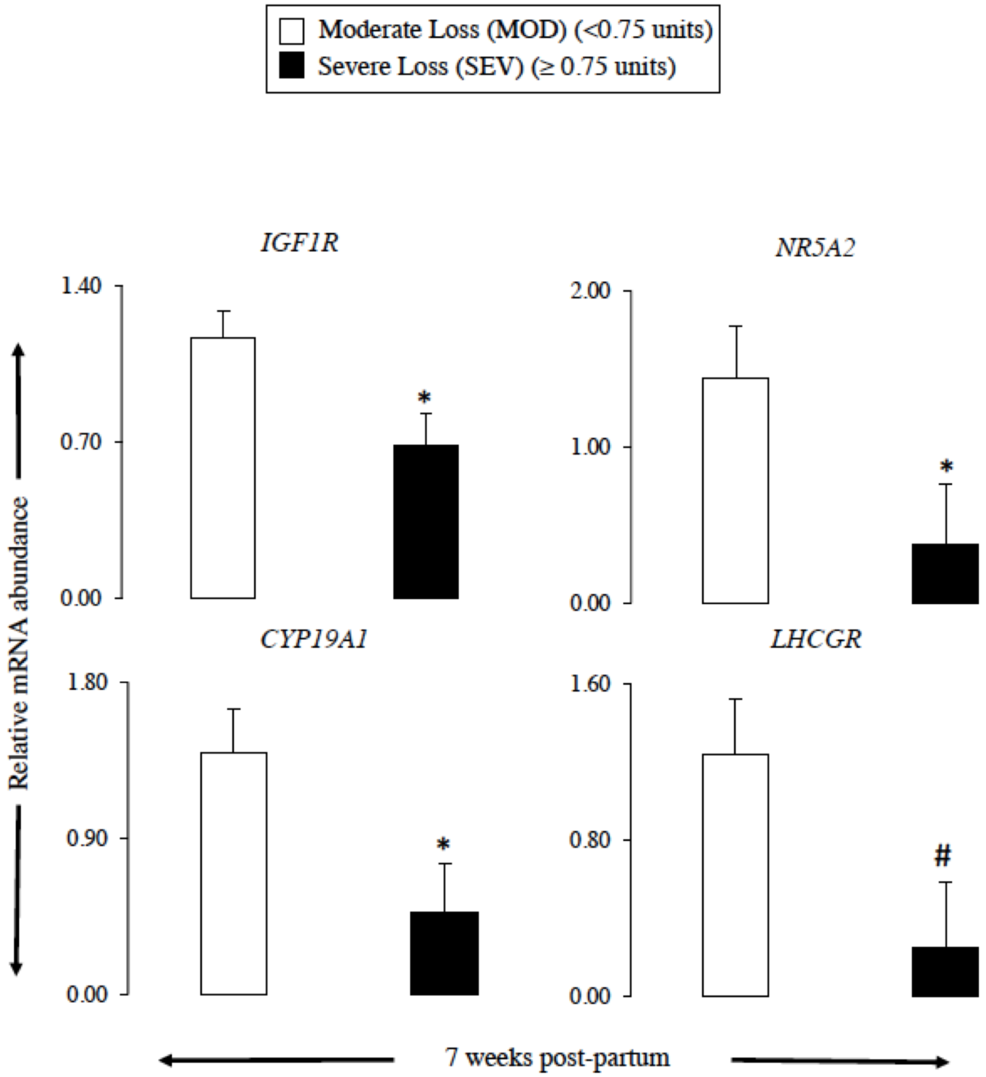
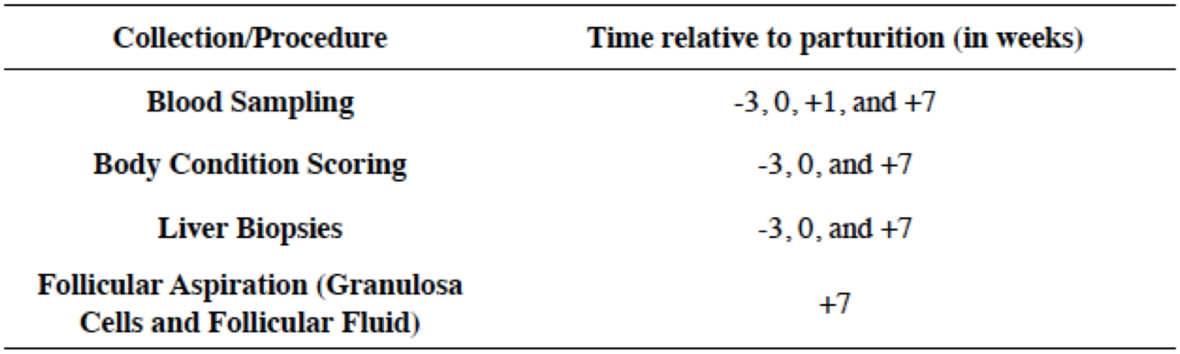


Figure 4.7.4: Transcript abundance (SQ) of *CYP19A1, NR5A2, IGF1R,* and *LHR* in granulosa cells of cows experiencing Moderate (MOD) or Severe (SEV) body condition loss from 3 weeks pre-partum to 7 weeks post-partum. Granulosa cells were collected in week 7 post-partum. Transcript abundance of each gene was normalized to reference genes *ACTB, L19*, and *CYCLOPHILIN*. Significant differences between groups are marked with the superscript * (*P*<0.05), while tendencies for differences are marked with the superscript # (*P*<0.1). All data are expressed as a mean ± S.E.M.



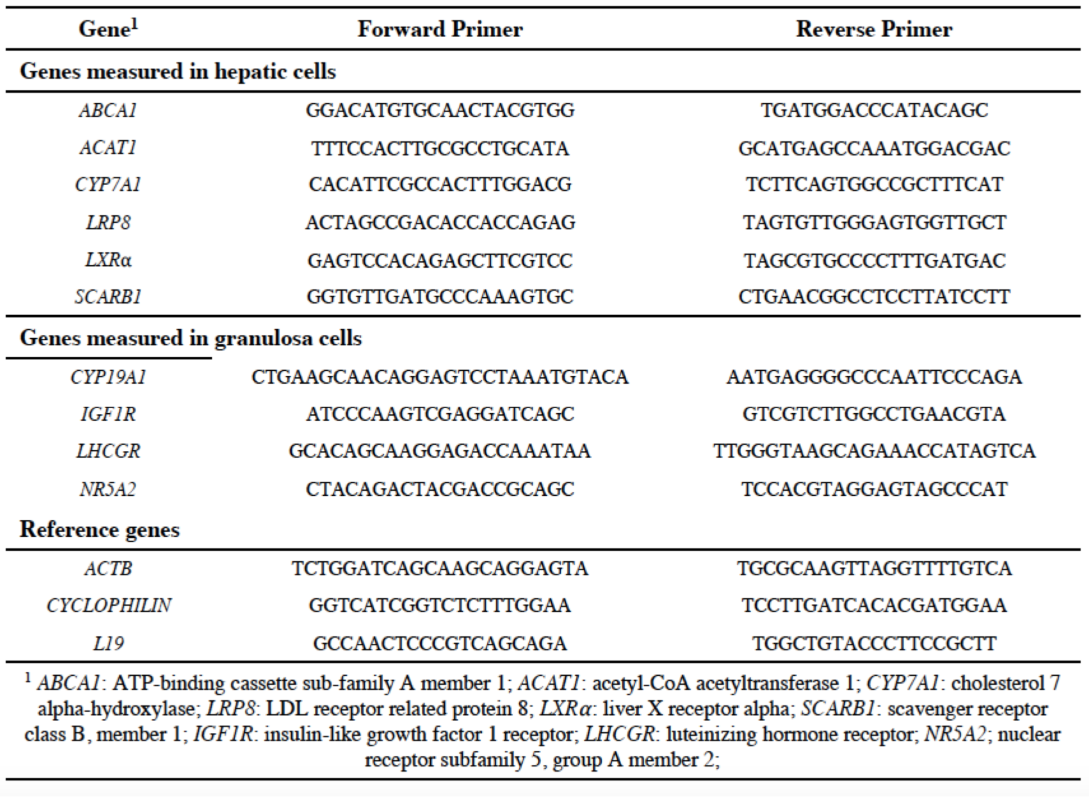
4.8 Tables

Table 4.8.1: Collection time-points for blood sampling, body condition scoring, liver biopsies, and follicular aspiration from 3 weeks pre-partum to 7 weeks post-partum.



Collection/Procedure	Time relative to parturition (in weeks)
Blood Sampling	-3, 0, +1, and +7
Body Condition Scoring	-3, 0, and +7
Liver Biopsies	-3, 0, and +7
Follicular Aspiration (Granulosa Cells and Follicular Fluid)	+7

Table 4.8.2: Primers used in real-time PCR experiments. All primers were purchased from Integrated DNA technologies (Skokie, U.S.A).



Gene[1]	Forward Primer	Reverse Primer
Genes measured in hepatic cells		
ABCA1	GGACATGTGCAACTACGTGG	TGATGGACCCATACAGC
ACAT1	TTTCCACTTGCGCCTGCATA	GCATGAGCCAAATGGACGAC
CYP7A1	CACATTCGCCACTTTGGACG	TCTTCAGTGGCCGCTTTCAT
LRP8	ACTAGCCGACACCACCAGAG	TAGTGTTGGGAGTGGTTGCT
LXRα	GAGTCCACAGAGCTTCGTCC	TAGCGTGCCCCTTTGATGAC
SCARB1	GGTGTTGATGCCCAAAGTGC	CTGAACGGCCTCCTTATCCTT
Genes measured in granulosa cells		
CYP19A1	CTGAAGCAACAGGAGTCCTAAATGTACA	AATGAGGGGCCCAATTCCCAGA
IGF1R	ATCCCAAGTCGAGGATCAGC	GTCGTCTTGGCCTGAACGTA
LHCGR	GCACAGCAAGGAGACCAAATAA	TTGGGTAAGCAGAAACCATAGTCA
NR5A2	CTACAGACTACGACCGCAGC	TCCACGTAGGAGTAGCCCAT
Reference genes		
ACTB	TCTGGATCAGCAAGCAGGAGTA	TGCGCAAGTTAGGTTTTGTCA
CYCLOPHILIN	GGTCATCGGTCTCTTTGGAA	TCCTTGATCACACGATGGAA
L19	GCCAACTCCCGTCAGCAGA	TGGCTGTACCCTTCCGCTT

[1] *ABCA1*: ATP-binding cassette sub-family A member 1; *ACAT1*: acetyl-CoA acetyltransferase 1; *CYP7A1*: cholesterol 7 alpha-hydroxylase; *LRP8*: LDL receptor related protein 8; *LXRα*: liver X receptor alpha; *SCARB1*: scavenger receptor class B, member 1; *IGF1R*: insulin-like growth factor 1 receptor; *LHCGR*: luteinizing hormone receptor; *NR5A2*; nuclear receptor subfamily 5, group A member 2;

Table 4.8.3: Plasma concentrations of glucose, BHBA, and haptoglobin in weeks 3 pre-partum, week of parturition, and weeks 1 and 7 post-partum from cows losing Moderate (MOD) or Severe (SEV) units of body condition score throughout the sampling period. Changes between the groups over the entire sampling period are marked with *P* values, where a *P*<0.05 was considered statistically different. All data are expressed as a mean ± S.E.M.

		Weeks Relative to Parturition								**P-value**
Parameter	**Group**	**-3**	**0**	**1**	**7**	**SEM (-3)**	**SEM (0)**	**SEM (1)**	**SEM (7)**	**Group**
Glucose, Mmol/L	MOD	3.78	3.51	3.22	3.54	0.12	0.12	0.12	0.12	0.56
	SEV	3.79	3.92	3.22	3.38	0.13	0.13	0.15	0.13	
BHBA, *μ*mol/L	MOD	377.07	594.51	614.40	440.85	112.35	112.35	112.35	112.35	0.22
	SEV	370.56	625.06	851.64	685.19	121.51	121.51	141.71	121.51	
Haptoglobin, g/L	MOD	0.36	0.59	0.76	0.14	0.19	0.19	0.19	0.19	0.35
	SEV	0.18	0.32	0.49	0.28	0.21	0.21	0.24	0.21	

Table 4.8.4: Relative mRNA abundance (SQ) of genes analyzed and unaltered in hepatic tissue of cows experiencing moderate (MOD) or severe (SEV) body condition loss from 3 weeks pre-partum to 7 weeks post-partum. Hepatic cells were collected at 3 weeks pre-partum, week of parturition and 7 weeks post-partum. Transcript abundance of each gene was normalized to week 3 pre-partum and reference genes *ACTB*, and *L19*. All data are expressed as a mean ± S.E.M.

		Weeks Relative to Parturition						**P-value**
Gene	**Group**	**-3**	**0**	**7**	**SEM (-3)**	**SEM (0)**	**SEM (7)**	**Group**
Cholesterol transport								
ABCA1	**MOD**	1.01	1.33	0.83	0.19	0.19	0.18	0.87
	SEV	0.99	1.22	0.83	0.23	0.22	0.21	
Cholesterol esterification								
ACAT1	**MOD**	1.01	0.78	1.15	0.17	0.16	0.17	0.49
	SEV	1.00	1.15	1.05	0.21	0.22	0.19	
Cholesterol level sensor								
LXRα	**MOD**	0.94	1.44	1.07	0.23	0.23	0.23	0.73
	SEV	0.92	0.97	1.39	0.26	0.28	0.25	
Lipoprotein receptors								
SCARB1	**MOD**	1.01	1.83	2.16	0.34	0.30	0.35	0.32
	SEV	0.95	1.39	1.81	0.38	0.37	0.41	
LRP8	**MOD**	1.00	2.53	1.36	0.45	0.45	0.42	0.67
	SEV	1.01	1.09	1.67	0.54	0.52	0.48	

CONNECTING STATEMENT 2

In the second study, we evaluated the change in BCS of multiparous dairy cows over a 10-week period starting from three weeks pre-partum to seven weeks post-partum. Cows were separated into two groups based on body condition loss: moderate (MOD) or severe (SEV). SEV cows had a higher BCS at time of parturition and a tendency for higher levels of circulating blood NEFAs during the trial period. In addition, we found that SEV cows had higher levels of circulating GGT suggesting compromised liver function. The hepatic tissue of SEV cows showed higher transcript abundance of *CYP7A1*, an important enzyme in cholesterol and bile acid homeostasis. The follicular microenvironment showed several differences which we have now linked with elevated lipid mobilization, including lower levels of follicular fluid estradiol and IGF1, in line with lower expression of *CYP19A1, NR5A2, IGF1R*, and *LHCGR* in granulosa cells of SEV cows compared to MOD cows. Ovarian dysfunction is among the primary reasons for poor reproductive success and thus, increased culling rates on Canadian dairy farms. We have established that higher body condition loss in the aforementioned timeline is expected to increase fluctuations in liver function and in the metabolic profile, which we suggested has a negative impact in the development and maturation of the dominant follicle. To further understand the underlying mechanisms of decreased fertility among dairy cattle we aimed to investigate signaling cascades in the ovulating follicle.

We directed our attention towards the ERK1/2 pathway as it has been well-established to play an imperative role in ovulation of the mouse (Fan et al., 2009, Siddappa et al., 2015). Additionally, *in vitro* studies of livestock follicles have suggested drastic changes in the follicular microenvironment and in the gene expression profile of granulosa and theca cells in the presence of ERK1/2 inhibitors (Tajima et al., 2005, Ryan et al., 2008).

Our previous two studies have shown that BHBA level are highest in multiparous cows at time of breeding (Chapter III) and that cows losing more body condition tend to have higher levels of circulating NEFAs in early lactation (Study IV). Although limited amounts of research have addressed the relationship between metabolites and ERK1/2 signaling, it has been shown that BHBA can signal through the ERK1/2 pathway to regulate GHRH synthesis in the rat hypothalamus (Fu et al., 2015a). Furthermore, high doses of NEFAs added to bovine adrenal cell cultures have shown to decrease ERK1/2 activity and increase *CYP17A1* expression, thus androgen production (Bellanger et al., 2012). However, the mechanisms underlying metabolite signaling in the bovine ovulatory follicle have not been defined. In addition, the role of ERK1/2 signaling in the ovulatory follicle has not been investigated *in vivo*.

Therefore, in the next and final study, we examined the role of ERK1/2 in bovine ovulation using next-generation sequencing. We performed follicular injection of the dominant follicle of cattle by using the ERK1/2 signaling inhibitor, PD0325901, which was later accompanied by GnRH stimulation to initiate an LH surge (3 groups: Vehicle, Vehicle + GnRH, PD0325901 + GnRH). We collected follicular fluid to evaluate the hormone and metabolic profile and theca cells for transcript analysis by qPCR, and granulosa cells for both RNA-sequencing and qPCR validation.

CHAPTER V

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ERK1/2-dependent gene expression in the bovine ovulating follicle.

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5.1 Abstract

Ovulation is triggered by gonadotropin surge-induced signaling cascades. To study the role of extracellular signal-regulated kinase 1/2 (ERK1/2) in bovine ovulation, we administered the pharmacological inhibitor, PD0325901, into the preovulatory dominant follicle by intrafollicular injection. Four of five cows treated with 50μM PD0325901 failed to ovulate. To uncover the molecular basis of anovulation in ERK1/2-inhibited cows, we collected granulosa and theca cells from Vehicle and PD0325901 treated follicles. Next-generation sequencing of granulosa cell RNA revealed 285 differentially expressed genes between Vehicle and PD0325901-treated granulosa cells at 6h post-GnRH. Multiple inflammation-related pathways were enriched among the differentially expressed genes. The ERK1/2 dependent LH-induced genes in granulosa cells included *EGR1, ADAMTS1, STAT3* and *TNFAIP6*. Surprisingly, PD0325901 treatment did not affect *STAR* expression in granulosa cells at 6h post-GnRH. Granulosa cells had higher STAR protein and theca cells had higher levels of *STAR* mRNA in ERK1/2-inhibited follicles. Further, both granulosa and theca cells of ERK1/2-inhibited follicles had higher expression of *SLC16A1*, a monocarboxylate transporter, transporting substances including β-hydroxybutyrate across the plasma membrane. Taken together, ERK1/2 plays a significant role in mediating LH surge-induced gene expression in granulosa and theca cells of the ovulating follicle in cattle.

5.2 Introduction

The process of ovulation is dependent upon the luteinizing hormone (LH) surge to elicit a plethora of signaling cascades required for the release of a fertilizable oocyte and formation of a corpus luteum (CL) needed for pregnancy (Bearden HJ, 2004, Thatcher, 2017). Granulosa and theca cells of the ovulating follicle respond to the hormonal triggers, which in turn lead to their morphological and functional differentiation. The LH-dependent changes in the gene expression program is required for follicle rupture, cumulus cell expansion, oocyte maturation, and luteinization (Duggavathi and Murphy, 2009).

The LH-surge has been shown to activate, in granulosa cells, signaling pathways including: phosphatidylinositide 3-kinase (PI3K/AKT), cAMP/Protein kinase A (PKA) and the extracellular signal-regulated kinase 1 and 2 (ERK1/2), also referred to as mitogen-activated protein kinase 3/1 (Tajima et al., 2005, Ryan et al., 2007, Ryan et al., 2008, Sayasith et al., 2008, Fukuda et al., 2009, Sayasith and Sirois, 2015). The ERK1/2 pathway is well-researched in the mouse model, whereby both pharmacological inhibition *in vivo* (Siddappa et al., 2015) and *in vitro* (Su et al., 2006), and a granulosa cell conditional knockout mouse model (Fan et al., 2009) have demonstrated the pivotal role of the ERK1/2 pathway in ovulatory processes. A number of pharmacological inhibitors (U0126, PD98059, and PD0325901) have been developed to impede ERK1/2 signaling, where PD0325901 was shown to have the least off-target effects and thus, making it the ideal candidate (Wauson et al., 2013). Nonetheless, all three inhibitors have been used to evaluate the role of ERK1/2 signaling in theca and/or granulosa cell cultures across multiple species including mouse(Su et al., 2002), bovine(Ryan et al., 2008), rat(Seger et al., 2001, Tajima et al., 2003), and human(Tajima et al., 2003, Henriquez et al., 2017).

Mouse studies have revealed that the absence of ERK1/2 signalling reduces the expression of LH-regulated genes including: the early growth response 1 (*Egr1)* transcription factor, as well as genes required for follicular rupture (a disintegrin and metalloproteinase with thrombospondin motifs 1 (*Adamts1)* and prostaglandin-endoperoxide synthase 2 (*Ptgs2)*), cumulus expansion (pentraxin 3*(Ptx3)* and TNF alpha induced protein 6 *(Tnfaip6)*), oocyte maturation (amphiregulin (*Areg)*) and luteinization markers (steroidogenic acute regulatory protein *(Star)* and cytochrome P450 family 11 subfamily a member 1 *(Cyp11a1)*) (Su et al., 2006, Fan et al., 2009, Siddappa et al., 2015).

Evaluation of ERK1/2 signaling in ovulatory-sized follicles in livestock species has been predominantly performed in cows, where granulosa and theca cells were pharmacologically treated *in vitro* by one of the aforementioned inhibitors. Furthermore, the experiments involved the collection of abattoir ovaries where theca and granulosa cells were isolated and treated with gonadotropins in the presence or absence of an ERK1/2 inhibitor for 15 minutes to 24h (Tajima et al., 2005, Ryan et al., 2008, Fukuda et al., 2009, Sayasith et al., 2013c, Sayasith and Sirois, 2014, Sayasith et al., 2014, Sayasith and Sirois, 2015). For example, pharmacological inhibition of ERK1/2 signaling with U0126 was performed in cultured bovine granulosa cells from abattoir ovaries (follicles between 8 and 12mm in diameter were selected). The following ovulatory genes were revealed to be down-regulated when cultured in the presence of forskolin (to induce the LH-surge) and U0126: *ADAMTS1, CXCR4, ADAM71, VNN2*, and *RGS2* (Sayasith et al., 2013b, Sayasith et al., 2013c, Sayasith and Sirois, 2014, Sayasith et al., 2014, Sayasith and Sirois, 2015). While treatment of bovine theca cells with PD98059 *in vitro* resulted in increased *STAR* expression and thus, enhanced progesterone production(Tajima et al., 2005). Although these bovine studies have demonstrated a key role for ERK1/2 in regulation of select LH-regulated genes including *ADAMTS1* and *STAR* in granulosa and theca cells, the global impact of ERK1/2 signalling in bovine ovulation remains to be investigated.

Based on the aforementioned studies, we hypothesized that in the absence of ERK1/2 signaling LH-regulated genes downstream ERK1/2 would be differentially expressed leading to aberrant ovulation in cows. Therefore, our objective was to determine the role of ERK1/2 in bovine ovulation by means of developing a dynamic *in vivo* model, where follicular wave synchronized cows were subjected to intrafollicular injection of PD0325901 to abolish ERK1/2 signaling specifically in the ovulatory follicle. Moreover, by use of a novel approach of next generation sequencing, we performed RNA-sequencing to identify global changes in gene expression of granulosa cells of the ovulatory follicle exposed to PD0325901 and thus, gain a greater understanding of fertility in the bovine species.

5.3 Results

5.3.1Inhibition of ERK1/2 signaling abolishes ovulation in cattle

First, we tested the impact of inhibition of ERK1/2 signaling on ovulation in cows. The dominant follicle of the synchronized follicular wave was treated by intrafollicular injection with either a Vehicle or ERK1/2 signaling inhibitor, PD0325901 thirty minutes before GnRH treatment. Transrectal ultrasonography five days after the GnRH treatment revealed that all cows treated with Vehicle, 1μM and 10μM doses of PD0325901 successfully ovulated, while only one of five cows treated with 50μM PD0325901 ovulated, however this cow had low levels of circulating progesterone, suggesting her CL was not functional (Figure 1). Additionally, we measured plasma levels of progesterone on day 5 after GnRH treatment. Cows treated with 10μM or 50μM had significantly lower levels of progesterone compared to Vehicle treated counterparts ($P<0.05$; Figure 5.8.1). Therefore, we used 50μM PD0325901 for all further experiments to investigate the molecular basis of anovulation in ERK1/2 inhibited ovulatory follicles in cattle.

Inhibition of ERK1/2 signaling in bovine granulosa cells by 50μM PD0325901 was confirmed by protein analysis. At 6h post-GnRH, there was lower abundance of phospho-ERK1/2 in granulosa cells of the ovulatory follicle treated with PD0325901 compared to those of follicles treated with Vehicle ($P<0.01$; Figure 5.8.2).

5.3.2 Differentially expressed genes (DEGs) in granulosa cells

Having established that ERK1/2 activity is indispensable for ovulation, we next performed the second experiment to determine the ERK1/2 dependent gene expression in the bovine ovulatory follicle. We performed RNA-seq analysis on granulosa cells collected from the dominant follicles of the cows in three treatment groups: Vehicle, Vehicle + GnRH, and PD0325901 + GnRH. The reads aligned at over 86% to the bovine genome and the principle component analysis (PCA) showed that the transcriptomes of the three treatment groups separated out into three unique clusters (Supplementary Figure 1 and Supplementary Table 1). We then performed pairwise differential genes expression analyses - first by comparing Vehicle + GnRH vs Vehicle to determine LH-induced genes and then by comparing PD0325901 + GnRH vs Vehicle + GnRH to determine ERK1/2 dependent genes. The pairwise differential analyses using the DESeq2 R package showed that 2121 genes were differentially expressed between Vehicle + GnRH and Vehicle groups and 285 genes were differentially expressed between PD0325901 + GnRH and Vehicle + GnRH (>1 or <-1 log2fold change, q-value <0.01; Figure 5.8.3 A and B). Of the 2121 LH-regulated DEGs from the first comparison, 1426 genes were upregulated and 695 genes were downregulated in Vehicle + GnRH granulosa cells relative to those of Vehicle group (a full list is available in Supplementary Table 2). The top ten upregulated genes were *IL1B*, *TGM3, CD69,*

CXCL8, SDS, IL1RN, CAPN6, CCL4, S100A12, and *CXCR1* and the top ten downregulated genes included *B3GALT2, GPR142, SLC30A2, HAND2, PLPPR1, ANKRD34B, GPR88, ANK3, HCN1,* and *ACSBG1* (Figure 5.8.4). Of the 285 ERK1/2-dependent DEGs from the second comparison, 33 genes were upregulated and 252 genes were downregulated in PD0325901+ GnRH granulosa cells relative to those of Vehicle + GnRH group (a full list is available in Supplementary Table 3). The top ten upregulated genes were *KCNJ5, CDH8, NDST3, AOX1, AGTR2, SSTR2, RLN3, NOS2, CYCS*, and *MMP15 CXCR1,* and the top ten downregulated genes included *CASS4*, *IL1B*, *GPR84*, *CXCL8*, *TNF*, *CSF1*, *MEFV*, *IL1RN*, *NLRP3*, and *CSF3R* (Figure 5.8.4). In addition, there were 210 DEGs that were common between the two pairwise comparisons (Supplementary Table 4). Interestingly, the majority (196) of these 210 genes were upregulated in Vehicle + GnRH (LH-induced genes) and downregulated in PD0325901 + GnRH (ERK1/2-induced genes) granulosa cells. On the other hand, six genes were downregulated in Vehicle + GnRH (LH-inhibited genes) and upregulated in PD0325901 + GnRH (ERK1/2-inhibited genes) granulosa cells. Finally, only seven genes were upregulated in Vehicle + GnRH (LH-induced genes) followed by another upregulation in PD0325901 + GnRH (ERK1/2-inhibited genes) granulosa cells and only one gene was downregulated in Vehicle + GnRH (LH-inhibited gene) followed by a downregulation in PD0325901 + GnRH (ERK1/2-induced genes) granulosa cells.

5.3.3 Gene ontology (GO) and pathway analysis of the DEGs

The DEGs were associated with 14 and 13 PANTHER classes (biological processes (BP)) for the comparison of Vehicle + GnRH vs Vehicle and PD0325901 + GnRH vs Vehicle + GnRH, respectively. Many of these BPs were overlapping between comparisons and include immune system processes and metabolic processes (Figure 5.8.5 A and B). To determine the pathways that were affected in the pairwise comparisons we used the DAVID tool to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. A table containing the list of top regulated pathways (P<0.05) can be found in the Supplementary Table 5. A number of inflammatory pathways were altered between the two comparisons including amongst others the tumor necrosis factor (TNF) signaling pathways.

5.3.4 Inhibition of ERK1/2 signaling does not inhibit global transcription in granulosa cells

Although intrafollicular administration of PD0325901 led to abrogation of ovulation, not all genes were differentially expressed in granulosa cells of the treated follicles. Using RT-qPCR, we confirmed that the relative levels of *LHR* transcript were similar in granulosa cells of all three treatment groups (P>0.05; Figure 5.8.6). Relative mRNA abundance of *FSHR* was lower (P<0.05; Figure 5.8.6) in granulosa cells from the dominant follicles of both Vehicle and PD0325901 in GnRH-stimulated cows compared to those in cows that were not stimulated with GnRH. GnRH-induced increase in mRNA levels of *PAPPA* and *SCARB1* occurred in granulosa cells of both Vehicle and PD0325901 treated follicles (P<0.05; Figure 5.8.6). Similar differential expression trend for these genes was also observed in RNA-seq data (Table 5.9.1).

5.3.5 Expression of LH-induced ovulatory genes in granulosa cells is reduced in the absence of ERK1/2 signaling

A multitude of genes are involved in follicle rupture, cumulus cell expansion, oocyte maturation, and luteinization, all of which are required for successful ovulation(Duggavathi and Murphy, 2009). As previously mentioned, our bioinformatics analysis revealed that 196 genes were upregulated in Vehicle + GnRH (relative to Vehicle group) and downregulated in PD0325901 + GnRH (relative to Vehicle + GnRH group) granulosa cells (Figure 5.8.4). From the list of 196 genes, we used RT-qPCR analysis to confirm the results of five genes (*EGR1, ADAMTS1, TNFAIP6, STAT3,* and *TIMP1*) known to be LH-induced based on studies performed in rodent(Carletti and Christenson, 2009, Fan et al., 2009, Siddappa et al., 2015), primate(Murphy et al., 2016), and cattle (Christenson et al., 2013, Shrestha et al., 2015).

Granulosa cells in Vehicle + GnRH cows had higher levels of *EGR1* mRNA relative to Vehicle cows, whereas granulosa cells of PD0325901 + GnRH cows had lower *EGR1* mRNA abundance relative to Vehicle + GnRH cows (P<0.05; Figure 5.8.7). Similar mRNA abundance trend among cows of the three treatment groups was observed for *ADAMTS1, TNFAIP6*, *STAT3*, and *TIMP1* genes, wherein GnRH-induced increase in their mRNA levels was abolished by PD0325901 treatment (P<0.05 & P<0.1; Figure 5.8.7). Similar differential expression trend for these genes was also observed in RNA-seq data (Table 5.9.1).

5.3.6 Expression of LH-induced ovulatory genes in theca cells is altered in the absence of ERK1/2 signaling

Successful ovulation and formation of a corpus luteum are dependent on functional and morphological changes in both granulosa and theca cells in response to the LH-surge (Murayama et al., 2012, Christenson et al., 2013). Therefore, we sought to further analyze the impact of PD0325901 on the gene expression profile in bovine theca cells by comparing GnRH–stimulated theca cells in the absence or presence of PD0325901. Similar to bovine granulosa cells, relative mRNA abundance of *TIMP1* was significantly decreased in theca cells of the ovulatory follicles from the PD0325901 + GnRH group compared to the Vehicle + GnRH group ($P<0.05$; Figure 5.8.8 A). Unlike in bovine granulosa cells, relative abundance of *ADAMTS1* and *STAT3* transcripts was higher in theca cells from the PD0325901 + GnRH follicles compared to the Vehicle + GnRH follicles ($P<0.05$; Figure 8B). Lastly, relative transcript levels of *EGR1* and *TNFAIP6* were unaltered between the two groups ($P>0.05$; Figure 5.8.8 C).

5.3.7 Inhibition of ERK1/2 signaling increases STAR abundance in granulosa and theca cells

STAR is a luteinization marker expressed in bovine granulosa and theca cells(Pescador et al., 1996, Bao et al., 1998). Mouse studies have demonstrated that LH-induced *Star* expression in granulosa cells is dependent on ERK1/2 signaling (Su et al., 2006, Fan et al., 2009). As expected, our RNA-seq data showed an increase in *STAR* transcript abundance in Vehicle + GnRH vs Vehicle granulosa cells (Table 5.9.1). Surprisingly, there was no difference in the levels of *STAR* between PD0325901 +GnRH and Vehicle + GnRH granulosa cells (Table 5.9.1). Likewise, RT-qPCR analysis showed that there was no reduction in *STAR* mRNA levels in PD0325901 + GnRH granulosa cells when compared to Vehicle + GnRH granulosa cells (Figure 5.8.9 A). To confirm these transcript data, we used immunoblot analysis, which revealed PD0325901 + GnRH granulosa cells had higher levels of STAR protein than Vehicle + GnRH granulosa cells ($P<0.01$; Figure 5.8.9 B and C). Using the same cell lysates, we observed lower abundance of EGR1 in PD0325901 + GnRH granulosa cells ($P<0.05$; Figure 5.8.9 B and C). Similar to granulosa cells, relative mRNA abundance of *STAR* was higher in PD0325901 + GnRH theca cells ($P<0.05$; Figure 5.8.9 D). Nonetheless, levels of progesterone in the follicular fluid collected at 6h post-GnRH were similar between the two groups: 696.38 ± 193.82 ng/mL in Vehicle + GnRH and 827.80 ± 173.36ng/mL in PD0325901 + GnRH ($P>0.05$).

5.3.8 ERK1/2 appears to regulate uptake of beta-hydroxoybutyric acid in granulosa and theca cells

We found that in RNA-seq data solute carrier family 16 member A1 (*SLC16A1*; also known as monocarboxylate transporter-1) was one of the 33 upregulated genes in PD0325901 + GnRH granulosa cells (Table 5.9.1). Multiple studies have shown that cellular transport of β-hydroxybutyric acid (BHBA) occurs through SLC16A1 (Muller et al., 2002, Fu et al., 2015b). We explored further the expression of *SLC16A1* in pre-ovulatory follicles as BHBA has been shown to increase proliferation and decrease steroidogenesis in cultured granulosa cells(Vanholder et al., 2006).We confirmed upregulation of *SLC16A1* in PD0325901 + GnRH granulosa cells by RT-qPCR analysis (Figure 5.8.10). Likewise, mRNA abundance of *SLC16A1* was significantly higher in theca cells collected from the dominant follicles of PD0325901 + GnRH compared to Vehicle + GnRH ($P<0.01$; Figure 5.8.10). No difference in the concentration levels of BHBA (0.45 ± 0.08mmol/L Vehicle + GnRH and 0.34 ± 0.07mmol/L PD0325901 + GnRH) and glucose (73.75 ± 17.4 mg/dL Vehicle + GnRH and 62.50 ± 17.4mgl/dL PD0325901 + GnRH) in follicular fluid was observed between the two groups of cows ($P>0.05$).

5.4 Discussion

In dairy cattle, anovulation on average persists in nearly 20% of dairy cows beyond the voluntary waiting period of breeding at 60 days in milk (Gumen et al., 2003). Detailed understanding of ovarian physiology is crucial to develop therapeutic and management strategies to enhance reproductive performance of lactating cows and to ensure sustainable dairy farming. Successful ovulation is dependent on the trigger elicited through the LH surge which in turn activates a cascade of signalling pathways, among others the ERK1/2 pathway(Siddappa et al., 2015). This study is novel in its approach in using an *in vivo* model to investigate the role of ERK1/2 in bovine ovulation. We have shown for the first time in a monoovulator species, that the intrafollicular treatment with an inhibitor of ERK1/2 pathway (50μM PD0325901) abrogates ovulation in cattle. Using next generation sequencing we were able to generate large data sets of transcript changes in bovine granulosa cells, which allowed us to confirm that anovulation is caused by downregulation of genes required for ovulation. To our knowledge, we are among few groups to analyze the transcriptome of bovine granulosa cells through RNA-sequencing (Walsh et al., 2012, Li et al., 2016a).

Our pairwise analysis of Vehicle + GnRH vs Vehicle cows revealed 2121 LH-regulated genes in granulosa cells of ovulating follicles. The immune system processes was one of the biological processes that was found to be regulated by the LH surge. Furthermore, the KEGG pathway analysis suggested that the JAK-STAT, TNF, and MAPK signaling pathways were among the pathways enriched among LH-regulated genes. All these biological processes and the pathways have been linked to inflammatory processes in multiple cell types (Newton and Dixit, 2012, Kaplan, 2013). Ovulation is an inflammation-like process and in line with this the genes involved in inflammation, such as *STAT3* and *TNFAIP6* (Espey, 1980, Richards et al., 2002, Sayasith et al., 2008, Ndiaye et al., 2016) were upregulated in response to the LH surge. Pairwise comparison of PD0325901+ GnRH vs Vehicle + GnRH cows showed that similar pathways were enriched among ERK1/2-dependent LH-regulated genes. These overall pairwise comparisons demonstrate that the ERK1/2 pathway regulates ovulation, similar to the mouse, by altering LH-regulated gene expression in granulosa cells of the ovulating follicle in cattle.

Some of the LH-induced gene were *SCARB1*, *PAPPA*, *EGR1*, *ADAMTS1*, *TNFAIP6, STAT3*, and *TIMP1*, which were also confirmed by RT-qPCR analyses. It has been previously shown that the expression of *SCARB1*, which is responsible for cellular uptake of high-density lipoproteins in steroidogenic tissues, increases as the bovine follicle size increases, using abattoir-collected ovaries (Argov et al., 2004). Moreover, *Scarb1-null* mice are infertile despite normal ovarian morphology, estrus cycles, progesterone levels, and number of ovulated follicles(Trigatti et al., 1999). However, the ovulated oocytes are dysfunctional and preimplantation embryos die shortly after mating, which may be caused due to abnormal lipoprotein metabolism (Trigatti et al., 1999, Miettinen et al., 2001). Elsewhere, *PAPPA* expression has been shown in granulosa cells of the dominant and also in the ovulating follicle, suggesting that pathways downstream of LH may mediate *PAPPA* expression in cattle(Luo et al., 2011). Additionally, *Pappa* knockout mice exhibit compromised fertility due to aberrant expression of steroidogenic genes, including *STAR*(Nyegaard et al., 2010). Our data along with these observations show that *SCARB1* and *PAPPA* may play critical roles in bovine ovulation. However, neither *SCARB1* nor *PAPPA* were downregulated in ERK1/2 inhibited granulosa cells. Similar lack of effect of ERK1/2 inhibition on *Scarb1* and *Pappa* expression was shown in a mouse study(Siddappa et al., 2015). Therefore, these studies demonstrated that LH induction of *SCARB1* and *PAPPA* expression does not require

ERK1/2 signaling and alternative signaling such as by the PKA pathway could be involved in their regulation (Luo et al., 2011).

Comparing GnRH + PD0325901 to GnRH + Vehicle granulosa cells showed that many of the LH-induced genes, such as *EGR1, ADAMTS1, TNFAIP6, STAT3,* and *TIMP1*, were downregulated in ERK1/2 inhibited granulosa cells. Importantly, these genes were also downregulated in the absence of ERK1/2 signaling in granulosa cells of mice(Fan et al., 2009, Siddappa et al., 2015). First, EGR1 plays an important role related to proliferation, differentiation, apoptosis, and gene regulation. Mice with *Egr1* deletion are sterile due to both ovarian and pituitary abnormalities (Topilko et al., 1998). In bovine ovulating follicles, *EGR1* is induced in granulosa cells at 6h post-human chorionic gonadotropin (hCG) and suggested to play an additional role in prostaglandin biosynthesis pathway(Sayasith et al., 2006). Our group previously reported that in mice, *Egr1* regulates *Ptgs2* expression by binding to its promoter(Siddappa et al., 2015). Second, *Adamts1*-null mice experience subfertility (Shindo et al., 2000), which was attributed to aberrant extracellular matrix remodelling of the follicle wall required for ovulation(Brown et al., 2010). In bovine granulosa cells *in vitro*, treatment with forskolin and ERK1/2 inhibitor (U0126) decreased *ADAMTS1* expression(Sayasith et al., 2013b). Third, *TNFAIP6* expression is induced between 4h and 8h post-hCG in rat granulosa cells(Yoshioka et al., 2000) and *Tnfaip6*-deficient mice are sterile due to abnormal remodelling of the cumulus extracellular matrix (Fulop et al., 2003). In cattle, *TNFAIP6* expression is up-regulated in the preovulatory follicle starting at 6h post-hCG(Sayasith et al., 2008). Moreover, this same group revealed by *in vitro* bovine granulosa cell culture that the PKA pathway was responsible for *TNFAIP6* promoter activity and transcription. However, they also confirmed that the activator protein-1 (AP-1) transcription factor was indispensable for *TNFAIP6* expression(Sayasith et al., 2008). Previous work has suggested that ERK1/2 may phosphorylate the transcription factor AP-1(Sharma and Richards, 2000). In Supplementary Table 3, we show that FOS like 1, *AP-1* transcription factor subunit (ENSBTAG00000006194) is downregulated in GnRH + PD0325901 vs GnRH + Vehicle granulosa cells, providing a justification for the decrease in *TNFAIP6* expression. Fourth, TIMP1 is a key regulator of matrix metalloproteinases activity and production, and *Timp1*-null mice have shortened estrus period and altered serum levels of progesterone and estradiol (Nothnick, 2000), yet surprisingly, these mice are fertile suggesting that there are redundant mechanisms that compensate for Timp1 (Nothnick et al., 1997). Lastly, STAT3 is a transcription factor involved in cytokine mediation and *Stat3*-null mice are embryonic lethal confirming the wide-spread expression of the gene (Takeda et al., 1997). Mice that are deficient of the progesterone receptor, which are infertile, experience a significant decrease in *Stat3* expression in ovaries(Akison et al., 2018). Thus, STAT3, a regulator of inflammation, may play an important role in ovulation. On the contrary, there was an increase in *STAT3* expression in ERK1/2 inhibited theca cells. This difference in ERK1/2 regulation of STAT3 in response to ERK1/2 inhibition between granulosa and theca cells is not surprising. In fact, ERK1/2 appears to have different regulatory effect on *STAT3* expression and function in different cell types. Inhibition of ERK1/2 has been shown to increase *STAT3* expression in human hepatoma cell lines (Tian and An, 2004) and cardiomyocytes(Li et al., 2004). In oral squamous cell carcinoma cells, it was shown that in the absence of ERK1/2 signaling, there was a decrease in phosphorylated -serine STAT3, but an increase in phosphorylated -tyrosine STAT3(Gkouveris et al., 2014).

The cow is an excellent model to study the impact of signaling pathways in theca cells, which is difficult in the murine model(Fortune et al., 2009, Young and McNeilly, 2010). Christenson et al(Christenson et al., 2013) have put together a list of differentially expressed genes comparing 0h and 21h GnRH-induced LH surge in both bovine granulosa and theca cells. This group identified *TIMP1* as being upregulated by LH in theca cells. Our data show that *TIMP1* expression in theca cells was downregulated as in granulosa cells of cows from the PD0325901 + GnRH vs Vehicle + GnRH. Similar to *STAT3*, *ADAMTS1* expression increased in ERK1/2 inhibited theca cells. Previous studies have shown that *ADAMTS1* has been detected in bovine theca cells and is increased by 24h post-GnRH (Willis et al., 2017), while *STAT3* has only yet been localized in theca cells of antral follicles in swine (Wen et al., 2006). Also, expression of *EGR1* and *TNFAIP6* was not affected by the inhibitor treatment in theca cells. *EGR1* expression has been previously detected in bovine theca cells and induced by 6h post-hCG (Sayasith et al., 2006, Guerrero-Netro et al., 2017), while *TNFAIP6* mRNA abundance has been detected in both bovine and equine theca cells (Sayasith et al., 2007, Hatzirodos et al., 2017). Theca cell transcriptome in bovine remains poorly studied, however these results contribute to the field of knowledge addressing signaling between follicular cell compartments.

The proper function of steroidogenic genes is crucial for ovulation and the formation of the corpus luteum. Of the multiple steroidogenic enzymes, STAR is the rate limiting enzyme and it facilitates the transport of intracellular cholesterol to the inner mitochondrial membrane for the onset of steroidogenesis and thus, progesterone synthesis. Mice deficient of *Star* have been shown to experience impaired ovulation (Ishii et al., 2002, Stocco et al., 2007). Murine studies have shown that in the absence of ERK1/2 signaling there is a decrease in *Star* abundance and circulating progesterone concentrations(Su et al., 2006, Fan et al., 2009). On the contrary, immortalized steroidogenic granulosa cells and primary rat and human granulosa cells show an increase in *STAR* expression and progesterone production when cells were subjected to ERK1/2 inhibition (Seger et al., 2001, Tajima et al., 2003). Nonetheless, another study has shown that ERK1/2 inhibition attenuated *STAR* expression and progesterone synthesis in primary cell cultures of human granulosa cells (Fang et al., 2016). In our *in vivo* bovine model, the LH-induced increase in *STAR* mRNA granulosa cells was not abolished by ERK1/2 inhibition. In fact, protein abundance was higher in both granulosa and theca cells of GnRH + PD0325901. In line with our data, treatment of bovine theca cells with PD98059 (ERK1/2 inhibitor) for 24h increased *STAR* mRNA and protein abundance and progesterone production *in vitro* (Tajima et al., 2005, Ryan et al., 2008).

RNA-sequencing of granulosa cells provided a diverse list of differentially expressed genes between different comparisons. One of the up-regulated genes in GnRH + PD0325901 granulosa cells was *SLC16A1*, which was further confirmed by RT-qPCR in both granulosa and theca cells. To our knowledge, this is the first study to report the presence of *SLC16A1* in both bovine granulosa and theca cells. Although limited data exists on the role of *SLC16A1*, it has been demonstrated that in the anterior pituitary cells of dairy cows cellular uptake of BHBA occurred through SLC16A1(Fu et al., 2015b). Moreover, elevated levels of BHBA reduced PKA activity, which subsequently prevented growth hormone and prolactin transcription and secretion from the pituitary cells (Fu et al., 2015b). Dairy cows during early lactation have elevated levels of circulating BHBA (McArt et al., 2012). This ketone has been described as a source of energy used under conditions of starvation (Laeger et al., 2010). Previous research has shown that high levels of BHBA increased proliferation, but decreased estradiol and progesterone production in bovine granulosa cells (Vanholder et al., 2006). As the preovulatory LH surge terminates proliferation and induces progesterone synthesis in granulosa cells, it is plausible that SLC16A1 may play a role in the process through regulation of BHBA uptake. Therefore, our data indicate that ERK1/2 mediates the LH-driven reduction in BHBA uptake to bring about luteinisation during ovulation.

In conclusion, ovulation in cattle, similar to the mouse, is dependent on proper ERK1/2 signaling, as a number of genes in granulosa cells required for different cascades in the ovulatory process are dysregulated in its absence. In contrast to mice, ERK1/2 inhibition increased STAR protein abundance in granulosa and theca cells. Similarly, genes such a *SLC16A1* were also elevated in granulosa cells lacking ERK1/2 signaling in both cell types. Moreover, some genes that were downregulated in response to ERK1/2 inhibition in granulosa cells were upregulated in theca cells. Taken together, ERK1/2 plays a significant role in transducing downstream signaling of LH surge during ovulation in cattle.

5.5 Materials and Methods

5.5.1 Cows and follicular synchronization

All beef cows in this study were used in accordance with procedures approved by the Ethics and Animal Welfare Committee of the Federal University of Santa Maria, Brazil. Follicular wave synchronization was performed by first (Day - 9) inserting an intravaginal device with 1g progesterone (DIB; Intervet/Schering-Plough, Brazil), and intramuscular injection of 2 mg estradiol benzoate and 500μg Sodium Cloprostenol. On day 0, the intravaginal device was removed and the ovaries were examined by transrectal ultrasonography using an 8MHz linear-array transducer (AquilaVet® scanner, Pie Medical, the Netherlands), where cows having a follicle with a diameter >12mm were subject to intrafollicular treatment described below.

5.5.2 Experimental design

We conducted two experiments to establish the role of ERK1/2 activity on ovulation in cattle. **Experiment 1:** We first tested whether or not inhibition of ERK1/2 activity in an ovulatory follicle would inhibit ovulation and formation of corpus luteum. As demonstrated by a study comparing three ERK1/2 pathway inhibitors(Wauson et al., 2013), unlike other inhibitors (U0126 and PD98059), PD0325901 does not appear to have off-target effects, thus making it an ideal ERK1/2 signaling inhibitor. Our group has previously shown that intraperitoneal administration of a single dose of PD0325901 in mice using a concentration of 25 μg/g body weight led to abrogated ovulation in mice without cytotoxicity(Siddappa et al., 2015). Since intraperitoneal administration of the inhibitor in cows is not feasible, we sought to inhibit ERK1/2 activity by intrafollicular administration of PD0325901 (Selleckchem, Houston, Texas, USA).

Experiment 1 was performed to determine the effective dose for inhibition of ovulation through dose trial. The dominant follicle of the synchronized follicular wave in separate groups of cows was treated on the morning of Day 0 with either a Vehicle control (Phosphate-buffered saline with DMSO; N=5), or three different doses of PD0325901 (1μM (N=5), 10μM (N=5), or 50μM (N=5)). The diameter of the dominant follicle of each cow was measured by ultrasonography prior to injection. The accurate injection volume to obtain a final intrafollicular concentration of 1μM, 10μM, or 50μM was determined based on the linear regression model equation for the follicular volume as previously described (Ferreira et al., 2007): $V= -685.1 + 120.7D$, where V corresponds to the estimated follicular volume and D to the diameter of the follicle to be injected. The inhibitor was initially dissolved in dimethyl sulfoxide (DMSO) (Fisher Scientific, Saint-Laurent, QC, Canada). Thirty minutes following Vehicle or PD0325901 treatment (Day 0), cows were stimulated with an intramuscular dose of gonadotropin releasing hormone (GnRH) (100μg gonadorelin acetate from Profertil®, Tortuga, Brazil) to induce the endogenous LH surge and thus, ovulation. It is well established that the peak of the endogenous LH surge occurs at 1h after GnRH treatment(Luo et al., 2011). Five days after GnRH treatment, a blood sample was collected from the coccygeal vein for plasma progesterone analysis and ultrasonography was performed to assess the presence of a corpus luteum. Progesterone concentrations were determined with an electrochemiluminescence immunoassay (Roche, Brazil) in a commercial lab (Laboratorio Pasin, Santa Maria, RS, Brazil). See Figure 5.8.11 for experimental design.

Experiment 2: A second study was conducted to determine the molecular basis of ERK1/2 regulation of ovulation. We used the effective dose of 50μM PD032901, which abrogated ovulation, to achieve ERK1/2 inhibition. Follicular wave synchronization and intrafollicular treatments were performed as described above. We collected ovaries from each cow at a specific developmental stage of the dominant follicle by colpotomy under caudal epidural anesthesia using 80 mg lidocaine chlorhydrate (4 ml 2% lidocaine) as previously described (Gasperin et al., 2012). Ovary collection times included 0h and 6h relative to GnRH treatment representing before and after LH surge. The three groups of cattle involved in ovary collection included: 1) 0h relative to GnRH treatment (Vehicle) (N=3) – these cows were not treated with GnRH, but ovaries were collected at the expected time of GnRH treatment during the follicular wave synchronization protocol; 2) 6h post-GnRH treatment with intrafollicular Vehicle (Vehicle + GnRH) (N=6) and 3) 6h post-GnRH treatment with intrafollicular PD0325901 (PD0325901 + GnRH) (N=5). See Figure

5.8.11 for experimental design. Upon collection, all ovaries were washed with a 0.9% saline solution followed by follicular puncture of the dominant follicle to collect samples for downstream analysis. Granulosa cells were collected through flushing of the follicle with phosphate buffered saline (PBS) for protein and gene expression analysis, followed by theca cell collection for gene expression analysis via dissection of the follicular wall. Follicular fluid was also collected. All samples, were stored in liquid nitrogen until return to the laboratory, where they were stored at -80°C until further processing.

5.5.3 RNA extraction and library preparation for RNA-sequencing of granulosa cells

To purify total RNA from granulosa cells we used the commercially available kit NucleoSpin® RNA Plus (Machery-Nagel, D-Mark Biosciences, ON, Canada) according to the manufacturer's protocol. All samples, were quantified by absorbance at 260nm using the NanoDrop 20000 spectrophotometer (Thermo Fischer Scientific Inc., Waltham, MA, USA). Total RNA was sent to the Functional Genomics Platform of McGill University and Génome Québec Innovation Centre, Canada for cDNA library preparation and sequencing with Illumina HiSeq 2500 PE 125, using 250ng as input material. Only top-quality samples were prepared for sequencing, whereby a minimum RNA integrated Number (RIN) of 8.5 was accepted and library quality and quantity was confirmed using a bioanalyzer at Genome Quebec Research Center (N=2-3 samples/treatment group). A total of 8 samples were selected based on quality assessment: Vehicle (N=2), Vehicle +6hGnRH (N=3) and PD0325901 + 6hGnRH (N=3) and they were run together on one lane. The RNA-Seq data used in this study are available from GEO data repository (GSE121588). Supplementary Table 1 and Supplementary Figure 1 provide details on RNA-seq quality.

5.5.4 Bioinformatics analysis: read cleaning and genome alignment

FastQC v0.11.5 was used to visualize the quality of the sequence reads(Bioinformatics, 2016). These reads were cleaned using the software Trimmomatic v0.36, where illumina adapters and reads with average PHRED scores below 20 and lengths below 63 base pairs (bp) were removed(Bolger et al., 2014). The FastQC software revealed clean per tile sequence and per base sequence quality. Quality of a representative sample can be seen in Supplementary Figure 1A and 1B. The index of the bovine genome, *Bos taurus* genome B.tau 4.6.1, was built using Bowtie v2.2.1.0 and the cleaned reads were aligned to the genome using TopHat v2.0.11 with the following modifications to the default settings: final read alignments having more than 3 mismatches were removed, 100 base pairs (bp) as the expected inner distance between mate pairs, 50bp as the standard deviation for the distribution on inner distances between mate pairs, 5 as an anchor length, 1 as the maximum number of mismatches in the anchor, 50 as the minimum intron length, 2 as the number of threads to align reads, 3 as the segment alignment mismatches, 20 as a minimum intron length found during split-segment search(John Hopkins University, 2016). Finally, the number of reads mapped to each gene were counted using HTSeq v0.6.1(Anders et al., 2015).

5.5.5 Differentially expressed genes (DEGs)

R studio v3.3.0 was used for statistical analysis of granulosa cell sequencing data, where R packages were used to determine differently expressed genes (DEGs) between treatment groups. The counts were normalized using the estimateSizeFactors and variance were stabilized using varianceStabilizingTransformaton commands in the statistical package DESeq2 v1.12.3(Love et al., 2014). A principle component analysis (PCA) was used to detect treatment clustering (Supplementary figure 1C). DESeq2 was used to build a list of DEGs by a negative binomial generalized linear model with the following criteria: Log2Fold Change of >1 or <-1 and a Benjamini-Hochberg false discover rate (FDR) /q-value <0.01. Heatmaps were constructed using the R package Pheatmap v1.0.8 and a Venn diagram for pairwise comparisons of DEGs within treatments was assembled with the Bioinformatics & Evolutionary Genomics website (http://bioinformatics.psb.ugent.be/webtools/Venn/).

5.5.6 Gene ontology and pathway enrichment

The identified DEGs were analyzed in the PANTHER classification system (13.1) to determine enriched biological systems. For functional annotation clustering and enrichment score representation of Gene Ontology (GO), we used the Data-base for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 to generate the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

5.5.7 RT-qPCR of granulosa and theca cells

Total RNA from granulosa cells was extracted as previously described. Total RNA from theca cells was purified using TRizol® (Thermo Fisher Scientific [Life Technologies, Inc.], Burlington, ON, Canada) according to the manufacturer's instructions. All samples, were quantified by absorbance at 260nm using the NanoDrop 20000 spectrophotometer (Thermo Fischer Scientific Inc., Waltham, MA, USA). Complementary DNA was synthesized from 250ng of total RNA using the iScript cDNA Synthesis kit (Bio-Rad, Mississauga, Canada) using the following temperature program: 25°C for 5 min (Priming), 46°C for 20 min (Reverse transcription) and 95°C for 1 min (Reverse transcription inactivation).

A larger number of granulosa cells samples was used to perform RT-qPCR analysis compared to RNA-seq: Vehicle (N=3), Vehicle + GnRH (N=6) and PD0325901 + GnRH (N=4). Gene expression of *CYP17A1* was used as a marker for theca cell contamination and thus, affected granulosa cell samples were removed from the analysis (one sample from PD0325901 + GnRH group was removed). The mRNA abundance of theca cells was compared between two treatment groups, Vehicle + GnRH (N=6) and PD0325901 + GnRH (N=5). All RT-qPCR assays were performed using previously described protocols (Schuermann et al., 2018). Relative transcript abundance for each gene of interest was calculated by dividing their respective starting quantity (SQ) values by the mean SQ values of three reference genes (*ACTB, L19*, and *CYCLOPHILIN*). The primer sequences of transcripts measured in this study can be found in Supplementary Table 6, where primer design was performed using the NCBI Primer-BLAST. If variants of a gene are present, the primers were designed to include all variants.

5.5.8 Protein isolation and immunoblot

Protein from granulosa cells was isolated using the AllPrep® DNA/RNA/Protein kit (Qiagen, Mississauga, ON, Canada), followed by a dilution in Laemmli buffer (Bio-Rad Laboratories, Mississauga, ON, Canada) and boiled at 95°C for 5 min. Protein extracts were resolved by polyacrylamide electrophoresis (10% gel) and transferred to nitrocellulose membranes. Blocking after the transfer was performed for 1.5h using 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T). All membrane fractions were incubated with their respective primary antibodies (STAR, Phospho-ERK1/2 and EGR1) overnight at 4°C. Next, membranes were washed with TBS-T (4X 7 min) followed by incubation with secondary antibody for 1.5 hours at room temperature. The Immun-Star Western Chemi luminescent Kit (Bio-Rad) and Chemidoc Analyzer were used to detect immunoblotted proteins. The membrane was stripped twice for detection of Total-ERK1/2 and β-actin using a stripping buffer (10% SDS, 0.5 M Tris-HCl, milliQ grade water and 2-mercaptoehtanol). We established ERK1/2 activity by determining the abundance of phosphorylated isoform of ERK1/2 relative to its total isoform. All antibodies used in the experiment have been validated in multiple species. The references for the studies that used these antibodies in the bovine ovary are given in Supplementary Table 7 with additional information pertaining to the required concentration and the purchasing information for each antibody. Image Lab Software from Bio-Rad was used to quantify protein abundance. The images of the immunoblots in Figures 2 and 9 were cropped and thus, uncropped images are available in Supplementary Figure 2.

5.5.9 Follicular fluid analysis

Progesterone concentrations were determined with an electrochemiluminescence immunoassay (Roche, Brazil) in a commercial lab(de Campos et al., 2017). Glucose and beta-hydroxybutyric acid (BHBA)/ketone levels were measured with the FreeStyle Optium Neo Blood Glucose and Ketone Monitoring System from Abbott (available from local pharmacies; Abbott Laboratories, Limited).

5.5.10 Statistical analysis for RT-PCR, immunoblot, and follicular fluid analysis

Analysis of data was performed using SAS University Edition (SAS Institute). Data that did not follow a normal distribution (Shapiro-Wilk test) were log-transformed. Differences between means were tested with the Tukey-Kramer honestly significant difference test or by Student's t-test. Data are presented as the mean ± SEM and significant differences were designated by $P<0.05$ and a tendency was marked by $P<0.1$.

5.6 Acknowledgements

The authors would like to thank Dr. Sarah Kimmins for the use of facilities. In addition, the authors would like to thank Dr. Tanya Copley for concept teaching of bioinformatics analysis. YS was supported by the Fonds de Recherche du Québec-Nature et Technologies (FRQNT), Réseau Québécois and Reproduction (RQR)-CREAT Scholarship and Department of Animal Science Graduate Excellence Fellowship and Travel Awards. This work was supported by the Natural Science and Engineering Research Council (NSERC) of Canada and FRQNT to RD.

5.7 Author Contributions

Y.S.: contributed to ideas, designed experiments, took part in the field work, collected, assembled, analyzed, and interpreted data, prepared figures and wrote manuscript; M.T.R.: took part in the field work, collected and analyzed data and prepared figures; B.G. & R.F.: contributed to ideas, took part in the field work, and collected and assembled data; J.F.: took part in the field work; E.M.: assembled and interpreted data; P.B.G. & V.B.: contributed to ideas, collected and interpreted data; R.D.: conceived the study, designed experiments, analyzed data, and edited manuscript.

5.8 Figures

Figure 5.8.1: Effect of intrafollicular administration of the MEK inhibitor, PD0325901 on ovulation in cattle. All cows were subjected to follicular-wave synchronization and were treated with an ultrasound-guided intrafollicular administration of a Vehicle or different doses of PD0325901 30 minutes prior to intramuscular administration of GnRH. The number of cows ovulating in response to PD0325901 treatment are given in the table. Ultrasonography was used to identify the presence of a corpus luteum (CL). Progesterone levels in plasma samples collected five days after ovulation are presented in the graph. Bars with different letters are significantly different P<0.05.

Treatments	**Vehicle**	**PD0325901**		
		1μM	10μM	50μM
# of cows ovulated	5/5	5/5	5/5	1/5

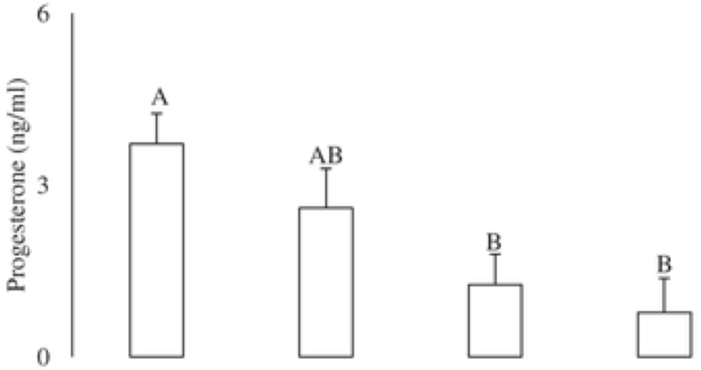


Figure 5.8.2: Inhibition of ERK1/2 activity in granulosa cells of ovulating follicles by an intrafollicular administration of PD0325901. Protein abundance of ERK1/2 phosphorylation in bovine granulosa cells collected from the dominant follicles of GnRH stimulated cows, which were challenged with a Vehicle control or 50μM PD0325901. Quantification by densitometry are presented in the graph. The blot was cropped and it was first used to quantify the presence of Phospho-ERK1/2 and then stripped to quantify for the presence of Total-ERK1/2.** denotes significant difference of Phospho-ERK1/2 normalized against Total-ERK1/2 between GnRH stimulated cows, which were challenged with a Vehicle control or 50μM PD0325901, where P<0.01.

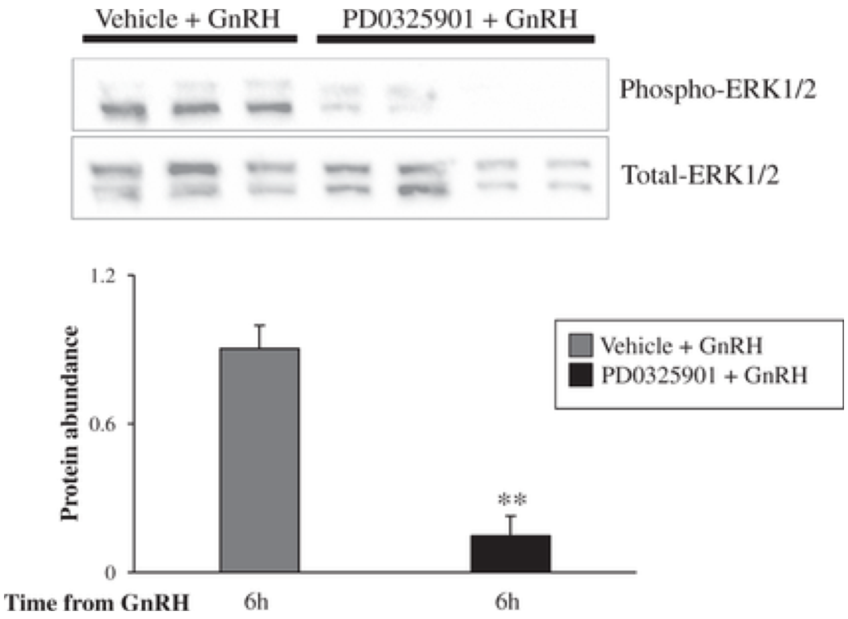


Figure 5.8.3: Heatmaps displaying differentially expressed genes (DEGs) in bovine granulosa cells for pairwise comparisons. A. Heatmap of 2121 DEGs in granulosa cells collected from cows prior to (Vehicle; n=2) or 6h after GnRH stimulation (Vehicle + GnRH; n=3). B. Heatmap of 285 DEGs in granulosa cells collected at 6h post-GnRH from cows that were pre-challenged, at 30 minutes prior to GnRH, with vehicle (Vehicle + GnRH; n=3) or MEK inhibition (PD0325901 +GnRH; n=3). The data from rlog transformed counts with high values and low values shown in red tones and blue tones, respectively. Significantly different genes were determined based on the statistical parameters: log2fold change <-1 and >1 with a q-value <0.01.

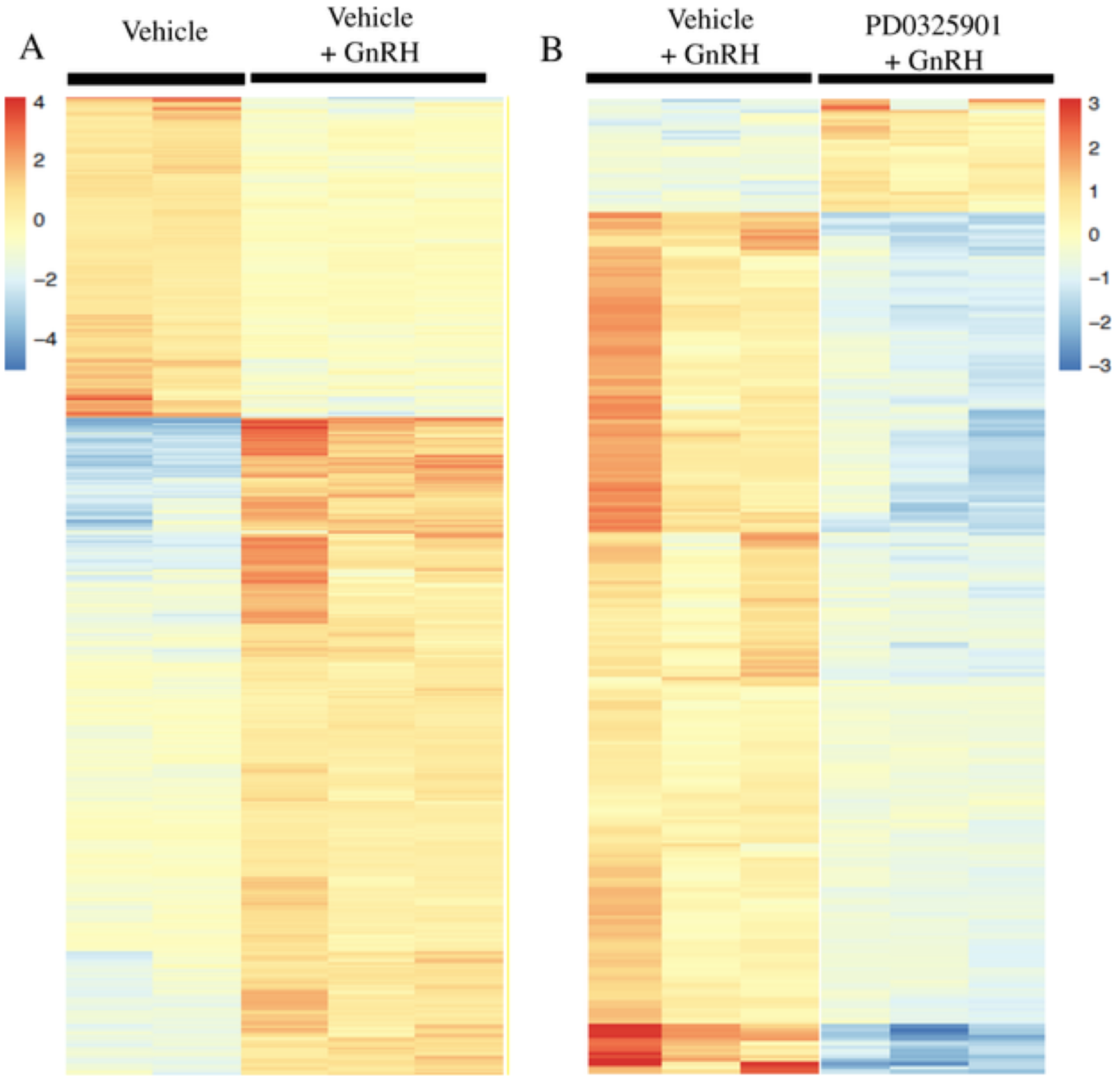
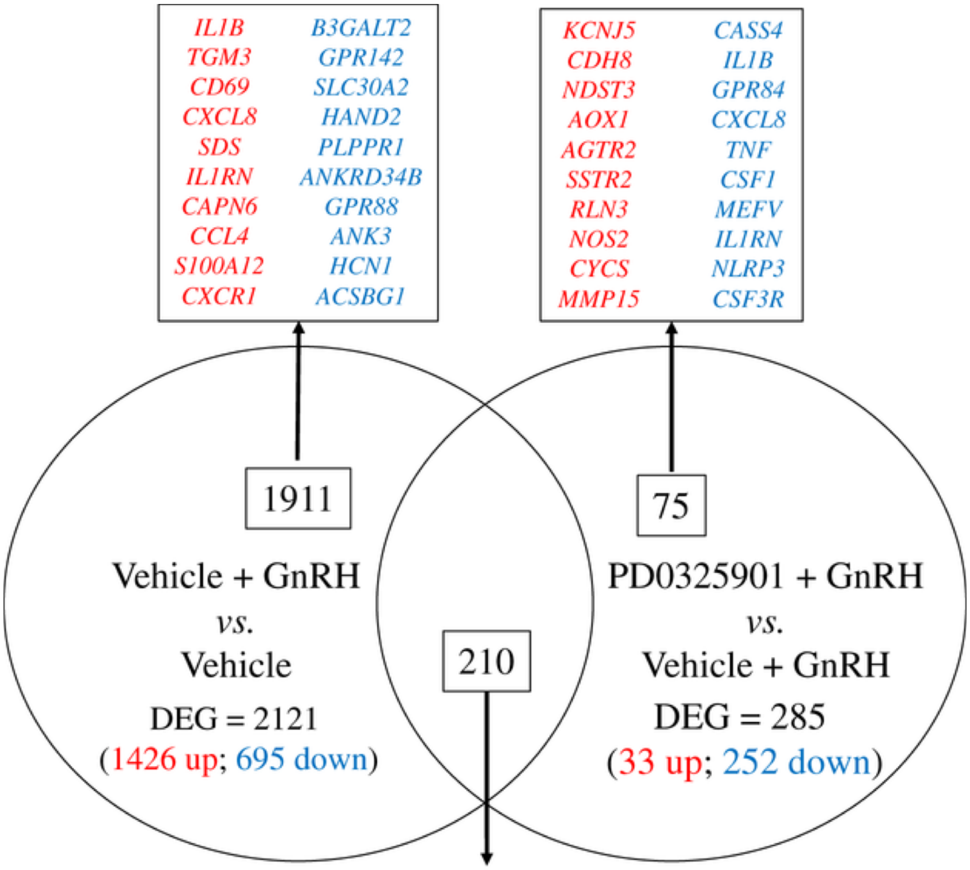


Figure 5.8.4: Venn diagram of differentially expressed genes (DEGs) for two pairwise comparisons: Vehicle +GnRH vs. Vehicle and PD0325901 + GnRH vs. Vehicle + GnRH. Top 10 up-regulated (red) and down-regulated (blue) DEGs are listed below for each specific comparison based on Log2FoldChange. The 210 DEGs that were common in both comparisons are represented with their respective direction of regulation in each comparison.



# of genes	Vehicle + GnRH	PD0325901 + GnRH	Inference
196	up	down	LH-induced ERK1/2-induced
6	down	up	LH-inhibited ERK1/2-inhibited
7	up	up	LH-induced ERK1/2-inhibited
1	down	down	LH-inhibited ERK1/2-induced

Figure 5.8.5: PANTHER GO-slim analysis. Pie-charts showing proportions of DEGs involved in biological processes of granulosa cells comparing Vehicle + GnRH to Vehicle (A) and PD032901 + GnRH to Vehicle + GnRH (B).

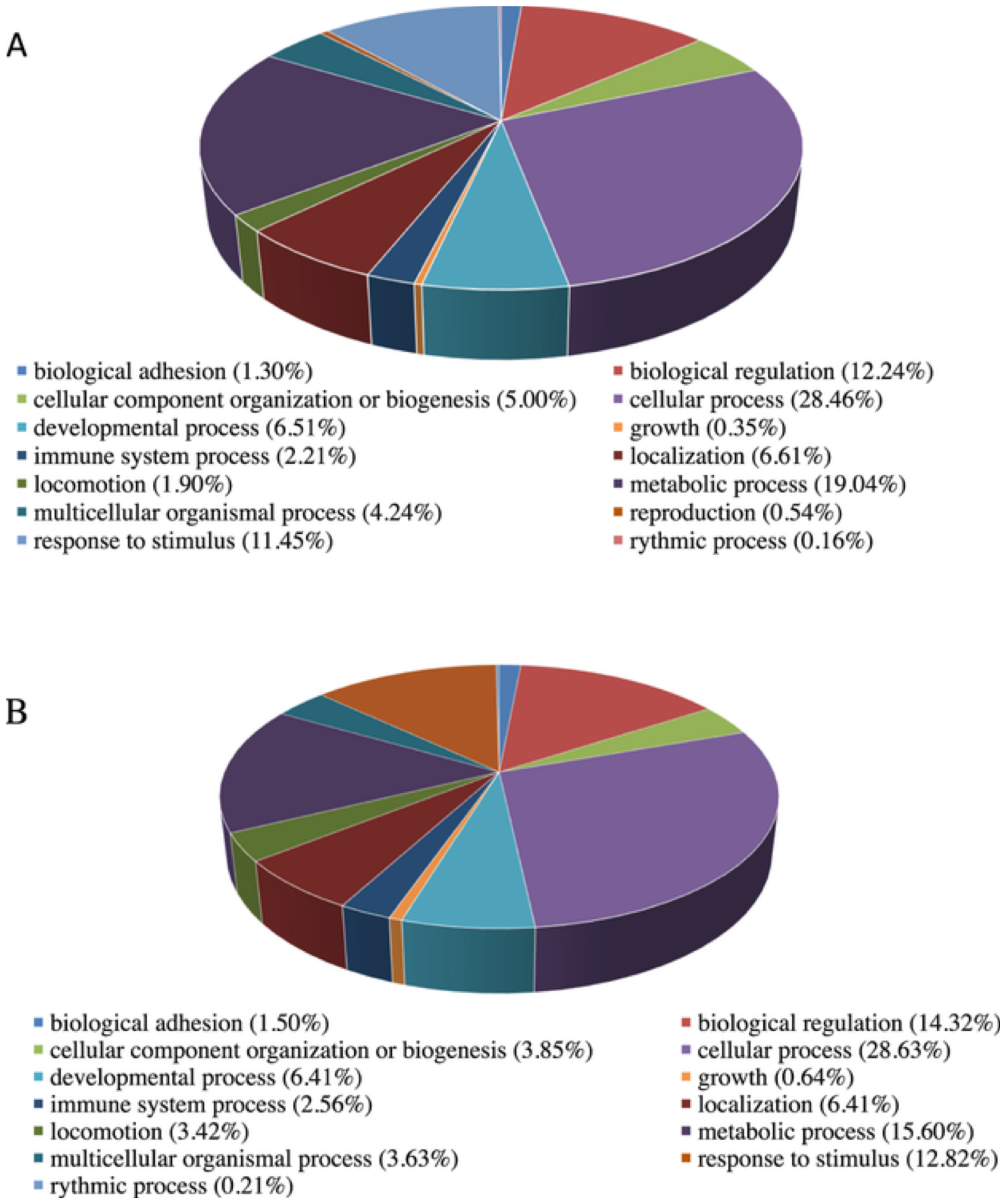


Figure 5.8.6: The MEK inhibitor PD0325901 treatment does not alter the global gene transcription system in bovine granulosa cells. Relative mRNA abundance of *LHR, FSHR, SCARB1,* and *PAPPA* in granulosa cells of cows in Vehicle, Vehicle + GnRH and PD0325901 + GnRH groups (N=3-6/time-point). Cows were subject to follicular-wave synchronization and were challenged with Vehicle or PD0325901 30 minutes prior to intramuscular administration of GnRH. Granulosa cell were collected from groups of individual cows at 0h and 6h relative to GnRH stimulus. Transcript abundance of each gene was normalized to reference genes *ACTB, L19*, and *CYCLOPHILIN*. All data are expressed as a mean ± S.E.M, where different letters represent differences at $p<0.05$ after a one-way ANOVA.

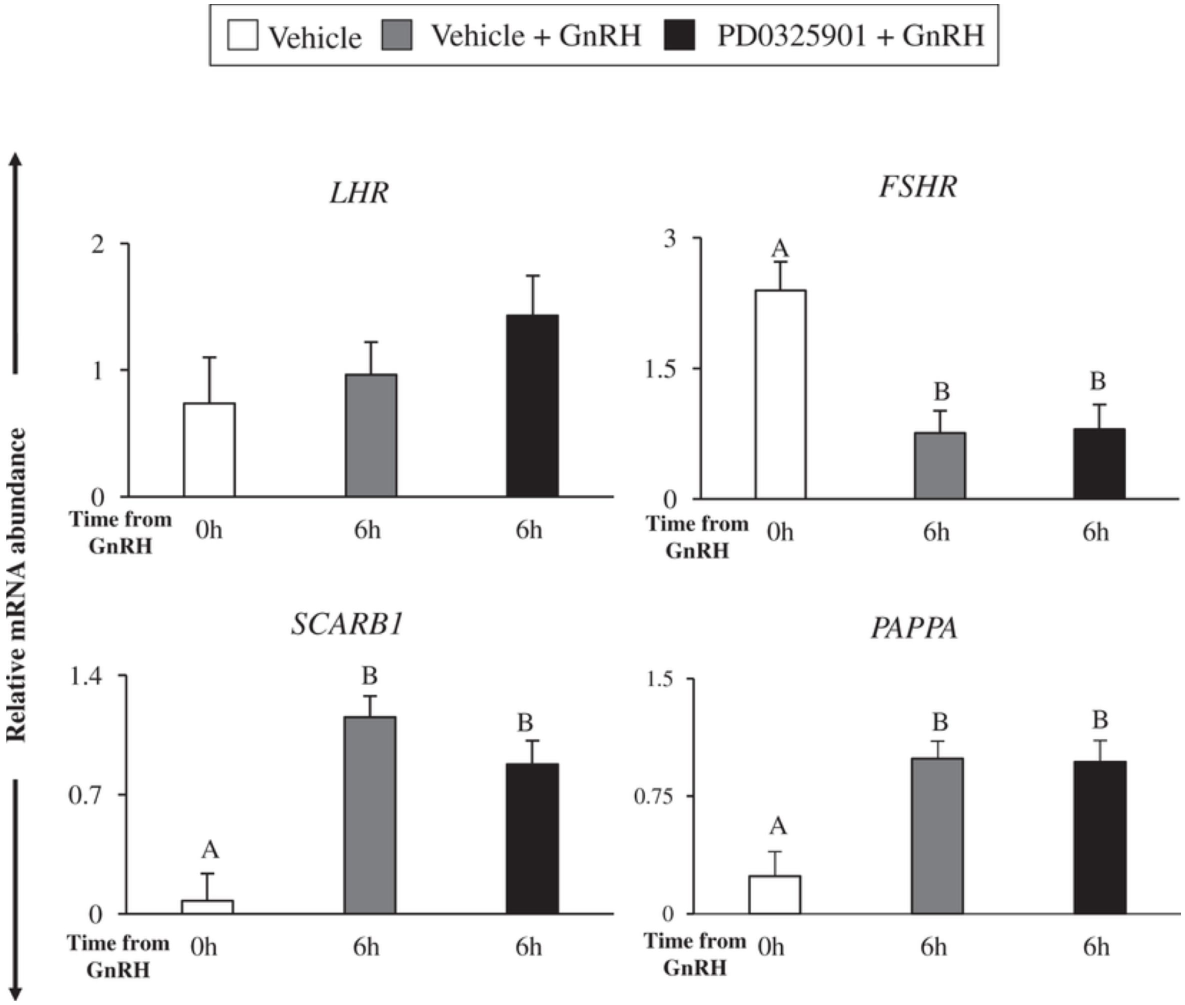


Figure 5.8.7: Intrafollicular injection of PD0325901 reduces the relative mRNA abundance of LH-induced ovulatory genes in granulosa cells. Relative mRNA abundance of *EGR1, ADAMTS1*, *TNFAIP6, STAT3*, and *TIMP1* in granulosa cells of cows in Vehicle, Vehicle + GnRH and PD0325901 + GnRH groups (N=3-6/time-point). Cows were subject to follicular-wave synchronization and were challenged with Vehicle or PD0325901 30 minutes prior intramuscular administration to GnRH. Granulosa cell were collected from groups of individual cows at 0h and 6h relative to GnRH stimulus. Transcript abundance of each gene was normalized to reference genes *ACTB, L19*, and *CYCLOPHILIN*. All data are expressed as a mean ± S.E.M, where different letters (A&B) represent differences at p<0.05 and C represents tendency for differences (P<0.1) after a one-way ANOVA.

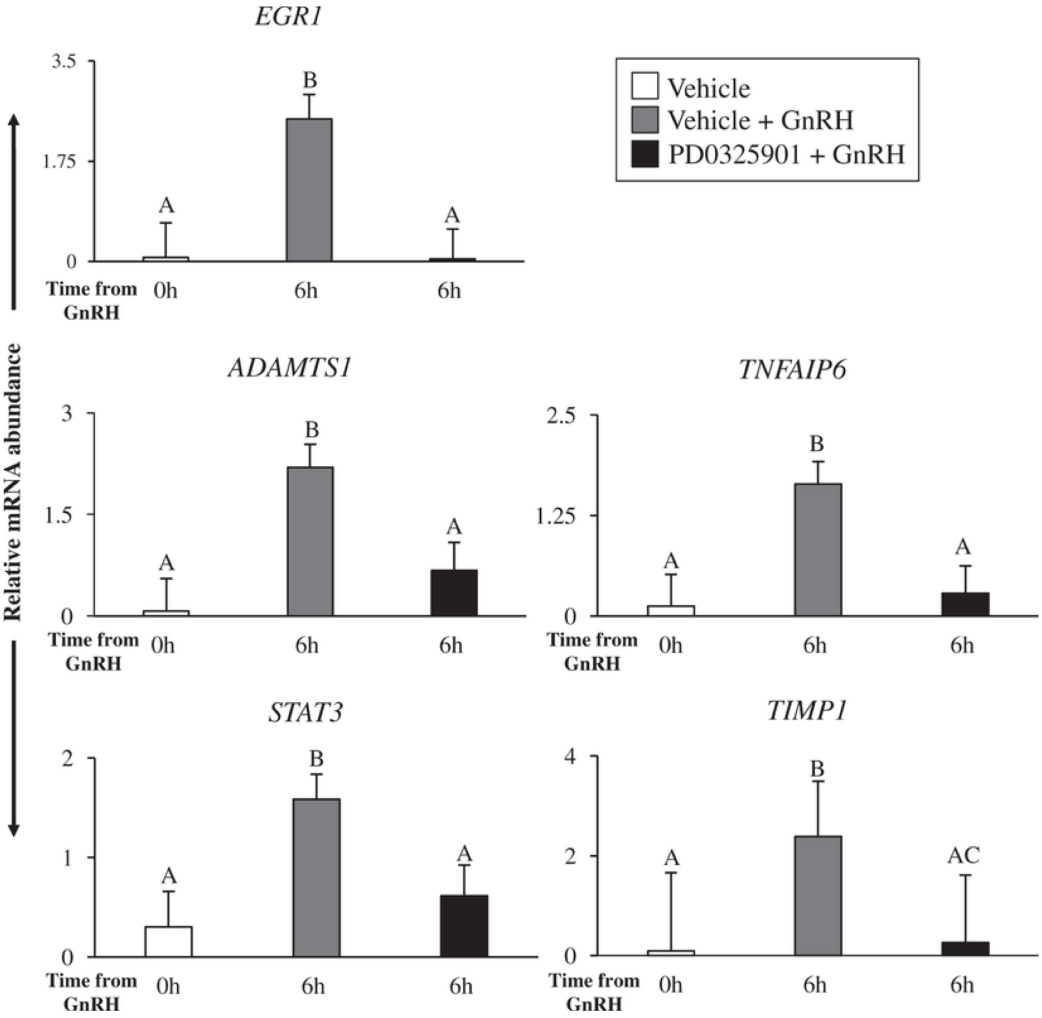


Figure 5.8.8: The impact of ERK1/2 pathway inhibition by PD0325901 on gene transcription in bovine theca cells. In the presence of PD0325901 following GnRH stimulation *TIMP1* is reduced in theca cells (A), while *ADAMTS1* and *STAT3* are induced (B). In bovine theca cells, *EGR1* and *TNFAIP6* remain unchanged between Vehicle + GnRH and PD0325901 + GnRH. Data were normalized to reference genes *ACTB, L19*, and *CYCLOPHILIN* (C). All data are expressed as a mean ± S.E.M, where * represents $p<0.05$.

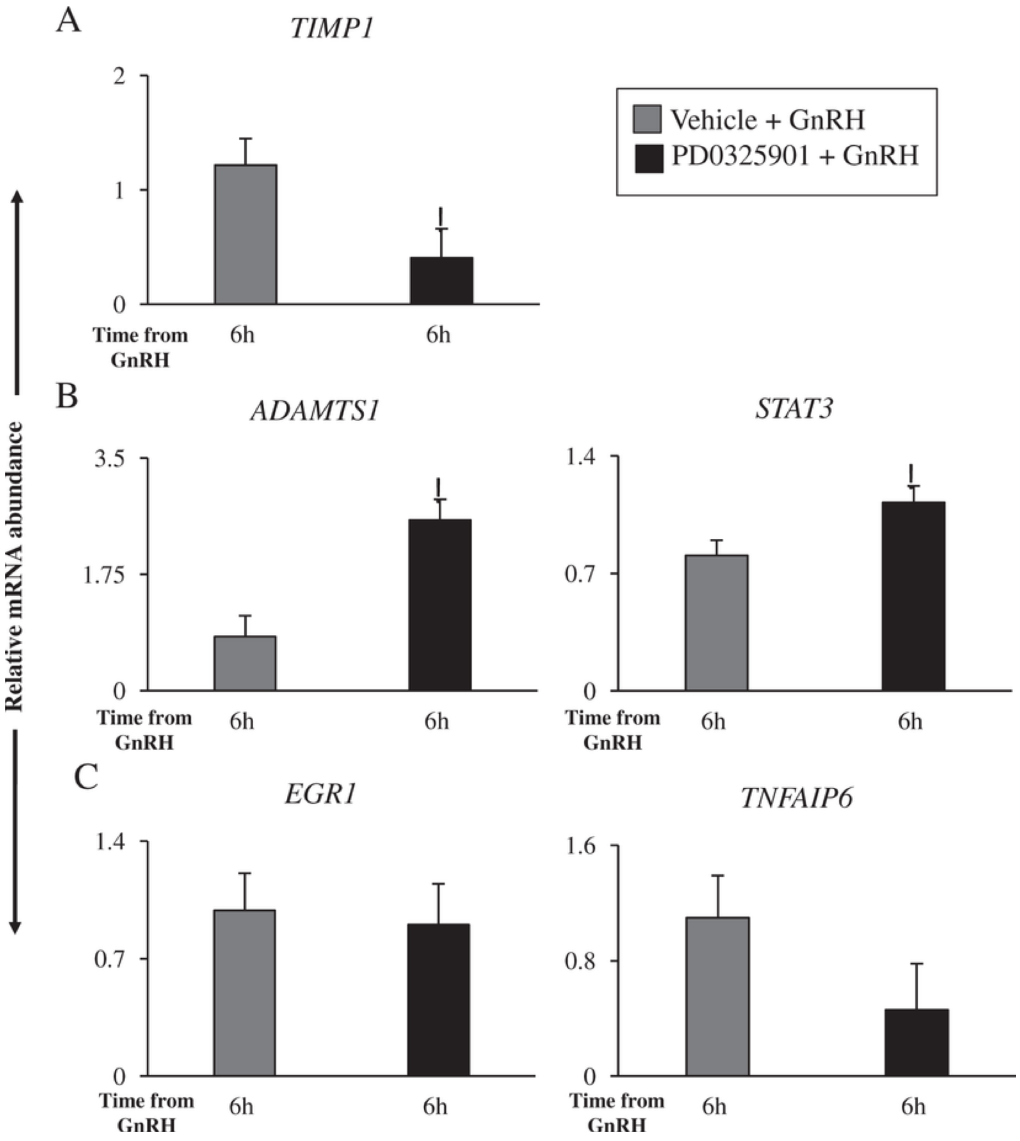


Figure 5.8.9: The impact of ERK1/2 pathway inhibition by PD0325901 on the transcription and translation of the steroidogenic enzyme STAR. A. Relative mRNA abundance of *STAR* comparing Vehicle + GnRH and PD0325901 + GnRH. B. STAR protein abundance by immunoblot in bovine granulosa cells subject to PD032590. EGR1 was used as a time-point control and ACTINB was used as a loading control. The blot was cut and cropped to analyze all three proteins by antibodies on the same blot. C. Densitometry reveals quantitative abundance of STAR protein abundance in GnRH stimulated bovine granulosa cells in the presence of PD0325901. D. RT-qPCR was performed on theca cells collected from cattle subject to GnRH treatment in the presence or absence of PD0325901 to establish the expression pattern of *STAR*. Transcript data were normalized to reference genes *ACTB, L19*, and *CYCLOPHILIN*. All data are expressed as a mean ± S.E.M, where letters (A&B) and * represents $p<0.05$ and ** represents $p<0.01$.

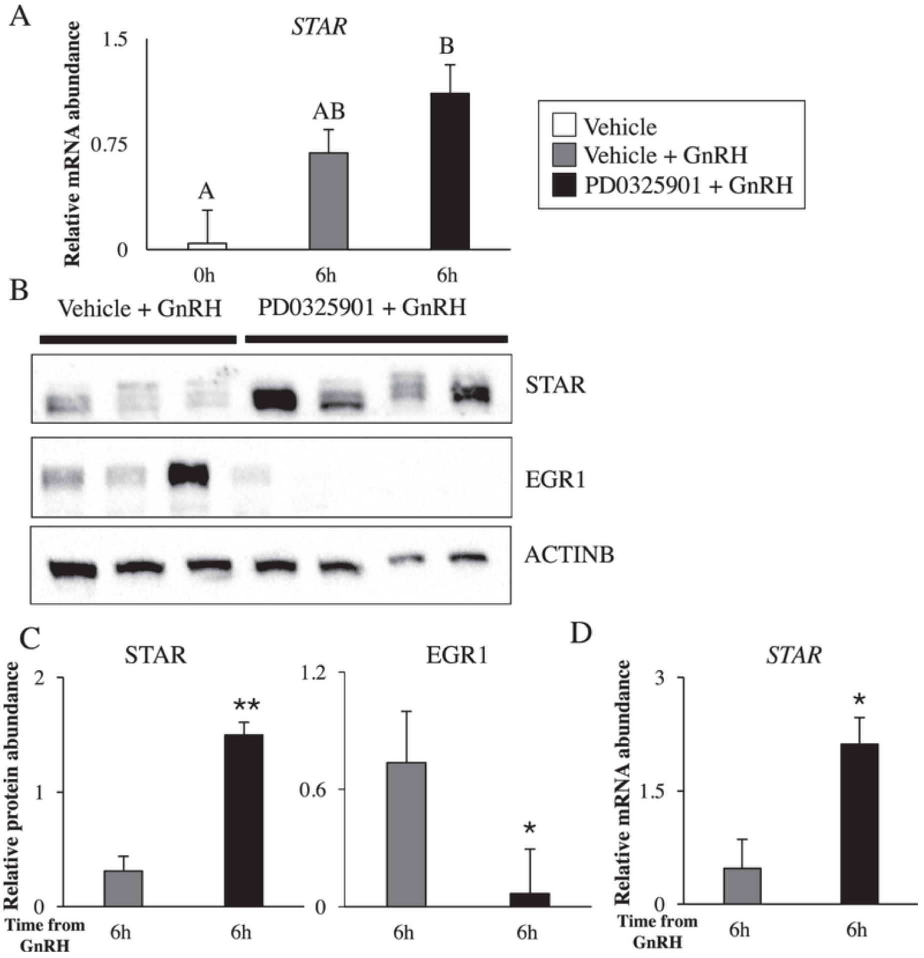


Figure 5.8.10: Intrafollicular administration of PD0325901 impact on SLC16A1. PD0325901 induces the relative mRNA abundance of solute carrier family 16 member 1 (*SLC16A1*), a suggested transporter of beta-hydroxybutyric acid, in both granulosa and theca cells. Transcript data were normalized to reference genes *ACTB, L19*, and *CYCLOPHILIN*. All data are expressed as a mean ± S.E.M, where different letters represents $p<0.05$ and ** represents $p<0.01$.

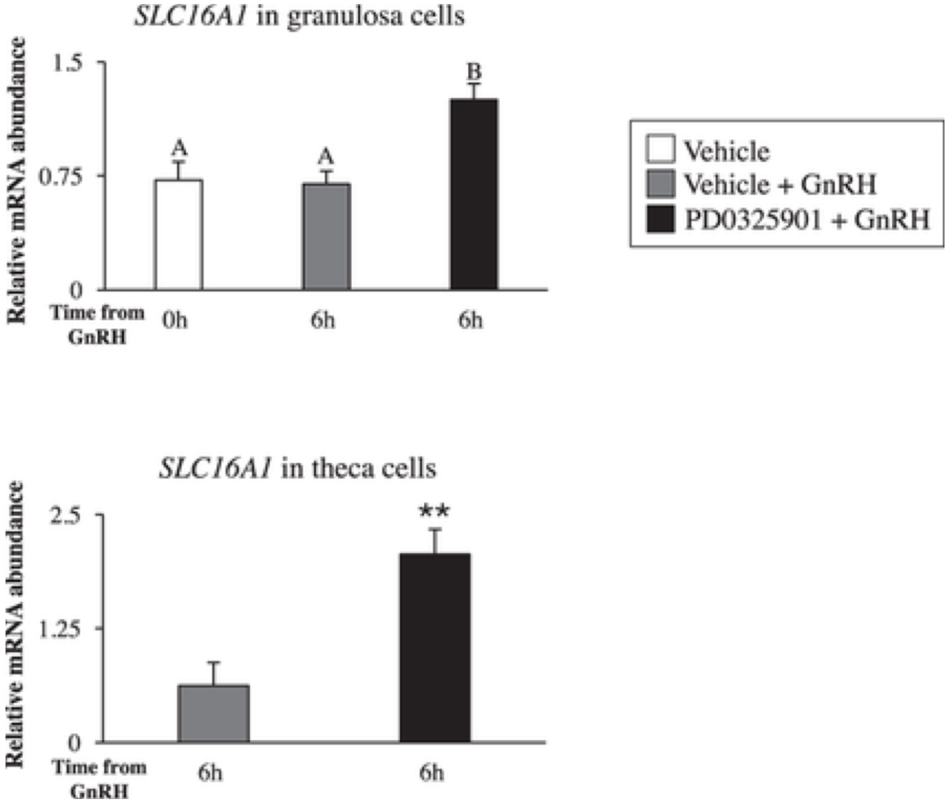
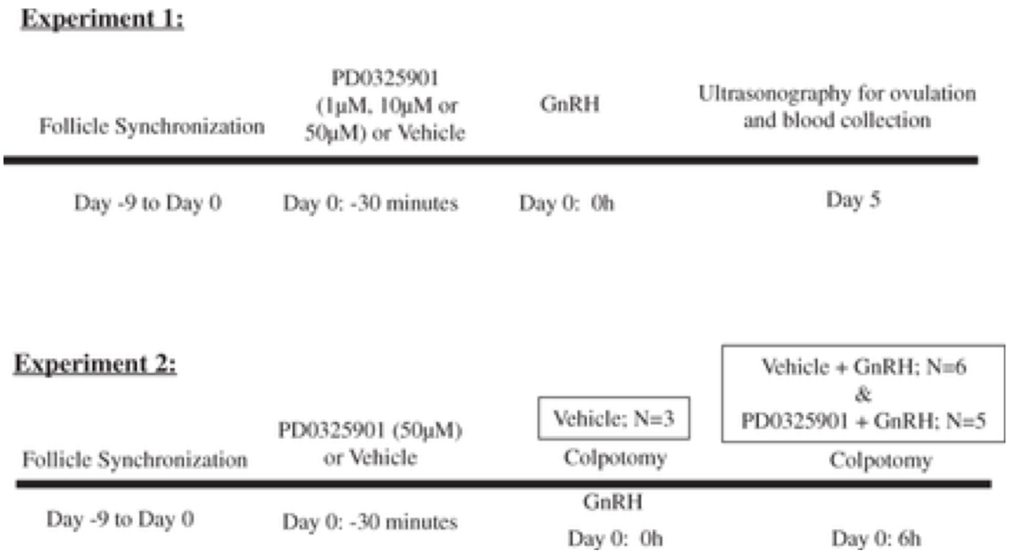
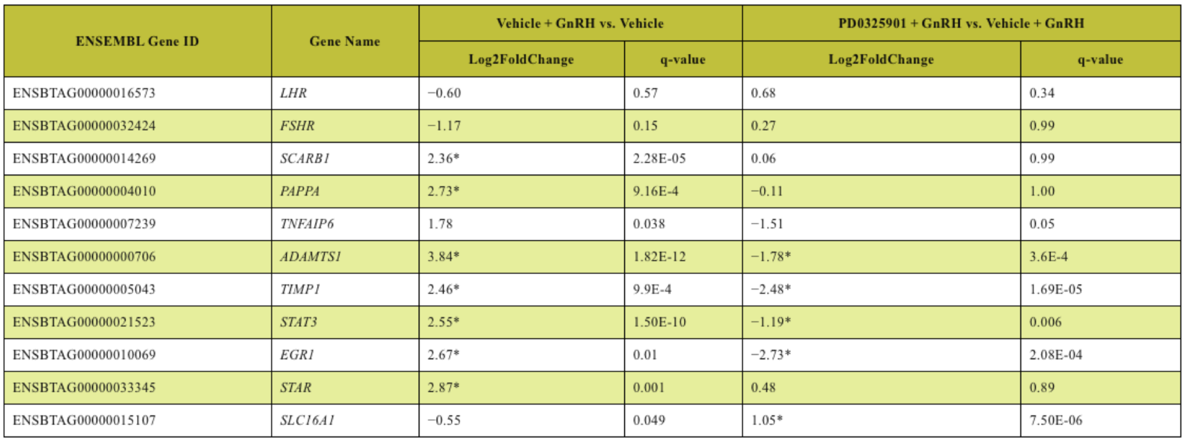


Figure 5.8.11: Experimental design for experiments 1 and 2. Timeline, treatment, and sample collection method for each experiment.



5.9 Tables

Table 5.9.1: Transcript abundance of 11 genes in bovine granulosa cells comparing Vehicle + GnRH to Vehicle and PD0325901 + GnRH to Vehicle + GnRH based on bioinformatics analysis. * q<0.01.



ENSEMBL Gene ID	Gene Name	Vehicle + GnRH vs. Vehicle		PD0325901 + GnRH vs. Vehicle + GnRH	
		Log2FoldChange	q-value	Log2FoldChange	q-value
ENSBTAG00000016573	*LHR*	−0.60	0.57	0.68	0.34
ENSBTAG00000032424	*FSHR*	−1.17	0.15	0.27	0.99
ENSBTAG00000014269	*SCARB1*	2.36*	2.28E-05	0.06	0.99
ENSBTAG00000004010	*PAPPA*	2.73*	9.16E-4	−0.11	1.00
ENSBTAG00000007239	*TNFAIP6*	1.78	0.038	−1.51	0.05
ENSBTAG00000000706	*ADAMTS1*	3.84*	1.82E-12	−1.78*	3.6E-4
ENSBTAG00000005043	*TIMP1*	2.46*	9.9E-4	−2.48*	1.69E-05
ENSBTAG00000021523	*STAT3*	2.55*	1.50E-10	−1.19*	0.006
ENSBTAG00000010069	*EGR1*	2.67*	0.01	−2.73*	2.08E-04
ENSBTAG00000033345	*STAR*	2.87*	0.001	0.48	0.89
ENSBTAG00000015107	*SLC16A1*	−0.55	0.049	1.05*	7.50E-06

5.10 Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-34015-4

CHAPTER VI

PUTTING IT ALL TOGETHER

6.1 GENERAL DISCUSSION AND CONCLUSIONS

For a dairy producer, the golden ticket is a herd of cows that produce an abundance of milk, has calm temperaments with remarkable conformation traits (udder, legs, rump, etc..), never suffer from illness, remain in the herd for several lactations, and of course transmit all favourable traits. Working towards developing such an astonishing herd requires exceptional genetics, comfort, and nutritional management, in addition to the ability of cows to be fertile. As is well-documented in this thesis and in the literature, modern lactating cows experience sub-fertility, which in part is linked to the ability of a cow to overcome the transition period (Staples et al., 1990, Walsh et al., 2007). The onset of lactation is an energetically demanding time, where feed intake does not sufficiently support maintenance and milk production, where the cow is dependent on lipid mobilization to meet energy requirements (Drackley, 1999). A state of negative energy balance can persist for many weeks in early lactation, directly and indirectly impeding reproductive performance (Wankhade et al., 2017). As reproduction is the number one reason cows are involuntarily culled from Canadian farms (Centre, 2018b), the topic merits additional investigation, specifically with regards to ovarian function.

In livestock, the release of a fertilizable oocyte is orchestrated through a number of signaling cascades including ERK1/2, which are gonadotropin-dependent (Fortune, 1994, Ryan et al., 2008). Moreover, the competence of an oocyte may be influenced by the follicular microenvironment, through proper maturation and function of granulosa and theca cells, as well as the metabolites and hormones present in the follicular fluid (Lucy et al., 1992b, Bender et al., 2010, Sanchez et al., 2014). In dairy cattle, it is imperative to consider the length of time required for a follicle to grow and reach ovulatory size, which is estimated to be 80-100 days (Britt, 1992). In modern North American dairy farms, a voluntary waiting period (VWP) of 60 days in milk is typical before first attempt at breeding (Miller et al., 2007). Therefore, the ovulatory follicle for first attempt at breeding is developing in the time frame coinciding with the transition period into early lactation met with drastic changes in metabolic profiles shown to be reflected in the follicular fluid (Britt, 1992, Lucy et al., 2014).

Considered a minimally invasive procedure, blood collection for profiling of metabolites and hormones from dairy cows provides an effective alternative to follicular fluid aspiration, as the former normally reflects the condition of the latter. A number of metabolites (NEFA, BHBA, cholesterol, triglycerides, bile acids, etc..) and hormones (estradiol, progesterone, IGF1, etc..) have been previously measured concurrently in blood and follicular fluid of dairy cows (Leroy et al., 2004b, Bender et al., 2010, Sanchez et al., 2014). These studies have clearly established that for example, follicular fluid BHBA levels are nearly identical, while total cholesterol is approximately 45% (Leroy et al., 2004b) and total bile acids are approximately 70% (Sanchez et al., 2014) of the levels found in blood. This information provided a solid base for our first study, where we compared plasma metabolic indicators and oxidative stress markers of primiparous cows, multiparous cows, and nulliparous heifers in the weeks leading up to the planned time of breeding.

Dairy heifers are more fertile than lactating cows, as shown by measurements of reproductive markers including reduced pregnancy rates in lactating cows (Balendran, 2008). Elsewhere, concurrent analysis of the follicular fluid from dominant follicles and/or preovulatory follicles and the blood profiles comparing heifers and lactating cows showed drastic changes in metabolite and hormone concentrations and composition. Lactating cows have higher levels of NEFAs (specifically, palmitic and stearic acid), BHBA, total bile acids, and total cholesterol and lower levels of IGF1 and glucose (Bender et al., 2010, Sanchez et al., 2014). However, metabolic indicators and oxidative stress markers analyzed in plasma of primiparous cows, multiparous cows, and nulliparous heifers in the weeks leading up to timed breeding remained poorly addressed. Ultimately, the results of our first study provided a window into the follicular microenvironment of the developing follicles for dairy cattle in different physiological states in the weeks leading up planned time of breeding. Not surprisingly, we showed that primiparous and multiparous cows tend to have lower pregnancy rates than heifers. As for metabolic profiles, we showed that in the week prior to anticipated time of breeding the plasma levels of BHBA were a staggering 2-fold higher in multiparous cows (~50 days in milk) compared to nulliparous heifers. This difference alone may have an important impact on granulosa cell function specifically in multiparous cows. It has been shown that granulosa cells collected from cows with elevated plasma BHBA levels at planned time of breeding and also, bovine granulosa cells treated with elevated levels of BHBA (1.5mM) have impaired function (Vanholder et al., 2006, Girard et al., 2015b). Lactating cows had higher plasma concentrations of total cholesterol, HDL, LDL, total bile acids, FRAP and lower plasma concentrations of glucose, triglycerides, and VLDL than heifers. We speculate that by planned time of breeding lactating cows and more specifically multiparous cows have a follicular milieu composed of the aforementioned changes, which acting together may hinder the developmental competence of follicles. After establishing that the metabolic milieu of heifers and lactating cows is drastically different, we went on to perform our second study to evaluate the molecular basis of unfavourable adaptation to early lactation using specifically multiparous cows as our experimental subjects.

A number of factors may impede a cow's ability to successfully adapt to early lactation including the degree of *change* in BCS experienced during the transition period (Carvalho et al., 2014b, Akbar et al., 2015). Evaluating the change in BCS has become a helpful management tool for dairy producers to visually monitor the degree of lipid mobilization of individual cows. Similar to previous studies, in our second study we showed that minimizing the loss of BCS in the transition period to planned time of breeding provides favourable development of a dominant follicle. Excess body condition loss, which we defined as ≥ 0.75 BCS loss, from 3 weeks pre-partum to 7 weeks post-partum has dire consequences on the overall ovarian health of dairy cattle. Previous studies have shown that the pre-partum dairy cow should maintain a BCS between 3.0 to 3.5 (Drackley and Cardoso, 2014, Puppel and Kuczynska, 2016). We emphasize this recommendation in order to prevent excess BCS loss, since cows that lost ≥ 0.75 BCS also had a higher BCS during the week of parturition compared to cows losing <0.75 BCS. During the sampling period, cows experiencing excess body condition had higher levels of plasma NEFAs (tendency) and GGT, establishing excess lipid mobilization and decreased liver function, respectively. Interestingly, high GGT levels have been linked with fatty liver, an economically compromising disease (Sevinc, 2001). In line with higher GGT levels, cows losing excess BCS had increased hepatic *CYP7A1* expression, a gene involved in bile acid synthesis (Wooton-Kee et al., 2010, Lionarons et al., 2016). Although *CYP7A1* mRNA transcript abundance naturally increases at the onset of lactation (Schlegel et al., 2012, Liang et al., 2017), we suggest that this change is more pronounced in cows experiencing excess BCS loss.

Most strikingly, the compromised metabolic environment of cows losing ≥ 0.75 BCS drastically impeded follicle development as evidenced by low concentrations of follicular fluid IGF1 in the dominant follicle at 7 weeks post-partum. We previously established that lactating cows are already plagued with lower follicular fluid IGF1 compared to heifers (Sanchez et al., 2014), a difference which is of even greater importance during excess BCS loss. Lower plasma IGF1 levels have been associated with decreased follicular competence, estrogen-inactive dominant follicles, anovulation, lower conception rates to first service and longer interval to commencement of luteal activity (Beam and Butler, 1997, 1999, Patton et al., 2007, Castro et al., 2012, Dupont et al., 2014). In addition, IGF1 contributes to the induction of *LHCGR, IGF1R,* and *CYP19A1* in granulosa cells follicle (Spicer and Echternkamp, 1995, Beam and Butler, 1999, Mani et al., 2010, Rawan et al., 2015), important in maintaining follicle dominance and drastically lower in cows experiencing excess BCS loss. We predict that the lower granulosa mRNA transcript abundance of *NR5A2* also contributes to attenuation of *CYP19A1* gene expression (Sahmi et al., 2014). The altered granulosa cell profile further explains the decrease in follicular fluid estradiol levels, a hormone known to be higher in dominant versus subordinate follicles (Fortune, 1994). Taken together, cattle experiencing greater body condition loss during the transition period leading to planned time of breeding had an unfavourable follicular microenvironment preventing proper acquisition and development of the dominant follicle. Alas, we propose that the release of a fertilizable oocyte in dairy cattle is dependent upon alleviating metabolic imbalances, through reduced loss of BCS.

In addition to the impact of the transition period on reproductive success, further understanding of sub-fertility in dairy cows requires a deeper investigation directed towards signaling of the preovulatory follicle. Therefore, in our third and final study, we addressed the role of ERK1/2 signalling in the bovine ovulatory follicle. This pathway has previously been shown to be imperative in ovulation in the mouse model (Fan et al., 2009, Siddappa et al., 2015). Cows were subject to follicular injection of the dominant follicle with 50μM of PD0325901, an ERK1/2 signaling inhibitor, followed by GnRH administration to induce an LH surge. Cows subjected to the aforementioned treatment failed to ovulate as evidenced by lack of a corpus luteum and lower levels of circulating progesterone. To understand the mechanisms behind failed ovulation, we investigated the gene expression profile of granulosa and theca cells of the preovulatory follicle at 6h post-GnRH in the presence or absence of PD0325901. This study was novel as it is the first of its kind to study the role of ERK1/2 *in vivo* in the cow and subsequently, analyze granulosa cells from a pharmacological inhibition study by RNA-sequencing. Pathway analysis among granulosa cell RNA-sequencing revealed an important role for immune processes in ERK1/2-dependent LH-regulated genes.

Similar to mice treated with PD0325901 not all genes that were LH-induced are dependent on ERK1/2 signaling as observed by unaltered levels of LH-induced *SCARB1* and *PAPPA* expression (Siddappa et al., 2015). Although both genes play a role in female fertility(Trigatti et al., 1999, Nyegaard et al., 2010), they are not regulated by ERK1/2 signaling. In contrast, *EGR1*, *ADAMTS1*, *TNFAIP6, STAT3*, and *TIMP1* transcript abundance in granulosa cells are all LH-induced and ERK1/2 dependent. All of which shown to be required for female fertility. Surprisingly, species differences do exist, where in the mouse, abrogation of ERK1/2 signaling attenuates *Star* expression (Fan et al., 2009), while the opposite has been observed in *in vitro* ERK1/2 signalling inhibitor treated bovine theca cells, human granulosa cells and immortalized rat granulosa cells (Seger et al., 2001, Tajima et al., 2003, Tajima et al., 2005). We showed that PD0325901 treatment did not affect *STAR* expression in granulosa cells at 6h post-GnRH. However, granulosa cells did have elevated STAR protein abundance and theca cells had higher levels of *STAR* mRNA transcript abundance in ERK1/2-inhibited follicles. Lastly, our RNA-sequencing data revealed an increase in *SLC16A1* gene expression in ERK1/2-inhibited follicles, which was confirmed by qPCR and also observed in theca cells. We predict that this gene, never before identified in the bovine ovary, may be involved in BHBA uptake by granulosa and theca cells, as suggested in other cell types as a BHBA transporter (Muller et al., 2002, Fu et al., 2015b). Therefore, ERK1/2 signaling may mediate the reduction in BHBA uptake in preovulatory follicles, a metabolite known to hinder granulosa cell function (Vanholder et al., 2006, Girard et al., 2015b).

In conclusion, poor fertility exhibited by modern dairy cows negatively affects the economical sustainability of the herd (Delgado, 2018). Reproductive issues represent the principal reasons cows are involuntary culled, which in turn affects the overall longevity of cows in herds (Valacta, 2018). The average cow only begins financially contributing to the operation in the 3rd lactation (Pellerin et al., 2014), and on Quebec herds only approximately 40% of cows are in their 3rd or higher lactation (Valacta, 2018). Increasing reproductive longevity is essential for the success of each and every dairy farm, where adequate management during the transition period and the voluntary waiting period is imperative as shown by the research presented in this thesis. By demonstrating that 1) the metabolic profile is drastically different between heifers and lactating cows, 2) cows losing severe BCS loss hinders follicle development, and 3) ERK1/2 is pivotal for ovulation, we have contributed to the understanding of the underling mechanisms affecting ovarian function in cattle. These data provide additional pieces to the puzzle of infertility, which in the future can be incorporated into therapeutic transition period management strategies.

6.2 FUTURE PERSPECTIVES

Increasing dairy cow longevity on Canadian dairy herds by improving reproductive management through a deeper understanding of ovarian physiology is imperative. The studies presented in this thesis have contributed to the knowledge behind sub-fertility in response to difficulty in adaptation to early lactation and the pivotal role of ERK1/2 signaling in bovine ovulation. The results can be used to develop additional experiments.

Of particular interest from the results of the first study, we observed distinct metabolic and oxidative stress profiles between multiparous cows, primiparous cows, and nulliparous heifers. As lipoproteins are essential for steroidogenic processes, their presence in the follicle is crucial, and their proportions were different between the three groups of animals based on the results of the first study. Therefore, analysis of granulosa cell function and steroidogenesis (gene expression and hormone analysis) in cultured granulosa cells treated with lipoproteins (HDL and LDL) at different concentrations and ratios would be interesting. A similar study can be performed with different levels of BHBA, since a previous *in vitro* study showed alterations in granulosa cell proliferation and estradiol and progesterone concentrations without investigating the direct impact on gene expression of granulosa cells (Vanholder et al., 2006). Furthermore, others have concluded that high levels of blood BHBA can have detrimental effects on follicular dynamics and reduce reproductive performance (Ospina et al., 2010b, Girard et al., 2015b). One could also investigate the profiles of bile acids in the three groups of animals. Bile acids can act as signaling molecules (Shapiro et al., 2018) and therefore, it would be interesting to study the direct effects of bile acids on granulosa cell functions. It would be beneficial to establish the concentration and profile of individual NEFAs during the weeks leading up to planned time of breeding. This would then establish if the NEFAs previously identified in cultured bovine granulosa cells (palmitic acid and stearic acid) to alter cell proliferation and steroidogenesis may be present in greater concentrations as the number of parities increase (Vanholder et al., 2005).

In the second study, we showed that excess BCS loss impedes development of the dominant follicle. The underlying mechanisms leading to poor dominant follicles appear to be linked with lower concentrations of follicular fluid IGF1, and granulosa cell expression of *CYP19A1, IGF1R,* *NR5A2*, and *LHGCR,* consequently decreasing estradiol production. Performing RNA-seq on granulosa cells as done in the third study will be necessary to establish the gene expression program and mechanisms of ovarian dysfunction in cows losing moderate and severe BCS. Knowledge from both the first and second studies could contribute to the development of targeted nutritional strategies by fine-tuning energy and fibre content in rations from the pre-partum period to the period leading up to planned time of breeding. Ultimately, we would like to mimic a metabolic profile in lactating cows similar to the one in heifers, by minimizing the drastic metabolic fluctuations observed and prevent overconditioning during the dry period.

We will have to further investigate some genes regulated by ERK1/2 signalling in bovine follicles. Of particular interest is *SLC16A1*, which was both increased in granulosa and theca cells of pre-ovulatory follicles when ERK1/2 signaling was abrogated. It would be interesting to test the hypothesis that *SLC16A1* is involved in BHBA uptake by bovine granulosa and theca cells. First, cultured bovine granulosa and theca cells treated with a pharmacological inhibition of *SLC16A1* by pCMBS amongst other currently available inhibitors is a viable option (Muller et al., 2002) or even a more targeted approach of siRNA can be used. Next, we could add different concentrations of BHBA to the cultures and establish if BHBA can alter steroidogenesis. Again, using an *in vitro* approach, we can further our investigation into the role of *EGR1* transcription factor speculated to be downstream the ERK1/2 signaling pathway and identify the genes it regulates in bovine granulosa cells. Overall, the three studies performed in this thesis open the door to a better understanding of bovine reproduction.

CHAPTER VII

APPENDIX: PROGRESSIVE DAIRYMAN → CANADA

7.1.1 Text published in Progressive Dairyman → Canada (October 2017)

It's not easy getting older: Heifers vs. lactating dairy cows as they transition

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The dairy cow is quite an extraordinary creature. Time and time again the record for highest milk production is broken, most recently by My Gold from Ever-Green-View Farms in Wisconsin with a mind-boggling 77,480 pounds (35,144 kilograms) of milk produced in a 365-day period (Bullvine, 2017b). Without a doubt, the biological limit for milk production by our dairy cows has not been reached and we can look forward to this record being challenged sooner rather than later. However, not as easily broken is another record, one which honours the cow producing the greatest amount of milk over her lifetime. To this day, Gillette Emperor Smurf from Ontario holds on to this title, whereby she produced 478,163 pounds (216,891 kilograms) of milk by the time she reached 15 years of age, a record which has stood since 2012 (Records, 2012). Although, fascinating, it is simultaneously unfortunate that no dairy cow could dethrone Smurf in the past five years.

In a nutshell, this tells us that cows might be producing astonishing volumes of milk, but they are simply unable to realize their full potential of the number of lactations. In addition to economics, this also raises important ethical questions. Do modern dairy cows achieve their full potential of lifetime calf and milk production? Is it reasonable to propose that cow longevity is the key factor that deserves attention when it comes to running a sustainable and profitable dairy operation? Table 7.2.1 provides a summary of our typical Canadian dairy farms. Does this table represent an ideal scenario? Well, not exactly and for that reason the focus of research is directed towards decreasing age at first calving, increasing the number of cows making it to subsequent lactations and decreasing the percentage of cows leaving the herd due to poor reproductive performance, all without drastically affecting their production performance. We need to address these parameters if we wish to see our cows last just a little bit longer.

At McGill University's annual Centre for Research in Reproduction and Development Research Day, held in May, a guest speaker (Dixon, 2017) said, ”Life is short, but fertility is shorter.” This is obviously and undeniably true, but when it comes to cows we must modify this quote to: “Life is shorter, because fertility is short.” Overall, fertility is viewed as a hot topic, especially considering that issues in reproductive performance are the predominant reasons cows are being culled (Centre, 2016). It is no secret that the transition period, defined as three weeks pre-calving to three weeks post-calving (Drackley, 1999), is viewed as a challenging time for both the cow and the producer. The producer will do his or her best to get the cow off to a solid start at the onset of lactation and get her ready for breeding within 80 to 100 days in milk. Nonetheless, this period comes with numerous challenges. It is well-established that transition into early lactation is characterized by drastic physiological changes leading to negative energy balance often followed by metabolic stress (Drackley, 1999). In severe cases, cows will go on to suffer from diseases such as ketosis, displaced abomasum and milk fever, all of which can result in costly losses in milk production or even death (Goff and Horst, 1997). Often at the onset of lactation, a cow can easily be the victim of abnormally decreased dry matter intake, increased fat mobilization, disease, a weakened immune system or liver damage, which can have a snowball effect and take a toll on subsequent reproductive performance (Drackley, 1999). Of course, a plethora of studies have and continue to investigate vitamin (LeBlanc et al., 2002, Duplessis et al., 2014, Pontes et al., 2015), mineral (Sordillo, 2013) and protein (Zhou et al., 2016a, Zhou et al., 2016b) supplementation, energy formulation (Janovick et al., 2011, Mann et al., 2015), modifications of the dry period length (Jolicoeur et al., 2014, Weber et al., 2015) and the body condition score around calving (Bernabucci et al., 2005, Carvalho et al., 2014b) among others. So far, there is no one silver bullet to increase cow longevity.

Ultimately, enhancing our understanding beyond the current knowledge of physiology during the transition period will help us formulate right hypotheses to test by scientific experiments and eventually develop more strategies to manage metabolic imbalance of lactating dairy cows. On that note, we must remember that the extent of transition period and metabolic alterations do change as cows get older. Simply put, heifers and cows differ greatly from a metabolic standpoint when it comes to the period leading up to breeding. Our heifers rarely give us breeding troubles, while our older cows come with some added challenges, especially depending on their success during the transition period. As they say, “little kids, little problems; big kids, big problems”. I suppose one analogy we can use is to compare our dairy cattle to car brands. First, we have our 13- to 15-month-old heifers, these are our Honda Civic models, a nice car for first time drivers; it works well and can tolerate a small fender bender here and there. But as soon as these heifers enter their first transition period, makes and models start to change. Of course, you will have some cows that will maintain their trouble-free status, but the spectrum of makes increases. Now you will start dealing with the Fords, Volkswagens, Mercedes, Audis, Ferraris, Teslas, Porsches and Lamborghinis. With so many different makes in the barn, producers have quite the task ahead of them to meet everybody's individualized needs. Therefore, it is important to clarify different but specific types of metabolic alterations that occur during transition period.

Blood chemistry has been used for decades to analyze the health status of cows, where a wide-range of metabolites and hormones can be measured to assess immune function, liver health and energy status. Ketone bodies, most notably, Beta-hydroxybutyric acid (BHBA), can easily be measured on-farm and provide producers with the likelihood of ketosis, where elevated levels (>1.2mmol/L) report a sign that cows are using their body fat stores as an energy source instead of obtaining energy from their diet (Drackley, 1999).

Our group in the Animal Science Department at McGill University has compared the metabolic profile from the blood of cattle at different stages of production: heifers (13 to 15 months old) vs. cows entering their first lactation vs. cows entering their second or greater lactation. This paints a colourful picture of animals experiencing the transition period versus "non-stressed" heifers. If we compare the BHBA levels three weeks pre-calving to 12 weeks post-calving, overall, older cows have higher levels of BHBA during this time when compared to cows entering their first lactation (Figure 7.3.1). Furthermore, if we look at seven weeks post-calving, a time right before breeding these cows, and compare to heifers (blood collected at a time right before they are to be bred by about 15 months of age). We can see that overall the BHBA levels for older cows are still very high even past the transition period, when heifers have significantly lower levels of BHBA. As cows get older, milk production increases which would suggest they have an increased need for energy in the form of feed or lipid mobilization (Figure 7.3.2). Heifers are not investing any energy other than for maintenance and breeding and will therefore not depend on mobilizing fat tissue. If cows are not experiencing any drastic health issues during the transition period that can severely hinder milk production, it is well established that mature cows will produce more milk than first lactation cows and will really shine and be most profitable by the fourth lactation

(Pellerin et al., 2014). This is a solid reason for keeping our cows around for more than one lactation, especially considering that the Canadian dairy cow only starts making a profit during the second lactation (Murray, 2013). However, this means we must make sure our cows are getting the most out of their diet, especially in the form of optimal dry matter intake and energy to decrease fat mobilization and improve energy used for milk production and subsequent reproduction.

Energy in the form of glucose levels (Figure 7.3.3) provides a striking example of differences between lactating cows and heifers during the weeks leading up to the breeding period. Glucose requirements are elevated in lactating cows for milk synthesis and very low levels are associated with metabolic stress (Leroy et al., 2008, Sanchez et al., 2014). Energy in the form of glucose is a component for immune regulation and helps in maintaining an important population of immune cells necessary for overall "body clean-up" (Pithon-Curi et al., 2004, Graugnard et al., 2012). Moreover, it has been suggested that high levels of BHBA, which represent an alternate source of energy, can be used by cows, but can simultaneously alter normal glucose metabolism (Zarrin et al., 2017). Ultimately, cows are at an automatic disadvantage in terms of energy in the form of glucose for immune function when compared to heifers, perhaps influencing overall physiological preparation for the onset of breeding. Therefore, our management must be altered depending on the stage of production (Reynolds, 2005).

It is good to consider the metabolic parameters in blood circulation as they are reflected in the ovarian follicular fluid (Sanchez et al., 2014). The latter is an environment that supports growth of the healthy egg (oocyte) with adequate developmental potential, which can have implications well beyond fertilization including the development of the fetus and its eventual production performance as an adult. In fact, the follicle that will be ovulating (releasing the egg into the uterus for fertilization), at the first attempted breeding, requires approximately 11 to 14 weeks to grow (Britt, 2008). If we back track, the ovulating follicle would be developing during the transition period when metabolites are severely altered; this is not the case for heifers. It comes to show the importance of adapting overall management of our cows, depending on their age and stage of production to increase overall longevity, by providing a smooth transition period. More fundamental and applied research is necessary so that we can develop individualized approach to take good care of our Ferraris and give Smurf a run for her money.

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Table 7.2.1 What is happening on our dairy farms?

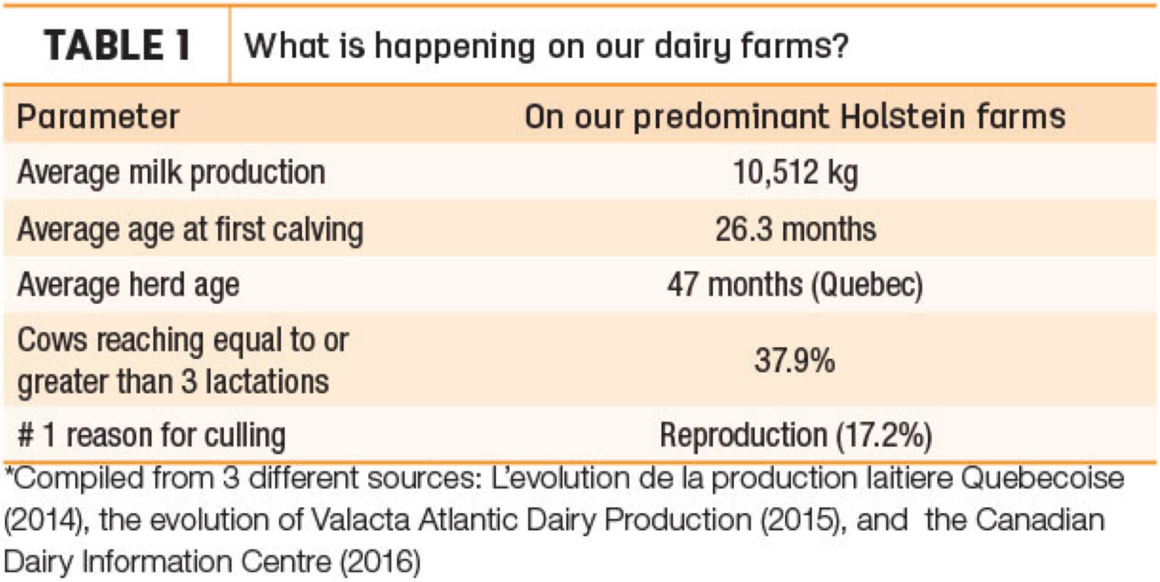


TABLE 1 What is happening on our dairy farms?

Parameter	On our predominant Holstein farms
Average milk production	10,512 kg
Average age at first calving	26.3 months
Average herd age	47 months (Quebec)
Cows reaching equal to or greater than 3 lactations	37.9%
# 1 reason for culling	Reproduction (17.2%)

*Compiled from 3 different sources: L'evolution de la production laitiere Quebecoise (2014), the evolution of Valacta Atlantic Dairy Production (2015), and the Canadian Dairy Information Centre (2016)

Figure 7.3.1: A look at ketones at different stages of production –BHBA beta-hydroxybutyric acid

FIGURE 1 A look at ketones at different stages of production – BHBA beta-hydroxybutryic acid (unpublished data, May 2017)

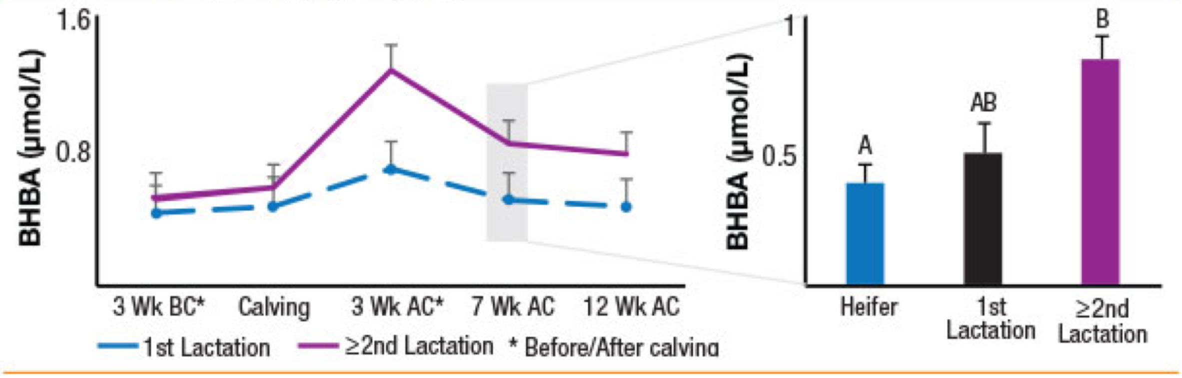


Figure 7.3.2: Changes of milk production among lactations

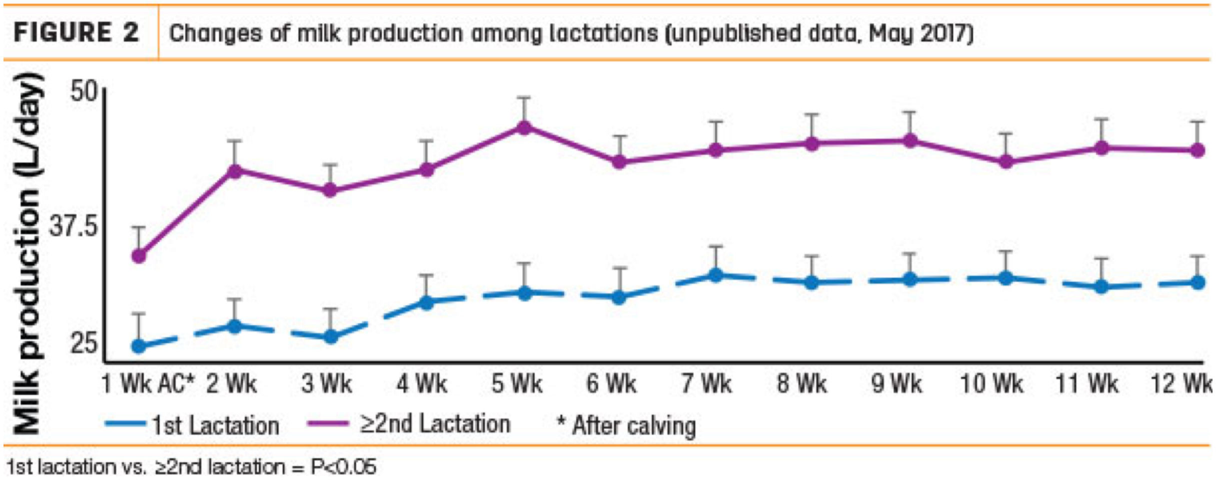
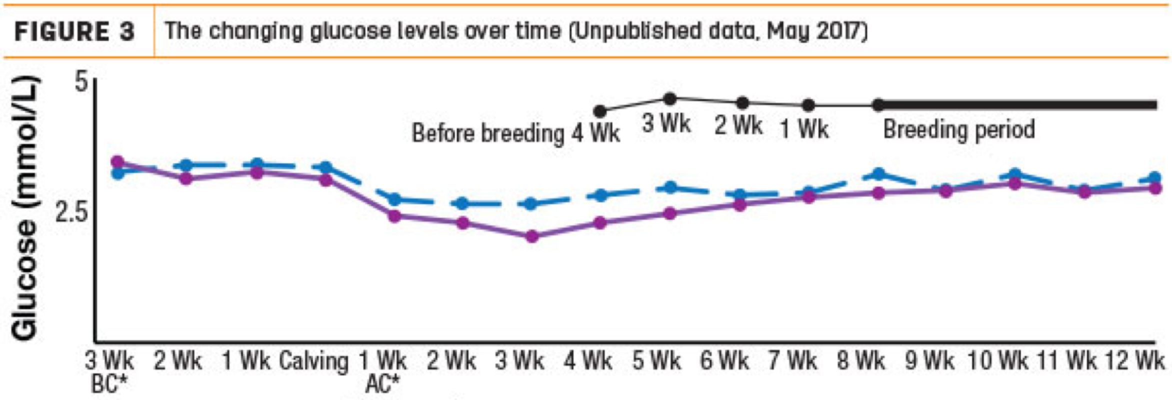


Figure 7.3.3: The changing glucose levels over time



CHAPTER VIII

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