Effect of cow's metabolic status on the epigenome profile of oocytes and embryos

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Mikhaël Poirier

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Dedicated to Berinjela

Abstract

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In lactating cows, intensive genetic selection for improved milk production has been associated with reduced reproductive capacity, leading to increased early embryo loss. During post-calving lactation, the cow's dietary intake is outmatched by the energy needed for biological processes causing the cow to enter a state of negative energy balance, associated with a perturbed metabolism. This thesis project aimed to investigate the impact of this transient metabolic stress on the epigenetic profile of genes involved in developmental competency of gametes and embryos in lactating cows. To do so, oocytes were collected during early and mid postpartum period from metabolically profiled multiparous cows as well from nulliparous heifers. Comparatively, *in vitro* derived early cleaving embryos were cultured *in vivo* in lactating cows as well as heifers to morula stage and collected through uterine flushing. Epigenetic profile of both samples was assessed by whole genome bisulfite sequencing.

Metabolic profiling revealed that selected lactating cows exhibited significantly higher levels of non-esterified fatty acids and beta-hydroxybutyrate during early post-partum and embryo transfer than nulliparous heifers. Accordingly, genome-wide hypomethylation of early post-partum oocytes was observed, and mid postpartum oocytes genomic feature methylation was similar to heifer's oocytes. Inversely, embryos from lactating cows were generally hypermethylated when compared to heifers. Further methylation profiling of early postpartum oocytes revealed 32,990 differentially methylated regions in early postpartum oocytes overlapping genes involved in metabolic pathways, carbon and fatty acid metabolism. Similarly, embryos grown in lactating cows revealed 13,383 differentially methylated regions in genes involved in metabolic and fatty acid biological processes, as

well as trophoblast invasion and embryo implantation. Both oocytes and embryos collected from metabolically stressed animals revealed differentially methylated regions of genes involved in lipid metabolic processes, and imprinted genes putatively acquired during maturation that must be maintained during embryo development.

Taken together, these studies demonstrate that transient metabolic stress associated with early lactation influences epigenetic status of oocytes and embryos of genes involved in developmental competency. Genes found in signaling pathways provide novel candidates for effector supplementation of these signaling molecules both during oocyte maturation and embryo culture to ultimately improve reproductive processes in lactating cows.

Einfluss des Stoffwechselstatus der Milchkuh auf das epigenetische Profil von Eizellen und Embryonen.

Bei laktierenden Kühen führt eine intensive genetische Selektion auf eine verbesserte Milchproduktion zu einer verminderten Fortpflanzungsfähigkeit, die mit einem erhöhten frühen Embryoverlust verbunden ist. Während der Hochlaktation nach der Kalbung wird der Nährstoffbedarf der Kuh durch die Energie, die für biologische Prozesse benötigt wird, versorgt. Das führt dazu, dass die Kuh in einen Zustand der negativen Energiebilanz kommt, was wiederum mit einem abnormalen Metabolisum verbunden ist. Diese Arbeit zielte darauf ab, die Auswirkungen dieser transienten metabolischen Belastung auf das epigenetische Profil von Gameten und Embryonen bei laktierenden Kühen zu untersuchen und Genomregionen zu identifizieren, die durch den metabolischen Stress bei Milchkühen anfällig für epigenetische Veränderungen sind. Dazu wurden Eizellen während der frühen und mittleren postpartum Perioden von metabolisch auffälligen multiparösen Kühen sowie von nulliparen Färsen entnommen. Im Vergleich dazu wurden *in vitro* gewonnene frühe, sich entwickelnde Embryonen *in vivo* in laktierende Kühe sowie Färsen bis zum Morula-Stadium kultiviert und durch Uterusspülung gesammelt. Das epigenetische Profil der Eizellen und Embryonen aus beiden Experimenten wurde mittels der Genom Bisulfit Sequenzierung untersucht.

Das metabolische Profiling ergab, dass die ausgewählten laktierenden Kühe signifikant höhere Werte von nicht veresterten Fettsäuren und Beta-Hydroxybutyrat aufwiesen als die Färsen während des frühen post-partum und Embryotransfers. Dementsprechend wurde eine genomweite Hypomethylierung der frühen post-partum Eizellen beobachtet und es zeigte sich, dass das genomische Methylierungsprofil der späten post-partum Eizellen ähnlich zu den Eizellen von Färsen war. Umgekehrt waren Embryonen von laktierenden

Kühen generell hypermethytliert im Vergleich zu den Färsen. Das weitere Methylierungs-Profiling der frühen post-partum Eizellen ergab 32.990 differentiell methylierte Regionen. Dabei konnten Gene ermittelt werden die an verschiedenen Stoffwechselwegen wie dem Kohlenstoff- und Fettsäure-Stoffwechsel beteiligt sind. Ebenso zeigten Embryonen die sich in laktierenden Kühen entwickelten 13.383 unterschiedlich methylierte Genregionen, die sowohl an biologischen Stoffwechsel- und Fettsäureprozessen als auch an der Trophoblasteninvasion und der Embryoimplantation beteiligt sind. Sowohl Eizellen als auch Embryonen, die von metabolisch gestressten Tieren entnommen wurden, zeigten unterschiedlich methylierte Regionen bei Genen die am Fettstoffwechsel beteiligt sind sowie geprägte Gene, die vermutlich während der Reifung erworben wurden und die während der Embryoentwicklung erhalten werden müssen.

Zusammenfassend zeigten diese Studien, dass transienter metabolischer Stress in der frühen Laktation den epigenetischen Status von Eizellen und Embryonen in Genregionen beeinflusst, die an der Entwicklungskompetenz beteiligt sind. Gene, die in diesen Signalwegen gefunden wurden, sind neue Kandidaten für zukünftige Untersuchung zur Entwicklung von molekularen oder epigenetischen Marker die auf verschiedene Umweltfaktoren einschließlich dem metabolischen Stresses bei Milchkühen sensitiv reagieren.

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List of Abbreviations

ACO1/2   Aconitase 1/2

ADCY1/3/7/9 Adenyl cyclase 1/3/7/9

ADD1   Adducin 1

AGAP1   GTP-Binding And GTPase-Activating Protein 1

AKT   Protein kinase B

AM095   LPA receptor antagonist

APIP   APAF1 Interacting Protein

ARID1A   AT-Rich Interaction Domain 1A

ART   Assisted reproduction technique/technology

BCS   Body condition score

bDNMT3L   Bovine DNA (cytosine-5)-methyltransferase 3-like

BHB, B-OHB   Beta-hydroxybutyric acid, Beta-hydroxybutyrate

BRCC3   BRCA1/BRCA2-Containing Complex Subunit 3

BWe   Body weight energy

Ce   Concentrate energy

CH   Cyclic heifers

ChIP   Chromatin immunoprecipitation

CIDR   Controlled internal drug release

CpG/CG   Cytosine-(phosphate) phosphodiester link- Guanine dinucleotide

CRYBG1   Crystallin Beta-Gamma Domain Containing 1

DIM   Days in milk

DMI   Dry matter intake

DNMTS   DNA methyltransferases

DMR(s)   Differentially methylated region(s)

DNA   Deoxyribonucleic acid

dpp   day post-partum

EB   Energy balance

EGA	Embryo(nic) genom(e/ic) activation
EGFR	epidermal growth factor receptor
EM	Embryonic lineages
Epp	Early postpartum
ESPN	Espin
EX	Extra-embryonic lineages
FDPS	Farnesyl Diphosphate Synthase
FDR	False discovery rate
FSH	Follicle stimulating hormone
GH	Growth hormone
GDF-9	Growth and differentiation factor 9
GGN	Gametogenetin
GNAS	Guanine Nucleotide Regulatory Protein
HCN4	Hyperpolarization Activated Cyclic Nucleotide Gated Potassium Channel 4
ICM	Inner cell mass
IDH3A	Isocitrate Dehydrogenase (NAD(+)) 3 Alpha
IFN-τ	Interferon-Tau
IGF-1	Insulin-like growth factor 1
IGF2	Insulin-like growth factor 2
IGF2R	Insulin-like growth factor 2 receptor
INSR	Insulin receptor
ITGA2B	Integrin alpha-IIb
ITGB4/8	Integrin beta 4/8
IVF	In vitro fertilization
KEGG	Kyoto encyclopedia of genes and genomes
KLF9	Kruppel Like Factor 9
LAMB1	Laminin Subunit Beta 1
LH	Luteinizing hormone

LINE   Long Interspersed Elements

LMNA   Laminin A/C

lncRNAs   Long non-coding RNAs

LPAR1   Lysophosphatidic Acid Receptor 1

LTRs   Long terminal repeats

MAP4K3   Germinal Center Kinase-Related Protein Kinase

MDFIC   MyoD Family Inhibitor Domain-Containing Protein

MEST   Mesoderm specific transcript

miRNAs   Micro RNAs

miRISC miRNA-induced silencing complex

MJ   Megajoule

Mpp   Mid postpartum

MYe   Milk yield energy

ncRNAs   Non-coding RNAs

NEB   Negative energy balance

NEFA(s)   Non-esterified fatty acid(s)

NFKB1   Nuclear Factor Kappa B Subunit 1

NLRP5   NLR Family Pyrin Domain Containing 5

OPU   Ovum Pick-Up

p53   Tumor Protein P53

PALLD   Palladin, Cytoskeletal Associated Protein

PAK2/3   Serine/threonine-protein kinase PAK 2/3

PAX 9   Paired Box 9

PCA   Principal component analysis

PCSK5   Proprotein Convertase Subtilisin/Kexin Type 5

PDPK1   Phosphoinositide-dependent kinase-1

PEB   Positive energy balance

PEG10   Paternally Expressed Gene 10

PGC(s)	Primordial germ cell(s)
PGF2α	Prostaglandin F2 alpha
PI3KCD	Phosphoinositide-3-Kinase C
PKC	Protein kinase C
PPARd	Peroxisome Proliferator Activated Receptor Delta
PRDM1	PR Domain Zinc Finger Protein 1
PRKACB	Protein kinase A catalytic subunit beta
PRKCB	Protein Kinase C Beta
PRKD2	Serine/threonine-protein kinase D2
PRKCB/G	Protein kinase C beta/gamma type
PROM1	Prominin 1
PTMs	Post-translational modifications
qPCR	Real-time polymerase chain reaction
RECK	Reversion Inducing Cysteine Rich Protein With Kazal Motifs
RNA	Ribonucleic acid
RRBS	Reduced representation bisulfite sequencing
SEM	Standard error of the mean
SINE	Short interspersed nuclear elements
sncRNAs	small non-coding RNAs
SNRPN	Small nuclear ribonucleoprotein polypeptide N
SOD1	Superoxide Dismutase 1
STK10	Serine/Threonine Kinase 10
SYDE1	Synapse Defective Rho GTPase Homolog 1
TCA	Tricarboxylic acid
TE	Trophectoderm
TNIK	NCK-interacting protein kinase
TRAF2	TNF receptor-associated factor 2
TRPM4/7	Transient receptor potential cation channel subfamily M member 4/7

TTC7B	Tetratricopeptide Repeat Domain 7B
TU	Transcriptional units
UTR	Untranslated region
WGBS	Whole genome bisulfite sequencing
Zap70	Zeta-chain-associated protein kinase 70

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Chapter 1: General overview

1.1   General introduction

Dairy production has drastically evolved in the past decades in terms of both scale and intensity. One managing trend that contributed to the increased scale of milk production was the growing demand of housed systems. Most developed in North America, where 82.2% of dairy cows are housed (National Animal Health Monitoring System 2010), it is currently increasing in Europe, from less than 10% to 30% in Holland and 16% to 70% in just 15 years in Denmark (Reijs 2013). This type of husbandry permitted the implementation of robotic milking, custom rationing, control of climate and increased herd size over pasture based systems. Coupled with intensive selection and breeding, housing systems have dramatically increased milk yield per cow (Butler 2003) and reduced the nutritional and metabolic stress seen in pasture system cattle (Kolver and Muller 1998; Bargo et al. 2002; Boken et al. 2005; Fontaneli et al. 2005; Kay et al. 2005; Vance et al. 2012). Dairy industry goals dictate that a cow should produce a calf per year in order to remain economically viable in terms of milk production and feed efficiency (Ioannidis and Donadeu 2016). Yet, for more than 20 years there has been a decline in terms of reproductive performance in these high producing animals (Foote 1996; Butler 2000; Roche et al. 2000; Royal et al. 2000; López-Gatius 2003; Roche et al. 2011).

While the conception rate in heifers has consistently hovered around 70% (Foote 1975; Pursley et al. 1997b), it dwindled in lactating cows from over 50% in the 1960s (Casida 1961; Mares et al. 1961) to less than 40% in recent decades regardless of time of insemination post partum (Pursley et al. 1997a; Washburn et al. 2002; Butler 2003). While cows have to sustain pregnancy to ensure their optimal milk production, studies have shown them to have weaker signs of oestrus, delayed interval to first ovulation, higher embryo loss and

lower pregnancy rates (Roche et al. 2011). Still, even cows with proper ovarian cycle resumption and high fertilization rate when using optimally timed artificial insemination show impared gamete and conceptus quality (Diskin and Morris 2008). As a matter of fact, oocyte and embryo quality seems to be at the heart of the problem, as 40% of early embryos do not survive within 2 weeks of conception, an additional 20% of loss is compounded 28 days post insemination and finally, 5% of fetuses are lost during late pregnancy (Wathes et al. 2008).

From an evolutionary perspective, the antagonistic relation between milk yield and reproductive resumption is a sound one, since monovulatory animals must either prioritize either the survival of their current offspring or secure their reproductive ability for future progeny (Knight 2001). Breeding for both production and reproductive traits has been proven difficult, as the mean heritability of reproductive traits range from 0.02 to 0.05 (Berry et al. 2014). Thus, the intensive selection of production traits in the dairy cow has exacerbated this trade-off relationship and has given place to animals that experience drastic transient physiological and metabolic changes that must both support high milk production and maintain viable reproductive function. A considerable body of research has sought to understand the relation between the physiology of milk production and reproductive function. The emerging landscape appears to be a multifactorial problem, of which metabolism as well as oocyte and embryo quality has been at the center.

1.2   Physiological and immunological challenges of the lactating cow

High yielding cows are expected to gestate around 3-4 months after calving to maintain economic viability (de Vries 2006). During this period, a wide range of physiological and tissue remodeling events must be resolved while, at the same time, intensive milk

production is desired. This transition period in the dairy cow is defined as the 6 week periparturient window where intense allocation of energetic resources is redirected to the mammary gland (Roche et al. 2018), which is coupled with decreased dry matter intake (DMI) up to 30% in transition cows (Bertics et al. 1992; Hayirli et al. 2002). The inability to properly transition to lactation after pregnancy can impact reproductive function. For example, the energetic needs in glucose, fatty acids and amino acids to make 30 kg of milk 4 days post-partum (dpp) are 2.7, 4.5 and 2.0 fold higher, respectively, than what is required 4 days prior to calving (Bell 1995). To meet these nutrient needs, the cow goes under extensive mobilization of fat tissue, with increased lipolysis associated body conditioning score (BCS) and body weight decrease during the first 2 to 4 months (Roche et al. 2009), reaching nadir around 30-40 dpp (Figure 1.1, Roche et al. 2018). Both *in vivo* and *in vitro* studies have associated BCS loss with negative pregnancy outcome and live weight gain with positive pregnancy outcome (Buckley et al. 2003; Roche et al. 2007). Association of this tissue mobilization during the first 5 weeks of lactation has been shown to occur regardless of nutrition regimen (McCarthy et al. 2007; Roche et al. 2006; Roche 2007; Roche et al. 2009). Interestingly, feed restriction and a more severe milking regimen in lactating cows revealed negative trends in energy balance status for the first 2 weeks of post-partum where they were monitored (P=0.079, Fenwick et al. 2008). Whilst the energy demands for milk production, uterine repair, and cycle resumption outmatch the dry matter intake (DMI), the cow will enter a period of negative energy balance (NEB). Taylor et al. have shown that cows with pronounced adipose tissue mobilization during this period can take up to 20 weeks to regain a positive energy balance (n = 177, Taylor et al. 2003), while other studies suggest that this happens 4-5 weeks after calving (n = 29 Figure 1.2, McGuire et al. 2004; n =

26, Moallem et al. 2000). In other cases, cows have continued body fat mobilization by week

5-8 (n = 297, Tamminga et al. 1997; n = 8, Block et al. 2001).
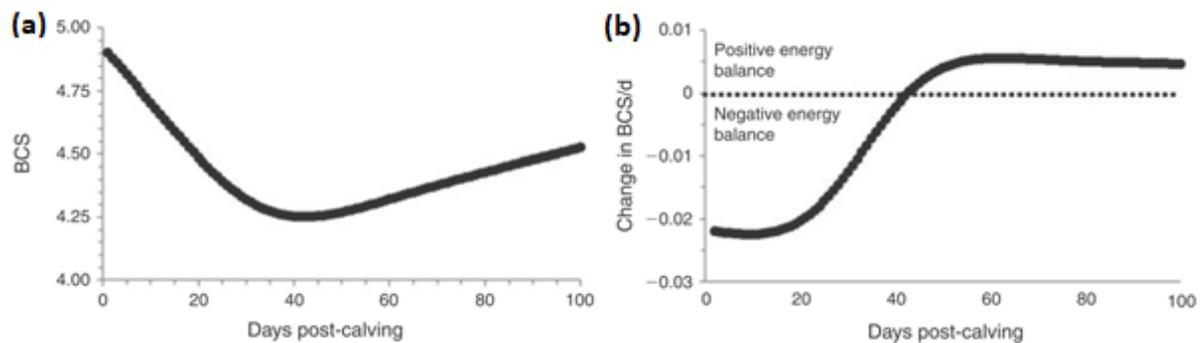
Figure 1.1 Early lactation BCS profile (a) and BCS daily variation (b). Cows exit NEB at 42

dpp, and experience the highest BCS variation during 10 dpp (Adapted from Roche et al.

2018).

Lactating and non-lactating cows were found to have significantly lower bodyweight and

BCS compared to heifers (Forde et al. 2015). In some instances, cows that had a high BCS

loss in the first three weeks post-partum had lower pregnancy rates through

synchronization (Carvalho et al. 2014). This homeorhetic process is associated with an

altered metabolite profile which is thought to play a role in cow fertility, both short term

and long term (Beam and Butler 1997). Cows experiencing these metabolic changes have

been associated with reduced estrus, delayed ovulation, lower conception rates and high

embryonic mortality (Opsomer et al. 1998; Bilodeau-Goeseels and Kastelic 2003; Rhodes et

al. 2003; Mann et al. 2006; Santos et al. 2009). NEB should then be considered a transient

metabolic state that can impact animal health and, subsequently, fertility beyond the time

during which it happened based on the genetic merit, feed intake and milk yield of the cow.
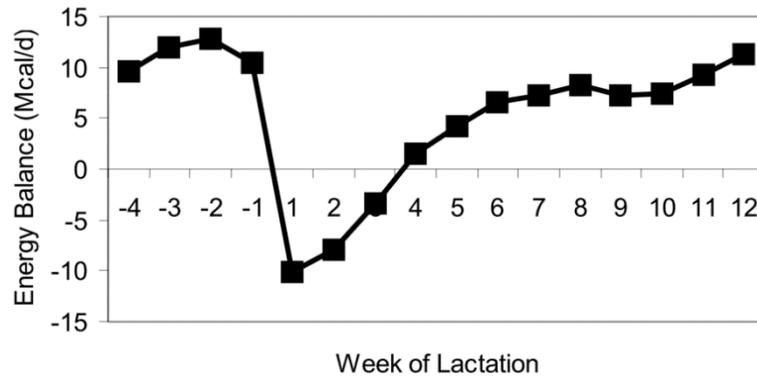
Figure 1.2 Energy balance of cows (n = 29) in early lactation (McGuire et al. 2004). Cows

are expected to exit negative energy balance by week 4 of lactation.

The transition period associated with NEB is sensitive for the well-being of a cow, as it

exposes them to metabolic and infectious diseases (Drackley 1999; van Saun 2016), which

will majorly affect the productive and reproductive performance of the cow further on

(Ferguson 2005). Poor transition is associated with milk loss, where peak milk yield

decreases up to 9 kg which can results in 907 to 1,814 kg of lost milk over the lactation

period (Wankhade et al. 2017). Post-calving physiological strain on the cow also impacts its

immunity, where cows experience metabolic disorders such as milk fever, fatty liver

syndrome and ketosis in incidences up to 16.8% in dairy herds (Correa et al. 1990; Jordan

and Fourdraine 1993). Moreover, mammary gland infection and resulting inflammation such

as mastitis and edema, as well as uterine complications like retained placenta, metritis and

endometritis are also in higher incidences (Correa et al. 1990; Jordan and Fourdraine 1993).

It appears that metabolic changes, not lactation, are the main factors of poor immunity of

the cows, as studies with mastectomized cows showed reduced leukocyte function after

calving in similar fashion than lactating cows (Nonnecke et al. 2003). Multiple studies link

the higher metabolic concentrations of non-esterified fatty acids (NEFA) and beta-

hydroxyburate (BHB) with occurrence of metabolic and infectious diseases and their

reciprocity, as cows with metabolic diseases such as ketosis were at higher risk of

developing infectious diseases like metritis (Sordillo and Mavangira 2014). An i*n vitro* study

found high NEFA concentration to have an impact on cell viability and necrosis of bovine

polymorphonuclear leukocytes, linking the role of NEFA in the inflammatory response of the

lactating cows (Scalia et al. 2006). This is reflected in the altered uterine immunity of

lactating cows, where cows with high pre-partum NEFA and high post-partum BHB were at

higher risk of endometritis (Giuliodori et al. 2013). It has been hypothesized that these

inflammatory signals are somewhat normal during the first 3-4 days of calving to properly

transition into a metabolically active state, but that delayed resolving of these signals could

lead to abnormal productivity, health and fertility (Bradford et al. 2015). Taken together,

these findings suggest that the early lactating cow is undergoing specific physiological,

metabolic and immunological challenges that play a role in their ability to maintain a healthy

weight and immune system, which in turns affects productive, health and reproductive

traits.

1.3   Metabolism of the dairy cow

1.3.1   *Lipid Metabolism – non-esterified fatty acids and beta hydroxybutyrate*

Intensive genetic selection for milk production has led to large and metabolically active

cows (Berry et al. 2016), and the event of parturition indicates the initiation of a cascade of

metabolic changes occurring rapidly. As it reduces movement and feed intake prior to

calving, energy demands for glucose, amino acids and fatty acids surge to ensure proper

lactation (Bauman and Currie 1980). Lipolysis of adipose tissue releases NEFA in the blood

stream which are then taken by the liver where oxidation will occur regardless of energy

balance status (Vernon et al. 1995). NEB has been shown to exacerbate lipolysis during the onset of lactation (Sordillo and Raphael 2013; Bell and Bauman 1997) with associated elevated NEFA blood concentrations. Oxidation of NEFA will provide carbon dioxide to generate energy, and partial oxidation will produce ketone bodies like BHB for use in the liver and other tissues as energy substrates in the citric acid cycle (Holtenius and Holtenius 1996). Dairy herd data revealed that sustained elevated concentrations of NEFA and BHB during the first 30 days in milk (DIM) are found to be associated with increased incidence of preparturient diseases such as displaced abomasum, clinical ketosis and metritis (n = 1318, NEFA = 0.57 mM, BHB = 0.96 mM Ospina et al. 2010). In a study following 500 lactating cows, circulating NEFA were shown to increase 1 week prior to calving and start to decrease after week 4 (Wathes et al. 2007). Similarly, another study found levels of NEFA to be low in heifers (0.2 mM) compared to lactating and non-lactating cows, where non-lactating cows returned to basal levels as early as day 10 postpartum but lactating cow took approximately 42 days to recover (Forde et al. 2015). Calving to first service and first service to pregnancy was increased and diminished, respectively, in cattle undergoing fatty acid concentrations of >1.0 mM in early lactation (Raboisson et al. 2014). Fenwick et al. (2008) observed significant NEFA serum concentrations differences in mild and severe NEB (Table 1, P = 0.007) cows who had differential feeding and milking regimen, indicating differential tissue mobilization in those animals.

In regard to ketone bodies, BHB is the most abundant metabolite in circulating blood and is considered a good indicator of fatty acid oxidation and ketosis. Ketosis is a metabolic disease associated with the failure to fully oxidize fatty acids present at different acuity.

Table 1.1 NEFA and BHB concentration ranges/thresholds found in literature.

Source	Animal Status	Time of measurement	Energy Balance status	NEFAs range/threshold [mM]	BHB range/threshold [mM]	Additional Remarks
Fenwick et al., 2008	Lactating cow	14 ± 0.7 dpp	Severe NEB	1.41 ± 0.136	3.71 ± 0.201	Differential feeding and milking between groups
			Mild NEB	0.55 ± 0.216	0.59 ± 0.097	
Forde et al., 2015	Lactating cow	-14 to 84 dpp	NEB	0.8 (0 dpp) - 0.3 (49 dpp)	~0.6	
	Non-milked cow	-14 to 84 dpp		0.6 (0 dpp) - 0.1 (35 dpp)	0.55 (-7 dpp) - 0.3 (7 dpp)	Recovered ~ 10dpp
	Heifer	-14 to 84 dpp		0.2	~0.3	Basal levels
Bender et al., 2010	Lactating cow	80.9 ± 2.98 dpp	NEB		0.60 (~81dpp)	CIDR stimulated
	Heifer				0.41 (~81dpp)	
Maillo et al., 2012	Lactating cow	-14 to 95 dpp	NEB	0.51 (21 dpp) - 0.15 (70 dpp)	0.68 (17dpp) - 0.47 (87dpp)	Recovered at 49 dpp
	Non-milked cow	-14 to 95 dpp		0.50 (0 dpp) - 0.05 (80 dpp)	0.50 (39dpp) - 0.29 (73dpp)	
Matoba et al., 2012	Lactating cow	-14 to 80 dpp	NEB	0.8 (14 dpp) - 0.3 (42 dpp)	1.1 (28dpp) - 0.66 (49dpp)	
Ospina et al., 2010	Lactating cow	0 to 30 dpp		0.57	0.96	Threshold associated with displaced abomasa, clinical ketosis and metritis
Girard et al., 2015	Lactating cow	60 dpp	Severe NEB	-	1.102 ± 0.174	EB Status grouped according to high and low BHB concentrations
	Lactating cow	60 dpp	Mild NEB	-	0.645 ± 0.094	
Wathes et al., 2009	Lactating cow	14 ± 0.4 dpp	Severe NEB	1.4 ± 0.14	3.7 ± 0.2	Differential feeding and milking between groups
			Mild NEB	0.3 ± 0.05	0.5 ± 0.09	

While the clinical ketosis is observed at BHB blood concentrations of 2mM, at least 50% of all dairy cows will experience subclinical ketosis during the first month of lactation with BHB concentrations of 1.2 to 1.4 mM (Wathes et al. 2007; Duffield et al. 2009; Gordon et al. 2013). Ketosis is thought to palliate to the increasing demand of glucose as glucose concentrations only drop for a short period around 1-2 weeks postpartum (Roche et al. 2018). However, severe ketosis has been associated with a number of metabolic diseases such as endometritis, mastitis and ovarian cysts, which impact reproductive efficiency (Raboisson et al. 2014; Shin et al. 2015). Forde et al. (2015) found that heifers had stable low BHB concentrations and lactation increased BHB levels, where dried cows returned to basal levels by 10 dpp and lactating cows never returned to basal levels for the duration of the study (up to 14 dpp) (Forde et al. 2015). A Wathes et al. (2007) study on 500 lactating cows found BHB levels to increase 1 week prior to calving to 6 weeks postpartum. Interestingly, blood BHB levels of 1.4 mM were associated with increased calving to first service and decreased pregnancy rates from first service (Raboisson et al. 2014). Fenwick showed significant differential BHB serum levels in severe NEB and mild NEB ($P < 0.001$), consistent with their findings in NEFA concentrations. Taken together, these studies show that the lactating cow has a specific lipid metabolism that plays a role in reproductive capacity.

1.3.2   *Hormone homeorhesis*

As nutrient demands increase to secure lactation, the growth hormone (GH) – insulin – Insuling-like Growth Factor-1 (IGF-1) – glucose pathway is altered (Lucy et al. 2001). Prior to calving, insulin resistance is increased in adipose tissue and shows an increased sensitivity to lipolytic agents (Bell 1995) which redirect nutrient flow to the placenta. Upon calving, the

increased energy demand for milk production, intensified by genetic selection, brings a decline in insulin levels (Bonczek et al. 1988; Taylor et al. 2003). IGF-1 is thought to be the mediator of GH driven milk synthesis in the mammary gland (Etherton and Bauman 1998). IGF-1 is usually released from the liver because of GH coupling to its receptor, and will in turn act as a negative feedback on the pituitary gland to regulate GH release. NEB downregulation of GH receptor ultimately reduces circulating IGF-1 concentrations, which, coupled with low insulin concentrations, both increase lipolysis and gluconeogenesis (Lucy et al. 2001). Levels of IGF-1 may not return to pre-calving levels for up to 12 weeks in some cows (Taylor et al. 2004; Fenwick et al. 2008) and has been associated with the increased calving to conception interval, reduced pregnancy outcome (Wathes et al. 2003, 2003; Taylor et al. 2004) and found to alter follicle gonadotropin sensitivity and growth (Figure 1.3; Garnsworthy et al. 2008). Moreover, a feed restriction study on lactating cows 2 weeks post-partum demonstrated that plasma concentrations of IGF-1 were lower in cows experiencing severe negative energy balance, while it was recovered after the first week in mild NEB cows, but no difference in GH concentrations was observed (Wathes et al. 2007; Fenwick et al. 2008). Higher IGF-1 blood concentrations have been found in heifers compared to lactating and non-milked cows post-calving, although in the non-lactating group, IGF-1 levels were recovered around day 84 post-partum (Forde et al. 2015). Moreover, changes in IGF-1 have been noted in the oviduct and endometrium, linking IGF-1 with implantation (Fenwick et al. 2008; Wathes et al. 2009).

NEB has been found to delay ovarian activity through impaired luteinizing hormone (LH) as well as follicle stimulating hormone (FSH) in follicular response, which in turn deregulates LH pulsatility and suppresses follicular estradiol production (Diskin et al. 2003). Another

hormone essential for the establishment of pregnancy is progesterone, as the bovine embryo will rely on the oviductal production of progesterone up until day 19 (Spencer et al. 2016). It has been proposed that low pregnancy rates are partially due to the late onset of progesterone rise as well as its low concentration during the luteal phase (Mann and Lamming 2001). Indeed, normal levels of progesterone in a healthy uterine environment will stimulate the embryo to produce interferon-tau, which in turn will inhibit the secretion of endometrial estrogen and transcription of the oxytocin receptor to ultimately prevent prostaglandin F2 alpha (PGF2α) production and luteolysis (Mann and Lamming 2001). As such, Villa-Godoy et al. (1988) demonstrated that NEB cows will have lower progesterone during the first three ovarian cycles, and lower than nulliparous heifers, even with larger luteal tissue (Villa-Godoy et al. 1988). Moreover, blood concentrations of progesterone have been found to impact the transcriptome signature of the endometrium (Forde et al. 2009), potentially influencing the embryo-maternal cross-talk happening before implantation (Mamo et al. 2012). Some higher producing cows have shown greater progesterone metabolism (Wiltbank et al. 2006), although this is not always the case (Green et al. 2012). Early production of progesterone increased developmental rates *in vivo*, a result that has not been confirmed *in vitro* (Lonergan 2011). It appears that the lactating cow has lowered hormonal levels profile, utlimatelyaffecting its reproductive capability.

1.4   Reproductive function of the lactating cow

1.4.1   Follicular development – oocyte quality

During the metabolically adverse lactating period, the cow may resume ovulation and follicular development. The interval to first ovulation in postpartum cows is an important metric for reproduction (Petersson et al. 2007). The preovulatory follicle can take from 40 to

90 days to grow from early antral status to ovulation (Fair 2010). In parallel, the oocyte will grow until the follicle reaches a diameter of 3 mm, and will plateau while the follicle continues to grow until it reaches a preovulatory size ranging from 15 to 20 mm in diameter (Fair 2003). Given the best outcome, a cow will ovulate first around 20-30 dpp giving time for 1 to 2 estrous cycles and a first service time of approximately 60 days, still falling within the calf per year goal (Dohoo 1983). Interestingly, only half of postpartum cows ovulate their first growing follicle, leaving the other half to be anovular cows that show estrus yet are not ovulating (Beam and Butler 1998; Wiltbank et al. 2011). The lactation window is sensitive to the NEB and may affect follicular and luteal development, ultimately compromising the ovulating follicle of the first service and oocyte quality (Figure 1.3). Multiple studies following the hormonal profile of lactating cows are also linking NEB with irregular cycles, longer interval to pregnancy and reduced conception rate (Taylor et al. 2003; Wathes et al. 2003). This includes delays to first ovulation and prolonged corpus luteum, which can then result in subsequent ovulation delays. These gaps are associated with uterine diseases like metritis and it has been shown that approximately half the cows in a dairy herd go through estrus irregularities (Taylor et al. 2003).

When follicular recruitment occurs regardless of NEB status, the animal's poor body condition score can result in lower ovulation rates (Beam and Butler 1999). Interestingly, follicle size is diminished during NEB, but follicles growing after NEB nadir were found to have bigger size and growth as well as increased estradiol production, making them more likely to ovulate (Beam and Butler 1999). Additionally, concentrations of IGF-1 in the follicle mirror the circulating concentration, where they are known to fall after the first week postpartum (Beam and Butler 1999; Wathes et al. 2007; Taylor et al. 2004). This could

contribute to delayed first ovulation by impeding LH pulsatility, which in turn extends oocyte maturation, follicle dominance and ultimately leading to ovulation of an aged oocyte similar to post-ovulatory ageing (Mihm et al. 1994; Lopez et al. 2004; Llewellyn et al. 2007). The notion that the follicular milieu provides the necessary growth environment to the oocyte, affecting its quality and therefore the subsequent embryo quality and development, is not new (McNatty 1978; Rizos et al. 2002). Snijder et al. (2000) demonstrated this concept *in vitro* by finding that a lower proportion of high genetic merit for milk production oocytes cleaved and developed into viable blastocysts compared to those from average genetic merit animals (Snijders et al. 2000). Interestingly, in a study investigating oocyte developmental competence in NEB cows, the number of oocytes aspirated from OPU was lower in lactating cows at day 43 pp compared to heifers, while cleavage and blastocyst rates did not differ (Rizos et al. 2005).

Follicular milieu is the result of blood plasma input crossing the follicular blood barrier, as well as granulosa and theca cell secretion (Gosden et al. 1988; Fortune 1994). Granulosa cells from cows with elevated blood BHB levels at day 60 (Severe NEB = 1.102 mM vs mild NEB = 0.645 mM) were found to have differential transcription profiles, supporting that NEB has an impact on follicular dynamics (Girard et al. 2015). Leroy et al. demonstrated a good correlation between blood and follicular concentrations of NEFA and BHB and *in vitro* maturation rates, where elevated NEFA concentrations were found to diminish oocyte maturation, fertilization and blastocyst developmental rates (Leroy et al. 2004; Leroy et al. 2005). Subsequent efforts have tried to assess bovine oocyte quality through fatty acids characterization, with lipid composition and accumulation in oocytes playing a role in their development (Zeron et al. 2001; Leroy et al. 2005; Aardema et al. 2011). A study reported

that follicular NEFA concentration was lower in the dominant follicle, where subordinate follicles mirrored the level of plasma concentration (Renaville et al. 2010). This finding has been more recently challenged by Bender et al. who observed no significant differences in NEFA and BHB between dominant and subordinate follicles of controlled internal drug release (CIDR) progesterone-stimulated lactating cows (average DIM = 80.9 ± 2.98) and heifers, and when comparing blood serum, the study found that BHB concentration in lactating cows was higher than in heifers (0.60 vs 0.41 mM, Bender et al. 2010). High levels of saturated fatty acids in lactating cows suggested that oocytes were at higher risk of lipid accumulation, which was previously found to have an impact on embryo quality (Abe et al. 1999; Reis et al. 2003).

Studies have postulated that NEB in high yielding dairy cows has carryover effects on fertility by affecting oocyte developmental competence and ultimately lower conception rates (Snijders et al. 2000). During maturation, the oocyte is accumulating RNA transcripts and proteins which have an important role on the outcome of the pregnancy (van den Hurk and Zhao 2005). Therefore, even if fertilization is successful, adverse growth conditions will influence pregnancy outcomes. As such, Van Hoeck demonstrated that 24 h maturation of oocytes under high NEFA concentrations associated with lipolysis and NEB resulted in altered gene expression and function, most notably of pathways involved in lipid and carbohydrate metabolism (van Hoeck et al. 2011; van Hoeck et al. 2015). This is consistent with Britt's carry over hypothesis that claims that the developmental competence of the oocyte, in conjunction with the steroidogenic capacity to produce estrogen and progesterone during the follicular growth prior to ovulation, is reliant on the biochemical environment of the cow (Britt 1992). The time taken by an oocyte to grow, mature and

ovulate is concordant with the time to first ovulation in the lactating cow (Lucy 2003; Leroy et al. 2005). Interestingly, lactating cows artificially inseminated at 140 dpp had similar conception rates to embryo transferred cows (Sartori et al. 2006). All in all, the metabolically divergent early lactating cows appear to place their oocytes in a disadvantageous developmental milieu compared to heifers, which can explain the observed phenotypic differences in fertility.

1.4.2   Embryos development and quality

As it has been suggested that NEB might affect oocyte competence (Vanholder et al. 2005), some researchers have supported the idea of using embryo transfer to circumvent low oocyte competency and improve reproductive success (Lucy 2007). While some higher pregnancy rates were observed in lactating cows following embryo transfer compared to artificial insemination (Putney et al. 1989; Drost et al. 1999; Rutledge 2001; Vasconcelos et al. 2006; Demetrio et al. 2007), a nefarious metabolic profile has also been shown to alter the receptivity of the reproductive tract and, by extension, embryo quality (Sirard et al. 2006). Indeed, day 5 embryos recovered from lactating cows around 30-90 dpp had poorer quality and viability compared to embryos collected from dried cows and heifers (Sartori et al. 2002). This early developmental period, up to day 4-5, takes place in the oviduct, where gamete transport, sperm capacitation, fertilization and early embryonic development happen (Hunter 2003; Latham and Schultz 2001). The transition period in cattle also include uterine involution, which involves endometrial tissue repair, myometrial contraction and bacterial clearance. This process has been described to take 30-50 days to complete (Gier and Marion 1968) and occurs in parallel with follicular activity. If these processes are not completed, return to normal cyclicity will be affected and uterine reception of the

conceptus may be compromised, resulting in low pregnancy rates (Sheldon et al. 2006). Non-surgical uterine flushing of lactating cows revealed that 50% of embryos could not survive past day 7 (Cerri et al. 2009; Sartori et al. 2010). Feed restriction studies in lactating cows demonstrated that severe NEB cattle demonstrated higher amounts of segmented cells but lower mononuclear cells, indicative of stronger uterine inflammation post calving with reduced conception rates (Wathes et al. 2007) as well as delayed uterine repair (Wathes et al. 2009). It has also been found that inflammatory uterine conditions such as endometritis increased conception interval by 15 days on average and reduced conception rates under 150 days by 31% (Bonnett and Martin 1995). Interestingly, lactating cows exposed to different feed and milking regimen to induce mild and severe NEB did not show significantly different gravid uterine horn size, and both groups were still in uterine repair at this stage (2nd week of post-partum). It is curious to note that although energy balance values and NEFA and BHB concentrations were significantly higher in severe NEB groups compared to mild NEB groups, milk yield and dominant follicles size were similar, possibly suggesting that NEB must be sustained well into lactation to have an impact, or that NEB might influence fertility through molecular rather than physiological changes (Fenwick et al. 2008).

In parallel, the preimplantation embryo undergoes quite drastic DNA activation and replication stages, which could make it particularly vulnerable to failing oviductal support and might contribute to early embryo losses (Rizos et al. 2010; Maillo et al. 2012; Matoba et al. 2012). Indeed, embryo quality was found to be impaired in NEB cows after fertilization (Sartori et al. 2002; Santos et al. 2004; Leroy et al. 2005). *In vitro* embryos transferred at the 2 cell stage in heifers were recovered at day 7 in greater number, with a higher blastocyst

rate similar to full *in vitro* culture (34%) than in lactating cows (18%, (Rizos et al. 2010)).

Since heifers may behave differently than non-milked cows, subsequent *in vivo* oviductal

flushes highlighted that elevated NEFA and BHB concentrations in lactating cows affected

the oviductal support of blastocyst development compared to age-matched dried cows at

60 days post-partum, an impact not observed in elongation rates of embryos of animals at

90 dpp (Maillo et al. 2012).
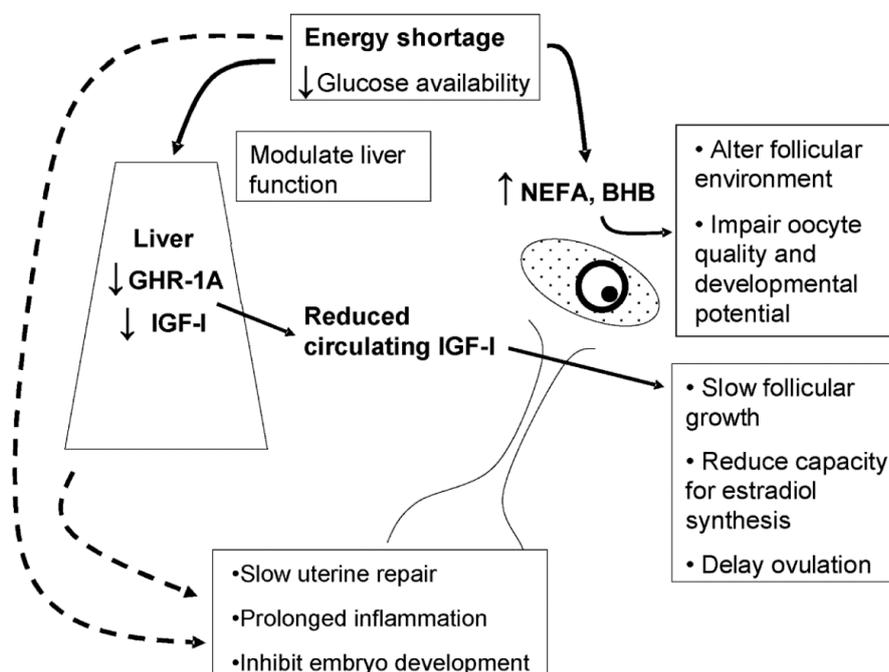
Figure 1.3 Schematic representation of the endocrine relationship between negative energy balance and reproductive function (Adapted from Wathes et al. 2007).

The same types of blastocyst development and elongation experiments were performed in

lactating and non-lactating cows, and lower rates of development were found in both cases

(Berg et al. 2010). This is further supported by a metabolite study of oviductal fluid NEFA

concentrations which were found to mirror plasma concentrations in slaughterhouse cows

(0.29 ± 19 mM vs 0.31 ± 0.14 mM, Jordaens et al. 2017). This impact of metabolite

concentrations on embryo developmental rates has been further demonstrated through *in vitro* work, where elevated NEFA in culture media affect embryo development negatively and alter gene transcription levels (van Hoeck et al. 2015). Overall, in addition to poor oocyte quality, the lactating cow seems to have lower reproductive function due to the suboptimal oviductal and uterine condition it provides to the conceptus.

1.5   Epigenetic regulation of gene expression

Due to the evident role of environmental and metabolic factors on gamete and embryo quality mentioned above, the lactating cow is an interesting model to study the effect of homeorhetic metabolic stress on the biomolecular status of oocytes and embryos resulting in lower developmental competence. One concept associated with this is the epigenetic developmental origin of health and disease. In association with the Barker hypothesis, epigenetics aims to link gestational stresses that could predispose the offspring to disease. Since phenotypical expression is reliant on gene expression, mechanisms regulating this gene expression become of specific interest when explaining the possible inheritance of traits induced by environmental stresses. While some conditions can regulate gene expression, like availability of RNA transcription machinery, transcription factors or bioavailability of necessary substrates like nucleotides, other conditions aim to organize the availability of DNA sequences to be transcribed. Such mechanisms are defined as epigenetic regulation of gene expression, as they are DNA modifications that do not alter its genetic code.

Epigenetic regulatory mechanisms of DNA expression are divided into 3 main categories: post-translational modification of histone protein and chromatin modeling, RNA based mechanisms and DNA methylation.

1.5.1   Post-translational modifications of histone proteins and chromatin remodeling

In eukaryotes, chromatin consists of DNA and histone proteins. Histone proteins H2A, H2B, H3 and H4 make an octamer which is wrapped in DNA, called a nucleosome. Histone protein H1 links nucleosome units together, and plays a role in the condensation of chromatin and by extending its accessibility to transcription machinery that dictates gene expression. This ability of condensing the DNA can be modulated through post-translational modifications (PTMs) of the amino acid residues tails of the histones, namely methylation, acetylation and phosphorylation. PTMs can regulate gene expression by influencing chromatin structure, disrupting the binding of proteins which interact with chromatin, and attracting effectors to the chromatin (Berger 2007; Kouzarides 2007). Transcription is associated with a high level of histone acetylation, where activators are thought to recruit histone acetylases and repressors recruit deacetylases. Recent work using chromatin immunoprecipitation (ChIP) coupled with array or sequencing demonstrates that certain modifications are associated with transcription and other with repression of genes. For example, trimethylation of lysine residue 9 on H3 (H3K9me3) has been associated with promoter and gene body regions of repressed genes while trimethylation of lysine residue 4 on the same histone has been associated with promoters of transcribed genes (Mikkelsen et al. 2007; Spivakov and Fisher 2007). This variation of modification within specific histones shows the regulatory action of histones and their post-transcriptional modifications into regulating gene expression. Additionnally, BHB has been showed to act as a histone deacetylase inhibitor in mice (Shimazu et al., 2013) which has yet to be proven in cattle. However, differential gene expression of genes associated with ketolysis has been found in bovine cell culture, as well

as differentiall expression of PPARA in cumulus cells after *in vitro* maturation in elevated concentrations of BHB (Sangalli et al., 2018). Taken together, the metabolic stress occurring durling lactation could modulate gene expression through chromatin regulators.

1.5.2   RNA-based mechanisms

Initially, the genome was thought to comprise the ensemble of protein coding genes, with non-coding sequences considered superfluous. Nevertheless, non-coding infrastructural RNAs were already found to be involved in translation and splicing, like transfer RNAs and ribosomal RNAs (Boyle 2008). With the advent of next-generation sequencing, an estimate of 90% of the human genome was found to be transcribed into RNAs (Djebali et al. 2012; Hangauer et al. 2013), leading to the classification of different non-coding RNAs (ncRNAs) of various sizes and conformations (Amaral and Mattick 2008) into small (sncRNAs) and long (lncRNAs) and both have been shown to modulate gene expression, possibly through different mechanisms.

LncRNAs are over 200 nucleotide in size and share some similar features of protein transcripts, such as 5' cap, polyadenylated tail and introns (Carninci et al. 2005) and similar promoting regions with coding transcripts as well as antisense transcripts (Kapranov et al. 2007; Harrow et al. 2012). LncRNA can act as signals, guides and scaffolds of expression regulatory machine. For example, Dum lncRNA has been shown to recruit DNA methyltransferases (DNMTs) to the promoter of the Dppa2 gene, enabling silencing of its expression through methylation (Wang et al. 2015). Alternatively, lncRNA HOX transcript antisense RNA knock-down prevented the guidance of polycomb repressive complex 2 to the Homeobox D locus, thus enabling its expression (Rinn et al. 2007). LncRNAs have also been shown to recruit transcription machinery like RNA polymerase II (Miao et al. 2018).

While lncRNAs have been shown to regulation gene expression through post-transcriptional expression (Bernard et al. 2010), more than 50% of mRNA are thought to be regulated by micro RNAs (miRNAs; Patil et al. 2014). A member of the sncRNAs family approximately 21 nucleotide in size, miRNAs have emerged as crucial post-transcriptional regulators of gene expression. MicroRNAs bind to a miRNA-induced silencing complex (miRISC) which targets mRNAs and either provoke translational repression or deadenylation and ultimately degradation (Carthew and Sontheimer 2009; Fabian et al. 2010). This complex recognizes the 3 prime untranslated region of target mRNAs (Krol et al. 2010). While the recognition is not perfect, the seed sequence of the miRNA (nucleotide 2-8) gives good insight on the recognition range of the miRNA. In addition, some miRNA can be transcribed in both directions, leaving multiple seed sequences and ultimately different gene regulation (Tyler et al. 2008). Some miRNAs have been shown to participate in negative feedback loops, down regulating the transcription of their initial target, demonstrating the ability of miRNA to regulate pre- and post-translational gene expression (Kim et al. 2009b). Interestingly, some miRNA have been described to be differentially expressed during lactation, where overexpression miR-30b caused reduced size of alveolar lumen and delayed uterine involution as well (Le Guillou et al., 2012). In cattle, 56 mirNA were found to be differentially exressed in mammary gland tissue between lactation and non-lactation periods (Li et al., 2012). In similar fashion, miRNA bta-miR-15a was found to modulate the expression of growth hormone receptor in mammary epithelial cells as well as decreasing cell viability and lactation (Li et al., 2012), further suggesting that miRNAs may also regulate gene expression during early lactation.

1.5.3   *DNA methylation*

DNA methylation is a reversible addition of a methyl group at the 5' position of the cytosine residue most commonly located next to a guanosine nucleotide through a phosphodiester bond, defined as a CpG site (Weber and Schübeler 2007). CpG sites vary across the genome and CpG rich regions are defined as CpG islands, which are irregularly spread across the genome and are thought to play a major role in gene expression of genes that are widely expressed across tissues (Nafee et al. 2008). The DNMTs family containing four members in mammals performs these modifications, which regulate gene expression through the inhibition of DNA recognition by protein machinery as well as favoring binding of other protein members (Delcuve et al. 2009; Prokhortchouk and Defossez 2008). DNMT1 maintains DNA methylation following DNA replication (Kim et al. 2009a), while DNMT3a/b are involved in *de novo* methylation (Hervouet et al. 2009). While the methylation function of DNMTs are well characterized, demethylation is thought to occur through either deactivation of the DNMTs (Kim et al. 2009a) or by slow passive demethylation through cell division with no maintenance of the methylation marks (Gibney and Nolan 2010). Similarly, oncogenic transcription factors have been shown to recruit DNMT and silence gene expression through methylation of their promoter (Brenner et al. 2005; Wang et al. 2005). Growing evidence shows positive correlation in methylation of gene body with gene expression (Ball et al. 2009) and methylation of the first exon is more indicative of gene expression than methylation of the promoter (Brenet et al. 2011). These findings support the notion DNA methylation can both inhibit and permit gene expression depending on its position in the genome (Anastasiadi et al. 2018), encouraging the use of whole genome analysis techniques to identify epigenome changes and their potential link to gene expression rather than enrichment based methods focusing on CpG rich regions only. In relation with epigenetic gene expression regulation occurring in lactating cows, a study

demonstrated that cows suffering from mastitis exhibited differential methylation of the aS1-casein-encoding gene promoter (Vanselow et al. 2006), which was similarly confirmed using a different bacterial-induced mastitis (Molenaar et al. 2009). These variations of epigenetic regulators during lactation demonstrate that this period after calving remains a strong metabolic stress that could modulate gene expression.

1.6 Epigenetic sensitivity during the reproductive process

In an attempt to increase the gene drive of high genetic merit cattle, major advances in assisted reproduction techniques, namely embryo transfer and cloning, have permitted transmission of specific phenotypic profile of animals. Unfortunately, their success rate has been tainted with reports of developmental failure such as early embryos losses and increased occurrence of large offspring syndrome (Dean et al. 2003; Suzuki et al. 2009; Smith et al. 2015). Multiple crucial steps occur during embryo development, most notably DNA demethylation, DNA replication and embryonic genome activation (EGA) (Beaujean et al. 2004; Fulka et al. 2004; Niemann et al. 2007). These processes can be hindered by the neighbouring oviductal lumen fluid and ultimately modify the progeny's metabolism or body function persisting well into birth, puberty and maturity (Sinclair and Singh 2007). In other words, transient metabolic stresses occurring during perimplantation could cause heritable changes and predispose the calf to diseases (Chavatte-Palmer et al. 2008). One way by which this mechanism could alter gene expression patterns could be altered is through epigenetic marks remodeling such as DNA methylation (Lillycrop and Burdge 2012).

1.6.1   DNA methylation dynamics in reproduction

Eukaryotic cells have their chromatin condensed to varying degree, to permit gene expression and repression of specific DNA sequences. Alteration in the proteins involved in this condensation, such as histone modifications, as well as the DNA itself, are named epigenetic modifications or marks, and can be inherited, acquired and/or maintained. As each mechanism plays its role in regulating gene expression, DNA methylation has the ability to be preserved through cell divisions making it an interesting model to study inheritable phenotypes. DNA methylation is usually associated with gene repression, and that repressed state is maintained through DNA replication and mitosis (Weber and Schübeler 2007). These mitotically or meiotically conserved profiles (defined as 'epigenomes') are not permanent, but rather can be acquired at very specific stages of development, and play a part in lineage and tissues-specific gene expression (Law and Jacobsen 2010; Kota and Feil 2010; Reik 2007). It is the case in mammals, where the epigenome is reset during germ line formation to permit development and differentiation for the next generation (Figure 1.4, Reik et al. 2001). This new acquisition of the epigenetic profile is also accompanied with some variations, induced by both intrinsic and environmental factors (Feil and Fraga 2012). While the biological reasons for intrinsic factors to modify DNA methylation remain unknown (Bjornsson et al. 2008; Wong et al. 2010), a growing field of environmental epigenetics has focused on the environmentally induced mechanisms between DNA methylation or histone modification changes and their resulting phenotypic profile. DNA methylation is crucial for developmental processes such as embryonic genome activation, gene expression maintenance after mitosis and tissue differentiation (Law and Jacobsen 2010; Reik 2007; Borgel et al. 2010). To date, little is known about spatial guidance of DNA methylation in the genome and how environmental and metabolic stress may affect it. Research in this particular field of epigenetics remains of

interest due to the potential long-term phenotypic effect that could occur despite aggressive genetic selection in cattle.

Animal models have outlined the role of nutritional and environmental exposures during development and how it induces phenotypical changes in the epigenome (Rosenfeld 2010). The most studied model is the agouti mouse viable yellow allele, which possesses a retrotransposon upstream of the agouti gene. Briefly, when the retrotransposon is unmethylated, the agouti gene is abnormally expressed, granting the mouse a yellow coat color, and induces obesity and diabetes (Waterland and Jirtle 2003). Inversely, when the retrotransposon is methylated, the wild-type agouti mice are mottled or brown with no metabolic issues. Differential dietary intake of folate and compounds affecting the methionine cycle in gestating mice was found to shift the allele expression of the developing offspring but not the mother, resulting in healthier offspring (Waterland and Jirtle 2003). Similar work has been reproduced in ruminants, where ewes that were fed reduced intake of folate, methionine and vitamin B12 had offspring with health problems like insulin resistance related to hypomethylation of CpG islands (Sinclair et al. 2007). In humans, studies on the Dutch hunger wintersof 1945-46 show that famine during the peri-conceptional period led to unfavourable metabolic phenotypes in children like predisposition to diabetes, possibly caused by hypomethylation of a differentially methylated region (DMR) of the imprinted gene insulin like growth factor 2 (IGF2, Heijmans et al. 2008). Interestingly, exposure to famine later during gestation (at least 10 weeks before birth) did not show such adverse methylation. This demonstrates that the early gestational period is when the conceptus is especially sensitive to epigenetic disturbances induced by differential nutrition and metabolic variations.

1.6.2   DNA methylation in oocyte maturation

The oocyte undergoes major methylation remodeling before it can be fertilized. Originally, the ovarian reserve emerges during embryonic development in mammals, where primordial germ cells (PGCs) will migrate to the gonads to populate the genital ridge. Before migration, the DNA of PGCs is thought to be highly methylated and undergoes extensive demethylation when PGCs migrate, where even imprinted genes are demethylated as well (Hackman et al. 2010; Simon et al. 2011). In the mouse, this demethylation occurs by embryonic day 14, when the PGCs then enter meiotic arrest (Figure 1.4 - Reik et al. 2001). In mammals, the remethylation of the germ line takes place after birth during oocyte growth. At this time, reprogramming is needed to properly acquire epigenetic imprinting marks, but also synthesis and accumulation of RNA transcripts, resumption and completion of meiosis and other epigenetic modifications (Fair 2010). Genomic imprints are epigenetic marks that are maintained only on one parental allele, giving place to parent specific expression of genes that are mostly involved in embryo development (Ferguson-Smith 2011). After maturation, the oocyte's DNA is thought to be highly methylated, compared to somatic cells. Such acquisition of methylation marks has been described in bovine oocytes, where genomic imprints establishment was dependent on oocyte size, giving temporal and morphological insight on the maturation process (O'Doherty et al. 2012). Improper acquisition of genomic imprints, however, has been associated with developmental diseases in humans like Beckwith-Wiedemann syndrome (Bliek et al. 2009) and Prader-Willi syndrome (Buiting 2010) and neonatal disease in cattle (Brisville et al. 2011). Moreover, data from multiple species indicating abnormal imprinting in oocytes derived from superovulation and *in vitro* maturation (IVM) suggests that the environment in which the oocyte matures is vital for

proper epigenetic programming (Sato et al. 2007; Fortier et al. 2008; Lee et al. 2008; Katz-Jaffe et al. 2009 Stouder et al. 2009). This abnormal epigenetic status could very well affect the transcriptome profile, as *in vivo* and *in vitro* matured bovine oocytes were found to have significantly different RNA expression signatures (Kues et al. 2008). This proper programming may result in the timely expression of oocyte-specific growth factors and glycoproteins influencing the oocyte growth and developmental competence of the oocyte ovulated during the NEB window (Fair 2010). Moreover, a post-ovulatory ageing *in vitro* experiment found that extended maturation (48 h) of oocytes resulted in differences in methylation status of bovine demethyltransferase 3-like (bDNMT3L), involved in *de novo* methylation during gametogenesis, in oocytes and embryo derived from this extended maturation (Heinzmann et al. 2015). A genome-wide study using reduced representation bisulfite sequencing (RRBS) of bovine oocytes matured *in vivo* vs *in vitro* revealed a considerable amount of differentially methylated regions (DMRs), of which some were imprinted genes (Jiang et al. 2018).
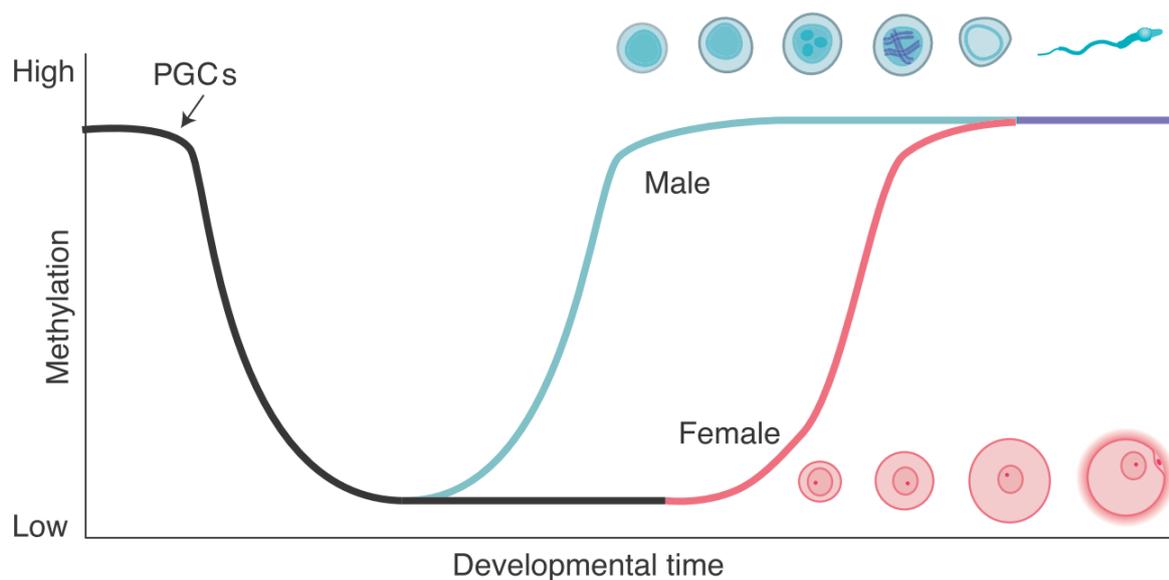
Figure 1.4 Expected methylation status of gamete over developmental time in mammals (From Reik et al. 2001).

O'Doherty demonstrated that imprinted gene methylation marks were irregular in oocytes originating from early NEB cows, as well as oocytes matured in elevated NEFA concentrations, highlighting the potential adverse effects of the NEB metabolism on the oocyte's epigenetic landscape (O'Doherty et al. 2014). Taken together, this suggests that oocytes are sensitive to methylation during growth and maturation environment, in addition to increased NEFA concentrations found in blood serum of lactating cows.

1.6.3   *DNA methylation in embryo development*

In mammalian development, there are periods of genome-wide reprogramming of the epigenetic landscape *in vivo*. Two highly differentiated mature gametes fertilize and undergo massive reprogramming to enable cell division and future differentiation. In mouse, the paternal genome undergoes genome-wide demethylation before DNA replication occurs (Oswald et al. 2000; Mayer et al. 2000), while genomic imprints and some repeat DNA sequence methylation marks are maintained (Figure 1.5; Reik et al. 2001) (Oswald et al. 2000; Lane et al. 2003). In bovine, extensive demethylation occurs during cleaving up to the eight cell stage (Dean et al. 2001; Jiang et al. 2018). When extensive demethylation is complete, remethylation takes place at different stages depending on the species. In mice, *de novo* methylation takes place at the inner cell mass of the expanded blastocysts, whereas in bovine occurs during the 8- to 16- cell stage division, in concordance with major EGA (Reik et al. 2003). Differential methylation of the germ line will give place to the trophectoderm (TE) and the inner cell mass (ICM), where their global DNA methylation differences are conserved between mouse, bovine, sheep and rabbit, which indicates its importance for

lineage differentiation (Manes and Menzel 1981; Santos et al. 2002; Reik et al. 2003; Santos et al. 2003; Beaujean et al. 2004; Santos and Dean 2004). Indeed, bovine embryonic tissues would appear to require more complex epigenetic control due to their higher differential potential compared to their trophectoderm counterpart (Santos et al. 2003; Santos et al. 2004). A possible reason for the embryo and the oocyte high susceptibility to epigenetic alterations is the degree of pluripotency of these cells. Indeed, embryonic stem cells have been shown to be sensitive to *in vitro* culture conditions (Khosla et al. 2001; Dean et al. 1998; Doherty et al. 2000), much like pre-implantation embryos which can show abnormal imprinted gene DNA methylation (Khosla et al. 2001; Young et al. 2001). No such variation has been reported in differentiated cell cultures where chromatin is more stable and less active, suggesting the involvement of DNA methylation regulators in early embryo development and the higher susceptibility in partly differentiated cells to external stresses (Feil and Fraga 2012). Interestingly, human embryos issued from ART have a higher incidence of diseases linked with abnormal imprinting (Hirasawa and Feil 2010). Additionally, bovine embryos derived from somatic cell nuclear transfer appear to have a hypermethylated genome compared to *in vitro* fertilized (IVF) embryos, indicative of the limited reprogramming ability of the oocyte (Zhang et al. 2016).
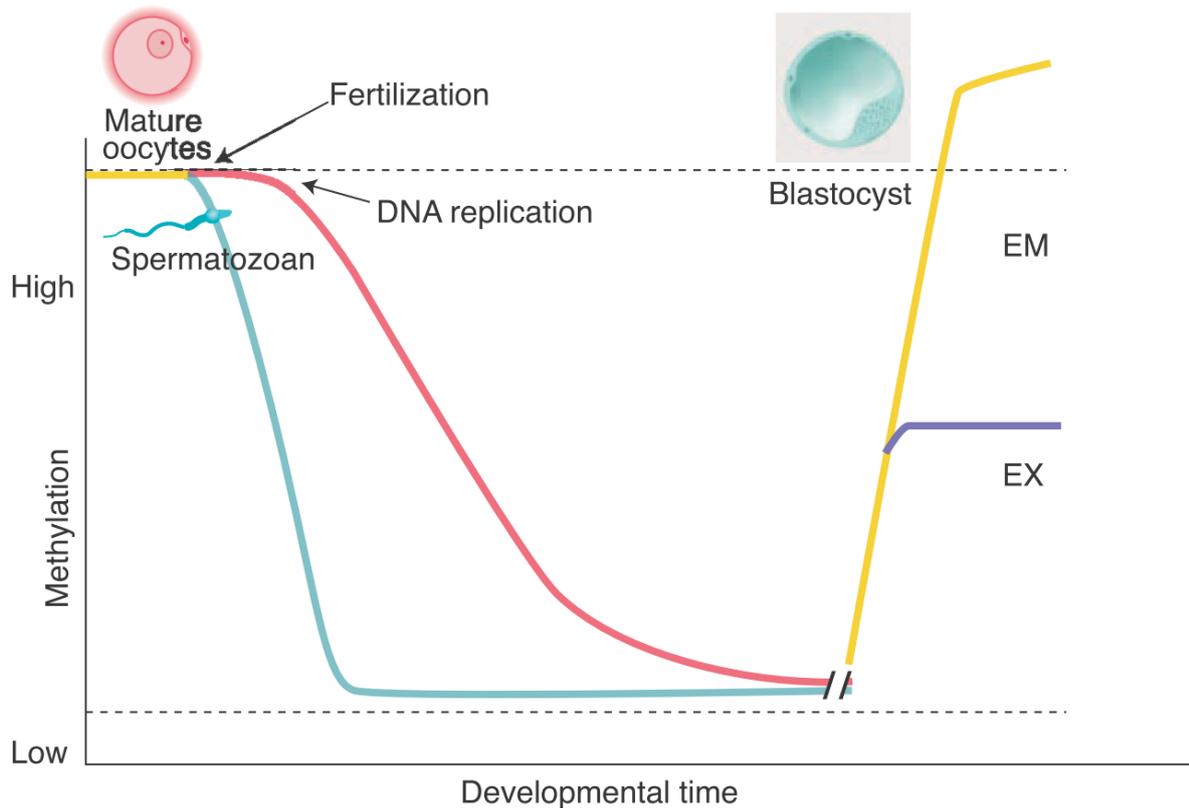
Figure 1.5 Expected methylation status of the fertilized oocyte over cell division reaching embryonic (EM) and extra-embryonic (EX) lineages at blastocyst stage (From Reik et al., 2001).

Canovas et al. introduced supplementation of oviductal fluids during porcine embryo culture in order to alleviate the DNA methylation changes found in *in vitro* produced embryos, highlighting the differential impact of growth culture on epigenetic stability (Canovas et al., 2017). Similarly, supplementation of oviductal fluids at different developmental stages before bovine EGA revealed hyper- and hypomethylation changes compared to control blastocysts, highlighting the plasticity of the epigenome to differential culture conditions at the time of embryonic genome activation (Barrera et al. 2017). Likewise, DMRs were found in bovine embryos grown *in vitro* until different stages prior to embryonic genome activation and transferred to recipients until blastocyst stage (Salilew-Wondim et al. 2015;

Salilew-Wondim et al. 2018). Inversely, Salilew et al. found DMRs of flushed *in vivo* embryos at different stages prior to EGA and cultured *in vitro* to blastocyst stage, reflecting the stage specific impact of suboptimal culture conditions on the epigenetic landscape of bovine embryos. Furthermore, *in vitro* studies have shown that elevated NEFA levels during early bovine blastocyst development reshape DNA methylation, most specifically genes involved in metabolism and development (Desmet et al. 2016). Taken together, these findings highlight the impact of adverse culture conditions associated with *in vitro* production of embryos and NEB in the oviduct on the epigenetic landscape of the pre-implantation embryo.

1.7   Hypothesis and objectives

The lactating cow, by its specific physiology upon parturition, is undergoing extensive fat mobilization to supply milk production energy demands. As a result, increase of metabolites such as NEFA and BHB are found in the blood and reach internal reproductive organs and modulate their function. Oocytes and embryos are thus exposed to abnormal growing conditions, ultimately jeopardizing their chance at establishing successful pregnancy. It has been shown that oocytes collected from lactating cows had variably methylated genomic imprinted genes. From *in vitro* work, abnormal growth conditions have been found to modulate the methylation of conceptuses, potentially impairing their developmental competency. Taken together, we hypothesize the following:

1.7.1   Hypothesis

We expect that negative energy balance characterized by body weight loss and abnormal blood concentrations of BHB and NEFA will impact the epigenetic landscape of oocytes and embryos, by modulating the methylation of genes involved in developmental competency.

1.7.2   Objectives

To do so, we divided the research into 2 objectives:

- Investigate the DNA methylome landscape of oocytes derived from cows in a NEB state postpartum.

- Investigate the DNA methylation profile of embryos grown in NEB oviductal conditions.

The following section sets out to explain how the following were achieved.

1.8   Material and methods

To reach the main goals of this thesis, several experiments were performed following specific protocols explain in depth in the respective chapters of this dissertation. Here, we list the general overview of the technical methods employed.

1.8.1   Experimental design, animals and ethics

Animal handling was carried out in accordance to the 2015 German law of protection (TierSchG & TierSchVersV). Experimental protocols performed on cows in this study were approved by state office for Nature, Environment and Consumer protection of North Rhine-

Westphalia, Germany (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Deutschland). Blood samples collections, ovum pick up (OPU) and endoscopic transfer procedures were approved under license number 84-02.04.2015.A139, 84-02.04.2014.A500, and 84-02.04.2015.A083, respectively.

1.8.1.1   Cows for oocyte collection

The first objective was to assess the impact of NEB on the methylation landscape of oocytes. To do so, 30 Holstein-Friesian cows were monitored on the farm for oocyte collection during which they had their body weight, feed intake, milk yield, and milk composition measured for up to 15 weeks post-partum. At week 5 post-partum, unstimulated cows of varied parity had their follicles of size 3 to 8 mm aspirated in both ovaries using ovum pick-up procedure. This collection went on weekly until week 10 pp where cows were put back in the breeding program. In parallel, this ovum pick up procedure was performed weekly, for 5 weeks, on 8 unmonitored nulliparous sexually mature heifers, aged 59 to 73 weeks at the start of collection, as acting control. Blood sampling was performed at each OPU collection regardless of animal and its serum was isolated with centrifugation was frozen for further analysis. Oocytes were denuded and frozen at -80 °C until further use.

1.8.1.2   Cows for embryo collection

The second objective of this thesis was to investigate the impact of the NEB uterus on the epigenetic landscape of developing embryos. Therefore, 26 Holstein-Friesian cows were monitored on the farm for laparoscopic oviductal embryo transfer. The cows had their body weight, feed intake, milk yield and milk composition measured up to 15 week post-partum. When uterine involution was complete, cows were synchronized. Presumptive zygotes or

early cleaving embryos were transferred in the oviduct of these cows exhibiting corpus luteum, having responded to the synchronization treatment. 1 week later, embryos were flushed using phosphate buffer saline and frozen at -80 °C until further use. In parallel, this oviductal embryo transfer was performed in 5 nulliparous sexually mature heifers, aged 76 to 102 weeks at the time of transfer, as control. At the time of transfer and flushing, blood was sampled and its serum was frozen until further use. Retrieved embryos were frozen in groups of 5 or 4 according to their morphological developmental stage frozen and put through a whole genome bisulfite sequencing pipeline.

1.8.2   NEB profiling and sample selection

To ensure study of cows exhibiting adverse metabolic conditions due to post-calving lactation, cows were selected based on their weight measurements. If cows gained weight immediately following post-partum, they were removed from further analysis. Next, cows had their energy balance progression monitored, to ensure that cows experience NEB early in lactation. Energy balance values where calculated using the following formula based on the equation of the German Society of Nutrition Physiology (GfE, 2001),

$$EB = DMIe + Ce - BWe - MYe$$

where the energy balance (EB; MJ NE_L/day) is the result of dry matter intake energy (DMIe, MJ NE_L / day), calculated by

$$DMIe = DMi * DMr * DMe$$

,where the daily matter intake (DMi, kg/day) is multiplied by the dry matter ratio (DMr; DM kg / PMR kg) and the dry matter energy (DMe, MJ NE_L / kg). This is added to the concentrate energy fed to the cows (Ce, MJ NE_L / day) which is calculated using daily

concentrate weight (kg / day) multiplied by concentrate energy (MJ NE$_L$ / kg) . From

this,maintenance of body weight energy (BWe) energy is calculated using

$$BWe = 0.293\,BW^{0.75}$$

Where body weight maintenance energy (BWe, MJ NE$_L$ / day) is found using daily body

weight (BW, kg) and subtracted along with daily milk yield energy (MYe, MJ NE$_L$ /day), which

is calculated using milk weight and composition (fat, protein and lactose percentages)with

the following formula:

$MYe = (0.39 * fat\% + 0.24 * protein\% + 0.17 * lactose\% + 0.07)(MJ\ NE_L / kg) * milk\ (kg/day)$

.

1.8.2.1   Oocyte pooling

Cows that had early positive energy balance values were excluded, given that their

metabolite profile was consistent with their energy balance status. To confirm this, blood

serums were analyzed for NEFA and BHB concentrations at the time of collection as

previously described (Frieten et al. 2017) and were selected according to thresholds found

in literature (Fenwick et al. 2008; Girard et al. 2015). If 2 out of the three values (EB, NEFA

and BHB) were indicative of negative energy balance, the sample was kept for pooling. At

later collection points, if 2 out of three values were indicative of positive energy balance,

the sample was kept for pooling, given they showed signs of negative energy balance prior

to collection. Out of this, 11 animals had their early lactation (week 5-6 pp) collections

points kept, and 7 animals had their mid lactation (week 9-10 pp) collection points kept for

oocyte analysis. Heifers had 40 collection points kept for oocyte analysis, as no energy

profiling was done. Oocytes were pooled in groups of 2 to 4 animals with varied parity, totaling a range of 19 to 63 oocytes per replicates.

1.8.2.2   Embryo pooling

A similar profiling was performed in lactating cows undergoing embryo transfer. Animals were selected based on their weight as described above. However, only animals that showed negative energy balance prior to week 6 were selected for further analysis, with metabolite analysis being used as characterization and not selection. This gave a total of four cows left for methylation profiling and 5 heifers were used for control. One tube of four to five morulae per animal were submitted as is to methylome profiling without pooling.

1.8.3   *Library preparation and sequencing*

To investigate the methylation profiles of oocytes and embryos we used whole genome bisulfite sequencing. The sample was lysed using proteinase K and bisulfite treated using EZ DNA Direct methylation kit (Zymo research). Once treated, library preparation was performed on the samples using Pico methyl seq kit (Zymo research) with some adjustments (Chapter 2/3). Libraries had their quality assessed using fragment migration with High sensitivity DNA chip, and quantity assessed using KAPA Biosystems™ library quantification kit for oocytes and Qubit fluorimetry with the Qubit ssDNA assay kit for embryos. Libraries were sequenced in multiplex using TruSeq v3 chemistry.

1.8.4   *Bioinformatics analysis*

First, raw sequencing data was quality assessed using FastQc. Reads were then trimmed and filtered again using Trimmomatic for oocytes and TrimGalore! for embryos and FastQc. Trimmed reads were aligned to the bovine genome UMD 3.1 using bowtie and Bismark.

Aligned reads were then deduplicated using deduplication function in Bismark. Methylation information was extracted using Bismark and imported to SeqMonk for visualization and analysis. Normalization, quantification and statistical comparisons of methylation were done in SeqMonk.

1.8.4   Statistical analysis

Characterization values, such as body weight loss, nadir average, and average daily milk yield/day were calculated and presented as mean ± S.E.M. Energy balance average comparisons between early and mid lactation were performed using an ANOVA. Metabolite concentrations between mid, early lactation and heifers for oocytes and lactating cows and heifers for embryos were performed with mixed model using random animal effect and fixed group effect. Methylation data was normalized using match distribution quantile quantification tool in seqmonk for oocytes. Differentially methylated regions were found using logistic regression ratio test of methylated and unmethylated calls in replicate sets and were considered significant when the value of adjusted $p < 0.05$. Gene ontology and pathways enrichment analysis was performed with NetworkAnalyst3.0 using $p < 0.05$ for relevant pathways.

1.9   Results

1.9.1   Energy balance profiling and metabolic profiling during lactation for oocytes and embryos

As expected, the cows selected for the study described in chapter 2 exhibited a physiological and metabolic status different than heifers. Early postpartum cows averaged -19.75 ± 3.5 MJ/day at the time of collection (average day post partum: 37.1 ± 1.4), compared to mid

postpartum cows (average day post partum: 65 ± 1.5, -0.76 ± 3.15 MJ/day). This was supported by the levels of blood metabolites measured at these collection points, which averaged above the threshold used from the literature in both metabolites for early postpartum oocytes (Chapter 2, Figure 2.1). Similarly, mid post-partum oocytes averaged metabolite levels under the threshold and significantly different than early post-partum oocytes. As for nulliparous heifers, their blood metabolite concentrations averaged well under the threshold and significantly different than early and mid postpartum collection point, with the exception of beta-hydroxybutyrate, where levels for heifers were lower, but not significantly different than midpostpartum cows. As for cows used for embryo collection, selected cows experienced negative energy balance average values of 25.7 ± 5.6 MJ/day prior to week 5 postpartum. At the time of *in vivo* embryo culture, energy balance values averaged -7.8 ± 6.0 MJ/day. Similarly, blood metabolite levels were significantly higher in cows than heifers, with averages being higher than threshold range mentioned in previous literature for lactating cows (Figure 3.1).

1.9.2   *Epigenetic landscape of oocytes and embryos*

To investigate the impact of metabolic stress on epigenetic marks of bovine gametes and embryos, whole-genome bisulfite sequencing was performed on oocytes and embryos. Sequencing of oocytes yielded a range of 64,957,486 to 247,797,579 methylation calls across all datasets, with a genomic coverage at 1x varying from 49.7% and 94.6% and a resulting coverage depth ranging from 1x to 11x. Subsequently, global CpG methylation levels were 61.3%, 68.9% and 69.2% in early postpartum, mid postpartum and cyclic heifers, respectively (Chapter 2). In embryos, sequencing of pools of morulae yielded between 18,883,661 and 71,945,138 CpG methylation calls between datasets with a coverage depth

of 0.69x to 2.63x associated with a genomic coverage at 1x spanning 20.1% to 82.2% of the genome. Overall CpG methylation averages ranged from 33.1 ± 0.9 % in lactating cows to 31.3 ± 1.9 % in nulliparous heifers.

Early postpartum oocytes displayed lower methylation of genomic features compared to mid postpartum and heifer groups (Table 2.1). Inversely, morulae grown in lactating cows exhibited an overall slight, though not significant, hypermethylation of genomic features when compared to morulae from heifers, with the exception of CpG islands, promoter regions and transcriptional units (Figure 3.2). To investigate more precise variations in the genome, quantification of bovine imprinted genes body regions revealed methylation of 12 and 11 imprinted genes in early postpartum oocytes to be significantly different ($p < 0.05$) than mid postpartum and heifer, respectively (Figure 2.2), which was not observed between mid postpartum and heifer oocytes. A similar variation of imprinted genes was not quantified between morulae from lactating cows and heifers, indicative of smaller variations.

1.9.3   *Differentially methylated regions in oocytes and embryos*

To adequately quantify the epigenetic variations occurring under metabolic stress, the genome was combined into 150 CpG windows in both oocytes and embryos. This yielded a total of 357,863 windows in oocytes and 336,378 windows in embryos. 98.7% and 92,6% of these respective windows were quantifiable across all oocytes and embryo datasets, respectively, and were used for quantification. Principal component analysis of these windows distribution revealed early postpartum oocytes to have great variation inside their replicates when compared to mid postpartum and heifer oocytes, indicative of a greater sensitivity to metabolic stress (Figure 2.3b). In embryos, samples from lactating cows

clustered together more tightly than morulae from heifers (Figure 3.3). Methylation distribution of these windows was consistent with previous genomic feature quantification where early postpartum oocytes had a smaller proportion of windows with methylation ranges between 80-100% (Figure 2.3a) than mid postpartum and heifers oocytes. Similarly, embryos from lactating cows had a greater distribution of windows ranging from 45-60% methylation than embryos from heifers (Figure 3.4a). After quantification, logistic regression with Benjamini-Hochberg correction ($p < 0.05$) was used to list differentially methylated regions (DMRs) between conditions. In oocytes, early postpartum oocytes had the highest amount of DMRs above 10% methylation difference, totalling 99,307 and 118,179 DMRs when compared to mid postpartum and heifer oocytes, respectively (Figure 2.4a). Cross referencing these lists gave a total of 84,356 DMRs exclusive to early postpartum oocytes, 1,142 mid postpartum specific DMRS and 2,290 DMRs for heifer specific DMRs. Such variation was smaller in embryos, with a total of 13,383 DMRs in both hyper and hypomethylated regions (Figure 3.5a).

1.9.4   Functional and pathway enrichment analysis of differentially methylated regions

Differentially methylated regions were filtered against multiple lists of genes known to be involved in developmental competency. As such, 34 early postpartum specific DMRs were found to overlap known bovine imprinted genes. One DMR overlapping the MEST upstream region of the gene body region was found overlapping both a CpG island and exon in all oocyte conditions (Table 2.2). A smaller variation of these genes was found in embryos, with 5 DMRs overlapping 3 different bovine genomic imprints (Table 3.4). Additionally, 13 DMRs in embryos were found overlapping genes involved in embryo implantation (Table 3.4

mouse annotation, Gene ontology term GO:0007566). DMR lists submitted to gene

enrichment pathway analysis revealed 10,114 genes in early postpartum oocytes specific

DMRs to be involved in metabolic pathways, carbon metabolism, fatty metabolism as well

as genes involved in focal adhesion and actin skeleton (Figure 2.5). As for embryos, DMRs

were found to be involved in 3,260 genes in signaling pathways, pluripotency and metabolic

pathways (Figure 3.6), in addition to multiple biological processes like lipid homeostasis and

metabolic, fatty acid metabolic and biosynthetic processes (Figure 3.7).

1.10   References

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Chapter 2: Metabolism-associated genome-wide epigenetic changes in bovine oocytes

during early lactation

Metabolism-associated genome-wide epigenetic changes in bovine oocytes during early lactation

Mikhael Poirier, Michael Hoelker, Tsige Hagos, Dessie Salilew Wondim, Samuel Gebremedhn, Franca Rings, Christiane Neuhoff, Karl Schellander, Dawit Tesfaye*.

Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn, Bonn, Germany

*Corresponding author information

Dawit Tesfaye

Institute of Animal Science

Animal Breeding and Husbandry Group

University of Bonn

Endenicher Allee 15 Bonn, Germany

Email: dtes@itw.uni-bonn.de

Phone: +4922872286

Fax: +49228732284

2.1   Abstract

Dietary intake in early lactating cows is outmatched by milk production and the cows undergo negative energy balance, resulting in a perturbed blood metabolism and poor reproductive function due to impaired ovulation and increased embryo loss. We hypothesize that oocytes from lactating cows undergoing transient metabolic stress exhibit differential epigenetic profile crucial for developmental competency. To investigate this, we collected oocytes from metabolically profiled cows at early postpartum, mid postpartum and characterized their epigenetic landscape using whole genome bisulfite sequencing compared to heifers. Early postpartum cows had a deficient metabolism with significantly lower energy balance and significantly higher concentrations of non-esterified fatty acids and beta-hydroxybutyrate than mid postpartum animals and heifers. Accordingly, 32,990 early postpartum specific differentially methylated regions (DMRs) were found overlapping genes involved in metabolic pathways, carbon metabolism and fatty acid metabolism, suggesting the epigenetic regulation of metabolism in early postpartum oocytes. DMRs found overlapping CpG islands and exons of imprinted genes such as MEST and GNAS in early postpartum oocytes suggest that early lactation metabolic stress may affect imprint acquisition which could explain early embryo losses. This holistic approach introduces new avenues about the genetic background governing the link between metabolic stress and reproductive outcome of oocytes.

2.2   Introduction

In previous decades, genetic selection for improved milk production in lactating cows has come with the price of increased early embryo losses, longer calving intervals, and high frequency of services per pregnancy[1]. In feed based systems, the cow's dietary intake is more often than not outmatched by the biological processes involved post parturition, mainly milk production and uterine repair. Cows experience fat mobilization, ketosis and a surge in hormone biosynthesis which ultimately changes their metabolic status[2]. Non esterified fatty acids (NEFA) and ketone bodies such as β-hydroxybutyrate (BHB) have been found in higher levels during the post-partum period and have been associated with increased incidence of endometritis[3], making them interesting indicators for pregnancy outcome. Even though their production level peak around week 2 post-partum[4], blood serum levels can be detected well into post-partum and still be an indicator for predisposition of ketosis and other periparturition diseases[5,6]. These metabolites appear to cross the blood-follicle barrier in ovaries as they have been described in matching elevated concentrations in developing follicles[7]. As the bovine ovarian cycle is resumed around day 10 post partum (pp), the oocytes growing within pre-antral follicles are exposed to the circulating elevated metabolites in the follicular environment[8]. During a period of 60 to 80 days, the oocyte is not only growing in size, but also accumulating the necessary gene transcripts, protein and epigenetic marks necessary to sustain embryo survival[9].

While no significant morphological and phenotypical changes were observed in oocytes derived from lactating cows pre and post day 42 pp[10], various studies have described the methylation dynamic changes occurring during oocyte maturation and fertilization. Overall methylation levels in fully matured oocytes were higher than their germinal vesicle stage counterpart[11] and an extensive demethylation in fertilized oocytes is expected to occur prior

to embryonic genome activation[12]. Moreover, it has been shown that imprinted genes in oocytes showed hypermethylation depending on their time of collection post-partum[13]. *In vitro* studies have also shown that concentrations of fatty acids similar to what is experienced post-partum in lactating dairy cows impaired maturation rate, fertilization, cleavage and blastocyst formation rates[7]. Using in vitro studies, we have shown how maturation conditions may have an impact on methylation marks on embryos prior to embryo genomic activation[14,15]. Taken together, it is possible to hypothesize that the abnormal metabolism of the lactating cow will impact the methylation status of oocytes, ultimately impairing their developmental competence. We report for the first time the genome wide methylation profile of in vivo oocytes collected from cows experiencing negative energy balance, using whole genome bisulfite sequencing. This holistic approach should bring new insights on epigenetic-regulatory mechanisms governing the oocyte developmental competency of post-partum lactating cows.

2.3   Results

2.3.1   *Early postpartum cows are metabolically divergent compared to mid postpartum and heifers*

In order to assess the association of divergent metabolic status with the epigenome of oocytes in lactating cows throughout post-partum, an initial metabolic profiling of multiparous cows was done. Thirty cows were followed during the first 15 weeks of calving to assess their energy status. First, cows were followed by weight measurements to exclude cows gaining weight. Accordingly, a single cow started to gain weight in its first week of post-partum, associated with low milk production and was excluded from the experiment. We then profiled cows based on their energy balance status calculated from farm data and the

blood metabolite analysis of both NEFA and BHB following thresholds obtained from previous studies[5,6], linking these concentrations with mild negative energy balance. When collection points met 2/3 criteria, the sample was assigned as being collected during negative energy balance, and when 2/3 were not meeting the criteria, the samples were considered to be from positive energy balance status. After this selection, 11 cows met the negative energy balance criteria in early postpartum (Epp; w5-6 pp), and 7 animals met the positive energy balance selection during mid postpartum (Mpp; w9-10 pp). These cows reached their weight nadir around 37.6 ± 3.9 dpp and lost on average 50.4 ± 4.4 kg from the beginning of parturition (Table 2.1), averaging a daily loss of 1.5 ± 0.18 kg/day.

In addition, these cows produced an average daily milk yield of 37.7 ± 1.5 kg/day, with a concurring daily negative energy balance average of -25.5 ± 5.0 MJ/day prior to oocyte collection. During collection of Epp oocytes, cows maintained a negative energy balance average (-19.75 ± 3.5 MJ/day). Interestingly, Mpp collection points of selected cows still exhibited negative energy balance on average, although very close to positive energy balance with a standard error of the mean overlapping positive energy balance values (-0.76 ± 3.15). Epp cows still had a significantly lower energy balance values at the time of oocyte collection when compared to Mpp cows ($p < 0.001$), indicating that these cows experience early lactation energy balance deficit and recuperate towards positive energy balance at later stages in lactation.

Blood metabolite analysis revealed similar patterns, with average levels of NEFA above the threshold[5] in Epp samples and significantly different than the levels found in Mpp and control heifers (CH) blood serum (Figure 2.1, $p < 0.05$). The same pattern was found in BHB levels, where Epp BHB serum levels were averaging above the threshold and Mpp BHB serum levels were on average under the threshold and closer to heifers. Animals selected for

early lactation negative energy balance on average exhibited significantly higher levels of both NEFA and BHB (Figure 2.1, p < 0.05) compared to Mpp and heifers, whereas Mpp animals only had NEFA levels significantly higher than heifers (p< 0.05). Blood metabolite analysis during ovum pick-up procedures revealed that heifers were not metabolically challenged, and their metabolite concentrations were significantly different from the Epp negative energy balance animals but not significantly different than Mpp animals (Figure 2.1). These results indicate that, although not all cows experience negative energy balance, selection of cows based on their body weight loss as well as their energy balance reveal oocytes collected at metabolically divergent status during post-partum, with mid postpartum animals resembling metabolically non-challenged cyclic heifers. Early postpartum cows were experiencing a significantly lower energy balance status as well as increased circulating blood NEFA and BHB metabolites than mid postpartum cows and cyclic heifers.

2.3.2   The epigenetic landscape of early postpartum oocyte genomic features diverges from mid postpartum and heifer oocytes

Following the different metabolic profile of early and mid postpartum and cyclic heifers, three pools of oocytes from each condition were submitted to genome-wide methylation profiling. Unique read alignment in samples varied from 64,957,486 to 247,797,579 reads and the corresponding CpG coverage at 1x was from 49.7% to 94.6%, with a coverage depth ranging from 1x to 11x. Global CpG methylation percentages were 61.3% ± 4.6, 68.9% ± 1.0 and 69.2% ± 0.1 in early postpartum, mid postpartum and cyclic heifers, respectively. When looking at the methylation over genomic features (Table 2.1), there is consistency in the level of methylation across features, with similar methylation in features across groups. Early postpartum oocytes showed lower methylation levels of all genomic features when

compared to the mid postpartum and heifers groups. Genomic features methylation levels of CpG islands, promoters and transcriptional units were lower than the global CpG methylation across the genome in all groups (Table 1). As such, high global CpG methylation averages seem to be caused due to the high methylation levels of repetitive elements like LINE, SINE and LTR elements (Table 1).

Although overall methylation levels for entire gene bodies in the Epp group was on average lower than Mpp and CH, gene bodies of genomic imprinted genes revealed a higher methylation status in most of the genes compared to their metabolically healthy counterparts. Epp oocyte methylation levels of gene bodies of imprinted genes were significantly different from 12 and 11 bovine imprinted genes in Mpp and CH groups, respectively (Figure 2, $p < 0.05$). Interestingly, Mpp genomic imprints did not significantly differ from the cyclic heifer oocytes, which could indicate that, coupled with the global and genomic feature methylation level distribution, the methylation profile of mid postpartum oocytes resembles the heifer epigenetic landscape.

As the whole gene body may be large for adequate characterization of differentially methylated regions, a more detailed analysis was performed by binning the genome into 150 CpG windows. Of the 357,863 windows created from the 9 datasets, 98.7% of these windows were quantified across all datasets. An overall representation of the methylation levels of these windows and genome view of the respective replicates is shown in figure 3A, of which a slightly greater representation of 60-80% methylation in the distribution of methylation windows for the mid postpartum samples is observed.

From these windows, differentially methylated regions (DMRs) were identified by logistic regression replicate testing with a >10% methylation change between conditions and

corrected with Benjamini-Horchberg at p < 0.05. PCA clustering of these windows is shown in Figure 3B, with a high degree of clustering in mid postpartum and heifer samples, but not for early postpartum samples. This is supported by the high degree of DMRs found in comparison of Epp with CH groups and Epp with Mpp groups (Figure 4A). Additionally, the Epp group had stronger methylation changes, with approximately 10% of its DMRs being between 25% to 100% methylation changes in both hypermethylated and hypomethylated DMRs (Figure 4C). Pairwise comparisons revealed DMRs that were overlapping gene body regions in all comparisons where the top 20 most differentially methylated regions are listed in figure 4B (Supp. table S2.2-4). Additionally, list of DMRs from each pairwise comparisons were analyzed for gene ontology pathway enrichment, with clusters of genes involved in metabolic pathways, hormone signaling pathways and cellular structure pathways (Supp. table S2.5-7).

Pairwise comparison lists of DMRs were cross combined to identify the changes that were exclusive to each biological condition. A total of 2290 DMRs were found to be exclusive to CH when compared to Epp and Mpp, of which 1014 and 1183 were found to be hyper- and hypomethylated in CH compared to Epp and Mpp, respectively, despite their diverging metabolic profile (Figure 4A). Of these DMRs, 705 CH specific DMRs were overlapping genes. This proportion of DMRs in gene regions seems a little higher than what was found initially in the genome (gene bodies representing 27.04% of windows in the genome) but this increase was also found in DMRs found in all biological conditions (Supp. table S2.1). A notable increase was found in the proportion of DMRs overlapping CpG islands, where Epp specific DMRs had 1.99 fold increased representation. This overrepresentation was found to be 3.08 fold (Supp. table S2.1) when looking into the 405 DMRs that were shared across all groups (Figure 4A), indicating that a proportion of methylation changes observed across all groups is

found to be overlapping CpG island regions of the genome. As for Mpp specific DMRs, 1142 DMRs were found, the smallest variation from the three conditions. The bulk of changes observed in DMRs stem from Epp specific DMRs, totaling 84,356 DMRs. Of these, 32,990 were overlapping gene regions, corresponding to 39.1% of the genome features overlapping gene bodies, a 12.1% increase from the normal representation of this feature (Supp. table S2.1). Consistent with the dispersed PCA clustering of replicates, early postpartum oocytes experience a greater magnitude of DNA methylation changes than their mid postpartum counterpart.

2.3.3   Global gene body methylation differences exclusive to early postpartum oocytes are involved in metabolic processes

To gain an insight on the functional relevance of these changes, DMRs found overlapping gene regions were selected for enrichment pathway and gene ontology analysis. When looking at mid postpartum oocytes from recuperating cows, a list of 265 DMRs overlapping genes showed low enrichment in biological processes such as immune response, down-regulation of DNA binding transcription, fatty acid oxidation and biosynthetic process (Supp. Fig. 1). These pathways were linked through the presence of phosphoinositide-dependent kinase-1 (PDPK1), Serine/threonine-protein kinase D2 (PRKD2), protein kinase C beta type (PRKCB) , TNF receptor-associated factor 2 (TRAF2) and NCK-interacting protein kinase (TNIK) genes, which are involved in immune response and fatty acid processing (Supp. table S2.9). The low enrichment of DMRs over the number of putative target genes could indicate their small variation on biological processes occurring in Mpp oocytes. On the other hand, CH specific DMRs yielded a better list of enriched KEGG pathways, with 517 genes involved in actin skeleton regulation, focal adhesion and adherens junction, as well as Ras and Hippo signalling pathways. Genes connecting these pathways included Serine/threonine-protein

kinase PAK 3 (PAK3), Integrin alpha-IIb (ITGA2B), Integrin beta-8 (ITGB8) and epidermal growth factor receptor (EGFR) (Supp. table S2.10).

As for DMRs found to be specific to early postpartum oocytes, a total of 10,114 genes were involved across multiple pathways, most notably enriched in metabolic pathways (761 genes), carbon metabolism (79 genes), fatty acid metabolism (42 genes) and amino acid biosynthesis (52 genes). Structural genes involved in focal adhesion and actin skeleton were also found ($p < 0.05$, FDR < 0.004, Supp. table S2.8). The low amount of DMRs specific to mid postpartum and heifer oocytes, as well as the low degree of gene enrichment in biological processes and pathways, compared to the high number of DMRs and enrichment in early postpartum oocytes indicate that metabolic stress experienced during early lactation may confer a high variation of the methylome, possibly impairing their metabolism and structure.

2.3.4   *Differentially methylated regions exclusive to early postpartum metabolically stressed oocytes are found in gene bodies of genomic imprints*

Since genomic imprints have an impact on the ultimate developmental competency of the offspring with improper imprinting status being associated with diseases, we found DMRs overlapping the genes of selected imprints (Figure 2). Only one DMR was found across all conditions, overlapping the MEST gene, upstream of the coding sequence (Figure 6). This DMR also overlapped a CpG island region, of which separate quantitation revealed it to be also differentially methylated in Epp oocytes, but not in Mpp and CH oocytes. Around 33 additional neighbouring DMRs were found to be differentially methylated only in Epp oocytes (Table 2.2) with a majority of them being hypermethylated compared to Mpp and CH, of which 13 DMRs were found to overlap CpG islands and 22 DMRs were found to overlap exons of those genes, indicating that these neighbouring CpG islands and gene body

regions are sensitive to metabolic stresses. Interestingly, a cluster of 7 adjacent Epp specific DMRs were hypermethylated compared to CH and Mpp oocytes with 6 of these DMRs overlapping two CpG rich regions inside the GNAS gene body, spanning 11.8 kbp (Table 2.2). Taken together, the methylation variations observed in gene bodies of imprinted genes in early postpartum oocytes lay out an interesting landscape to investigate the impact of metabolic stress over developmental competency of oocytes.

2.4   Discussion

The follicular milieu of the oocyte is crucial for its proper maturation and acquisition of epigenetic marks. These will dictate its ability to sustain fertilization and develop to the offspring stage, and altering its profile during *in vitro* maturation has been linked to reduced developmental capability[7,16]. As such, the lactating cow has been previously described to exhibit an alternate metabolic profile associated with phenotypical changes regarding folliculogenesis and ovulation, ultimately resulting in poor reproductive quality[17]. To our knowledge, this study is the first to demonstrate that cows which undergo negative energy balance exhibit differentially methylated regions across the genome in genes involved in metabolism and development. To gain molecular insights into what lactation induced stress during post-partum has on its gamete population during follicular waves, we profiled cows undergoing negative energy balance both physiologically and metabolically and evaluated the epigenetic landscape of subordinate oocytes population during (week 5-6 pp) and exiting (week 9-10 pp) exposure to metabolic stress. Physiologically, we obtained oocytes at a time when the cow has resumed its ovarian cycle even though estrous signals are somehow silent[18], and oocytes around a period when the cow has usually recuperated from post-partum ailments, like uterine involution and body weight loss, and is ready to be inseminated for reproduction[19]. Using nulliparous heifer oocytes, we were able to

characterize what defines subordinate oocytes that are grown in unchallenged metabolic conditions and compare epigenetic patterns with oocytes that are or were exposed to metabolic stress.

Indeed, 97% of cows experience a weight loss prior to oocyte collection, with only one cow gaining weight immediately post-partum which produced negligible amounts of milk and was removed from further analysis. Therefore, most of the cows in the present experiment were behaving consistently with negative energy balance and weight loss of lactating cows as it has been reported in other studies[4,20]. Furthermore, cows were selected on additional criteria like energy balance and metabolic assessment. A previous report outlines that cows exit negative energy balance around 41.5 days in milk[21]. Interestingly, cows selected for the current study still experienced negative energy balance around this time period, while exhibiting overall negative energy balance at the time of mid postpartum (week 9-10 pp). Even if cows exhibited an average negative energy balance, their metabolic levels had significantly reduced to be close to the levels of unchallenged heifers. This suggests that, though Mpp cows return to metabolically basal NEFA and BHB concentrations, the animals used in this study were still physiologically challenged. A previous study described cows to exit the metabolically challenging period at d42 based on body condition score increase, while NEFA and BHB values return to normal levels preparturition[10]. However, body weight, insulin and glucose levels of these cows remained low after d42, suggesting that these could be more accurate indicators of energy balance, which would correspond to the energy balance averages found in the cows presented in this study. Nevertheless, we found significantly different averages for energy balance, NEFA and BHB, indicating that cows do recuperate from negative energy balance during lactation, although perhaps at a more delayed rate than suggested by afore-named literature.

Our study revealed that the early postpartum period has a stronger metabolic impact on cows than mid postpartum time points, which metabolically resembled cyclic heifers. We subsequently set out to characterize the epigenetic impact of this metabolic stress on oocytes. As such, methylation profiles of pools of Epp oocytes were highly heterogeneous compared to Mpp and CH oocytes, indicating the high impact variability of early lactation on oocytes methylation status. Animals and follicle-specific effects of metabolic stress on the oocyte epigenome are not ruled out. Future research on cow individuals and follicle specific sensitivity to stress will explain the basis for such variation in response to metabolic status. Nevertheless, average quantification of genomic features exhibited similar differences in Epp oocytes compared to Mpp and CH oocytes, with global methylation levels being lower in Epp samples and differences between features being conserved across all biological conditions. Interestingly, methylation levels found on CpG islands were overall lower than other features, as it has been previously observed in bovine oocytes[22]. A similar methylation level of CpG islands and differential clustering within certain conditions has also been described in whole genome bisulfite sequencing of pig embryos, although levels of global methylation between oocytes and embryos are not comparable, as the latter is undergoing extensive demethylation[23]. This study showed ratios of methylation in gene features spread similarly across blastocysts groups, indicating a similar organisation of the genome in all conditions. In the present study, overall organization of genomic feature methylation of Epp oocytes was conserved when comparing Mpp and CH oocytes, with a slight hypomethylation in both comparisons.

At closer inspection, compartmentalizing the genome in fixed CpG windows revealed specific regions to be differentially methylated. Subsequent clustering analysis revealed a greater fold of variation in DMRs specific to Epp oocytes. Functionally, gene ontology revealed that

fatty acid metabolism, oxidation and degradation were involved in Epp, Mpp and CH oocytes, respectively. A previous report has outlined the different fatty acid uptake during early embryo cleavages, with embryos failing to develop beyond the 4-cell stage having a significantly higher concentration of saturated fatty acid[24]. Coupled with previous reports of developmental failure in oocytes matured in high NEFA concentration, fatty acid oxidation and degradation found in Mpp and CH oocytes could suggest a metabolic direction of fatty acid metabolism to favor developmental competence. In Epp specific DMRs, 18 genes were found to be differentially methylated and involved in more than 4 pathways with multiple genes being isozymes or similar in structure. Of these, IDH3A is involved in tricarboxylic acid (TCA) cycle metabolism in the nuclei of early cleavage embryos prior to zygotic embryo activation[25], suggesting its role in early developmental competency. Also, PI3KCD is involved in follicle growth, where PIK3CD null mice have been found to be subfertile, with fewer growing follicles and reduced response to gonadotropins stimulation[26]. DMRs were also found in genes involved in cross-talk between bovine oocyte and surrounding cumulus cell including ACO1 and ACO2, both involved in carbon metabolism, and over expressed in oocytes co-cultured with cumulus cells[27]. Also, isoforms of protein kinase B (AKT2/3) were found to be differentially methylated in Epp oocytes, which is known to impact meiosis through the organization of microtubules in mice, subsequently influencing fertilization outcomes[28].

DMRs specific to Mpp oocytes were also found to be involved in various reproductive processes. Of these, protein kinase C (PKC) was previously involved in improving maturation, where stimulation of PKC resulted in an increase in pronuclear formation and a faster meiotic resumption rates in bovine oocytes[29]. Another protein kinase, 3-phosphoinositide-dependent protein kinase-1 (PDK1) was found to be crucial to maintain survival of primordial

follicles in mice, where PDK1 knockout in mice oocytes cause infertility and premature ovarian aging[30]. Taken with the remaining DMRs from Mpp oocytes, the results from this work evidenced the effect of metabolic stress on gene regulatory mechanisms explaining failure associated with embryo loss at the time of first service post calving. Additionally CH specific DMRs might give us clues on methylome of oocytes under normal conditions which can easily be associated with developmental competency. Genes such as integrin beta 8 were found to be downregulated during in vitro maturation of porcine oocytes, although its influence on developmental competency remains to be proven[31]. Lysophosphatidic acid receptor (LPAR1) might modulate COC quality, as supplementation of LPA during maturation improved expression of oocyte quality markers follistatin and growth and differentiation faction 9 (GDF-9), although no improved d7 blastocyst rates were observed[32]. Moreover, epidermal growth factor receptor (EGFR) and FYN kinase were found to play a role in the completion of meiosis[33,34]. Although gene expression validation studies need to be performed to further confirm the scale and direction of these methylation differences, our results indicate that metabolic status of post calving cows have a significant influence on the activity of genes associated with developmental compete of oocytes.

Previous reports have linked the impact of postpartum NEFA concentration with differential imprint acquisition in post-partum oocytes[13], where differential methylation levels were screened in imprinted genes previously described along their gene bodies, notably MEST, IGF2R and SNRPN. Although not at the same positions, we report specific DMRs found in gene bodies of these imprints and others, indicating early lactation metabolic stress has a similar impact on imprint acquisition postpartum. Similarly, MEST, a gene that is expressed in the paternal alleles in the mesoderm and its differentiated lines, was found to be dynamically methylated in mice, in the upstream promoter region of a CpG island similar to

the one we observed[35]. Normally, fully methylated in the maternal germ line, MEST was found to be differentially methylated in fully grown and freshly ovulated oocytes, while hypermethylated in oocytes cultured *in vitro*, outlining the dynamic nature of imprints acquisition and sensitivity to different growth conditions. Inversely to overall global methylation levels, DMRs overlapping imprinted gene bodies were of higher methylation levels in early postpartum compared to late postpartum and heifers in 28 of the 34 DMRs listed. Additionally, numerous studies report the positive correlation of transcribed gene body regions methylation with its expression, indicating that silencing the methylation of gene bodies is associated with upregulation of expression[36–38]. This suggests that imprints are overall hypermethylated in early postpartum oocytes, modulating their expression regardless of putative imprint status. Further validation with gene expression analysis in all conditions is needed to confirm the functional relevance of the epigenetic modifications of these regions.

In conclusion, we report here the genome wide methylation profiling of oocytes retrieved from cows of metabolically divergent conditions, where we have found relevant biological processes that could be involved in the reproductive limitations of lactating cows. We demonstrate that although metabolically recuperated, cows do exhibit negative energy balance mid postpartum, but differential methylation was mainly pronounced in oocytes from cows at an early stage of postpartum. Nevertheless, the afore-presented holistic approach lists an array of new DMRs in the bovine genome to investigate the relationship between metabolic stress and developmental capacity. Future studies will aim to validate the correlation between gene body methylation and gene expression to confirm how these genes are involved in bovine embryo developmental failure associated with early postpartum metabolic stress.

2.5   Material and Methods

2.5.1   Animal handling, oocyte and blood collection from lactating cows postpartum and cyclic heifers.

Animal handling was carried out in accordance to the 2015 German law of protection (TierSchG & TierSchVersV). Experimental protocols performed on cows in this study were approved by state office for Nature, Environment and Consumer protection of North Rhine-Westphalia, Germany (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Deutschland). Blood samples collections and ovum pick up (OPU) procedure were approved under license number 84-02.04.2015.A139 and 84-02.04.2014.A500, respectively. Thirty Holstein Friesian cows and eight Holstein Friesian nulliparous cyclic heifers were used for this experiment. Lactating cows were monitored for daily body weight, mixed ration and concentrate intake, milk yield and milk composition recorded until 15 weeks post-partum. Starting at week 5 post-partum, oocytes were collected from cows using transvaginal ultrasound-guided ovum pick-up (OPU). Using an ultrasound probe with a cannula connected to an aspiration pump, subordinate follicles of size smaller than 8 mm were aspirated. Once collected, oocytes were denuded using hyaluronidase and snap frozen. This procedure was repeated weekly until week 10 post-partum. This OPU procedure was also performed in 8 nulliparous heifers for 5 consecutive weeks. In parallel to OPU, 20 mL of blood were taken from each animal for blood metabolite analysis. Serum was separated and frozen at -80 $^{\circ}$C for future analysis.

2.5.2   Energy balance assessment of cows and oocyte selection pooling

Body weight curves for all cows were drawn to ensure lack of weight gain in early post-partum phase. Cows experiencing immediate weight gain post-partum were removed from

further analysis to ensure selection of cows exclusively undergoing negative energy balance post calving. Weekly energy balance average was calculated to assess energy status using the following equation based on previous literature (GfE, 2001, Hailay, 2019)

$$EB = DMIe + Ce - BWe - MYe$$

where the energy balance (EB) is the result of daily dry matter intake energy (DMI), plus the daily concentrate energy (Ce) and subtracting the maintenance of body weight energy (BWe, BWe = 0.293 BW ^ 0.75) and milk yield energy (MYe), which is calculated using milk weight and composition : (0.39 × fat% + 0.24 × protein% + 0.17 × lactose% + 0.07) × kg milk). . Additionally, blood metabolite analysis of β-OHB and NEFA was done spectrophotometrically (HORIBA, Montpellier, France) for both β-OHB (Kit #RB1008, Randox Laboratories, Crumlin, United Kingdom) and NEFA (#434–91795, Wako Chemicals GmbH, Neuss, Germany) following a previously described protocol[39]. Serum NEFA and BHB values over thresholds identified in literature[5,6] were associated with negative energy balance. Accordingly, 3 phenotypes were selected: oocytes from early postpartum, (Epp; w5-6 pp), oocytes from mid postpartum, (Mpp; w9-10 pp) and oocytes from cyclic heifers (CH) as not metabolically challenged status. Oocytes were pooled into biological triplicates for each phenotype (Epp, n = 62; Mpp, n = 64; CH, n = 181) for bisulphite conversion.

2.5.3   *Oocyte DNA bisulfite conversion and isolation*

Oocytes in each triplicate were lysed and bisulfite treated using the EZ-DNA Methylation-Direct Kit (Zymo Research, Freiburg, Germany) following manufacturer's instructions with some modifications. Oocytes were initially digested in 10 μL of digestion buffer (Zymo Research) and 1 μL of proteinase K (Zymo Research), where digestion reactions were scaled up according to the volume of starting sample (up to 27 μL), to a limit of 3-fold at 50 °C for

20 min. Resulting digested samples were split according to the previous lysate volume, up to 3 reactions and were bisulfite converted using the provided CT conversion reagent (Zymo Research) at 98 °C for 8 minutes and a final incubation at 64 °C for 3.5 h. Conversion reactions were bound to collection columns, desulphonated and washed according to manufacturer's directives. After this purification, reaction replicates were eluted using 8 µL of the elution buffer provided in the Pico Methyl-Seq Library Prep Kit™ (Zymo Research). The resulting bisulfite treated DNA was used for library preparation.

2.5.4   *Library preparation for whole genome bisulfite sequencing*

Sequencing libraries were prepared from the resulting bisulfite treated DNA of pooled oocytes using the Pico Methyl-Seq Library Prep Kit™ (Zymo Research, Freiburg, Germany) according to manufacturer's recommendations with some additional modifications. Briefly, bisulfite treated DNA was pre-amplified with a primer concentration reduced to 20uM to prevent primer-dimer in the final library. Following this, DNA was purified using a DNA binding buffer to sample ratio of 5 to 1 during all purification steps, to increase fragment recovery. Following purification, pre-amplified DNA was further amplified for 10 PCR cycles during the amplification step of the library preparation as per manufacturer's recommendation. The resulting reaction was purified and further amplified with index primers. The resulting PCR product was purified and its fragment size and quality was assessed using the Agilent High Sensitivity DNA assay with a Bioanalyzer (Agilent, Waldbronn, Germany). Library quantification was assessed by qPCR using the KAPA Biosystems™ library quantification kit (Roche, Mannheim, Germany). Libraries were sequenced single-end for 114 cycles on an Illumina HiSeq 2500 using TruSeq v3 chemistry (Illumina GmbH, Munich, Germany). Raw sequencing data was demultiplexed according to

index primers added during library preparation and available on the European Nucleotide

Archive (Array Express accession number E-MTAB-8191) and used for further processing.

2.5.5   Raw sequence data processing

Raw sequence data was subjected to quality control with FastQC v0.11.6[40] and subsequently

trimmed with Trimmomatic 0.36[41] with hard trimming of 10 bp on each end of the fragment

to reduce base composition bias of the reads as per the aligner's recommendations, with the

following parameters: SE –threads 8 ILLUMINACLIP:TruSeq3-SE.fa:2:30:20 MAXINFO:83:1

LEADING:20 TRAILING:20 CROP:100 HEADCROP:10 MINLEN:20. Trimmed sequences were

verified on FastQC again and mapped to the bisulfite converted bovine genome generated

from the reference bovine genome (Ensembl release 93) by the Bismark tool[42]. Trimmed

sequences were aligned using the Bismark v0.19.0 tool with the Bowtie 2 aligner[43] with the

single-end read default aligning parameters. After alignment, duplicate sequences were

removed and CpG methylation calling was extracted using deduplication and methylation

extraction modules of the Bismark tool. Methylation coverage output files were imported

into SeqMonk v1.44.0[44] for further analysis.

2.5.6   DNA methylation quantitative analysis

For general quantitation of genome features between conditions, probes spanning genome

features had their overall ratio of methylated counts over demethylation counts measured.

CpGs islands, promoters, transcriptional units, LINE, SINE and LTRs probes were delimited

using Ensembl release 93 genome annotation and methylation levels were calculated using

the ratio of methylated CpGs over unmethylated CpGs. Bovine specific genomic imprinted

probes were selected using the gene imprint list[45]. Promoters were set to span 1000 bp

upstream of the whole gene body, where transcription units (TU) covered 500 bp downstream of the annotated start of the gene body.

For unbiased analysis, the genome was binned in 150 CpGs windows using the read position probe generator and selecting 1 minimum read count to include position and 150 valid positions. Windows were generated across all replicates for a total of 357,863 windows in all 9 datasets. Percentage methylation of the 150 CpG site binned windows was calculated using the bisulphite methylation over features quantitation pipeline with a minimum count of one to include position and 20 minimum observations to include a feature. To enable logistic regression comparison of quantitated windows, a value filter was applied to every window that had a value between 0 and 100 across all 9 datasets. The resulting total number of windows when filtered was 353,294 and these windows were quantitated again using the bisulphite methylation over features pipeline. This quantitation was subsequently normalized across all data sets using the match distribution quantitation tool. Differentially methylated regions calculated through logistic regression were submitted to gene ontology and KEGG pathway enrichment analysis using network analyst[46].

2.5.7   Statistical analysis

When comparing energy balance status, data from biological conditions were compared using ANOVA, where a $p < 0.05$ was found to be statistically significant. Data are presented as mean ± SEM. To compare metabolite profile averages between biological conditions, a mixed model using a random effect for each animal and a fixed effect for the group was used, where $p < 0.05$. For differential methylation analysis, a logistical regression for replicate statistical test was used and corrected using Benjamini-Hochberg of $p < 0.05$ with a

false discovery rate of 5%. For gene ontology analysis, gene ontology enrichment was filtered either with p-value < 0.05 or adjusted p-value < 0.05.

2.6   Acknowledgments

2.7   Author contributions

MP conducted the experiment, performed sample processing and sequencing, data analysis, interpreting the results, drafting and writing the manuscript. MH was involved in ovum-pick up, blood collection and design of the experiment. THH was involved in ovum pick-up and blood collection. DSW was involved in the data processing and reviewing the manuscript. SG was involved in the design of the experiment. FR was involved in oocyte handling and freezing. CN was involved in design of the experiment. KS designed and supervised the experiment and reviewed the manuscript. DT was responsible for designing the experiment, acquisition of funds and reviewing of the manuscript. All authors approved the final version of the paper.

2.8   Competing interests

The authors declare no competing interests.

2.9   Data availability

Raw sequencing files are available at the European Nucleotide Archive under accession number E-MTAB-8191

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2.11   Figure legends

Figure 2.1 Blood metabolite concentrations [mM] of NEFA and BHB from cows selected during early postpartum (Epp), mid postpartum (Mpp) and heifers (CH). Dotted lines represent threshold of NEB found in literature (NEFA = 0.55 mM Fenwick, 2008; BHB = 0.65 mM Girard, 2015), where letters show significant differences between groups and $p < 0.05$). Error bars represent the standard error of the mean.

Figure 2.2 Methylation levels of genomic imprints gene bodies from early postpartum (Epp), mid postpartum (Mpp) and heifers (CH). Letters represent significance between groups where $p < 0.05$.

Figure 2.3 Methylation level distribution across the genome across samples. (a) Methylation levels of 150 CpG probes across the genome of the three groups of oocytes (Epp, Mpp and CH). (b) Principal Component Analysis of the DNA methylation distribution in early pp (Epp, blue), mid pp (Mpp, green), and heifers (CH, red) with their corresponding average (black).

Figure 2.4 DNA methylation analysis of oocytes derived from early postpartum (Epp), mid postpartum (Mpp) and heifers. (A) Venn diagram of DMRs obtained by logistic regression (10% methylation difference, adjusted $p < 0.05$), with number of hyper- and hypomethylated DMRs in each comparison, where 405 DMRs are found to be shared across all conditions (center). (B) Top 20 differentially methylated regions overlapping gene (GSy = gene symbol) body regions and their methylation changes in all three comparisons. (C) Fold methylation changes found in hyper- (right) and hypomethylated (left) DMRs from pairwise comparisons.

Figure 2.5 Functional relevance of early postpartum (Epp) specific DMRs. (a) Top pathways from the KEGG pathway database involved with the DMRs found in the early postpartum group with their pathway enrichment increase. (b) KEGG database pathway network association with DMRs from the early postpartum groups and their interaction. DMRs/TGs: Ratio of differentially methylated regions over the number of genes targeted by the pathway. Pathways shown have adjusted p-values < 0.05.

Figure 2.6 DMRs around the MEST locus. (a) 150 CpG windows DMRs overlapping the MEST gene body region. (b) Methylation quantification of the CpG island overlapping the MEST gene body. Methylation percentages are found in the bars, where * represents significant (adjusted $p < 0.05$) methylation differences either in early postpartum values or across all conditions.

2.12 Tables

Table 2.1 Methylation percentages of genome features in bovine oocytes derived from early postpartum (Epp), mid postpartum (Mpp) cows and cyclic heifers (CH).

Genomic features	Methylation (%)		
	Epp	Mpp	CH
CpG Islands	26.1	30.2	29.9
Promoters	36.5	41.9	41.6
Transcriptional units	34.4	41.1	40.9
Gene bodies	61.0	64.8	64.7
Intergenic	52.2	58.9	58.5
LINE1	67.8	74.1	73.7
LTR	63.1	71.2	70.7
SINE	67.7	78.4	77.8
Global	61.3	68.9	69.2

Table 2.2 List of early postpartum oocyte specific DMRs overlapping imprinted gene bodies.

Gene	Probe location	Methylation level (%)			Overlapping CpG island	Overlapping exon
		Epp	Mpp	CH		
PEG10	Chr4:11911539-11912583	67.52	35.40	33.34	+	+
MEST	Chr4:95066705-95067686	64.18*	34.63*	45.52*	+	+
	Chr4:95067710-95072935	74.69	61.76	56.91	-	+
NAP1L5	Chr6:37509075-37510923	71.47	45.96	43.66	-	+
IGF2R	Chr9:97639143-97641473	74.90	84.92	89.40	-	-
	Chr9:97654566-97658868	83.48	71.02	68.94	-	+
	Chr9:97658884-97661928	68.46	40.84	39.89	+	-
	Chr9:97664415-97669428	79.96	56.80	54.21	+	+
	Chr9:97669459-97672435	76.73	58.01	55.43	-	-
	Chr9:97677102-97678968	75.23	50.88	47.24	-	-
	Chr9:97683111-97685609	82.86	62.47	68.34	-	+
	Chr9:97696872-97698360	84.14	67.99	64.95	-	+
	Chr9:97698361-97700386	82.20	68.84	64.51	-	-
	Chr9:97723665-97725285	88.60	76.54	75.85	-	+
	Chr9:97729941-97731789	85.35	75.30	71.92	-	-
	Chr9:97731832-97733485	73.48	52.29	51.05	+	+
GNAS	Chr13:58017810-58022193	60.92	49.16	50.34	-	+
	Chr13:58028217-58033182	73.78	41.85	42.32	+	+
	Chr13:58033183-58034298	69.10	39.73	34.80	+	-
	Chr13:58034299-58035180	70.20	46.54	41.68	+	-
	Chr13:58035201-58036679	73.48	55.78	49.28	+	-
	Chr13:58036729-58037912	63.27	34.61	28.17	+	-
	Chr13:58037929-58040073	76.55	46.12	45.39	+	-
	Chr13:58040227-58046005	64.63	53.93	52.90	-	-
	Chr13:58047147-58048143	19.79	37.44	30.89	+	+
NNAT	Chr13:67118471-67119734	77.60	40.88	46.39	-	+
	Chr13:67119735-67122534	73.18	60.18	60.95	-	+
SNRPN	Chr21:13703-24837	70.60	37.27	44.83	-	+
	Chr21:24838-26023	66.22	34.92	32.17	+	+
TSSC4	Chr29:49836440-49837857	74.90	87.95	87.48	-	+

ASCL2	Chr29:49957329-49959108	60.98	46.72	45.26	+	+
IGF2	Chr29:50045707-50047790	28.20	39.23	39.61	-	+
	Chr29:50061341-50062955	43.98	61.46	64.07	+	+
	Chr29:50064682-50065982	43.86	80.23	80.77	+	+

* denotes a single DMR differentially methylated in all conditions. + and - denote presence or absence of overlap, respectively.

2.13 Figures
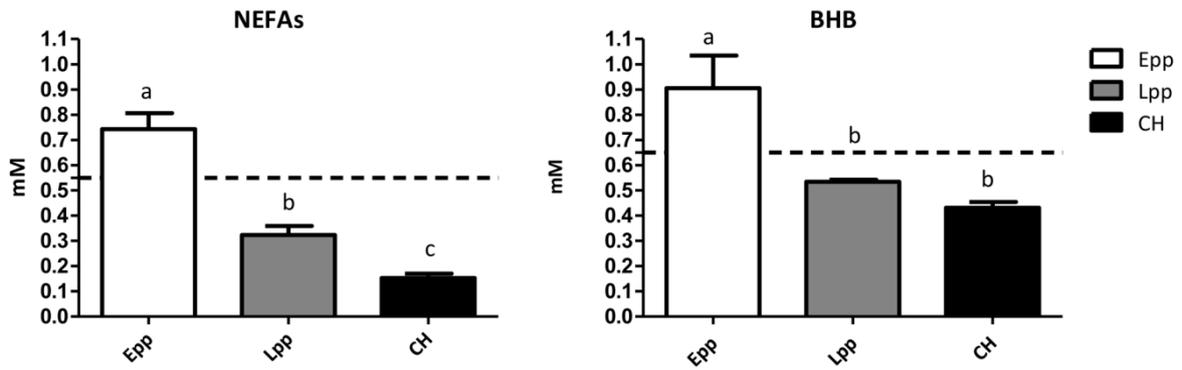
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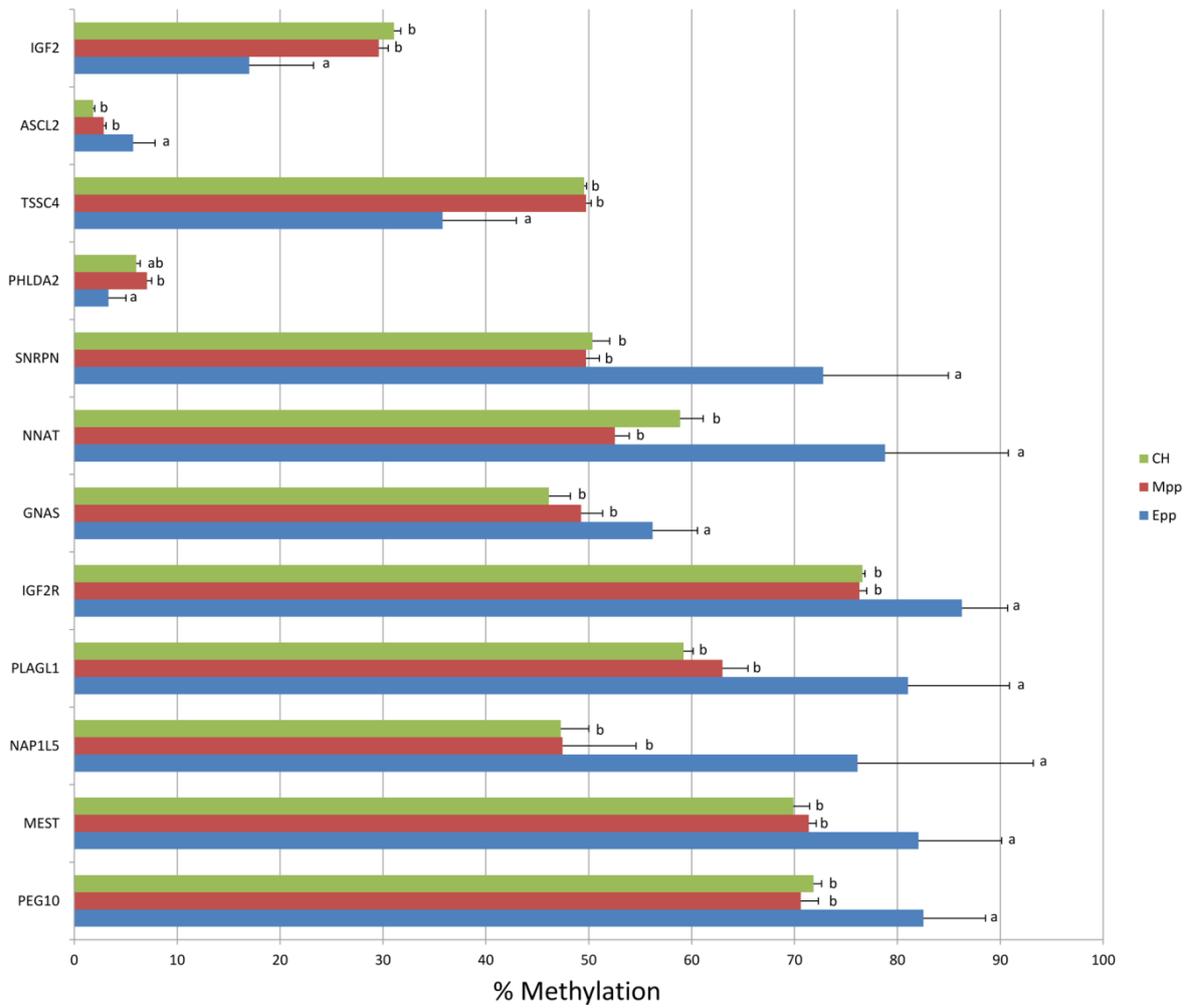
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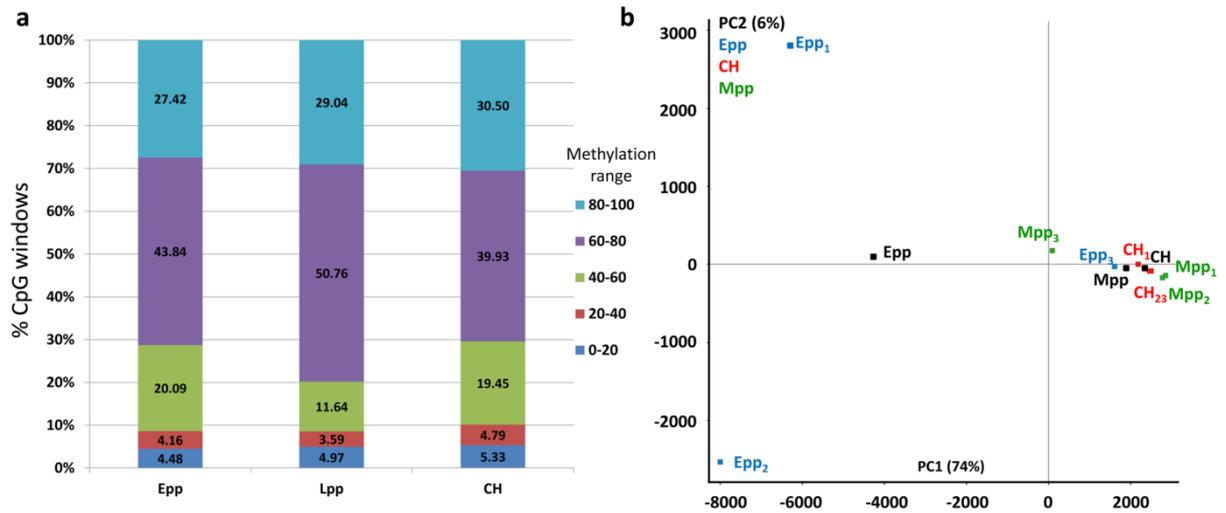
Figure 2.3

Figure 2.4

a

b
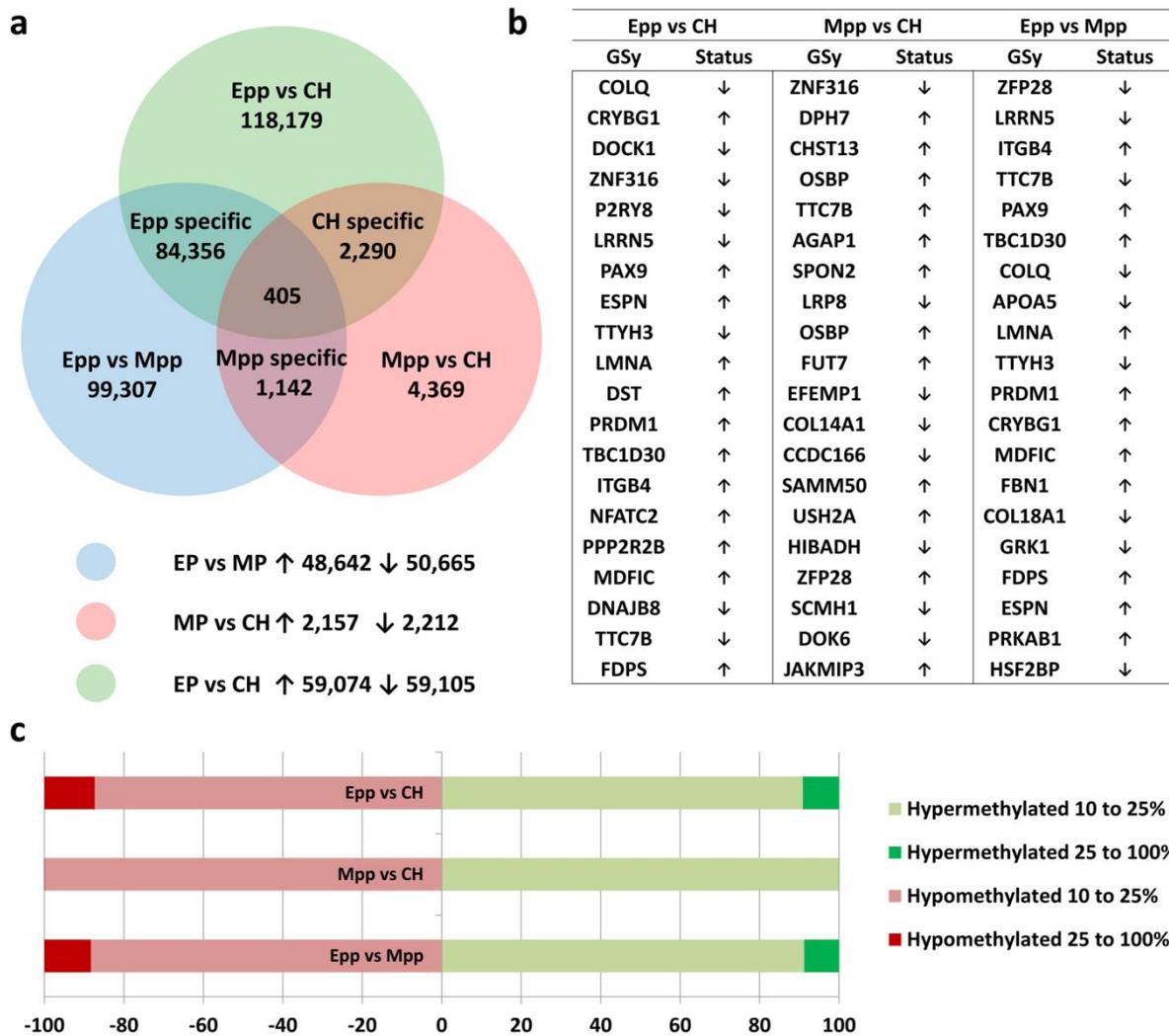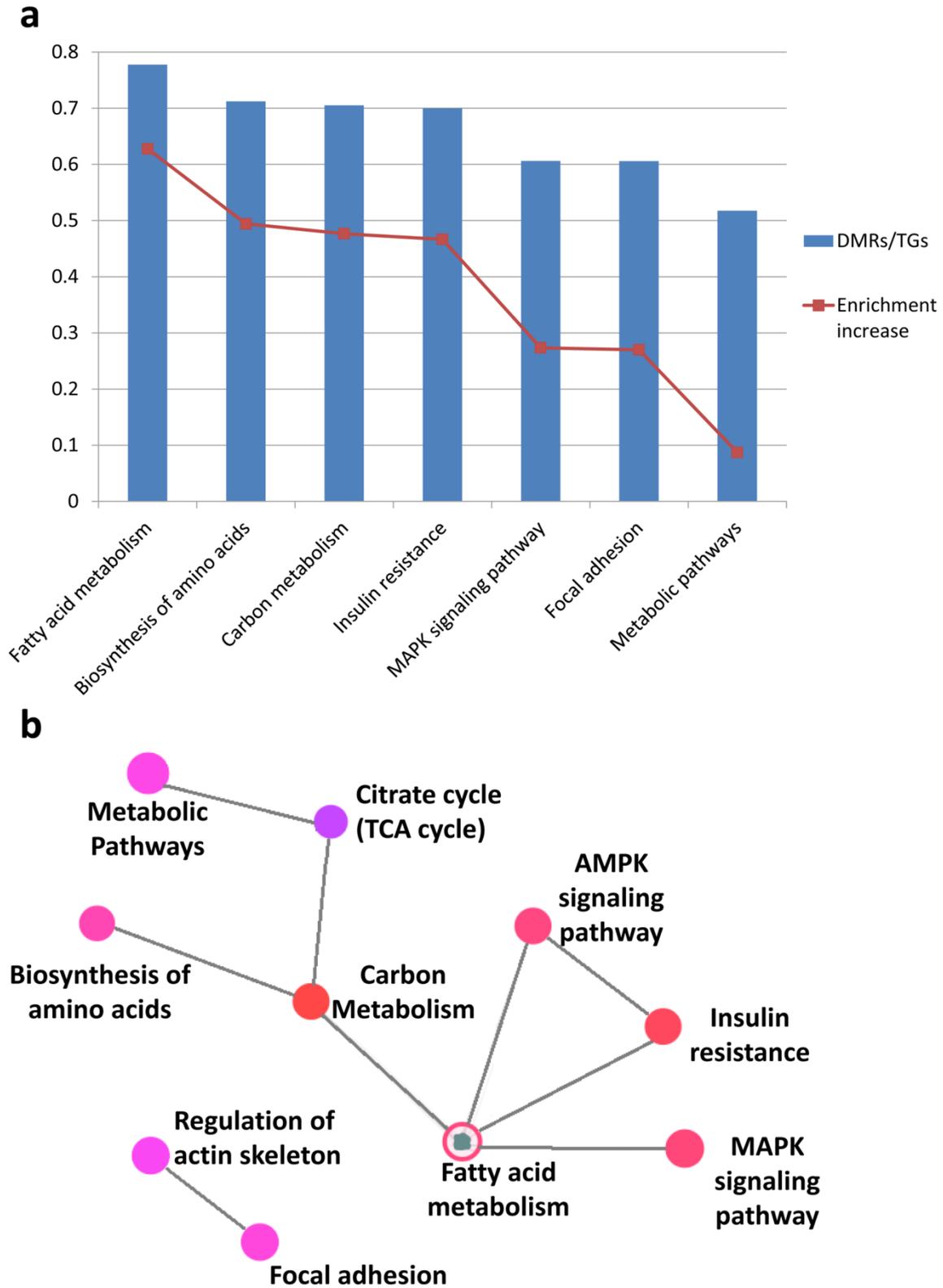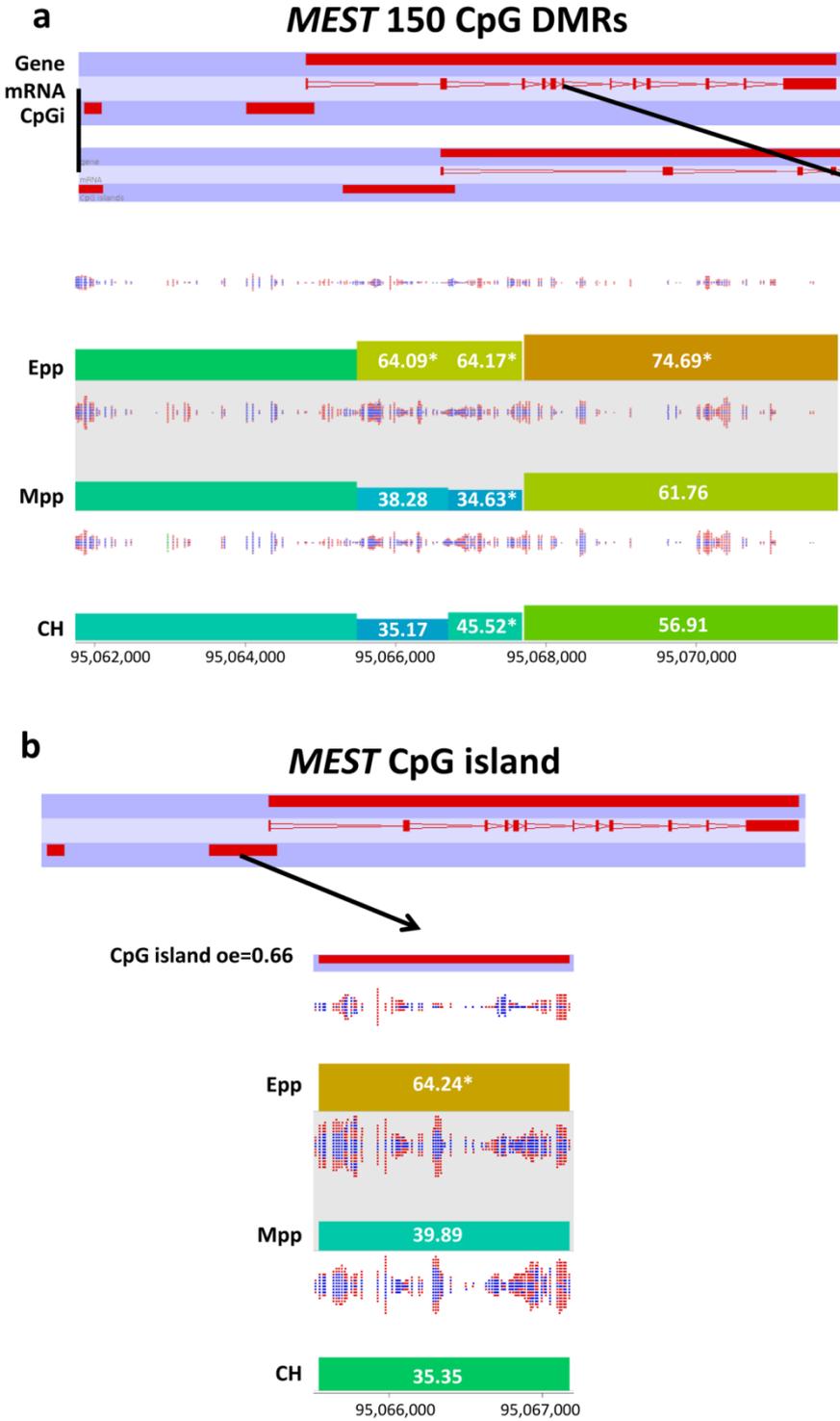
Figure 2.5

Figure 2.6

2.14 Supplemental figures

a

b
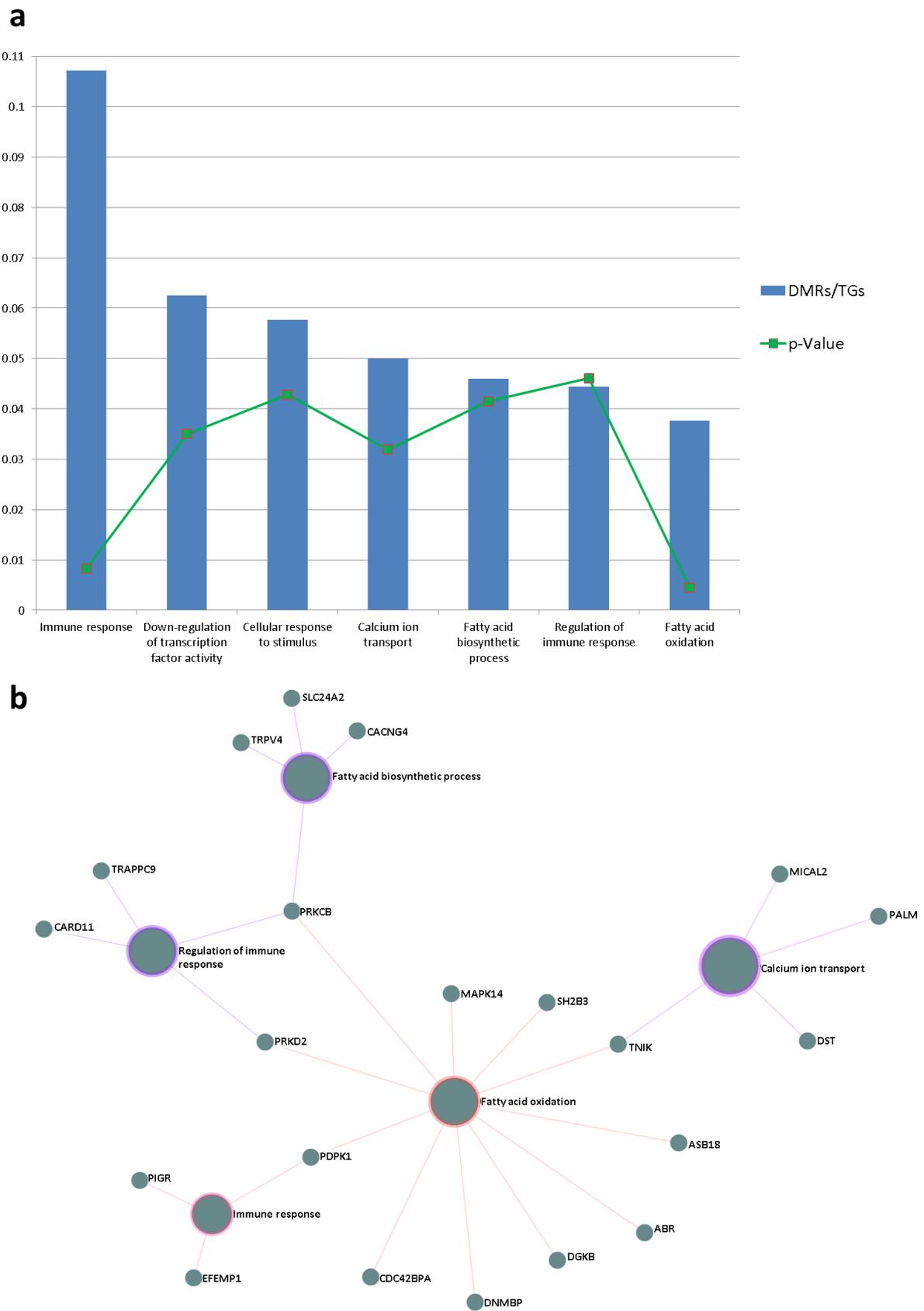
Supplemental figure S2.1 Functional relevance of mid postpartum (Mpp) specific DMRs. (a) Top enriched biological processes involved with the DMRs found in the mid postpartum group. (b) Biological processes network association with DMRs from the mid postpartum groups and their interaction. DMRs/TGs: Ratio of differentially methylated regions over the number of genes targeted by the pathway. Pathways shown have p-values < 0.05.
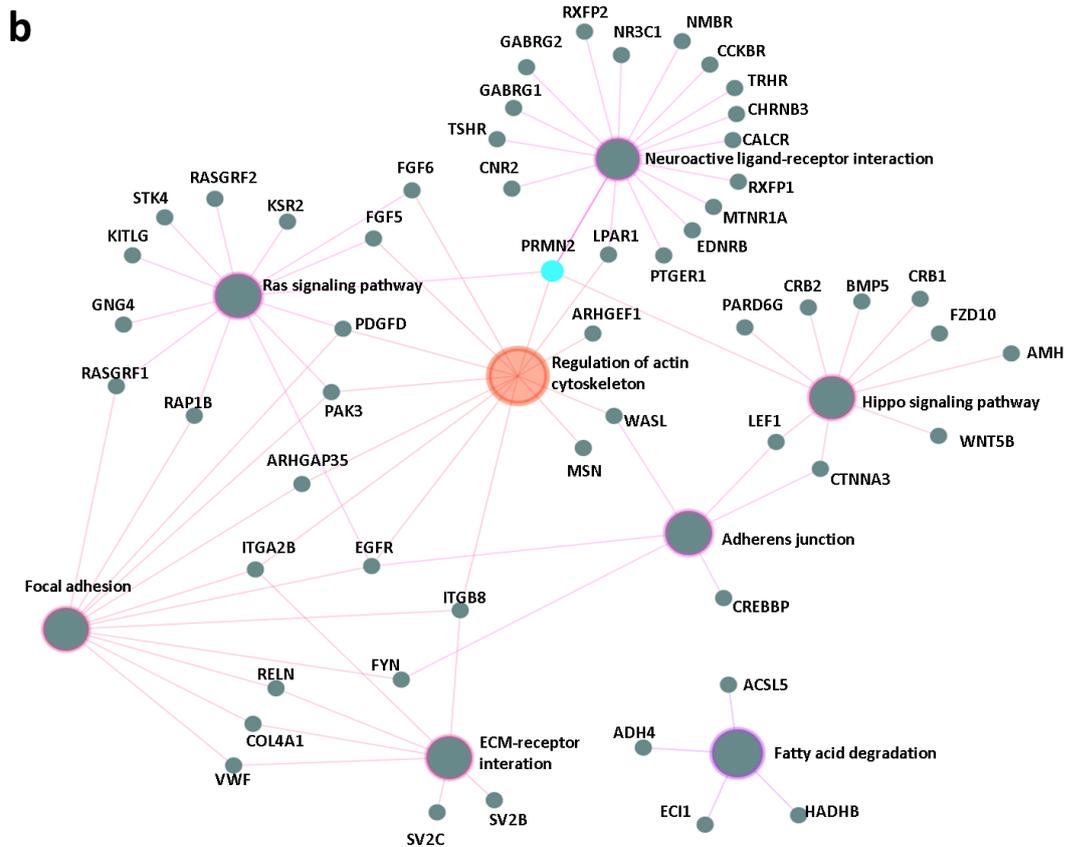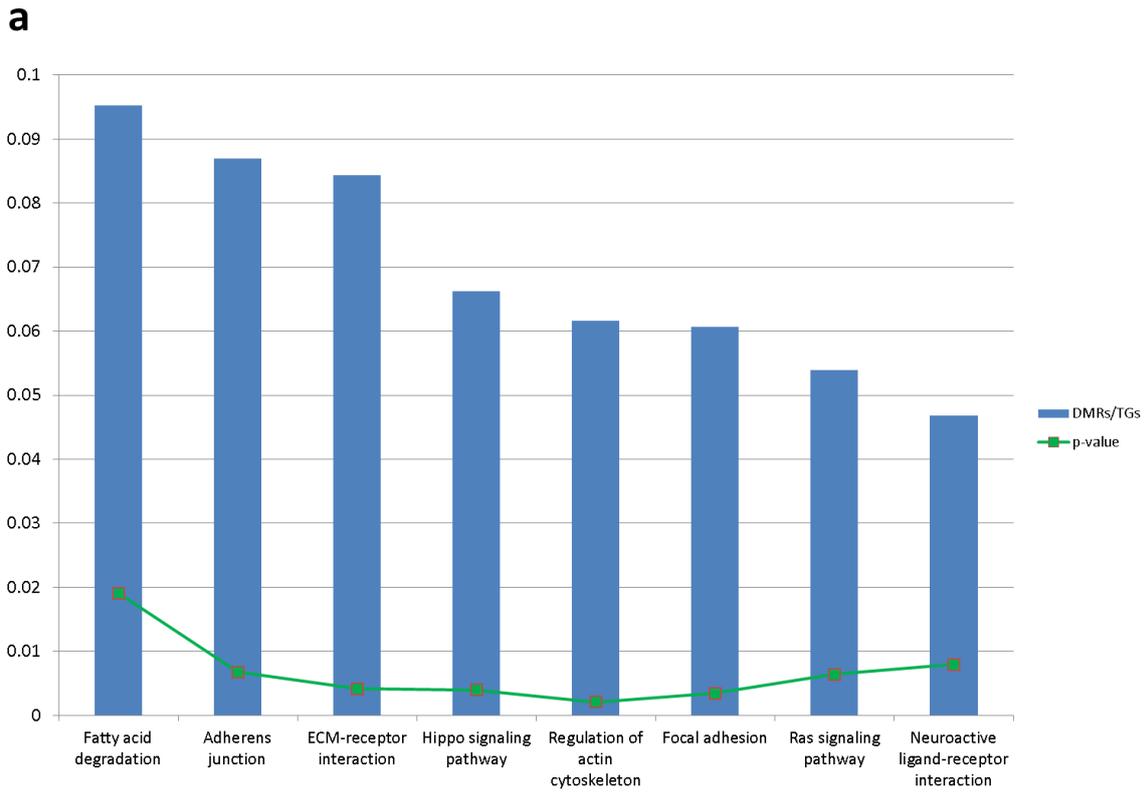
Supplemental figure S2.2 Functional relevance of cyclic heifer (CH) specific DMRs. (a) Top enriched KEGG pathways involved with the DMRs found in the CH group. (b) KEGG pathway network association with DMRs from the CH group and their interaction. DMRs/TGs: Ratio of

differentially methylated regions over the number of genes targeted by the pathway. Pathways shown have p-values < 0.05.

2.15 Supplemental tables

Supplemental table S2.1 Representation of 150 CpG windows across genome features and across DMRs found from different biological conditions. Number of total DMRs is found in parentheses in the column titles, where other values in the table between parentheses represent the ratio of DMRs found in the feature over the total number of DMRs.

Features	Global (535294)	Epp specific DMRs (84536)	Mpp specific DMRs (1142)	CH specific DMRs (2290)	Intersecting DMRs (405)
CpG Islands	48425(9.05)	15190(18.01)	162(14.19)	219(9.56)	113(27.90)
Promoters	29696(5.55)	8095(9.60)	54(4.73)	124(5.41)	23(5.68)
Transcriptional units	28237(5.28)	8337(9.88)	62(5.43)	119(5.20)	30(7.41)
Gene bodies	144738(27.04)	32990(39.11)	359(31.44)	745(32.53)	171(42.22)
Intergenic	243089(45.41)	60092(71.24)	875(76.62)	1724(75.28)	274(67.65)
LINE	292425(54.63)	65627(77.80)	942(82.49)	1976(86.29)	258(63.70)
LTR	196387(36.69)	40393(47.88)	711(62.26)	1425(62.33)	152(37.53)
SINE	314445(58.74)	73613(87.26)	1001(87.65)	2094(91.44)	300(74.07)

Supplemental table S2.2 Genome position of top 20 DMRs found from Epp vs Mpp pairwise comparison.

Gene symbol	Probe position	Δ Methylation (%)	Status	Overlapping CpG island	Overlapping exon
ZFP28	Chr18:64077039-64077551	-63.42	↓	+	+
LRRN5	Chr16:2185780-2186884	-59.2	↓	+	+
ITGB4	Chr19:56475217-56476048	58.17	↑	+	+
TTC7B	Chr10:103185185-103186088	-57.85	↓	+	+
PAX9	Chr21:47297087-47297648	57.78	↑	+	+
TBC1D30	Chr5:49221516-49222688	57.48	↑	+	-
COLQ	Chr1:154222779-154223725	-57.22	↓	+	-

APOA5	Chr15:27870402-27872378	-57.09	↓	+	+
LMNA	Chr3:14713931-14715243	56.4	↑	+	+
TTYH3	Chr25:41287360-41288246	-56.1	↓	+	+
PRDM1	Chr9:44358494-44359780	56.03	↑	+	+
CRYBG1	Chr9:43937179-43937875	55.98	↑	+	+
MDFIC	Chr4:53852118-53855692	55.8	↑	-	+
FBN1	Chr10:61877459-61879596	55.17	↑	+	+
COL18A1	Chr1:147038286-147039069	-54.91	↓	+	+
GRK1	Chr12:90768909-90769860	-54.63	↓	+	+
FDPS	Chr3:15361358-15362795	54.6	↑	+	+
ESPN	Chr16:47767113-47767816	54.47	↑	+	-
PRKAB1	Chr17:58007687-58009447	54.22	↑	+	+
HSF2BP	Chr1:146435387-146436351	-54.05	↓	+	+

Arrows represent Hyper(↑) and hypo(↓) methylation. + and - denote presence or absence of overlap, respectively.

Supplemental table S2.3 Genome position of top 20 DMRs found from Mpp vs CH pairwise comparison.

Gene symbol	Probe position	Δ Methylation (%)	Status	Overlapping CpG island	Overlapping exon
ZNF316	Chr25:39013931-39014396	-42.58	↓	+	+
DPH7	Chr11:105578300-105579199	25.64	↑	+	-
CHST13	Chr22:61124384-61124871	23.21	↑	+	+
OSBP	Chr15:84135149-84137876	21.41	↑	-	+
TTC7B	Chr10:103212984-103214076	20.79	↑	+	-
AGAP1	Chr3:115869755-	20.45	↑	+	-

	115872729				
SPON2	Chr6:109224515-109225210	20.2	↑	+	+
LRP8	Chr3:93536104-93536525	-19.3	↓	+	-
OSBP	Chr15:84114728-84115425	19.1	↑	+	+
FUT7	Chr11:106202040-106202641	19.09	↑	+	+
EFEMP1	Chr11:38384797-38402126	-18.95	↓	-	-
COL14A1	Chr14:84023702-84030759	-18.72	↓	-	+
CCDC166	Chr14:2253903-2254475	-18.51	↓	+	+
SAMM50	Chr5:114924811-114928308	18.28	↑	-	+
USH2A	Chr16:20284161-20294382	17.96	↑	-	+
HIBADH	Chr4:68973235-68985038	-17.9	↓	-	-
ZFP28	Chr18:64077039-64077551	17.85	↑	+	+
SCMH1	Chr3:105705228-105716820	-17.63	↓	-	-
DOK6	Chr24:7709706-7715585	-16.97	↓	-	-
JAKMIP3	Chr26:51499042-51500800	16.88	↑	+	+

Arrows represent Hyper(↑) and hypo(↓) methylation. + and - denote presence or absence of overlap, respectively.

Supplemental table S2.4 Genome position of top 20 DMRs found from Epp vs CH pairwise comparison.

Gene symbol	Probe position	Δ Methylation (%)	Status	Overlapping CpG island	Overlapping exon
COLQ	Chr1:154222779-154223725	-61.88	↓	+	-
CRYBG1	Chr9:43937179-43937875	61.13	↑	+	+
DOCK1	Chr26:47000942-47001742	-60.45	↓	+	+
ZNF316	Chr25:39013931-39014396	-59.48	↓	+	+
P2RY8	ChrX:148802468-148804545	-59.39	↓	+	+

Gene	Location	Value	Methylation	Overlap 1	Overlap 2
LRRN5	Chr16:2185780-2186884	-59.31	↓	+	+
PAX9	Chr21:47297087-47297648	59.29	↑	+	+
ESPN	Chr16:47767113-47767816	58.98	↑	+	-
TTYH3	Chr25:41287360-41288246	-58.38	↓	+	+
LMNA	Chr3:14713931-14715243	58.21	↑	+	+
DST	Chr23:3596328-3597712	58.21	↑	+	-
PRDM1	Chr9:44358494-44359780	57.86	↑	+	+
TBC1D30	Chr5:49221516-49222688	57.84	↑	+	-
ITGB4	Chr19:56475217-56476048	57.71	↑	+	+
NFATC2	Chr13:80067749-80069013	57.54	↑	+	-
PPP2R2B	Chr7:60098873-60099804	57.19	↑	+	+
MDFIC	Chr4:53852118-53855692	57.08	↑	-	+
DNAJB8	Chr22:60039879-60040464	-56.55	↓	+	+
TTC7B	Chr10:103185185-103186088	-56.28	↓	+	+
FDPS	Chr3:15361358-15362795	56.18	↑	+	+

Arrows represent Hyper(↑) and hypo(↓) methylation. + and - denote presence or absence of overlap, respectively.

Supplemental table S2.5 Top 20 enriched pathways from Epp vs Mpp pairwise comparison DMRs.

					Epp vs Mpp				
Hypermethylated					Hypomethylated				
Pathway	Total	Hits	P value	FDR	Pathway	Total	Hits	P value	FDR
Axon guidance	179	104	1.29E-06	3.09E-04	Neuroactive ligand-receptor interaction	363	106	1.01E-09	3.18E-07
Fatty acid metabolism	54	39	2.27E-06	3.09E-04	Insulin secretion	85	34	2.54E-07	3.99E-05
Inositol phosphate metabolism	73	49	3.75E-06	3.09E-04	Calcium signaling pathway	197	60	1.02E-06	1.07E-04
Insulin resistance	110	68	4.86E-06	3.09E-04	Complement and coagulation cascades	87	30	4.10E-05	3.22E-03
Metabolic pathways	1470	671	4.92E-06	3.09E-04	Adrenergic signaling in cardiomyocytes	144	43	5.72E-05	3.59E-03
Autophagy - animal	133	79	7.72E-06	3.51E-04	ECM-receptor interaction	83	28	1.13E-04	5.94E-03
AMPK signaling pathway	123	74	7.83E-06	3.51E-04	Cell adhesion molecules (CAMs)	158	45	1.35E-04	6.05E-03
Carbon metabolism	112	68	1.17E-05	4.61E-04	Central carbon metabolism in cancer	61	22	2.03E-04	7.73E-03
Peroxisome	84	52	5.90E-05	2.06E-03	Inflammatory mediator regulation of TRP channels	103	32	2.22E-04	7.73E-03
mTOR signaling pathway	154	86	7.96E-05	2.50E-03	Aldosterone synthesis and secretion	96	30	3.08E-04	9.66E-03
Thyroid hormone signaling pathway	113	66	8.76E-05	2.50E-03	Bile secretion	84	27	3.66E-04	1.05E-02
Phosphatidylinositol signaling system	99	58	2.05E-04	4.96E-03	cGMP-PKG signaling pathway	167	45	5.24E-04	1.30E-02
Glucagon signaling pathway	99	58	2.05E-04	4.96E-03	Rap1 signaling pathway	210	54	5.39E-04	1.30E-02
Aldosterone synthesis and secretion	96	56	3.07E-04	6.88E-03	Cytokine-cytokine receptor interaction	323	76	8.48E-04	1.85E-02
Apelin signaling pathway	140	77	3.52E-04	7.37E-03	Oxytocin signaling pathway	152	41	8.90E-04	1.85E-02
Focal adhesion	198	104	3.81E-04	7.48E-03	Sphingolipid signaling pathway	120	34	9.43E-04	1.85E-02
Biosynthesis of amino acids	73	44	5.01E-04	9.25E-03	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	68	22	1.13E-03	1.98E-02
MAPK signaling pathway	292	146	5.61E-04	9.79E-03	AGE-RAGE signaling pathway in diabetic complications	103	30	1.13E-03	1.98E-02
Wnt signaling pathway	159	85	6.14E-04	1.01E-02	PI3K-Akt signaling pathway	373	85	1.22E-03	1.98E-02
Adipocytokine signaling pathway	72	43	7.59E-04	1.19E-02	MAPK signaling pathway	292	69	1.28E-03	1.98E-02

FDR = False discovery rate adjusted P-value

Supplemental table S2.6 Top 20 enriched pathways from Mpp vs CH pairwise comparison DMRs.

					Mpp vs CH				
Hypermethylated					Hypomethylated				
Pathway	Total	Hits	P value	FDR	Pathway	Total	Hits	P value	FDR
Platelet activation	120	10	2.27E-04	4.66E-02	Mucin type O-glycan biosynthesis	31	5	3.08E-04	9.68E-02
Focal adhesion	198	13	2.97E-04	4.66E-02	ECM-receptor interaction	83	7	1.17E-03	1.84E-01
Adherens junction	69	7	6.23E-04	5.41E-02	Focal adhesion	198	10	5.54E-03	4.48E-01
Morphine addiction	92	8	9.29E-04	5.41E-02	Arginine biosynthesis	19	3	5.71E-03	4.48E-01
Gastric acid secretion	74	7	9.50E-04	5.41E-02	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	68	5	1.06E-02	6.23E-01
Pancreatic secretion	97	8	1.03E-03	5.41E-02	Neuroactive ligand-receptor interaction	363	14	1.19E-02	6.23E-01
Glycosaminoglycan biosynthesis - heparan s	24	4	1.51E-03	6.76E-02	Leukocyte transendothelial migration	113	6	2.36E-02	7.70E-01
Regulation of actin cytoskeleton	211	12	1.78E-03	6.99E-02	Protein digestion and absorption	113	6	2.36E-02	7.70E-01
Vascular smooth muscle contraction	133	9	2.07E-03	7.22E-02	Fluid shear stress and atherosclerosis	145	7	2.39E-02	7.70E-01
Gap junction	90	7	2.97E-03	9.32E-02	Axon guidance	179	8	2.45E-02	7.70E-01
Salivary secretion	93	7	3.57E-03	9.73E-02	Osteoclast differentiation	133	6	4.67E-02	1.00E+00
MAPK signaling pathway	292	14	3.72E-03	9.73E-02					
Pathways in cancer	539	21	5.08E-03	1.18E-01					
Ras signaling pathway	241	12	5.27E-03	1.18E-01					
Glycosaminoglycan biosynthesis - chondroit	20	3	8.27E-03	1.67E-01					
ErbB signaling pathway	85	6	9.31E-03	1.67E-01					
Insulin secretion	85	6	9.31E-03	1.67E-01					
Proteoglycans in cancer	198	10	9.58E-03	1.67E-01					
Axon guidance	179	9	1.41E-02	2.24E-01					
Arrhythmogenic right ventricular cardiomyc	68	5	1.46E-02	2.24E-01					

FDR = False discovery rate adjusted P-value

Supplemental table S2.7 Top 20 enriched pathways from Epp vs CH pairwise comparison DMRs.

					Epp vs CH				
Hypermethylated					**Hypomethylated**				
Pathway	Total	Hits	P value	FDR	Pathway	Total	Hits	P value	FDR
AMPK signaling pathway	123	81	2.83E-07	8.88E-05	Neuroactive ligand-receptor interaction	363	120	7.00E-12	2.20E-09
Carbon metabolism	112	74	7.58E-07	9.82E-05	Calcium signaling pathway	197	69	1.74E-08	2.73E-06
Autophagy - animal	133	85	1.00E-06	9.82E-05	Complement and coagulation cascades	87	34	4.96E-06	4.68E-04
MAPK signaling pathway	292	166	1.25E-06	9.82E-05	Bile secretion	84	33	5.97E-06	4.68E-04
Fatty acid metabolism	54	40	3.85E-06	2.42E-04	Gastric acid secretion	74	29	2.28E-05	1.20E-03
Axon guidance	179	107	4.78E-06	2.50E-04	Insulin secretion	85	32	2.30E-05	1.20E-03
mTOR signaling pathway	154	93	1.10E-05	4.94E-04	Inflammatory mediator regulation of TRP channels	103	36	4.76E-05	2.13E-03
Metabolic pathways	1470	705	1.87E-05	7.33E-04	Adrenergic signaling in cardiomyocytes	144	46	6.14E-05	2.41E-03
Insulin resistance	110	69	2.59E-05	9.04E-04	Cell adhesion molecules (CAMs)	158	49	8.33E-05	2.91E-03
Inositol phosphate metabolism	73	49	2.96E-05	9.29E-04	ECM-receptor interaction	83	30	9.91E-05	3.11E-03
Adipocytokine signaling pathway	72	48	4.67E-05	1.33E-03	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	68	25	2.72E-04	7.75E-03
Glucagon signaling pathway	99	62	7.05E-05	1.84E-03	Aldosterone synthesis and secretion	96	32	3.33E-04	8.71E-03
Ubiquitin mediated proteolysis	140	83	8.17E-05	1.97E-03	Central carbon metabolism in cancer	61	22	8.40E-04	1.97E-02
Thyroid hormone signaling pathway	113	69	8.99E-05	2.01E-03	Amoebiasis	101	32	9.04E-04	1.97E-02
Mitophagy - animal	66	44	9.61E-05	2.01E-03	Dilated cardiomyopathy	89	29	9.40E-04	1.97E-02
Synaptic vesicle cycle	78	50	1.50E-04	2.79E-03	cGMP-PKG signaling pathway	167	47	1.29E-03	2.53E-02
Focal adhesion	198	111	1.55E-04	2.79E-03	Oxytocin signaling pathway	152	43	1.82E-03	3.36E-02
Apelin signaling pathway	140	82	1.60E-04	2.79E-03	Thyroid hormone synthesis	74	24	2.68E-03	4.46E-02
Longevity regulating pathway	90	56	2.00E-04	3.31E-03	Vascular smooth muscle contraction	133	38	2.70E-03	4.46E-02
Hippo signaling pathway	151	87	2.25E-04	3.37E-03	Endocrine and other factor-regulated calcium reabsorption	43	16	2.89E-03	4.53E-02

FDR = False discovery rate adjusted P-value

Supplemental table S2.8 Epp DMRs found in genes involved in 4 enriched pathways or more.

Gene	Probe location	Methylation level (%)			Status	Overlapping CpG island	Overlapping exon
		Epp	Mpp	CH			
PFKL	Chr1:145767673-145770175	19.42	33.69	32.39	↓	+	+
	Chr1:145774464-145776828	61.21	83.76	87.46	↓	-	+
	Chr1:145776850-145779411	74.98	86.30	91.91	↓	-	+
	Chr1:145779426-145781259	66.27	86.25	92.11	↓	-	+
	Chr1:145781260-145783956	66.46	89.63	88.78	↓	-	+
	Chr1:145783957-145785527	64.56	87.45	92.22	↓	+	+
	Chr1:145785536-145786923	70.98	90.13	90.67	↓	+	+
	Chr1:145786940-145789182	65.58	82.78	83.15	↓	-	+
	Chr1:145789230-145791488	39.52	75.87	79.85	↓	-	+
	Chr1:145791500-145794125	76.58	91.01	90.07	↓	-	+
	Chr1:145794126-145796714	76.37	88.21	86.63	↓	-	+
IDH1	Chr2:96954864-96961626	68.85	50.43	52.15	↑	+	+

Gene	Location						
PFKM	Chr5:32333489-32338204	61.43	34.12	33.35	↑	-	+
ACO2	Chr5:113089283-113094947	55.35	38.54	38.28	↑	-	-
	Chr5:113094948-113100376	77.07	55.45	55.59	↑	-	-
	Chr5:113121520-113124398	79.48	67.26	66.43	↑	-	+
	Chr5:113127679-113130873	84.51	65.94	65.24	↑	-	+
	Chr5:113130874-113133472	88.69	71.43	72.93	↑	-	+
	Chr5:113136929-113138803	78.16	53.33	54.12	↑	-	+
ACO1	Chr8:11481878-11487141	66.19	86.93	87.21	↓	-	+
PCK2	Chr10:20876501-20878643	27.87	7.41	6.41	↑	+	+
PFKP	Chr13:45500574-45503606	79.62	60.37	60.02	↑	-	+
	Chr13:45507277-45509440	70.69	87.78	87.21	↓	-	-
	Chr13:45509441-45511316	65.17	80.74	75.79	↓	-	-
	Chr13:45535916-45538816	57.01	77.01	80.80	↓	-	-
	Chr13:45542581-45546149	69.10	87.82	88.74	↓	-	+
IDH3B	Chr13:52971134-52978988	40.39	24.21	25.78	↑	-	+
	Chr13:52978999-52984951	71.11	57.00	54.81	↑	-	+
PCK1	Chr13:59143112-59146204	52.62	83.43	83.92	↓	-	+
	Chr13:59146207-59148861	67.05	86.09	84.49	↓	-	+
	Chr13:59148975-59152175	53.90	75.79	79.11	↓	-	+
AKT3	Chr16:34122944-34133277	61.24	36.67	35.58	↑	+	+
PIK3CD	Chr16:44681918-44684465	63.86	88.48	90.11	↓	-	+
	Chr16:44687424-44693392	61.40	74.84	81.04	↓	-	+
AKT2	Chr18:49929248-49931503	87.22	74.62	73.24	↑	-	+
	Chr18:49931514-	72.55	47.42	47.13	↑	-	-

	49934097						
	Chr18:49937119-49939269	85.10	73.36	65.49	↑	-	-
	Chr18:49939270-49941514	82.56	64.67	62.27	↑	-	-
	Chr18:49941523-49944066	78.03	59.77	60.74	↑	-	-
	Chr18:49944067-49948027	67.99	49.76	47.07	↑	-	-
PIK3R1	Chr20:11333875-11338183	56.75	11.59	9.29	↑	-	+
	Chr20:11338452-11343689	85.69	69.53	65.95	↑	-	-
	Chr20:11348337-11354279	76.76	64.39	62.58	↑	-	+
	Chr20:11354289-11360068	81.06	70.04	67.81	↑	-	-
IDH2	Chr21:21909700-21912779	70.42	81.64	83.97	↓	-	+
	Chr21:21912780-21917427	60.80	74.53	74.82	↓	-	-
IDH3A	Chr21:31060474-31063708	21.07	35.30	35.73	↓	+	+
PC	Chr29:45517201-45517966	76.21	92.53	94.14	↓	+	+
	Chr29:45538960-45541063	70.88	46.00	46.36	↑	-	-
	Chr29:45558765-45562136	66.78	54.26	52.49	↑	-	-
	Chr29:45562138-45564642	65.16	29.58	24.72	↑	-	+
	Chr29:45570805-45575028	78.27	67.75	65.16	↑	-	-
	Chr29:45575029-45578566	78.19	62.24	60.61	↑	-	-
	Chr29:45606014-45608788	69.75	52.38	51.11	↑	-	-
IDH3G	ChrX:39921361-39924847	63.74	47.58	45.16	↑	+	+

Arrows represent Hyper(↑) and hypo(↓) methylation. + and - denote presence or absence of overlap, respectively.

Supplemental table S2.9 Mpp DMRs found in genes involved in 2 enriched pathways or more.

Gene	Probe location	Methylation level (%)			Status	Overlapping CpG island	Overlapping exon
		Epp	Mpp	CH			
TNIK	Chr1:96945997-96956506	76.50	88.65	73.39	↑	-	-
SNCA	Chr6:36374217-36389051	83.05	68.99	79.17	↓	-	-
PRKD2	Chr18:54228444-54232544	77.88	64.79	77.86	↓	+	+
PDPK1	Chr25:2088443-2090204	88.25	73.69	84.98	↓	-	+
PRKCB	Chr25:21921612-21928810	71.58	83.43	73.34	↑	-	+

Arrows represent Hyper(↑) and hypo(↓) methylation. + and - denote presence or absence of overlap, respectively.

Supplemental table S2.10 – CH DMRs found in genes involved in 4 enriched pathways or more.

Gene	Probe location	Methylation level (%)			Status	Overlapping CpG island	Overlapping exon
		Epp	Mpp	CH			
ITGB8	Chr4:29193599-29210230	71.9	72.3	85.6	↑	-	+
KITLG	Chr5:18373493-18381704	84.0	82.8	71.7	↓	-	+
RAP1B	Chr5:45384375-45397348	76.9	77.9	64.1	↓	-	-
FGF6	Chr5:106160273-106164039	53.3	63.9	75.0	↑	-	-
WNT5B	Chr5:108644032-108646126	69.8	78.0	90.8	↑	-	+
	Chr5:108649355-108650714	57.2	80.6	93.4	↑	-	+
LEF1	Chr6:18447933-18451443	81.2	78.4	67.7	↓	-	+
FGF5	Chr6:96736576-96745271	81.1	75.6	65.0	↓	-	-
LPAR1	Chr8:102098891-102106568	79.4	78.6	67.1	↓	-	-
FYN	Chr9:3912846	80.6	76.9	66.2	↓	-	-

	8-39141776						
COL4A1	Chr12:88964423-88965712	70.9	59.7	49.2	↓	-	-
PDGFD	Chr15:4520059-4536469	76.8	74.6	63.0	↓	-	-
FZD10	Chr17:47932240-47936312	57.7	55.1	45.0	↓	+	+
ARHGAP35	Chr18:5449225954493644	80.6	69.8	57.1	↓	-	-
ITGA2B	Chr19:4479836544801286	36.5	41.9	53.4	↑	-	+
EGFR	Chr22:1041964-1046978	83.6	84.1	72.7	↓	-	-
CTNNA3	Chr28:2346829123485680	88.0	86.1	73.8	↓	-	-
	Chr28:2406225324075120	63.7	62.5	74.3	↑	-	-
PAK3	ChrX:6459491464607101	77.4	69.1	57.3	↓	-	+

Arrows represent Hyper(↑) and hypo(↓) methylation. + and - denote presence or absence of overlap, respectively.

Chapter 3: Genome-wide epigenetic changes on bovine morula associated with metabolic

status of recipients

Genome-wide epigenetic changes on bovine morula associated with metabolic status of recipients

Mikhael Poirier[1], Dawit Tesfaye[1]*, Dessie Salilew Wondim[1], Samuel Gebremedhn[1], Franca Rings[1], Vita Havlicek[2], Urban Besenfelder[2], Christiane Neuhoff[1], Karl Schellander[1], Michael Hoelker[1].

[1]Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn, Bonn, Germany

[2]Institute of Animal Breeding and Genetics, University of Veterinary Medicine Vienna, Vienna, Austria.

*Corresponding author information

Dawit Tesfaye
Institute of Animal Science
Animal Breeding and Husbandry Group
University of Bonn
Endenicher Allee 15 Bonn, Germany
Email: dtes@itw.uni-bonn.de
Phone: +4922872286
Fax: +49228732284

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3.1   Abstract

Early lactation in cattle is associated with negative energy balance, alterations of steroid expression and their receptors, and an abnormal metabolic profile that reduces reproductive success through increased early embryo losses. We hypothesize that embryos grown in oviducts of metabolically stressed cattle display an abnormal epigenetic profile of genes that are involved in developmental competency and implantation. To demonstrate this, we collected morulae derived from *in vitro* derived early cleavage embryos transferred laparoscopically in the oviduct of negative energy balance lactating cows having completed uterine involution and characterized their epigenome using whole genome bisulfite sequencing compared to embryos grown in nulliparous heifers. Selected lactating cows exhibited weight loss and a significantly different metabolic profile of non-esterified fatty acids and beta-hydroxybutyrate when compared to heifers. Accordingly, 13,383 differentially methylated regions (DMRs) were found in lactating cows overlapping genes involved in metabolic pathways and fatty acid biological processes, in hormone signaling pathways involved in oxidative stress and pathways regulating pluripotency of stem cells, as well as placentation and vascularization. DMRS overlapping genes and CpG regions of imprinted genes PEG10, MEST and IGF2R as well as genes involved embryo implantation suggest that negative energy balance induced metabolic stress might affect imprint maintenance and subsequently implantation leading to embryo losses. This genome wide approach gives new insight into the relationship between transient metabolic stress and epigenetic gene regulation on pregnancy outcome.

3.2   Introduction

Genetic selection of performance traits in dairy cattle, most notably milk production, has long been associated with a decline in reproductive function [1]. Lactating cows encounter a number of reproductive failures such as delayed ovulation, delayed uterine involution, extended calving interval and increased number of services per pregnancy [2]. Upon calving, the cow's energy needs outmatch its dietary intake, namely for increased milk production and successful resumption of its ovarian cycle. This period of negative energy balance (NEB) has been associated with abnormal blood metabolite concentrations that mirror poor oocyte quality, and studies have demonstrated that differential *in vitro* maturation conditions strongly impacts oocyte quality [3]. Interestingly, oocyte collected from lactating cows showed no noticeable morphological changes when compared to non-milked cows, with comparable blastocyst development rates, putting to question the notion that oocyte quality is at the heart of developmental competency [4]. Nevertheless, lactating cows experience higher levels of early embryo loss, with embryo transfer linking lower blastocyst rates in lactating cows compared to their dried counterparts [5]. This may suggest that oviductal environment could play a more important role in developmental capacity than previously suspected. Other reports have demonstrated the differential expression of the IGF system in the oviduct of lactating cows as well as a different metabolite profile of oviductal fluid at different cycle stages [6, 7], indicating that oviductal environment is sensitive to hormone profile and lactation-induced metabolic stress.

We previously demonstrated the impact of stage specific *in vitro* culture before and after genome activation of embryo on the epigenetic landscape, showing that embryos are sensitive to their environment during early stages of development [8]. Similarly, metabolically divergent animals exhibit different oviductal fluid compositions that may

impact the survival of the conceptus and its gene regulation [9]. We hypothesize that the transient metabolic challenges induced by negative energy balance during early lactation will alter the epigenetic status of embryos regardless of gamete origin, resulting in adiverging methylation profile of genes involved in developmental competency. To do so, we profiled the weight changes and energy profiles of lactating cows, and metabolically profiled the blood serum of these cows as well as nulliparous heifers as a gold standard of fertility. Once cows have been selected to be metabolically divergent from heifers, *in vitro* produced early embryos were transferred into both groups and resulting morulas were collected. Their epigenetic landscape was profiled using whole genome bisulfite sequencing to identify differentially methylated regions sensitive to metabolic stress. This comprehensive approach allowed us to shed some light in the epigenetic regulation of early embryo loss in lactating cows.

3.3   Results

3.3.1   *Physiologic and metabolic profiling of post-partum lactating cows and nulliparous heifers*

To characterize the impact of lactation on the epigenome of embryos, multiparous cows were profiled for their energy status. Initially, twenty-six cows were followed the first 15 weeks after calving to assess their energy status. Initially, cows were monitored through body weight measurements to exclude cows immediately gaining weight. As such, 9 cows started gaining weight upon calving, and were excluded from further analysis. Additionally, cows had their energy balance status assessed to ensure cows were experiencing NEB at least 5 weeks post-partum, of which a further 11 animals were excluded.

Following this selection, 4 lactating cows were kept for blood metabolite profiling and epigenetic analysis. These cows reached an average weight nadir at 45.3 ± 15.4 dpp (n=4, S.E.M.) and lost on average 58.8 ± 14.3 kg, with an average daily weight loss of 1.5 ± 0.3 kg per day. These cows produced a daily average of 34.9 ± 3.0 kg of milk prior to transfer (average time postpartum: 62 dpp), indicating their proper milk production. Accordingly, these cows experienced an average NEB of -25.7 ± 5.6 MJ/day prior to week five post-partum. During *in vivo* culture of the embryos, cow energy balance status averaged -7.8 ± 6.0 MJ/day (n=3, S.E.M.), indicating that these cows experience early lactation energy balance deficiency and tend to recuperate towards uterine involution and time of first service.

Furthermore, blood metabolite profiling revealed lactating cows and nulliparous heifers to be metabolically different as expected (Figure 3.1). Both at the time of transfer and flushing, average NEFA serum concentrations for cows were significantly different from heifers (p<0.05), with cows average level being above the threshold level associated with mild NEB as suggested in previous literature [10]. Interestingly, levels of BHB in lactating cows on average were slightly under the previously described threshold, although still different than levels found in heifers (p <0.05) which can be expected from cows having completed uterine involution and ready for servicing. Notwithstanding, weight, energy balance and metabolic profiling indicates that the cows used for transfer experienced negative energy balance and were exhibiting significantly different metabolic profiles from nulliparous heifers.

3.3.2   *Epigenetic landscape characterization*

In parallel to the profiling described above, early embryos derived *in vitro* were transferred into the oviduct of the lactating cows and nulliparous heifers, and pools of morulae were then collected at day 7 post-fertilization for bisulfite treatment and whole genome

sequencing. Unique CpGs obtained from alignment in each dataset ranged from 18,883,661

and 71,945,138 CpG methylation calls, with a coverage depth varying between 0.69x to

2.63x. Global CpG methylation average ranged from 33.1 ± 0.9 % in lactating cows and 31.3 ±

1.9 % in nulliparous heifers. Quantification of the genome features was similar between both

conditions (Figure 3.2), suggestive of smaller variations. The lowest levels of methylation

were associated with upstream and downstream regions of transcription start site, such as

promoters and transcriptional units, as well as 5'UTR regions, indicating gene expression

profiles were possibly linked to post embryonic gene activation lineage development.

Inversely, repetitive elements such as SINE, LINE and LTR but also intronic regions and 3'UTR

features seem to make up most of the global methylation levels found in morulas. Although

not significant, there seems to be an overall slight hypermethylation of genomic features in

lactating cows, with the exceptions of promoters.

As variations between whole gene features may be too small to draw comparisons between

morulas collected from both conditions, a window based approach was taken to adequately

derive differentially methylated regions. The genome was split into 150 CpG wide windows,

totaling 336,378 windows from all 9 datasets, and 92.6% of these windows were quantitated

for further characterization. Consistent with small methylation changes observed in genomic

features between conditions, principal component analysis of all quantitated windows

across replicates revealed clustering of cow samples together close with heifers, though

heifers spanned a wider variation between replicates and did not overlap with cow

replicates, indicating different methylation profiles (Figure 3). Furthermore, total

methylation distribution of these windows was consistent with genome feature

quantitation, where the scatter plot of all windows shows a strong linear relationship

between conditions with a slight hypermethylation shift towards cow replicates (Figure

3.4a). According to the methylation distribution of these windows, the bulk of methylation observed in these changes is accounted by an increase in windows with methylation ranging from 45% and up when compared to heifers (Figure 3.4b).

Once windows were quantified, differentially methylated regions were outlined using logisitic regression replicate statistical test between conditions after Benjamini-Horchberg correction with an adjusted p value < 0.05 (Figure 3.5a). To ensure wide variation between conditions, a further selection of DMRs with a minimum variation of 10% was applied to DMRs calculated, totaling 13,383 DMRs (Figure 5a). Of these, 11,612 DMRs made up the hypermethylated DMRs with the remaining 1,771 DMRs consisting of hypomethylated DMRs between lactating cows and heifers (Figure 3.5b). The distribution of methylation changes between hyper- and hypomethylated DMRs was similar, with a slightly higher representation of DMRs above 20% difference in hypomethylated DMRs. Nevertheless, the top 20 DMRs from each hyper and hypomethylated DMRs are comprised within a similar range (Table 3.1).

Of all DMRs found in morulas collected from lactating cows and heifers, 3,557 were found to overlap exons which corresponded to 26.6% of the total DMRs found (Table 3.2). Although this is similar to the ratio of windows quantitated overlapping exons in the genome (24.3%), this ratio was slightly increased when looking at hypomethylated DMRs which increased to 34.7%, indicating an overrepresentation of hypomethylated changes found in gene bodies (52.9% of hypomethylated DMRs). Interestingly, this was also found in CpGs islands, where 33.3% of hypomethylated DMRs overlapped them, compared to 10.3%, 8.1% and 4.3% of quantitated windows, total DMRs and hypermethylated DMRs, respectively. Taken together, this indicates that although some specific hypomethylated changes are found in DMRs overlapping CpG islands and exons, genome features quantitation, PCA clustering and

distribution of methylation changes in DMRs indicate that morulas grown in lactating cows exhibit consistent global hypermethylation when compared to morulas grown in nulliparous heifers.

3.3.3   *Pathway and biological enrichment analysis*

To further describe methylation changes occurring during embryo development under metabolic stress, 3,260 DMRs found overlapping genes which fit against gene ontology terms. First, DMRs were screened against 12 known bovine imprinted genes. Only 5 DMRs were found, overlapping PEG10, MEST and IGF2R genes, of which only DMRs found in PEG10 and IGF2R were overlapping CpG islands (Table 3.3). A similar screen was performed on a list of known human imprinted genes, where 34 DMRs were found overlapping 15 human imprinted genes (Supplemental Table 1). Of these, imprinting status of 12 genes is unknown in bovine, suggesting possible new relationships between metabolic stress and imprinting-involved embryo development. Similarly, DMRs overlapping genes were filtered against a list of genes involved in embryo implantation (mouse annotation, GO:0007566). From this, 13 DMRs were found to overlap 11 genes, with the PCSK5 gene region containing 3 DMRs. Three DMRs overlapping the CpG islands of SYDE1, GGN and NLRP5 genes were also overlapping a CpG island (Table 3.3) were hypomethylated and all remaining DMRs were hypermethylated in lactating cows. Considering broader biological processes, DMRs found were also screened against a list of genes involved in *in utero* embryo development (mouse annotation, GO:0001701). A total of 187 DMRs were found overlapping 117 genes, of which 24 were overlapping CpG islands as well (Supp table 2).

Beyond targeted filtering of all DMRs in specific gene ontology lists, DMRs found overlapping genes were also submitted to enrichment pathway analysis. When looking at KEGG pathway

database enrichment, a total of 3,260 genes found from the DMRs information were involved in multiple signaling pathways, such as thyroid hormone (27 genes) and GnRH (25 genes) signaling pathways, pluripotency regulation (29 genes), NF-Kappa B signaling (24 genes) and metabolic pathways (259 genes; Figure 3.6). Some of the genes that are involved in more than 5 pathways included protein kinase C subunits Beta and Gamma (PRKCB/G), RAC-gamma serine/threonine-protein kinase (AKT3), adenylyl cyclases 1,3,7,9 (ADCY1/3/7/9) involved in involved in signaling pathways, Protein kinase A catalytic subunit beta (PRKACB) and epidermal growth factor receptor (EGFR) involved in hormone synthesis and signaling, and NFKB1 involved in inflammation signaling (Supplemental Table 3.4).

When looking at biological processe enrichment, multiple processes involving lipids were enriched, including lipid homeostasis (15 genes) and metabolic process (31 genes), as well as fatty acid metabolic and biosynthetic processes (21 and 33 genes, respectively) and also fatty acid oxidation (97 genes, Figure 3.7; Supplemental table 3.5). Genes that were the most involved in these processes ranged from melastatin family members (TRPM4/7), cellular differentiation genes like Hyperpolarization Activated Cyclic Nucleotide Gated Potassium Channel 4 (HCN4), Serine/Threonine-Protein Kinase 2 (PAK2), as well as genes involved in cellular growth, apoptosis and embryo development, such as Germinal Center Kinase-Related Protein Kinase (MAP4K3) and Insulin receptor (INSR, Supplemental table 6). Taken together, our analysis shows the presence of DMRs in bovine imprinted genes that are known to impact embryo development and in unverified regions obtained from already known human imprinted genes. Moreover, DMRs were found overlapping genes not only involved in metabolic pathways and fatty acid metabolism, but also in pluripotency regulation and hormone signaling. These results indicate that metabolic stress experienced

by lactating cows at the time of first service impact the epigenetic status of embryos compromising their developmental competency and successive pregnancy outcomes.

3.4   Discussion

The oviductal environment is vital for proper maternal-embryo communication to ensure proper gene expression and blastocyst development. The adverse oviductal conditions during early lactation has been shown to affect embryo quality and viability [11, 12]. The impact of negative energy balance on oocyte quality being increasingly characterized, additional studies investigating the impact of *in vivo* culture environment regardless of gamete origin remains crucial. Phenotypic research has been carried out, where *in vitro* produced zygotes transferred to lactating cows displayed lower blastocyst rates than in nulliparous heifers [5]. Coupled with previous work demonstrating that embryos grown *in vitro* display differential methylation compared to *in vivo* grown blastocysts [8], we present the genome wide methylation profile of embryos grown in lactating cows compared to nulliparous heifers. To investigate the effect of adverse early post-partum lactation metabolism on embryo development, we profiled cows undergoing negative energy balance prior to uterine involution and ready for first service. Using embryos grown in nulliparous heifers as control, we were able to compare them to embryo grown in metabolically adverse cows.

As such, 56% of cows in this experiment experienced weight loss prior to embryo transfer and behaved according to previously described negative energy balance animals [4], the rest being excluded. Previous report states that cows exit NEB around week 7 post-partum [13]. However, we observed 9 animals exhibiting positive energy balance by week 6 post-partum, leaving 7 animals with successful transfers and a subsequent 4 cows for analysis. Compared

to heifers, of which all five animals yielded sufficient embryos post flushing, embryos grown in lactating cows resulted in lower developmental rates, as previously described [14]. Additionally, blood metabolite concentrations around the time of *in vivo* culture in cows were significantly different than heifers, indicating the different physiological and metabolic status during embryo growth. Non-esterified fatty acid and beta-hydroxybutyrate levels are known to peak around week 2-3 post-partum [4, 15] but can sometimes be sustained until week 6 [16]. Here, concentrations of both NEFA and BHB around the time of transfer in our lactating cows were above a threshold associated with mild NEB [10]. These elevated concentrations of blood metabolites that are significantly higher than those found in nulliparous heifers and above established energy balance threshold suggests that lactating cows at time of servicing still exhibit impaired metabolism, perhaps more sustained than previously found in literature.

Having demonstrated the transient perturbed metabolic conditions experienced by the lactating cow, we have investigated the effect of metabolic stress on the embryo epigenome. Initial profiling of genomic features revealed a slight hypermethylation in all features of morulas grown in lactating cows, with the exception of CpG islands, promoters and transcriptional units, suggesting a potentially similar gene expression profile. Global methylation levels (~35%) were higher in both groups compared to previously reported methylation of early and compact morula (~25%; 17) which could be explained in part by the use of whole genome bisulfite sequencing compared to reduced-representation bisulfite sequencing (RRBS). As such, the global methylation levels, coupled with levels of promoters, CpG islands, exons and introns were similar to previously RRBS profiled compact morulae. Further methylation quantitation of the genome using 150 CpG site windows revealed some clustering of replicates within their respective groups, with no overlap between conditions

through principal component analysis, suggesting that overall hypermethylation of global methylation is contained within biological replicate variability. Although embryo pooling variation effect could not be ruled out, we quantified genomic features and global methylation profile of morula grown in single animals with different metabolic status, which resulted in an overall hypermethylation of the genome in lactating cows.

Previous studies have focused on the plasticity of imprinted genes in bovine embryos, with the focus on the impact of cellular reprogramming occurring in somatic cell nuclear transfer and imprint maintenance and acquisition. Embryos derived from this method exhibit low survivability [19] and aberrant methylation patterns of imprinted genes like Small Nuclear Ribonucleoprotein Polypeptide N [20], as well as the imprinting control region of H19/Insulin Growth Factor 2 (IGF2) and Insulin growth factor 2 receptor (IGF2R; 21), outlining the plasticity and developmental risk of improper methylation status of these genes. Additional studies in pre-implantation embryos demonstrated that DNA methylation and establishment of these imprints was the most variable around blastocyst stage, gradually stabilizing by implantation period, suggesting that proper acquisition of these genes are vital for successful implantation [22, 23]. Although not at the same positions, we reveal five DMRs found in three imprinted genes, PEG10, MEST and IGF2R, of which PEG10 and IGF2R were associated with CpG islands in the gene. Similarly, DMRs found in MEST and PEG10 overlap previous DMRs found in oocytes derived from NEB lactating cows, where hypermethylation was observed in early post-partum cows when compared to nulliparous heifers (Poirier et al, 2019). This suggests that MEST and PEG10 is sensitive during both oocyte maturation and culture *in vivo*.

Functionally, PEG10 is involved in placentation, evidenced by embryonic lethality due to placental defects in PEG10 knockout mice [24]. In earlier stages, PEG10 is crucial for proper

trophoblast differentiation into placental lineages [25] and also proliferation and invasion in

mice [26], and has been shown to be downregulated toward blastocyst stages in bovine [27].

MEST was shown to be downregulated after embryo genomic activation in bovine, and its

hypermethylation can lead to Silver-Russel syndrome in humans and associated with altered

growth in mice [28, 29]. IGF2R has been shown to be involved in fetal growth control [30],

where its ablation resulted in overgrowth in mice [31], and in bovine as well, where single

nucleotide polymorphism present in IGF2R was associated with abnormal body size traits

[32]. Taken together, these results show that embryo exposed to adverse metabolic

conditions *in vivo* can showcase differential methylation in gene regions of imprinted control

of growth, differentiation and placentation, suggesting that plasticity of genes in these

processes may explain early embryonic losses in high producing dairy cows. Further

validation of gene expression and investigation of DMRs overlapping known human

imprinted genes is needed to confirm and expand on the functional relevance of imprint

control of these regions on embryo development.

Additionally, we derived a list of DMRs overlapping genes previously involved in embryo

implantation, giving insight on the changes occurring in the early development bovine

embryo and its resulting early embryo loss under metabolic stress. As some genes in this list

were involved with the recipient's ability to establish proper cross-talk to the embryo to

permit implantation, through reduced progesterone sensitivity (KLF9; [33]), improper luteal

formation (SOD1; [34]) and endometrial epithelium proliferation and decidualization

(ARID1A; [35]), the impact of these genes in the developing embryos remains somewhat

questionable. Nevertheless, DMRs overlapped genes involved in the embryo's ability to

develop, achieve implantation and proper organ development. Genes such as

Gametogenetin (GGN) and Laminin b1 (LAMB1) have been proven to be crucial in early

implantation, with knock-out mice not being able to develop past blastocyst stage and failed to establish basement membranes, respectively [36, 37]. Additionally, some genes were involved in development of vascularization, heart development and placentation. PPARd-null mice have shown reduced placental size and loosened placenta-decidua interface and flooding of blood in the placental space [38, 39]. Similarly, RECK null mice also exhibited arrested vasculogenesis and reduced tissue integrity, while conditional deletion of PSCK5 resulted in abnormal cardiogenesis [40]. While mice exhibit different embryo kinetics than bovine embryos, DMRs found in these genes involved in implantation might give insight in the abnormal blastocyst development occurring in pre-implantation embryos grown in metabolic stress environment.

When looking for functionally relevant pathway enrichments in genes with DMRs, multiple signaling pathways were found, notably in hormone signaling and oxidative stress, as well as metabolic pathways involved in fatty acid metabolism. While lipid content might not be associated with lower blastocyst rates, the differences in embryo quality from embryo grown *in vivo* and *in vitro* have been associated with lipid accumulation caused from serum supplementation to culture media, ultimately affecting their cryotolerance [14, 41, 42]. As it was previously reported, fatty acid uptake was associated with failure to develop beyond the 4-cell stage [43]. The oocytes in this study being fertilized in serum-free media, the oviductal milieu of lactating cows seemed to have an impact on the regulation of genes involved in fatty acid metabolism. Moreover, 18 genes were found to be differentially methylated in more than 7 of the pathways presented in KEGG pathway enrichment analysis.

NF-KB1 (7 pathways) has been shown to be involved in oxidative stress response occurring in embryo production associated with elevated reactive oxygen species, possibly indicating that oviduct conditions at the time of servicing in lactating cows could be associated with higher

oxidative stress, as experienced in *in vitro* culture [44]. Other genes found have been involved in the maintenance and proliferation of stem cells, like AKT serine/threonine kinase 3 (12 pathways), where its inhibition led to cell cycle arrest and apoptosis through accumulation of p53 in human embryonic stem cells [45], and Protein Kinase C Beta (PRKCB; 15 pathways), which is actively involved in transcription regulation in undifferentiated stem cells [46]. Other genes found are involved in hormone based signalling and growth, like epidermal growth factor receptor (EGFR; 8 pathways), of which its ligand EGF has been used extensively to improve blastocyst rate in *in vitro* bovine embryo production [47, 48]. In lactating cows, EGF profile has been found to be perturbed in 70% and of repeat-breeder and 40% high-producing cows, of which treatment with high doses of estradiol benzoate seemed to recover the pregnancy rates when compared to cows with an abnormal EGF profile [49]. At the embryo level, EGF was found to increase trophoblast proliferation through Ras activation [50].

Although further validation is needed, this expected abnormal steroid hormone profile of the lactating cow might result in a differential expression of its receptor, which is found to be differentially methylated in our morulae grown in metabolically-stressed lactating cows, and by extension modulating the response to existing concentrations of EGF in the oviduct and impair successful implantation and placentation. Taken together, these variations in the methylation levels observed in genes involved in KEGG pathway suggest that adverse metabolic conditions in lactating cows change the methylation profile of morulae affecting their fatty acid metabolism, response to oxidative stress, regulation of differentiation and cell growth.

Similarly, DMRs in our two groups of morulae were also found overlapping genes involved in multiple processes like fatty acid oxidation, metabolic and biosynthetic processes, further

confirming the impact of metabolic stress on lipid metabolism in the embryo supported from pathway enrichment analysis. Genes involved in multiple processes identified in our analysis were found to be implicated in cell proliferation and apoptosis, embryonic lethality and maintenance of pluripotency. Zeta-chain associated protein kinase-70 (Zap70; 5 processes) knock-out mouse embryonic stem cells shows sustained self-renewal but impaired differentiation [51]. Serine/threonine-protein kinase 10 (STK10, 5 processes) has been show to suppress NF-KB activity and promote apoptosis [52]. Other genes such as transient receptor potential ion channel subfamily M, member 7 and 4 (TRPM7, 6 processes; TRPM4, 5 processes) were found to cause embryonic lethality in mice by day 7 when knocked-out [53] and impact migration capacity of mouse embryonic fibroblasts [54]. Interestingly, insulin receptor gene was involved in multiple processes and insulin has been linked with negative energy balance in lactating cows, where its levels will increase compared to non-lactating cows [4]. Diets leading to higher levels of insulins led to reduced concentration of INSR in healthy follicles, pointing out the regulatory impact of insulin on its receptor [55]. *In vitro* research on the supplementation of insulin during embryo development was not definitive on the improvement of blastocyst development, but higher number of cells was described in insulin supplemented culture of embryos [56]. Moreover, cyclic nucleotide-gated channel 4 (HCN4), was found to be involved in the pacemaker function of heart in embryonic mice, with full knock-out mice dying during development [57]. At a broader level, one could speculate that genes differentially methylated in processes like trophoblast invasion, placentation, vascularization and heart function could lead the early developing embryo to fail to implant. While additional validation of these methylation changes and whether they are translated with differential expression and maintained until implantation still needs to

be performed, our analysis clearly indicates that lactation-induced metabolic stress modulates the epigenetic pattern of embryos during culture.

In conclusion, we characterized here the genome wide methylation changes occurring in embryos grown in metabolically divergent recipient oviducts, where biological pathways and processes that could be involved in early embryo loss of high yielding diary cows were found to be differentially methylated. We demonstrated that even at time of first service, after uterine involution, cows still exhibit divergent metabolite concentrations which results in an overall genomic hypermethylation of embryos when compared to those grown in heifers. From these changes, multiples DMRs were described and potentially have a play in the relationship of metabolic stress and early embryo loss. Further validation work will aim to link these changes with transcriptional and translational changes as well as maintenance of these changes to different developmental stages to elucidate the role of these genes in embryo loss caused by metabolic stress.

3.5   Material and Methods

3.5.1   *Animal handling, embryo and blood collection from lactating cows postpartum and cyclic heifers.*

Experimental protocols performed on cows in this study were approved by state office for Nature, Environment and Consumer protection of North Rhine-Westphalia, Germany (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Deutschland). 26 Holstein Friesian cows (average parity: 2.15) and 5 nulliparous cyclic Holstein-Friesian heifers were used for this experiment. Lactating cows were monitored for daily body weight, feed and concentrate intake, milk yield and milk composition recorded until 15 weeks post-partum. During post-partum, uterine involution of these cows was assessed by rectal

palpation around day 40 postpartum. Upon involution, cows were synchronized for transfer using previously described procedures [58, 59]. In parallel, 5 nulliparous heifers were synchronized in similar fashion. Presumptive zygotes were transferred to animals in groups of approximately 150 embryos and were recovered at day 7 of development by uterine flushing. At the time of embryo transfer and flushing, cows had their blood collected (20 mL) in parallel for blood metabolite analysis. Serum was separated using centrifugation and frozen at -80 ºC for future analysis. Blood sample collections and endoscopic embryo transfer procedures were approved under license number 84-02.04.2015.A139 and 84-02.04.2015.A083, respectively.

3.5.2 *Energy balance assessment of cows*

Body weight measurements for cows were profiled daily up to 15 weeks post partum to remove animals that were not losing weight during the early post-partum phase. Body weight curves for all cows were drawn to ensure lack of weight gain in early post-partum phase. Cows who gained weight upon calving were removed. Energy balance averages were derived from the following equation based from previous literature [69, 70]

$$EB = DMIe + Ce - BWe - MYe$$

where dry matter intake energy (DMIe) with concentrate energy (Ce) make the energy intake and body weight energy (BWe; BWe = 0.293 BW ^ 0.75) and milk yield energy (MYe), which is calculated using milk weight and composition : (0.39 × fat% + 0.24 × protein% + 0.17 × lactose% + 0.07) × kg milk), make up energy expenditure for the net energy balance (EB).

Furthermore, blood metabolite concentrations of β-OHB and NEFA were assessed spectrophotometrically for both metabolites (β-OHB; Kit #RB1008, Randox Laboratories, Crumlin, United Kingdom; NEFA, #434–91795, Wako Chemicals GmbH, Neuss, Germany)

following a previously described protocol [60]. From energy balance assessment, 2 phenotypes were assessed, lactating cows (4 groups of 5 morulas) and nulliparous heifers (5 groups of 5 morulas).

3.5.3   Embryo production and transfer

Oocytes were collected from ovaries from local slaughterhouse. To do so, cumulus-oocyte complexes (COCs) were aspirated from follicles of size between 2 and 8 mm. COCs were further matured and fertilized as previously described [8]. Briefly, matured COCs were co-incubated with sperm for 18 h, were denuded using hyalorunidase, washed in SOF-faf aa media supplied with 10% fetal calf serum. The embryos were kept in culture medium under mineral oil at 38.7 °C in 5% CO_2 in humidified air. Presumptive zygotes and early cleaving embryos were transferred in recipient animals (average time of transfer: 62 dpp) using transvaginal endoscopic tubal transfer technique [61]. Estrous synchronization was performed as previously described in our lab [59]. Embryos were collected at day 7 of the estrous cycle by uterine flushing [61]. After flushing, groups of 5 morulae were washed in PBS, snap-frozen according to cows and stored at -80 °C until further processing.

3.5.4   Morula DNA isolation and bisulfite conversion

Morula biological replicates for each condition were processed as described previously (ME) with some changes. Morulas were lysed and bisulfite converted using EZ-DNA Methylation-Direct^{TM} Kit (Zymo Research, Freiburg, Germany) according to manufacturer's instructions with some changes. Morulas were digested in single-step lysis reactions in 10 uL of digestion buffer and 1 uL of proteinase K for 20 min at 50 °C. Digested samples were then bisulfite converted with reconstituted CT conversion reagent for 8 minutes at 98 °C and a following incubation of 3.5 h at 64 °C. Conversions reaction was subsequently bound to collection

columns, desulphonated for 15 minutes and washed using manufacturer's guidelines. Purified samples were then eluted with 8 µL of the elution buffer from the Pico Methyl-Seq Library Prep Kit[TM] (Zymo Research) for downstream library preparation.

3.5.5   *Library preparation for whole genome bisulfite sequencing*

Eluted bisulfite treated DNA from groups of morulas were subjected to library preparation using the Pico Methyl-Seq Library Prep Kit[TM] (Zymo Research, Freiburg, Germany) with some changes to the manufacturer's guidelines. First, bisulfite treated DNA was pre-amplified using a low primer concentration (20 µM) to prevent primer-dimer. The resulting PCR product was purified using a 5:1 DNA binding buffer-to-sample ratio to ensure maximal sample recovery in all purification steps. The purified material was further amplified using 10 PCR cycles as recommended in the manufacturer's protocol. Following additional purification, the PCR product was further amplified with index primers to permit multiplexing sequencing. A final purification step was performed and library fragment size and quality was assessed using the Agilent High sensitivity DNA assay on the Bioanalyzer (Agilent, Waldbronn, Germany). Library concentration was quantified using a Qubit fluorimeter with the Qubit ssDNA assay kit (Invitrogen, Schwerte, Germany). Libraries were single-end sequenced for 114 cycles on an Illumina HiSeq 2500 using TruSeq v3 chemistry (Illumina GmbH, Munich, Germany). Raw sequencing data was demultiplexed and submitted to the European Nucleotide Archive (Accession number E-MTAB-8239) and used for further analysis.

3.5.6   *Raw sequence data processing*

Demultiplexed sequenced data was submitted to quality control using FastQC v0.11.6[62] and subsequently trimmed with TrimGalore! V0.4.5[63] with hard trimming of 8 bp on 5' end

and 4 bp on the 3' end of the fragment to reduce base composition bias of the reads, with the following parameters: --clip_R1 8 --three_prime_clip_R1 4. Trimmed sequences were validated again using FastQC and subsequently aligned to the bisulfite converted genome (Ensembl release 93) using the Bismark 0.19.0 tool[64] with the Bowtie 2 aligner[65] under default single-end alignment parameters. Upon alignement, duplicate sequences were excluded and CpG methylation information was extracted using deduplication and methylation extraction modules of the Bismark tool. Methylation coverage files (.cov) were imported into SeqMonk v1.45.4 for additional analysis[66].

3.5.7   *DNA methylation quantitative analysis*

To characterize the genomic features between conditions, features were quantitated across the genome using the overall ratio of methylated counts over demethylated counts enclosed in these features. CpG Islands, introns, exons, intergenic, LINE, SINE and LTR probes were outlined according to Ensembl genome annotation release 93. Promoters and transcriptional units were designed to span 1000 bp upstream and 500 bp downstream of transcription start site predicted through the eponine promoter prediction tool[67], respectively. 5' and 3' UTR regions were assigned to span 2kb upstream and downstream of gene body sequences, respectively.

For unbiased analysis, the genome was split into 150 CpG wide windows through the read position probe generator with a minimum of one read count including a position and 150 valid positions making up a probe. All windows were generated in all replicates resulting in a total of 336,378 windows across all 9 datasets. Methylation levels of windows were quantified using the bisulphite methylation over features quantitation pipeline using a minimum count of one to include position and 20 observations to include a feature. To

permit proper statistical analysis of differentially methylated probes, only probes containing a value between 0 and 100 across all replicates were kept for analysis. A total of 311,612 (92.6%) probes remained, which were requantitated with the same tool and parameters. Differentially methylated regions (DMRs) were calculated through logistic regression and submitted to KEGG pathway and gene ontology biological processes gene enrichments analysis with NetworkAnalyst 3.0 [68]. DMRs were filtered against lists of known bovine and human imprinted genes found previously (http://www.geneimprint.com/site/genes-by-species).

3.5.8   *Statistical analysis*

For weight loss, weight nadir, milk yield and energy balance status, data were presented as mean ± standard error of the mean (SEM). When comparing metabolite averages between conditions, a mixed model using a mixed effect for each animal and a fixed effect for the groups was used where $p < 0.05$. For differential methylation analysis, a logistical regression statistical for replicated data was performed and corrected with Benjamini-Hochberg of $p < 0.05$ with false discovery rate under 5%. Gene ontology enrichment analysis was performed with a p-value under 0.05.

3.6   Acknowledgments

3.7   Author contributions

MP conducted the experiment, performed sample processing and sequencing, data analysis, interpreting the results, drafting and writing the manuscript. MH was involved in embryo transfer, blood collection and design of the experiment. DSW was involved in reviewing the manuscript. SE was involved in the design of the experiment. FR was involved in embryo production, morula handling and freezing. VH performed transfer and flushing procedures. UB performed transfer and flushing procedures. CN was involved in design of the experiment. KS designed and supervised the experiment and reviewed the manuscript. DT was responsible for designing the experiment, acquisition of funds and reviewing of the manuscript. All authors approved the final version of the paper.

3.8   Competing interests

The authors declare no competing interests.

3.9   Data availability

Raw sequencing files are available at the European Nucleotide Archive under accession number E-MTAB-8239.

3.10   References

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3.11   List of figures
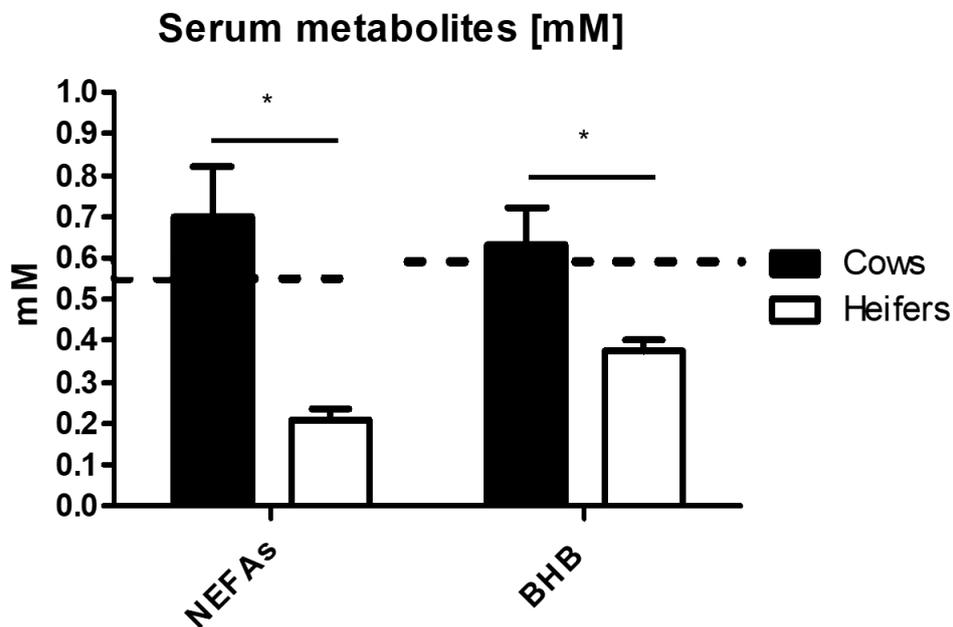
Figure 3.1 Blood metabolite concentrations [mM] of Non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) during *in vivo* culture in selected cows and heifers. Dotted lines represent threshold of NEB found in literature (NEFA = 0.55 mM; BHB = 0.59 mM) [10] to be associated with negative energy balance, where asterisks show significance between groups and p < 0.05. Error bars represent the standard error of the mean.
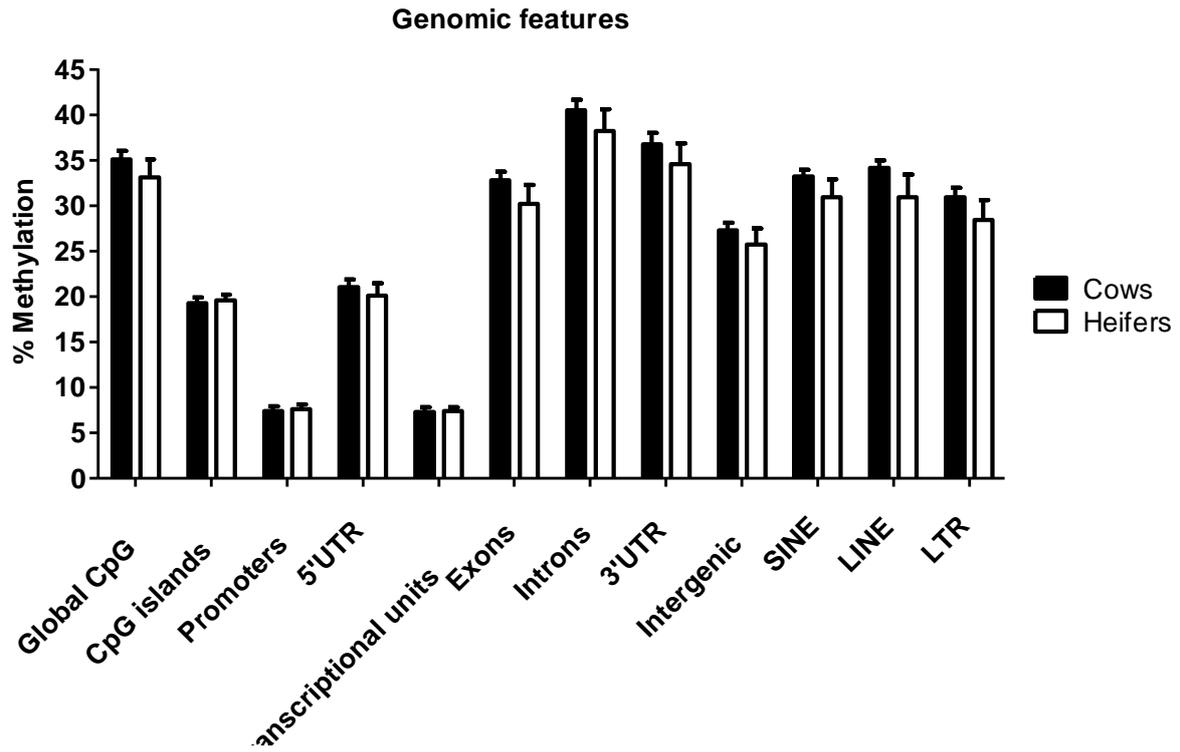
Figure 3.2 Methylation levels of genomic features from postpartum cows and heifers. Error bars represent standard error of the mean.
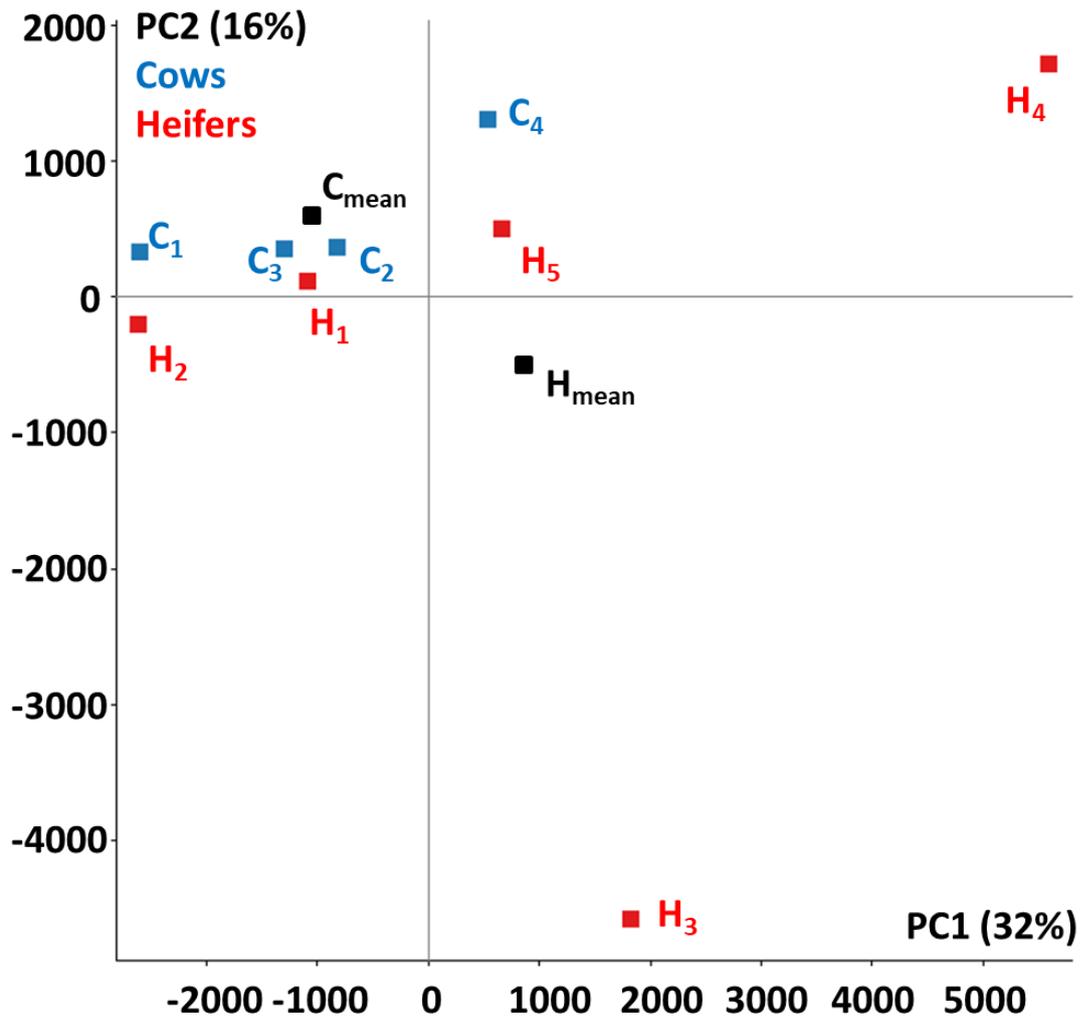
Figure 3.3 Principal Component Analysis of the 150 CpG windows DNA methylation distribution in cows (C1-4, blue) and heifers (H1-5, red) with their corresponding average (black).
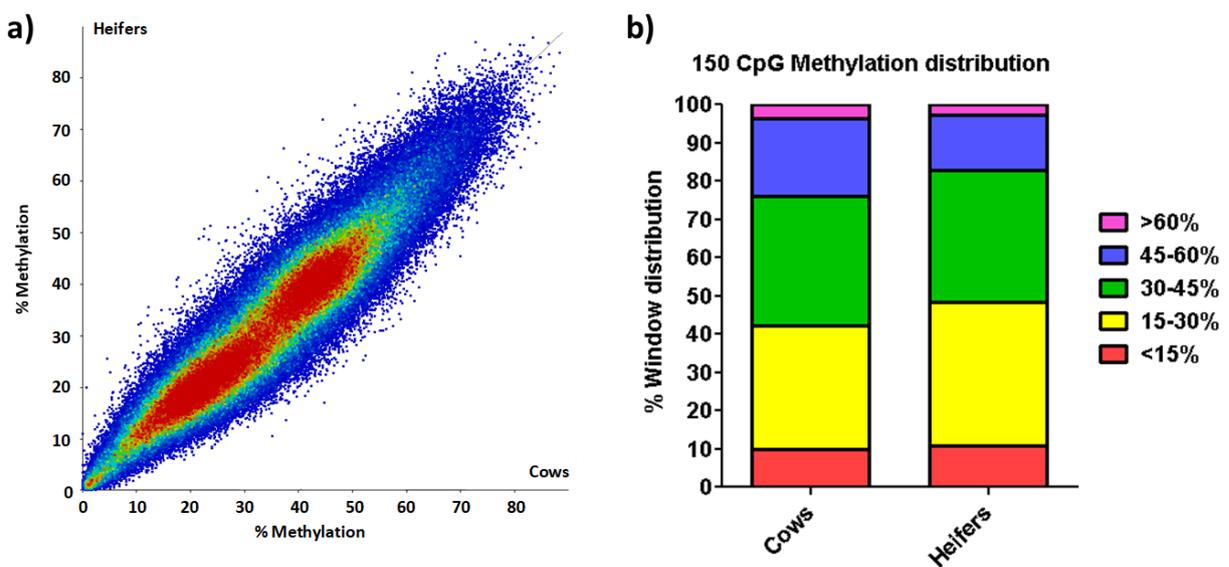
Figure 3.4 a) Scatter plot of all 150 CpG quantitated windows in cows and heifers. Color represents the frequency of methylation relationship in windows ranging from red to blue.

b) Window distribution methylation levels of 150 CpG probes across the genome of both groups.
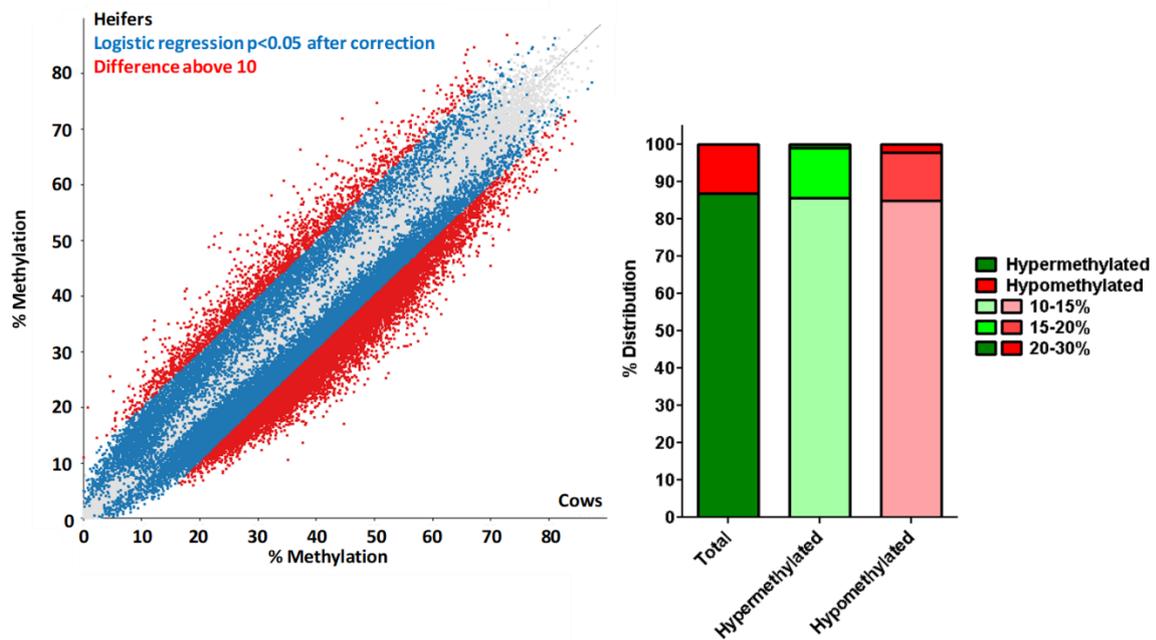
Figure 3.5 a) Scatter plot of differentially methylated regions (DMRs) found between cows and heifers using logistic regression test (blue, n= 32,895). DMRs with percentage above 10 used for further analysis are shown in red (n= 13,383). **b) Distribution of DMR methylation above 10% between cows and heifers.** Total bar represents the whole of DMRs > 10%, while hyper- (11,612 DMRs) and hypomethylated (1,771 DMRs) bars represent the DMRs distribution.
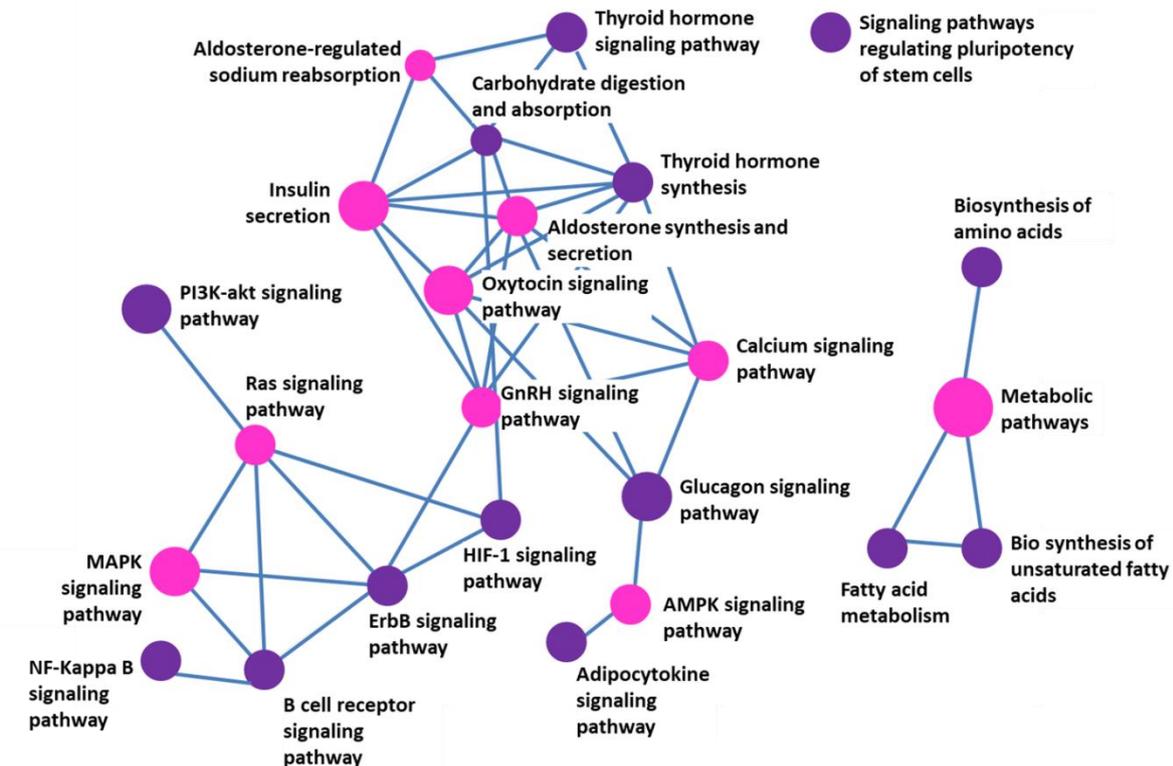
Figure 3.6 KEGG Pathway functional relevance of DMRs found between cows and heifers. Selected pathways from the KEGG pathway database enriched in DMRs found and their

network association with DMRs. Pathways shown have p-values <0.05 with a false discovery rate < 0.17.
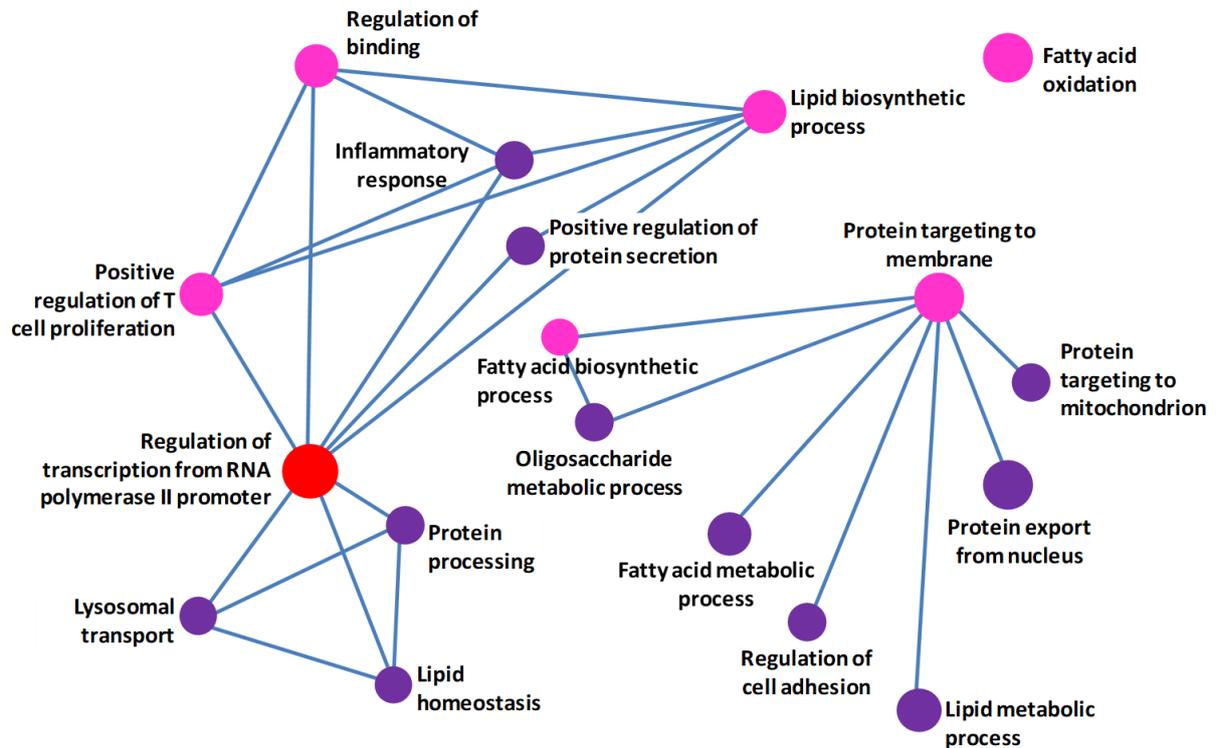
Figure 3.7 Biological processes functional relevance of DMRs found between cows and heifers. Selected biological processes from Gene Ontology enriched from DMRs and their network association with DMRs. Biological processes shown have p-values <0.05.

3.12 List of Tables

Table 3.1 Top 20 DMRs found in hypermethylated and hypomethylated DMRs between lactating cow and heifer morulas.

Hypermethylated			Hypomethylated		
Probe position	Gene symbol	Absolute difference	Probe position	Gene symbol	Absolute difference
Chr5:119590478-119591715	FAM19A5	25.7	Chr25:42524912-42525925	PRKAR1B	29.1
Chr8:897565-898966	PALLD	24.7	Chr29:44432505-44433543	EHBP1L1	28.2
Chr6:115987639-115990909	PROM1	24.6	Chr17:67465465-67467127	KIAA1671	27.3
ChrX:38766605-38774303	BRCC3	24.6	Chr23:50315726-50317352	SLC22A23	26.8
ChrX:112750504-112760396	MAGEB16	24.5	Chr12:89129897-89131289	COL4A2	25.2
Chr14:83204413-83207309	CHMP4C	23.8	Chr14:1918961-1919696	WDR97	24.3
Chr2:77020865-77029974	CNTNAP5	23.4	Chr1:155775271-155776126	PLCL2	23.5
Chr6:107989329-107995305	ADD1	22.9	Chr13:30069192-30070118	FAM171A1	23.5
Chr15:66209744-66219538	APIP	22.5	Chr21:16959925-16961308	KLHL25	23.4
Chr13:52890948-52891424	EBF4	22.2	Chr11:106340977-106342053	C9orf172	23.4
Chr18:16577599-16582489	ABCC12	22.2	Chr10:103252399-103253417	TTC7B	23.3
ChrX:130459185-130467294	SH3KBP1	21.8	Chr22:60492033-60493183	MGLL	22.8
Chr24:1248194-1255492	ATP9B	21.6	Chr18:10982524-10985526	CRISPLD2	22.1
Chr22:56218538-56220413	VGLL4	21.6	Chr3:116226279-116228111	AGAP1	21.9
Chr13:46660473-46665513	WDR37	21.5	Chr19:37983953-37985051	PHOSPHO1	21.9
Chr10:53340802-53349738	TCF12	21.4	Chr7:20566976-20568091	FEM1A	21.9
Chr25:1150003-1151670	CLCN7	21.2	Chr17:70719085-70720285	GAS2L1	21.8
Chr15:74683524-74691338	HSD17B12	21.1	Chr29:28711195-28712305	ROBO3	21.6
Chr6:109333537-109335252	MAEA	21.1	Chr29:44382295-44383131	LTBP3	21.6
Chr25:9996338-9999369	PRM2	21.0	Chr25:120656-121968	RHBDF1	21.0

Table 3.2 Distribution of total quantitated windows, total DMRs, hyper- and hypomethylated DMRs found overlapping genomic features. Numbers in parentheses represent the percentage over total quantitated windows or DMRs.

Windows overlapping	Global	Total DMRs	Hypermethylated DMRs	Hypomethylated DMRs
Total quantitated	311,612	13,383	11,612	1,771
CpG islands	32,144 (10.3)	1,090 (8.1)	500 (4.3)	590 (33.3)
Promoters	18,247 (5.9)	501 (3.7)	249 (2.1)	252 (14.2)
5'UTR	23,276 (7.5)	813 (6.1)	653 (5.6)	160 (9.0)
Transcriptional units	15,230 (4.9)	420 (3.1)	176 (1.5)	244 (13.8)
Gene bodies	125,793 (40.4)	5707 (42.6)	4,771 (41.1)	936 (52.9)
Exons	75,751 (24.3)	3,557 (26.6)	2,943 (25.3)	614 (34.7)
Introns	109,290 (35.1)	5,025 (37.5)	4,242 (36.5)	783 (44.2)
3'UTR	23,022 (7.4)	1,066 (8.0)	871 (7.5)	195 (11.0)
Intergenic	214,336 (68.8)	8,851 (66.1)	7,809 (67.2)	1,042 (58.8)
SINE	287,806 (92.4)	12,241 (91.5)	10,953 (94.3)	1,288 (72.7)
LINE	270,507 (86.8)	11,569 (86.4)	10,513 (90.5)	1,056 (59.6)
LTR	186,093 (59.7)	8,150 (60.9)	7,568 (65.2)	582 (32.9)

Table 3.3 List of DMRs found overlapping imprinted gene regions.

Probe position	Gene symbol	Absolute Difference	Methylation Cow	Methylation Heifers	Overlapping CpG island
Chr4:11911992-11913021	PEG10	17.2	43.5	26.3	+
Chr4:11918879-11919758	PEG10	11.3	38.6	49.8	+
Chr4:95068082-95073244	MEST	18.4	55.9	37.5	-
Chr9:97694035-97695102	IGF2R	10.5	73.9	63.4	+
Chr9:97716702-97718407	IGF2R	10.5	51.9	62.4	+

Table 3.4 List of DMRs found overlapping genes involved in embryo implantation.

Probe position	Gene symbol	Absolute Difference	Methylation Cow	Methylation Heifers	Overlapping CpG island
Chr1:3117612-3121476	SOD1	12.2	33.0	20.8	-
Chr2:126993399-127000266	ARID1A	11.7	45.2	33.5	-
Chr4:49315047-49324197	LAMB1	12.4	52.0	39.6	-
Chr7:9030349-9033230	SYDE1	11.9	26.8	38.7	+
Chr8:46882305-46889010	KLF9	11.8	51.3	39.5	-
Chr8:52296470-52305685	PCSK5	14.0	51.8	37.8	-
Chr8:52412219-52422817	PCSK5	10.4	50.2	39.8	-
Chr8:52678737-52687959	PCSK5	10.7	42.7	32.0	-
Chr8:60718312-60735740	RECK	11.7	45.5	33.9	-
Chr17:47334296-47340798	STX2	13.3	50.3	37.0	-
Chr18:48457897-48460266	GGN	14.1	33.8	47.9	+
Chr18:63672199-63673122	NLRP5	14.8	37.4	52.2	+
Chr23:9350667-9352656	PPARD	10.5	68.2	57.7	-

3.13 List of supplemental tables

Supplemental table 3.1 List of DMRs found overlapping imprinted genes in human and their respective imprint status in bovine.

Probe position	Gene symbol	Absolute Difference	Methylation Cow	Methylation Heifers	Imprint status in bovine
Chr3:77570928-77572856	DIRAS3	12.1	44.4	56.5	unknown
Chr4:5181623-5184870	GRB10	13.0	63.9	50.9	unknown
Chr4:5201948-5206305	GRB10	10.6	64.8	54.2	unknown
Chr4:5215274-5218142	GRB10	10.9	66.2	55.3	unknown
Chr4:5232681-5237575	GRB10	14.4	61.1	46.7	unknown
Chr4:5275472-5279516	DDC	13.1	26.0	39.1	unknown
Chr4:11911992-11913021	PEG10	17.2	43.5	26.3	imprinted
Chr4:11918879-11919758	PEG10	11.3	38.6	49.8	imprinted
Chr4:95068082-95073244	MEST	18.4	55.9	37.5	imprinted
Chr9:45554360-45571341	LIN28B	13.0	39.7	26.7	unknown
Chr10:82037821-82045732	SMOC1	10.7	48.1	37.4	unknown
Chr14:4666110-4668904	KCNK9	17.9	42.8	24.9	unknown
Chr14:4716792-4720547	KCNK9	13.8	32.1	18.4	unknown
Chr14:4733111-4736777	KCNK9	13.7	44.4	30.7	unknown
Chr21:2387997-2396761	UBE3A	11.2	40.0	51.2	unknown
Chr21:2912373-2915224	ATP10A	11.3	60.4	49.1	unknown
Chr21:2926705-2931157	ATP10A	12.0	53.3	41.3	unknown
Chr21:67281416-67282802	DLK1	18.1	40.6	22.5	unknown
Chr21:67425804-67427882	RTL1	18.3	26.5	44.8	unknown
Chr21:67427883-67429034	RTL1	16.6	36.4	53.0	unknown
Chr21:67429035-67430226	RTL1	15.5	37.8	53.2	unknown
Chr29:35215970-35222676	NTM	13.0	29.6	16.7	unknown
Chr29:35250703-35257731	NTM	13.0	31.5	18.4	unknown
Chr29:35276524-35280692	NTM	10.9	28.2	17.3	unknown
Chr29:35376108-35381804	NTM	11.7	32.8	21.1	unknown
Chr29:47935504-47938457	ANO1	10.6	27.2	16.6	unknown
Chr29:47963477-47965437	ANO1	14.2	30.5	44.6	unknown
Chr29:48004886-48007165	ANO1	13.3	34.8	21.5	unknown
Chr29:49122387-49123680	OSBPL5	10.6	68.0	78.6	not imprinted
Chr29:49123681-49126303	OSBPL5	15.5	53.4	69.0	not imprinted
Chr29:49126304-49129689	OSBPL5	13.5	46.0	59.6	not imprinted
Chr29:49129696-49131237	OSBPL5	10.6	56.1	66.6	not imprinted
Chr29:49131238-49132418	OSBPL5	13.5	53.3	66.8	not imprinted
Chr29:49134044-49136138	OSBPL5	10.2	56.9	67.0	not imprinted

Supplemental table 3.2 List of DMRs found overlapping genes involved in *in utero* embryo development.

Probe position	Gene symbol	Absolute Difference	Methylation Cow	Methylation Heifers	Overlapping CpG island
Chr6:107989329-107995305	ADD1	22.9	59.2	36.2	-
Chr17:74409710-74410803	SMPD4	20.0	61.0	81.0	+
Chr8:38171507-38184718	KDM4C	19.3	60.5	41.1	-
ChrX:107825276-107839160	USP9X	19.1	54.0	34.9	-
Chr14:59238107-59253831	ANGPT1	18.3	56.9	38.6	-
Chr19:15282348-15285158	NLE1	18.0	64.5	46.6	-
Chr12:90361063-90363262	ATP11A	17.8	76.6	58.8	-
ChrX:3792896-3802215	UBE2A	17.3	53.3	36.0	-
Chr13:32162748-32167642	ST8SIA6	17.3	55.9	38.6	-
Chr16:52221232-52225093	SLC35E2	17.1	63.6	46.5	-
Chr17:74407331-74408492	SMPD4	16.5	54.1	70.6	+
Chr10:19991149-19995727	HCN4	16.5	54.3	37.7	-
Chr13:80301811-80302601	SALL4	16.5	44.6	61.1	+
ChrX:18232771-18242700	PLAC1	16.5	57.0	40.5	-
Chr24:51033835-51043220	SMAD4	16.5	48.9	32.4	-
Chr6:89217383-89218720	ADAMTS3	16.4	25.2	41.6	+
Chr19:62573911-62579489	GNA13	16.3	40.9	24.6	+
ChrX:68634396-68641721	AMOT	16.2	35.6	19.5	+
Chr24:34879059-34884485	MIB1	16.0	59.7	43.8	-
Chr25:3304273-3308166	ADCY9	15.8	61.9	46.1	-
Chr5:96490882-96497915	GRIN2B	15.7	59.9	44.2	-
Chr12:90587938-	CUL4A	15.6	81.8	66.1	+

90588999					
Chr2:18293001-18296396	TTN	15.5	65.5	50.1	-
Chr17:73235139-73236621	SMARCB1	15.4	66.3	50.9	+
Chr11:77959199-77964376	APOB	15.4	48.0	32.6	-
Chr21:61542050-61547468	DICER1	15.3	56.2	40.9	-
Chr22:48799540-48811009	PBRM1	15.2	53.0	37.8	-
Chr21:28975304-28980904	TJP1	15.2	63.5	48.3	-
Chr19:23504623-23513471	RPA1	15.2	50.3	35.2	-
Chr14:61298057-61312586	ZFPM2	15.1	48.3	33.2	-
Chr2:73092428-73098578	GLI2	15.1	52.6	37.5	-
ChrX:3780300-3792895	UBE2A	14.9	52.5	37.7	-
Chr18:63672199-63673122	NLRP5	14.8	37.4	52.2	+
Chr14:65017923-65025834	GRHL2	14.8	36.8	22.0	-
Chr17:67889998-67893592	MYO18B	14.8	57.9	43.2	-
Chr19:48225910-48238230	TANC2	14.8	45.5	30.8	-
Chr6:89333423-89344807	ADAMTS3	14.7	48.1	33.4	-
Chr6:7752337-7754015	SEC24D	14.7	59.4	44.7	-
Chr25:344300-346301	AXIN1	14.7	19.2	33.9	+
Chr13:32109144-32120411	ST8SIA6	14.6	37.4	22.8	-
Chr15:37079145-37086458	SOX6	14.6	45.7	31.1	-
ChrX:108903081-108906608	BCOR	14.6	69.0	54.4	-
Chr7:8899230-8901593	BRD4	14.4	72.6	58.2	-
Chr28:41835553-41842707	BMPR1A	14.4	47.7	33.3	-
Chr17:53692069-53694232	NCOR2	14.3	62.4	48.1	-
Chr4:51397879-	WNT2	14.3	27.0	41.2	+

51398614					
Chr19:39230278-39233470	SP2	14.2	35.0	49.2	+
Chr22:5223227-5228653	TGFBR2	14.2	54.3	40.1	-
Chr5:96526690-96534241	GRIN2B	14.1	56.7	42.6	-
Chr22:37432357-37437764	PRICKLE2	14.0	36.0	50.1	-
Chr2:22868816-22881343	SP3	14.0	55.5	41.5	-
Chr19:34753428-34755810	EPN2	13.9	57.5	43.6	-
Chr22:30633292-30637856	FOXP1	13.9	51.8	37.9	-
Chr17:59090130-59096028	SUDS3	13.9	58.5	44.7	-
Chr14:61019966-61027553	ZFPM2	13.8	29.0	15.2	-
Chr19:35532844-35535648	FLCN	13.8	64.1	50.3	-
Chr5:86995267-87004064	SOX5	13.7	44.3	30.6	-
Chr4:51440724-51447264	WNT2	13.7	48.5	34.8	-
Chr2:4148667-4149750	HS6ST1	13.6	48.8	35.2	+
Chr2:26466731-26476632	UBR3	13.6	59.0	45.4	-
Chr23:27770189-27774006	POU5F1	13.6	12.6	26.1	+
Chr14:64945034-64954871	GRHL2	13.5	43.9	30.4	-
Chr13:32040722-32050145	ST8SIA6	13.4	26.4	39.7	-
Chr19:39199044-39204003	SP2	13.4	57.6	44.2	-
Chr24:20039633-20042434	CELF4	13.3	12.7	26.0	+
Chr13:49550522-49551869	BMP2	13.3	5.6	18.9	+
Chr10:88810508-88813391	ESRRB	13.2	36.6	23.4	-
Chr2:18125880-18132038	TTN	13.1	56.9	43.8	-
Chr15:37034553-37044237	SOX6	13.0	45.2	32.2	-
Chr18:48324309-	SPINT2	13.0	55.0	42.0	-

Location	Gene				
48328915					
ChrX:7615259-7631167	THOC2	13.0	53.9	40.9	-
Chr17:53563205-53569198	NCOR2	13.0	61.5	48.5	-
Chr5:96431984-96440109	GRIN2B	12.9	51.2	38.2	-
Chr14:61113458-61123951	ZFPM2	12.9	37.4	24.5	-
Chr26:41860848-41864307	FGFR2	12.9	66.3	53.4	-
Chr16:27817094-27820882	CAPN2	12.9	54.5	41.6	+
Chr11:104008422-104009797	NOTCH1	12.9	70.0	57.2	-
Chr22:922051-927894	EGFR	12.8	57.5	44.8	-
Chr14:38754917-38761858	RDH10	12.8	53.3	40.5	-
Chr29:36970993-36975183	ST14	12.8	13.4	26.2	+
Chr19:22579271-22582697	NXN	12.8	57.2	44.4	-
Chr11:78000343-78005635	APOB	12.7	44.8	32.1	-
Chr21:42142324-42153289	HECTD1	12.6	59.3	46.7	-
Chr10:19983131-19986790	HCN4	12.6	40.0	27.4	-
Chr12:90356361-90358598	ATP11A	12.6	61.4	48.8	-
Chr19:48390182-48396055	TANC2	12.6	60.1	47.5	-
Chr18:53411025-53412893	ERCC2	12.5	64.3	51.8	-
Chr16:1735165-1738182	ETNK2	12.5	60.5	48.0	-
Chr25:325016-326867	AXIN1	12.5	58.9	71.3	-
Chr2:108122609-108125102	SPEG	12.4	61.9	49.5	+
Chr11:88483788-88492814	KIDINS220	12.3	54.9	42.6	-
Chr19:34716834-34719565	B9D1	12.2	64.4	52.2	-
Chr23:4526818-4534788	BMP5	12.2	29.1	16.9	-
Chr2:18296413-	TTN	12.1	61.7	49.6	-

18301403

Chr7:8893281-8896483	BRD4	12.1	59.7	47.6	-
Chr2:39347895-39354233	ACVR1	12.1	53.9	41.8	-
Chr7:16236700-16238970	KEAP1	12.1	54.4	42.4	+
Chr14:64939081-64944818	GRHL2	12.0	24.3	12.2	-
Chr6:107977489-107982476	ADD1	12.0	52.4	40.4	-
Chr13:59500663-59505174	BMP7	12.0	58.8	46.8	-
Chr15:54233151-54238461	C2CD3	12.0	53.9	41.9	-
ChrX:13497416-13501936	OCRL	12.0	63.6	51.7	-
Chr19:22459186-22464803	NXN	12.0	31.8	19.8	-
Chr2:9747615-9757867	ITGAV	12.0	39.9	27.9	-
Chr6:60287596-60299311	SMIM14	12.0	38.8	26.9	-
Chr19:62564143-62566338	GNA13	11.9	44.9	32.9	-
Chr18:26386129-26394778	CNOT1	11.8	48.9	37.1	-
Chr18:8279239-8281228	CMIP	11.7	72.7	61.0	-
Chr24:19996177-19999404	CELF4	11.6	32.6	20.9	-
Chr29:36969954-36970992	ST14	11.6	11.4	23.1	+
Chr18:63427969-63429440	CNOT3	11.6	64.3	76.0	-
Chr14:28171121-28175412	CHD7	11.6	35.9	24.3	+
Chr26:41868082-41874182	FGFR2	11.6	48.0	36.4	-
Chr6:41575290-41587690	SLIT2	11.6	31.0	19.5	-
Chr28:35127928-35129929	ZMIZ1	11.6	59.7	71.3	-
ChrX:18219099-18232770	PLAC1	11.5	47.5	36.0	-
Chr2:18311205-18315595	TTN	11.5	58.9	47.4	-
Chr22:48780813-	PBRM1	11.5	54.0	42.5	-

48791340 Chr7:53208234-53218765	HBEGF	11.5	48.1	36.6	-
Chr19:35505545-35509584	COPS3	11.5	61.2	49.7	-
Chr2:72172360-72181241	EPB41L5	11.4	47.5	36.0	-
Chr18:8244691-8246873	CMIP	11.4	68.0	56.5	-
Chr17:67981736-67985827	MYO18B	11.4	54.8	43.3	-
Chr10:51628519-51638156	ADAM10	11.4	51.0	39.5	-
Chr19:49664517-49673696	BPTF	11.4	55.5	44.1	-
Chr2:26524417-26538894	UBR3	11.4	47.2	35.8	-
Chr11:15093261-15107683	BIRC6	11.4	55.6	44.2	-
Chr17:67893599-67898069	MYO18B	11.4	60.9	49.6	-
ChrX:7569759-7582049	THOC2	11.4	34.5	23.2	-
Chr5:28104392-28108921	ACVRL1	11.3	29.5	18.1	-
Chr5:30927590-30933140	KMT2D	11.3	41.6	52.9	+
ChrX:13470403-13483062	OCRL	11.3	54.1	42.8	-
Chr25:41167620-41168760	GNA12	11.3	77.9	66.6	+
Chr24:51023409-51033829	SMAD4	11.3	29.6	40.9	-
Chr6:7690287-7700169	SEC24D	11.3	56.5	45.2	-
Chr6:41388839-41405661	SLIT2	11.3	31.2	19.9	-
Chr26:41880333-41885616	FGFR2	11.2	51.4	40.2	-
Chr11:78029796-78036474	APOB	11.2	48.2	37.0	-
Chr19:22617862-22621729	NXN	11.2	31.0	42.2	-
Chr14:61351295-61362852	ZFPM2	11.2	33.1	22.0	-
Chr1:70092781-70095698	HEG1	11.2	54.7	43.5	-
Chr22:1040203-	EGFR	11.2	61.6	50.4	-

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Position	Gene				
Chr19:42886198-42889230	KAT2A	11.2	25.6	36.8	-
Chr16:37583973-37592755	ATP1B1	11.1	47.9	36.8	-
Chr17:67874563-67878368	MYO18B	11.1	59.1	48.0	-
Chr10:51616149-51628518	ADAM10	11.1	50.5	39.4	-
Chr12:90566600-90569110	CUL4A	11.1	64.5	53.3	-
Chr24:19977237-19981711	CELF4	11.1	38.9	27.8	-
Chr14:64933863-64939080	GRHL2	11.1	35.2	24.1	-
Chr12:20423478-20429201	RNASEH2B	11.1	44.0	32.9	-
Chr19:56768027-56775567	GRB2	11.0	51.4	40.4	-
Chr6:116216269-116219685	TAPT1	10.9	61.2	50.2	-
Chr19:22611303-22614916	NXN	10.9	41.2	52.1	-
Chr25:3294892-3300152	ADCY9	10.9	58.3	47.4	-
Chr26:31092280-31104970	MXI1	10.9	46.9	36.0	-
Chr10:88806314-88810507	ESRRB	10.9	17.1	28.0	-
ChrX:88567179-88579857	AR	10.8	40.0	29.1	-
Chr19:48175919-48187579	TANC2	10.8	47.4	36.6	-
Chr5:111190946-111194816	PDGFB	10.8	33.5	22.7	-
Chr14:17129789-17135201	MTSS1	10.8	59.1	48.3	-
Chr13:59505184-59509691	BMP7	10.6	43.9	54.5	-
Chr14:16974884-16980524	MTSS1	10.6	50.5	39.9	-
Chr5:68458333-68465678	CHST11	10.6	53.0	42.4	-
Chr12:20418404-20423477	RNASEH2B	10.6	30.9	20.3	+
Chr1:147769539-147776505	PCNT	10.5	54.3	43.8	-
Chr8:64278107-	ANKS6	10.5	57.2	46.7	-

64284077 Chr7:85374838-85386938	XRCC4	10.5	54.3	43.9	-
Chr24:19840447-19843518	CELF4	10.4	27.5	17.0	-
Chr10:51257079-51262459	CCNB2	10.4	55.7	45.2	-
Chr14:64905374-64910460	GRHL2	10.4	31.5	21.1	-
Chr6:18373499-18384728	LEF1	10.4	45.8	35.4	-
Chr9:98031713-98037316	PLG	10.4	20.2	30.6	-
Chr11:15016486-15026897	BIRC6	10.3	52.4	42.1	-
Chr11:88555621-88561087	KIDINS 220	10.3	53.7	43.4	-
Chr15:63654032-63658578	RCN1	10.3	52.6	42.4	-
Chr14:17036349-17043650	MTSS1	10.2	49.8	39.6	-
Chr2:22858361-22868812	SP3	10.2	43.2	32.9	-
Chr6:7732899-7737060	SEC24D	10.2	54.4	44.2	-
Chr6:116219686-116224440	TAPT1	10.2	56.8	46.6	-
Chr6:38076931-38088586	PKD2	10.2	45.5	35.2	-
Chr17:67859502-67864011	MYO18 B	10.2	43.9	54.0	-
Chr15:36696264-36714220	SOX6	10.1	36.9	26.7	-
Chr6:71403523-71409705	PDGFR A	10.1	34.1	24.0	-
Chr12:90370231-90371709	ATP11A	10.1	70.5	60.3	-
Chr24:51000625-51011494	SMAD4	10.1	44.3	34.2	-
Chr10:88253584-88256708	TGFB3	10.0	31.4	21.4	-
Chr6:60046746-60055776	WDR19	10.0	50.5	40.5	-

Supplemental table 3.3 All KEGG pathways enriched from DMRs overlapping gene regions.

Pathway	Total	Expected	Hits	P.Value	FDR
Axon guidance	179	27.4	51	4.05E-06	1.27E-03
Autophagy - animal	133	20.4	39	2.65E-05	4.16E-03
Gastric acid secretion	74	11.3	25	5.93E-05	5.28E-03
Morphine addiction	92	14.1	29	6.72E-05	5.28E-03
Circadian entrainment	100	15.3	30	1.39E-04	7.02E-03
Aldosterone synthesis and secretion	96	14.7	29	1.57E-04	7.02E-03
Adrenergic signaling in cardiomyocytes	144	22.1	39	1.78E-04	7.02E-03
Sphingolipid signaling pathway	120	18.4	34	1.79E-04	7.02E-03
Dopaminergic synapse	132	20.2	36	2.69E-04	8.46E-03
Insulin secretion	85	13	26	2.70E-04	8.46E-03
MAPK signaling pathway	292	44.7	67	3.04E-04	8.46E-03
Glycosaminoglycan biosynthesis - heparan sulfate / heparin	24	3.68	11	3.77E-04	8.46E-03
ABC transporters	60	9.19	20	3.89E-04	8.46E-03
Long-term depression	60	9.19	20	3.89E-04	8.46E-03
Platelet activation	120	18.4	33	4.04E-04	8.46E-03
Oxytocin signaling pathway	152	23.3	39	5.86E-04	1.13E-02
AMPK signaling pathway	123	18.8	33	6.53E-04	1.13E-02
Phosphatidylinositol signaling system	99	15.2	28	6.66E-04	1.13E-02
Aldosterone-regulated sodium reabsorption	37	5.67	14	6.90E-04	1.13E-02
GABAergic synapse	90	13.8	26	7.21E-04	1.13E-02
mTOR signaling pathway	154	23.6	39	7.71E-04	1.15E-02
Amoebiasis	101	15.5	28	9.40E-04	1.29E-02
Inositol phosphate metabolism	73	11.2	22	9.75E-04	1.29E-02
Lysine degradation	64	9.8	20	9.90E-04	1.29E-02
T cell receptor signaling pathway	103	15.8	28	1.31E-03	1.63E-02
Glutamatergic synapse	113	17.3	30	1.35E-03	1.63E-02
Wnt signaling pathway	159	24.3	39	1.48E-03	1.71E-02
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	20	3.06	9	1.52E-03	1.71E-02
Gap junction	90	13.8	25	1.67E-03	1.79E-02
Calcium signaling pathway	197	30.2	46	1.76E-03	1.79E-02
Choline metabolism in cancer	100	15.3	27	1.76E-03	1.79E-02
Focal adhesion	198	30.3	46	1.96E-03	1.92E-02
cGMP-PKG signaling	167	25.6	40	2.08E-03	1.95E-02

pathway					
Oocyte meiosis	116	17.8	30	2.11E-03	1.95E-02
Pancreatic secretion	97	14.9	26	2.38E-03	2.12E-02
Autophagy - other	33	5.05	12	2.45E-03	2.12E-02
Regulation of actin cytoskeleton	211	32.3	48	2.50E-03	2.12E-02
Type II diabetes mellitus	46	7.04	15	2.59E-03	2.14E-02
GnRH signaling pathway	93	14.2	25	2.73E-03	2.15E-02
Salivary secretion	93	14.2	25	2.73E-03	2.15E-02
Ras signaling pathway	241	36.9	53	3.32E-03	2.54E-02
Longevity regulating pathway	90	13.8	24	3.69E-03	2.72E-02
Metabolic pathways	1470	224	259	3.73E-03	2.72E-02
Phospholipase D signaling pathway	152	23.3	36	4.13E-03	2.94E-02
Mucin type O-glycan biosynthesis	31	4.75	11	4.57E-03	3.19E-02
Sphingolipid metabolism	49	7.5	15	5.10E-03	3.48E-02
Inflammatory mediator regulation of TRP channels	103	15.8	26	5.71E-03	3.76E-02
Pathways in cancer	539	82.5	104	5.75E-03	3.76E-02
Ubiquitin mediated proteolysis	140	21.4	33	6.33E-03	3.90E-02
Apelin signaling pathway	140	21.4	33	6.33E-03	3.90E-02
Cholinergic synapse	114	17.5	28	6.34E-03	3.90E-02
Small cell lung cancer	94	14.4	24	6.66E-03	4.02E-02
Insulin resistance	110	16.8	27	7.33E-03	4.34E-02
Rap1 signaling pathway	210	32.2	45	1.05E-02	6.05E-02
Thyroid hormone signaling pathway	113	17.3	27	1.06E-02	6.05E-02
HIF-1 signaling pathway	98	15	24	1.14E-02	6.38E-02
Purine metabolism	135	20.7	31	1.17E-02	6.45E-02
Synaptic vesicle cycle	78	11.9	20	1.20E-02	6.49E-02
Retrograde endocannabinoid signaling	152	23.3	34	1.28E-02	6.82E-02
Fatty acid metabolism	54	8.27	15	1.33E-02	6.97E-02
PI3K-Akt signaling pathway	373	57.1	73	1.37E-02	7.04E-02
Thyroid hormone synthesis	74	11.3	19	1.39E-02	7.04E-02
cAMP signaling pathway	225	34.5	47	1.43E-02	7.14E-02
Serotonergic synapse	116	17.8	27	1.50E-02	7.35E-02
NF-kappa B signaling pathway	101	15.5	24	1.64E-02	7.94E-02
AGE-RAGE signaling pathway in diabetic complications	103	15.8	24	2.07E-02	9.85E-02
Adipocytokine signaling pathway	72	11	18	2.14E-02	1.00E-01
ECM-receptor interaction	83	12.7	20	2.34E-02	1.08E-01

Endocrine and other factor-regulated calcium reabsorption	43	6.58	12	2.45E-02	1.12E-01
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	68	10.4	17	2.49E-02	1.12E-01
Dilated cardiomyopathy	89	13.6	21	2.56E-02	1.13E-01
Adherens junction	69	10.6	17	2.85E-02	1.24E-01
ErbB signaling pathway	85	13	20	2.97E-02	1.28E-01
Biosynthesis of unsaturated fatty acids	30	4.59	9	3.11E-02	1.32E-01
B cell receptor signaling pathway	75	11.5	18	3.16E-02	1.32E-01
Hippo signaling pathway	151	23.1	32	3.19E-02	1.32E-01
Protein digestion and absorption	113	17.3	25	3.36E-02	1.35E-01
Carbohydrate digestion and absorption	40	6.13	11	3.39E-02	1.35E-01
Nicotine addiction	40	6.13	11	3.39E-02	1.35E-01
Acute myeloid leukemia	66	10.1	16	3.78E-02	1.48E-01
Signaling pathways regulating pluripotency of stem cells	137	21	29	4.02E-02	1.54E-01
Proximal tubule bicarbonate reclamation	22	3.37	7	4.05E-02	1.54E-01
Chagas disease (American trypanosomiasis)	115	17.6	25	4.07E-02	1.54E-01
Progesterone-mediated oocyte maturation	88	13.5	20	4.16E-02	1.55E-01
Glucagon signaling pathway	99	15.2	22	4.24E-02	1.56E-01
Vascular smooth muscle contraction	133	20.4	28	4.59E-02	1.65E-01
Neurotrophin signaling pathway	122	18.7	26	4.65E-02	1.65E-01
Biosynthesis of amino acids	73	11.2	17	4.67E-02	1.65E-01
Hedgehog signaling pathway	47	7.2	12	4.69E-02	1.65E-01
Bile secretion	84	12.9	19	4.82E-02	1.68E-01
Prostate cancer	95	14.5	21	4.89E-02	1.69E-01

Supplemental table 3.4 All genes involved in selected enriched pathways from DMRs found to be overlapping genes.

Pathway	Genes
Aldosterone synthesis and secretion	ATP1B3, ATP1A4, ATP1A2, PRKACB, ADCY1, CREB3L2, ITPR2, CAMK2D, GNAQ, PRKD3, PRKCE, ADCY3, PLCB1, KCNK9, PDE2A, ATP1B1, NPPA, ADCY7, PRKCG, CACNA1G, ITPR1, CACNA1D, ATP2B2, ITPR3, ADCY9, PRKCB, CAMK2G, KCNJ5,

ATP2B3

Insulin secretion	ATP1B3, RAPGEF4, ATP1A4, ATP1A2, PRKACB, ADCY1, CREB3L2, CAMK2D, GNAQ, KCNN2, ADCY3, PLCB1, RIMS2, ABCC8, ATP1B1, ADCY7, TRPM4, PRKCG, GIP, CACNA1D, ITPR3, ADCY9, PRKCB, RYR2, CAMK2G, KCNMA1
MAPK signaling pathway	PAK2, IL1RAP, RASA2, MAP3K6, RPS6KA1, PRKACB, KITLG, PTPRR, CACNG2, FGF6, FGF23, MAPK8IP2, NFKB1, PDGFRA, FGF5, MAPK10, FGFR3, CACNA1A, INSR, FGFR4, EFNA2, FGF1, NTRK2, MAP3K4, RPS6KA2, MAP2K5, RASGRP1, PLA2G4F, MAX, TGFB3, RPS6KA5, SOS1, MAP4K3, CACNA1B, FLT1, CACNB2, MAP3K8, ANGPT4, PDGFD, AKT3, CACNA1E, RAPGEF2, TAOK3, NFATC3, PRKCG, CACNG7, NLK, MAP2K4, CACNA1G, MAP3K3, GRB2, CACNG4, FGF18, EGFR, TGFBR2, FLNB, CACNA1D, NFATC1, PRKCB, CACNG3, GNA12, FGFR2, IKBKB, RASGRP2, IKBKG, RPS6KA3
Oxytocin signaling pathway	PRKACB, PIK3CG, ADCY1, CACNG2, ITPR2, CAMK2D, GNAQ, MAP2K5, PLA2G4F, ADCY3, ROCK2, PLCB1, CACNB2, MYLK2, NFATC2, GUCY1A2, NPPA, GUCY1B3, GUCY1A3, PRKAB1, ADCY7, NFATC3, RYR1, PRKCG, CACNG7, PIK3R5, CACNG4, EGFR, ITPR1, CACNA1D, ITPR3, NFATC1, ADCY9, PRKCB, CACNG3, RYR2, CAMK2G, KCNJ5
AMPK signaling pathway	ADIPOQ, PIK3CB, PPP2R3A, CAB39, CREB3L2, PPARGC1A, TBC1D1, SCD5, RAB8A, INSR, PPP2CA, PPP2R2A, PPP2R5E, RAB10, TSC1, CAB39L, FOXO1, PFKFB3, PFKP, PCK1, HNF4A, AKT3, MTOR, PRKAB1, ACACB, ACACA, STRADA, RPTOR, PPP2R5C, TSC2, PDPK1, PPP2CB
Aldosterone-regulated sodium reabsorption	ATP1B3, PIK3CB, ATP1A4, ATP1A2, INSR, ATP1B1, PRKCG, NEDD4L, SLC9A3R2, PDPK1, SCNN1B, PRKCB, KCNJ1
Calcium signaling pathway	PDE1A, PRKACB, ADCY1, ITPR2, CAMK2D, PDGFRA, CACNA1A, HTR4, GNA14, GNAQ, ITPKA, TACR1, ADCY3, CACNA1B, HTR2A, PLCB1, MYLK2, STIM1, P2RX3, CACNA1E, SLC25A31, ATP2A2, PLCG2, ADCY7, RYR1, ITPKC, SLC8A2, SPHK2, PRKCG, NOS2, CACNA1G, EGFR, ITPR1, CACNA1D, ATP2B2, ITPR3, GNAL, ADCY9, PRKCB, ORAI2, HTR7, RYR2, CAMK2G, ATP2B3, PHKA1, PHKA2
GnRH signaling pathway	PRKACB, ADCY1, ITPR2, CAMK2D, MAPK10, HBEGF, GNAQ, MAP3K4, PLA2G4F, SOS1, ADCY3, PLCB1, FSHB, ADCY7, PLD2, MAP2K4, MAP3K3, GRB2, EGFR, ITPR1, CACNA1D, ITPR3, ADCY9, PRKCB, CAMK2G

Ras signaling pathway	PAK2, RASA2, PIK3CB, PRKACB, KITLG, GRIN2B, FGF6, FGF23, NFKB1, PDGFRA, FGF5, MAPK10, FGFR3, INSR, GNG7, FGFR4, EFNA2, FGF1, NTRK2, MLLT4, RASGRP1, PLA2G4F, GNG2, ZAP70, SOS1, ABL1, FLT1, RASA3, ANGPT4, PDGFD, AKT3, GNB1, KSR2, PLCG2, PAK4, PRKCG, KSR1, PLD2, GRB2, FGF18, EGFR, SYNGAP1, EXOC2, RALBP1, PRKCB, HTR7, FGFR2, IKBKB, ETS1, RASGRP2, IKBKG, PAK3
Metabolic pathways	GART, ATP5J, CPOX, ST3GAL6, ATP6V1A, UMPS, DGKG, PLCH1, GYG1, PIK3CB, PDE9A, LSS, HLCS, PDE1A, PDE11A, GAD1, CERS6, GALNT13, LCT, CPS1, CHPF, EXTL1, MAN1C1, HMGCL, SDHB, ADCY10, UCK2, PI4KB, PRUNE1, DPYD, KYAT3, PIGK, PDE4B, SCP2, TXNDC12, CTPS1, TRIT1, AGXT, ISPD, PIK3CG, NT5C3A, ADCY1, AASS, TBXAS1, AGK, GSTK1, TPK1, CHPF2, NDUFA12, GALNT6, CYP27B1, HAL, PAH, ST8SIA1, MGST1, ATP6V1E1, NAGA, NDST3, NDST4, UGT8, ELOVL6, HADH, CYP2U1, ADH7, GBA3, UGDH, PPAT, SCD5, IDUA, BST1, QDPR, ISYNA1, PGLS, CERS4, ACSBG2, CHSY3, HSD17B4, PDE6A, NDST1, GALNT10, MAT2B, GALNTL6, EXTL3, HACD4, GLDC, GDA, PSAT1, B4GALT1, SPTLC1, ALDOB, ECHDC1, AK9, ARG1, SYNJ2, AGPAT4, GCNT4, ARSB, BHMT, ADPGK, ITPKA, PLA2G4F, PYGL, CA12, MTHFD1, FUT8, GALNT16, POMT2, SPTLC2, INPP4A, MGAT4A, MTHFD2, HAAO, ACYP2, ST3GAL5, GGCX, UGP2, GALNT14, ADCY3, ATP6V1C2, AK1, ASS1, POMT1, AK8, SARDH, UAP1L1, DGKH, MTMR6, PCCA, PLCB1, DHTKD1, PFKFB3, PFKP, UCKL1, PCK1, LPIN3, PIGT, ACOT8, CYP24A1, PYCRL, NAPRT, ST3GAL1, CA8, RDH10, EXT1, GUCY1A2, ACAT1, PAFAH1B2, TREH, GALNT18, SMPD1, RRM1, INPPL1, PDE2A, CAT, APIP, HSD17B12, EXT2, ACAD8, ETNK2, EPRS, KMO, PLCH2, NADK, INPP4B, MGST2, GUCY1B3, GUCY1A3, AACS, ATP6V0A2, ACACB, MTMR3, UPB1, PI4KA, SMPD4, PLCG2, ACSF3, ADCY7, SMPD3, ITPKC, SPHK2, MTMR4, ACACA, NOS2, PIGS, PLD2, ADPRM, SHMT1, PHOSPHO1, ACLY, TK1, ACOX1, AK6, PLPP1, NDUFS4, MOCS2, CHSY1, CERS3, MAN2A2, CYP1A2, STT3B, GLB1, XYLB, SUCLG2, FHIT, GMPPB, QARS, HMGCLL1, GCLC, GNMT, PLA2G7, ELOVL5, GPLD1, B4GALT6, LPIN2, ACAA2, PIGN, HAGH, ADCY9, PMM2, XYLT1, ACSM1, NDUFAB1, WBSCR17, PAPSS2, CYP26C1, PDE6C, NT5C2, ENO4, ACADSB, ASAH1, MTMR7, TUSC3, GPAT4, POMK, HGSNAT, CSGALNACT1, GALNT2, MTR, HKDC1, SGPL1, MAT1A, ME3, ALG8, PC, NADSYN1, TALDO1, B4GALNT4, OCRL, IDS, MTM1, MTMR1, GUCY2F, EBP, MAOA, OTC, PCYT1B, CTPS2, PIGA
Thyroid hormone signaling pathway	ATP1B3, PIK3CB, ITGAV, WNT4, ATP1A4, ATP1A2, PRKACB, SLC16A10, ESR1, FOXO1, PLCB1, NCOA2, AKT3, ATP1B1, MTOR, MED13L, PLCG2, PRKCG, MED13, NCOR1, MED24, THRA, TSC2, PDPK1, PRKCB, THRB

HIF-1 signaling pathway	PIK3CB, CAMK2D, NFKB1, INSR, IFNGR1, FLT1, PFKFB3, ANGPT4, AKT3, NPPA, MTOR, PLCG2, PRKCG, NOS2, STAT3, EGLN3, EGFR, BCL2, PRKCB, ENO4, HKDC1, CAMK2G, CYBB
Fatty acid metabolism	SCP2, ELOVL6, HADH, SCD5, ACSBG2, HSD17B4, HACD4, ACAT1, HSD17B12, ACACA, ACOX1, ELOVL5, ACAA2, ACADSB
PI3K-Akt signaling pathway	ITGB5, PIK3CB, PPP2R3A, COL6A6, ITGAV, FN1, COL4A3, LPAR3, JAK1, COL6A3, CDK6, PIK3CG, LAMB1, CREB3L2, KITLG, EIF4B, FGF6, FGF23, NFKB1, SPP1, PDGFRA, FGF5, FGFR3, INSR, GNG7, FGFR4, EFNA2, PPP2CA, FGF1, PPP2R2A, NTRK2, GNG2, PPP2R5E, SOS1, TSC1, FLT1, COL4A1, COL4A2, LAMA5, PCK1, ANGPT4, PTK2, IL7, YWHAZ, PDGFD, AKT3, MTOR, GNB1, LAMB3, PIK3R5, CSF3, RPTOR, GRB2, FGF18, ITGA1, OSMR, PPP2R5C, EGFR, ITGA9, LAMB2, PHLPP1, BCL2, TSC2, PDPK1, YWHAG, FGFR2, PPP2CB, IKBKB, IKBKG, COL4A6, COL4A5, LPAR4
Thyroid hormone synthesis	ATP1B3, ATP1A4, ATP1A2, PRKACB, ADCY1, CREB3L2, ITPR2, CANX, GNAQ, ADCY3, PLCB1, TG, ATP1B1, ADCY7, PRKCG, ITPR1, ITPR3, ADCY9, PRKCB
NF-kappa B signaling pathway	ERC1, NFKB1, TICAM1, PIAS4, CCL21, ZAP70, TNFSF11, PRKCQ, LY96, ATM, PLCG2, CARD14, MALT1, TNFRSF11A, BCL2, PRKCB, CARD11, IKBKB, PIDD1, CD40LG, IKBKG, EDA, EDA2R
Biosynthesis of unsaturated fatty acids	SCP2, ELOVL6, SCD5, HSD17B4, HACD4, HSD17B12, ACOT7, ACOX1, ELOVL5
Signaling pathways regulating pluripotency of stem cells	PIK3CB, ACVR1, WNT4, JAK1, WNT2, BMPR1B, SMARCAD1, FGFR3, WNT9A, FGFR4, ONECUT1, ESRRB, MEIS1, SMAD9, AKT3, ZFHX3, STAT3, GRB2, AXIN2, IL6ST, WNT7A, JARID2, SMAD4, AXIN1, PCGF5, FGFR2, KAT6A, BMPR1A
B cell receptor signaling pathway	PIK3CB, VAV3, NFKB1, SOS1, NFATC2, INPPL1, AKT3, PLCG2, NFATC3, NFKBIB, GRB2, NFATC1, MALT1, PRKCB, CARD11, IKBKB, IKBKG
Glucagon signaling pathway	PRKACB, CREB3L2, ITPR2, PPARA, CAMK2D, PPARGC1A, GNAQ, PYGL, FOXO1, PLCB1, PCK1, SIK2, AKT3, PRKAB1, ACACB, ACACA, ITPR1, ITPR3, CAMK2G, PHKA1, PHKA2
Biosynthesis of amino acids	CPS1, PAH, MAT2B, PSAT1, ALDOB, ARG1, ASS1, PFKP, PYCRL, SHMT1, ENO4, MTR, MAT1A, PC, TALDO1, OTC
Adipocytokine signaling pathway	ADIPOQ, PPARA, NFKB1, PPARGC1A, MAPK10, ACSBG2, PRKCQ, PCK1, AKT3, TNFRSF1B, MTOR, PRKAB1, ACACB, NFKBIB, STAT3, IKBKB, IKBKG
Carbohydrate digestion and absorption	ATP1B3, PIK3CB, LCT, ATP1A4, ATP1A2, AKT3, ATP1B1, CACNA1D, PRKCB, HKDC1
ErbB signaling pathway	PAK2, PIK3CB, CAMK2D, MAPK10, HBEGF, SOS1, ABL1, PTK2, AKT3, MTOR, PLCG2, PAK4, PRKCG, MAP2K4, GRB2, EGFR, PRKCB, CAMK2G, PAK3

Supplemental table 3.5 Biological processes enrichment of DMRs found to be overlapping genes.

Pathway	Total	Expected	Hits	P.Value	FDR
Regulation of transcription from RNA polymerase II promoter	498	95.6	158	5.60E-12	3.70E-09
Fatty acid oxidation	292	56	97	5.95E-09	1.97E-06
Cytokine-mediated signaling pathway	62	11.9	31	4.35E-08	9.60E-06
Lipid biosynthetic process	374	71.8	112	2.39E-07	3.96E-05
Androgen receptor signaling pathway	54	10.4	26	1.35E-06	1.54E-04
Positive regulation of T cell proliferation	126	24.2	47	1.40E-06	1.54E-04
Protein targeting to membrane	374	71.8	105	1.43E-05	1.35E-03
Regulation of binding	83	15.9	32	3.04E-05	2.49E-03
Fatty acid biosynthetic process	87	16.7	33	3.39E-05	2.49E-03
Anatomical structure morphogenesis	91	17.5	33	9.57E-05	5.84E-03
Calcium ion transport	80	15.3	30	9.71E-05	5.84E-03
Regulation of cell growth	43	8.25	19	1.53E-04	8.47E-03
Behavior	48	9.21	20	2.74E-04	1.39E-02
Carbohydrate transport	146	28	45	4.76E-04	2.25E-02
Response to hypoxia	105	20.1	34	8.61E-04	3.80E-02
Cellular lipid metabolic process	13	2.49	8	9.26E-04	3.83E-02
Lipid homeostasis	35	6.72	15	1.11E-03	4.31E-02
Mitotic spindle organization	88	16.9	29	1.48E-03	5.43E-02
Striated muscle contraction	20	3.84	10	1.85E-03	5.95E-02
Oligosaccharide metabolic process	17	3.26	9	1.89E-03	5.95E-02
Lipid catabolic process	17	3.26	9	1.89E-03	5.95E-02
Microtubule-based movement	106	20.3	33	2.13E-03	6.25E-02
Regulation of immune response	90	17.3	29	2.17E-03	6.25E-02
Endosomal transport	71	13.6	24	2.50E-03	6.66E-02
Lipid metabolic process	99	19	31	2.58E-03	6.66E-02
Regulation of cell adhesion	87	16.7	28	2.62E-03	6.66E-02
Regulation of I-kappaB kinase/NF-kappaB cascade	15	2.88	8	3.21E-03	7.87E-02
Immune response	28	5.37	12	3.43E-03	8.12E-02
Protein processing	46	8.83	17	3.63E-03	8.30E-02
Lysosomal transport	62	11.9	21	4.40E-03	9.71E-02
Microtubule_based process	36	6.91	14	4.73E-03	1.01E-01
Humoral immune response	48	9.21	17	5.99E-03	1.24E-01
Cellular response to stimulus	52	9.98	18	6.25E-03	1.25E-01
Fatty acid metabolic process	64	12.3	21	6.65E-03	1.30E-01
Regulation of MAP kinase activity	3	0.576	3	7.06E-03	1.33E-01
Epithelial cell differentiation	34	6.52	13	7.55E-03	1.39E-01
Smooth muscle contraction	91	17.5	27	1.04E-02	1.84E-01

Regulation of mitosis	108	20.7	31	1.05E-02	1.84E-01
Establishment of localization	21	4.03	9	1.09E-02	1.85E-01
Monocarboxylic acid transport	67	12.9	21	1.17E-02	1.94E-01
Microtubule organizing center organization	52	9.98	17	1.44E-02	2.30E-01
ER to Golgi vesicle-mediated transport	6	1.15	4	1.46E-02	2.30E-01
Protein targeting to mitochondrion	69	13.2	21	1.65E-02	2.55E-01
RNA biosynthetic process	26	4.99	10	1.75E-02	2.63E-01
MRNA processing	19	3.65	8	1.82E-02	2.68E-01
Macromolecule biosynthetic process	252	48.4	62	1.88E-02	2.71E-01
ER-nucleus signaling pathway	4	0.767	3	2.42E-02	3.40E-01
Inflammatory response	80	15.3	23	2.47E-02	3.41E-01
Regulation of DNA binding	47	9.02	15	2.59E-02	3.49E-01
Interferon-gamma production	10	1.92	5	2.77E-02	3.58E-01
Positive regulation of protein secretion	10	1.92	5	2.77E-02	3.58E-01
Regulation of body fluid levels	24	4.6	9	2.81E-02	3.58E-01
Tyrosine phosphorylation of STAT protein	232	44.5	56	3.47E-02	4.20E-01
M phase of mitotic cell cycle	57	10.9	17	3.52E-02	4.20E-01
Protein export from nucleus	995	191	213	3.53E-02	4.20E-01
Response to carbohydrate stimulus	53	10.2	16	3.62E-02	4.20E-01
Positive regulation of MAP kinase activity	2	0.384	2	3.68E-02	4.20E-01
Regulation of JNK cascade	2	0.384	2	3.68E-02	4.20E-01
Regulation of translational initiation	41	7.87	13	3.85E-02	4.28E-01
Striated muscle tissue development	37	7.1	12	3.88E-02	4.28E-01
Positive regulation of cysteine type endopeptidase activity involved in apoptotic process	42	8.06	13	4.63E-02	4.96E-01
RNA export from nucleus	26	4.99	9	4.68E-02	4.96E-01
CAMP-mediated signaling	34	6.52	11	4.76E-02	4.96E-01
Spindle organization	59	11.3	17	4.79E-02	4.96E-01

Supplemental table 3.6 All genes involved in selected enriched biological processes from DMRs found to be overlapping genes.

Pathway	Genes
Lipid biosynthetic process	AK1, FGFR3, GRK5, IKBKB, PRKCG, PRKG1, ROCK2, PDGFRA, PI4KA, PRKACB, PRKCB, ADRBK1, CSNK1A1, PI4KB, FGFR4, ACVR1, EIF2AK2, BMPR1A, FGFR2, BMPR1B, PFKFB3, EGFR, INSR, FLT1, RPS6KA5, MERTK, ZAP70, AK9, TK1, TEC, CLK3, NTRK2, PRKCQ, PTK2, PRKCE, PFKP, NLK, MAP4K3, IP6K2, STAT3, RPS6KA3, GAK, LMTK2, TYK2, GRK4, PRKDC, NADK, PAK2, XYLB, EPHB4, EPHA8, PIK3CB, RPS6KA2, ADPGK, DGKG, EPHA4, ITK, NEK10, NOL9, SPEG, SMG1, ATM, CSNK1G1, STK33, MAP3K15, PIK3CG, BLK, CAMK2D, SPHK2, ETNK2, MYLK2, RPS6KA1, IP6K1, UCKL1, ITPKC, MAST3, RIPK2, PAK3, ACVRL1, TGFBR2, MAST2, LRRK2, DCLK2, PAPSS2, FER, ADCK3, JAK1, DGKH, MAPK10, PRKD3, EPHA7, PLK2, SIK2, PAK4, KALRN, ABL1, UCK2, MAP3K6, MOB1A, HKDC1, ITPKA, CDK10, CIT, TPK1, AKT3, MTOR, EPHA6, EPHA5, MAP2K6, AK6, PIK3C2G
Positive regulation of protein secretion	HRG, EGFR, PRKCE, PDGFD, EPHA7
Positive regulation of T cell proliferation	FGFR3, GRK5, CAMK2G, PDGFRA, FGFR4, EIF2S1, EIF2AK2, FGFR2, EGFR, INSR, FLT1, ZAP70, CLK3, ULK3, PTK2, WNK2, NLK, MAP3K3, LMTK2, PAK2, TRPM7, EPHB4, NEK6, EPHA8, MINK1, EPHA4, SMG1, DAPK3, STK10, ATM, STK33, CAMK2D, MYLK2, DDR2, TAOK3, LRRK2, MYO3A, FER, STK26, PKDCC, SIK2, TNIK, DAPK1, ABL1, CLK1, ULK2, MTOR
Inflammatory response	PDGFRA, EGFR, INSR, FLT1, ZAP70, TEC, NTRK2, TYK2, LCP2, GFRA2, EPHB4, EPHA8, EPHA4, ITK, AHI1, BLK, DDR2, FER, JAK1, EPHA7, EPHA6, EPHA5

Regulation of transcription from RNA polymerase II promoter	APP, FGFR3, GRK5, IKBKB, PRKCG, PRKG1, ROCK2, ADAM10, CAMK2G, GUCY2F, PDGFRA, PRKACB, PRKCB, ST3GAL1, ADRBK1, CSNK1A1, FGFR4, ACVR1, EIF2AK2, BMPR1A, CASK, FGFR2, BMPR1B, CSNK1G3, EGFR, INSR, BIRC5, FLT1, AKAP13, RPS6KA5, MERTK, ZAP70, GAS6, TEC, CLK3, NTRK2, NEK3, PRKCQ, ULK3, PTK2, WNK1, WNK2, SIK3, PRKCE, NLK, PIK3R4, MAP4K3, MAP3K3, RSRC1, FASTK, RPS6KA3, GAK, CDK6, MAP3K4, HIPK1, LMTK2, TYK2, GRK4, FASTKD3, PAK2, EIF2AK4, LRRK1, POMK, TRPM7, PTK7, STRADA, IRAK2, SCYL1, PHKA2, EPHB4, NEK6, EPHA8, PIK3CB, RPS6KA2, MINK1, EPHA4, ITK, NEK10, SPEG, ALPK1, DAPK3, STK10, MAP2K4, ATM, CSNK1G1, STK33, MAP3K15, PIK3CG, NEK8, BLK, CAMK2D, CDKL2, ADCK1, MYLK2, DDR2, RPS6KA1, PRKAB1, MAST3, RIPK2, PAK3, ACVRL1, TAOK3, FAM20C, TLK2, TGFBR2, MAP3K8, MAST2, LRRK2, MYO3A, DCLK2, FER, JAK1, TLK1, MAPK10, HUNK, BRSK1, TRIO, PRKD3, EPHA7, HIPK3, STK26, PLK2, PKDCC, SIK2, TNIK, CDK17, PAK4, KALRN, SMAD9, DAPK1, ABL1, TTBK2, CLK1, PXK, MAP3K6, EIF2A, BMP2, KSR2, ULK2, NEK7, CDK10, CIT, PDGFB, RNASEL, AKT3, PTPRA, PDPK1, KSR1, MTOR, MAP2K5, EPHA6, PHKA1, EPHA5, IP6K3, WNK4, MAP2K6, PIK3C2G
Regulation of binding	FGFR3, PDGFRA, FGFR4, FGFR2, EGFR, INSR, FLT1, MERTK, ZAP70, TEC, CLK3, NTRK2, PTK2, EFEMP1, LMTK2, PTK7, EPHB4, EPHA8, EPHA4, DDR2, RIPK2, FER, EPHA7, PKDCC, ABI1, ABL1, CLK1, PDGFB, EPHA6, EPHA5, MAP2K6
Lipid homeostasis	MAP4K3, PAK2, STRADA, CCDC88C, MINK1, STK10, PAK3, TAOK3, MAP3K8, STK26, TNIK, PAK4, MAP3K6, MAP2K6
Lysosomal transport	BIRC5, SAE1, MAP4K3, CDK5RAP3, SDCBP, PAK2, STRADA, MINK1, DAPK3, STK10, PAK3, TAOK3, MAP3K8, TTC28, STK26, TNIK, PAK4, MAP3K6, AATF, MAP2K6
Protein processing	INSR, AXIN1, MAP4K3, CAB39, PAK2, STRADA, MINK1, STK10, KIF14, PAK3, TGFBR2, MAP3K8, STK26, TNIK, PAK4, PDGFB

Fatty acid oxidation	ZP4, GUCY1A3, MC1R, RGS7, RGS9, ADCY1, ADCY7, PRKCG, ROCK2, GUCY2F, PRKCB, GUCY1B3, RASA3, PLCB1, ARHGEF12, AKAP13, RPS6KA5, ZAP70, ARHGAP29, TEC, GNA13, ARHGEF4, PRKCQ, ARHGEF2, WNK1, PRKCE, NLK, CHN2, ASB7, MAP4K3, CHN1, WSB2, MAP3K3, PLCL2, ITSN1, SDCBP, RPS6KA3, ARHGEF11, NFATC1, CAB39, MAP3K4, RASGRP2, TYK2, LCP2, MYO9B, PLCH1, PLCH2, IRAK2, GNA12, ABR, RPS6KA2, DGKG, PDZD8, PREX2, DEPDC5, ADCY10, VAV3, ITK, ADCY9, DAPK3, UNC13A, RGS14, RASGRP1, RASA2, ARHGEF6, RPS6KA1, MAST3, TLK2, ADCY3, MAST2, LRRK2, ASB4, DCLK2, JAK1, RAPGEF4, TLK1, DGKH, HUNK, BRSK1, PRKD3, SIK2, TNIK, SMAD4, DAPK1, DEPDC1B, GUCY1A2, SOCS6, KSR2, DEPTOR, SH3BP5, CIT, PDPK1, KSR1, TULP4, DCDC2, PLCG2
Fatty acid biosynthetic process	, ITPR2, ITPR3, CAMK2G, CORO1A, PRKCB, CACNA1B, CACNA1G, CACNA1A, CACNG2, ITPR1, CACNB2, ANXA6, CACNA1E, CACNA1D, ATP2B3, MICU1, ATP2C2, TRPM7, SARAF, CACNG4, PKD2, CAMK2D, SLC8A2, RYR2, CACNG3, CYP27B1, CACNG7, TRPV4, ATP2A2, ATP2B2, RYR1, TRPM4
Oligosaccharide metabolic process	CACNA1A, CACNG2, NFASC, CACNG4, GRM7, SPTBN4, CACNG3, CACNG7
Regulation of cell adhesion	CHRND, GABRA3, GABRB1, CACNA1G, KCNMA1, KCNK2, CACNA1A, CACNG2, CHRNA1, NEDD4L, KIF5B, CNGA4, GABRP, GABRR1, GABRA5, KCNH6, GRIK3, LRRK2, GLRA2, KCNH5, ATP1A4, HCN1, GABRB2, GRIK2, HCN4, TRPM4, HCN2
Protein targeting to membrane	CYBB, CHRND, CLIC5, ITPR2, ITPR3, KCNA4, KCNQ3, SCN5A, ATP6V1A, GABRA3, GABRB1, SLC4A4, CACNA1B, CACNA1G, GUCY1B3, KCNMA1, KCNK2, CACNA1A, ATP5J, CACNG2, SLC4A1, ITPR1, CACNB2, ATP6V0A2, CHRNA1, CACNA1E, KCNN2, TRPC5, SLCO3A1, SLC5A10, CACNA1D, SLCO4C1, ATP2B3, KCNG2, WNK1, CLDN10, SLC26A11, MICU1, SLC4A9, CLCN4, CLCN7, TRPM7, ATP1A2, SARAF, CNGA4, GABRP, CACNG4, SLC22A1, ATP1B1, GABRR1, SLC12A6, KCNS1, SLC9A7, CLCN3, GABRA5, KCNK10, KCNAB1, KCNH6, SFXN5, GRIA1, PKD2, P2RX3, GRIK3, ATP1B3, SLC9A6, SCNN1B, SLCO5A1, SLC9A9, KCNB2, SLC9A4, RYR2, GLRA2, GRIN2B, KCNH5, HCN1, CACNG3, SLC5A11, KCNJ1, KCNB1, KCNK9, KCNA2, CACNG7, TRPV4, KCND2, ATP2A2, GABRB2, TRPM3, ATP2B2, KCNJ16, KCNIP4, SLC39A13, GRIK2, SLC39A11, SLC24A3, ASIC2, HCN4, ASIC3, SLC17A6, RYR1, TRPM4, HCN2, SCN10A, TRPM5, WNK4

Protein export from nucleus	AP3D1, ABCA4, TAP2, ABCC1, CHRND, CLIC5, IGF2R, ITPR2, ITPR3, KCNA4, KCNQ3, MYO1C, RAB3B, RLBP1, SCN5A, ATP6V1A, GABRA3, GABRB1, SLC4A4, SLC6A6, STXBP1, CACNA1B, CACNA1G, GUCY1B3, BICD2, KCNMA1, KCNK2, CACNA1A, ATP5J, CACNG2, SLC4A1, ITPR1, CACNB2, MTCH2, ATP6V0A2, SLC35A2, CHRNA1, CACNA1E, SYNE1, TRPC5, SLC6A1, SLCO3A1, FYTTD1, SLC5A10, ATG4A, CACNA1D, SLCO4C1, ABCA9, ATP2B3, SLC25A19, SEC24D, ABCA10, OSCP1, ABCA3, KCNG2, CLDN10, SLC25A17, MVB12A, SLC26A11, AP1G1, MICU1, SLC28A3, AFM, ABCC10, SLC4A9, NAPG, AP3M2, TMCO3, NUP93, SVOP, OSBP2, SLC2A6, ABCG1, SLC29A1, CLCN4, CHP1, SDCCAG3, SLC25A21, CLCN7, UNC50, SLC6A12, TRPM7, PEX5, ATP1A2, AP2A2, SARAF, SLC6A9, CNGA4, MICALL1, SLC25A45, DDX19A, GABRP, CACNG4, SLC22A1, SNX4, ATP1B1, SIL1, ABCC2, GABRR1, SLC15A1, SLC12A6, KCNS1, VPS35, ACTN4, SLC9A7, CLCN3, SLC23A3, RALBP1, GABRA5, PITPNM2, SLC7A11, KCNK10, KCNAB1, AQP6, KCNH6, SLC6A5, ABCC12, RHBDF1, GRIA1, PKD2, P2RX3, SLC26A8, ABCB11, KDELR2, SLC25A46, BBS4, SLC4A11, GRIK3, OSBPL5, SLC6A20, ATP1B3, SLC9A6, SCNN1B, DHX40, MTM1, ABCB7, G3BP1, CLVS2, SEC23B, SLCO5A1, ABCA1, SLC25A43, SLC9A9, KCNB2, SLC9A4, ABCA6, RYR2, GLRA2, GRIN2B, KCNH5, RAB7B, HCN1, SV2C, CACNG3, SLC16A13, ABCC8, SLC5A11, KCNJ1, VPS4B, SLC7A1, KCNB1, KCNK9, KCNA2, SLC6A7, CACNG7, TRPV4, KCND2, ATP2A2, GABRB2, AP1S3, TRPM3, JAKMIP1, SLC25A31, SYNPR, SLC2A13, STX8, DYNC1I1, ATP2B2, KCNJ16, SNX24, SEC61A2, KCNIP4, SLC25A26, SLC39A13, GRIK2, SLC25A13, SLC39A11, RAB4A, SLC6A11, SLC9B2, PITPNA, SLC26A7, SLC44A2, ASIC2, ACBD3, HCN4, ASIC3, SLC25A53, SLC17A6, RAB8A, RYR1, TRPM4, HCN2, SCN10A, TRPM5, ABCA2, ABCA13, ABCC9, OSBPL1A, ABCB4, SLC6A19, SLC15A5
Lipid metabolic process	KCNA4, KCNQ3, KCNMA1, KCNK2, KCNN2, NSF, KCNG2, ATP1A2, CNGA4, ATP1B1, KCNS1, KCNAB1, KCNH6, ATP1B3, TSC1, KCNB2, KCNH5, ATP1A4, HCN1, ABCC8, KCNJ1, VPS4B, KCNB1, KCNA2, KCNJ5, KCND2, KCNJ16, KCNIP4, HCN4, HCN2, ABCC9
Fatty acid metabolic process	SCN5A, SLC4A4, SLC5A10, SLC13A2, ATP1A2, ATP1B1, SLC9A7, SLC4A11, ATP1B3, SLC9A6, SCNN1B, SLC9A9, SLC9A4, ATP1A4, SLC5A11, SLC9B2, ASIC2, HCN4, ASIC3, SLC17A6, SCN10A
Protein targeting to mitochondrion	TMCO3, ATP13A2, TRPM7, SLC22A1, ANO6, SLC9A7, P2RX3, ANO1, SLC9A6, STIM1, SLC9A9, SLC9A4, TRPM3, ATP7A, TMEM63C, SLC9B2, HCN4, CALHM3, ASIC3, TRPM4, TRPM5

Chapter 4: General Discussion

The aim of this PhD thesis was to characterize the variation of developmental competency in high-yielding cows when exposed to metabolically adverse conditions induced by negative energy balance during post-partum lactation in oocytes and embryos at the epigenetic level. To do so, a whole genome epigenetic profile was performed on oocytes collected from lactating cows experiencing negative energy balance at early and late post-partum time points as well as control heifers using next-generation sequencing. In order to investigate the role of oviductal metabolic conditions on embryo epigenetic plasticity, *in vivo* culture of embryo in the oviduct of metabolically profiled lactating cows and heifers was performed followed by whole genome bisulfite sequencing. It was found that, during early lactation, cows exhibit significant different metabolic status at different stages postpartum compared to heifers. From these metabolically divergent animals, a general hypomethylation was observed in genome features of oocytes during early postpartum when compared to mid postpartum cows and heifers. Inversely, overall genomic features were found to be hypermethylated in embryos from lactating cows compared to heifers. Further characterization of differentially methylated regions (DMRs) in the genome revealed highest variation DMRs in genes involved in DNA binding, embryo development and metabolic pathways in both oocytes and embryos. This chapter discusses the differences and similarities of both experiments.

4.1   Energy balance profiling

In the first experiment, result analysis demonstrated that oocytes are sensitive to metabolic conditions in a time specific manner, which results in different methylation profiles. Cows during week 5-6 postpartum had a significantly increased metabolic profile of NEFA and BHB compared to week 9-10, consistent with mid-postpartum recuperation of negative energy balance in lactating cows. Even though NEFA levels of mid-postpartum cows were

significantly different from heifers, such difference was not found in BHB. Since ketone bodies like BHB are a byproduct of incomplete fatty oxidation, it appears that NEFA might be a better indicator of overall lipomobilization and by extension negative energy balance in the cow, and overall metabolic variability between animals. A previous study found that a higher ratio of cows were experiencing lipomobilization through NEFA screening than cows experiencing ketosis with BHB screening, further supporting the idea that NEFA provide a broader indication of the lipomobilization physiological status than BHB reading (González et al., 2011).

We found a similar profile when investigating embryos cultured in cattle, where lactating cows exhibited averages of BHB over a lower threshold than observed in cows used for oocyte collection, while maintaining NEFA level above the original threshold. Additionally, one should note that while negative energy balance is a transient metabolic stress in the lactating cows, positive energy balance is not experienced in the same magnitude during lactation. As cows are differentially fed with concentrates based on their milk production, feed efficiency in the cow aims to reach a net balance of zero MJ/day, and so recuperation from negative energy balance is more accurate term than cows reaching positive energy balance. Accordingly, both set of cows selected at mid-postpartum points or time of transfer did not reach an average positive energy balance, but remained rather closer to net balance of zero MJ/day. Interestingly, some cows experienced immediate weight gain upon post-partum, which one could speculate they could be associated with an unperturbed metabolic profile. Although following these cows in the breeding program to assess their reproductive ability is crucial, investigating the oocyte methylation profile of such animals would expose the epigenome of gametes in animals resilient to this transient metabolic stress and could be used to improve animal selection. Nevertheless, coupled with body weight assessment and

energy balance profiling, all animals selected exhibited weight loss and negative energy balance prior to week 5 and had their metabolite significantly different from heifers at the time of collection and transfer, indicating different metabolic profiles in both cows used for oocyte and embryo collection.

4.2   Global methylation characterization

Upon selection of animals exhibiting metabolic stress, global methylation characterization was performed from sequencing data in oocytes and embryos. To assess variability of samples, PCA clustering of quantitation was performed. This PCA clustering showed heterogeneity between conditions and extreme variation within conditions of oocytes, where oocytes from mid postpartum and heifers were clustered together and early postpartum oocytes clustered far apart. In the case of embryos, PCA clustering of samples reveal a close relation between samples, even if samples from separate conditions did not overlap each other, indicative of smaller differences to be observed between conditions. Still, principal components accounted for 80% and 48% of the variation in oocytes and embryos, which suggest that single-cell sequencing may be a great avenue to investigate further variations within the conditions not accounted for in embryos. The characterization of methylation features looked similar across features in the genome and indicated an overall hypomethylation in early oocytes when comparing with late and heifer oocytes. Global methylation levels were found to be higher than some previously reported studies, ranging from ~50% and 53.8% in WGBS human oocytes of different developmental stages (Okae et al., 2014; Yu et al., 2017), ~30 to 40% in RRBS bovine oocytes (Jiang et al., 2018) and ~30% in WGBS bovine oocytes (Duan et al., 2019). While differences in RRBS CpG methylation levels of features have been shown to be lower than WGBS derived methylation, with as much as an 11% difference (Doherty and Couldrey, 2014), the

methylation levels observed in our oocytes do not account for such a high variation. As most of these studies filter out CpG sites with less than five reads, this could possibly remove some methylation information data still present in our analysis. Further sequencing of our samples to permit such filtering of coverage depth could further characterize the methylation status of these oocytes with respect to other findings in the literature. In embryos, methylation of features revealed a slight hypermethylation found in morulae grown in lactating cows when compared to heifers, which was further reflected in the number of hypermethylated DMRs compared to hypomethylated DMRs in lactating cows. The global average CpG methylation was constant with levels observed in literature, where morulae from lactating cows and heifers (33.1 and 31.3%, respectively) were hypermethylated compared to 8 cell embryos and similar to 16 cell embryos, early and compact morulae, indicative of post-embryonic genome activation methylation changes observable in our morulae (Duan et al., 2019; Jiang et al., 2018). Similarly, Jiang et al. report demethylation occurring from morula to blastocyst, suggesting that methylation levels are the highest at the morula stage. The results presented in this thesis seem to show that morulae grown in heifers can demethylate properly towards blastocyst stage methylation profile, with morulae grown in cows "lagging" behind to demethylate genes involved in proper development. Two mechanisms are thought to be involved in demethylation of genes upon fertilization, a post cellular division "passive" demethylation based on non-maintenance of epigenetic marks by DNMT1, and an active demethylation occurring through the T5-methylcytosine hydroxylases (TET) family of enzymes (Wu and Zhang, 2017). The investigation of the downregulation or inefficiency of such machinery in morulae grown under metabolic stress could be of great interest to shed some light into the pressure of lactation on basic molecular machinery involved in embryo development. Although the

methylation changes are somewhat smaller than observed in oocytes, our results suggest that growth in metabolically stressed conditions induces methylation changes across the genome. Nevertheless, methylations levels in genomic features were similar across all biological conditions in both oocytes and embryos, indicating smaller gene-specific changes. Even accounting for the variability of follicle size during collection and potential animal-specific variation in embryo transfer, the statistical filter post normalization found differentially methylated regions across conditions, indicating the deep variation occurring in these oocytes and embryos were induced by metabolic stress.

4.3   Pairwise comparison of differentially methylated regions

After derivation of DMRs through statistical testing, the highest variation of DMRs associated with genes was investigated. When combing through the list of the 20 highest methylation differences observed in DMRs of pairwise comparisons in conditions, most of the variation occurring in early postpartum oocytes was shared with both mid postpartum and heifer oocytes. 12 of the top 20 DMRs shared hyper- and hypomethylated states in both comparisons, indicative of the strong methylation changes occurring in early lactation metabolic stress. These genes were found to be most involved in structural integrity (ESPN, CRYBG1, FDPS, ITGB4 and LMNA) but also in the regulation of transcription (PAX 9, PRDM1 and MDFIC). As examples, Espin (ESPN) is a mammalian homolog of the Forked gene, which was involved in oocyte symmetry and polarization in the drosophila through organization of microtubules (Baskar et al., 2019). Similar genes were involved in the membrane composition of oocytes, where Farnesyl Diphosphate Synthase (FDPS) was previously found to modulate the cholesterol content of oocytes through oocyte-regulated crosstalk with cumulus cells in mice (Su et al., 2008). Laminin A (LMNA) was also found to mediate spindle assembly in mice oocytes and exhibit a dynamic localization during meiosis, further

supporting its role in oocyte structural integrity (Nikolova et al., 2017). Another structural gene which was differentially methylated in our samples was integrin beta-4 (ITGB4), of which its homologs have been characterized at the membrane surface of both pig and bovine ooplasm, demonstrating its role in sperm-oocyte interaction (Linfor and Berger, 2000; Pate et al., 2007).

When compared with embryos, only one of top 20 DMRs was found in all conditions (TTC7B) and one was found in late-postpartum when compared to heifer oocytes (AGAP1). The shared functional role of Tetratricopeptide Repeat Domain 7B TTC7B in oocytes and embryo remains vague, as its main function is to regulate plasma membrane identity through differential localization of phosphatidylinositol 4-phosphate (Nakatsu et al., 2012). As for GTP-Binding And GTPase-Activating Protein 1 AGAP1, its functional role is to modulate cell membrane signaling, while its shared role in oocytes and embryos remains to be identified (Xia et al., 2003). Even if the most differentially methylated regions in oocytes and embryos were not overlapping similar genes, top DMRs in embryo were also involved in cell structural integrity, with genes such as ADD1 and PALLD, which were found to be involved in cytoskeleton organization (Fukata et al., 1999; Royal et al., 2000), and BRCC3 to modulate spindle assembly and DNA damage response (Yan et al., 2015). While other genes were differentially methylated in oocytes involved in transcription, differentially methylated genes involved in cell differentiation and apoptosis were found in embryos grown in lactating cows. Genes like APIP and PROM1 were involved in cell death regulation and differentiation, and apoptosis suppression, respectively, (Kang et al., 2014; Takenobu et al., 2011). A previous study demonstrated that lower blastocyst developmental rate was observed in lactating versus non lactating cows, while embryos which reached blastocyst stage shared a similar cell number. These differentially methylated gene regions suggest that metabolic stress

induced by negative energy balance may impact structural integrity and increase apoptosis at the morula stage, which will eventually lower the blastocyst rate of lactating cows, as previously observed (Maillo et al., 2012). Further *in vitro* assessment of differential methylation of these structural genes through embryo culture in similar metabolic conditions occurring during negative energy balance and investigation of arrested embryos during culture is needed to confirm this proposition. Nevertheless, these results demonstrate that the 20 highest variations of DMRs found in oocytes and embryos are involved in transcriptional and structural integrity changes, which can compromise their ability to undergo fertilization and preserve proper blastocyst quality through maintenance of high cell number.

4.4   Targeted gene differentially methylated region analysis

Though investigating the most differentially methylated regions provides interesting insights on the impact of metabolic stress on developmental capacity, observation of functionally relevant DMRs that have lower methylation changes is pertinent to describe changes occurring in the epigenome during this stress. Observation of differential methylation of known bovine imprints in early lactating oocytes suggested once more that metabolic stress is associated with epigenetic changes influencing developmental competency. First, multiple DMRs were found to be differentially methylated in early lactating oocytes, suggesting an impaired acquisition of these genes during early lactation. A single DMR overlapping the upstream region of MEST, coincidentally overlapping a CpG island, shows that metabolic stress impairs the methylation status of regulatory regions of those genes even before fertilization occurred in all metabolic conditions. Additionally, multiple DMRs were found to overlap CpG rich-regions, further indicating differential imprint regulation acquisition prior to fertilization in oocytes. Interestingly, we found DMRs in embryos to overlap three

imprinted bovine genes that were also previously found in our oocytes sample group, which

demonstrates a reoccurrence of this sensitivity to stress at both developmental stages. One

DMR overlapping the MEST gene was found upstream of the coding sequence, overlapping

the previously found DMR in all oocyte conditions. Similarly, DMRs found in the PEG10 gene

and IGF2R were also overlapping CpG islands in embryos, indicative of potential control

regions in their expression similar to what was found in oocytes. Functionally, MEST is

involved in placental growth and is necessary for embryo development, as the embryonic

knock-out is lethal in mice (Kobayashi et al., 1997). A previous report demonstrated that

MEST was downregulated upon embryo genomic activation in cattle (Jiang et al., 2015), yet

our DMR was found to be hypermethylated in early postpartum oocytes and embryos from

lactating cows, suggesting its increased inaccessibility by the protein machinery in oocytes

and embryos grown in lactating cows during metabolic stress. Further validation of this

region with regards to gene expression could give us better understanding of the topography

of epigenetic control occurring in this gene. Techniques interrogating chromatin accessibility

in the cell like chromatin immunoprecipitation or nucleosome occupancy assays coupled

with high throughput sequencing have been shown to function at the single cell scale

(Grosselin 2019; Small 2017) and could detail the relation between DNA methylation of

genes identified in this project and their availability to transcription machinery. In a similar

vein, modulation of these DMRs through gene editing through CRISPR-Cas9 electroporation

of oocytes followed by fertilization and monitoring subsequent development could also

elucidate the functional relevance of aforementioned regions with regards to expression and

developemental competency in bovine gametes.

Additionally, DMRs found in embryos were also found to be involved in embryo implantation

as some genes have been shown to take crucial part in blastocyst development, as well as

trophoblast invasion (Jamsai et al., 2013; Kim et al., 2015). Coupled with genes which were found to be differentially methylated in processes like vascularization and placentation, we propose that morulae grown in metabolically challenged conditions are unable to properly establish the blood nutrient network necessary for elongation and successful implantation. Previous works have shown hormone-caused modulations of genes involved in implantation occurring at the morula-to-blastocyst transition having maintained changes to the implantation stage (Tríbulo et al., 2019). In a similar fashion, supplementation of effectors of these genes found in our study during *in vitro* embryo development could confirm their role during trophoblast invasion and their impact in later developmental stages, and subsequently their role in developmental competency.

4.5 Pathway enrichment analysis

After looking at the most differentially methylated regions and genes functionally established in developmental competency, gene ontology enriched pathway analysis was performed to investigate the functional role of the ensemble of DMRs found in oocytes and embryo derived in different metabolic status. The highest variation of changes occurred in early lactation oocytes, of which the resulting pathway analysis was the most enriched of all three conditions, with 10,114 genes involved. A lower magnitude of changes was found in embryos, with 13,383 DMRs found, of which 3,260 overlapped genes in pathway analysis. Interestingly, similar parts of pathways were found in different conditions in oocytes, with genes involved in fatty acid metabolism, oxidation, and degradation respectively. As fatty acid uptake is known to impair early embryo development and is associated with developmental arrest (Haggarty, 2010), investigating the expression of genes involved in these pathways during *in vitro* maturation would shed some light on the homeostasis occurring when exposed to different fatty acids concentration, as well as fatty acid uptake by

the maturing oocyte. While this has been performed to some extent (Desmet et al., 2016), array based methods are limited in the number of probes they offer, hence giving a partial picture of methylation and transcriptomic profile. Using whole genome bisulfite sequencing, we listed *in vivo* observed genes that may not have been observed through such a platform and that could easily be further validated using deep-sequencing methods such as pyrosequencing. Additionally, multiple genes were found to be involved in meiosis and structural like microtubule organization changes prior to fertilization. As no morphological changes between early lactation and late-postpartum oocytes have been observed (Matoba et al., 2012), we suggest that metabolic stress might induce expression changes involved in structural integrity of oocytes that do not show readily observable phenotypic changes. Other metabolic stresses, such as subclinical mastitis, have been shown to influence ovulation rates, oocyte quality, and cumulus cell transcription (Santos et al., 2018). *In vitro* DMRs-associated validation of gene expression through transcriptomic profiling or protein immunofluorescence of oocytes exposed to metabolic stress could give us a better assessment than oocyte quality grading into small morphological changes occurring under metabolic stresses.

Moreover, DMRs found in mid-portpartum oocytes could be involved in oocyte quality, as Lysophosphatidic acid receptor 1 was shown to be differentially methylated and was previously indirectly associated with follistatin expression through supplementation of LPA during maturation (Boruszewska et al., 2014). As heat stress has been shown to modulate follicular follistatin expression in dominant follicles (Vanselow et al., 2016), our findings suggest a cross-talk happening in follicles at the time of first service, improving chances of fertilization compared to early postpartum oocytes. These combined features show a specific regulating region of gene expression that should be investigated further. Deletion of

these DMRs found in the oocytes using gene editing technologies and subsequent expression validation of these DMRs in in vitro matured oocytes under similar metabolic conditions could further explain their role in oocyte maturation. Additional work using supplementation of LPAR1 inhibitor, AM095, during *in vitro* maturation of oocytes and assessment of post-fertilization development of these oocytes could further link its role to developmental competency (Lee et al., 2019). Nevertheless, whole genome bisulfite sequencing of oocytes from lactating cows revealed that metabolic stress induced by negative energy balance is involved with methylation changes of genes both involved in bovine metabolism and embryo development.

DMRs found between morulae grown in lactating cows and heifers were involved in embryo development and implantation. Furthermore, pathway enrichment analysis revealed genes differentially methylated in fatty acid metabolism as was previously described in our oocyte sample analysis. As the initial fertilization of these embryos occurs in serum free media, this suggests that the oviductal milieu of lactating cows might supplement adverse effectors modulating fatty acid metabolism compared to heifers. Moreover, DMRs found were involved in cell proliferation, apoptosis and NF-kB signaling pathway, giving new candidates genes for metabolically induced changes in response to oxidative stress not yet described in the bovine embryo development. Other genes were involved in hormonal response during embryo development, notably EGFR and INSR, which have both been shown to modulate embryo developmental rates and higher cell number *in vitro* through the use of their respective ligand (Laskowski et al., 2016; Lonergan et al., 1996; Sakagami et al., 2012). While validation of the expression of these genes must be completed to confirm the findings described above, we propose that growth of embryo under metabolically stress oviduct

conditions results in modulation of gene expression through differential epigenetic profile maintenance in early embryo development.

While we listed above the functional pathway involved in metabolic induced stress on the epigenetic profile of oocytes and embryos separately, comparison of both epigenetic profiles was also performed to give a broader view of the overall sustained changes induced by adverse growth conditions. While methylation level comparison is unadvisable, since the epigenetic profile undergoes extensive demethylation upon fertilization, comparisons of genes differentially methylated at the time of observation in oocytes and embryos gave us a qualitative insight on maintained functional changes under metabolic stress. As such, comparative gene lists of early postpartum oocytes and embryos revealed 2,515 shared genes to be involved in lipid metabolic processes and cellular lipid metabolic processes (7 processes; 261 genes), gene expression and regulation of gene expression (3 processes; 183 genes) and structural processes like cytoskeleton organization and microtubule and spindle based processes (4 processes; 85 genes) (Annex Table 4.1). Such magnitude of shared changes was not observed in mid postpartum or heifer oocytes with morulae with 109 and 216 shared genes, respectively. Of these shared differences between early postpartum oocytes and morulae, 24 genes were found to be involved in more than 5 biological processes (Annex Table 4.2). Of these, 3 isoforms of protein kinase PAK (PAK2/3/4) were differentially methylated, and some PAKs are crucial for oocyte maturation completion in xenopus oocytes (Cau et al., 2000) as well as vascularization and extra-embryonic tissue development in mice, causing embryo lethality in some cases (Kelly and Chernoff, 2012; Tian et al., 2009). While the functional link of these 24 genes during oocyte maturation and embryo development remains to be elucidated, they indicate sustained methylation sensitivity in both gametes and embryos of similar biological processes. Gene expression

validation of genes described is required to validate methylation similarities observed between oocytes and embryos, nevertheless these genes could provide novel candidates for how sustained epigenetic changes might impact developmental competency at both stages of development in cattle.

4.6   Conclusion and future perspectives

In conclusion, this PhD thesis aimed to characterize the epigenetic changes induced by negative energy balance during lactation on genes that could be involved in developmental competency. From this, we demonstrated that early lactation induces great variations in the methylation profile of oocytes, with changes in genes involved in metabolic pathways, fatty acid metabolism, and also focal adhesion and regulation of actin skeleton, implying structural modulation in oocytes. Moreover, differential methylation in bovine imprinted genes of early lactation oocytes suggests an improper acquisition of methylation status of genes necessary for proper embryo development. As for embryo, our results demonstrate that embryos grown in metabolically stressed conditions were overall hypermethylated when compared to their heifer counterparts, with differentially methylated regions in genes involved in embryo development, placentation, trophoblast invasion, oxidative stress response and hormone modulated signaling. Taken together, these findings will help us design new studies to better understand the relationship between metabolic stress and epigenetic plasticity during development. First, gene expression validation of regions found both in oocytes and morulae should be performed to confirm these findings. To do this, a great body of research has been focusing on mimicking early embryo development, from insights on cattle oviductal fluid composition to 3-D culture of the oviduct and uterine wall (Chen et al., 2017). *In vitro* modeling of the metabolic stressors experienced during early lactation is of great value to confirm repeatability of such findings. Similarly, *in vitro*

functional studies of these genes in early embryo development using gene editing technology such as CRISPR-Cas 9 mediated deletion or interference could identify which of these candidate genes are crucial checkpoints of developmental competency in bovine. In this same vein, functional investigation of these genes at further developmental stages might confirm changes observed at earlier times during embryo development and what gene must be properly activated in a stage-sensitive manner. Ultimately, this future work should aim to link epigenetic sensitivity to metabolic stress to observable phenotypes during development.

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Chapter 5: General summary

Bovine reproductive processes like folliculogenesis, oocyte maturation, and embryo development and implantation are complex regulated biological processes that can be perturbed by multiple ailments, whether they are physiological, immunological or metabolic. All gene products and metabolites needed for successful completion of these processes must be present in a time and tissue specific manner. It falls to reason that the lactating cow is a great model to investigate the impact of metabolism on the plasticity of gametes and embryos, and of reproductive subfertility as a whole. A growing body of evidence has demonstrated transcriptional differences and epigenetic sensitivity of certain genes occuring in gametes and embryos exposed to adverse metabolite concentrations associated with negative energy balance induced metabolic stress during lactation. Since this transient metabolic state has been linked to lower reproductive function both at the follicular and uterine level, with diminished estrous capability and early embryo losses, the investigation genome-wide modulation of epigenetic marks should give us insights into what genes are associated with these reproductive limitations. As such, this PhD thesis has followed 2 possible avenues from which the lactating cow may exhibit lower fertility, either through the production of epigenetically unstable oocytes exposed to adverse follicular conditions (chapter 2) or through the sensitivity of the epigenetic profile of embryos grown in oviducts exposed to adverse metabolic conditions (chapter 3).

In chapter 2, a total of 30 Holstein-Friesian multiparous cows were monitored following calving for energy balance profiling. Starting at week 5 post-partum, lactating cows had their oocytes collected from follicles sized between 3-8 mm, with ovum pick-up together with blood collection until week 10 post-partum. Oocyte and blood was also collected from 8 nulliparous cyclic heifers for 5 consecutive weeks. Oocytes were pooled according to cow weight loss, energy balance and blood metabolite profiling into 3 distinct phenotypes; early

post-partum (11 cows; week 5-6 dpp) lactation associated with negative energy balance, mid post-partum (7 cows; week 9-10 dpp) lactation associated with recuperation from negative energy balance and metabolically unchallenged nulliparous heifers (8 heifers) as controls. Oocyte were then submitted to whole genome bisulfite library preparation and sequencing using next generation sequencing technology. Data analysis of methylated sequences with calculations of differentially methylated regions was done using Bismark alignment tool with the SeqMonk mapped sequence analysis tool. Results demonstrated that selected early lactation cows exhibited significantly different energy balance values at time of collection (-19.75 ± 3.5 MJ/day) compared to mid postpartum cows (-0.76 ± 3.15 MJ/day), indicative of different physiological status ($p < 0.001$). Metabolic analysis revealed a similar pattern, where non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB) were found to be significantly different in early lactating cows than in mid post-partum animals ($p < 0.05$). Similarly, early and mid-postpartum animals had significantly different concentrations of NEFA than nulliparous heifers ($p < 0.05$), but not for BHB, where only early lactation had significantly different concentrations than nulliparous heifers ($p < 0.05$). DNA methylation quantitation demonstrated that early postpartum oocytes showed lower methylation levels of all genomic features when compared to the mid postpartum and heifers groups, which was further supported by methylation of gene bodies of imprints, where early post-partum exhibited significantly different levels of methylation in 12 and 11 bovine imprinted genes when compared to mid postpartum and heifers, respectively. A more detailed comparison of the genome revealed 32, 990 significant differentially methylated regions (DMRs) exclusive to early post-partum oocytes overlapping gene body regions, considerably higher than mid-postpartum (265 DMRs) and heifers (705 DMRs). Functional pathway analysis of these DMRs revealed fatty acid metabolism, oxidation and degradation pathways were involved in early

postpartum, mid postpartum and heifer oocytes, respectively, suggesting that different

sections of fatty acid metabolism might be solicitated during lactation. Early post-partum

oocytes revealed DMRs in genes known to impact cumulus-oocyte cross-talk (ACO1-ACO2)

and genes involved microtubules organization during meiosis (AKT2/3). Similarly, early post-

partum oocytes showed DMRs overlapping 10 bovine imprinted genes to be involved in

development. Of these, a DMR overlapping MEST and a CpG island was found to be

differentially methylated in all three conditions suggesting its sensitivity to metabolic stress.

Therefore, differentially methylated regions exclusive to metabolic status supports the

notion that metabolic stress can impact epigenetic status in oocytes, as well as in genes that

could govern developmental competency of these oocytes.

In chapter 3, embryos cultured *in vivo* to morula stage from *in vitro* fertilized zygotes were

collected from lactating and nulliparous cows, to investigate the impact of metabolically

stressed oviductal environment on epigenetic status of embryos. Similarly to chapter 2,

lactating cows were profiled for weight and energy balance, but were also estrous

synchronized following uterine involution. Zygotes were transferred in lactating cows (n=26)

and heifers (n=5) and collected through uterine flushing at day 7 post-transfer while blood

collection was performed at both time points for metabolic assessment. Morulae from

respective animals were pooled in groups of 5, and blood metabolic profiling was performed

similarly as chapter 2. 4 cows and 5 heifers were selected for further epigenetic processing

of morulae done similarly in chapter 2. Selected lactating cows demonstrated an average

energy balance of -25.7 ± 5.6 MJ/day prior to week five post-partum. This level decreased to

-7.8 ± 6.0 MJ/day (n=3, S.E.M.) at the time of *in vivo* embryo culture, indicating a slow

recuperation of energy balance status. As such, concentrations of NEFA and BHB were

significantly different at the time of transfer and flush in lactating cows than heifers (p <

0.05) further confirming the metabolic differences showcased by lactating cows. Consequently, 13,383 DMRs were found in lactating cows compared to heifers, with 11,612 being hypermethylated and 1,771 hypomethylated. Of these, only 5 were found overlapping imprinted genes like PEG10, MEST and IGF2R. A further 13 DMRs were found overlapping annotated genes involved in embryo implantation, like PCSK5 with 3 DMRs. Pathway analysis of DMRs revealed genes involved in metabolic, signaling and pluripotency pathways as well as processes like lipid homeostasis and fatty acid oxidation. Interestingly, genes involved in placentation and trophoblast invasion were found to be differentially methylated, shedding light on the relation of metabolic stress and early embryo loss.

In summary, the work presented in this thesis give new insights on the influence of post-calving lactation induced metabolic stressed cows on the proper epigenetic maintenance in oocytes, prior to ovulation, and embryo, prior to implantation. Differentially methylated regions found in genes involved in maturation processes of oocytes and trophoblast development support the notion that metabolism perturbs reproduction efficiency of the lactating cow both in gametes and embryos. These epigenetic signatures can pave the way into identifying what gene expression profiles are needed for proper developmental competency during early lactation, and recoup early embryo loss occurring in high yield dairy cows. The findings described in this project bring us closer to understanding the link between transient physiological stresses and their impact on reproduction, and ultimately, its potential place within the developmental origins of health and disease

Annex

Annex table 1 List of biological processes enriched by shared differentially methylated regions between early postpartum oocytes specific DMRs and embryos DMRs.

Pathway	Gene ontology term	Total	Expected	Hits	P.Value	FDR
Regulation of transcription from RNA polymerase II promoter	GO:0006357	498	73.5	132	2.13E-12	1.41E-09
Cytokine_mediated signaling pathway	GO:0019221	62	9.15	30	3.37E-10	1.12E-07
Fatty acid oxidation	GO:0019395	292	43.1	83	8.69E-10	1.92E-07
Positive regulation of T cell proliferation	GO:0042102	126	18.6	43	3.90E-08	6.45E-06
Protein targeting to membrane	GO:0006612	374	55.2	90	9.32E-07	1.21E-04
Regulation of binding	GO:0051098	83	12.3	30	1.10E-06	1.21E-04
Lipid biosynthetic process	GO:0008610	374	55.2	89	1.77E-06	1.67E-04
Calcium ion transport	GO:0006816	80	11.8	27	1.62E-05	1.34E-03
Fatty acid biosynthetic process	GO:0006633	87	12.8	27	8.64E-05	6.35E-03
Endosomal transport	GO:0016197	71	10.5	23	1.38E-04	8.42E-03
Cellular lipid metabolic process	GO:0044255	13	1.92	8	1.43E-04	8.42E-03
Androgen receptor signaling pathway	GO:0030521	54	7.97	19	1.53E-04	8.42E-03
Anatomical structure morphogenesis	GO:0009653	91	13.4	27	2.00E-04	1.02E-02
Lipid homeostasis	GO:0055088	35	5.17	14	2.41E-04	1.14E-02
Oligosaccharide metabolic process	GO:0009311	17	2.51	9	2.57E-04	1.14E-02
Mitotic spindle organization	GO:0007052	88	13	26	2.81E-04	1.16E-02
Lysosomal transport	GO:0007041	62	9.15	20	3.93E-04	1.53E-02
Smooth muscle contraction	GO:0006939	91	13.4	26	5.00E-04	1.79E-02
Regulation of I-kappaB kinase/NF-kappaB cascade	GO:0043122	15	2.21	8	5.40E-04	1.79E-02
Microtubule_based movement	GO:0007018	106	15.7	29	5.40E-04	1.79E-02
Protein processing	GO:0016485	46	6.79	16	5.78E-04	1.82E-02
Lipid metabolic process	GO:0006629	99	14.6	27	8.72E-04	2.62E-02
Response to hypoxia	GO:0001666	105	15.5	28	1.03E-03	2.81E-02
Protein export from nucleus	GO:0006611	995	147	181	1.05E-03	2.81E-02
Carbohydrate transport	GO:0008643	146	21.6	36	1.06E-03	2.81E-02

Immune response	GO:0006955	28	4.13	11	1.33E-03	3.39E-02
Lipid catabolic process	GO:0016042	17	2.51	8	1.55E-03	3.80E-02
Fatty acid metabolic process	GO:0006631	64	9.45	19	1.67E-03	3.96E-02
Regulation of cell growth	GO:0001558	43	6.35	14	2.58E-03	5.88E-02
Behavior	GO:0007610	48	7.09	15	2.89E-03	6.37E-02
Regulation of cell adhesion	GO:0030155	87	12.8	23	3.15E-03	6.65E-02
Regulation of MAP kinase activity	GO:0043405	3	0.443	3	3.21E-03	6.65E-02
Microtubule-based process	GO:0007017	36	5.32	12	4.12E-03	8.27E-02
Striated muscle contraction	GO:0006941	20	2.95	8	5.32E-03	1.04E-01
Microtubule organizing center organization	GO:0031023	52	7.68	15	6.66E-03	1.26E-01
Epithelial cell differentiation	GO:0030855	34	5.02	11	7.62E-03	1.40E-01
Tyrosine phosphorylation of STAT protein	GO:0007260	232	34.3	48	8.50E-03	1.52E-01
Regulation of immune response	GO:0050776	90	13.3	22	1.03E-02	1.79E-01
Inflammatory response	GO:0006954	80	11.8	20	1.09E-02	1.85E-01
Cell maturation	GO:0048469	426	62.9	80	1.21E-02	2.00E-01
Regulation of translational initiation	GO:0006446	41	6.05	12	1.29E-02	2.08E-01
Regulation of mitosis	GO:0007088	108	15.9	25	1.32E-02	2.08E-01
Monocarboxylic acid transport	GO:0015718	67	9.89	17	1.56E-02	2.36E-01
Inorganic anion transport	GO:0015698	115	17	26	1.57E-02	2.36E-01
M phase of mitotic cell cycle	GO:0000087	57	8.42	15	1.61E-02	2.36E-01
Cell morphogenesis involved in differentiation	GO:0000904	63	9.3	16	1.84E-02	2.65E-01
Humoral immune response	GO:0006959	48	7.09	13	1.91E-02	2.68E-01
Intrinsic apoptotic signaling pathway in response to DNA damage	GO:0008630	90	13.3	21	2.00E-02	2.68E-01
Protein targeting to mitochondrion	GO:0006626	69	10.2	17	2.07E-02	2.68E-01
RRNA processing	GO:0006364	186	27.5	38	2.14E-02	2.68E-01
Positive regulation of MAP kinase activity	GO:0043406	2	0.295	2	2.18E-02	2.68E-01

Regulation of JNK cascade	GO:0046328	2	0.295	2	2.18E-02	2.68E-01
Cytoplasm organization	GO:0007028	16	2.36	6	2.18E-02	2.68E-01
Spindle organization	GO:0007051	59	8.71	15	2.19E-02	2.68E-01
Chemical homeostasis	GO:0048878	5	0.738	3	2.54E-02	3.06E-01
Protein modification process	GO:0036211	21	3.1	7	2.64E-02	3.11E-01
Protein modification by small protein conjugation	GO:0032446	45	6.64	12	2.67E-02	3.11E-01
Viral reproduction	GO:0050792	17	2.51	6	2.96E-02	3.38E-01
Negative regulation of biosynthetic process	GO:0009890	13	1.92	5	3.20E-02	3.59E-01
Cellular response to stimulus	GO:0051716	52	7.68	13	3.58E-02	3.95E-01
Regulation of DNA binding	GO:0051101	47	6.94	12	3.68E-02	3.99E-01
Striated muscle tissue development	GO:0014706	37	5.46	10	3.78E-02	4.04E-01
Negative regulation of MAP kinase activity	GO:0043407	23	3.4	7	4.28E-02	4.48E-01
Monovalent inorganic cation transport	GO:0015672	43	6.35	11	4.39E-02	4.48E-01
Translational initiation	GO:0006413	202	29.8	39	4.48E-02	4.48E-01
Pattern specification process	GO:0007389	59	8.71	14	4.52E-02	4.48E-01
Leukocyte migration	GO:0050900	6	0.886	3	4.54E-02	4.48E-01
Centrosome organization	GO:0007098	10	1.48	4	4.74E-02	4.62E-01
Meiotic cell cycle	GO:0051321	65	9.6	15	4.87E-02	4.66E-01

Annex table 2 All genes involved in selected enriched pathways from DMRs found to be overlapping genes shared between early postpartum specific DMRs and embryos.

Pathway	Genes
Regulation of transcription from RNA polymerase II promoter	APP, FGFR3, GRK5, IKBKB, PRKCG, PRKG1, ADAM10, CAMK2G, GUCY2F, PDGFRA, PRKACB, PRKCB, ST3GAL1, ADRBK1, CSNK1A1, FGFR4, EIF2AK2, CASK, FGFR2, CSNK1G3, EGFR, INSR, BIRC5, FLT1, AKAP13, RPS6KA5, MERTK, ZAP70, GAS6, CLK3, NTRK2, NEK3, PRKCQ, ULK3, PTK2, WNK1, SIK3, PRKCE, NLK, PIK3R4, MAP3K3, RSRC1, FASTK, RPS6KA3, GAK, CDK6, MAP3K4, LMTK2, PAK2, EIF2AK4, LRRK1, PTK7, STRADA, IRAK2, SCYL1, PHKA2, EPHB4, NEK6, EPHA8, RPS6KA2, MINK1, EPHA4, ITK, NEK10, SPEG, ALPK1, DAPK3, STK10, MAP2K4, CSNK1G1, STK33, MAP3K15, PIK3CG, NEK8, BLK, CDKL2, ADCK1, MYLK2, DDR2, RPS6KA1, PRKAB1, MAST3, RIPK2, PAK3, ACVRL1, TAOK3, FAM20C, TLK2, TGFBR2, MAP3K8, MAST2, LRRK2, MYO3A, FER, HUNK, BRSK1, TRIO, PRKD3, EPHA7, STK26, PKDCC, SIK2, TNIK, PAK4, KALRN, SMAD9, DAPK1, ABL1, TTBK2, CLK1, PXK, MAP3K6, BMP2, KSR2, ULK2, NEK7, CDK10, CIT, PDGFB, AKT3, PDPK1, KSR1, MTOR, MAP2K5, EPHA6, PHKA1, EPHA5, IP6K3, WNK4, MAP2K6, PIK3C2G
Cytokine-mediated signaling pathway	ARHGEF12, AKAP13, ARHGEF4, ARHGEF2, ALS2CL, ITSN1, ARHGEF11, FGD2, FGD3, MYO9B, ABR, PREX2, VAV3, ARHGEF7, PLEKHG1, ARHGEF37, ARHGEF10L, ARHGEF26, FARP1, ARHGEF6, SOS1, ARHGEF9, TRIO, FGD6, FGD5, KALRN, FGD1, ARHGEF10, MCF2L2
Fatty acid oxidation	GUCY1A3, MC1R, RGS7, RGS9, ADCY1, ADCY7, PRKCG, GUCY2F, PRKCB, GUCY1B3, RASA3, PLCB1, ARHGEF12, AKAP13, RPS6KA5, ZAP70, ARHGAP29, GNA13, ARHGEF4, PRKCQ, ARHGEF2, WNK1, PRKCE, NLK, CHN2, CHN1, WSB2, MAP3K3, PLCL2, ITSN1, SDCBP, RPS6KA3, ARHGEF11, NFATC1, CAB39, MAP3K4, RASGRP2, LCP2, MYO9B, PLCH1, PLCH2, IRAK2, GNA12, ABR, RPS6KA2, DGKG, PDZD8, PREX2, DEPDC5, ADCY10, VAV3, ITK, ADCY9, DAPK3, UNC13A, RGS14, RASGRP1, ARHGEF6, RPS6KA1, MAST3, TLK2, MAST2, LRRK2, ASB4, RAPGEF4, HUNK, BRSK1, PRKD3, SIK2, TNIK, SMAD4, DAPK1, GUCY1A2, KSR2, DEPTOR, SH3BP5, CIT, PDPK1, KSR1, TULP4, DCDC2, PLCG2

Positive regulation of T cell proliferation	FGFR3, GRK5, CAMK2G, PDGFRA, FGFR4, EIF2S1, EIF2AK2, FGFR2, EGFR, INSR, FLT1, ZAP70, CLK3, ULK3, PTK2, NLK, MAP3K3, LMTK2, PAK2, EPHB4, NEK6, EPHA8, MINK1, EPHA4, SMG1, DAPK3, STK10, STK33, MYLK2, DDR2, TAOK3, LRRK2, MYO3A, FER, STK26, PKDCC, SIK2, TNIK, DAPK1, ABL1, CLK1, ULK2, MTOR
Protein targeting to membrane	CYBB, CHRND, CLIC5, ITPR2, ITPR3, KCNA4, KCNQ3, SCN5A, ATP6V1A, GABRA3, GABRB1, SLC4A4, CACNA1B, CACNA1G, GUCY1B3, KCNMA1, KCNK2, CACNA1A, ATP5J, CACNG2, SLC4A1, ITPR1, CACNB2, ATP6V0A2, CHRNA1, CACNA1E, KCNN2, TRPC5, SLCO3A1, SLC5A10, CACNA1D, ATP2B3, KCNG2, WNK1, CLDN10, SLC26A11, MICU1, SLC4A9, CLCN4, ATP1A2, SARAF, CACNG4, SLC22A1, ATP1B1, SLC12A6, KCNS1, SLC9A7, CLCN3, GABRA5, KCNK10, KCNAB1, KCNH6, SFXN5, GRIA1, P2RX3, GRIK3, SLC9A6, SCNN1B, SLC9A9, KCNB2, SLC9A4, GLRA2, GRIN2B, KCNH5, HCN1, CACNG3, KCNJ1, KCNB1, KCNK9, KCNA2, CACNG7, TRPV4, KCND2, GABRB2, TRPM3, ATP2B2, KCNJ16, KCNIP4, GRIK2, SLC39A11, SLC24A3, ASIC2, HCN4, ASIC3, SLC17A6, RYR1, HCN2, SCN10A, TRPM5, WNK4
Regulation of binding	FGFR3, PDGFRA, FGFR4, FGFR2, EGFR, INSR, FLT1, MERTK, ZAP70, CLK3, NTRK2, PTK2, EFEMP1, LMTK2, PTK7, EPHB4, EPHA8, EPHA4, DDR2, RIPK2, FER, EPHA7, PKDCC, ABL1, CLK1, PDGFB, EPHA6, EPHA5, MAP2K6
Lipid biosynthetic process	AK1, FGFR3, GRK5, IKBKB, PRKCG, PRKG1, PDGFRA, PI4KA, PRKACB, PRKCB, ADRBK1, CSNK1A1, FGFR4, EIF2AK2, FGFR2, PFKFB3, EGFR, INSR, FLT1, RPS6KA5, MERTK, ZAP70, AK9, CLK3, NTRK2, PRKCQ, PTK2, PRKCE, PFKP, NLK, IP6K2, STAT3, RPS6KA3, GAK, LMTK2, PRKDC, PAK2, XYLB, EPHB4, EPHA8, RPS6KA2, DGKG, EPHA4, ITK, NEK10, NOL9, SPEG, SMG1, CSNK1G1, STK33, MAP3K15, PIK3CG, BLK, SPHK2, ETNK2, MYLK2, RPS6KA1, UCKL1, ITPKC, MAST3, RIPK2, PAK3, ACVRL1, TGFBR2, MAST2, LRRK2, FER, ADCK3, PRKD3, EPHA7, SIK2, PAK4, KALRN, ABL1, UCK2, MAP3K6, MOB1A, HKDC1, ITPKA, CDK10, CIT, TPK1, AKT3, MTOR, EPHA6, EPHA5, MAP2K6, PIK3C2G
Calcium ion transport	UGT8, BFSP2, CAPZB, PTK2, CAMSAP1, ELMO2, SH3KBP1, ARHGAP10, ZFHX3, MAEA, CAP2, PDZD8, PACSIN1, PSTPIP2, MICAL3, EPB41L3, DPYSL2, BRWD3, DST, MAST2, DOCK1, TNIK, ZFHX4, ABLIM3, FMNL2, FGD1

Fatty acid biosynthetic process	ITPR2, ITPR3, CAMK2G, CORO1A, PRKCB, CACNA1B, CACNA1G, CACNA1A, CACNG2, ITPR1, CACNB2, ANXA6, CACNA1E, CACNA1D, ATP2B3, MICU1, ATP2C2, SARAF, CACNG4, SLC8A2, CACNG3, CYP27B1, CACNG7, TRPV4, ATP2B2, RYR1
Endosomal transport	APP, FSHR, CNTN1, CAPZB, CHL1, CAMSAP1, MICALL2, VAPA, MICALL1, EPHA8, LAMB1, LAMB2, UBE4B, EFHD1, GDPD5, PQBP1, STMN2, PTPRM, BICDL1, MECP2, SEMA7A, ATXN10, MATN2
Cellular lipid metabolic process	TSC2, RALGAPA2, GARNL3, SIPA1L2, AMOT, ARHGAP26, RAP1GAP2, SIPA1L1
Androgen receptor signaling pathway	PRKG1, ADAP1, RASA3, PYCARD, NTRK2, CHN1, EPHA4, VAV3, RASGRP1, TSC1, OCRL, RAB3GAP2, IQGAP2, SIPA1L1, SYNGAP1, FGD1, MTOR, EPHA5
Anatomical structure morphogenesis	CORO1A, CAPZB, ELMO1, SDCBP, PAK2, DAAM1, FLII, STRADA, ABR, IQSEC1, MTSS1, DIAPH3, EPB41L5, PAK3, AMOT, ARHGAP26, SSH1, PAK4, ABL1, FLNB, ABLIM3, PALLD, DAAM2, FMNL2, MOB2, FGD1
Lipid homeostasis	PAK2, STRADA, CCDC88C, MINK1, STK10, PAK3, TAOK3, MAP3K8, STK26, TNIK, PAK4, MAP3K6, MAP2K6
Oligosaccharide metabolic process	CACNA1A, CACNG2, NFASC, CACNG4, GRM7, SPTBN4, CACNG3, CACNG7
Mitotic spindle organization	GAP43, PLXNA4, CRMP1, CHL1, SLIT1, SEMA3C, NFASC, APBB2, KIF5B, EPHA8, UNC5D, LAMB2, EPHA4, NTN1, CDH4, DPYSL2, NRCAM, KLF7, EXT1, SMAD4, BMP7, EFNA2, TTC8, MATN2, EPHA5, SLIT2
Lysosomal transport	BIRC5, SAE1, CDK5RAP3, SDCBP, PAK2, STRADA, MINK1, DAPK3, STK10, PAK3, TAOK3, MAP3K8, TTC28, STK26, TNIK, PAK4, MAP3K6, AATF, MAP2K6
Smooth muscle contraction	DSC1, CDH17, PCDH17, CDH13, CLSTN1, PCDH7, DCHS1, CADM2, PCDHA12, CELSR3, PCDH19, CDH4, FREM2, CADM1, PTPRM, ROBO1, CDH7, DSCAML1, CDH20, CDH22, CD226, KIRREL3, FAT4, CRTAM, CDH11
Regulation of I-kappaB kinase/NF-kappaB cascade	CACNA1A, CHN1, SSH2, EPHA4, WNT7A, KIF13B, SSH1, SIPA1L1,
Microtubule-based movement	PRKCG, GABRA3, GABRB1, HCRTR1, CACNA1B, CACNA1G, CACNA1A, HTR4, CACNB2, CACNA1E, SLC6A1, SSTR5, SDCBP, SLC6A12, GIPC1, GABRA5, DLGAP1, UNC13A, HCRTR2, GRIA1, P2RX3, GRM7, NRXN2, HTR7, MECP2, GABRB2, GRIK2, SLC6A11
Protein processing	INSR, AXIN1, CAB39, PAK2, STRADA, MINK1, STK10, KIF14, PAK3, TGFBR2, MAP3K8, STK26, TNIK, PAK4, PDGFB,

Lipid metabolic process	KCNA4, KCNQ3, KCNMA1, KCNK2, KCNN2, KCNG2, ATP1A2, ATP1B1, KCNS1, KCNAB1, KCNH6, TSC1, KCNB2, KCNH5, ATP1A4, HCN1, ABCC8, KCNJ1, KCNB1, KCNA2, KCNJ5, KCND2, KCNJ16, KCNIP4, HCN4, HCN2, ABCC9
Response to hypoxia	C3, FGF1, EGFR, INSR, AXIN1, GAS6, PTK2, AXIN2, RSPO1, GPRC5B, WDFY2, BRAT1, RASGRP1, FBXO18, LRRK2, EPHA7, ATG14, TNIK, ABL1, SEMA7A, FAM129A, BMP2, SEMA4D, PRR5, MOB2, MTOR, PELI2
Protein export from nucleus	AP3D1, ABCA4, TAP2, ABCC1, CHRND, CLIC5, IGF2R, ITPR2, ITPR3, KCNA4, KCNQ3, MYO1C, RAB3B, RLBP1, SCN5A, ATP6V1A, GABRA3, GABRB1, SLC4A4, SLC6A6, STXBP1, CACNA1B, CACNA1G, GUCY1B3, BICD2, KCNMA1, KCNK2, CACNA1A, ATP5J, CACNG2, SLC4A1, ITPR1, CACNB2, ATP6V0A2, SLC35A2, CHRNA1, CACNA1E, SYNE1, TRPC5, SLC6A1, SLCO3A1, SLC5A10, ATG4A, CACNA1D, ABCA9, ATP2B3, SLC25A19, SEC24D, ABCA10, OSCP1, ABCA3, KCNG2, CLDN10, SLC25A17, MVB12A, SLC26A11, AP1G1, MICU1, SLC28A3, AFM, ABCC10, SLC4A9, NAPG, TMCO3, NUP93, SVOP, OSBP2, SLC2A6, ABCG1, SLC29A1, CLCN4, SDCCAG3, SLC25A21, UNC50, SLC6A12, PEX5, ATP1A2, SARAF, SLC6A9, MICALL1, SLC25A45, DDX19A, CACNG4, SLC22A1, SNX4, ATP1B1, SIL1, ABCC2, SLC15A1, SLC12A6, KCNS1, ACTN4, SLC9A7, CLCN3, SLC23A3, GABRA5, PITPNM2, SLC7A11, KCNK10, KCNAB1, AQP6, KCNH6, SLC6A5, ABCC12, RHBDF1, GRIA1, P2RX3, SLC26A8, ABCB11, KDELR2, SLC25A46, SLC4A11, GRIK3, OSBPL5, SLC6A20, SLC9A6, SCNN1B, MTM1, ABCB7, G3BP1, CLVS2, ABCA1, SLC25A43, SLC9A9, KCNB2, SLC9A4, ABCA6, GLRA2, GRIN2B, KCNH5, RAB7B, HCN1, SV2C, CACNG3, SLC16A13, ABCC8, KCNJ1, SLC7A1, KCNB1, KCNK9, KCNA2, SLC6A7, CACNG7, TRPV4, KCND2, GABRB2, TRPM3, JAKMIP1, SLC25A31, SYNPR, STX8, DYNC1I1, ATP2B2, KCNJ16, SNX24, KCNIP4, GRIK2, SLC25A13, SLC39A11, SLC6A11, SLC9B2, PITPNA, SLC26A7, SLC44A2, ASIC2, HCN4, ASIC3, SLC25A53, SLC17A6, RAB8A, RYR1, HCN2, SCN10A, TRPM5, ABCA2, ABCA13, ABCC9, OSBPL1A, ABCB4, SLC6A19
Carbohydrate transport	ITGAV, CORO1A, PDGFRA, FGFR4, FLT1, ZAP70, PTK2, LAMA5, ELMO2, PTPRK, ELMO1, SH3KBP1, PAK2, PTK7, STRADA, DCHS1, TNS3, MYO18A, WWC1, VAV3, HBEGF, PSTPIP2, ADAMTS12, RHBDF1, NFATC2, GOLPH3, BRAT1, PAK3, FER, DOCK1, LRRC16A, PAK4, ABL1, LCP1, PDPK1

Immune response	PIGR, EGFR, PTK2, GAREM1, EFEMP1, HBEGF, KIF16B, SOS1, FAM83B, ABL1, PDPK1
Lipid catabolic process	AR, PPARA, PPARD, STAT3, HNF4A, THRA, RORA, THRB
Fatty acid metabolic process	SCN5A, SLC4A4, SLC5A10, SLC13A2, ATP1A2, ATP1B1, SLC9A7, SLC4A11, SLC9A6, SCNN1B, SLC9A9, SLC9A4, ATP1A4, SLC9B2, ASIC2, HCN4, ASIC3, SLC17A6, SCN10A

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