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ANIMAL NUTRITION:
NEW STRATEGIES FOR NUTRITIONAL OPTIMIZATION

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LIST OF ABBREVIATIONS

AA	Amino acids
AMBP	Alpha-1-microglobulin/bikunin precursor
BCS	Body condition score
BHBA	β-hydroxybutyrate
CACT	Carnitine- acylcarnitine translocase
CE	Cholesteryl ester
CPT1	Carnitine palmitoyltransferase I
CPT2	Carnitine palmitoyltransferase II
DM	Dry matter
DMI	Dry matter intake
DRTC	Days relative to calving
ER	Endoplasmic reticulum
FA	Fatty acids
G6PC	Glucose-6-phosphatase
HMBS	Hydroxymethylbilane synthase
HSL	Hormone-sensitive lipase
LCAS	Long-chain acyl-CoA synthetase
LCFA	Long-chain fatty acids
LDL	Low density lipoprotein
MCM	Methylmalonyl-CoA mutase
MTTP	Microsomal triglyceride transfer protein
MY	Milk yield (MY)
NEB	Negative energy balance
NEFA	Nonesterified fatty acids
NE_L	Net energy for lactation
NFC	Non-fiber carbohydrate
OAA	Oxaloacetate
PC	Pyruvate carboxylase
PCK	Phosphoenolpyruvate carboxykinase
PCK1	Cytosolic phosphoenolpyruvate carboxykinase
PCK2	Mitochondrial phosphoenolpyruvate carboxykinase
PCoAC	Mitochondrial propionyl-CoA carboxylase

PEP	Phosphoenolpyruvate
PGF2α	Prolactin
PKA	Protein kinase A
PLIN	Perilipin
PPARA	Peroxisome proliferator-activated receptors α
PPARB	Peroxisome proliferator-activated receptors β
PPARG	Peroxisome proliferator-activated receptors y
PPARs	Peroxisome proliferator-activated receptors
PPLIN	Phosphorylated
PtdCho	Phosphatidylcholine
RPC	Rumen-protected choline
SAM	S-adenosyl methionine
SCFA	Short- chain fatty acids
TAGs	Triglycerides
TCA	Tricarboxylic acid cycle
TMR	Total mixed ratio
VFA	Volatile fatty acid
VLDL	Very low density lipoproteins
18S	18S ribosomal

ABSTRACT

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This dissertation focuses on the impact of nutrition to modulate and optimize milk production, blood metabolites and liver metabolism. Specifically, the main aim was to elucidate the effects of rumen protected choline (RPC) supplementation to lactating dairy cows on production, metabolic health and hepatic gene expression, for which two different studies were performed.

The first study, a meta-analysis of the effect of RPC supplementation on milk yield (MY), nonesterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA) in lactating dairy cows was performed in order to obtain an overall view of the effect of rumen protected choline. Rumen protected choline supplementation has been reported to have a positive effect on milk yield and metabolic health in lactating dairy cows. In light of this, a meta-analysis has been performed in order to elucidate the effect of choline chloride supplemented as RPC on both milk yield and selected blood metabolites. For this purpose, 21 peer-reviewed articles published from 1985 to 2016 were selected. This systematic review was carried out to evaluate the effects of RPC supplementation on MY, NEFA, and BHBA. Results obtained showed positive effects of RPC supplementation on MY in lactating dairy cows. The studies selected for this meta- analysis supplemented choline chloride in a range from 6.25 to 50g/d and milk production increase averaged 2.14±1.86 kg/d. Meta-regression on the dose- response relationship between dietary RPC and MY was significant. When NEFA and BHBA were evaluated, no overall effect was detected. Moderator analysis revealed that all outcomes, MY, NEFA and BHBA, were not significantly affected by the mode of choline supplementation (blended vs. topdressed).

In the second study, the mechanism beyond the metabolic changes due to RPC was investigated, with emphasis on hepatic gluconeogenesis, lipid oxidation and transport that occur during the transition period; particularly, the interaction of RPC and dietary energy concentration was tested and the expression of selected hepatic genes was analyzed. Hepatic gluconeogenic and oxidation genes were

studied during the transition from late pregnancy to early lactation dependent upon RPC supplementation during the periparturient period, and prepartum energy intake. Controlling prepartum energy intake or supplementing RPC during the periparturient period, are two strategies to optimize hepatic metabolic function. At -48 days relative to calving (DRTC), multiparous Holstein cows were assigned to either a controlled (1.40 Mcal of NEL/kg DM; CE) or high (1.63 Mcal NEL/kg DM; HE) energy prepartum diet with or without RPC (top-dressed daily from -21 to +21 DRTC). Postpartum diets only differed in addition vs absence of RPC. Liver tissue biopsy samples were collected at -14, +7, +14, and +21 DRTC for RNA isolation and cDNA generation (n=16/treatment). Six genes involved in gluconeogenesis, lipid oxidation and lipid transportation were selected. Results obtained indicate that an increase in the expression of pyruvate carboxylase mRNA was reduced in cows receiving RPC after calving, which suggests that RPC had improved energy status and carbohydrate metabolism in the liver and reduced the need for pyruvate carboxylase. RPC supplementation decreased PCK1 in HE+RPC probably due to higher oxidation of increased circulating NEFA that translated to increased oxidative capacity of the TCA cycle. Moreover, this change can help to maintain the oxaloacetate pool. No effect of RPC supplementation was observed with regard to CPT1A, which is involved in fatty acid transportation. On the other hand, PPARA and MTTP were affected by RPC treatment, indicating that RPC supplementation may have modulated FA transport and oxidation.

In light of the two studies performed, RPC can improve milk production through lactation. Additionally, RPC supplementation may support and increase hepatic oxidative capacity.

CHAPTER 1. LITERATURE REVIEW

Introduction

The world cattle inventory in 2016 was at 971.482 million head. According to the Food and Agriculture Organization (FAO) there were 271m dairy cows in the world in 2013, and approximately 54% of the world's dairy cows were raised by the top 10 countries. In the last three decades, world milk production has increased by more than 50 percent, from 500 million tonnes in 1983 to 769 million tonnes in 2013 (source FaoStat). World milk production is forecasted to grow by 1.6 percent in 2016 to reach 816 million tonnes (Hallam et al., 2016). Further details about the world dairy market are reported in Table 1.1.

Table 1.1 World dairy market at glance (Hallam et al., 2016).

Year	**WORLD BALANCE**		**SUPPLY AND DEMAND INDICATORS Per caput food consumption:**		**FAO DAIRY PRICE INDEX (2002-2004=100)**
	Total milk production	Total trade	World (kg/yr)	Trade share of prod. (%)	Jan-May over 2015 and 2016
2014	789.1	72.1	108.6	9.1	224
2015 estim	802.8	72.2	109.2	9	160
2016 f'cast	816	73.2	109.8	9	135
Change: 2016 over 2015	1.6	1.5	0.5	-0.2	-23.6

Over the last thirty years milk production has increased. This improvement is not only the result of an increased demand for milk, but it is also linked to a progress in animal genetics. From the beginning, breeding programs have focused on the genetic improvement of production traits, such as milk yield (Oltenacu and Broom, 2010). Milk yield per cow has more than doubled in the previous 40 years and some cows now produce more than 20,000 kg of milk per lactation (Oltenacu and Broom, 2010; USDA, 2016: https://www.nass.usda.gov/Charts_and_Maps/Milk_Production_and_Milk_Cows/mmlkpercow.php

). Data from National Milk Records in the UK show an increase in average milk yield of dairy cows of about 200 kg per year from 1996 to 2002, and 50% of the progress in milk yield has been attributed to genetics (Pryce and Veerkamp 2001). The situation is similar in the US where between 1957 and 2007 the average milk production per cow increased by 5,997 kg, with 3,390 kg of this increase (or 56%) due to genetics (VanRaden, 2004). However, increase in milk yield has also implied the presence of some detrimental effects. Indirect genetic selection responses for an increased risk of metabolic disorders seem to be related to selection focused on increased milk production. Associations between increased milk production and increased risk of metabolic disorders, as well as with decreased fertility (Sordillo, 2016), are well documented, but less is known about the biological mechanisms behind these relationships (Oltenacu and Broom, 2010).

Selection for increased milk production has led to a high energy requirement and, consequently, a reliance on body reserves to support early lactation (Pryce et al., 2016). In the first third of the lactation cycle, until energy intake catches up with energy requirements, high-producing cows enter a state of negative energy balance (NEB; Suthar et al., 2013). Selection for increased milk production also changes the partitioning of available energy by diverting a larger proportion of it to tissue, specifically to the mammary gland, that has a high energy demand. Selection for greater milk yield also increases feed intake but, the genetic correlation between milk yield and feed intake is only 0.46 to 0.65 (Veerkamp, 1998), which may contribute to the NEB observed in early lactation.

In early lactation, energy needs for dairy cows can be better met by adopting specific nutritional strategies to satisfy nutrient requirements. Nutrition is a key factor in the performance, health, and welfare of dairy cattle (NRC, 2001). Dairy cattle nutrition can be defined broadly as the use of components of feeds for the processes of maintenance, growth, reproduction, lactation, and health. Applied nutrition is the selection and proportioning of feedstuffs and ingredients to supply the correct amounts and balance of nutrients required for optimal productive and reproductive performance. Fundamental nutrition is the series of biochemical reactions used in the body during the assimilation

and processing of nutrients to meet the physiological needs of the animal. Fundamental and applied nutrition are equally important in determining optimal feeding and management strategies for dairy cattle health and production (Drackley et al., 2006). Until the mid-1980s, most of the increase in milk yield was the result of improved management, in particular of a better application of nutritional standards and an improved quality of roughage (Oltenacu and Broom, 2010). Optimal nutritional strategies for periparturient dairy cows are not yet clearly defined. The genetic potential for milk production by dairy cows has outpaced our current ability to feed and manage the cows to achieve maximum production and concurrently minimize health disorders (Burkholder, 2000; Litherland, 2006). And optimal feeding and management of dairy cows towards maximum milk production and minimum health disorders should match the genetic potential for milk production currently achieved in these animals.

Transition Period

The transition period is defined as 21 days before parturition through 21 days postpartum (Grummer, 1995; Drackley, 1999), and is one of the most challenging periods in the life cycle of dairy cows. The transition period includes three different phases: pre-partum, partum, and post-partum. These phases are characterized by different physiological stages, as animals move from pregnancy to lactation (Table 1.2). Dairy cows experience several metabolic adaptations, in glucose, fatty acid and mineral metabolism to support fetal growth and milk production. During the third trimester nutrient requirements of the fetus increase dramatically reaching maximal level three weeks prepartum (Esposito et al., 2014). Additionally, maternal energy requirements in late gestation increase by 30% to 50%. Early studies conducted by Reynolds et al. (1986) determined that fetal metabolic rate, represented as weight-specific oxygen consumption, is twofold higher than that of the dam. The evidence for increased oxygen consumption is supported by a greater uptake of glucose and amino acids, main sources of carbon and nitrogen, respectively, required for fetal growth and metabolism (Bell, 1995). Uterine glucose uptake during late gestation accounts for half of maternal glucose

supply. Insufficient glucose availability leads to increased catabolism of amino acids. The amino acid requirement also dramatically increases prepartum to support protein synthesis and deposition in fetal tissues. Thus, the maternal metabolism needs to accommodate the increasing demand of nutrients by the fetus. Consequently, changes in carbohydrate, protein and lipid metabolism occur during late pregnancy (Bell, 1995).

The most challenging adaptation during the periparturient period is the increased glucose requirement due to fetal growth and then lactation. There is an increase in hepatic gluconeogenesis as well as a decrease in glucose use by peripheral tissues. This metabolic shift is characterized by increased fatty acid mobilization from the adipose tissue, and increased amino acid mobilization from muscle.

Around parturition, a change in the metabolic status of cows occurs together with a hormonal status shift, from maintenance of pregnancy to milk production. Hormonal status plays a key role in the metabolic regulation of adaptation. In particular, the hormones implied are progesterone, estrogen, prolactin ($PGF_{2\alpha}$) and insulin. Among them, progesterone is predominant and important in maintaining pregnancy. In pregnant cows, plasma progesterone concentration increases until 250 days of gestation, when its level is around 7-8 ng/ml. As animals approach calving, plasma progesterone concentration decreases to about 3-4 ng/ml. Progesterone concentration falls to undetectable levels postpartum (Goff and Horst, 1997). On the other hand, in pregnant cows, plasma estrogen concentration increases rapidly before calving from a concentration about 2000 pg/ml at 7 days before calving to a peak concentration around 4000 and 6000 pg/ml at calving (Goff and Horst, 1997). Synthesis of estrogen by the placenta is stimulated by the secretion of fetal cortisol. Therefore, the fetus modulates progesterone and estrogen concentration since 30 days before calving. Twenty-four to thirty-six hours before parturition, plasma prolactin concentration increases and peaks at parturition. Prolactin plays an important role in luteolysis and inhibition of progesterone synthesis by the uterus. Prolactin, the primary lactogenic hormone, is responsible for the rapid increase in colostrum synthesis before parturition and milk production. Finally, insulin concentration shifts with

the cow physiological state, i.e. it decreases during milk production, making it low after calving (Lomax et al., 1979). Consistent with other works (Reist et al., 2003; Hammon et al., 2009; Weber et al., 2016), that reported decreased plasma insulin concentration after calving compared with pre-partum. In fact, lactating dairy cows, especially during the transition period, are characterized by a state of insulin resistance. Specifically, in lactating cows the majority of glucose utilization occurs through noninsulin-dependent pathways such as the mammary gland (Bell, 1995; Bauman, 2000; Ingvartsen and Andersen, 2000; Weber et al., 2016). Glucose, as a lactose precursor, is one of the most important substrates for milk synthesis. Consequently, the mammary gland utilizes high amounts of glucose. Hypoglycemia can also occur during early lactation, when energy intake does not meet energy needs for lactogenesis and maintenance. In light of this, decreased insulin concentration is a part of the homeorhetic metabolic regulation (Weber et al., 2016) in energy metabolism. However, insulin and NEB increase responsiveness of lipolysis and nonesterified fatty acids (NEFA) mobilization (Bell, 1995).

Table 1.2 Metabolic changes during the transition period, before and after parturition.

Metabolic pathway	**Tissue/organ**	**Prepartum**	**Afterpartum**
Gluconeogenesis	Liver	↓	↑
Ketogenesis	Liver	↓	↑
Glycolisis	Liver	↓	↓
Lipogenesis	Adipose tissues	↓	↓
TAG esterification	Adipose tissues	↓	↓
Lipolysis	Adipose tissues	↑	↑
Protein synthesis	Skeletal muscle	↓	↓
Protein degradation	Skeletal muscle	↑	↑

↓: decrease ↑: increase

Excessive NEB led animals to be more susceptible to metabolic disorders, a poor future reproductive performance, and infections (Bell, 1995; Drackley, 1999; Esposito et al., 2014; Kay et al., 2015).

Health and productivity may be compromised during the transition period. Over the last two decades, interest in nutrition and management has been emphasized to improve dairy cows welfare (Drackley et al., 2005). In light of this, several research groups have focused their attention and interest on

describing the transition period and excellent reviews on the topic have been published (Bell, 1995; Goff and Horst, 1997; Grummer, 1995; Drackley, 1999; Overton and Waldron, 2004; Ingvartsen, 2006; Singh and Singh, 2016).

During the transition period, cows primarily undergo a sudden increase in nutrient requirements for milk production, while dry matter intake (DMI) simultaneously decreases resulting in NEB. The physiological and endocrine changes occurring can make dairy cows more susceptible to metabolic disorders. During late gestation, changes in the endocrine status and decreases in DMI influence their metabolism, leading to mobilization of fat from adipose tissue (NRC, 2001) into the bloodstream in the form of nonesterified fatty acids (NEFA). Triglycerides (TAGs) are hydrolyzed to NEFA in the adipose tissue by the activation of hormone-sensitive lipase (HSL) through the protein kinase A cascade (White, 2015). Once in the bloodstream, NEFA can be absorbed and metabolized either by maternal and fetal tissues or by the mammary gland to synthetize milk fat (Adewuyi et al., 2005; Palmquist et al., 2006). Additionally, the liver takes up NEFA in proportion to their supply in blood (Pullen et al., 1989; Reynolds et al., 2003). The liver cannot completely oxidize NEFA released during the transition period as the oxidative capacity of the tricarboxylic acid cycle (TCA) is exceeded. Therefore, in early lactation, cows accumulate NEFA as TAGs within the liver when large amounts of NEFA are released from adipose tissue into the circulation (Emery et al., 1992). Furthermore, the formation of ketone bodies acetone, acetoacetate, and β-hydroxybutyrate (BHBA) as a direct consequence of the partial oxidation of fatty acids represents an integral part of the ruminant intermediary metabolism; they can provide an important energy source to peripheral tissues when glucose concentration is reduced (Duffield, 2000). However, when ketone bodies are elevated they can induce ketosis (Overton et al., 2004). Ketosis and fatty liver are the most frequent metabolic diseases that occur during the transition period (Sundrum, 2015). The hepatic mechanism of bovine fatty liver and ketosis is not completely understood. However, it is clear that regulation of TAG

uptake and storage, lipolysis in adipose tissue, and oxidation through mitochondrion, play a key role in the onset and progression of these disorders (White, 2015).

Regulation of periparturient feed intake and negative energy balance

Dry matter intake is fundamentally important in nutrition because it establishes the amount of nutrients available to an animal for health and production (NRC, 2001). In ruminants, regulation of feed intake is affected by multiple factors related to physiological state, presence of metabolic disorders, hormonal changes, stress, management, etc. Despite several investigations on DMI (Colucci et al., 1982; Russell et al., 1992; Allen, 2000; Krizsan et al., 2014; Kuhla et al., 2016), the mechanisms behind feed intake regulation are not yet completely understood.

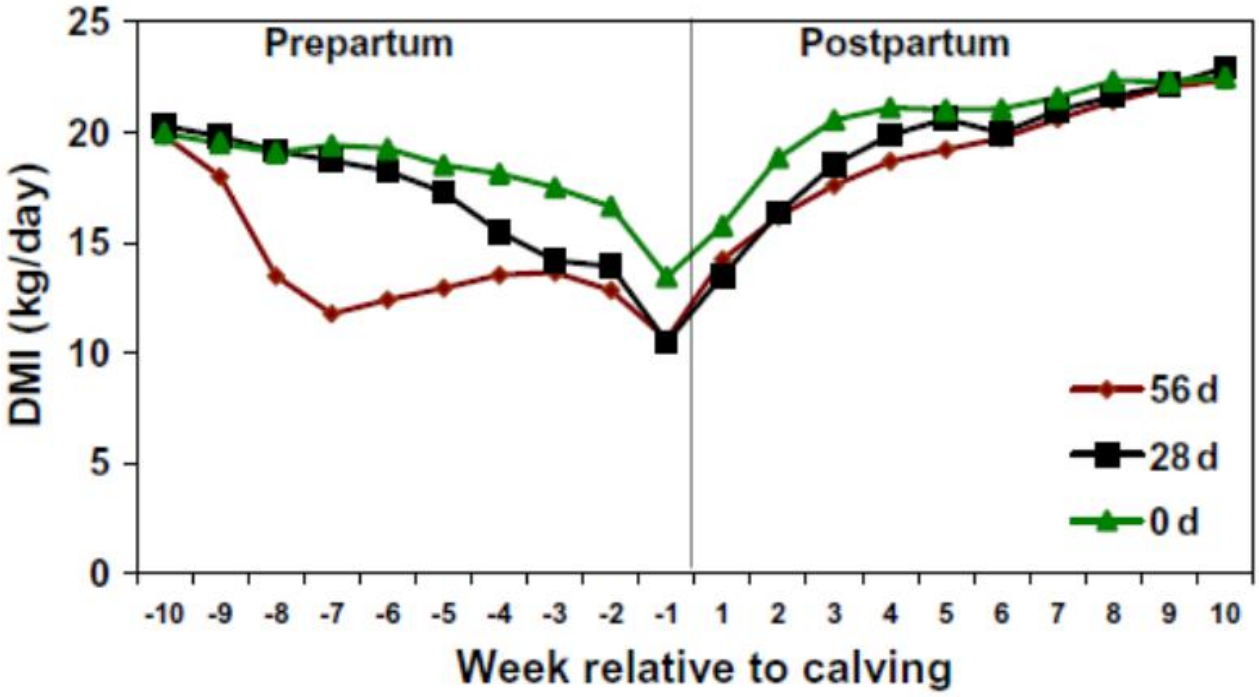

Figure 1.1 Dry matter intake of cows with different dry period lengths (Rastani et al., 2005) http://www.sciencedirect.com/science/article/pii/S0022030205727685

During late gestation, changes in endocrine status and decreases in DMI, can further influence the animal metabolism and lead to mobilization of fat from the adipose tissue and glycogen from the liver (NRC, 2001).

Parturition is one of the most challenging periods for dairy cows, due to the dramatic endocrine changes implied. From late gestation to early lactation, insulin and growth hormone increase (Kunz

et al., 1985). During late gestation, estrogen, primarily of placental origin, increases in plasma, and at calving, it decreases immediately (Chew et al., 1979). The high circulating estrogen concentration may be one factor that contributes to decreased DMI around parturition (Grummer, 1993), although regulation of DMI in periparturient cows is multifaceted and far from being understood (Grummer et al., 2004). A few days prior to calving and during the first week of lactation, demand for amino acids (AA), fatty acids (FA), and net energy dramatically increases. During this time frame, compared to the demand of the gravid uterus during late pregnancy, the requirement for glucose increases threefold, for AA twofold and for FA approximately fivefold (Bell, 1995). Mammary gland requirement for energy increases three times compared to the uterus (Miller, 1991). Thus, with the initiation of milk synthesis after calving, a rapid increase in milk production greatly increases the

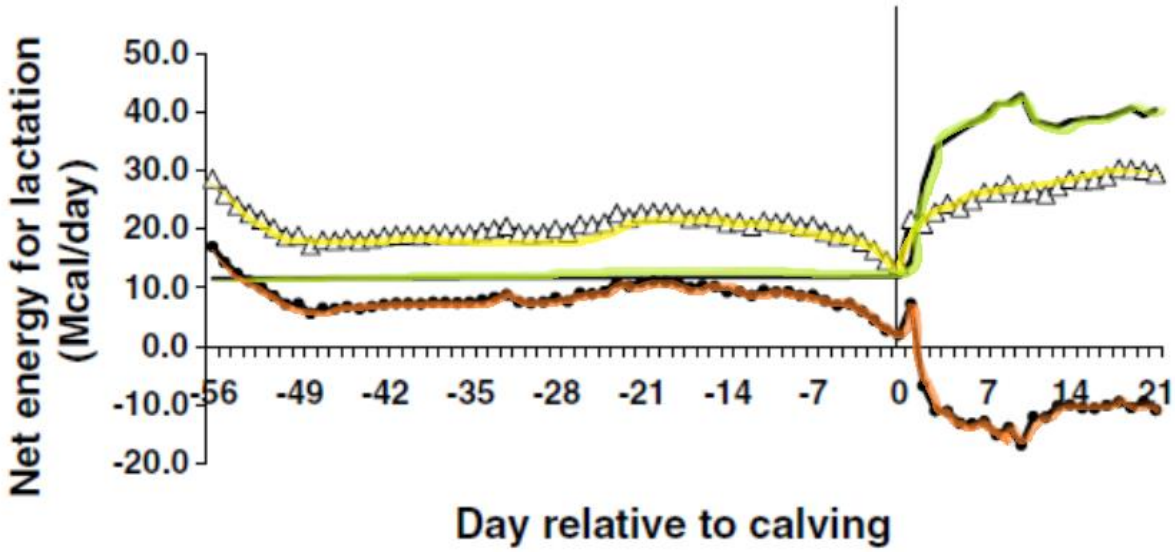

Figure 1.2 Energy (Mcal NEl/day) required (green) and consumed (yellow), and energy balance (orange) for cows during an experiment. Cows were fed a diet containing 1.50 Mcal/kg DM during the far-off dry period and a 'close-up' diet containing 1.69 Mcal/kg DM during the final 4 weeks prior to calving. After calving, the lactation diet contained 1.71 Mcal/kg, the minimal increase relative to the close-up diet is due to greater DMI following calving (Grummer, 2008) http://www.sciencedirect.com/science/article/pii/S109002330700425X.

demand for glucose for milk lactose synthesis at a time when DMI in depressed (Drackley et al., 2005). Feed intake reduction begins in the week prior to parturition (Bertics et al., 1992, Figure 1.1), resulting in a decrease in energy intake at a critical point in time. Reduction of DMI has been estimated to be 30-35% prior to calving, which is achieved during the last week prior to calving (Hayirli et al., 2002). During the last two weeks of gestation, dairy cow metabolism must

accommodate the increased energy demand by the fetus, and, from the last week of gestation, by the mammary gland. However, the energy status is compromised due to a decline of the feed intake. The concept of energy balance and energy requirements is essential, since the different physiological stages have different energy requirements (Figure 1.2). Energy balance is calculated as energy requirements for maintenance and milk production minus dietary energy intake, generally measured as megacalories (Mcal) of NE_L. Dietary energy density recommendations are different for dry cows (1.25 Mcal NE_L/kg DM) compared with transition cows (1.62 Mcal NE_L/kg DM; NRC, 2001). However, the increased dietary energy density does not meet the energy required in early lactation, due to insufficient DMI. Increasing energy concentration up to 1.70 Mcal NE_L/kg DM prepartum may help compensate the NEB experienced by early lactation cows. Rabelo et al. (2005) fed cows before calving with high energy density diet (1.70 Mcal NE_L/kg DM) and low energy density diet (1.58 Mcal of NE_L/kg), showing that animal fed the high energy density diet had a more positive energy balance during the prepartum period. However, these high energy diets may not contain adequate fiber to support rumen health and function. Dairy cows commonly experience a NEB of 5 to 10 Mcal/day postpartum.

Besides the several endocrine and metabolic changes, due to the start of lactation, cows generally experience a major diet shift as well. This sudden change in diet may not let the rumen environment have adequate time to adjust (Dirksen et al., 1985). Ruminants, through the fermentation of dry matter (DM), are able to produce volatile fatty acid (VFA). During the early postpartal period, propionate derived from rumen fermentation is insufficient to meet the glucose requirement due to low DMI (Drackley et al., 2001). In order to meet glucose requirements, glucose can be synthesized from different sources including dietary amino acids, skeletal muscle (breakdown), and glycerol from mobilized body fat (Reynolds et al., 2003). Glucose, however, is not the only nutrient needed to meet requirements during the first weeks of lactation. Dairy cows mobilize large quantities of fatty acids from adipose tissue, resulting in increased circulating concentrations of NEFA (Overton, 2004) that

are a major source of energy to the cows during this period. The concentration of NEFA in blood reflects the degree of adipose tissue TAG mobilization (Pullen et al., 1989). Therefore, when NEB increases, more NEFA are released from body fat and their blood concentration increases. Moreover, stressors and poor nutritional management, causing decreases in voluntary DMI, will result in large increases in NEFA immediately after calving (Bertoni et al., 1998; Drackley, 1999).

Lipid metabolism and fatty liver during the transition period

One of the most common postpartum metabolic disorders that can affect dairy cows is fatty liver. This disorder results from excessive adipose mobilization almost partially due to insufficient feed intake to support energy for maintenance and milk production (Grummer, 1993). Several main metabolic functions occur in the liver; they are particularly important as it controls the absorption and synthesis of endogenous nutrients that are supplied to the rest of the body (Reynolds et al., 1994). Liver adaptation to metabolic changes due to the different life cycle stages of dairy cows, together with defects in timing or amplitude of those metabolic changes, can increase susceptibility in transition cows to disorders such as ketosis and hepatic lipidosis (Goff and Horst, 1997; Drackley, 1999). This adaptation, which is a complex system, occurs gradually and varies among cows (Jorritsma et al., 2003). Furthermore, not only the etiology of hepatic fat accumulation during the transition period, but also the metabolic changes behind these disorders are not yet completely understood though several studies have been conducted to clarify their complexity (Overton and Piepenbrink, 1999; Gummer, 2012; Gummer, 2008).

Fatty liver or hepatic steatosis is a condition that occurs when the rate of NEFA uptake by the liver exceeds the rate of TAG disappearance via hydrolysis or export as a constituent of very low density lipoprotein (VLDL; Grummer, 1993). It is well known that decreases in voluntary DMI around parturition result in cows entering a state of NEB. During this period of NEB, to come closer to meeting the energy requirement and to support milk production, triglycerides are mobilized from adipose stores (Dole, 1956; Emery et al., 1992).

Thus, the first mechanism by which cows compensate for NEB is to mobilize TAGs. At this time, lipolysis occurs predominantly in the adipose tissue. TAG synthesized there are rapidly hydrolyzed to NEFA and glycerol. Hormone-sensitive lipase catalyzes the hydrolysis of fatty acids generating 2-monoglycerol which is subsequently hydrolyzed by monoglyceride lipase to yield glycerol and free fatty acids (Fredrikson et al., 1986; Holm, 2003). Hormone-sensitive lipase is activated through the protein kinase A (PKA) cascade, which phosphorylates HSL (Ahmadian et al., 2007). Phosphorylation results in increased hydrolytic activity (Fredrikson et al., 1981), translocation of HSL from the cytosol to the lipid-droplet surface, and enhanced TAGs breakdown in the cell (Holm, 2003; White, 2015; Koltes and Spurlock, 2011; Locher et al., 2011). In addition to the hydrolytic action of HSL, perilipin (PLIN) may translocate HSL to the lipid droplet allowing the lipase access to its substrate (Miyoshi et al., 2006; Sztalryd et al., 2003). Perilipin is a lipid-droplet-associated protein; its phosphorylation increases the rate of lipolysis (Ahmadian et al., 2007; White, 2015) and plays a key role during the transition period (Koltes and Spurlock, 2011). On the other hand, adipose triglyceride lipase, which is important for lipolysis in other species, is down-regulated in dairy cows during the transition period (Koltes and Spurlock, 2011; White, 2015).

Perilipin is known as a target of PKA phosphorylation, which is increased in early lactation. The abundance of phosphorylated PLIN (PPLIN) in adipose tissue increased from 5 to 21 day in milk (DIM; Koltes and Spurlock, 2011). The abundance of PPLIN in adipose tissue was greater in cows in early lactation (5–14 DIM) versus cows in late lactation (176–206 DIM) and a greater abundance of phosphorylated HSL was detected in the adipose tissue during early versus mid lactation, indicating greater lipolytic activity in the adipose tissue (Elkins and Spurlock, 2009). In the same study, a positive correlation between the abundance of PPLIN and NEFA concentration was reported during both early and mid lactation. Accordingly, the presence of PPLIN allows HSL access to the lipid droplet increasing the rate of lipolysis and the release of NEFA. Consequently, free fatty acids are released into the bloodstream and can be absorbed by other organs to meet the energy

requirements of the cow (Gibbons et al., 2000; Gilham and Lehner, 2004; Ahmadian et al., 2007). Blood NEFA concentrations increase at calving and can be metabolized by several tissues, including the liver and the mammary gland for energy production and fat synthesis (White, 2015). Circulating NEFA provides energy to tissues, but high NEFA concentration can be toxic (Drackley et al., 2001). NEFA concentrations exceeding 0.7 mmol/l for more than seven days after calving are an indicator of excessive NEB or health problems (Bertics et al., 1992; Vazquez-Anon et al., 1994; Grum et al., 1996; Duffield et al. 2009). Similarly to increased NEFA, BHBA concentration also increases during the first two weeks of lactation. Levels above 1.2 mmol/L are considered indicative of ketosis (Oetzel, 2013). Nonesterified fatty acids and β-hydroxybutyrate are the two main metabolites that are direct indicators of NEB and metabolic disorders such as ketosis (Chapinal et al. 2011; Krempaský et al., 2014).

In the liver, NEFA are β-oxidized to acetyl-CoA. Acetyl-CoA, can be completely oxidized through the TCA cycle to provide energy; partially oxidized through ketogenesis to ketone bodies; re-esterified as TAG for packaging as VLDL for export from the liver (minimal in ruminants), or re-esterified for TAG storage as liver TAG (White, 2015). Thus, ketone body production and TAG deposition in the liver increase when the availability of acetyl-CoA exceeds the oxidative capacity of the TCA cycle. This scenario leads to the onset of ketosis and fatty liver syndrome. Similarly to NEFA, ketone bodies can be used as a fuel source by several tissues (i.e. heart, brain, liver, and mammary tissue). However, excessive ketone bodies can negatively affect animal health and productivity. Clinical hyperketonemia is defined as blood BHBA concentration >3.0 mmol/L (McArt et al., 2011; White, 2015) while subclinical ketosis, hyperketonemia without clinical symptoms of ketosis, is characterized by serum BHBA levels ranging from between 1.0 and 1.4 mmol/L to 2.9 mmol/L (Duffield et al., 1998; Iwersen et al., 2009; Rollin et al., 2010; Suthar et al., 2013). The incidence of subclinical ketosis is considered to be 15% to 60% while clinical ketosis occurs in 2% to 15 % of cows (Duffield et al., 2000; Duffield et al., 2009). Ketosis and hepatic lipidosis in

periparturient cows are closely associated, often developing simultaneously. In the case of fatty liver, the incidence in cows during the first three weeks of lactation is around 60% (Drackley, 1999; Bobe et al., 2004; Suthar et al., 2013).

Increased NEFA uptake by the liver, limited oxidation of hepatic fatty acids and low export of TAG as VLDL combine together to result in TAG accumulation in the liver (White, 2015). Fatty acid oxidation and ketogenesis are likely the major routes for removal of excess fat from the liver.

Hepatic Metabolism

Fatty acid oxidation

Hepatic metabolism, principally fatty acid metabolism, has been largely studied to understand the mechanisms underlying metabolic disorders that can affect cows during the transition period. Several pathways, and enzymes, are involved in mitochondrial β-oxidation, VLDL assembly, peroxisomal β-oxidation and esterification, and TAG accumulation in liver parenchyma. Nonesterified fatty acids, taken up by the liver from the bloodstream, can have different fates that are represented by these pathways. Thus, mitochondria β-oxidation is the first step by which NEFA are metabolized inside the hepatocyte. Before being oxidized in the mitochondria, fatty acids must be transported from the cytosol to the organelle by carrier proteins. The flux of long chain fatty acids (LCFA) into the mitochondrial matrix is mediated by the carnitine-acylcarnitine translocase system (Figure 1.3), which consists of three carrier proteins: carnitine palmitoyltransferase I (CPT1), carnitine-acylcarnitine translocase (CACT), and carnitine palmitoyltransferase II (CPT2), each with a different mitochondrial localization (McGarry and Brown, 1997; Kerner and Hoppel, 2000; Al-Trad et al., 2009). Briefly, CPT1 catalyzes the transesterification of acyl-CoA LCFA, formed by long-chain acyl-CoA synthetase (LCAS), to fatty acyl-carnitine. Carnitine-acylcarnitine translocase, translocate fatty acyl-carnitine to mitochondria, allowing LFCA to cross the inner mitochondrial membrane. Once inside the mitochondria, fatty acyl-carnitine is reconverted to acyl-CoA by CPT2 (Kerner and Hoppel, 2000). Then, within the mitochondria, it can be β-oxidized to acetyl-CoA. Carnitine

palmitoyltransferase I represents a key regulatory site controlling the flux through β-oxidation (Kerner and Hoppel, 2000). Acetyl-CoA can be completely oxidized to carbon dioxide through the TCA cycle to provide energy. Furthermore, through ketogenesis, acetyl-CoA can be incompletely oxidized to ketone bodies. This conversion to ketone bodies results in the formation of less ATP per mole of fatty acid oxidized, so complete oxidation conserves more energy (ATP). CPT1 in ruminants is inhibited by methylmalonyl- CoA, an intermediate in gluconeogenesis (Brindle et al., 1985). Increased expression of CPT1 is associated with an increased rate of hepatic β-oxidation, as well as

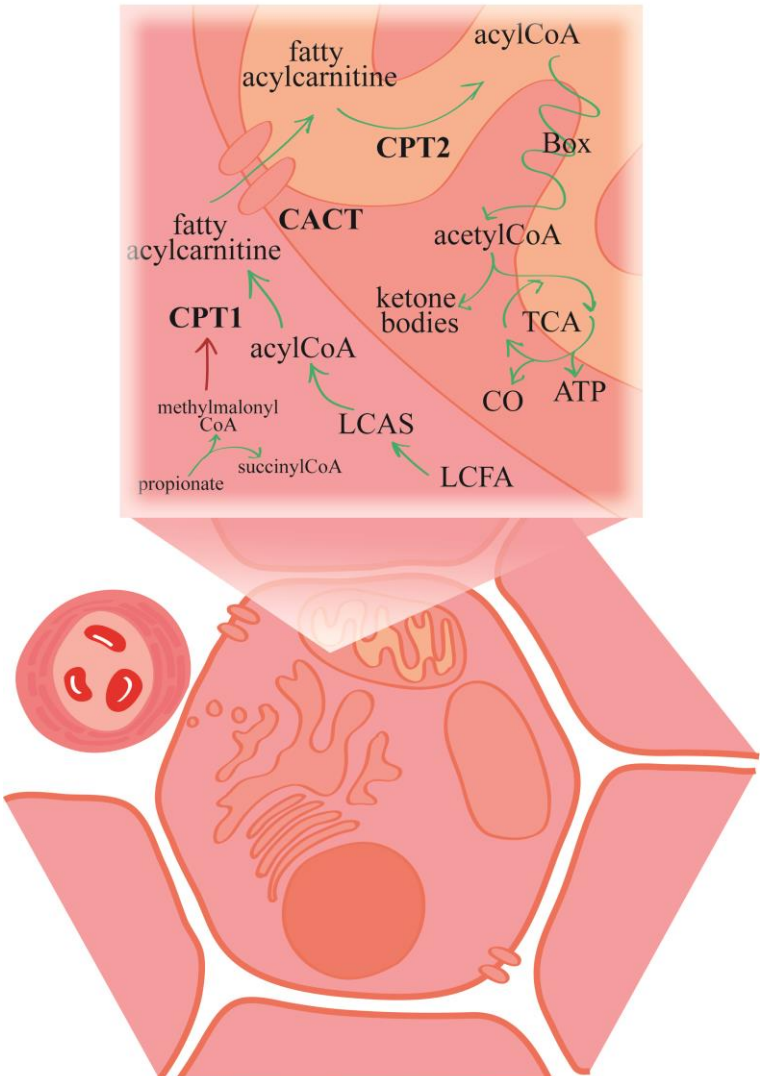

Figure 1.3 Hepatic metabolism, Carnitine-acylcarnitine translocase system. Three carrier proteins mediate flux of long chain fatty acids (LCFA) into the mitochondrial matrix: carnitine palmitoyltransferase I (CPT1), carnitine-acylcarnitine translocase (CACT), and carnitine palmitoyltransferase II (CPT2). CPT1 catalyzes the transesterification of acyl-CoA LCFA, formed by long-chain acyl-CoA synthetase (LCAS).

with a decreased accumulation of TAG (Stefanovic-Racic et al., 2008). In light of this, upregulation of CPT1 is important to prevent lipid-related metabolic disorders in dairy cows (Drackley, 1999).

Peroxisomal metabolism

Microbodies, also called peroxisomes, are subcellular organelles present in most cells of the body. Peroxisomes have several functions since they are involved in many metabolic pathways, including various aspects of the lipid metabolism. Their functions include: i) degradative oxidation (e.g., β-oxidation of very long chain fatty acids, dicarboxylic acids, leukotrienes, bile acid intermediates and cholesterol side chains, and both α- and β-oxidation of 3-methylbranched chain fatty acids); ii) synthesis of either glycerolipids or plasmalogens; iii) formation of bile acids, dolichol, and cholesterol; iv) catabolism of purines, polyamines, and amino acids, and the detoxification of reactive oxygen species such as hydrogen peroxide, superoxide anions, and epoxides (Subramani, 2004; Singh, 1997; De Duve and Baudhuin, 1966). Long-chain fatty acids can be oxidized through mitochondrial β-oxidation as well as through a similar pathway that occurs in peroxisomes (Overton and Piepenbrink, 1999). Peroxisomal β-oxidation shows steps similar to mitochondrial oxidation with some exceptions. The first dehydrogenase step occurring in the mitochondria is replaced by an

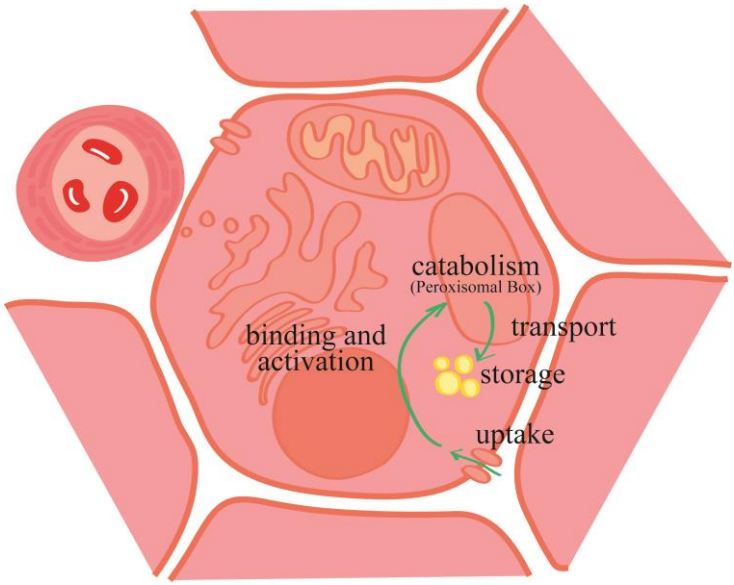

Figure 1.4 Hepatic metabolism, Peroxisome proliferator-activated receptors alpha (PPARA), regulate hepatic fatty acid oxidation

oxidase step (acyl-CoA oxidase) in the peroxisome, resulting in the formation of hydrogen peroxide instead of NAD+ (Drackley, 2000).

Peroxisome proliferator-activated receptors (PPARs) are nuclear lipid-activated receptors that control several genes in different pathways of lipid metabolism (Figure 1.4), including fatty acid transport, uptake by cells, intracellular binding and activation, and catabolism (β-oxidation) or storage (Desvergne and Wahli, 1999). Three related PPAR isotypes have been identified in mouse, rat, hamster, and humans (Xing et al., 1995; Dreyer et al., 1992). These PPAR isotypes were named PPARα (PPARA), PPARβ (PPARB), and PPARγ (PPARG). Particularly, isotypes α and γ are involved in the regulation of hepatic fatty acid oxidation and adipose tissue insulin sensitivity (Weber et al., 2013; Desvergne et al., 2006; Alaynick, 2008). During fasting and starvation, PPARA is upregulated, as well as in cows induced to develop ketosis early post-partum (Loor et al., 2007). Furthermore, greater PPARA expression in ruminant liver is induced by increased NEFA blood concentration and its expression and activity promotes increased β-oxidation (Alaynick, 2008), during the periparturient period and fasting-induced ketosis (Loor et al., 2007; Loor et al., 2005; Goselink et al., 2013; Janovick et al., 2004).

Microsomal transfer protein, VLDL packaging and TAG export

The microsomal triglyceride transfer protein (MTTP) was originally isolated from the microsomal fraction of bovine liver (Wetterau and Zilversmit, 1984). Microsomal triglyceride transfer protein expression is restricted to the liver and intestine, the only two tissues that express large amounts of apoB (Hussain et al., 2012). Microsomal triglyceride transfer protein accelerates the transport of triglyceride, cholesteryl ester (CE), and phosphatidylcholine (PtdCho) from the endoplasmic reticulum (ER) to the site of VLDL packaging (Gruffat et al., 1996; Love et al., 2015). The graphical representation of the MTTP mechanism is reported in Figure 1.5. Microsomal triglyceride transfer protein plays a key role in lipid transportation from the endoplasmic reticulum membrane to apoB particles in the lumen of the ER where it is involved in VLDL assembly (Wetterau et al., 1997). The

major constituents of VLDL are apolipoproteins (ApoB-100), phospholipids (derived from PtdCho), free cholesterol, TAG, cholesterol esters. Apolipoproteins and MTTP are essential for hepatic synthesis of VLDL, a complex process that involves several pathways and genes. Apolipoproteins synthesis occurs in the ER where MTTP transfers lipid from the inner leaflet of the ER membrane to ApoB (Gordon et al. 1995). Thus, MTTP is an important factor for ApoB translocation across the ER membrane and for the post-translational addition of triglyceride to ApoB (Alexander et al. 1976, Rustaeus et al. 1998, Wang et al. 1997, Du et al. 1996). Microsomal triglyceride transfer protein is also necessary for adding CE to the nascent ApoB (Gordon, 1997; Wetterau et al., 1997) and MTTP overexpression results in increased VLDL ApoB secretion by the liver (Lin et al, 1994; Tietge et al., 1999).

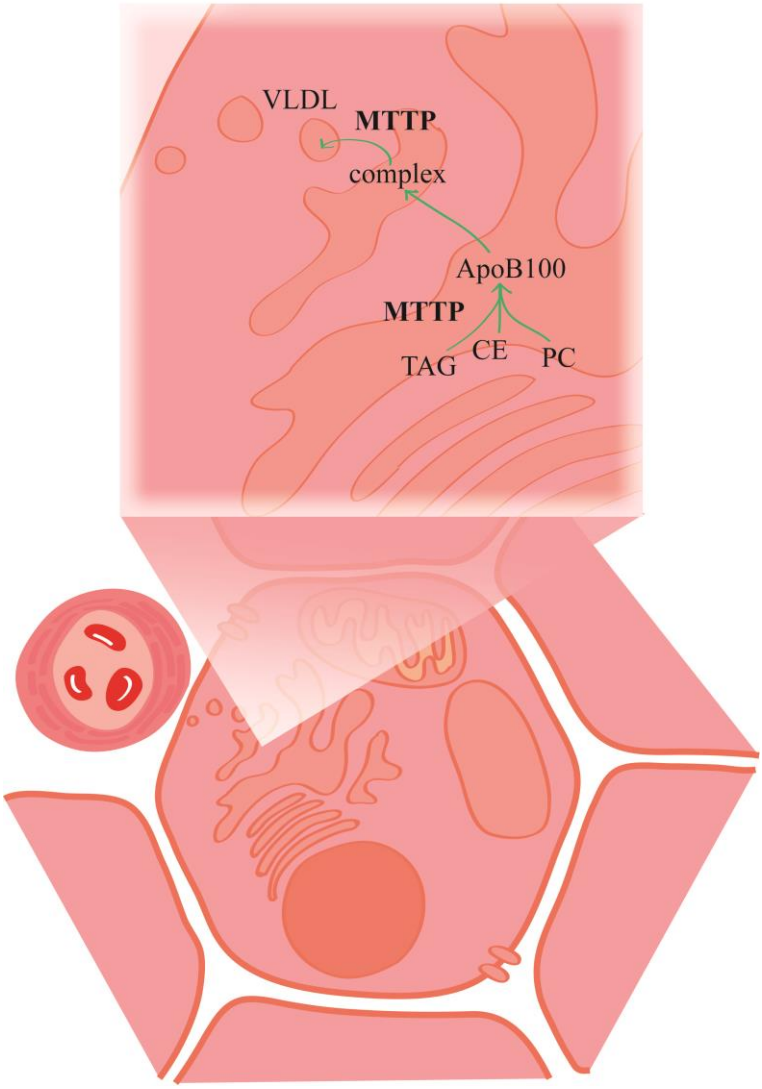

Figure 1.5 Hepatic metabolism, microsomal triglyceride transfer protein (MTTP) accelerates the transport of triglyceride (TAG), cholesteryl ester (CE), and phosphatidylcholine (PC) from the endoplasmic reticulum (ER) to the site of VLDL packaging

Despite the clear mechanism of MTTP effect, there is no definite evidence that MTTP does play a role in ruminant liver metabolism. The expression of MTTP is regulated by nutritional factors; Lin et al. (1994) have fed Syrian Golden hamsters with high fat (20% wt/wt hydrogenated coconut oil) and high sucrose diet (60% sucrose) reporting an increased MTTP expression, about 55% compared with the control group. In the same study, it was observed a positive correlation between hepatic MTTP and VLDL and low density lipoprotein (LDL) cholesterol or TAG levels, indicating that MTTP is involved in VLDL synthesis. This finding is not consistent with other researches in ruminants (Bremmer et al., 2000). From this study, they measured MTTP mass and mRNA abundance around calving, reporting no significant correlation between MTTP mass or mRNA and liver TAG or plasma NEFA. This result suggests that MTTP does not play a key role in the etiology of fatty liver in dairy cows around calving. Another work has suggested that MTTP might be deficient or inactive in ruminant liver (Gruffat-Mauty et al., 1999). However, the mechanism by which MTTP is involved in VLDL packaging in ruminants is not completely understood.

Hepatic Gluconeogenesis

Glucose is an essential fuel in energy metabolism of the mammalian cells (Cárdenas et al., 1998; Aschenbach et al., 2010). Among mammals, the role played by glucose is somehow different such as in the case of ruminants versus non-ruminants. Gluconeogenesis, during the early life stage of ruminants, is very similar to that in monogastric species (Leat, 1970; Baldwin et al., 2004). The digestive adaptation in developing ruminants has been largely studied (Van Soest, 1994; Harmon, 1993; Baldwin et al., 2004). These adaptations consist essentially in a shift of metabolism caused by the development of rumen. Precisely, animals in this phase move from preruminant to functional ruminant; consequently, glucose metabolism changes to meet these digestive adaptations. The digestive adaptations that occur during the transition from a preruminant to an adult ruminant are characterized by a shift from intestinally absorbed glucose, LFCA, and milk-derived amino acids to ruminally absorbed short-chain fatty acid (SCFA), ketones, amino acids from feed and microbial sources, and other dietary compounds (Baldwin et al., 2004). Developing ruminants move from a

digestive condition very similar to “monogastric digestion” to a ruminant digestion involving microbial fermentation into the rumen. As fermentation increases through rumen bacteria, less carbohydrates are available, which triggers changes in liver metabolism from glycolysis to gluconeogenesis pathway (Leat, 1970; Baldwin et al., 2004). Glucose disposal is lower in ruminants than in non-ruminants. Ruminants are characterized by high production and absorption of VFA, used to make glucose through gluconeogenesis. Propionate, valerate and isobutyrate are the main VFA glucogenic precursors for net synthesis of glucose (Aschenbach et al., 2010; Bergman, 1990; Reynolds et al., 2003; Larsen and Kristensen, 2009). The primary glucose-producing organ is the liver (Brockman and Laarveld, 1986). The estimation of VFA contribution to net liver glucose in dairy cows has been studied in numerous researches (Lomax and Baird, 1983; Reynolds et al., 2003; Doepel et al., 2009; Larsen and Kristensen, 2013). These studies show that from 44 to 78% of VFA contribute to liver gluconeogenesis, with propionate as the major source (90-95% of VFA supply for glucose production). The VFA uptake capacity by the liver is different according to the physiological stage of the animals. Generally, before parturition when cows are not in NEB, the liver takes up 60-74% of propionate, 16-26% of L-lactate, 3–5% of alanine, 5–6% of valerate and isobutyrate, 0.5–3% of glycerol, and 8–11% of other amino acids (Reynolds et al., 2003; Larsen and Kristensen, 2013; Aschenbach et al., 2010). However, after parturition, hepatic glucose output becomes twice than that of the prepartum output. Post-partum hepatic propionate uptake does not account for all glucose synthesis. Consequently, after calving, hepatic uptake of endogenous gluconeogenic precursors, such as alanine, lactate, and glycerol, increases.

An additional factor that modulates the uptake of gluconeogenesis precursors by the liver is their availability. The conversion of propionate to glucose increases when cows are fed a high concentrate diet compared with a high forage diet. Aiello et al. (1984) used radiolabeled [1-^{14}C] propionate to determine the conversion rate of propionate to glucose in liver slices finding that it was greater for cows fed high concentrate diets compared with those fed low concentrate diets. Other studies

conducted on sheep and goat hepatocytes during starvation (Lomax et al., 1986) and feed restriction (Armentano et al., 1991) showed a decreased conversion of propionate to glucose compared with *ad libitum* fed animals. In light of this, energy deficiency and its association with lipid mobilization led the liver to utilize different gluconeogenic precursors such as glycerol and lactate (Lomax et al., 1986; Drackley et al., 2001). Additionally, Overton et al. (1998) found that the conversion of propionate to glucose was higher at 1 and 21 day postpartum (19 and 29% respectively) compared to 21 day prepartum. They also found a positive correlation between the conversion of propionate to glucose with fat-free NE_L intake. During early lactation, the capacity to convert propionate to glucose is a more efficient use of propionate, and is modulated by nutrient supply and energy balance (Drackley et al., 2001). In light of these findings, synthesis of glucose around parturition is higher compared with prepartum synthesis (Drackley et al., 2001), probably due to the demands of lactation (Bell and Bauman, 1997).

Lactate and glycerol are also gluconeogenic precursors, though they are not major precursors. They do play an important role during feed restriction and lactation of dairy cows (Lomax and Baird, 1983). Lactate utilization for gluconeogenesis is greater during late gestation compared to early lactation (Baird et al., 1983). The explanation for this different utilization of gluconeogenic precursors is attributed to the release of lactate by the gravid uterus and muscle during late gestation (Bell, 1995). During energy deficiency, the main gluconeogenic precursors are L-lactate, glycerol, and alanine (Aschenbach et al., 2010). Cows during late pregnancy to early lactation are characterized by decreased plasma glucose, insulin, and blood amino acids, and an increased plasma glucagon concentration (Hammon et al., 2009; Reynolds et al., 2003). The decrease in blood glucose and amino acids are related to an increased demand for these substrates by the gravid uterus for fetal growth and by the mammary gland for milk synthesis.

For a successful transition through late pregnancy and early lactation, the liver increases in size by 3.5% (Reynolds et al., 2004), and enzymatic activity changes as well (Drackley et al., 2001). Hepatic

carbon homeostasis must be preserved during periparturient period. The concepts of anaplerosis and cataplerosis are essential to understand and describe the biological reactions related to the flux of carbons in the hepatocytes. The carbon flux is mainly related to the maintenance of TCA cycle intermediates, especially during the periparturient period when adipose tissue lipolysis and hepatic TAG uptake, hepatic oxidation and storage are increased. The main substrate and the supply of carbons utilized for gluconeogenesis are propionate, lactate and amino acids, and glycerol (Aschenbach et al., 2010). Propionate enters the TCA cycle through succinate, thereby providing carbons. Precisely, propionate transported to the liver is converted to propionyl CoA through mitochondrial propionyl-CoA carboxylase (PCoAC); then, methylmalonyl-CoA mutase (MCM) catalyses the carboxylation reaction of propionyl CoA to (S)-methylmalonyl CoA that, when converted, can be isomerized to succinyl-CoA by MCM (Aschenbach et al., 2010). Lactate and amino acids (one of the main glucogenic amino acids is alanine) are converted to pyruvate in the cytosol, then to oxaloacetate (OAA) or acetyl-CoA to enter the TCA cycle. Lactate dehydrogenase converts lactate to pyruvate, while alanine is deaminated via transaminase to yield pyruvate. Pyruvate carboxylase (PC) catalyses the carboxylation of pyruvate to oxaloacetate. The TCA cycle is an important key point to provide substrate for the gluconeogenesis (Figure 1.6). Phosphoenolpyruvate carboxykinase (PCK) converts OAA from the TCA cycle to phosphoenolpyruvate (PEP) that can be converted to glucose through gluconeogenesis or serve as an acetyl-coA acceptor in the TCA cycle (Aschenbach et al., 2010).

Liver glucose-6 phosphatase (G6PC) is a key enzyme in the glucose synthesis that catalyzes the last reaction of glycogenolysis and gluconeogenesis, glucose-6-phospate to glucose (Minassian et al., 1999; Foster et al., 1997; Mithieux, 1997). It has been shown that the activity of G6PC is related to the lack of glucokinase, an enzyme necessary to take up glucose from blood and store it inside the hepatocytes as glucose 6-phosphate which can be utilized in glycolysis, glycogen synthesis, and other synthesis pathways (Seoane et al., 1999). However, cows exhibit low hepatic glycolysis due to the lack of glucokinase as would be expected due to the high rates of gluconeogenesis (Aschenbach et al., 2010; Donkin, 2016).

Two isoforms of PCK are known, the mitochondrial isozyme (PCK2) and the cytosolic isozyme (PCK1). The mitochondrial form of PCK controls the entry of lactate into the TCA cycle and converts OAA to PEP in the cytosol (Watford et al., 1981; Aschenbach et al., 2010; White, 2015). To be converted to PEP, OAA must be transported out of the mitochondrion in the form of malate. When

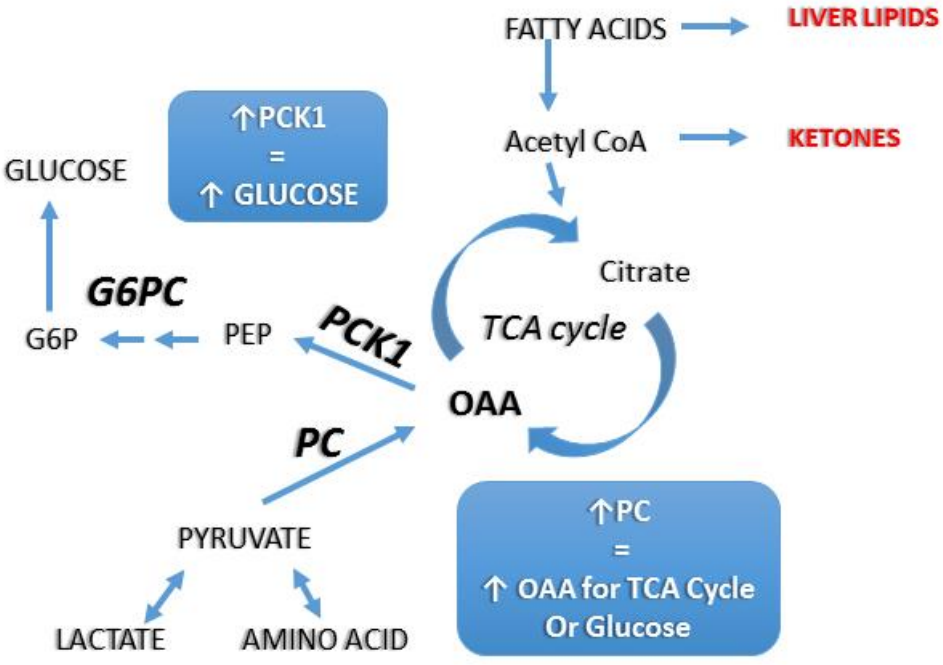

Figure 1.6 Anaplerosis and Cataplerosis of oxaloacetate in the TCA cycle.

out of the mitochondrion, malate is reoxidized to OAA in the cytosol by NAD malate dehydrogenase (Hanson and Reshef, 1997). Therefore, PC and PCK2 regulate the entry of lactate into gluconeogenesis (Velez and Donkin, 2006; White et al., 2009); PC and PCK1 regulate the entry of

amino acids, while PCoAC, MCM, and PCK1 regulate the entry of propionate. Coenzyme B12 is the prosthetic group of Methylmalonyl-CoA Mutase which converts methylmalonyl-CoA to succinyl-CoA. Vitamin B12 can clearly play an important role to enhance or reduce gluconeogenesis. An early study conducted by Peters and Elliot (1983) showed that vitamin B12 supplementation in sheep increased gluconeogenesis from propionate. However, in bovine species, the exact mechanism beyond MCM and PCoAC regulation is not clear. Coordination between PCoAC and PCK has been observed during fatty liver (Murondoti et al., 2004) and during the transition period (Hammon et al., 2005). Cytosolic phosphoenolpyruvate carboxykinase expression can also be regulated by propionate supply. Effects on PCK1 expression have been reported under specific nutritional interventions (e.g. monensin supplementation) that may affect propionate production. Likewise, Greenfield et al. (2000) and Karcher et al. (2007) found increased expression of PCK in dairy cows when feed intake was increased. By contrast, feed restriction does not alter PCK mRNA abundance or activity; PCK (PCK1) is actively regulated by hormones and nutrients at the transcriptional level (Taylor et al., 1971; Smith and Walsh, 1982; Hanson and Reshef, 1997; Velez and Donkin, 2005, Zhang et al., 2016). In contrast to PCK regulation, feed restriction increases PC expression while somatotropin administration increases PCK expression but not PC expression (Velez and Donkin, 2005; Velez and Donkin, 2004; White et al., 2009). Pyruvate carboxylase plays a crucial role in stimulating glucose synthesis by promoting the use of endogenous precursors for gluconeogenesis when feed intake is compromised (e.g. during the transition period) (White, 2015). On the other hand, PCK1 promotes and controls gluconeogenesis when feed intake is adequate (Aschenbach et al., 2010). Clearly, these two genes are characterized by differential pattern of regulation. If so, upregulation of PCK without gluconeogenic precursors supply can lead to depression of the oxidative capacity of the TCA cycle, because OAA is drained from the cycle for gluconeogenesis. The consequence of this occurrence is an increase in ketones synthesis, induced by the decreased capacity to completely oxidize acetyl-CoA. The balance of cataplerosis and anaplerosis is a critical factor in maintaining the oxidative capacity of the TCA cycle. Anaplerosis replenishes the pools of OAA in the TCA cycle though PC

activity, while cataplerosis describes reactions involved in the disposal of OAA by PCK activity (Owen et al., 2002). Consequently, if the pool of TCA cycle intermediates is adequate due to anaplerosis, then cataplerosis is possible. However, when in the TCA cycle no intermediates are available because of increased gluconeogenesis and energy demands, cataplerosis and anaplerosis become unbalanced. The transition period is characterized by this imbalance, induced by the combination of a limited DMI and a lipid mobilization that increase NEFA and TAG circulation. Nonesterified fatty acids are converted to acetyl-CoA, which is oxidized through the TCA cycle (Figure 1.6). However, when NEFA are elevated, the capacity to completely oxidize acetyl-CoA exceeds the capacity of the TCA cycle, specifically OAA, to completely oxidize all NEFA. Therefore, acetyl-CoA is partially oxidized to ketone bodies or re-esterified to TAG, ultimately increasing acetate, acetoacetate, and BHBA synthesis, and TAG infiltration in the liver (Grummer, 1993; Drackley et al., 2001; van Knegsel et al., 2005; White, 2015).

The input-output equilibrium of precursors for the TCA cycle is essential to maintaining sufficient OAA for acetyl-CoA oxidation (van Knegsel et al., 2005; White et al., 2012; White, 2015). However, this balance can be compromised by various physiological and nutritional challenges. Around parturition, hepatic expression of PC increases thereby increasing oxidative capacity (Greenfield et al., 2000; Loor et al., 2006). At calving, mitochondrial and peroxisome oxidation increases, which is essential for complete or partial oxidation of NEFA. However, when the oxidative capacity is exceeded, the alternative metabolic fate of acetyl-coA is ketogenesis or esterification (Drackley, 1999). Upregulation of PCK1 post calving increases the use of OAA for glucose production (Aschenbach et al., 2010; Karcher et al., 2007; Carvalho et al., 2011). Ketotic cows are characterized by an upregulation of the hepatic genes involved in cytokine signaling, inflammation, oxidation (including PC), and esterification. On the other hand, genes involved in gluconeogenesis are downregulated, especially PCK (Loor et al., 2007).

PC and PCK balance are the key control point to shift net carbon flux towards glucose production. The coordination between these genes is essential to optimizing hepatic metabolism, especially during the periparturient period. Investigating the impact of dietary changes on the regulation of these genes is pivotal in order to define the best nutrition practices for transition dairy cows.

Nutritional strategies to reduce metabolic disorders-fatty liver

Optimal nutritional strategies for the periparturient dairy cows are not yet clearly defined. However, some nutritional strategies have been proposed to alleviate metabolic disorders during the transition period. Negative energy balance, feed intake reduction, increased body lipid mobilization, hormonal changes, raised blood NEFA and ketones concentration, and liver lipid accumulation are the main metabolic challenges that can lead to metabolic disorders. In order to relieve these conditions, several nutritional approaches have been adopted to better meet glucose demand and decrease TAG liver accumulation. The main proposed solutions consist of a diet formulation apt to increase energy density and the inclusion of additives to modify metabolism and reduce liver TAG accumulation.

The National Research Council (NRC) establishes nutrient and energy density recommendations for dry, lactating and pregnant cows. Formulation of two different diets for the dry period and prepartum transition period of the cows is recommended. In fact, energy requirements for these periods are different. Energy density of 1.25 Mcal NE_L/kg DM is suggested as adequate for meeting the energy requirements for far-off dry cows. However, this energy level is suggested to be inadequate during the final two weeks prepartum, when 1.62 Mcal NE_L/kg DM is recommended. Increasing dietary energy density provides more energy that can be used for maintenance and gestation (NRC, 2001). Increasing the supplementation of non-fiber carbohydrate (NFC) pre-partum may be beneficial for ruminal microorganisms as well, since cows will receive high concentrate diets after parturition (Sundrum, 2015). Diets characterized by higher NFC content promotes the development of ruminal papillae for adequate absorption of VFA by rumen wall (Rabelo et al 2003). In a study conducted by

Knegsel and colleagues (2007) cows were fed on glucogenic (starch) or lipogenic diets; results showed that NEB and fat mobilization were lower in cows fed the gluconeogenic diet.

However, as several studies showed (Hayirli et al., 2002; Grummer and Rastani, 2003; Grummer, 2008), DMI decreases 30-35% prior to calving. Hayirli et al. (2002) reported that DMI is extremely mutable in pre-partum cows. Decreased DMI can compromise energy status pre-calving (Grummer, 2008). Cows fed with fat during the transition period may show lower DMI depression. Many studies (Grummer and Carroll, 1991; Chilliard, 1993; Allen et al. 1995) proved that feeding fat prepartum did not affect DMI. However, fat supplementation does increase plasma NEFA concentration. These results may be due to the increased incorporation of the fatty acids into lipoprotein, which ultimately can be released as NEFA.

Adipose lipolysis, TAG export from the liver and fatty acid oxidation can be modulated by several dietary interventions and nutrients such as ionophores (monensin), propylene glycol, and choline. Ionophores are produced by a variety of actinomycetes and can modulate rumen fermentation Methane production is reduced, the proportions of acetate and butyrate are decreased, while propionate is increased. Early studies determined propionate production increased 50 to 75% above control (Van Maanen et al., 1978) and methane production decreased by 30% (Schelling, 1984; Mackintosh, et al., 1997). However, the utilization of ionophores as feed additives for dairy cows has been banned in Europe. Monensin was previously authorized in the European Union as a feed additive to improve growth and feed efficiency in cattle, except for lactating cows (Council Regulation (EC) No 1831/2003). Its use in cattle was phased out as of January 2006, while is still used for prevention and control of coccidiosis.

Propylene glycol administration has a potential impact on insulin response and fatty acid mobilization from adipose tissue. Rumen microbial degradation of propylene glycol produces propionate (Clapperton and Czerkawski, 1972); thus, blood propionate concentration increases stimulating an increase in blood insulin concentration (Kristensen and Raun, 2007). Insulin reduces lipolysis in

adipose tissue, reducing NEFA released from adipocytes (West and Passey, 1967; Chung et al., 2009). This supplement is able to increase glucose and insulin prepartum, and reduce NEFA, total liver lipids and blood ketones postcalving (Grummer, 2008; Studer et al., 1993).

Choline is a micronutrient that can enhance VLDL export and has been proposed as a limiting nutrient for lactating dairy cows (Erdman, 1991). Choline is classified as a vitamin, although, it is not a vitamin in a traditional sense because it is not a part of an enzyme system, and is required in gram rather than milligram amounts as for true vitamins (NRC, 2001; Pinotti, 2012). Choline can be synthetized endogenously and it is not involved as a co-factor in enzymatic reactions. The ability to synthetize choline does not mean it is a non-essential nutrient. Choline can be metabolized to betaine and further to S-adenosyl methionine (SAM), serving a methyl donor function. Beyond methyl donor function, choline plays a major role as a constituent of membrane phospholipids as PtdCho. Phosphatidylcholine is required for synthesis and release of VLDL by the liver (Pinotti, 2002). In light of its important function in lipid metabolism for synthesis and release of VLDL by the liver, choline supplementation has been adopted in transition dairy cows. Choline deficiency is considered as a major cause of fatty liver accumulation (Figure 1.7). Yao and Vance (1990) showed that choline deficiency in rats resulted in a pronounced increase in TAG liver content. Rumen protected choline (RPC) fed as supplementation form to transition cows tended to decrease TAG accumulation in liver slices in vitro (Piepenbrink and Overton, 2003). In an in vivo trial, Cooke et al. (2007) demonstrated that choline supplementation significantly reduced plasma NEFA and liver TAG concentration (16.7 and 9.3 mg/mg DNA for control and choline- supplemented cows). In this experiment, far off dry cows were fed restricted (to 30% of maintenance requirements for energy) for 10 days to induce fatty liver; cows received 0 or 15 g of rumen protected choline. Several research groups (Erdman and Sharma, 1991; Piepenbrink and Overton, 2003; Elek et al., 2012; Goselink et al., 2013; Leiva et al., 2015) have evaluated the efficacy of choline during the transition period utilizing different

concentrations of rumen protected choline. Elek et al. (2008) supplemented 25 g choline/day to transition cows for 21 days prepartum, and 50 g/day postpartum.

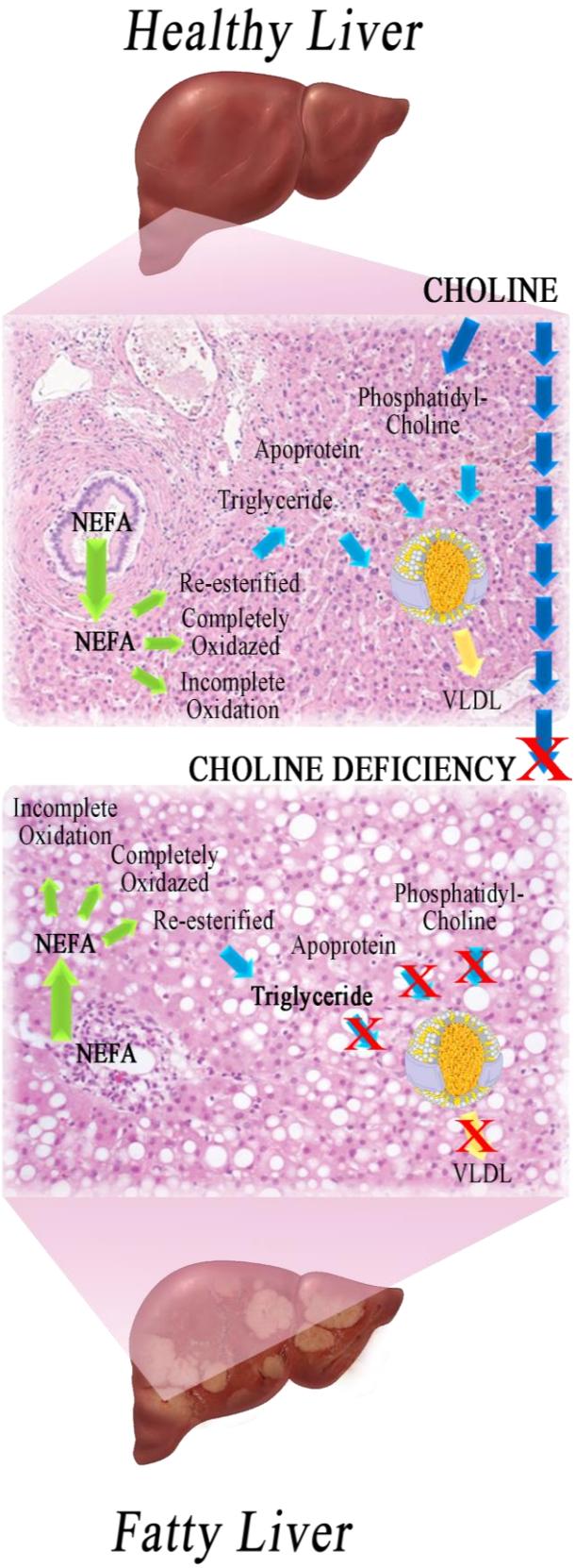

Figure 1.7 Hepatic metabolism, action of choline at liver level. Healthy liver box represents NEFAs taken up by the liver; when absorbed, NEFAs are: i) completely oxidized to provide energy; ii) incompletely oxidized to ketones; iii) or re- esterified to Triglyceride. Triglyceride, Apoprotein and Phosphatidylcholine are the main constituents of very low-density lipoprotein (VLDL). Choline as a constituent of membrane phospholipids as phosphatidylcholine plays an important role on VLDL synthesis by the liver. Choline deficiency is considered as a major cause of fatty liver accumulation.

Results from this experiment showed lower liver lipid concentration at day 7 and 35 in the RPC group compared to the control group receiving no supplement. Consistently with other research (Zenobi et al., 2016), choline supplementation (15 g/day) tended to increase milk yield during the first 100 days of lactation by 2.4 Kg/day (Scheer et al., 2002). In a study conducted by Pinotti et al. (2003), cows were fed 20 g/day of choline from 14 days pre-partum to 30 days postpartum resulting in increased milk production compared with unsupplemented cows (2.9 Kg/day). Several studies report that choline decreases plasma NEFA concentration and NEFA to cholesterol ratio in plasma at calving (Overton, 2005).

In light of these findings, choline supplementation during the transition period may reduce plasma NEFA, liver TAG accumulation and increase milk production.

Conclusions and Future Directions

One of the most challenging periods in the life cycle of dairy cows is represented by the transition period, characterized by different physiological stages requiring several metabolic adaptations in glucose and fatty acid metabolism to support fetal growth and milk production. Therefore, an effective monitoring of the transition period management is extremely important to breeders.

In nutrition and physiology research, biology and management of the transition period are crucial (Overton and Waldron, 2004). It was pointed out that several metabolic disorders that can affect dairy cows postpartum are strictly related to the diet fed during the transition period (Curtis et al., 1985). However, the increase of metabolic diseases is connected to NEB. Thus, alleviating NEB during the transition period is a top priority in dairy nutrition (Sun et al., 2016). On the other hand, understanding the mechanisms beyond these metabolic changes is necessary. In particular, in the last few years, the

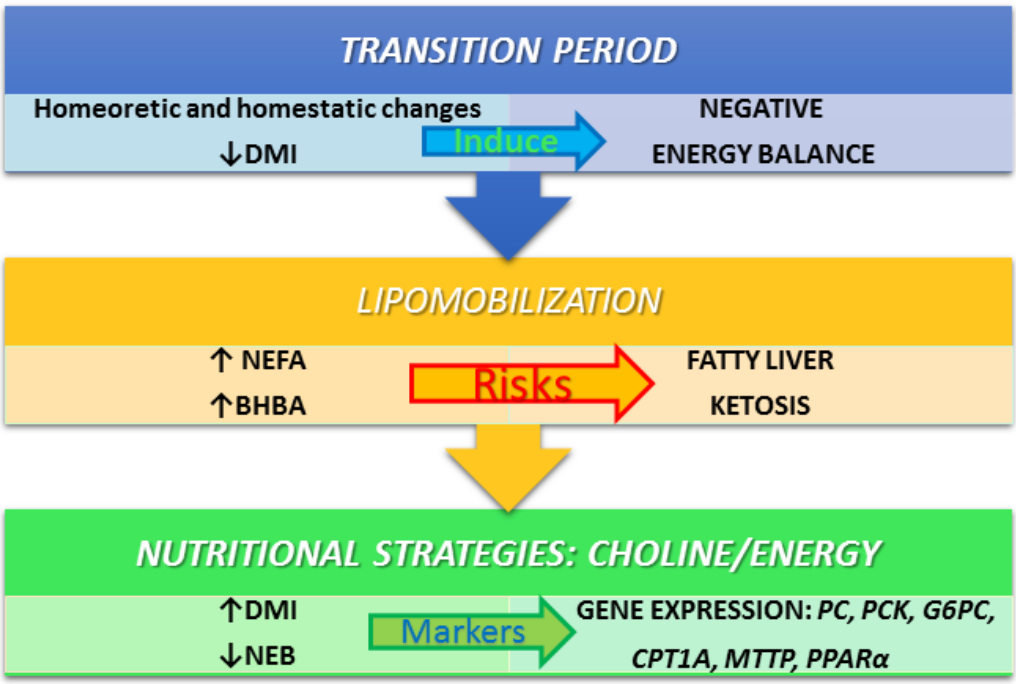

Figure 1.8 Summary of transition period, problematic and nutritional strategies.

application of nutrigenomics, i.e. the effects of nutrition on gene expression, has become essential to understand these mechanisms. Dietary compounds can affect gene expression directly or indirectly via interactions with transcription factors (Figure 1.8).

In light of this, the first aim of the present dissertation was to elucidate the effects of supplementation of rumen protected choline on milk yield and metabolic health in lactating dairy cows, adopting a meta-analysis approach. The second aim was to evaluate the effects of controlling prepartum energy intake or supplementing rumen-protected choline during the periparturient period on the regulation of hepatic gluconeogesis and oxidation in the transition to lactation. These data may provide new insights on how choline can modulate hepatic gluconeogenesis and lipid traffic, as well as evidence for possible strategies targeted at prevention and/or treatment of metabolic disorders in dairy cows.

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CHAPTER 2. EFFECT OF RUMEN-PROTECTED CHOLINE SUPPLEMENTATION ON MILK YIELD, NEFA AND BHBA IN LACTATING DAIRY COWS: A META-ANALYSIS

Abstract

Rumen-protected choline (RPC) supplementation has been reported to have an effect on milk yield and metabolic health in lactating dairy cows. In light of this, a meta-analysis based on 21 publications published from 1985 to 2016 was carried out in order to evaluate the effects of choline chloride supplemented in a rumen-protected form on milk yield (MY), nonesterified fatty acids (NEFA) and β-Hydroxybutyrate (BHBA). The inputs for meta-analysis were: means, standard error/standard deviation and P-value for each variable used. The effect sizes were calculated using Hedges' g approach. The random-effect model was used to determine the overall weighted mean difference. Methods of administration (blended or topdressed) were included as categorical characteristic moderators, while continuous variables (choline dose, g/d; pre-partum treatment, days) were examined as covariates using random effects meta-regression. The heterogeneity of effect size through trials was tested by P values, Cochrane Q, and I2 statistic. Data analysis was performed using Prometa Software Version 2 (INTERNOVI s.a.s., Cesena, FC, Italy). Results obtained showed positive effects ($P < 0.05$) of RPC supplementation on milk yield in lactating dairy cows. Choline chloride supplementation in a rumen-protected form ranged from 6.25 to 50g/d, and the average milk yield response was 2.14±1.86 kg/d (overall random effect). Meta-regression on dose- response relationship between dietary RPC and MY was significant ($P < 0.05$; intercept -0.01; slope 0.03). When NEFA and BHBA were considered, no effect of RPC supplementation was observed ($P > 0.05$), as confirmed by heterogeneity test ($P > 0.05$). Moderator analysis revealed that all outcomes, MY, NEFA and BHBA were not affected by the RPC administration method (blended vs. topdressed). Considering these outcomes, supplementation of RPC would therefore seem to be essential for optimizing milk yield in dairy cows, while its effect on NEFA and BHBA needs further investigation.

Key words Rumen-protected choline, dairy cow, meta- analysis

Introduction

Recent advance in nutrition have established that choline is an essential nutrient for mammals when a sufficient supply in methionine and folates is not available in the diet. Vitamin B12 is also involved in this process. The dynamic interactions between these nutrients introduced the concept of choline as vitamin-like compound. Choline can be considered according its two different functions: as choline per se, for which choline moiety is required, and as a methyl donor. Choline per se plays a major role in lipid metabolism, particularly in lipid transport and trafficking, as a lipotropic agent. Choline is also an important source of labile methyl groups for the biosynthesis of other methylated compounds. Based on this second function, choline and methionine are interchangeable, as sources of methyl groups. Accordingly, choline occupies a key position between energy and protein metabolism in mammals (Pinotti, 2012).

However, choline and methyl group metabolism in ruminants is different. In adult ruminants, choline is extensively degraded in the rumen - thus, dietary choline does not significantly contribute to the choline body pool - while methyl group metabolism is generally conservative, with a relatively low rate of methyl catabolism and an elevated rate of de novo synthesis of methyl groups via the tetrahydrofolate system. This can be exacerbated in lactating dairy ruminants, whose dietary availability of choline is nearly non-existent, output of methylated compounds in milk is high, and methionine, as well as other sources of methyl groups, is likely to be in short supply, especially at the onset of lactation. In light of this, the hypothesis that choline can be a limiting nutrient for milk production, has been formulated and tested in several studies (Erdman and Sharma 1991; Erdman, 1994; Hartwell et al. 2000; Piepenbrink and Overton 2003; Pinotti et al. 2003; Scheer et al. 2002; Xu et al., 2006; Zahra et al., 2006; Elek et al., 2008; Davidson et al., 2008). To date, though choline requirement in dairy cows is still unknown (NRC, 2001), there are several indications that higher choline availability (by feeding rumen-protected choline, RPC) can have a favorable effect on milk production (Erdman and Sharma 1991; Erdman, 1994; Hartwell et al. 2000; Piepenbrink and Overton 2003; Pinotti et al. 2003; Scheer et al. 2002). In fact, numerous narrative reviews aiming at

summarizing and addressing the variable response to RPC supplementation on milk production are based on such indications (Grummer, 2012; Pinotti et al., 2002; Brüsemeister and Südekum, 2006; Pinotti, 2012). Narrative reviews are typically performed by the best experts in a given field. However they suffer from two main limitations, i) subjectivity, i.e. study selection, weight assigned to the single study, interpretation of findings for each study review; ii) difficulty to manage a huge volume of information, i.e. author may be able to synthetize data from few studies, however when the treatment effect is considered it can vary from study to study. In this respect, in 2010, a meta-analysis was conducted by Sales and coworkers (2010) in order to quantify the effects of dietary RPC on production traits of dairy cows. Dry matter intake (kg/d), milk yield (kg/d), milk fat (% and kg/d), and milk protein (% and kg/d) were evaluated as dependent variables in models. Accounting for the experiment as a random effect, DMI, milk yield, milk protein content, and milk protein yield could adequately be related to levels of dietary rumen-protected choline chloride by a logistic model. Marginal responses in milk yield decreased from 131.5 to 0.037 g of milk/g of dietary rumen-protected choline chloride when supplementation increased from 6 to 50 g/d. From estimated values for the metabolizable Met supplied by diets, it appears that dietary rumen-protected choline chloride functions as a methyl donor to spare Met for milk protein synthesis. However, the authors also stated that more accurate input data on Met status of diets are needed to confirm this. Within the range of 6 to 50 g/d of rumen-protected choline chloride, milk fat content decreased linearly at a rate of 0.00339% for a 1g/d increase in dietary rumen-protected choline chloride. This illustrates that dietary rumen-protected choline chloride has no effect on milk fat content. Numerous physiological and dietary factors probably related to the responses obtained with dietary rumen-protected choline supplementation, and the precise mechanism of choline action in lactating dairy cows warrants further investigation. In Sales at al., (2010) however, the number of studies varied from 7 for milk fat and protein contents to 11 studies for milk yield, whereas plasma metabolites were not considered. In light of this, the present meta-analysis investigates the effect of RPC supplementation not only on

milk yield, but also on selected plasma metabolites, namely NEFA and BHBA, in lactating dairy cows.

Materials and Methods

Data Sources

A literature search and a screening process were conducted using Web of Science and Google Scholar search engines to identify studies evaluating the influence of rumen-protected choline supplementation on milk production and plasma metabolites of dairy cows. The search criteria included studies published in English or Italian from January 1985 to May 2016. In order to create a data set of choline-related articles the following keywords were adopted: "rumen-protected choline" or "choline cows" or "choline transition period". A manual review of the reference list of the selected articles was conducted to identify additional articles for their possible inclusion. The literature search focused on articles published in peer-reviewed journals for the methodological accuracy of the studies.

Study selection

Selection criteria included: 1) data collected from January 1985 to May 2016; 2) study presented in a peer-reviewed journal including proceeding papers; 3) study published in English or Italian language to extract all relevant information; 4) dietary choline supplemented in rumen-protected form. Twenty studies (Table 2.1) were identified that satisfied the required criteria. As the aim of our study was to evaluate the effect of rumen-protected choline administration on milk yield and plasma metabolites in dairy cows, we considered and recorded experimental trials accounting for different breeds, trial length and doses of choline in a rumen-protected form, and considering milk yield and plasma blood metabolites.

The outcomes evaluated and included in the meta-analysis were: milk yield, blood NEFA and BHBA concentration, while the methods of analysis for NEFA and BHBA determination were not considered.

Table 2.1 Characteristics of included trials to evaluate the effect of rumen-protected choline supplementation on milk yield (MY), Nonesterified fatty acids (NEFA), β-hydroxybutirate (BHBA) in lactating dairy cows.

Study	Country	Dose, g/d of choline chloride	Mode of application	Cow/treatment, n	DIM	Day of Prepartum treatment, n	Outcome used
Abeni et al. 2007	Italy	0-25	Topdressed	11	0-35	21	MY
Ardalan et al. 2010	Iran	0, 13.8	Topdressed	10	0-140	28	MY
Ardalan et al. 2011	Iran	0, 13.8	Topdressed	10	0-70	28	MY, NEFA, BHBA
Chung et al. 2009	US	0, 12.5, 6.25	Topdressed	6	41-51	-	MY, NEFA, BHBA
Davidson et al. 2008	US	0, 8.8	Blended		21-91	-	MY, NEFA BHBA
Primiparus				8			
Multiparus				12			
Deuchler et al. 1998	US	0, 50	Blended	5	Mid-lactation	-	MY
Elek et al. 2008	Hungary	0, 50	Topdressed	16	0-60	21	MY
Elek et al. 2012	Hungary	0, 50	Topdress	16	0-60	21	NEFA, BHBA
Emanuele et al. 2007	US	0, 18	-	254, 253	217-287	-	MY
Erdman and Sharma 1991	US	0, 15, 30, 45	Blended	12	0-147	0	MY, NEFA,
Janovick Guretzky et al. 2006	US	0, 15	Topdressed	16, 5	0-21	21	MY
Leiva et al. 2015	Brazil	0, 18.8	Topdressed	11, 12	0-45	21	MY, NEFA BHBA
Piepenbrink and Overton 2003	US	0, 11.25, 15, 18.75	Topdressed	12	0-63	21	MY, NEFA, BHBA
Pinotti et al. 2003	Italy	0, 20	Topdressed	13	0-30	21	MY, NEFA, BHBA

Pinotti et al. 2004	Italy	0, 20	Blended	15	0-30	21	MY, NEFA, BHBA
Pinotti et al. 2015	Italy	0, 20	Topdressed	6	28-91	-	MY, NEFA, BHBA
Rahmani et al. 2014	Iran	0, 22.5	Topdressed	8	35-63	-	MY
Suksombat et al. 2011	Thailand	0, 20, 40	-	8	32-102	-	MY, NEFA, BHBA
Toghdory et al. 2007	Iran	0, 6.25, 12.5	-	8	Mid/late-lactation	-	MY
Xu et al. 2006_exp1	China	0, 7.5	Topdressed	7	0-21	7	MY
Xu et al. 2006_exp2	China	0, 11.25, 22.5,33.75	Topdressed	9	0-15	7	MY
Zahra et al. 2006	Canada	0, 14	Topdressed	45	0-28	21	MY, NEFA, BHBA

Data extraction

The template drafted for data extraction included: number of animals per treatment group, mean, and P-value or standard deviation for the variables considered in the meta-analysis (i.e. MY, NEFA, BHBA). If the standard deviation was not published, either it was estimated from the standard error or the data were excluded. Other factors that influenced the outcomes of interest were considered in the data extraction process, including type of study (factorial or not; multiple or single dose), treatment dose (g/die), type of rumen-protected choline (commercial brand), prepartum treatment duration (days), mode of supplementation (topdressed or blended), publication year, publication type (journal article or conference presentation), publication country. Data for all the analyses measured in each study were extracted and entered into a spreadsheet.

Statistical analysis

A meta-analysis was conducted on the extracted outcomes using ProMeta2 (INTERNOVI s.a.s., Cesena, FC, Italy). A random-effects model was used for each parameter to estimate the effect size (ES), 95% confidence interval (CI), and statistical significance of ES.

When studies are gathered from the published literature, the use of random effects model is suggested (Borenstein et al., 2009). Random-effects Meta-analysis estimates the mean of a distribution of effects. Study weights are more balanced under the random-effects model than under the fixed-effect model. Furthermore, the standard error of the summary effect and, as a consequence, the confidence intervals for the summary effect are wider under the random-effects model than under the fixed-effect model (Borenstein et al., 2009). The ES estimate analysis was conducted using the Hedges' g approach. Based on conventional standards, effect sizes of g equal to 0.20, 0.50, and 0.80 were considered small, medium, and large, respectively (Cohen, 1988). Heterogeneity between studies was assessed using the Cochrane Q test and I2 score. The χ2-based Cochran's Q statistic and the I2 statistic were used to quantify evaluated heterogeneity (Higgins & Thompson 2002). The I^2 statistic yields results ranging from 0 to 100% and value higher than 50% represents substantial heterogeneity (Higgins et al. 2003). Publication bias was investigated using "trim and fill" procedure (Duval & Tweedie, 2000). This method uses an iterative procedure to remove the most extreme small studies from the positive side of the funnel plot, re-computing the effect size at each interaction until the funnel plot is symmetric about the (new) effect size (Borenstein et al., 2009). Categorical characteristic (mode of application) was treated as a moderator, while continuous characteristics (dose of choline and prepartum treatment duration) were examined as covariates using random effects of meta-regression.

Results

Study selection

The flowchart of the study selection process is shown in Figure 2.1. The literature search on the influence of rumen-protected choline supplementation on milk production and plasma metabolites of dairy cows, conducted using Web of Science and Google Scholar search engines, identified 363 publications. After the screening analysis, 284 studies were removed for the following reasons: language (n=9), irrelevant outcomes or topics (n=256) and reviews (n=19). Subsequently, a full-text assessment was performed on twenty-seven studies and six studies were removed. Of these, one study applied feed restriction, one study combined the supplementation of RPC with another micro-nutrient, two studies did use choline in unprotected form and two studies did not report raw means and SD or SE. After applying exclusion criteria, 19 studies were selected. Two additional articles were added manually searching in references. A total of 21 studies were included in the meta-analysis. Some studies included different trials. Different trials from the same study have been considered as single different study in this meta-analysis. The characteristics of included trials are reported in Table 2.1. The meta-analysis has considered the follow inputs: milk yield (MY), nonesterified fatty acids (NEFA), β-hydroxybutyrate (BHBA). Of the selected trials, 10 reported MY, NEFA and BHBA means; 10 studies reported MY means; one reported MY and NEFA means; and one study reported NEFA and BHBA means. The selected trials were characterized by two methods of RPC supplementation, namely topdressed and blended. From the selected studies, 15 adopted topdressed method, of these one blended RPC with 0.5 Kg of total mixed ratio (TMR), one study blended RPC with 0.5 Kg of concentrate; 3 adopted blended methods, and 4 did not report their supplementation method. Dose of choline chloride in a rumen-protected form (g/d) ranged from a minimum of 6.25 g/d to a maximum of 50 g/d. The study conducted by Janovick et al. (2006) included a breed (Jersey) different from Holstein.

Studies by Xu et al. (2006) and Davison et al. (2008) divided cows by parity (primiparous or multiparous). Davison and collaborators provided separate means for primiparous and multiparous cows, while Xu's study reported two experiments where only multiparous cows were considered separately, providing separate means for all cows (composed of primiparous or multiparous) and multiparous cows.

Several studies included prepartum RPC treatment. Nine different commercial products were selected for RPC supplementation (Table **2.2**). The study conducted by Deuchler et al. (1998), Davidson et al. (2008), and Xu et al. (2006) did not reported the name of the product used. The percentage of choline chloride inclusion for these products ranged from 18, 8 to 50 % wt/wt.

Figure 2.1 Meta-analysis flow-chart—flow diagram demonstrating studies selection for meta-analysis (PRISMA diagram).

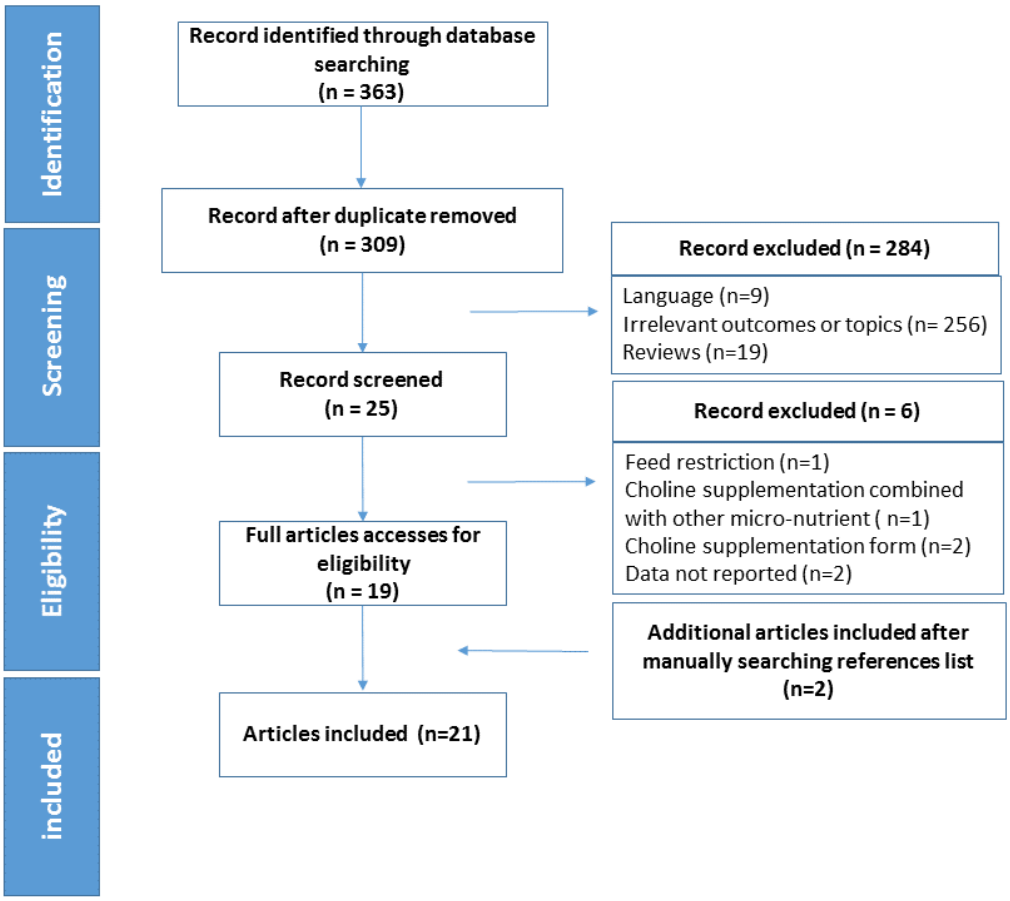


Table 2.2 Description of commercial rumen-protected choline products used in the different trials.

Study	Choline Chloride % (wt/wt)	Product/Manufacturer
Abeni et al. 2007	50	11Sta-Chol (Ascor Chimici, Italy)
Ardalan et al. 2010	24	COL24 (Soda Feed Ingredients, Monaco, France)
Ardalan et al. 2011	24	COL24 (Soda Feed Ingredients, Monaco, France)
Chung et al. 2009	50	Pro-Choline 50 (Probiotech Inc., Saint-Hyacinthe, Quebec, Canada)
Davidson et al. 2008	–	–
Deuchler et al. 1998	–	–
Elek et al. 2008	25	Norcol-25 (Nordos Italy, Bussolengo, Italy)
Elek et al. 2012	25	Norcol-25 (Nordos Italy, Bussolengo, Italy)
Emanuele et al. 2007	25	Reashure (Balchem Corp., Slate Hill, NY)
Erdman and Sharma 1991	–	Showa Denko (KK, Yokyo, Japan)
Janovick Guretzky et al. 2006	25	Reashure (Balchem Corp., Slate Hill, NY)
Leiva et al. 2015	18.8	CholiPearl, Kemin Agrifoods South America, Indaiatuba, São Paulo, Brazil
Piepenbrink and Overton 2003	25	Reashure (Balchem Corp., Slate Hill, NY)
Pinotti et al. 2003	40	Overcholine 45% (Coated Ascor Chimici, Forli, Italy)
Pinotti et al. 2004	40	Overcholine 40% Coated, Ascor Chimici, Forlì, Italy
Pinotti et al. 2015	25	Reashure (Balchem Corp., Slate Hill, NY)
Rahmani et al. 2014	25	Reashure (Balchem Corp., Slate Hill, NY)
Toghdory et al. 2007	25	Capshure choline Balchem Corp., Slate Hill, NY
Xu et al. 2006	–	–
Zahra et al. 2006	25	Reashure (Balchem Corp., Slate Hill, NY)

Meta-analysis of Rumen-protected choline supplementation on lactating cows

The results obtained in the present meta-analysis are summarized in Table 2.3; the graphical representations, as forest plot of rumen-protected choline effects on MY, NEFA and BHBA are reported in Figure 2.2, Figure 2.3, Figure 2.4, respectively. A significant effect of RPC on MY was observed (ES=0.52; 95% C.I. 0.31-0.72; $P < 0.001$). The heterogeneity test was significant for the same traits (I^2 >50%; $P < 0.001$). Concerning the effect of RPC supplementation on lactating dairy cows on plasma metabolites, neither NEFA nor BHBA were affected by the treatment (P >0.05). In this case no heterogeneity was observed for both NEFA and BHBA (I^2=0.00; $P > 0.05$).

Table 2.3 Summary statistic Overall (random-effects model) and Test of Heterogeneity.

					Test of Heterogeneity	
	No. of treatment means	ES	P value	SE	I^2	P value
MY	32	0.52	<0.001	0.11	57.08	<0.001
NEFA	20	0.001	0.99	0.09	0.00	0.48
BHBA	17	-0.10	0.27	0.10	0.00	0.91

Figure 2.2 Forest plot of rumen-protected choline effects on milk yield (MY).

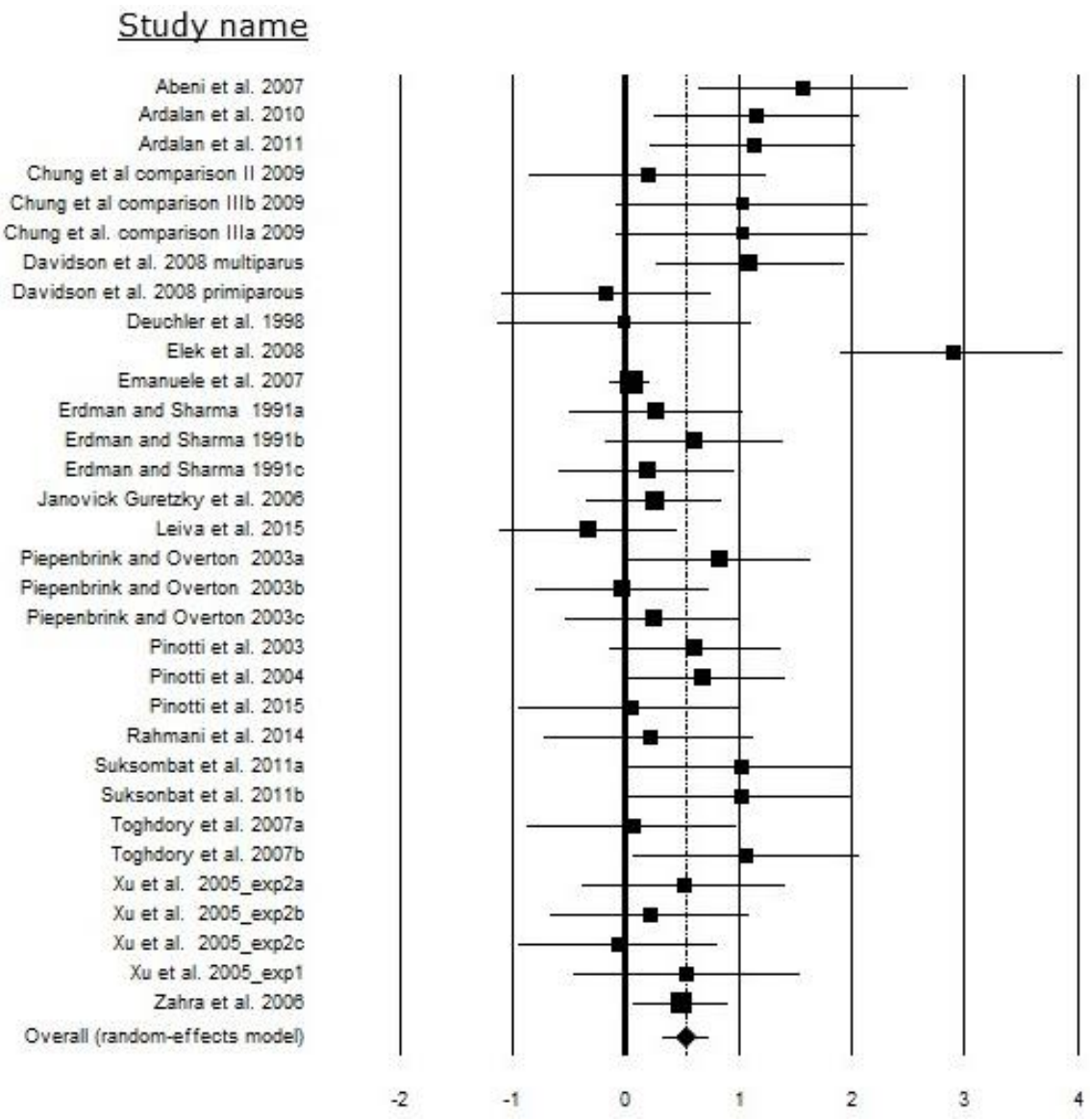


Figure 2.3 Forest plot of rumen- protected choline effects on nonesterified fatty acids (NEFA).

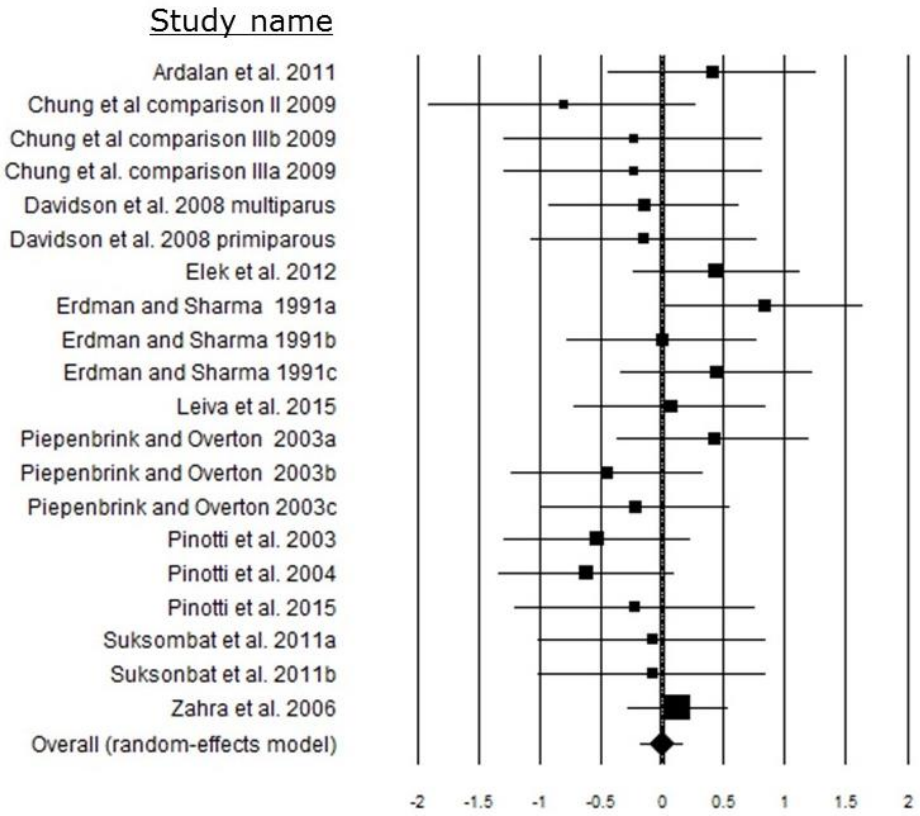
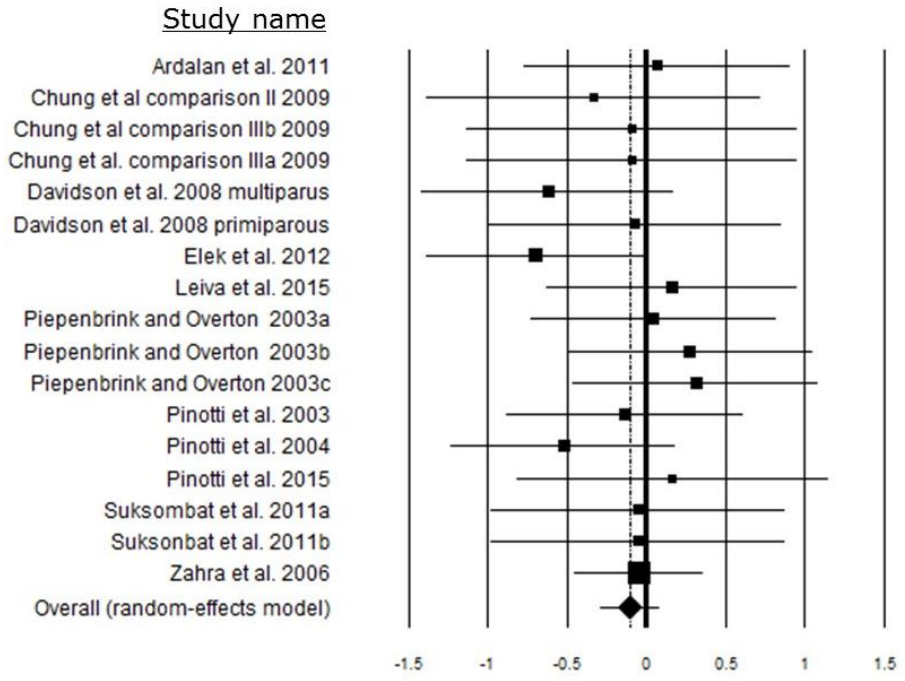


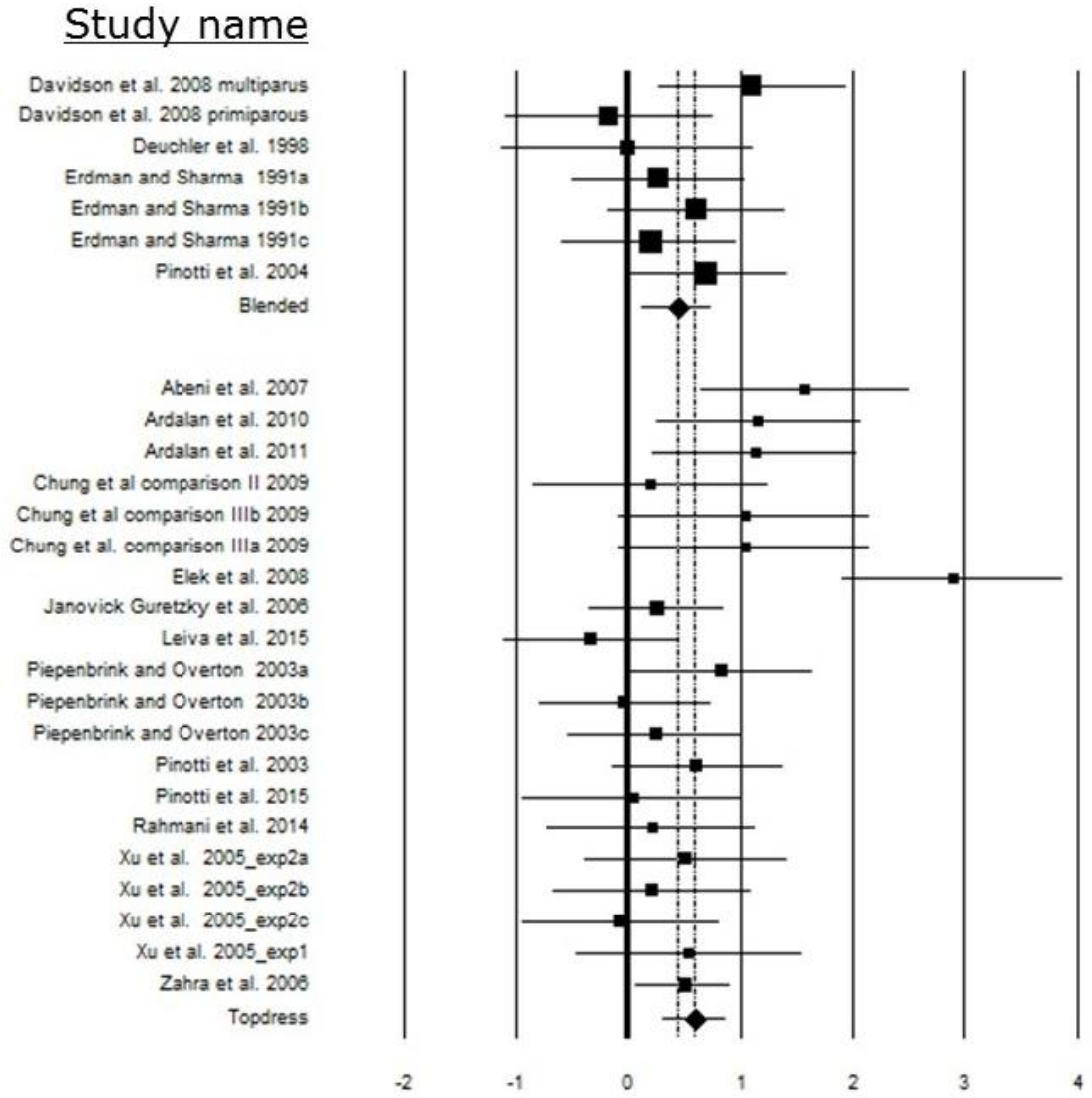
Figure 2.4 Forest plot of rumen- protected choline effects on β-hydroxybutyrate (BHBA).



Moderator analysis

The methods of administration of the treatment (RPC), blended vs. topdress, were included as a qualitative moderator in the analysis. All parameters considered, namely MY, NEFA and BHBA, were not affected by the methods of administration of the treatment ($P > 0.05$). The graphical representation of results obtained for MY moderator analysis is shown in Figure 2.5 as representative example.

Figure 2.5 Moderator analysis of milk yield (MY), blended vs topdress.



Meta-regression

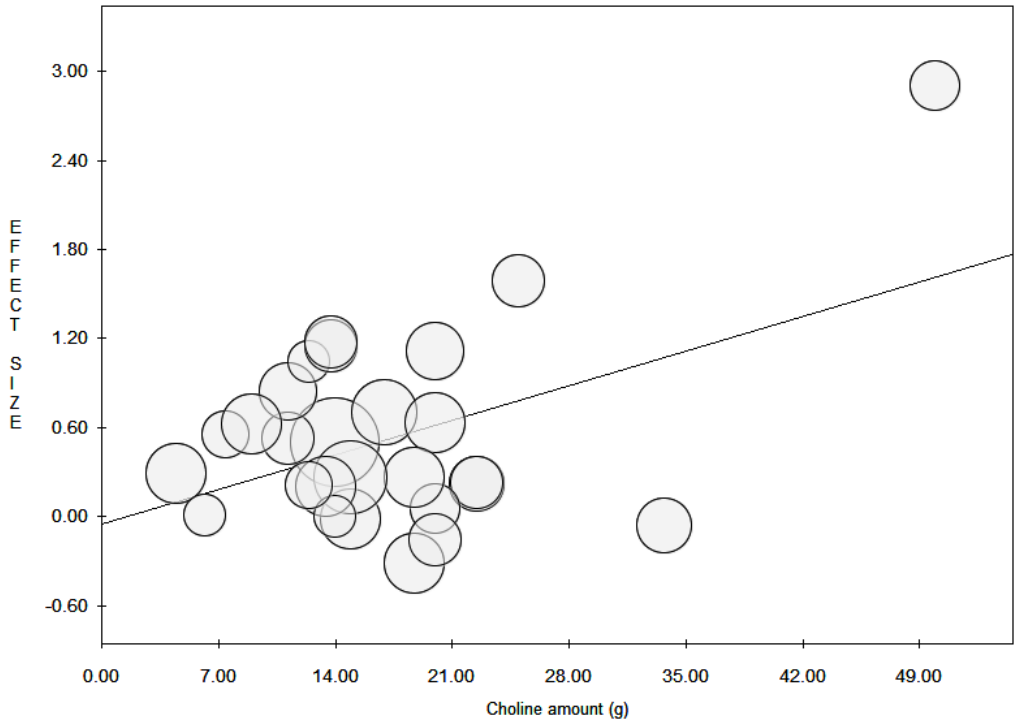
A further step in the meta-analysis was to investigate by meta-regression the possible effects of two continues variables on MY, NEFA and BHBA, namely the dose of choline chloride in the rumen-protected form and the length of its administration (Days of administration before calving),. The meta-regression results are reported in Table 2.4.

Table 2.4 Meta-regression of rumen-protected choline and prepartum treatment.

	No. of treatment means	Intercept	Slope	P value
MY	32			
Choline amount (g)		-0.01	0.03	0.01
Prepartum treatment (day)		0.35	0.02	0.11
NEFA	20			
Choline amount (g)		-0.03	0	0.877
Prepartum treatment (day)		-0.03	0	0.808
BHBA	17			
Choline amount (g)		0.16	-0.01	0.046
Prepartum treatment (day)		-0.13	0	0.84

A positive association between choline dose and MY was observed (slope= 0.03; intercept -0.01; $P < 0.01$); its graphical representation is reported in Figure 2.6. When prepartum treatment duration and MY were considered, a tendency ($P = 0.11$) was observed. By contrast, no association (Table 2.4) among choline dose, prepartum treatment duration and plasma NEFA concentration was detected. In the case of plasma BHBA concentration, a significant ($P < 0.05$) interaction was observed with the choline amount (slope= -0.01; intercept 0.16). However, no interaction between BHBA plasma levels and prepartum treatment was observed.

Figure 2.6 Meta-regression plot of milk yield (MY).



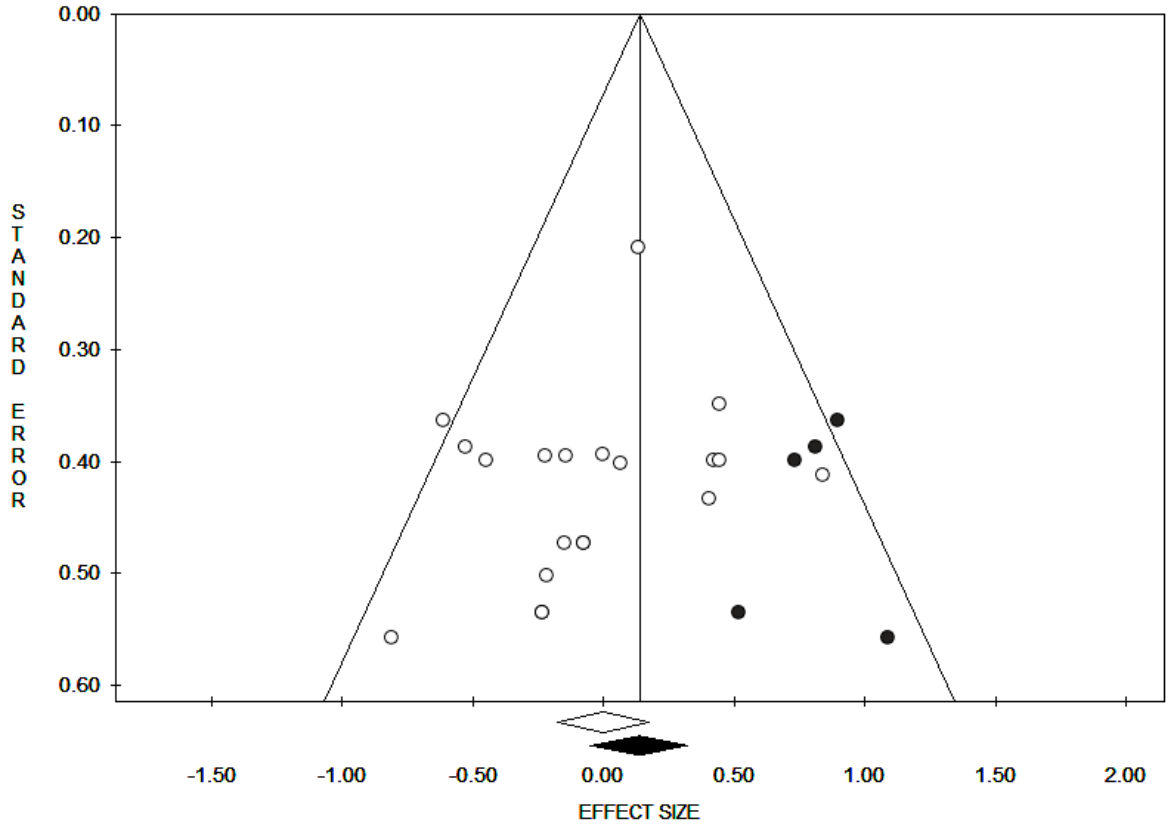
Publication bias of Rumen-protected choline supplementation on lactating cows

The Trim and Fill method did not evidence any bias for MY and BHBA. By contrast, a moderate bias was observed in the case of NEFA. The adjusted values, reported in Table 2.5, did not affect the results. Graphical representation of ES adjustment is shown in Figure 2.7.

Table 2.5 Adjusted results from "Trimm and fill" method of the overall effect size for random effect model.

Overall effect size	No. of treatment means	ES	P value	SE
MY	32			
observed		0.52	<0.001	0.11
estimated		0.52	<0.001	0.11
*trimmed study = 0				
NEFA	20			
observed		-0.001	0.99	0.09
estimated		0.14	0.16	0.10
*trimmed study = 5				
BHBA	17			
Observed		-0.10	0.3	0.10
Estimated		-0.10	0.3	0.10
*trimmed study = 0				

Figure 2.7 Funnel plot for the association between RPC supplementation and blood nonesterified fatty acids (NEFA) concentration; solid diamond represents estimated ES of RPC supplementation on blood NEFA (♦), empty diamond represents observed ES of RPC supplementation on blood NEFA (◊).



Discussion

Results obtained in the present meta- analysis showed a positive effect ($P < 0.05$) of RPC supplementation on milk yield in lactating dairy cows. These results are in line with several studies and reviews (Overton, 2005; Grummer et al., 2012; Pinotti, 2012; Brüsmeister and Südekum, 2006; Pinotti et al., 2010) reporting a positive effect of RPC supplementation on MY. These results were obtained in presence of a significant heterogeneity among different studies, which should guarantee a quite wide range of variability among the studies included. This is in line with the inputs in the analysis: of the 21 works considered in the present meta-analysis, thirteen reported a significant effect of choline on milk yield, while nine studies reported no effect of choline supplementation on the same variable.

Although the method used in the present study does not provide a direct measurement of the MY response (kg/d) to RPC administration, from the trials considered, rumen- protected choline supplementation was able to increase milk yield by 2.14±1.86 Kg/d. However, this result must be considered with caution since it represents an estimation obtained from the raw dataset. In spite of that, these figures seems to be in line with another report published by Grummer (2012) reporting that RPC supplementation is able to increase milk yield by 2.2 Kg/d.

Of course, the method proposed in the present work does not allow a precise evolution of the possible mechanism of action of RPC in lactating dairy cows. The link between choline supplementation and milk response has been mainly attributed to the metabolic interchangeability of choline and methionine, in the sense that both can furnish labile methyl groups. This interchangeability was investigated in goats by Emmanuel and Kennelly (1984), who estimated that 6% of the choline pool derives from methionine and that approximately 28% of methionine is used for choline synthesis via the pathway for the de novo biosynthesis of the choline moiety involving sequential methylation of phosphatidylethanolamine. Subsequent studies (Pinotti at al., 2012; Xue and Snoswell, 1985; Snoswell and Xue, 1987; Robinson et al., 1984) supported this hypothesis that nowadays is still the

most reported. A further step in the meta-analysis was to test the dose-response effect of choline on MY. In order to elucidate this possible connection a meta-regression analysis was performed (Table 2.4; Figure 2.6). Results obtained indicated that choline dose significantly affects milk response. The overall mean of all trials considered, was 14 g/d of choline chloride. Considering data reported for each single study, thirteen trials reported a significant effect of choline on MY, where the dose of choline chloride utilized varied from a minimum dose of 6.25 g/d up to a maximum of 50 g/d with an average around 15.71±9.60 g/d. Considering the minimum and maximum dose of choline indicated in the studies in the present meta- analysis, a tendency for a beneficial effect on MY by choline supplementation was observed by Chung at al. (2009) even in the presence of the minimum level of choline chloride (6.25g/d). Likewise, a significant effect of choline supplementation was observed in a study performed by Elek et al. (2008) where the maximum choline chloride amount (50 g /d) was implied. The highest response on milk yield (6.4 kg/d) obtained by supplementing 50 g/d of choline chloride in multiparous cows occurred in a study carried out by Davidson et al. (2008), which was not true in primiparous cows. Conversely, a trial conducted by Erdman and Sharma (1991) on a group including both primiparous and multiparous cows showed a lower milk yield response supplementing 13.5 g/d of choline chloride. Although the meta-analysis we performed did not include parity as moderator (only one study, Davison et al. (2008), provided separated data according to parity groups), the latter two cases exemplify how data from different studies can differ in terms of response. Thus, it can be speculated that the dose-response effect can be hidden by other variables in different studies. A further aspect to be considered is the stage of lactation monitored in the two studies, namely early lactation by Davison et al. (2008) and from mid to late lactation by Erdman and Sharma (1991).

Despite these discrepancies, the results herein presented seem to be in line with Pinotti et al. (2010) meta-analysis indicating that milk yield can only increase when a dose higher than 10 g/d of choline chloride in a rumen-protected form is supplemented.

When prepartum treatment was considered in meta- regression, milk yield response did not reach statistical significance, even though a tendency was observed (P = 0.11). This is a key aspect that can be very important from different points of view. The effect of choline depends (i) on the period when supplementation is started, (ii) the duration of supplementation, (iii) the level of supplementation and (iv) the efficacy in protecting choline from rumen degradation. For instance, Pinotti (2012), Shahsavari et al. (2016) seem to indicate that RPC can be more effective when supplementation starts before parturition and is protracted to the onset of lactation. This hypothesis appears in line with the present results even though they recorded only a tendency in effectiveness.

The dose-response of choline was not the only moderator considered by the present meta- analysis that also considered the method of its administration. The different studies reported two different forms for supplemented choline, i.e. blended or topdressed. The former means that the animals receive any nutrient supplementation embedded in their diet. In this case, RPC was mixed with the total mixed ratio (TMR) composed by roughage and concentrate ingredients. The latter means that the animals are given choline supplementation in a small amount of feed added on top of the TMR or of the usual ratio. Data from moderator analysis suggested that the mode of supplementation (blended vs. topdress) did not affect milk production response (Figure 2.5). This aspect may be due to the small sample size (number of trials considered in each class) and the large variability, highlighted by the heterogeneity test (I^2= 57.08; $P < 0.001$).

With regard to the metabolic health markers investigated in this meta-analysis, it is well known that the highest plasma NEFA concentration occurs near the time of calving as a consequence of hormonal changes, reduction of feed intake and increase in energy requirements to support lactation (Grummer, 2010). During the first 3 weeks of lactation, the major source of energy needed for milk synthesis comes from NEFA. A situation caused by the decrease of feed intake that leads to this metabolism shift. NEFA, once taken up by the liver, can be completely oxidized to provide energy, partially oxidized to ketone bodies (BHBA) or re-esterified to TAG that can be stored in the hepatocytes or

exported from the liver to other tissues. Excessive NEFA concentration results in higher production of ketone bodies that can cause ketosis disease during the transition period. The role of choline in alleviating this condition is due to the fact that it is involved, as phosphatidylcholine, in the synthesis of VLDL that can export liver TAG to other tissues. In light of the role of choline on liver lipid metabolism, it might be expected that animals receiving RPC supplementation be characterized by lower blood NEFA and BHBA concentration (Pinotti, 2012). In spite of this, data provided by the present meta- analysis suggest that choline supplementation did not alter blood NEFA and BHBA concentration. However, a positive association between choline amount and BHBA was observed, indicating that choline supplementation decreased plasma BHBA concentration. In light of this, three trials reported a significant effect of choline supplementation on either NEFA or BHBA or on both. The study performed by Albeni et al. (2007) showed that choline supplementation tended to reduce blood NEFA and increased blood BHBA concentration. Elek et al. (2012) in their investigation found that plasma NEFA was not affected by RPC supplementation, while plasma BHBA concentration decreased when choline was supplemented. Pinotti et al. (2004) evaluated a positive effect on NEFA reduction by choline administration, but no significant effects on BHBA. The majority of the studies considered in this meta- analysis reported same results on blood BHBA concentration. These contradictory results can suggest that RPC supplementation may not directly affect the lipid metabolism from adipose tissue and consequently the plasma NEFA concentration.

Conclusions

The aim of this study was to evaluate the choline response on milk production and on changes in blood metabolites in lactating dairy cows. Accordingly, a meta-analysis was performed to summarize the effects of rumen-protected choline chloride supplementation in lactating dairy cows through studies and to investigate the factors explaining potential response heterogeneity.

Overall, these data indicate that RPC supplementation in lactating dairy cows improves milk production. About 62% of the trials included in this meta- analysis reported a significant increase in milk production, confirming the hypothesis that choline can improve milk yield. Moreover, dose-response relationship between dietary RPC and MY was significant, indicating that rumen-protected choline chloride can have a dose-dependent effect on milk yield response. However, these data are in contrast with the initial hypothesis that RPC can decrease blood NEFA and BHBA concentrations. No significant effect of rumen- protected choline chloride was observed for both NEFA and BHBA. Combining the observation of MY, NEFA and BHBA, it can be speculated that the significant MY increase may have masked an improvement of the metabolic profile.

This meta-analysis provides additional information regardless blood metabolite response to RPC supplementation. In addition, another meta-analysis conducted on RPC supplementation in lactating dairy cows reported only milk-production but no blood-metabolite response.

Eventually, results from studies conducted on RPC supplementation show inconsistent and call for further investigation in this field.

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CHAPTER 3. HEPATIC OXIDATION IS RESPONSIVE TO PREPARTUM ENERGY AND PERIPARTUM RUMEN PROTECTED CHOLINE SUPPLEMENTATION

Abstract

Controlling prepartum energy intake or supplementing rumen-protected choline (RPC) during the periparturient period, are two strategies to optimize hepatic metabolic function. The objective of this study was to examine the regulation of hepatic gluconeogesis and oxidation during the transition to lactation. At -48 days relative to calving (DRTC), multiparous Holstein cows were assigned to either a controlled (1.40 Mcal of NE_L/kg DM; CE) or high (1.63 Mcal NE_L/kg DM; HE) energy prepartum diet with or without RPC (top-dressed daily from -21 to +21 DRTC). Postpartum diets only differed by addition of RPC. Liver tissue biopsy samples were collected at -14, +7, +14, and +21 DRTC for RNA isolation and cDNA generation (n=16/treatment). Quantitative PCR was performed and mRNA abundance was normalized to reference genes. Data were analyzed by Proc Mixed (SAS 9.4, 2009) with repeated measures in a model that accounted for the main effects of RPC, energy, DRTC, and corresponding 2-way and 3-way interactions, and the random effect of cow (energy×choline). When interactions were significant ($P \leq 0.05$), energy×choline means were separated by Tukey's and time interactions were separated within timepoint by slice. Data are presented as least squares means + SE, arbitrary units (AU). Pyruvate carboxylase (PC) expression increased ($P \leq 0.05$) after calving. There was an energy×choline ($P \leq 0.05$) and choline DRTC ($P \leq 0.05$) interaction where RPC increased PC expression at -14 and +7 DRTC. There was no interaction ($P > 0.1$) of prepartum energy and DRTC. Expression of cytosolic phosphoenolpyruvate carboxykinase (PCK1) was greatest ($P \leq 0.05$) at +14 and lowest at -14 and +7 DRTC (1.62a, 0.75b, and 0.62b + 0.16 AU, respectively). Expression of PCK1 was decreased ($P \leq 0.05$) in cows fed HE+RPC compared with other treatments (0.57b, 1.00ab, 1.26a, 1.21a + 0.09 AU; HE+RPC, HE, CE+RPC, CE). Expression of glucose-6-phosphatase was increased ($P \leq 0.05$) at +14 and +21 DRTC, and decreased (energy×choline; $P \leq 0.05$) in cows fed the CE+RPC (1.36 vs. 2.32, 2.33, 2.24 + 0.17 AU; CE+RPC, CE, HE+RPC, HE). Expression of carnitine palmitoyltransferase 1A was greatest at +21 DRTC ($P \leq 0.05$) but was

unaltered ($P > 0.1$) by energy or choline. The transcription factor PPARA was increased ($P \leq 0.05$) in CE+RPC (1.35 vs. 0.86, 0.68, 0.90 + 0.08 AU; CE+RPC, CE, HE+RPC, HE). Microsomal triglyceride transfer protein (MTTP) was increased ($P \leq 0.05$) at +14 and +21 DRTC. Increased PC peripartum with RPC, across energy treatments, may support increased oxidative capacity at calving. Decreased PCK1 in HE+RPC may serve to increase oxidation of increased circulating NEFA by maintaining the oxaloacetate pool.

Key words Gluconeogenesis, TCA cycle, transition cow

Introduction

The term ‘transition dairy cow’ was introduced in the 1990s and refers to cows during the period from approximately 3 weeks before calving to 3 weeks after calving (Grummer, 1995; Drackley, 1999). The transition to lactation is one of the most metabolically challenging periods for dairy cows. Homeorhetic changes, negative energy balance (NEB), and increased risk for metabolic and reproductive disorders can affect dairy cattle during this period (White, 2015). Insufficient feed intake leads many animals to develop a severe NEB due to the high energy requirement of lactation and insufficient feed intake (Block et al., 2001; Drackley et al., 2001; Grummer et al., 2004). To meet their energy requirements during the first weeks of lactation, dairy cows mobilize fatty acids from adipose tissue, resulting in increased nonesterified fatty acids (NEFA) in the blood steam (Overton and Piepenbrink, 1999). Nonesterified fatty acids are largely metabolized by the hepatic tissue involving several pathways. Once taken up by the liver, NEFA are converted to acetyl-CoA units. Acetyl-CoA can be completely oxidized through the tricarboxylic acid cycle (TCA), incompletely oxidized through ketogenesis, or fatty acids can be re-esterified to triglycerides (TAGs) and exported from the liver as very-low density lipoprotein (VLDL), or stored as liver lipids (Grummer, 1993; White, 2015). VLDL secretion is relatively low in ruminants. The rapid increase in NEFA and their uptake by the liver could predispose animals to hepatic lipidosis and ketosis (Kleppe et al., 1988).

Several approaches to prevent fatty liver have been studied and can be subdivided into three main categories: decrease TAG lipolysis in adipose tissue reducing blood NEFA concentration; increase complete hepatic oxidation of NEFA increasing the flux of carbons into TCA cycle; increase VLDL packaging to export TAG from the liver (Overton and Piepenbrink, 1999). The most common practices to prevent hepatic lipid accumulation are the development of diet formulations with increased energy density and the inclusion of feed additives to reduce the likelihood of hepatic TAG accumulation (Grummer, 2008). One among the widely adopted practices to prevent fatty liver is the supplementation of rumen protected choline (RPC) during the transition period. Moreover, inclusion

of choline in dry cows' diets might enhance VLDL secretion. Choline availability in ruminants is hampered by the loss of dietary choline by extensive microbial degradation (Sharma and Erdman, 1989), which means that supplements should be industrially protected against ruminal degradation. Choline is classified as a vitamin-like compound (NRC, 2001; Pinotti, 2012) that has a variety of functions in mammalian metabolism. Choline is a part of Phosphatidylcholine (PtdCho), the predominant phospholipid contained in membranes; a component of acetylcholine, a neurotransmitter; and is involved in methyl metabolism as a betaine precursor (Overton, 2005).

Consistent with recent research, RPC supplementation to dairy cattle may improve the rate of VLDL synthesis and thereby decrease TAG accumulation in the liver (Grummer, 2008; Cooke et al., 2007; Zom et al., 2011), increase milk production (Elek et al., 2008) and increase milk protein production (Zom et al., 2011). Synthesis of VLDL requires TAG, phospholipids (PtdCho), cholesterol esters, microsomal triglyceride transfer protein (MTTP), and apolipoproteins including apolipoprotein B100 (Bernabucci et al., 2004). Goselink et al. (2013) observed a decrease in TAG liver content with RPC supplementation, hypothesizing that RPC supplementation may enhance VLDL synthesis by upregulating hepatic *MTTP* expression, which promotes VLDL assembly in the endoplasmic reticulum (Wetterau et al., 1997).

Increased glucose demand and insufficient intake during early lactation, lead dairy cows' metabolism to undergo metabolic and endocrine adaptation. In order to support these changes, several hepatic energy metabolism adaptations occur at the beginning of lactation including stimulation of gene expression of transcription factors and enzymes involved in glucose production, FA oxidation, and ketogenesis in the liver (Greenfield et al., 2000; Loor et al., 2005).

As reported before, feeding strategies for the dairy cattle during transition may impact production in the ensuing lactation through changes in the availability and type of gluconeogenic precursors, and key reactions in glucose metabolism (Greenfield et al., 2000). Gluconeogenesis is a key pathway to

meet the high glucose requirements of lactation. In ruminants, propionate produced by ruminal fermentation is the principal substrate for hepatic gluconeogenesis, accounting for 50 to 60% of total glucose entry in fed animals (Lomax and Baird, 1983). Voluntary feed intake reduction during the periparturient period (Bertics et al., 1992; Coppock et al., 1971; Grummer et al., 1995; Hernandez-Urdaneta et al., 1976; Johnson and Otterby, 1987; Vazquez-Anon et al., 1994), leads to a shift of metabolism to the utilization of different glucose precursors in place of propionate. Lactate, glycerol, and amino acids contribute a greater percentage of total glucose synthesis in an energy insufficient state (Danfaer et al., 1995; Donkin and Armentano, 1993; Lomax and Baird, 1983; Reynolds et al., 1988).

Pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PCK) are limiting enzymes involved in gluconeogenesis and formation of oxaloacetate (Greenfield et al., 2000). Glucose 6-phosphate, the last step of gluconeogenesis, may also play an important role for glucose synthesis in ruminants. Liver metabolism is challenged by plasma NEFA as a result of the mobilization of fat in early lactation to compensate for NEB. Genes belonging to Peroxisome proliferator-activated receptors (PPAR) family of ligand-activated nuclear transcription factors can be activated by NEFA. Specifically, isoform PPARA is involved in expression of genes involved in lipid oxidation, ketogenesis, and gluconeogenesis in the liver (Goselink et al., 2013). CPT1A is responsible for the regulation of mitochondrial β-oxidation in the liver, and is also a known target of PPARA in mice, humans (Rakhshandehroo et al., 2010) and dairy cattle (Loor et al., 2005; van Dorland et al., 2009). Loor et al. (2005) found that mRNA expression of several hepatic genes involved in lipid metabolism is changed during the transition period. Despite this knowledge, the mechanisms of hepatic regulation of the metabolism in dairy cows and for the beneficial effects of RPC and energy intake in periparturient dairy cattle are still not fully understood. The aim of this study was to examine the regulation of hepatic gluconeogesis and oxidation genes during the transition to lactation dependent upon RPC supplementation during the periparturient period, and prepartum energy intake.

Materials and Methods

This experiment was in collaboration with the University of Florida and all experimental procedures involving animals were approved by the University of Florida Animal Care and Use Committee. Ninety-three non lactating multiparous Holstein cows (n = 93) were grouped by expected calving date and randomly assigned to treatment groups. Diets were provided as total mixed ration (TMR) *ad libitum* using Calan gates. Prepartum diets were arranged in a 2 by 2 factorial design (n = 16 per group). The animals at -48 days relative to calving (DRTC) were randomly assigned to one of four treatment groups, controlled energy (CE, 43% wheat straw) or high energy (HE, 58% corn silage) prepartum diet with or without RPC. Net energy for lactation (Mcal/Kg of DM) was 1.40 and 1.63 for the control and high energy prepartum diet, respectively. Cows in the RPC group received 60 g of a RPC source daily (ReaShure, Balchem Corp., New Hampton, NY) that was mixed with ground corn and dried molasses in a 30:56:14 ratio (as-is basis) and top-dressed onto the TMR fed to individual cows. Cows assigned to the 0 g/d RPC treatment received a top-dressing of ground corn and dried molasses in an 80:20 ratio (as-is basis). The experimental treatments with RPC were applied from -21 to +21 DRTC. Animals were followed through 15 weeks postpartum.

Liver tissue collection and selection of cows for gene transcript analysis

Liver biopsies were performed at -14, 7, 14, and 21 DIM using a stainless steel percutaneous liver biopsy tool (Aries Surgical, Davis, CA). An ultrasound imaging on the right flank (Aloka SSD-500 equipped with a 3.5-MHz convex transducer, Aloka Co., Tokyo, Japan) was used to determine the optimal intercostal liver biopsy location. Following clipping of the hair, cleaning of the area with povidone iodine scrub and 70% isopropyl alcohol, local anesthesia (10 mL of 2% lidocaine), and 1.5 cm incision, liver tissue (0.5 to 1.5 g wet weight) was collected with 1 to 2 insertions. Liver tissue was rinsed with sterile saline, sliced into three sections, transferred into three cryovials, snap-frozen in liquid N, and then stored at -80^{0}C until analysis. A subset of cows from each treatment group (n=16) was selected for gene transcript analysis at the University of Wisconsin-Madison. Selection

criteria included: 1. availability of liver tissue from all four biopsy time points; 2. prepartum biopsy at least five days prior to calving (i.e. cow did not calve more than 10 days early); 3. no chronic health conditions that resulted in severe reductions in DMI.

RNA extraction, real time RT-PCR, and primer evaluation

Total RNA was isolated using Trizol reagent (Invitrogen), following the manufacturer's instructions, and quantified by absorbance at 260 nm using a Synergy Hybrid Spectrophotometer (BioTek, Winooski, VT). To eliminate DNA contamination, the isolated RNA was treated with DNase I and further purified using an RNeasy Mini Kit (Qiagen, Thousand Oaks, CA). Cleaned samples (0.5 µg) were reverse transcribed to cDNA (n=16/treatment) using iScript reverse transcriptase kit (Bio-Rad Laboratories, Inc., Hercules, CA). Abundance of mRNA transcripts was determined using real-time PCR, SsoFast EvaGreen supermix (Bio-Rad Laboratories, Inc., Hercules, CA) and primers described in Table 1. A 100 µL RNA pool was formed by combining equal quantities of total RNA from all cows and all sampling times. A cDNA pool was formed by combining an equivalent quantity of cDNA from each sample and used to generate a 1 to 4 dilution series standard curve.

The abundance of transcripts was determined relative to the cDNA pool. Nuclease-free water served as the no template control for the assay. Before analyzing samples, primers were evaluated and a single PCR product was verified using the following protocol: 1 cycle at 95 °C for 3 min; 45 cycles at 95 °C for 5 s, 55 °C for 5 s; and a melt curve from 65 °C to 95 °C by 0.5 °C increment for 3 s. The abundance of the pyruvate carboxylase (PC), cytosolic phosphoenolpyruvate carboxykinase (PCK1), glucosephosphatase (G6PC), carnitine palmitoyltransferase 1A (CPT1A), peroxisome proliferator-activated receptor alpha (PPARA), microsomal triglyceride transfer protein (MTTP), mRNA were determined using SYBR Green-based real-time quantitative PCR. Primers for reference and target genes are presented (Table 3.1).

Samples were analyzed in triplicates using the following reaction: 1 cycle at 95 °C for 3 min; 45 cycles at 95 °C for 5 s, 55 °C for 5 s. Only reaction efficiencies that were between 90 and 110% based

on standard curve of pooled samples were used for further analysis. Mean values for transcripts evaluated were normalized to the arithmetic mean of mRNA abundance of two reference genes within each sample: hydroxymethylbilane synthase (HMBS) and alpha-1-microglobulin/bikunin precursor (AMBP). Because 18S ribosomal (18S), which was also tested as reference gene, was influenced by RPC, energy, and time, it was excluded from the analyses. The use of the arithmetic mean of HMBS and AMBP for data normalization was verified by the lack of effect of treatment on the threshold cycle (Ct) and differences Ct values of less than 1 between groups. Data are expressed as arbitrary units of mRNA adjusted for HMBS and AMBP abundance.

Table 3.1 Primers used for Reference and Target genes.

		Primer sequence (5'-3')	
Gene	GeneBank accession	Forward	Reverse
	Reference gene		
AMBP	NM_173989.3	ACTGTCAAGCTCTATGGGCG	CCTCTGTCGGGCATTGTGAA
HMBS	NM_001046207.1	GATGGGCAACTGTACCTGACT	TGGTTTGCATGGTGTCTTGC
	Gluconeogenesis-related gene		
PC	NM_177946.4	CCACGAGTTCTCCAACACCT	TTCTCCTCCAGCTCCTCGTA
PCK1	NM_174737.2	AGGGAAATAGCAGGCTCCAGGAAA	CACACGCATGTGCACACACACATA
G6PC	NM_001076124.2	TGATGGACCAAGAAAGATCCAGGC	TATGGATTGACCTCACTGGCCCTCTT
	FA oxidation-related gene		
CPT1A	NM_001304989.1	TTCGCGATGGACTTGCTGTA	TTTCCTCCCGGTCCAGTTTG
	Nuclear receptor-related gene		
PPARA	NM_001034036.1	ACAAAGCCTCTGGCTACCAC	AGCTTCAGCCGAATCGTTCT
	VLDL packaging-related gene		
MTTP	NM_001101834.1	ACCTGTGCTCCTTCATCTAATTCAT	GCTAGCCAGGCCTCTCTTGA

Statistical Analysis

Data were analyzed using Proc Mixed of SAS 9.4 (SAS Inst. Inc., Cary, NC). Analysis accounted for the fixed effects of energy, choline, DRTC, associated two- and three-way interactions and the random effect of cow within treatment (EnergyxCholine). Repeated measures were used and a power (POW) correlation covariance structure SP was used since the biopsy timepoints were unequally spaced. Means were considered different when $P < 0.05$ and tended to differ if $0.05 < P \leq 0.10$. Tukey-Kramer studentized adjustments were used to separate treatment means within the two-way interactions. Within significant three-way interactions, slice option was used to separate means within a specific DRTC. Results are reported as least squares means and standard errors of the means.

Results

Results for feed intake (DMI), body condition score (BCS), nonesterified fatty acids (NEFA), β-hydroxybutyrate (BHBA), glucose, and concentration of liver triacylglycerol (liverTAG) are presented elsewhere (Zenobi et al., 2016) but also briefly given below. In order to better interpret the data, the results from DMI, BCS, NEFA, BHBA, and glucose content were considered for the transition period (three weeks before and 3 weeks after parturition); while liver TAG, milk yield (MY) and milk composition were considered for the experimental period (from -14 to +21 DRTC).

Feed intake and body condition score

A DRTC effect ($P \leq 0.05$) was detected, during the transition period, for dry matter intake (DMI). High energy (HE) tended to increase pre-partum DMI compared to the CE fed group (11.29 vs 10.42, $0.05 < P \leq 0.10$); an Energy effect (E) on post-partum was also detected ($P \leq 0.05$), where cows fed HE diet had greater DMI compared to CE group (18.84 vs. 17.74 Kg/d). A tendency for Choline (C) treatment to increase DMI during the transition period was detected ($0.05 < P \leq 0.10$), whereas cows fed RPC tended to have greater DMI during the transition period (18.73 vs 17.85 Kg/d).

DRTC affected BCS during the pre-partum period ($P \leq 0.05$); an interaction between ExDRTC was detected for the same period ($P \leq 0.05$). Cows that received the HE diet tended to have greater DMI at -49 DRTC compared to CE group. There was a DRTC and Choline effect for BCS during the post-partum period ($P \leq 0.05$); cows that received RPC supplementation had higher BCS at +14 and +21 DRTC compared with the unsupplemented group.

Nonesterified fatty acids, β-hydroxybutyrate, Glucose and Liver Triacylglycerol Concentration

There was an Energy (E) effect for NEFA concentrations in plasma pre-partum. Cows that received the HE diet had a greater level of NEFA compared to the CE group (503.68 vs 451.88 μEq/L, $P \leq 0.05$). A DRTC effect was detected post-partum ($P \leq 0.05$).

There was a DRTC effect on BHBA concentration. During the post-partum period, an Energy effect was detected for BHBA concentrations in plasma. Cows that received the HE diet had higher BHBA concentration compared to the CE group (5.28 vs 4.36 mg/dL, $P \leq 0.05$). There was also an interaction between ExDRTC that tended to be significant ($0.05 < P \leq 0.10$). At +7 and +21 DRTC cows that received the HE diet had greater BHBA concentration compare to the CE group. There was a significant interaction between CxDRTC ($P \leq 0.05$); cows fed RPC tended to have greater BHBA concentration at +21 DRTC.

A tendency for an Energy effect during the pre-partum was detected for glucose concentration. Cows fed the HE diet tended to have greater glucose concentration compared to the CE group (64.18 vs 66.27 mg/dL, $0.05 < P \leq 0.10$). An interaction between ExDRTC was detected pre-partum, where HE group had greater glucose concentration at -7 DRTC compared to the CE group (67.33 vs 63.58 mg/dL, $P \leq 0.05$). There was also an interaction between ExC. A DRTC effect was detected during the post-partum period ($P \leq 0.05$).

Liver TAG concentration was affected by DRTC, increasing during the whole transition period ($P \leq 0.05$). A significant interaction between ExDRTC was detected, with the HE group having a greater liver TAG concentration at +21 DRTC (9.24 vs 7.38 %DM, $P \leq 0.05$) and a tendency to be greater at +7 DRTC (8.89 vs 6.59 %DM, P $0.05 < P \leq 0.10$) compared to the CE diet. Conversely, RPC had no overall effect on hepatic TAG (Control=5.73 vs. RPC=5.89 %DM) though an interaction among ExCxDRTC was detected ($P \leq 0.05$).

Milk yield, Lactose, Fat and Protein

DRTC affected MY the first three weeks of lactation ($P \leq 0.05$). Cows fed RPC had greater MY compared to the noRPC group (38.54 vs 35.68 Kg/d, $P \leq 0.05$). There was no interaction between CxDRTC. However, considering the effect slices there was a tendency at +7 DRTC and a significant effect at +14 and +21 DRTC, where RPC group had a greater MY through the transition period. There was also an interaction between ExDRTC, on MY during the first three weeks of lactation.

Milk lactose increased through the transition period ($P \leq 0.05$). There was ExCxDRTC interaction on milk lactose content ($P \leq 0.05$).

Milk fat and milk protein (Kg) were affected by DRTC ($P \leq 0.05$). A significant effect of choline on milk fat was detected such that cows fed RPC had greater milk fat at + 14 and +21 compared to the noRPC group DRTC (1.76 vs 1.59 and 1.31 vs 1.20 Kg/d, $P \leq 0.05$). There was also a significant effect of choline on milk protein. Cows fed RPC had greater milk fat at + 7 and tended to be greater at +14 and+21 DRTC compared to noRPC group (1.31 vs 1.20 Kg, $P \leq 0.05$).

Gene expression

Table 3.2 shows the effect of high energy (HE) and controlled energy (CE) with (CE+RPC, HE+RPC) or without (CE, HE) choline treatment on expression of key enzyme involved in gluconeogenesis, fatty acid oxidation, nuclear receptor and VLDL packaging metabolism in liver from -14 to 21 DRTC. The graphical representation of the main effects of energy or choline treatments is presented in Figure 3.1 and Figure 3.2, respectively. The two-way interaction between Energy or Choline and DRTC are shown in Figure 3.3 and Figure 3.4. Figure 3.5 is the graphical representation of the three-way interaction Energy x Choline x DRTC.

Gluconeogenesis-related genes

PC was increased as the transition period progressed ($P \leq 0.05$) and was greatest at +7, +14 and +21 DRTC ($P \leq 0.05$). Energy and supplementation of RPC did not alter PC abundance. An ExC interaction was detected ($P \leq 0.05$), but it was not possible to separate means within day with Tukey's. There was a significant CxDRTC interaction ($P \leq 0.05$). The RPC group had a greater PC mRNA abundance at -14 and +7 DRTC compared to the noRPC group. On the other hand, at +21 DRTC cows that received RPC had lower PC expression compared to the noRPC group. The ExCxDRTC interaction was not significant ($P > 0.1$); however, the slices effect denoted a significant effect at +21 ($P \leq 0.05$) and a tendency was detected at +7 DRTC ($0.05 < P \leq 0.10$).

PCK1 was increased during the transition period and was greatest ($P \leq 0.05$) at +14 and +21 DRTC. A significant effect of Energy ($P \leq 0.05$) was detected. The HE group had lower PCK1 expression compared to the CE group. There was also a significant effect of Choline ($P \leq 0.05$), such that RPC treatment decreased PCK1 expression. An ExC interaction was detected ($P \leq 0.05$). PCK1 expression for HE+RPC group was 2-fold lower compared to the CE+RPC group (0.57 vs 1.26, $P \leq 0.05$). A significant ExDRTC interaction was detected. The HE group had lower mRNA abundance at +21 DRTC compared to the CE group (0.29 vs 1.81, $P \leq 0.05$). The ExCxDRTC interaction tended to be significant ($0.05 < P \leq 0.10$). At -14 DRTC CE+RPC group had greatest mRNA abundance compared to the other groups, while at +21 DRTC PCK1 expression increased for the HE group compared to the CE group.

G6PC was increased across the transition period and was greatest ($P \leq 0.05$) at +14 and +21 DRTC. G6PC expression was decreased in the CE group (1.84 vs 2.29, $P \leq 0.05$) compared with HE and RPC had lower mRNA abundance compared to noRPC group (1.85 vs 2.28, $P \leq 0.05$).

An ExC interaction was detected ($P \leq 0.05$). The CE+RPC group had lower G6PC mRNA abundance compared to the other groups. There was a significant interaction between ExDRTC ($P \leq 0.05$) The HE group had a greater mRNA abundance compared to CE group. There was also a significant interaction between CxDRTC ($P \leq 0.05$) with the RPC group having a lower G6PC expression compared to the noRPC group. An ExCxDRTC interaction was detected ($P \leq 0.05$); with a significant slice effect ($P \leq 0.05$) at -14, +14 and 21 DRTC. At +14 and +21 DRTC CE+RPC treatment had a lower mRNA abundance compared to the other treatments.

Table 3.2 Effect of high energy (HE) and controlled energy (CE) with (CE+RPC, HE+RPC) or without (CE, HE) choline treatment on expression of key enzyme involved in gluconeogenesis, fatty acid oxidation, nuclear receptor and VLDL packaging metabolism in liver from -14 to 21 DRTC. Energy (E), choline (C), DRTC (D). Data are shown as least squares means and SEM. a-b Means with different superscripts are significantly different (ExC $P \leq 0.05$).

						P-Value						
Gene	HE	HE+RPC	CE	CE+RPC	SEM	E	C	DRTC	ExC	ExD	CxD	ExCxD
Gluconeogenesis-related gene												
PC	2.47	1.90	2.08	2.53	0.21	0.59	0.78	<0.05	<0.05	0.40	<0.05	0.15
PCK1	1.00	0.57	1.21	1.26	0.08	<0.05	<0.05	<0.05	<0.05	<0.05	0.12	0.08
G6PC	2.24	2.33	2.32	1.36	0.16	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
FA oxidation-related gene												
CPT1A	2.97	2.45	2.98	2.97	0.21	0.20	0.20	<0.05	0.21	0.18	<0.05	<0.05
Nuclear receptor-related gene												
PPARA	0.91	0.68	0.87	1.35	0.08	<0.05	0.11	0.47	<0.05	0.33	<0.05	<0.05
VLDL packaging-related gene												
MTTP	1.71	1.03	1.86	1.38	0.19	0.19	<0.05	<0.05	0.60	0.59	<0.05	0.92

FA oxidation-related gene

CPT1A expression was increased at ($P \leq 0.05$) at +7 and -21 DRTC. Energy and choline did not alter CPT1A expression ($P > 0.1$). An CxDRTC interaction was detected. There was also an ExCxDRTC interaction ($P \leq 0.05$); in particular, at +7 DRTC CE+RPC group had greater mRNA abundance compared to the other groups ($P \leq 0.05$), but at +21 DRTC had lower CPT1A expression compared to the other treatments ($P \leq 0.05$). There was also a significant difference at +14 DRTC ($P \leq 0.05$) where HE group had greater gene expression compare to HE+RPC group.

Nuclear receptor-related gene

PPARA was not affected by DRTC ($P > 0.1$). Energy increased PPARA expression ($P \leq 0.05$) and C did not affect mRNA abundance ($P > 0.1$). There was a significant interaction between ExC ($P \leq 0.05$), and the CE+RPC group had greater mRNA abundance compared to the other treatments. There was also a significant interaction between CxDRTC ($P \leq 0.05$). At +7 DRTC RPC group had greater PPARA expression (1.31 vs 0.71, $P \leq 0.05$), but at +21 the RPC group had lower PPARA abundance compared to the noRPC group (0.97 vs 1.08, $P \leq 0.05$). ExCxDRTC was significant ($P \leq 0.05$). Significant slice effects within the 3-way interaction were detected ($P \leq 0.05$) at +7, +14 and +21 DRTC and a tendency was observed at -14 ($0.05 < P \leq 0.10$). Specifically, CE+RPC group had grater mRNA abundance at +7, +14 and +21 DRTC compared to the other groups, while at -14 HE had lower expression compared to the other treatments.

VLDL packaging-related gene

MTTP was affected by DRTC ($P \leq 0.05$). Energy tended to affect MTTP expression ($0.05 < P \leq 0.10$) and RPC supplementation decreased mRNA abundance ($P \leq 0.05$). There was no ExC or ExDRTC interaction ($P > 0.1$). A CxDRTC interaction was detected, where the RPC group had the lowest

mRNA abundance at +21 DRTC compared to noRPC group. There was no 3-way interaction ($P >$ 0.1).

Figure 3.1 Effect of high energy (HE) and controlled energy (CE) treatment on expression of PC, PCK1, G6PC, CPT1A, PPARA, MTTP in liver from -14 to 21 DRTC. Data are expressed as arbitrary units of mRNA adjusted for the arithmetic mean abundance of two reference genes (HMBS and AMBP). Data are shown as least squares means and SEM. Gray bars represent high energy (HE) treatment, striped bars represent controlled energy. Superscripts denote significance ($P \leq 0.05$).

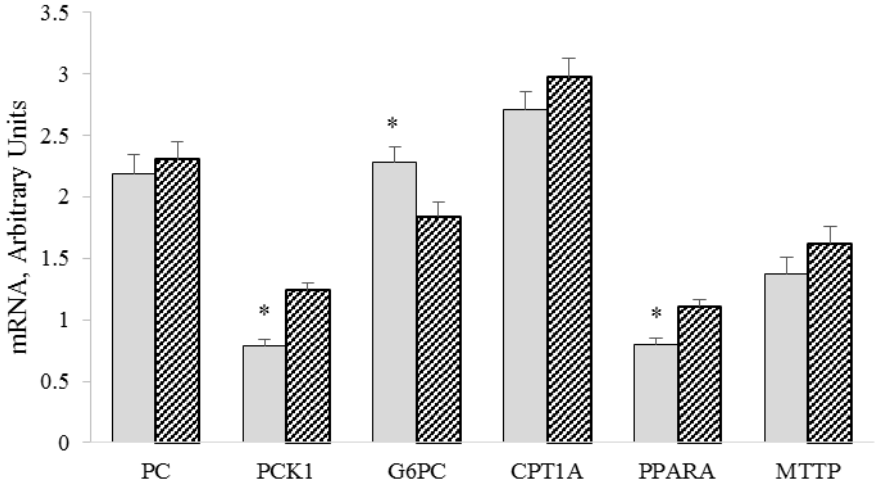


Figure 3.2 Effect of choline treatment on expression of PC, PCK1, G6PC, CPT1A, PPARA, MTTP in liver from -14 to 21 DRTC. Data are expressed as arbitrary units of mRNA adjusted for the arithmetic mean abundance of two reference genes (HMBS and AMBP). Data are shown as least squares means and SEM. Solid bars represent choline treatment, open bars represent no choline. Superscripts denote significance ($P \leq 0.05$).

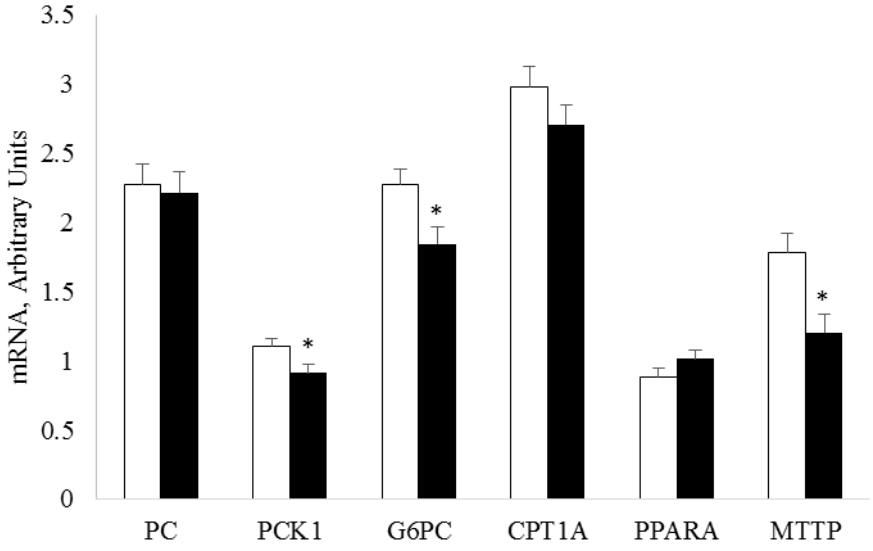


Figure 3.3 Effect of choline treatment on expression of hepatic PC (panel A), PCK1 (panel B), G6PC (panel C), CPT1A (panel D), PPARA (panel E), MTTP (panel F), from -14 to 21 DRTC. Asterisks denote days, within the two-way interactions (CxDRTC), that differ ($P \leq 0.05$) by slice effect. Letters indicate difference between treatment groups within specific days using Tukey ($P \leq 0.05$); asterisk, but no letters, indicates inability to separate means within day with Tukey ($P > 0.05$).

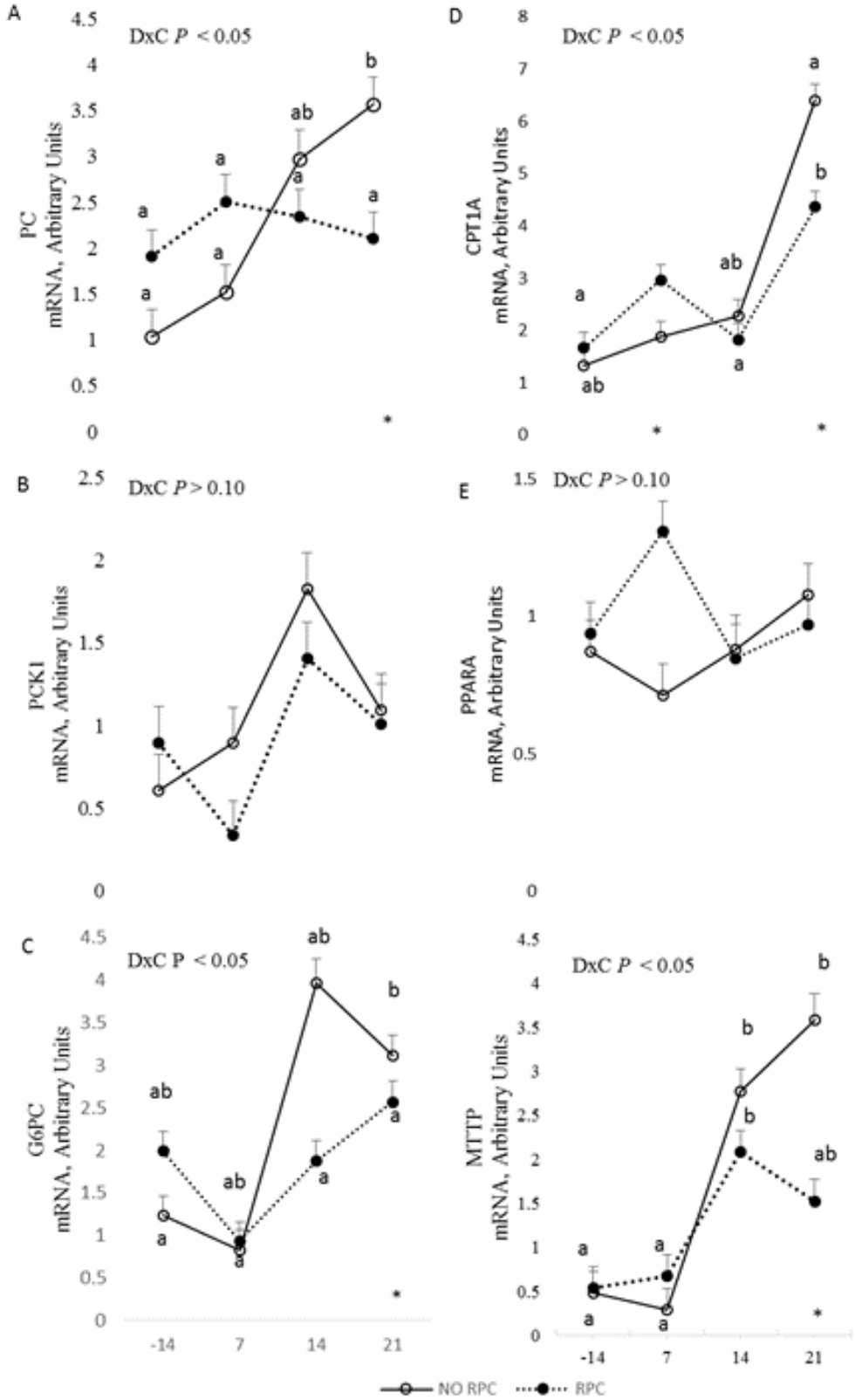


Figure 3.4 Effect of high energy (HE) and controlled energy (CE) on expression of hepatic PC (panel A), PCK1 (panel B), G6PC (panel C), CPT1A (panel D), PPARA (panel E), MTTP (panel F), from -14 to 21 DRTC. Asterisks denote days, within the two-way interactions (ExDRTC), that differ ($P \leq 0.05$) by slice effect. Letters indicate difference between treatment groups within specific days using Tukey ($P \leq 0.05$); asterisk, but no letters, indicates inability to separate means within day with Tukey ($P > 0.05$).

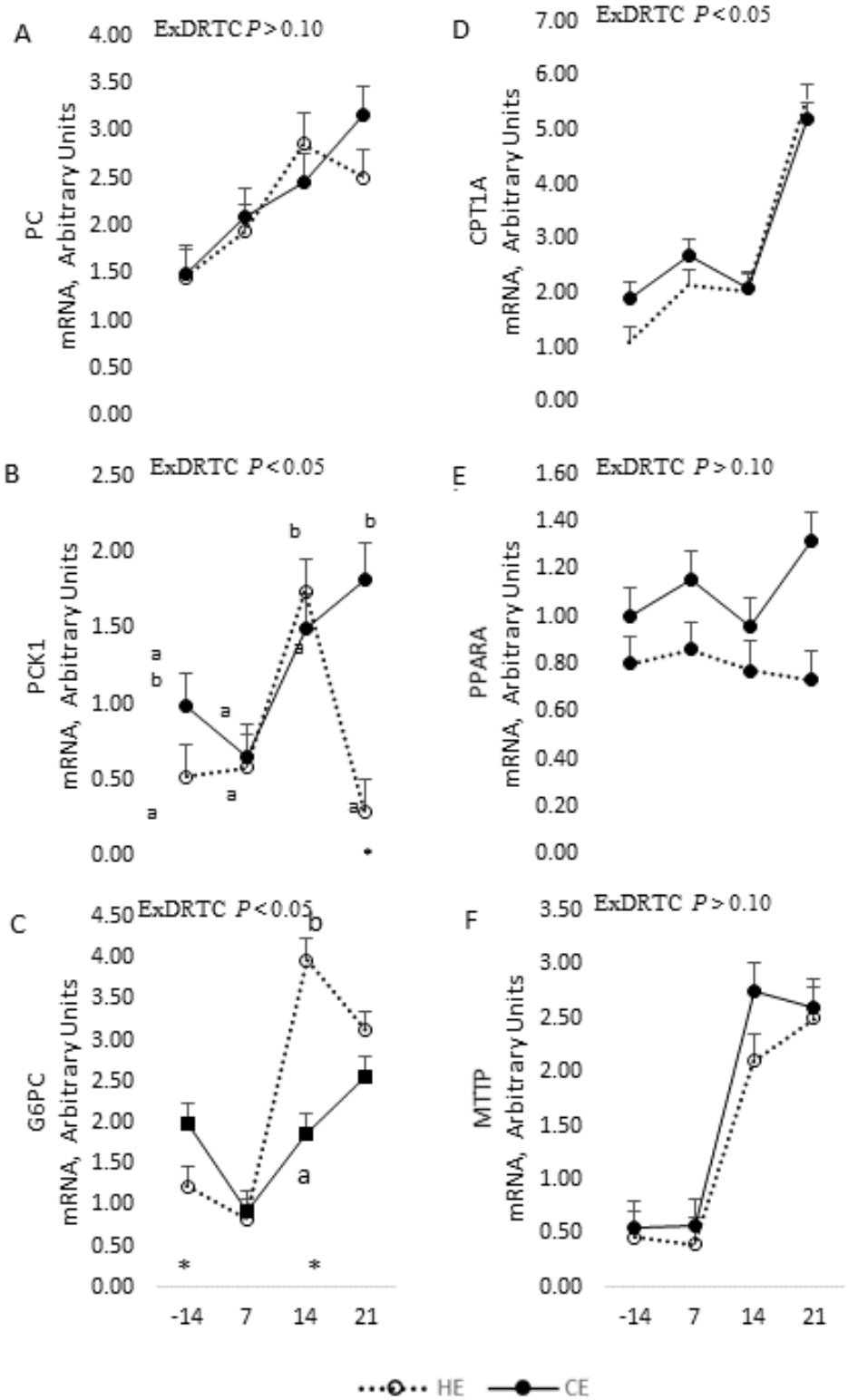
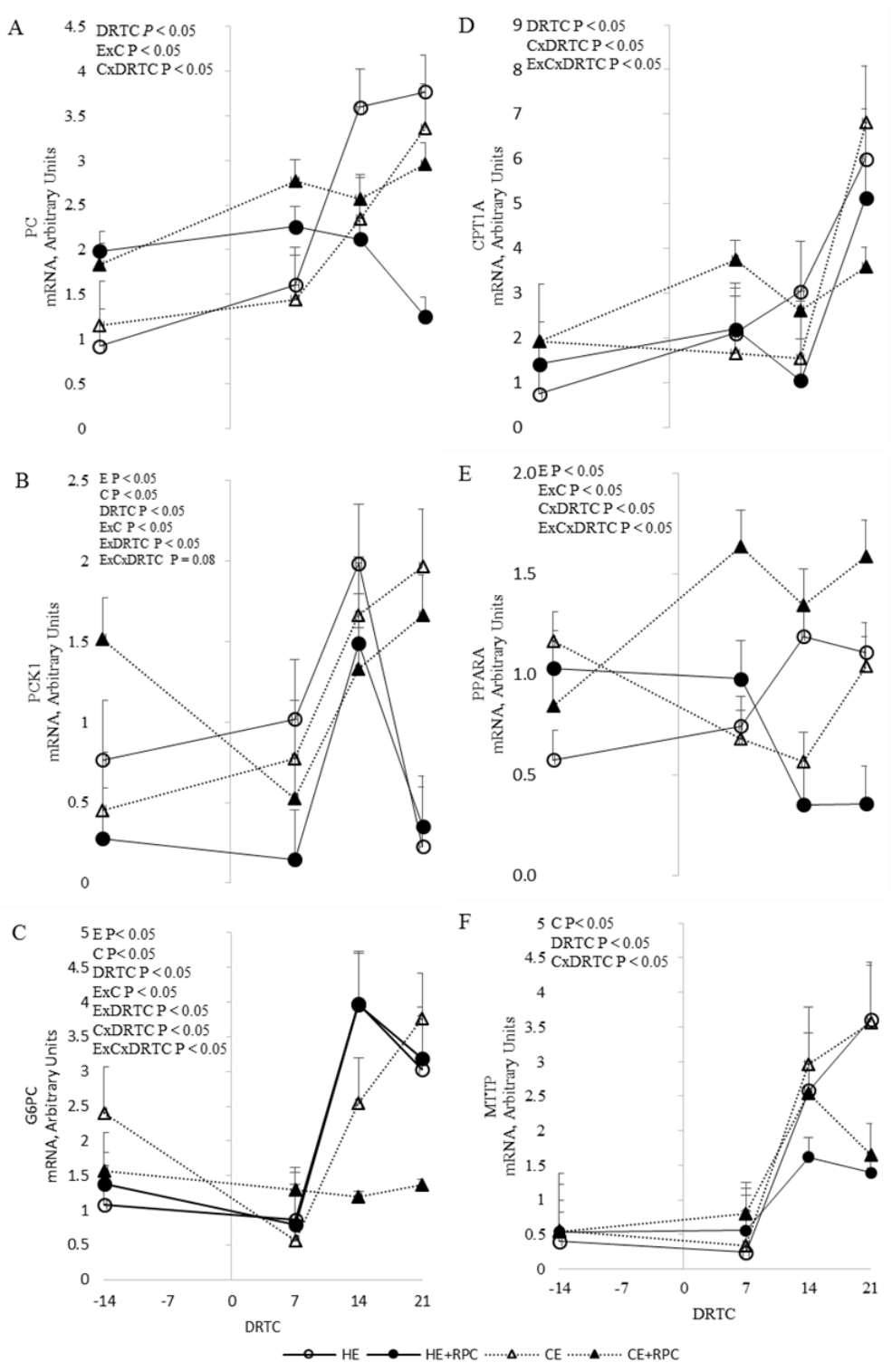


Figure 3.5 Effect of high energy (HE) and controlled energy (CE) with (CE+RPC, HE+RPC) or without (CE, HE) choline treatment on expression of hepatic CPT1A (panel A), PPARA (panel B) and G6PC (panel C) from -14 to 21 DRTC. Asterisks denote days, within the three-way interactions, that differ ($P \leq 0.05$) by slice effect. Letters indicate difference between treatment groups within specific days using Tukey ($P \leq 0.05$); asterisk, but no letters, indicates inability to separate means within day with Tukey ($P > 0.05$)**.**



Discussion

The purpose of this study was to examine the regulation of hepatic gluconeogenesis and oxidation during the transition period. In the present study, data on plasma concentrations of metabolites and production were used to describe metabolic function and correlation of those data with mRNA abundance of hepatic genes**.** Gene expression data provide only a snapshot of information regarding the quantity of a given transcript in a cell, but its analysis help determine the mechanisms behind the functional parameters measured in this study.

Changes in mRNA Abundance of Gluconeogenic Enzymes

We analyzed mRNA expression of 3 genes involved in gluconeogenesis in order to evaluate the oxidative capacity of the cell in response to prepartum energy and peripartum RPC supplementation. Pyruvate carboxylase, phosphoenolpyruvate carboxykinase and glucose 6-phosphatase play crucial roles in bovine hepatic gluconeogenesis to ensure a high glucose output during early lactation for milk synthesis (Baird et al., 1968; Donkin, 1999; Aschenbach et al., 2010). Gene expression of PC, PCK2, and G6PC increased immediately after parturition, whereas PCK1 mRNA increased 2 weeks after parturition (Weber et al., 2013). Indeed, transition dairy cows have increased PC mRNA and activity at calving (Greenfield et al., 2000). According to our results, PC expression increased during across the transition period and was greatest at +7, +14 and +21 DRTC compared to the pre-partum period, while PCK1 and G6PC expression increased at +14 DRTC. Supplementation of RPC decreased expression of PCK1 and G6PC, a significant interaction between choline treatment and DRTC was detected. Specifically, RPC decreased PC expression at +21 DRTC; on the other hand, G6PC abundance decreased not only at +21 DRTC, but also at +14 DRTC. A significant effect of energy treatment and ExDRTC interaction was detected. High energy treatment negatively affected PCK1 expression at +21 DRTC. The increase in expression of PC was reduced in cows receiving RPC after calving especially at +21 DRTC, suggesting that RPC improves energy status and carbohydrate metabolism in the liver and reduces the need for PC (Goselink et al., 2012). On the other hand, increased PC peripartum with RPC, across energy treatments, may support increased oxidative

capacity at calving. Pyruvate carboxykinase is important in gluconeogenesis and formation of oxaloacetate, and expression is upregulated around parturition in cows with lower energy status or high liver fat content (Loor et al., 2007; Hammon et al., 2009; Castro et al., 2012). Increased PC mRNA abundance and activity during the periparturient period may reflect the necessity for adaptation to the source and availability of gluconeogenic precursors. Similarly, decreased PCK1 in HE may serve to increase oxidation of circulating NEFA by maintaining the oxaloacetate pool as there was a tendency for HE to increase. No effect of treatments was observed on plasma NEFA concentration. However, when energy effect was considered a tendency to increment plasma NEFA concentration was detected for HE group. Reduced DMI is associated with elevated plasma NEFA concentrations around parturition in dairy cows (Ingvartsen and Andersen, 2000; Drackley et al., 2001; Hammon et al., 2009) and NEFA may stimulate hepatic PC gene expression by activating PC promoter 1 (White et al., 2011). In our study DMI decreased before parturition and increased after +7 DRTC, which coincide with the decrease in NEFA concentration. Furthermore, a RPC supplementation decreased BHBA concentration 7 days before parturition and at time zero. TAG liver content increased across the transition period, by 4 fold from -14 to +7 DRTC, but there was no effect of choline on TAG liver content.

Findings in transition and early lactating dairy cows suggest that greater choline availability can improve not only milk production (Erdman and Sharma, 1991; Hartwell et al., 2000; Pinotti et al., 2003), but also lipid metabolism (Piepenbrink and Overton, 2003; Pinotti et al., 2003), methyl group metabolism (Baldi and Pinotti, 2006) and choline secretion in milk (Deuchler et al., 1998; Elek et al., 2008; Pinotti et al., 2003; Pinotti et al, 2004). Consistent with other research and Grummer (2012), feeding RPC to transition cows increased DMI and milk, protein and fat yield. Our results support this finding as choline significantly increased milk yield. Milk components (expressed as percentage) were not affected by feeding RPC to transition dairy cows. There was a significant effect for three-way interaction among ExCxDRTC for G6PC, ($P \leq 0.05$), there was also a tendency for PCK1 (0.05

$< P \leq 0.10$). The three-way interaction among energy, choline and day relative to calving is due to the fact that reflect the effect of choline, energy and DRTC alone as main effect.

Changes in mRNA Abundance of Hepatic Enzymes of the FA oxidation, Nuclear receptor and VLDL packaging

During the transition period, when negative energy balance is enhanced, nonesterified fatty acids are mobilized from adipose stores (Dole, 1956; Emery et al., 1992). During the transition to lactation and associated surge in plasma NEFA, rate of blood flow increases (Lomax and Baird, 1983). This combination results in an increase uptake of NEFA into hepatic tissue in postpartum dairy cows (Emery et al., 1992; Reynolds et al., 2003). Aside from being utilized by the mammary gland, fatty acids are taken up by the liver and oxidized to acetyl-CoA units with four possible fates: complete oxidation through the TCA cycle, incomplete oxidation through ketogenesis (production of ketone bodies i.e., acetoacetate, acetone, and BHBA), re esterification into TAG, which can then either be sequestered in internal stores or be released into the circulation as TAG-rich, VLDL (Drackley et al., 2006; Grummer, 2008). When available acetyl-CoA exceeds the capacity of the TCA cycle, there is increased production of ketone bodies and deposition of TAG, leading to the onset of ketosis and fatty liver syndrome. Fatty liver occurs when the rate of hepatic acid esterification exceeds the rate of TAG disappearance via hydrolysis plus export as a constituent of VLDL. In order to understand hepatic VLDL synthesis, lipid oxidation, ketogenesis, gluconeogenesis and mitochondrial β-oxidation in liver, we selected MTTP, PPARA and CPT1A to analyze. According to our results, MTTP and CPT1A increased through the transition period, except on PPARA that remained unchanged. CPT1A expression started to increase one week after calving, having the maximum expression at +21 DRTC; MTTP had greater expression at +14 and +21 DRTC. In our study, PPARA and PPARγ served as master regulators of hepatic fatty acid oxidation and adipose tissue insulin sensitivity (Mandard et al., 2004; Desvergne et al., 2006). The pivotal role of PPARA in preventing liver TAG accumulation has been clearly shown in non-ruminants (George and Liddle, 2008; Seo et al., 2008). Consistent with recent research, feeding RPC to transition cows increased expression of

the PPARα/δ gene that is involved in regulatory loops of FA oxidation and transport (Loor et al., 2007; Goselink et al., 2012). On the other hand, Goselink (2013) reported no effect of RPC treatment neither on PPARA expression nor on CPT1A mRNA abundance. We detected a significant interaction between means for CxDRTC; RPC affected CPT1A and increased PPARA one week after parturition, whereas MTTP expression decreased three weeks after parturition. There was a significant three-way interaction among ExCxDRTC, such that PPARA was increased in CE+RPC (1.35 vs. 0.86, 0.68, 0.90 +- 0.08; CE+RPC, CE, HE+RPC, HE), while expression of carnitine palmitoyltransferase 1A increased at +21 DRTC. PPARA expression was highest for CE+RPC treated cows at +7 DRTC. On the other hand, HE+RPC had lower expression of this gene. The expression of PPARA in ruminant liver is mainly induced by plasma NEFA levels, as shown in dairy cows during the periparturient period (Loor et al., 2005) and during fasting-induced ketosis (Loor et al., 2007), which is in agreement with our results. Even though we detected a choline effect, there are several unclear patterns that need to be investigated.

Microsomal triglyceride transfer protein transports neutral lipids from the cytosol to the lumen of the endoplasmic reticulum and plays a critical role in coordinating the assembly of VLDL in the liver and chylomicrons in the intestine of non-ruminants (Vazquez-Anon et al., 1994). Hepatic MTTP activity may be an important factor in determining the rate of VLDL secretion in dairy cows and the severity of fatty liver at calving (Bremmer et al., 2000a, 2000b). Several studies reported the beneficial effect of RPC on MTTP expression (Goselink et al., 2012) promoting VLDL synthesis in the ER and reducing the accumulation of TAG in hepatic tissue (Wetterau et al., 1997). Despite the fact that we detected a significant effect for choline treatment, there is a lack of a consistent pattern. In agreement with the conclusion of Bremmer et al. (2000a, 2000b), microsomal triglyceride protein probably does not play a role in the etiology of fatty liver that occurs in dairy cows at calving and RPC supplementation does not appear to affect FA transport into the mitochondria.

RPC had no effect on mRNA abundance for CPT1A (Goselink et al., 2012; Chandler et al 2015). This suggests that the transport of long chain FA into mitochondria is not affected by the supplementation of choline to transition cows (Loor et al., 2005, van Dorland et al., 2009).

Conclusions

Overall, these data indicate a different response on RPC and energy treatments on our gene of interest. The increase in expression of the pyruvate carboxylase gene was reduced in cows receiving RPC after calving, suggesting that RPC improved energy status and carbohydrate metabolism in the liver and reduced the need for pyruvate carboxylase. On the other hand, increased PC around parturition in RPC receiving cows, across energy treatments, may support increased oxidative capacity of the TCA cycle at calving. Decreased PCK1 in HE may serve to increase oxidation of increased circulating NEFA by maintaining the oxaloacetate pool. Plasma NEFA concentration tended to increase in high energy receiving cows, suggesting that NEFA may stimulate hepatic PC gene expression.

The expression of PPARA in ruminant liver is mainly induced by plasma NEFA levels, as shown in dairy cows during the periparturient period and during fasting-induced ketosis, which is in agreement with our results. Consistently, we detected an increased expression of PPARA on CE+RPC at 7 DRTC. Pre- and post-partum glucose, BHBA and NEFA concentration in plasma of RPC cows did not differ significantly compared to noRPC group. However, the significant milk-yield response for RPC group may have masked an improvement of the metabolic profile. The significant increases in milk yield in RPC-treated cows of our experiment are in line with the observations reported in our meta- analysis (Chapter 2), where thirteen studies selected reported a significant effect of RPC supplementation on milk yield. In the present study, RPC supplementation increased milk yield, milk fat yield (Kg/d), milk protein yield (Kg/d) at the onset of lactation. These results may suggest that choline supplementation can improve milk production, lipid and methyl group metabolism in transition dairy cows, and its effect is protracted to onset of lactation.

Rumen protected choline treatment did no effect TAG liver content, probably duo to the fact that MTTP expression decreased after parturition. MTTP and CPT1A may be important factors

in determining the rate of VLDL secretion and transport of long chain FA into mitochondria, and RPC treatment can enhance these activities. However, our results shows that RPC supplementation did not alter MTTP and CPT1A expression, which suggests that RPC supplementation might not have affected FA transport into mitochondria. Even though we detected a choline effect on several genes of interest, there are several unclear patterns that need to be investigated.

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SUMMARY AND CONCLUSIONS.

Nutritional strategies for lactating dairy cows, with particular emphasis on the transition period are not clearly defined. However, several nutritional approaches have been proposed to alleviate metabolic disorders that can affect lactating dairy cows. Controlling energy intake or supplementing rumen-protected choline, especially during the periparturient period, are possible strategies for the optimization of the hepatic metabolic function. The main aim of this dissertation was to clarify the mechanism of rumen protected choline (RPC) supplementation in lactating dairy cows on milk production, metabolic health and hepatic gene expression. Firstly, a meta-analysis was performed in order to obtain an overall view of the effects of rumen-protected choline. Secondly, two distended nutritional strategies, controlled energy intake and rumen- protected choline supplementation during the transition period, were tested in order to elucidate the mechanisms beyond hepatic gluconeogenesis, lipid oxidation and transport, testing and measuring the abundance of selected hepatic genes.

Overall, data presented in this dissertation indicate that rumen protected choline supplementation significantly increases milk production both in meta-analysis (chapter 2) and in vivo trial (chapter 3). It was possible to detect the effects of choline supplementation, in a rumen-protected form, on different periods of lactation, i.e. the entire lactation period considered in the meta-analysis and the transition period, a specific period of lactation, considered in in vivo trial.

When plasma NEFA and BHBA were considered, data provided by the meta-analysis indicated that RPC supplementation in lactating dairy cows did not affect the plasma metabolites. Consistently, data provided by the in vivo trial confirmed these findings, i.e. pre- and post-partum glucose, NEFA and BHBA concentrations in plasma of RPC cows did not differ significantly. However, the significant milk-yield response for RPC group may have masked an improvement of the metabolic profile.

With regard to the effect of controlled energy and RPC, the supplementation during the transition period on hepatic gene expression was also evaluated. Generally, gene expression data provided only a snapshot of the information regarding the quantity of a given transcript in a cell, but its analysis helped to determine the mechanisms behind the functional parameters measured in this study. Gene expression data indicated a different response on RPC and energy treatments. The expression of pyruvate carboxylase decreases after calving in cows receiving RPC supplementation, which suggests that RPC improves energy status and carbohydrate metabolism in the liver. On the other hand, increased PC peripartum with RPC, through energy treatments, may support increased oxidative capacity at calving. Decreased PCK1 in HE may serve to increase oxidation of increased circulating NEFA by maintaining the oxaloacetate pool. Plasma NEFA concentration was increased by high-energy treatment; a tendency that was detected in our study, suggesting that NEFA may stimulate hepatic PC gene expression. RPC treatment did no effect TAG liver content, probably because MTTP expression decreased after parturition. MTTP and CPT1A may be important factors in determining the rate of VLDL secretion and transport of long chain FA into mitochondria, and RPC treatment can enhance these activities. However, our results shows that RPC supplementation did not alter MTTP and CPT1A expression, which can suggest that RPC supplementation might not have affected FA transport into mitochondria. Additionally, no effect of RPC supplementation was detected on PPARA abundance indicating that RPC might not have modulated FA oxidation and transport. Combining the results from the two studies presented in this dissertation, it can be concluded that:

i. Even though we detected consistent results with regard to milk production, plasma NEFA and BHBA concentrations in both studies, further investigation is required to answer the question why NEFA and BHBA were not altered by RPC supplementation;

ii. Several genes of interest involved in hepatic gluconeogesis, lipid oxidation and transport, responded to RPC supplementation. However, several unclear patterns need further investigation, specifically those genes involved in the FA transport into mitochondria as well as in the regulation of mitochondrial and peroxisome β-oxidation in the liver.