ROLE OF PROPIONATE AND UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION ON HEPATIC METABOLISM AND FEEDING BEHAVIOR IN DAIRY COWS

By

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ABSTRACT

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The negative energy balance of dairy cows in the postpartum period is exacerbated by a suppression in appetite. Metabolic diseases resulting from negative energy balance can decrease milk production, health and fertility, increasing risk of culling. Research suggests that feeding behavior is linked to the oxidation of fuels in the liver and that an increase in oxidation of fuels causes satiety. Propionate derived from the fermentation of starch in the rumen is a major glucose precursor in dairy cows but can also cause hypophagia. Because propionate is anaplerotic, it stimulates the oxidation of fuels in the liver, likely causing the hypophagic effects. In order to determine the role of anaplerotic metabolites and uncouplers of oxidative phosphorylation on hepatic metabolism and feeding behavior in dairy cows, 6 studies were conducted. First, using 8 dairy cows in a duplicated 4x4 Latin square design, the effect of feed status (before or after access to feed) on the hepatic extraction and metabolism of propionate over 20 min was determined. The results indicated that hepatic extraction and metabolism of propionate is very rapid, and that metabolite concentrations were greater after compared with before feeding. Additionally, potential bottlenecks of propionate metabolism were identified. Secondly, a study using hepatic explants incubated in [$^{13}C_3$]sodium propionate was conducted to test the effects of 3 different concentrations of propionate (1, 2 or 4 mM) on propionate metabolism over 60 min. An increased concentration of propionate in the range from 1 – 4 mM resulted in the conversion of propionate to acetyl CoA, likely through pyruvate, and that the acetyl CoA was incorporated into the TCA cycle for oxidation. Additionally, metabolism of

[$^{13}C_3$]propionate occurred rapidly with significant increases of ^{13}C enrichment detected within 0.5 min for many metabolites. Furthermore, uncoupling the electron transport chain should affect the metabolism of propionate and the feeding behavior of dairy cows as a result of inefficient oxidation of fuels. The effects of two uncouplers of oxidative phosphorylation, 2,4-dinitrophenol methyl ether (DNPME) and sodium salicylate (SAL), on feeding behavior in dairy cows were determined in 2 experiments. Treatment with DNPME and SAL decreased eating rate in dairy cows over the first 4 h following access to feed. Additionally, DNPME increased meal length over the first 4 h following access to feed. Lastly, an experiment was conducted to determine the effects of DNPME and SAL on metabolism and oxidation of propionate over 60 min using hepatic explants from 8 dairy cows. Neither DNPME nor SAL increased oxidation. The DNPME treatment did not alter metabolism of propionate except for an increase of propionate converted to succinyl CoA, however, SAL decreased glucose synthesis from propionate. From this research, we conclude that hepatic metabolism of propionate occurs rapidly and is likely having effects on feeding behavior within minutes as well. As such, the importance of short-term metabolism in regard to feeding behavior and dry matter intake should be considered in future research. Understanding the mechanisms for metabolic control of feed intake will lead to development of novel nutritional or pharmacological approaches to increase energy intake, health, and milk yield of dairy cows; thus, improving nutrient utilization and sustainability of the dairy industry.

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KEY TO ABBREVIATIONS

α-KG	α-ketoglutarate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BCS	Body condition score
BHB	β-hydroxybutyrate
BUN	Blood urinary nitrogen
BW	Body weight
CoA	Coenzyme A
CON	Control
CP	Crude protein
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
DNP	2,4-dinitrophenol
DNPME	2,4-dinitrophenol methyl ether
DRF	Dose relative to feeding
ETC	Electron transport chain
EXP	Experiment
FAD	Flavin adenine dinucleotide
GABA	γ-aminobutyric acid
GC-MS	Gas chromatography – mass spectrometry

GTP	Guanosine-5'-triphosphate
HOT	Hepatic oxidation theory
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
NAD	Nicotinamide adenine dinucleotide
NDF	Neutral-detergent fiber
NEFA	Non-esterified fatty acids
OAA	Oxaloacetate
OM	Organic matter PA Propionic acid
OXPHOS	Oxidative phosphorylation
PP	Postpartum
ROS	Reactive oxygen species
SAL	Salicylate
TCA CYCLE	Tricarboxylic acid cycle
TMR	Total-mixed ration

CHAPTER 1: LITERATURE REVIEW

Optimizing efficiency and milk production of dairy cows is a high priority in the dairy industry in order to maximize farm profits and improve sustainability. The Journal of Dairy Science recently publishing a variety of reviews and celebrated over a century of research (Volume 100, Issue 12, 2017). Researchers have explored improving efficiency and milk production through a variety of avenues including management, genetics, reproduction and nutrition. One area of interest within this goal is to improve dry matter intake (DMI) in dairy cows during the transition period. This review will focus on hepatic metabolism of propionate and its relation to dry matter intake in dairy cows during the transition period.

THE IMMEDIATE POSTPARTUM PERIOD IN DAIRY CATTLE: DEFINITION AND CHALLENGES

The transition period spans the three weeks prior to parturition to three weeks post parturition and has been referred to as the "final frontier" by Drackely (1999). Dairy cows in the immediate postpartum (PP) period are in a physiological state known as a negative energy balance (NEB), i.e. expending more energy than consuming, due to the onset of lactation (Bauman and Currie, 1980). Along with increased energy demands as a result of lactation, maintenance energy requirements for lactating dairy cows are higher as well partially due to an increase in organ growth (Smith and Baldwin, 1974). Smith and Baldwin (1974) estimate that liver energy requirements increased by approximately 850 kcal/d in lactating dairy cows compared with dry cows based only on increase in organ size, but that this value may be higher as a result of increased metabolic function during lactation. Additionally, dairy cows in the PP period typically

are more insulin resistant and have lower circulating plasma insulin concentrations, likely to ensure that adipose tissue is mobilized for energy and milk fat synthesis, subsequently sparing glucose for lactose production (Bell, 1995). As such, in order to satisfy energy demands, dairy cows mobilize body tissues, particularly adipose tissue, resulting in a lipolytic state with higher plasma concentrations of non-esterified fatty acids (NEFA; Allen and Piantoni, 2013). Fatty acids are used my various tissues including the liver. The liver extracts NEFA in proportion to its concentration in plasma and rate of blood flow (Emery et al., 1992). The liver then either exports the fatty acids as very low-density lipoproteins, stores the fatty acids, which can lead to fatty liver (Bobe et al., 2004), or catabolize the fatty acids to acetyl CoA (Emery et al., 1992). Acetyl CoA, in turn, can either be oxidized for energy or converted to, and ultimately exported as, ketone bodies (Wieland and Weiss, 1963; White, 2015), as noted by an increased concentration of plasma β-hydroxybutyrate (BHB; Allen and Piantoni, 2013).

Negative energy balance has been associated with increased risk of developing metabolic disorders, and decreased milk production and lower fertility when dairy cows are unable to adapt effectively (Herdt, 2000; Butler, 2003; Ospina et al., 2010). As such, the success of dairy cows transitioning into lactation is likely related to the concept of metabolic flexibility, the ability of the body to respond or adapt to changes in metabolic demand (Goodpaster and Sparks, 2017). However, a suppression in appetite during the transition period further exacerbates the NEB (Allen and Piantoni, 2013); thus, improving dry matter intake (DMI) during the transition would reduce the risk of developing metabolic disorders. Therefore, developing new management and feeding strategies to improve DMI during the PP period would improve the sustainability of the dairy industry.

TRICARBOXYLIC ACID CYCLE AND OXIDATIVE PHOSPHORYLATION

The tricarboxylic acid (TCA) cycle acts as a focus point of metabolism that provides entries and exits for various metabolic pathways while oxidizing fuels for energy. Oxidation of fuels and synthesis of ATP through oxidative phosphorylation (OXPHOS) is dependent on the interrelationship between the TCA cycle and the electron transport chain (ETC; Figure A1.1), which are both located within the mitochondrion. Oxidation in the TCA cycle is affected by entry of TCA intermediates (anaplerosis), availability of acetyl CoA, and redox state (Allen and Piantoni, 2013). Furthermore, ATP synthesis from the oxidation of acetyl CoA is dependent on the proton motive force (pmf) generated by the ETC (Stock et al., 1999).

Acetyl CoA is derived from the catabolism of a variety of fuels (lactate, amino acids, fatty acids, etc.), but its principle source in the liver of dairy cows in the PP period is from mitochondrial β-oxidation of NEFA (Allen, 2014). Each round of mitochondrial β-oxidation yields 1 mole of acetyl CoA, 1 mole of $FADH_2$, and 1 mole of mitochondrial NADH. Therefore, each round of mitochondrial β-oxidation yields 1 mole of acetyl CoA and 4.3 moles of ATP when using the assumptions listed in Kennedy (2014), i.e. 3.7 protons are required to generate 1 mole of ATP through ATP synthase (Watt et al., 2010).

Increased ATP concentration and $NADH/NAD^+$ are strong inhibitors of the TCA cycle in order to prevent the cycle from needlessly producing energy. Within the TCA cycle are 3 non-equilibrium enzymes: citrate synthase, isocitrate dehydrogenase and alpha-ketoglutarate dehydrogenase (Williamson and Cooper, 1980). Increased ATP concentration inhibits all 3 enzymes. Additionally, increased concentrations of NADH inhibits isocitrate dehydrogenase; increased concentrations of succinyl CoA inhibits α-ketoglutarate dehydrogenase; and increased

concentrations of citrate inhibits citrate synthase (Williamson and Cooper, 1980). Although these enzymes are regulators of the TCA cycle, the major regulator of TCA cycle flux is the ratio of $NADH/NAD^+$ (Williamson and Cooper, 1980), which affects enzymes like malate dehydrogenase, α-ketoglutarate dehydrogenase, isocitrate dehydrogenase and other enzymes that use NAD^+ or NADH as a cofactor in the mitochondrion.

NADH and $FADH_2$ are reducing equivalents that donate electrons and protons to the ETC. The donation of electrons is responsible for the pumping of protons into the inner mitochondrial membrane space and are used by ATP synthase for the synthesis of ATP from ADP and inorganic phosphate (P_i). Therefore, the moles of NADH and $FADH_2$ produced/consumed within a metabolic pathway contribute to the total moles of ATP synthesized by the ETC. For example, oxidation of 1 mole of acetyl CoA in the TCA cycle results in the production of 3 moles of NADH, 1 mole of GTP, 1 mole of $FADH_2$, and 2 moles of carbon dioxide (CO_2) per mole of acetyl CoA, theoretically resulting in the synthesis of 10.5 moles of ATP per mole of acetyl CoA oxidized (Kennedy, 2014). Assuming that the acetyl CoA is derived from β-oxidation, 14.8 moles of ATP are generated from a round of β-oxidation coupled to acetyl CoA oxidation by the TCA cycle. The CO_2 produced from the oxidation of acetyl CoA in the TCA cycle is a source of mitochondrial bicarbonate that can stimulate OXPHOS via the stimulation of soluble adenylyl cyclase (Acin-Perez et al., 2009). However, reducing equivalents may not be immediately oxidized by the ETC, and thus, the lag between the production and oxidization by the ETC of reducing equivalents may result in an accumulation of reducing equivalents in the mitochondrion and delay in ATP synthesis (Allen and Piantoni, 2013).

The ETC uses a transmembrane electrochemical proton gradient to provide energy for ATP synthase. However, this system is inherently inefficient due in part to proton leak. Proton

leak occurs when protons from the mitochondrial intermembrane space (higher concentration) cross the inner mitochondrial membrane to the mitochondrial matrix (lower concentration) without synthesizing ATP (Brand et al., 1994). In rats, proton leak has been estimated to account for as much as 23% of total basal metabolic rate (Rolfe et al., 1999) and that about 4% of basal metabolic rate is from proton leak in the liver (Brand et al., 1994). Nobes et al. (1990) estimated that 20 - 40% of basal oxygen consumption by isolated hepatocytes from rats goes towards proton leak, and subsequent heat production, instead of ATP synthesis. However, the mitochondrion can adjust the P/O ratio to meet the second-by-second demands of ATP in cells (i.e. the ratio increases when ATP demand increases and vice-versa) through multiple forms of regulation like alternating proton leak (Brand et al., 1994). As such, regulation of proton leak can be advantageous as it would allow for a rapid increase in ATP production independent of an increase in oxidation of fuels (Boss et al., 2000).

Divakaruni and Brand (2011) classified proton leak as either continuous (basal) or regulated (inducible proton conductance through uncoupling proteins). Basal proton leak is not fully understood; however, the amount of adenine nucleotide translocase present is thought to be the major contributor to basal proton leak (Brand et al., 2005; Divakaruni and Brand, 2011). Uncoupling proteins have been found in a variety of tissues and thought to participate in thermogenesis and help protect cells from oxidative damage caused by reactive oxygen species (ROS) as discussed by Divakaruni and Brand (2011). Reactive oxygen species are produced primarily from the ETC (Turrens, 2003), and can either serve as signaling molecules or oxidizing agents that can cause cell death (Mailloux and Harper, 2011). However, St-Pierre et al. (2002) reported that production of ROS is more apparent in heart and skeletal tissue than liver tissue in

rats, likely because the ETC complexes are more active in heart and skeletal tissue than liver tissue in mice (Kwong and Sohal, 2000).

One of the most potent mitochondrial uncouplers of OXPHOS is the protonophore 2,4-dinitrophenol (DNP). DNP was used as a weight loss drug in the 1930s due to its ability to increase basal metabolic rate (Grundlingh et al., 2011). Although DNP was banned by the US Food and Drug Administration in 1938 (FDA, 1938; ATSDR, 1995), DNP can still be bought in large quantities over the internet for industrial uses as well as by body builders (Grundlingh et al., 2011). However, interest exists in targeting DNP in order to counter obesity and fatty liver disease (Blaikie et al., 2006; Perry et al., 2013). When DNP was fed at a dose of 100 mg/kg to broilers, feed intake increased (Toyomizu et al., 1992); however, no research has examined the effect of DNP on feed intake in any ruminant model. Perry et al. (2013) identified 2,4-dinitrophenol methyl ether (DNPME) as a liver targeted form of DNP with a similar uncoupling potency as DNP, which allows for liver focused experiments.

Another uncoupler of OXPHOS, though not as potent as DNP, is salicylate (Brody and Fouts, 1956; Penniall, 1958). Like DNP, salicylate is a protonophore (Haas et al., 1985). A well-known member of salicylate compounds is aspirin (sodium acetylsalicylate), which is hydrolyzed to salicylate in the liver (Needs and Brooks, 1985). Therefore, unsurprisingly, administration of aspirin induces uncoupling (Brody and Fouts, 1956; Mehlman and Tobin, 1972), though at a lower potency than dosing with sodium salicylate (Brody and Fouts, 1956). Brody and Fouts (1956) reported that relative to control, rat liver mitochondria were uncoupled 99% with sodium salicylate but 72% with sodium acetylsalicylate.

Unlike DNP, salicylates have been used in ruminant research. Dairy cows treated with intramuscular injections of acetylsalicylate over the first 5 days PP had a higher milk yield at the

peak of lactation compared with control, but also a greater loss in BCS (Bertoni et al., 2004). Farney et al. (2013a,b) reported similar results when they treated dairy cows with sodium salicylate through drinking water for the first 7 days PP. Farney et al. (2013a) observed a significant decrease in energy balance and significant increase in plasma NEFA and milk fat yield at 3 weeks of lactation for dairy cows treated with sodium salicylate compared with control, however, plasma insulin concentration did not differ between treatment groups at 3 weeks of lactation. Dairy cows with a parity of 3 or more had a higher 305-d milk yield and 305-d milk fat yield, and also tended to have a higher 305-d milk protein yield with sodium salicylate treatment compared with control (Farney et al., 2013b). Dry matter intake was increased when acetylsalicylate was provided intramuscularly (Bertoni et al., 2004), but DMI did not differ or tended to decrease when salicylate was provided orally (Carpenter et al., 2018). Furthermore, Carpenter et al. (2018) reported that treating dairy cows with salicylate resulted in dairy cows eating fewer but longer meals. However, rumen fermentation and digestion can be inhibited by salicylate (Carpenter et al., 2017), which may explain the contrary results of salicylates on DMI. Salicylates also decreased plasma glucose concentrations in early lactation dairy cows (Bertoni et al., 2004; Farney et al., 2013a). Recently, Montgomery et al. (2019) reported that dairy cows dosed with sodium salicylate through drinking water for the first 7 d PP had a tendency to decrease glucose turnover rate by 25% compared with control cows, but transcription abundance of hepatic pyruvate carboxylase or glucose-6-phosphatase did not differ between treatments.

PROPIONATE: HEPATIC METABOLISM AND RELATION TO FEEDING BEHAVIOR

Propionate is the primary gluconeogenic precursor and Wiltrout and Satter (1972) estimated that 61% of propionate contributes to glucose production in lactating dairy cows based on infusions with [2-^{14}C]sodium propionate. However, this may be an underestimate due to inadequate correcting for carbon transfer with isotopic tracers and the true value may be closer to 95% (Armentano, 1992). Yet, Reynolds et al. (2003) calculated that the maximal potential for net contribution of propionate to net glucose release by the liver was 56% in dairy cows in the PP period based on net propionate removal by liver and net glucose release by the liver. Ruminants must synthesize the majority of their glucose because little to no glucose is absorbed from dietary sources. Furthermore, extraction of glucose by the liver and use of glucose as an energy source would be expected to be negligible (Stangassinger and Giesecke, 1986) particularly because hexokinase and glucokinase are low in ruminants compared with non-ruminants (Ballard, 1965). Hexokinase and glucokinase phosphorylate glucose to glucose-6-phosphate, which prevents transport of glucose out of the liver.

Propionic acid is primarily produced from the fermentation of starch in the rumen of ruminants; however, its production rate is highly variable depending upon its source, processing method and other feeds included in the diet as reviewed by Ørskov (1986). Starch is usually supplied through cereal grains in dairy rations, such as corn. However, dairy cows in the PP period have a suppressed appetite and research has shown that propionate has a hypophagic effect when dosed directly (Gualdrón-Duarte and Allen, 2018b; Maldini and Allen, 2018) or produced by fermentation of starch (Oba and Allen, 2003; Albornoz and Allen, 2018). Dairy

cows in the PP period fed a more-fermentable starch source had decreased DMI compared with dairy cows fed a less-fermentable starch source (Albornoz and Allen, 2018). This change was even more apparent when the more-fermentable starch source was included at a 28% diet starch concentration compared with a 22% diet starch concentration (Albornoz and Allen, 2018). In a similar study, Oba and Allen (2003) estimated that propionate production in the rumen was higher with a more-fermentable starch source compared with a less-fermentable starch source in early lactation dairy cows. Therefore, the decrease in DMI is likely related to propionate production in the rumen. Additionally, when fed a starch concentration at 32% of diet, a more-fermentable starch source fed to dairy cows in early lactation decreased DMI by decreasing meal size, suggesting rapid absorption and metabolism of propionate from the diet within a meal (Oba and Allen, 2003).

Propionate is associated with hypophagia in ruminants, particularly dairy cows in the PP period and early lactation (Stocks and Allen, 2012; Gualdrón-Duarte and Allen, 2018b; Maldini and Allen, 2018). The hypophagic effect of propionate is likely connected to the liver because hepatic vagotomy removed this effect in sheep (Anil and Forbes, 1988). Additionally, the liver efficiently extracts propionate (Baird et al., 1980; Reynolds et al., 2003) and other tissues use propionate minimally (Brockman, 2005). Therefore, the liver metabolizes the majority of propionate. However, Stocks and Allen (2012) reported that hepatic acetyl CoA concentration was positively related to the hypophagic effects of propionate, suggesting that acetyl CoA is integral to the hypophagic effects observed from propionate. As such, dairy cows that are in a lipolytic state, such as cows in the PP period, are more likely to be prone to the hypophagic effects of propionate (Allen, 2014) because dairy cows in the PP period have a higher concentration of hepatic acetyl CoA than late-lactation cows (Piantoni et al., 2015). Allen et al.

(2009) proposed that the oxidation of acetyl CoA through the TCA cycle is connected to the hypophagic effects observed for some anaplerotic metabolites, like propionate. Additionally, Maldini and Allen (2018) reported that a faster rate of propionic acid infusion into the rumen increased meal size and decreased meal frequency compared with a slower rate, possibly from a saturation of propionate metabolism in the liver; yet both infusions decreased daily DMI to a similar extent compared with control (no infusion). These results suggest that the rate of extraction and metabolism of propionate within the time frame of a meal is another important factor affecting the hypophagic effects of propionate.

Glycerol, lactate, and propionate are all 3-carbon glucose precursors with anaplerotic capabilities; however, their hepatic metabolism differs substantially. Both glycerol and lactate initially enter the gluconeogenic pathway within the cytosol whereas propionate initially enters the TCA cycle in the mitochondrion. Because gluconeogenesis is typically upregulated in dairy cows in the PP period and glycerol enters as glyceraldehyde-3-phosphate, the carbons of glycerol are more likely to be shunted towards glucose synthesis, and thus, likely have lower anaplerotic capabilities and hypophagic effects compared with propionate (Gualdrón-Duarte and Allen, 2017). Indeed, isomolar and isoenergetic infusions of propionic acid decreased DMI more than glycerol when infused into the abomasum (Gualdrón-Duarte and Allen, 2017).

Conversely, hepatic lactate can be converted to pyruvate for gluconeogenesis and/or oxidation, and, as pyruvate, can enter the TCA cycle as oxaloacetate through the enzyme pyruvate carboxylase (EC 6.4.1.1; Greenfield et al., 2000). Research has shown that transcription of pyruvate carboxylase increases during the transition period (Greenfield et al., 2000) and that serum NEFA may upregulate its expression (White et al., 2011). Therefore, lactate may have an anaplerotic role in the liver (Baird et al., 1980; Gualdrón-Duarte and Allen, 2018b). However,

isoenergetic abomasal infusions of propionic acid decreased DMI 24% compared with control whereas lactic acid decreased DMI 14% compared with control (Gualdrón-Duarte and Allen, 2018b); and thus, the hypophagic effects are not as potent as propionate. The hypophagic effects of lactate are likely not as potent as propionate for a variety of reasons. First, liver extraction of lactate has been estimated to be approximately 30% in lactating cows (Baird et al., 1980) whereas liver extraction of propionate has been estimated to be approximately 85% (Reynolds et al., 1988). Secondly, Baird et al. (1980) reported that infusion of sodium propionate into the mesenteric-vein of lactating dairy cows decreased hepatic extraction of lactate. Therefore, extraction of lactate during and/or immediately after a rapidly fermentable starch meal may decrease the extraction of lactate by the liver. Lastly, the capacity for lactate conversion to glucose is about half of the capacity of propionate in bovine calf hepatocytes (Armentano, 1992) and Reynolds et al. (2003) calculated that the maximal potential for net contribution of lactate to net glucose release by the liver was 21% in dairy cows in the PP period.

Once the liver extracts propionate, propionate ultimately enters the TCA cycle as succinyl CoA and stimulates oxidation by replenishing TCA cycle intermediates and/or is shunted to the cytosol as malate for glucose production through gluconeogenesis. Assuming a mole of propionate will complete one cycle of the TCA cycle and using the assumptions from Kennedy (2014), approximately 8.5 moles of ATP are produced from the oxidation of a mole of acetyl CoA when a mole of propionate is converted to a mole of oxaloacetate that is used for condensation with acetyl CoA. Conversely, a mole of glucose produced from 2 moles of propionate would cost 0.8 moles of ATP if propionate went directly to glucose synthesis and exported from the mitochondrion as malate, again using the assumptions from Kennedy (2014). As such, depending on the flux of propionate towards glucose synthesis, TCA cycling, or other

paths of metabolism, ATP production/utilization resulting from propionate metabolism could vary considerably.

Labeling of propionate, ^{14}C or ^{13}C, has been used in dairy cows to research gluconeogenesis and propionate metabolism (Knapp et al. 1992; Donkin and Armentano, 1995; Zhang et al., 2015). Metabolism of propionate is well-defined, so the labeling patterns of [$^{13}C_3$]propionate are known (Kelleher, 1986; Previs and Kelley, 2015). Typically, the annotation of [M+n] is used to denote the number of carbons labeled within a metabolite. For example, [$^{13}C_3$]propionate is expected to result in [M+3] propionyl CoA since no carbons are removed or gained in the reaction. Additionally, molecules that differ only in isotopic composition are known as isotopologues. However, placement of labeled carbons within a metabolite can differ. Isotopomers are molecules with the same number of each isotopic atom (i.e. same isotopologue) but their position within the species differs. Nuclear magnetic resonance spectroscopy is necessary for isotopomer identification but isotopologues can be identified using mass spectrometry because of the mass difference.

[$^{13}C_3$]propionate enters the TCA cycle as [M+3] succinyl CoA and either exports to the cytosol as malate or remains in the TCA cycle, which would result in [M+3] labeling of TCA cycle intermediates except for α-ketoglutarate which may be [M+2] depending on the position of the ^{13}C label (Figure A1.2A). Although no net gain of carbon is present in the cycling of the TCA cycle, the carbons oxidized as CO_2 from isocitrate and α-ketoglutarate are not the carbons originating from acetyl CoA (Previs and Kelley, 2015). Therefore, unlabeled acetyl CoA can dilute the labeling of the TCA cycle intermediates to [M+2] depending on positioning of the ^{13}C of the [M+3] metabolites. Furthermore, labeling above [M+3] citrate would require incorporation of labeled acetyl CoA. Exportation and conversion to phosphoenol pyruvate (PEP) would expect

to result in [M+3] or [M+2] PEP, which in turn, would either result in [M+2]/[M+3] glucose or [M+2]/[M+3] pyruvate (Previs and Kelley, 2015). Pyruvate can enter the TCA cycle as [M+2]/[M+3] oxaloacetate (Figure A1.2B; Previs and Kelley, 2015) or as [M+1]/[M+2] acetyl CoA (Figure A1.2C).

Along with propionate, acetate and butyrate are also produced from rumen fermentation in dairy cows. Infusion of acetate into the mesenteric vein of steers had no effect on feed intake, but propionate decreased or inhibited feed intake (Elliot et al., 1985). Furthermore, infusion of propionate into the portal vein of sheep limited feed intake whereas acetate and butyrate had no effect (Anil and Forbes, 1980). However, a study conducted by Gualdrón-Duarte and Allen (2018a) found that acetic acid infused into either the rumen or abomasum of dairy cows in the PP period decreased DMI compared with control. Metabolism of acetate by the liver is minimal because bovine liver has minimal amounts of mitochondrial and cytosolic acetyl CoA synthetase, which is necessary for activation of acetate to acetyl CoA for metabolism (Ricks and Cook, 1981). Therefore, the hypophagic effect observed with infusion of acetic acid is likely not related to the liver. Although butyrate can contribute to hepatic oxidation, net metabolism is likely less than propionate because ruminal production of butyrate is lower (Balch and Rowland, 1957), butyrate is not extracted by the liver as efficiently (approximately 68%; Reynolds et al., 1988) and butyrate is preferentially oxidized by ruminal epithelia (Weigland et al., 1975). Additionally, butyrate enters the TCA cycle as acetyl CoA in the liver (Leng and Annison, 1963). As such, it is not anaplerotic and does not stimulate oxidation, but rather, contributes to the existing pool of acetyl CoA. Therefore, although other metabolites may induce hypophagia, propionate has a more pronounced effect likely resulting from different hepatic metabolism.

HEPATIC OXIDATION THEORY

Allen et al. (2009) proposed that the liver controls feeding behavior through the hepatic oxidation of fuels, referred to as the Hepatic Oxidation Theory (HOT) of control of feed intake. The liver is in a position to be able to sense the energy status of the animal and enact necessary changes to feed intake because it 1) receives fuels from both endogenous and exogenous sources and 2) has first pass at nutrients absorbed from the rumen and digestive tract via the portal vein (Allen, 2014). Energy status is a balance between the rate of synthesis and utilization of high-energy phosphate bonds and can be defined with the equation $\frac{\text{ATP}+0.5\text{ADP}}{\text{ATP}+\text{ADP}+\text{AMP}}$ (Allen and Piantoni, 2013). Friedman and coworkers showed that hepatic ATP concentration and energy status affected feeding behavior in rats wherein a decrease in ATP concentration and/or energy status caused an increase in feed intake (Rawson et al., 1994; Friedman et al., 1999). However, Ji et al. (2000) observed that a change in hepatic energy status, not ATP concentration, was necessary to elicit a change in feeding behavior.

Because fructose metabolism can result in the sequestering of inorganic phosphate, and thus, hinder ATP production, Yair and Allen (2017) infused dairy cows in the PP period with either fructose or a mixture of fructose and inorganic phosphate. Yair and Allen (2017) reported that infusion of fructose to dairy cows in the PP period increased DMI compared with an infusion of a mixture of fructose and inorganic phosphate; suggesting that, like in non-ruminants, hepatic ATP production and energy status is related to feed intake in dairy cows in the PP period (Yair and Allen, 2017). Additionally, Piantoni et al. (2015) observed that dairy cows in the PP period and late lactation had higher DMI over 4 h when hepatic acetyl CoA and plasma NEFA concentration decreased more rapidly compared with dairy cows that had a slower decrease in

these metabolites. Plasma insulin concentration also increased over 4 h in those dairy cows, suggesting that plasma insulin decreased plasma NEFA, which in turn, likely decreased hepatic acetyl CoA concentration; subsequently decreasing hepatic oxidation and increasing feed intake compared with those cows that had more hepatic acetyl CoA available for oxidation (Piantoni et al., 2015).

Research in rats suggests that a satiety signal is carried from the liver to the brain via vagal afferents and affecting feeding behavior (Langhans et al., 1985; Langhans and Scharrer, 1987). The firing rate of vagal afferents may stimulate or inhibit appetite such that a decrease in energy status increases the firing rate, increasing feeding whereas an increase in energy status decreases the firing rate, decreasing feeding (Allen, 2104). Therefore, normal fluctuations in hepatic energy status within days might stimulate both hunger and satiety depending upon the balance between energy production and utilization (Allen, 2104). Metabolic reactions occur very rapidly, and therefore, oxidation of fuels and production of ATP likely varies greatly over minutes, affecting feeding behavior (Allen et al., 2009).

Daily DMI, which is a function of meal size and frequency, consists of 4 main parts: 1) number of meals eaten per day; 2) amount of feed eaten per meal; 3) length of meal; and 4) length of time between meals (Allen, 2014). Therefore, understanding the mechanism(s) influencing feeding behavior within a meal are vital to increasing DMI. Meals are eaten within minutes and the HOT provides a mechanism that acts on this minute time scale. For example, the HOT explains the hypophagic effects observed in propionate infusions and high starch diets with rapidly fermentable starch sources in dairy cows in the PP period. Propionate and other anaplerotic fuels increase the oxidation of acetyl CoA in the TCA cycle; thus, increasing the production of ATP (Allen et al., 2009). Because dairy cows in the PP period have increased

plasma NEFA concentration, more acetyl CoA is available for oxidation in the liver. Therefore, an increase in propionate leads to greater hepatic oxidation, which leads to an increase in energy status causing satiety. However, production of ATP and energy status in the liver is affected by a variety of factors (stage of lactation, oxidation of fuels, presence of anaplerotic fuels, proton leak, etc.) and the contribution of these factors on feeding behavior in dairy cows should be further evaluated in ruminants. Nevertheless, the HOT is an appealing model because it integrates nutrient digestion and metabolism from exogeneous and endogenous sources with feeding behavioral effects (Allen et al., 2009; Allen, 2014). Furthermore, it provides a mechanism that could be used to help improve energy intake and partitioning in lactating dairy cows through nutritional or pharmaceutical approaches; thus, improving health, production and nutrient utilization in lactating dairy cows (Allen, 2014). Yet, further research needs to be conducted within lactating dairy cows with focus on a within-meal time-scale to elucidate propionate metabolism *in vivo*, anaplerotic and oxidative contributions of propionate metabolism, effects of uncouplers of OXPHOS on feeding behavior in lactating dairy cows, and anaplerotic and oxidative contributions of propionate metabolism in the presences of uncouplers of OXPHOS.

OBJECTIVE AND AIMS

The overall objective of this dissertation was to determine the role of propionate and uncouplers of oxidative phosphorylation on hepatic metabolism and feeding behavior in dairy cows. The central hypothesis of this dissertation is that feed intake in dairy cows is affected by the oxidation

of fuels in the liver where an increase in oxidation of fuels will stimulate satiety. This dissertation addresses this hypothesis under two specific aims:

Aim 1. Determine the short-term effects of hepatic propionate metabolism.

Two experiments were conducted to address Aim 1 (Ch. 2 and 3) in which metabolism of propionate was examined within 60 min of its introduction *in vivo* or in liver explants. In Ch. 2, potential short-term metabolic bottlenecks of hepatic propionate metabolism in the liver of dairy cows in the postpartum period were identified and the effect of feeding status (before or after meals) on these bottlenecks was examined. We hypothesized that rapid propionate absorption results in metabolic bottlenecks and that these bottlenecks are greater after feeding due to partial saturation of the propionate metabolism pathway from diet-derived propionate. In Ch. 3, the temporal effects of increasing concentrations of propionate on propionate metabolism in liver tissue of dairy cows in the postpartum period was examined. We hypothesized that the majority of propionate will be used for synthesis of glucose, but that increasing the concentration of propionate will result in an increase of the carbons of propionate being converted to acetyl CoA for oxidation in the TCA cycle.

Aim 2. Determine the effects of uncoupling the electron transport chain on liver metabolism and feeding behavior of ruminants.

Two experiments were conducted to address Aim 2 (Ch. 4 and 5). In Ch. 4, the effects of uncouplers of OXPHOS, DNPME and sodium salicylate, on feeding behavior in lactating dairy cows were examined. We hypothesized that uncouplers of OXPHOS will increase meal size and length, possibly increasing DMI. In Ch. 5, the effects of uncouplers of OXPHOS, DNPME and

sodium salicylate, on the metabolism and oxidation of propionate in liver explants from dairy cows in the postpartum period were examined. We hypothesized that uncoupling of the electron transport chain would increase anaplerosis by propionate as evidenced by increased ^{13}C enrichment of TCA cycle intermediates and increased oxidation.

This dissertation is anticipated to provide a better understanding of mechanisms controlling DMI within the short-term period of meals in dairy cows during the postpartum period that may lead to new methods of altering energy intake and partitioning in lactating dairy cows through changes in diet; subsequently minimizing health disorders and improving energy balance, milk yield, and efficiency of nutrient utilization.

APPENDIX

Figure A1.1. Diagram of the electron transport chain. Protons enter the intermembrane space through the actions of complex I, III and IV. Complex II donates electrons to the electron transport chain but does not actively pump protons. Complex II is also succinate dehydrogenase in the tricarboxylic acid cycle. Protons pass through the ATP synthase, causing the ATP synthase to rotate and synthesize ATP from ADP + inorganic phosphate.

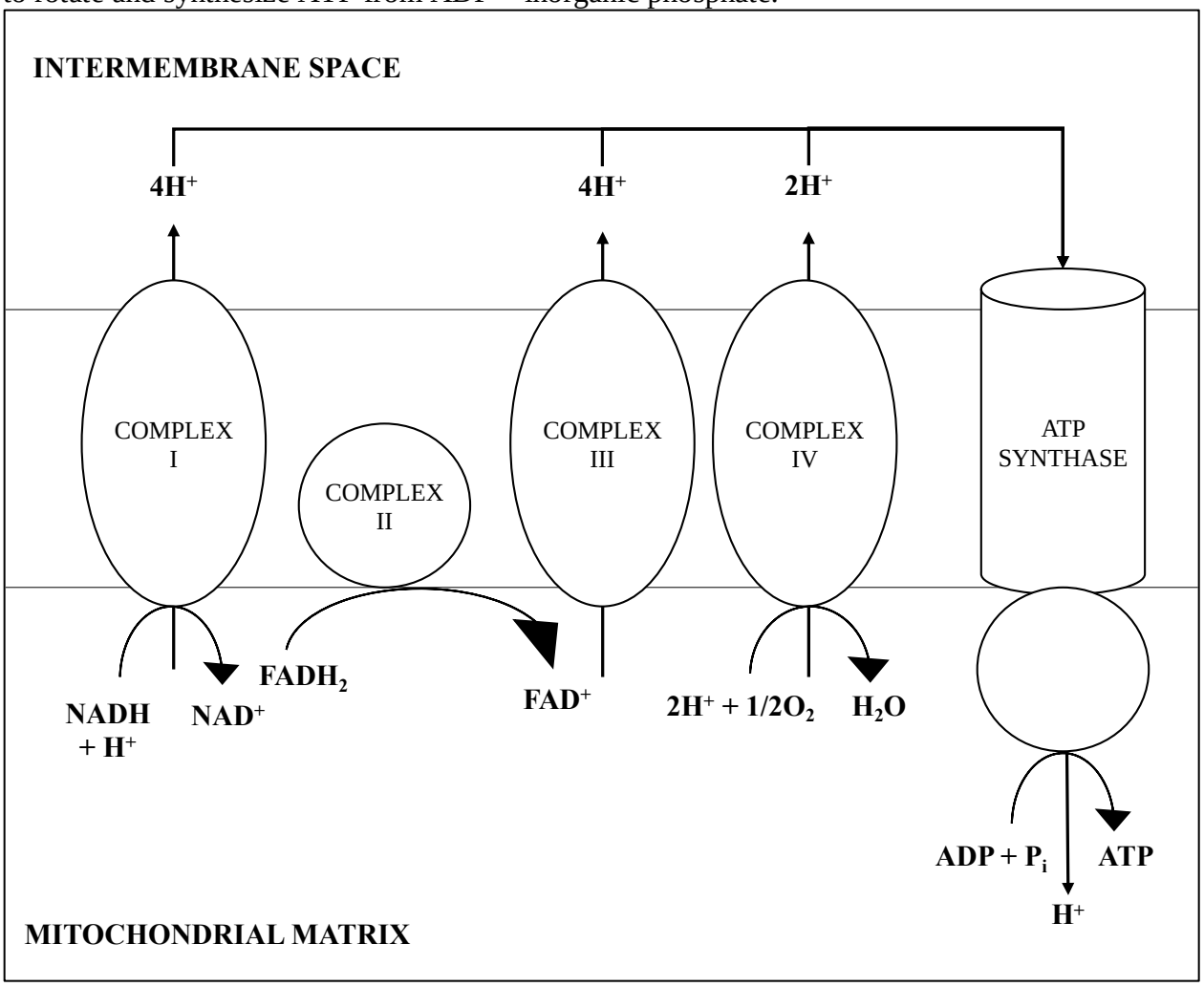


Figure A1.2. Examples of [$^{13}C_3$]propionate labeling during propionate metabolism when: propionate metabolism is directed towards gluconeogenesis and anaplerosis (A); propionate is converted to pyruvate and enters the TCA cycle as oxaloacetate (B); and propionate is converted to pyruvate and enters the TCA cycle as acetyl CoA (C). Each circle represents a carbon within the molecule. Dark circles represent ^{13}C-label and open circles represent no labeling. One complete circle of the TCA cycle is shown per figure. Because of the chemical structure of fumarate ($HO_2CCH=CHCO_2H$), placement of the label can either be found in the 1 (A and C) or 4 (B) position.

Figure A1.2A.

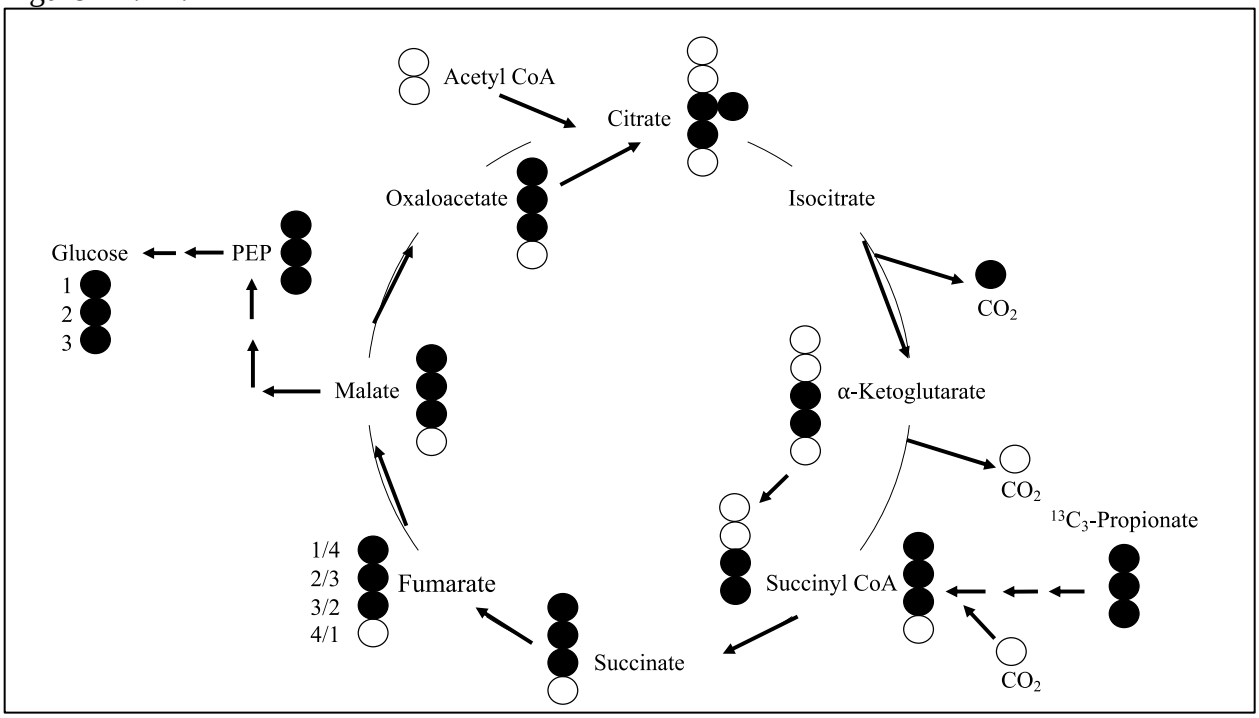


Figure A1.2 (cont'd)

Figure A1.2B.

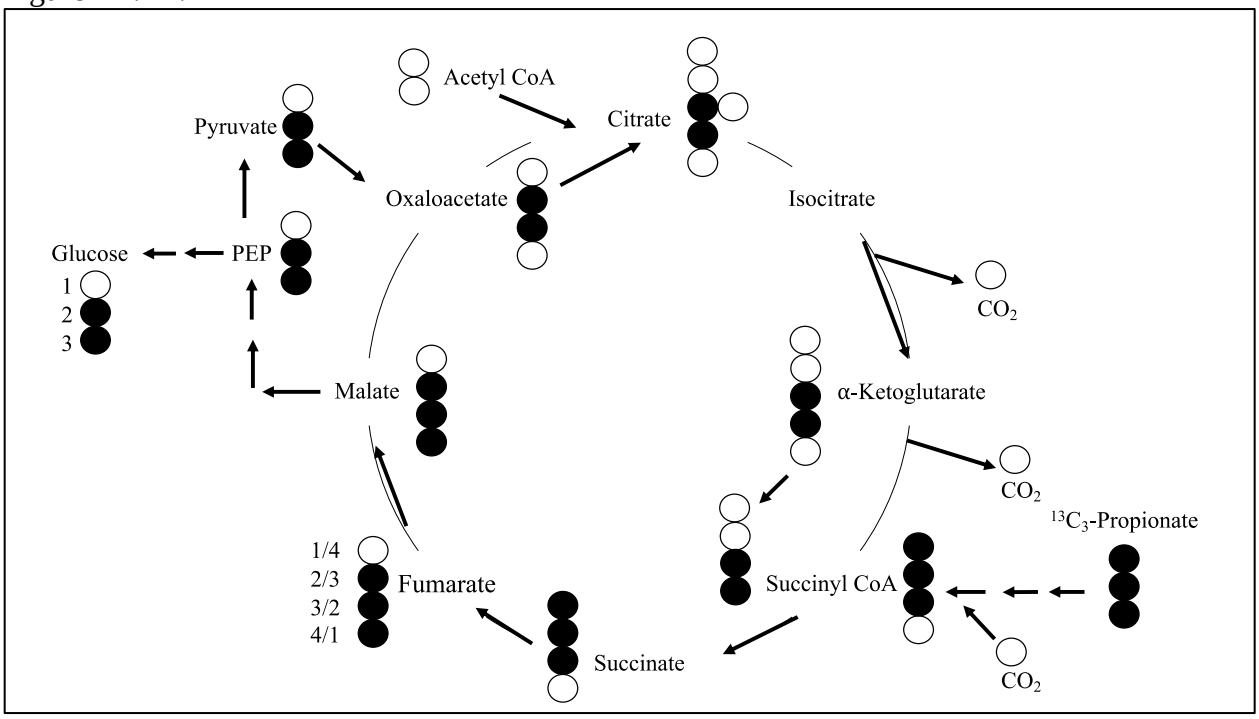
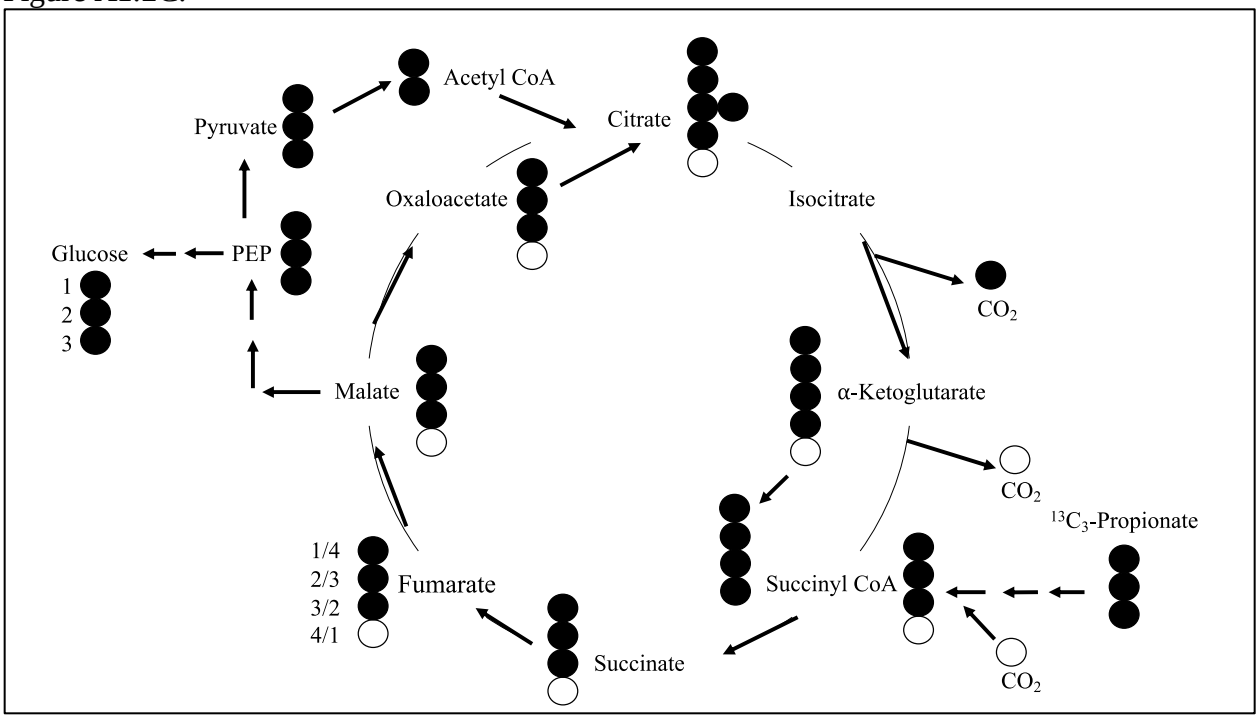


Figure A1.2C.



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CHAPTER 2: HEPATIC METABOLISM OF PROPIONATE RELATIVE TO MEALS FOR COWS IN THE POSTPARTUM PERIOD

ABSTRACT

Our objective was to identify potential short-term metabolic bottlenecks of propionate metabolism in the liver of dairy cows in the postpartum (PP) period and the effect of feeding status on these bottlenecks. We observed that propionate is rapidly absorbed from the rumen and extracted by the liver. We also observed that propionate metabolism occurred quickly (within 20 min) and we identified potential metabolic bottlenecks of propionate metabolism. Specifically, we identified propionyl CoA synthetase and succinate dehydrogenase as potential metabolic bottlenecks of metabolism.

For a full text of this work see: K.M. Kennedy and M.S. Allen. 2019. Hepatic metabolism of propionate relative to meals for cows in the postpartum period. J. Dairy Sci. 102: 7997-8010. https://doi.org/10.3168/jds.2018-15907

APPENDICES

APPENDIX A

EFFECTS OF A PULSE DOSE OF PROPIONIC ACID TO THE RUMEN ON METABOLIC RESPONSE IN LACTATING DAIRY COWS DURING THE POSTPARTUM PERIOD

ABSTRACT

Our objective was to determine potential metabolic bottlenecks for propionate metabolism in the liver of dairy cows and if the sampling time points of -1, 10 and 20 min relative to dosing are appropriate for detecting bottlenecks. Six multiparous dairy cows (14 -18 d postpartum; BCS of 3.25 ± 0.36 (mean ± SD)) were used in a crossover design. Cows received a pulse dose to the rumen of either 2.0 moles/500 mL of propionic acid (PA) or 500 mL of water (CON). Plasma and liver samples were collected -1, 10 and 20 min relative to dosing. Plasma propionate concentration was much greater for PA than CON at 10 min but declined rapidly. The PA treatment did not affect hepatic propionyl CoA, but increased the hepatic concentrations of methylmalonyl CoA, succinyl CoA, succinate, fumarate, malate, pyruvate and lactate. Potential metabolic bottlenecks were identified at malate dehydrogenase and succinate dehydrogenase. The PA treatment decreased plasma non-esterified fatty acid concentration, which likely decreased plasma and hepatic BHB concentration and tended to decrease acetyl CoA concentration. The accumulation of methylmalonyl CoA and succinyl CoA concentrations suggest other potential metabolic bottlenecks. Methylmalonyl CoA conversion to succinyl CoA requires vitamin B_{12} as a cofactor and its supplementation may improve efficiency of propionate metabolism by alleviating that bottleneck. These results are in agreement with our hypothesis that potential metabolic bottlenecks of propionate metabolism can be identified in the liver of transition dairy cows within the time-frame of a meal.

INTRODUCTION

During the postpartum (PP) period, cereal grains containing starch are often substituted for forage in diets in order to increase energy intake in dairy cows that are currently in a negative energy balance. Propionate, the major precursor for gluconeogenesis in ruminants, is produced primarily from fermentation of starch in the rumen but propionate causes hypophagic effects in ruminants (Allen, 2000). Feeding behavior is likely affected by the hepatic oxidation of fuels, which can vary greatly over minutes (Allen et al., 2009). Oxidation within a meal is likely stimulated by propionate because of its rapid production and absorption (Benson et al., 2002), efficient extraction by the liver (Baird et al., 1980; Reynolds et al., 2003), and replenishment of TCA intermediates, which stimulates oxidation of fuels (Allen et al., 2009). Furthermore, a rapid ruminal infusion rate of propionic acid in dairy cows in the PP period delayed satiety compared with a slower rate of infusion, possibly because of metabolic bottlenecks resulting from the saturation of enzymes and depletion of cofactors (Maldini and Allen, 2018).

Most research focused on the metabolic effects of propionate on liver metabolism in ruminants either used cells derived from bovine calves and/or were conducted over a time period of hours (Aiello et al., 1989; Donkin and Armentano, 1995; Zhang et al., 2015). However, the effects of propionate metabolism within the timeframe of meals have not been examined.

Our objective was to determine if metabolic bottlenecks in the liver were present and if the sampling time points of -1, 10 and 20 min relative to dosing were appropriate for observing these bottlenecks. We hypothesized that plasma propionate would be elevated and that we would observe elevated concentrations of CoA compounds and TCA cycle intermediates associated with propionate metabolism that may signify a potential metabolic bottleneck. Additionally, we hypothesized that these effects will be observed within 20 min of administration of a pulse dose

of propionic acid to the rumen.

METHODS AND MATERIALS

The Institutional Animal Care and Use Committee at Michigan State University approved all animal procedures for this experiment.

Animals, Housing, and Diets

Six multiparous Holstein dairy cows (14-18 d PP; mean BCS of 3.25 ± 0.36) were used in this experiment. Cows were housed in individual tie-stalls for the duration of the experiment and milked twice daily in the milking parlor. Cows were fed a TMR once daily at 110% of expected intake and received a common experimental diet from parturition through the end of the experiment. The experimental ration was formulated to contain 28% NDF, 23% forage NDF, 25% starch, and 18.5% CP on a DM basis and consisted of corn silage, alfalfa silage, alfalfa hay, dry ground corn, soybean meal, soybean hulls and a premix of protein supplement, minerals, and vitamins (Table B2.1). Feed offered was adjusted daily for individual animals.

Experimental Design and Treatments

A crossover design was used for the experiment and animals were blocked based on parturition date. Treatments were a pulse dose of either 2 moles/500 mL of propionic acid (food grade, 99.5%, Kemin Industries Inc., Des Moines, IA) or 500 mL of water (control; CON) into the rumen via rumen cannula. Cows were blocked from feed 1 h before treatment. Each block was randomly allocated to a treatment sequence in which each animal received each treatment once and treatment was administered once per day. A rest day was provided between treatment

days to minimize risk of carryover effects.

Data and Sample Collection

Animals were transferred to a surgery room at the Michigan State University Dairy Cattle Teaching & Research Center (East Lansing) for administration of treatment, and liver and blood collections. Animals were fitted with jugular catheters prior to the start of treatments. Catheters were maintained by flushing the lines twice daily with a sterile 3.5% sodium citrate solution. Blood samples were collected from the jugular catheter at -1, 10 and 20 min relative to dosing. Blood was sampled and harvested for plasma as described by Kennedy and Allen (2019). Plasma was frozen at −20°C until analysis. Liver biopsies were performed according to Bradford and Allen (2005) and sampled at -1, 10 and 20 min relative to dosing. Samples were flash frozen in liquid nitrogen, stored on dry ice for transportation, and then stored at -80°C until further analysis.

Analysis of Samples

Plasma. Plasma was analyzed for concentrations of propionate, glucose, insulin, non-esterified fatty acids (NEFA), BHB, acetate and lactate according to methods described by Kennedy and Allen (2019).

Liver. Liver samples were analyzed for citrate, isocitrate, succinate, fumarate, malate, pyruvate, lactate, BHB, glutamate, oxaloacetate (OAA) and α-ketoglutarate by GC-MS and for acetyl CoA, propionyl CoA, succinyl CoA, and methylmalonyl CoA by liquid chromatography – tandem mass spectrometry (LC-MS/MS) as described by Kennedy and Allen (2019) except

samples were suspended in 500 µL of 1% formic acid in water. All samples for GC-MS and LC-MS/MS were evaporated to dryness using a Savant SC110 SpeedVac Concentrator (Savant Instruments, Inc., Holbrook, NY).

Gas Chromatography – Mass Spectrometry. Samples were prepared and derivatized by methoximation and silylation for GC-MS as described by Kennedy and Allen (2019) except 10 µL of O-methylhydroxylamine reagent solution (40 mg/1 mL of pyridine; Sigma Aldrich, St. Louis, MO, USA) was added to each sample. Analysis and quantification of peaks were conducted with MassLynx Mass Spectrometry Software (version 4.1, Waters Corporation, Milford, MA) as described by Kennedy and Allen (2019). GC-MS analysis was performed as described by Kennedy and Allen (2019) except selected ion monitoring was not used. The oven program was: initial temperature of 40°C and held for 1 min, programmed at 40°C/min to 110°C and held for 1 min, then programmed at 40°C/min to 170°C, then programmed at 10°C/min to 300°C for a total run time of 18.25 min per sample.

Liquid Chromatography – Tandem Mass Spectrometry. Samples were prepared for LC-MS/MS as described by Kennedy and Allen (2019). Analysis and quantification of peaks were conducted with MassLynx Mass Spectrometry Software (version 4.1, Waters Corporation, Milford, MA) as described by Kennedy and Allen (2019). LC-MS/MS analysis was performed as described by Kennedy and Allen (2019). Mass spectrometry analysis was performed using multiple reaction monitoring (MRM) for the target compounds as described by Kennedy and Allen (2019) except that propionyl CoA was monitored using 824.1 m/z as the precursor and 135.2 m/z as the product.

Statistical Analysis

Data was analyzed with a mixed model in JMP Pro 13 (version 13.2, 2016; SAS Institute Inc., Cary, NC). Prior to statistical analysis, Box-Cox transformation analyses were applied to each response variable to determine necessary transformations on the data. Block, cow, period, and treatment were classified as nominal variables and time was classified as a continuous variable. All liver and plasma metabolites were analyzed using the following model:

$$Y_{ijklm}=\mu+B_i+C_j(B_i)+P_k(B_i)+T_l+S_m+T_lS_m+S_mS_m+ T_lP_k(B_i)+T_lS_mS_m+e_{ijklm},$$

where Y_{ijklm} = the response variable; μ = overall mean; B_i = random effect of block *i*; $C_j(B_i)$ = random effect of cow *j* nested within block; $P_k(B_i)$ = random effect of period *k* nested within block; T_l = fixed effect of treatment *l*; S_m = fixed effect of sampling time *m*; T_lS_m = interaction of treatment and sampling time; S_mS_m = the quadratic effect of time; $T_lP_k(B_i)$ = interaction of treatment and period nested within block; $T_lS_mS_m$ = interaction of treatment with the quadratic effect of time; and e_{ijklmn} = residual.

Treatment effects were declared significant at $P \leq 0.05$ and tendencies for treatment effects were declared at $P \leq 0.10$. Interactions were declared significant at $P \leq 0.10$ and tendencies for interactions were declared at $P \leq 0.15$. Data transformed are noted in the tables and figures along with the transformation used for statistical analysis. All transformed data has been back-transformed for interpretation. Percent differences were calculated by back-transforming the least square means from the statistical models, subtracting the baseline from the time point of interest, dividing that number by the baseline and multiplying it by 100.

RESULTS AND DISCUSSION

Treatment effects on plasma metabolite and hormone concentrations are summarized in Table B2.2 and treatment by time interactions are shown in Figure B2.1. Treatment effects on hepatic metabolite concentrations are summarized in Table B2.3 and treatment by time interactions are shown in Figure B2.2.

Plasma propionate concentration peaked at 10 min and returned towards baseline at 20 min with PA compared with CON (Figure B2.1A; interaction, $P = 0.03$). The rapid increase and subsequent decrease in plasma propionate concentration was consistent with research showing efficient extraction of propionate by the liver (Baird et al., 1980; Reynolds et al., 2003). Additionally, the increase in plasma propionate concentration at 10 min suggests that propionate administered to the rumen is rapidly absorbed. However, a lack of difference in hepatic propionyl CoA concentration with PA compared with CON ($P = 0.63$) suggests that the activation of propionate to propionyl CoA is unlikely to be a metabolic bottleneck. Conversely, CON decreased hepatic methylmalonyl CoA concentration over time (Figure B2.2A; interaction, $P = 0.01$) and decreased concentration at 10 and 20 min compared with baseline (pairwise tests, $P < 0.05$), however, PA did not affect concentration over time (pairwise tests, $P > 0.10$). Additionally, PA tended to increase succinyl CoA concentration at 20 min compared with 10 min (pairwise test, $P = 0.07$) whereas CON tended to decrease hepatic succinyl CoA concentration at 20 compared with baseline (Figure B2.2B; pairwise test, P = 0.09). A lack of change in methylmalonyl CoA compared with a decrease in control and tendency to increase in succinyl CoA suggests that the enzymes associated with these metabolites are potential metabolic bottlenecks. Vitamin B_{12} is a cofactor for methylmalonyl CoA mutase (EC 5.4.99.2), the enzyme that converts methylmalonyl CoA to succinyl CoA. Previous research with vitamin

B_{12} has shown that injections of folate and vitamin B_{12} increased expression of methylmalonyl CoA mutase in the liver (Preynat et al., 2010) and tended to increase whole-body glucose flux and milk production in dairy cows (Preynat et al., 2009). Additionally, Wang et al. (2018) reported that injections of vitamin B_{12} increased DMI in transition cows. As such, increased supplementation of vitamin B_{12} may help increase the rate at which propionate is metabolized in the liver.

The PA treatment increased hepatic succinate concentration over time (Figure B2.2C; interaction, $P = 0.04$) with an increase at 20 min compared with baseline (pairwise test, $P = 0.01$). Thus, another potential metabolic bottleneck may be succinate dehydrogenase (EC 1.3.5.1), the enzyme responsible for converting succinate to fumarate. Because succinate dehydrogenase is strongly inhibited by OAA (Das, 1937) and malate (Dervartanian and Veeger. 1965; Ackrell et al., 1974), the accumulation in succinate concentration may be related to the increase in malate concentration with PA over time (Figure B2.2E; interaction, $P = 0.04$). The PA treatment increased malate at 10 and tended to increase at 20 min compared with baseline (pairwise tests, $P = 0.02$ and $P = 0.07$, respectively). Although PA increased malate concentration, PA had no effect on OAA concentration compared with CON ($P = 0.34$). Therefore, a metabolic bottleneck likely occurred at malate dehydrogenase (EC 1.1.1.37), the enzyme responsible for conversion between malate and OAA. Furthermore, PA increased hepatic fumarate concentration at 10 and 20 min compared with baseline (Figure B2.2D; pairwise tests, $P < 0.05$) possibly from the accumulation of malate in the liver caused by either a decrease in the rate of the reaction of fumarate to malate or conversion of malate to fumarate. However, mitochondrial and cytosolic metabolites were not separated for analysis and malate is present in both compartments.

The PA treatment increased hepatic pyruvate concentration compared with CON (Figure B2.2G; interaction, $P = 0.03$). Although baseline values were significantly different ($P < 0.01$), PA increased pyruvate concentration at 10 and 20 min compared with baseline (pairwise tests, $P = 0.01$ and $P = 0.02$, respectively) whereas CON had no differences among time points ($P > 0.20$). Additionally, mean lactate concentration increased with PA compared with CON ($P = 0.01$) and had a tendency to increase over time (Figure B2.2H; $P = 0.14$), however, both PA and CON increased at 10 and 20 min compared with baseline (pairwise tests, $P < 0.01$ for all) and baseline concentrations differed between treatments ($P < 0.01$). Although PA increased plasma lactate concentration compared with CON (Figure B2.1G; interaction, $P < 0.01$), the increase in plasma lactate concentration could have resulted either from a release of the accumulated hepatic lactate or a decrease in hepatic uptake of lactate (Baird et al., 1980). The increase in pyruvate concentration suggests that excess carbons from propionate metabolism are contributing to gluconeogenesis and that another potential bottleneck may be associated with pyruvate. However, because multiple enzymes are associated with pyruvate metabolism, we are unable to determine which enzyme(s) may be the metabolic bottleneck from our experimental results. Nevertheless, PA increased plasma glucose concentration compared with CON (Figure B2.1B; interaction, $P < 0.01$), consistent with synthesis of glucose from propionate.

The PA treatment increased plasma insulin concentration compared with CON (Figure B2.1C; interaction, $P < 0.01$) with a peak at 10 min (2,276% increase relative to baseline). The increase in plasma propionate concentration likely stimulated the increase in plasma insulin concentration (Horino et al., 1968; Bines and Hart, 1984). The increase in insulin concentration, in turn, is likely responsible for the decrease in plasma NEFA concentration with PA compared with CON (Figure B2.1D; interaction, $P < 0.01$). Furthermore, a tendency for a decrease in

acetyl CoA concentration ($P = 0.08$) and decreases in both plasma BHB concentration (Figure B2.1E; interaction, $P < 0.01$) and hepatic BHB concentration (Figure B2.2F; interaction, $P = 0.03$) with PA compared with CON are likely the result of the decrease in plasma NEFA concentration (Stocks and Allen, 2014; Piantoni et al., 2015), however, baseline values of BHB tended to differ between treatments which should be taken into consideration.

A dose of 2 moles/500 mL of propionic acid was chosen in order to challenge the dairy cow with hopes of identifying potential bottlenecks of propionate metabolism in the liver. Measurable changes in hepatic concentrations were apparent by 10 min relative to a pulse dose of propionic acid, supporting that our sampling time points are appropriate to observe hepatic metabolism within a meal. However, the rapid change for most plasma metabolites and hormones at 10 min was unexpected, and as such, additional sampling time points of blood may help to determine systematic effects of propionate on the measured metabolites and hormones.

CONCLUSIONS

We identified methylmalonyl CoA mutase, succinate dehydrogenase, and malate dehydrogenase as potential metabolic bottlenecks associated with propionate metabolism in the liver. Additionally, changes in hepatic metabolite concentrations occurred quickly, and thus, the proposed time sampling at -1, 10 and 20 min relative to dosing was appropriate. Changes in measured plasma metabolite and hormone concentrations also occurred quickly, however, additional sampling points and a longer sampling period may be beneficial to determine the systemic effects of propionate on select plasma metabolites and hormones. Along with identifying potential metabolic bottlenecks, this pilot study also supports that metabolism of

propionate occurs rapidly and that feeding behavior and dry matter intake are likely affected by individual meals throughout a day.

ACKNOWLEDGEMENTS

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APPENDIX B

Figure B2.1. Effects of a ruminal pulse dose of 2 moles of propionic acid (PA) or water (CON) on plasma propionate[1] (A), glucose (B), insulin[1] (C), non-esterified fatty acids[1] (NEFA; D), BHB[1] (E), acetate[1] (F), and lactate[1] (G) over the first 20 min in dairy cows in the postpartum period. Associated *p*-values are located in Table B2.2. Data was transformed for statistical analysis and back-transformed for interpretation. Treatment differences within sampling time point denoted by ***** ($P < 0.05$) and tendencies are denoted by † ($P \leq 0.10$). Differences within treatments among time points are discussed in the text.

[1]Data log-transformed for statistical analysis and back-transformed for interpretation

Figure B2.1A.

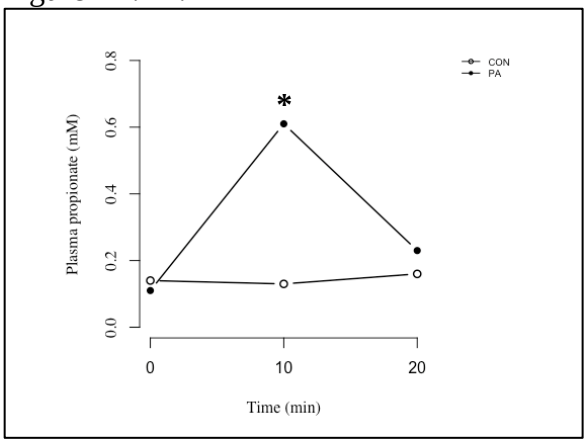


Figure B2.1C.

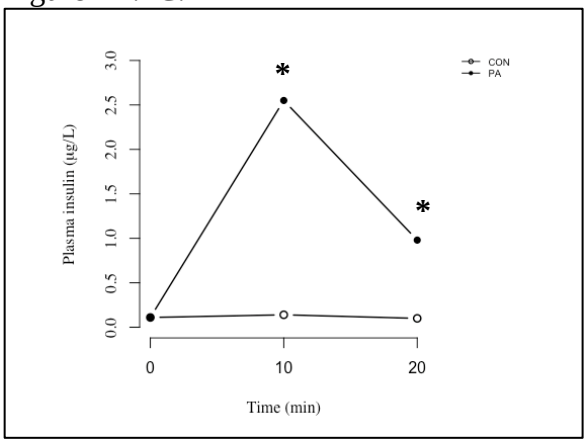


Figure B2.1B.

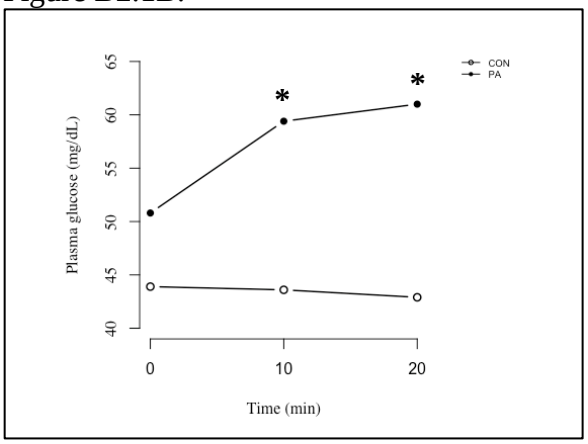


Figure B2.1D.

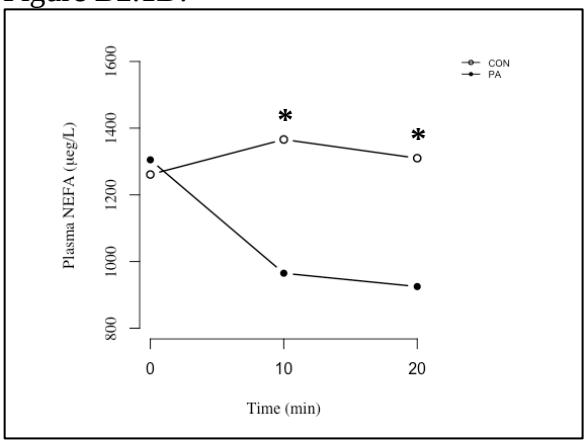
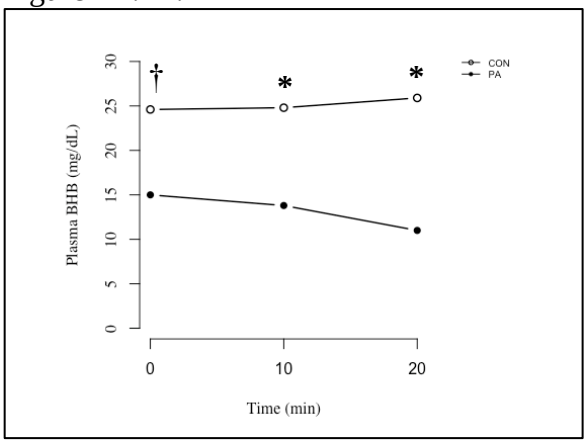


Figure B2.1 (cont'd)

Figure B2.1E.



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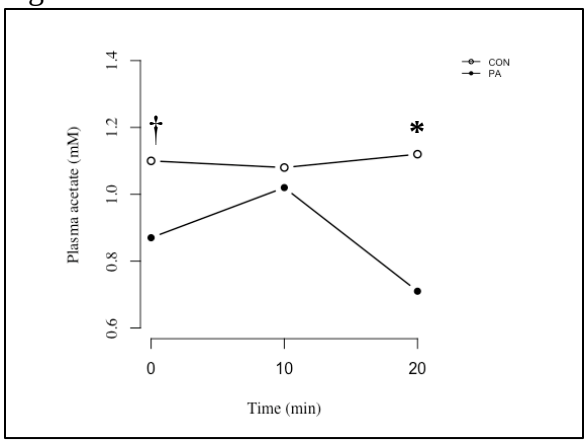


Figure B2.1G.

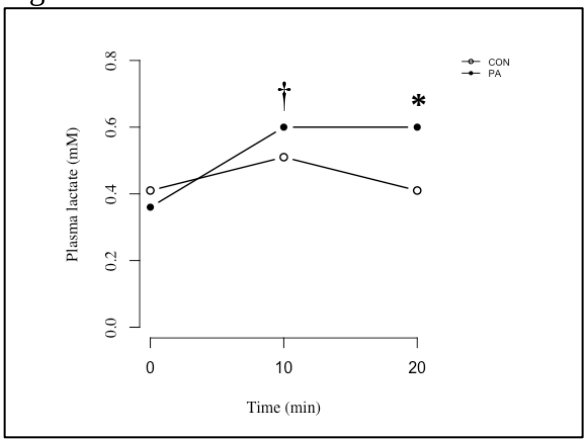
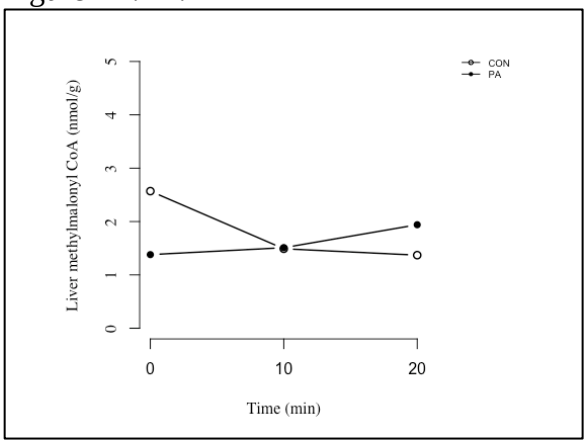


Figure B2.2. Effects of a pulse dose of 2 moles of propionic acid (PA) or water (CON) to the rumen on hepatic concentrations of methylmalonyl CoA[1] (A), succinyl CoA[2] (B), succinate[2] (C), fumarate[2] (D), malate[2] (E), BHB[2] (F), pyruvate[2] (G) and lactate[3] (H) over the first 20 min in dairy cows in the postpartum period. Associated *p*-values are located in Table B2.3. Data was transformed for statistical analysis and back-transformed for interpretation. Treatment differences within sampling time point denoted by ***** ($P < 0.05$) and tendencies are denoted by † ($P \leq 0.10$). Differences within treatments among time points are discussed in the text.

[1]Data transformed using square root for statistical analysis and back-transformed for interpretation
[2]Data log-transformed for statistical analysis and back-transformed for interpretation
[3]Data transformed using the inverse of the square root for statistical analysis and back-transformed for interpretation

Figure B2.2A.



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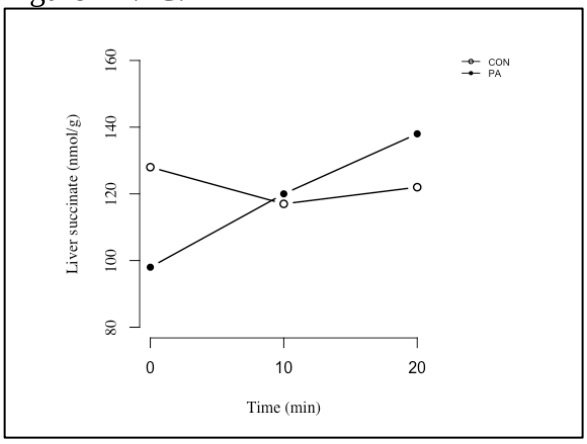
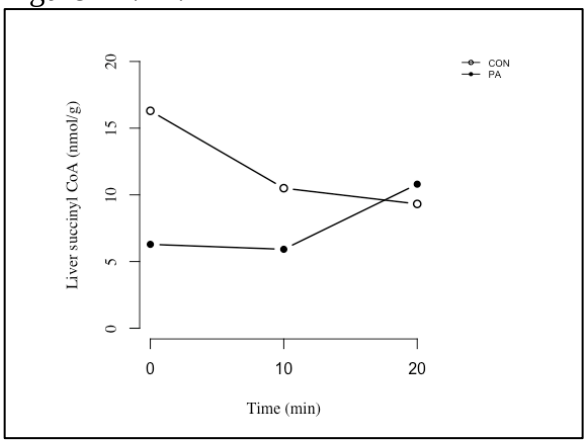


Figure B2.2B.



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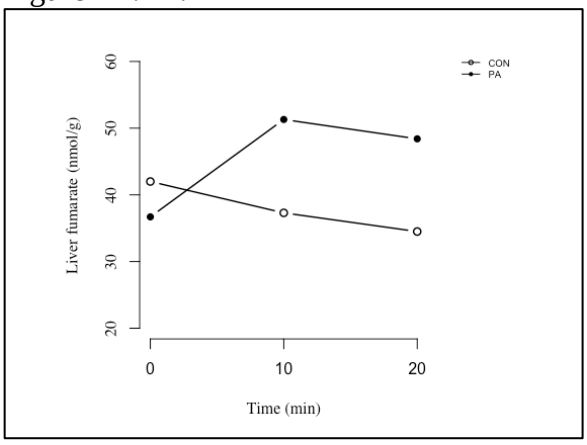
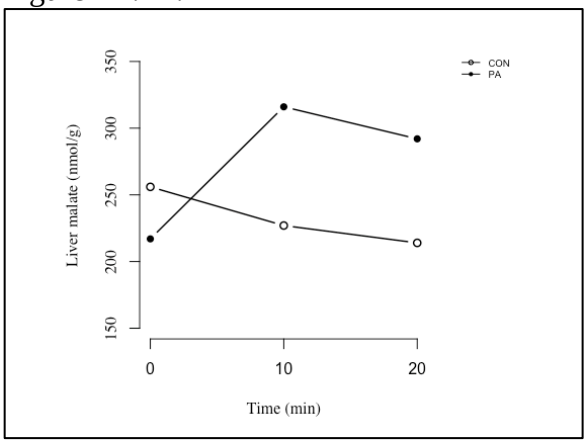


Figure B2.2 (cont'd)

Figure B2.2E.



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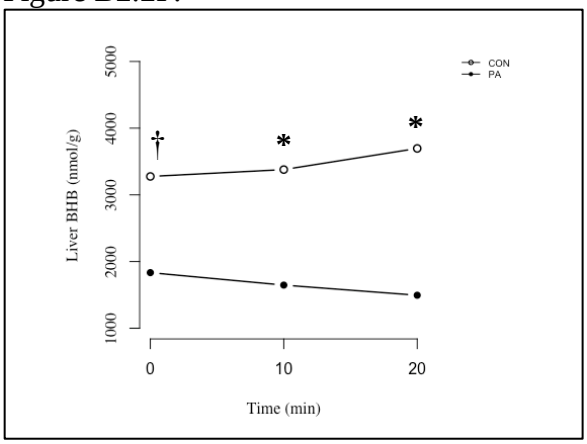

Figure B2.2G.

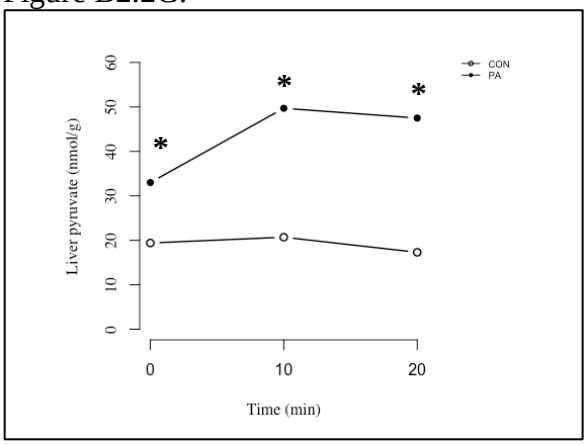

Figure B2.2H.

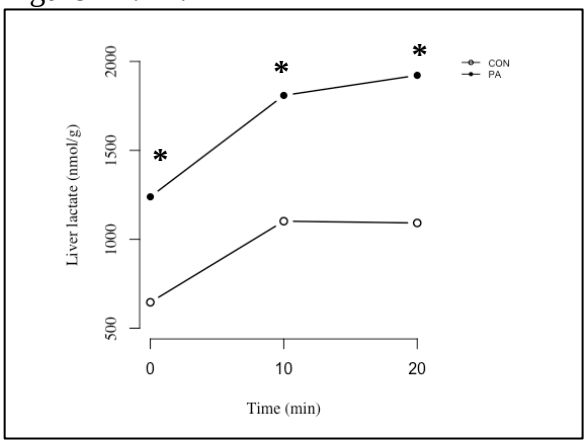

Table B2.1. Ingredients and nutrient composition of experimental diet (% of dietary DM except for DM)

Item	%
Ingredient	
Corn silage	29.3
Ground corn	19.5
Soybean meal	19.1
Alfalfa silage	14.2
Alfalfa hay	11.3
Vitamin and mineral mix[1]	4.2
Soybean hulls	2.4
Nutrient composition	
DM	59.3
NDF	28.0
forage NDF	23.3
Starch	25.0
CP	18.5

[1]Vitamin and mineral mix contained 25.6% NaCl, 10.0% Ca, 2.0% Mg, 2.0% P, 30 mg/kg Co, 506 mg/kg of Cu, 20 mg/kg I, 2,220 mg/kg Fe, 2,083 mg/kg Mn, 15 mg/kg Se, 2,025 mg/kg Zn, 300 kIU/kg vitamin A, 50 kIU/kg vitamin D, and 1,500 kIU/kg vitamin E.

Table B2.2. Effect of ruminal pulse dose of 2 moles of propionic acid (PA) compared with water (control; CON) on plasma metabolites and hormones over the first 20 min in dairy cows in the postpartum period.

				P-value				
Metabolite	CON	PA	SEM	TRT[1]	ST[1]	TRT*ST	ST*ST	TRT*ST*ST
Propionate (mM)[2]	0.13	0.61	0.38	0.04	<0.01	0.03	<0.01	<0.01
Glucose (mg/dL)	43.6	59.4	4.40	0.02	<0.01	<0.01	0.12	0.18
Insulin (μg/L)[2]	0.14	2.55	0.28	<0.01	<0.01	<0.01	<0.01	<0.01
NEFA (μeq/L)[1,2]	1366	965	0.11	0.05	0.01	<0.01	0.42	0.04
BHB (mg/dL)[2]	24.8	13.8	0.30	0.05	<0.01	<0.01	0.14	0.03
Acetate (mM)[2]	1.08	1.02	0.08	0.57	0.07	0.04	0.02	<0.01
Lactate (mM)[2]	0.51	0.60	0.09	0.10	<0.01	<0.01	<0.01	0.73

[1]SEM = standard error of the mean; TRT = treatment; ST = sampling time; NEFA = non-esterified fatty acid

[2]Data log-transformed for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.

Table B2.3. Effect of ruminal pulse dose of 2 moles of propionic acid (PA) compared with water (control; CON) on hepatic metabolites and select couplets over the first 20 min in dairy cows in the postpartum period.

				P-value				
Metabolite[1]	CON	PA	SEM[2]	TRT[1]	ST[1]	TRT*ST	ST*ST	TRT*ST*ST
Propionyl CoA[3]	4.55	5.88	0.42	0.63	0.63	0.65	<0.01	0.92
Methylmalonyl CoA[3]	1.49	1.51	0.22	0.98	0.34	0.01	0.25	0.58
Succinyl CoA[4]	10.5	5.92	0.64	0.62	0.96	0.02	0.22	0.67
Succinate[4]	117	120	0.13	0.91	0.11	0.04	0.79	0.54
Fumarate[4]	37.3	51.3	0.15	0.17	0.68	0.02	0.28	0.18
Malate[4]	227	316	0.18	0.15	0.60	0.04	0.31	0.18
Oxaloacetate[5]	30.6	17.8	0.05	0.34	0.51	0.36	0.67	0.27
Citrate[3]	42.7	79.7	1.62	0.23	0.56	0.27	0.12	0.77
Isocitrate[4]	2.20	3.99	0.34	0.13	0.43	0.23	0.89	0.29
α-ketoglutarate[4]	232	218	0.39	0.93	0.91	0.82	0.86	0.33
Glutamate[3]	1532	1568	6.50	0.97	<0.01	0.88	0.70	0.85
Acetyl-CoA[3]	13.6	5.50	0.48	0.08	0.90	0.44	0.02	0.11
BHB[4]	3379	1647	0.22	0.04	0.56	0.03	0.78	0.84
Pyruvate[4]	20.7	49.7	0.12	<0.01	0.22	0.03	0.06	0.55
Lactate[5]	1102	1809	0.00	0.01	<0.01	0.14	<0.01	0.20

[1]Metabolite values reported in nanomoles/g.
[2]SEM = standard error of the mean; TRT = treatment; ST = sampling time
[3]Data transformed using square root for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.
[4]Data log-transformed for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.
[5]Data transformed using the inverse of the square root for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.

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CHAPTER 3: EFFECTS OF INCREASING PROPIONATE CONCENTRATION ON SHORT-TERM METABOLISM IN LIVER EXPLANTS FROM DAIRY COWS IN THE POSTPARTUM PERIOD

ABSTRACT

Our objective was to determine the temporal effects of increasing supply or availability of propionate on propionate metabolism in liver tissue of dairy cows in the postpartum (PP) period. A total of 6 dairy cows (primiparous: n=3, 9.00 ± 1.00 (mean ± SD) d PP and multiparous: n=3; 4.67 ± 1.15 d PP) were biopsied for liver explants in a block-design experiment. Explant treatments consisted of 3 concentrations of [$^{13}C_3$]sodium propionate at 1, 2 or 4 mM. Explants were incubated in 2 mL of Media 199 supplemented with 1% bovine serum albumin, 0.6 mM oleic acid, 2 mM sodium L-lactate, 0.2 mM sodium pyruvate, and 0.5 mM L-glutamine at 38°C and sampled at 0.5, 15 and 60 min. Increasing the concentration of [$^{13}C_3$]propionate increased total ^{13}C% enrichment of propionyl CoA, succinyl CoA, succinate, fumarate, malate, and citrate with time. Treatment did not affect total ^{13}C% enrichment of hepatic glucose or acetyl CoA but total ^{13}C% enrichment increased with time for both. Whereas increased concentration of propionate did not result in greater labeling of acetyl CoA, increases in ^{13}C% enrichment of [M+4] citrate and [M+5] citrate indicate propionate conversion to acetyl CoA and subsequent entry of acetyl CoA into the TCA cycle in dairy cows in the PP period. However, dairy cows in the PP period are in a lipolytic state and have an elevated hepatic acetyl CoA concentration. Therefore, this research supports that propionate will contribute to the acetyl CoA pool despite elevated concentrations of hepatic acetyl CoA during the PP period. Propionate is a major volatile fatty acid produced by the rumen of dairy cows and a major glucose precursor.

Therefore, by understanding hepatic propionate metabolism, we are able to better understand and improve feeding strategies and management of dairy cows in the PP period.

INTRODUCTION

Dairy cows in the immediate postpartum (PP) period (~first 3 weeks) experience negative energy balance from increased demands for milk production (Bauman and Currie, 1980). These animals exhibit a suppression in appetite (Allen and Piantoni, 2013), exacerbating the negative energy balance. In order to increase energy intake, cereal grains containing starch are frequently substituted for fiber sources in the diet of early lactation cows. Propionic acid produced from starch fermentation is a primary glucose precursor in dairy cows. However, propionate has hypophagic effects on dairy cows in the PP period (Allen, 2000) and decreases DMI to a greater extent than other glucose precursors such as lactate (Gualdrón-Duarte and Allen, 2018) and glycerol (Gualdrón-Duarte and Allen, 2017).

The metabolic pathway of propionate in the liver of ruminants is well-established. However, the majority of work examining propionate metabolism in ruminants was conducted in cell/explant cultures from calves (Aiello et al., 1989, Donkin and Armentano, 1995), goats (Aiello and Armentano, 1987; Demigné et al., 1991) or cows not in the PP period (Drackley et al., 1991; Knapp et al., 1992; Zhang et al., 2015). Dairy cows in the PP period have higher concentrations of hepatic acetyl CoA compared with late-lactation dairy cows (Piantoni et al., 2015) in which the majority of this pool is derived from hepatic β-oxidation as a consequence of higher concentrations of circulating plasma free-fatty acids. Additionally, gluconeogenesis and pyruvate carboxylase, the enzyme responsible for converting pyruvate to oxaloacetate, are

upregulated during this stage of lactation (Greenfield et al., 2000; Aschenbach et al., 2010). Therefore, the majority of propionate is expected to be used for glucose synthesis and/or stimulate the oxidation of acetyl CoA. However, propionate might contribute to the pool of acetyl CoA as well. As part of gluconeogenesis, propionate exits the TCA cycle as malate, converts to oxaloacetate, and then converts to phosphoenolpyruvate. Phosphoenolpyruvate can either continue through gluconeogenesis to synthesize glucose or convert to pyruvate. Pyruvate, in turn, can be decarboxylated to acetyl CoA through the pyruvate dehydrogenase complex. Although elevated acetyl CoA and ATP concentrations inhibits the pyruvate dehydrogenase complex, a recent study incubated liver slices from early lactation dairy cows (~12 DIM) with labeled pyruvate and unlabeled propionate for 2 h and reported an increase in flux of pyruvate to acetyl CoA (Garcia et al., 2015). In a previous study, potential metabolic bottlenecks of propionate metabolism, such as succinate dehydrogenase and enzymes sensitive to NADH concentrations, were identified within 20 min of a pulse dose of propionic acid, including an increase in pyruvate concentration (Kennedy and Allen, 2019a; *in press*). Understanding the short-term hepatic metabolism of propionate in dairy cows in the PP period may help to improve feeding strategies during this period.

Our objective was to determine the direct, short-term temporal effects of increasing concentrations of propionate on propionate metabolism in liver tissue of dairy cows in the PP period. We hypothesized that the majority of propionate will be used for synthesis of glucose, but that increasing the concentration of propionate will result in an increase of propionate carbon converted to acetyl CoA for oxidation in the TCA cycle.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at Michigan State University approved all animal experimental procedures for this experiment.

Experimental Design, Treatments and Animal Management

Six dairy cows (3 primiparous and 3 multiparous) were used in a block-design experiment. Cows were housed in tie-stalls at the Michigan State University Dairy Cattle Teaching and Research Center. Animals were fed a common ration formulated to contain 27% NDF, 23% forage NDF, 25% starch and 18.4% CP that consisted of corn silage, ground corn, soybean meal, alfalfa silage, alfalfa hay, soybean hulls and a premix of minerals and vitamins. Primiparous animals were 9.00 ± 1.00 (mean ± SD) d PP at the time of biopsy and had a BCS of 3.08 ± 0.30 and BW of 618 ± 74.9 kg. The multiparous animals (≥ 3 parity) were 4.67 ± 1.15 d PP at the time of biopsy and had a BCS of 3.50 ± 0.65 and BW of 755 ± 73.9 kg. Treatments consisted of 3 concentrations of [$^{13}C_3$]sodium propionate (Sigma Aldrich, 99 atom % ^{13}C, St. Louis, MO, 490636) at 1, 2 or 4 mM and sampled at 0.5, 15 and 60 min. Treatment concentrations were chosen to bracket the 2.5 mM propionate concentration used in previous experiments (Donkin and Armentano, 1995; Zhang et al., 2015).

Data Collection and Analysis

Prior to the liver biopsy, animals were moved to a surgery room at the Michigan State Dairy Cattle Research and Teaching Center (East Lansing, MI). Liver was biopsied prior to the morning feeding at approximately 0800 h. Cows were blocked from feed for approximately 30

min prior to biopsy and 1 cow was biopsied per day. Liver tissue (~300-400 mg) for the experiment was biopsied as described by Kennedy and Allen (2019a). Tissue was rinsed in sterile ice-cold saline to remove excess blood and then placed in ice-cold supplemented Media 199 (Sigma Aldrich, St. Louis, MO, M2154) for immediate transport to the laboratory. Explants were prepared from biopsied tissue as described by Zhang et al. (2015). Briefly, explants were incubated in 2 mL of Media 199 supplemented with 1% bovine serum albumin (GenDEPOT, Katy, TX, A0100), 0.6 mM oleic acid (Sigma Aldrich, St. Louis, MO, O3008), 2 mM sodium L-lactate (Sigma Aldrich, St. Louis, MO, L7022), 0.2 mM sodium pyruvate (Sigma Aldrich, St. Louis, MO, P2256), 0.5 mM L-glutamine (Sigma Aldrich, St. Louis, MO, G3126) and the treatment amount of [$^{13}C_3$]propionate in 20 mL glass scintillation vials (Research Products International, Mt. Prospect, IL, 121002). Prior to addition of tissue, the media was equilibrated to 38°C. Vials were flushed with 95% O_2:5% CO_2, capped, and then gently agitated at 40 strokes per minute at 38°C in a Precision™ Reciprocal Shaking Bath (Thermo Fisher Scientific, Waltham, MA) until the designated sampling time. Samples were then removed from the media with sterile tweezers, rapidly blotted (~10 s) on Kimwipes™ (Kimtech Science, Kimberly-Clark Professional, Roswell, GA), placed in 2 mL screw-cap tubes with O-ring caps (Thomas Scientific; Swedesboro, NJ, 1213G01) and dropped in liquid nitrogen. Samples were stored at -80°C until analysis. Tissue not used for explants was frozen in liquid nitrogen as described and used as a baseline (0 min) and correction factor for isotope natural abundance.

Explants were analyzed for citrate, isocitrate, succinate, fumarate, malate, pyruvate, lactate, glutamate, oxaloacetate (OAA), α-ketoglutarate and glucose by GC-MS and for acetyl CoA, propionyl CoA, succinyl CoA, and methylmalonyl CoA by liquid chromatography – tandem mass spectrometry (LC-MS/MS). Samples were prepared for mass spectrometry analysis

and analyzed on GC-MS and LC-MS/MS using the methods described by Kennedy and Allen (2019b) except additional m/z values were monitored to account for complete ^{13}C tracer incorporation into the metabolites ([M + n]). Samples analyzed on GC-MS were derivatized by methoximation and silylation as described by Kennedy and Allen (2019a). Area quantification of peaks were integrated using MassLynx Mass Spectrometry Software (version 4.1, Waters Corporation, Milford, MA). Naturally low abundance of isocitrate caused data to be undetectable and/or unreliable with higher isotopologues so the data were not included. Additionally, an unknown mass at [M+2] for OAA and α-ketoglutarate interfered with those isotopologues by masking their abundance, including unlabeled samples, so the data were not included. Quantified area was corrected using the recorded weight of each explant (g wet weight). Limit of detection (LoD) was calculated (Armbruster and Pry, 2008) and undetectable values were assigned a value of LoD/2 (Hornung and Reed, 1990). Total % of ^{13}C enrichment of each metabolite was calculated as modified from Wang and Jones (2014) using the following equation:

$$^{13}C\%\ \text{enrichment} = \frac{\bar{M}_L - \bar{M}_U}{13.00335 - 12} \times 100$$

where $\bar{M}_L$ is the weighted average isotopologue mass of the labeled metabolite, $\bar{M}_U$ is the weighted average isotopologue mass of the unlabeled metabolite, and the denominator is the difference between ^{13}C and ^{12}C atomic masses. This equation is a simple representation of stable isotope labeling and calculates the total percent increase of ^{13}C over its natural abundance that is present within a metabolite (i.e. it does not distinguish between the distribution of isotopologues within the metabolite).

Additionally, the isotope enrichment, expressed as molar percent, was calculated for the isotopologues [M+3] propionyl CoA, [M+2] pyruvate, [M+1] acetyl CoA, [M+2] acetyl CoA, [M+3] citrate, [M+4] citrate, [M+5] citrate, [M+2] glutamate, [M+3] glutamate, [M+2] glucose, and [M+3] glucose as follows: 1) integrated areas/g were normalized to [M+0] as a percentage; 2) multiplied by a correction matrix based on the stable isotope distribution of each compound in unlabeled samples (Fernandez et al., 1996); and 3) each isotopologue was divided by the total isotopologue abundance (Wilson et al., 2017). The enrichment of each isotopologue is relative to the mass distribution of all the isotopologues in the metabolite and is on a scale of 0 to 100%. The isotopologues [M+2] pyruvate, [M+1] acetyl CoA, [M+2] acetyl CoA, [M+3] citrate, [M+4] citrate, and [M+5] citrate were examined because they are key points in determining if acetyl CoA was synthesized from propionate and entered the TCA cycle for oxidation. Because of known labeling patterns of labeled propionate (Kelleher, 1986; Previs and Kelley, 2015), an increase in [M+2] pyruvate and/or [M+3] pyruvate is expected which would result in [M+1] acetyl CoA and/or [M+2] acetyl CoA through the pyruvate dehydrogenase complex. The labeled acetyl CoA will then condense with [M+2]/[M+3] oxaloacetate to produce [M+4] or [M+5] citrate. Because the carbons from acetyl CoA are not oxidized during the initial turn of the TCA cycle, unlabeled acetyl CoA will dilute labeled citrate; as such an enrichment greater than [M+3] citrate would be unexpected from the anaplerosis of [$^{13}C_3$]propionate in ruminants. Therefore, enrichment of [M+4] citrate or [M+5] citrate would suggest labeled pyruvate entering the TCA cycle via labeled acetyl CoA. All [M + n] of glutamate from propionate results from flux of the TCA cycle as the labeling must have derived from α-ketoglutarate through glutamate dehydrogenase (Garcia et al., 2015; Zhang et al., 2015).

Statistical Analysis

All data were analyzed with mixed models (PROC MIXED) and repeated measures using SAS software (version 9.4, 2013; SAS Institute Inc., Cary, NC). Prior to statistical analysis, Box-Cox transformation analyses were applied to each response variable to determine necessary transformations on the data using JMP Pro (version 13.2, 2016; SAS Institute Inc., Cary, NC). Total ^{13}C% enrichment of each metabolite and isotope enrichment of select isotopologues except for CoA compounds (LC-MS/MS analysis) were analyzed with the following model:

$$Y_{ijklm} = \mu + C_i(P_j \times R_k) + P_j + R_k + T_l + S_m + T_lS_m + T_lP_j + T_lR_k + P_jS_m + T_lS_mP_j + e_{ijklm},$$

where Y_{ijklm} = the response variable; μ = overall mean; $C_i(P_j \times R_k)$ = random effect of cow *i* nested within parity *j* and run *k*; P_j = fixed effect of parity *j*; R_k = fixed effect of run *k*; T_l = fixed effect of treatment *l*; S_m = fixed effect of sampling time *m*; T_lS_m = interaction of treatment and sampling time; T_lP_j = interaction of treatment and parity; T_lR_k = interaction of treatment and machine run; P_jS_m = interaction of parity and sampling time; $T_lS_mP_j$ = interaction of treatment, sampling time and parity; and e_{ijklm} = residual. Run was defined as the group of samples analyzed on a mass spectrometer within a single session (GC-MS). CoA compounds were analyzed with the same model as above except there were no run effects as all data was analyzed during the same run.

Time was used in the repeated statement to account for repeated measures and the subject was defined as treatment by either cow nested within parity by run interaction (GC-MS analyses) or cow nested within parity (LC-MS/MS analysis). The covariance structure was first-order Toeplitz and denominator degrees of freedom were estimated by using the Kenward-Roger

option in the MODEL statement. Paired differences were determined using the diff option with a Tukey adjustment in PROC MIXED.

Treatment effects and interactions were declared at $P \leq 0.05$ and tendencies for treatment effects were declared at $P \leq 0.10$. Data transformed are noted in the tables and figures along with the transformation used for statistical analysis. All transformed data has been back-transformed for interpretation.

RESULTS

Total ^{13}C% Enrichment

All metabolites had highly significant increases in total ^{13}C% enrichment over time (Figure A3.1; $P < 0.0001$). An increase in total ^{13}C% enrichment between baseline and 0.5 min was observed for propionyl CoA, methylmalonyl CoA, succinate, fumarate, citrate, glucose and acetyl CoA (Table A3.1; pairwise tests, $P \leq 0.01$). No significant treatment by run interactions were detected ($P > 0.15$).

Increased concentrations of [$^{13}C_3$]propionate increased total ^{13}C% enrichment of succinate, fumarate, malate, citrate, glutamate, pyruvate and lactate over time (Table A3.2; Figure A3.1; interaction, $P \leq 0.01$). Propionyl CoA had a tendency (Figure A3.1A; $P = 0.08$) and succinyl CoA had a significant (Figure A3.1C; $P = 0.04$) treatment by parity by time interaction. However, parity groups did not differ among treatments at each time point for propionyl CoA, but a significant difference between parity groups was detected with the 2 mM treatment at 60 min for succinyl CoA (primiparous: 116% vs. multiparous: 208% enrichment; pairwise test, $P = 0.01$).

Treatment tended to interact with parity for total ^{13}C% enrichment of lactate ($P = 0.09$) and multiparous cows tended to have greater total ^{13}C% enrichment of lactate compared with primiparous cows with the 2 mM treatment (1.96 vs. 1.19% enrichment; pairwise test, $P = 0.10$). Multiparous cows had increased total ^{13}C% enrichment of lactate with the 2 mM and 4 mM treatments compared with 1 mM treatment (1 mM: 0.88% vs. 2 mM: 1.96% or 4 mM: 2.13% enrichment; pairwise tests, $P \leq 0.01$). Primiparous cows had increased total ^{13}C% enrichment of lactate with the 4 mM compared with 1 mM treatment (1.74 vs. 0.84% enrichment; pairwise test, $P < 0.01$).

Concentration of [$^{13}C_3$]propionate did not affect total ^{13}C% enrichment of methylmalonyl CoA, glucose or acetyl CoA (Table A3.2; $P > 0.30$). A main effect of [$^{13}C_3$]propionate concentration on total ^{13}C% enrichment of propionyl CoA, succinate, fumarate, malate, citrate, pyruvate and lactate was detected ($P \leq 0.05$). Concentration of [$^{13}C_3$]propionate tended to increase total ^{13}C% enrichment of glutamate ($P = 0.07$) and the 2 mM treatment tended to increase total ^{13}C% enrichment compared with the 1 mM treatment (pairwise test, $P = 0.06$).

^{13}C% Enrichment of Isotopologues

All isotopologues increased in ^{13}C% enrichment over time (Figure A3.2; $P < 0.0001$) except for [M+2] acetyl CoA. No significant treatment by run interactions were detected ($P > 0.25$).

Effect of different concentrations of [$^{13}C_3$]propionate on ^{13}C% enrichment of isotopologues are shown in Table A3.3. ^{13}C% enrichment of [M+2] pyruvate, [M+1] acetyl CoA, [M+3] citrate, [M+4] citrate, [M+5] citrate, [M+2] glutamate and [M+3] glutamate had significant treatment by time interactions (Figure A3.2; $P \leq 0.05$) in which increasing

concentration of [$^{13}C_3$]propionate increased ^{13}C% enrichment over time for all but [M+1] acetyl CoA. ^{13}C% enrichment of [M+3] citrate tended to be greater with the 4 mM than 2 mM treatment at 60 min (pairwise test, $P = 0.07$). Treatment, time and parity tended to interact for ^{13}C% enrichment of [M+3] propionyl CoA (Figure A3.2A; $P = 0.08$) and [M+3] glucose (Figure A3.2J; $P = 0.07$). However, parity did not differ among treatments at each time point for [M+3] propionyl CoA, but multiparous cows had greater ^{13}C% enrichment of [M+3] glucose than primiparous cows with the 2 mM and 4 mM treatments at 60 min (pairwise tests, $P < 0.01$).

Treatment interacted with parity for ^{13}C% enrichment of [M+2] glucose ($P = 0.01$) and treatment tended to interact with parity for ^{13}C% enrichment of [M+2] pyruvate and [M+3] glucose ($P = 0.06$ for both). ^{13}C% enrichment of [M+2] glucose did not differ between parity within each treatment (pairwise tests, $P > 0.10$). Multiparous cows had greater ^{13}C% enrichment of [M+2] pyruvate with the 2 mM treatment than primiparous cows (2.54 vs. 1.75%; pairwise test, $P = 0.03$). Multiparous cows had greater ^{13}C% enrichment of [M+3] glucose than primiparous cows with the 2 mM (0.16 vs. 0.07%; pairwise test, $P = 0.01$) and 4 mM treatments (0.16 vs. 0.08%; pairwise test, $P = 0.02$).

DISCUSSION

Total ^{13}C% enrichment of acetyl CoA did not increase with increased concentrations of [$^{13}C_3$]propionate, however, ^{13}C% enrichment of acetyl CoA did increase over time compared with baseline. Furthermore, although no significant treatment or time effects were detected for [M+2] acetyl CoA enrichment, [M+4] citrate and [M+5] citrate enrichment significantly increased over time and with increased treatment concentrations. Significant [M+3] citrate

enrichment would be expected as [$^{13}C_3$]propionate feeds into the TCA cycle but labeling beyond [M+3] citrate would require labeled acetyl CoA. Enrichment of pyruvate and [M+2] pyruvate enrichment significantly increased with treatments of [$^{13}C_3$]propionate over time; indicating that propionate was converted to pyruvate. Because ATP citrate lyase and malic enzyme are negligible in ruminants (Hanson and Ballard, 1967;Young et al., 1969), the labeled acetyl CoA is likely derived from labeled pyruvate. However, the pyruvate dehydrogenase complex is tightly regulated (Williamson and Cooper, 1980) and likely synthesized acetyl CoA at a rate not affected by the concentration of the pyruvate pool, particularly since pyruvate carboxylase is upregulated during this stage of lactation (Greenfield et al., 2000). This could partially explain the lack of treatment effect detected for enrichment of acetyl CoA when incubated with a concentration of propionate from 1 – 4 mM. Conversely, Garcia et al. (2015) reported an increase in the relative activity of the pyruvate dehydrogenase complex versus pyruvate carboxylase when liver slices from early lactation cows were incubated with a pyruvate and propionate treatment compared with a pyruvate only treatment for 2 h. Notably, Garcia et al. (2015) did not measure enrichment of acetyl CoA directly but estimated [M+2] acetyl CoA enrichment from glutamate enrichment.

In addition to its anaplerotic capabilities, propionate is also a primary glucose precursor in dairy cows. Estimates of propionate contribution to glucose synthesis range from 61 – 95% (Wiltrout and Satter, 1972; Armentano, 1992). However, enrichment of intracellular hepatic glucose was approximately 4% at its greatest enrichment and no treatment or treatment by time effects were detected. Similar to total ^{13}C% enrichment of glucose, [M+3] glucose enrichment increased over time, but enrichment did not exceed 1%. However, [M+3] glucose enrichment was higher in multiparous than primiparous animals at higher [$^{13}C_3$]propionate concentrations.

Notably, we measured the ^{13}C enrichment of the accumulated, free glucose located within the hepatic tissue and not the extracellular labeling of glucose, but free glucose is released from hepatic tissue for use of other tissues. The biologically active form of glucose, glucose-6-phosphate, prevents glucose from exporting out of the tissue, but Rukkwamsuk et al. (1999) reported an increase in glucose-6-phosphatase (EC 3.1.3.9) activity within 0.5 week after partition in dairy cows. An increase in glucose-6-phosphatase activity suggests that phosphate will be liberated from glucose-6-phosphate to form glucose, which in turn, suggests an increase in the release of glucose from hepatic tissue. Therefore, the appearance of the isotope in the intracellular pool does not likely affect the overall labeling of glucose because of its rapid release from the tissue. Furthermore, the enzymes glucokinase and hexokinase (EC 2.7.1.2 and EC 2.7.1.1, respectively), which phosphorylates glucose to glucose-6-phosphate, is minimal to non-existent in the liver of dairy cows (Ballard, 1965). As such, recycling and absorption of extracellular glucose is likely minimal or negligible as well. Consequently, glucose from extrahepatic sources (extracellular) and from intracellular synthesis is not expected to be stored in hepatic tissue of dairy cows in the immediate PP period. We suspect that because extracellular glucose represents a sink for [^{13}C]propionate we were unable to observe changes in glucose. The lack of enrichment indicates a rapid shunting of glucose out of the tissue and little or no capacity to retain newly synthesize free glucose.

Although propionate is the primary glucose precursor in dairy cows, its contribution slightly decreased in the immediate PP period (approximately 60%), despite increased propionate uptake and glucose output, and lactate, also a gluconeogenic precursor, contributed approximately 25% to glucose synthesis during the PP period (Aschenbach et al. 2010). Because lactate was present in our media, glucose synthesis from lactate may have also diluted the

observed enrichment of free glucose. Additionally, liver glycogen is approximately 8% on a DM basis for dairy cows 7 – 14 DIM (Vazquez-Anon et al., 1994), and so, glycogen in the liver may have been broken-down to glucose and diluted the labeled glucose synthesized from propionate. Therefore, along with rapid of shunting glucose out of the hepatic tissue, the fraction of free glucose synthesized from propionate may have been diluted from other gluconeogenic precursors and glycogen.

Our treatment concentrations were chosen to bracket the 2.5 mM propionate concentration used in previous experiments, which suggests that a concentration of 2.5 mM of propionate for a 2 h incubation supplies enough propionate for steady-state to be achieved (Donkin and Armentano, 1995; Zhang et al., 2015). The labeling of all measured pools within 15 mins of introduction of explants supports that steady-state enrichment was achieved in this experiment as well. Although our lowest treatment was 1 mM of [$^{13}C_3$]propionate, Reynolds et al. (2003) stated that 795 mmol/h of propionate was measured from net portal drained viscera and that plasma flow through the portal vein was 1287 L/h for dairy cows 11 d PP on a 24% starch diet. Therefore, approximately 0.62 mM of plasma propionate are present in the portal vein for these dairy cows. A similar concentration (0.44 mM) was reported by Bergman et al. (1966) for sheep consuming a full ration and infused with ~25 mmol/h of propionic acid into the rumen vein. Both of these circulating concentrations are below our lowest treatment concentration (1 mM), suggesting that hepatocytes likely have greater capacity for propionate metabolism beyond physiological levels as suggested by Armentano (1992).

Enrichment of metabolites increased as concentration of [$^{13}C_3$]propionate increased for the majority of measured metabolites from 1 – 4 mM, which suggests an increase in anaplerosis at higher concentrations of [$^{13}C_3$]propionate in the range of 1 – 4 mM. The observed increase in

enrichment of glutamate supports increased anaplerosis by propionate and suggests that α-ketoglutarate was enriched as well, as glutamate can be synthesized from α-ketoglutarate via the glutamate dehydrogenase reaction (EC 1.4.1.2). This reaction oxidizes NADH to NAD^+, which in turn, replenishes the supply of NAD^+ that is used as a cofactor in many reactions within and associated with the TCA cycle.

Additionally, enrichment of metabolites associated with propionate metabolism increased with time as expected and significant detection of enrichment was present for all metabolites by 15 min. Interestingly, ^{13}C enrichment of some metabolites appears to occur soon after introduction of explants to the incubation media as propionyl CoA, methylmalonyl CoA, succinate, fumarate, citrate, glucose and acetyl CoA had significant increases in total ^{13}C% enrichment between baseline and exposure to [$^{13}C_3$]propionate enriched media for ~0.5 min independent of [$^{13}C_3$]propionate concentration. The 0.5 min samples were originally proposed baseline samples that were taken by adding an explant to each treatment media and then immediately removing the tissue from the media, blotting it and freezing it in liquid nitrogen as previously described. However, rapid incorporation of the stable tracer was observed for some of the measured metabolites and therefore, these samples were unsuitable as baseline values. This rapid incorporation of labeling could be related to a variety of factors such as enzyme activity, enzyme concentration, and the depletion and/or saturation of the cofactor pools.

Propionate is a major volatile fatty acid produced by the rumen of dairy cows and a major glucose precursor, yet research suggests it can decrease dry matter intake during the transition period (Allen, 2000). Dry matter intake is important for transition cow success and is often considered on a daily basis. Equally important are the daily meals that comprise DMI. Metabolic reactions proceed quickly (within seconds and minutes) and dynamic changes are occurring

within a system when studied on a minute time scale. Therefore, by understanding the short-term metabolism of nutrients we can begin to imagine new ideas and strategies to improve and manage nutrition in dairy cows to minimize the metabolic stress associated with transition to lactation. Further research is needed to understand the extent of and scenarios in which propionate contributes to acetyl CoA production both in *in vitro* and *in vivo* model.

CONCLUSIONS

Despite high concentrations of hepatic acetyl CoA in dairy cows in the PP period, our results indicate the propionate will convert to acetyl CoA and enter the TCA cycle for oxidation. Additionally, the contribution of propionate to acetyl CoA increases as concentrations of propionate increase within a 1 – 4 mM range. Lastly, the accumulated fraction of free glucose synthesized from propionate in hepatic tissue was not affected by the concentration of propionate.

ACKNOWLEDGEMENTS

This work is supported by AFRI NIFA Fellowships Grant Program (2017-67011-26042 / 1010850) from the USDA National Institute of Food and Agriculture. We also thank D. G. Main, R. Albornoz, G. Maldini, A. Meade and R. West (all from Michigan State University), as well as the staff of the Michigan State University Dairy Cattle Field Laboratory (East Lansing) and Michigan State University Mass Spectrometry and Metabolomics Core (East Lansing) for their assistance in this experiment.

APPENDIX

Figure A3.1. Effects of different concentrations of [$^{13}C_3$]propionate (1 mM, square; 2 mM, circle; or 4 mM, triangle) on total ^{13}C% enrichment above natural abundance of hepatic propionyl CoA (A), methylmalonyl CoA (M-CoA; B), succinyl CoA (C), succinate[1] (D), fumarate[2] (E), malate (F), citrate[2] (G), glutamate[2] (H), pyruvate[3] (I), lactate[1] (J), glucose[4] (K), and acetyl CoA[3] (L) over 60 min. Total ^{13}C% enrichment is the percent increase of ^{13}C within each metabolite above the natural abundance. Time points are baseline (0 min), 0.5, 15 and 60 min. Graphs are presented as highest level of significant or tendency for significant interaction and associated *p*-values can be found in Table A3.2. Time effects are presented at the bottom of each graph with a letter in order of baseline/0.5/15/60 min and different letters define significant difference from each other ($P \leq 0.05$). Treatment by time interactions at each time point were examined for differences among treatments. Letters are presented in the order of 1/2/4 mM treatments and different letters define significant difference from each other ($P \leq 0.05$). Significant results from pairwise tests for the 3-way interactions are described in the text.

[1]Data transformed using square root (n+ 1) for statistical analysis and back-transformed for interpretation
[2]Data transformed using log (n + 1) for statistical analysis and back-transformed for interpretation
[3]Data transformed using square root (n + 2) for statistical analysis and back-transformed for interpretation
[4]Data transformed using log (n + 2) for statistical analysis and back-transformed for interpretation

Figure A3.1A.

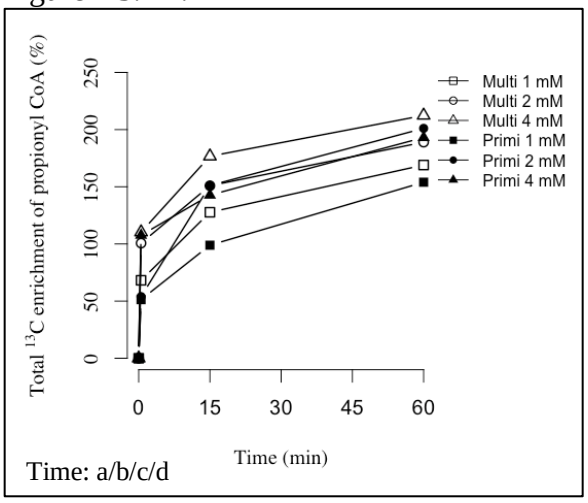


Figure A3.1B.

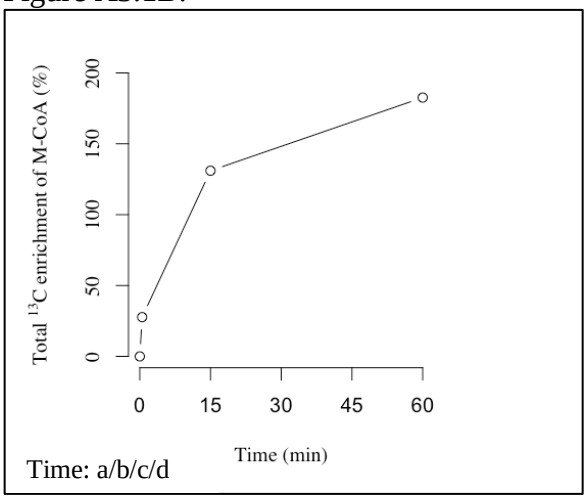
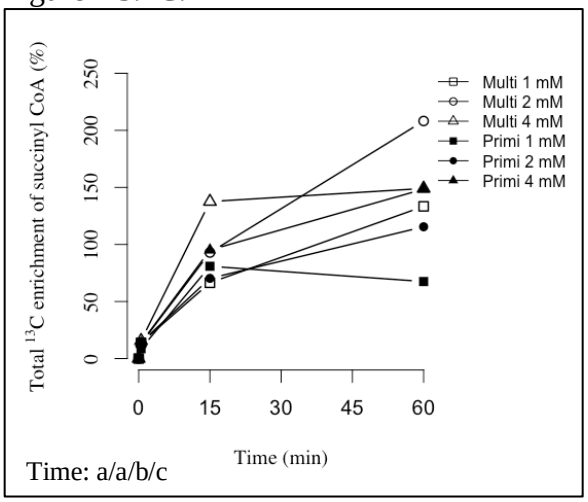


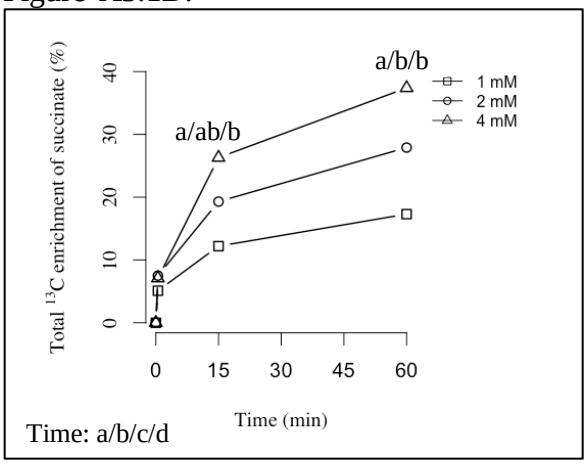
Figure A3.1 (cont'd)

Figure A3.1C.



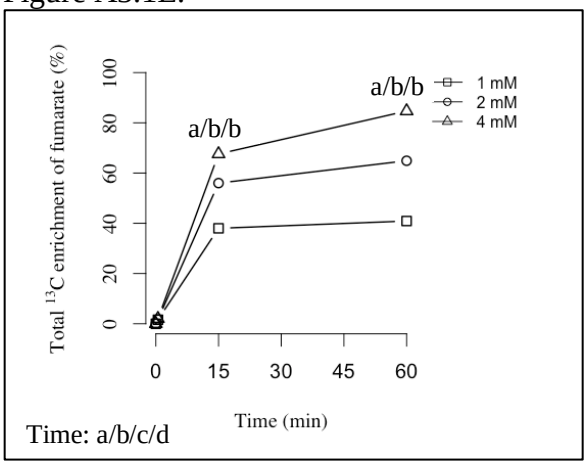
Time: a/a/b/c

Figure A3.1D.



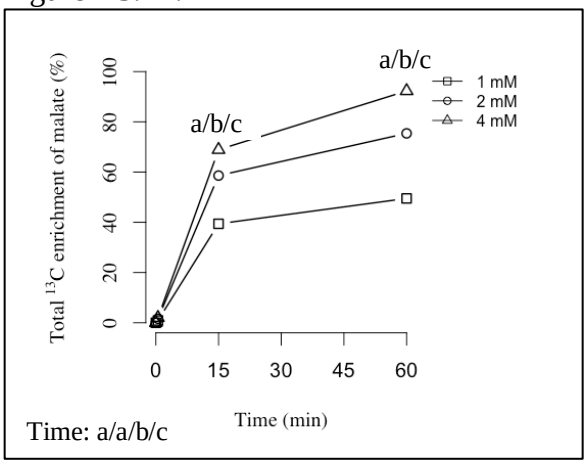
Time: a/b/c/d

Figure A3.1E.



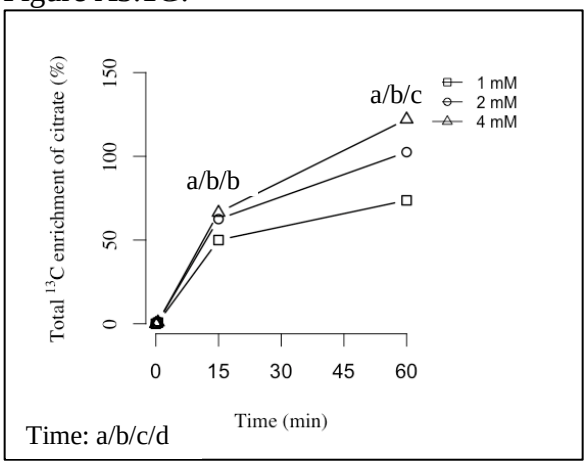
Time: a/b/c/d

Figure A3.1F.



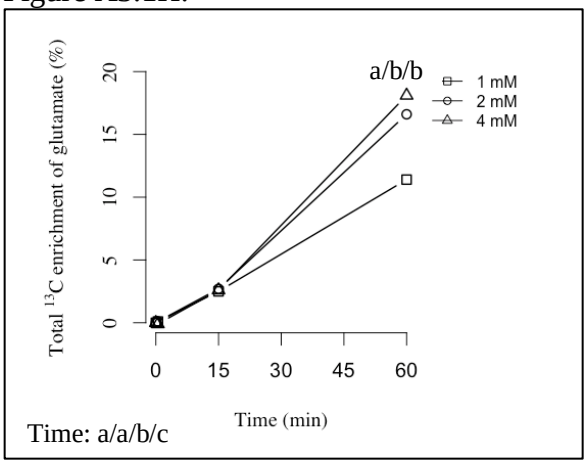
Time: a/a/b/c

Figure A3.1G.



Time: a/b/c/d

Figure A3.1H.



Time: a/a/b/c

Figure A3.1 (cont'd)

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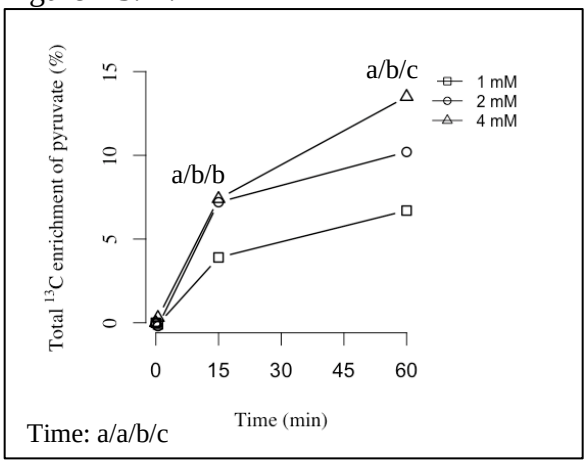

Time: a/a/b/c

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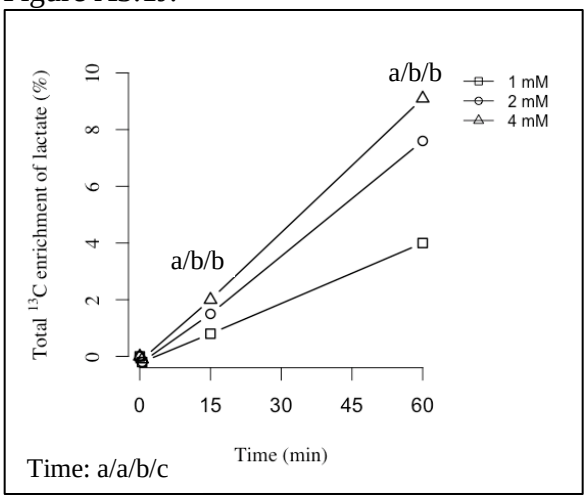

Time: a/a/b/c

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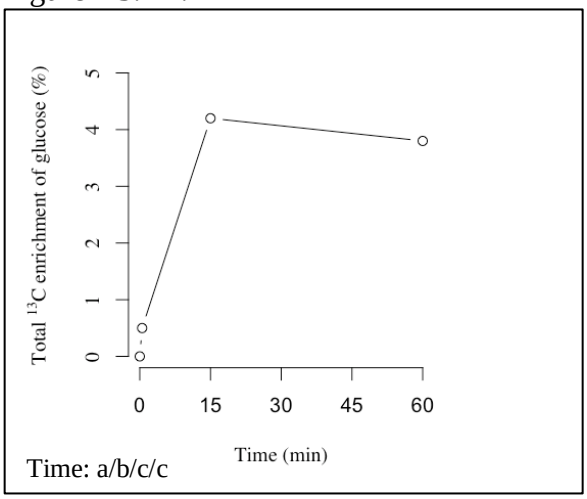
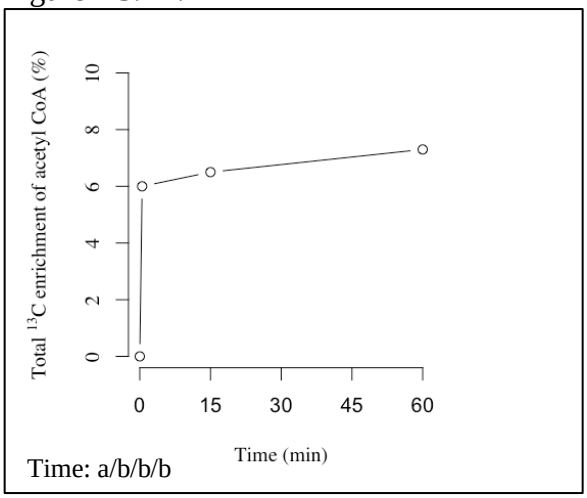


Time: a/b/c/c

Figure A3.1L.

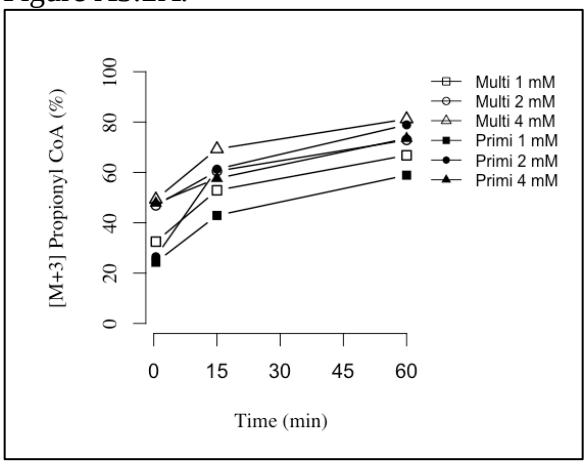


Time: a/b/b/b

Figure A3.2. Effects of different concentrations of [$^{13}C_3$]propionate (1 mM, square; 2 mM, circle; or 4 mM, triangle) on ^{13}C% enrichment of hepatic [M+3] propionyl CoA (A), [M+1] acetyl CoA (B), [M+2] pyruvate (C), [M+3] citrate (D), [M+4] citrate (E), [M+5] citrate (F), [M+2] glutamate[1] (G), [M+3] glutamate[1] (H), [M+2] glucose (I), and [M+3] glucose[2] (J) over 60 min. The enrichment of each isotopologue is relative to the mass distribution of all the isotopologues in the metabolite (molar percent) and is on a scale of 0 to 100%. Time points are 0.5, 15 and 60 min. Graphs are presented as highest level of significant or tendency for significant interaction and associated *p*-values can be found in Table A3.3. All graphs had significant time effects that differed among each time point ($P < 0.01$) except ^{13}C% enrichment of [M+1] acetyl CoA which had no time effect ($P = 0.55$). Treatment by time interactions at each time point were examined for differences among treatments. Letters are presented in the order of 1/2/4 mM treatments and different letters define significant difference from each other ($P \leq 0.05$). Significant results from pairwise tests for the 3-way interactions are described in the text.

[1]Data transformed using log (n + 1) for statistical analysis and back-transformed for interpretation
[2]Data transformed using inverse (n + 1) for statistical analysis and back-transformed for interpretation

Figure A3.2A.



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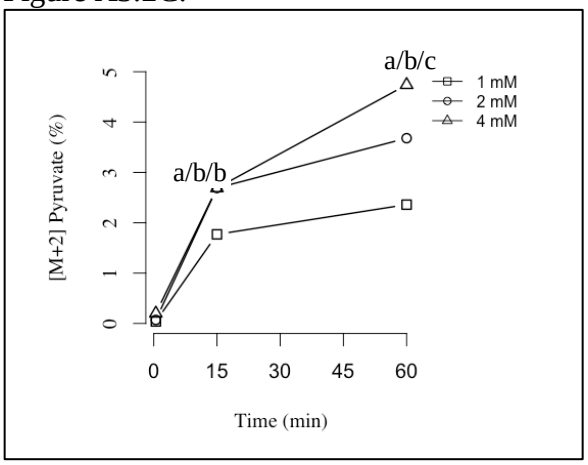

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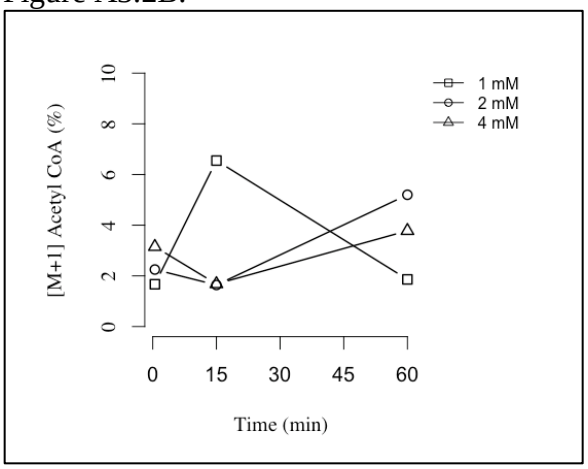

Figure A3.2D.

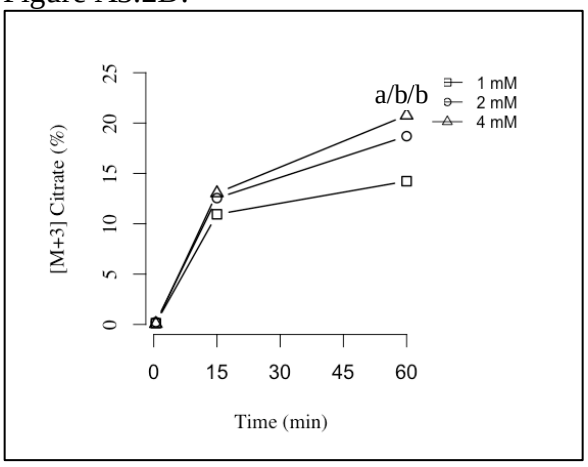

Figure A3.2 (cont'd)

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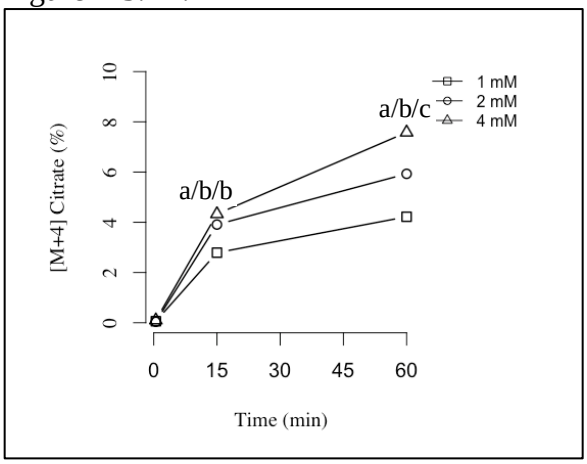

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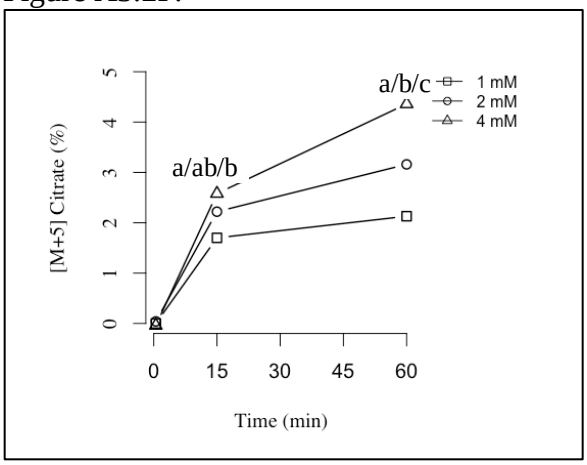

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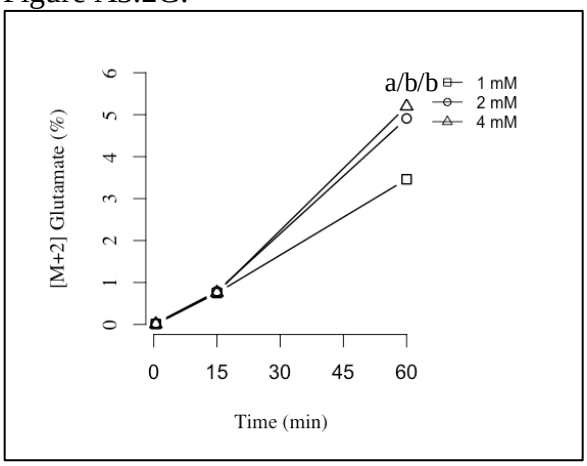

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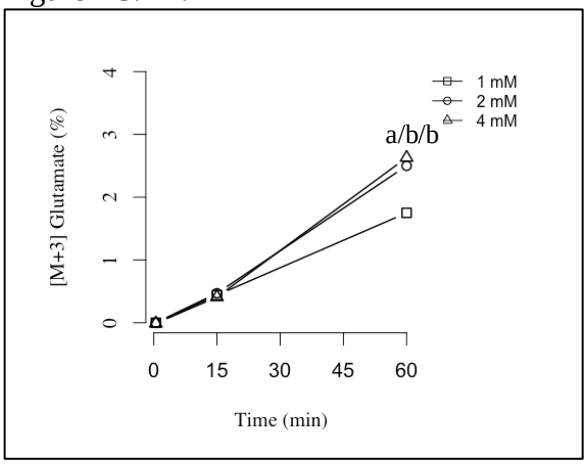

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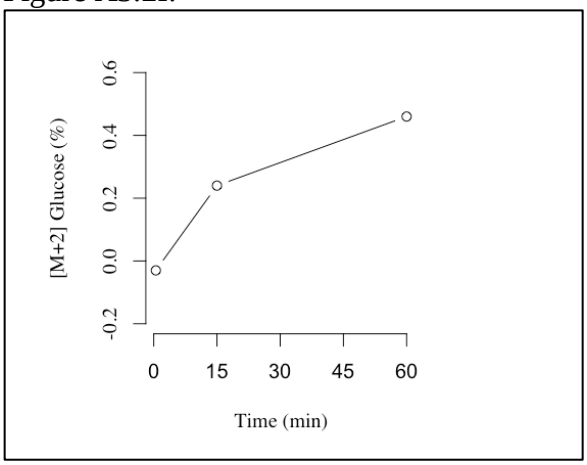

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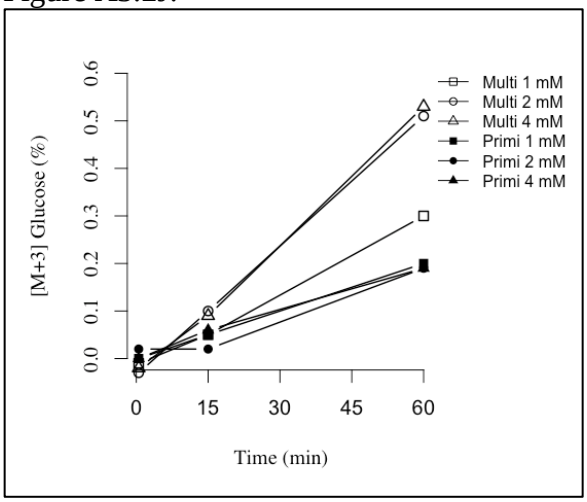

Table A3.1. Difference of total ^{13}C% enrichment above natural abundance of select hepatic metabolites with significant sampling time effect (Table A3.2; P <0.0001) assuming 0% total ^{13}C enrichment (baseline, 0 min) and after incubation of explant for approximately 0.5 min in [$^{13}C_3$]propionate.

Metabolite[1]	0 min	0.5 min	*P*-value[2]
Propionyl CoA	0.00	82.0	<.0001
Methylmalonyl CoA	0.00	27.7	0.01
Succinyl CoA	0.00	13.2	0.36
Succinate[3]	0.00	6.52	<.0001
Fumarate[4]	0.00	1.74	<.0001
Malate	0.00	1.50	0.76
Citrate[3]	0.00	0.91	0.01
Glutamate[4]	0.00	0.01	1.00
Pyruvate[5]	0.00	0.00	1.00
Lactate[3]	0.00	-0.18	0.31
Glucose[6]	0.00	-0.52	<0.01
Acetyl CoA[5]	0.00	6.03	<.0001

[1]Total percent of ^{13}C enrichment above natural abundance
[2]Pairwise comparisons were adjusted with Tukey's method.
[3]Data transformed using square root (n + 1) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.
[4]Data transformed using log (n + 1) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.
[5]Data transformed using square root (n + 2) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.
[6]Data transformed using log (n + 2) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.

Table A3.2. Effects of 3 different concentrations of [$^{13}C_3$]propionate (1, 2 or 4 mM) on total ^{13}C% enrichment of select hepatic metabolites over 60 min.

	Concentration of [$^{13}C_3$]propionate				*P*-value				
Metabolite[1]	1 mM	2 mM	4 mM	SEM	Trt[2]	Time[2,3]	Trt*Time	Trt*Parity	Trt*Time*Parity
Propionyl CoA	83.6[a]	106[b]	118[c]	3.97	<.0001	<.0001	<0.01	0.76	0.08
Methylmalonyl CoA	85.6	79.6	90.8	5.53	0.34	<.0001	0.27	0.91	1.00
Succinyl CoA	46.3[a]	64.3[b]	70.0[b]	5.43	<0.01	<.0001	0.01	0.41	0.04
Succinate[4]	7.08[a]	10.9[b]	13.6[c]	0.12	<.0001	<.0001	<0.01	0.36	0.67
Fumarate[5]	7.07[a]	9.01[b]	10.5[c]	0.03	<.0001	<.0001	<.0001	0.11	0.17
Malate	22.4[a]	33.9[b]	40.8[c]	1.30	<.0001	<.0001	<.0001	0.30	0.35
Citrate[4]	19.5[a]	25.6[b]	28.3[c]	0.08	<.0001	<.0001	<.0001	0.15	0.75
Glutamate[5]	1.61	1.90	1.79	0.03	0.07	<.0001	<0.01	0.49	0.15
Pyruvate[6]	2.18[a]	3.36[b]	4.18[c]	0.06	<.0001	<.0001	<.0001	0.56	0.69
Lactate[4]	0.86[a]	1.56[b]	1.93[c]	0.04	<.0001	<.0001	<.0001	0.09	0.34
Glucose[7]	0.89	0.89	1.00	0.08	0.79	<.0001	0.90	0.22	0.53
Acetyl CoA[6]	4.23	4.48	4.87	0.17	0.77	<.0001	0.86	0.30	0.18

[a-c]Means within a row with different superscripts differ ($P \leq 0.05$)

[1]Total percent of ^{13}C enrichment above natural abundance

[2]SEM = standard error of mean; Trt = treatment, Time = sampling time

[3]Sampling time points are baseline, 0.5, 15 and 60 min

[4]Data transformed using square root (n + 1) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.

[5]Data transformed using log (n + 1) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.

[6]Data transformed using square root (n + 2) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.

[7]Data transformed using log (n + 2) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.

Table A3.3. Effects of 3 different concentrations of [$^{13}C_3$]propionate (1, 2 or 4 mM) on ^{13}C% enrichment of select hepatic metabolite isotopologues over 60 min.

	Concentration of [$^{13}C_3$]propionate				*P*-value				
Metabolite[1]	1 mM	2 mM	4 mM	SEM[2]	Trt[2]	Time[2,3]	Trt*Time	Trt*Parity	Trt*Time*Parity
Propionyl CoA [M+3]	46.4[a]	57.8[b]	63.1[b]	2.01	<.0001	<.0001	0.54	0.73	0.08
Pyruvate [M+2]	1.39[a]	2.15[b]	2.54[c]	0.11	<.0001	<.0001	<.0001	0.06	0.65
Acetyl CoA [M+1]	3.36	3.03	2.87	1.14	0.92	0.55	0.05	0.16	0.57
Acetyl CoA [M+2]	1.65	2.09	2.92	0.58	0.26	0.90	0.12	0.11	0.40
Citrate [M+3]	8.43[a]	10.5[b]	11.3[c]	0.36	<.0001	<.0001	<.0001	0.12	0.43
Citrate [M+4]	2.36[a]	3.30[b]	4.00[c]	0.17	<.0001	<.0001	<.0001	0.31	0.67
Citrate [M+5]	1.28[a]	1.81[b]	2.30[c]	0.09	<.0001	<.0001	<.0001	0.22	0.47
Glutamate [M+2][4]	0.99[a]	1.22[ab]	1.22[b]	0.03	0.03	<.0001	0.02	0.22	0.48
Glutamate [M+3][4]	0.58	0.73	0.72	0.03	0.05	<.0001	0.02	0.29	0.48
Glucose [M+2]	0.19	0.23	0.25	0.03	0.22	<.0001	0.13	0.01	0.35
Glucose [M+3][5]	0.09	0.11	0.12	0.01	0.09	<.0001	0.22	0.06	0.07

[a-c]Means within a row with different superscripts differ ($P \leq 0.05$)

[1]Enrichment of each isotopologue is relative to the mass distribution of all the isotopologues in the metabolite (molar percent) and is on a scale of 0 to 100%

[2]SEM = standard error of mean; Trt = treatment; Time = sampling time

[3]Sampling time points are 0.5, 15 and 60 min.

[4]Data transformed using log (n + 1) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.

[5]Data transformed using inverse (n + 1) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.

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CHAPTER 4: THE EFFECT OF UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION ON THE FEEDING BEHAVIOR OF LACTATING DAIRY COWS

ABSTRACT

Our objective was to determine the effects of uncouplers of oxidative phosphorylation on feeding behavior of lactating dairy cows. Two experiments were conducted to evaluate the effects of 2,4-dinitrophenol methyl ether (DNPME) and sodium salicylate (SAL) on the feeding behavior of lactating cows. Feeding behavior was recorded for the first 4 h after access to feed (approximately 15 min after treatment) for both experiments by a computerized data acquisition system. Meal size was not affected by DNPME or SAL over the first 4 h after access to feed compared with their respective controls. However, DNPME and SAL decreased eating rate over the first 4 h after access to feed compared with their respective controls. Additionally, DNPME increased meal length and tended to decrease the number of meals over the first 4 h after access to feed compared with control.

For a full text of this work see: K.M. Kennedy and M.S. Allen. 2019. The effect of uncouplers of oxidative phosphorylation on the feeding behavior of lactating dairy cows. J. Dairy Sci. 102: (In press). https://doi.org/10.3168/jds.2019-16567

CHAPTER 5: EFFECT OF UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION ON METABOLISM OF PROPIONATE IN LIVER EXPLANTS FROM DAIRY COWS

ABSTRACT

Our objective was to determine the effects of uncouplers of oxidative phosphorylation on the metabolism of propionate in liver tissue of dairy cows in the postpartum (PP) period. A total of 8 primiparous dairy cows (5.88 ± 2.83 (mean ± SD) DIM) were biopsied for liver tissue for explants in 2 block-design experiments. Treatments for experiment 1 were 10 μM of 2,4-dinitrophenol methyl ether (DNPME) or propylene carbonate (vehicle control). Treatments for experiment 2 were 5 mM of sodium salicylate or no treatment (control). Explants were incubated in 2.5 mM of [$^{13}C_3$]propionate with treatments and sampled at 0.5, 15 and 60 min. Additionally, 0.25 μCi of [2-^{14}C]propionate was added to 2.5 mM ^{12}C-propionate vials to measure production of $^{14}CO_2$ from propionate. Treatment with DNPME increased total ^{13}C% enrichment of succinyl CoA (55.7 vs. 38.5%) but had no other effects on measured metabolites compared with control. Treatment with SAL increased total ^{13}C% enrichment of succinate (10.91 vs. 8.84%) and tended to decrease total ^{13}C% enrichment of malate (15.4 vs. 17.7%) compared with control. Additionally, SAL tended to decrease total ^{13}C% enrichment of glucose (1.99 vs. 2.53%) and decreased glucose synthesis from propionate compared with control. Neither DNPME nor SAL increased oxidation compared with their respective controls. Treatment with DNPME appeared to have minimal effects on hepatic propionate metabolism while treatment with SAL may impair glucose synthesis from propionate.

INTRODUCTION

The negative energy balance of dairy cows in the postpartum period is exacerbated by the suppression of appetite (Allen and Piantoni, 2013); increasing the risk for metabolic diseases as well as decreasing milk production and impairing fertility (Herdt, 2000; Butler, 2003). To combat the negative energy balance, energy density of diets is increased by substituting starch-containing cereal grains for forages. Propionic acid produced by the fermentation of starch is a primary glucose precursor for ruminants but has been shown to have hypophagic effects (Gualdrón-Duarte and Allen, 2018; Maldini and Allen, 2018). The hypophagic effects of propionate are likely from stimulating hepatic oxidation according to the hepatic oxidation theory (HOT; Allen et al., 2009).

As a test of the HOT, we previously injected dairy cows with uncouplers of oxidative phosphorylation that we expected to decrease the capacity of the liver to efficiently oxidize fuels (Kennedy and Allen, 2019). Two different uncouplers of oxidation phosphorylation (OXPHOS) were used in that study, 2,4-dinitrophenol methyl ether (DNPME) and sodium salicylate (SAL). Perry et al. (2013) showed that DNPME is preferentially extracted by the liver and converted to 2,4-dinitrophenol (DNP). Both uncouplers are protonophores and decrease the proton-motive force necessary for ATP synthesis by the electron transport chain. Kennedy and Allen (2019) showed that intrajugular injection of 10 mg/kg $BW^{0.75}$ of DNPME increased meal length and decreased eating rate (meal size/meal length) over the first 4 h of meals compared with control. Intrajugular injection of 50 mg/kg BW of SAL decreased the eating rate over the first 4 h of meals compared with control (Kennedy and Allen, 2019). However, short-term effects (≤ 1 h) of these uncouplers on hepatic metabolism in bovines are unknown.

Our objective was to determine the effects of uncouplers of oxidative phosphorylation, DNPME and SAL, on the metabolism of propionate in liver explants from dairy cows in the postpartum period. We hypothesized that uncoupling the electron transport chain would increase anaplerosis by propionate as evidenced by increased ^{13}C enrichment of TCA cycle intermediates and increased oxidation.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at Michigan State University approved all animal procedures for this experiment.

Experimental Design, Treatments and Animal Management

Eight primiparous dairy cows were used in 2 block-design experiments. Animals were housed in tie-stalls at the Michigan State University Dairy Cattle Teaching and Research Center (East Lansing). They were fed a common ration formulated to contain 32% NDF, 23% forage NDF, 22% starch and 18.4% CP that consisted of corn silage, ground corn, soybean meal, alfalfa silage, alfalfa hay, and a premix of minerals and vitamins. Cows were 5.88 ± 2.83 (mean ± SD) DIM at the time of biopsy and had a BCS of 3.01 ± 0.71 and BW of 585 ± 77.1 kg. Two experiments (Exp. 1 and Exp. 2) were conducted simultaneously using the liver tissue biopsied from these cows and used the same techniques for data collection and analysis.

Exp. 1. Treatments of either 20 μL of the DNPME solution (10 μM of DNPME dissolved in propylene carbonate (vehicle) per vial; Alfa Aesar, Haverhill, MA, B21542) or 20 μL of propylene carbonate (vehicle, control; Sigma Aldrich, St. Louis, MO, 310328) were added to

1.98 mL of the media for a total volume of 2 mL per vial. Treatment dose was based on Perry et al. (2013) who incubated liver mitochondria from rats in 0, 3, 6 or 12.5 μM of either DNPME or DNP and measured oxygen consumption rate.

Exp. 2. Treatments were either 20 μL of the sodium salicylate solution (5 mM of sodium salicylate dissolved in media (vehicle) per vial; Sigma Aldrich, St. Louis, MO, S2679) added to 1.98 mL of media for a total volume of 2 mL per vial or no treatment (control; CON) for a total volume of 2 mL per vial. Treatment dose was based on Jeffrey and Smith (1959) who incubated isolated mitochondria from rats in 5 mM sodium salicylate and measured oxygen and inorganic phosphate uptake.

Data Collection and Analysis

Prior to the liver biopsy, animals were moved to a surgery room at the Michigan State Dairy Cattle Research and Teaching Center. Liver was biopsied prior to the morning feeding at approximately 0800 h. Cows were blocked from feed for approximately 30 min prior to biopsy and 1 cow was biopsied per day. Liver tissue (~300-400 mg) for the experiment was biopsied as described in Ch. 3. Tissue was rinsed in ice-cold sterile saline to remove excess blood and then placed in ice-cold supplemented Media 199 (Sigma Aldrich, St. Louis, MO, M7528) for immediate transport to the laboratory. Explants were prepared from biopsied tissue as described by Zhang et al. (2015) and incubated as described in Ch. 3. Explants were incubated in 2 mL of Media 199 supplemented with 1% bovine serum albumin (GenDEPOT, Katy, TX, A0100), 0.6 mM oleic acid (Sigma Aldrich, St. Louis, MO, O3008), 2 mM sodium L-lactate (Sigma Aldrich, St. Louis, MO, L7022), 0.2 mM sodium pyruvate (Sigma Aldrich, St. Louis, MO, P2256), and 0.5 mM L-glutamine (Sigma Aldrich, St. Louis, MO, G3126) and either 2.5 mM of [$^{13}C_3$]sodium

propionate (Sigma Aldrich, 99 atom % ^{13}C, St. Louis, MO, 490636) for ^{13}C metabolite analysis or 2.5 mM of ^{12}C-sodium propionate (Sigma Aldrich, St. Louis, MO, P1880) for $^{14}CO_2$ analysis in 20 mL glass scintillation vials (Research Products International, Mt. Prospect, IL, 121002) for 0.5, 15 or 60 min. Tissue not used for explants was frozen in liquid nitrogen and used as a baseline (0 min) and correction factor for isotope natural abundance.

Treatment Concentrations Analysis. Explants were prepared and analyzed for DNP, salicylate and inorganic phosphate by GC-MS as described in Kennedy and Allen (2019). Samples were derivatized by methoximation and silylation as described by Kennedy and Allen (2019). Non-incubated tissue for each cow was analyzed and used as a baseline (0 min) concentration for treatment and control samples.

^{13}C Metabolite Analysis. Explants were prepared and analyzed for citrate, isocitrate, succinate, fumarate, malate, pyruvate, lactate, glutamate, oxaloacetate (OAA), α-ketoglutarate and glucose by GC-MS and for acetyl CoA, propionyl CoA, succinyl CoA, and methylmalonyl CoA by liquid chromatography – tandem mass spectrometry (LC-MS/MS) as described by in Ch. 3. Area quantification of peaks were integrated using MassLynx Mass Spectrometry Software (version 4.1, Waters Corporation, Milford, MA). Naturally low abundance of isocitrate caused data to be undetectable and/or unreliable with higher isotopologues so the data was not included. Additionally, an unknown mass at [M+2] for OAA and α-ketoglutarate interfered with those isotopologues by masking their abundance, including unlabeled samples, so the data were not included. Total % of ^{13}C enrichment of each metabolite was calculated as modified from Wang and Jones (2014) using the following equation:

$$^{13}\text{C\% enrichment} = \frac{\bar{M}_L - \bar{M}_U}{13.00335 - 12} \times 100$$

where $\bar{M}_L$ is the weighted average isotopologue mass of the labeled metabolite and $\bar{M}_U$ is the weighted average isotopologue mass of the unlabeled metabolite, and the denominator is the difference between ^{13}C and ^{12}C atomic masses. This equation is a simple representation of stable isotope labeling and calculates the total percent increase of ^{13}C over its natural abundance that is present within a metabolite (i.e. it does not distinguish between the distribution of isotopologues within the metabolite). The isotope enrichment, expressed as molar percent, was calculated for the isotopologues [M+3] propionyl CoA, [M+2] pyruvate, [M+1] acetyl CoA, [M+2] acetyl CoA, [M+3] citrate, [M+4] citrate, [M+5] citrate, [M+2] glutamate, [M+3] glutamate, [M+2] glucose, and [M+3] glucose as described in Ch. 3. The enrichment of each isotopologue is relative to the mass distribution of all the isotopologues in the metabolite and is on a scale of 0 to 100%.

$^{14}CO_2$ Analysis. Prior to the addition of the explant, 0.25 μCi of [2-^{14}C]sodium propionate (American Radiolabeled Chemicals, Inc., St. Louis, MO, ARC 0281A) was added to each vial. Upon addition of the explant, vials were flushed with 95% O_2:5% CO_2, capped with rubber stoppers with a hanging center-well containing filter paper, and then shaken at 40 strokes per minute at 38°C in a Precision™ Reciprocal Shaking Bath (Thermo Fisher Scientific, Waltham, MA) until the designated sampling time. At sampling, 0.2 mL of phenethylamine (Sigma Aldrich, St. Louis, MO, 241008) was added to the hanging center-well and 0.5 mL of 1 M perchloric acid was added to the media to stop metabolic reactions. Vials remained closed for an additional hour prior to transferring the filter paper to a clean 20 mL scintillation vial. Twenty mL of Ecolite(+)™ Scintillation Cocktail (MP Biomedicals, Solon, OH, 0188247501) was added to each vial and shaken. Vials were read in a Packard Tri-Carb™ 2100TR Liquid Scintillation Analyzer (PerkinElmer Life Sciences, Boston, MA) set to measure ^{14}C radioactivity. Explant

tissue was weighed (mg) prior to disposal for weight correction. Nanomoles of $^{14}CO_2$ produced from [2-^{14}C]propionate was calculated as follows:

$$\text{nmol/vial} = \frac{\text{Measured } ^{14}\text{C DPM - Blank DPM}}{(\text{specific activity of propionate DPM}/\mu\text{mol of propionate per vial})}.$$

Values are reported as nmol/mg/h of $^{14}CO_2$ produced from [2-^{14}C]propionate.

Statistical Analysis

All data were analyzed with mixed models (PROC MIXED) and repeated measures using SAS software (version 9.4, 2013; SAS Institute Inc., Cary, NC). Prior to statistical analysis, Box-Cox transformation analyses were applied to each response variable to determine necessary transformations on the data using JMP Pro (version 13.2, 2016; SAS Institute Inc., Cary, NC).

Hepatic concentrations of metabolites, total ^{13}C% enrichment of metabolites and ^{13}C% enrichment of specific isotopologues for both experiments were analyzed with the following model:

$$Y_{ijkl} = \mu + C_i(R_j) + R_j + T_k + S_l + T_kS_l + T_kR_j + e_{ijkl},$$

where Y_{ijkl} = the response variable; μ = overall mean; $C_i(R_j)$ = random effect of cow *i* nested within run *j*; R_j = fixed effect of run *j*; T_k = fixed effect of treatment *k*; S_l = fixed effect of sampling time *l*; T_kS_l = interaction of treatment and sampling time; T_kR_j = interaction of treatment and run; and e_{ijkl} = residual. Time was used in the repeated statement to account for repeated measures and the subject was defined as treatment by cow nested within run interaction.

Run was defined as the group of samples analyzed on a mass spectrometer (GC-MS or LC-MS/MS) within a single session. The covariance structure was first-order Toeplitz and denominator degrees of freedom were estimated by using the Kenward-Roger option in the MODEL statement. Paired differences were determined using the diff option in PROC MIXED.

Production of $^{14}CO_2$ from [2-^{14}C]propionate (nmol/mg/h) for both experiments was analyzed with the following model:

$$Y_{ijk}=\mu + C_i + T_j + S_k + T_jS_k + e_{ijk},$$

where Y_{ijkl} = the response variable; μ = overall mean; C_i = random effect of cow *i*; T_j = fixed effect of treatment *j*; S_k = fixed effect of sampling time *k*; T_jS_k = interaction of treatment and sampling time; and e_{ijkl} = residual. Time was used in the repeated statement to account for repeated measures and the subject was defined as treatment by cow. The covariance structure was first-order Toeplitz and denominator degrees of freedom were estimated by using the Kenward-Roger option in the MODEL statement. Paired differences were determined using the diff option in PROC MIXED.

Treatment effects and interactions were declared significant at $P \leq 0.05$ and tendencies were declared at $P \leq 0.10$. Data transformed are noted in the tables and figures along with the transformation used for statistical analysis. All transformed data has been back-transformed for interpretation.

RESULTS

Exp. 1: DNPME

The DNPME treatment increased DNP concentration compared with CON (Table A5.1; 7.55 vs. 6.18 nmol/g; $P = 0.05$) and DNP concentrations increased with time (Figure A5.1A; $P = 0.01$), but no significant treatment by time interaction was detected ($P = 0.68$). The DNPME treatment tended to increase inorganic phosphate concentration compared with CON (9.67 vs. 8.98 μmol/g; $P = 0.08$) but no significant treatment by time interaction was detected ($P = 0.69$). Inorganic phosphate concentrations initially increased when introduced to the media but then decreased over time (Figure A5.1B; $P < 0.01$).

The DNPME treatment did not affect production of $^{14}CO_2$ from [2-^{14}C]propionate compared with control (Table A5.2; 0.08 vs. 0.02 nmol/mg/h; $P = 0.37$) nor were any time effects ($P = 0.18$) or treatment by time effects ($P = 0.44$) detected.

The DNPME treatment increased total ^{13}C% enrichment of succinyl CoA (Table A5.3; $P = 0.01$) but the total ^{13}C% enrichment of all other measured metabolites had no treatment or treatment by time effects. All metabolites increased total ^{13}C% enrichment over time (Figure B5.1; $P < 0.05$) and significant effects of time are reported within each figure. Total ^{13}C% enrichment of glutamate tended to increase at 0.5 min compared with baseline (0.10 vs. 0.00%; pairwise test, $P = 0.08$). Total ^{13}C% enrichment of acetyl CoA tended to increase at 60 min compared with 15 min (9.14 vs. 1.69%; pairwise test, $P = 0.10$).

The DNPME treatment did not affect ^{13}C% enrichment of the selected isotopologues compared with CON (Table A5.4; $P > 0.15$). ^{13}C% enrichment of all selected isotopologues

increased over time (Figure B5.2; $P \leq 0.05$) except for ^{13}C% enrichment of [M+1] acetyl CoA ($P = 0.54$).

Exp. 2: SAL

The SAL treatment increased salicylate concentration compared with CON (Table A5.1; 380 vs. 7.33 nmol/g; $P < 0.0001$) and increased salicylate concentrations over time compared with CON (Figure A5.2A; $P < 0.0001$). The SAL treatment had no treatment or treatment by time effects on inorganic phosphate ($P > 0.10$) but inorganic phosphate concentration increased over time compared with baseline (Figure A5.2B; $P < 0.0001$).

The SAL treatment had no effect on the production of $^{14}CO_2$ from [2-^{14}C]propionate compared with CON (Table A5.2; 0.08 vs. 0.06 nmol/mg/h; $P = 0.57$) nor any significant treatment by time effects ($P = 0.48$). Production of $^{14}CO_2$ from [2-^{14}C]propionate decreased from 15 min to 60 min (0.13 to 0.02 nmol/mg/h; $P = 0.01$).

The SAL treatment increased total ^{13}C% enrichment of succinate (Table A5.3; $P = 0.04$) and tended to increase total ^{13}C% enrichment of succinate over time compared with CON (Figure A5.3B; interaction, $P = 0.09$). The SAL treatment tended to decrease total ^{13}C% enrichment of succinyl CoA, malate and glucose compared with CON ($P < 0.10$) and interacted with time to affect total ^{13}C% enrichment of succinyl CoA, fumarate and malate (Figure A5.3; $P \leq 0.05$). All metabolites increased in total ^{13}C% enrichment over time (Figure B5.3; $P < 0.0001$) except for acetyl CoA ($P = 0.09$), which tended to increase at 60 min compared with 15 min (6.68 vs. 1.56%; pairwise test, $P = 0.07$).

The SAL treatment decreased ^{13}C% enrichment of [M+3] citrate, [M+2] glucose, and [M+3] glucose compared with CON (Table A5.4; $P \leq 0.01$). The SAL treatment tended to

decrease ^{13}C% enrichment of [M+2] glucose compared with CON over time (Figure A5.4A; interaction, $P = 0.08$) and decreased ^{13}C% enrichment of [M+3] glucose compared with CON over time (Figure A5.4B; interaction, $P < 0.01$). In addition, labeling of all isotopologues increased over time (Figure B5.4; $P < 0.05$) except for ^{13}C% enrichment of [M+1] acetyl CoA ($P = 0.54$).

DISCUSSION

Exp. 1: DNPME

Although treatment increased DNP concentration and tended to increase inorganic phosphate concentration, no treatment by time effects were detected. The detected DNP may have resulted from the DNPME being metabolized only on the surface exposed to the media and the treatment did not penetrate into the inner portion of the tissue. In support of this, no increase in oxidation was detected as expected, which is contrary to our hypothesis and results reported by Perry et al. (2013) who reported an increase in oxygen consumption for DNPME comparable with DNP. Other researchers have also observed an increase in the rate of oxidation with DNP (Ehrenfest and Ronzoni, 1933; Hall et al., 1933; Katyare et al., 1971), however, Loomis and Lipmann (1948) argue that concentrations of DNP between 50 and 200 μM prevented phosphorylation without affecting, or slightly stimulating oxidation. However, these concentrations are greater than the 10 μM of DNPME used in our experiment. Additionally, DNPME did not increase inorganic phosphate compared with CON as expected. An increase inorganic phosphate was expected because DNP is known to liberate phosphate (Lardy and Wellman, 1953; Katyare et al., 1971).

The concentration of DNP for control samples is not at 0 nmol/g because GC-MS scanned for fragment masses using select ion monitoring, which measures fragment masses indiscriminately. Although the heaviest/most reliable peak fragment of DNP was selected for monitoring (241.1 m/z), the liver is a complex matrix and this fragment mass is not very heavy compared to other compounds. Consequently, other compounds not related to DNP are likely to have a similar fragment mass. Therefore, DNP concentration of control was never expected to be 0 nmol/g as some measurement of the selected mass was expected from sources other than DNP.

An increase in ^{13}C% enrichment of all measured metabolites and isotopologues over time, except for [M+1] acetyl CoA, confirms that propionate was extracted and metabolized by the liver explants. However, DNPME had little effect on metabolism of propionate in this experiment as only an increase in total ^{13}C% enrichment of succinyl CoA was observed. Kennedy and Allen (2019) reported no significant differences among the TCA cycle intermediates in liver samples biopsied 4 h after lactating dairy cows were given an intrajugular injection of 10 mg/kg $BW^{0.75}$ of DNPME compared with control, which is in agreement with our results except for the noted increase in enrichment of succinyl CoA. However, the current results show the enrichment of succinyl CoA synthesized from [$^{13}C_3$]propionate and not the total concentration of succinyl CoA, and thus, the difference in results for the two studies might be from the synthesis of succinyl CoA from sources other than propionate. Furthermore, the lack of treatment effect on ^{13}C% enrichment of acetyl CoA suggests that DNPME does not divert propionate towards acetyl CoA production to a greater extent than CON.

Exp. 2: SAL

The increase in salicylate concentration over time by SAL compared with CON shows that salicylate was extracted by the tissue. However, contrary to our hypothesis, no difference in CO_2 production was observed between treatments. This result is in contrast to Brody and Fouts (1956) who measured a decrease in inorganic phosphate uptake and increase in oxygen uptake, resulting in a decreased P/O ratio in rats. The SAL treatment had no effect on inorganic phosphate compared with CON, which is also in disagreement with Brody and Fouts (1956) who reported that salicylate should liberate inorganic phosphate like DNP. Others have also reported a decrease in the P/O ratio with a concentration of 5 mM sodium salicylate in rat liver mitochondria *in vitro* (Jeffrey and Smith, 1959; Charnock et al., 1962).

A significant increase in total ^{13}C% enrichment of measured metabolites, except for acetyl CoA (tendency), and select isotopologues over time, except for [M+1] acetyl CoA (no effect), confirms that propionate was extracted and metabolized by the liver explants. Kennedy and Allen (2019) reported an increase in acetyl CoA concentration with a 50 mg/kg BW intrajugular injection of sodium salicylate compared with a control. However, the cows in Kennedy and Allen (2019) were in a lipolytic state so the acetyl CoA pool is likely primarily from β-oxidation of free fatty acids. This is in agreement with our results in which total ^{13}C% enrichment of acetyl CoA did not differ between SAL and CON; suggesting that propionate is not diverted towards acetyl CoA production with SAL, similar to the DNPME treatment in Exp. 1.

The SAL treatment decreased total ^{13}C% enrichment of succinyl CoA compared with CON at 60 min, in contrast to SAL tending to increase succinyl CoA concentration compared with control *in vivo* (Kennedy and Allen, 2019), which might have been the result of succinyl

CoA synthesis from sources other than propionate. The observed decrease in total ^{13}C% enrichment of succinyl CoA suggests that synthesis of succinyl CoA from propionate might be impaired. However, the increase in total ^{13}C% enrichment of succinate at 60 min with SAL compared with CON and decrease in succinyl CoA with SAL compared with CON at 60 min suggests that succinyl CoA was converting to succinate. An increase in total ^{13}C% enrichment of succinate and decrease in total ^{13}C% enrichment of fumarate with SAL supports that a time delay is occurring within the propionate metabolism pathway. Because succinate and fumarate are the reactant and product, respectively, of the succinate dehydrogenase reaction, salicylate is likely negatively impacting this enzyme, as has been previously reported (Kaplan et. al, 1954; Miyahara and Karler, 1965). The decrease in total ^{13}C% enrichment of malate at 15 min is likely a direct result of the decrease in total ^{13}C% enrichment of fumarate at 15 min, and consequently, the tendency for a decrease in total ^{13}C% enrichment of malate may also have caused the decrease in the ^{13}C% enrichment of [M+3] citrate detected with SAL compared with CON. Although no difference in succinate concentration was noted with salicylate treatment by Kennedy and Allen (2019), salicylate has a short half-life (50 min in cattle; Short et al., 1991) and sampling occurred 4 h post-treatment whereas this study focused on metabolism within an hour.

A tendency for decrease in total ^{13}C% enrichment of glucose and a decrease in [M+2] glucose and [M+3] glucose enrichment with SAL suggests that synthesis of glucose from propionate is impaired by salicylate. This is consistent with results reported by Farney et al. (2013) who reported a decrease in plasma glucose concentration at 7 d postpartum with oral administration of salicylate in drinking water to dairy cows for the first 7 DIM. Bertoni et al. (2004) also reported a decrease in plasma glucose concentration over the first week of lactation

when a daily intramuscular injection of lysine acetylsalicylate was administered to dairy cows in the first 5 DIM. However, a single pulse dose of salicylate had no effect on plasma glucose concentration (Kennedy and Allen, 2019) possibly because changes in gluconeogenesis were too minute to detect in the general circulation. Recently, Montgomery et al. (2019) reported glucose turnover rate tended to decrease with administration of salicylate in drinking water to dairy cows for the first 7 DIM but no change in transcription abundance of pyruvate carboxylase or glucose-6-phosphatase in the liver was detected. Our results suggest that SAL does not interfere with absorption of propionate or conversion of propionate to pyruvate, despite a tendency for a decrease in total ^{13}C% enrichment of malate, as neither total ^{13}C% enrichment of propionyl CoA nor pyruvate differed with SAL compared with CON.

Overall

Unexpectedly, a decrease in CO_2 production from 15 min to 60 min was observed in Exp. 2 across treatments and a numerical decrease was observed in Exp. 1. Incubations with [$^{13}C_3$]propionate detected an increase in enrichment in TCA cycle intermediates over time; suggesting the tissue was not dying nor nutrient supply diminishing over the sampling time. Overall, measurement of $^{14}CO_2$ remained near background across treatments and experiments; as such, it is more likely that the techniques used for measuring $^{14}CO_2$ with our experimental design were not sensitive enough to detect differences.

In summary, neither DNPME nor SAL increased oxidation compared with their respective controls, contrary to our hypothesis. The DNPME treatment did not alter metabolism of propionate compared with CON except for an increase in total ^{13}C% enrichment of succinyl CoA. The SAL treatment increased total ^{13}C% enrichment of succinate and tended to decrease

total ^{13}C% enrichment of malate and glucose compared with CON. Additionally, SAL decreased glucose synthesis from propionate compared with CON. However, we did not observe an increase in total ^{13}C% enrichment of TCA cycle intermediates as hypothesized for either experiment. Future work should examine the effects of uncouplers of OXPHOS with focus on metabolites associated with propionate metabolism and other fuels used by the liver of dairy cows both in the short-term and long-term.

CONCLUSIONS

The SAL treatment decreased synthesis of glucose from propionate *in vitro*, however, the mechanism responsible for the decreased production of glucose is unknown. Conversely, DNPME had no measurable effects on propionate metabolism, possibly because of inadequate absorption and metabolism of DNPME by the explant.

ACKNOWLEDGEMENTS

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APPENDICES

APPENDIX A

Figure A5.1. Hepatic concentration of 2,4-dinitrophenol[1] (DNP; A) and inorganic phosphate (phosphate; B) from liver explants from dairy cows in the postpartum period for Experiment 1. Explants were incubated in [$^{13}C_3$]propionate with treatments of either 10 μM of 2,4-dinitrophenol methyl ether (DNPME) or propylene carbonate (control; CON) and sampled at 0 (baseline), 0.5, 15 and 60 min. Graphs are presented as highest level of significant or tendency for significant interaction and associated *p*-values can be found in Table A5.1. Time effects are presented at the bottom of each graph with a letter in order of 0/0.5/15/60 min and different letters define significant difference from each other ($P \leq 0.05$).

[1]Data transformed using inverse square root for statistical analysis and back-transformed for interpretation

Figure A5.1A.

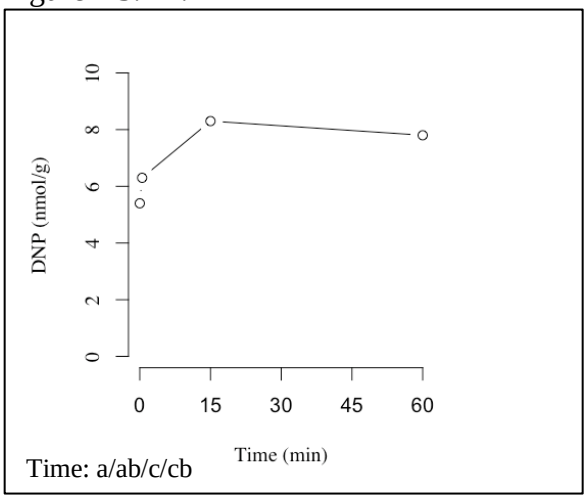


Figure A5.1B.

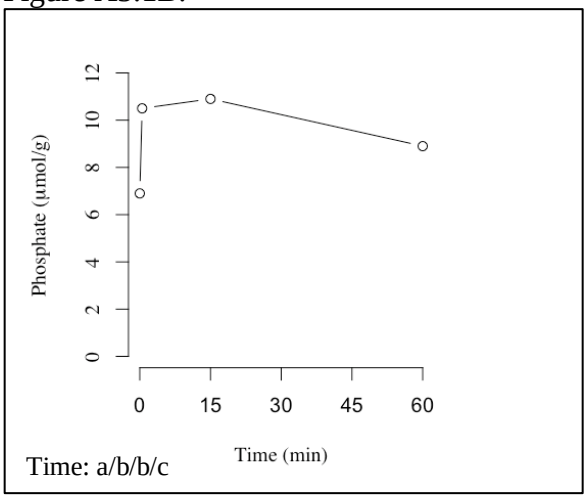


Figure A5.2. Hepatic concentration of salicylate (C) and inorganic phosphate (phosphate; D) from liver explants from dairy cows in the postpartum period for Experiment 2. Explants were incubated in [$^{13}C_3$]propionate with treatments of either 5 mM of sodium salicylate (SAL) or no treatment (control; CON) and sampled at 0 (baseline), 0.5, 15 and 60 min. Graphs are presented as highest level of significant or tendency for significant interaction and associated *p*-values can be found in Table A5.1. Time effects are presented at the bottom of each graph with a letter in order of 0/0.5/15/60 min and different letters define significant difference from each other ($P \leq 0.05$). Treatment by time interactions significantly different from each other at each time point are denoted by * ($P \leq 0.05$). Data were transformed using log for statistical analysis and back-transformed for interpretation.

Figure A5.2A.

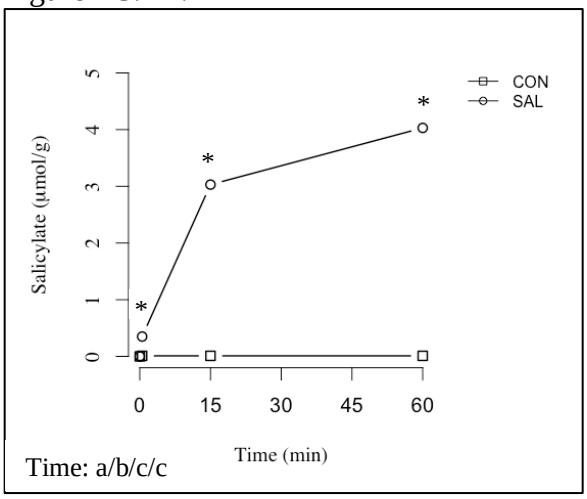


Figure A5.2B.

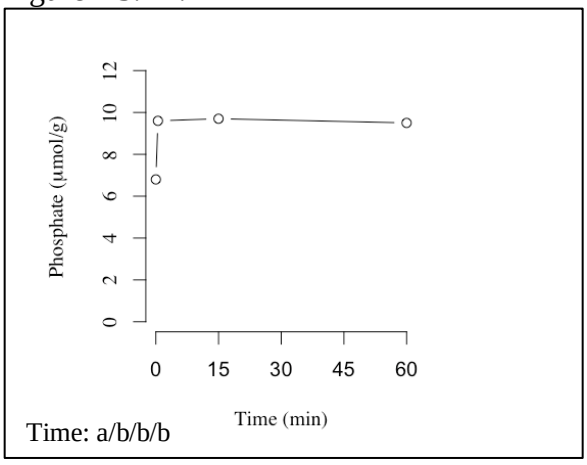


Figure A5.3. Total ^{13}C% enrichment of metabolites of hepatic succinyl CoA (A), succinate[1] (B), fumarate[1] (C), and malate[2] (D) from liver explants from dairy cows in the postpartum period for Experiment 2. Total ^{13}C% enrichment is the percent increase of ^{13}C within each metabolite above the natural abundance. Explants were incubated in [$^{13}C_3$]propionate with treatments of either 5 mM of sodium salicylate (SAL) or no treatment (control; CON)and sampled at baseline (no enrichment, 0), 0.5, 15 and 60 min. Graphs are presented as highest level of significant or tendency for significant interaction and associated *p*-values can be found in Table A5.3. Time effects are presented at the bottom of each graph with a letter in order of 0.5/15/60 min and different letters define significant difference from each other ($P \leq 0.05$). Treatment by time interactions significantly different from each other at each time point are denoted by * ($P \leq 0.05$).

[1]Data transformed using square root (n+1) for statistical analysis and back-transformed for interpretation
[2]Data transformed using square root (n+3) for statistical analysis and back-transformed for interpretation

Figure A5.3A.

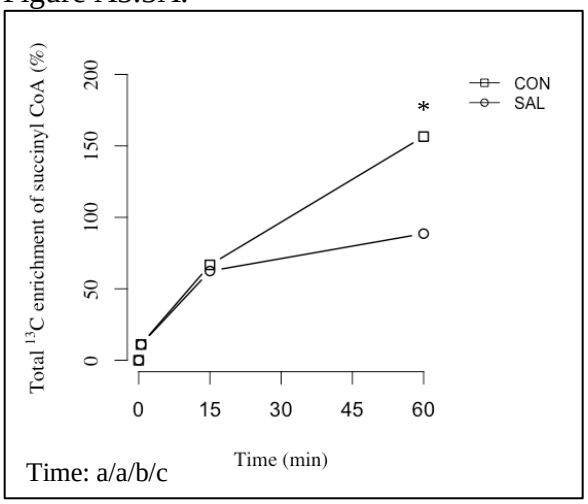


Figure A5.3C.

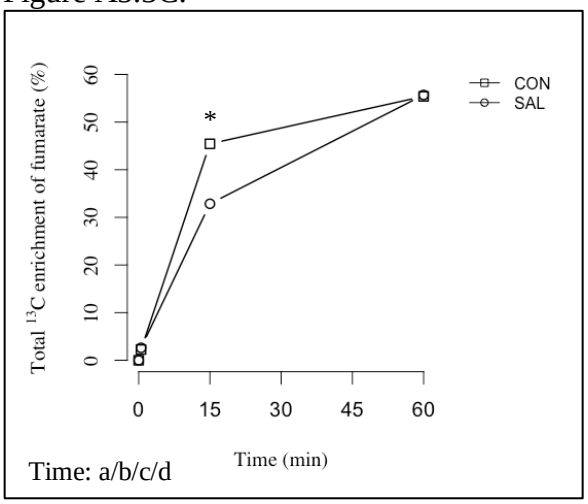


Figure A5.3B.

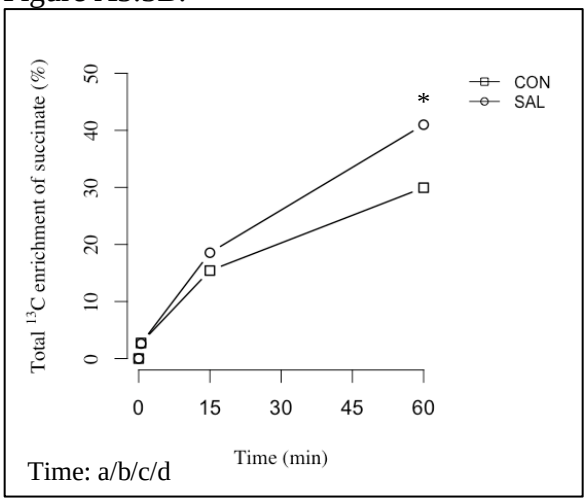


Figure A5.3D.

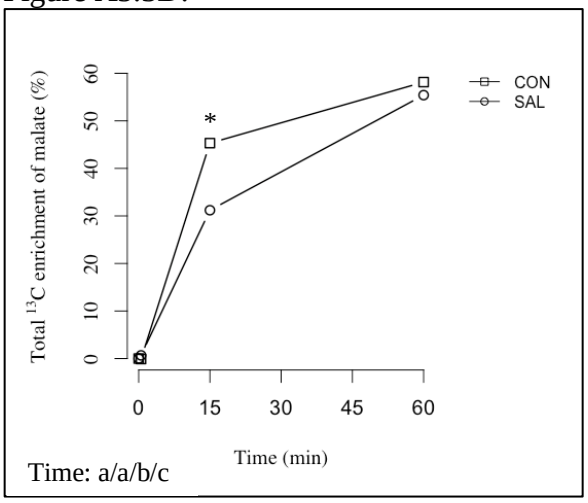


Figure A5.4. ^{13}C% enrichment of hepatic [M+2] glucose (A) and [M+3] glucose[1] (B) from liver explants from dairy cows in the postpartum period for Experiment 2. Explants were incubated in [$^{13}C_3$]propionate with treatments of either 5 mM of sodium salicylate (SAL) or no treatment (control; CON) and sampled at 0.5, 15 and 60 min. The enrichment of each isotopologue is relative to the mass distribution of all the isotopologues in the metabolite (molar percent) and is on a scale of 0 to 100%. Graphs are presented as highest level of significant or tendency for significant interaction and associated *p*-values can be found in Table A5.4. Time effects are presented at the bottom of each graph with a letter in order of 0.5/15/60 min and different letters define significant difference from each other ($P \leq 0.05$). Treatment by time interactions significantly different from each other at each time point are denoted by * ($P \leq 0.05$) and tendencies are denoted by † ($P \leq 0.10$).

[1]Data transformed using inverse (n+1) for statistical analysis and back-transformed for interpretation

Figure A5.4A.

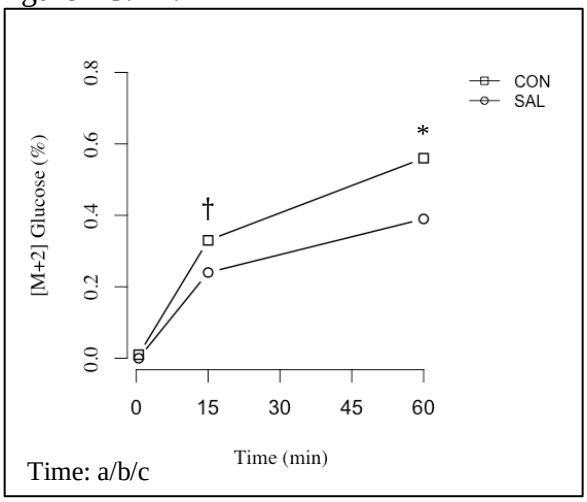


Figure A5.4B.

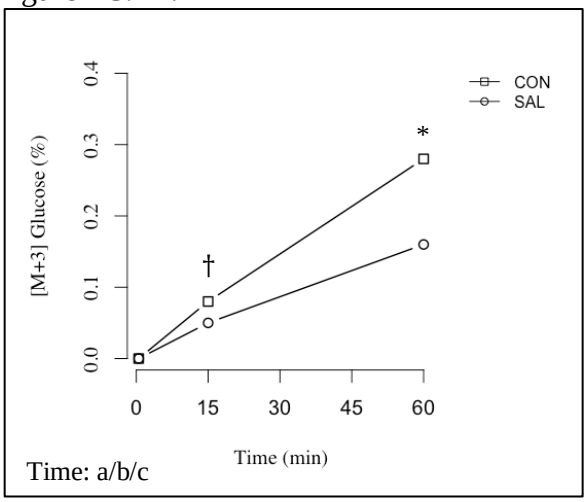


Table A5.1. Liver concentrations of metabolites from liver explants from dairy cows in the postpartum period.

Metabolite[1]				*P*-value		
Exp. 1[3]	CONTROL	DNPME	SEM[2]	Treatment	Time	Treatment*Time
DNP (nmol/g)[2,5]	6.18	7.55	0.02	0.05	0.01	0.68
Phosphate (μmol/g)[2]	8.98	9.67	0.49	0.08	<.0001	0.69
Exp. 2[4]	CONTROL	SAL	SEM	Treatment	Time	Treatment*Time
Salicylate (nmol/g)[6]	7.33	380	0.14	<.0001	<.0001	<.0001
Phosphate (μmol/g)[6]	8.85	8.76	0.05	0.80	<.0001	0.21

[1]nmol/g

[2]SEM = standard error of the mean; DNP = 2,4-dinitrophenol; Phosphate = inorganic phosphate

[3]Explants were incubated for up to 60 min in 2.5 mM of [$^{13}C_3$]propionate with treatments of either 10 μM of 2,4-dinitrophenol methyl ether (DNPME) or propylene carbonate (control)

[4]Explants were incubated for up to 60 min in [$^{13}C_3$]propionate with treatments of either 5 mM of sodium salicylate (SAL) or no treatment (control).

[5]Data transformed using inverse square root for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.

[6]Data transformed using log for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.

Table A5.2. Production of $^{14}CO_2$ from [2-^{14}C]sodium propionate from liver explants from dairy cows in the postpartum period.

	CONTROL		TREATMENT			*P*-value		
$^{14}CO_2$ produced from propionate (nmol/mg/h)	15 min	60 min	15 min	60 min	SEM[1]	Treatment	Time	Treatment*Time
Exp. 1[2,3]	0.04	0.00	0.15	0.01	0.04	0.37	0.18	0.44
Exp. 2[4,5]	0.16	0.01	0.10	0.02	0.03	0.42	<.0001	0.84

[1]SEM = standard error of the mean

[2]Explants were incubated for up to 60 min in 2.5 mM of ^{12}C-propionate and 0.25 μCi of [2-^{14}C]propionate with treatments of either 10 μM of 2,4-dinitrophenol methyl ether (DNPME) or propylene carbonate (control)

[3]Data transformed using log (n+1) for statistical analysis and back-transformed for interpretation purposes. SEM reported in transformed scale.

[4]Explants were incubated for up to 60 min in 2.5 mM of ^{12}C-propionate and 0.25 μCi of [2-^{14}C]propionate with treatments of either 5 mM of sodium salicylate (SAL) or no treatment (control).

[5]Data transformed using inverse (n+1) for statistical analysis and back-transformed for interpretation purposes. SEM reported in transformed scale.

Table A5.3. Total ^{13}C% enrichment of metabolites from liver explants from dairy cows in the postpartum period.

Metabolite[1]				*P*-value		
Exp. 1[3]	CONTROL	DNPME	SEM[2]	Treatment	Time	Treatment*Time
Propionyl CoA	89.7	93.8	5.25	0.42	<.0001	0.90
Methylmalonyl CoA	76.0	79.2	6.57	0.67	<.0001	0.28
Succinyl CoA	38.5	55.7	5.18	0.01	<.0001	0.37
Succinate[5]	11.1	10.7	0.08	0.62	<.0001	0.45
Fumarate	37.7	37.5	1.35	0.86	<.0001	0.89
Malate	36.4	35.9	1.32	0.70	<.0001	0.80
Citrate	25.2	24.1	1.43	0.47	<.0001	0.74
Glutamate[7]	0.60	0.56	0.01	0.40	<.0001	0.95
Pyruvate[6]	2.88	2.95	0.13	0.84	<.0001	0.97
Lactate[5]	1.84	1.85	0.05	0.93	<.0001	0.99
Glucose	1.85	2.05	0.26	0.53	<.0001	0.46
Acetyl CoA	2.64	4.13	1.59	0.51	0.03	0.95
Exp. 2[4]	CONTROL	SAL	SEM	Treatment	Time	Treatment*Time
Propionyl CoA	88.7	92.8	5.91	0.42	<.0001	0.84
Methylmalonyl CoA	92.0	98.2	5.50	0.41	<.0001	0.96
Succinyl CoA	58.6	40.5	7.76	0.08	<.0001	0.05
Succinate[5]	8.84	10.91	0.11	0.04	<.0001	0.09
Fumarate[5]	17.3	15.5	0.10	0.12	<.0001	0.02
Malate[6]	17.7	15.4	0.11	0.06	<.0001	0.01
Citrate[8]	8.41	7.26	0.06	0.11	<.0001	0.62
Glutamate[8]	1.46	1.37	0.03	0.35	<.0001	0.21
Pyruvate[6]	4.05	3.90	0.13	0.74	<.0001	0.96
Lactate[5]	1.41	1.53	0.04	0.46	<.0001	0.80
Glucose	2.53	1.99	0.37	0.10	<.0001	0.47
Acetyl CoA	2.95	3.22	1.76	0.89	0.09	0.69

[1]Total ^{13}C enrichment of metabolite above the natural abundance (%)

[2]SEM = standard error of the mean

[3]Explants were incubated for up to 60 min in 2.5 mM of [$^{13}C_3$]propionate with treatments of either 10 μM of 2,4-dinitrophenol methyl ether (DNPME) or propylene carbonate (control).

[4]Explants were incubated for up to 60 min in [$^{13}C_3$]propionate with treatments of either 5 mM of sodium salicylate (SAL) or no treatment (control)
[5]Data transformed using square root (n+1) for statistical analysis and back-transformed for interpretation purposes. SEM reported in transformed scale.
[6]Data transformed using square root (n+3) for statistical analysis and back-transformed for interpretation purposes. SEM reported in transformed scale.
[7]Data transformed using inverse square root (n+1) for statistical analysis and back-transformed for interpretation purposes. SEM reported in transformed scale.
[8]Data transformed using log (n+1) for statistical analysis and back-transformed for interpretation purposes. SEM reported in transformed scale.

Table A5.4. ^{13}C% enrichment of select metabolite isotopologues from liver explants from dairy cows in the postpartum period.

Metabolite[1]				*P*-value		
Exp. 1[3]	CONTROL	DNPME	SEM[2]	Treatment	Time	Treatment*Time
Propionyl CoA [M+3][5]	55.8	57.4	205	0.46	<.0001	0.90
Pyruvate [M+2]	2.12	2.27	0.21	0.37	<.0001	0.46
Acetyl CoA [M+1]	0.57	3.18	1.43	0.21	0.54	0.27
Acetyl CoA [M+2]	2.01	1.28	1.58	0.75	0.05	0.68
Citrate [M+3]	4.87	4.79	0.31	0.78	<.0001	0.43
Citrate [M+4]	2.68	2.44	0.15	0.18	<.0001	0.32
Citrate [M+5][6]	1.23	1.32	0.04	0.58	<.0001	0.55
Glutamate [M+2][7]	0.33	0.31	0.03	0.54	<.0001	0.94
Glutamate [M+3][8]	0.15	0.14	0.03	0.27	<.0001	0.84
Glucose [M+2]	0.24	0.26	0.02	0.39	<.0001	0.23
Glucose [M+3][7]	0.06	0.06	0.00	0.49	<.0001	0.37
Exp. 2[4]	CONTROL	SAL	SEM	Treatment	Time	Treatment*Time
Propionyl CoA [M+3]	51.1	53.7	2.43	0.26	<.0001	0.47
Pyruvate [M+2]	2.47	2.28	0.18	0.36	<.0001	0.67
Acetyl CoA [M+1]	2.05	1.37	1.75	0.72	0.54	0.77
Acetyl CoA [M+2]	1.08	1.99	1.20	0.45	0.04	0.66
Citrate [M+3]	9.65	8.12	0.49	0.01	<.0001	0.15
Citrate [M+4]	3.36	3.03	0.29	0.18	<.0001	0.68
Citrate [M+5]	2.29	2.31	0.18	0.92	<.0001	0.68
Glutamate [M+2][9]	0.82	0.76	0.03	0.41	<.0001	0.18
Glutamate [M+3][7]	0.39	0.38	0.01	0.58	<.0001	0.25
Glucose [M+2]	0.30	0.21	0.03	<0.01	<.0001	0.08
Glucose [M+3][7]	0.11	0.07	0.01	<0.01	<.0001	<0.01

[1]Enrichment of each isotopologue is relative to the mass distribution of all the isotopologues in the metabolite (molar percent) and is on a scale of 0 to 100%

[2]SEM = standard error of the mean

[3]Explants were incubated for up to 60 min in 2.5 mM of [$^{13}C_3$]propionate with treatments of either 10 μM of 2,4-dinitrophenol methyl ether (DNPME) or propylene carbonate (control).

[4]Explants were incubated for up to 60 min in [$^{13}C_3$]propionate with treatments of either 5 mM of sodium salicylate (SAL) or no treatment (control)

[5]Data transformed using square (n+1) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.
[6]Data transformed using square root (n+1) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.
[7]Data transformed using inverse (n+1) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.
[8]Data transformed using inverse sqrt (n+1) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.
[9]Data transformed using log (n+1) for statistical analysis and back-transformed for interpretation. SEM reported in transformed scale.

APPENDIX B

Figure B5.1. Total ^{13}C% enrichment of metabolites of hepatic propionyl CoA (A), methylmalonyl CoA (M-CoA; B), succinyl CoA (C), succinate[1] (D), fumarate (E), malate (F), citrate (G), glutamate[2] (H), pyruvate[3] (I), lactate[1] (J), glucose (K), and acetyl CoA (L) from liver explants from dairy cows in the postpartum period for Experiment 1. Total ^{13}C% enrichment is the percent increase of ^{13}C within each metabolite above the natural abundance. Explants were incubated in [$^{13}C_3$]propionate with treatments of either 10 μM of 2,4-dinitrophenol methyl ether (DNPME) or propylene carbonate (control; CON) and sampled at baseline (no enrichment, 0), 0.5, 15 and 60 min. Time effects are presented at the bottom of each graph with a letter in order of baseline/0.5/15/60 min and different letters define significant difference from each other ($P \leq 0.05$).

[1]Data transformed using square root (n+1) for statistical analysis and back-transformed for interpretation
[2]Data transformed using inverse square root (n+1) for statistical analysis and back-transformed for interpretation
[3]Data transformed using square root (n+3) for statistical analysis and back-transformed for interpretation

Figure B5.1A.

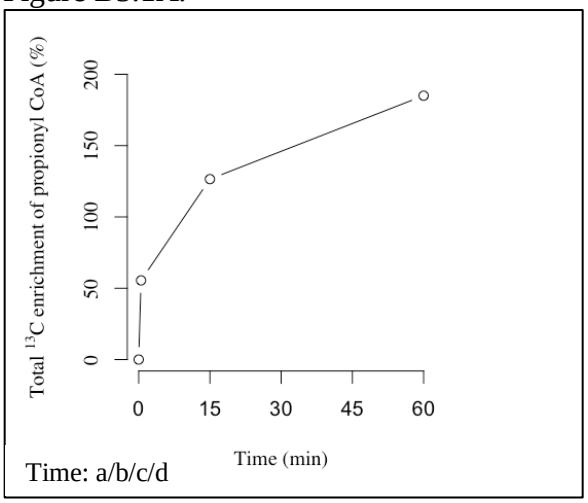


Figure B5.1C.

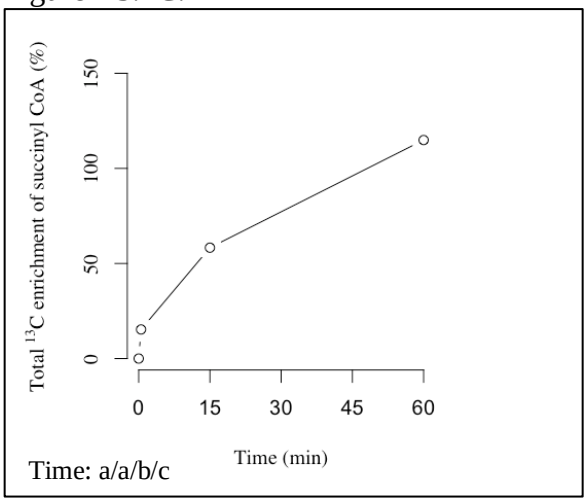


Figure B5.1B.

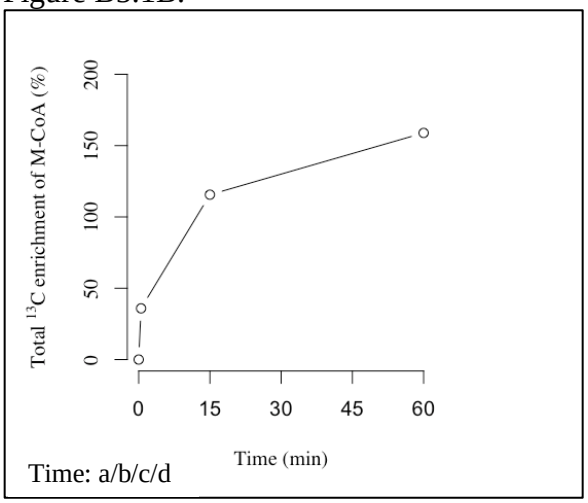


Figure B5.1D.

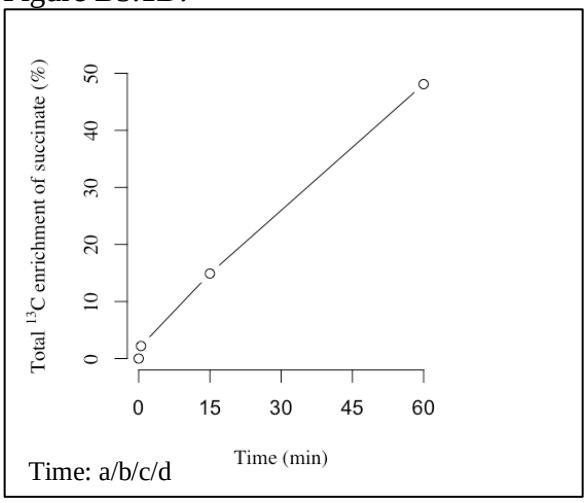


Figure B5.1 (cont'd)

Figure B5.1E.

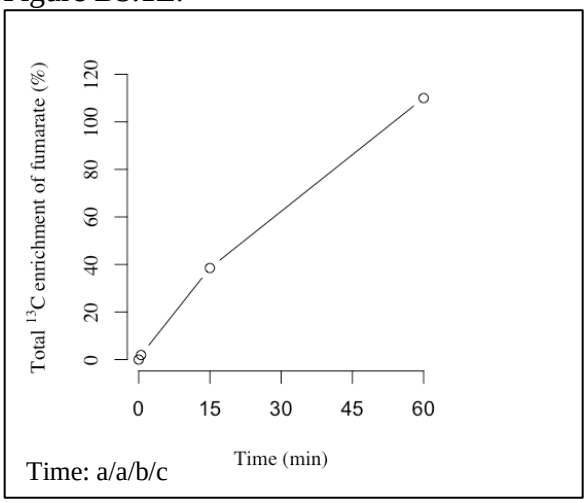


Figure B5.1F.

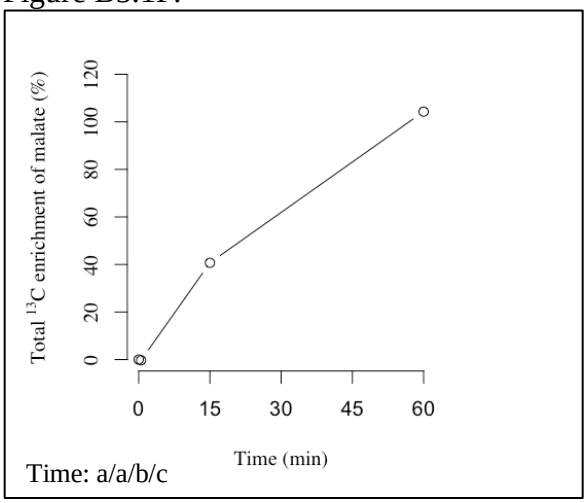


Figure B5.1G.

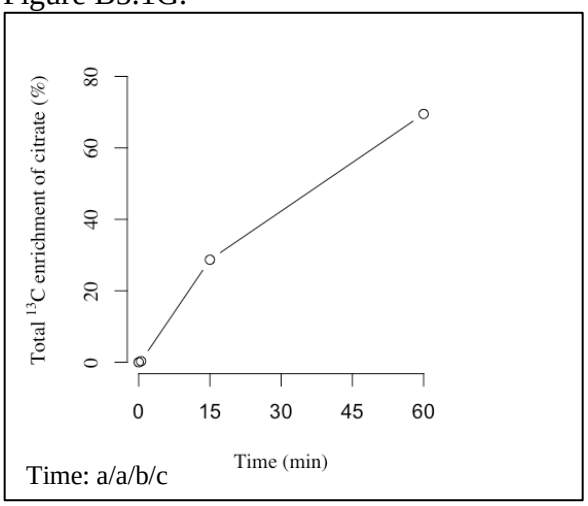
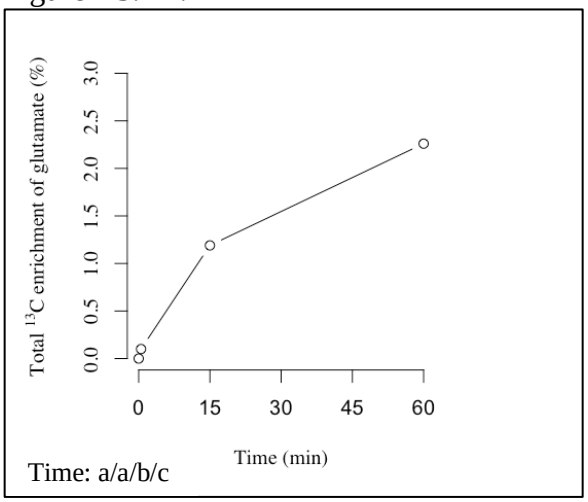


Figure B5.1H.



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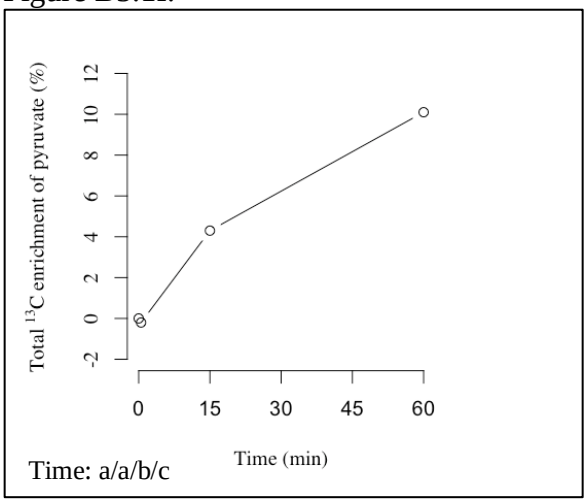


Figure B5.1J.

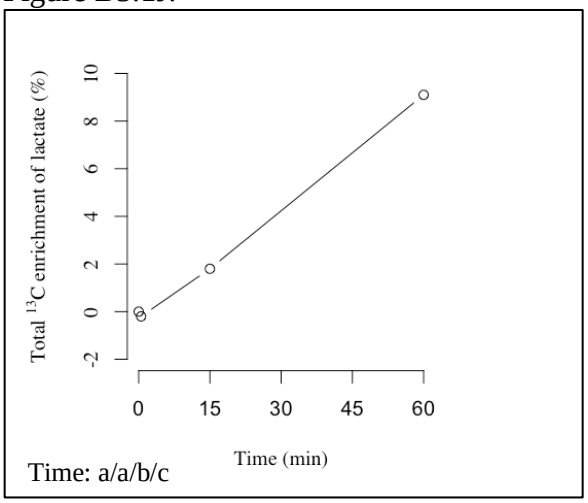
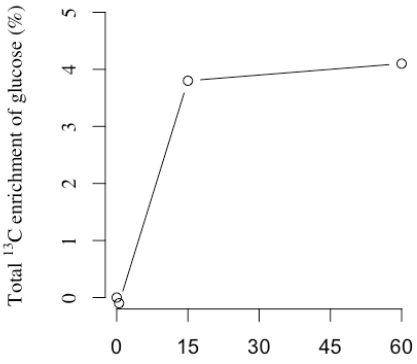


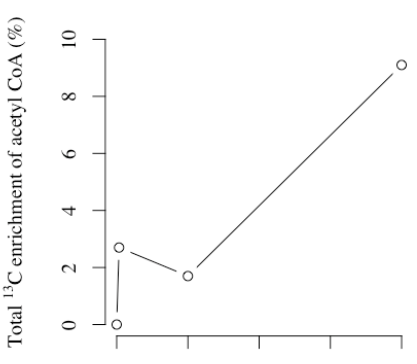
Figure B5.1 (cont'd)

Figure B5.1K.



Time: a/a/b/b

Figure B5.1L.



Time: a/ab/ab/b

Figure B5.2. ^{13}C% enrichment of hepatic [M+3] propionyl CoA1 (A), [M+2] pyruvate (B), [M+2] acetyl CoA (C), [M+3] citrate (D), [M+4] citrate (E), [M+5] citrate[2] (F), [M+2] glutamate[3] (G), [M+3] glutamate[4] (H), [M+2] glucose (I), and [M+3] glucose[3] (J) from liver explants from dairy cows in the postpartum period for Experiment 1. The enrichment of each isotopologue is relative to the mass distribution of all the isotopologues in the metabolite (molar percent) and is on a scale of 0 to 100%. Explants were incubated in [$^{13}C_3$]propionate with treatments of either 10 μM of 2,4-dinitrophenol methyl ether (DNPME) or propylene carbonate (control; CON) and sampled at 0.5, 15 and 60 min. Time effects are presented at the bottom of each graph with a letter in order of 0.5/15/60 min and different letters define significant difference from each other ($P \leq 0.05$).

[1]Data transformed using square (n+1) for statistical analysis and back-transformed for interpretation
[2]Data transformed using square root (n+1) for statistical analysis and back-transformed for interpretation
[3]Data transformed using inverse (n+1) for statistical analysis and back-transformed for interpretation
[4]Data transformed using inverse square root (n+1) for statistical analysis and back-transformed for interpretation

Figure B5.2A.

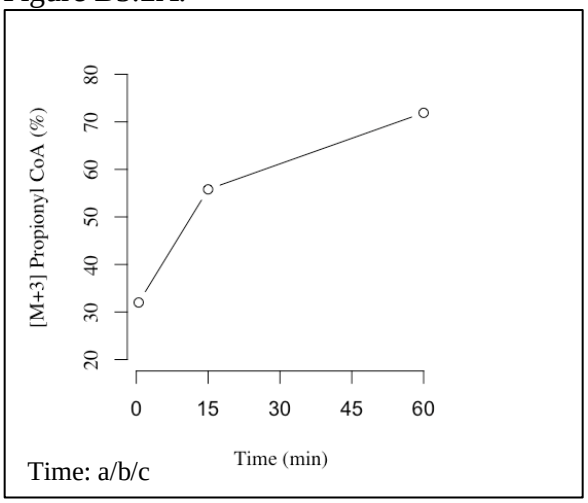


Figure B5.2C.

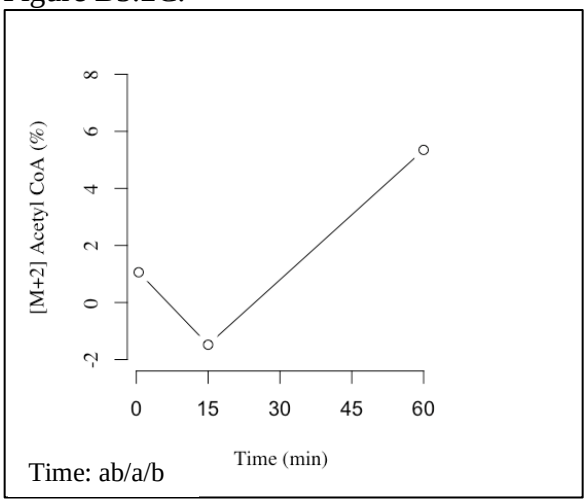


Figure B5.2B.

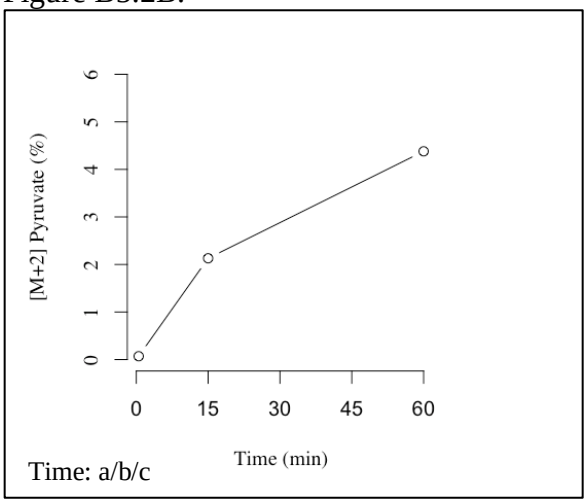


Figure B5.2D.

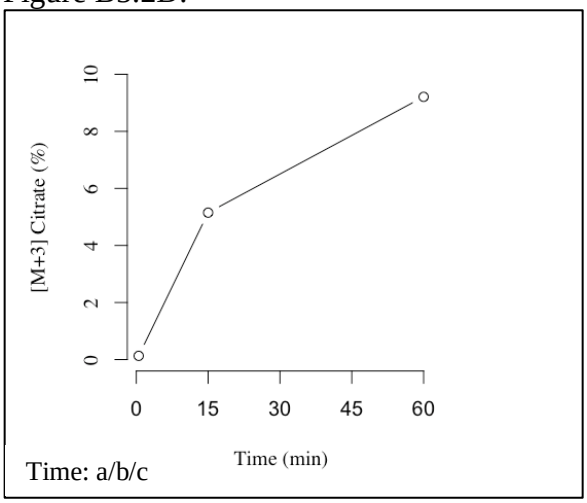


Figure B5.2 (cont'd)

Figure B5.2E.

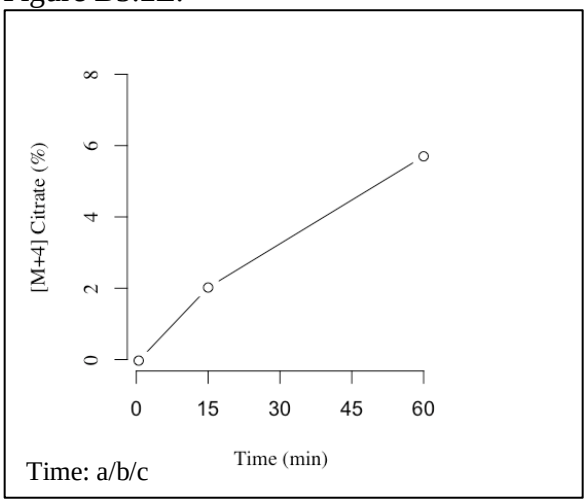


Figure B5.2F.

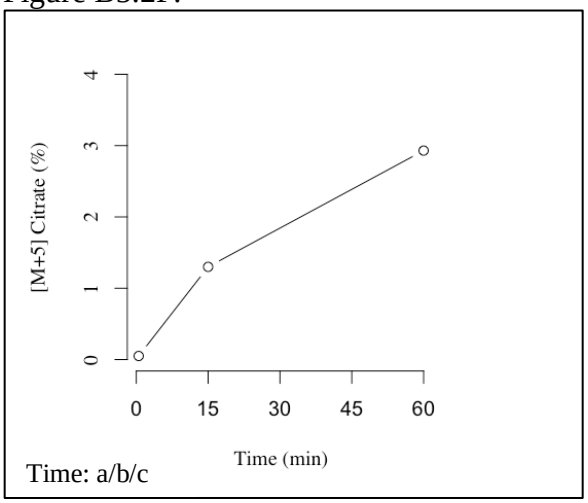


Figure B5.2G.

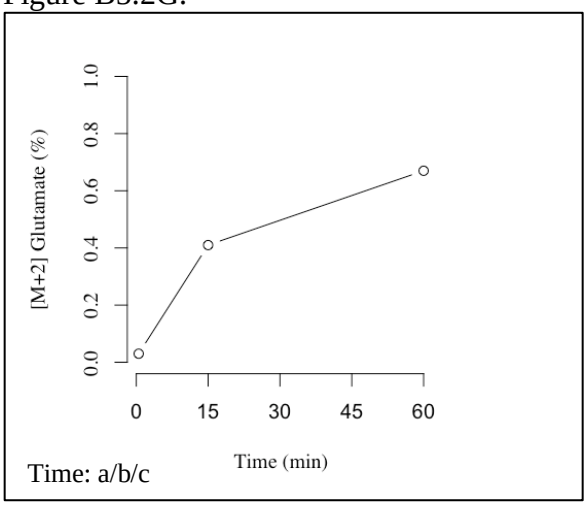


Figure B5.2H.

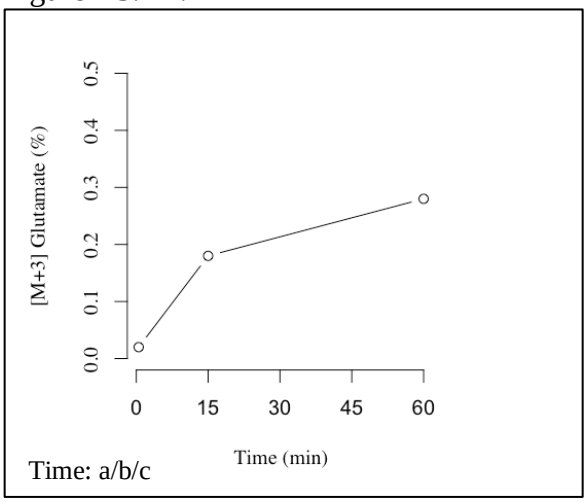


Figure B5.2I.

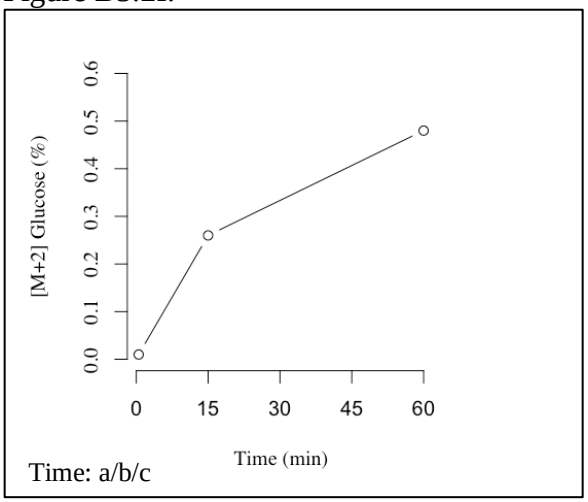


Figure B5.2J.

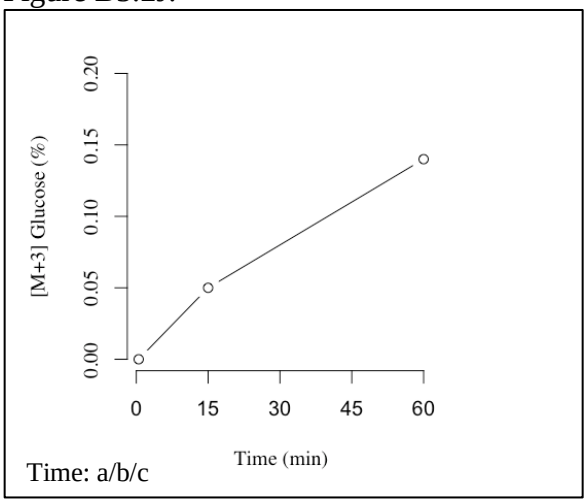


Figure B5.3. Total 13C% enrichment of metabolites of hepatic propionyl CoA (A), methylmalonyl CoA (M-CoA; B), citrate[1] (C), glutamate[1] (D), pyruvate[2] (E), lactate[3] (F), glucose (G), and acetyl CoA (H) from liver explants from dairy cows in the postpartum period for Experiment 2. Total ^{13}C% enrichment is the percent increase of ^{13}C within each metabolite above the natural abundance. Explants were incubated in [$^{13}C_3$]propionate with treatments of either 5 mM of sodium salicylate (SAL) or no treatment (control; CON)and sampled at baseline (no enrichment, 0), 0.5, 15 and 60 min. Time effects are presented at the bottom of each graph with a letter in order of baseline/0.5/15/60 min and different letters define significant difference from each other ($P \leq 0.05$).

[1]Data transformed using log (n+1) for statistical analysis and back-transformed for interpretation
[2]Data transformed using square root (n+3) for statistical analysis and back-transformed for interpretation
[3]Data transformed using square root (n+1) for statistical analysis and back-transformed for interpretation

Figure B5.3A.

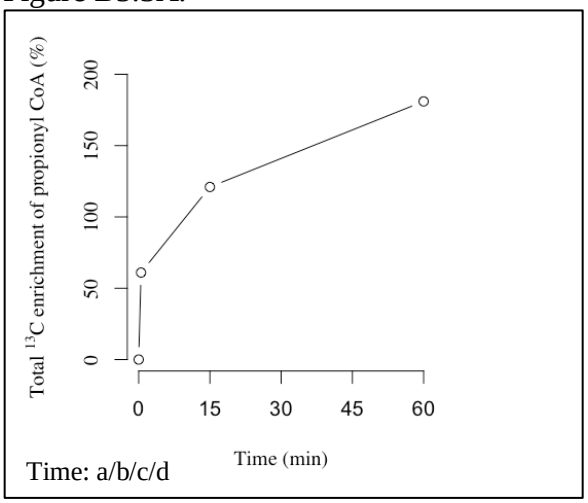


Figure B5.3C.

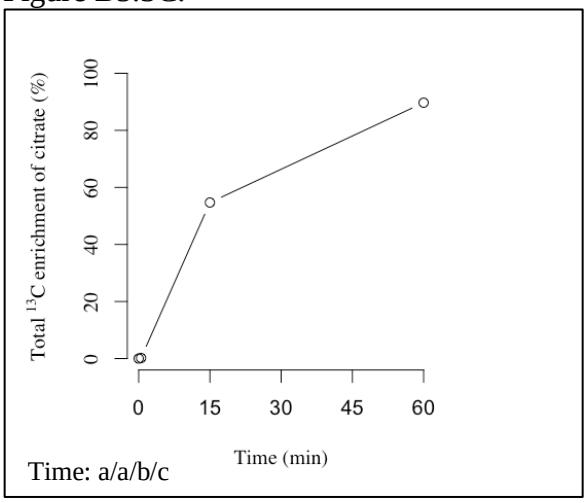


Figure B5.3B.

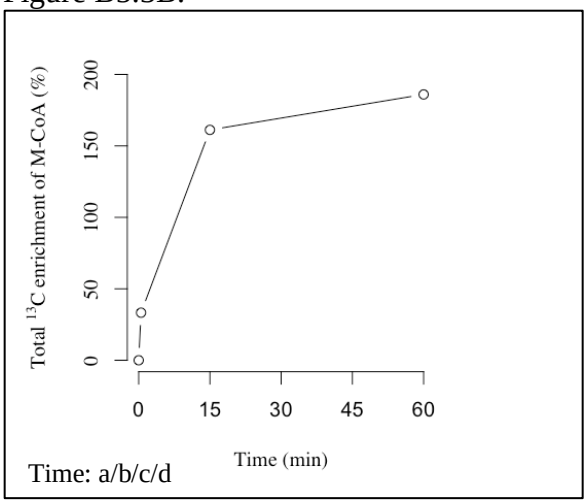


Figure B5.3D.

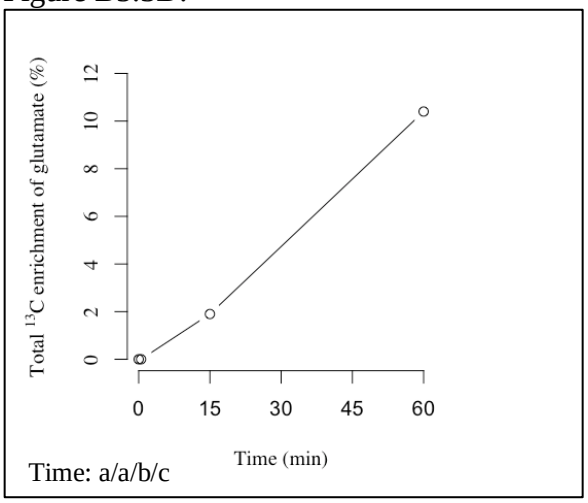
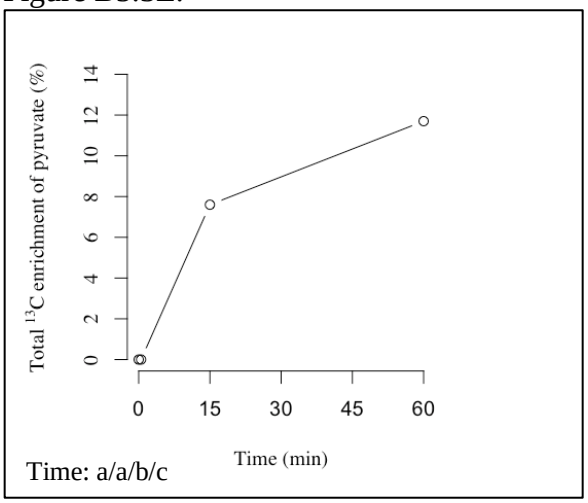


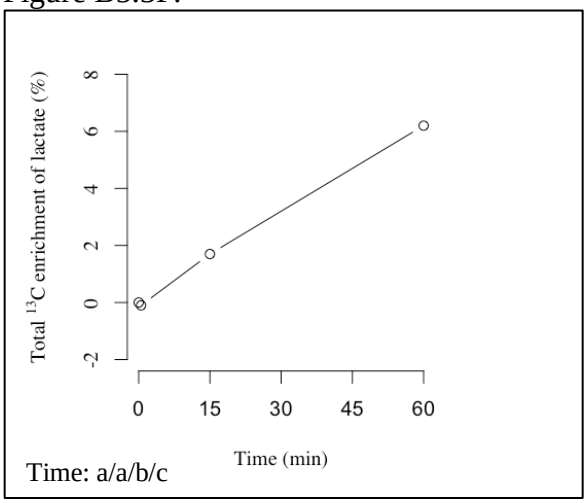
Figure B5 (cont'd)

Figure B5.3E.



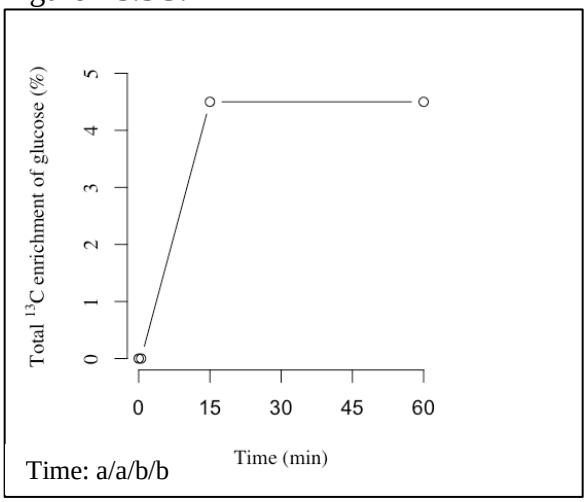
Time: a/a/b/c

Figure B5.3F.



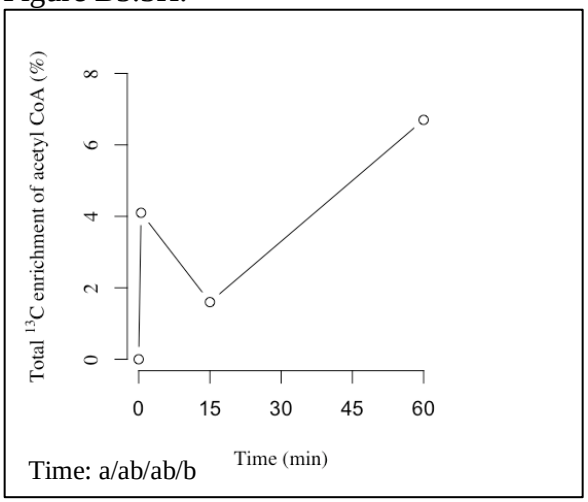
Time: a/a/b/c

Figure B5.3G.



Time: a/a/b/b

Figure B5.3H.



Time: a/ab/ab/b

Figure B5.4. ^{13}C% enrichment of hepatic [M+3] propionyl CoA (A), [M+2] pyruvate (B), [M+2] acetyl CoA (C), [M+3] citrate (D), [M+4] citrate (E), [M+5] citrate (F), [M+2] glutamate1 (G), and [M+3] glutamate[2] (H) from liver explants from dairy cows in the postpartum period for Experiment 2. The enrichment of each isotopologue is relative to the mass distribution of all the isotopologues in the metabolite (molar percent) and is on a scale of 0 to 100%. Explants were incubated in [$^{13}C_3$]propionate with treatments of either 5 mM of sodium salicylate (SAL) or no treatment (control; CON) and sampled at 0.5, 15 and 60 min. Time effects are presented at the bottom of each graph with a letter in order of baseline/0.5/15/60 min and different letters define significant difference from each other ($P \leq 0.05$).

[1]Data transformed using log (n+1) for statistical analysis and back-transformed for interpretation
[2]Data transformed using inverse (n+1) for statistical analysis and back-transformed for interpretation

Figure B5.4A.

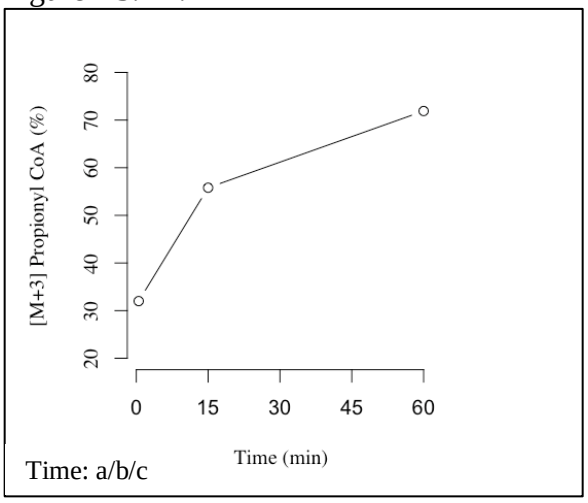


Figure B5.4C.

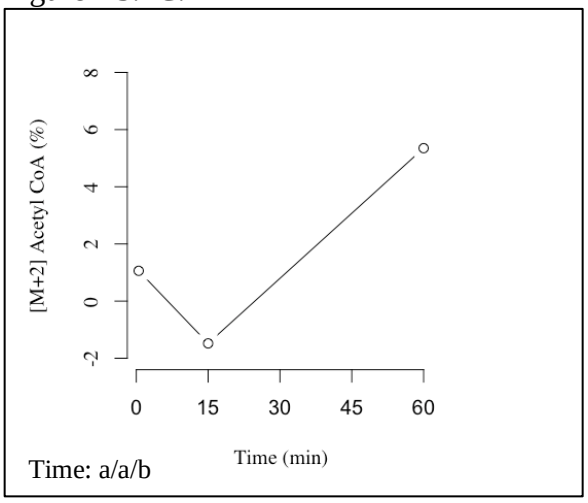
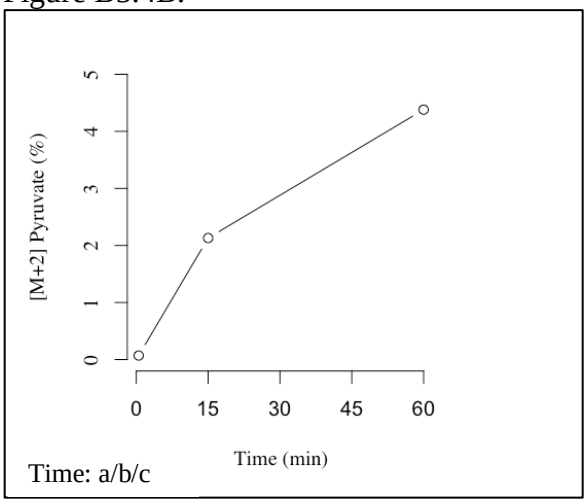


Figure B5.4B.



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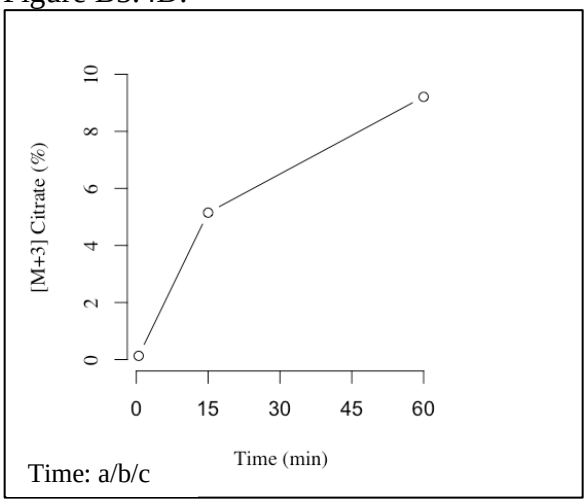
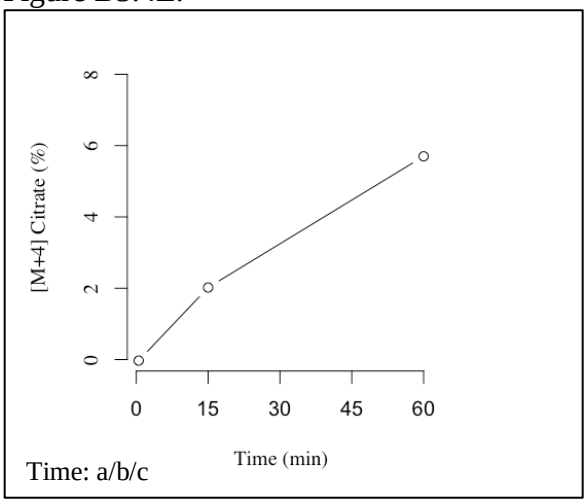


Figure B5.4 (cont'd)

Figure B5.4E.



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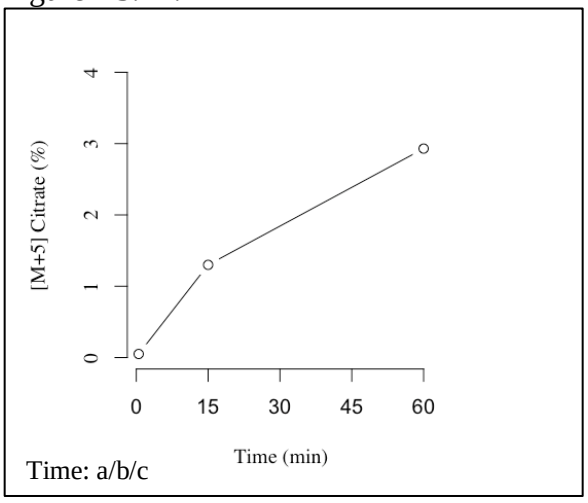

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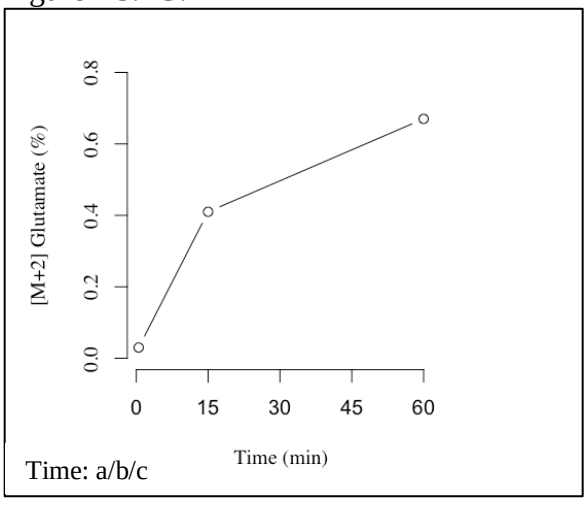

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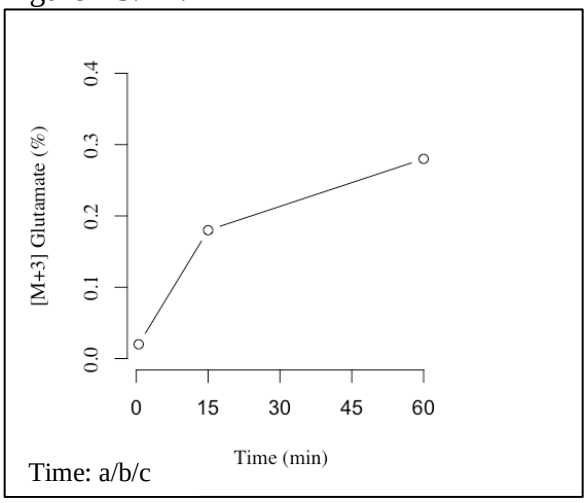

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CHAPTER 6: CONCLUSIONS

The overall objective of this dissertation was to determine the role of propionate and uncouplers of oxidative phosphorylation on hepatic metabolism and feeding behavior in dairy cows. The central hypothesis of this dissertation is that feed intake in dairy cows is affected by the oxidation of fuels in the liver where an increase in oxidation of fuels will stimulate satiety. This dissertation addressed this hypothesis under two specific aims: 1) determine the short-term effects of hepatic propionate metabolism; and 2) determine the effects of uncoupling the electron transport chain on liver metabolism and feeding behavior of ruminants.

Two experiments were conducted to address Aim 1 (Ch. 2 and 3) in which metabolism of propionate was examined within 60 min of its introduction *in vivo* or *in vitro*. In Ch. 2, potential short-term metabolic bottlenecks of hepatic propionate metabolism in the liver of dairy cows in the postpartum period were identified and the effect of feeding status (before or after meals) on these bottlenecks was examined. We concluded that: 1) absorption of propionate from the rumen into the bloodstream, and subsequent extraction by the liver, was very rapid; 2) both the metabolism and the metabolic effects of propionate were rapid, occurring within the timeframe of a meal; 3) that metabolic bottlenecks for propionate metabolism were present and that these potential metabolic bottlenecks, such as propionyl CoA carboxylase and succinate dehydrogenase, might affect feeding behavior; and 4) that feeding status affected metabolite concentrations and response to propionate with metabolite concentrations generally higher after than before feeding. In Ch. 3, the temporal effects of increasing concentrations of propionate on propionate metabolism in liver tissue of dairy cows in the postpartum period was examined. We concluded that: 1) the accumulated fraction of free glucose synthesized from propionate in hepatic tissue was not affected by the concentration of propionate. 2) although ^{13}C enrichment of

acetyl CoA was unaffected by propionate concentration from 1 – 4 mM, increased ^{13}C enrichment of [M+4] citrate and [M+5] citrate suggested that pyruvate derived from propionate was converted to acetyl CoA and entered the tricarboxylic acid (TCA) cycle for oxidation; 3) increased concentrations of propionate resulted in increased ^{13}C enrichment of most metabolites; and 4) that metabolism of propionate occurred very quickly with a measurable increase in ^{13}C enrichment observable within 0.5 min for many metabolites and within 15 min for all metabolites.

Two experiments were conducted to address Aim 2 (Ch. 4 and 5). In Ch. 4, the effects of uncouplers of oxidative phosphorylation (OXPHOS), 2,4-dinitrophenol methyl ether (DNPME) and sodium salicylate (SAL), on feeding behavior in lactating dairy cows were examined. We concluded that: 1) neither SAL nor DNMPE significantly affected daily DMI or increased meal size over the first 4 h following access to feed; 2) DNPME increased meal length over the first 4 h following access to feed; and 3) SAL and DNPME decreased eating rate over the first 4 h following access to feed. In Ch. 5, the effects of uncouplers of OXPHOS, DNPME and SAL, on the metabolism and oxidation of propionate in liver explants from dairy cows in the postpartum period were examined. We concluded that: 1) DNPME did not alter metabolism of propionate except for an increase of propionate converted to succinyl CoA; 2) SAL decreased glucose synthesis from propionate; and 3) neither DNPME nor SAL increased production of $^{14}CO_2$ from propionate, possibly due to inadequate sensitivity of our techniques for measuring $^{14}CO_2$. Background radioactive counts were very similar to our radioactive samples, so determining differences in treatments were difficult.

We improved our understanding about the hepatic metabolism of propionate with the research conducted in this dissertation. Changes in metabolism occurred rapidly (within 20 min)

and we were able to identify potential enzymes within the hepatic propionate metabolism pathway that may be bottlenecks of metabolism within this time-frame that merit further investigation. Changes in plasma parameters occurred rapidly as well as evident by a peak in plasma propionate and insulin within 5 min of a pulse dose of 1.5 moles of propionic acid to the rumen. Furthermore, we provided support that at increased concentrations of propionate in a range from 1 – 4 mM, propionate, likely through pyruvate, incorporates into the TCA cycle as acetyl CoA.

This research was the first to examine the effects of DNPME on metabolism and feeding behavior in dairy cows. Research with sodium salicylate had been conducted with the focus primarily on production responses from an immunological perspective (Farney et al. 2013; Carpenter et al., 2018; Montgomery et al., 2019). Our research examined the short-term effects of salicylate on feeding behavior and hepatic propionate metabolism, which had not previously been studied in ruminants. From this research we observed a decrease in synthesis of glucose from propionate with treatment of salicylate, which supports the observed decrease in plasma glucose concentration observed by Farney et al. (2013).

The use of uncouplers of OXPHOS to study metabolism and feeding behavior could be beneficial to improving our general understanding of the physiology of dairy cows throughout the different stages of lactation. Particularly, their use could help to study the metabolic flexibility capabilities of dairy cows by purposely increasing oxidation requirements and studying the compensatory oxidation mechanisms enacted, which could have direct benefits to understanding and/or manipulating energy partitioning and nutrient utilization. One enzyme of interest that likely influences metabolic flexibility is mitochondrial soluble adenylate cyclase. Mitochondrial soluble adenylate cyclase (sAC) has been identified as a link between the electron

transport chain and TCA cycle (Acin-Perez et al., 2009) and acts as a modulator of oxidative phosphorylation (Valsecchi et al., 2014). Zippin et al. (2013), using sAC-knockout mice, reported that glucose tolerance and insulin secretion was impaired in the absence of sAC. However, the role of sAC in dairy cow physiology has not been researched. Research into sAC contribution to feeding behavior, glucose synthesis from propionate, hormonal release, energy partitioning and milk production could lead to improved strategies of managing dairy cows.

Hypophagic effects of propionate have been well-documented (Allen, 2000) and metabolism of propionate in the liver is related to feed intake (Anil and Forbes, 1988). Dry matter intake is a function of: 1) number of meals eaten per day; 2) amount of feed eaten per meal; 3) length of meal; and 4) length of time between meals (Allen, 2014). Previous research has shown that propionate decreases meal frequency and meal size (Maldini and Allen, 2018), meaning that the effects of propionate are occurring within the time frame of a meal. Maldini and Allen (2018) reported that, contrary to their hypothesis, a faster rate of propionic acid infusion into the rumen increased meal size and decreased meal frequency compared with a slower rate and suggest that the faster rate of infusion saturated propionate metabolism in the liver; thus, causing longer meals and more time between meals. This dissertation has provided support that metabolism of propionate occurs very quickly and likely related to the rapid changes in feeding behavior characteristically observed upon its infusion into the rumen. Because propionate is a major glucose precursor and routinely produced in the rumen of ruminants as propionic acid, it is important to improve our understanding of its metabolism in order to maximize feed intake and milk production.

Many other metabolites and hormones act on the liver to influence metabolism besides propionate. Although this dissertation focused on hepatic propionate metabolism, the hepatic

oxidation theory proposed by Allen et al. (2009) encompasses oxidation of all fuels utilized by the liver as well as hormones that may affect the rate and efficiency of metabolism. Therefore, further research should be conducted focusing on the effects of various fuels and hormones on liver metabolism within a meal.

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