Abstract

DRAYTON, JOSEPHINE BENTLEY. Methylated Medium- and Long-Chain Fatty Acids as Novel Sources of Anaplerotic Carbon for Exercising Mice. (Under the direction of Dr. Jack Odle and Dr. Lin Xi.)

We hypothesized that methylated fatty acids (e.g. 2-methylpentanoic acid (2MeP), phytanic acid or pristanic acid) would provide a novel source of anaplerotic carbon and thereby enhance fatty acid oxidation, especially under stressed conditions when tricarboxylic acid (TCA) cycle intermediates are depleted. The optimal dose of 2MeP, hexanoic acid (C6) and pristanic acid for increasing *in vitro* [1-^{14}C]-oleic acid oxidation in liver or skeletal muscle homogenates from fasted mice was determined using incremental doses of 0, 0.25, 0.5, 0.75, or 1.0 mM. The 0.25 mM amount maximally stimulated liver tissue oxidation of [1-^{14}C]-oleate to $^{14}CO_2$ and [^{14}C]-acid soluble products (ASP; $P < 0.05$). Similar incubations of 0.25 mM 2MeP, C6, palmitate, phytanic acid, or pristanic acid or 0.1 mM malate or propionyl-CoA were conducted with liver or skeletal muscle homogenates from exercised or sedentary mice. *In vitro* oxidation of [1-^{14}C]-oleic acid in liver homogenates with 2MeP increased mitochondrial $^{14}CO_2$ accumulation ($P <0.05$), but no change in [^{14}C]-ASP accumulation as compared to C6 ($P > 0.05$). Phytanic acid treatment increased [^{14}C]-ASP accumulation in liver tissue as compared to palmitate ($P < 0.05$). Exercise increased [^{14}C] accumulations ($P < 0.05$). Results were consistent with our hypothesis that methyl-branched fatty acids (2-MeP, phytanic and pristanic acids) provide a novel source of anaplerotic carbon and thereby stimulate *in vitro* fatty acid oxidation in liver and skeletal muscle tissues.

Next, serum concentrations of β-hydroxybutyrate (BHB) were measured in mice at 0, 1, 2 and 4 h after gavaging 0.110 mL (~13 umol/kg body weight) of one of three triglycerides: tri-hexanoate (t-C6), tri-2-methylpentanoate (t-2MeP) or t-C6 + t-2MeP (mixed). Serum concentrations of BHB peaked after one hour; however, the peak from the mixed triglyceride was less than from t-C6 by 49% (2.52 versus 4.927 mM, respectively; $P < 0.05$). No change in BHB was observed from t-2MeP ($P < 0.05$). After two hours, BHB levels decreased in the t-C6 and mixed gavaged mice, but were not different from one hour concentrations ($P < 0.05$). After four hours, mice dosed with t-C6 still experienced elevated BHB levels as compared to t-2MeP ($P < 0.05$). Consistent with our hypothesis, an oral bolus of t-C6 induced ketogenesis whereas t-2MeP did not.

Finally, 47 mice were blocked for body weight and randomly allocated to a basal control diet or the basal diet with an additional 8% soybeanoil, 8% t-2MeP, 8% t-C6 or mixed diet (4% t-C6 + 4% t-2MeP). Over the course of 9 weeks, feed intake and body weights were measured weekly. At the end of the trial, tissue weights and serum were collected. Feed intake was not affected by dietary treatment ($P > 0.05$). However, by week 3, mice fed t-2MeP experienced decreased body weights ($P < 0.05$). By week 4, all mice fed medium-chain triglycerides weighed less than soyoil fed mice ($P < 0.05$). Mice fed t-2MeP had increased liver weights ($P < 0.05$). Mice fed t-2MeP had elevated fasting blood urea nitrogen, alkaline phosphatase and BHB but decreased triglyceride levels as compared to soyoil or t-C6 fed mice ($P < 0.05$). Serum albumin and nonesterified fatty acids were decreased in t-2MeP fed mice as compared to soyoil fed mice ($P < 0.05$).

We found that feeding t-2MeP decreased body weight gain, increased liver weight and affected serum metabolite profiles as compared to t-C6 or soyoil feeding ($P < 0.05$). Overall, these results are consistent with our hypothesis that the anaplerotic structure of methyl-branched fatty acids affects metabolism and produces a unique physiological response.

Methylated Medium- and Long-Chain Fatty Acids as Novel Sources of Anaplerotic Carbon for Exercising Mice

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Biography

Josie is from Baltimore, Maryland. She received a Bachelor of Science degree in Animal Science with an emphasis in companion animals from the University of California, Davis in 2006. While there, she worked in a nutrition lab and became interested in nutritional biochemistry. In 2010, Josie began working towards a Master of Science degree, studying Animal Science and Nutrition under the guidance of Dr. Jack Odle and Dr. Lin Xi. Josie will be pursuing a Doctor of Veterinary Medicine degree at the University of Minnesota in the fall of 2012.

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Chapter I: Literature Review

Triglyceride and fatty acid structure.

A triglyceride is composed of three fatty acid molecules, each attached to one of the three carbons in a glycerol molecule (Figure 1; 1). For example, triglyceride 2-methylpentanoate (t-2MeP) contains only 2-methylpentanoic acid (2MeP) fatty acids and a glycerol molecule. Unesterified, 2MeP is a methyl-branched medium chain fatty acid (MCFA; Figure 2). The methyl-branch is a methyl group attached at the 2-carbon of the fatty acid. While it is similar to hexanoic acid (C6), in that it has six carbon molecules, it has a carbon chain length of only five carbons, which makes it an odd chain fatty acid (2). This shorter carbon chain makes medium chain triglycerides (MCT) such as tri-2-methylpentanoate (t-2MeP) or tri-hexanoate (t-C6) more hydrophilic and moderately polar, which increases their solubility in water and biological fluids as compared to long chain triglycerides (LCT; 3). LCT are composed of long chain fatty acids attached to one of the three carbons in a glycerol molecule (1). For example, pristanic acid (3,7,11,15-tetramethylhexadecanoic) acid and pristanic (2,6,10,14-tetramethylpentadecanoic) acid are similar to 2MeP in that they are methyl-branched fatty acids, however, their longer chain length means they make up LCT instead of MCT. This difference in chain length causes differences in the catabolism of LCT as compared to MCT (1). For example, the higher solubility of MCT allows them to be more quickly digested and absorbed than LCT (2). In fact, MCFA are absorbed more quickly than glucose (3); however, at 8 kcal/kg, they are almost as energy dense as long chain fatty acids (LCFA; 4).

Lipid digestion and absorption.

Lipid digestion begins in the stomach, as food is already mixed in the mouth with lingual lipase, which the stomach's acidic pH activates (6). Gastric lipase is secreted from the chief cells in the stomach (6). Lipases act to hydrolyze the fatty acid-glycerol ester bond (7). Gastric lipase acts preferentially on MCT (6) therefore, some MCFA are even absorbed through the stomach (5). In the small intestine, the much shorter chain length of MCT permits much faster and more complete hydrolysis by pancreatic lipase (7). Even so, whole MCT can diffuse into enterocytes and then be enzymatically hydrolyzed within the cell or travel via the lymph as a MCT (8). The polar nature of MCFA allows them to incorporate into emulsions and diffuse across the unstirred water layer more easily than LCFA which moreover escapes any negative feedback of absorption (3). In all, MCT are digested and absorbed prior to and more completely than LCT in the small intestine (8).

Long chain triglyceride digestion is more gradual and does not significantly begin until it reaches the small intestine (7). This is a consequence of LCT's hydrophobicity which causes them to be less soluble and require emulsification for the significant portion of their digestion (7). Digestion within the lumen of the small intestine is mostly a combination of emulsification and enzymatic hydrolysis (7). Bile contains bile salts which, due to their amphipathic nature, act as an emulsifier in the small intestine (7). Bile, along with second amphipath lysolecithin, gives pancreatic lipase and colipase better access to LCT ester bonds (6;7) within the coarse emulsion droplet. Because pancreatic lipase acts on the sn-1 and sn-3

bonds of a triglyceride, 2-monoacylglycerides and nonesterified fatty acids (FFA) are the primary products of LCT digestion; however, rearrangement and complete triglyceride hydrolysis do occur (5;7). Still, long chain 2-monoacylglycerides and FFA are non-polar products (7). As such, they must be incorporated into emulsions for absorption (7). In the small intestine, coarse emulsions are slowly broken down so that FFA and 2-monoacylglycerides may be extracted into a mixed micelle (6). Lysolecithin is produced when pancreatic phospholipase A_1 and A_2 hydrolyze lecithins to lysolecithins and a fatty acid (6). This highly amphipathic component of mixed micelles allows them to cross the unstirred water layer at the boarder of the microvillus (6). Subsequently, the products of LCT hydrolysis may diffuse into the enterocyte (8).

In the enterocyte, LCFA are bound by binding proteins (6). These binding proteins increase absorption and direct fatty acids to the endoplasmic reticulum where they can be activated and re-esterified through the monoacylglyceride pathway (5). In the monoacylglyceride pathway, activated fatty acyl-CoAs are sequentially added to a dietary 2-monoacylglyceride molecule (6). Additionally, a minor pathway also acts to completely re-esterify a glycerol molecule to a LCT in the α-glycerol-phosphate pathway (6). Subsequently, LCT are packaged into chylomicrons which are secreted from the enterocyte into the lymphatic system (6). Chylomicrons are 50-500 nm in size, and will expand in response to increased lipid intake (6). Their large size requires them to be transported in the lymphatic system to the thoracic duct and then to the liver as well as some extrahepatic tissue (6). Medium chain fatty acids, however, are not re-esterified within the enterocyte (8). Instead, MCFA can diffuse across the endothelial wall, enter the portal vein, and be quickly

transported to the liver as FFA in the portal vein (8). Some MCT have been identified in the lymphatic system after absorption; however, this is a minor fate of the MCT that diffuse into the enterocyte (8).

Medium chain fatty acids are transported independently of chylomicrons and diffuse directly into the blood instead of travelling through the lymph (Figure 3; 3). Their swift absorption and transport delays chylomicron formation; consequently, MCT feeding inhibits the absorption of LCFA (1).

Medium chain fatty acids are transported from the intestine in the portal blood (Figure 3; 8). Thus, they are more directly transported to the liver and absorbed to a greater extent by the liver than LCFA entry into circulation via the lymphatic system (2). Once in the hepatocyte, MCFA may be elongated to LCFA and re-esterified, but this is a very minor fate of MCFA as they are poor substrates for re-esterifying enzymes (1). Primarily, MCFA are quickly catabolized as they can cross the mitochondrial membrane independently of carnitine palmitoyltransferase (CPT)-I (Figure 4; 9; 13).

Dietary lipids in chylomicrons, or endogenous lipids in lipoproteins, will interact with lipoprotein lipase (LPL) on cell walls (7). This enzyme will hydrolyze triglycerides and phospholipids for absorption (6;7). Fatty acids will either diffuse into the cell or be transported across the cell membrane using a fatty acid transport protein. The antedated arrival of MCFA will augment their preferential use for energy by the tissue, especially the liver (2).

Beta-oxidation.

Once MCFA are in the mitochondrial matrix, an initial activation step must occur before cycles of β-oxidation may proceed (Figure 5; 10). This activation will most likely occur as medium-chain acyl-CoA synthetase converts the MCFA to medium chain fatty acyl-CoA, as described in the oxidation of heptanoate by Roe *et al.* (11). The acyl-CoA may then be oxidized by medium-chain acyl-CoA dehydrogenase to 2-enoyl-CoA (11). In this step, a double bond is formed between the 2 and the 3 carbon of the fatty acyl-CoA (10). Next, 2-enoyl-CoA hydratase hydrates the C2-C3 double bond, producing a 3-hydroxyacyl-CoA (10). Then, 3-hydroxyacyl-CoA dehydrogenase produces 3-ketopentanoyl-CoA by converting the hydroxy group to a keto group (10). Lastly, short-chain 3-ketoacyl-CoA thiolase may clip two fatty acid chain carbons from the carboxyl end of the fatty acid molecule (11). With straight chain fatty acids like hexanoic acid, this action would typically produce an acetyl-CoA and a fatty acyl-$CoA_{(n-2)}$ molecule with each cycle (10). While this may occur in catabolism of 2MeP-CoA, a methyl-branch at the 2-carbon of the 2MeP chain in combination with an odd chain length of 5 carbons results in the production of two 3-carbon propionyl-CoA molecules rather than the normal product of 2-carbon acetyl-CoA, in much the same way that odd MCFA and methyl-branched LCFA have been shown to produce propionyl-CoA (Figure 1;11;12).

If absorbed into the hepatocyte, LCFA are either oxidized or re-esterified for transport as a triglyceride to other organs in the body (1). However, in other tissues like skeletal muscle tissue, LCFA will be destined for mitochondrial β-oxidation (10). After

entering the cell, LCFA are bound by fatty acid binding proteins and then activated to fatty acyl-CoAs by enzyme long-chain acyl-CoA synthetase in the outer mitochondrial membrane (Figure 4; 13). However, long chain fatty acyl-CoAs cannot cross the mitochondrial membrane and must be converted to an acylcarnitine by enzyme CPT-I, which also transports the acylcarnitine across the outer mitochondrial membrane (13). Acylcarnitines can cross the inner mitochondrial membrane using transporter carnitine-acylcarnitine translocase, so named because it exchanges a carnitine molecule for an acylcarnitine molecule (13). A second inner membrane enzyme, CPT II will regenerate the fatty acyl-CoA molecule using free CoASH in the mitochondrial matrix (13).

Regulation of β-oxidation.

Enzyme CPT-I is inhibited by elevated levels of malonyl-CoA which are synthesized by the first step of fatty acid synthesis; acetyl-CoA carboxylase (ACC; Figure 6; 10). This occurs, first, as excess acetyl-CoA in the mitochondrion is converted to citrate in the first step of the tricarboxylic acid (TCA) cycle (10). However, instead of continuing through the TCA cycle, the excess citrate molecules are shuttled out of the mitochondrion and into the cytosol via a tricarboxylate carrier (10). Citrate will be converted back to acetyl-CoA by cytosolic adenosine triphosphate (ATP)-citrate lyase and then carboxylated to malonyl-CoA by ACC (10). Malonyl-CoA will act to inhibit CPT I and thus decrease LCFA oxidation (13) but will also be used for cholesterol synthesis (1), fatty acid synthesis or fatty acid elongation (10).

This sensitivity to β-oxidation flux prevents futile cycling of fatty acid oxidation and fatty acid synthesis or ketogenesis in the liver as inhibition of CPT-I effectively inhibits β-oxidation (Figure 6; 13). Sensitivity to malonyl-CoA will vary under different physiological conditions (10; 13). For example, molecules that have been shown to increase membrane fluidity have also been shown to increase CPT-I sensitivity to malonyl-CoA (10). The affinity of CPT-I for acyl-CoA molecules can decrease when the liver is in a ketogenic state (13). Malonyl-CoA synthesis via ACC is increased due to carbohydrate feeding (14). This occurs as there is an increased glycolytic flux to acetyl-CoA, which will be converted to citrate in the first step of the TCA cycle, exported to the cytosol and converted to malonyl-CoA (10).

There is a much lower rate of citrate export from the mitochondrion in the extrahepatic tissues like heart and skeletal muscle (10). As such, CPT-I is much more sensitive to malonyl-CoA in these tissues (10). A decreased rate of export and increased sensitivity supports the fact that skeletal muscle does not participate in fatty acid synthesis (10). Nevertheless, carbohydrate feeding has also been shown to downregulate CPT-I activity even though fatty acids are a primary source of fuel for skeletal muscle tissue (15).

While CPT-I is the primary regulator of β-oxidation flux, regulation of β-oxidation can also occur at the individual enzymes (Figure 5; 10). In fact, elevated levels of acetyl-CoA not only inhibit CPT-I but also inhibit the last enzyme in the β-oxidation cycle, 3-ketoacyl-CoA thiolase (10). The first enzyme in β-oxidation, acyl-CoA dehydrogenase has a high affinity for acyl-CoA's and for 2-enoyl-CoA's and as such, can be inhibited by increased 2-

enoyl-CoA molecules (10). The 3-ketoacyl-CoA esters that are produced also act to inhibit short, medium and long acyl-CoA dehydrogenases (10). Therefore, acyl-CoA dehydrogenase can be inhibited by negative feedback of later β-oxidation enzyme products (10). Enzymes 3-hydroxyacyl-CoA dehydrogenase and 2-enoyl-CoA hydratase are regulated by negative feedback of their individual products (10). Because these enzymes are successive steps in β-oxidation, inhibition of the latter can induce product inhibition of the former (10). As mentioned, 3-ketoacyl-CoA can also act to inhibit acyl-CoA dehydrogenases, and therefore, will also concertedly inhibit the first step of β-oxidation when 3-ketoacyl-CoA and/or 3-hydroxyacyl-CoA are at inhibitory concentrations (10).

The enzymes of β-oxidation can be additionally regulated by the proportion of acetyl-CoA to free CoASH in the mitochondrion (16). Depletion of CoASH will inhibit CPT-II and 3-ketoacyl-CoA thiolase (16). This depletion will also inhibit other CoASH-requiring enzymes like pyruvate dehydrogenase, branched chain ketoacid dehydrogenase and α-ketoglutarate dehydrogenase (16). Redox regulation of β-oxidation also occurs when the respiratory chain is inhibited, ATP/adenosine diphosphate (ADP) ratio is high, during anoxia or as a normal synchronizing function of the liver (16).

Peroxisomal fatty acid oxidation.

Peroxisomes are subcellular organelles that oxidize fatty acids through α- and β-oxidation, synthesize etherphospholipids and detoxify glyoxylate (17). Peroxisomal glyoxylate detoxification is important in humans as enzyme alanine glyoxylate aminotransferase is only present in the peroxisome (17). It is important in etherphospholipid

synthesis for the same reason; strictly peroxisomal enzymes dihydrooxyacetone phosphate acyltransferase and dihydrooxyacetone phosphate synthase catalyze the first two reactions in etherphospholipid biosynthesis, respectively (17). The remainder of etherphospholipid biosynthesis occurs in the endoplasmic reticulum (17).

Peroxisomes oxidize several types of LCFA through the β-oxidation pathway; such as very LCFA, pristanic acid, and di- and tri-hydroxycholestanoic acid (17). Unlike other LCFA like palmitic acid, naturally-occurring branched LCFA phytanic acid and pristanic acid are primarily oxidized in the peroxisome (17). Phytanic acid is a metabolite that is produced during microbial degradation of chlorophyll (17). As such, humans will typically ingest phytanic acid when eating ruminant products like dairy and meat (17). Pristanic acid is produced from the α-oxidation of phytanic acid (Figure 7; 17). Alpha-oxidation occurs exclusively in the peroxisomes and is a necessary step in the oxidation of phytanic acid (12). Phytanic acid must be α-oxidized to pristanic acid because a branch point at the 3-position of the molecule prevents fatty acyl-CoA dehydrogenase from accessing the β-carbon of phytanic acid (12). Alpha-oxidation typically begins as phytanoyl-CoA synthetase activates phytanic acid to phytanoyl-CoA (12). Next, phytanoyl-CoA 2-hydroxylase hydroxylates phytanoyl-CoA to 2-hydroxyphytanoyl-CoA (12). This molecule is next cleaved by 2-hydroxyphytanoyl-CoA lyase to pristanal and formyl-CoA (12). This formyl-CoA molecule is typically non-enzymatically hydrolyzed to CoASH and formic acid, the latter of which is exported out of the peroxisome (12). Pristanal dehydrogenase converts pristanal to pristanic acid (12).

Hence, when pristanic acid is chain-shortened by one carbon in α-oxidation, it allows all of the methyl-branches to fall in order for β-oxidation (12). Within the peroxisome, pristanic acid will be activated by an acyl-CoA synthetase to pristanoyl-CoA so that it may go through three rounds of β-oxidation (12). Peroxisomal fatty acid β-oxidation is similar to mitochondrial fatty acid β-oxidation and after three β-oxidation cycles, pristanic acid will produce a net total of two acetyl-CoA's, one propionyl-CoA and one 4,8-dimethylnonanoyl-CoA which then may be transported to the mitochondrion for further oxidation using either a carnitine dependent or carnitine independent mechanism (12;17; Figure 7). However, at the mitochondrion, 4,8-dimethylnonanoyl-CoA will be taken up by only the CPT transport system (12). Therefore, β-oxidation of methyl-branched LCFA is more regulated than that of methyl-branched MCFA as high glycolytic flux can redirect these molecules for storage in adipocytes (14). In fact, it has been shown that rats on a phytol-rich diet will accumulate significant phytanic acid stores at 15-20% of total fatty acids in blood and liver (17).

Ketogenesis.

Unregulated hepatic fatty acid catabolism, such as in the case of unbranched MCFA, will produce excessive amounts of acetyl-CoA (9; 13; 19). The acetyl-CoA molecules will enter the TCA cycle and proceed through the first step of condensing with oxaloacetate in formation of citrate and free CoASH (Figure 8; 20). However, the TCA cycle is a carbon cycle with regenerating intermediates so when the cycle substrate outstrips the intermediates it becomes imbalanced (21). For example, the acetyl-CoA molecule is a two-carbon molecule and in the process of a complete cycle, there are two carbon dioxide (CO_2) molecules

produced from cycle reactions with no overall change in the cycle upon completion (21). So, when unregulated MCFA oxidation overwhelms the TCA cycle capacity, it begins to redistribute incompletely oxidized acetyl-CoA molecules to other tissues using ketone bodies (2;13;19).

The hepatocyte metabolism shifts towards to ketogenesis as eventually the TCA cycle will be functionally impaired (22). Acetyl-CoA will bind up free CoASH; consequently; this shift in acetyl-CoA:CoASH will inhibit fatty acid oxidation enzyme 3-ketoacyl-CoA thiolase and TCA cycle α-ketoglutarate dehydrogenase (22; 23). The liver mitochondrion thus switches from complete oxidation to CO_2 to ketone body synthesis (Figure 8; 24). The first step of ketone body synthesis requires two acetyl-CoA molecules (24). Acetyl-CoA acetyltransferase condensates these molecules to form acetoacetyl-CoA which may be converted to 3-hydroxy-3-methylgluratyl-CoA (HMG-CoA) via enzyme 3-hydroxy-3-methyl-gluratyl-CoA synthase (HMG-CoA synthase; 24). With continued ketone body synthesis, some acetoacetate is converted to β-hydroxybutyrate (BHB) by β-hydroxybutyrate dehydrogenase or acetoacetate can be nonenzymatically converted to acetone and exported into circulation for extrahepatic tissue use (24).

Measurement of triglyceride and fatty acid metabolism in animal models.

Triglyceride absorption can be measured *in vivo* using a clearance test (2;25). In this experiment, an animal is gavaged with a measured amount of the TG. The TG may or may not be emulsified. This should be taken into consideration with the study as emulsification will increase the rate of digestion and absorption (7;26). Subsequently, blood samples may be

collected for lipid profile and metabolite analyses across a time course. These data may be used to indicate peak rates of absorption and metabolism (2;25).

In vitro fatty acid oxidation of specific fatty acids can also be measured either directly or indirectly using radiolabeled fatty acids (2;9;19;27). If directly measured, [^{14}C]-complexed fatty acids are used as a tracer of fatty acid metabolism (2;9;19;27). If indirectly measured, a nonspecific labeled fatty acid such as [1-^{14}C]-oleic acid can be used as an indicator of oxidation rates under different treatment conditions (28). Labeled oxidation products are collected; $^{14}CO_2$ from complete oxidation and [^{14}C]-acid-soluble products (ASP) comprised of ketone bodies and TCA cycle intermediates (TCAI; 19). Subsequently, [^{14}C]-ASP can be analyzed for individual components (19).

Anaplerosis.

The unique metabolic fate of MCT feeding has been used to treat neurological and metabolic diseases (1). Medium chain triglyceride feeding has also been shown to improve neonatal survivability in swine and also alleviate symptoms in humans with fatty acid oxidation enzyme deficiencies (5;9;11). In piglet studies, decreasing chain length has proven to improve digestion and absorption time, even within the MCT class (i.e. t-hexanoate > t-octanoate; 28; 29). This discovery has been used to enhance premature infant nutrition as these characteristics of MCT improve digestibility (1). As such, Lin *et al.* (1996) found that *in vitro* hepatic oxidation of tri-heptanoate contributed 85% more TCAI carbon, 12% more BHB carbon but the same amount of acetate carbon as compared to t-octanoate (19). They

concluded this effect was a result of the anaplerotic products of odd chain MCFA metabolism (19).

Anaplerosis (Greek: ana-, up; perotikos, to fill) is a word used to describe the reactions which contribute to the TCAI in balancing with cataplerosis, or the removal of TCAI (Figure 9; 30). Cataplerosis occurs for a variety of reasons, one of which is inadvertent cell membrane leakage, as indicated by the release of creatine kinase (11). Cataplerosis, just from membrane leakage, depletes 1-2% of the TCAI pool per minute (22). Thus, the need for carbohydrate or amino acid derived intermediates only occurs when TCAI have leaked excessively and crippled energy production (22). The term cataplerosis technically also encompasses pathways which draw off TCAI for other biosynthetic purposes in the cell (22). While this could be a concern in some physiological conditions (i.e. diabetes), this type of cataplerosis is not the primary concern of anaplerosis during exercise (31). As such, the need for both anaplerotic carbohydrate and fatty acids for energy production is memorably described in the adage, "fat burns in the flame of carbohydrate" (32). Here, it is clear that fat cannot be completely oxidized for energy, or burn, without carbohydrate intermediates (22). The initial step of using acetyl-CoA as a TCA cycle substrate clearly demonstrates the need for sustainable intermediates, or instead, anaplerosis (22). Here, acetyl-CoA is condensated with intermediate oxaloacetate (OAA; 20). This reaction, catalyzed by citrate synthase, produces citrate and releases free coenzyme A (20). With a full turn of the TCA cycle, OAA is completely regenerated and ready for another acetyl-CoA molecule (20). However, there are several points in the TCA cycle where inadequate TCAI, excess substrates and/or

regulation may put the cell into a ketogenic state (19;33). Such changes happen quickly in a TCAI pool of only 1-2 umol/g tissue with a flux of 1-2 umol acetyl-CoA/g.minute (22).

Anaplerotic reactions.

The TCA cycle is an indispensable step in energy metabolism as it represents the convergence of protein, lipid and carbohydrate catabolism into one energy pathway (21). In fact, according to Baldwin and Krebs, the TCA cycle is the quintessential "process in which an overall chemical change is brought about by a cyclic reaction cycle", a process also known as a metabolic cycle (21). The TCA cycle is a, "cyclic reaction cycle" because at the end of it, the reactants necessary for accepting another acetyl-CoA molecule are regenerated (21). There are several anaplerotic reactions that may occur which feed net carbon into the TCA cycle (Figure 9). The first is at TCAI α-ketoglutarate (22). Here, glutamine dehydrogenase and aminotransferases can interconvert glutamine with α-ketoglutarate (22). The second anaplerotic reaction in the TCA cycle is at succinyl-CoA (22). Firstly, anaplerosis can be useful here as a depletion of CoASH will cause α-ketoglutarate to be inhibited (23). Under stressed conditions, CoASH is likely to be depleted and so α-ketoglutarate dehydrogenase is replaced by the anaplerotic reactions of the methylmalonyl pathway (Figure 10; 23). The methylmalonyl pathway typically begins with enzyme propionyl-CoA carboxylase (22). Otherwise, it will begin with acetyl-CoA synthase which will feed propionate into propionyl-CoA (23). Propionyl-CoA carboxylase will carboxylate the propionyl-CoA to (D)-methylmalonyl-CoA using biotin, bicarbonate and ATP while releasing ADP, P_i and H^+ (34). Then, (D)-methylmalonyl-CoA is converted to (L)-

methylmalonyl-CoA by D-methylmalonyl-CoA racemase (34). Finally, (L)-methylmalonyl-CoA is converted to succinyl-CoA by the enzyme methylmalonyl-CoA mutase using cofactor adenosylcobalamin (34). The resultant succinyl-CoA may enter the TCA cycle as an exogenously-derived TCAI, thus sparing endogenous glycogen and protein stores during physiological stress (35;36). Propionyl-CoA precursors include; branched-chain amino acids, propionate from intestinal fermentation, odd-chain fatty acids and odd chain ketone bodies (22). The purine nucleotide cycle has been indicated to feed into fumarate; however, at a very low rate (37;38). Additionally, phosphoenolpyruvate feeds into OAA via phosphoenolpyruvate decarboxylase (38). The final anaplerotic pathway feeds pyruvate into both malate and OAA via malic enzyme and pyruvate carboxylase, respectively (22;38). The pathways that feed into pyruvate have been indicated to be especially important in anaplerosis as they most directly provide the anaplerotic carbon required for the initiation of the TCA cycle (33). Additionally, these pyruvate-generating pathways seems to be the most crucially limited during stressed conditions that are associated with increased glucagon levels (33).

The primary purpose of the TCA cycle is to provide ATP for the body (21). Thus, when the skeletal muscle's need for ATP increases, as in the case of moderate to intense exercise, the TCA cycle flux has been shown to increase as well (39). This flux has been shown to increase by approximately 60-fold (40;41). While the change in flux is massive compared to the change in total TCAI levels, the TCAI concentrations also increased by approximately 4-fold in response to increased ATP demand (41). For example, Gibala *et al.* showed that TCAI pool intermediates doubled after 60% $VO_{2\ max}$ one-legged kicking for ten

minutes, while TCA cycle flux increased by approximately 70% (41). Subjects immediately continued exercise at 100% $VO_{2\ max}$ and TCAI were measured through exhaustion (41). Gibala was able to calculate a curvilinear relationship between the increased TCAI and TCA cycle flux which may partially result from TCA cycle "spans" (41). When summed TCAI are separated out into a "first span" of citrate to α-ketoglutarate and "second span" from succinate to oxaloacetate, there is a distinct difference between span concentrations (41). Gibala shows the similar concentrations of first and second spans at rest, and even during a moderate intensity 60% $VO_{2\ max}$ exercise test (41). Differences were seen when exercise was increased to 100% $VO_{2\ max}$ (41). During high intensity exercise, increases in TCAI levels occurred almost exclusively in the "second span" TCAI (41). Gibala *et al.* suggests this may be a result of the large amount of pyruvate oxidation occurring at the onset and during exercise (41). A study by Sahlin *et al.* indicates that TCAI decrease from their initially expanded state during exercise (38). TCA cycle substrate, on the other hand, is plentiful even at exhaustion (38). They suggest that the decreased TCAI levels (seen after the initial expansion to 4.41±0.23 after 5 minutes at 70% $VO_{2\ max}$) to 3.33±029 after 40 minutes and finally to 2.83±0.27 mmol/kg dry weight at exhaustion are a result of decreased carbohydrate availability during glycogen depleted fatigue as skeletal muscle glycolysis was estimated to have decreased by approximately 40% in the last 35 minutes of exercise (38).

Differences between rodent and human exercise physiology.

Gibala *et al.* attributed the species-specific differences in skeletal muscle TCAI pool size to three possible sources; skeletal muscle contraction intensity, fiber recruitment order or

fiber type composition (39). They found differences could be attributed to fiber type composition (39). Their samples of human skeletal muscle were comprised of human vastus lateralis muscle, which is made of 51.4% type I, 34.4% type IIa, and 14.3% type IIb fibers (42). Rodent hindlimb muscle samples, as those from Aragon *et al.* are mostly glycolytic skeletal muscle fibers (mostly type IIx/d) with lower mitochondrial levels and accordingly fatigue more quickly (37;43). This composition difference may contribute to the oxidative capacity differences in the two species because type I fibers contain higher levels of citrate and malate than type II do, both at rest and during exercise (39).

Furthermore, rodents may spare skeletal muscle glycogen to a greater extent than humans (44). Terjung *et al.* showed that after exhaustive exercise, rat liver glycogen stores were depleted while skeletal muscle glycogen was not (44). This is in contrast to human skeletal muscle fuel selection where depletion of skeletal muscle glycogen antedates exhaustion (44).

Medium chain triglyceride feeding may affect glycogen utilization. In an experiment by Aoki *et al.* it was shown that feeding high levels of MCT to mice spared skeletal muscle glycogen after one hour of exercise at 65% $VO_{2\ max}$. However, liver glycogen levels were lower in MCT fed animals as compared to controls (45). This difference between skeletal muscle and liver glycogen utilization may result from one of the aforementioned factors.

Evaluation of exercise endurance in mice.

Mouse exercise performance may be evaluated using a treadmill (46). This method is advantageous in that it allows for the evaluation of performance at a specific and uniform

exercise intensity and length (47). Variations in treadmill running capacity have been identified among mouse breeds (47). The C57BL/6J mouse strain was chosen for its middling aerobic capacity among popular strains of laboratory mice (i.e. Balb/c > SWR/J > CBA/J > C57L/J > C3H/HeJ > C3Heb/FeJ > C57Bl/6J > AKR/J > DBA/2J > A/J; 48). Mice are motivated to run by using aversion stimulus (47). Mice are acclimated to the treadmill days before their endurance test so as to ensure an exhaustion response is evaluated as opposed to a fear response (47). Sedentary controls are placed on a non-moving treadmill to eliminate stress hormone differences between treatments (47). Mice are nocturnal, so exercise tests are performed during their dark cycle with only red lights (47). Endurance test protocols vary depending on exercise intensity and parameters being evaluated (47). However, in tests of exercise exhaustion, treadmill speed is incrementally increased (47).

Mouse exercise performance may also be evaluated using a swimming test (49). This method is advantageous in that the materials are cheaper and swimming can be considered a clearer test of endurance time; however, this absolute response is potentially a response to fear and exhaustion (50). Continuous swimming may be encouraged by adding a current to the swimming pool. This addition has been shown to increase precision (50). Adding weight to the tail also encourages continuous swimming (47).

Regulation of fuel selection during exercise.

Despite the straightforward nature of TCA cycle's primary goal, its centrality in whole body metabolism means that changes in TCA cycle flux can affect physiological status (22). Exercise intensity and duration have a significant effect on the proportion and source of

fatty acids or carbohydrate used for energy in skeletal muscle tissues (15; 33; 51). During low to moderate intensity exercise (25-65% $VO_{2\ max}$) in humans, plasma FFA contribute the greatest proportion of energy to skeletal muscle tissue (51). After 30 minutes of exercise at 65% $VO_{2\ max}$, skeletal muscles have been shown to increase fat oxidation rates above that at either 25 or 85% $VO_{2\ max}$ (51). However, this is mostly a result of increased endogenous fat and carbohydrate oxidation (51). Nevertheless, after two hours of exercise at 65% $VO_{2\ max}$ plasma FFA have been shown to supply 90% of the fat being oxidized and as such, there is an increase in reliance on exogenous energy stores (51). It is suggested the endogenous stores are used to maintain the skeletal muscle tissues until FFA can be sufficiently released by the adipose tissue (51). However, plasma FFA are the predominant energy source only during low to moderate intensity prolonged exercise (51). When exercise intensities increase to 85% $VO_{2\ max,}$ after 30 minutes FFA release from adipose tissue is decreased, while plasma glucose levels are elevated and maintain the exogenous energy contribution (51). Plasma FFA are generated either from the diet or as the liver and adipose tissues send FFA bound to albumin and in triglyceride-containing lipoproteins to the skeletal muscle, respectively (52). These lipoproteins are bound by LPL and fatty acids are hydrolyzed from the lipoprotein and used by the skeletal muscle tissue (52). Muscle fiber type is related to fuel selection (52). Type I skeletal muscle fibers use plasma FFA for energy during prolonged exercise. As such, they have high concentrations of mitochondria and high LPL activity (52). Skeletal muscle fiber type IIa predominantly uses endogenous TG (52). These skeletal muscle fibers have low LPL activity (52). Glycolytic muscle fiber type IIb is recruited when exercise intensity is increased even further (52).

Fatty acid oxidation in the skeletal muscle tissues is regulated by epinephrine, norepinephrine and insulin (3;33;51). As exercise time prolongs and/or increases, circulating epinephrine levels have been shown to increase and increase lipolysis in adipose tissue (51). Insulin has been shown to decrease LCFA oxidation by more than 40% and concomitantly increase glucose release and oxidation (14). It is suggested this is mediated through the increase in acetyl-CoA from glycolytic flux causing inhibition of CPT-I, decreased activity of LPL and decreased levels of CoASH in the mitochondrion (14;15;52). This regulation may impair energy production and actually lead to depletion of glycogen stores in prolonged exercise (3;14;33).

Anaplerotic methyl-branched fatty acids.

The anaplerotic characteristic of methyl-branched fatty acids is due to their structures (Figure 1). For example, synthetic 2MeP consists of a five-carbon chain with a methyl branch on the 2-carbon. This methyl branch makes an enormous difference in the products that result from the oxidation of 2MeP. 2MeP, like all MCFA, is characteristically rapidly oxidized in the mitochondrial matrix of a cell (2). This rapid oxidation is due to the fact that MCFA may bypass CPT-I regulation of fatty acid entry into the mitochondrion and enter relatively unregulated β-oxidation (13). With straight chain MCFA hexanoic acid (C6), this action would typically produce an acetyl-CoA and a fatty acyl-$CoA_{(n-2)}$ molecule with each cycle (11). While this does occur in catabolism of 2MeP-CoA, a branch point at the 2-carbon of 2-MeP chain in combination with an odd chain length of 5 carbons results in the production of

two 3-carbon propionyl-CoA molecules rather than the normal product of 2-carbon acetyl-CoA, much in the same way as long branched chain fatty acid pristanic acid (36;12).

Anaplerotic methyl-branched LCFA are naturally-occurring chlorophyll derivatives; phytanic acid and pristanic acid (Figure 1; 12). Pristanic acid is actually a metabolite of phytanic acid produced during α-oxidation (12). Phytanic acid must be α-oxidized to pristanic acid because a branch point at the 3-position of the molecule prevents fatty acyl-CoA dehydrogenase from accessing the β-carbon of phytanic acid (12). However, when pristanic acid is chain-shortened by one carbon in α-oxidation, it allows all of the methyl-branches to fall in order for β-oxidation (12). Pristanoyl-CoA proceeds through β-oxidation in much the same way as any fatty acid; except it produces a total of one isobutyryl-CoA, three propionyl-CoA, and three acetyl CoA molecules (12). The isobutyryl-CoA and propionyl-CoA molecules can be converted to succinyl-CoA through the anaplerotic methylmalonyl pathway (53). Additionally, phytanic acid can act as a retinoid X receptor and phytanic acid and pristanic acid may act as peroxisome proliferator-activated receptor-α agonists (54). Thus, phytanic and pristanic acids have the added effect of bioactivity as they may contribute to lipid homeostasis (54). In all, once methyl-branched fatty acids have been catabolized to propionyl-CoA, they may enter the anaplerotic methylmalonyl pathway (Figure 10; 11;34;36). In this way, methyl-branched odd chain fatty acids have the potential to stimulate fat oxidation via anaplerotic release of inhibitory mechanisms in the fat oxidation pathway (22;36).

Toxicological evaluation of medium chain triglycerides.

As aforementioned, the difference in methyl-branched odd chain MCFA feeding is evident in the catabolic production of propionyl-CoA instead of acetyl-CoA (11;36). Thus, with t-2MeP feeding, unregulated β-oxidation of branched chain fatty acids produces excessive propionyl-CoA molecules instead of excessive acetyl-CoA molecules, and thus a very different physiological effect due to the difference in catabolites (13;11). Propionyl-CoA overload, or propionic acidemia, can theoretically occur with odd chain fatty acid feeding (11). However, it has been shown that with odd chain feeding in humans at 30% of the calories over 27 months, propionyl overload was not indicated as urinary metabolites 3-hydroxypropionate, propionylglycine and methylcitrate did not approach levels associated with propionyl acidemia (11).

The toxicological effects of MCT have been thoroughly investigated. In a 30-day rat oral gavage study, rats experienced no adverse effect of up to 21.3 mL/kg body weight per day of a MCT bolus except temporarily (on days 5-7) where rats experienced decreased food intake and diarrhea (55). When rats were administered MCT at 5% of the diet for 3 months, no adverse effects were observed (55). However, when very young male rats (weanling) were fed a MCT-LCT mixture at 15% of the diet for three months, they experienced lower relative and absolute liver weights and increased feed intake (55). They experienced no other toxicologically significant changes (55).

In some cases, diets high in MCT have been shown to decrease weight gain and fat deposition (49;55;56). The decreased energy density of MCFA, their inability to be directly

stored in the body and the thermogenic energy expenditure associated with catabolism and disposal of MCFA all have been attributed for this difference in energy metabolism of MCT versus LCT (55). A review of toxicological studies found no toxicologically significant effects of feeding very high levels of MCT in the diet (11.2%) for six weeks, except for 20% decreased weight gain and 23% decreased fat deposition (55). In acute human studies, feeding 1 g MCT/kg body weight changed fatty acid profiles but did not produce any toxic effects. In a second, feeding approximately 95 g MCT caused increased levels of serum FFA, but no other effects (55). In chronic human studies, when patients were fed MCT as the sole source of fat at 40% of diet calories, cholesterol levels were decreased (as compared to a butter treatment, but not as compared to a corn oil treatment) and temporary nausea and fullness on days 3-4 of the 10 week crossover study (55). In another chronic study, non-obese males were fed 40% of calories as MCT in meals consisting of 150% of energy requirements (55). No toxic effects were detected (55). However, fasting serum cholesterol was decreased in control LCT fed males but not MCT fed males (55). Furthermore, fasting serum TG were three times higher with MCT feeding (55). Thus, it is indicated that energy balance influences the effects of MCT (55). Regardless, MCT were non-toxic in all study subjects (55).

In this study, we hypothesized that methylated fatty acids (e.g. 2MeP, phytanic acid or pristanic acid) would provide a novel source of anaplerotic carbon and thereby enhance fatty acid oxidation, especially under stressed conditions when TCA cycle intermediates are depleted. We believed these glucogenic fatty acids will enhance fatty acid oxidation by supplementing TCA cycle intermediates through the methylmalonyl pathway instead of

supplementing TCA cycle substrate acetyl-CoA, as seen with ketogenic fatty acids like C6 or palmitate.

Figures

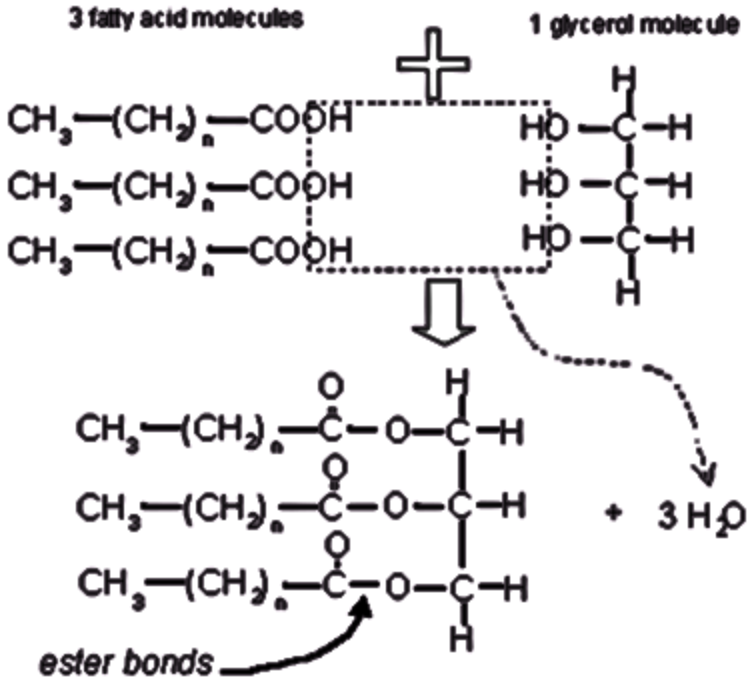

Figure 1 Triglyceride formation (56).

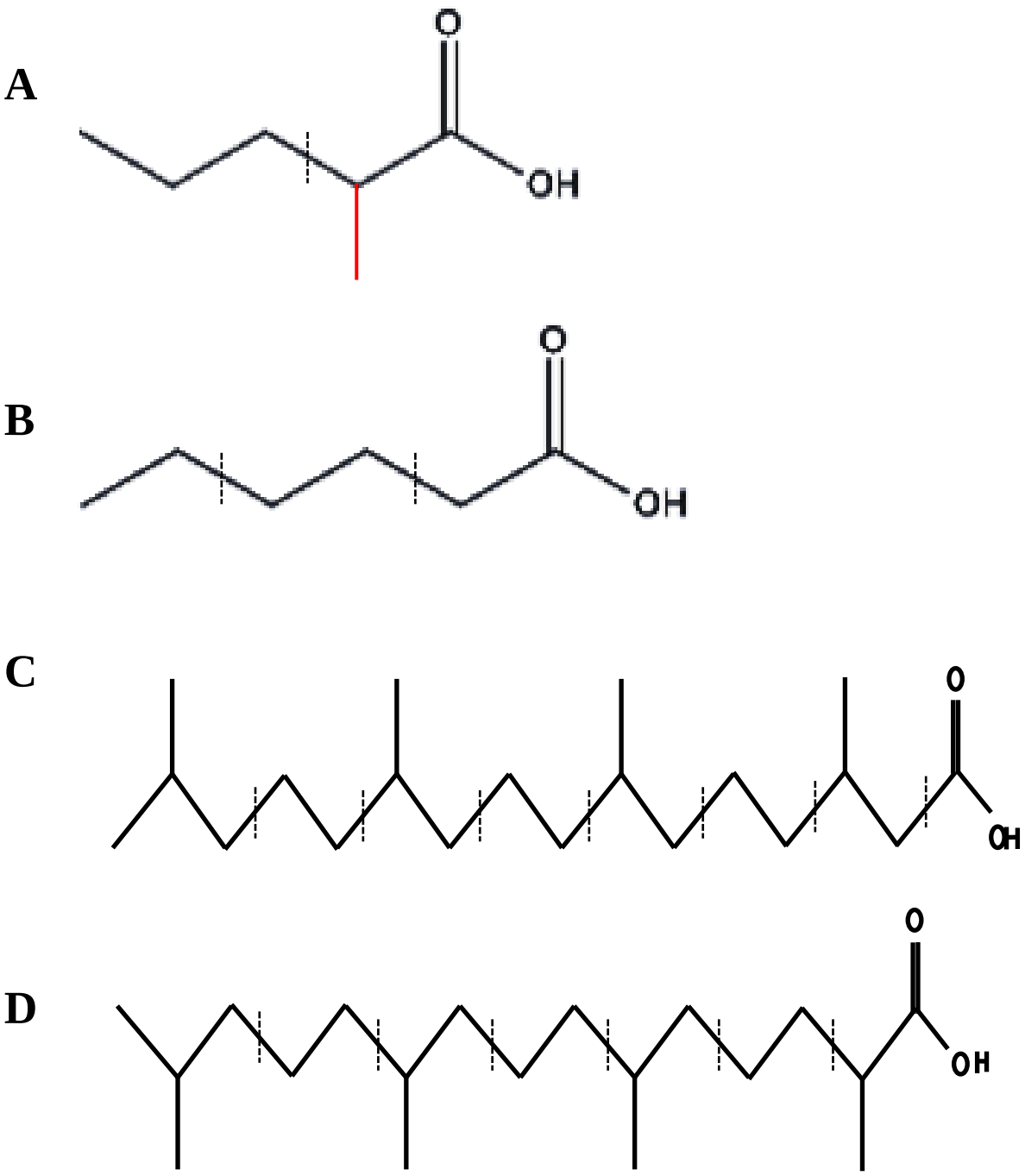

Figure 2 Straight chain and anaplerotic fatty acids. A: 2-methylpentanoate; (C6); B: hexanoic acid; C: phytanic acid; D: pristanic acid. Fatty acid oxidation cleavage is indicated with dashed lines.

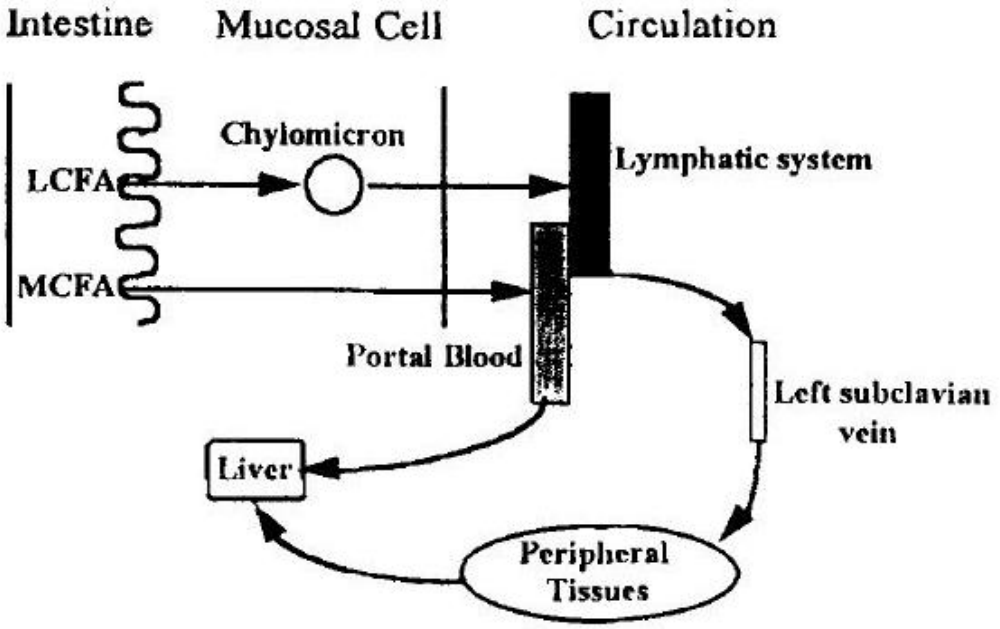

Figure 3 Summary of dietary MCFA & LCFA absorption & transportation (57).

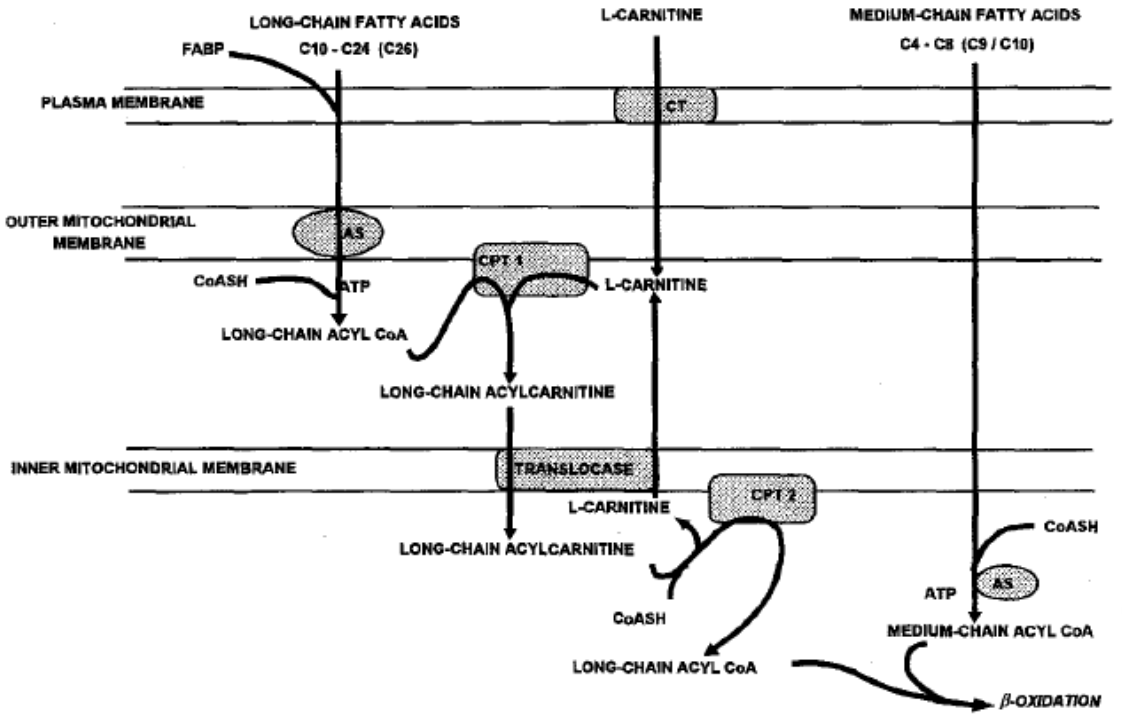

Figure 4 Fatty acid transport across the mitochondrial membrane (9). FABP: fatty acid binding protein; CT: carnitine transporter; AS: acyl-CoA synthetase; translocase: carnitine-acylcarnitine translocase.

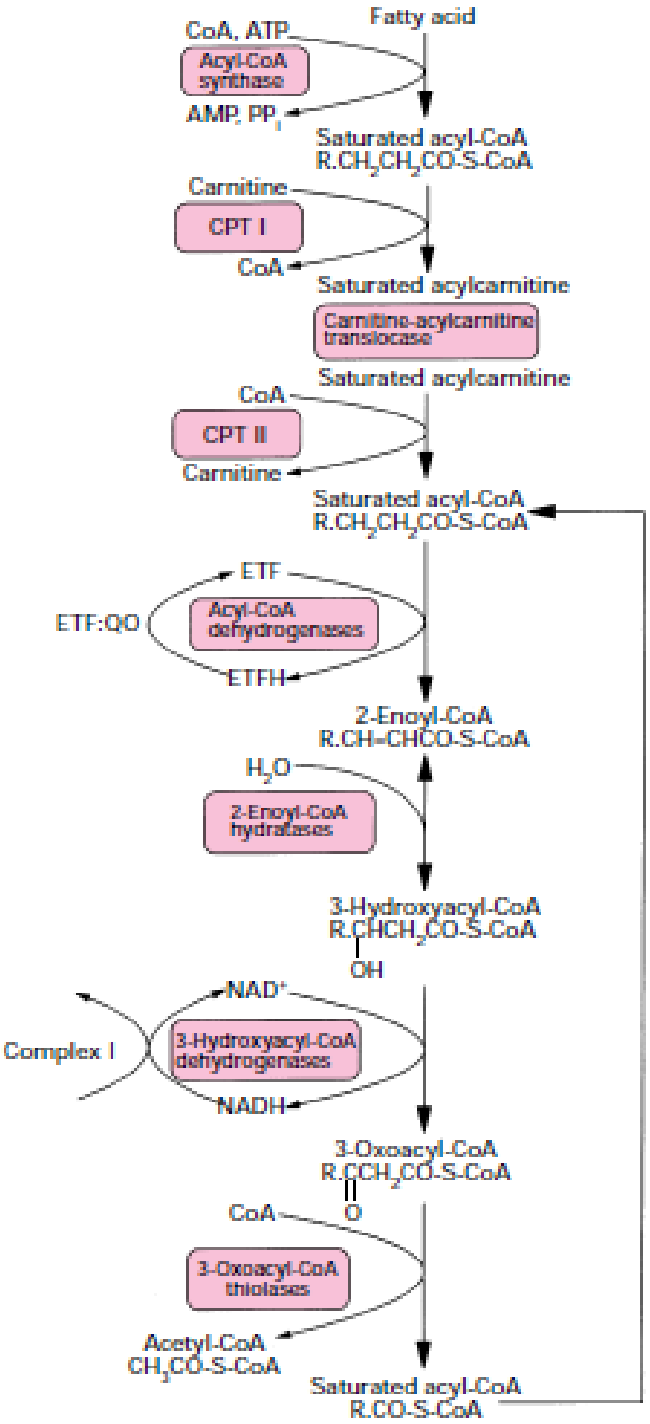

Figure 5 The pathway of mitochondrial β-oxidation (10). ETF: electron transport flavoprotein; ETF:QO: ubiquinone oxireductase; ETFH: reduced ETF.

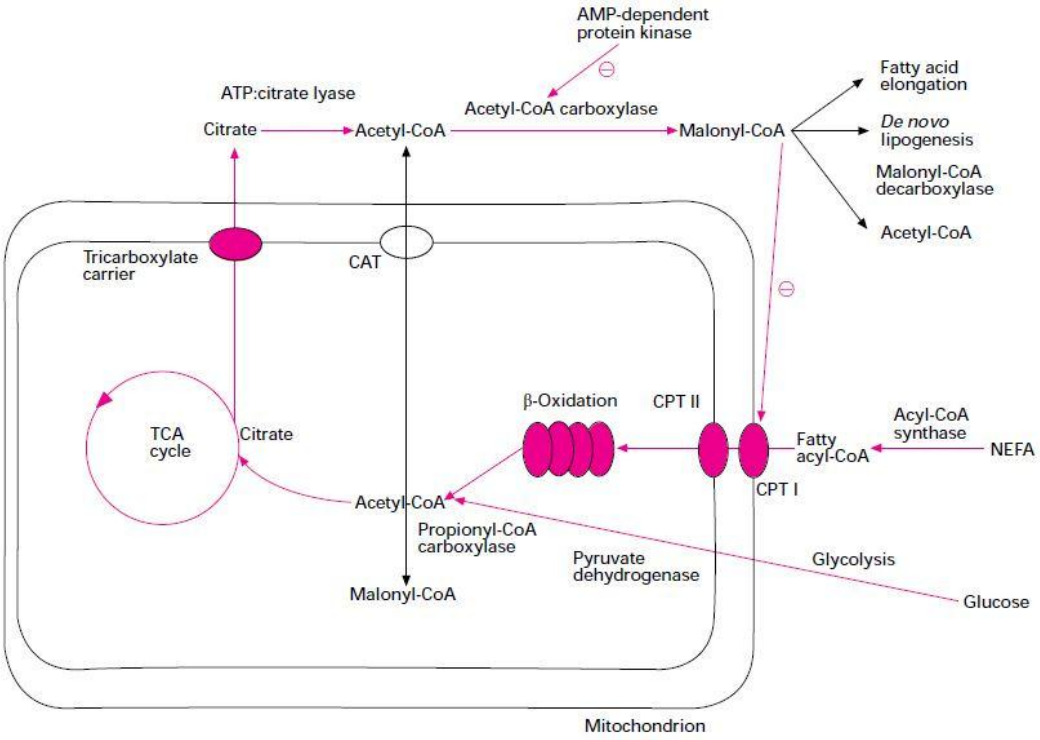

Figure 6 Malonyl-CoA regulation of CPT-I (10). NEFA: non-esterified fatty acid.

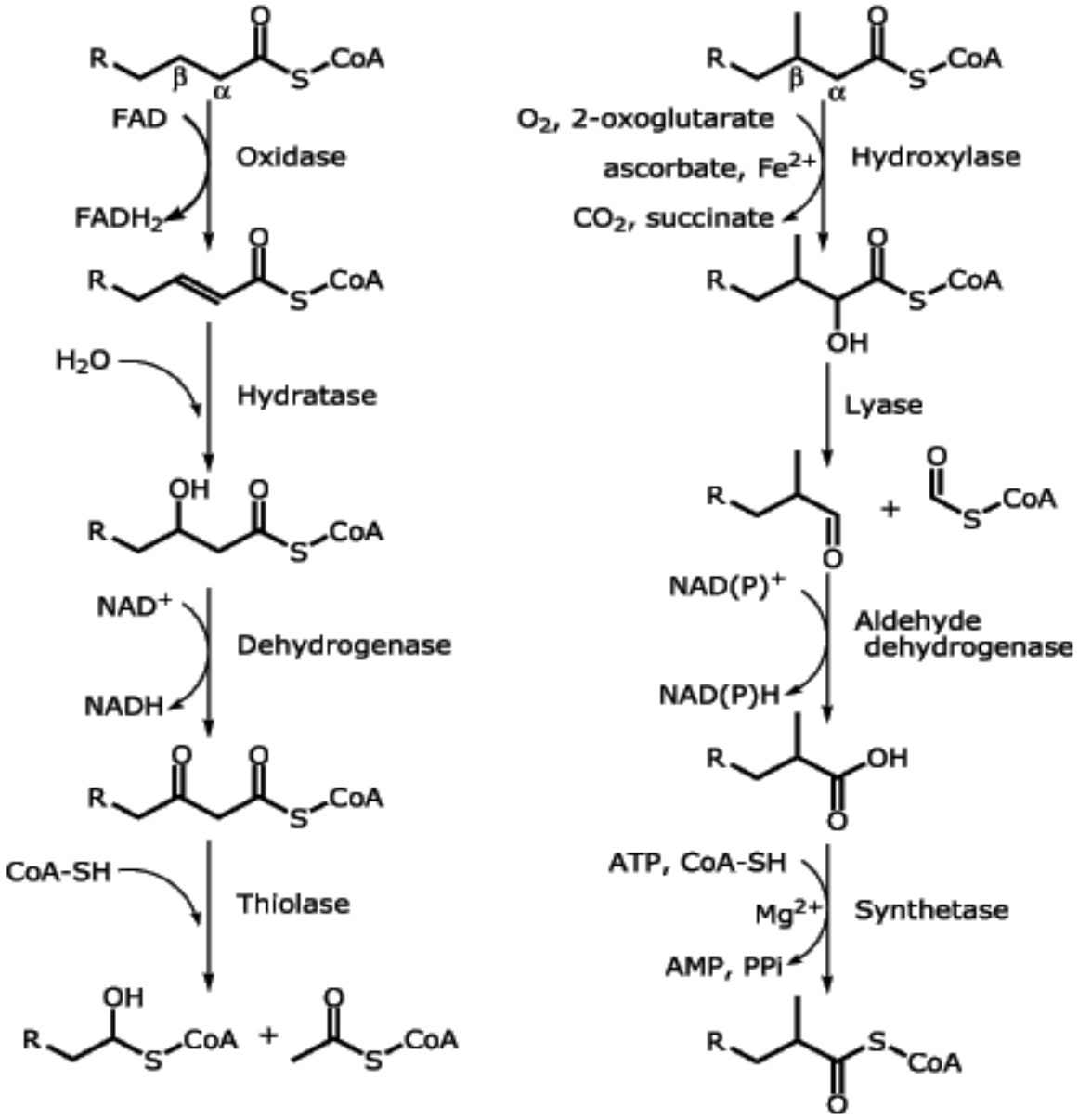

Figure 7 A comparison of α- and β-oxidation. Left: α-oxidation; Right: β-oxidation (17).

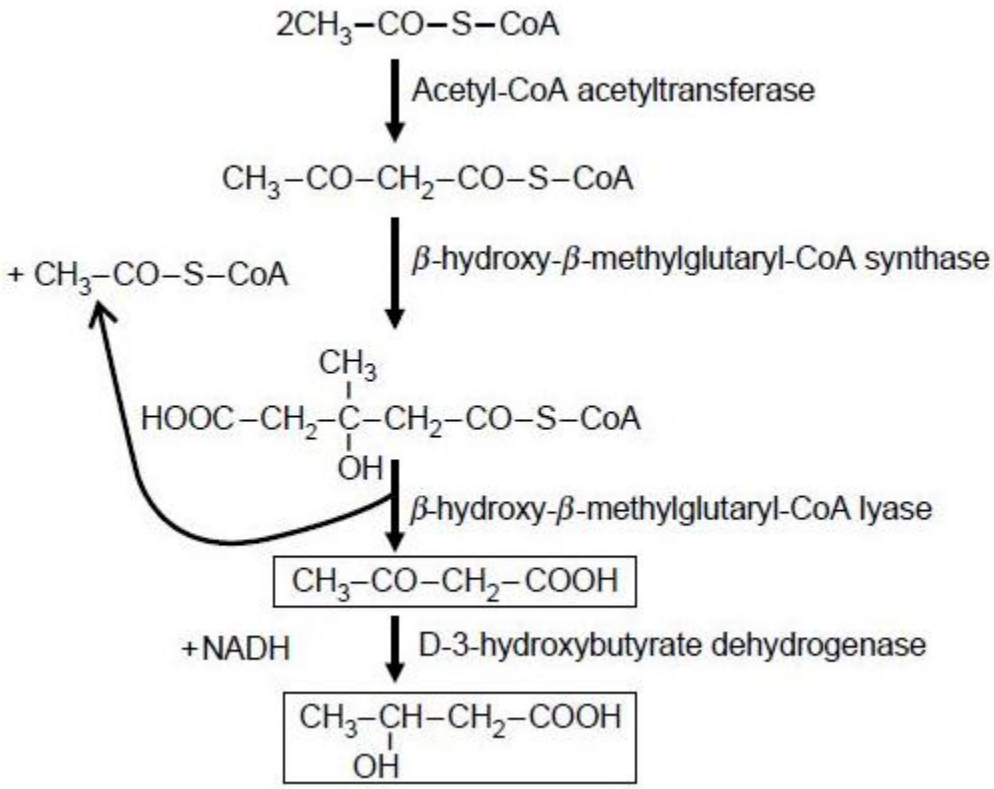

Figure 8 The ketone body synthesis pathway (24).

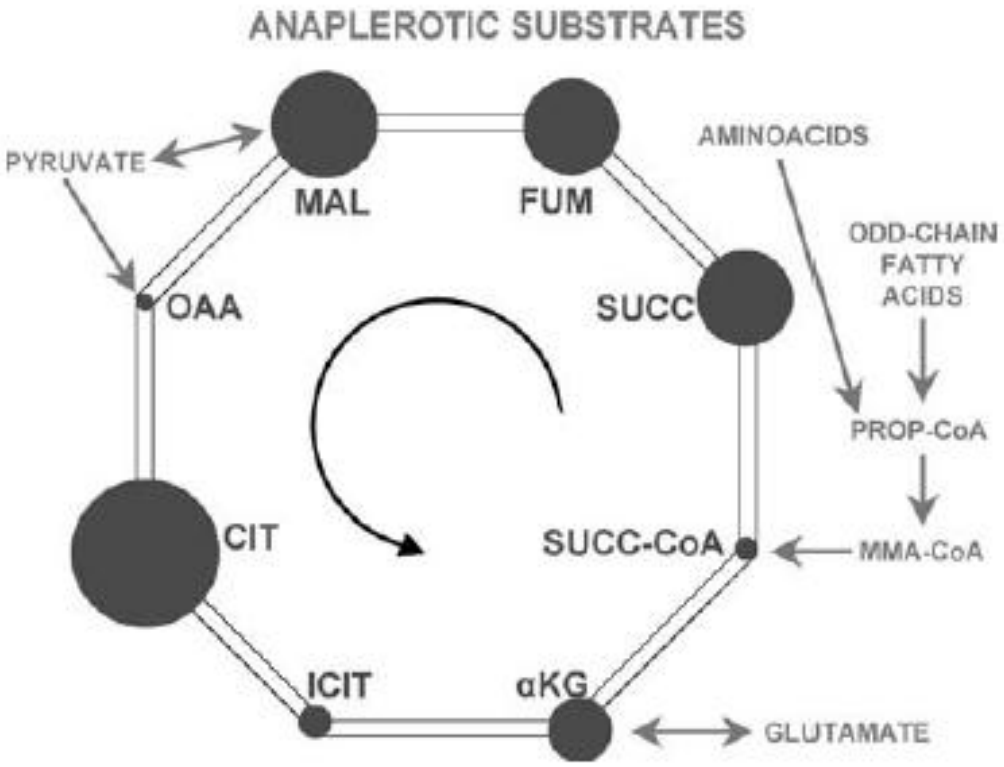

Figure 9 Anaplerotic pathways for the TCA cycle (11).

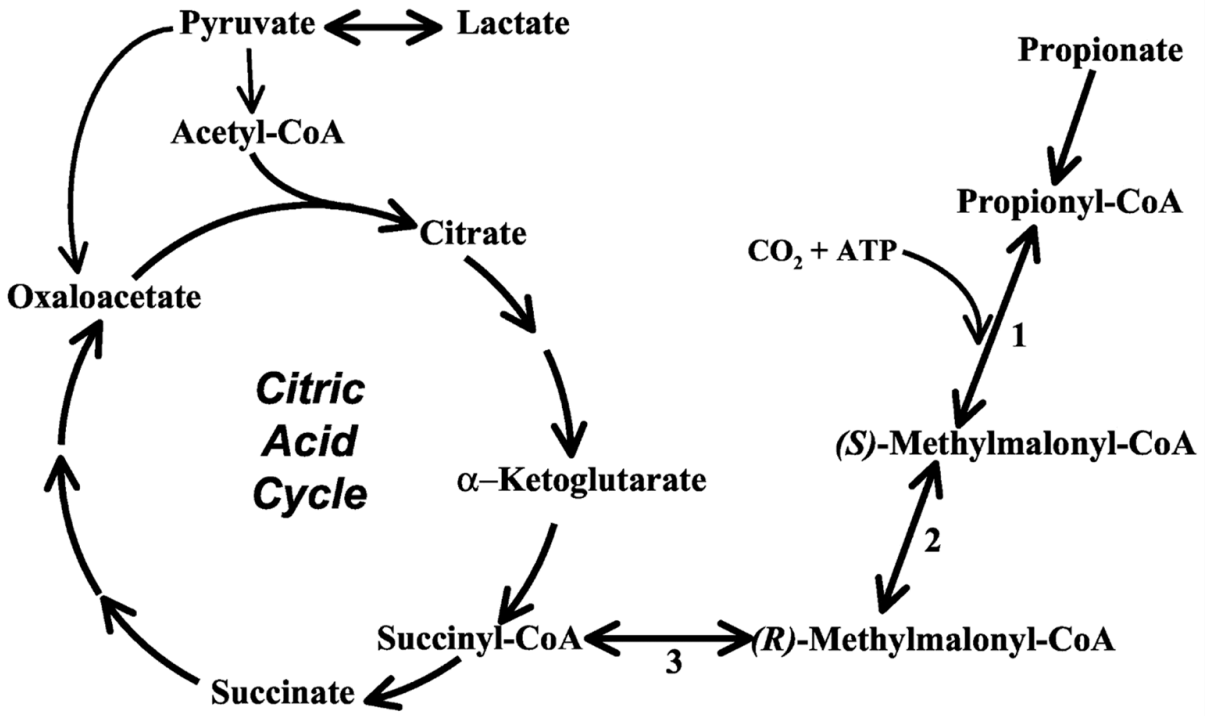

Figure 10 The methylmalonyl pathway (56). 1: propionyl-CoA carboxylase; 2: methylmalonyl-CoA racemase, 3: methylmalonyl-CoA mutase (57).

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Chapter II: Methyl-branched fatty acids provide anaplerotic carbon to stimulate *in vitro* fatty acid oxidation in exercised or sedentary mice.

Abstract

The purpose of this study was to evaluate the ability of anaplerotic methyl-branched fatty acids to enhance *in vitro* fatty acid oxidation by liver and skeletal muscle tissues from exercised or sedentary mice. Incubations of 0.25 mM 2-methylpentanoic acid (2MeP), hexanoic acid (C6), palmitate, phytanic acid, or pristanic acid or 0.1 mM malate or propionyl-CoA were conducted with liver and skeletal muscle homogenates from exercised or sedentary mice. *In vitro* oxidation of [1-^{14}C]-oleic acid in liver homogenates with 2MeP led to increased mitochondrial $^{14}CO_2$ accumulation (P <0.05), but no change in [^{14}C]-acid soluble products (ASP) accumulation as compared to C6 (P > 0.05). Phytanic acid treatment increased [^{14}C]-ASP accumulation in liver tissue as compared to palmitate (P < 0.05). Exercise increased [^{14}C] accumulations (P < 0.05). Results were consistent with our hypothesis that methyl-branched fatty acids (2-MeP, phytanic and pristanic acids) provide anaplerotic carbon to the tricarboxylic acid cycle and thereby stimulate *in vitro* fatty acid oxidation in liver and skeletal muscle tissues, especially in tissues from exercised mice.

Keywords: medium chain fatty acid; methyl-branched fatty acid; odd-chain fatty acid; fatty acid oxidation.

Introduction

During exercise, skeletal muscle switches from using approximately 40% nonesterified fatty acids and 60% carbohydrate to 60% fatty acids and 40% carbohydrate (1). However, with the increase in exercise intensity, the skeletal muscle's rate of plasma nonesterified fatty acids uptake cannot sustain the increased rates of fat oxidation (1). As a result, the skeletal muscle begins hydrolyzing endogenous triglycerides for energy (1).

Fatty acid oxidation must be maintained at an optimal rate for energy production, and during normal physiological conditions at rest this is not a challenge (2;3). Beta-oxidation combusts fatty acids efficiently, being regulated primarily by the carnitine palmitoyltransferase (CPT) system (2). However, during extended or intense exercise, the true limitation to energy production is not due to impairment of fatty acid catabolism, but instead to an inability to effectively use the acetyl-CoA produced by β-oxidation (4). This limitation is due to insufficient TCA (tricarboxylic acid) cycle intermediates (TCAI) which puts a secondary inhibition on fatty acid oxidation (2;4). The synergism between carbohydrate and fatty acid metabolism for energy production is memorably described in the adage, "fat burns in the flame of carbohydrate" (5) where it is clear that fat cannot be completely oxidized for energy, or burned, without carbohydrate intermediates. The need for carbohydrate or amino acids derived intermediates was first described by the term anaplerosis (Greek: ana-, up; perotikos, to fill) to describe reactions which add to the TCAI (6). The TCA cycle's eight TCAI are referred to as, "catalytic intermediates" because they are completely regenerated through a turn of the TCA cycle (7). At rest, these molecules

remain at very low concentrations (8;9). This was evidenced by Aragon and Lowenstein's research indicating a total TCAI level of 1.44±0.21 umol/g dry weight in resting rat skeletal muscle (8), and confirmed in human skeletal muscle by Gibala *et al.*'s research showing TCAI concentrations of 1.39±0.11 mmol/kg dry weight (9). However, according to Gibala, from rest to 10 minutes of exercise, TCAI levels increased to 2.88 ± 0.31 mmol/kg dry wt, and increased to 5.38 ± 0.31 mmol/kg dry wt at exhaustion (9). These increases corresponded with approximately 70-fold and 100-fold increases in TCA cycle flux, respectively (9). Thus, despite the straightforward nature of the TCA cycle's primary function, its centrality in whole body metabolism requires flexibility in the TCAI in response to shifts in physiological state (7). For example, under stressed conditions of fasting or exercise, metabolic substrate channeling decreases anaplerosis of the TCA cycle (7). This switch is a problem in the face of cataplerosis or high energy demands (7;10). In cataplerosis, 2-carbon molecules stall at the TCA cycle for complete oxidation to carbon dioxide (CO_2) because TCAI are leaked from the cell membrane (7). Cataplerosis depletes 1-2% of the TCAI pool per minute and can be indicated by creatine kinase in the serum (7). The resulting stalling of the TCA cycle causes hepatic ketogenesis (10). Thus, the flux of β-oxidation is feedback inhibited as there is an increased need for the disposal of incompletely oxidized fatty acids (2). As such, during stressed, low-energy conditions, cataplerosis may impair the TCA cycle's capacity to oxidize acetyl-CoA molecules for energy (7).

We hypothesize that methyl-branched fatty acids (namely, 2MeP, phytanic and pristanic acids) will provide a novel source of supplementary TCAI (via propionyl-CoA) and will thereby stimulate fatty acid oxidation by liver and skeletal muscle tissues. We further

hypothesized that the effects would be greater in tissues derived from mice that were exercised to exhaustion at a submaximal running intensity.

Materials & Methods

Experimental design.

Liver and skeletal muscle tissue homogenates were used to determine the specific tissue-level and subcellular-level metabolic effects of methyl-branched fatty acids as compared to unbranched fatty acids (i.e., hexanoate or palminate), malate and propionyl-CoA controls. First, a dose-response experiment was conducted to elucidate the optimal medium chain fatty acid (MCFA) concentration. Then, in the second experiment, a long chain fatty acid (LCFA) pristanic acid dose response was elucidated. Last both long and medium chain fatty acids were applied to tissue samples from sedentary or exercised animals at a selected concentration of 0.25 mM. Tissue metabolism was measured as total fatty acid oxidation using labeled [1-^{14}C]-oleic acid as described previously (18; 19; 20; 21) as modified below. The MCFA dose response experiment was repeated four times (n=4). The LCFA dose response experiment was conducted once (n=1). The exercised versus non-exercised animal tissue sample experiment was repeated five times (n=4). All experiments were approved by the Institutional Animal Care and Use Committee (IACUC).

Tissue homogenates.

In the MCFA dose response experiment, four 5-7 week old male C57BL/6J mice from North Carolina State University (NCSU) laboratories were fasted for 24 hours prior to euthanasia and tissue sampling. The 24 hour fast has been shown to deplete skeletal muscle glycogen levels to 66% of fed glycogen levels and liver glycogen levels to 1.2% of that in fed rats (11). Two days prior to euthanasia and tissue sampling, in the LCFA dose response

experiment, and in the exercised versus non-exercised animal tissue sample experiment, eight mice (5-7 week old C57BL/6J mice, Jackson Laboratories, Bar Harbor, ME) were given a treadmill orientation consisting of a 10 min run at 8 m/min. Then on the day of the experiment, four mice were exercised to exhaustion using a treadmill (Exer 3/6M, Columbus Instruments) set to a 20° incline with an initial speed of 12 m/minute. The speed was increased by 1 m/min 2, 5, 10, 20, 30, 40, 50, and 60 min after the initiation of the exercise to achieve exhaustion. Methods of exercise exhaustion were modified from Pederson *et al.* (12). Mice were determined to be exhausted after tolerating 5 seconds on the shock grid instead of resuming treadmill running (13). Mice ran for approximately 1.5 h. Sedentary mice were paired mice from the same cage (four mice per cage; two were placed in the exercised treatment group; two in the sedentary treatment group). Sedentary mice were subject to similar conditions as those of the exercised mice. To decrease differences in treatment group stress responses during the exercises treatment, sedentary mice were placed on a treadmill which was turned off.

Liver and hindlimb skeletal muscle tissues were harvested from each mouse and homogenized using methods modified from Yu *et al.* (14). Samples were pooled according to tissue type in homogenate buffer to ensure sufficient tissue for a complete experimental replicate. Homogenate buffer contained 220 mM mannitol, 70 mM sucrose, 2 mM HEPES (hydroxyethyl piperazineethanesulfonic acid) and 0.1 mM disodium EDTA (ethylenediaminetetraacetic acid) adjusted to pH 7.2 at 4 ℃ using potassium hydroxide (KOH; 14). Liver tissues were homogenized on ice in homogenate buffer at a 1:7 (w/w) ratio and skeletal muscle tissues were homogenized at a 1:4 ratio. Tissues were gently

homogenized using 3 passes of a manual glass homogenizer (7mL All-Glass Tenbroeck, Kontes/Kimble Chase).

Total fatty acid oxidation: collection of $^{14}CO_2$ & [^{14}C]-acid soluble products.

In the MCFA dose response experiment, duplicates of 0.25 mL tissue homogenate were aliquoted to 25 mL Erlenmeyer flasks containing incremental amounts of 0, 0.25, 0.5, 0.75, or 1.0 mM treatment fatty acid in substrate buffer, with or without addition of 50 µM antimycin A (A8674, Sigma-Aldrich, St. Louis, MO) together with 10 µM rotenone (R8875, Sigma-Aldrich, St. Louis, MO) mitochondrial oxidation inhibitor (15). These mitochondrial inhibitors allow quantification of the first round of peroxisomal β-oxidation of [1-^{14}C] oleic acid (15). Thus, the mitochondrial oxidation rate is calculated as the difference between total (uninhibited) and peroxisomal (inhibited) oxidation rates. Treatments were 2MeP (W275409, Sigma-Aldrich, St. Louis, MO), hexanoic acid (C6; 153745 Sigma-Aldrich, St. Louis, MO), 0.1 mM malate (M1000, Sigma-Aldrich, St. Louis, MO) or 0.1 mM propionyl-CoA (P5397, Sigma-Aldrich, St. Louis, MO; 15). Treatments were complexed with bovine serum albumin (BSA) in a 5:1 ratio. Each treatment contained one pair of duplicate flasks. The experiment was replicated four times (n=4).

The MCFA dose response experiment was conducted using substrate buffer containing 50 mM sucrose, 150 mM tris-hydrochloride (tris-HCl), 20 mM dipotassium phosphate (K_2HPO_4), 10 mM magnesium chloride hexahydrate ($MgCl_2$•$6H_20$), 2 mM disodium EDTA, 1 mM carnitine, 0.2 mM coenzyme A (CoA), 2 mM nicotinamide adenine

dinucleotide (NAD), 10 mM ATP and 0.0022 mM vitamin B12, adjusted to pH 7.4 at 4 ℃ using KOH (14).

First, 0.25 mL of liver or skeletal muscle homogenate was added to designated flasks and allowed to pre-incubate in a 35°C shaking water bath for 30 minutes. This allowed for antimycin A/rotenone inhibition of the mitochondria. Fatty acid oxidation measurement was initiated by the addition of 0.25 mL of 1mM [1-^{14}C]-oleic acid (0297, American Radiolabeled Chemicals, St. Louis, MO) complexed 5:1 with BSA in substrate buffer to each flask. Immediately, the sample was gassed with a O_2/CO_2 (5/95%) mixture and the Erlenmeyer flask stopper replaced by a stopper with a suspended microfuge $^{14}CO_2$ collection tube containing 0.5 mL ethanolamine. After incubating in a 35°C shaking water bath for 30 minutes, flask fatty acid oxidation was terminated with addition of 0.5 mL 3.5 M perchloric acid (15).

The $^{14}CO_2$ capture was complete after 2 hours at 25°C. Individual vials of $^{14}CO_2$ were quantified using liquid scintillation counting. $^{14}CO_2$ was used as a measure of completely combusted products. Flask contents were centrifuged so that supernatant would be separated from perchloric acid precipitated protein and unmetabolized [1-^{14}C]-oleic acid. Supernatant was collected for [^{14}C]-ASP quantification using liquid scintillation counting. [^{14}C]-ASP was used as a measure of incompletely oxidized products such as ketone bodies and TCA cycle intermediates (TCAI). Blanks were collected by immediately adding perchloric acid to designated flasks after adding tissue homogenate. Blank corrected radioactivity of $^{14}CO_2$ + [^{14}C]-ASP was used as measures of [1-^{14}C]-oleic acid catabolism (15).

The LCFA dose response experiment was conducted in the same manner as described above except with incremental amounts of 0, 0.25, 0.5, 0.75, or 1.0 mM pristanic acid (P6617, Sigma-Aldrich, St. Louis, MO) in substrate buffer (14). Antimycin A together with 10 µM rotenone mitochondrial oxidation inhibitors were applied only at the 0.25 mM pristanate level (15).

The exercised versus non-exercised animal tissue sample experiment was also conducted in the same manner except fatty acid treatments were applied at 0.25 mM concentrations only. Treatments were 2MeP, C6, sodium palmitate (P9767, Sigma-Aldrich, St. Louis, MO), phytanic acid (P4060, Sigma-Aldrich, St. Louis, MO), pristanic acid, 0.1 mM malate or 0.1 mM propionyl-CoA.

The LCFA dose response experiment and exercised versus non-exercised animal tissue sample experiment were conducted using a substrate buffer contained 50 mM sucrose, 150 mM tris-HCl, 20 mM K_2HPO_4, 10 mM $MgCl_2 \cdot 6H_20$, 2 mM disodium EDTA, 1 mM carnitine, 0.2 mM CoA, 2 mM NAD, 10 mM ATP, 25 mM potassium carbonate ($KHCO_3$), 0.0022 mM vitamin B12, and 0.0037 mM biotin adjusted to pH 7.4 at 4 ℃ using KOH (14;16). All reported values were normalized to tissue protein content. Tissue protein content was quantified using the biuret method (17).

Oxygen consumption.

In the third experiment, tissue oxygen consumption rates from exercised and non-exercised animal tissues were measured using a biological oxygen monitor (YSI 5300 Biolog O_2 monitor). Air saturated water (8.6 mg O_2/L) at 25°C was used as a standard and set to

100%. Measurements were made with 0.125 mL exercised and non-exercised liver homogenates and 0.25 mL exercised and non-exercised skeletal muscle homogenates. Samples were brought up to 3 mL in substrate buffer and measured over the course of 30 and 45 minutes, respectively.

Statistical analysis.

Both MCFA and LCFA dose response data were analyzed according to a completely randomized block design blocked by replicate. Analysis of variance using the mixed model procedure of SAS (version 9.2, SAS Inst. Inc., Cary, NC) was used to calculate least-squares means (LSM) and standard error of means (SEM).

The experiment using exercised versus non-exercised animal tissue samples was analyzed using the SAS general linear model procedure to calculate least-squares means (LSM) and standard error of means (SEM). Statistical analyses were analyzed according to a 7 x 2 completely randomized block design (seven fatty acid treatments x exercised or sedentary), blocked by replicate. Replicate-by-exercise state was treated as a random effect.

In all experiments, comparisons were adjusted using Tukey's test. Results were considered significant when $P < 0.05$ and trends were considered when $P < 0.10$.

Results

In vitro fatty acid oxidation response to increasing doses of fatty acids in skeletal muscle and liver tissues.

Liver tissue *in vitro* $^{14}CO_2$ accumulation from oxidation of [1-^{14}C]-oleic acid was maximized with a 0.25 mM dose of either C6 or 2MeP (Table 1; Figure 1A; $P < 0.05$). Furthermore, 2MeP was shown to bolster whole tissue $^{14}CO_2$ accumulation above that of treatment with C6 at all treatment concentrations (Figure 1A; $P < 0.0001$). There was a trend in fatty acid-by-dosage interaction oxidation measurements seen in increasing difference between 2MeP and C6 total fatty acid oxidation levels with increasing dosages (Table 1; $P = 0.065$). At 0.25 mM, 2MeP stimulated total liver tissue $^{14}CO_2$ accumulation to 204% that of C6. At 0.5 mM, 2MeP stimulated fatty acid oxidation to 234% of its C6 counterpart, 267% at 0.75 mM and 290% at 1.0 mM. Liver tissue [^{14}C]-ASP *in vitro* fatty acid oxidation rates were not different between treatments (Figure 1B; $P > 0.05$).

The percentage of liver tissue *in vitro* fatty acid oxidation occurring in mitochondria was greater with treatment of 0.25 mM 2MeP than 0.25 mM C6 (70.9 versus 56.1%, respectively; Figure 3B; $P < 0.05$). This apparent shift to mitochondrial fatty acid oxidation was driven by an overall increase in mitochondrial $^{14}CO_2$ accumulation in 2MeP-treated liver tissue as compared to tissues treated with C6 (Table 1; $P < 0.0001$). As such, in 2MeP-treated liver tissue, 99.1% of the total accumulative $^{14}CO_2$ came from mitochondrial oxidation while only 73.6% of the total accumulated $^{14}CO_2$ came from mitochondrial oxidation in C6 treated tissues. However, the percentage of mitochondrial $^{14}CO_2$ accumulation occurring *in vitro* in

liver tissue did not differ between treatments for all other dosages ($P > 0.05$). This pattern also carried to the total ($^{14}CO_2$ + [^{14}C]-ASP) percent of liver fatty acid oxidation contributed by mitochondrial oxidation (Figure 3B).

The $^{14}CO_2$ peroxisomal *in vitro* fatty acid oxidation in liver tissue did not differ between C6 and 2MeP (Table 1; $P = 0.902$). However, 2MeP peroxisomal [^{14}C]-ASP and total ^{14}C accumulation trended toward increased fatty acid oxidation above C6 treatment effects ($P = 0.057$; $P = 0.058$, respectively). Correspondingly, the percent of total [^{14}C]-ASP contributed by peroxisomal oxidation was greater with 2MeP than C6 treatment ($P < 0.05$).

Medium chain fatty acid accumulative skeletal muscle tissue oxidation rates were not different from one another (Table 2; $P > 0.05$). There was one exception; peroxisomal [^{14}C]-ASP accumulation. However, there were no differences between 2MeP and C6 treatment effects in the peroxisomal [^{14}C]-ASP accumulation ($P = 0.492$). Consecutive dosage oxidation rates did not differ from one another ($P > 0.05$). There was no interaction between fatty acid treatment and dosage ($P > 0.05$).

The addition of pristanic acid to liver tissue homogenates incrementally decreased ^{14}C total ($^{14}CO_2$ + [^{14}C]-ASP) accumulation (Table 3; Figure 4). From 0 mM to 0.75 mM pristanic acid, $^{14}CO_2$ accumulation decreased from 6.10 to 1.25 umol/h/mg protein. The [^{14}C]-ASP accumulation decreased from 33.9 to 15.2 umol/h/mg protein. However, the decreased accumulation of [^{14}C]-ASP from 0 to 0.25 mM pristanic acid was negligible (33.9 to 33.8 umol/h/mg protein). This was in comparison to positive control liver tissue treated with 0.1 mM malate in the same experiment. Malate treatment slightly decreased $^{14}CO_2$

accumulation as compared to the 0 mM pristanic acid treatment while increasing [^{14}C]-ASP production ($^{14}CO_2$ accumulation: 5.83 umol/h/mg protein; [^{14}C]-ASP accumulation: 49.5 umol/h/mg protein). However, the decrease in $^{14}CO_2$ accumulation resulting from the addition of 0.25 mM pristanic acid was 6.74-fold that of the 0.1 mM malate treatment.

The effect of methyl-branched fatty acids on in vitro fatty acid oxidation in liver and skeletal muscle tissues from sedentary and exercised mice.

There were no fatty acid treatment-by-exercise interactions evident in the oxidation rates of liver or skeletal muscle tissue samples (Table 4; Table 5; $P > 0.05$), except in the measurement of peroxisomal total $^{14}CO_2$ oxidation in skeletal muscle tissue (Table 5; $P < 0.05$). Therefore, treatment and fatty acids main effects were presented in graphs.

Treatment with 2MeP increased *in vitro* liver tissue total ($^{14}CO_2$ + [^{14}C]-ASP) fatty acid oxidation as compared to C6 control (12.4 vs 7.81 umol/h/mg protein accumulated $^{14}CO_2$, respectively, Figure 5; $P < 0.05$). However, the accumulations of [^{14}C]-ASP are not different between liver tissue treated with 2MeP or C6 (Figure 5B). Thus, there was an increase in [1-^{14}C]-oleic acid oxidation and complete combustion in the TCA cycle as the total level of oxidized products increased.

Treatment with 2MeP shifted liver mitochondrial fatty acid oxidation products to 26.9% $^{14}CO_2$. This was in contrast to 17.7% with C6 treatment (Table 4). This shift was a result of 2MeP's 1.52-fold increase in mitochondrial fatty acid oxidation and complete combustion over C6. There was no interaction between fatty acid treatment and exercise ($P > 0.05$); however, exercise increased the percent mitochondrial $^{14}CO_2$ accumulation (Figure 3;

$P < 0.05$). There were no significant differences between fatty acid treatment effects on total ($^{14}CO_2$ + [^{14}C]-ASP) percent mitochondrial accumulation (Figure 7; $P > 0.05$). Malate-treated liver percent total mitochondrial oxidation was the only exception as it was significantly lower than that of all other treatments ($P < 0.05$).

There were no effects of methyl-branched long chain fatty acids on $^{14}CO_2$ accumulation in the liver tissue (Table 4; Figure 5A; $P > 0.05$); however, in ^{14}C ($^{14}CO_2$ + [^{14}C]-ASP) whole liver tissue accumulation, phytanic acid was increased over the palmitate control (Table 4; Figure 5C; $P < 0.05$) while pristanic acid was almost identical to the palmitate control ($P = 0.983$). These differences between phytanic acid and palmitic acid and lack thereof between pristanic acid and palmitic acid were also seen in [^{14}C]-ASP accumulation rates (Figure 5B). However, these differences did not translate into differences in mitochondrial and peroxisomal oxidation rates, but instead just an overall increase in oxidation (Figure 7B).

Interestingly, there was a trend towards increased percent total ($^{14}CO_2$ + [^{14}C]-ASP) mitochondrial oxidation in pristanate treated skeletal muscle tissues as compared to palmitate treated skeletal muscle tissues ($P = 0.091$) while phytanate treated tissues were the same as palmitate treated tissues (Figure 7A; $P = 0.119$). Skeletal muscle LCFA oxidation was not different between treatments ($P > 0.05$). While there was an effect of fatty acid in several of the measurements of oxidation, the individual treatments were not different between methyl-branched fatty acids and their respective controls (Table 5; $P > 0.05$). Furthermore, there

were no differences in skeletal muscle tissue accumulation of $^{14}CO_{2,}$ [^{14}C]-ASP, or total ($^{14}CO_2$ + [^{14}C]-ASP).

In vitro oxygen consumption rates of liver and muscle tissues from sedentary and exercised mice.

In vitro oxygen consumption rates of liver tissue from exercised mice tended to be lower than liver tissue from sedentary mice (Table 6; Figure 4; $P = 0.125$). Skeletal muscle tissue oxygen consumptions, however, were almost identical between the sedentary and exercised treatments ($P = 0.848$).

Discussion

In vitro fatty acid oxidation response to increasing doses of fatty acids in skeletal muscle and liver tissues.

The anaplerotic characteristic of methyl-branched and odd chain fatty acids is in their structures. For example, 2MeP consists of a five-carbon chain with a methyl branch on the 2-carbon. This methyl branch, combined with an odd chain length, makes an enormous difference in the products that result from the oxidation of 2MeP. Like all MCFA, 2MeP is characterized by rapid oxidation in the mitochondrial matrix. This rapid oxidation is due to the fact that MCFA may bypass CPT-I regulation of fatty acid entry into the mitochondrion (2). From there, it can be assumed that 2MeP proceeds through β-oxidation, like heptanoate except with only one round of β-oxidation instead of two. If so, 2MeP would be activated by matrix medium-chain acyl-CoA synthetase to 2-methylpentanoate-CoA and thus begins β-oxidation (18). In the last step of β-oxidation, short-chain 3-ketoacyl-CoA thiolase may clip two fatty acid chain carbons from the carboxyl end of the fatty acid molecule (18). With straight MCFA like C6, this action would typically produce an acetyl-CoA and a fatty acyl-$CoA_{(n-2)}$ molecule with each cycle (19). While this does occur in catabolism of 2-methylpentanoyl-CoA, a branch point at the 2-carbon of 2-methylpentanoic acid chain in combination with an odd chain length of 5 carbons results in the production of two 3-carbon propionyl-CoA molecules rather than the normal product of 2-carbon acetyl-CoA (13). This production of propionyl-CoA has been proven as Roe *et al.* has identified labeled propionylcarnitine produced in association with labeled heptanoate treatment *in vitro* (13).

Once methyl-branched fatty acids (whether medium or long chain) have been catabolized to propionyl-CoA, they may enter the anaplerotic methylmalonyl pathway. Here, enzyme propionyl-CoA carboxylase converts propionyl-CoA to (D)-methylmalonyl-CoA using biotin, bicarbonate and ATP while releasing ADP, P_i and H^+. Then, (D)-methylmalonyl-CoA is converted to (L)-methylmalonyl-CoA by D-methylmalonyl-CoA racemase. Finally, (L)-methylmalonyl-CoA is converted to succinyl-CoA by enzyme methylmalonyl-CoA mutase using cofactor adenosylcobalamin (20). Succinyl-CoA may enter the TCA cycle as an exogenously-derived TCAI and act anaplerotically to reinvigorate the TCA cycle. Yu *et al.* was able to show anaplerosis of 6-10% of the TCA cycle using labeled pentanoate and decreased endogenous production of propionyl-CoA (21). They suggest the exogenous source of propionyl-CoA allows the tissue to spare protein (21). Fushiki *et al.* has suggested MCT feeding can minorly increase skeletal muscle glycogen after an exercise endurance test (0.83 ± 0.13 mg/g skeletal muscle versus long chain triglyceride feeding: 0.69 ± 0.11 mg/g muscle, $P > 0.05$; 22). Additionally, Beckers *et al.* has suggested that feeding a diet that may counteract the anti-lipolytic effects of carbohydrate feeding may increase endurance time (23).

A 0.25 mM methyl-branched fatty acid dosage was chosen for the evaluation of their effects on *in vitro* fatty acid oxidation of [1-^{14}C]-oleic acid in liver and skeletal muscle tissues from exercised or sedentary mice. This dosage was chosen based on preliminary data showing that 0.25 mM optimally stimulates *in vitro* fatty acid oxidation of [1-^{14}C]-oleic acid in liver tissue from fasted mice (Table 1, Figure 1). In this incremental treatment evaluation, *in vitro* fatty acid oxidation was elevated in all 2MeP treatment levels as compared to C6 in

liver tissue ($P < 0.05$). In fact, it was at least two fold that of C6 for all treatment levels. The differences between 2MeP and C6 capacity to stimulate *in vitro* fatty acid oxidation increased in a dose-response manner. These data agree with data from Odle *et al.* indicating *in vitro* hepatocyte oxidation rates are stimulated by more than 40% with odd-chain MCFA treatment as compared to treatment with even-chain MCFA (24).

The maximally-stimulated fatty acid oxidation occurring in liver tissues treated with 2MeP resulted from increased mitochondrial fatty acid oxidation. This increased mitochondrial fatty acid oxidation was identified in the increase of total and percent mitochondrial fatty acid oxidation (Table 1, Figure 3B). Thus, while there was a trend towards increased peroxisomal [^{14}C]-ASP and total ($^{14}CO_2$ + [^{14}C]-ASP) accumulations, significantly increased mitochondrial $^{14}CO_2$ and [^{14}C]-ASP accumulations indicates the mitochondrion is the primary location of 2MeP's action to stimulate both fatty acid oxidation and combustion. Tetrick *et al.* reported concurring data with labeled fatty acid *in vitro* oxidation studies (25). They used labeled nonanoyl-CoA to trace labeled propionyl-CoA oxidation products. They found 75-84% of propionyl-CoA from nonanoyl-CoA β-oxidation is combusted to CO_2 in the TCA cycle (25). They, and Odle *et al.*, suggest the differences in oxidation rates between odd- and even-carbon fatty acids may stem from propionyl-CoA's ability to enter into the TCA cycle at succinyl-CoA and thus increase TCA cycle flux as an anaplerotic substrate (24;25). Tetrick *et al.* observed this phenomenon in skeletal muscle tissue (24). However, we did not observe differences in skeletal muscle tissue.

There were no differences between skeletal muscle fatty acid oxidation treatments (Table 2, Figure 2). There was no overall effect of fatty acid treatment or dose on the skeletal muscle tissue fatty acid oxidation ($P = 0.423$; $P = 0.809$, respectively). Additionally, there were no differences between 2MeP and C6 treatment effects ($P > 0.05$). There was no interaction between fatty acid treatment and dosage in any of the oxidation measurements ($P > 0.05$).

Anaplerotic methyl-branched LCFA are naturally-occurring chlorophyll derivatives; phytanic (3,7,11,15-tetramethylhexadecanoic) acid and pristanic acid (2,6,10,14-tetramethylpentadecanoic) acid. Pristanic acid is actually a metabolite of phytanic acid produced during α-oxidation (26). Phytanic acid must be α-oxidized to pristanic acid because a branch point at the 3-position of the molecule prevents fatty acyl-CoA dehydrogenase from accessing the β-carbon of phytanic acid (26). However, when pristanic acid is chain-shortened by one carbon in α-oxidation, all of its methyl-branches fall in order for β-oxidation (26). Within the peroxisome, pristanic acid will be activated by an acyl-CoA synthetase to pristanoyl-CoA so that is may go through three rounds of β-oxidation (26). These three rounds will produce a net total of two acetyl-CoA's, one propionyl-CoA and one 4,8-dimethylnonanoyl-CoA which then may be transported to the mitochondrion for further oxidation using either a carnitine dependent or a carnitine independent mechanism (26). However, at the mitochondrion, 4,8-dimethylnonanoyl-CoA will be taken up by only the CPT transport system (26). Therefore, methyl-branched LCFA oxidation is more regulated than that of a MCFA as its transport can be inhibited by high levels of acetyl-CoA or ketone bodies and instead stored in the body (2;3).

The incremental decreases in fatty acid oxidation associated with increased levels of pristanic acid treatment were observed in decreases in both $^{14}CO_2$ and [^{14}C]-ASP accumulations (Table 3, Figure 4). However, $^{14}CO_2$ accumulation was decreased to a greater extent than those observed in [^{14}C]-ASP accumulations. In fact, the entirety of suppressed fatty acid oxidation between 0 and 0.25 mM pristanic acid stemmed from decreases in $^{14}CO_2$ accumulation. It is possible pristanic acid competed with oleic acid for oxidation and thus diluted labeled CO_2 collection. Competition, combined with, and resulting in, increase in ketone bodies and citrate activated β-oxidation negative feedback that would further decrease fatty acid oxidation, and agrees with the observed delayed [^{14}C]-ASP depression (25). This phenomenon has been previously observed in phytanic acid oxidation lagging at 82% of that of phytanic acid (27). In conclusion, 0.25 mM was chosen as the best treatment level for measurements of [1-^{14}C]-oleic acid fatty acid oxidation rates in response to treatment with methyl-branched LCFA.

The effect of methyl-branched fatty acids on in vitro fatty acid oxidation in liver and skeletal muscle tissues from sedentary and exercised mice.

Methyl-branched LCFA or MCFA may act as a novel source of anaplerotic carbon when TCAI are diminished. We propose that the structuring of these fatty acids may allow for β-oxidation to produce anaplerotic carbon. This is a novel product for β-oxidation, which traditionally is thought to produce TCA cycle substrate (19). The anaplerotic structure of methyl-branched fatty acids may improve *in vitro* fatty acid oxidation in liver and skeletal muscle tissues from exercised mice.

Treatment with 2MeP increased levels of $^{14}CO_2$ accumulation from β-oxidation of [1-^{14}C]-oleic acid in liver tissue almost 2-fold as compared to treatment with C6 (Figure 5A). However, the accumulations of [^{14}C]-ASP were not different between 2MeP and C6; this indicated ketone body levels in the tissue were not increasing as a result of increased fatty acid oxidation (Figure 5B). Instead, 2MeP was increasing fatty acid utilization flux as a whole by increasing the complete combustion of [1-^{14}C]-oleic acid as more products went completely to $^{14}CO_2$. This effect indicated the two propionyl-CoA molecules resulting from β-oxidation of 2MeP were in fact augmenting energy production in the tissue. This idea was proposed by Yu *et al.* using labeled pentanoate and Tetrick *et al.* using labeled nonanoyl-CoA to trace labeled propionyl-CoA through oxidation. Tetrick found 75-84% and as aforementioned, Yu found 6-10% of propionyl-CoA from nonanoyl-CoA and pentanoate, respectively, in the TCA cycle (21;25). They, and Odle *et al.*, suggest the differences in oxidation rates between odd- and even-carbon fatty acids may stem from propionyl-CoA's ability to enter into the TCA cycle at succinyl-CoA and thus increase TCA cycle flux as anaplerotic substrate (21;24;25). Thus, two propionyl-CoA molecules are produced from β-oxidation of 2MeP and then are converted, through the methylmalonyl pathway, into succinyl-CoA to supplement the TCA cycle (18). This finding corroborates with Russell *et al.* who indicated propionyl-CoA can improve TCA cycle functionality by two mechanisms; first, as an anaplerotic molecule (28). Propionyl-CoA may assist in anaplerosis beyond the negative feedback that may occur at α-ketoglutarate dehydrogenase as it has been shown to helpfully redistribute TCAI beyond α-ketoglutarate dehydrogenase (28). Secondly, propionyl-CoA does not compete with other fatty acid products (i.e. acetyl-CoA) for entry

into the TCA cycle at citrate synthase (28). Acting as an anaplerotic molecule in the second half of the TCAI (i.e. beyond the α-ketoglutarate dehydrogenase reaction) allows the cycle to continue functioning even when a lack of CoASH may have created a negative feedback at α-ketoglutarate dehydrogenase (28). Another mechanism of relieving inhibition of β-oxidation was proposed by Sidossis *et al.* as they observed increased β-oxidation in response to octanoate supplementation (29). They propose that the inhibition of fatty acid oxidation observed at high exercise intensities is a result of glycolytic inhibition (29). They demonstrate that octanoate oxidation is not inhibited by the glycolytic inhibition (29). Therefore, with octanoate supplementation, glycolysis is increased; these products may act as a TCA cycle substrate or anaplerotic molecule. Meanwhile, fat oxidation continues without LCFA inhibition. This combination of carbohydrate and lipid influx may be more sustainable than a more absolute switch to carbohydrate.

These findings were confirmed by the identification of increased mitochondrial fatty acid oxidation with 2MeP treatment (Table 4). Interestingly, $^{14}CO_2$ made up only 17.7% of the products with treatment of C6 but increased more than 50% with treatment of 2MeP to 26.9%. This was aligned with an increase in absolute $^{14}CO_2$ accumulation in the liver tissue (mitochondrial + peroxisomal oxidation) with 2MeP over C6 treatment (Figure 5A). Such a shift in oxidation products indicated the TCA cycle was more efficient. Overall, this increased efficiency was seen first as ketone body levels stayed the same between 2MeP and C6 treatments, as evidenced in constant mitochondrial and total absolute [^{14}C]-ASP accumulation. Then second, increased efficiency was evident as a greater percentage of β-oxidation products completely combusted in the mitochondria, as seen in increased levels of

$^{14}CO_2$. Identification of the specific components of [^{14}C]-ASP products will give a more clear indication of oxidation final products. If the effects of feeding odd chain MCFA result from the same anaplerotic mechanism described by Odle *et al.*, then we expect to observe a similar increase in TCA cycle intermediates relative to ketone bodies as observed by Lin *et al.*'s analysis of ASP from hepatocytes treatment with heptanoate versus octanoate (24;30).

While the methyl-branched MCFA treatment differences were seen in only $^{14}CO_2$ accumulation, methyl-branched LCFA treatment differences were evident in the [^{14}C]-ASP and total ($^{14}CO_2$ + [^{14}C]-ASP) accumulations of liver tissue (Figure 5B; Figure 5C). Still, palmitate and pristanic acid treatment effects did not differ in any of the measurements of oxidation ($P > 0.05$; Figure 5). However, phytanic acid treated liver tissue [^{14}C]-ASP and total ($^{14}CO_2$ + [^{14}C]-ASP) fatty acid oxidation rates were increased from those treated with palmitate (Figure 5; $P < 0.05$). Thus, phytanic acid increases whole tissue liver fatty acid oxidation. This occurrence may be attributed to the structure of phytanic acid which produces an even number of acetyl-CoA and propionyl-CoA molecules during β-oxidation. Thus, there would be an even contribution to both TCA cycle substrate and intermediates. More β-oxidation-ready pristanic acid should be more efficiently used by the liver tissue as it has been shown to be oxidized 82% faster than phytanic acid (27). In this case, it is also possible that pristanic acid may have out-competed some of the [1-^{14}C]-oleic acid for oxidation and combustion in the TCA cycle, thus leading to the decreased values. Less efficiently metabolized phytanic acid may compete for the TCA cycle to a lesser degree since it must go through peroxisomal α-oxidation and some peroxisomal β-oxidation prior to entering mitochondrial β-oxidation (26).

Skeletal muscle fatty acid oxidation rates did not differ between treatments ($P > 0.05$) and there were no fatty acid treatment-by-exercise interactions evident in the skeletal muscle tissue samples ($P > 0.05$), except in the measurement of peroxisomal total $^{14}CO_2$ oxidation in skeletal muscle tissue (Table 5; $P < 0.05$). However, there was a trend towards differences between pristanic and palmitic treatment effects in percent of oxidation occurring in the mitochondrion ($P = 0.091$) while phytanic acid treated tissues were the same as palmitate treated tissues (Figure 7A; $P = 0.119$). This trend is intriguing as it raises the possibility of pristanic acid being more quickly used to enhancing fatty acid oxidation in the mitochondrion. It appears that while pristanic acid may be more directly affecting mitochondrial oxidation, it is not increasing TCA cycle flux as effectively as it increases fatty acid oxidation. This is seen as [^{14}C]-ASP appears to contribute the major portion of the increase in pristanic acid-treated total ^{14}C accumulation in the liver tissue of exercised mice as compared to palmitate treatment. However, this is only slightly below [^{14}C]-ASP accumulation of tissues treated with anaplerosis positive-control propionyl-CoA. The size of this difference is diminished with the probable dilution of [1-^{14}C]-oleic acid oxidation due to pristanic acid oxidation products of equal part propionyl-CoA and acetyl-CoA. Tetrick *et al.* observed a similar dilution effect (25;26).

Malate treatment sufficiently stimulated fatty acid oxidation to indicate that treatments do not affect skeletal muscle fatty acid metabolism with the same activity as liver fatty acid metabolism. These differences may be attributed to liver and skeletal muscle metabolism efficiencies in mice where the liver tissue status will more directly correlate with physiological stress level (31). It may also stem from a disproportionate collection of

glycolytic skeletal muscle fibers instead of oxidative skeletal muscle fibers which have disproportionate concentrations of mitochondria, and thus disproportionate rates of fatty acid oxidation (32). Even beyond this, it must be noted that the mitochondrial density, capacity, function and stimulation will differ between liver and skeletal muscle tissues. As such, it may be of little value to compare oxidation rates across tissues. Another potential source of error may stem from the exercise protocol used in this study as it was later proven inadequate to reach 80% $VO_{2\ max}$; a requirement for accurate induction of exhaustion (12;22). Additionally, mice in this study were not fasted prior to exhaustion, perhaps accounting for the differences in exercise protocols (12).

Limitation to total fatty acid oxidation: collection of $^{14}CO_2$ & [^{14}C]-acid soluble products.

The approximately 20 min delay between the exercise treatment and the liver and skeletal muscle tissue dissections may have allowed the tissues to redistribute metabolites or recover from the exercise treatment. This time delay is in addition to the fact that the skeletal muscle tissue was not contracting during the oxidation measurements.

In vitro oxygen consumption rates of liver and skeletal muscle tissues from sedentary and exercised mice.

In fact, the rate of oxygen consumption in liver from exercised mice was 65% that of liver from sedentary mice. These data are in agreement with literature values indicating that mitochondrial respiration efficiency is decreased in the post-exercise state (33).

Conclusions

Methyl-branched fatty acids may act as a novel source of anaplerotic carbon in liver tissues from fasted or exercised mice. Methyl-branched fatty acids may act as a novel source of anaplerotic carbon as they produce carbohydrate-like molecules during β-oxidation (21;25;30). The anaplerotic carbohydrate-like molecule, propionyl-CoA, may enter the TCA cycle via methylmalonyl pathway conversion to succinyl-CoA (25;28). Medium chain fatty acid 2MeP further acts to increase fatty acid oxidation as it does not interfere with the oxidation of other fatty acids (25). It achieves this by not producing any acetyl-CoA (18). The production of only anaplerotic carbon allows 2MeP to avoid TCA cycle competition with acetyl-CoA produced from other fatty acids (25). It also allows 2MeP to avoid contributing to the acetyl-CoA pool, thus decreasing the risk of ketone body-induced inhibition of β-oxidation (29), which would be activated in a stressed physiological state like exercise (29). Thus, fatty acid oxidation is improved as acetyl-CoA molecules are more efficiently oxidized and lead to release of β-oxidation inhibition at CPT-I and 3-ketoacyl-CoA thiolase (2;19). Our data agrees with this suggestion as we observed that 2MeP increased liver tissue mitochondrial $^{14}CO_2$ accumulation, but did not affect [^{14}C]-ASP accumulation differently from C6. This shift in $^{14}CO_2$ accumulation is promising as it indicates 2MeP indeed may indeed enhance β-oxidation efficiency, especially in liver tissue from exercised animals. Methyl-branched MCFA 2MeP augmented fatty acid oxidation and combustion as it does not compete with other fatty acid catabolites (i.e. acetyl-CoA) for combustion in the TCA cycle; instead, it solely supports activation of oxidation as an anaplerotic molecule. Methyl-branched LCFA phytanic and pristanic acids may also act as

naturally-derived novel sources of anaplerotic carbon (26). These LCFA hold promise as it was shown that phytanic acid increases *in vitro* liver tissue fatty acid oxidation. In conclusion, methyl-branched fatty acids improve *in vitro* fatty acid oxidation in liver tissues from fasted or exercised mice.

Further investigation into the value of methyl-branched MCFA and LCFA may more clearly unveil the value of methyl-branched fatty acids in tissues from physiologically stressed individuals. Similar studies using labeled fatty acid treatments during oxidation measurements will allow for the elucidation of exact relations between oxidation and energy production and specific identification of their final end products.

Figures

Table 1 Dose-response effects of medium-chain fatty acid on *in vitro* [1-^{14}C]-oleic acid oxidation (µmol/(h.mg protein)) in liver tissue of fasted C57BL/6J mice[1].

	Fatty acid, mM													*P*-value		
Measures of oxidation, umol/(h.mg protein)[2]	C6 0	C6 0.25	C6 0.5	C6 0.75	C6 1	2MeP 0	2MeP 0.25	2MeP 0.5	2MeP 0.75	2MeP 1	Malate 0.1	Prop-CoA 0.1	SEM	FA	Dose	FA x Dose
Total CO_2	9.83	11.1	8.03	7.89	7.58	8.47	22.6	18.8	21.1	22	17.8	13.2	2.85	<0.0001	0.121	0.065
Total ASP	62.7	46.7	50.8	49.6	53.2	58.8	54.6	53.7	51.7	55.9	61.9	70.2	3.95	0.227	0.081	0.688
Total Total	72.6	57.8	58.8	57.5	60.7	67.3	77.2	72.5	72.8	77.9	79.7	83.4	4.76	0.001	0.808	0.094
Mitochondrial CO_2	7.02	8.71	5.84	5.92	5.6	8.23	22.4	18.6	20.9	21.7	17.3	14	3.16	<0.0001	0.106	0.099
Mitochondrial ASP	29.1	24.4	33.1	25.2	32.1	28.4	32.1	25.2	20.3	25.3	29.9	39.9	5.15	0.322	0.643	0.482
Mitochondrial Total	36.1	33.1	38.9	31.1	37.7	36.6	54.5	43.7	41.2	47	47.3	53.9	5.19	0.013	0.381	0.282
Peroxisomal CO_2	0.30	0.19	0.21	0.17	0.23	0.24	0.17	0.22	0.23	0.25	0.37	0.36	0.07	0.985	0.652	0.897
Peroxisomal ASP	33.6	22.3	17.8	24.3	21.1	30.4	22.5	28.6	31.4	30.6	33.7	35.3	4.26	0.157	0.124	0.327
Peroxisomal Total	33.9	22.5	18	24.5	21.3	30.7	22.7	28.8	31.6	30.9	34.1	35.7	4.26	0.157	0.12	0.322
% Mitochondrial CO_2	71.7	73.6	72.5	73.3	71.4	74.0	99.1	98.8	99.0	98.9	84.9	86.0	14.6	0.083	0.865	0.895
% Mitochondrial ASP	56.0	52.3	66.9	43.1	65.2	53.4	59.4	51.4	48	52.5	56.0	59.1	8	0.701	0.357	0.443
% Mitochondrial Total	57.8	56.1	66.7	53.9	65.9	58.5	70.9	62.6	62.1	65.1	63.1	63.6	5	0.436	0.307	0.256
% Peroxisomal CO_2	3.3	1.4	2.8	1.8	3.6	26.0	0.9	1.2	1.0	1.1	2.6	1.6	7.1	0.745	0.283	0.350
% Peroxisomal ASP	44.0	47.7	33.1	56.9	34.8	46.6	40.6	48.6	52	47.5	44	41	7.9	0.701	0.357	0.443
% Peroxisomal Total	38.5	39.1	29.2	43	31.1	41.5	29.1	37.4	37.9	34.9	35.1	34.5	4.6	0.997	0.218	0.228
Total % CO_2	13.3	18	12.6	18.8	11.2	10.8	29.2	24.1	27.7	26.5	19.1	15.1	3.1	0.0003	0.004	0.077
Total % ASP	86.8	81.4	87.4	81.2	88.9	89.2	70.8	75.9	72.3	73.5	80.9	84.9	3.1	0.0003	0.004	0.077
Mitochondrial % CO_2	16.7	23.6	11.4	33.5	11.9	19.2	40.8	38.1	45.5	41.4	28.7	23.1	9.1	0.009	0.144	0.503
Mitochondrial % ASP	83.3	76.4	88.6	66.5	88.1	80.8	59.2	61.9	54.5	58.6	71.3	76.9	9.1	0.009	0.144	0.503
Peroxisomal % CO_2	1.4	1.1	1.3	0.8	1.5	1.4	1.2	1.1	1	1.0	2.6	2.0	0.5	0.643	0.858	0.935
Peroxisomal % ASP	98.6	98.9	98.7	99.2	98.6	98.6	98.8	98.9	99	99	98.4	98	0.5	0.643	0.858	0.935

[1] Data are presented as LSM ± SEM, n=4 replicates with 4 pooled mice per replicate. Percent values may not be additive due to calculation of LSM.

[2] Samples were derived from 24 hour food-deprived mice. Peroxisomal fatty acid oxidation rates were obtained by adding a mitochondrial inhibitor to the oxidation incubations. Mitochondrial fatty acid oxidation rates were calculated as the difference between total (uninhibited oxidation rates) and peroxisomal (inhibited) oxidation rates, see materials and methods above.

Table 2 Dose-response effects of medium-chain fatty acid on *in vitro* [1-^{14}C]-oleic acid oxidation (µmol/h.mg protein)) in skeletal muscle tissue of fasted C57BL/6J mice[1].

	Fatty acid, mM													*P*-value		
Measures of oxidation, umol/(h.mg protein)[2]	C6 0	0.25	0.5	0.75	1	2MeP 0	0.25	0.5	0.75	1	Malate 0.1	Prop-CoA 0.1	SEM	FA	Dose	Fa x Dose
Total CO_2	0.17	0.25	0.15	0.10	0.20	0.28	0.08	0.16	0.12	0.15	0.26	0.43	0.10	0.628	0.809	0.668
Total ASP	8.24	2.36	2.57	2.16	1.91	2.73	3.35	2.22	2.56	2.19	2.51	6.39	1.95	0.454	0.405	0.463
Total Total	8.41	2.61	2.72	2.26	2.11	3.02	3.43	2.38	2.68	2.35	2.77	6.82	1.94	0.423	0.373	0.485
Mitochondrial CO_2	0.10	0.21	0.10	0.03	0.07	0.02	0.05	0.08	0.09	0.06	0.21	0.28	0.06	0.499	0.763	0.478
Mitochondrial ASP	6.15	0.59	0.89	0.43	0.14	0.88	1.2	0.22	0.78	0.14	1.55	0.81	1.81	0.673	0.369	0.473
Mitochondrial Total	6.24	0.80	1.0	0.46	0.22	0.9	1.3	0.31	0.87	0.20	1.77	1.08	1.8	0.652	0.369	0.462
Peroxisomal CO_2	0.16	0.06	0.05	0.10	0.14	0.29	0.14	0.08	0.03	0.19	0.11	0.13	0.09	0.727	0.369	0.84
Peroxisomal ASP	2.09	1.77	1.9	2.15	2.49	1.86	2.25	2	2.58	2.72	0.858	7.46	0.46	<0.0001	0.536	0.939
Peroxisomal Total	2.25	1.82	1.94	2.26	2.63	2.14	2.39	2.08	2.61	2.91	0.946	7.61	0.48	<0.0001	0.526	0.967
%Mitochondrial CO_2	50	61.5	55.5	31.7	55.7	34	40.8	64.9	71.2	52.4	53.9	58	16.9	0.975	0.874	0.41
%Mitochondrial ASP	56.7	42.5	40.5	17.6	10.9	46.4	36.6	12.1	24	14	64.2	15.5	14.2	0.097	0.072	0.759
%Mitochondrial Total	58.3	43.8	42.5	19.6	18.3	43.5	38.3	18.9	51	16.5	63.4	40.9	12.2	0.497	0.106	0.231
%Peroxisomal CO_2	100	81.3	44.5	90.8	69.3	90.5	100	35.1	28.8	80.8	100	49.5	91.6	0.573	0.409	0.588
%Peroxisomal ASP	43.3	57.5	63.7	57.4	76.6	53.6	65.6	62.9	51	62.1	39.4	54.8	9.1	0.584	0.196	0.638
%Peroxisomal Total	45	57.3	61.7	100	100	56.8	69.5	81.1	100	100	36.6	59.1	100	0.563	0.116	0.931
Total %CO_2	19.3	16.6	7.58	29.1	28.3	14.3	4.37	30.5	30.1	29.8	16.7	28.2	11.2	0.747	0.383	0.608
Total %ASP	80.8	83.4	92.4	70.9	71.7	85.7	95.6	69.5	69.9	70.2	83.4	71.8	11.2	0.747	0.383	0.608
Mitochondrial %CO_2	18.3	21.5	35.8	9.4	59.4	3.97	29.8	44.8	34.3	34.6	15.6	34.5	17.3	0.799	0.26	0.623
Mitochondrial %ASP	81.7	78.5	64.2	65.6	40.6	96	45.2	55.2	65.7	40.4	83.6	64.7	18.1	0.725	0.15	0.755
Peroxisomal %CO_2	7.7	27.2	26	4.5	16.5	38.1	30.2	25.7	2.1	6.6	32.9	0	12.2	0.237	0.217	0.538
Peroxisomal %ASP	67.3	72.8	74	95.5	83.5	62	69.8	74.3	97.9	93.4	67.9	100	11.3	0.219	0.049	0.968

[1] Data are presented as LSM ± SEM, n=4 replicates with 4 mice pooled per replicate. Percent values may not be additive due to calculation of LSM.

[2] Samples were derived from 24 hour food-deprived mice. Mitochondrial fatty acid oxidation rates were calculated as the difference between total (uninhibited oxidation rates) and peroxisomal (inhibited) oxidation rates, see materials and methods above.

Table 3 Dose-response effects of pristanic acid on *in vitro* [1-^{14}C]-oleic acid oxidation (µmol/(h.mg protein)) in liver tissue of exercised C57BL/6J mice[1].

	Measurement of oxidation, umol/(h.mg protein)[2]																			
Pristanic acid dose, mM	Total			Mitochondrial			Peroxisomal			%Mitochondrial			%Peroxisomal			Total		Mitochondrial		Peroxisomal	
	CO_2	ASP	Total	CO_2	ASP	Total	CO_2	ASP	Total	CO_2	ASP	Total	CO_2	ASP	Total	$\%CO_2$	%ASP	$\%CO_2$	%ASP	$\%CO_2$	%ASP
0	6.10	33.9	40.0													15.3	84.8				
0.25	4.28	33.8	38.1	4.06	27.8	31.9	0.217	5.98	6.20	94.9	82.2	83.8	5.07	17.7	16.3	11.2	88.8	94.9	82.2	5.07	17.7
0.5	3.88	26.7	30.6													12.7	87.3				
0.75	1.25	15.2	16.5													7.60	92.4				

[1] Data are presented as means, n=1 replicate with 4 mice pooled per replicate. 0.25 mM palmitate effects for the same experiment were; CO_2: 4.36; ASP: 38.3; Total: 42.7 µmol/(h.mg protein).

[2] Samples were derived from exercised mice. Mitochondrial fatty acid oxidation rates were calculated as the difference between total (uninhibited oxidation rates) and peroxisomal (inhibited) oxidation rates, see materials and methods above.

Table 4 Effect of methylated fatty acids on *in vitro* [1-^{14}C]-oleic acid oxidation (µmol/(h.mg protein)) in liver tissue of sedentary (Sed) or exercised (Ex) C57BL/6J mice[1].

Oxidation measurement, umol/(h.mg protein)[2]	Fatty acid, mM															*P*-value		
	C6		2MeP		Palmitate		Phytanic		Pristanic		Malate		Propionyl-CoA		SEM	FA	Ex	FA x Ex
	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex				
Whole CO_2	6.96	8.67	11.8	13.1	3.55	4.65	5.01	6.62	4.13	4.85	7.66	9.74	10.1	12.4	1.47	<.0001	0.143	0.998
Whole ASP	39.1	43.1	39.3	46.8	34.8	40.8	44.4	52.1	37.7	43.1	41.1	49.5	59.2	63.6	3.03	<.0001	0.0004	0.985
Whole Total	46.1	51.7	51.1	59.9	38.3	45.5	49.4	58.7	41.8	48	48.7	59.2	69.3	76.1	3.75	<.0001	0.0003	0.994
Peroxisomal CO_2	0.51	0.67	0.56	0.57	0.30	0.49	0.41	0.55	0.38	0.47	0.66	0.80	0.63	0.84	0.09	0.0004	0.006	0.944
Peroxisomal ASP	6.78	8.64	9.38	12.5	6.25	7.41	7.66	10.1	6.08	8.02	16.4	19.9	14.5	19.3	2.87	0.0002	0.088	0.997
Peroxisomal Total	7.32	9.31	9.94	13	6.55	7.89	8.07	10.6	6.46	8.49	17	20.7	15.2	20.1	2.93	0.0002	0.080	0.997
Mitochondrial CO_2	6.42	8	11.3	12.5	3.25	4.17	4.63	6.07	3.75	4.38	7.01	8.94	9.51	11.6	1.41	<.0001	0.069	0.998
Mitochondrial ASP	32.4	34.4	29.9	34.4	28.5	33.4	36.7	42	31.6	35.1	24.7	29.6	44.7	44.4	3.34	0.0001	0.128	0.979
Mitochondrial Total	38.8	42.4	41.2	46.9	31.8	37.6	41.3	48.1	35.3	39.5	31.7	38.6	54.2	56	3.46	<.0001	0.0099	0.989
Mitochondrial %CO_2	16.7	18.8	27.8	26.1	10.3	11	11.4	12.6	11.3	11.2	40.8	29.8	17.2	19.8	5.4	0.0001	0.762	0.894
Mitochondrial %ASP	83.3	81.2	72.2	73.9	89.7	89	88.6	87.4	88.7	88.8	59.2	70.2	82.8	80.2	5.4	0.0001	0.762	0.894
Peroxisomal %CO_2	15.1	16.6	23	21	9.25	10.1	10.3	11.2	10.2	10.1	19.5	16.7	13.8	15.1	2.3	<.0001	0.946	0.940
Peroxisomal %ASP	84.9	83.4	77	79	90.8	89.9	89.7	88.8	89.8	89.9	80.5	83.4	86.2	84.9	2.3	<.0001	0.946	0.940
%Mitochondrial CO_2	92.6	93.2	95.2	95.7	91.9	90.4	91.7	92.1	91	91.7	92.5	92.9	94.1	94	1.1	0.002	0.803	0.947
%Mitochondrial ASP	82.9	81	76	74.7	82	82.3	82.5	81.4	83.1	82.2	54.4	64.1	76.3	72.4	5.1	0.0001	0.873	0.807
%Mitochondrial Total	84.4	83.1	81	79.8	82.9	83.2	83.5	82.7	84	83.2	63.6	69.5	79.2	76.3	3.6	<.0001	0.954	0.928
%Peroxisomal CO_2	7.4	6.8	4.9	4.3	8.1	9.6	8.3	7.9	9.0	8.3	7.5	7.1	5.9	6.0	1.1	0.002	0.803	0.947
%Peroxisomal ASP	17.1	19	24	25.3	18	17.7	17.5	18.6	17	17.8	47.6	35.9	23.7	27.6	5.1	0.0001	0.873	0.807
%Peroxisomal Total	15.6	16.9	19.1	20.2	17.1	16.8	16.5	17.3	16	16.8	36.4	30.5	20.8	23.7	3.6	<.0001	0.954	0.928

[1] Data are presented as LSM ± SEM, n= 4. Percent values may not be additive due to calculation of LSM.

[2] Mitochondrial fatty acid oxidation rates were calculated as the difference between total (uninhibited oxidation rates) and peroxisomal (inhibited) oxidation rates, see materials and methods above. There was no interaction of fatty acid x exercise, $P > 0.05$.

Table 5 Effect of methylated fatty acids on *in vitro* [1-^{14}C]-oleic acid oxidation (µmol/(h.mg protein)) in skeletal muscle tissue of sedentary (Sed) or exercised (Ex) C57BL/6J mice[1].

	Fatty acid, mM															*P*-value		
Oxidation measurement, umol/(h.mg protein)[2]	C6		2MeP		Palmitate		Phytanic		Pristanic		Malate		Propionyl-CoA		SEM	FA	Ex	FA x Ex
	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex				
Whole CO_2	1.24	1.69	1.42	1.84	1.06	1.06	1.06	1.73	0.99	1.1	2.19	3.29	1.81	1.63	0.27	0.001	0.56	0.7
Whole ASP	10.7	5.22	12.8	5.58	10.6	3.73	11.4	4.32	10.1	4.28	14.5	6.37	20.8	5.87	1.24	0.011	<.0001	0.156
Whole Total	11.9	6.91	14.2	7.42	11.7	4.79	12.5	6.06	11.1	5.38	17	9.66	22.6	7.51	1.32	0.003	<.0001	0.164
Peroxisomal CO_2	0.02	0.05	0.03	0.04	0.01	0.01	0.06	0.03	0.01	0.02	0.02	0.02	0.28	0.02	0.05	0.337	0.313	0.285
Peroxisomal ASP	3.9	3.36	4.49	3.3	3.13	2.41	3.69	2.96	2.58	2.15	4.29	3.33	6.61	3.48	0.34	<.0001	0.0001	0.109
Peroxisomal Total	3.92	3.4	4.51	3.34	3.15	2.42	3.75	2.99	2.59	2.17	4.31	3.35	6.89	3.5	0.32	<.0001	0.415	0.035
Mitochondrial CO_2	1.22	1.65	1.39	1.8	1.04	1.05	1	1.7	0.97	1.08	2.18	3.27	1.72	1.61	0.27	0.002	0.076	0.73
Mitochondrial ASP	6.79	1.86	8.27	2.28	7.49	1.32	7.75	1.37	7.5	2.14	10.6	3.05	14.2	2.39	1.17	0.163	<.0001	0.452
Mitochondrial Total	8.01	3.51	9.66	4.08	8.53	2.37	8.75	3.07	8.47	3.22	12.7	6.32	15.7	4.01	1.23	0.055	<.0001	0.483
Mitochondrial %CO_2	36.3	38.2	26.8	26.6	26.1	42.5	26	49.7	20.4	28.3	29.9	39.5	24.2	32.4	4.2	0.026	0.075	0.238
Mitochondrial %ASP	63.7	61.8	73.3	63.4	73.9	57.6	74.1	50.3	79.6	71.7	70.1	60.5	110	67.6	11	0.175	0.007	0.568
Peroxisomal %CO_2	13.9	19.8	13.6	20.7	13.5	18.2	13.5	25.1	12.7	17.1	17.9	25.4	11	16.9	1.2	0.0006	<.0001	0.387
Peroxisomal %ASP	86.2	80.2	86.4	79.4	86.6	81.9	86.5	74.9	87.3	82.9	82.1	74.6	89.9	83.1	1.2	0.0006	<.0001	0.387
%Mitochondrial CO_2	97.8	94.5	96.9	97.1	98	92.6	96.1	96.5	97.8	97	98.8	95.4	73.3	93.4	4.7	0.353	0.751	0.542
%Mitochondrial ASP	39.8	37.1	48.9	42.8	49.8	33.5	47.2	32	61.7	50.5	55.9	46.7	44.3	37.6	3.0	0.001	0.0002	0.681
%Mitochondrial Total	50.7	49.1	57.3	54.4	57.6	44.4	54.7	48.4	67.5	58.9	65.5	59.8	44	46.8	3.0	0.0009	0.033	0.624
%Peroxisomal CO_2	2.2	5.5	3.1	2.9	2	7.4	3.9	3.5	2.2	3	1.2	4.6	139	6.6	25.7	0.419	0.384	0.457
%Peroxisomal ASP	60.2	63	51.1	57.2	50.2	66.5	52.8	68	38.3	49.5	44.1	53.3	55.7	62.4	3.0	0.001	0.574	0.681
%Peroxisomal Total	49.4	50.9	42.7	45.6	42.4	55.6	45.3	51.6	32.5	41.1	34.5	40.2	56	53.2	3.0	0.0009	0.768	0.624

[1] Data are presented as LSM ± SEM, n= 4. Percent values may not be additive due to calculation of LSM.

[2] Mitochondrial fatty acid oxidation rates were calculated as the difference between total (uninhibited oxidation rates) and peroxisomal (inhibited) oxidation rates, see materials and methods above. There was no interaction of fatty acid x exercise, $P > 0.05$.

Table 6 Effect of exercise on *in vitro* oxygen consumption rates in liver and skeletal muscle tissue from sedentary or exercised C57BL/6J mice[1].

Tissue[2]		Rate[3]	
		umol O2/mg tissue/min	SEM
Liver	Exercised	0.028	0.006
	Sedentary	0.043	0.006
Muscle	Exercised	0.008	0.006
	Sedentary	0.006	0.006

[1] Data are presented as LSM ± SEM, n= 5-7.
[2] Samples were derived from exercised or sedentary mice. Measures of oxidation are expressed as µmol O_2/mg protein/minute.
[3] Tissues: $P = 0.0009$; Exercise: $P = 0.338$; Tissue x Exercise: $P = 0.225$.

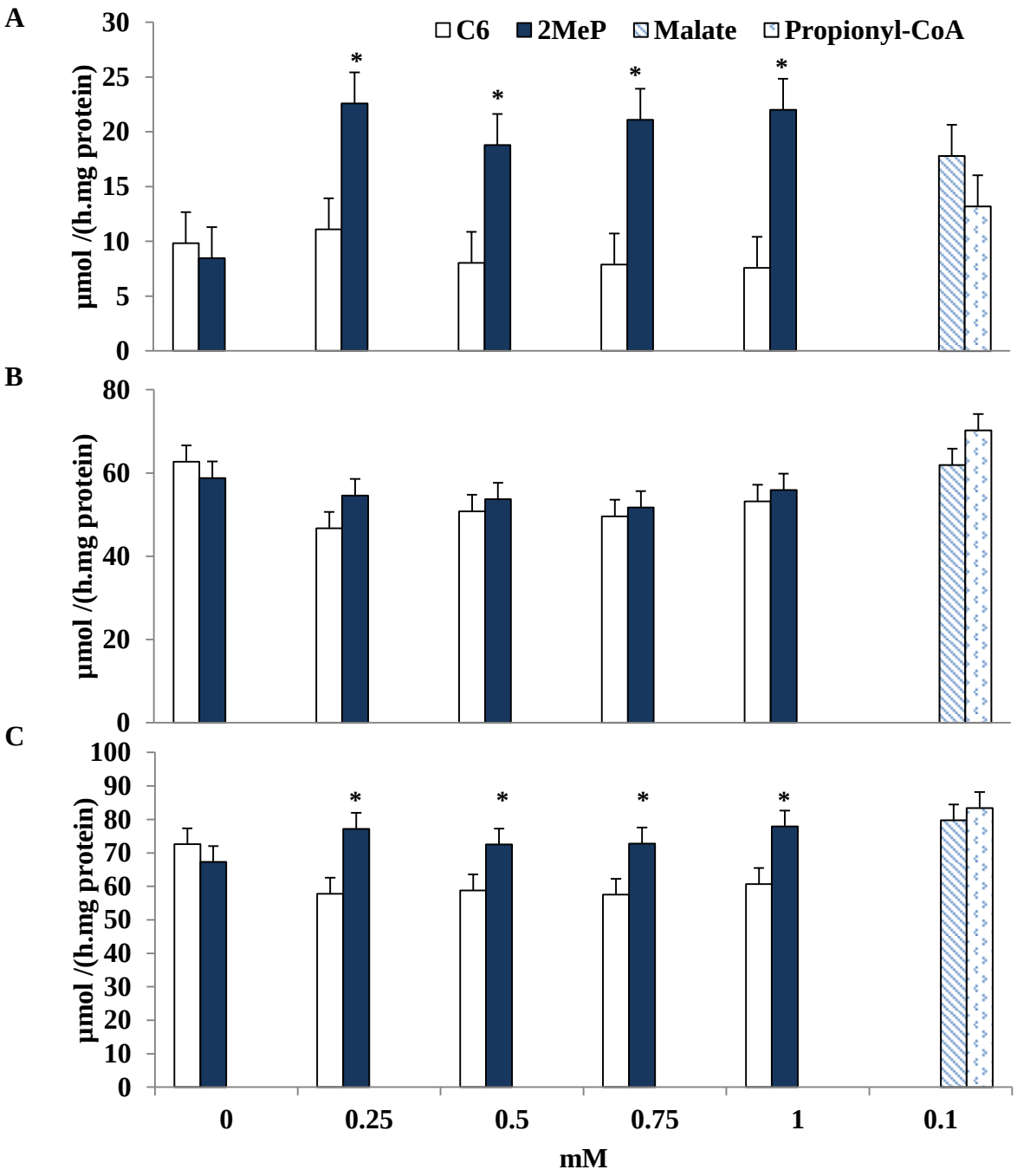

Figure 1 Dose-response effects of medium-chain fatty acids (mM) on *in vitro* [1-^{14}C]-oleic acid oxidation (µmol/(h.mg protein)) in liver tissue of fasted C57BL/6J mice. Measure of total liver tissue ^{14}C accumulation; A: CO_2; B: ASP; C: Total (CO_2 + ASP). Data are presented as LSM ± SEM. Samples were derived from 24-h fasted mice, n=4.
*Bar differs from C6 at indicated dose, $P < 0.05$.

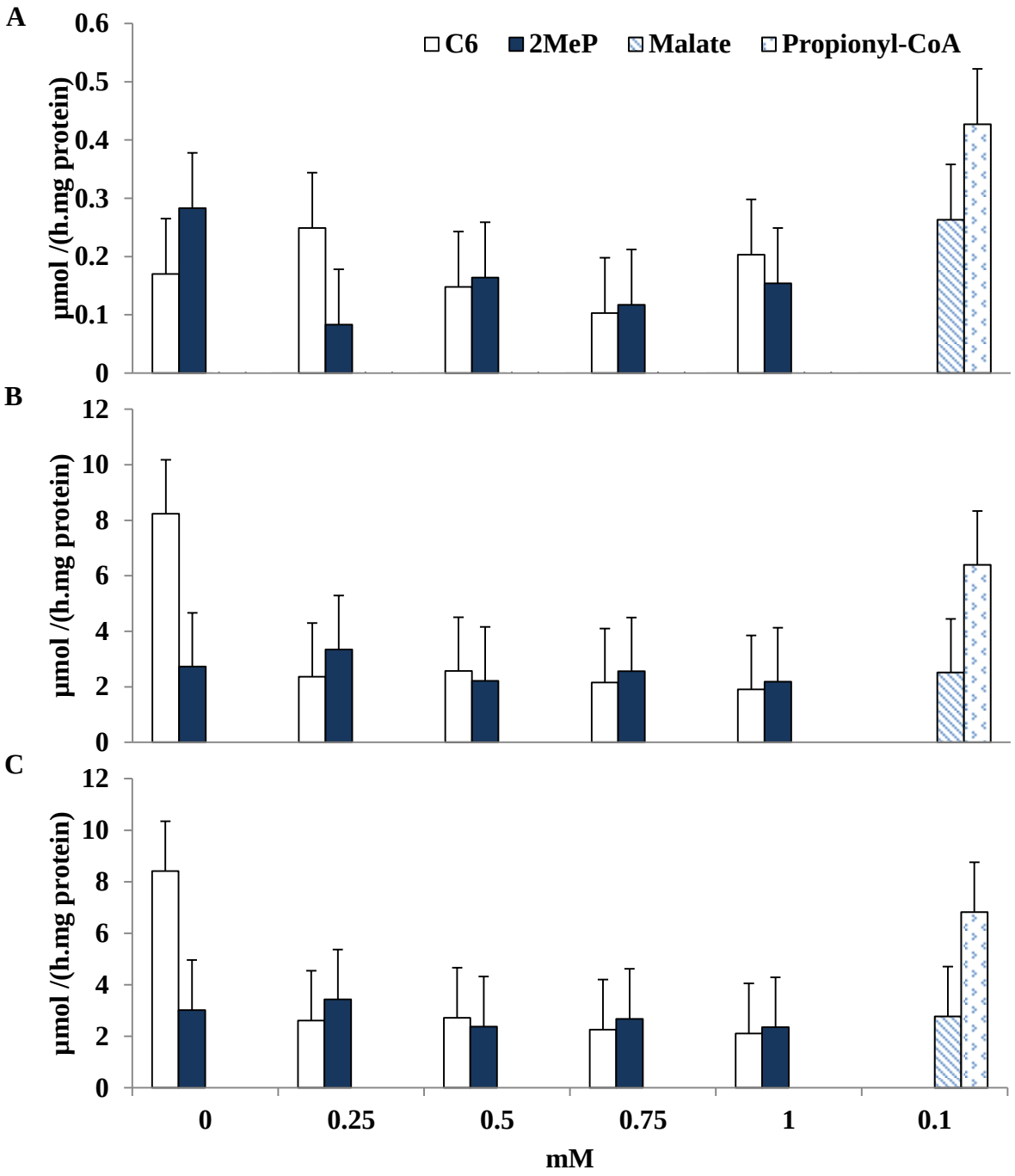

Figure 2 Dose-response effects of medium-chain fatty acids (mM) on *in vitro* [1-^{14}C]-oleic acid oxidation (µmol/(h.mg protein)) in skeletal muscle tissue of fasted C57BL/6J mice. Measure of total skeletal muscle tissue $^{14}CO_2$ accumulation; A: CO_2; B: ASP; C: Total (CO_2+ASP). Data are presented as LSM ± SEM. Samples were derived from 24-h fasted mice, n=4.
*Differs from C6 at indicated dose, $P < 0.05$.

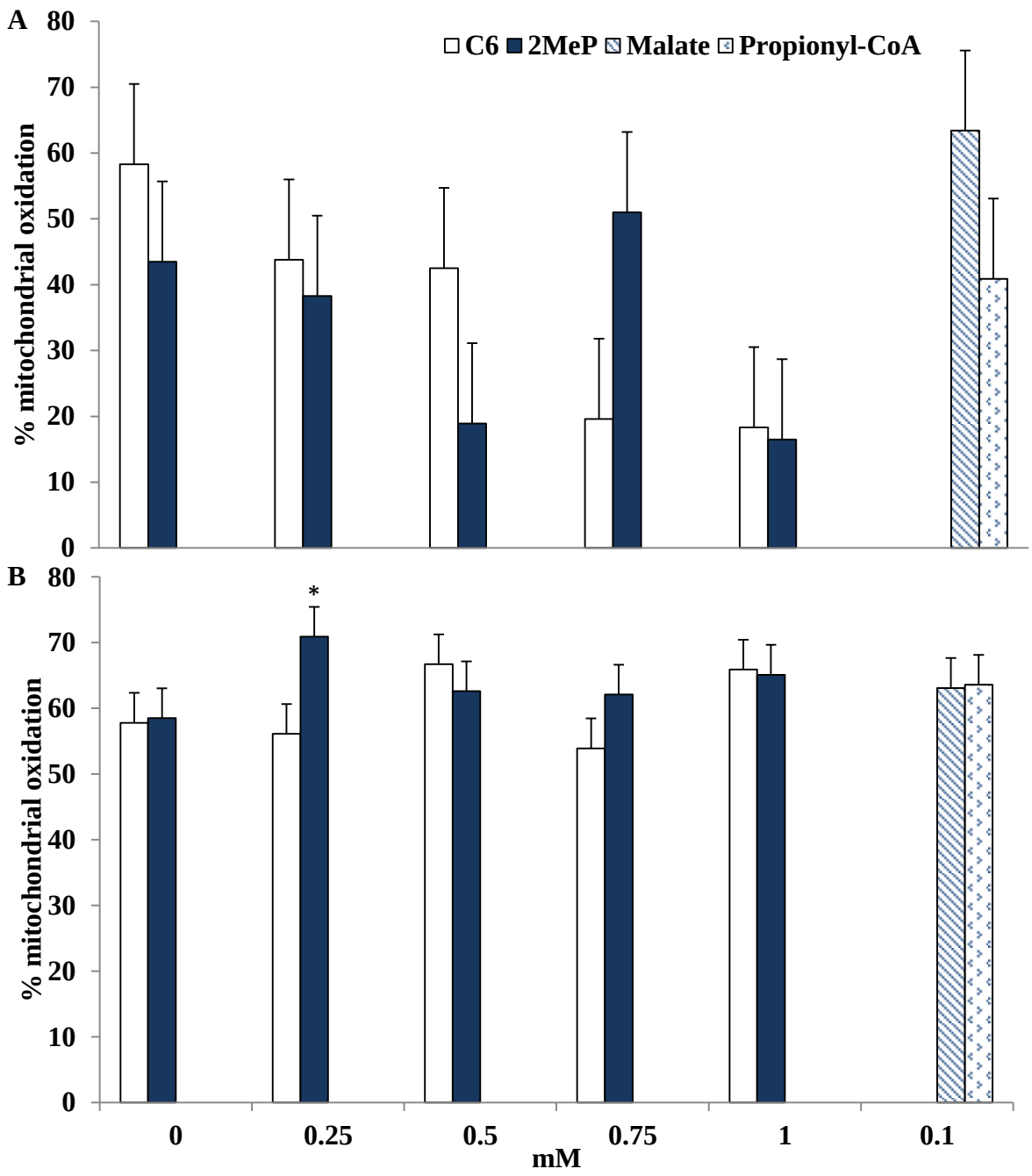

Figure 3 Dose-response effects of medium-chain fatty acids on *in vitro* [1-^{14}C]-oleic acid oxidation (µmol/(h.mg protein)) in tissues of fasted C57BL/6J mice. Measurement of % mitochondrial oxidation (CO_2+ASP); A: Skeletal muscle B: Liver. Mitochondrial fatty acid oxidation rates were calculated as the difference between total (uninhibited oxidation rates) and peroxisomal (inhibited) oxidation rates, see materials and methods above. Data are presented as LSM ± SEM. Samples were derived from 24-h food-deprived mice, n=4. *Bar differs from C6 at indicated dose, $P < 0.05$.

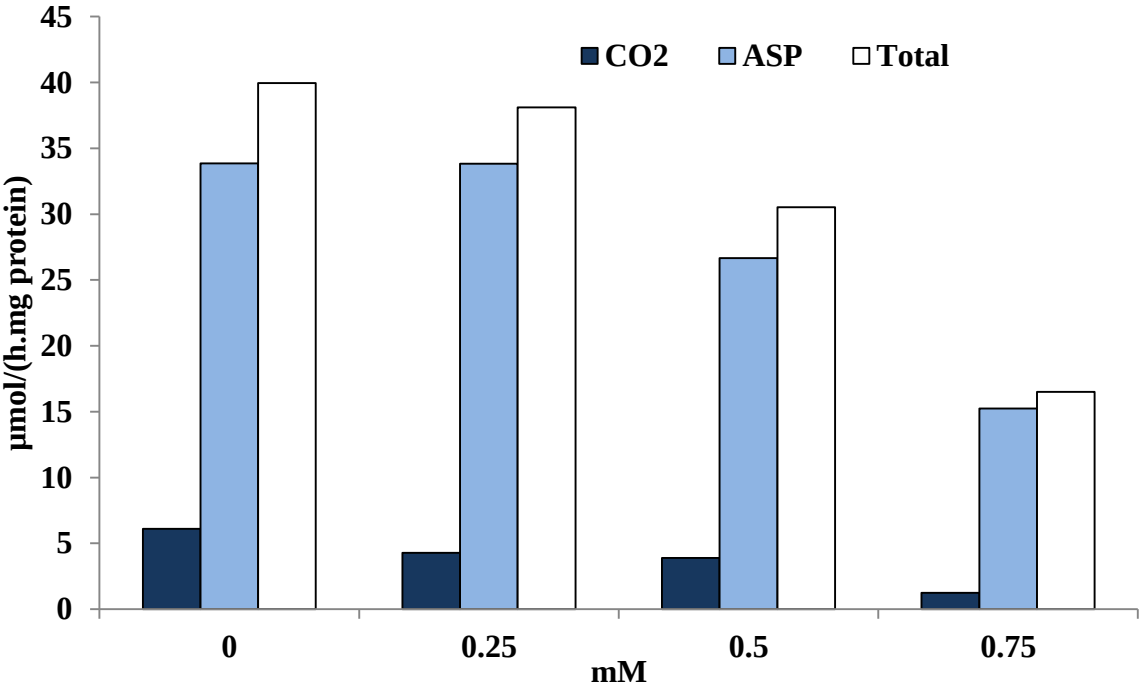

Figure 4 Dose-response effects of pristanic acid (mM) on *in vitro* [1-^{14}C]-oleic acid oxidation (µmol/(h.mg protein)) in liver tissue of exercised C57BL/6J mice. Measures of CO_2, ASP and total (CO_2 + ASP) liver tissue ^{14}C accumulation. Data are presented as means of duplicate flasks, n=1. 0.25 mM palmitate effects for the same experiment were; CO_2: 4.36; ASP: 38.3; Total: 42.7 µmol/(h.mg protein).

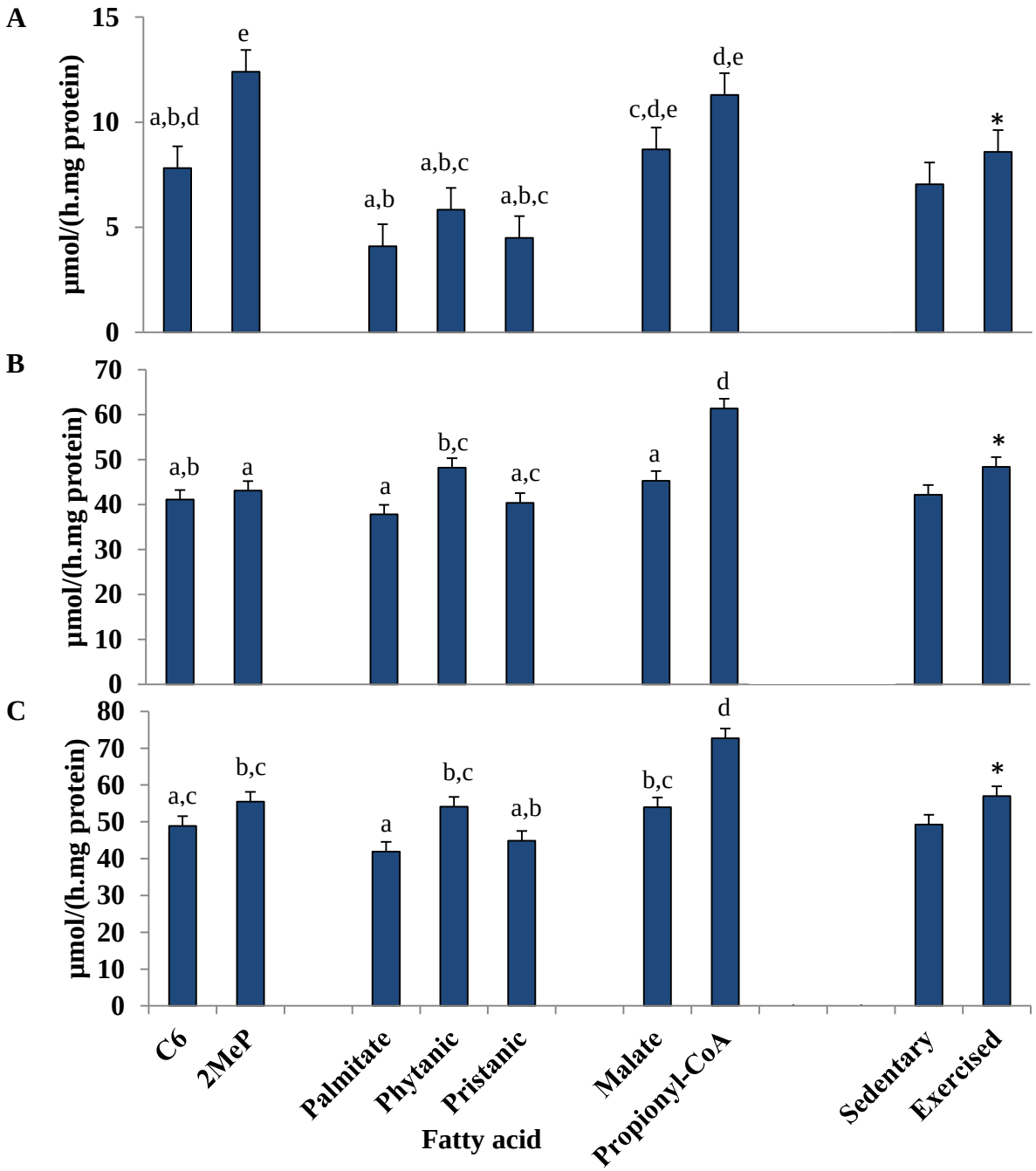

Figure 5 Effects of methyl-branched fatty acids on *in vitro* [1-^{14}C]-oleic acid oxidation (µmol/(h.mg protein)) in liver tissue of C57BL/6J mice. Measurement of whole liver tissue total ^{14}C accumulation; A: CO_2; B: ASP; C: Total (CO_2 + ASP). Data are presented as LSM ± SEM, n=4/fatty acid treatment, n=8/exercise treatment. Samples were derived from either sedentary or exercised mice. There was no fatty acid by exercise interaction, $P > 0.05$. ^{a,b,c,d,e} Bars lacking a common letter differ, $P < 0.05$.

* Differs from sedentary control, $P < 0.05$.

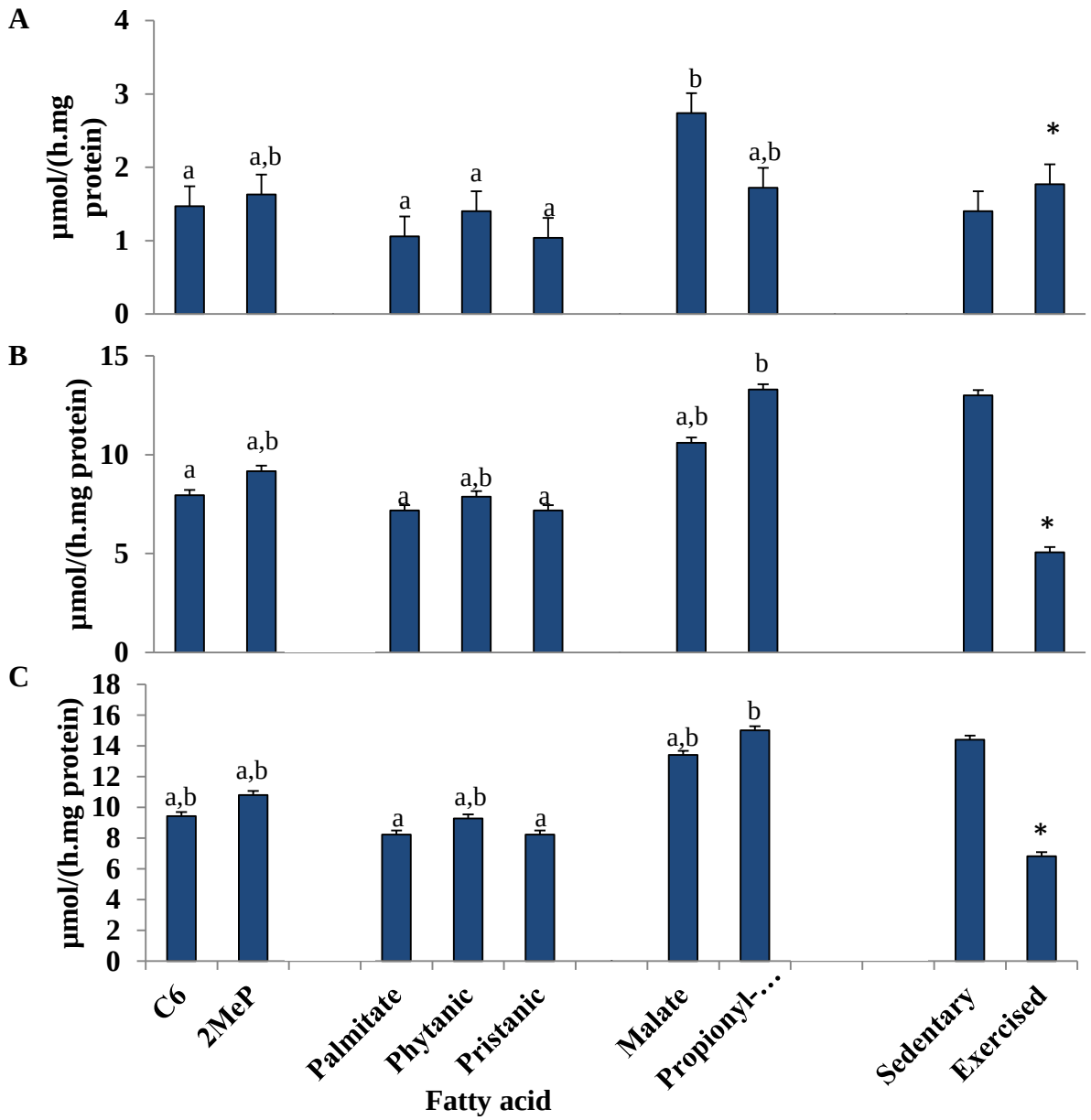

Figure 6 Effects of methyl-branched fatty acids on *in vitro* [1-^{14}C]-oleic acid oxidation (µmol/(h.mg protein)) in skeletal muscle tissue of C57BL/6J mice. Measurement of whole skeletal muscle tissue ^{14}C accumulation; A: CO_2; B: ASP; C: Total (CO_2 + ASP). Data are presented LSM ± SEM, n=4/fatty acid treatment, n=8/exercise treatment. Samples were derived from either sedentary or exercised mice. There was no fatty acid by exercise interaction, $P > 0.05$.

[a,b] Bars lacking a common letter differ, $P < 0.05$.

* Differs from sedentary control, $P < 0.05$.

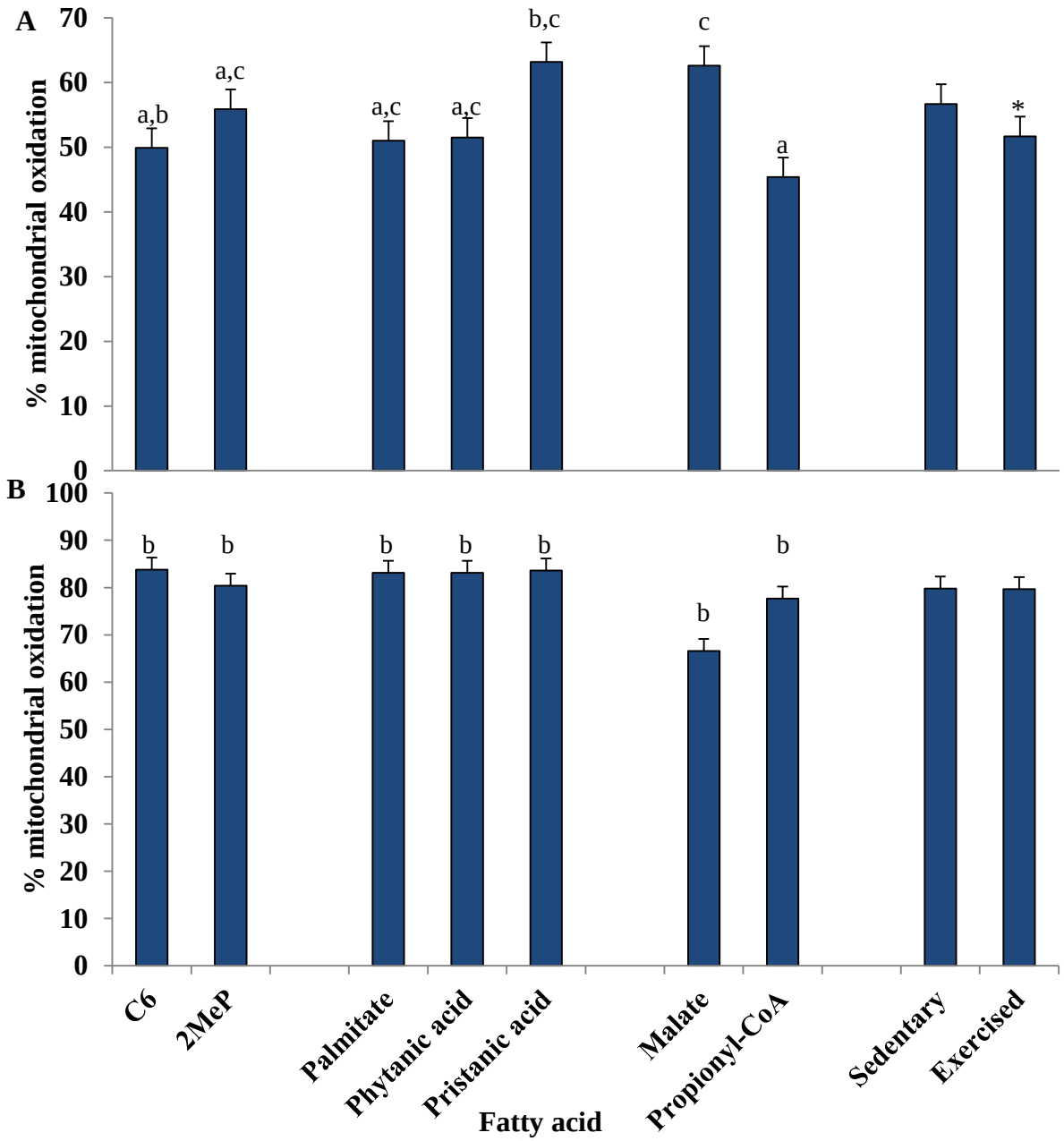

Figure 7 Effects of fatty acids on *in vitro* [1-^{14}C]-oleic acid oxidation (µmol/(h.mg protein)) in tissues of sedentary or exercised C57BL/6J mice. Measurement of % mitochondrial oxidation (CO_2+ASP); A: Skeletal muscle B: Liver. Mitochondrial fatty acid oxidation rates were calculated as the difference between total (uninhibited oxidation rates) and peroxisomal (inhibited) oxidation rates, see materials and methods above. Data are presented as LSM ± SEM, n=4/fatty acid treatment, n=8/exercise treatment. There was no interaction between fatty acid and treatment, $P > 0.05$.

[a,b,c] Bars lacking a common letter differ, $P < 0.05$.

* Differs from sedentary control, $P < 0.05$.

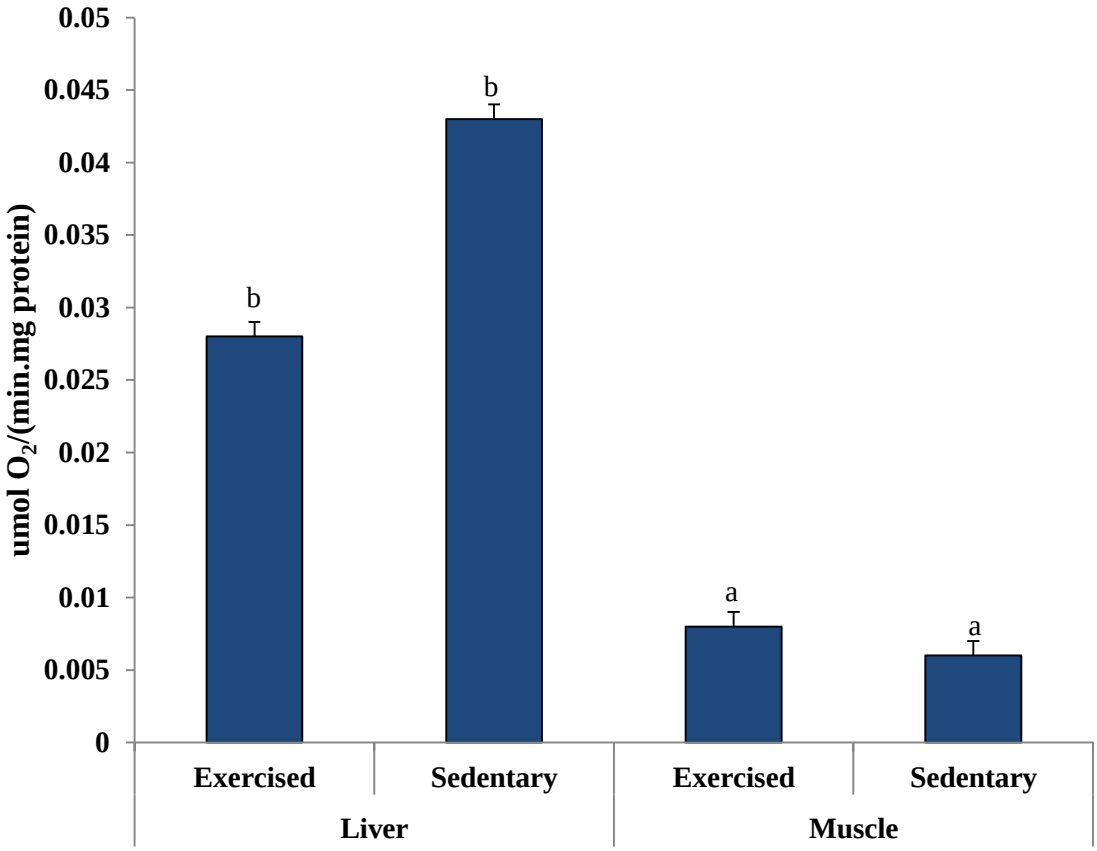

Figure 8 *In vitro* oxygen consumption rates in liver and skeletal muscle tissues from sedentary or exercised C57BL/6J mice. Tissues were obtained from sedentary or exercised mice, n=5-7.
[a,b] Bars lacking a common letter differ, $P < 0.05$. Tissues: $P = 0.0009$; Exercise: $P = 0.338$; Tissue x Exercise: $P = 0.225$.

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Appendices

Appendix A: Effects of dietary methyl-branched medium-chain fatty acids on growth, hematology, hepatology and exercise capacity of C57BL/6J mice.

Abstract

The aim of this study was to evaluate the effects of feeding methyl-branched medium chain triglycerides (MCT) as compared to straight-chain MCT or long chain triglycerides (LCT). First, serum concentrations of β-hydroxybutyrate (BHB) were measured in mice at 0, 1, 2 and 4 h after gavaging 0.110 mL (~13 umol/kg body weight) 90% pure triglyceride mixtures: tri-hexanoate (t-C6), tri-2-methylpentanoate (t-2MeP) or t-C6 + t-2MeP (mixed). Serum BHB was highest at 1 h in the t-C6 and the mixed dosed mice. The peak BHB appearance in mice given mixed was less than t-C6 by 49% ($P < 0.05$). In a second trial, 47 mice were assigned to a soy-based dietary treatment; soyoil, t-2MeP, t-C6 or mixed. Over 9 weeks, mice were evaluated for feed intake and body weight. Feed intake was not affected by diet ($P > 0.05$). However, by week 3, mice fed t-2MeP experienced decreased body weights ($P < 0.05$). By week 4, all MCT fed mice weighed less than soyoil ($P < 0.05$). Mice fed t-2MeP had increased liver weights ($P < 0.05$). Mice fed t-2MeP had elevated serum urea nitrogen, alkaline phosphatase, BHB and decreased triglycerides as compared to soyoil or t-C6 fed mice ($P < 0.05$).

We found that feeding methyl-branched MCT t-2MeP decreased body weight but increased liver weight as compared to soyoil feeding ($P < 0.05$). Non-branched MCT t-C6, however, only decreased body weight as compared to soyoil feeding ($P < 0.05$). Feeding t-2MeP affected serum metabolite profiles as compared to soyoil or t-C6 ($P < 0.05$). These

results were consistent with our hypothesis that the anaplerotic structure of methyl-branched fatty acids affects metabolism and produces a unique physiological response.

Keywords**:** Medium-chain triglyceride, odd-chain triglyceride, β-hydroxybutyrate, toxicological.

Introduction

A triglyceride is composed of three fatty acid molecules, each attached to one of the three carbons in a glycerol molecule (1). For example, triglyceride 2-methylpentanoate (t-2MeP) contains only 2-methylpentanoic acid (2MeP) fatty acids and a glycerol molecule. Unesterified, 2MeP is a methyl-branched medium chain fatty acid (MCFA). The methyl-branch is a methyl group attached at the 2-carbon of the fatty acid. While it is similar to hexanoic acid (C6), in that it has six carbon molecules, it has a carbon chain length of only five carbons, which makes it an odd chain fatty acid (2). This shorter carbon chain makes MCT such as 2MeP or C6 more hydrophilic and moderately polar, which increases their solubility in water and biological fluids as compared to LCT (3). The higher solubility of MCT allows them to be more quickly digested, absorbed and transported (2;3). In fact, MCFA are absorbed more quickly than glucose (3); however, at 8 kcal/kg, they are almost as energy dense as long chain fatty acids (LCFA; 4).

Once in the hepatocyte, MCFA may be elongated to LCFA and re-esterified, but this is a very minor fate of MCFA as they are poor ligands for re-esterifying enzymes (1). Primarily, MCFA are quickly catabolized as they can cross the mitochondrial membrane independently of carnitine palmitoyltransferase (CPT)-I (5;6). In the mitochondrial matrix, MCFA can be activated by medium-chain acyl-CoA synthetase to MCFA-CoA and finally begin β-oxidation (7).

Unregulated fatty acid catabolism, as seen in the relatively unregulated β-oxidation of unbranched MCFA, will produce large amounts of acetyl-CoA (7). However, the capacity of

the TCA cycle cannot keep up with unregulated β-oxidation and the TCA cycle will eventually run out of OAA and be forced to shunt the excess acetyl-CoA to ketone bodies; thus, the ketogenic nature of even chain MCFA (7;8). However, it has been observed that TCA cycle function and energy production can be rescued by the simultaneous administration of both anaplerotic molecules and MCT (1;7;9;10). It has been suggested this occurs through the actions of anaplerosis and circumvention of fatty acid oxidation inhibition, respectively (7;9).

Feeding MCT has been shown to affect circulating lipid profiles, decrease serum triglyceride concentrations and decrease circulating cholesterol levels (7;11;12). In acute studies, high levels of circulating MCFA will cross the blood brain barrier and have been associated with narcoleptic symptoms and death (12;13;14). However, dangerous concentrations of MCFA in the body cannot be achieved from MCT in a food matrix and only have been demonstrated in acute studies (12).

Because feeding even chain MCT is associated with ketone body production, ketoacidosis may be a concern (2;8). However, ketoacidosis was not observed with even or odd chain MCT feeding in humans (7). Tissues have been shown to increase ketone body utilization with increasing concentrations of ketone bodies, thus even with high administration or intake of MCFA, levels are limited in the blood (15).

In some cases, diets high in MCT have been shown to decrease weight gain and fat deposition (11). The decreased energy density of MCFA as compared to LCFA, their inability to be directly stored in the body and the thermogenic energy expenditure associated

with catabolism and disposal of MCFA have been attributed for this difference in energy metabolism of MCT versus LCT (1;11). A review of toxicological studies found no toxicologically significant effect of feeding very high levels of MCT in the diet (20% for 10-12 months), except those on the MCT diet gained 15% less weight and had lowered serum cholesterol levels (12). In chronic human studies, when patients were fed MCT as the sole source of fat at 40% of calories in the diet, cholesterol levels were decreased (as compared to a butter treatment, but not as compared to a corn oil treatment) and temporary nausea and fullness were experienced on days 3-4 of the 10 week crossover study (12). In another chronic study, non-obese males were fed MCT as 40% of calories in the diet in diets fed at 150% of energy requirements (12). Here, no toxic effects were detected (12). However, fasting serum cholesterol was decreased in control LCT fed males but not in MCT fed males (12). Furthermore, fasting serum triglycerides were three times higher with MCT feeding (12). Nevertheless, MCT were determined to be nontoxic (12).

In this study, we evaluated the growth, hepatological and hematological effects of feeding methyl-branched odd chain MCT and non-branched even chain MCT in the diet. We evaluated their individual effects and synergistic effects as compared to a soyoil diet. We hypothesized that feeding methyl-branched odd chain MCT t-2MeP in the diet will influence biological parameters and exercise endurance time.

Materials & Methods

Triglyceride metabolism test.

Mice were managed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC). Male 6-week old C57BL/6J mice from Jackson Laboratories were gavaged (Straight 22Gx1in. Stainless Steel Animal Feeding Bio-medical Needles, Popper & Sons, Inc., New Hyde Park, New York) with a 0.110 mL (~13 umol/kg body weight) bolus of 90% tri-hexanoate (t-C6), t-2MeP, or a mixture of both. Triglyceride synthesis was adapted from the methods of Wheeler *et al.* (Appendix B; 16). This was calculated to be an appropriate volume for mice weighing about 20 grams (17). After 0, 1, 2, 3, or 4 hours, mice were anesthetized using 4% isoflurane in oxygen and a terminal cardiac blood draw was performed, followed by cervical dislocation. Serum was separated in serum separator tubes (BD Microtainer SST clear/amber), aliquoted, and stored at -80°C until analysis. Serum was analyzed for β-hydroxybutyrate (BHB; β-hydroxybutyrate Assay Kit, Biovision), n= 3/treatment timepoint.

Effects of dietary methyl-branched medium chain triglyceride 2-methylpentanoate, dietary straight chain medium chain triglyceride hexanoate and the effect of both on hematology, hepatology and exercise endurance in mice.

Experimental design.

Mice were managed according to protocols approved by the IACUC. Male 7-week old C57BL/6J mice from Jackson Laboratories were blocked for body weight and then

randomly allocated to a soy-based dietary treatment; soybean oil diet (10% SBO), tri-2-methylpentanoate diet (8% t-2MeP), tri-hexanoate diet (8% t-C6) or mixed diet (4% t-2MeP + 4% t-C6). Experimental MCT synthesis was adapted from the methods of Wheeler *et al.* and pelleted diets were formulated by Harlan laboratories (Appendix B; 16). Mice were housed four per cage and ear punches were used for individual identification within a cage.

Feed intake and body weights were measured weekly throughout the trial (Compact Scale 5000, Ohaus, Parsippany, New Jersey). Feed intake normalized to metabolic rate was calculated as follows. The average daily body weight gain of each cage, expressed on a per mouse basis was adjusted to a metabolic body weight value using Kleiber's law: (body weight, kg)$^{0.75}$, n=3/treatment (18). Average daily feed intake, calculated as a cage's average daily feed intake expressed on a per mouse basis, was divided by the metabolic body weight value. Feed efficiency was calculated using the cage's average daily feed intake expressed on a per mouse basis. This value was calculated as intake value divided by the average daily body weight gain of each cage, expressed on a per mouse basis, n=3/treatment.

Exercise endurance tests.

Mice were evaluated for innate exercise endurance capacity at the beginning of the trial using a treadmill endurance time test. Two days prior to this endurance test, mice were acquainted with the treadmill with a 10 minute run at 8 m/min. The custom treadmill was set to a 20° incline with an initial speed of 12 m/minute. The speed was increased by 1 m/min 2, 5, 10, 20, 30, 40, 50, and 60 minutes after the initiation of the exercise to achieve exhaustion. Methods of exercise exhaustion were modified from Pederson *et al.* (19). Mice were

determined to be exhausted after tolerating 5 seconds on the shock grid (Small Animal Shocker, Coulbourn Instruments, Whitehall, Pennsylvania) instead of resuming treadmill running (20). Proper shock grid function at 1.6 mA was verified using a multimeter (Multimeter 20 Series III, Fluke, Everett, WA). Proper treadmill speed was verified using a tape measure and a timer.

Mice were evaluated for exercise endurance time after 4 weeks, 6 weeks and 8 weeks on the experimental diets. At the 4 and 8 week endurance tests, mice were exercised on a treadmill according to the methods used for initial endurance evaluation. The 6 week endurance test consisted of a swimming endurance test modified from methods described by Fushiki *et al.* (15). The swimming endurance test was performed in a custom current pool modified from the Matsumoto variable-speed swimming pool (21). The current motor output was set to a rate of ~4 L/minute.

Sample collection.

After nine weeks on the diets, mice were euthanized for blood and tissue samples. Mice were euthanized according to protocols approved by the IACUC. Prior to euthanasia, mice were fasted for either 8 or 24 hours. Mice were anesthetized using 4% isoflurane in oxygen and a terminal cardiac blood draw was performed followed by cervical dislocation. Blood collections were performed in replicate order so as to reduce collection delay bias (22).

Serum was separated in serum separator tubes (Microtainer SST clear/amber, Becton, Dickinson, & Co., Franklin Lakes, New Jersey), aliquoted and placed on ice for later

analysis. Serum was analyzed for BHB (β-hydroxybutyrate Assay Kit, Biovision, Milpitas, California) and a clinical chemistry panel was performed by Antech Diagnostics (Morrisville, NC). Clinical chemistry testing was performed using Olympus reagents and the Olympus 640e clinical chemistry analyzer (Beckman Coulter, Inc. Brea, CA). Tests performed by the Olympus included: albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea nitrogen (BUN), cholesterol (CHOL), creatinine (CREA), creatine phosphokinase (CPK), glucose (GLU), total bilirubin (TBIL), total protein (TP) and triglycerides (TG). Non-esterified fatty Acids (FFA), when treated with acyl-CoA synthetase in the presence of adenosine triphosphate (ATP), Mg and Coenzyme A (CoASH), form thiol esters, adenosine monophosphate (AMP) and pyrophosphate. Acyl-CoA is oxidized by acyl-CoA oxidase to produce hydrogen peroxide which in the presence of peroxidase allows the oxidative condensation of 3-metyl-N-ethyl-n-(B-hydroxy-ethyl)-aniline with 4-aminoantipyrine to form a purple colored adduct. Nonesterified fatty acids were also analyzed using the Olympus 640 and WAKO Diagnostics reagents (WAKO Chemicals, Richmond VA). Globulin levels were calculated as the difference between TP and ALB. Epididymal fat pads, lungs, heart, spleen, liver, hindlimb skeletal muscle and kidneys were also weighed. Hindlimb skeletal muscles were collected; these muscles are primarily composed of soleus, extensor digitorum longus, tibialis anterior and gastrocnemius muscles (23).

Statistical analysis.

For the triglyceride metabolism test, analysis of variance using the mixed model procedure of SAS (version 9.2, SAS Inst. Inc., Cary, NC) was used to calculate least-squares means (LSM) ± standard error of means (SEM). Comparisons were adjusted using Tukey's test. Differences were considered significant when $P < 0.05$ and trends were considered when $P < 0.10$.

For the feeding trial, data were analyzed according to a completely randomized block design, blocked for replicate number. Initial weight was used as a covariate for body weight analyses. Analysis of variance using the mixed model procedure of SAS was used to calculate LSM ± SEM. Comparisons were adjusted using Tukey's test. Differences were considered significant when $P < 0.05$ and trends were considered when $P < 0.10$.

Results

The effect of acute methyl-branched triglyceride treatment on serum BHB concentrations at different time points.

One hour after gavage, the mice treated with t-2MeP had lower serum BHB levels than mixed triglyceride or t-C6 (Figure 1; $P < 0.05$). Additionally, mice gavaged with t-2MeP had serum BHB levels not different from zero hour mice ($P < 0.05$), while those gavaged with mixed triglyceride or t-C6 had elevated BHB concentrations after one hour. Mice gavaged with mixed triglyceride also had lower serum BHB levels than those gavaged with t-C6 ($P < 0.05$).

Serum BHB levels of mice gavaged with t-2MeP did not significantly change from time zero to one hour, two hours or four hours after gavage (Figure 1; $P > 0.05$). However, two hours after gavage, mixed triglyceride treated mice experienced decreased BHB concentrations and consequently were no longer different from t-2MeP or mixed triglyceride treatments ($P > 0.05$). Still, mixed triglyceride gavaged mouse BHB levels did not differ between one and two hours after gavage ($P > 0.05$). The t-C6 gavaged mice also experienced decreases in serum BHB levels at the two hour time point. This decrease did not change the effect of t-C6 between the one hour and two hour time points ($P > 0.05$). However, the effect of t-C6 two hours after gavage no longer differed from that of mixed triglyceride gavaged mice one hour after gavage ($P > 0.05$).

Four hours after gavage, t-2MeP showed no increase or decrease in BHB levels (Figure 1; $P > 0.05$). Mice gavaged with t-C6, however, did significantly differ from mice

gavaged with t-2MeP four hours after gavage ($P < 0.05$). At this time point, BHB levels in the mice treated with t-C6 decreased from but were not different than concentrations at the two hour time point ($P > 0.05$). Still, t-C6 four hour serum BHB levels were no longer different from mixed triglyceride gavaged mice at the one and two hour time points ($P > 0.05$).

Feed intake of mice fed a methyl-branched triglyceride based diet over the course of nine weeks.

Treatment differences in feed intake occurred only during the first week of the experiment (Figure 2; $P < 0.05$). During the first week, mice on the t-2MeP diet ate less food than mice on any of the other treatments ($P < 0.05$). Additionally, mice on the t-C6 diet and the mixed diet ate less food than mice on the soyoil diet ($P < 0.05$), but not different amounts from one another ($P > 0.05$). For the remainder of the experiment, there was no effect of diet on feed intake ($P > 0.05$). The results were almost identical when expressed per kilogram of metabolic body weight ($kg^{0.75}$; Figure 3; 18).

Body weights of mice fed a methyl-branched triglyceride based diet over the course of nine weeks.

At the start of the experiment, there were no differences in average treatment group body weights (Figure 4). This level starting point can be seen in week one body weights ($P > 0.05$) as there was not yet an effect of diet on body weight ($P = 0.896$). However, by week two, there was a trend in treatment effect on body weights ($P = 0.057$). Mice in the t-2MeP dietary treatment group weighed less than mice on the soyoil or mixed diets ($P < 0.05$). The

t-C6 treatment group weights were intermediate between the mixed treatment group weight and the t-2MeP treatment group weight, but did not different from any of the treatment groups ($P > 0.05$).

In weeks 3-9, there was an effect of diet on mouse body weight (Figure 4; $P < 0.05$). At week three, t-2MeP fed mice weighed less than all other treatments ($P < 0.05$). At week three, none of the other treatment groups differed in body weight ($P > 0.05$). By week four, t-C6 fed mice were still heavier than t-2MeP fed mice ($P < 0.05$), however, they were also lighter than soyoil or mixed diet fed mice ($P < 0.05$). Mice in the soyoil and mixed diet treatments continued to not differ in body weights ($P < 0.05$). During week five, mice fed t-2MeP weighed less than mice fed the mixed or soyoil diets ($P < 0.05$). However, body weights of mice on the t-C6 diet did not differ from that of mice on the t-2MeP diet ($P > 0.05$). Additionally, t-C6 treatment group body weights were not different from mixed ($P > 0.05$), which were not different from soyoil ($P > 0.05$). At week six, soyoil fed mice weighed more than any of the other treatment group ($P < 0.05$). The body weights of t-C6 and mixed diet fed mice did not differ from one another ($P > 0.05$), however, 2-MeP fed mice weighed less than any other treatment group ($P < 0.05$). At week seven, mice on the soyoil diet did not weigh more than mixed diet fed mice ($P > 0.05$). The body weights of mixed diet fed mice did not differ from that of t-C6 fed mice ($P > 0.05$). However, t-2MeP fed mice were lighter than mice in other treatment groups ($P < 0.05$). By week eight, soyoil fed mice weighed more than any other treatment group ($P < 0.05$). The t-C6 and mixed diet fed mice did not differ in body weights ($P > 0.05$). The t-2MeP fed mice were lighter than all other treatment groups ($P < 0.05$). At week nine, soyoil fed mice continued to weigh more than any other treatment

group ($P < 0.05$). The t-C6 and mixed fed mice did not differ in body weight ($P > 0.05$). The t-2MeP fed mice continued to weigh less than all other treatment groups ($P < 0.05$).

Feed conversion efficiency of mice fed a methyl-branched triglyceride based diet over the course of nine weeks.

The effect of diet on feed conversion efficiency was only evident in the first week of the feeding trial (Figure 5; $P = 0.004$). All other weeks were too variable to detect any effect of diet on feed conversion efficiency ($P > 0.05$).

Tissue weights of mice fed a methyl-branched triglyceride treatment diet for nine weeks.

Absolute and relative liver weights of t-C6 fed mice were less than those of t-2MeP or mixed diet fed mice (Table 1; Table 2; $P < 0.05$). When expressed on an absolute basis, soy-fed mouse liver weights were not different from any of the treatments and there were no differences between liver weights from mice fed t-2MeP and mixed diets (Table 1; $P > 0.05$). However, when expressed relative to body weight, t-2MeP fed mice liver weights were significantly heavier than any other treatment (Table 2, $P < 0.05$).

Diet not have an overall effect on absolute or relative skeletal muscle tissue weight ($P = 0.901$; $P = 0.378$, Table 1; Table 2; respectively). Diet did not affect absolute or relative epididymal fat pad weights ($P > 0.05$). Also, there was only a trend for the effect of diet on epididymal fat pad weight ($P = 0.082$), which was nonexistent when data were expressed on a relative basis ($P = 0.439$). Diet did not affect absolute or relative lung weights ($P > 0.05$) and diet main effects on lung weights were not evident ($P = 0.969$; $P = 0.157$, respectively).

Diet did not affect absolute or relative spleen weights ($P > 0.05$) and diet main effects on spleen weights were not significant ($P = 0.244$, $P = 0.897$, respectively).

Mice fed soyoil or t-C6 diets had heavier hearts than mice fed t-2MeP (Table 1; $P < 0.05$). Heart weights from mice fed the mixed diet did not differ from any other treatment and there were no differences between heart weights of mice fed soyoil or t-C6 ($P > 0.05$). However, when heart weights were expressed relative to body weight, diet did not have an effect on heart weight and the effect of diet on heart weight was not evident (Table 2; $P = 0.157$).

Mice fed the soyoil diet had heavier kidneys than mice fed the t-2MeP diet (Table 1; $P < 0.05$). Kidney weights from mice fed the t-C6 diet or the mixed diet did not differ from any other treatments ($P > 0.05$). However, when kidney weights were expressed relative to body weight, there were no differences among dietary effect on kidney weights (Table 2; $P > 0.05$). Furthermore, diet did not have an overall effect on kidney weights ($P = 0.153$).

Fasting serum metabolites of mice fed a methyl-branched triglyceride treatment diet for nine weeks.

Kidney function tests.

Feeding the t-2MeP diet elevated fasting serum BUN in mice, as compared to soyoil-fed mice (Table 3, $P < 0.05$). The t-C6 and mixed diets did not have an effect on BUN as compared to the soyoil diet ($P > 0.05$). The effects of mixed triglyceride and 2-MeP diets did not differ from one another ($P > 0.05$). Fasting serum CREA was not different between

treatments ($P > 0.05$), and there was no overall effect of diet on fasting serum CREA ($P = 0.482$).

Biliary function tests.

Total bilirubin did not differ between treatments and the effect of diet were not significant (Table 3; $P = 0.549$). Fasting serum ALP was highest in mice fed t-2MeP; as such, it was different from soyoil fed ALP levels (Table 3; $P < 0.05$). Alkaline phosphatase was also elevated in mixed triglyceride fed mice as compared to soyoil fed mice ($P < 0.05$). Additionally, there were no differences between ALP in soyoil and t-C6 fed mice or t-2MeP and mixed triglyceride fed mice ($P > 0.05$).

Liver enzyme tests.

Due to large variation, ALT and AST were not significantly different among treatments and there was no effect of diet on ALT or AST concentrations (Table 3; $P = 0.403$; $P = 0.552$, respectively). However, reported mixed diet treatment ALT levels were more than four times those of the soyoil treatment group (mixed: 360 versus soy: 87.9 ± 133 U/L). The mixed diet increased reported AST levels to over 2.5-times those of the soyoil fed mice (mixed: 645 versus soyoil 242 ± 216 U/L).

Serum components.

Fasting serum TP did not differ between treatments ($P > 0.05$), and there was no effect of diet on TP (Table 3; $P = 0.220$). Fasting serum ALB was higher in t-2MeP than soyoil fed mice ($P < 0.05$). Mixed and t-C6 fed mice did not differ from any other treatment

($P > 0.05$). Globulin levels were not different between treatment groups ($P > 0.05$) and there was no overall effect of diet on globulin ($P = 0.224$). The albumin/globulin ratio of t-C6 and t-2MeP fed mice was different from that of soyoil fed mice ($P < 0.05$), but they did not differ from each other ($P > 0.05$). The albumin/globulin ratio of the mixed diet mice was not different from any of the other treatments ($P > 0.05$).

Due to large variation, CK was not significantly different between treatments and there was no effect of treatment on CK concentrations (Table 3; $P = 0.841$). Fasting serum GLU was not different among treatments (Table 3; $P > 0.05$), and there was no overall effect of diet on fasting serum GLU ($P = 0.558$).

Fasting serum BHB levels were not different between soyoil and t-C6 fed mice (Table 3; $P > 0.05$). They were also not different between mixed and t-C6 or t-2MeP fed mice ($P > 0.05$). However, BHB levels were significantly lower in soyoil or t-C6 mice than t-2MeP fed mice ($P < 0.05$).

Serum lipid profile.

Fasting serum CHOL was not affected by dietary treatment ($P > 0.05$) and there was no overall effect of diet on CHOL (Table 3; $P = 0.121$). Fasting serum TG levels were not different between soyoil and t-C6 fed mice or t-2MeP and mixed diet fed mice ($P > 0.05$). However, t-2MeP or mixed triglyceride fed mice had lower TG levels than soyoil or t-C6 fed mice ($P < 0.05$). Fasting serum FFA levels were not significantly different between t-2MeP, mixed triglyceride and t-C6 fed mice ($P > 0.05$). Nonesterified fatty acid levels in mice fed t-

C6 or mixed diets were not different from soyoil fed mice ($P > 0.05$); however, T-2MeP fed mice had decreased FFA than soyoil fed mice ($P < 0.05$).

Exercise endurance of mice after feeding a methyl-branched triglyceride treatment diet for 4, 6 and 8 weeks.

Diet did not have an effect on exercise endurance in the first treadmill endurance test ($P = 0.622$), the swim test ($P = 0.235$), or the second treadmill endurance test (Figure 6; $P = 0.603$).

Discussion

The effect of acute methyl-branched triglyceride treatment on serum BHB concentrations at different time points.

Mouse serum BHB concentrations were associated with ingestion of t-C6 in a dose-response manner (Figure 1). This effect was evidence when mice gavaged with a bolus of t-2MeP (0% t-C6) had BHB concentrations that were 18 and 36% of BHB levels from mice gavaged with a 100% and 50% t-C6 triglyceride bolus, respectively, at one hour post-gavage. Thus, at peak BHB serum levels (i.e. time point one), mice gavaged with the mixed gavage containing 50% t-C6 and 50% t-2MeP had serum BHB levels 51% of those gavaged with a completely t-C6 bolus of triglyceride.

The association between even chain MCFA dose and resulting serum BHB concentrations has been previously observed in piglets dosed with fatty acids octanoate (C8:0), nonanoate (C9:0) or decanoate (C10:0) (2). In this study, piglets dosed with even chain MCFA had BHB levels elevated above those of piglets dosed with odd chain fatty acids for over four hours (8). The same differences in BHB levels four hours post-gavage were observed in this study (Figure 1). Odle et al. suggested these differences were a result of chain length differences between C8:0 and C9:0 MCT and differences in catabolic fates of even versus odd chain MCFA (8). Tetrick *et al.* dosed macaques with water, C8:0, C9:0 or C10:0 and evaluated plasma fatty acid and serum BHB concentration (24). They suggest these differences could not be attributed solely to chain length effect (24). They postulated

the unexpectedly low BHB levels in C9:0 treated animals most likely resulted from odd-chain fatty acid anaplerosis (24).

The dose-response effect was less clearly seen two hours post-gavage (Figure 1). At this time point, BHB levels of t-2MeP gavaged mice increased incrementally (0.896 to 1.01 mM) and t-C6 treated mouse BHB levels decreased at a greater rate than those gavaged with a mixed triglyceride bolus. At the two hours post-gavage time point, the t-2MeP gavaged mice had BHB levels that were 27% and 45% of t-C6 and mixed gavaged mice, respectively. Thus, BHB concentrations in mixed treatment mice were 59% of those in t-C6 treatment mice.

The accelerated decrease in BHB concentrations observed with t-C6 treatment continued at the four hour time point (Figure 1). After four hours, t-2MeP treated mouse BHB concentrations reached 34% of t-C6 treated mice. However, the dramatic 24% decrease in BHB concentrations of t-C6 treated mice from peak concentration to one hour post-peak concentrations (i.e. from time point one to time point two) was decelerated in the next concentration reading. This concentration reading showed the change over two hours, from time point two to time point four. At time point two, BHB levels were not different from that of mixed triglyceride gavaged mouse BHB levels at time point one. Thus, it is interesting to find their percent decrease per hour was similar (mixed from one hour to two hours decreases 12% from 2.52 to 2.53 mM; t-C6 from two hours to four hours decreases 14% per hour from 3.76 to 2.65 mM over the course of two hours). It may have been the molar concentration of

BHB that drove clearance rates with accelerated clearance occurring at peak concentrations. This phenomenon was also observed by Odle *et al.* (8).

If BHB concentrations do in fact indicate catabolism of t-C6, this experiment showed catabolites from orally dosed t-C6 mice peaked during the first hour after consumption in mice (Figure 1). The assumed indication of t-C6 oxidation rate is characteristic of MCT and has been previously observed by Odle *et al.* (8). Furthermore, Odle *et al.* has observed correlated peaks in serum MCFA and BHB concentrations after gavage (2); however, further evaluation of serum fatty acid concentrations from this experiment would be required for conclusive clearance curves.

Feed intake of mice fed a methyl-branched triglyceride based diet over the course of nine weeks.

Mouse feed intake was affected by dietary treatment only during the first week on the diet (Figure 2; Figure 3). During the first week, the mice on the soyoil diet ate more than any of the other treatments ($P < 0.05$). Additionally, the mice on the t-C6 and mixed diets ate more than the mice on the t-2MeP diet ($P < 0.05$). The decreased intake by mice on MCT diets may have been a result of gastrointestinal upset and/or abdominal fullness as similar responses have been observed in previous MCT feeding trials (12).

Interestingly, instead of t-2MeP treatment group feed intake increasing back to the other groups, the feed intake of the mice on the soyoil diet decreased so that it was no longer different from any other treatment group's intake (Figure 2; Figure 3; $P > 0.05$). The feed intake of the mice on the t-C6 and mixed diets also decreased to where they were not

different from the feed intake of the mice on the t-2MeP diets ($P > 0.05$). As such, it is possible that the mice on the MCT diets were compelled to immediately decrease food intake during week one due to a taste aversion while a different factor like energy density of the isoenergetic diets may have caused more gradual decreases in the feed intake of all other treatment groups by week two (Appendix B). Additionally, there was a small zig-zag pattern in feed intake, indicating that intake may have been affected by the freshness of the food as it was only topped off or completely replaced every other week.

Body weights of mice fed a methyl-branched triglyceride based diet over the course of nine weeks.

Overall, body weights increased over the course of the 9-week trial (Figure 4). Treatment average body weights were the same at the onset of the trial, but dietary treatments affected treatment group body weight as early as week two. During week two, mice fed the t-2MeP diet lost weight and weighed less than mice on the soyoil diet ($P < 0.05$). At this point, the trend of treatment effect on weight began to manifest ($P = 0.057$). During weeks 3-9, treatment effects on body weight were evident as the t-2MeP fed mice continued to weigh less than the soyoil fed mice for the remainder of the trial ($P < 0.05$). During weeks 4-9, mice on the t-C6 diet weighed less than mice on soyoil diet ($P < 0.05$). Mice fed the mixed diet also weighed less than mice on the soyoil diet on weeks 4-9, except for weeks 5 and 7. The effect of t-C6 and mixed diet feeding almost never differed from one another ($P > 0.05$), except on week 4 where the mice on the t-C6 diet weighed less than the mice on the mixed

diet ($P < 0.05$). A decrease in body weight gain has been previously observed with MCT feeding and therefore our results are consistent with the literature (1;4;11).

Feed conversion efficiency of mice fed a methyl-branched triglyceride based diet over the course of nine weeks.

The variation in feed conversion efficiency was such that differences could not be elucidated in weeks 2-9 (Figure 5; $P > 0.05$). This may be a result of the fact that feed intake values were measured on the unit of cage, while body weights were measured on the unit of individual animal. While calculations were performed using mean cage body weight, the differences in individual animal intakes, not shown in the measurement unit of cage, most likely caused the variability.

Differences in feed efficiency were observed during the first week of the experiment (Figure 5; $P < 0.05$). While the effects of t-C6, soyoil and mixed diets on feed conversion efficiency were not different from one another ($P < 0.05$), the feed conversion efficiency of the mice on the t-2MeP diet was different from all other treatments at a value of -48.8 ($P < 0.05$). This number perhaps results from the overall weight loss in the t-2MeP fed mice during the first week of the experiment (Figure 4).

Tissue weights of mice fed a methyl-branched triglyceride based diet for nine weeks.

When liver tissue weights were expressed as a percent of body weight, feeding t-2MeP significantly increased liver weight relative to body weight (Table 2). In the best case scenario, this is a response to increased liver enzyme synthesis; however, it is also possible

this increase is a result of fatty liver, edema or necrosis, as suggested by Atshaves *et al.* (25). Further liver histology may reveal a definite prognosis. Increased *de novo* fatty acid synthesis would be required with the MCT based diets since MCFA cannot be stored in the body. Shinohara *et al.* fed rats a diet high in either LCT (7%), MCT (5%), or a LCT+MCT mixture (7%) for four weeks (11). As compared to LCT feeding, MCT feeding upregulated lipogenic enzyme activity (fatty acid synthase; ATP-citrate lyase; glucose-6-phosphate dehydrogenase) and lipogenic signaling (SREBP-1; 11). It also increases liver TG concentrations as compared to LCT feeding (11). Increased *de novo* fatty acid synthesis may cause fatty liver disease in individuals fed a high MCT diet (11). Animals fed MCT, however, did not differ from LCT fed mice in liver weights (11). It is possible this difference reveals sometime after four weeks of feeding as Fushiki *et al.* did observe increased liver weights after six weeks of feeding 8% MCT to mice (15).

Dietary treatment did not affect absolute or relative skeletal muscle tissue or epididymal fat pad weights (Table 1; Table 2). This may result from either no effect or inconsistent tissue dissection. However, Fushiki *et al.* found no effect of a MCT diet on gastrocnemius or quadriceps muscles (15). Shinohara *et al.* also found no effect of MCT feeding on epididymal fat pad weights (11). They postulated that the increased energy expenditure observed in the MCT-fed rats was overcome by upregulated *de novo* hepatic fatty acid synthesis (11). Fushiki *et al.*, however, did see significant increases in both perirenal and epididymal fat pad weights (15). In this experiment, dietary treatment did not affect absolute or relative lung or spleen weights (Table 1; Table 2). Fushiki *et al.* also observed no effect of dietary treatment on spleen weights (15).

Feeding t-2MeP decreased absolute heart and kidney weights (Table 1). However, this effect was not evident when tissue weights were adjusted for body weights (Table 2). Thus, it is more likely that a smaller heart and kidneys are required for a smaller body size. No differences in relative heart or kidney weights were observed by Fushiki *et al.* (15).

Fasting serum metabolites of mice fed a methyl-branched triglyceride based diet for nine weeks.

Kidney function tests.

Fasting BUN was elevated in t-2MeP fed mice as compared to t-C6 and soyoil fed mice (Table 3; $P < 0.05$). All other dietary treatments did not have an effect on BUN as compared to soyoil ($P > 0.05$). Because urea is released by the kidneys, it can act as an indicator of pre-renal, renal or post-renal function, especially as urea nitrogen levels will not elevate until 70-75% of kidney has decreased functionality (22). Thus, although BUN levels of t-2MeP fed mice (31.5 ± 1.5 mg/dL) were within the normal literature ranges (31 ± 2.5 mg/dL; 26), BUN levels were deemed abnormal as they differed from control fed animals (23.9 ± 1.5 mg/dL). Fasting serum CREA levels were not affected by dietary treatment and there was no overall effect of diet on CREA levels ($P > 0.05$). However, these reported values were far below normal ranges found in the literature (0.8 ± 0.3; 26). The clinical chemistry analyzer used to measure CREA levels was not the optimal method as many reported values were below the limit of detection for the assay and may have contributed to the unreliability of the data. However, other literature found mouse CREA levels more congruent with our results while using high performance liquid chromatography (HPLC;

0.104 mg/dL). HPLC is considered the gold-standard in CREA quantification (27). If kidney damage had occurred, BUN and CREA would increase in concert (22). If protein catabolism was elevated, then CREA would also be increased in the serum (28). There were no detectable increases in CREA; this indicates dietary treatment did not increase protein catabolism. Since BUN was elevated in t-2MeP fed mice, it was concluded that t-2MeP feeding may impair kidney function.

Biliary function tests.

Total bilirubin levels did not differ between treatments ($P > 0.05$) and there was no overall effect of diet on TBIL (Table 3; $P = 0.211$). As such, there was a large amount of variation in reported results. Also, samples were at or below the lower limit of normal ranges found in the literature (0.2-0.6 mg/dL; 26). The t-2MeP and mixed fed mice had ALP levels elevated above those of soyoil fed mice (178 and 127; versus 54.7 ± 16.1 U/L; Table 3; $P < 0.05$). However, t-C6 fed mice (88.2 ± 16.1 U/L) only differed from t-2MeP fed mice ($P < 0.05$). Furthermore, the soyoil fed mice had ALP concentrations below those found in the literature; while t-2MeP fed mice ALP was elevated outside of the normal range reported in the literature (88-145 U/L; 26). Regardless, the increase in ALP in mixed triglyceride and t-2MeP fed mice indicates the mixed triglyceride and t-2MeP diets may have induced extrahepatic cholestasis (22). However, the TBIL results do not give a positive confirmation to this indication (29).

Liver enzyme tests.

Alanine aminotransferase and AST levels were not significantly different among treatments (Figure 3; $P > 0.05$) and there was no overall effect of diet on ALT or AST ($P = 0.403$; $P = 0.552$). As such, there was a large amount of variation in reported results. The mixed diet mice, however, had reported ALT levels far above normal literature ranges (360 ±133 U/L versus 20-88 U/L, 26). They also had reported AST levels far above the normal literature ranges (645 ± 216 U/L versus 45-176 U/L, 26). Increased ALT and AST levels are usually associated with hepatic necrosis (22).

Serum components.

Total protein levels were not different among treatments ($P > 0.05$) and there was no overall effect of diet on TP (Figure 3; $P = 0.220$). Feeding t-2MeP elevated ALB levels as compared to feeding the soyoil diet ($P < 0.05$). All other treatments were not different from one another ($P > 0.05$). Since it is made only by the liver, hypoalbuminemia indicates liver disease, inflammation absorptive/digestive disease. It may also be associated with kidney or intestinal disease associated with protein loss; hyperalbuminemia, however, indicates dehydration (22). This is perhaps a result of the food-deprivation period. Globulin levels were not different among treatments ($P > 0.05$) and there was no overall effect of diet on globulin levels ($P = 0.224$). Albumin/globulin ratios of t-2MeP and t-C6 fed mice were not different from each other ($P > 0.05$), but were both elevated as compared to soyoil fed mice ($P < 0.05$), again indicating possible dehydration (22), however, this is unexpected from a standard 8 hour fast.

Creatine kinase levels were not significantly different among treatments ($P > 0.05$) and there was no overall effect of diet on CK (Table 3; $P = 0.841$). As such, there was a large amount of variation in reported results. The large variation in CK may be due to the release of CK during cardiac puncture (28). However, it should also be noted that CK levels in the literature were also highly variable. Schell *et.al.* reported an average value of 187 ± 109 U/L using cardiac puncture (26). With similarly aged mice and the same strain (C57/BL6), Jackson laboratories reported an average value of 601 U/L (30). Regardless, the reported CK level in mixed fed mice was 43% decreased from that of soyoil fed mice and could indicate reduced skeletal muscle mass (22); however, skeletal muscle weights did not differ between the mixed and soyoil fed groups ($P > 0.05$). Elevated CK may also point to cell membrane leakage, or cataplerosis (7). Thus, the reported 24% decrease in CK levels in mixed triglyceride fed mice may have resulted from a decrease in cataplerosis. Additionally, the lack of differences in CK values indicate the smaller hearts observed in the t-2MeP fed mice did not cause adverse effects on the heart tissue.

Fasting serum GLU levels were not affected by dietary treatment and there was no overall effect of diet on GLU levels (Table 3; $P > 0.05$). Overall, fasting blood GLU levels were lower than normal levels reported in the literature (100 mg/dL; 26), but were not different from each other.

Serum lipid profile.

Beta-hydroxybutyrate levels were increased in t-2MeP and mixed triglyceride fed mice as compared to soyoil fed mice (Table 3; $P < 0.05$); however, t-2MeP and mixed

treatment effects did not differ from one another ($P > 0.05$). Beta-hydroxybutyrate levels in mice fed the t-C6 diet did not differ from either soyoil or mixed fed mice ($P > 0.05$) but were lower than t-2MeP fed mice ($P < 0.05$). These data support conclusions drawn by Fushiki *et al.*; the chronic feeding of even chain MCT (i.e. t-C6) upregulated ketone body utilizing enzymes like 3-oxo acid CoA-transferase in skeletal muscle tissue (15). If in fact digested and absorbed at the same rate as t-C6, mice on the t-2MeP diet would see fewer ketone bodies than the t-C6 or even soyoil fed mice (see above, "the effect of a methyl-branched triglyceride gavage on serum BHB concentrations at different time points"; Figure 1). Thus, the mice on these diets would not have upregulated ketone body utilization enzymes and would not be able to handle ketone bodies produced during the food deprivation period as well as mice on the ketogenic t-C6 diet would.

Concertedly, mice fed the soyoil diet had higher FFA concentrations as compared to t-2MeP fed mice ($P < 0.05$). These results also indicate that t-2MeP treatment effects may source from the glucogenic nature of t-2MeP fatty acid oxidation products. Here, propionyl-CoA is produced and fed into the TCA cycle and some may exit the TCA cycle at malate for glucose synthesis, but can never be fed into acetoacetate synthesis. As such, the tissue would be less likely to reach acetyl-CoA concentrations which would inhibit fatty acid oxidation and may even produce some carbohydrate. The lack of MCT t-C6 treatment effect on FFA is in agreement with data from Shinohara *et al.* indicating even chain MCT feeding has no effect on FFA concentrations (11). However, these results may simply stem from the FFA assay used. It has been previously indicated this assay is not optimized for evaluation of

MCFA (data not shown) and it therefore may have missed the small amount of, if any, C5 fatty acids still circulating in the 2-MeP fed mice.

Cholesterol levels were not different among treatments (Table 3; $P > 0.05$) and there was no overall effect of diet on CHOL ($P = 0.121$). These data are also in agreement with results from Shinohara *et al.* and only contribute to the summary by Traul *et al.*'s of the controversial effects of TG chain length on serum cholesterol levels (11;12).

The t-2MeP diet lowered circulating TG levels as compared to the soyoil or t-C6 diets (Table 3; $P < 0.05$). However, the t-C6 treatment group did not differ from the soyoil fed group; thus, the mechanism by which previously shown even chain MCT feeding in mice decreases serum TG is debatable, or just was not elucidated in this study. These findings disagree with data from Shinohara *et al.* indicating chronic feeding of even chain MCT reduces serum TG (11). Previously, the TG lowering effects have been attributed to the fact that MCT are only minorly incorporated into TG until TG synthesis enzymes are upregulated (1;11). The mechanism by which t-2MeP feeding lowers circulating TG may be the same as indicated in its ability to decrease circulating FFA levels; that is, its glucogenic structure. Since propionyl-CoA, a carbohydrate-like molecule, is produced from the catabolism of this molecule, it would not inhibit fatty acid oxidation and could only feed into gluconeogenesis.

Overall, it should be noted that different methods of blood collection have been shown to induce vast differences in blood parameter outcomes (26). Additionally, age and sex affect blood serum parameters (28). Lastly, mice have lower CREA, ALB, and TP levels

than humans and therefore extrapolation of these mouse-modeled finding to human outcomes should be made with caution (28).

Exercise endurance of mice after feeding a methyl-branched triglyceride treatment diet for 4, 6 and 8 weeks.

Diet had no effect on exercise endurance time (Figure 6; $P > 0.05$). This may be due to the fact that diet in fact did not have an effect on exercise endurance. However, it is also possible that differences in dietary treatment effects were simply not detectable with the endurance tests that were used.

In the case of the exercise endurance test, there are several potential sources of error. Firstly, the treadmill used to evaluate endurance time was designed for rats. The treadmill was clearly not designed for animals with only a 20-30 g body mass, therefore the gap between the treadmill belt and the shock grid was such that mice could perch on one shock bar and not complete the shock grid circuit. This gap also created a problem wherein the mice would purposefully slip between the treadmill belt and the shock grid so as to exit the endurance test. This gave them and whatever other mice were perching on a single shock grid bar a break in running. Aluminum foil was wrapped around the bars closest to the treadmill belt, but it seemed to diminish the mice's response and it was suggested that it may have shorted out the shock grid altogether.

During the second treadmill endurance test, an additional wire was added to the shock grid to prevent mice from falling through the shock grid space and remove the need for aluminum foil. Still, the mice did not seem to respond to the shock grid. Thus, it is possible

that while the shock grid amperage was checked, it may not have been conducting as well as other treadmills set to the same amperage.

It was finally determined the exercise protocol that was used was inadequate to sufficiently evaluate exercise exhaustion in mice. The exercise protocol was adapted from Pederson *et al.* where the treadmill was set at a 20° incline and exercise intensity was ramped from an initial belt speed of 12 m/min to a final speed of 19 m/min (19). Previously, this speed has been shown to be sufficient for exhaustive exercise as it corresponds to about 84% $VO_{2\ max}$ when the treadmill is level (31). However, the mice in the Pederson trial were fasted prior to the exercise test (19). This difference may have contributed to the lack of endurance evaluation observed in this exercise test (Figure 6A; Figure 6C; 19). Furthermore, Fushiki *et al.* evaluated the effects of MCT feeding on exercise endurance time at 50-60% $VO_{2\ max}$ (15). An exercise intensity of 65% $VO_{2\ max}$ has been shown to maximally stimulate skeletal muscle fat oxidation (32), so it is also possible that the exercise protocol was too intense for appropriate evaluation of fatty acid effects on exercise endurance. Overall the treadmill shock grid prevented the evaluation of the exercise protocol or endurance time in the mice as most of them would refuse to run from the beginning of the exercise trial which resulted in vast variability as mice would intermittently run; adding up to a "endurance" time of over 4 hours.

The swimming endurance test was also not run optimally for evaluation of exercise endurance time in mice. For the pool size used in this experiment, Matsumoto *et al.* recommended running the swim pool motor at a rate equal to or greater than 4-5 L/min (21).

As such, the rate of this pool was set to 4 L/min. However, due to the observed risk of drowning, barriers were implemented in the swimming pool to prevent the creation of whirlpools. Even at this speed, mice were observed to either float or sink. It is possible this problem may have been overcome with training prior to the swim test. Overall, the exercise endurance tests were unsuccessful in evaluating the exercise capacity of mice in this trial.

Conclusions

Mouse serum BHB concentrations are correlated with ingestion of t-C6 in a dose-response manner. BHB concentrations peaked one hour after gavage with t-C6. Further investigation of the exact time course of t-C6 digestion and absorption should be performed to achieve definitive correlation between MCT ingestion, MCFA in the blood, and BHB catabolites.

The t-2MeP diet increased BUN levels, indicating t-2MeP may have caused impaired kidney function (22). However, dietary treatment did not have an effect on CREA. These CREA results do not corroborate with the elevated BUN implication of impaired kidney function.

Feeding t-2MeP was shown to increase relative liver weights. This increase may have resulted from either increased enzyme synthesis for *de novo* fatty acid synthesis, or liver disease resulting from increased fatty acid synthesis or biliary obstruction (11;22;25;29). However, ALT and AST enzyme levels did not indicate liver disease developed from any of the dietary treatments. Histology of liver tissue samples will give a more definitive indication of diet effect on liver status.

Feeding t-2MeP elevated serum ALP levels; thus, this TG may have induced extrahepatic cholestasis (29). However, dietary treatment did not have an effect on TBIL. Thus, the TBIL results do not support indications of cholestasis from the ALP test.

Feeding t-2MeP increased BHB concentrations. It is not clear why this occurred. It may have resulted from an increase in ketone bodies during the food deprivation period. If this occurred, then mice on the t-C6 dietary treatment would have upregulated ketone body utilizing enzyme and may have been better able to use the circulating ketone bodies (15). However, mice on the t-2MeP would not normally encounter elevated ketone bodies and thus would not have the same capacity to catabolize ketone bodies. Feeding t-2MeP decreased FFA concentrations in the 2-MeP fed mice. However, this decrease may have been a result of methodology and not a true decrease in FFA. There was no effect of dietary treatment on cholesterol levels, which is congruent with some studies on the effects of MCT on cholesterol and contrasts with conclusions from other studies (12).

These results are congruent with our hypothesis that a diet containing t-2MeP affects metabolism differently than other MCT or LCT. These differences in metabolism are believed to be a result of t-2MeP's unique structure. As a methyl-branched odd chain MCT, mitochondrial oxidation of t-2MeP results in glucogenic propionyl-CoA molecules instead of ketogenic acetyl-CoA molecules (7). We believe the resulting anaplerotic molecules enhance fatty acid oxidation.

On the other hand, there is a reason for moderation in metabolism as constructing glucogenic lipid and bypassing fatty acid oxidation regulation may have detrimentally affected physiological functions. The effect of nonalcoholic fatty liver disease on hepatic TCA cycle function provides an established description of the negative effects of excessive anaplerosis (33). Sunny *et al.* observed that with dosing of anaplerotic propionate, there is an

increase in futile pyruvate cycling (33). This occurs as propionate enters the TCA cycle as succinyl-CoA, but due to high levels, is exported via pyruvate kinase or malic enzyme and then back into the TCA cycle via pyruvate carboxylase or phosphoenolpyruvate carboxykinase (33). They indicate this futile cycling leads to increased energy demands (33). They also indicate the increase in mitochondrial oxidation causes oxidative stress and hepatic injury (33). Nevertheless, the results from this study indicate that feeding t-2MeP in the diet is nontoxic. However it should be noted that some serum metabolites were affected by dietary treatment, which may indicate some detrimental effects of feeding t-2MeP. Further serum and tissue analysis may confirm or deny these confounding indicators.

Figures

Table 1 Fasting tissue weights of mice fed soyoil, t-C6, t-2MeP or mixed (t-C6+t-2MeP) diets for 9 weeks[1].

	Diet					
Tissue, mg[2]	Soyoil	t-2MeP	t-C6	Mixed	SEM	*P*-value
Liver	961 [a,b]	1033 [b]	890 [a]	998 [b]	31	0.011
Skeletal muscle	1747 [a]	1676 [a]	1698 [a]	1758 [a]	90	0.901
Epididymal fat pad	421 [a]	295 [a]	361 [a]	264 [a]	43.7	0.082
Lungs	125 [a]	125 [a]	123 [a]	122 [a]	5.6	0.969
Heart	133 [a]	109 [b]	136 [a]	128 [a,b]	5.79	0.006
Spleen	67.5 [a]	56.5 [a]	64.9 [a]	59.6 [a]	4.1	0.244
Kidneys	324 [a]	294 [b]	312 [a,b]	314 [a,b]	7.22	0.045

[1] Data are presented as LSM ± SEM, n=9-12 mice/treatment. LSM in a row lacking a common letter differ, $P < 0.05$.
[2] Samples were derived from 8 or 24 hour food-deprived mice. No effect of time by dietary interaction was detected, $P > 0.05$.

Table 2 Fasting tissue weights per gram of body weight from mice fed soyoil, t-C6, t-2MeP or mixed (t-C6+t-2MeP) diets for 9 weeks[1].

	Diet					
Tissue, mg/100g body weight[2]	Soyoil	t-2MeP	t-C6	Mixed	SEM	*P*-value
Liver	0.39 [a,b]	0.47 [c]	0.36 [a]	0.42 [b]	0.01	<0.0001
Skeletal muscle	0.70 [a]	0.76 [a]	0.68 [a]	0.74 [a]	0.03	0.378
Epididymal fat pad	0.15 [a]	0.14 [a]	0.15 [a]	0.11 [a]	0.02	0.439
Lungs	0.05 [a]	0.06 [a]	0.05 [a]	0.05 [a]	0.002	0.157
Heart	0.05 [a]	0.05 [a]	0.06 [a]	0.05 [a]	0.002	0.231
Spleen	0.03 [a]	0.03 [a]	0.03 [a]	0.03 [a]	0.002	0.897
Kidneys	0.13 [a]	0.13 [a]	0.12 [a]	0.13 [a]	0.002	0.153

[1] Data are presented as LSM ± SEM, n=9-12 mice/treatment. LSM in a row lacking a common letter differ, $P < 0.05$.
[2] Samples were derived from 8 or 24 hour food-deprived mice. No effect of time by dietary interaction was detected, $P > 0.05$.

Table 3 Fasting serum metabolites of mice fed soyoil, t-C6, t-2MeP or mixed (t-C6+t-2MeP) diets for 9 weeks[1,3].

		Diet					
Metabolite[2]		Soyoil	t-C6	t-2MeP	Mixed	SEM	*P*-value
Urea Nitrogen	*mg/dl*	23.9 [a]	25.5 [a]	31.5 [b]	29.5 [a,b]	1.5	0.009
Creatinine	*mg/dl*	0.097 [a]	0.139 [a]	0.097 [a]	0.111 [a]	0.022	0.482
Glucose	*mg/dl*	73.9 [a]	67.4 [a]	80.5 [a]	79.6 [a]	7.27	0.558
Alkaline Phosphatase	*U/l*	54.7 [a]	111 [a,b]	178 [c]	127 [b,c]	16.05	0.0007
Alanine aminotransferase	*U/l*	87.9 [a]	121 [a]	81.3 [a]	360 [a]	133	0.403
Aspartate aminotransferase	*U/l*	242 [a]	328 [a]	301 [a]	645 [a]	216	0.552
Total bilirubin	*mg/dl*	0.168 [a]	0.356 [a]	0.127 [a]	0.192 [a]	0.211	0.549
Creatine kinase	*U/l*	422 [a]	312 [a]	356 [a]	240 [a]	139	0.841
Total protein	*g/dl*	4.85 [a]	4.70 [a]	5.15 [a]	5.06 [a]	0.161	0.220
Albumin	*g/dl*	2.71 [a]	2.82 [a,b]	3.15 [b]	2.99 [a,b]	0.089	0.014
Globulin	*g/dl*	2.14 [a]	1.88 [a]	2.00 [a]	2.14 [a]	0.090	0.224
Albumin/Globulin		1.27 [a]	1.52 [b]	1.558 [b]	1.45 [a,b]	0.052	0.004
B-hydroxybutyrate	*mM*	1.60 [a]	2.01 [a,b]	4.04 [c]	3.74 [b,c]	0.269	<.0001
Cholesterol	*mg/dl*	103 [a]	80.8 [a]	100 [a]	107 [a]	7.61	0.121
Triglyceride	*mg/dl*	94.2 [b]	83.5 [b]	39.4 [a]	48.4 [a]	7.76	0.0004
Nonesterified fatty acids	*mmol/l*	1.63 [b]	1.51 [a,b]	1.05 [a]	1.19 [a,b]	1.35	0.011

[1] Data are presented as LSM ± SEM, n=6 mice per diet.
[2] Samples were derived from 8 hour food-deprived mice.
[3] LSM in a row lacking a common letter differ, $P < 0.05$.

Table 4 Feed intake, weight gain and feed efficiency of mice fed soyoil, t-C6, t-2MeP or mixed (t-C6+t-2MeP) diets for 9 weeks [1].

	Diet					
	Soyoil	t-2MeP	t-C6	Mixed	SEM	*P*-value
Intake	3.06	2.68	2.85	2.93	0.177	0.529
Weight	28.6	25.1	26.6	27.0	0.522	0.0002
Efficiency	22.8	1.36	-22.0	25.9	28.0	0.616

[1] Data are presented as LSM ± SEM, n=9-12 mice/treatment.

[2] Samples were derived from 8 or 24 hour food-deprived mice. No effect of time by dietary interaction was detected, $P > 0.05$.

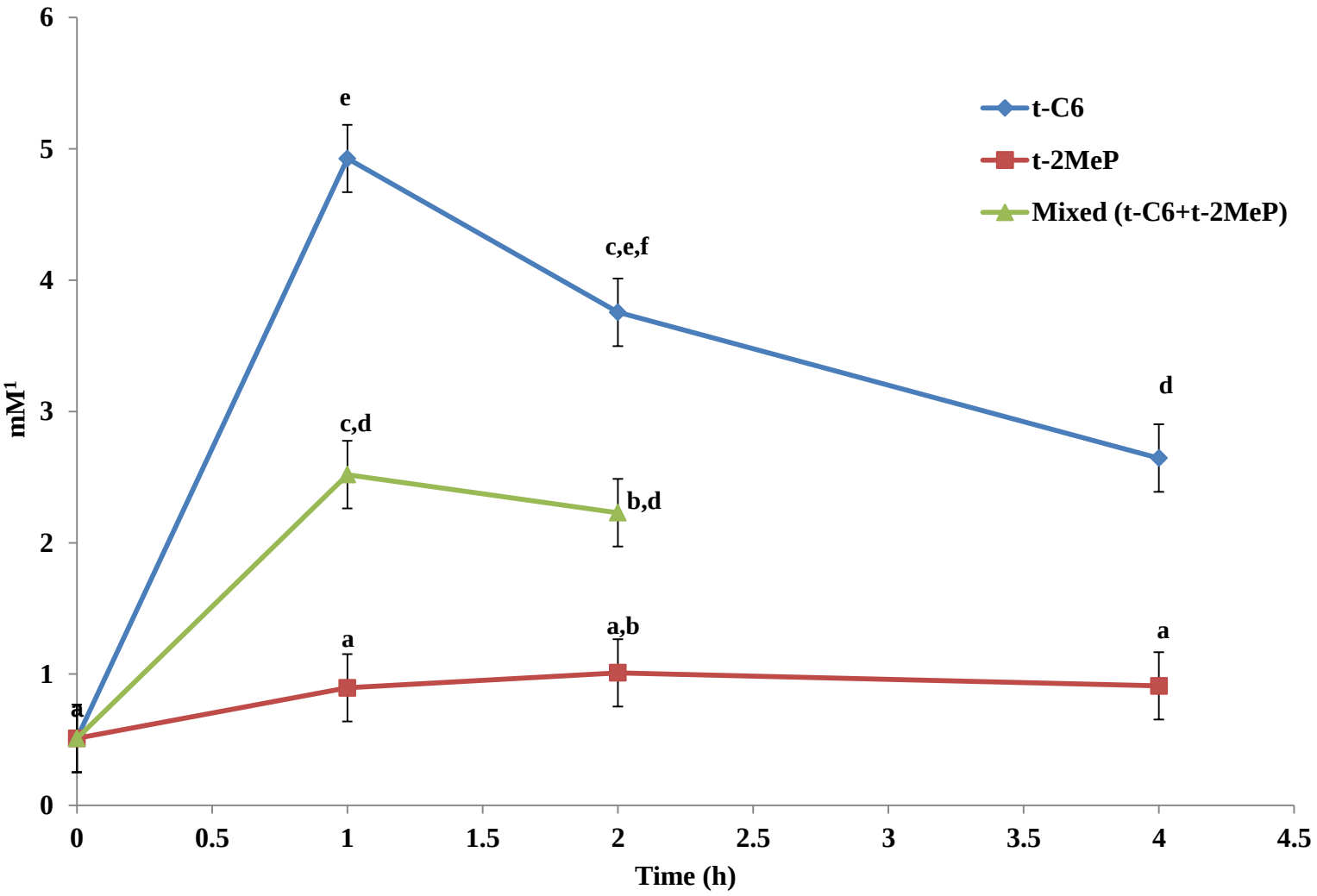

Figure 1 Serum β-hydroxy butyrate levels of mice gavaged with t-C6, t-2MeP or mixed (t-C6 + t-2MeP). Data are presented as LSM ± SEM, n=3/treatment timepoint.

[1] Serum β-hydroxy butyrate (BHB)

[a,b,c,d,e,f] Data points lacking a common letter differ, $P < 0.05$.

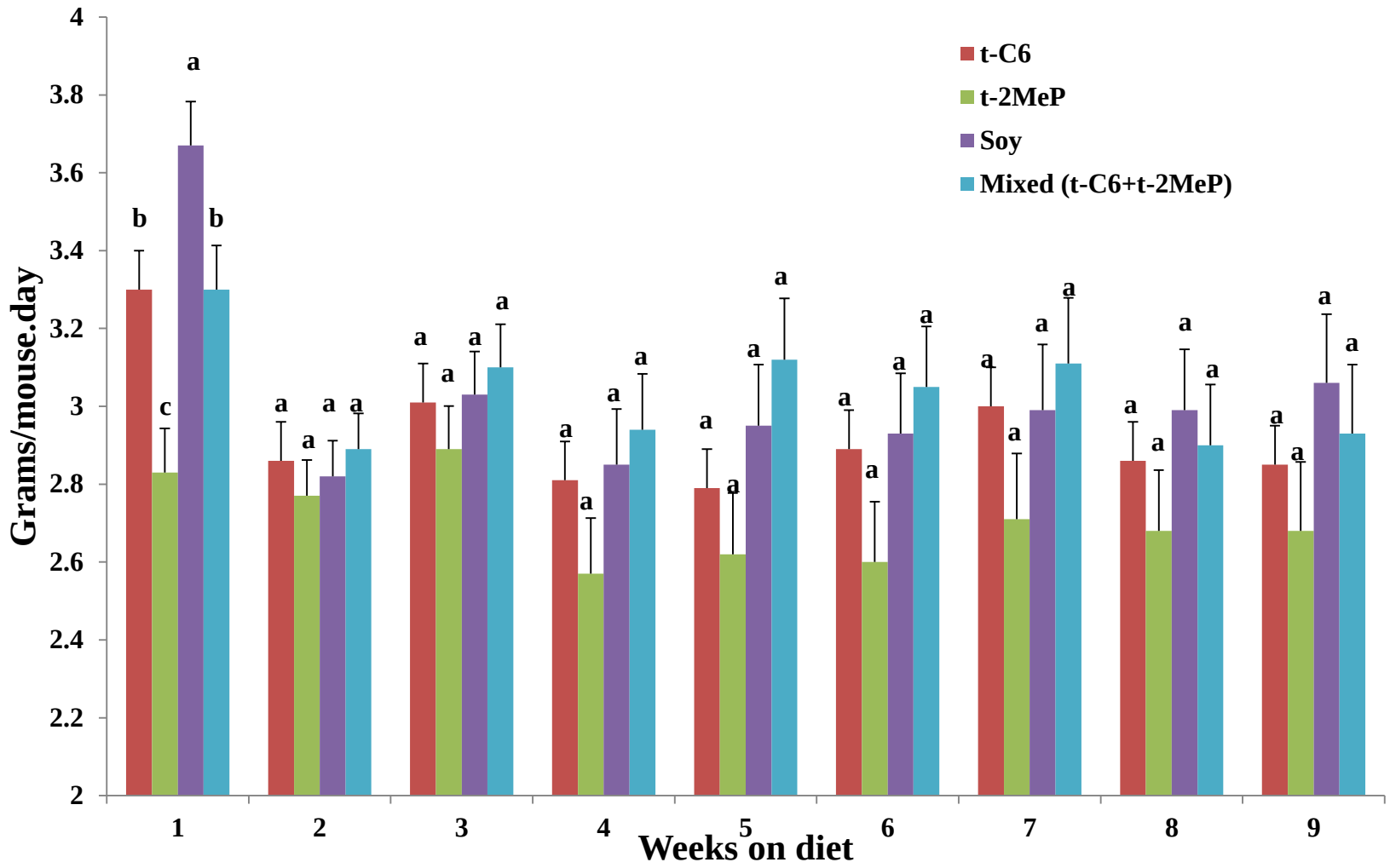

Figure 2 Feed intake of mice fed soyoil (soy), t-C6, t-2MeP or mixed (t-C6+t-2MeP) diet. Data are presented as LSM ± SEM, n=3/treatment. Data were collected as cage mean daily intake per mouse over a 7-day interval.
[a,b,c] LSM in a common timepoint lacking a common letter differ, $P < 0.05$.

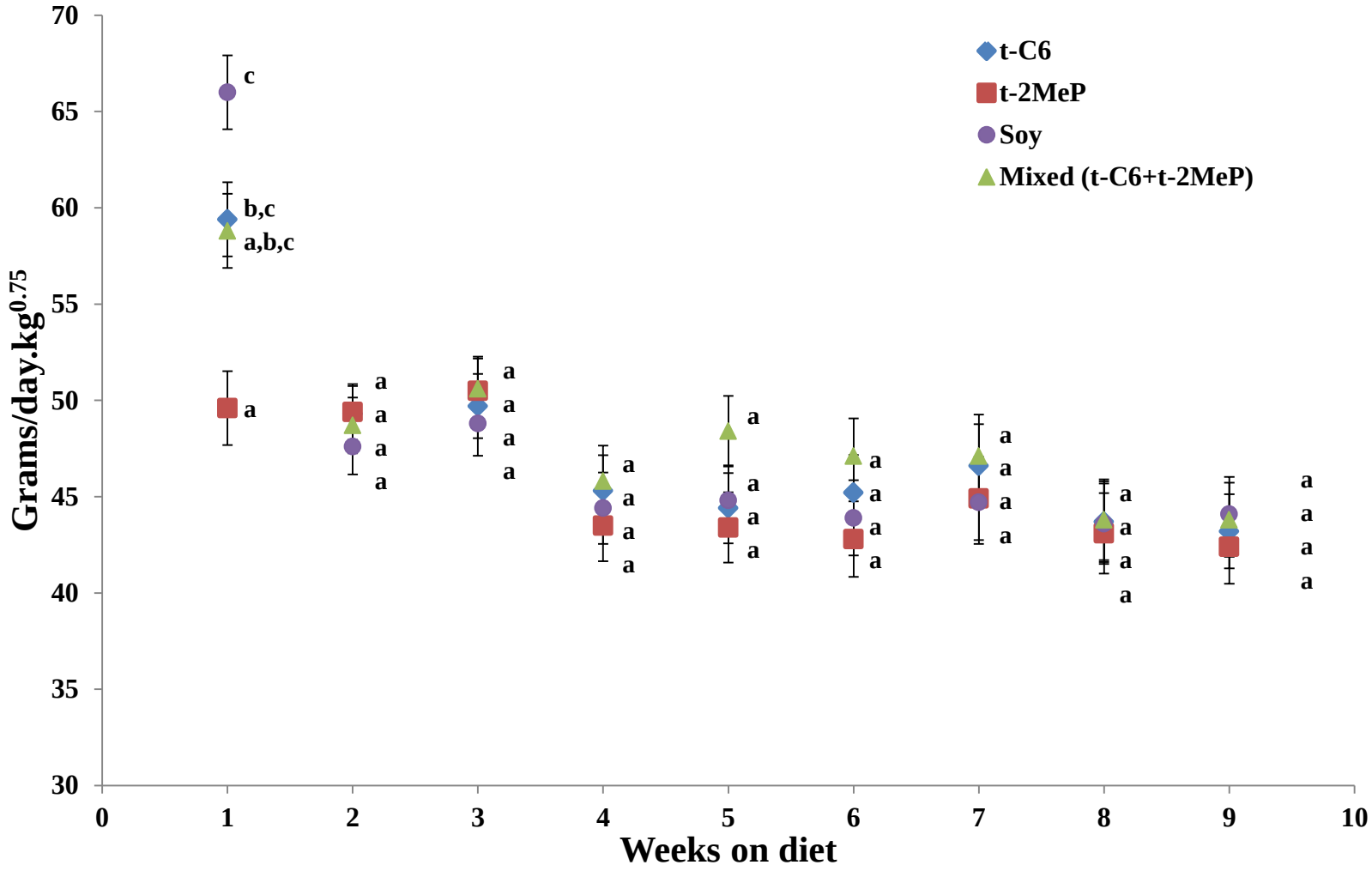

Figure 3 Adjusted feed intake expressed per unit of metabolic body weight ($kg^{0.75}$) of mice fed soyoil (soy), t-C6, t-2MeP or mixed (t-C6+t-2MeP) diet. Data are presented as LSM ± SEM, n=3/treatment. Data were collected as cage mean daily intake per mouse over a 7-day interval.

[a,b,c] LSM in a common timepoint lacking a common letter differ, $P < 0.05$.

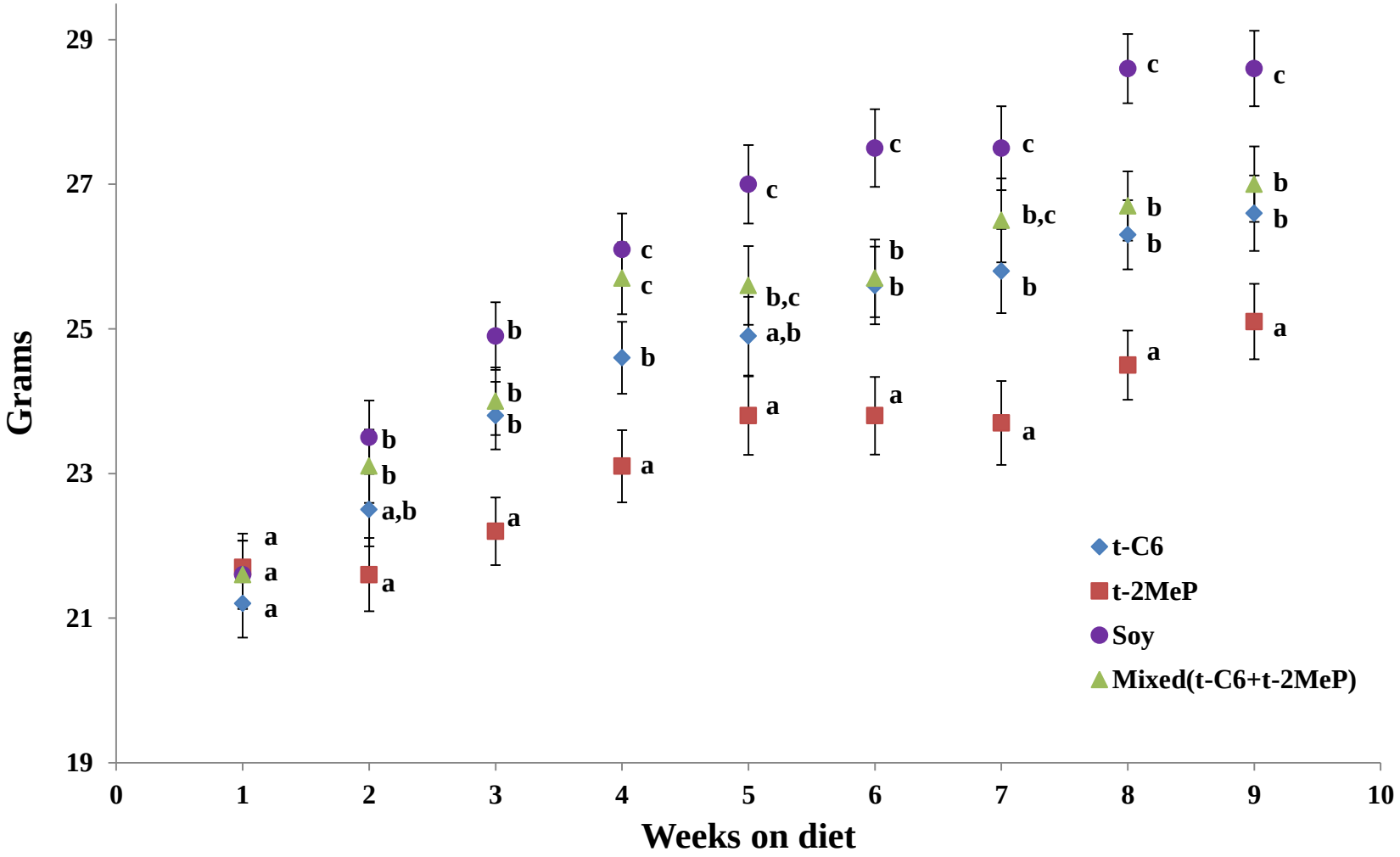

Figure 4 Body weight of mice fed soyoil (soy), t-C6. t-2MeP or mixed (t-C6 + t-2MeP) for 9 weeks. Data are presented as LSM ± SEM, n=11-12/treatment.

[a,b,c] LSM in a common timepoint lacking a common letter differ, $P < 0.05$.

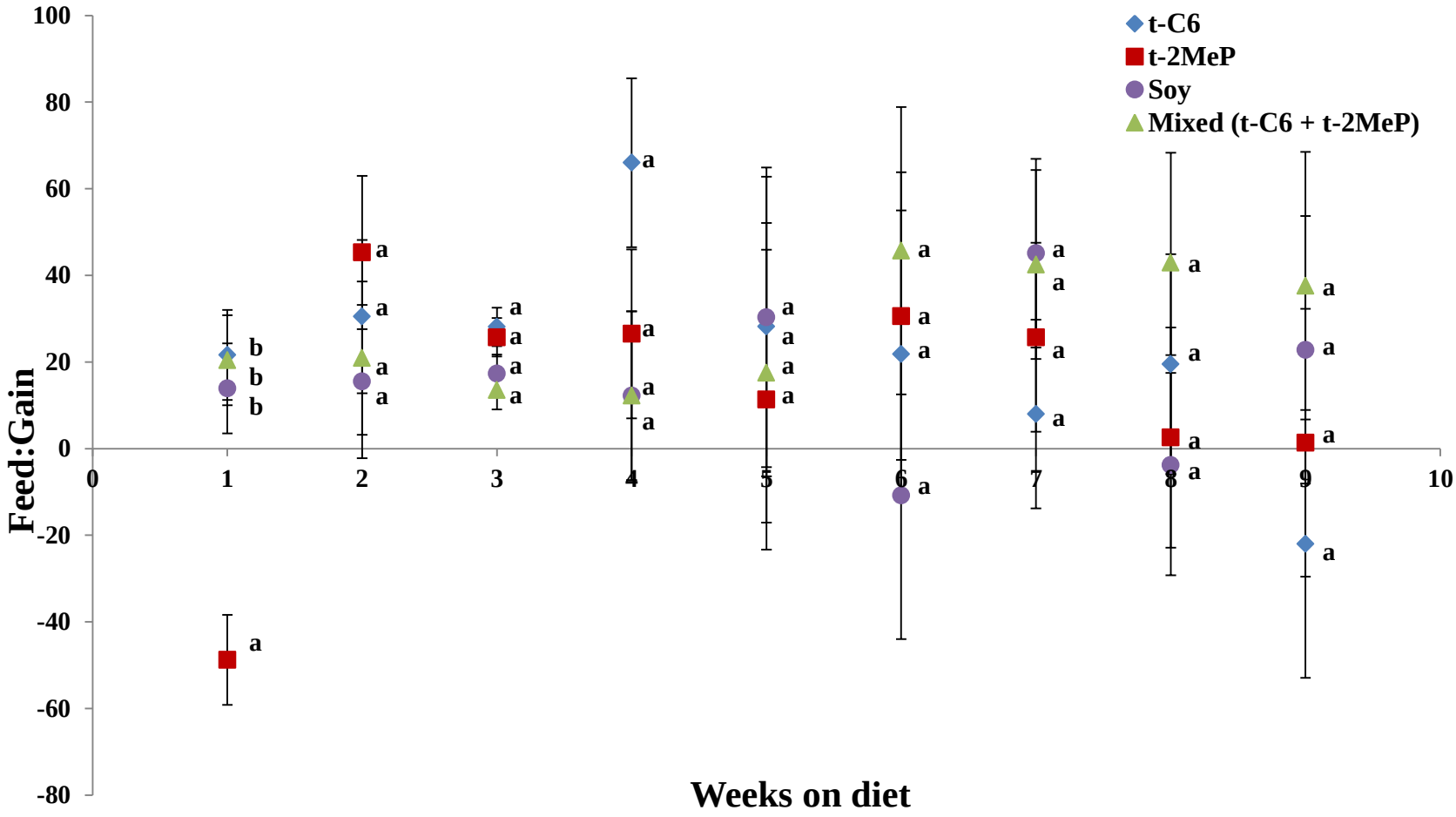

Figure 5 Feed conversion efficiency of mice fed soyoil (soy), t-C6, t-2MeP or mixed (t-C6+t-2MeP) for 9 weeks. Data were collected as cage mean daily intake per mouse over a 7-day interval. Feed: Gain was calculated as cage daily feed intake/cage body weight, expressed on a per mouse per day basis (see materials and methods).
Data are presented as LSM ± SEM, n=3 cages/treatment.
[a,b] LSM in a common week lacking a common letter differ, $P < 0.05$.

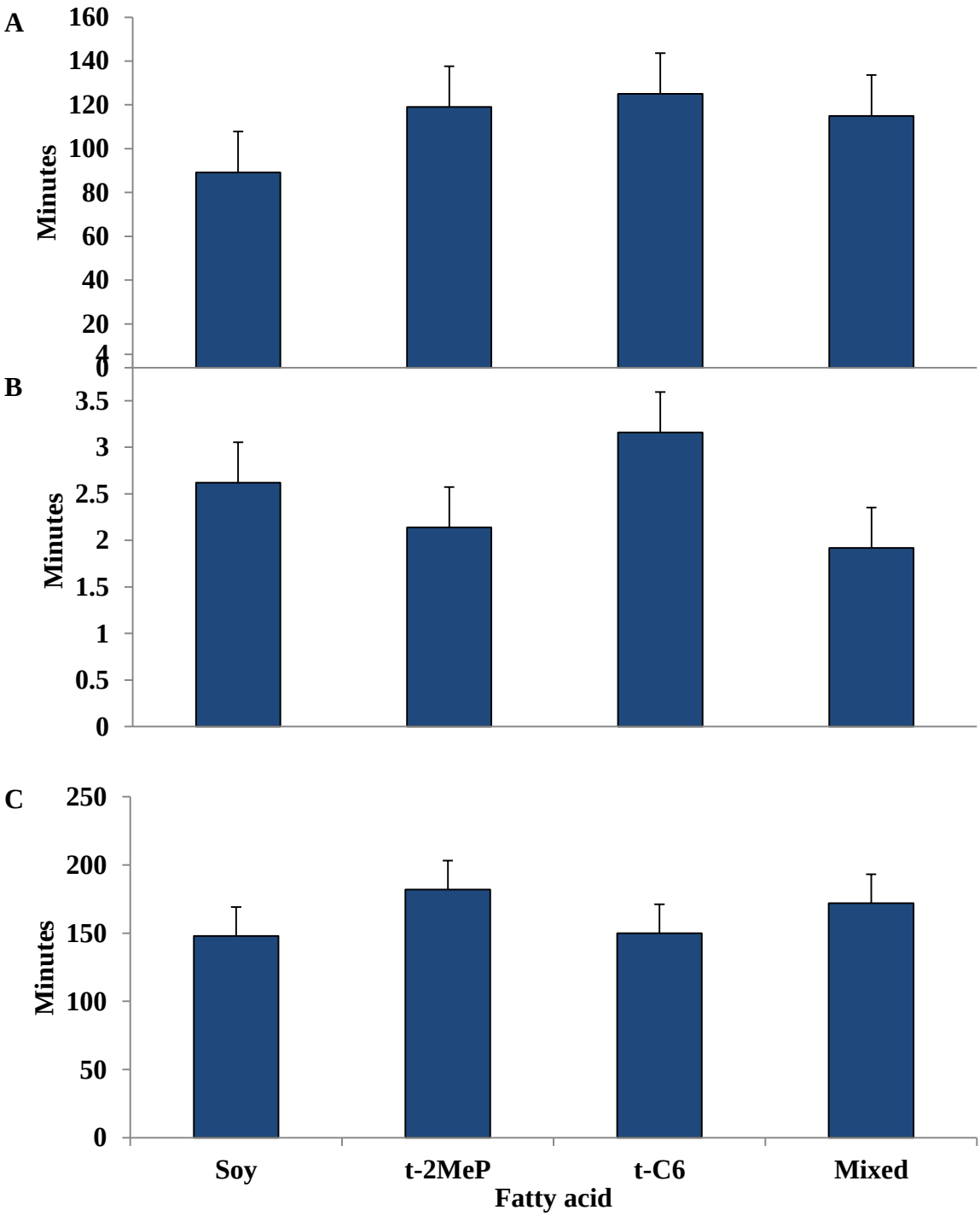

Figure 6 Time to exhaustion measured in mice fed soyoil (soy), t-C6, t-2MeP or mixed (t-C6+t-2MeP). A: Treadmill endurance test at week 4; B: Swimming endurance test at week 6; C: Treadmill endurance test at week 8. Data are presented as LSM ± SEM, n=11-12/treatment.

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Appendix B: Synthesis and analysis of 2-methylpentanoate and hexanoate triglycerides and experimental diets.

Treatment fatty acids hexanoic acid (120705000, Acros Organics, Geel, Belgium) and 2-methylpentanoic acid (13-77800; Penta International Corporation, Millburn-Short Hills, NJ) were condensated with glycerol into triglyceride form using Wheeler's methods for triglyceride synthesis (1). Esterification was catalyzed using p-toluenesulfonic acid (T35920 Sigma-Aldrich, St. Louis, MO). Fatty acid and glycerol proportions were calculated on a 1:3 molar basis, plus an additional 5% fatty acid added to the reaction flask. Catalyst p-toluenesulfonic acid was added at 1% of the glycerol and fatty acid combined amount.

Triglycerides were synthesized in a 500 mL boiling flask (294000, Kimble Chase, Vineland, NJ). The boiling flask was connected to a bent distillation adapter (205600-1420, Kimble Chase, Vineland, NJ). This was connected to a small stream of nitrogen attached to the hose connection. On the other side, it was attached to a claisen adapter (274200-0000, Kimble Chase, Vineland, NJ). The claisen adapter was plugged with a rubber stopper in the small upper joint and attached to a Graham water condenser (439000, Kimble Chase, Vineland, NJ). A small boiling flask (25276, Kimble Chase, Vineland, NJ) was attached to the lower joint. All joints were lubricated with mineral oil prior to triglyceride synthesis set up.

A stir bar was placed in the flask and the flask was placed in a large beaker of silicone oil on a hot plate. The hot plate stir function was turned on to the highest speed the flask would allow. The hot plate was turned to an initial temperature of 50 °C and a thermometer

was placed in the silicone oil to monitor temperature change. Once the silicone oil reached a steady temperature, the temperature on the hot plate was increased by 20 °C every time a steady temperature was observed until a final thermometer temperature of at least 115 °C was achieved. Water evaporation/condensation from the synthesis reaction could be monitored as an indicator of reaction completion. The hot plate was increased by 5 °C every time water evaporation/condensation into the small flask stopped until an increase in temperature did not produce water, or at the end of 3 days of synthesis above thermometer temperature of 115 °C. Thermometer readings were not allowed to exceed 135 °C to prevent free glycerol burning. It was observed that tri-hexanoate synthesis would tolerate upper limit temperatures (around 135 °C) better than tri-2-methylpentanoate synthesis. This may be a result of chain length differences between hexanoic acid and 2-methylpentanoic acid.

Triglycerides were analyzed for purity using high performance liquid chromatography (HPLC; 717 Autosampler, 996 Photodiode Array Detector, 600 Controller, Inline Degasser, Waters, Milford, Massachusetts) using a LiChrospher Si 100 (5µm) prepacked column (Hibar 50316; 2). Triglycerides were then purified to 100% purity using a potassium hydroxide (KOH)/ethanol extraction and HPLC verification (1).

Purification was performed by calculating the moles of fatty acid left and an equimolar amount of KOH was dissolved in 70% ethanol. This 15-20 mL solution was added to 125 mL of triglyceride solution in a separation flask, vigorously shaken, and allowed to separate. Two hours later, the triglyceride was separated from the upper ethanol phase, which was discarded. The triglyceride phase was washed with 15-20 mL of 70% ethanol without

KOH, vigorously shaken, and allowed to separate for two hours. The ethanol phase was discarded and the triglyceride phase was combined with 15-20 mL of water, vigorously shaken, and allowed to separate. The lower water phase was discarded. Two hours later the water purification step was repeated. Sodium sulfate was added and/or triglyceride was heated on a hotplate with a stir bar to remove trace amounts of water.

Mice were fed one of four experimental diets; a control soybean oil diet comprised of a Teklad modification of AIN-93G with 10% soybean oil (TD.110824), a 8% tri-2-methylpentanoate diet (TD.110826), a 8% tri-hexanoate diet (TD.110825) or mixed diet consisting of 4% tri-hexanoate and 4% tri-2-methylpentanoate (TD.110827). Diets were isocaloric and isonitrogenous purified diets varying only in dietary fat content (Harlan Laboratories Inc., Madison, Wisconsin; Table 1).

Figures

Table 1 Composition of experimental diets.

	Soyoil	**t-C6**	**t-2MeP**	**Mixed**
Ingredient		*g/Kg*		
Casein	200.0	200	200	200
L-Cysteine	3.0	3.0	3.0	3.0
Corn Starch	317.2	317.2	317.2	317.2
Maltodextrin	132.0	132.0	132.0	132.0
Sucrose	100.0	100	100	100
Soybean oil	100.0	20.0	20.0	20.0
Tri-hexanoate	0.0	80.0	0.0	40.0
Tri-2-methylpentanoate	0.0	0.0	80.0	40.0
Cellulose	100.0	100.0	100.0	100.0
Mineral Mix, AIN-93G-MX[1]	35.0	35.0	35.0	35.0
Vitamin Mix, AIN-93-VX[1]	10.0	10.0	10.0	10.0
Choline Bitartrate	2.5	2.5	2.5	2.5
TBHQ, antioxidant	0.2	0.2	0.2	0.2
Food color	0.1	0.1	0.1	0.1

[1] (Reeves, 1993)

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