# Effects of Maternal Folic Acid Supplementation on Skeletal Muscle Function and Metabolism in Male and Female CD-1 Mouse Offspring

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ABSTRACT

In 1998, folic acid (FA) fortification of all white flour, enriched pasta and cornmeal products became mandatory in Canada to reduce the risk of neural tube defects at birth. Furthermore, Health Canada and the Society of Obstetricians and Gynaecologists of Canada recommends all women take daily prenatal FA supplements in addition to FA-fortified foods during pregnancy, resulting in pregnant women being exposed to approximately 4 times higher FA during pregnancy than the current recommended guidelines. However, the influence of maternal FA supplementation on offspring development, specifically muscle, is currently unknown. Skeletal muscle is one of the most abundant tissues in the human body and is essential for locomotion and energy metabolism.Thus, the purpose of this study was to determine the effect of supplemental FA (4 times higher than normal dietary consumption), in utero and throughout suckling on muscle function and metabolism in male and female CD-1 mouse offspring. The major findings were ~25% faster contractions in EDL, characterized by a more rapid relaxation rate, and ~15% slower contractions, characterized by a reduced force development rate, in SOL among females in FA group, with no differences in contractile function seen between groups in males. Additionally, carbohydrate metabolism markers in the FA group decreased in SOL among females, whereas, carbohydrate and oxidative metabolism markers increased in EDL and SOL, respectively, among males. These findings suggest that exposure to folic acid supplementation *in utero* and throughout suckling programs skeletal muscle function and metabolism in a sex-specific manner.

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## CONTRIBUTIONS

This study was a subset of a larger study looking at the influence of a maternal folic acid supplemented diet on offspring skeletal health, performed by Dr. Sandra Sacco under the supervision of Dr. Wendy Ward. The concept of the project was developed by Dr. Wendy Ward. The author was actively involved in the *in vivo* aspects of the study including bi-weekly feedings, scanning the mice at 2, 4 and 6 months of age using micro-computed tomography, and tissue collection. Additionally, the experimental design, data collection of enzyme activity in males, analysis and interpretation were performed by the author. All aspects of muscle isolation for muscle function testing, stimulation and force assessments were performed by W. Gittings and J. Bunda. C. Giles performed the data collection of enzyme activity in females.

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ABBREVIATIONS

½ RT – half-relaxation time

$A^{vy}$ – agouti

ACh – acetylcholine

AChRs – acetylcholine receptors

ADP – adenosine diphosphate

AI – adequate intake

ANOVA – analysis of variance

ATP – adenosine triphosphate

β-HAD – 3-Hydroxyacyl-CoA dehydrogenase

$Ca^{2+}$ – calcium

CON – control AIN93G diet

COX – cytochrome c oxidase

CpG – cytosine-guanine dinucleotide

CPT – carnitine palmitoyltransferase

CS – citrate synthase

CSA – cross sectional area

+dP/d*t* – rate of force development

-dP/d*t* – rate of relaxation

DFE – dietary folate equivalents

DHPRs – dihydropyridine receptors

DNA – deoxyribonucleic acid

Dnmt1 – DNA methyltransferase 1

DRI – dietary reference intake

EDL – extensor digitorum longus

FA – folic acid supplemented AIN93G diet

FG – fast glycolytic

FOG – fast oxidative glycolytic

GLUT4 – glucose transporter 4

GR – glucocorticoid receptor

HK – hexokinase

IMF – intermyofibrillar

$L_o$ – optimal length

$L_f$ – optimal fibre length

MTHFR – methylenetetrahydrofolate reductase

$Na^+$ – sodium

NADPH – nicotinamide adenine dinucleotide phosphate

NADH – nicotinamide adenine dinucleotide + hydrogen

$P_i$ – inorganic phosphate

$P_o$ – peak tetanic force

$P_t$ – peak twitch force

$P_t$:$P_o$ – peak twitch-to-peak tetanus ratio

PFK – phosphofructokinase

PND – postnatal day

PPARα – peroxisome proliferator-activated receptor

RDI – recommended daily intake

RNA – ribonucleic acid

RyRs – ryanodine receptors

SAH – S-adenosylhomocysteine

SAM – S-adenosylmethionine

SERCA – sarco(endo)plasmic reticulum calcium ATPase

SO – slow oxidative

SOL – soleus

SR – sarcoplasmic reticulum

SS – subsarcolemmal

THF – tetrahydrofolate

TPT – time to peak tension

UL – tolerable upper intake level

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# CHAPTER 1.0 – GENERAL INTRODUCTION

## 1.1. Skeletal Muscle

Skeletal muscle is the one of the most abundant tissues in the human body comprising approximately 40% of total body weight in healthy individuals (Frontera & Ochala, 2015; Hopkins, 2006). Skeletal muscle is a contractile tissue essential for locomotion. Muscle is remarkable in its ability to convert chemical energy into mechanical energy to produce force with extreme efficiency (Frontera & Ochala, 2015). Additionally, skeletal muscle is an important metabolic tissue playing a major role in basal energy metabolism, glucose metabolism, oxidative phosphorylation, and thermogenesis (Frontera & Ochala, 2015).

### 1.1.1 Structure

Skeletal muscle is composed of many individual muscle fibres, also referred to as muscle cells, which run parallel to one another spanning the length of the muscle. Each muscle fibre is comprised of a sarcolemma, sarcoplasm, transverse (T)-tubules, sarcoplasmic reticulum (SR), mitochondria and protein. The sarcolemma is a three-layered cell membrane, which includes a lipid bilayer sandwiched between a basement membrane and a submembranous cytoskeletal network (Campbell & Stull, 2003; Ozawa, Nishino, & Nonaka, 2001). The two outer membranes are structurally tough layers protecting the lipid bilayer from injury, allowing it to remain intact, maintaining its structure and function. In addition to regulating metabolites, the sarcolemma is a unique membrane as it is excitable, thus, the sarcolemma is fundamental for skeletal muscle metabolism and initiating muscle contractions. The T-tubules are invaginations of the sarcolemma that conduct excitable signals into the cell (Frontera & Ochala, 2015). The T-

tubules are found sandwiched between terminal cisterna from the SR forming a triad **(**Al-Qusairi & Laporte, 2011; Sorrentino, 2004). The SR is a storage site for calcium ($Ca^{2+}$) dedicated to maintaining $Ca^{2+}$ homeostasis within the muscle fibre (Frontera & Ochala, 2015; Sorrentino, 2004). The mitochondria generate the energy required for muscle functions through adenosine triphosphate (ATP) synthesis (Frontera & Ochala, 2015, Hood, 2001). There are two types of mitochondria found in skeletal muscle, subsarcolemmal (SS) and intermyofibrillar (IMF). SS mitochondria represent approximately 10-15% of total mitochondria and are located beneath the sarcolemma, whereas, IMF mitochondria are embedded within the myofibrils (Hood, 2001). Each individual muscle fibre is composed of bundles of myofibrils containing myofilaments (proteins). The most abundant myofilaments, thin (actin) and thick (myosin), are arranged in repeated units of sarcomeres parallel to one another (Frontera & Ochala, 2015; Hopkins, 2006; Kaya et al., 2014). The sarcomere is the smallest contractile unit of an individual fibre that provides skeletal muscle with its characteristic striated appearance. Two other regulatory proteins, tropomyosin and the troponin complex, are critical to muscle contraction**.** The troponin complex is composed of three subunits: troponin T (attaches to tropomyosin), troponin I (inhibits actin-myosin binding), and troponin C (binds $Ca^{2+}$) (Gordon et al., 2000; Hopkins, 2006; Koubassova & Tsaturyan, 2011). Troponin T is attached to tropomyosin, which lies in the grooves between actin filaments (Gordon et al., 2001). At rest, troponin I attaches to the actin filament blocking the myosin-binding site, preventing contraction (Gordon et al., 2001; Gordon et al., 2000; Hopkins, 2006).

### 1.1.2 Contraction

For a contraction to initiate, an electrical impulse (action potential) travels down a motor neuron to the presynaptic region, or terminal nerve ending, opening voltage-gated $Ca^{2+}$ channels allowing an influx of $Ca^{2+}$ into the presynaptic region (Kaya et al., 2014; Tintignac et al., 2015). The influx of $Ca^{2+}$ triggers synaptic vesicle exocytosis and the subsequent release of the neurotransmitter, acetylcholine (ACh), into the synaptic region (Kaya et al., 2014; Tintignac et al., 2015). ACh travels across the synaptic region binding to nicotinic acetylcholine receptors (AChR) located in the postsynaptic region of the muscle fibre (Tintignac et al., 2015). The binding of ACh to AChR triggers the opening of voltage-gated sodium ($Na^{+}$) and potassium ($K^{+}$) channels located within the sarcolemma, causing a rapid influx of $Na^{+}$ into the cell and an efflux of $K^{+}$ ions out of the cell, depolarizing the cell (Hopkins, 2006; Kaya et al., 2014). A wave of depolarization (action potential) spreads across the sarcolemma and into the cell via the T-tubules, arriving at the triad (Frontera & Ochala, 2015; Hopkins, 2006; Schneider, 1994). The action potential causes a conformational change to the voltage sensing dihydropyridine receptors (DHPR) located within the T-tubules (Hopkins, 2006; Kaya et al., 2014; Ríos & Pizarro, 1991; Schneider, 1994). The conformational change of DHPR triggers the opening of the ryanodine receptor (RyR), which are $Ca^{2+}$ channels located on the terminal cisternae of the SR, causing an influx of $Ca^{2+}$ from the SR into the sarcoplasm of the cell (Hopkins, 2006; Imagawa et al., 1987; Kaya et al., 2014; Ríos & Pizarro, 1991; Schneider, 1994). In the sarcoplasm, $Ca^{2+}$ binds to troponin C causing a conformational change to troponin I, exposing the myosin-binding site on the actin filament (Gómez et al., 2006; Gordon et al., 2001; Gordon et al., 2000; Hopkins, 2006). An ATP molecule binds to the

myosin head in a rigor state leading to subsequent detachment of myosin from actin, initiating the cross-bridge cycle (Cooke, 1997; Koubassova & Tsaturyan, 2011; Llinas et al., 2015; Lymn & Taylor, 1971). Prior to myosin binding to a new actin filament, ATP is hydrolyzed by myosin ATPase, located on the myosin head, producing adenosine diphosphate (ADP) and inorganic phosphate ($P_i$) (Koubassova & Tsaturyan, 2011; Llinas et al., 2015; Lymn & Taylor, 1971). The myosin head weakly binds to actin with its hydrolysis by products (ADP + $P_i$) and the sequential release of $P_i$ triggers the transition from weak to strong myosin binding, resulting in the power stroke (Cooke, 1997; Koubassova & Tsaturyan, 2011; Llinas et al., 2015; Rayment et al., 1993). After the power stroke is complete, ADP is released and the cross-bridge is in a rigor state (Koubassova & Tsaturyan, 2011; Llinas et al., 2015). To prepare for the next cross-bridge cycle, ATP re-binds to the myosin head causing dissociation of myosin from actin. In order for relaxation to occur, $Ca^{2+}$ must be removed from the sarcoplasm and translocated back into the SR. Removal of $Ca^{2+}$ allows the tropomyosin-troponin I complex to block the myosin-binding site on actin, preventing contraction (Gordon et al., 2000; Hopkins, 2006; Koubassova & Tsaturyan, 2011). Re-uptake of $Ca^{2+}$ into the SR occurs through the sarco(endo)plasmic reticulum calcium ATPase (SERCA) pump located in the SR (Kaya et al., 2014; Stammers et al., 2015)

### 1.1.3 Muscle Fibre Types

Skeletal muscles are composed of different types of muscle fibres that are responsible for a wide variety of functions. Skeletal muscle fibre types are classified into three main categories based on their contractile and metabolic properties: type I (SO; slow oxidative), type IIa (FOG; fast oxidative glycolytic), type IIb, (FG; fast glycolytic),

and a hybrid of type IIa and IIb fibres characterized as type IId/x (Bär & Pette, 1988; Bottinelli & Reggiani, 2000; Delp & Duan, 1996; Termin et al., 1989).

Type I fibres are used for continuous low-intensity activities, such as maintaining posture and stability (Schiaffino, 2010). Type I fibres are rich in aerobic oxidative enzymes that rely on carbohydrates and lipids to produce ATP (Bottinelli & Reggiani, 2000; Rivero et al., 1998; Schiaffino, 2010). Type I fibres are able to transport substrates, such as glucose and fatty acids, across the sarcolemma at a faster rate than type II fibres, allowing for greater ATP synthesis (Bottinelli & Reggiani, 2000). In addition to increased substrate availability, type I fibres have high mitochondrial and myoglobin content and increased capillary density making them highly fatigue resistant and excellent for prolonged submaximal activities (Bottinelli & Reggiani, 2000; Schiaffino, 2010). Due to their high sensitivity to $Ca^{2+}$, type I fibres are the first to be recruited for activities (Bottinelli & Reggiani, 2000; Trinh & Lamb, 2006). Furthermore, type I fibres are characterized by long, slow contraction and relaxation times compared to type II fibres. The length and speed of muscle contraction depends on myosin ATPase activity (ATP hydrolysis rate) and the speed of $Ca^{2+}$ uptake by the SR, which is slow in type I fibres (Baylor & Hollingworth, 2003; Bottinelli & Reggiani, 2000; Schiaffino, 2010; Trinh & Lamb, 2006).

Type IIa fibres are used for prolonged anaerobic activities, such as walking (Schiaffino & Reggiani, 2011). Type IIa fibres contain aerobic oxidative and glycolytic enzymes, thus, they are fatigue resistant with the ability to participate in continuous activities (Rivero et al., 1998; Schiaffino, 2010). These fibres have a faster, shorter contraction and relaxation speed compared to type I fibres, due to their higher myosin

ATPase activity and quicker $Ca^{2+}$ uptake (Baylor & Hollingworth, 2003; Bottinelli & Reggiani, 2000; Trinh & Lamb, 2006).

Type IIb fibres are involved in high power, anaerobic activities for short periods of time, such as jumping (Bottinelli & Reggiani, 2000). Type IIb fibres depend on glycolytic enzymes that generate ATP rapidly, however, glycolysis depletes glycogen stores rapidly. Additionally, type IIb fibres have low mitochondria and myoglobin content and the lowest capillary density, making these fibres highly fatigable (Rivero et al., 1998; Schiaffino, 2010). Type IIb fibres have the fastest and shortest contraction and relaxation time with the greatest power output, however, type IIb fibres have the lowest $Ca^{2+}$ sensitivity, thus, these fibres are the last to be recruited (Baylor & Hollingworth, 2003; Bottinelli & Reggiani, 2000; Schiaffino, 2010; Trinh & Lamb, 2006).

Type IId/x fibres are also used in short duration, high power activities and rely on glycolysis for ATP synthesis (Schiaffino & Reggiani, 2011). Type IId/x fibres have contraction and relaxation times similar to IIb fibres, however, their resistance to fatigue is slightly greater than type IIb fibres (Schiaffino, 2010). Table 1.1 highlights the differences in physical, metabolic and contractile characteristics of each fibre type (I, IIa, IIx/d, IIb).

**Table 1.1** Physical, metabolic, and contractile characteristics of skeletal muscle fibres (Adapted from Fajardo, 2011; Zibamanzarmofrad, 2015).

| Characteristics | Type 1 | Type IIa | Type IId/x | Type IIb |
|---|---|---|---|---|
| Diameter | Small[1] | Intermediate[1] | Large[1] | Largest[1] |
| Glycolytic capacity | Low[2,3] | Intermediate[2,3] | High[2,3] | Highest[2,3] |
| Oxidative capacity | High[1,2,3] | High[1,2,3] | Intermediate[1,2,3] | Low[1,2,3] |
| Mitochondria | Abundant[2] | Abundant[2] | Few[2] | Fewer[2] |
| Myoglobin | High[2] | Intermediate[2] | Low[2] | Lowest[2] |
| Capillaries | Abundant[2.4] | Intermediate[2,4] | Sparse[2,4] | Rare[2,4] |
| Glycogen | Low[2,4] | Intermediate[2,4] | High[2,4] | Higher[2,4] |
| ATP synthesis | Aerobic[2,4] | Aerobic[2,4] | Anaerobic[2,4] | Anaerobic[2,4] |
| ATP consumption | Low[2,4] | Intermediate[2,4] | High[2,4] | Highest[2,4] |
| Myosin ATPase | Slow[2,4] | Intermediate[2,4] | Fast[2,4] | Fastest[2,4] |
| Motor unit size | Small[2] | Intermediate[2] | Large[2] | Largest[2] |
| Recruitment | Early[1,4] | Intermediate[1,4] | Late[1,4] | Later[1,4] |
| Contraction | Slow[2,4] | Fast[2,4] | Faster[2,4] | Fastest[2,4] |
| Relaxation | Slow[2,4] | Fast[2,4] | Faster[2,4] | Fastest[2,4] |
| Twitch | Long[2,4] | Short[2,4] | Shorter[2,4] | Shortest[2,4] |
| Resting [$Ca^{2+}$] | High[2,4] | Intermediate[2,4] | Low[2,4] | Lowest[2,4] |
| $Ca^{2+}$ uptake | Slow[2,5] | Intermediate[2,5] | Fast[2,5] | Fastest[2,5] |

Delp & Duan, 1996[1], Schiaffino & Reggiani, 2011[2], Rivero et al., 1998[3], Bottinelli & Reggiani, 2000[4], Baylor & Hollingworth, 2003[5]
$Ca^{2+}$: calcium; [$Ca^{2+}$]: calcium concentration

### 1.1.4 Mechanics

Force production in skeletal muscle is highly dependent on structural and biochemical alterations within the muscle fibre. Furthermore, fast and slow twitch muscle fibre compositions play a critical role in biochemical function. The interaction between the force generated by the muscle, muscle load, and the number of cross-bridge formations results in three types of muscle contractions: 1) concentric or shortening of the muscle during contraction; 2) isometric or no length change in the muscle during contraction and; 3) eccentric or lengthening of the muscle during contraction (Frontera & Ochala, 2015). Isometric contractions are useful for studying the basic mechanisms behind contraction and relaxation, such as rates of contraction and $Ca^{2+}$ release (Schiaffino & Reggiani, 2011), therefore, only the details of isometric contractions will be discussed further in this review.

As discussed previously, the cross-bridge cycle leads to a contractile response from muscle tissue. A twitch contraction occurs when a single stimulus at a low frequency (1 Hz) results in a single contraction and relaxation cycle of the muscle. Intracellular $Ca^{2+}$ transients and $Ca^{2+}$ sensitivity of muscle fibres determine the amplitude of the twitch force, thus, twitch force is an indicator of potential changes to $Ca^{2+}$signaling (Schiaffino & Reggiani, 2011). A tetanic contraction occurs when repeated and rapid stimuli at a greater frequency (150 Hz), results in amplified, sustained muscle contraction, indicating the muscle has reached peak force production. In contrast to a twitch contraction, tetanic contraction is determined by the number of active cross-bridges, as well as, peak $Ca^{2+}$ concentration in the cytoplasm (Schiaffino & Reggiani, 2011).

The length-tension relationship is the relationship between a muscle’s length and

the force it generates during an active contraction in an isometric state. Furthermore, the force a muscle generates is a reflection of the degree of overlap between thick and thin filaments (Close, 1972). Thus, the force generated during an isometric contraction depends on the length of the muscle. Peak force production occurs at the optimal length ($L_o$) of the muscle, which is characterized by a maximal overlap of thick and thin filaments, resulting in peak tetanic tension (Close, 1972). Additionally, biochemical alterations, such as $Ca^{2+}$ kinetics, have a direct impact on the contractile properties of skeletal muscle fibres. Isometric twitch fibres with faster $Ca^{2+}$ transients reach a greater peak at a quicker rate, characterized by a shorter time to peak tension (TPT, defined as the time (ms) it takes from the onset of an active contraction to the peak tension produced) and ½ relaxation time (½ RT, defined as the time (ms) it takes for the muscle to return to 50% of peak tension) and faster rates of force development (+dP/d*t*) and relaxation (-dP/d*t*) (Schiaffino & Reggiani, 2011) (Figure 1.1.A). The twitch:tetanus ($P_t$:$P_o$) ratio compares the two types of forces, peak twitch ($P_t$) and peak tetanic ($P_o$) tension, to one another. In comparison to slow twitch fibres, there is a higher $P_t$:$P_o$ ratio seen in fast twitch fibres. Moreover, intracellular $Ca^{2+}$ transients, peak cytoplasmic $Ca^{2+}$ concentration and $Ca^{2+}$ sensitivity are all greater in fast twitch fibre types (Schiaffino & Reggiani, 2011). Thus, fast twitch fibre types (extensor digitorum longus; EDL) are associated with a greater $P_t$ characterized by a shorter TPT and ½ RT, faster +dP/d*t* and -dP/d*t*, as well as, greater $P_o$, compared to slow twitch fibre types (soleus; SOL) (Figure 1.1B).

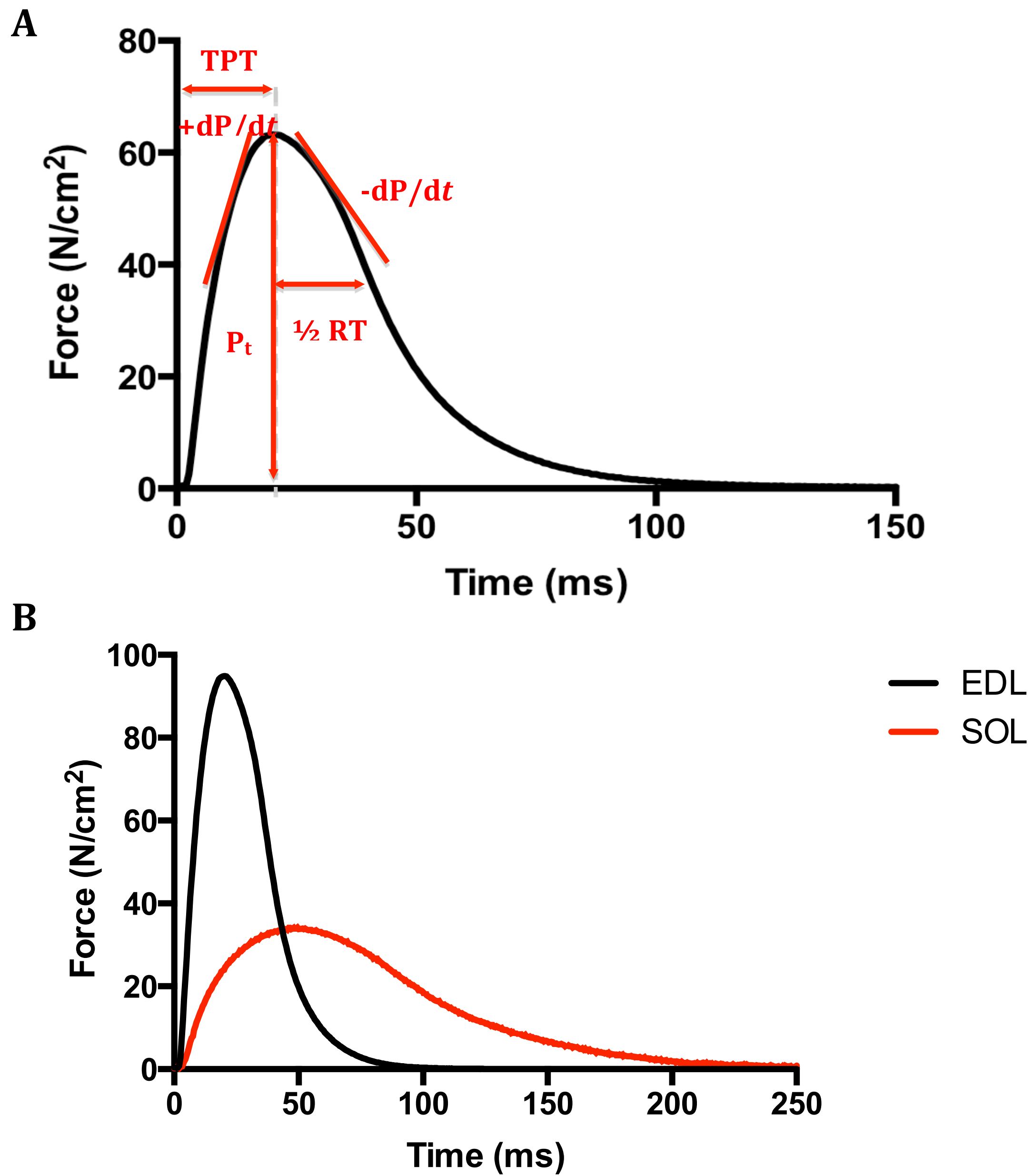


**Figure 1.1. A)** Representation of the isometric contractile parameters measured from the twitch force trace. **B)** Comparison of an isometric peak twitch force trace between glycolytic fast twitch muscle fibres (EDL) and oxidative slow twitch muscle fibres (SOL). Data taken from the current study. $P_t$, peak twitch; TPT, time to peak tension; ½ RT, half-relaxation time, +dP/d*t*, rate of force development; -dP/d*t,* rate of relaxation; EDL, extensor digitorum longus; SOL, soleus

#### 1.1.5 Metabolism

During skeletal muscle contraction, the majority of energy consumption is from molecular motors and ion pumps. The three major energy consuming ATPase's are myosin ATPase, $Ca^{2+}$ ATPase, and $Na^{+}/K^{+}$ ATPase, used for cross-bridge formation and maintaining ion homeostasis, respectively (Cornelius & Mahmmoud, 2003). The energy source required for cellular functions, including contractile activity, is ATP, which is produced via metabolic pathways. The main mechanisms that provide ATP to skeletal muscle are carbohydrate and lipid metabolism, and to a lesser degree protein metabolism (which will not be discussed further).

***i) Carbohydrate Metabolism***

Glycolysis (Figure 1.2) is the catabolic pathway that breaks down carbohydrates to produce energy (ATP) in the cytosol of muscle fibres. Glycolysis occurs under two conditions: 1) anaerobic (does not involve mitochondria) and; 2) aerobic (involves mitochondria). Anaerobic glycolysis is a 10-step pathway which converts glucose into two pyruvate molecules. Briefly, glucose molecules, which are generated by the breakdown of glycogen stores in skeletal muscle or transported across the sarcolemma via GLUT proteins, are broken down into two molecules of pyruvate, producing two ATP molecules during the process (Schiaffino & Reggiani, 2011). Glycolysis is initiated by the phosphorylation of glucose to glucose-6-phosphate, which is catalyzed by the enzyme hexokinase (HK) (Figure 1.2). Glucose-6 phosphate is converted into fructose-6-phosphate by the removal of one carbon via phosphoglucose isomerase. Phosphofructokinase (PFK) is the rate-limiting enzyme of glycolysis, which catalyzes the phosphorylation of fructose-6-phospate to yield fructose-1,6-biphosphate (Al Hasawi et

al., 2014; Nordenberg et al., 1981) (Figure 1.2). Through a series of additional subsequent reactions, two pyruvate, two NADH and two ATP molecules are produced. If the cell is deficient in substrates, pyruvate molecules are converted into lactate to aid in the regeneration of NAD to continue anaerobic glycolysis. However, if mitochondria are involved, pyruvate molecules are transported across the inner mitochondrial membrane and converted into acetyl-CoA by pyruvate dehydrogenase to undergo oxidative phosphorylation (Figure 1.3).

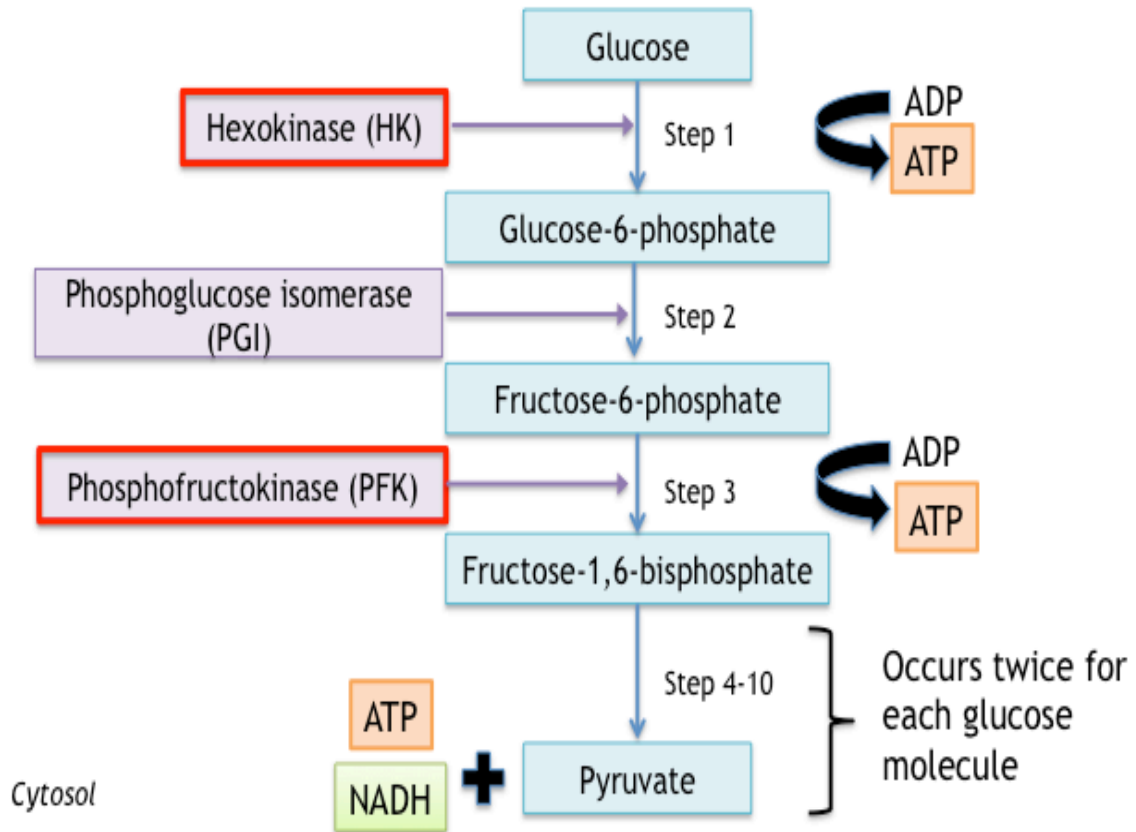


**Figure 1.2.** Glycolytic pathway in the cytosol highlighting (in red) two key enzymes, hexokinase (HK) and phosphofructokinase (PFK), that play an important regulatory role in the breakdown of carbohydrates into pyruvate molecules. ADP, adenosine diphosphate; ATP, adenosine triphosphate; NADH, nicotinamide adenine dinucleotide + hydrogen.

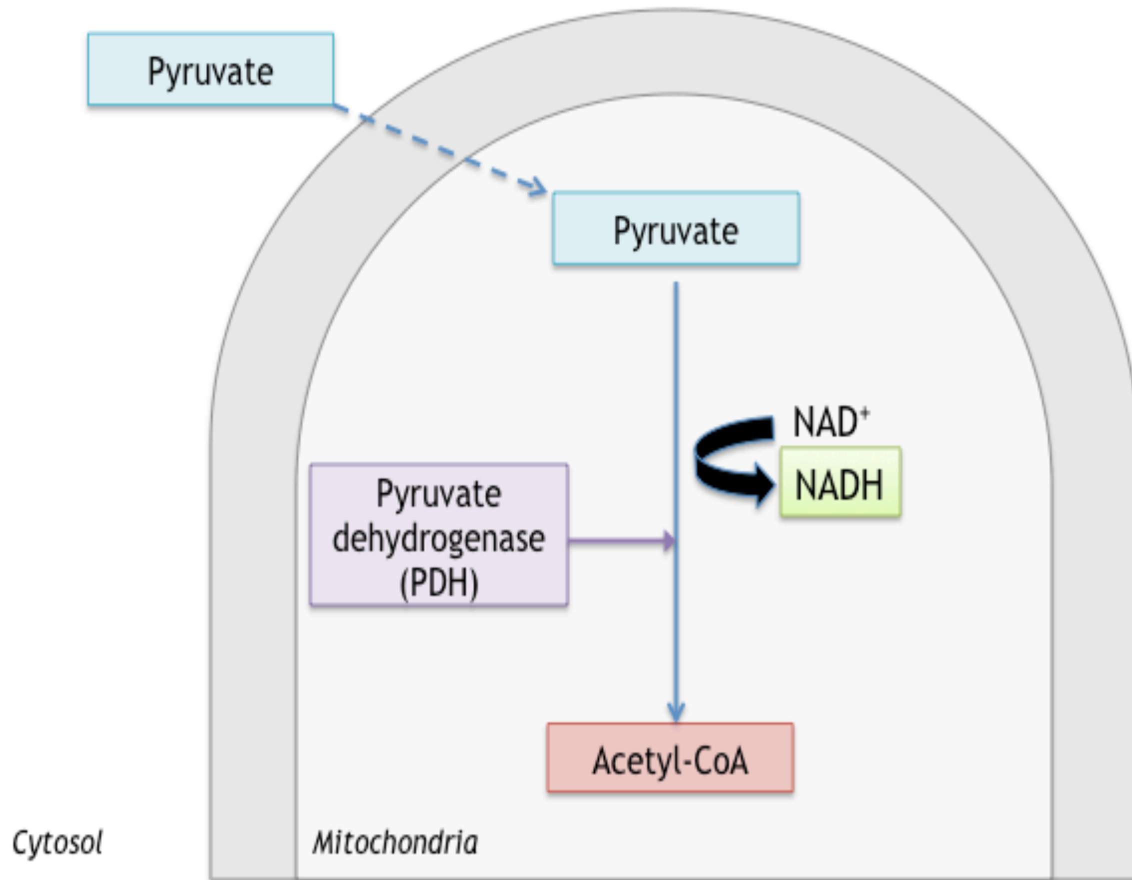


**Figure 1.3.** Pyruvate metabolism. Pyruvate is broken down into acetyl-CoA molecules via pyruvate dehydrogenase (PDH) in the mitochondria of the cell. $NAD^+$, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide + hydrogen.

### *ii) Lipid Metabolism*

Triglycerides are broken down into glycerol and three fatty acid molecules and used for oxidative metabolism. Glycerol becomes a substrate entering glycolysis, whereas, fatty acid molecules undergo β-oxidation producing acetyl-CoA. Initially, long-chain fatty acid molecules are converted to fatty acyl-CoA by long-chain fatty acyl-CoA synthetase (FACS) for transport across the outer and inner mitochondrial membranes (Gargiulo et al., 1999) (Figure 1.4). Long-chain fatty acyl-CoA molecules are transported from the cytosol into the mitochondria via carnitine acyltransferase, with carnitine palmitoytransferase (CPT) being the most often studied (Figure 1.4). CPT is the rate-limiting step in the transport of long-chain fatty acids into the mitochondrial matrix (Rakheja et al., 2002). Once the long-chain fatty acyl-CoA molecules have entered the mitochondrial matrix, they undergo β-oxidation, where each 2-carbon acyls bind with a CoA molecule, producing acetyl-CoA. 3-Hydroxyacyl-CoA dehydrogenase (β-HAD) is an enzyme that regulates the oxidation of long-chain fatty acids within the mitochondria by catalyzing the oxidation of L-3-hydroxyacyl CoA by $NAD^+$ (Aragao et al., 2014) (Figure 1.4). Therefore, β-HAD is a key enzyme in the β-oxidation of fatty acids.

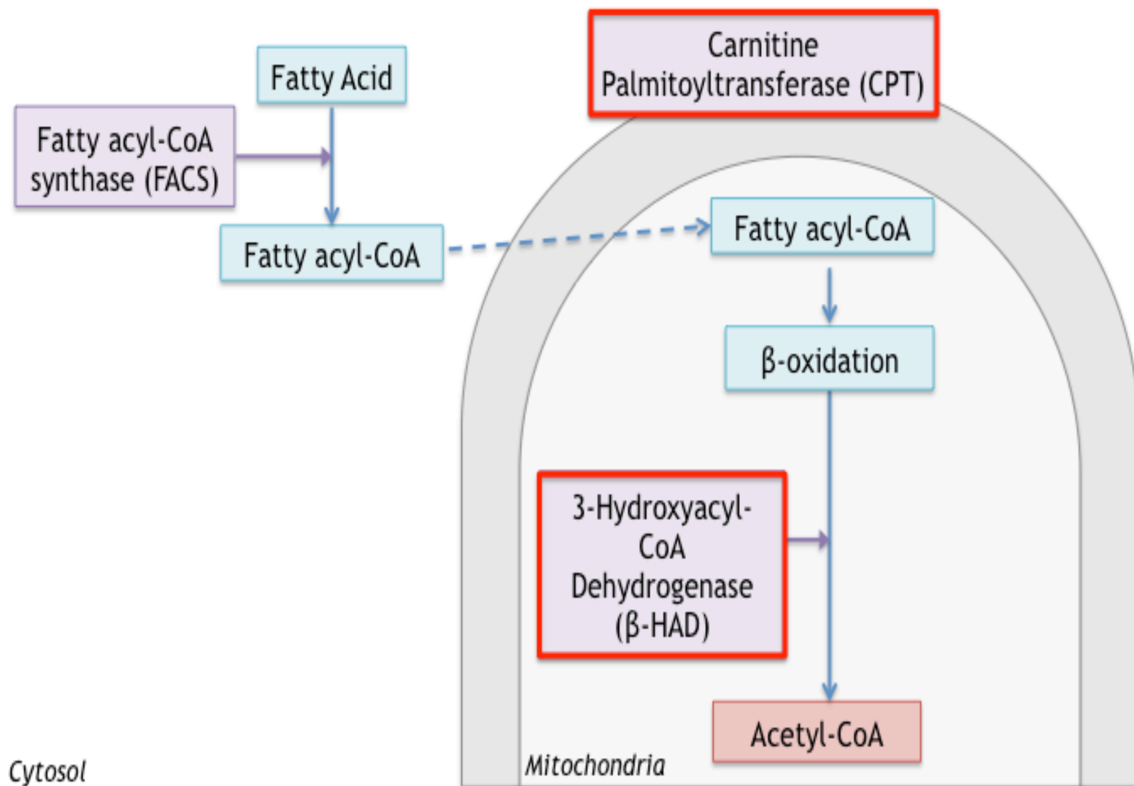


**Figure 1.4.** Schematic diagram representing the breakdown of lipids in the mitochondria of the cell. Carnitine palmitoyaltransferase (CPT) and 3-Hydroxyacyl-CoA dehydrogenase (β-HAD), are two key enzymes (highlighted in red) that play an important regulatory role in the breakdown of lipids into acetyl-CoA.

### *iii) Oxidative Phosphorylation*

Acetyl-CoA (derived from pyruvate and β-oxidation, as discussed previously) enters the tricarboxylic acid cycle (TCA cycle) by combining with oxaloacetate (the last intermediate of the TCA cycle) to produce citrate via citrate synthase (CS) (Figure 1.5). CS is a key marker of oxidative metabolism as it correlates with mitochondrial volume density in skeletal muscle and plays a key regulatory role in the TCA cycle (Aragao et al., 2014; Esteva et al., 2009). The citrate molecule then goes through a series of reactions, completing the TCA cycle as an oxaloacetate molecule, producing 3 NADH, 1 $FADH_2$, 2 $CO_2$ and 1 ATP molecule (Figure 1.5).

Coenzymes (NADH and $FADH_2$) transfer electrons to proteins in the electron transport chain (ETC), located on the inner mitochondrial membrane, converting energy to ATP (Figure 1.6). The energy from the transferred electrons pumps protons ($H^+$ ions) from the mitochondrial matrix to the intermembrane space, creating an electrochemical gradient. As the $H^+$ ions move back into the mitochondrial matrix, ATP formation is coupled to ATP synthase (Sazanov, 2015). Briefly, NADH dehydrogenase (complex I) is the first enzyme of the ETC which receives electrons from NADH and transfers them to ubiquinone (Coenzyme Q). Succinate dehydrogenase (complex II), the second enzyme of the ETC, is unique as it is also part of the TCA cycle. Complex II receives electrons ($FADH_2$) from succinate (a TCA cycle intermediate), which are then transferred to coenzyme Q (Sazanov, 2015). Coenzyme Q passes the electrons onto cytochrome $bc_1$ (complex III), which then transfers the electrons to cytochrome c oxidase (COX; complex IV) (Figure 1.6). COX is the terminal electron acceptor of the ETC which transfers electrons to oxygen producing water molecules, while pumping $H^+$ ions across the

mitochondrial membrane (Sazanov, 2015). COX is the rate-determining enzyme of the mitochondrial ETC, which is a major determinant of mitochondrial respiratory capacity in skeletal muscle. ATP synthase (complex V) is the final enzyme in the ETC pathway. As the $H^+$ ions move back into the mitochondrial matrix, complex V uses the energy stored from the proton gradient to drive the synthesis of ATP from ADP and phosphate ($P_i$) (Sazanov, 2015) (Figure 1.6).

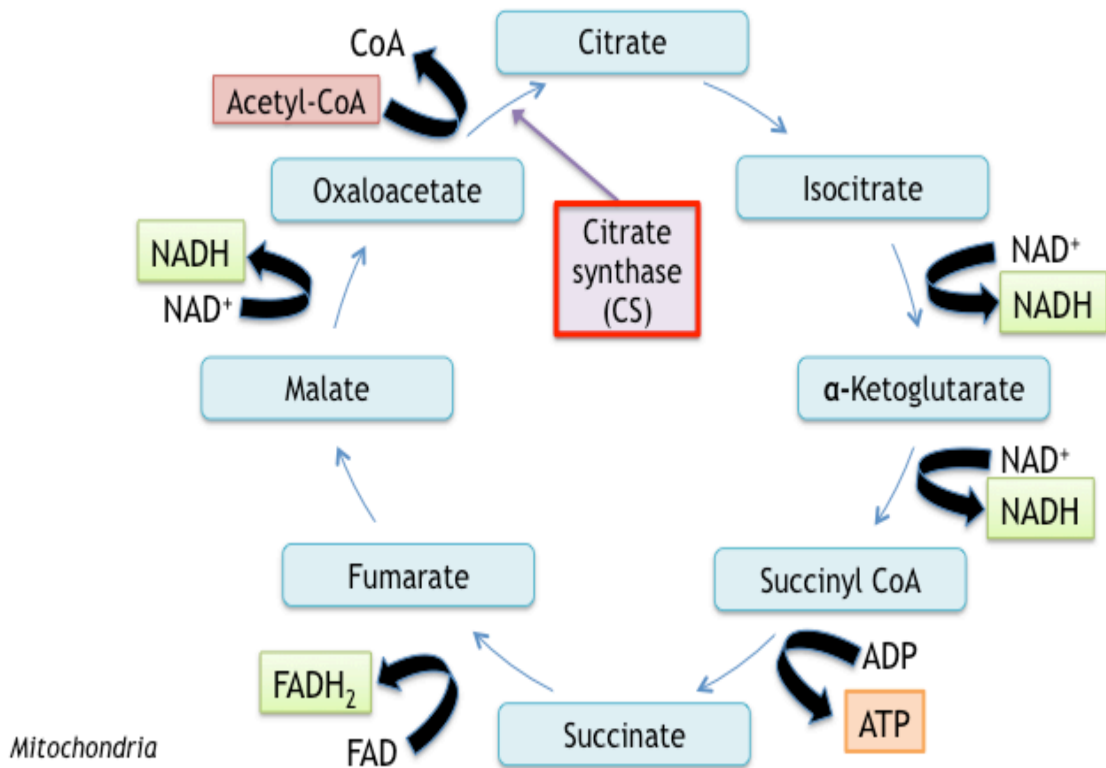


**Figure 1.5.** Tricarboxylic acid (TCA) cycle. Acetyl-CoA enters the TCA cycle combining with oxaloacetate via citrate synthase (CS), a key regulatory enzyme in the TCA cycle correlating to mitochondrial volume density. ADP, adenosine diphosphate; ATP, adenosine triphosphate; FAD, flavin adenine dinucleotide; $FADH_2$, flavin adenine dinucleotide + 2 hydrogen; $NAD^+$, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide + hydrogen.

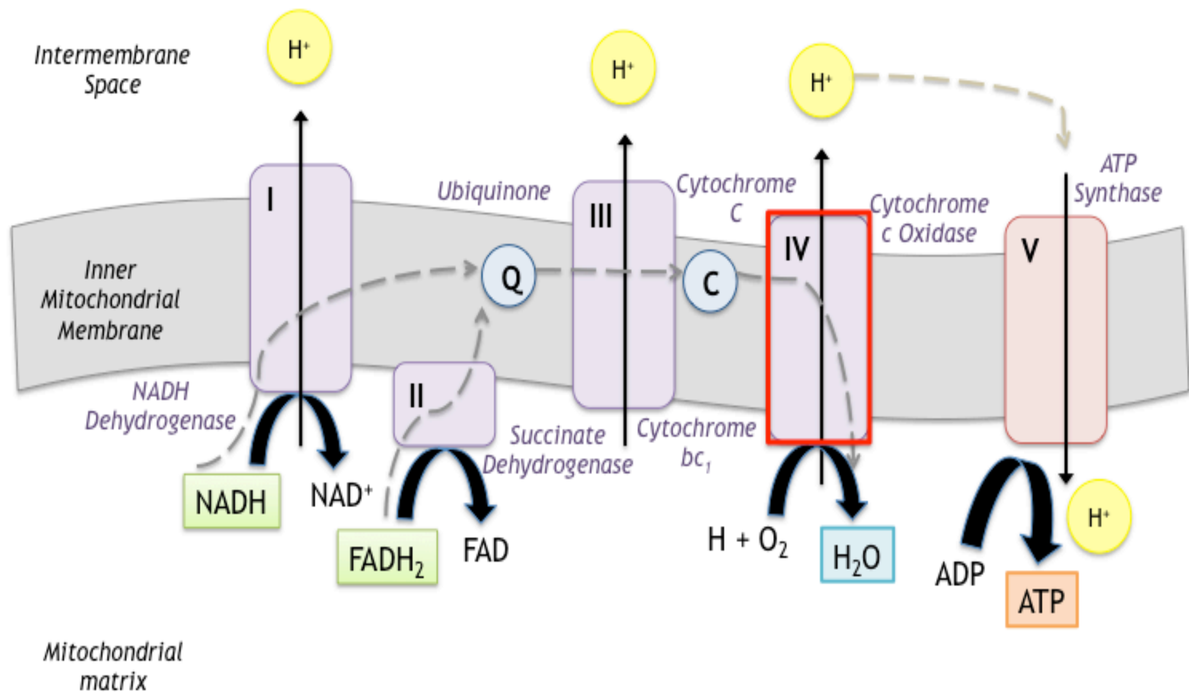


**Figure 1.6.** The Electron Transport Chain (ETC) located on the inner mitochondrial membrane. Electrons are transferred from protein to protein (Complex I – IV), releasing energy ($H^+$ ions) and driving ATP synthesis. Cytochrome c oxidase (COX; complex IV) is an important enzyme that is a major determinant of mitochondrial respiratory capacity in skeletal muscle. Q, ubiquinone, ADP, adenosine diphosphate; ATP, adenosine triphosphate; C, cytochrome C; FAD, flavin adenine dinucleotide; $FADH_2$, flavin adenine dinucleotide + 2 hydrogen; $H^+$, hydrogen ion**;** $H_2O$, water; $NAD^+$, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide + hydrogen; $O_2$, oxygen.

### 1.1.6 Sex Differences in Skeletal Muscle Function and Metabolism

It has been well documented there are sex differences in skeletal muscle morphology. Males have an overall greater muscle mass compared to females, which may be attributed to the larger cross-sectional area of individual muscle fibres seen in men compared to women (Haizlip et al., 2015; Lundsgaard & Kiens, 2014). Additionally, male and female mice express variations in fibre type composition within the same muscle. For example, males express 58% type IIa muscle fibres, whereas, females express only 36% in mice SOL (Haizlip et al., 2015). These differences are also seen in the plantaris (IIa - 16%, males; 37%, females) and tibialis anterior (IIa - 39%, males; 25%, females) muscles (Haizlip et al., 2015). Furthermore, myosin heavy chain (MHC) isoform expression varies between men and women, with women expressing a greater proportion of MHCI and fewer MHCIIa and MHCIIx in the vastus lateralis compared to men (Lundsgaard & Kiens, 2014).

MHC isoform expression is associated with fibre-type morphology, contractile function and enzymatic characteristics of skeletal muscle (Haizlip et al., 2015; Lundsgaard & Kiens, 2014). Maximal rates of force development and relaxation and contractile velocity is significantly lower in females compared to males which correlates with the greater proportion of slow twitch type I fibres found in female skeletal muscle tissue (Haizlip et al., 2015). Additionally, glucose and lipid metabolism are highly correlated with the enzymatic characteristics of muscle fibres. Glycolytic capacity appears to be greater in men compared to woman, characterized by a higher maximal activity of PFK and other carbohydrate related metabolic markers, which may be due to the greater percentage of type II fibres found in males (Lundsgaard & Kiens, 2014). In

contrast, women express higher levels of CPT-1 and increased β-oxidation capacity compared to men, which is likely attributed to the greater proportion of type I oxidative fibres found in female skeletal tissue (Lundsgaard & Kiens, 2014). Additionally, maternal protein restriction results in increased carbohydrate metabolism genes in skeletal muscle tissue of female, but not male, offspring, indicating sex-specific differences (Zheng et al., 2012). Due to the observed differences between sexes, the current study will examine males and females separately since responses to an intervention may be sex-specific.

**1.2. Nutritional Programming**

Nutritional programming refers to the concept that nutrients consumed *in utero* and through lactation can have profound long-term effects on a tissue's growth and metabolism such that differences in susceptibility to chronic diseases are observed at adulthood (Langley-Evans, 2009; Yan et al., 2013). Nutritional programming has been studied extensively in a cohort of term singletons that were exposed to the Dutch Winter Famine (1944-1945) *in utero* with daily rations below 1000 calories for a minimum of 13 weeks. The Dutch Winter Famine provided a unique and 'natural' opportunity for researchers to study the link between the development of chronic diseases in adulthood with a short, but severe, period of maternal undernutrition during pregnancy. Researchers found individuals exposed to the famine *in utero* were associated with an increased incidence of cardiovascular disease, metabolic disorders, obesity and mortality compared to unaffected individuals, independent of birth weight (Chmurzynska, 2010; Painter et al., 2006; Painter et al., 2005; van Abeelen et al., 2012), suggesting low birth weight is not the only predictor of poor health at adulthood. The Dutch Winter Famine is one example that suggests that maternal undernutrition creates and imbalance between nutrient supply

and fetal demands, and may have a long-lasting disruption in the health trajectory of the offspring. Additionally, researchers have shown that maternal undernutrition of specific micronutrients, such as folic acid, can be detrimental to the fetus (Finglas, 2003; Iyer & Tomar, 2009; Lindzon & O'Connor, 2007). However, the effect of maternal supplementation with folic acid during pregnancy and lactation, on offspring health is less understood.

### 1.2.1 Early Dietary Factors Affecting Skeletal Muscle Function and Metabolism

Mesenchymal stem cells are a subset of quiescent mononuclear satellite cells critical for muscle tissue development, growth and repair. During fetal development, stem cells are committed to a myogenic lineage via regulatory proteins, subsequently proliferating, differentiating and fusing to become mature muscle fibres (Woo et al., 2011; Yan et al., 2013). Skeletal muscle fibres develop prenatally, thus, muscle fibre numbers are determined prior to birth. Alternatively, muscle mass is increased due to fibre size (hypertrophy), not quantity, postpartum (Walter & Klaus, 2014; Woo et al., 2011). *In utero,* skeletal muscle growth and development is largely affected by the intrauterine environment, thus, factors, such as diet, can alter both skeletal muscle function and metabolism of offspring in adulthood via nutritional programming. Therefore, the remainder of this review will discuss the effects of nutritional programming from a maternal diet, during gestation and/or lactation, on offspring skeletal muscle tissue at adulthood.

### *i) Maternal Undernutrition*

It has been shown that maternal undernutrition (50% overall reduction in food intake) significantly reduced muscle mass, stem cell number and regenerative capacity after injury in offspring at 8 weeks of age (Woo et al., 2011). Thus, muscle fibre development, associated with stem cell quantity and activity, is highly sensitive to nutrient availability during rapid periods of growth, such as fetal development. Additionally, previous research has shown protein restriction throughout gestation reduced body weight and muscle mass in rat offspring at weaning (Desai et al., 1996). Moreover, protein restriction through cross-fostering immediately after birth showed a similar effect (Desai et al., 1996), indicating exposure to nutritional insults *in utero* and during suckling are both critical time periods that can affect offspring postnatal skeletal muscle development

Maternal protein restriction, during pregnancy and lactation, has shown DNA hypermethylation at the glucose transporter type 4 promotor region (GLUT4) resulted in increased GLUT4 expression, as well as regulatory genes associated with carbohydrate metabolism, and silenced peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) expression in skeletal muscle of female offspring at 18 months of age (Zheng et al., 2012; Zeng et al., 2013). PGC-1α stimulates mitochondrial biogenesis, regulates skeletal muscle fibre type determination (promoting more oxidative type 1 fibres), and regulates carbohydrate and lipid metabolism, playing a significant role in the regulation of energy metabolism (Liang & Ward, 2006), and GLUT4 is a transporter protein essential for glucose homeostasis (Liang & Ward, 2006; Huang & Czech, 2007). Additionally, maternal folate deficiency results in decreased skeletal muscle size and

number of male offspring in a swine model immediately after birth (Li et al., 2013). Gene chip analysis indicated metabolic shifts in offspring of folate deficient sows. Genes related to carbohydrate (HK) and lipid metabolism (CPT) were significantly down-regulated in folate deficient offspring compared to controls (Li et al., 2013).

#### *ii) Maternal Overnutrition*

Maternal over-nutrition in animal models, due to diets high in fat (40% energy from fat) or Westernized junk food diets (processed foods high in high, sugar and salt), during pregnancy and lactation, have shown decreased muscle mass due to reduced muscle fibres in offspring at 10 - 12 weeks of age (Bayol et al., 2009; Bayol et al., 2005; Walter & Klaus, 2014). Muscle fibres and adipocytes are derived from mesenchymal stem cells, which are then committed to a myogenic or adipogenic lineage via regulatory proteins during prenatal development (Yan et al., 2013). Maternal overnutrition in animal models have shown decreased muscle fibres and increased adipocytes resulting in increased intramuscular fat in offspring skeletal muscle (Yan et al., 2013). Furthermore, these studies have shown contractile properties are impaired (reduced twitch and tetanic tensions), reducing muscle force (Bayol et al., 2009; Walter & Klaus, 2014). Lastly, skeletal muscle glucose and fatty acid metabolism and cellular respiration rates were shown to be impaired, decreasing substrate availability and, in turn, affecting muscle function (Bayol et al., 2005; Claycombe et al., 2015; Walter & Klaus, 2014). Taken together, these studies indicate offspring skeletal muscle function and metabolism are responsive to suboptimal maternal diets, through both over- and undernutrition. However, there is a paucity of studies relating to folic acid supplementation and skeletal muscle.

## 1.3. Nutritional Programming and Folate

### 1.3.1 Folate and Folic Acid

Folate is a water-soluble B vitamin ($B_9$) fundamental for human health and development. Humans are unable to synthesize folate *de novo*, thus, it must be supplied through the diet to meet the recommended daily requirements (Lindzon & O'Connor, 2007; Lucock, 2000). Folate is a generic term that refers to two forms: a) the naturally occurring form found in foods and b) the synthetic form, folic acid, used in food fortification and dietary supplements (Blancquaert et al., 2010; Institute of Medicine, 1998; Iyer & Tomar, 2009). Folic acid, also referred to as pteroylmonoglutamic acid, is the most oxidized and stable form of folate, consisting of a fully oxidized aromatic pteridine ring attached to a *p*-aminobenzoic acid molecule and a glutamic acid residue (Finglas, 2003; Guéant et al., 2013; Institute of Medicine, 1998; Lindzon & O'Connor, 2007; Lucock, 2000; Scott, 1999). The chemical structure of naturally occurring folate is similar to folic acid, however, there are two distinct structural differences. First, the aromatic pteridine ring of natural folate is reduced and second, they are pteroylpolyglutamates, thus, they contain additional glutamic acid molecules (1-6) linked to the glutamate (Finglas, 2003; Institute of Medicine, 1998; Lucock, 2000; Scott, 1999). However, the focus of this thesis will be on the synthetic folic acid form as this form is most commonly used in the literature.

### 1.3.2 Function of Folic Acid

Folic acid plays a fundamental role in DNA and RNA biosynthesis, DNA repair, and DNA methylation processes required for normal metabolism and regulation. Thus, adequate folic acid intake is essential for normal cell division and maintaining genome

integrity (Bailey & Gregory, 1999; Guéant et al., 2013). Folic acid primarily functions as a coenzyme in one-carbon transfer reactions necessary for amino acid and nucleic acid metabolism (Bailey & Gregory, 1999; Gregory, 2001). During periods of rapid growth and development, such as pregnancy, it is critical folic acid intake is increased due to the significant rise in one-carbon transfer reactions necessary for rapid cell division (Iyer & Tomar, 2009). The main one-carbon reactions that occur within the cell are: 1) the interconversion of serine and glycine; 2) the catabolism of histidine to glutamic acid; 3) the synthesis of purine; 4) the synthesis of thymidylate and; 5) the synthesis of methionine via remethylation of homocystine (Blancquaert et al., 2010; Gregory, 2001; Guéant et al., 2013; Lucock, 2000). A detailed explanation of the intracellular folic acid metabolism and the methionine synthesis pathway will be discussed next due to the fundamental role it plays in nutritional programming and DNA methylation, however, the details of the other one-carbon reactions will only be discussed as needed.

### 1.3.3 Intracellular Folic Acid Metabolism

The folic acid cycle begins as peripheral tissues take up circulating 5-methyltetrahydrofolate (THF). Intracellularly, 5-methyl THF is demethylated to THF and subsequently converted to the polyglutamate form via methionine synthase (from the methionine synthesis reaction) and polyglutamate synthase, respectively (Figure 1.7). Folic acid is unable to be stored or participate in one-carbon transfer reactions unless it is in the reduced polyglutamate THF form (Crider et al., 2012; Finglas, 2003; Kelly et al., 1997). Additionally, unmetabolized folic acid taken up by peripheral tissues is reduced to dihydrofolate and subsequently reduced to THF by dihydrofolate reductase. THF is then converted to 5,10-methylene THF and immediately reduced to 5-methyl THF via

methylenetetrahydrofolate reductase (MTHFR), for a new folate cycle to begin (Crider et al., 2012; Finglas, 2003; Kelly et al., 1997; Wright et al., 2007; Figure 1.7). This reaction is integral to methionine synthesis and S-adenosylmethionine (SAM)-mediated methylations. As 5-methyl THF is demethylated to THF, the methyl group from 5-methyl THF is transferred to homocysteine by methionine synthase, converting homocysteine to methionine (Crider et al., 2012; Kelly et al., 1997). Methionine is subsequently converted to SAM, a versatile coenzyme and major methyl donor for DNA methylation. DNA methylation occurs as methyltransferase transfers a methyl group from SAM to a cytosine residue on a DNA strand. During this process, SAM is demethylated to S-adenosylhomocysteine (SAH), which is then hydrolyzed to homocysteine to begin a new methionine synthesis and SAM-mediated methylation cycle (Chmurzynska, 2010; Crider et al., 2012; Lucock, 2000; Figure 1.7).

Additionally, THF, regenerated from the methionine synthesis reaction, is used in the formation of 5,10-methylene THF and 10-formyl THF used for the synthesis of thymidylate and purine, respectively (Bailey & Gregory, 1999; Chmurzynska, 2010). Briefly, in the thymidylate synthesis reaction, 5,10-methyene THF donates a methyl group to deoxyuridylate via thymidylate synthesis, converting it to thymidylate for DNA synthesis (pathway not shown). Additional dietary nutrients are also necessary to maintain methionine synthase reactions. These nutrients include: vitamin $B_6$ (purine synthesis), vitamin $B_{12}$ (methionine synthase function), choline (precursor to betaine), and riboflavin (MTHFR stability), however, they will not be discussed further in this review.

SAM and SAH play a fundamental role in the regulation of intracellular folic acid metabolism and methionine synthesis. High concentration of SAM inhibits MTHFR,

reducing synthesis of 5-methyl THF and thereby reducing the methylation of homocysteine to methionine. In contrast, low concentrations of SAM activate MTHFR activity, increasing 5-methyl THF synthesis and homocysteine methylation. Furthermore, high concentrations of SAH inhibit methyltransferase activity, resulting in DNA hypomethylation. Thus, hydrolysis of SAH to homocysteine is critical to maintain DNA methylation rates (Crider et al., 2012).

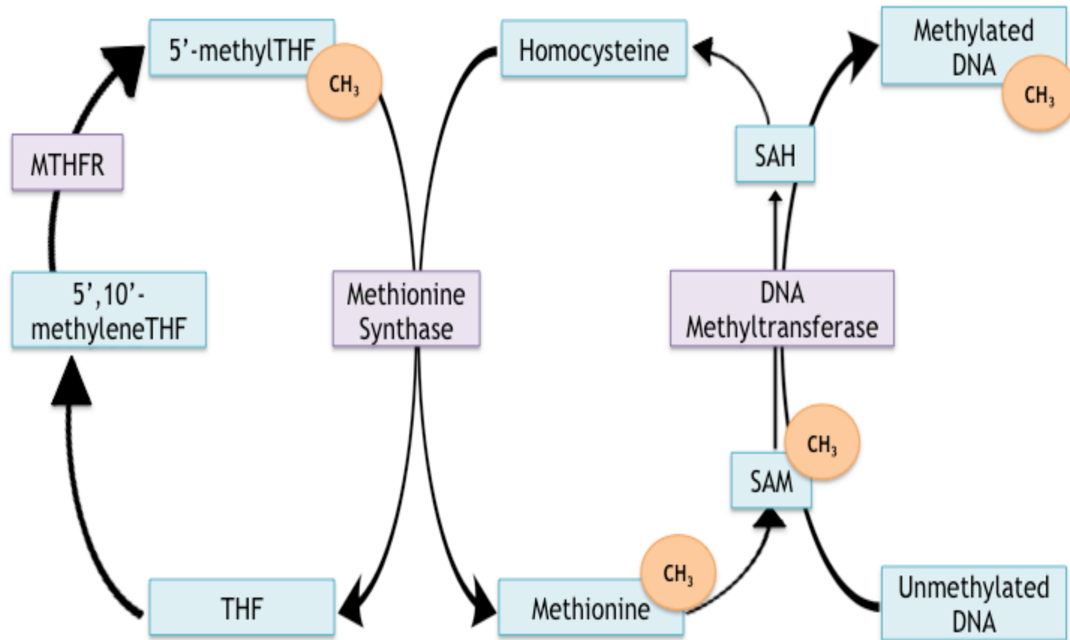


**Figure 1.7** Schematic diagram of intracellular folic acid metabolism involving DNA methylation. $CH_3$, methyl group; MTHFR, methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate (Saint, 2016).

### 1.3.4 Epigenetics

Epigenetics refers to alterations in gene expression (phenotype) that occur without alterations in DNA sequence (genotype) (Chmurzynska, 2010). Suboptimal maternal nutrition during pregnancy and lactation can cause epigenetic alterations of the fetal genome, affecting the long-term health and development of offspring (Langley-Evans, 2009; Wu et al., 2004). The mechanism by which nutritional programming affects tissue development has not been well established. However, it is hypothesized epigenetic alterations, such as DNA methylation, may play a role.

#### *i) DNA Methylation*

Methyl donors, derived from folic acid and methionine consumed in the diet, as well as, betaine, vitamin B12 and choline, are necessary for DNA methylation. Thus, early nutrition may influence adult phenotype through epigenetic alterations. DNA methylation is a post-replication modification of genomic DNA that alters gene expression. Fetal development may alter DNA methylation patterns, resulting in stable changes in gene expression that can be maintained throughout an individuals' lifespan.

DNA methylation patterns, established by cytosine methylation at specific sites along DNA strands, mostly develop *in utero* (Crider et al., 2012; Lillycrop et al., 2005; Waterland & Jirtle, 2003). DNA methylation occurs at the 5' position of cytosine within a cytosine-guanine (CpG) dinucleotide. DNA methyltransferase (Dnmt1) catalyzes DNA methylation by transferring a methyl group from a methyl donor, such as SAM, to the cytosine residue (Chmurzynska, 2010; Crider et al., 2012; Wu et al., 2004). SAM is a universal methyl donor in methyl transfer reactions synthesized from methionine via one-carbon metabolism reactions, as discussed previously (section 1.3.3. *Intracellular Folic*

*Acid Metabolism*). Methylation of CpG rich clusters, referred to as CpG islands located at the promotor regions of DNA strands, are associated with repressed gene transcription, whereas, hypomethylation of CpG islands results in increased transcriptional activity (Chmurzynska, 2010; Crider et al., 2012; Lillycrop et al. 2005). The mechanisms by which DNA methylation silences gene transcription are not fully understood, however, two potential mechanisms have been proposed: 1) DNA methylation directly inhibits gene transcription by binding to the promotor regions of DNA, blocking transcriptional factors, such as RNA polymerase (an enzyme essential for initiating gene transcription) from binding; and 2) methyl-CpG-binding domain proteins translocate binding to methylated CpGs and subsequently recruit transcriptional silencing complexes, such as histone deacetylase, resulting in chromatin condensation and consequently repressed gene transcription (Crider et al., 2012; Klose & Bird, 2006; Lillycrop et al. 2005; Yan et al., 2013). DNA methylation is a reversible process, thus, demethylation (removal of a methyl group) can up-regulate gene expression by relaxing chromatin to activate gene transcription (Chmurzynska, 2010; Crider et al., 2012).

***ii) Folic Acid and DNA Methylation***

Lillycrop et al. (2005) showed rat offspring of mothers fed a protein-restricted diet (containing 1 mg folic acid/kg diet) during pregnancy and lactation, expressed lower levels of methylation and higher expression of the peroxisome proliferator-activated receptor (PPARα) and glucocorticoid receptor (GR) genes in the liver. Supplementation of a protein-restricted diet with supplemental folic acid (5 mg folic acid/kg diet) negated hypomethylation of PPARα and GR genes. Moreover, a similar phenomenon has been shown in the agouti ($A^{vy}$) mouse model. The $A^{vy}$ gene locus is responsible for pigment

colouration of hair follicles, thus, epigenetic variation of the $A^{vy}$ locus results in a wide variation of coat colours (Chmurzynska, 2010). Hypomethylation of the $A^{vy}$ gene locus results in overexpression of the gene consequently producing a yellow coat (agouti phenotype), as well as, a tendency towards obesity, whereas, hypermethylation of the gene produces a brown coat (pseudoagouti phenotype) (Cooney et al., 2002). Maternal methyl-supplemented diets, containing folic acid (5mg folic acid/kg diet), betanine, vitamin B12, and choline, during pregnancy and lactation, increased hypermethylation at seven $A^{vy}$ pseudoexon 1A sites and the prevalence of pseudoagouti phenotype, while subsequently lowering obesity in offspring agouti mice at 3 weeks of age (Cooney et al., 2002; Waterland & Jirtle, 2003; Wolff et al., 1998). A follow-up study compared the effects of maternal dietary supplementation (4.3mg folic acid/kg diet) on DNA methylation in bisphenol A-treated agouti mice exhibiting hypomethylation at 9 CpG sites upstream of the $A^{vy}$ pseudoexon promoter (Dolinoy et al., 2007) Maternal supplementation with folic acid counteracted the DNA hypomethylating effect of bisphenol A, restoring the pseudoagouti phenotype (Dolinoy et al., 2007). Together, these studies indicate maternal dietary methyl donors have strong effects on DNA methylation and phenotype of offspring, which may affect long-term health.

DNA methylation, through methyl donors, such as folic acid, is thought to affect the commitment of mesenchymal stem cells to a myogenic lineage during the developmental process (Yan et al., 2013). However, the mechanisms underlying methyl supplementation and gene modification have yet to be fully elucidated in response to maternal diet, specifically in regard to skeletal muscle development. To our knowledge, there are no studies related to folic acid supplementation and skeletal muscle, however, a

study examined the effects of maternal folic acid deficiency on skeletal muscle offspring in a swine model (Li et al., 2013). Muscle fibre size and number was reduced in folic acid deficient piglets. Furthermore, folic acid deficiency impacted muscle gene transcription and expression, extensively down-regulating lipid-metabolism related genes and associated metabolic pathways, such as glucose and protein metabolism, in offspring (Li et al., 2013). This provides evidence that folic acid undernutrition affects the skeletal muscle transcriptome of offspring, which may be through epigenetic alterations.

## 1.4. Food Folate and Supplemental Folic Acid

### 1.4.1 Dietary Sources

Folate is present in a variety of food sources at varying amounts. The main dietary sources of folate are liver, dark green leafy vegetables (spinach, broccoli, asparagus), legumes (beans and chickpeas), citrus fruits (oranges), fermented dairy products (yogurt, cheese, milk), and yeast (Table 1.2) (Blancquaert et al., 2010; Finglas, 2003; Iyer & Tomar, 2009; Lucock, 2000; Winkels et al., 2007). Furthermore, breads, pastas, and breakfast cereals fortified with folic acid provide a significant contribution to the diet (Finglas, 2003; Winkels et al., 2007). Breakfast consisting of two medium eggs (~75 $\mu$g), 1 piece of toast (~100 $\mu$g), 1 medium banana (~25 $\mu$g) and 1 cup of orange juice (~100 $\mu$g) will provide approximately 250 $\mu$g of folic acid (Table 1.2). Lunch consisting of a salad with 1 cup of spinach (~300 $\mu$g), 1 avocado (~150 $\mu$g), 8 strawberries (~100 $\mu$g), and ½ cup of chickpeas (~200 $\mu$g) will provide approximately 750 $\mu$g of folic acid (Table 1.2). Lastly, dinner consisting of 1 cup of pasta (~200 $\mu$g) with ½ a cup of broccoli (~100 $\mu$g) and a side Caesar salad with ½ a cup of romaine lettuce (~50 $\mu$g) will provide approximately 350 $\mu$g of folic acid (Table 1.2). Thus, pregnant and lactating woman can

consume approximately 3-4 times (1800-2400 $\mu$g) the RDA of folic acid through daily dietary intake (~1350 $\mu$g) and supplemental use (400-1000 $\mu$g) (Table 1.3).

**Table 1.2** Food folate content of common foods from the Canadian Nutrient File, 2010.

| Food Source | Serving Size | Folate ($\mu$g) (DFE) |
|---|---|---|
| *Vegetables* | | |
| Edemame/soybeans, cooked | 125 mL (½ cup) | 255 |
| Spinich, cooked | 125 mL (½ cup) | 139 |
| Asparagus, cooked | 6 spears | 139 |
| Broccoli, cooked | 125 mL (½ cup) | 89 |
| Brussels sprouts, cooked | 4 sprouts | 85 |
| Lettuce (Romaine) | 250 mL (1 cup) | 80 |
| Potato, white, with skin, baked | 1 medium | 66 |
| *Fruits* | | |
| Avacado | ½ fruit | 81 |
| Orange Juice | 125 mL (½ cup) | 58 |
| Orange | 1 fruit | 48 |
| *Grain Products†* | | |
| Pasta, egg noodles, enriched, cooked | 125 mL (½ cup) | 138 |
| Pasta, white, enriched, cooked | 125 mL (½ cup) | 113 |
| Bread, white | 1 slice | 106 |
| Instant cereal, hot, oats | 250 mL (1 cup) | 87 |
| Muffin, oat bran | ½ muffin | 79 |
| *Meat and Alternatives* | | |
| Liver (lamb), cooked | 75 g (2½ oz) | 300 |
| Lentils, cooked | 175 mL (¾ cup) | 265 |
| Chickpeas, cooked | 175 mL (¾ cup) | 209 |
| Beans, kidney, cooked | 175 mL (¾ cup) | 170 |
| Beans, navy, cooked | 175 mL (¾ cup) | 146 |
| *Milk and Alternatives* | | |
| Eggs | 2 large | 76 |
| Cheese, brie* | 50 g (1.7oz) | 32 |
| Yogurt, plain* | 175 mL (¾ cup) | 21 |
| Milk 2%* | 250 mL (1 cup) | 13 |
| *Other* | | |
| Yeast extract spread (vegemite or marmite) | 30 mL (2 Tbsp) | 371 |

$\mu$g: micrograms; DFE: Dietary Folate Equivalents
* Folate level increases if fermented (Iyer & Tomar, 2009)
† Fortified food products may contain higher folate content than reported (Shakur et al., 2009)

### 1.4.2 Absorption

After consumption, food folates (polyglutamates) are hydrolyzed by the brush border of the mucosal cells in the stomach to the monoglutamate form, via folate conjugase (Halsted, 1991; Institute of Medicine, 1998; Wright et al., 2007). Monoglutamates are absorbed by reduced folate carriers or proton-coupled folate transporters in the mucosal cells of the jejunum (proximal small intestine) (Guéant et al., 2013; Visentin et al., 2014; Zhao, Matherly & Goldman, 2009). At concentrations below 280 $\mu$g, monoglutamates are absorbed by an active pH-dependent process, however, at concentrations above 280 $\mu$g, monoglutamates can be absorbed by a passive diffusion process, indicating folic acid uptake is saturable (Finglas, 2003; Kelly et al., 1997; Wright et al., 2007). Prior to entry into the hepatic portal vein, folic acid is reduced to tetrahydrofolate (THF) and subsequently methylated to 5-methyltetrahydrofolate (5-methyl-THF) (Kelly et al., 1997; Scott, 1999; Wright et al., 2007). The liver removes a large amount of folic acid from the hepatic blood circulation before it enters the systemic plasma circulation. The liver either stores the folic acid, approximately 50% of total folate is stored in the liver, or it is released in the bile where it enters the enterohepatic cycle to be recirculated (Finglas, 2003; Lucock, 2000; Wright et al., 2007). The primary form of folic acid found in the systemic circulation is 5-methyl-THF, however, if pharmacological amounts (>280 $\mu$g) of folic acid are ingested, unmetabolized folic acid may also be present in circulation (Finglas, 2003; Kelly et al., 1997). Circulating folic acid is then transported to peripheral tissues by folate transport systems to be used for various functions, discussed previously (Section 1.3.1 *Function of Folate*).

### 1.4.3 Bioavailability

Bioavailability is defined as the proportion of an ingested nutrient that is absorbed and can be used for metabolic processes (Blancquaert et al., 2010; Winkels et al., 2007). Multiple factors play a role in the bioavailability of folates, such as folate structure and physiological influences. Naturally occurring food folates, such as polyglutamates, have a bioavailability of approximately 50% (Institute of Medicine, 1998; Lindzon & O'Connor, 2007; Winkels et al., 2007). This may be due to the additional hydrolysis step necessary for polyglutamates prior to absorption. Moreover, studies have shown individuals with a less active form of folate conjugase have a lower folate status than those with higher folate conjugase activity (Devlin et al., 2000; Gregory, 2001).

When consumed alone, supplemental folic acid has a bioavailability of 100%, however, when consumed with food the bioavailability decreases to approximately 85%. Absorption of monoglutamates are dependent on intestinal pH levels, which can be altered by various foods or drugs, however, little is known regarding nutritional interactions that affect folate bioavailability (Gregory, 2001; Tamura & Stokstad, 1973). Moreover, evidence has shown active absorption is maximized at a pH of 6.3 and rapidly declines between pH 6.3 and 7.6 (Finglas, 2003; Russell et al., 1979).

### 1.4.4 Recommended Daily Requirements

Erythrocyte folate is an indicator of the long-term status of folate and is reflective of tissue folate stores, whereas, plasma homocysteine levels are indicative of the amount folate present in the blood to donate methyl groups for the conversion of homocysteine to methionine (Institute of Medicine, 1998). Taken together, erythrocyte folate and plasma homocysteine biomarkers were used to determine the Recommended Dietary Allowance

(RDA) of folic acid. The adequate intake (AI) of folic acid is determined from the observed average intake of infants exclusively fed human milk (Institute of Medicine, 1998) (Table 1.3).

***i) General Recommendations for Adults***

The current RDA of folate intake for adults is 400 $\mu$g/day of dietary folate equivalents (DFE) (Table 1.3) (Institute of Medicine, 1998). DFEs adjust for the lower bioavailability of naturally occurring folate compared to folic acid found in fortified foods and supplements, which will be discussed later (Section 1.4.3 *Absorption*). One DFE is equivalent to 1 $\mu$g of food folate, 0.6 $\mu$g of folic acid fortified food, and 0.5 $\mu$g of folic acid consumed on an empty stomach (Institute of Medicine, 1998). The Tolerable Upper Intake Level (UL) of folate (folic acid only) is 1000 $\mu$g/day for adults (Institute of Medicine, 1998).

***ii) Recommendations for Pregnancy***

The RDA for pregnant and lactating woman is 600 and 500 $\mu$g/day, respectively (Institute of Medicine, 1998) (Table 1.3). To reduce the risk of neural tube defects at birth, Health Canada recommends all women take a daily prenatal folic acid supplement containing 400 $\mu$g of folic acid, in addition to their dietary folate intake, three months prior to pregnancy and throughout the pregnancy (De Wals et al., 2007; Health Canada, 2009; Ray, 2004). Moreover, the Society of Obstetricians and Gynaecologists of Canada recommends woman with an increased risk for neural tube defects, characterized by a family or personal history of neural tube defects or folate sensitive abnormalities, take a daily prenatal folic acid supplement containing 1000 $\mu$g of folic acid in addition to their dietary folate intake (SOGC Clinical Practice Guideline, 2015). Additionally, in 1998,

folic acid fortification (150 - 200 $\mu$g/100g) of all white flour, enriched pasta and cornmeal products became mandatory in Canada to reduce the risk of neural tube defects at birth (De Wals et al., 2007; Lindzon & O'Connor, 2007; Ray, 2004; Shakur et al., 2009; Wright et al., 2007). The Flour Fortification Policy was estimated to increase an individual's dietary folate intake by a minimum of 100 $\mu$g/day (Jägerstad, 2012; Ray, 2004; Shakur et al., 2009). After flour fortification was implemented, the prevalence rate of neural tube defects at birth decreased by approximately 50% in Canada (Castillo-Lancellotti et al., 2013; De Wals et al., 2007; Jägerstad, 2012). Furthermore, the prevalence of folate inadequacy among the Canadian population remains low (<20%) since mandatory folic acid fortification (Shakur et al., 2010). Moreover, with the prevalent use of prenatal supplements, pregnant woman in Canada have high maternal folate concentrations of 2400 and 2800 nmol/L during early pregnancy and delivery, respectively (Plumptre et al., 2015). Additionally, unmetabolized folic acid is detectable in >90% of maternal blood due to the high level of supplemental folic acid intake (Plumptre et al., 2015). High maternal folate concentrations may affect the long-term health outcomes of offspring, such as skeletal muscle function and metabolism, thus, further investigation is warranted due to the mandatory folic acid fortification and prevalent supplemental use due to Health Canada's current recommendations for woman of childbearing age.

**Table 1.3** Recommended dietary allowance (RDA) of folate by age group (Adapted from Health Canada, 2010).

| **Age** | ***μ*g/day (DFE)** |
|---|---|
| Birth to 6 months | 65* |
| 7-12 months | 80* |
| 1-3 years | 150 |
| 4-8 years | 200 |
| 9-13 years | 300 |
| 14+ years | 400 |
| Pregnancy | 600 |
| Lactation | 500 |

*Adequate Intake (AI)
*μ*g: micrograms; DFE: Dietary Folate Equivalents

**1.5. Statement of the Problem**

Skeletal muscle is an important contractile and metabolic tissue essential for locomotion and cellular energy metabolism. Evidence suggests maternal nutrition, such as high-fat diets, protein restriction, methyl supplemented diets, and folic acid deficiency diets, during pregnancy and lactation can cause epigenetic alterations of the fetal genome, affecting the long-term growth and development of offspring skeletal muscle (Cooney et al., 2002; Langley-Evans, 2009; Li et al., 2013; Waterland & Jirtle, 2003; Wolff et al., 1998; Wu et al., 2004; Zeng et al., 2013). Maternal overnutrition in animal models, due to high fat diets, during pregnancy and lactation have shown impaired skeletal muscle contractile and metabolic properties, characterized by reduced twitch and tetanic tension, glucose and fatty acid metabolism, and cellular respiration rates, affecting overall muscle function in offspring at 12 weeks of age (Bayol et al., 2005; Claycombe et al., 2015; Walter & Klaus, 2014). There is evidence maternal methyl-supplemented diets, such as folic acid supplementation, during pregnancy and lactation, modulate DNA methylation and phenotype of offspring (Cooney et al., 2002; Dolinoy et al., 2007; Lillycrop et al., 2005; Waterland & Jirtle, 2003; Wolff et al., 1998). Additionally, maternal folate deficiency results in significant down-regulation of carbohydrate (HK) and lipid (CPT1) metabolism gene expression in male offspring, as indicated through gene chip analysis (Li et al., 2013).

However, there is limited information regarding the influence of maternal supplemental folic acid diet on offspring skeletal muscle growth and development, characterized by contractile and metabolic function, through nutritional programming. Additionally, sex-specific responses to methyl-supplemented diets on skeletal muscle

function and metabolism has not been studied. Thus, the purpose of this thesis was to examine if supplemental folic acid from preconception through lactation programs skeletal muscle function in male and female offspring at adulthood by 1) examining twitch characteristics of representative slow (SOL) and fast (EDL) twitch muscles and 2) measuring maximal enzyme activities of carbohydrate and lipid metabolism.

### 1.6. Objectives

The overall objective of this study was to determine if maternal supplemental folic acid diet, throughout pregnancy and lactation, programs skeletal muscle tissue function and metabolism in male and female offspring. The specific objectives of this study were to determine if adult offspring (at 6 months of age) exposed to maternal supplemental folic acid diet throughout pregnancy and lactation had differences in:

1) Contractile function characterized by peak twitch tension, peak tetanic tension, half-relaxation time, peak time to relaxation, twitch-to-tetanus ratio, rate of force development and rate of relaxation, in SOL and EDL muscle and;

2) Metabolic function measured by carbohydrate (hexokinase; HK and phosphofructokinase; PFK), lipid (carnitine palmitoyltransferase; CPT and 3-Hydroxyacyl-CoA dehydrogenase; β-HAD), and oxidative (citrate synthase; CS and cytochrome C oxidase; COX) regulators in EDL and SOL muscle tissue.

### 1.7. Hypothesis

Six-month-old male and female offspring exposed to maternal supplemental folic acid diet, *in utero* and during suckling, compared to recommended folic acid diet will have:

1) Increased contractile function reflected by a greater peak twitch and tetanic tension, faster half-relaxation time and peak time to relaxation, a higher twitch-to-tetanus ratio, and a faster rate of force development and rate of relaxation, in SOL and EDL muscle.

2) Higher metabolic enzyme activity measured by greater HK, PFK, CPT, β-HAD, CS and COX activity in EDL and SOL muscle tissue.

# CHAPTER 2.0 – METHODS

## 2.1. Animals and Diet

This study was a subset of a larger study looking at the influence of maternal folic acid supplemented diet on offspring skeletal health. Briefly, at 2, 4, and 6 months of age, the right hindlimb of a subset of mice and the abdominal region of an additional subset of mice was irradiated to assess bone structure and body composition, respectively, using micro-computed tomography. For that reason, skeletal muscle contractile outcomes were assessed in the left hindlimb, as it was not irradiated during the study. This avoided any potential effects due to irradiation. Skeletal muscle enzyme activity outcomes were assessed in both the left and right hindlimbs of mice used to assess body composition, as muscles were not irradiated during the study. To allow for time involved with longitudinal scans of the hindlimb, mice were studied in three consecutive batches. Each batch of mice received the same treatment throughout the study, and was approximately equal size (Figure 3.1 & 3.2). Sample sizes for each group (CON & FA) and outcome (contractile & enzymatic activity) varied due to death of offspring at birth, during scanning procedures, or for unknown reasons over the period of the study. Additionally, some force traces during contractile function were lost due to technological issues. All experimental procedures complied with the Canadian Council on Animal Care and were approved by the Brock University Animal Care Committee (Olfert et al., 1993).

Female CD-1 mice (*n*=31) were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada) at 5 weeks of age and housed 4-5 per cage at 22 ± 1°C with a 12:12 h light-dark cycle. Mice were housed in groups to provide environmental enrichment, since mice are social animals that benefit from interactions, and enhance

their overall well-being (Olfert et al., 1993). After a 5-day adaptation period, mice were randomized to receive control (CON; modified AIN93G diet, 2 mg folic acid/kg diet**;** *n*=15**)** or supplemented folic acid **(**FA; modified AIN93G diet, 8 mg folic acid/kg diet; *n*=16) diet. Mice were given access to food and water *ad libitum*. The AIN93G diet is a 'healthy' diet specifically used in nutritional science research to support the nutritional needs of growing, pregnant and lactating rodents (Reeves, Nielsen & Fahey, 1993). The CON diet was modified with alcohol-extracted 'vitamin-free' casein. This was done to ensure any naturally occurring folic acid present in casein was removed so any observed effects in offspring could be attributed to the known higher quantity of folic acid in the diet, and not due to the intake of additional nutrients. The amount of folic acid in the CON diet (2 mg/kg diet) was to represent the basal dietary requirements for growing, lactating and/or pregnant rodents. The FA diet was identical to the CON diet except it contained a higher level (8 mg/kg diet) of folic acid (Table A.1). The purpose of increasing folic acid in the diet to 4 times higher than the CON diet was to mimic the amount of folic acid pregnant and lactating mothers consume through folic acid fortification and prenatal supplement use (Health Canada, 2009). Diets were isocaloric with 19.4% energy from protein, 63.8% energy from carbohydrate, and 16.7% energy from fat (TD.06706 & TD.140396; Harlan Teklad, Missisauga, ON).

Food intake was determined by weighing, recording and subtracting the amount of food remaining from the previous feeding. The total food intake per cage was then divided by the number of days since the last recording, and the number of mice in the cage, to provide an estimated daily food consumption for each mouse. Food intake and

body weight was recorded biweekly and weekly, respectively (Denver Instrument Scale, MXX-5, Bohemia, NY, USA**).**

**2.2. Experimental Design**

At 10 weeks of age, female mice were mated harem-style with male CD-1 mice (*n*=14) of similar age (Figure 2.1). Male mice were obtained from Charles River Laboratories at 9 weeks of age and housed singly prior to breeding. Male mice were fed CON diet and water, *ad libitum,* prior to mating, when they were switched to the female's respective diets (CON or FA) (Figure 2.1). Each male mouse was bred with two females from each intervention group, with the exception of one male bred with three females (CON), to ensure equal paternal influence among groups. Once pregnancy was confirmed (~1 week), determined by an increase in body weight greater than normal weekly weight gain, the female mice were housed singly until delivery, remaining on their respective diets throughout pregnancy (~3 weeks) and lactation (3 weeks). Offspring remained with the mother until weaning on postnatal day (PND) 21. Litter weights were recorded on PND 9, 16, and 21 (Denver Instrument, MXX-5, Bohemia, NY, USA).

At PND 21, offspring were weaned to CON diet and housed 5 per cage, per gender (Figure 2.1). Because there were often more than 5 females and 5 males per litter, offspring that were followed to adulthood were selected according to their body weight at weaning. Specifically, 5 female and 5 male offspring with median body weights, within the range of body weights per gender, per litter, were selected. The offspring were subsequently ear tagged to ensure proper identification of each animal throughout the study. Food intake was recorded biweekly, while body weight was recorded once weekly for the duration of the study.

At 6 months of age, offspring were euthanized after an overnight fast. Only one offspring per litter, per gender was reported, representing an *n*=1for each outcome, to ensure the results were not biased due to over-representation of each litter ("litter effect"; Wainwright, 1998). One mouse per gender was used for *in vitro* skeletal muscle contractile experiments using the left hindlimb (discussed next) and two mice per litter, per gender were pooled using both hindlimbs for enzyme assays (Figure 2.1).

The mice were anaesthetized using isoflurane gas inhalant (3-5% delivery rate) until an adequate depth of anaesthesia, confirmed by the absence of the withdraw reflex to a toe-pinch, was reached. Soleus (SOL; 31% type I, 49% type IIa, 12% type IId/x, 3% type IIb) and extensor digitorum longus (EDL; <1% type I, 10% type IIa, 24% type IId/x, 56% type IIb) (Bloemberg & Quadrilatero, 2012) were surgically removed from both hindlimbs, immediately frozen in liquid nitrogen and stored at -80°C until further analysis. The mice were euthanized via exsanguination.

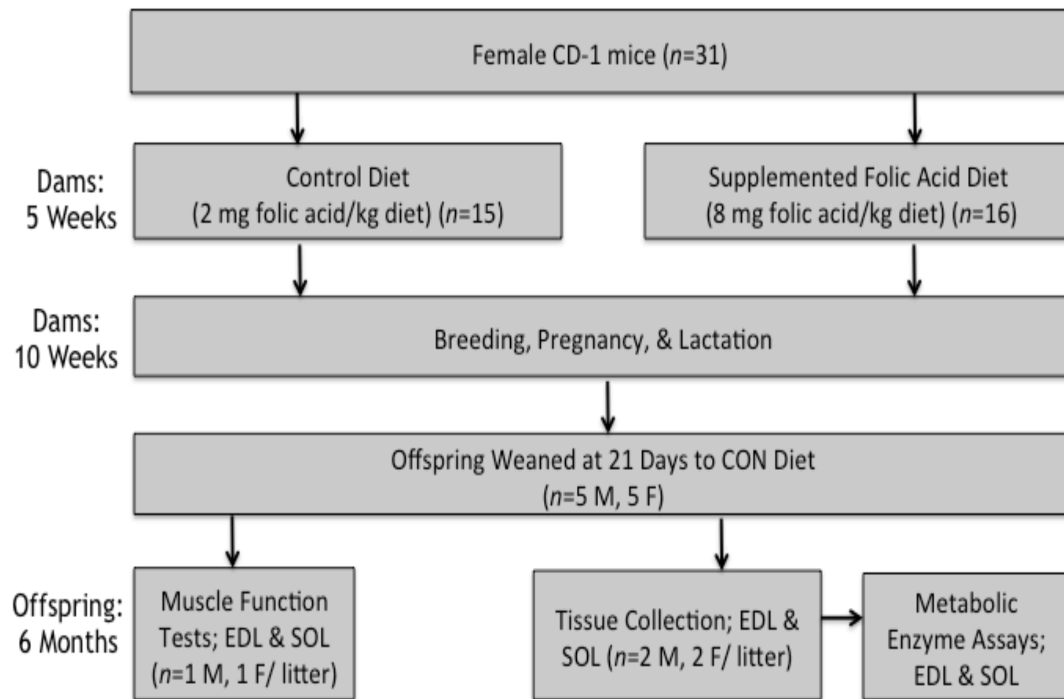


**Figure 2.1.** Flow chart of the experimental design. CON, control diet; EDL, extensor digitorum longus; SOL, soleus; M, male; F, female (Saint, 2016).

## 2.3. In Vitro Muscle Preparation

### 2.3.1 Muscle Isolation

Once mice were under adequate depth of anaesthesia, the skin of the lower hindlimb was gently peeled back to expose the musculature of interest. The EDL and SOL were surgically removed from the left hindlimb once both proximal and distal tendons were secured with suture. To accomplish this, the distal tibialis anterior tendon was cut to expose the distal tendons of the EDL, which extend along the metatarsals. The tibialis anterior muscle was dissected away to uncover the EDL muscle underneath. Distal to the myotendon junction, the EDL tendon was secured using a non-absorbable braided 4-0 silk suture. A small incision lateral to the knee was made to the biceps femoris muscle to expose the proximal tendon of the EDL, which was then lashed with suture and the muscle removed by cutting the tendon proximal to the suture.

To remove the SOL muscle, the distal Achilles tendon was cut from the calcaneus and the triceps surae muscles were peeled back away from the tibia. The SOL muscle was located on the underside of the gastrocnemius muscle and separated from the posterior compartment muscles using blunt scissors. At the distal myotendon junction of the SOL (attached to the Achilles tendon), sutures were tied and the tendon was cut inferior to the suture knot. The proximal tendon of the SOL, located on the posterior surface of the tibia, was sutured and cut superior to the suture knot prior to removal of the muscle. Isolated muscles were initially left to equilibrate under slight tension in a resting bath for 30 minutes that contained cooled (4-5 °C) and oxygenated Tyrode's solution to ensure viability until required (MacPherson et al., 2013).

### 2.3.2 Mounting Muscles to the Apparatus

From each animal, EDL and SOL were mounted into separate experimental apparatus (1200a *In Vitro* Muscle Testing System, Aurora Scientific Inc.). Muscles were mounted to the dual-mode servomotor (Model 305B) by clamping the distal tendon to the fixed electrode assembly and lashing the proximal tendon to the lever arm of the servomotor via a hooked non-compliant stainless steel wire. The muscles were suspended in a jacketed organ bath containing oxygenated (95% $O_2$ and 5% $CO_2$) Tyrode's solution maintained at 25.0 ± 0.1 °C with a pH of approximately 7.4 (Lännergren et al., 2000). Tyrode's solution is a physiological saline solution that approximates the physiological conditions of the muscle *in vivo* and provides the essential substrates necessary for cell metabolism and muscle contraction. The composition of the buffered solution (in mM) was: 121 NaCl; 5 KCl; 24 $NaHCO_3$; 0.4 $NaH_2PO_4$; 0.5 $MgCl_2$; 1.8 $CaCl_2$; 5.5 D-glucose; and 0.1 EDTA. During brief and/or intermittent stimulation (i.e. low duty cycle), viability of intact EDL and SOL muscles can be maintained at 25 °C due to adequate oxygen diffusion, thus, the Tyrode's solution was maintained at this temperature (Barclay, 2005). The muscles were stimulated using flanking platinum electrodes driven by a Model 701B biphase stimulator (Aurora Scientific Inc.). Data acquisition was performed using Aurora Scientific Inc. 600a software (Version 1.60). All contractile experiments for each muscle type were collected from the same apparatus to ensure there was no additional variability introduced between samples.

## 2.4. Isometric Contractile Experiment Protocol

### 2.4.1 Determination of Supramaximal Stimulus Conditions

Once mounted, left EDL and SOL were briefly stimulated supramaximally at 150 Hz to remove any possible compliance in the suture/tendon connection before subsequent experimental procedures. Complete muscle recruitment was ensured by setting the current of the stimulation above the threshold required to achieve maximal twitch force. Once supramaximal stimulus conditions were established, the muscles were left to return to baseline conditions (MacPherson et al., 2013).

### 2.4.2 Determination of Optimal Length

Optimal length ($L_o$) was defined as the muscle length at which peak twitch force was produced. Following the equilibrium period, $L_o$ was determined by stimulating the muscle at various resting tensions (MacPherson et al., 2013). Initially, the resting tension of the muscle was removed and stimulated by a single electrical pulse (1 Hz) to elicit a twitch response. Resting tension was subsequently increased at 1.0 mN intervals while a single stimulus was applied at each length. The muscle length at which maximal active twitch force (total force – passive force) was reached was determined as $L_o$. Muscle length ($L_o$) was measured visually between the myotendon junctions using digital Vernier calipers (SOL) and a horizontal stereo zoom microscope (EDL) and was used as the reference length for all subsequent procedures.

### 2.4.3 Peak Twitch and Peak Tetanus Force

At $L_o$, a single stimulation pulse was applied to the muscle to produce a maximal twitch response (forces measured in mN). Additionally, TPT and ½ RT were calculated (in ms) from the force-time trace. TPT was calculated from the time the twitch

contraction began to the time peak tension was produced. ½ RT was calculated from the time it takes to the muscle to reach 50% of peak tension from the time peak tension was produced. +dP/d*t* and –dP/d*t* were measured by taking the maximal positive and negative slopes, respectively, over a 17-point consecutive spread, from the differentiated force-time trace. Subsequently, peak tetanic force ($P_o$) was determined by brief (500 ms) supramaximal stimulation (150 Hz for EDL and 100 Hz for SOL) (MacPherson et al., 2013). The twitch:tetanus ratio ($P_t$:$P_o$) was later calculated from the two force amplitudes.

**2.4.4 Specific Tension Calculation**

To control for variations in cross sectional area (CSA) between each muscle, muscle forces were normalized to CSA. The CSA for each muscle was determined by dividing the mass of the muscle (g) by the product of its optimum fibre length ($L_f$) and density of mammalian muscle (1.06 mg/mm$^3$) (Equation 2.1) (Liu et al., 2005; Mendez & Keys, 1960). $L_f$ was calculated by multiplying $L_o$ by the fibre length to muscle length ratio previously reported for each muscle type: 0.44 for EDL and 0.71 for SOL (Brooks & Faulkner, 1988). Next, $P_t$ and $P_o$ were divided by muscle CSA to obtain normalized peak twitch and tetanic forces (Equation 2.2).

$$CSA\ (mm^2) = \frac{Muscle\ mass\ (g)}{(L_f \times 1.06\ mg/mm^3)}$$

**Equation 2.1.** Cross sectional area equation.

$$Specific\ tension\ (g/mm^2) = \frac{Peak\ tetanic\ or\ twitch\ force\ (nM)}{CSA\ (mm^2)}$$

**Equation 2.2.** Specific twitch and tetanic force equation.

## 2.5. Metabolic Enzyme Assays

### 2.5.1 Muscle Tissue Preparation

Whole muscle tissue of EDL and SOL was weighed (~18mg) and added to a pre-chilled 2mL glass homogenizer containing homogenization buffer (50 mM Tris-HCL; pH 7.8) to a final concentration of 20:1 (v/w). The sample was homogenized using glass-on-glass manual homogenization. All metabolic enzyme activities were measured using an Ultraspec 2100 Pro spectrophotometer (Biochrom, Cambridge, UK) at 25 ± 1$^{o}$C.

### 2.5.2 Enzyme Activity

***i) Hexokinase***

Carbohydrate metabolism was measured by hexokinase (HK; EC 2.7.1.1) activity as previously outlined by Thompson & Cooney (2000) with the following modifications. The reaction buffer included 40 mM triethanolamine-HCl (pH 7.6), 5 mM EDTA, 7.5 mM $MgCl_2$, 0.42 mM NADP, 2.5 mM ATP, 10 µg (1.67 U/mL) glucose-6-phosphate dehydrogenase, 2.22 mM D(+)-glucose, and 50 µL homogenate. The reaction was initiated by the addition of D(+)-glucose. The appearance of NADPH was used to determine HK activity at 340 nM (millimolar extinction coefficient: $\varepsilon_{340}$=6.22).

***ii) Phosphofructokinase***

Phosphofructokinase (PFK; EC 2.7.1.11) activity was measured to determine carbohydrate metabolism. The details of the assay were previously described by Passonneau & Lowry (1993) with the following modifications. The reaction buffer contained 38.3 mM Tris-Base (pH 8.0), 2 mM $MgCl_2$, 10 mM $K_2HPO_4$, 0.375 mM NADH, 1 mM 5'-AMP, 2 U/mL α-glycerophosphate dehydrogenase, 62.4 U/mL triosephosphate isomerase, 1.61 U/mL aldolase, 1 mM ATP, 2.5 mM fructose-6-

phosphate, and 10 μL homogenate. The addition of fructose-6-phosphate initiated the reaction. Activity was measured by the change in absorbance of NADH to $NAD^+$ at 340nm (millimolar extinction coefficient: $\varepsilon_{340}$=6.22).

***iii) Carnitine Palmitoyltransferase***

Lipid metabolism was measured by carnitine palmitoyltransferase (CPT; EC 2.3.1.21) activity as previously described by Bieber et al. (1972) with the following modifications. The reaction buffer contained 76.6 mM Tris-HCl (pH 8.0), 1.1 mM EDTA, 0.24mM DTNB, 0.08 mM palmitoyl-CoA, 1.1 mM L-carnitine, and 15 μL of homogenate. Additionally, the homogenate was subjected to three freeze-thaw cycles to ensure mitochondrial membrane ruptured. The enzyme reaction rates were determined by measuring change in absorbance of DTNB to TNB at 412 nm (millimolar extinction coefficient: $\varepsilon_{412}$=13.6).

***iv) 3-Hydroxyacyl-CoA Dehydrogenase***

β-oxidation of fatty acids was measured by 3-Hydroxyacyl-CoA dehydrogenase (β-HAD; EC 1.1.1.35) activity as previously described by Bass et al., (1969) with the following modifications. The reaction buffer contained 103.2 mM triethanolamine-HCL, 5 mM EDTA, 0.225 mM NADH, 0.1 mM acetoacetyl-CoA, and 20 μL of homogenate. The reaction was initiated with the addition of acetoacetyl-CoA. The enzyme reaction rates were determined by the change in absorbance of NADH to $NAD^+$ at 340 nm (millimolar extinction coefficient: $\varepsilon_{340}$=6.22).

***v) Citrate Synthase***

Citrate synthase (CS; EC 2.3.3.1) activity was used as a marker to measure the presence of intact mitochondria as per Srere (1969) with the following modifications. The

reaction buffer contained 0.1 mM DTNB, 0.5 mM acetyl-CoA, 0.5% Triton X-100, 0.5 mM oxaloacetate, and 2.5 µL of homogenate. Additionally, three freeze-thaw cycles were performed to the homogenate to ensure rupture of the mitochondrial membrane. Addition of oxaloacetate initiated the reaction. Change in absorbance of DTNB to TNB at 412 nM determined CS activity (millimolar extinction coefficient: $\varepsilon_{412}$=13.6)

***vi) Cytochrome C Oxidase***

Mitochondrial respiratory capacity was measured via cytochrome c oxidase (COX; EC 1.9.3.1) activity as previously described (Cooperstein & Lazarow, 1950), with the following modifications. The reaction buffer contained 18.3 mM phosphate buffer (pH 7.4), 3.0% Tween 20, 0.04 mM cytochrome c, and 10 µL of homogenate. The homogenate underwent three freeze-thaw cycles to ensure mitochondrial membrane was ruptured. Addition of cytochrome C initiated the reaction. Enzymatic rates were determined at 550 nm (millimolar extinction coefficient: $\varepsilon_{550}$=21.84).

**2.6. Statistical Analysis**

All values are expressed as means ± standard errors (SEM). Male and females were examined separately for all analysis. For offspring, repeated measures two-way ANOVA, followed by a Tukey post-hoc analysis was used to analyze body weight, with offspring ages and maternal diet (CON and FA) used as the two factors. Independent sample t-tests were conduced to establish differences between diet (CON and FA) in muscle function characteristics ($P_t$, $P_o$, ½ RT, TPT, $P_t$:$P_o$, +dP/d*t*, -dP/d*t*), as well as, metabolic enzyme activities (HK, PFK, CPT, β-HAD, CS and COX) for male and female offspring. Additionally, contractile function data was normalized to peak twitch force and independent sample t-tests were conduced to establish differences between diet (CON

and FA) in muscle function characteristics. Statistical significance was determined at $p \leq 0.05$. All statistical analyses were performed using IBM SPSS Statistics (version 23, SPSS INC, Chicago, IL).

# CHAPTER 3.0 – RESULTS

## 3.1. Body Weight

At 6 months of age, final body weights of the folic acid group did not differ from controls within males (Figure 3.1) and females (Figure 3.2). There were no diet or diet x time interaction effects on body weight throughout the duration of the study in male or female mice (Figure 3.1 and Figure 3.2). As expected, there was an effect of time ($p<0.001$) as the mice were growing (Figure 3.1 and Figure 3.2). Additionally, final body weights of male and females did not differ between groups when mice were separated based on outcome (contractile function or metabolic activity) at 6 months of age (data not shown).

## 3.2. Anthropometric Muscle Properties

In both male and female mice, EDL mass (mg), length (mm) and CSA ($cm^2$) did not significantly differ between control and folic acid groups (Table 3.1; Table 3.2). In males, SOL length and mass did not differ from controls for the folic acid group, however, CSA in the folic acid group significantly decreased by 10% ($p<0.05$; Table 3.1). In females, SOL mass and CSA were similar between diets but muscle length significantly decreased by ~3% ($p<0.05$) with folic acid compared to controls (Table 3.2).

## 3.3. Isometric Contractile Function

Representative twitch force traces for the control and folic acid diets are presented in Figure 3.3 and Figure 3.4 for both males and females, respectively. Force traces that were closest to the average twitch parameters across each group were chosen to be representative force traces. In males, the isometric contractile characteristics for EDL and SOL did not significantly differ between control and folic acid groups (Table 3.3; Figure

3.3). In female mice, the groups did not differ in EDL and SOL, with the exception of increased TPT (15%) in SOL and decreased ½ RT (25%) and –dP/d*t* (28%) in EDL of the folic acid group (Table 3.4; Figure 3.4).

### 3.4. Maximal Enzyme Activity

In males, CS and HK activity increased by 24% and 13% ($p<0.05$) in SOL and EDL, respectively (Table 3.5; Figure 3.5; Figure 3.6). In female mice, with the exception of significantly decreased HK activity in the FA group by ~12% ($p<0.05$) compared to controls in the SOL, there was no effect on maximal enzyme activities (Table 3.6; Figure 3.7; Figure 3.8).

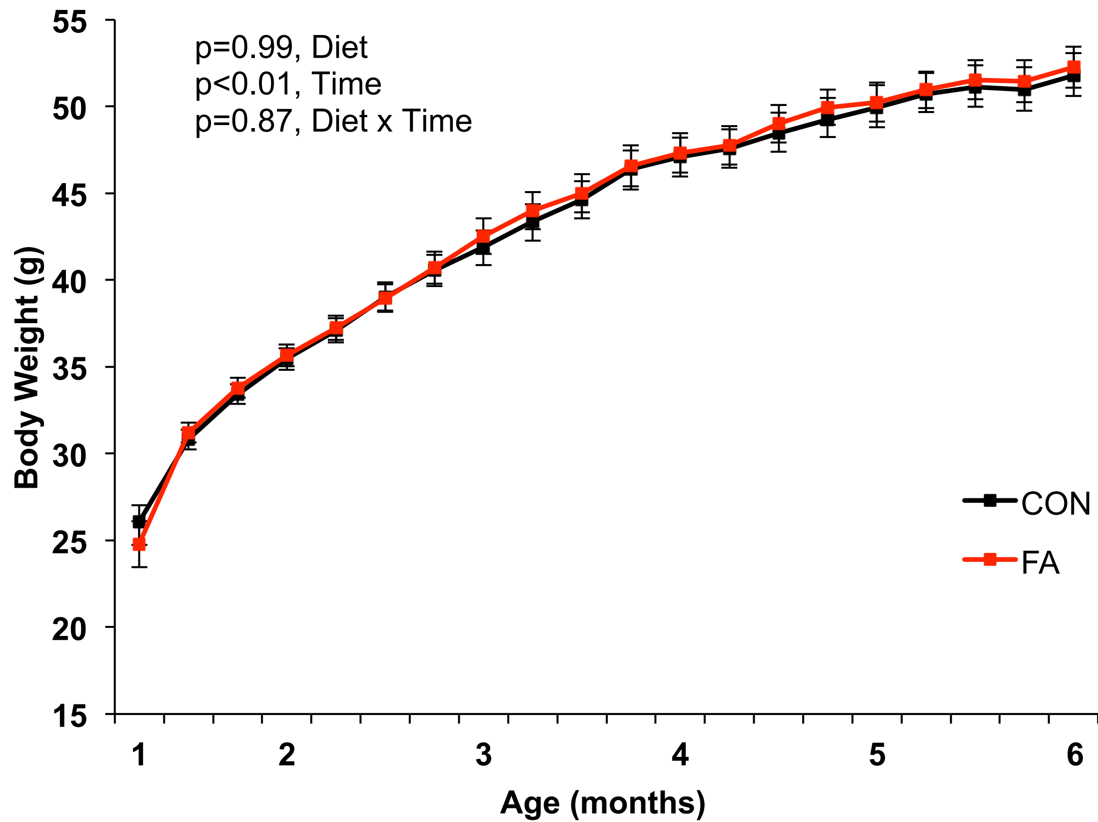


**Figure 3.1.** Body weight in grams of male offspring. Values are mean ± SEM. CON, n=24 (n=10 for contractile function; n=14 for enzymatic activity); FA, n=28 (n=8 for contractile function; n=20 for enzymatic activity). CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet.

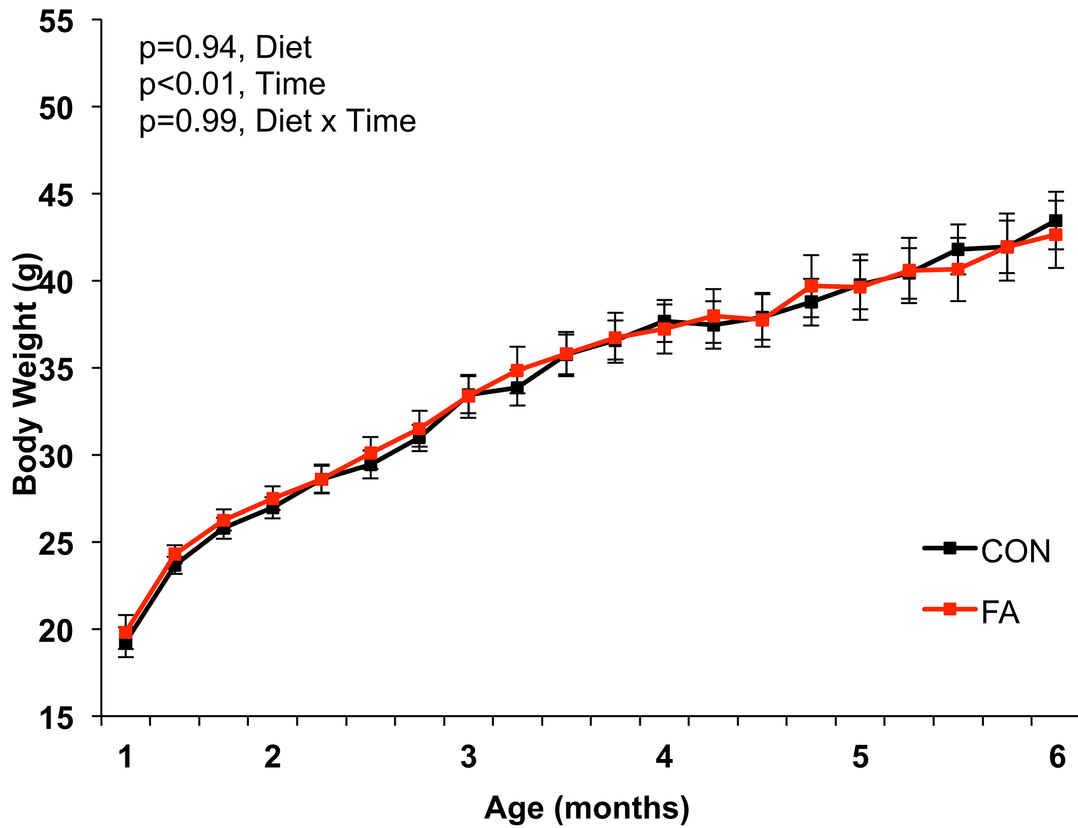


**Figure 3.2.** Body weight in grams of female offspring. Values are mean ± SEM. CON, n=29 (n=9 for contractile function; n=20 for enzymatic activity); FA, n=28 (n=8 for contractile function; n=20 for enzymatic activity). CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet.

**Table 3.1.** Effect of a supplemental folic acid diet on male offspring CD-1 mice soleus (SOL) and extensor digitorum longus (EDL) muscle properties.

| | **CON** | **FA** |
|---|---|---|
| ***SOL*** | | |
| Mass (mg) | 13.6 ± 0.7 | 12.7 ± 0.4 |
| Length (mm) | 13.3 ± 0.2 | 13.5 ± 0.1 |
| CSA ($mm^2$) | 1.38 ± 0.05 | 1.25 ± 0.03* |
| ***EDL*** | | |
| Mass (mg) | 14.2 ± 0.5 | 14.0 ± 0.4 |
| Length (mm) | 13.6 ± 0.2 | 13.8 ± 0.2 |
| CSA ($mm^2$) | 2.24 ± 0.07 | 2.19 ± 0.07 |

Values are mean ± SEM. CON, n=9; FA, n=7; * Significantly different from CON. CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet; SOL, soleus; EDL, extensor digitorum longus; CSA, cross-sectional area.

**Table 3.2.** Effect of a supplemental folic acid diet on female offspring CD-1 mice soleus (SOL) and extensor digitorum longus (EDL) muscle properties.

| | **CON** | **FA** |
|---|---|---|
| ***SOL*** | | |
| Mass (mg) | 12.2 ± 0.5 | 12.1 ± 0.6 |
| Length (mm) | 13.4 ± 0.2 | 13.0 ± 0.1* |
| CSA ($mm^2$) | 1.21 ± 0.06 | 1.24 ± 0.06 |
| ***EDL*** | | |
| Mass (mg) | 12.3 ± 0.4 | 12.0 ± 0.5 |
| Length (mm) | 13.8 ± 0.1 | 13.3 ± 0.2 |
| CSA ($mm^2$) | 1.91 ± 0.05 | 1.93 ± 0.09 |

Values are mean ± SEM. CON, n=9; FA, n=8; * Significantly different from CON. CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet; SOL, soleus; EDL, extensor digitorum longus; CSA, cross-sectional area.

**Table 3.3.** Effect of a supplemental folic acid diet on male offspring CD-1 mice soleus (SOL) and extensor digitorum longus (EDL) muscle isometric contractile function.

| | **CON** | **FA** |
|---|---|---|
| ***SOL*** | | |
| $P_t$ (mN) | 32 ± 3 | 32 ± 2 |
| $P_o$ (mN) | 229 ± 19 | 224 ± 18 |
| $P_t$/CSA (mN/cm$^2$) | 2.3 ± 0.1 | 2.8 ± 0.2 |
| $P_o$/CSA (mN/cm$^2$) | 16.5 ± 1.1 | 17.9 ± 1.2 |
| $P_t$:$P_o$ | 0.142 ± 0.002 | 0.157 ± 0.013 |
| TPT (ms) | 41.2 ± 2.3 | 44.8 ± 3.3 |
| ½ RT (ms) | 51.8 ± 6.1 | 56.8 ± 7.5 |
| +dP/d*t* (mN/ms) | 1.80 ± 0.17 | 2.03 ± 0.09 |
| -dP/d*t* (mN/ms) | 0.49 ± 0.09 | 0.54 ± 0.07 |
| ***EDL*** | | |
| $P_t$ (mN) | 84 ± 9 | 93 ± 5 |
| $P_o$ (mN) | 463 ± 27 | 459 ± 21 |
| $P_t$/CSA (mN/cm$^2$) | 3.7 ± 0.3 | 4.2 ± 0.2 |
| $P_o$/CSA (mN/cm$^2$) | 20.6 ± 0.7 | 20.9 ± 0.6 |
| $P_t$:$P_o$ | 0.179 ± 0.012 | 0.204 ± 0.014 |
| TPT (ms) | 18.6 ± 0.4 | 18.2 ± 0.4 |
| ½ RT (ms) | 21.4 ± 1.6 | 22.1 ± 1.0 |
| +dP/d*t* (mN/ms) | 7.27 ± 0.78 | 8.04 ± 0.52 |
| -dP/d*t* (mN/ms) | 3.37 ± 0.40 | 3.75 ± 0.25 |

Values are mean ± SEM. CON, n=7; FA, n=9; CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet; SOL, soleus; EDL, extensor digitorum longus; $P_t$, peak twitch force; $P_o$, peak tetanic force; CSA, cross-sectional area; $P_t$:$P_o$, twitch to tetanus ratio; TPT, time-to-peak tension; ½ RT, half relaxation time; +dP/d*t*, rate of contraction; -dP/d*t*, rate of relaxation.

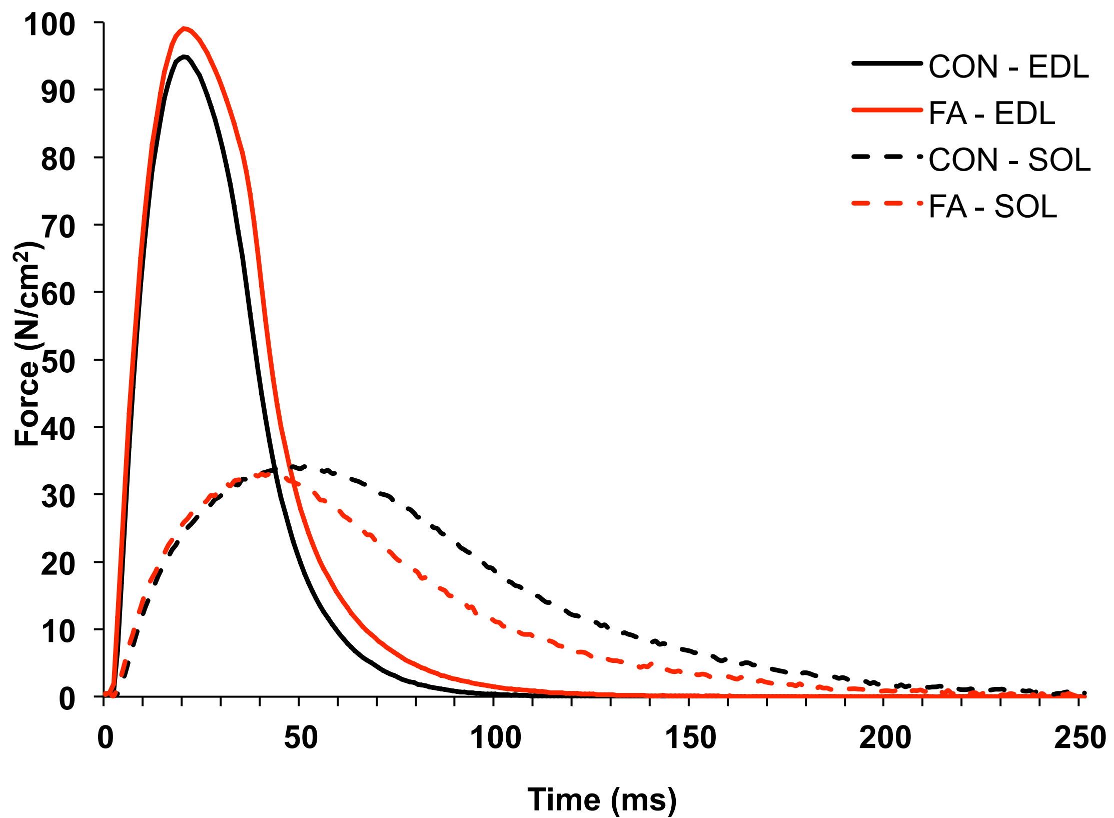


**Figure 3.3.** Representative isometric peak twitch force trace of male mice extensor digitorum longus and soleus muscles from control and folic acid groups. EDL, extensor digitorum longus; SOL, soleus; CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet.

**Table 3.4.** Effect of a supplemental folic acid diet on female offspring CD-1 mice soleus (SOL) and extensor digitorum longus (EDL) muscle isometric contractile function.

| | CON | FA |
|---|---|---|
| ***SOL*** | | |
| $P_t$ (mN) | 33 ± 2 | 30 ± 4 |
| $P_o$ (mN) | 242 ± 14 | 199 ± 25 |
| $P_t$/CSA (mN/cm$^2$) | 2.6 ± 0.2 | 2.4 ± 0.2 |
| $P_o$/CSA (mN/cm$^2$) | 18.9 ± 1.5 | 15.8 ± 1.5 |
| $P_t$:$P_o$ | 0.149 ± 0.01 | 0.156 ± 0.01 |
| TPT (ms) | 40.8 ± 0.9 | 46.9 ± 2.3* |
| ½ RT (ms) | 49.0 ± 2.9 | 52.1 ± 1.6 |
| +dP/d*t* (mN/ms) | 1.74 ± 0.13 | 1.44 ± 0.17 |
| -dP/d*t* (mN/ms) | 0.47 ± 0.04 | 0.44 ± 0.05 |
| ***EDL*** | | |
| $P_t$ (mN) | 74 ± 4 | 73 ± 8 |
| $P_o$ (mN) | 388 ± 23 | 340 ± 33 |
| $P_t$/CSA (mN/cm$^2$) | 3.9 ± 0.1 | 3.8 ± 0.4 |
| $P_o$/CSA (mN/cm$^2$) | 20.2 ± 0.8 | 17.5 ± 1.3 |
| $P_t$:$P_o$ | 0.193 ± 0.007 | 0.217 ± 0.020 |
| TPT (ms) | 18.3 ± 0.3 | 18.5 ± 0.5 |
| ½ RT (ms) | 24.1 ± 1.2 | 18.1 ± 0.7* |
| +dP/d*t* (mN/ms) | 6.41 ± 0.37 | 6.16 ± 0.73 |
| -dP/d*t* (mN/ms) | 2.23 ± 0.15 | 3.09 ± 0.38* |

Values are mean ± SEM. CON, n=9; FA, n=8; * denotes significance CON; CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet; SOL, soleus; EDL, extensor digitorum longus; $P_t$, peak twitch force; $P_o$, peak tetanic force; CSA, cross-sectional area; $P_t$:$P_o$, twitch to tetanus ratio; TPT, time-to-peak tension; ½ RT, half relaxation time; +dP/d*t*, rate of contraction; -dP/d*t*, rate of relaxation.

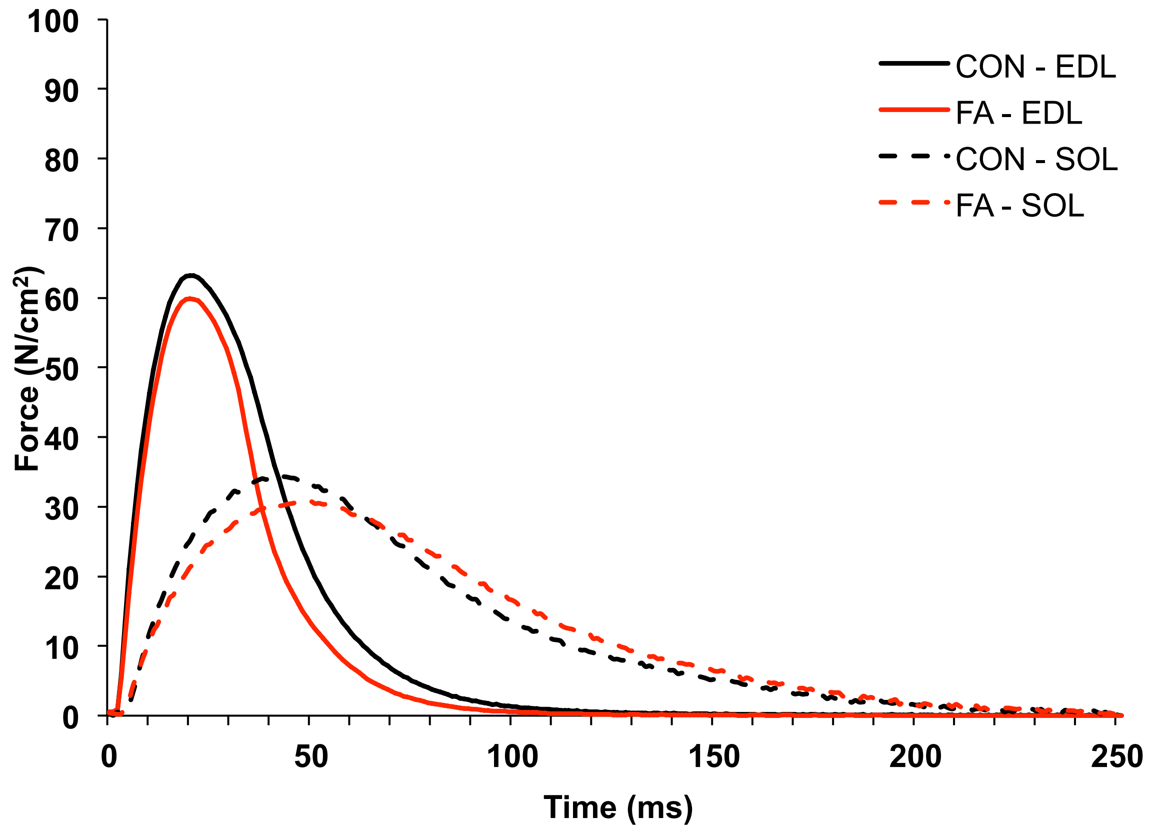


**Figure 3.4.** Representative isometric peak twitch force trace of female mice extensor digitorum longus and soleus muscles from control and folic acid groups. EDL, extensor digitorum longus; SOL, soleus; CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet.

**Table 3.5.** Effect of a supplemental folic acid diet on male offspring CD-1 mice soleus and extensor digitorum longus muscle maximal enzyme activity (μmol/min/g of wet wt. tissue).

| | **CON** | **FA** |
|---|---|---|
| ***SOL*** | | |
| *Carbohydrate* | | |
| HK | 5.6 ± 0.2 | 6.1 ± 0.2 |
| PFK | 27.2 ± 0.6 | 27.8 ± 1.1 |
| *Lipid* | | |
| CPT | 0.67 ± 0.09 | 0.84 ± 0.22 |
| β-HAD | 26.9 ± 1.2 | 26.9 ± 1.7 |
| *Oxidative* | | |
| CS | 30.5 ± 2.3 | 37.7 ± 2.5* |
| COX | 37.3 ± 4.7 | 34.4 ± 3.0 |
| ***EDL*** | | |
| *Carbohydrate* | | |
| HK | 3.4 ± 0.1 | 3.9 ± 0.2* |
| PFK | 44.5 ± 1.2 | 47.2 ± 1.2 |
| *Lipid* | | |
| CPT | 0.26 ± 0.05 | 0.49 ± 0.12 |
| β-HAD | 11.0 ± 0.9 | 13.6 ± 1.1 |
| *Oxidative* | | |
| CS | 24.1 ± 1.8 | 22.7 ± 1.4 |
| COX | 31.8 ± 3.5 | 26.5 ± 3.8 |

Values are mean ± SEM. $n = 10$; CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet; SOL, soleus; EDL, extensor digitorum longus; HK, hexokinase; PFK, phosphofructokinase; CPT, carnitine palmitoyltransferase; β-HAD, 3-hydroxyacyl-CoA dehydrogenase; CS, citrate synthase; COX, cytochrome c oxidase.

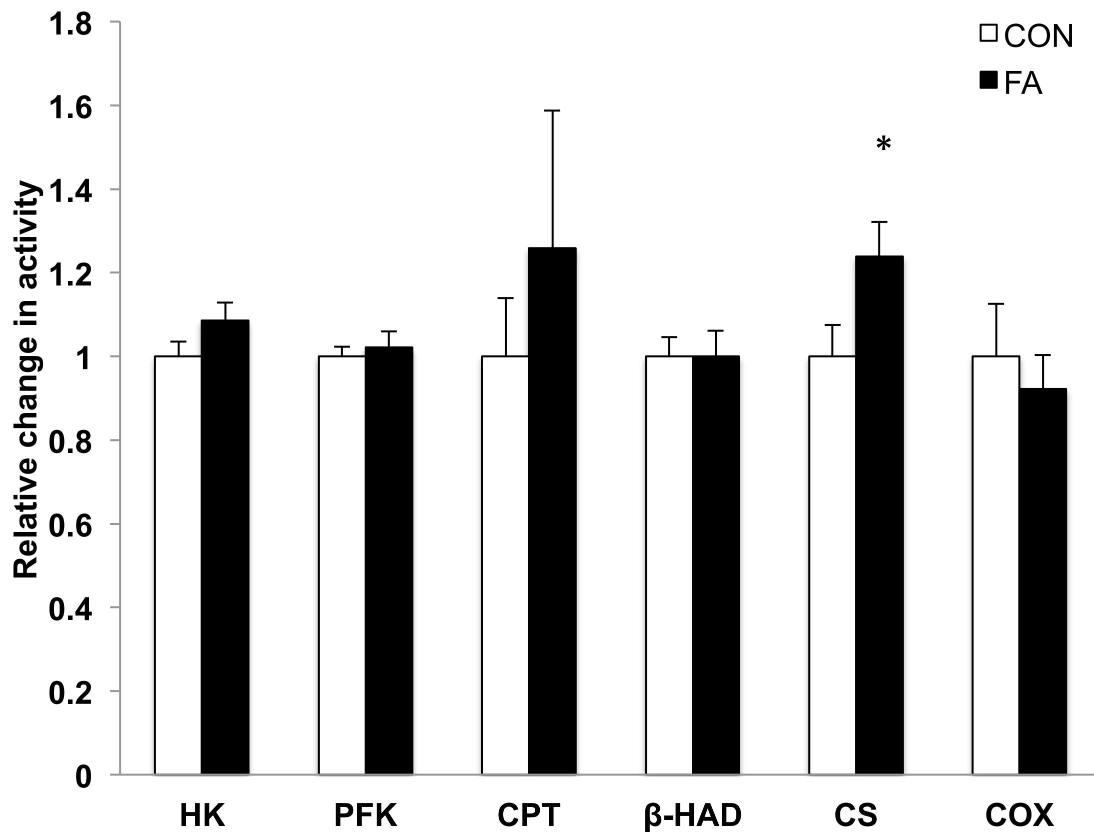


**Figure 3.5.** Relative change in maximal enzyme activity in male mice soleus (SOL) muscles. Values are mean ± SEM. *n* = 10; * Significantly different from CON. CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet; HK, hexokinase; PFK, phosphofructokinase; β-HAD, β-hydroxyacyl-CoA dehydrogenase; CPT, carnitine palmitoyltransferase; CS, citrate synthase; COX, cytochrome c oxidase.

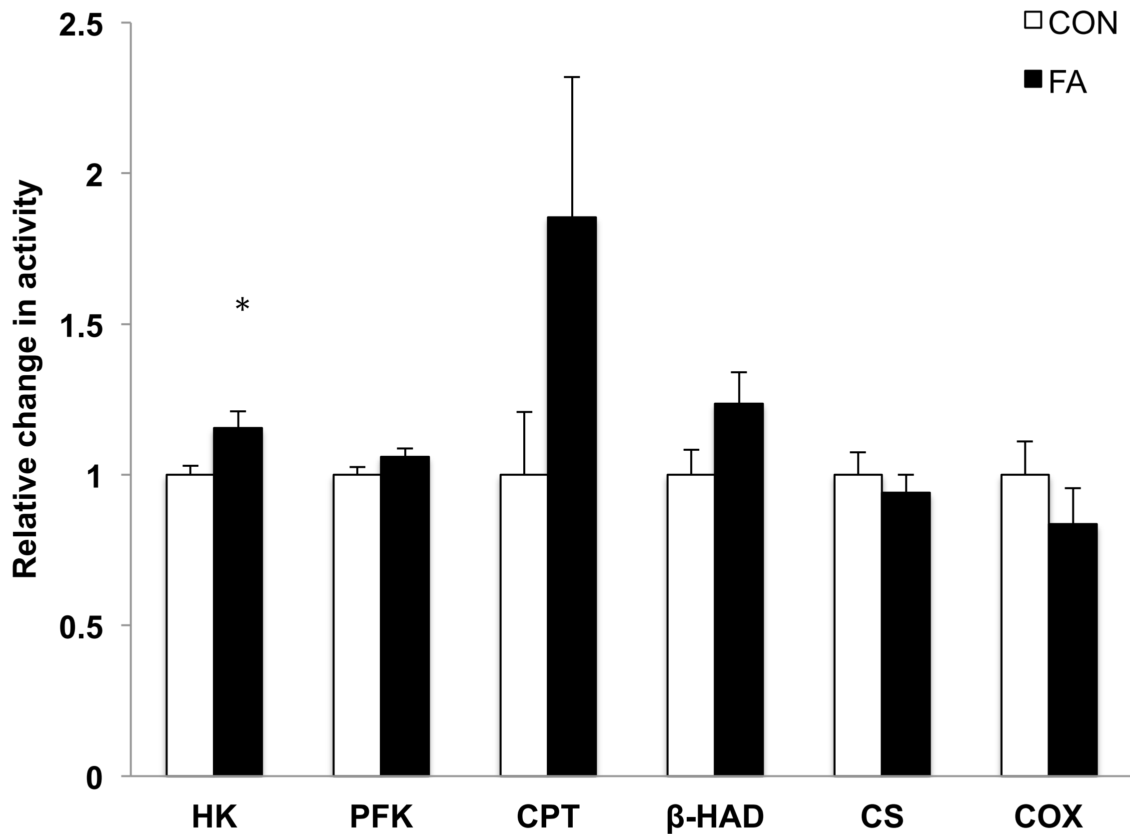


**Figure 3.6.** Relative change in maximal enzyme activity in male mice extensor digitorum longus (EDL) muscles. Values are mean ± SEM. $n = 10$; * Significantly different from CON. CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet; HK, hexokinase; PFK, phosphofructokinase; β-HAD, β-hydroxyacyl-CoA dehydrogenase; CPT, carnitine palmitoyltransferase; CS, citrate synthase; COX, cytochrome c oxidase.

**Table 3.6.** Effect of a supplemental folic acid diet on female offspring CD-1 mice soleus (SOL) and extensor digitorum longus (EDL) muscle maximal enzyme activity (μmol/min/g of wet wt. tissue).

| | CON | FA |
|---|---|---|
| ***SOL*** | | |
| *Carbohydrate* | | |
| HK | 5.0 ± 0.1 | 4.4 ± 0.2* |
| PFK | 20.4 ± 0.8 | 19.0 ± 0.7 |
| *Lipid* | | |
| CPT | 0.90 ± 0.09 | 0.77 ± 0.13 |
| β-HAD | 12.9 ± 0.9 | 11.4 ± 0.4 |
| *Oxidative* | | |
| CS | 21.1 ± 1.0 | 19.6 ± 0.8 |
| COX | 29.0 ± 1.1 | 28.9 ± 0.9 |
| ***EDL*** | | |
| *Carbohydrate* | | |
| HK | 2.9 ± 0.1 | 3.0 ± 0.1 |
| PFK | 46.5 ± 2.6 | 47.2 ± 1.7 |
| *Lipid* | | |
| CPT | 0.34 ± 0.04 | 0.44 ± 0.05 |
| β-HAD | 5.0 ± 0.3 | 5.1 ± 0.4 |
| *Oxidative* | | |
| CS | 19.9 ± 0.7 | 17.4 ± 1.3 |
| COX | 26.7 ± 1.1 | 28.2 ± 1.3 |

Values are mean ± SEM. $n$ = 10; CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet; SOL, soleus; EDL, extensor digitorum longus; HK, hexokinase; PFK, phosphofructokinase; CPT, carnitine palmitoyltransferase; β-HAD, 3-hydroxyacyl-CoA dehydrogenase; CS, citrate synthase; COX, cytochrome c oxidase.

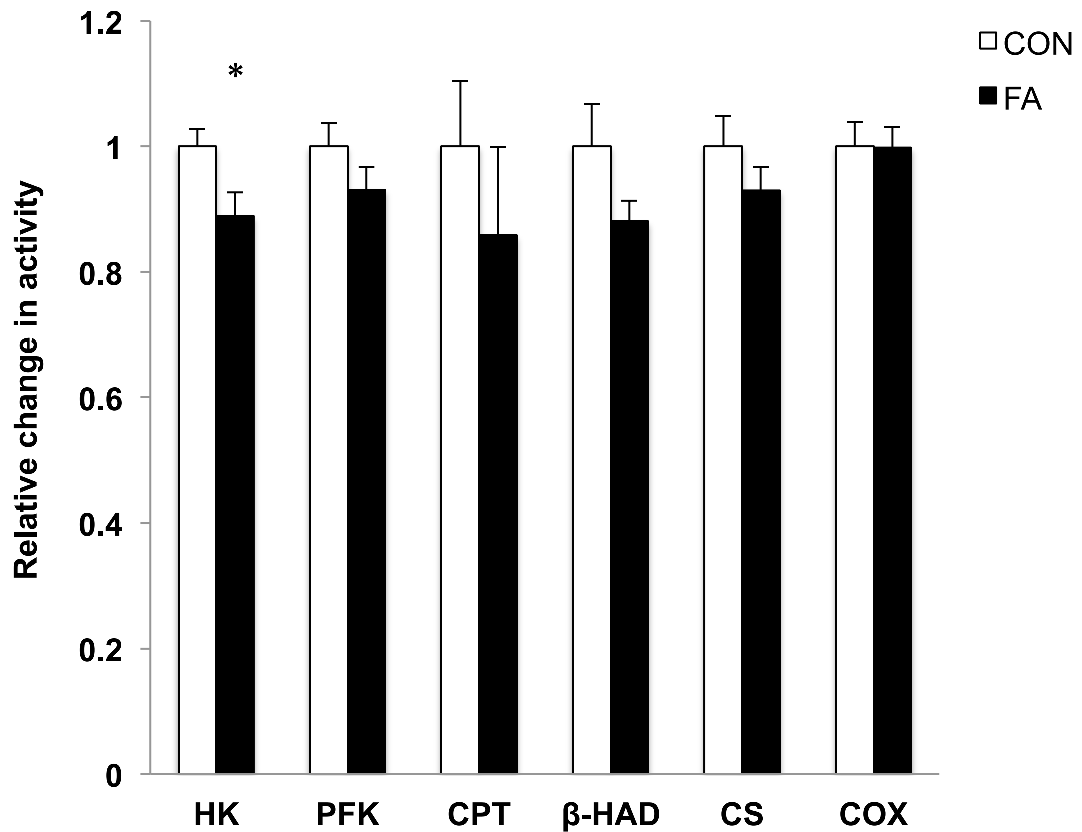


**Figure 3.7.** Relative change in maximal enzyme activity in female mice soleus (SOL) muscles. Values are mean ± SEM. *n* = 10; * Significantly different from CON. CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet; HK, hexokinase; PFK, phosphofructokinase; β-HAD, β-hydroxyacyl-CoA dehydrogenase; CPT, carnitine palmitoyltransferase; CS, citrate synthase; COX, cytochrome c oxidase.

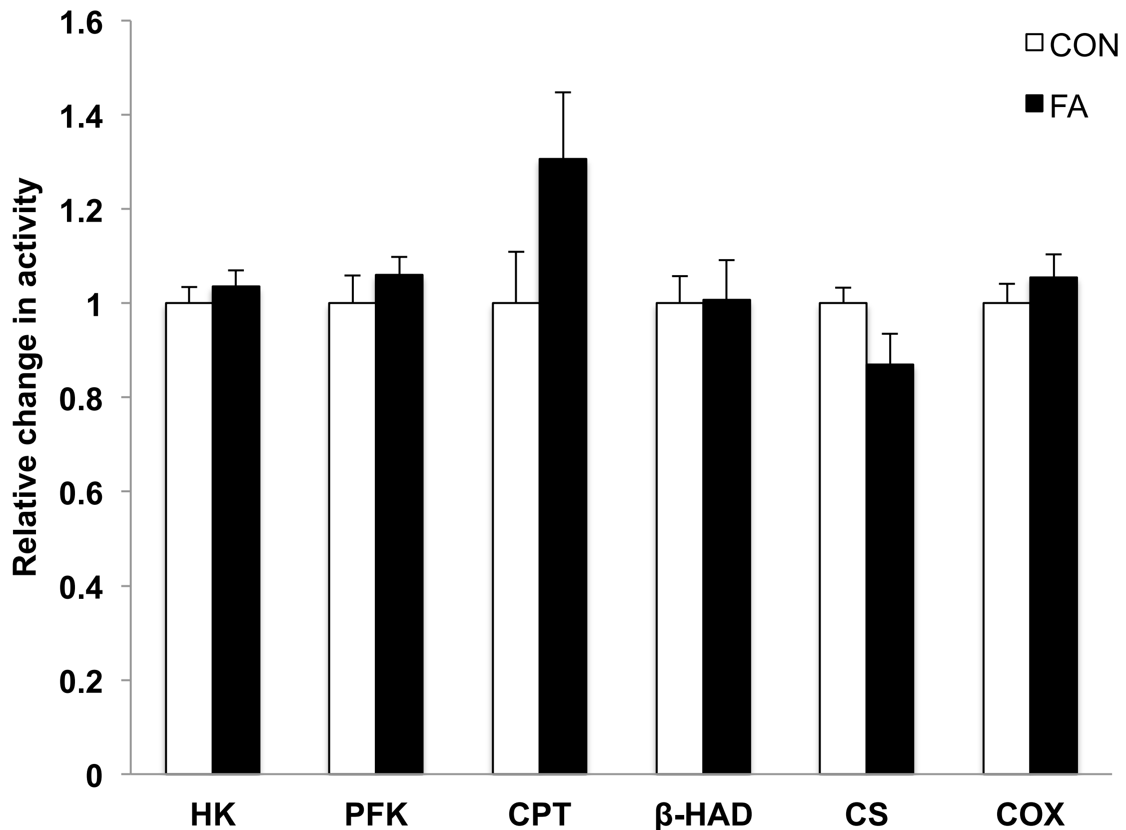


**Figure 3.8.** Relative change in maximal enzyme activity (μmol/min/g of wet wt. tissue) in female mice extensor digitorum longus (EDL) muscles. Values are mean ± SEM. *n* = 10; CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet; HK, hexokinase; PFK, phosphofructokinase; β-HAD, β-hydroxyacyl-CoA dehydrogenase; CPT, carnitine palmitoyltransferase; CS, citrate synthase; COX, cytochrome c oxidase.

# CHAPTER 4.0 – DISCUSSION

The present study investigated the effect of maternal exposure to supplemental folic acid diet (4 times higher than normal dietary levels), from preconception through lactation, on muscle function and metabolism in male and female CD-1 mice. Folic acid is a methyl donor that affects DNA methylation and is important in the relationship between maternal nutrition and fetal growth and development. A study examining the effects of maternal folic acid deficiency on skeletal muscle offspring in swine model found lipid-metabolism related genes were down-regulated through gene chip analysis (Li et al., 2013). However, to our knowledge this is the first study to examine the long-term effects of early life exposure to maternal folic acid supplementation on offspring muscle function and metabolism. The major results of this study were that exposure to folic acid 1) increased TPT in SOL and decreased ½ RT and –dP/d*t* in EDL in females with no contractile differences seen in males; 2) decreased HK activity in the SOL among females; and 3) increased HK activity and CS activity in the EDL and SOL, respectively, in males.

## 4.1. Males

### 4.1.1 Isometric Contractile Function

Maternal folic acid supplementation had no impact on male offspring SOL mass, length or contractile function compared to controls. Furthermore, there was no difference between offspring EDL mass, length, cross-sectional area (CSA) or contractile function between groups. However, male offspring SOL CSA, after exposure to maternal folate supplementation, was reduced by ~10% compared to controls. At 6 months of age, body weight did not differ between groups, thus, the smaller muscle CSA seen in the folic acid

group is likely not due to overall body weight. In contrast, the decrease in SOL CSA is nearly proportional to the overall smaller mass (~8%) seen in the folic acid group compared to controls. Although the difference of SOL mass was not statistically significant ($p=0.3$) between groups (potentially due to an underpowered sample size (ex. $n$=8-10/group; power = 0.52 versus a sufficient 0.80)), the differences seen in CSA may be attributed to this. A sample size of 19 per group would be needed for a power of 0.8.

Skeletal muscle mass is determined by the number and size of individual muscle fibres (Frontera & Ochala, 2015). The formation of new muscle fibres (myogenesis) occurs prenatally, whereas, postnatal skeletal muscle development occurs due to an increase in muscle fibre size (Yan et al., 2013). Thus, prenatal development of muscle fibres plays a critical role on the overall growth and development of adult skeletal muscle. DNA methylation, through methyl donors, such as folic acid, is thought to affect the commitment of mesenchymal stem cells to a myogenic lineage during the developmental process (Yan et al., 2013). Myogenic differentiation factor D (MyoD) plays a major role in regulating muscle differentiation. MyoD binds monocyte enhancer factor 2 (MEF2), a transcription factor expressed in oxidative tissues essential for oxidative metabolism, to metabolic genes increasing gene expression for myogenesis (McGee & Hargreaves, 2010; Zheng et al., 2010). Hypermethylation of histone residues suppresses the transcriptional activity of MyoD and MEF2, in turn inhibiting myogenesis (McGee & Hargreaves, 2010; Yahi et al., 2006). In contrast, hypomethylation of histone residues promotes transcriptional activity, initiating myogenesis (Yahi et al., 2006). Thus, the decrease in SOL CSA from maternal supplemental levels of folic acid may be explained by the inhibition of offspring skeletal muscle myogenesis during prenatal development

due the suppression of MyoD and MEF2. Furthermore, SOL may have been impacted by the high maternal levels of folic acid more than EDL because MEF2 is mainly expressed in oxidative tissues and SOL muscle expresses a higher percentage of oxidative type I fibres (~31%) compared to EDL (<1%) (Bloemberg & Quadrilatero, 2012).

Previous research has shown protein restriction throughout gestation reduced body weight and muscle mass in rat offspring at weaning (Desai et al., 1996). Moreover, protein restriction through cross-fostering immediately after birth showed a similar effect on offspring body weight and muscle mass at weaning (Desai et al., 1996), indicating early life exposure to nutritional insults can greatly affect postnatal skeletal muscle development. In rodents, changes in fibre type and myosin heavy chain (MHC) isoform composition of skeletal muscle occur during early postnatal development. In early life, fast twitch fibre types, such as EDL, progressively accumulate MHCII isoforms (IIa, IId/x, & IIb), whereas, slow twitch fibre types (SOL) progressively transform type IIa fibres into type I fibres (Schiaffino & Reggiani, 2011). Overall, type II muscle fibres are larger in size with a higher glycolytic capacity compared to oxidative type I fibres (Haizlip et al., 2015). Therefore, the differences in male offspring SOL CSA between the two groups may be attributed to a shift in fibre types toward smaller, oxidative type I fibres that occur during early life exposure to maternal supplemental folic acid. However, previous research has shown type II fibres are more susceptible to nutritional insults resulting in greater atrophy and fibre loss compared to slow twitch oxidative fibres (Matsakas & Patel, 2009). Thus, the 10% decrease in SOL CSA may be due to a loss of larger glycolytic type II fibres, rather than a switch in fibre types. Future research should examine other hindlimb muscles with variant fibre type compositions, such as plantaris

(<1% type I, 19% type IIa, 22% type IId/x, 47% type IIb) or tibialis anterior (1% type I, 18% type IIa, 45% type IId/x, 25% type IIb) muscles (Bloemberg & Quadrilatero, 2012), to confirm these findings.

### 4.1.2 Maximal Enzyme Activity

Maternal folic acid supplementation did little to alter the metabolic activity in SOL and EDL muscles among male offspring with the exception of CS and HK, respectively. The maximal activity of CS in SOL muscles increased by 24%, whereas, the maximal activity of EDL HK increased by 13% in male offspring exposed to a supplemental folic acid diet *in utero* and during early life compared to control. Previous studies have shown that hypermethylation results in decreased expression of energy producing genes, such as PGC-1α, CPT and HK, in turn decreasing lipid and carbohydrate metabolism in mice cardiac muscle (Czubryt et al., 2003; McGee & Hargreaves, 2010). Moreover, PGC-1α expression is silenced by DNA hypermethylation in skeletal muscle of female offspring exposed to a maternal low protein diet (Zeng et al., 2013). PGC-1α stimulates mitochondrial biogenesis, regulates skeletal muscle fibre type determination (promoting more oxidative type 1 fibres), and regulates carbohydrate and lipid metabolism, playing a significant role in the regulation of energy metabolism (Liang & Ward, 2006).

In contrast to previous research, the current study found offspring SOL CS maximal activity significantly increased when exposed to a maternal supplemental folic acid diet, which may be due to discrepancies in the literature. For example, although hypermethylation occurs with a maternal low protein diet and supplemental levels of folic acid, it is possible each nutrient may have varying effects on gene expression (Czubryt et

al., 2003; McGee & Hargreaves, 2010; Zeng et al., 2013). Additionally, responses to DNA methylation may vary between the different types of muscle tissues examined (ex. cardiac vs. skeletal muscle), as each muscle type has a unique function. Lastly, sex-specific epigenetic differences may occur since over 3,000 genes are differentially expressed between male and female skeletal muscle (Welle et al., 2008).

Additionally, maximal activity of EDL HK significantly increased in offspring exposed to a maternal supplemental folic acid diet. HK is a key protein involved in carbohydrate metabolism and glucose uptake. Zheng et al. (2012) found *in utero* maternal protein restriction results in methylation at the GLUT4 promotor region increasing GLUT4 expression, as well as, regulatory genes associated with carbohydrate metabolism (ex. MEF2) in skeletal muscle tissue of female offspring. GLUT4 is a transporter protein that plays a critical role in regulating glucose homeostasis through glucose uptake (Huang & Czech, 2007). Thus, the findings from the current study, in regards to EDL HK activity, are similar to that of Zheng et al. (2012), however, comparisons being made should be done with caution. As mentioned previously, discrepancies between the current studies findings and the literature are the gender of the rodent and the dietary interventions used. HK and CS are mediators at the beginning of the glycolytic and TCA cycle, respectively, therefore, changes in subsequent metabolic markers may not have been seen due to a potential homeostatic regulation of these pathways.

## 4.2. Females

### 4.2.1 Isometric Contractile Function

#### *i) SOL*

Female offspring SOL length was 3% less with maternal folic acid supplementation compared to controls. However, there was no differences seen between SOL mass or CSA. Additionally, exposure to maternal folic acid supplementation resulted in a ~15% increase in time-to-peak tension in SOL compared to control. Conversely, time-to-peak tension (TPT) was no longer statistically significant when twitch kinetics were normalized to force produced, however, rate of force development (+dP/d*t*) significantly decreased by 10% in the folic acid group compared to control (Table A.3).

The smaller length seen in the folic acid group is likely not attributed to biological significance, as mass and CSA is a larger indicator of force production (Matsakas & Patel, 2009). Thus, a potential explanation for the differences in contractile function could be due to a shift in fibre type composition or another mechanism, such as SERCA (discussed further in detail). Slow twitch fibres are characterized by a longer TPT and slower +dP/d*t* compared to fast twitch fibres (Schiaffino & Reggiani, 2011). Additionally, TPT and shortening velocity decreases in female rat SOL as type II fibres progressively transform into type I fibres during the first 3 months of life (Close, 1964). Thus, the findings from this study could be indicative of a switch to a greater proportion of type I oxidative fibres from type II glycolytic fibres in male and female SOL muscles.

#### *ii) EDL*

Maternal folic acid supplementation had no impact on offspring EDL mass, length or CSA compared to controls among females. In contrast, exposure to maternal folic acid supplementation resulted in a 25% increase in half-relaxation time (½ RT) and 28% increase in maximal rate of relaxation (-dP/d*t*) in offspring EDL compared to control. Furthermore, when normalizing the twitch kinetics to force produced, ½ RT was not statistically significant but statistical differences seen with -dP/d*t* persisted (Table A.3).

A shorter ½ RT and more rapid –dP/d*t* are indicative of a greater proportion of fast twitch glycolytic fibres (Schiaffino & Reggiani, 2011), thus, EDL appears to be impacted differently than the SOL among females. However, these differences could be explained due to the differential developmental rates of muscles during gestation as previous research has shown nutritional interventions affects all muscles differently (Matsakas & Patel, 2009). Moreover, type II fibres appear to be more susceptible to nutritional insults. Undernutrition results in greater fibre loss and atrophy of type II muscle fibres, however, it is unknown how overnutrition affects each fibre type (Matsakas & Patel, 2009). Given that mouse EDL is predominately composed of type II fibres (~90%), it is possible the high levels of maternal folic acid may have impacted type II fibres in EDL, in turn, impacting the contractile properties of the muscle.

Additionally, there are multiple isoforms of type II muscle fibres in mice (IIa, IIx/d, IIb). EDL contains more type IId/x (24%) and type IIb (56%) fibres, whereas, SOL is predominately type IIa (49%) (Bloemberg & Quadrilatero, 2012), thus, the differential response to maternal supplemental folic acid between EDL and SOL may be explained by the differences of type II isoform composition. As mentioned previously, future research

should examine additional mice hindlimb muscles with different fibre type compositions (ex. plantaris and tibialis anterior) to further understand these findings.

#### 4.2.2 Maximal Enzyme Activity

***i) SOL***

Maternal folic acid supplementation did little to alter the metabolic activity in SOL muscles among female offspring with the exception of HK. The maximal activity of HK in offspring SOL muscles that were exposed to a supplemental folic acid diet *in utero* and during early life exposure decreased by 12% compared to control. Previous studies have shown that hypermethylation results in decreased expression of PGC-1α, CPT and HK, in turn decreasing lipid and carbohydrate metabolism in mice cardiac muscle (Czubryt et al., 2003; McGee & Hargreaves, 2010). In contrast, a study found *in utero* maternal protein restriction resulted in increased GLUT4 expression, as well as, regulatory genes associated with carbohydrate metabolism in skeletal muscle tissue of female offspring (Zheng et al., 2012). Thus, there are discrepancies in the literature in regards to hypermethylation and its effect on metabolic markers in muscle tissue, due to the nutritional intervention used and the type of muscle tissue examined.

TPT and +dP/d*t* increased in SOL muscles among females, indicating a potential shift in fibre type composition to more oxidative slow twitch fibres, thus the decrease in maximal HK activity could be due to a decrease in fast twitch glycolytic muscle fibres, as HK plays a key role in glycolysis. However, metabolic markers of lipid and oxidative metabolism showed no significant influence from supplemental levels of folic acid, therefore, it is difficult to determine the biological significance of these findings with no changes in other skeletal muscle metabolic markers.

***ii) EDL***

Although there were differences seen in ½ RT and -dP/d*t* between groups in the EDL, maternal folic acid supplementation did not alter the metabolic activity in offspring EDL muscles among females. This may suggest alternate pathways are being utilized for the changes seen in the contractile data, such as sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase (SERCA). SERCA's main function is to facilitate muscle relaxation by pumping calcium ions from the cytosol into the SR lumen which re-establishes calcium homeostasis necessary for muscle contraction (Periasamy & Kalyanasunaram, 2007). Moreover, SERCA's function can be characterized by its activity and efficiency. SERCA activity is measured by the amount of calcium translocated per unit of time, whereas, SERCA efficiency is measured by the ratio of calcium ions transferred per hydrolyzed ATP (Lervik et al, 2012; Periasamy & Kalyanasunaram, 2007). Although SERCA can bind up to two calcium ions per ATP molecule, previous studies have calculated SERCA's efficiency to be only 12% (Lervik et al, 2012), since the ratio of calcium ions transferred per hydrolyzed ATP, varies under physiological conditions. This is a result of calcium slippage (calcium released prematurely) or calcium leakage due to the concentration of calcium in the SR lumen being too high (Reis et al, 2001; Yu & Inesi, 1995).

In adult skeletal muscle, two main isoforms of SERCA are expressed that are muscle and fibre type specific: SERCA1a and SERCA2a (Periasamy & Kalyanasundaram, 2007). SERCA1a is expressed with type II MHC isoforms (IIa, IId/x, and IIb) in fast twitch muscles (ex. EDL), whereas, SERCA2a is predominately found with type I MHC isoforms in slow twitch muscles, such as the SOL (Fajardo et al., 2013;

Periasamy & Kalyanasundaram, 2007). Fast twitch muscle fibres produce quick, repetitive contraction cycles, thus, SERCA1a is present at a greater density and displays a higher ATPase activity, compared to SERCA2a, to ensure calcium is sequestered rapidly (Murphy et al., 2009; Szentesi et al, 2001). In contrast, SERCA2a displays decreased calcium leakage compared to SERCA1a in fatigued skeletal muscles, which helps to maintain fatigue resistance in slow twitch muscle fibres (Macdonald & Stephenson, 2006). Furthermore, similar to skeletal muscle fibres, SERCA isoforms are highly plastic and can switch during development (Periasamy & Kalyanasundaram, 2007). However, the regulatory processes initiating SERCA isoform switches is not understood, thus, future research should aim to quantify SERCA and measure SERCA maximal activity in mice offspring skeletal muscle exposed to a maternal supplemental folic acid diet to further explain these findings.

### 4.3. Conclusion

In summary, this study was the first to examine the effects of a maternal supplemental folic acid diet, from preconception through lactation, on offspring skeletal muscle function and metabolism. Our findings suggest sex-specific differences of offspring skeletal muscle contractile function and metabolism to a maternal supplemented folic acid diet may occur. Contractile function was affected in female, but not male offspring, which may be attributed to shifts in fibre types. Furthermore, EDL and SOL muscles were affected differently in female offspring. SOL appeared to become even more slow twitch, characterized by a longer TPT and +dP/d*t*, whereas, the opposite occurred in the EDL, becoming more fast twitch, characterized by a more rapid ½ RT and –dP/d*t*. Additionally, carbohydrate metabolic markers (HK) significantly decreased in the SOL in the folic acid group, whereas, there were no significant differences seen in metabolic markers in the EDL among females. Moreover, in the males, oxidative markers (CS) increased in the SOL, whereas, carbohydrate metabolic markers (HK) increased in the EDL in the folic acid groups. Therefore, future research is necessary to understand the mechanisms associated with maternal supplemental levels of folic acid and how it affects male and female offspring skeletal muscle at young adulthood.

### 4.4. Implications of Research

In 1998, folic acid fortification of all white flour, enriched pasta and cornmeal products became mandatory in Canada to reduce the risk of neural tube defects at birth. Additionally, Health Canada recommends all women take a daily prenatal folic acid supplement containing 400 $\mu$g of folic acid, in addition to their dietary folate intake, three months prior to pregnancy and throughout the pregnancy (De Wals et al., 2007; Health

Canada, 2009; Ray, 2004). Moreover, the Society of Obstetricians and Gynaecologists of Canada recommends women with an increased risk for neural tube defects take a daily prenatal folic acid supplement containing 1000 $\mu$g of folic acid (SOGC Clinical Practice Guideline, 2015). The current RDA for pregnant and lactating woman is 600 $\mu$g/day and 500 $\mu$g/day, respectively (Institute of Medicine, 1998). Thus, mandatory folic acid fortification and prevalent supplemental use due to Health Canada's current recommendations for women of child-bearing age, results in pregnant women being exposed to 4 times higher folic acid during pregnancy.

Folic acid is a methyl donor that affects DNA expression and is important in the relationship between maternal nutrition and fetal growth and development, therefore, high maternal folate concentrations may affect the long-term health outcomes of offspring, such as skeletal muscle function and metabolism. Based on the findings of this study, skeletal muscle function and metabolism is affected by *in utero* and early exposure to supplemental levels of folic acid, with sex-specific differences. This research will provide further insight into the impact of high maternal folate concentrations on the long-term health and development of exposed offspring, which is important due to the lack of evidence on offspring skeletal muscle function and metabolism in the literature and the increased levels of maternal consumption. A previous study has shown high multivitamin intake (10-fold increase in folic acid) during pregnancy results in increased expression of an obesogenic phenotype in male and female rat offspring (Szeto et al., 2009), thus, although the findings from this study do not show detrimental implications on offspring skeletal muscle function and metabolism from a maternal supplemental folic acid diet in

rodents at adulthood, future studies in human populations are necessary to confirm high levels of maternal folic acid intake are not detrimental to the offspring.

## 4.5. Strengths and Limitations

### 4.5.1 Strengths

This research is novel in that, to our knowledge, it is the first to examine the impact of a maternal folic acid diet on offspring skeletal muscle function and metabolism into adulthood. A previous study has looked at the effects of maternal folic acid deficiency on skeletal muscle offspring in a swine model (Li et al., 2013), however, the long-term effects of offspring skeletal muscle into adulthood, from exposure to supplemental levels of folic acid *in utero* or in early life has not been studied. Due to the prevalent use of supplements and mandatory folic acid fortification in Canada, it is important to understand the long-term health effects of folic acid on various tissues in adulthood. Moreover, this research is the first to investigate a wide array of maximal enzyme activities in skeletal muscle tissue representing key enzymes of carbohydrate, lipid and oxidative metabolism as a result of a maternal supplemental folic acid diet. With this information we are able to examine a variety of metabolic pathways that may be altered in offspring due to high maternal levels of folic acid.

There are several strengths associated with the design of the study, such as the duration of the study. Previous research in bone has shown CD-1 mice reach young adulthood becoming fully developed at 4 months of age (Ward et al., 2007). Additionally, skeletal muscle tissue has fully developed in rodents by 3 months of age (Close, 1964). Thus, 6 months of age ensures the mice are fully developed but are not yet showing “aging” effects (Brooks & Faulkner, 1988). It is important species are fully developed,

surpassing the growth and developmental stages, to truly detect a nutritional programming effect. Measuring the outcomes at 6 months of age allowed for examination of the sustained effects of a maternal supplemental diet on offspring skeletal muscle into adulthood. Another strength of the study design, was that only one offspring per litter, per gender was reported for each outcome to ensure the results were not biased due to over-representation of each litter ("litter effect"; Wainwright, 1998). It is expected individual offspring within each litter are more similar to one another then offspring from another litter, thus, studying only one offspring per litter removes any potential bias and appropriately represents each dam randomized to the control or folic acid diets.

A major strength of this study was the chosen diets. The folic acid diet was identical to the control diet (2 mg folic acid/kg diet) except it contained a higher level of folic acid (8 mg folic acid/kg diet). The amount of folic acid in the control diet was to represent the basal dietary requirements for growing, pregnant and/or lactating rodents. The purpose of increasing folic acid in the diet to 4 times higher was to mimic the amount of folic acid pregnant and lactating mothers consume through folic acid fortification and prenatal supplement use (Health Canada, 2009). Additionally, the diets were modified with alcohol-extracted 'vitamin-free' casein to ensure any naturally occurring folic acid present within the protein was removed. Overall, this guaranteed that any observed effect on offspring skeletal muscle was truly attributed to increased levels of folic acid consumption, and not due to the intake of additional nutrients.

### 4.5.2 Limitations

A limitation of the study was the small sample size. The primary outcome of this study was skeletal muscle contraction. The current study was underpowered (~0.5 vs a sufficient 0.8). In order to obtain a power of 0.8 for the primary outcome of contractile function, 19 mice per group were necessary, however, due to the logistics of the current study, this was not feasible. A smaller sample size can show significant differences that are not seen with larger sample sizes of sufficient power. Thus, the results from this study should be interpreted with caution.

Another limitation of the study was that the mice (dams) were housed 4-5 per cage up until pregnancy was confirmed. From a social and ethical perspective this is considered a strength in order to enhance the animals overall well-being (Olfert et al., 1993). Housing multiple animals together may result in less accurate food intake measurements for each individual dam, however, there were no differences seen in body weight between groups suggesting this did not contribute to differences seen in offspring skeletal muscle function and metabolism at young adulthood. Additionally, maternal and offspring blood concentrations of folate were not collected during pregnancy or lactation to determine folate status. Previous studies have found women who consume supplemental levels of folic acid (1000 $\mu$g) during pregnancy and lactation, in addition to their dietary intake, have significantly higher blood folate concentrations during pregnancy and at birth, as well as, higher milk folate concentrations postpartum compared to nonusers (Mackey & Picciano, 1999; Plumptre et al., 2015). Moreover, umbilical cord blood from woman taking folic acid supplements during late pregnancy resulted in significantly higher blood folate concentrations compared to those who were

not (Plumptre et al., 2015). Thus, knowing the levels of folate concentrations in maternal circulation, *in utero* and during early life exposure, may help to understand the levels of folic acid associated with long-term skeletal muscle health outcomes of offspring. Future research should aim to replicate this study quantifying blood concentrations of folate in mothers during pregnancy and lactation and offspring at birth and throughout suckling.

Secondly, fibre typing was not measured directly. It is known skeletal muscle is a highly adaptable tissue that responds to environmental and physiological stimuli, such as nutritional status, by shifting fibre type composition (Matsakas & Patel, 2009). Therefore, it can be speculated the effects seen from a maternal supplemental folic acid diet on offspring skeletal muscle contractile function could be attributed to shifts in fibre types. Additionally, slack tests or fatigue indices were not conducted which would have been a good surrogate to determine potential shifts in fibre types, as faster fibre types are associated with faster shortening velocities (Edman, 1979).

Lastly, no mechanisms were examined. DNA methylation, through methyl donors such as folic acid, influences many genes critical to offspring skeletal muscle development. The mechanisms underlying methyl supplementation and gene modification have yet to be fully elucidated in response to maternal diet in skeletal muscle. Understanding the potential pathways of DNA methylation on skeletal muscle tissue could help determine whether changes in contractile function was due to signalling of metabolic pathways or as a result of the utilization of different pathways, such as $Ca^{2+}$ signalling via SERCA.

## 4.6. Future Research

Future research should aim to identify the underlying biological pathways and mechanisms affected by methyl-donor supplementation, such as folic acid, during pregnancy and lactation on offspring skeletal muscle tissue. Previous research has found folate deficiency during pregnancy and lactation resulted in significant down regulation of carbohydrate and lipid metabolism genes through the peroxisome proliferator-activated receptor (PPAR) signalling pathway of skeletal muscle in male swine offspring (Li et al., 2013). Additionally, maternal methyl-donor deficiency has been shown to impair fatty acid oxidation through decreased expression of PPAR-α and PGC-1α of cardiac tissue in rat offspring (Guéant et al., 2013). In contrast, overexpression of PPAR-α has been shown to be involved in increased lipid metabolism and reduced glucose metabolism, whereas, PGC-1α stimulates mitochondrial biogenesis and regulates muscle fibre type determination (promoting oxidative type I fibres) and fatty acid oxidation in rodent skeletal muscle fibres (Burri et al., 2010; Guéant et al., 2013; Matsakas & Patel, 2009). Thus, in elucidating these mechanisms, it is essential to understand how skeletal muscle tissue is altered by *in utero* and early life exposure to maternal supplemental folic acid.

Additionally, SERCA is an alternate pathway that may be utilized to explain the differences seen in the contractile data. SERCA's main function is to facilitate muscle relaxation by pumping calcium ions from the cytosol into the SR lumen, which is necessary for muscle contraction (Periasamy & Kalyanasunaram, 2007). In adult skeletal muscle, two main isoforms of SERCA are expressed that are fibre type specific: SERCA1a and SERCA2a (Periasamy & Kalyanasundaram, 2007). Although the structure is similar between the two isoforms, they are functionally different due to their co-

expression with different MHC isoforms. For example, SERCA1a is expressed with fast type II MHC isoforms (IIa, IId/x, and IIb), whereas, SERCA2a is predominately found with slow type I MHC isoforms (Fajardo et al., 2013; Periasamy & Kalyanasundaram, 2007). Additionally, SERCA1a is typically present at greater densities displaying higher ATPase activity, compared to SERCA2a. Thus, future research should aim to quantify SERCA via Western blots and measure SERCA maximal activity in mice offspring skeletal muscle exposed to a maternal supplemental folic acid diet to further explain these findings.

Also, future research should characterize skeletal muscle fibre types in offspring exposed to a maternal supplemental folic acid diet compared to control-fed rodents *in utero* and during early life exposure. Skeletal muscle displays high plasticity due to the adaptive changes of muscle fibres in response to a variety of stimuli, including diet (Matsakas & Patel, 2009). Thus, methyl donors, such as folic acid, may alter the fibre type composition of various skeletal muscle tissue, consequently affecting contractile function and metabolism. Additionally, a variety of muscle types, such as soleus (predominately type I fibres), extensor digitorum longus (predominately type II fibres) and plantaris (mix of type I and II fibres), should be characterized to determine whether specific muscle types are affected more than others. Western blot analysis for the contractile protein MHC is a technique that quantifies MHC isoform expression, in turn, defining fibre type composition of skeletal muscle tissue (Galpin et al., 2012; Quinn et al., 1995). Additionally, multicolour immunofluorescence analysis is a more precise technique for determining muscle fibre type composition as it allows for quantification of multiple MHC isoforms simultaneously (Bloemberg & Quadrilatero, 2012).

It is documented there are over 3,000 genes that are differentially expressed between male and female skeletal muscles, suggesting gender differences may be seen due to hypermethylation (Welle et al., 2008). Additionally, male and female mice express variations in fibre type composition within the same muscle. For example, males express 58% type IIa muscle fibres, whereas, females express only 36% in mice SOL (Haizlip et al., 2015). These differences are also seen in the plantaris (IIa - 16%, males; 37%, females) and tibialis anterior (IIa - 39%, males; 25%, females) (Haizlip et al., 2015). Moreover, MHC isoform composition is highly correlated with contractile function, especially the rate of force development and rate of relaxation, as well as, the enzymatic makeup of skeletal muscle tissue (Haizlip et al., 2015). Therefore, future research should examine sex-specific differences of offspring skeletal muscle fibre type composition, contractile function, and metabolism to a maternal supplemented folic acid diet.

Lastly, future research should aim to study the effects of a maternal folic acid supplemented diet on offspring skeletal muscle tissue in humans. The results from this study indicate high levels of maternal folic acid does not appear to have significant implications on offspring skeletal muscle function and metabolism at young adulthood in rodents, however, the results from this study cannot be directly applied to a human population. Although human and rodent skeletal muscle tissue have similar characteristics, future work is necessary in human populations to confirm these findings.

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## APPENDIX A: Supplementary Results

**Table A.1** Dietary Composition of Control and Folic Acid Supplement Diets.

| **Ingredient** | **CON** | **FA** |
|---|---|---|
| *Formula (g/kg)* | | |
| Folic Acid | 0.002 | 0.008 |
| Casein, "Vitamin-Free" Test | 200 | 200 |
| L-Cystine | 3 | 3 |
| Corn Starch | 397.5 | 397.5 |
| Maltodextrin | 132 | 132 |
| Sucrose | 100 | 100 |
| Soybean Oil | 70 | 70 |
| Cellulose | 50 | 50 |
| Mineral Mix | 53 | 53 |
| Vitamin Mix | 20 | 20 |
| Choline Bitartrate | 3.5 | 3.5 |
| TBHQ, antioxidant | 0.014 | 0.014 |
| *Energy Contribution (% kcal)* | | |
| Protein | 19.4 | 19.4 |
| Carbohydrate | 63.8 | 63.8 |
| Fat | 16.7 | 16.7 |
| *Relative Quantity (% by weight)* | | |
| Protein | 18.3 | 18.3 |
| Carbohydrate | 60.1 | 60.1 |
| Fat | 7.0 | 7.0 |
| *Total Energy (kcal/g)* | 3.8 | 3.8 |

CON: control modified AIN93G diet (TD.06706; Harlan Teklad); FA: folic acid supplemented AIN93G diet (TD.140396; Harlan Teklad); TBHQ: tertiary butylhydroquinone

**Table A.2.** Effect of a supplemental folic acid diet on male offspring CD-1 mice soleus (SOL) and extensor digitorum longus (EDL) muscle isometric contractile function normalized to force.

| | **CON** | **FA** |
|---|---|---|
| ***SOL*** | | |
| TPT/force (ms/mN) | 1.28 ± 0.12 | 1.35 ± 0.13 |
| ½ RT/force (ms/mN) | 1.83 ± 0.32 | 1.68 ± 0.21 |
| +dP/d*t*/force | 0.055 ± 0.001 | 0.054 ± 0.002 |
| -dP/d*t*/force | 0.016 ± 0.002 | 0.014 ± 0.002 |
| ***EDL*** | | |
| TPT/force (ms/mN) | 0.22 ± 0.02 | 0.20 ± 0.01 |
| ½ RT/force (ms/mN) | 0.25 ± 0.03 | 0.22 ± 0.02 |
| +dP/d*t*/force | 0.087 ± 0.001 | 0.087 ± 0.001 |
| -dP/d*t*/force | 0.040 ± 0.002 | 0.041 ± 0.002 |

Values are mean ± SEM. CON, n=9; FA, n=8; * denotes significance CON; CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet; SOL, soleus; EDL, extensor digitorum longus; TPT, time-to-peak tension; ½ RT, half relaxation time; +dP/d*t*, rate of contraction; -dP/d*t*, rate of relaxation.

**Table A.3.** Effect of a supplemental folic acid diet on male offspring CD-1 female soleus (SOL) and extensor digitorum longus (EDL) muscle isometric contractile function normalized to force.

| | CON | FA |
|---|---|---|
| ***SOL*** | | |
| TPT/force (ms/mN) | 1.34 ± 0.10 | 1.71 ± 0.21 |
| ½ RT/force (ms/mN) | 1.51 ± 0.10 | 1.92 ± 0.25 |
| +dP/d*t*/force | 0.053 ± 0.001 | 0.048 ± 0.001* |
| -dP/d*t*/force | 0.014 ± 0.001 | 0.015 ± 0.001 |
| ***EDL*** | | |
| TPT/force (ms/mN) | 0.25 ± 0.01 | 0.28 ± 0.03 |
| ½ RT/force (ms/mN) | 0.33 ± 0.03 | 0.28 ± 0.03 |
| +dP/d*t*/force | 0.086 ± 0.001 | 0.085 ± 0.002 |
| -dP/d*t*/force | 0.031 ± 0.001 | 0.043 ± 0.003* |

Values are mean ± SEM. CON, n=9; FA, n=8; * denotes significance CON; CON, 2mg folic acid/kg diet; FA, 8mg folic acid/kg diet; SOL, soleus; EDL, extensor digitorum longus; TPT, time-to-peak tension; ½ RT, half relaxation time; +dP/d*t*, rate of contraction; -dP/d*t*, rate of relaxation.

## APPENDIX B: Laboratory Procedures

### *Part I: Sample Preparation*

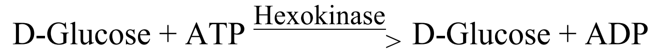
*Homogenization Buffer:*

- 50 mM Tris-HCl
- pH 7.8
- *store at -4°C*

*Homogenization Protocol:*

1. Weigh muscle tissue sample and add it to pre-chilled 2 mL glass homogenizer.
2. Add homogenization buffer to achieve a 20:1 (w/v) dilution.
3. Manually homogenize on ice (~5 min).
4. Transfer sample to eppendorf.

***Part II: Hexokinase Assay (EC: 2.7.1.1 // NADPH ε: 6.22)***



| | Volume | Substance | Stock | Final |
|---|---|---|---|---|
| | 765 µL | Triethanolamine-HCl (pH 7.6) + EDTA + $MgCl_2$ | 50 mM buffer + 5.32 mM EDTA + 7.98 mM $MgCl_2$ | 5 mM EDTA + 7.5 mM $MgCl_2$ |
| | 30 µL | NADP (in fresh buffer) | 14 mM | 0.42 mM |
| | 125 µL | ATP (in fresh buffer) | 20 mM | 2.5 mM |
| | 10 µL | Glucose-6-phosphate dehydrogenase (in distilled water) | 1 mg/mL | 10 µg (~1.67 U/mL) |
| | 50 µL | Sample | | |
| **Initiate Reaction** | 20 µL | D(+)Glucose (in fresh buffer) | 111 mM | 2.22 mM |
| **FINAL** | 1 mL | | | |

*Buffer:*
- 50 mM Triethanolamine-HCl
- 5.32 mM EDTA
- 9.80 mM $MgCl_2$
- pH 7.6
- *store at -4°C*

*Protocol:*
1. Prepare buffer solution.
2. Prepare and vortex reagent solutions:
    a. 0.5 mL of 14 mM NADP in buffer
    b. 2 mL of 20 mM ATP in buffer
    c. 2 mL of 111 mM D(+)Glucose in buffer
    d. 1 mg/mL of Glucose-6-phosphate dehydrogenase in distilled water
    ** Note: Glucose-6-phosphate dehydrogenase dissolved in deionized water can be aliquoted and stored at -20°C for 2 months*
3. Pipette 1 mL of buffer into a 1 mL cuvette.
4. Wipe down cuvette with Kim wipe and place into the spectrophotometer to reference absorbance at 340 nm.

5. Set up reaction kinetics program:
    a. Temperature: 25°C
    b. Wavelength: 340 nm
    c. Time: 15 min
6. Pipette reagents into 1 mL cuvette in the following order:
    a. 765 µL buffer
    b. 30 µL NADP
    c. 125 µL ATP
    d. 10 µL Glucose-6-phosphate dehydrogenase
    e. 50 µL sample
7. Invert, wipe down cuvette with Kim wipe and place into spectrophotometer.
8. Run the assay for ~2 minutes to get a background reading.
9. After 2 minutes, add 20 µL of D(+)Glucose to initiate the reaction, invert and wipe down with Kim wipe before placing back into spectrophotometer
    * *Ensure this is done quickly as this starts the reaction*
10. Record the slope of the background reading (between 0-2 minutes).
11. Record the linear portion of the slope after the initiation of the reaction.

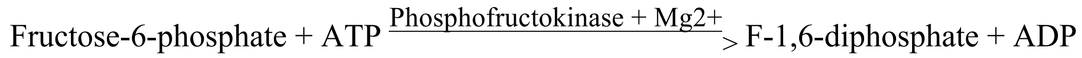
*References:*

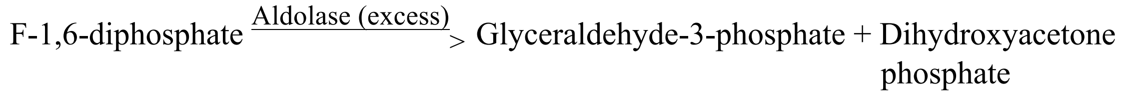
Bass, A., Brdiczka, D., Eyer, P., Hofer, S., & Pette, D. (1969). Metabolic differentiation of distinct muscle types at the level of enzymatic organization. *European Journal of Biochemistry, 10*(2), 198-206.

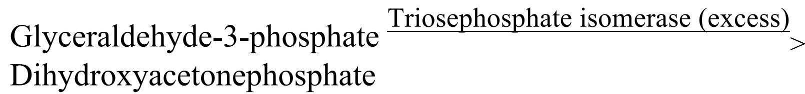
Thompson, A., & Cooney, G. (2000). Acyl-CoA inhibition of hexokinase in rat and human skeletal muscle is a potential mechanism of lipid-induced insulin resistance. *Diabetes, 49*(11), 1761-1765.

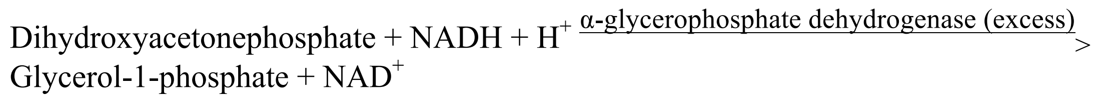
***Part III: Phosphofructokinase Assay (EC: 2.7.1.11 // NADH ε: 6.22)***

Coupled Enzyme Assay System


Fructose-6-phosphate + ATP —Phosphofructokinase + Mg2+→ F-1,6-diphosphate + ADP


F-1,6-diphosphate —Aldolase (excess)→ Glyceraldehyde-3-phosphate + Dihydroxyacetone phosphate


Glyceraldehyde-3-phosphate —Triosephosphate isomerase (excess)→ Dihydroxyacetonephosphate


Dihydroxyacetonephosphate + NADH + $H^+$ —α-glycerophosphate dehydrogenase (excess)→ Glycerol-1-phosphate + $NAD^+$

| | **Volume** | **Substance** | **Stock** | **Final** |
|---|---|---|---|---|
| | 769 µL | Tris-Base (pH 8.0) + $MgCl_2$ + $K_2HPO_4$ | 50 mM buffer + 2.08 mM $MgCl_2$ + 10.38 mM $K_2HPO_4$ | 2 mM $MgCl_2$ + 10 mM $K_2HPO_4$ |
| | 25 µL | NADH (in fresh buffer) | 15 mM | 0.375 mM |
| | 100 µL | 5'AMP (in fresh buffer) | 10 mM | 1 mM |
| | 20 µL | α-Glycerophosphate Dehydrogenase | 100 U/mL | 2 U/mL |
| | 2 µL | Triosephosphate isomerase | 31 201.8 U/mL | 62 U/mL |
| | 4.2 µL | Aldolase | 384 U/mL | 1.61 U/mL |
| | 10 µL | Sample | | |
| | 20 µL | ATP (in fresh buffer) | 50 mM | 1 mM |
| **Initiate Reaction** | 50 µL | Fructose-6-phosphate (in fresh buffer) | 50 mM | 2.5 mM |
| **FINAL** | 1.0002 mL | | | |

*Buffer:*

- 50 mM Tris-Base
- 2.62 mM $MgCl_2$
- 13.09 mM $K_2HPO_4$
- pH 8.0
- *store at -4°C*

*Protocol:*

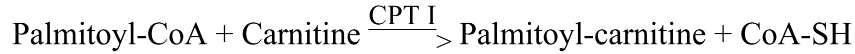
1. Prepare buffer solution.
2. Prepare and vortex reagent solutions:
    a. 0.5 mL of 15 mM NADH in buffer
    b. 2 mL of 10 mM AMP in buffer
    c. 0.5 mL of 50 mM ATP in buffer
    d. 0.5 mL of 50 mM Fructose-6-phosphate in buffer
3. Pipette 1 mL of buffer into a 1 mL cuvette.
4. Wipe down cuvette with Kim wipe and place into the spectrophotometer to reference absorbance at 340 nm.
5. Set up reaction kinetics program:
    a. Temperature: 25°C
    b. Wavelength: 340 nm
    c. Time: 15 min
6. Pipette reagents into 1 mL cuvette in the following order:
    a. 769 µL buffer
    b. 25 µL NADP
    c. 120 µL AMP
    d. 20 µL α-Glycerophosphate dehydrogenase
    e. 2 µL Triosephosphate isomerase
    f. 4.2 µL Aldolase
    g. 10 µL sample
    h. 20 µL ATP

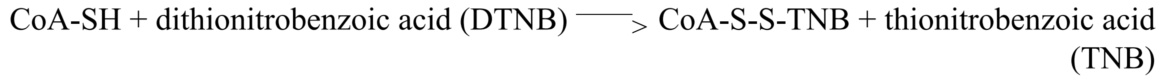
    * *Add ATP immediately following the addition of the sample to stabilize PFK*
    * *α-Glycerophosphate dehydrogenase, Triosephosphate isomerase, and aldolase were added in excess to show maximal enzymatic activity of PFK*
    * *$Mg^{2+}$ concentration was kept higher than ATP concentration to form Mg-ATP complex and decrease the presence of ATP molecules that may inhibit PFK activity*
7. Invert, wipe down cuvette with Kim wipe and place into spectrophotometer.
8. Run the assay for ~2 minutes to get a background reading.
9. After 2 minutes, add 50 µL of fructose-6-phosphate to initiate the reaction, invert and wipe down with Kim wipe before placing back into spectrophotometer
    * *Ensure this is done quickly as this starts the reaction*
10. Record the slope of the background reading (between 0-2 minutes).
11. Record the entire slope after the initiation of the reaction.

*Reference:*

Passonneau, J., & Lowry, O. (1993). A collection of enzyme assays. In *Enzymatic Analysis.* Humana Press, New York), pp. 292-293.

***Part IV: Carnitine Palmitoyltransferase Assay (EC: 2.3.1.21 // TNB ε: 13.6)***


Palmitoyl-CoA + Carnitine $\xrightarrow{\text{CPT I}}$ Palmitoyl-carnitine + CoA-SH


CoA-SH + dithionitrobenzoic acid (DTNB) $\longrightarrow$ CoA-S-S-TNB + thionitrobenzoic acid (TNB)

| | Volume | Substance | Stock | Final |
|---|---|---|---|---|
| | 165 µL | Tris-HCl (pH 8.0) + EDTA | 116 mM buffer + 1.28 mM EDTA | 1.1 mM EDTA |
| | 25 µL | DTNB (in fresh buffer) | 2.4 mM | 0.24 mM |
| | 20 µL | Palmitoyl-CoA (in water) | 1 mM | 0.08 mM |
| | 25 µL | L-Carnitine (in fresh buffer) | 11 mM | 1.1 mM |
| **Initiate Reaction** | 15 µL | Sample | | |
| **FINAL** | 250 µL | | | |

*Buffer:*
- 116 mM Tris-HCl
- 1.28 mM EDTA
- pH 8.0
- *store at -4°C*

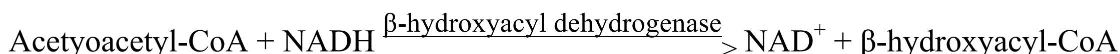
*Protocol:*
1. Prepare buffer solution.
2. Prepare and vortex reagent solutions:
   a. 4 mL of 2.4 mM DTNB in buffer
   b. 2 mL of 11 mM L-Carnitine in buffer
   c. 30 µL of 1 mM Palmitoyl-CoA in distilled water
      ** Note: Palmitoyl-CoA dissolved in deionized water can be aliquoted and stored at -20°C for 2 months*
3. Pipette 250 mL of buffer into a 250 mL cuvette.
4. Wipe down cuvette with Kim wipe and place into the spectrophotometer to reference absorbance 412.
5. Set up reaction kinetics program:
   a. Temperature: 25°C
   b. Wavelength: 412 nm
   c. Time: 15 min
6. Pipette 40 µL of sample into an Eppendorf tube.

7. Freeze-thaw the sample in liquid nitrogen 3 times to ensure rupture of the mitochondria.
   ** Final thaw is done immediately prior to adding to reaction*
8. Pipette reagents into 250 mL cuvette (Cuvette A) in the following order:
   a. 165 µL buffer
   b. 25 µL DTNB
   c. 20 µL Palmitoyl-CoA
   d. 25 µL L-Carnitine
9. Pipette reagents into 250 mL cuvette (Cuvette B) in the following order:
   a. 190 µL buffer *(L-carnitine is replaced by buffer)*
   b. 25 µL DTNB
   c. 20 µL Palmitoyl-CoA
10. Invert, wipe down cuvettes with Kim wipe and place into spectrophotometer.
11. Run the assay for ~2 minutes to get a background reading before initiating the reaction.
12. After 2 minutes, add 15 µL of sample into each cuvette (A & B) to initiate the reactions, invert and wipe down with Kim wipe before placing back into spectrophotometer
    ** Ensure this is done quickly as this starts the reaction*
13. Record the slope of the background reading (between 0-2 minutes) for cuvette A and B.
14. Record the first 2 minutes of the slope after the initiation of the reaction for cuvette A and B.

*Reference:*

Bieber, L., Abraham, T., & Helmrath, T. (1972). A rapid spectrophotometric assay for carnitine palmitoyltransferase. *Analytical Biochemistry, 50*(2), 509-518.

***Part V: 3(β)-Hydroxyacyl-CoA Dehydrogenase (EC: 1.1.1.35 // NADH ε: 6.22)***




| | Volume | Substance | Stock | Final |
|---|---|---|---|---|
| | 215 µL | Triethanolamine-HCl (pH 7.0) + EDTA | 120 mM buffer + 5.55 mM EDTA | 5 mM EDTA |
| | 10 µL | NADH (in fresh buffer) | 5.625 mM | 0.225 mM |
| | 20 µL | Sample | | |
| **Initiate Reaction** | 5 µL | Acetoacetyl-CoA (in water) | 5 mM | 0.1 mM |
| **FINAL** | 250 µL | | | |

*Buffer:*

- 120 mM Triethanolamine-HCl
- 5.55 mM EDTA
- pH 7.0
- *store at -4°C*

*Protocol:*

1. Prepare buffer solution.
2. Prepare and vortex reagent solutions:
    a. 2 mL of 5.625 mM NADH in buffer
    b. 20 µL of 5 mM Acetoacetyl-CoA in distilled water
    ** Note: Acetoacetyl-CoA dissolved in deionized water can be aliquoted and stored at -20°C for 3 months*
3. Pipette 250 mL of buffer into a 250 mL cuvette.
4. Wipe down cuvette with Kim wipe and place into the spectrophotometer to reference absorbance at 340 nm.
5. Set up reaction kinetics program:
    a. Temperature: 25°C
    b. Wavelength: 340 nm
    c. Time: 15 min
6. Pipette reagents into 250 mL cuvette in the following order:
    a. 215 µL buffer
    b. 10 µL NADH
    c. 20 uL sample
7. Invert, wipe down cuvette with Kim wipe and place into spectrophotometer.
8. Run the assay for ~2 minutes to get a background reading before initiating the reaction.

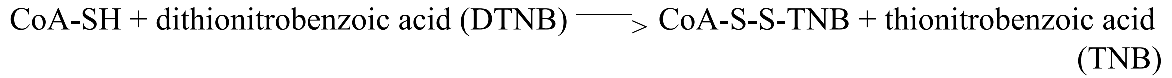
9. After 2 minutes, add 5 μL of acetyoacetyl-CoA to initiate the reaction, invert and wipe down with Kim wipe before placing back into spectrophotometer.
   * *Ensure this is done quickly as this starts the reaction*
10. Record the slope of the background reading (between 0-2 minutes).
11. Record the first 45 seconds of the slope after the initiation of the reaction.

*Reference:*

Bass, A., Brdiczka, D., Eyer, P., Hofer, S., & Pette, D. (1969). Metabolic differentiation of distinct muscle types at the level of enzymatic organization. *European Journal of Biochemistry, 10*(2), 198-206.

***Part VI: Citrate Synthase Assay (EC: 2.3.3.1 // DTNB ε: 13.6)***

Oxaloacetate + Acetyl-CoA —Citrate Synthase→ Citrate + CoA-SH



CoA-SH + dithionitrobenzoic acid (DTNB) ——→ CoA-S-S-TNB + thionitrobenzoic acid (TNB)

| | Volume | Substance | Stock | Final |
|---|---|---|---|---|
| | 200 µL | $dH_2O$ | | |
| | 10 µL | DTNB (in buffer) | 2.5 mM | 0.1 mM |
| | 12.5 µL | Acetyl-CoA (in water) | 10 mM | 0.5 mM |
| | 12.5 µL | Triton X-100 (in water) | 10% (w/v) | 0.5% |
| | 2.5 µL | Sample | | |
| **Initiate Reaction** | 12.5 µL | Oxaloacetate (in buffer) | 10 mM | 0.5 mM |
| **FINAL** | 250 µL | | | |

*DTNB Buffer:*
- 1.0 M Tris-HCl
- pH 8.1
- *store at -4°C*

*Oxaloacetate Buffer:*
- 0.1 M Tris-HCl
- pH 8.1
- *store at -4°C*

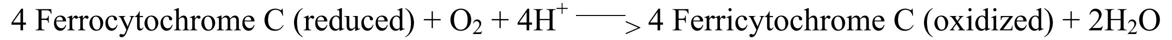
*Protocol:*
1. Prepare buffer solution.
2. Prepare and vortex reagent solutions:
   a. 4 mL of 2.5 mM DTNB in DTNB buffer
   b. 4 mL of 10 mM oxaloacetate in oxaloacetate buffer
   c. 20 µL of 10% (w/v) Triton X-100 in distilled water
   d. 20 µL of acetyl-CoA in distilled water
   ** Note: Acetyl-Coa dissolved in deionized water can be aliquoted and stored at -20°C for 2 weeks or -80°C for 6 months*
3. Pipette 10 µL of sample into an Eppendorf tube.

4. Freeze-thaw the sample in liquid nitrogen 3 times to ensure rupture of the mitochondria.
   * *Final thaw is done immediately prior to adding to reaction*
5. Pipette 250 mL of distilled water into a 250 mL cuvette.
6. Wipe down cuvette with Kim wipe and place into the spectrophotometer to reference absorbance at 412 nm.
7. Set up reaction kinetics program:
   a. Temperature: 25°C
   b. Wavelength: 412 nm
   c. Time: 15 min
8. Pipette reagents into 250 mL cuvette in the following order:
   a. 200 μL distilled water
   b. 10 μL DTNB
   c. 12.5 μL Acetyl-CoA
   d. 12.5 μL Triton X-100
   e. 2.5 μL sample
9. Invert, wipe down cuvette with Kim wipe and place into spectrophotometer.
10. Run the assay for ~2 minutes to get a background reading before initiating the reaction.
11. After 2 minutes, add 12.5 μL of oxaloacetate to initiate the reaction, invert and wipe down with Kim wipe before placing back into spectrophotometer
    * *Ensure this is done quickly as this starts the reaction*
12. Record the slope of the background reading (between 0-2 minutes).
13. Record the linear portion of the slope after the initiation of the reaction.

*Reference:*

Srere, P. (1969). Citrate synthase. In *Methods in Enzymology,* ed Lowenstein JM. (Academic, New York), pp. 305.

***Part VII: Cytochrome C Oxidase Assay (EC: 1.9.3.1 // Cytochrome C ε: 29.5)***



4 Ferrocytochrome C (reduced) + $O_2$ + $4H^+$ ⟶ 4 Ferricytochrome C (oxidized) + $2H_2O$

| | **Volume** | **Substance** | **Stock** | **Final** |
|---|---|---|---|---|
| | 610 µL | Phosphate Buffer (pH 7.4) | 30 mM | |
| | 300 µL | Tween 20 | 10% | 3% |
| | 10 µL | Sample | | |
| **Initiate Reaction** | 80 µL | Cytochrome C (in fresh buffer) | 0.5 mM | 0.04 mM |
| **FINAL** | 1 mL | | | |

*Buffer:*

- 26.4 mM potassium phosphate dibasic
- 9.9 mM potassium phosphate monobasic
- pH 7.4
- *store at -4°C*

*Protocol:*

1. Prepare buffer solution.
2. Prepare and vortex 1 mL of 0.5 mM cytochrome C in buffer
3. Pipette 250 mL of distilled water into a 250 mL cuvette.
4. Wipe down cuvette with Kim wipe and place into the spectrophotometer to reference absorbance at 550 nm.
5. Add 240 µL of distilled water and 10 µL of cytochrome C to a 250 mL cuvette.
6. Test absorbance at 550 nm and 565 nm.
7. Calculate the absorbance ratio of 550 nm/565nm.
   ** Ratio should be between 8-11*
   ** If cytochrome C solution is too oxidized (ratio above 11), add a pinch of sodium hydrosulfite to reduce it*
   ** If cytochrome C solution is too reduced (ratio below 8), slowly bubble oxygen into the solution to oxidize it*
8. Set up reaction kinetics program once the ratio is between 8-11:
   a. Temperature: 25°C
   b. Wavelength: 550 nm
   c. Time: 15 min
9. Pipette 15 µL of sample into an Eppendorf tube.

10. Freeze-thaw the sample in liquid nitrogen 3 times to ensure rupture of the mitochondria.
    * *Final thaw is done immediately prior to adding to reaction*
11. Pipette reagents into 1 mL cuvette in the following order:
    a. 610 µL buffer
    b. 300 µL Tween 20
    c. 10 µL sample
12. Invert, wipe down cuvette with Kim wipe and place into spectrophotometer.
13. Run the assay for ~2 minutes to get a background reading before initiating the reaction.
14. After 2 minutes, add 80 µL of cytochrome C to initiate the reaction, invert and wipe down with Kim wipe before placing back into spectrophotometer
    * *Ensure this is done quickly as this starts the reaction*
15. Record the slope of the background reading (between 0-2 minutes).
16. Record the first 45 seconds of the slope after the initiation of the reaction.

*Reference:*

Cooperstein, S., & Lawarow, A. (1950). A microspectrophotometric method for the determination of cytochrome oxidase. *Journal of Biological Chemistry, 189*(2), 665-670.