Practical and representative methods to assess skeletal muscle redox status in an ageing population.

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by

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Abstract

The aged population is growing globally which has led to an increase in research on healthy ageing. Skeletal muscle health and function is a key component of maintaining overall health and independence as we age. It is thought that a deterioration in muscle health is caused by a combination of increased oxidative stress, physical inactivity, dysfunction in metabolism and a progressive loss of protection against such stressors.

This thesis presents an overview of redox balance response to high intensity intermittent exercise (HIIE) in blood and skeletal muscle with age and the antioxidant and metabolic effects of coenzyme Q10 (CoQ) supplementation. A cell culture toolkit was also developed to allow for pre-screening of pharmacological compounds to help limit the need for skeletal muscle biopsies in potentially vulnerable populations, such as the aged.

Using a biochemical exercise mimic, it was shown that intermittent exposure to an exercise stimulus provided the greatest increase in ROS production in C2C12 skeletal muscle cells. This protocol was then replicated in a human-based experiment which altered redox balance in young healthy males above and beyond any disruption caused by the skeletal biopsy procedure itself. The efficacy of CoQ supplementation was subsequently tested in the same exercise and biopsy protocol. Consumption of 300 mg CoQ per day increased blood and muscle CoQ concentration sufficiently in young and aged populations and this provided some protection against exercise-induced increases in oxidative stress in aged blood and muscle.
A cell culture toolkit was developed and used to assess the impact of CoQ on ageing and exercise in C2C12 cells. CoQ provided limited impact on exercise-induced markers of oxidative stress but, it appeared to reduce antioxidant capacity in older cells. This highlights the importance for further research into the impact of chronic CoQ supplementation, combined with exercise, on the beneficial adaptations to training in an aged population.
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Completing a PhD in exercise physiology was always a dream of mine, but something I didn’t think I would be able to achieve. I am incredibly proud for making it through this challenging and difficult journey to accomplish something that I hold with the greatest respect. The knowledge I have gained throughout my journey has fuelled my desire to keep learning and researching! I have met some wonderful people and made some lifelong friends along the way who have helped shape the kind of researcher and scientist I have become and I would like to thank them all.

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I dedicate this thesis to my parents Kev and Di and my husband Adam.
Abbreviations:

°C  Degrees centigrade
%   Percentage
8-OHdG  8-hydroxy 2’-deoxyguanosine
CAT  Catalase
CoQ  Coenzyme Q10
DHE  Dihydroethidium
DNA  Deoxyribonucleic acid
GPX  Glutathione peroxidase
GSH/GSSG  Glutathione (reduced/oxidised)
HBSS  Hanks’ balanced salt solution
H₂O₂  Hydrogen peroxide
HIIE  High intensity intermittent exercise
HO-1  Heme oxygenase-1
HR   Heart rate
HSP  Heat shock protein
kg   Kilogram
LC-MS  Liquid chromatography mass spectrometry
LOOH  Lipid hydroperoxides
ml   Millilitre
mM  Millimole
mmol/L  Millimole per litre
mRNA  Messenger RNA
mtRNA  Mitochondrial RNA
O₂⁻*  Superoxide anion
PBMC  Peripheral blood mononuclear cell
RNA  Ribonucleic acid
RNS  Reactive nitrogen species
ROS  Reactive oxygen species
RPE  Rate of perceived exertion
SOD  
Superoxide dismutase

µl  
Microlitre

µg  
Microgram

µmol  
Micromole

VO₂max  
Maximal oxygen uptake (ml O₂/kg/min)
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Chapter 1 - Literature Review
1.1 Introduction

Across the world, the number of people aged 60 years and above was estimated to be 600 million in the year 2000, a figure expected to increase to 1.2 billion by 2025 and 2 billion by 2050 (World Health Organisation, 2009). An ageing population has brought a rise in research focussed on improving the quality of life and independence with ageing. Ageing is an inevitable and irreversible process and a variety of theories have been developed to explain its key causes (Jin, 2010). Programmed based theories include; the Longevity Theory (Davidovic et al., 2010), where ageing is associated with the switching on and off of genes, the Endocrine Theory (van Heemst, 2010) which proposes ageing is as a result of hormonal changes over the lifespan, and the Immunological Theory (Cornelius, 1972), which suggests that ageing is as a result of immunological decline over time. Damage and error-based theories of ageing include; the Rate of Living theory (Brys, Vanfleteren, & Braeckman, 2007) where lifespan is inversely related to the rate of basal metabolism, the Cross-linking Theory (Bjorksten & Tenhu, 1990) which proposes that slowing down of bodily processes is caused by an accumulation of cross-linked, damaged proteins and the Free Radical Theory of Ageing (Harman, 2006), which suggests that ageing is caused by oxidative cellular damage (Cobley, Sakellariou, Husi, & McDonagh, 2019; Fulle et al., 2004; Ji, 2001; Liochev, 2013a) dysfunction of metabolism (Ferguson, Mockett, Shen, Orr, & Sohal, 2005; Viña, Borras, Abdelaziz, Garcia-Valles, & Gomez-Cabrera, 2013) and a progressive loss of protective mechanisms (Bori et al., 2012; Pansarasa, Bertorelli, Vecchiet, Felzani, & Marzatico, 1999; Pellegrino et al., 2011). This, results in an increased risk of age-related diseases (D’Arca, Caporali, Mitic, Davalli, & Lauriola, 2016; Muller, Lustgarten, Jang, Richardson, & Van
Ageing is associated with a decline in function of both the cardiovascular and musculoskeletal systems. Advancing age can have a negative impact upon muscle mitochondrial function and adaptation, and a reduced antioxidant capacity to protect against rising reactive oxygen species (ROS) levels (Cobley, Moul, Burniston, Morton, & Close, 2015). A decrease in cellular defence with ageing ultimately leads to cellular deterioration (Gianni, Jan, Douglas, Stuart, & Tarnopolsky, 2004). An increase in ROS can also be caused by compromised cell signalling and reduced ATP production that is experienced with age (Fulle et al., 2004; Guescini et al., 2017; Ji, 2015; Marzetti et al., 2008).

1.2 Skeletal muscle and ageing

Skeletal muscle mass and function deteriorate with age (Cruz-Jentoft et al., 2014; Fulle et al., 2004; Pansarasa et al., 1999) and is termed sarcopenia when this loss becomes debilitating (Landi et al., 2017). Sarcopenia may affect between 5 - 13% of 60 - 70 year olds, and 11 - 50% of those over the age of 80 (Morley, 2008). Interventions to limit age-related muscle loss and improve quality of life could have a large impact on not only the individual but on the population, and also associated healthcare costs. In 2000, the annual cost of muscle deterioration-associated healthcare in the US was approximately $18.5 billion (Janssen, Shepard, Katzmarzyk, & Roubenoff, 2004) estimates in the UK are currently unknown, but are expected to rise with the ageing population.

Sarcopenia is a complex process involving degradation in numerous processes including a reduction in type IIb muscle fibre number and subsequent increase in
type I fibres (McIntire & Hoffman, 2011), reduced mitochondrial ATP production (Marzetti et al., 2008), inadequate nutrition (Miquel, 2001), physical inactivity (Radak, Chung, Koltaí, Taylor, & Goto, 2008), increased inflammation (Phillips & Leeuwenburgh, 2005), neuromuscular junction decline (Walston, 2012) and oxidative damage (Guescini et al., 2017). Sarcopenia is believed to be the result of both intrinsic changes in circulating concentrations of hormones and redox status at a cellular level, and extrinsic factors, such as diet and physical activity (Carmeli, Coleman, & Reznick, 2002).

1.2.1 Muscle fibre type composition changes with age

Skeletal muscle fibres fall into three categories; type I, type IIa and type IIb (Brooke & Kaiser, 1974). The categories separate the fibres into their predominant metabolic preference. Type I fibres are also known as slow oxidative fibres which use aerobic metabolism to produce slow, low power contractions over a long duration and are more fatigue resistant, compared with other fibres. Type I fibres are usually found in higher concentrations in postural muscles and have a greater density of mitochondria to allow for a larger oxidative capacity. Type IIa muscle fibres use a combination of oxidative and glycolytic metabolism to provide moderately-fast contractions while predominantly using aerobic metabolism, but have the capacity to shift towards more glycolytic respiration, however, they fatigue quicker than type I fibres. Type IIb fibres use anaerobic respiration to produce explosive and powerful contractions. These fibres fatigue more rapidly than other fibre types.

With age there is a significant loss of skeletal muscle fibres, with a higher loss in type IIb muscle fibres (McIntire & Hoffman, 2011), leading to a reduced capacity for explosive power and strength (Faulkner, Brooks, & Zerba, 1995; Lexell, 1995;
Örlander, Kiessling, Larsson, Karlsson, & Aniansson, 1978; Verdijk et al., 2007). Adequate skeletal muscle mass, in addition to strength and power, is essential to maintain functional capacity and a good quality of life. Severe loss of type IIb fibres is a classic symptom of sarcopenia, one possible cause of which is dysregulation of the opening of the permeability transition pore (PTP), leading to muscle cell apoptosis (Baumgartner et al., 2009; Lemasters, Theruvath, Zhong, & Nieminen, 2009). In addition to the change in fibre number and distribution, with age, the intramuscular lipid content increases (Landi, Marzetti, Martone, Bernabei, & Onder, 2013; Vina et al., 2014). This reduces the contractility of the skeletal muscle (Kent-Braun, Ng, & Young, 2000) and therefore the amount of power that can be generated.

1.3 Redox alterations with age

Ageing skeletal muscle is impacted greatly by a shift in redox balance due to increased ROS production and a reduced antioxidant defence, although it is currently unknown if there is an upper limit to ROS production with age. The role of ROS in skeletal muscle ageing has been researched for many years (Davies, Quintanilha, Brooks, & Packer, 1982; Dillard, Litov, Savin, Dumelin, & Tappel, 1978; Fisher-Wellman & Bloomer, 2009). It has been well established that skeletal muscle of older organisms has greater damage to lipids, proteins and DNA than their younger counterparts (Vasilaki et al., 2006), causing a redox imbalance.

Redox balance is a term used to describe the balance of oxidation and reduction reactions that occur as a part of metabolism and include the balancing of multiple redox reactions, including; glutathione in the reduced and oxidized form (GSH/GSSG), further described in section 1.7.1.2, nicotinamide adenine
dinucleotide in the oxidized and reduced form (NAD+/NADH), a reduction in this ratio is thought to limit the leakage of O₂⁻ from complex I of the ETC (Sakellariou, Jackson, & Vasilaki, 2014), and nicotinamide adenine dinucleotide phosphate in the oxidized and reduced form (NADP+/NADPH). Upon activation, NADPH is oxidized into NADP+ releasing two electrons which in turn reduce two molecules of oxygen to form two molecules of superoxide (Halliwell, 2006); this system is further explained in section 1.5.2. Redox status can also be classified as the difference between ROS generation and antioxidant protection within the body (Halliwell, 2007). The focus of this thesis will be on ROS control of redox balance. The main functions of ROS are described in Table 1-1.

Table 1-1 Biologically relevant ROS and their main functions. Modified from Radak, 2000, Finaud et al., 2006.

<table>
<thead>
<tr>
<th>Radical species</th>
<th>Molecular Formula</th>
<th>Main effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide radical</td>
<td>O₂⁻</td>
<td>Lipid peroxidation and oxidation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protein oxidation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNA damage</td>
</tr>
<tr>
<td>Ozone</td>
<td>O₃</td>
<td>ROS generation</td>
</tr>
<tr>
<td>Singlet oxygen</td>
<td>¹O₂</td>
<td>LDL oxidation</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>OH⁺</td>
<td>Lipid oxidation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protein oxidation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNA damage</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>H₂O₂</td>
<td>Generates hydroxyl radical</td>
</tr>
<tr>
<td>Hypocholrus acid</td>
<td>HOCl</td>
<td>Protein oxidation</td>
</tr>
</tbody>
</table>

Free radicals are an important group of ROS comprising of one or more unpaired electrons, which damage other molecules resulting in pairs for these electrons (Clarkson & Thompson, 2000). Antioxidants are biological components that scavenge free radicals by donating an electron to ROS, limiting damage caused
The body controls redox balance through a complex signalling process whereby an increase in ROS leads to an increase in antioxidants in an adaptive manner (Jackson, 2009; Jackson, 1999). In young, healthy individuals, this balance is well maintained, whereas overtraining, illness and ageing can cause an imbalance, resulting in oxidative damage when ROS production can no longer be compensated for by an increase in antioxidant protection (Sies, Berndt, & Jones, 2017). It has been suggested that antioxidant supplementation may be required in these compromised individuals.

The Free Radical Theory of Ageing was first introduced by Harman in 1954 and initially defined as the random accumulative and deleterious increase in damage caused by ROS with age. However this definition has continued to be modified (Harman, 2009; Liochev, 2013). Free radicals, in low to moderate levels, provide essential function in cell signalling and physiology and are especially important in the promotion of cell protection and adaptation (Jackson, 1999; Ji, 2002). With age this response diminishes and the negative effect of high ROS levels becomes more apparent.

With age, the efficiency of mitochondrial respiration progressively declines causing a higher ROS output for a comparatively lower ATP output (Chabi et al., 2008). Ageing induces an increase in $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ production from the mitochondria causing damage to lipids, proteins and DNA (Sohal & Sohal, 1991). Oxidative damage has been proposed as one of the main factors in age-related skeletal muscle loss and decline in function (Fanò et al., 2001; Pansarasa et al., 1999; Pansarasa, Felzani, Vecchiet, & Marzatico, 2002).

It is now accepted that low levels of ROS can induce beneficial adaptations of skeletal muscle to stimuli such as exercise (He et al., 2016; Zuo, Zhou, Pannell,
Ziegler, & Best, 2015). ROS have been shown to mediate adaptive responses such as increased glucose uptake, facilitation of mitochondrial biogenesis (Powers, Nelson, & Hudson, 2011) and initiation of secondary signal transduction processes (Daiber, 2010; Nathan & Cunningham-Bussel, 2013; Zuo & Pannell, 2015), all of which are fundamental to maintaining muscle function in older age. Exercise and increased physical activity are, therefore, often prescribed to prevent the negative impact of ageing on skeletal muscle.

1.3.1 Impact of exercise on ageing

Regular exercise has been shown to increase life-expectancy (Lee et al., 2012) and reduce risk for age-associated diseases such as; CVD, stroke and diabetes (Stavropoulos-Kalinoglou et al., 2013; Swift, Johannsen, Lavie, Earnest, & Church, 2014; Williams & Thompson, 2013). Exercise has been shown to delay, and to some extent prevent, the development of sarcopenia and associated frailty (Burton & Sumukadas, 2010; Cadore et al., 2014; Fulle et al., 2004; Landi et al., 2013; Lenk, Schuler, & Adams, 2010). The World Health Organisation reported that physical inactivity is one of the leading risk factors for global mortality (http://www.who.int/dietphysicalactivity/pa/en/). Exercise is known to increase muscle mass, although it is also understood that exercise can increase ROS production and mechanical damage. The beneficial adaptations following exercise may not be equal between young and aged populations (Drummond et al., 2008). This is thought to be due to the shift in redox balance towards that of increased damage with age.

Aged individuals that have maintained a regular exercise routine throughout life, have similar biochemical and redox muscle profiles to those of a younger population (Cobley et al., 2012). However, acute bouts of high intensity and
strenuous exercise can also result in muscle and oxidative damage that opposes the beneficial effects of exercise in older age (Aoi, Naito, & Yoshikawa, 2013). Aged individuals may respond differently to their younger counterparts when exercise stress is kept at the same relative intensity. This will be investigated within this thesis.

1.4 Reactive oxygen species and oxidative stress

Approximately 1-2% of oxygen consumed in oxidative respiration is converted to ROS, predominantly superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). This production increases with age due to a less efficient ETC (Ferguson et al., 2005; Joseph et al., 2012; A Vasilaki et al., 2006). ROS have the potential to damage proteins, lipids and DNA.

1.4.1 Superoxide Radical

The superoxide anion (O$_2^-$) is considered to be a primary radical; it is formed as an intermediate in almost all aerobic metabolism from the incomplete reduction of O$_2$ during electron transport (Powers and Jackson 2008). Superoxide is produced from electron ‘leakage’ in the mitochondria (Goncalves, Quinlan, Perevoshchikova, Hey-Mogensen, & Brand, 2015b), NADPH oxidases and xanthine oxidase (Sakellariou et al., 2014), and during ischemia/redistribution injury (Cooper, Vollaard, Choueiri, & Wilson, 2002). Superoxide is negatively charged and relatively membrane impermeable. Dismutation of O$_2^-$, both spontaneously and catalysed by superoxide dismutase (SOD), is a major source of H$_2$O$_2$.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Superoxide dismutase
1.4.2 Hydrogen peroxide

Hydrogen peroxide ($H_2O_2$) is a reactive compound that belongs to the family of ROS, however it is not a free radical by definition due to having no unpaired electrons. $H_2O_2$ is considered stable in comparison with other reactive species with a relatively long half-life. $H_2O_2$ is primarily generated through the dismutation of $O_2•−$ and xanthine oxidase (Kelley et al., 2010). $H_2O_2$ is membrane permeable and can diffuse out of the cell and inactivate enzymes through the oxidation of key thiol groups (Halliwell & Gutteridge, 2007). It is also able to generate the hydroxyl radical ($•OH$) from redox-active metal-catalysed reactions and interaction of $H_2O_2$ with haem peroxidases (Jackson, 2011).

1.4.3 Hydroxyl radical

Hydroxyl radicals ($•OH$) are some of the most reactive, and therefore dangerous, radical species in biological systems (Halliwell & Gutteridge, 2007). They are not membrane permeable, but due to their highly reactive nature they will react with most molecules within close proximity to their generation site (Matsuo & Kaneko, 2000). Thus, the damage caused by $•OH$ will be dependent upon its location. Hydroxyl radicals are generated through the interaction of $H_2O_2$ with reduced transition metal ions, primarily free iron ($Fe^{2+}$), which is known as the Fenton reaction.

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH + •OH$$
1.4.4 Nitric oxide

Nitric oxide (NO) is considered a primary radical as it is the first in a chain of radical reactions. It is synthesised from conversion of the amino acid, L-arginine, to citrulline by nitric oxide synthases (NOS), which is controlled by calcium (Ca\(^{2+}\)) and calmodulin (Davidson & Duchen, 2006). Nicotinamide adenine dinucleotide phosphate (NADPH) acts as a cofactor in this conversion. NO readily reacts with O\(_2^{-}\) to produce peroxynitrite (ONOO\(^{-}\)), and with oxygen to form nitric dioxide (NO\(_2\)). Aside from other radicals, NO is unreactive with most other molecules (Matsuo & Kaneko, 2000). NO has the potential to diffuse through membranes, and its intracellular production of NO can therefore cause extracellular damage and interact with extracellular radicals (Beckman & Koppenol, 1996).

1.5 Formation of reactive species

Reactive species in skeletal muscle are generated through a multitude of mechanisms. The production site and rate vary greatly and are dependent upon level of physical activity, activity type, ageing and disease state. Sites of ROS generation in skeletal muscle are summarised in Figure 1-1.
Mitochondrial sources of ROS

Mitochondrial production of ROS has received much attention and was thought to be the greatest contributor to skeletal muscle ROS production and as the main factor in the increase in oxidative stress during aerobic exercise (Powers & Jackson, 2008; Steinbacher & Eckl, 2015). During resting aerobic respiration, electrons are passed along a chain of complexes, moving from electron donors to acceptors through a series of redox reactions (Figure 1-2). At complex IV, cytochrome c oxidase, oxygen is reduced by four electrons and four hydrogen atoms. During this process, a small amount of oxygen is thought to “leak”, producing intermediates, including; O$_2^-$, H$_2$O$_2$ and OH$^+$ (Goncalves et al., 2015b; Ji, 1995; Quinlan et al., 2012). It is considered that the main site of O$_2^-$ production...
is from the miscommunication between complex I and II with complex III (Barja, 1999; Boveris & Cadenas, 1975; Sjödin, Westing, & Apple, 1990; Wang et al., 2014), which relies on CoQ to act as a transporter of electrons between the complexes (López-Lluch, Rodríguez-Aguilera, Santos-Ocaña, & Navas, 2010). It has been suggested that as much as 2-3% of the oxygen reduced during aerobic respiration may leak out of the muscle mitochondria to form \( \text{O}_2^- \), which is rapidly dismutated to form \( \text{H}_2\text{O}_2 \) (Chance, Sies, & Boveris, 1979). This rate is thought to increase with advancing age due to a loss of efficiency of the ETC, causing an increase in ROS production with ageing.

![Figure 1-2 – The electron transport chain. Arrows show the movement of electrons through complexes I - V. Abbreviations; Cyt. c, cytochrome c. (Adapted from; Spindler et al., 2009, von Bergen et al., 2009)](image)

For many years it has been thought that the more metabolically active the tissue, the higher the oxygen demand, resulting in an increased production of ROS. This miscommunication was thought to occur more frequently during exercise due to the increase in oxygen consumption (Astrand & Rodahl, 1986). More recently, however, it has been suggested that this may not be the case during exercise (Goncalves, Quinlan, Perevoshchikova, Hey-Mogensen, & Brand, 2015a; Sakellariou et al., 2014). An alteration in redox state as a result of contraction...
can lead to altered reduced mitochondrial NADH/NAD\textsuperscript{+} ratio (He et al., 2016). A reduction in this ratio is thought to limit the leakage of O\textsubscript{2}\textsuperscript{−} from complex I of the ETC (Sakellariou et al., 2014).

This adaptation appears to be present, but at a lower rate in ageing skeletal muscle (Viña, Salvador-Pascual, Tarazona-Santabalbina, Rodriguez-Mañas, & Gomez-Cabrera, 2016). The efficiency of antioxidant protection is reduced with ageing, in addition to an increase in ROS production (Jackson & Mcardle, 2016). Therefore, exercise can often increase ROS production above the level required for adaptation, leading to oxidative damage and redox imbalance (Close & Jackson, 2014; Deaton & Marlin, 2003; Radak et al., 2008). It is therefore suggested that dietary supplementation may help to counteract this over production of ROS in an exercising and aged population (Landi et al., 2017, 2013; Ryan et al., 2010). The combination of regular exercise and adequate nutrition, including antioxidant supplementation from both food and pharmacological sources, may limit the development of age-related sarcopenia (Calvani et al., 2013; Guescini et al., 2017; Landi et al., 2017).

1.5.2 Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes

Research has shifted from mitochondrial sources of ROS to focus on NADPH oxidases (NOX) as the greatest source of ROS in skeletal muscle (Sakellariou et al., 2014; Xia, Webb, Gnall, Cutler, & Abramson, 2003). NADPH oxidases are the only enzymes whose sole function is to produce ROS, in both disease and under physiological conditions (Altenhöfer et al., 2012; Cave et al., 2006). The NOX family consists of at least five members (NOX1-5) (Geiszt, 2006). Research in C2C12 skeletal muscle cells have identified the key homologues expressed in skeletal muscle are NOX1, NOX2 and NOX4 (Piao et al., 2005), of these, a clear
physiological role for NOX1 in skeletal muscle is unknown (Ferreira & Laitano, 2016). While the NOX2 isoform is closely associated with the extracellular production of superoxide (Sakellariou et al., 2014; Xia et al., 2003), which is rapidly converted by SOD to H$_2$O$_2$ (Okutsu et al., 2014; Pal, Basu Thakur, Li, Minard, & Rodney, 2013). The NOX4 homologue has been shown to produce O$_2^{-}$ and H$_2$O$_2$ (Nisimoto, Diebold, Cosentino-Gomes, Constantino-Gomes, & Lambeth, 2014; Zhang, Perino, Ghigo, Hirsch, & Shah, 2013) and is localised within skeletal muscle mitochondria (Sakellariou et al., 2014) and sarcoplasmic reticulum (Xia et al., 2003). NOX enzymes have also been identified on the muscle fibre plasma membrane (Whitehead, Yeung, Froehner, & Allen, 2010).

The production of ROS from NADPH oxidases and mitochondrial sources interact to promote the production of additional ROS from each other, causing ROS accumulation (Brandes, 2005; Daiber, 2010; Zorov, Juhaszova, & Sollott, 2014). There are many mechanisms involved in this ‘cross-talk’ between NOX and mitochondrial ROS production in most cell types, including smooth and cardiac muscle (Dikalov, 2011). However, there is little evidence of equivalent reactions in skeletal muscle. One suggested mechanism involves the mitochondrial ATP-sensitive sodium (K$^+$) channels. ROS produced from NOX stimulate the opening of the mitochondrial ATP-sensitive K$^+$ channel, leading to an opening of the permeability transition pore (PTP), subsequently elevating ROS production further and initiating apoptosis (Brandes, 2005; Powers & Jackson, 2008). Another potential mechanism when mitochondria initiate cross-talk with NO, leads to an increase in mitochondrial ROS activating redox-sensitive kinases which can upregulate the expression of NOX2 and NOX4 (Zhang et al., 2013). In addition, mitochondrial ROS oxidise the ryanodine receptor (RyR) leading to
an increase in Ca$^{2+}$ release, and subsequent accumulation, triggering NOX activation in non-skeletal muscle cells (Dikalov, 2011; Kröller-Schön et al., 2014). NADPH oxidases are responsible for the majority of ROS production in normal physiological conditions, but also during ischaemic redistribution (see section 1.5.3). NADPH oxidases are also a key producer of O$_2^-$ in phagocytes during the respiratory burst and are located on the plasma membranes of lymphocytes, phagocytes and macrophages (Bedard & Krause, 2007). This consideration is important when interpreting blood markers of ROS in relation to skeletal muscle, as these may show increases based on the localised production of ROS from NADPH oxidases which may not be representative of skeletal muscle ROS production. It is therefore important to consider the blood as a possible location of ROS production independently of skeletal muscle.

1.5.3 Ischemic redistribution

ROS can be produced though the process of ischemic redistribution following surgery, shock or high intensity exercise Figure 1-3 (Eltzschig & Collard, 2004; Finaud & Biologie, 2006; Thompson-Gorman & Zweier, 1990). During high intensity exercise, blood is shunted towards working muscles and away from other organs, causing hypoxia and ischemia in these tissues (Gourdin, Bree, & De Kock, 2009; Maxwell & Lip, 1997). Localised ischemia can also occur in muscle fibres which may not be used during specific exercises. During ischemia, ATP production is disturbed, causing an alteration in metabolism and an increase in intracellular Ca$^{2+}$ (Kleikers et al., 2012). This shift in metabolism has also been shown to increase the inflammatory response to exercise ( Förstermann, 2008; Maxwell & Lip, 1997).
On cessation of exercise, blood flow to these tissues increases to allow re-oxygenation and an additional increase in inflammatory response (Förstermann, 2008; Kleikers et al., 2012). This process is more pronounced in older people who are more likely to have a compromised, less efficient cardiovascular system, including an overall reduction in capillarisation of the skeletal muscle fibres (Coggan et al., 1992; Williams, Higgins, & Lewek, 2002), although the impact of this on oxidative capacity may be limited (Barnouin et al., 2017). During high intensity exercise, large amounts of ATP are consumed and oxidative metabolism becomes insufficient. Which may be a further source of ROS production during high intensity training (Kang, O’Moore, Dickman, & Ji, 2009). It is thought that the ROS generated through this pathway may contribute to the regulation of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a controller of exercise-induced mitochondrial biogenesis (Ji, Kang, & Zhang, 2016; Steinbacher & Eckl, 2015) and a key component of preserving adaptation to training in the aged.

![Figure 1-3 - Mechanism of ischemia-reperfusion injury (Sasaki and Joh, 2007)](image)
The third period of ROS production in ischemic redistribution is the post-redistribution phase. This is characterised by tissue adaptation and remodelling (Kleikers et al., 2012), suggesting a potential beneficial effect of ROS produced through ischemia-redistribution (Maulik & Das, 2002).

1.5.4 Respiratory burst

A single bout of high-intensity exercise may induce a secondary production of ROS. Intense exercise increases mechanical shear stress and metabolic disruption which can result in microvascular dysfunction, oedema and cell death (Pyne, 1994). This process initiates an inflammatory response, causing an influx of neutrophils and phagocytes (Toumi & Best, 2003). This is followed by a respiratory burst which can produce $O_2^-$, $H_2O_2$ and other ROS (Vollaard, Shearman, & Cooper, 2005). It is thought that the inflammatory and oxidative response to a single bout of extreme endurance or high intensity exercise can have a similar impact to that of an ischemic stroke or myocardial infarction (Mastaloudis, Leonard, & Traber, 2001).

1.6 Biochemical implications of reactive species

1.6.1 Lipid peroxidation

Oxidation of polyunsaturated fatty acids (PUFA) is termed lipid peroxidation. It is well established that lipid peroxidation consists of three main steps: initiation, propagation and termination (Figure 1-4) (Niki, 2009). Initiation occurs when free radicals (R•) remove hydrogen atoms from the methylene group of PUFA, forming a carbon-centred radical (L•). This radical reacts with $O_2$, forming a peroxyl radical (LOO•). These highly reactive species propagate a radical chain reaction, producing additional lipid peroxidation products by interacting with neighbouring fatty acids causing damage to cell membranes integrity, stability and fluidity.
The products produced from this reaction are highly dependent upon the location of the lipid peroxidation. Hydroperoxides (LOOH) are one of the most common and relatively stable products of this process, however in the presence of redox-active metals, iron and copper, LOOH can be reduced to form reactive peroxyl or alcoxyl radicals (Niki, 2009). The lipid peroxidation process can be terminated when two free radicals interact to form a non-radical. Antioxidants, vitamins C and E and CoQ can produce stable radicals which can also terminate lipid peroxidation (Ernster & Forsmark-Andrée, 1993; Matsuo & Kaneko, 2000; Stear et al., 2010).

As we age there is an increase in lipid peroxidation rates at rest and post-exercise (Sohal & Sohal, 1991; Sohal & Forster, 2007). This is thought to be caused by a reduction in antioxidant defence and an increase in ROS production (Doria, Buonocore, Focarelli, & Marzatico, 2012; McArdle, Pattwell, Vasilaki, Griffiths, &
Lipid peroxidation may also occur due to the rise in intramuscular lipids with age (Cruz-Jentoft & Landi, 2014; Domenech et al., 2018; Landi et al., 2017; Vina et al., 2014), which can increase the exposure of lipids to ROS produced within skeletal muscle.

1.6.2 Protein oxidation

Protein oxidation can occur in both the protein backbone and the amino acid side chains, of which cysteine and methionine are most susceptible to oxidation (Garrison, 1989; Shacter, 2000). Oxidation can result in the formation of protein cross-bridges, protein fragmentation and oxidation of amino acid residues (Doria et al., 2012). These modifications can interfere with inter and intra cellular signalling and organ function (Lushchak, 2007; Wong, Marcocci, Liu, & Suzuki, 2010).

Protein carbonyls (PCs) are the most common by-product of protein oxidative damage, produced during the oxidation of lysine, arginine, proline, threonine, glutamic acid and aspartic acid (Cheeseman & Slater, 1993; Dalle-Donne et al., 2006; Shacter, 2000). PC are considered to be stable products and a reliable measure of protein oxidation (Levine, 2002; Lushchak, 2007; Matsuo & Kaneko, 2000; Wong et al., 2010). They may also act as secondary messengers for signal transduction (Wong et al., 2010). The majority of oxidised proteins cannot be repaired and must therefore be removed, this is carried out by a proteasome which degrades the protein through proteolytic degradation (Rossi, Marzani, Giardina, Negro, & Marzatico, 2010). This process becomes less efficient with ageing, leading to an increase in oxidised proteins and a subsequent increase in ROS production (Reeg & Grune, 2015).
PC content increases rapidly in the last third of the lifespan (Levine, 2002) by which time, on average, one in three protein molecules carry a modification (Doria et al., 2012). Research by Pansarasa et al. (1999) found that PC content of skeletal muscle was significantly increased in men over 70 years compared with those younger than 40 years (Pansarasa et al., 1999). The accumulation of PC and subsequent protein modifications can vary in different muscles dependent upon their composition and function (Buonocore, Rucci, Vandoni, Negro, & Marzatico, 2011; Doria et al., 2012).

1.7 Antioxidants as a defence system

As previously discussed, (section 1.5.1), regular physical activity paired with adequate nutrition may slow or prevent age-related sarcopenia development. Exercise in an aged population still provides the beneficial adaptations to training (Viña et al., 2016), however, it can also increase ROS production beyond the capabilities of the antioxidant defence system. The antioxidant system works to prevent, reduce, intercept and repair damage caused during periods of increased ROS production (Powers & Jackson, 2008; Sies et al., 2017). It works directly by scavenging ROS, and indirectly by reducing oxidized molecules. The system is comprised of enzymatic and non-enzymatic antioxidants which work in coordination to provide a comprehensive level of protection.

1.7.1 Enzymatic antioxidants

The primary enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). Thioredoxins (TRx), glutaredoxins (GRx) and peroxiredoxins (PRx) also provide protection against oxidation, however, to a lesser extent than the three primary enzymes (Powers & Jackson, 2008).
1.7.1.1 *Superoxide dismutase*

Superoxide dismutase (SOD) was first discovered in 1969 (McCord & Fridovich, 1969) and is described as the first line of defence against superoxide radicals. Although \( \text{O}_2^- \)-radicals are not considered highly reactive, they can initiate a chain of radical reactions (Powers & Jackson, 2008). SOD are a family of metalloenzymes responsible for catalysing the one-electron dismutation of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) and oxygen (Hollander, Bejma, Ookawara, Ohno, & Ji, 2000; Malcolm J. Jackson & Mcardle, 2011). The metalloenzymes of SOD require a redox active transition metal to bind to its active site in order to catalyse the dismutation of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) and oxygen.

\[
\text{SOD} \quad \text{O}_2^- + \text{O}_2^- \rightarrow \text{O}_2 + \text{H}_2\text{O}_2
\]

1.7.1.2 *Glutathione peroxidase*

Glutathione peroxidase (GPx) comprises a group of five enzymes containing a selenium atom. They are located in the mitochondrial matrix and cytosol of mammalian cells. Each of the different isoforms of GPx reacts to different hydroperoxides which makes GPx an important cellular antioxidant capable of protecting cellular lipid membranes, proteins and DNA against oxidative damage (Matsuo & Kaneko, 2000). All isoforms of GPx catalyse the reduction of \( \text{H}_2\text{O}_2 \) or organic hydroperoxide (ROOH) to \( \text{H}_2\text{O} \) or alcohol (ROH), respectively. Most commonly this reaction uses reduced glutathione (GSH) as the electron donor, however, in some instances thioredoxin (TRx) or glutaredoxin (GRx) are used for electron donation (Holmgren et al., 2005). When GSH is used as the electron donor it is oxidized to glutathione disulphide (GSSG).
In order to provide a continual supply of electrons to GPx, GSH must be regenerated from GSSG within the cell. The reduction of GSSG is catalysed by the enzyme glutathione reductase.

1.7.1.3 Catalase

Catalase (CAT) is a haem-containing antioxidant enzyme located throughout mammalian cells with widespread biological functions (Kirkman & Gaetani, 2007). Its primary role as an antioxidant is to catalyse the breakdown of $H_2O_2$ to $H_2O$ and $O_2$ (Jackson, 2009). CAT requires iron as a cofactor to activate its role as a catalyst.

\[
\text{CAT} \quad 2H_2O_2 \quad \rightarrow \quad 2H_2O \quad + \quad O_2
\]

Catalase has a lower affinity for $H_2O_2$ than GPx, however, when $H_2O_2$ levels rise CAT becomes more significant in the decomposition of $H_2O_2$ to protect biological molecules (Halliwell & Gutteridge, 2007). In low concentrations of $H_2O_2$, mammalian CAT catalyses peroxidase-type reactions (Chance et al., 1979).

1.7.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants work alongside the enzymatic system to protect cellular components from damage caused by oxidative stress (Sies et al., 2017).
The non-enzymatic system includes both lipid soluble and hydrophilic antioxidants, some of which can be synthesised endogenously while others must be consumed through the diet (Halliwell, 1996; Halliwell & Gutteridge, 2007). The main non-enzymatic antioxidants' locations and actions are shown in Table 1-2.
<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Location</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E (α-tocopherol)</td>
<td>Lipids</td>
<td>Lipid peroxidation inhibition</td>
</tr>
<tr>
<td></td>
<td>Cells &amp; mitochondria membranes</td>
<td>Membrane stabilisation</td>
</tr>
<tr>
<td>Vitamin A (retinol)</td>
<td>Lipids</td>
<td>Lipid peroxidation reduction</td>
</tr>
<tr>
<td></td>
<td>Cell membranes</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (ascorbic acid)</td>
<td>Cytosol</td>
<td>α-tocopherol regeneration</td>
</tr>
<tr>
<td></td>
<td>Extracellular fluids</td>
<td>LDL protection</td>
</tr>
<tr>
<td>Glutathione</td>
<td></td>
<td>Substrate for GPX</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamins C &amp; E regeneration</td>
</tr>
<tr>
<td>Lipoic acid</td>
<td></td>
<td>Lipid peroxidation inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamins C &amp; E and cysteine regeneration</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Linked with glucids</td>
<td>Pro-oxidant enzymes inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pro-oxidant ions trapping</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LDL protection</td>
</tr>
<tr>
<td>Coenzyme Q10</td>
<td>Internal membrane of the mitochondria</td>
<td>Lipid peroxidation inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNA protection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin C &amp; E regeneration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LDL protection</td>
</tr>
<tr>
<td>Uric Acid</td>
<td></td>
<td>Pro-oxidant ions trapping</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Erythrocyte, haemoglobin, DNA and lipid protection</td>
</tr>
</tbody>
</table>

### 1.8 Coenzyme Q10 (CoQ)

Coenzyme Q10 (CoQ) is a lipid soluble, vitamin-like substance with antioxidant capabilities. It is a central component of the mitochondrial ETC and is located within the inner mitochondrial membrane. It transports electrons from complexes I and II to complex III for the production of ATP (López-Lluch et al., 2010). The
redox state of CoQ also determines the stability of complex I of the mitochondrial ETC (Guarás et al., 2016), which can determine ROS production in the mitochondria. CoQ is a key factor in the protection of lipid membranes and cellular components against oxidative stress and has the ability to regenerate α-tocopherol (vitamin E), another potent lipid antioxidant. CoQ has also been shown to limit PTP opening and therefore reduce dysregulated cell apoptosis (Guescini et al., 2017). Although humans produce CoQ endogenously, it is thought the production of CoQ reduces with age (Niklowitz et al., 2016; Sohal & Forster, 2007). Due to the multiple roles of CoQ that are closely linked with aspects of ageing, CoQ is the primary focus of this thesis.

1.8.1 Biosynthesis

CoQ is chemically designated 2,3-dimethoxy-5-methyl-6-decaprenyl-1, 4-benzoquinone (Overvad et al., 1999). CoQ is comprised of a tyrosine derived aromatic quinone ring and a superpreniiod tail which varies in length, consisting of ten subunits in humans, giving the name coenzyme Q10 (Ernster & Forsmark-Andrée, 1993). The tail length determines the motility, its capacity to be auto-oxidised and its ability to interact with cellular proteins, otherwise the tail bears no physiological function other than to anchor it to the cell membrane (Laredj, Licitra, & Puccio, 2014). CoQ is present in three forms, fully reduced ubiquinol, fully oxidized ubiquinone or ubisemiquinone radical (Figure 1-5).
Endogenous CoQ is the main source of CoQ in humans and is synthesised in the inner mitochondrial membrane through the mevalonate pathway, see Figure 1-6 below (Turunen, Olsson, & Dallner, 2004). Briefly, acetyl CoA condenses with acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA reductase is one of the key regulatory enzymes of cholesterol production and converts HMG-CoA to mevalonate which is phosphorylated in two steps to form famesyl pyrophosphate (FPP) via isopentyl pyrophosphate and FPP synthase (Grünler, Ericsson, & Dallner, 1994). FPP is the main substrate for the synthesis of cholesterol as well as CoQ. FPP is converted to CoQ through the rate limiting enzyme, trans-prenyltransferase, which forms the terminal part of CoQ biosynthesis.
In the circulation, ubiquinol forms part of LDL/VLDL particles and therefore plasma CoQ content is positively correlated to cholesterol levels (Littarru & Lambrechts, 2011). For this reason, plasma values of CoQ should be represented alongside cholesterol levels. The total pool of CoQ in humans is thought to be between 0.5 g and 1.5 g in an average adult male and is found at its highest concentrations in tissues with a higher energy demand such as heart, liver and skeletal muscle (Bhagavan & Chopra, 2006; Turunen et al., 2004). The distribution and redox state of CoQ in humans is shown in Table 1-3.
Table 1-3 - Concentration and redox state of CoQ in human tissues (Bhagavan and Chopra, 2006)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CoQ (nmol/g)</th>
<th>Redox state (% reduced)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>132</td>
<td>61</td>
</tr>
<tr>
<td>Kidney</td>
<td>77.0</td>
<td>75</td>
</tr>
<tr>
<td>Liver</td>
<td>63.6</td>
<td>95</td>
</tr>
<tr>
<td>Muscle</td>
<td>46.0</td>
<td>65</td>
</tr>
<tr>
<td>Brain</td>
<td>15.5</td>
<td>23</td>
</tr>
<tr>
<td>Intestines</td>
<td>13.3</td>
<td>95</td>
</tr>
<tr>
<td>Lungs</td>
<td>9.2</td>
<td>25</td>
</tr>
<tr>
<td>Plasma (µmol/l)</td>
<td>1.1</td>
<td>96</td>
</tr>
</tbody>
</table>

Average plasma CoQ levels are reported to be between 0.4 – 1.72 µmol/L for Caucasian males, the British average is at the lower end of the range, at 0.675 umol/L (Duncan et al., 2005). Concentration of CoQ within human skeletal muscle is reported to be 140 - 580 pmol/mg protein with a mean value of 241 pmol/mg protein (Duncan et al., 2005). Some studies have identified a correlation between the CoQ content of skeletal muscle and blood mononuclear cells, however, there appears to be no correlation between plasma and either skeletal muscle (Laaksonen, Jokelainen, Sahi, Tikkanen, & Himberg, 1995; Svensson et al., 1999) or blood mononuclear cells (Duncan et al., 2005).

1.8.2 CoQ with age

Total body CoQ concentration increases up to 20 years of age, then begins to decline and by the age of 80 it can be lower than at birth (Ernster & Forsmark-Andrée, 1993). In an animal model, premature ageing has been associated with an increased ubiquinol/ubiquinone ratio (Olgun, 2009) and a higher mtDNA mutation rate. Olgun (2009) proposed that the maintenance of the
ubiquinol/ubiquinone ratio should be considered to prevent premature ageing and reduce the risk for age-related diseases. CoQ supplementation has been shown to have potential positive effects in numerous pathological diseases such as cancers (Roffe, Schmidt, & Ernst, 2004), cardiovascular disease (Langsjoen, Langsjoen, Langsjoen, Willis, & Folkers, 1994), neuropathies (Folkers & Simonsen, 1995) and diabetes (Golbidi, Ebadi, & Laher, 2011). Most of these diseases increase with age and are accompanied by an increase in oxidative damage and impaired mitochondrial activity. The use of CoQ as a therapeutic agent with age needs to be further investigated, aspects of which are a focus of this thesis.

1.8.3 CoQ as an antioxidant

The majority of research to date has focussed on the ability of CoQ to protect lipids from peroxidation by preventing the initiation and propagation stages (Lee, Huang, Chen, & Lin, 2012; Littarru & Tiano, 2007; Pobezhimova & Voinikov, 2000). CoQ can act indirectly as an antioxidant by interactions with other antioxidants, primarily α-tocopherol. CoQ can prevent the potential pro-oxidant properties of α-tocopherol by reducing it to its non-radical form (Sohal, 2004). It is also oxidized preferentially and more rapidly than α-tocopherol (Stocker, Bowry, & Frei, 1991). In addition to the regeneration of α-tocopherol, CoQ is also involved in the regeneration of vitamin C (Crane, 2001).

CoQ may also play a role in the control of the plasma membrane redox system (PMRS) which regulates the NAD+/NADH ratio in cells, regulating cell bioenergetics (Larm et al., 1994). This becomes of increased importance in the ageing process, as regulation of cellular ATP production is reduced with age.
(Fulle et al., 2004; Ji, 2015) and the effect of PMRS is compromised with age (De Cabo et al., 2004).

1.8.4 CoQ and cell metabolism

CoQ is involved in ATP production and the concentration of CoQ is higher in Type II skeletal muscle fibres, due to the higher oxidative capacity and increased mitochondrial content of these fibres (Karlsson, Diamant, Folkers, & Lund, 1991). Approximately 50% of CoQ is located in the inner mitochondrial membrane with less in the cytosol and other organelles (Bhagavan & Chopra, 2006). CoQ is essential for aerobic respiration; it shuttles electrons from NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II) to the cytochrome system at complex III (Figure 1-2) (Crane, 2007; López-Lluch et al., 2010). CoQ is also involved in the shuttling of electrons across the mitochondrial membrane. Quinone reductase reduces CoQ in the presence of NADH to produce ubiquinol, this transfers electrons to NADH dehydrogenase which reduces extracellular electron acceptors on the surface of the plasma membrane (Turunen et al., 2004).

1.8.5 CoQ and the permeability transition pore (PTP)

The PTP is prone to opening and can allow macromolecules to enter the inner mitochondrial membrane, leading to mitochondrial swelling and dysfunction, and result in the activation of cell apoptosis (Di Lisa, Menabò, Canton, Barile, & Bernardi, 2001). As we age, the opening of the PTP becomes dysregulated, leading to an increase in cell apoptosis within the skeletal muscle. This may be a contributing factor to a reduction in skeletal muscle fibres with age. CoQ is thought to interact with the PTP by binding to the active site, preventing its opening (Littarru & Tiano, 2007; Papucci et al., 2003), and therefore may reduce the rate of apoptosis in the ageing skeletal muscle.
1.8.6 Other functions of Coenzyme Q10

CoQ has been shown to be cofactor in the function of uncoupling proteins (UCPs), specifically their role in fatty acid-dependant H⁺ transport (Echtay, Winkler, Frischmuth, & Klingenberg, 2001; Echtay, Winkler, & Klingenberg, 2000). It subtracts H⁺ from fatty acids and transports them to the fatty acid group of UCPs. CoQ exerts a protective effect by activating these proteins and reducing ROS production within the mitochondria (Beal, 2006; Beal, 2004; Xu et al., 2010).

1.8.7 CoQ bioavailability

Bioavailability of all dietary CoQ is very limited and it is believed that only ~6% of that which is orally ingested is absorbed through the small intestine and diffuses into blood (Bentinger, Tekle, & Dallner, 2010). After oral supplementation, there is an initial peak within a couple of hours following ingestion and a second peak at 24 hours, thought to be caused by enterohepatic re-circulation (Bhagavan & Chopra, 2006, 2007; Tomono, Hasegawa, Seki, Motegi, & Morishita, 1986). Orally ingested CoQ appears in the circulation as ubiquinol irrespective of the form it is ingested in (Hosoe et al., 2007; Kaikkonen, Nyyssönen, Tuomainen, Ristonmaa, & Salonen, 1999). The body readily interconverts the reduced and oxidized forms CoQ dependent upon the needs of the tissue. It is therefore suggested that ingestion of the reduced form is not necessarily required due to the redox action of CoQ (Bhagavan & Chopra, 2007). The ability to reduce CoQ to ubiquinol diminishes with age and disease (Ernster & Dallner, 1995; Tang, Miles, DeGrauw, Hershey, & Pesce, 2001), the cause and effect of this compromised ability is not yet fully understood. This is an area that will be investigated within this thesis.
1.9 Methods of research

Traditionally, blood has been used as a medium to measure markers of ageing and redox balance, often with the broad assumption that these results are reflective of skeletal muscle biochemistry (Fisher-Wellman & Bloomer, 2009; Souza-Silva et al., 2016; Wadley, Turner, & Aldred, 2016). However, no clear evidence has been presented establishing a link between blood markers and skeletal muscle redox balance. It has also been recently proposed that blood may act as a compartment of oxidative stress in and of itself (Fogarty et al., 2016).

In order to understand these combined continuums it is therefore necessary to use a muscle biopsy technique, especially following exercise. However, the impact of the biopsy procedure on redox balance and skeletal muscle damage is not known. Research relying solely on tissue sampling may not, therefore, be clearly representative of physiological conditions as the invasive biopsy procedure could trigger artefactual oxidative stress response in any sample beyond the initial baseline measurement. Analysis of both blood and skeletal muscle would allow for comparisons across the wider literature. It would also allow for direct comparison of the two tissue types’ antioxidant capacity and oxidative stress markers, although a representative, less-invasive method would also be beneficial.

Biopsy procedures are invasive and can often cause soreness and interfere with physical activity on subsequent days. These side effects may be more pronounced in an aged population due to compromised repair and protection of skeletal muscle (Dice, 1993; Fulle et al., 2004). It is, therefore, ethically important to find alternative methods for assessing redox balance and dietary interventions within the skeletal muscle in this population. As already stated, it is unclear if
blood is a suitable alternative for analysis of skeletal muscle redox status or if biopsies are required. However, if skeletal muscle biopsy use can be limited in an aged population and alternative methods can be used in the preliminary stages of research, this would be beneficial to the researchers and participants. A cell culture model may provide a suitable pre-screening tool for the development of a human research protocol and could allow extensive preliminary research to take place. There is a need, therefore, for the development of an appropriate cell culture model that can mimic exercise in skeletal muscle, which also causes changes in the redox balance of the muscle cells. An ageing model in cell culture will allow research to be expanded across a range of ages, in addition to providing comparison of young and aged cells under replicated conditions in cell culture.

1.10 Objectives

The main objectives of this thesis are;

- To assess the impact of multiple skeletal muscle biopsy procedures on redox balance of human blood and skeletal muscle samples
- To compare young and old population responses to HIIE and dietary intervention with the aim of reverting aged tissue redox balance towards those of their younger counterparts
- To develop a cell culture toolkit for the early assessment of dietary and pharmacological interventions, including an ageing and an exercise model.
Chapter 2 - General Methodology
2.1 Introduction

The details outlined in this chapter are indicative of the methods employed across two or more experimental chapters within this thesis. Specific methodologies will be detailed within each chapter.

2.2 Human experimental procedures

Ethical approval for all human based research was approved by the Faculty of Science and Engineering, The University of Hull, UK (Ref: FoSE/FEC/29.10.2015 and approved amendment 04.05.2016).

2.2.1 Anthropometric Measures

Participants body mass was measured using a balance scale (Vogel & Halke, Hamburg Germany) to the nearest 0.1 kg and height was measured using a wall-mounted stadiometer (Holtain Ltd, Crymych, Dyfed) to the nearest 0.1 cm. Participants wore sports kit, including shorts and t-shirt and were asked to remove footwear before measurements were taken.

2.2.2 Resting Heart Rate

Participants were fitted with a heart rate monitor chest strap (Polar, Electro Oy, Finland) which was linked by short-range telemetry to a wristwatch. Participants were asked to remain in a supine position and rest for 5 minutes. Heart rate was measured within the final 15 seconds of each minute of rest and the average value calculated.

2.2.3 Maximal Oxygen Uptake (VO$_{2\text{max}}$)

Following appropriate familiarisation to testing protocol and equipment, participants were given a standardised 5 min warm up at 50 watts (W) on an
electromagnetically-braked cycle ergometer (Daum electronic Gmbh, Furth, Germany). Once the warm-up was complete the participants performed a standard incremental step protocol from 80 W with a gradual increase of 20 W every two minutes. This gives the experience of cycling uphill. The test continued until the individual could not maintain a set cycling speed of over 60 rpm or signalled that they were volitionally exhausted. During the test, the participants were connected to an Oxycon Pro metabolic system (Jaegger, Hoechberg, Germany) which measured oxygen usage every minute, heart rate and breathing flow-rate. The participant’s vital outputs were continuously monitored, and the test was terminated if it was felt they were experiencing any unusual physiological stress in accordance with ACSM guidelines (e.g. chest pain, paleness, leg cramps and severe fatigue) (ACSM, 2017). Once completed, the participants were allowed time to cool down appropriately. VO2max was considered to be achieved when the respiratory exchange ratio was above 1.15 arbitrary units, heart rate was within 10 beats/min-1 of age predicted HR max (220 - age) and a plateau was reached in oxygen uptake and exercise intensity.

2.2.4 Rate of Perceived Exertion (RPE)

The Rate of Perceived Exertion (RPE) scale (Borg, 1982) was used as a reliable and valid measurement to quantify feelings of fatigue and exercise intensity during exercise testing. The scale ranges from 6-20 correlating with a rate of exertion from “No Exertion” to “Maximal Exertion”, respectively. The RPE scale was used throughout the VO2max test and also used during exercise testing to compare subjective feelings of exertion with individual physiological measurements.
2.3 Haematological Measures

2.3.1 Cannula Insertion & Removal

Participants were fasted overnight before blood sample collection, in an attempt to reduce the effect of dietary intake on the metabolic profile, including antioxidant status. Participants assumed a supine position and a tourniquet was applied 6 inches above the selected puncture site of the antecubital vein. The site was prepared with a 70% isopropanol swab (Uhs, Enfield, UK) and left for 30 seconds to air dry. A 20-gauge, single port cannula (Braun Introcan Safety 20 G Closed Catheter, Pennsylvania, USA) was inserted into the selected vein. When blood samples were not being drawn, a mandarin stylet (Braun Vasofix Stylet, Pennsylvania, USA) was inserted into the cannula to prevent coagulation.

On completion of the study, the cannula was removed immediately following the final blood sample collection. Gauze was applied firmly to the puncture site to ensure bleeding had stopped and the area was secured with a sterile dressing.

2.3.2 Blood Sample Collection

Samples were collected into either di-potassium ethylene diamine tetra-acetic acid (EDTA) or serum separation tube (SST) Vacutainers™ (Becton, Dickinson, Oxford, UK). The mandarin stylet was removed from the cannula and the Vacutainers attached to draw blood. After the blood draw was completed, a new stylet was placed into the cannula to prevent coagulation. A 4 ml sample was collected into one EDTA and 4 ml into one SST Vacutainers at each of the eight time points throughout the testing period. Samples were collected at baseline, after each of the three bouts of high intensity exercise and then at 30, 60, 120, and 180 min post-exercise.
Following collection, both tubes were immediately inverted 10 times and placed on ice. SST’s were left to clot in the dark for 10 minutes. All blood samples were centrifuged at 3000 g for 15 minutes and the supernatant removed. The serum and plasma supernatants were stored in duplicate, 1 ml aliquots in cryotubes and stored at -80°C.

2.4 Skeletal Muscle Biopsy Procedure

Skeletal muscle biopsies were taken by three qualified persons using the following procedure, which was overseen and controlled by myself to ensure replication. Three biopsies were taken from participants on each experimental day. The first sample was collected at baseline after an overnight fast, the second sample was taken immediately post-exercise and the final sample was taken at three hours post-exercise. Skeletal muscle biopsies were taken from the medial part of the vastus lateralis using an 11 G, single-use, ACECUT, automatic biopsy system (TSK Laboratory Europe, Oisterwijk, The Netherlands). The puncture site was identified by measuring directly between the anterior superior iliac spine and the top of the patella and marking at the half-way point. The muscle was palpated to ensure adequate muscle mass for sample collection and the area cleaned and shaved. For repeat biopsies, the puncture site was moved 3 cm distally to the previous biopsy site to minimized the influence of previous biopsies on muscle integrity and oxidative damage caused by inflammation (Smith, Kruger, Smith, & Myburgh, 2008).

The biopsy procedure was completed under local anaesthetic (5 ml, 2% Lidocaine, Braun, Melsungen, Germany), which was injected and then massaged into the biopsy area to ensure even distribution. A small incision was made in the participant’s skin with an 11G surgical grade scalpel (Swann-Morton,
Sheffield, UK). The sterile biopsy needle was then inserted perpendicular to the skin and a muscle sample captured. Once the needle was removed, gauze was applied with pressure to the biopsy site to prevent bleeding. The wound was then secured with Steri-strips (3M Steri-strips, St. Paul, USA) and a waterproof dressing securely fixed (MePore, Mölnlycke Healthcare Ltd., Dunstable, UK).

All muscle samples were placed into a cryotube, immediately flash frozen in liquid nitrogen and stored at -80°C until analysis.

2.5 Exercise Protocol

Due to the demands of the research requiring multiple skeletal muscle biopsies, an exercise protocol of shorter duration on a cycle ergometer was deemed most suitable compared to weight bearing modalities.

The exercise protocol used throughout the research was a modified version of the classic 4 x 4 High Intensity Intermittent Exercise (HIIE) protocol as described by Helgerud (Helgerud et al., 2007). Briefly, participants completed a five-minute incremental warm up starting at 50 W and increasing by 25 W each minute. This was followed by four minutes of high intensity exercise at 90 – 95% heart rate max (HR\textsubscript{max}), which was predetermined at their VO\textsubscript{2max} test. This was followed by 3 minutes of lower-intensity exercise at 70% of HR\textsubscript{max} and repeated three times. Participants were connected to an Oxycon Pro metabolic system. Heart rate and measurements of RPE were recorded at one-minute intervals and the resistance of the cycle ergometer was adjusted accordingly to ensure participant’s heart rate remained at the required level. Participants were continually monitored, and the test was terminated if it was felt that they were experiencing any unusual physiological stress.
2.6 Blood biochemical Analysis

2.6.1 Protein Measurement

The Bradford Assay (Bradford, 1976) was used to determine protein concentration to allow for normalisation of fluorescence data. The Bradford assay is a simple colourimetric technique which is highly a reproducible and sensitive way to quantify protein which allows for rapid and automatic processing of a large number of samples. Briefly, plasma and serum samples were defrosted on ice in the dark. A standard curve of bovine serum albumin (BSA) standard stock (20 mg/ml) diluted with phosphate buffered saline (PBS). This was prepared at concentrations of 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.4 mg/ml. Each standard was added in duplicate (5 µl per well). The plasma and serum samples were centrifuged (3000 x g for 5 minutes) and diluted 1:80 in PBS. Duplicates of 5 µl of the diluted sample were added to 195 µl Bradford reagent and incubated for 20 minutes in the dark. The absorbance at 595 nm was determined using a spectrophotometer (BMG Labtech, Ortenberg, Germany) for each sample and standard. A calibration curve of absorbance and protein concentration was plotted using the BSA standards and used to determine the protein content of samples.

2.6.2 Reactive Oxygen Species Detection in whole blood samples

Total ROS production was detected by Liquid Chromatography-Mass Spectrometry (LC-MS). For sample preparation, 200 µl of whole blood was removed from ETDA vacutainers, prior to centrifugation. This was added to DHE (10 µl DHE stock solution and 1730 µl deionised water) and control (10 µl DMSO and 1730 µl deionised water) and incubated for 15 minutes at 37°C. Two volumes
of methanol were added, and the samples were inverted before being stored at -20°C in order to deproteinate them.

2.6.3 Superoxide Detection ($O_2^{-}\cdot$)

Generation of whole blood $O_2^{-}\cdot$ was detected by LC-MS using a modified methodology as previously described in platelets and endothelial cells (Jones et al., 2016; Magwenzi et al., 2015). Protein was removed from the methanolic extracts (prepared in section 2.6.2) by centrifugation at 16,100 x g for 10 minutes at 4°C, with the supernatant retained. The supernatant was evaporated using centrifugal evaporation, without heating, for four hours (GenevacMiVac, Genevac, Ipswitch, UK), and the resulting residue was re-suspended in 60 µL water and 60 µL stabilization solution (33.3% methanol, 5 µmol/L fluorescein internal standard, 0.1 mmol/L diethylenetriaminepentaacetic acid (DTPA) and 5 mmol/L sodium ascorbate), and vortex-mixed before a final centrifugation (1600 x g for 5 minutes). The supernatant was removed and used for LC-MS analysis, detecting the superoxide reaction product, 2-hydroxy-ethidum+ (2-HE+), at an $m/z$ ratio of 330.3 (Shimadzu prominence LC20 quatenary pump and autosampler). Two solvents were used; 0.5% formic acid in water (A) and 0.5% formic acid in methanol (B) to achieve separation at a flow rate of 0.5 ml/min. The percentage of solvent A to solvent B throughout the protocol was as follows; 47% A for 3.35 min, reducing to 15% A by 8 min and remaining at 15% until 11.5 min after which it increases to 47% A until 18.75 min. The internal standard of fluorescein was used at an $m/z$ ratio of 333.0 and retention time of 11.25 minutes. Separation and detection were achieved using a CTO10 column oven (Shimadzu, Kyoto, Japan) set to 40°C, and a C18 column (Zorbax, Agilent Eclipse XDB, Santa Clara, USA).
2.6.4 Serum Lipid Hydroperoxide Detection (FOX assay)

Lipid hydroperoxides were measured spectrophotometrically using the Ferrous Oxidation of Xylenol orange (FOX) assay -1. As described by Jiang (Jiang, Hunt, & Wolff, 1992), this method quantifies the rapid peroxide-mediated oxidation of ferrous iron under acidic conditions and in the presence of Xylenol orange. These conditions lead to the formation of an oxidized iron-Xylenol orange chromophores which is measurable spectrophotometrically at 590 nm. This method is simple, reproducible and sensitive and is applied to lipid extracts of samples. A standard curve of known H$_2$O$_2$ concentrations between 0 – 5 µM was measured for comparison, 20 µl of H$_2$O$_2$ standards were added to 180 µl of FOX assay reagent.

The FOX assay reagent was prepared using an aqueous solution containing 250 µM ammonium ferrous sulphate, 100 µM Xylenol orange, 100 µM sorbitol and 25 mM of sulphuric acid (H$_2$SO$_4$) (all from Sigma-Aldrich Poole, UK). Serum samples were defrosted in the dark and on ice to reduce further oxidation. Once thawed, 2 µl of serum was added to 198 µl FOX reagent, creating a 1:10 dilution, in comparison to the standard curve. The samples were incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 550 nm against the standard curve.

2.6.5 Plasma Superoxide Dismutase Detection (SOD)

Total SOD activity was quantified using a modified SOD assay. A standard curve of SOD active units between 0 and 1 (U) in 500 mM Tris-HCl was measured for comparison, 10 µl of SOD standard was added to 20 µl NBT (Nitro Blue Tetrazolium, VWR International Ltd., Lutterworth, UK) and 170 µl of sample assay buffer.
The sample assay buffer was prepared using 3.4 ml of 500 mM Tris-HCl, 400 µl of 1 mM of DTPA and 400 µl of 20 mM Xanthine, 60 µl of Xanthine oxidase (equating to 0.06 active units per well) (All from VWR International Ltd., Lutterworth, UK). This was added immediately before the sample assay buffer was added to the plate. Plasma samples were defrosted in the dark on ice. Once thawed, samples were inverted 10 times and then centrifuged at 4°C (3000 x g for 5 minutes). Plasma samples were diluted 1:1000 in 500 mM Tris-HCl and 10 µl of the diluted sample was added to 20 µl of NBT and 170 µl of sample assay buffer. Absorbance was measured at 560 nm against the standard curve.

2.6.6 Plasma Protein Carbonyl Detection

Carbonyl groups of oxidized proteins were quantified using a modified spectrophotometric method as described by Mesquita (Mesquita et al., 2014). This method is quicker and reduces protein loss compared with more commonly used methods of detection using 2,4-dinitrophenylhydrazine (DNPH), such as that described by Levine (Levine et al., 1990). The modified method does not require protein precipitation, washing and protein resuspension. Plasma samples were defrosted on ice, in the dark. Once thawed, up to 1 mg protein sample was added to 100 µl of deionised water and 10 µl of 10 mM DNPH solution and incubated in the dark at room temperature for 10 minutes. After incubation, 50 µl of sodium hydroxide was added to each well and incubated for a further 10 minutes. Absorbance was measured at 450 nm against a blank (Magellan, Tecan, Infinite 2000 Pro, Zurich, Switzerland). Samples were normalised for protein content determined by the Bradford Assay (described in section 2.6.1).
2.6.7 Additional serum biochemical analysis

The ABX Pentra 400 autoanalyser (Horiba, Montpellier, France) was used to analyse serum creatine kinase (CK), uric acid (UA) and total cholesterol. Calibration and quality controls were completed prior to analysis in accordance with the manufacturer’s guidelines.

2.7 Muscle sample biochemical analysis

2.7.1 Muscle sample preparation

Skeletal muscle samples were removed from -80°C, weighed then immediately placed on wet ice for mechanical homogenisation (Griffiths tube, 60 mm fine ground section, VWR, International Ltd., Lutterworth, UK). Homogenisation buffer was prepared using an aqueous solution containing 320 mM sucrose, 1 mM EDTA and 10 mM Tris base (all VWR, International Ltd., Lutterworth, UK) at a pH of 7.4 at 4°C. The volume of buffer was calculated as 9 times the weight of the sample. Samples were manually homogenised on wet ice and the homogeniser was pre-cooled in wet ice for 15 minutes prior to homogenisation and the buffer was kept at 4°C. To keep the homogenisation standardized, the pestle was moved up and down in a slow, twisting motion 10 times, ensuring contact with the sample for five seconds for each repeated motion; it was then placed back on ice for two minutes in the dark, and this was repeated three times. The homogenate was then removed by pipette and stored in 1.5 ml cryotubes at -80°C until further analysis. The homogeniser was washed in buffer before homogenisation of each sample.
2.7.2 Protein Measurement

Muscle homogenate protein content was quantified using the Bradford assay as described in section 2.6.1. Muscle homogenates were centrifuged (3000 x g for 5 minutes) and added in duplicates of 2.5 µl to 197.5 µl Bradford reagent and incubated for 20 minutes in the dark.

2.7.3 Muscle Lipid Hydroperoxide Detection (FOX assay)

Muscle homogenate hydroperoxides were measured spectrophotometrically using the Ferrous Oxidation of Xylenol orange (FOX) assay, as described in section 2.6.4. A standard curve of H₂O₂ concentrations between 0 – 5 µM was measured for comparison. 20 µl of H₂O₂ standards was added to 180 µl of FOX assay reagent.

Muscle samples were deproteinated prior to analysis, 50 µl of muscle homogenate was added to 100 µl of 100% methanol and stored at -20°C overnight. The following day, muscle samples were defrosted in the dark on ice to reduce further oxidation. Once thawed, samples were centrifuged (3000 x g for 5 minutes at 4°C) and the supernatant removed. Then, 40 µl of muscle homogenate supernatant was added to 160 µl FOX reagent. The samples were incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 550 nm against the standard curve.

2.7.4 Muscle Protein Carbonyl Detection

Carbonyl groups of oxidized proteins were quantified using a modified spectrophotometric method as described in section 2.6.6. Muscle homogenates were defrosted on ice, in the dark. Once thawed, up to 1 mg protein sample was added to 100 µl of deionised water and 10 µl of 10 mM DNPH in 0.2 M phosphoric
acid solution and incubated in the dark at room temperature for 10 minutes. After
incubation, 50 µl of sodium hydroxide was added to each well and incubated for
a further 10 minutes. Absorbance was measured at 450 nm against a blank
(Magellan, Tecan, Infinite 2000 Pro, Zurich, Switzerland).

2.8 Skeletal muscle cell culture experimental procedures

Cells were cultured under aseptic conditions in a class 2 biosafety cabinet (Esco
Airstream, Singapore). Cells were incubated at 37°C and 5% CO₂ (Thermo
Scientific Heraeus, Leicestershire, UK) throughout the duration of culture. C2C12
mouse C3H muscle myoblasts were obtained from Sigma-Aldrich (Poole, UK).
C2C12 cells are a subclone of a myoblast line from normal adult C3H mouse leg
muscle and are appropriate to measure physiological and biochemical changes
within proliferation and differentiation of skeletal muscle (Yaffe & Saxel, 1977).
Reduction in serum content of cell culture medium induces cell differentiation
from single myoblasts into tubular, multinuclear myotubes (Burattini et al., 2004).

2.8.1 Medium Compositions

Cells were cultured in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM,
4.5 g/l glucose), 15% foetal calf serum (FCS), 1% L-glutamine, 1% penicillin-
streptomycin solution and 0.1% amphotericin B (Amp B) (All from Sigma-Aldrich,
Poole, UK).

Cell differentiation medium contained a reduced FCS content of 2%, but
otherwise remained the same as the standard culture medium detailed above.
Medium was pre-warmed to 37°C in a shaking water bath (Stuart, Bibby-Scientific,
Stone, UK) prior to use, and maintained at 37°C in a warming oven (VWR
International Ltd., Lutterworth, UK) when in use. Cell medium was refreshed every second day to remove waste products from the cell culture medium.

2.8.2 Defrosting of Frozen Cells

The cell ampoule was removed from liquid nitrogen storage and rapidly defrosted to 37°C in a shaking water bath until no ice crystals remained. Cells were transferred to a sterile 50 ml centrifuge tube and 10 ml of growing medium was added, the cells were centrifuged (1600 x g for 5 min), and the supernatant discarded to remove the DMSO from the storage medium. Cells were re-suspended in 2 ml of fresh growing medium and thoroughly pipette-mixed to form a homogenous suspension. Cells were counted (see Cell Count, section 2.8.5) and seeded into T75 corning culture flasks (VWR International Ltd., Lutterworth, UK) at a density of 2000/cm² in accordance with the supplier’s guidelines. Cells were cultured in 8 ml growing medium per T75 flask and incubated at 37°C and 5% CO₂ for 48 to 72 hours until cells reached 70-80% confluence. Cells were passaged in order to develop a stock of cryopreserved C2C12 cells.

2.8.3 Cryopreservation of Cells

C2C12 cell stocks were cryopreserved at passage number 16 (P16) and greater for future use. Cells were treated with trypsin-EDTA and counted (see Passage of Adherent cells, section 2.8.4 and Cell Counting, section 2.8.5). Cells were frozen in 1 ml cryovials at a concentration of 3 x 10⁶ / ml growing medium with the addition of 5% DMSO as a cryopreservative. C2C12 stocks were stored in liquid nitrogen.
**2.8.4 Passage of Adherent Cells**

Purchased cells were on passage 14 (P14) on arrival and are suitable for use up to P60; additional passages may cause resistance to apoptosis (Burattini et al., 2004). Passaging was completed once the cells reached 70-80% confluence to prevent the development of senescence and to allow continual cell growth, in accordance with the supplier’s guidelines (Sigma-Aldrich, Poole, UK).

Growing medium was removed from the flasks and discarded. The flasks were then washed twice with 7 ml of pre-warmed, sterile Phosphate Buffered Saline (PBS) solution (1 tablet added to 200 ml of double distilled water, 0.2 M, pH 7.2) to each flask to remove the growing medium, which can affect the action of trypsin. The PBS was then removed from the flask. The growing surface was covered with 3 ml of Trypsin-EDTA (Sigma-Aldrich, Poole, UK) and incubated at room temperature for 5-10 minutes, until detachment of the cells. Cell detachment was confirmed when viewed under a light microscope at 10 x magnification. Once detachment was confirmed, 6 ml of growing medium was added to each flask and washed down the growing surface to ensure all cells had collected in the suspension. Cell suspensions were collected from the flasks and combined into 50 ml centrifuge tubes and spun at 1600 x g for 5 minutes (MSE, London, UK). Following centrifugation, the supernatant was gently removed, and the cell pellet re-suspended in 1 ml of growing medium per original flask. The cell count was completed using an improved Neubauer haemocytometer (Hawksley Counting Chamber, Lancing, UK) (see Cell Count, 2.8.5) and seeded at 20 x 10^4 cells/ml in T75 corning culture flasks, or seeded into clear bottomed, TC-treated 96-well plates (VWR International Ltd., Lutterworth, UK) at a density of 0.32 x 10^4 cells/well for experimentation.
2.8.5 Cell Count

Once the cells had been re-suspended in growing medium, 50 µl of cell suspension was added to 50 µl of PBS and pipette mixed. 50 µl of the resulting solution was then transferred to a 1.5 ml Eppendorf tube and 50 µl of 0.4% Trypan blue solution (Sigma-Aldrich, Poole, UK) was added to determine cell viability. Once thoroughly mixed, 20 µl of this solution was pipetted under the improved Neubauer haemocytometer cover slip and viewed under a light microscope at 10 x magnification. Dark blue stained cells were deemed non-viable and only white cells were included in the cell count. Cells were counted from the four corner quadrants of the haemocytometer (Figure 2-1).

Figure 2-1 - Diagram of the counting chambers of Neubeur haemacytometer. Cells stained blue were classified as non-viable and not counted. Taken from Player, 2013.
To calculate the required volume of cell suspension for flasks the following equation was used:

**Volume required for cell suspension (ml) = Required cell number/Total cell number**

Flasks were required to be seeded at 20 x 10^4.

To calculate the required volume of cell suspension for 96-well plates:

**Volume required for cell suspension (ml) = (Required cell number x 15)/Total cell number**

96-well plates are seeded at 0.32 x 10^4 cells/cm^2. This equation includes a multiplication of the required cell number by 15 due to 15 ml of cell and growing medium solution was required for a 96-well plate.

2.8.6 *Experimental 96-well plates*

For experimentation, cells were seeded in 96 well plates with 200 µl growing medium per well. Once cells reached 70 - 80% confluence the medium was discarded and replaced with 200 µl per well of differentiation medium (2% FCS). The medium was changed every 24 - 48 hours. Cells reached ~100% confluence within 1-2 days and myotube formation at days 4-6, at which point the cells could be used for experimentation.

2.8.7 *Experimental Flasks*

For experimentation, cells were seeded at 20 x 10^4 cells/cm^2 with 8 ml growing medium. As described in Section 2.8.6, growing medium was replaced with 8 ml

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differentiation medium at 70-80% confluence and used for experimentation once myotubes were formed.

2.9 C2C12 experimental procedures

2.9.1 C2C12 Coenzyme Q10 Treatment

Coenzyme Q10 (Cambridge Biosciences, Cambridge, UK) was dissolved in 100% dimethylformamide (DMF) to form a stock solution which was frozen in 50 µl aliquots. For experimentation, CoQ stock was diluted with growing medium, to reduce the residual solvent to 0.01% v/v DMF and applied to the cells. Fresh treatment was made for each experimental day. During experimentation the control solution was prepared using 2% differentiation medium and 0.01% v/v DMF to account for the presence of the solvent.

2.9.2 C2C12 Biochemical Exercise Mimic

A biochemical exercise mimic treatment was applied to the cells, as previously described by Kurdiova (Kurdiova et al., 2014). The treatment contains 4 µM Forskolin in 0.01% DMSO and 0.5 µM Ionomycin in 0.01% ethanol in 2% growing medium, which has been shown to activate similar pathways as those activated in exercise. Thus, forskolin activates adenyl cyclase which catalases the conversion of ATP to cyclic adenosine monophosphate (cAMP), subsequently causing an increase in cAMP levels. cAMP activates the protein kinase A (PKA) pathway which is a key regulator of metabolism (Mihaylova & Shaw, 2011). Ionomycin is a calcium ionophore and is known to activate calcium and adrenergic signalling (Liu & Hermann, 1978; Morgan & Jacob, 1994), both of which are activated during exercise.
Cell differentiation medium was removed from the flasks and replaced with 8 ml of the biochemical exercise mimic per flask for a variety of exercise protocols, including; continuous, intermittent and consecutive days. To provide a control, flasks seeded at the same time point and from the same passage were treated with 8 ml of a control treatment containing 0.01% DMSO and 0.01% ethanol in 2% differentiation medium for the same time scale as the experimental cells. For the intermittent exercise protocols (further described in section 3.2.1), the biochemical treatment or control treatment medium was removed from the flask and replaced with 8 ml of 2% differentiation medium to wash the cells.

Once the exercise mimic protocols were complete, the treatment or control was removed and washed with 3 ml of either PBS or 100% methanol and removed. Methanol was used for cells being tested for LOOH and PBS for cells tested for PC and SOD. A further 1 ml of either PBS or methanol was added to the flask and cells were scraped from the surface using a 2-position cell scraper (Starstedt, Numbrecht, Germany) into 1.5 ml cryotubes and stored at -80°C until analysis.

2.10 C2C12 biochemical analysis

2.10.1 Lipid Hydroperoxide Detection (FOX assay)

Lipid hydroperoxides were measured spectrophotometrically using the Ferrous Oxidation of Xylenol orange (FOX) assay, as previously described in blood (Section 2.6.4). A standard curve of H₂O₂ concentrations between 0 – 5 µl was prepared, with 20 µl of H₂O₂ solutions added to 180 µl of FOX assay reagent.

The FOX assay reagent was prepared using an aqueous solution containing 250 µM ammonium ferrous sulphate, 100 µM Xylenol orange, 100 µM sorbitol and 25 mM of sulphuric acid (H₂SO₄) (all from Sigma-Aldrich Poole, UK).
Deproteinated cell samples in 100% methanol were defrosted on ice in the dark. Once thawed 20 µl of cell sample was added to 108 µl FOX reagent. The samples were incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 550 nm against the standard curve.

2.10.2 Superoxide Dismutase Detection (SOD)

Total SOD activity was quantified using a modified SOD Assay Kit based on Cayman Chemicals standard kit as previously described in blood (Section 2.6.5). Cell samples in PBS were defrosted in the dark on ice. Once thawed, samples were inverted and then centrifuged at 4°C (3000 x g for 5 minutes). Cell samples were diluted 1:1000 in 500 mM Tris-HCl and 10 µl of the diluted sample was added to 20 µl NBT and 170 µl of sample assay buffer. Absorbance was measured at 560 nm against the standard curve.

2.10.3 Protein Carbonyl Detection

Carbonyl groups of oxidized proteins were quantified using a modified spectrophotometric method as previously described (Section 2.6.6). Cell samples in PBS were defrosted on ice, in the dark. Once thawed, up to 1 mg protein sample was added to 100 µl of deionised water and 10 µl 10 mM DNPH solution and incubated in the dark at room temperature for 10 minutes. After incubation, 50 µl of sodium hydroxide was added to each well and incubated for a further 10 minutes. Absorbance was measured at 450 nm against a blank (Magellan, Tecan, Infinite 2000 Pro, Zurich, Switzerland).

2.10.4 Ageing process

Skeletal muscle myoblasts were passaged until a minimum of P30, but a maximum of P45. Previous research (Pronsato et al., 2013) demonstrated signs
of senescence in C2C12 cells with high passage numbers. Cell cultures of passage ≥30 were classified as ‘old’ and represented by the letter O. To ensure a distinct difference in cell age ‘young’ cells were classified between P16 – P22, and represented by the letter Y.

2.10.5 CoQ dose response

C2C12 myotube cultures were treated with a range of physiologically-relevant CoQ concentrations (1-1000 nM) for 24 h and analysed for total ROS by fluorescence to identify the most appropriate dose for use in the remaining research.

2.11 Statistical Analysis

Statistical analysis was completed using SPSS package – version 25 (Surrey, UK).

The alpha was accepted at P = < 0.05 and indicated by a * with all descriptive data sets expressed as a mean value ± standard deviations. Data were assessed for normal distribution using the Shapiro Wilks test. Outliers were highlighted from box-plots and homogeneity of variance was assessed using Levene’s test. Data were then analysed using an independent t-test for differences between conditions and using repeated measures ANOVA for analysis of differences over time.

Standardised effect size (SE) was calculated using \( \eta^2 \) for the ANOVA analysis and Cohen’s \( d \) for differences between conditions. The scales used for reporting these values are shown in Table 2-1.
Table 2-1 - Scales for defining standardised effect size for $\eta^2$ and Cohen's $d$.  

<table>
<thead>
<tr>
<th></th>
<th>$\eta^2$ (Cohen J, 1992)</th>
<th>Cohen's $d$ (Sawilowsky, 2009)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very small</td>
<td>--</td>
<td>0.1</td>
</tr>
<tr>
<td>Small</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Medium</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Large</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Very Large</td>
<td>--</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Chapters 4 and 5: The effect of exercise and repeated muscle biopsies and CoQ supplementation on markers of oxidative stress and antioxidant response in blood and skeletal muscle were determined using multiple repeated measures ANOVA. The data set was split for analysis, due to small sample sizes, into conditions (exercise vs. rest) and analysis over time, within each condition. Following significant interactions, between group markers were analysed using Bonferroni corrected paired t-tests. Within group differences were analysed using a one-way ANOVA.

Chapter 6: The effect of CoQ on redox balance of young and aged C2C12 skeletal muscle cells following biochemically simulated exercise was determined using Welch’s ANOVA due to violation of the homogeneity assumption required for a classic ANOVA. Following significant interactions, between group markers were analysed using Bonferroni corrected paired t-tests.
Chapter 3 - Optimisation of a biochemically simulated exercise protocol in C2C12 skeletal muscle cells.
CHAPTER 3 – Study 1 – Optimisation of a biochemically simulated exercise protocol in C2C12 skeletal muscle cells.

3.1 Introduction

Cell culture models have successfully been used to optimise research protocols for many years, allowing pilot testing in cells prior to human participant recruitment (Halliwell & Whiteman, 2004). This valuable tool limits the need for time consuming and expensive studies that can be difficult to recruit for in human subjects. A biochemical technique has been developed, which, when applied to skeletal muscle cells, can provide a stimulus similar to that of exercise (Egawa et al., 2008; Kurdiova et al., 2014; Sparks et al., 2011). The mimic contains forskolin and ionomycin. Forskolin activates adenylyl cyclase which catalyses the conversion of ATP to cyclic adenosine triphosphate (cAMP). cAMP is an important regulatory messenger required for cell communication and hormonal feedback. cAMP activates the protein kinase A pathway which regulates of glycogen and lipid metabolism. Ionomycin stimulates an increase in intracellular calcium. Calcium acts as a messenger and as an initiator of contraction within the skeletal muscle cells. Kurdiova (Kurdiova et al., 2014) found a doubling of PGC-1α mRNA following exposure to the mimic. Previous research by Sparks (Sparks et al., 2011) found an increase in mitochondrial volume following treatment with the mimic. These findings are consistent with those from human skeletal muscle following exercise. Further optimisation of this method will allow for exercise protocols to be first examined in cell culture, prior to invasive muscle biopsy research. This approach may be used to help refine the limits of a human experiment prior to recruiting participants. The previous experimental protocol used exposure to the chemical treatment of 1 hr, repeated on three consecutive days (Kurdiova et al., 2014). However, the human experimental model has often
varied in duration, intensity and frequencies of the chosen exercise model causing a lack of consistency within the main research literature (Alessio et al., 2000; Bloomer, Goldfarb, Wideman, McKenzie, & Consitt, 2005). This approach further highlights the need for an effective cell model to help reduce some of the inconsistencies with human based experiments and add additional environmental controls within experiments.

Methods to stress the cells in a simulation of exercise include, but are not limited to, a mechanical stretch method (Ward, Prosser, & Lederer, 2013) or a biochemical application of an exercise mimic (Kurdiova et al., 2014). Mechanical stretch provides a more physically relevant simulation of exercise on the muscle cells. However, this method requires specialist equipment, which can be costly, and the procedures can be very time consuming. Mechanical stretch methods also have a low level of reproducibility due to variations across the stretch surface (Ursekar et al., 2014). A biochemical exercise mimic allows for large quantities of sample to be tested in a short space of time, allowing for greater optimisation of cell culture protocols.

An optimised exercise cell culture model will be beneficial in research on ageing skeletal muscle health and potential pharmacological interventions. The development of a suitable exercise model could allow preliminary testing on skeletal muscle cells without the need for invasive skeletal muscle biopsy procedures. This is of greatest importance for research in vulnerable groups, such as aged or diseased populations.
3.1.1 Objectives:

- Identify a suitable exercise modality using a cellular model of biochemical exercise to elicit the greatest increase in oxidative stress with the potential to translate into human based research.

3.2 Materials and methods

Skeletal muscle cells were cultured under aseptic conditions as described in section 2.8. Cells were seeded at $0.32 \times 10^4$ cells per cm$^2$ in 96 well plates with 200 µl growing medium per well. Once the cells reached 70 - 80% confluence the medium was discarded and replaced with 200 µl per well of differentiation medium (2% FCS). The medium was changed every 24 - 48 hours. The cells reached ~100% confluence within 1-2 days and myotube formation at days 4-6, at which point the cells could be used for experimentation.

3.2.1 C2C12 Biochemical Exercise Mimic

A biochemical exercise mimic treatment was applied to the cells, as previously described (Kurdiova et al., 2014). The treatment contains 4 µM Forskolin in 0.01% DMSO and 0.5 µM Ionomycin in 0.01% ethanol in 2% growing medium, which has been shown to activate signalling pathways similar to those activated in exercise.

3.2.2 Biochemical exercise modality

Cell differentiation medium was removed from the 96-well plates and replaced with 200 µl of the biochemical exercise mimic medium per well for a variety of exercise protocols, including; continuous, intermittent and consecutive days. To provide controls, a column of wells next to each experimental column was treated with 200 µl per well of a control treatment containing 0.01% DMSO and 0.01% ethanol in 2% differentiation medium for the same time scale as the experimental
cells. For the intermittent exercise protocols, the biochemical treatment or control treatment was removed from the wells and replaced with 200 µl of 2% differentiation medium to wash the cells.

The cells were exposed to the exercise mimic under the following conditions; 1 hr per day on three consecutive days (3d x 1h), 1 hr of exposure followed by 1 hr of wash repeated three times on one day (1h x 3), 3 hr continuously on one day (3h), 1 hr continuously on one day (1h) and a 5 min exposure followed by 5 min of wash repeated three times on one day (5 min x 3). These protocols aimed to replicate repeated bouts across three days; two types of continuous exposure and two types of intermittent exposure to mimic five of the more popular exercise training schedules used in human based research.

3.2.3 Biochemical exercise dose

To optimise the dose of biochemical exercise treatment, the original dose (100%) was applied to the cells containing 4 µM Forskolin in 0.01% DMSO and 0.5 µM Ionomycin in 0.01% ethanol and compared with a reduced dose (50%) and a high dose (200%). The dose was assessed under the intermittent exercise mimic protocol 5 min x 3 and each dose was compared to its own control treatment containing 0.01% DMSO and 0.01% ethanol in 2% differentiation medium. The three doses were applied across the same 96-well plate to remove variance between plates. Plate layout was also altered for each experiment.

3.3 C2C12 biochemical analysis
3.3.1 Rate of ROs production by Fluorescence

Once the experimental protocols were complete, all treatment and controls were removed from the plate and washed with 200 µl per well of Hanks’ Balanced Salt Solution HBSS (VWR International Ltd., Lutterworth, UK). This wash was
immediately discarded and replaced with 200 µl per well of 20 µM dihydroethidium (DHE) solution. The solution contained 20 µl DHE (20 mM in DMSO, Sigma-Aldrich, Poole, UK) in 20 ml HBSS. The plate was immediately transferred to a multi-modal plate reader (BMG Labtech, Ortenberg, Germany) and fluorescence was recorded at an excitation wavelength of 544 nm, and an emission wavelength of 590 nm, every 3 minutes minute for 30 minutes.

Data were analysed with MARS Data Analysis Software (BMG Labtech, Ortenberg, Germany), using a kinetic calculation of the blank corrected data per minute, to account for change in fluorescence over the duration of the measurement.

3.3.2 Protein measurement

After completion of DHE analysis, the DHE solution was removed from the plates and 70 µl per well of the cell lysis reagent, Cellytic M (Sigma-Aldrich, Poole, UK) was added and left to incubate at room temperature for 15 minutes. Experimental plates were then transferred to the -20°C freezer for later analysis of protein content.

Experimental 96-well plates were removed from the freezer and warmed to room temperature for 30 minutes. A standard curve of Bovine Serum Albumin (BSA) standard stock (20 mg/ml) (VWR International Ltd., Lutterworth, UK) diluted with Cellytic M. This was prepared at concentrations of 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.4 mg/ml in sterile micro-centrifuge tubes. Each standard was added in duplicate (5 µl per well) to the first and last column of a clear-bottom 96-well plate. The cell lysates were mixed by pipetting, and 5 µl transferred from the lysed plate to the assay plate. To each well, 200 µl of Bradford assay reagent (Sigma-Aldrich, Poole, UK) was added and incubated for 20 minutes in darkness at room
temperature. The absorbance at 595 nm was determined using a spectrophotometer (BMG Labtech, Ortenberg, Germany) for each sample and standard. A calibration curve of absorbance and protein concentration was plotted using the BSA standards and used to determine the protein content of samples.

3.4 Statistical analysis

Data were assessed using SPSS. The alpha was accepted at \( P = < 0.05 \) and indicated by a *, all descriptive data sets are expressed as a mean value ± standard deviations. Standardised effect size (SE) was calculated using Cohen’s \( d \) for differences between conditions.

3.5 Results

To optimise the biochemical exercise mimic protocol, the concentration of the active reagents, and exposure to the stimulus were adapted to replicate different modalities of exercise.

3.5.1 Biochemical exercise modality

Biochemical exercise-mimicking increased ROS production in the intermittent 3 x 5 min (difference in means of 99% compared to control, \( SE = 1.16; \) very large, \( p = 0.154 \)) and continuous 3 hr (difference in means of 26% compared to control, \( SE = 0.66; \) medium) conditions (Figure 3-1), however, these were not significant. There was a decrease in ROS production with the 3 day x 1 h treatment (difference in means of 44% compared to control, \( SE = 2.5; \) very large). Only the 3 day x 1 h treatment was statistically significant (\( p = 0.012 \)). The 3 x 5 min intermittent protocol elicited the greatest increase in total ROS compared with the control, such that this mode of exercise was taken forward for the remaining research. However, it is also interesting to note a significant reduction in ROS
production following the 3-day x 1 hr protocol, which could indicate signs of early adaptive responses within the cells.

3.5.2 Biochemical stimulus dose

The results of the dose response using the 3 x 5 min intermittent exercise modality are shown in Table 3-1. A reduction of the concentration of Forskolin and Ionomyocin to 50% of the original dose (100%) was shown to reduce total ROS production (difference in means of 98% compared to controls, SE = 2.0; Very Large), whereas doubling the dose to 200% showed an increase compared with the original dose (100%), however this was not exponential and showed great variance in response (difference in means of 52% compared to controls, SE = 0.5; Medium). There was a large difference between the 50% and 200% doses (difference in means of 150% compared to controls, SE = 1.8; Very Large). To ensure a balance between an increased stress response and reduced variance, the original, 100% dose, was taken forward for the remaining research using the intermittent, 3 x 5 min, exercise modality.

Table 3-1 - Exercise mimic dose response. Total ROS as a percentage of control which is set to 100%. Values are means ± standard deviations.

<table>
<thead>
<tr>
<th>Percentage change from control (%)</th>
<th>50% dose</th>
<th>100% dose</th>
<th>200% dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>87 ± 10</td>
<td>185 ± 75</td>
<td>237 ± 117</td>
<td></td>
</tr>
</tbody>
</table>
3.6 Discussion

The main aim of the current study was to examine the feasibility of a biochemical exercise protocol in a cell culture model that could potentially translate into human experiments investigating exercise-induced oxidative stress and antioxidant capacity in skeletal muscle.

A suitable exercise protocol to maximise alterations in oxidative stress was identified. From which it was concluded that a previous dose of exercise mimic (Kurdiova et al., 2014) was appropriate in ensuring a reproducible increase in total ROS, detected by fluorescence, under the revised model of intermittent 3 x 5 min exposure. The original concentration, containing 4 µM Forskolin and 0.5
µM ionomycin, resulted in an increase in total ROS production of 85% compared with control, whereas a doubling of the concentration increased total ROS production further, but only slightly. This slight increase in total ROS with a concentration doubling was less reproducible and, the original dose was therefore accepted. However, the previous research used an application of the exercise mimic for only 1 hour a day, repeated for three days. Research in the current study suggests that this may not be the most effective modality for increasing oxidative stress in C2C12 cells, and that an intermittent protocol elicits a greater ROS response.

Human based experiments have yet to determine fully whether exercise frequency, intensity or duration is the major contributing factor in terms of increased oxidative stress. It is likely that a combination of all three factors plays a key role. One of the key questions before progressing to interventions-based experiments is the type of exercise that produces the highest and potentially most reproducible oxidative stress response. It was found that while moderate bouts of exposure had limited impact on total ROS output, a short, intermittent protocol of 3 x 5 min exposure increased total ROS production. High Intensity Intermittent Exercise (HIIE) has received a great deal of interest recently due to the promising nature of this style of exercise in providing beneficial adaptations to training (Wadley, Chen, Lip, Fisher, & Aldred, 2016), while remaining relatively time-efficient. It is well understood that exercise needs to be of high intensity (Bailey et al., 2004; Fogarty et al., 2011; Lawler et al., 1994), or of long duration (Bloomer, 2008; Bloomer et al., 2005), to elicit an oxidative stress response. HIIE provides high intensity, but due to its short duration, is more sustainable to maintain high intensity for a longer time. It is interesting to see that the cell culture model reflects this conclusion, since short, but repeated, exposure to an exercise
stimulus results in an increased oxidative stress response. This may make the intermittent protocol as the most appropriate modality of exercise to elicit the greatest oxidative stress response in future research.

Interestingly, it was also found that 1 hour exposure to the exercise mimic, repeated each day for three days caused a significant reduction in total ROS production. It is possible to speculate that this could potentially reflect early signs of adaptation to training. These initial results show promise for further research to investigate the impact of repeated bouts of biochemical exercise mimic exposure on adaptations to training in this cell culture model. This finding is consistent with research where repeated exercise bouts on consecutive days resulted in a modified redox response in rodents (Gomez-Cabrera et al., 2005; Vincent et al., 2000) and in humans (Egan & Zierath, 2013; Gibala, Little, Macdonald, & Hawley, 2012).

3.7 Conclusions

In the present study a cell culture model of biochemical exercise stimulus was evaluated. It was concluded that this protocol provides a structured toolkit for use in future research to investigate the impact of dietary and pharmacological agents on skeletal muscle, without the need for skeletal muscle biopsies. The potential for further research into the impact of repeated exposures to the exercise mimic as a possible model of training adaptations in cell culture was also identified.
Chapter 4 – The effect of HIIE on redox balance in blood and skeletal muscle.
CHAPTER 4 - Study 2 – The effect of HIIE on redox balance in blood and skeletal muscle.

4.1 Introduction

Skeletal muscle biopsies have previously been used in an attempt to determine redox status of exercising muscle (Burgomaster, Hughes, Heigenhauser, Bradwell, & Gibala, 2005; Cipryan, 2017; Cooke et al., 2008; Devries et al., 2008; Radák et al., 2003; Sahlin et al., 2010). However, there is limited research concerning the impact of the procedure itself or if muscle biopsies contribute to oxidative stress and inflammation, above that caused by exercise (Fogarty et al., 2013). A comparison between muscle biopsies taken under exercise and resting conditions is therefore required.

Although muscle biopsies are accepted as a gold standard for assessing muscle tissue directly, investigators have traditionally relied on analysis of blood samples to quantify the redox response to exercise (Fisher-Wellman & Bloomer, 2009; Souza-Silva et al., 2016; Wadley, Chen, et al., 2016). However, it is currently unclear if there is a correlation between the two tissue types. Only a limited number of studies have compared the same markers of redox status in both blood and skeletal muscle tissue samples (Child et al., 1999; Fogarty et al., 2013; Meydani et al., 1993; Parker, Trewin, Levinger, Shaw, & Stepto, 2018). Therefore, it is not yet known if blood based markers are reflective of muscle oxidative stress or if blood is a viable pathway for free radical generation in its own right (Nikolaidis et al., 2008).

To elicit an appropriate level of exercise-induced oxidative stress, it is accepted that exercise needs to be of either very high intensity (Bailey et al., 2004; Fogarty
et al., 2011; Lawler et al., 1994) or of sufficient duration (Bloomer, 2008), however, both of these approaches have their limitations. Traditional high intensity protocols, such as the Wingate test or a VO$_{2\text{max}}$ test allow for little control over relative intensity of the exercise between subjects, and ultra-endurance exercise can prove to be time-consuming in a research setting. Research has therefore, shifted towards high intensity intermittent exercise (HIIE) as an alternative to the two traditional models of exercise testing. This method allows for repeated bouts of high intensity exercise, producing an overall high output (Milanović, Sporiš, & Weston, 2015) in a shorter amount of time with a greater level of control over relative intensity. In Chapter 3 it was found that a 3 x 5 min exposure to a biochemical exercise mimic elicited the greatest increase in ROS production in comparison to continuous exposure or multiple exposures completed on subsequent days. Therefore the work from Chapter 3 has been redesigned to provide an exercise protocol to elicit the best chance of increasing oxidative stress.

4.1.1 Objectives:

The objectives of this study were:

- To quantify the impact of multiple skeletal muscle biopsies on markers of muscle damage, oxidative stress and antioxidant status following resting and exercising conditions.
- To assess the relationship between measurements of oxidative stress and antioxidant capacity in blood and tissue samples.
4.2 Methodology

4.2.1 Human subjects and experimental design

Nine (n=9) healthy, young (28 ± 4 years) males volunteered to participate in the cross-over study. All subjects completed a medical history questionnaire and consent forms (see appendices A and B) prior to participation. Participants were recreationally active, participating in a minimum of 3 recreational sessions per week, non-smokers and not taking any dietary supplements or medication. Ethical approval for all human based research was given by the Faculty of Science and Engineering, The University of Hull, UK (Ref: FoSE/FEC/29.10.2015).

4.2.2 Preliminary testing

Prior to experimental testing, anthropometric data was collected as described in section 2.2.1, and the results are shown in Table 4-1. A cycle ergometer test was deemed most appropriate to reduce the amount of weight bearing on the biopsied limb during exercise. Additionally, in comparison to running, cycling causes less muscle damage and a reduced amount of inflammation due to the modality of the exercise, which may interfere with ROS production.

Participants performed an incremental exercise test to exhaustion to elicit VO\textsubscript{2max}, as described in section 2.2.3. Participants returned to the laboratory for a familiarisation session and to assess the suitability of the HIIE protocol. The HIIE protocol required an average heart rate of 90 – 95% during three high intensity sections and for the average heart rate to drop to 70% during the lower intensity sections. The resistance of the cycle was programmed based on the heart rate achieved throughout the previously completed VO\textsubscript{2max}. Thus, if during the familiarisation session the participant’s heart rate did not fall within these
ranges, the resistance was gradually adjusted until the heart rate was within range. The adjusted resistance for high and lower intensity sections were then programed into the cycle ergometer for the main trials.

Table 4-1 Anthropometric data (n = 9). All values are means ± standard deviation.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>Body Mass (kg)</td>
<td>83 ± 14</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>181 ± 9</td>
</tr>
<tr>
<td>BMI (kg.cm(^{-2}))</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>(\text{VO}_{2\text{max}}) (ml.kg(^{-1}).min(^{-1}))</td>
<td>40 ± 8</td>
</tr>
<tr>
<td>Heart Rate Max (bpm)</td>
<td>178 ± 11</td>
</tr>
</tbody>
</table>

4.2.3 Experimental testing

Participants were instructed to refrain from strenuous exercise and not to consume alcohol or caffeine for 48 hours prior to testing. They reported to the laboratory after an overnight fast, although water could be consumed \textit{ad libitum}. Biometric data was collected, and a cannula fitted as described in section 2.3.1. Baseline blood and muscle samples were collected and processed as described in sections 2.3.2 and 2.4. Participants completed either a HIIE cycle ergometer protocol or remained rested in a supine position for a total of 23 minutes. During the HIIE protocol, participants completed a five-minute incremental warm-up starting at 50W, this was followed by four minutes of high intensity exercise at 90–95% heart rate max (HR\(_{\text{max}}\)), predetermined at their \(\text{VO}_{2\text{max}}\) test and familiarisation session. This was followed by 3 minutes of lower-intensity, recovery exercise at 70% of HR\(_{\text{max}}\), each set was repeated a total of three times. The structure of the HIIE protocol was based on previous research within our laboratory, which showed a greatest increase in ROS production following a 3 x 5 min intermittent protocol in C2C12 muscle cells. The high intensity sessions
were reduced compared to our cell protocol to improve completion rates by participants.

Following either the exercise protocol or rest period, participants were asked to remain within the laboratory for a further three hours, during which they could move freely and consume water as required. Participants returned for the cross-over condition a minimum of 6 weeks later to allow for a wash out period from the exercise and for skeletal muscle to recover. A schematic overview of the experimental day is outlined in Figure 4-1.

![Figure 4-1 - Main trial schematic. Where ▼ represents a muscle biopsy capture and ▼ represents a blood draw.](image)

**4.2.4 Haematology & skeletal muscle samples**

Blood was drawn from the cannula at baseline, after each bout of high intensity exercise then at 30, 60, 120 and 150 minutes post-exercise. Blood samples were collected into serum separation tubes (SST) for serum analysis and EDTA vacutainers for plasma analysis, inverted and stored on ice for further analysis.

Skeletal muscle biopsies were collected at baseline, immediately post-exercise and at 180 minutes post-exercise, using the protocol described in section 2.4
4.2.5 Biochemical analysis
4.2.5.1 Protein quantification

The Bradford Assay (Bradford, 1976) was used to determine protein concentration to allow for normalisation of data (see section 2.6.1).

4.2.5.2 Total reactive oxygen species (ROS)

Whole blood total ROS production was detected by fluorescence of DHE using the method of Kearney (Kearney et al., 2015) (see section 2.6.2).

4.2.5.3 Lipid hydroperoxides (LOOH)

Serum and skeletal muscle homogenate LOOH were measured spectrophotometrically using the Ferrous Oxidation of Xylenol orange (FOX) assay (see sections 2.6.4 and 2.7.3).

4.2.5.4 Protein Carbonyls (PC)

Plasma and skeletal muscle homogenate PC were quantified spectrophotometrically using the method described by Mesquita (Mesquita et al., 2014) (see sections 2.6.6 and 2.7.4).

4.2.5.5 Superoxide dismutase (SOD)

Total plasma SOD activity was quantified using xanthine oxidase as a ROS-generating system and NBT as a ROS detector to assess SOD activity against a standard curve of bovine SOD (see section 2.6.5).

4.2.5.6 Additional biochemical analysis

The ABX Pentra 400 auto-analyser (Horiba, Montpellier, France) was used to analyse serum creatine kinase (CK) and uric acid (UA). Calibration and quality
controls were completed prior to analysis in accordance with the manufacturer’s guidelines.

4.2.6 Statistical analysis

Data were assessed using SPSS as described in section 2.11. The alpha was accepted at \( P = < 0.05 \) and indicated by a *, all descriptive data sets are expressed as a mean value ± standard deviations. Standardised effect size (SE) was calculated using \( \eta^2 \) for the ANOVA analysis and Cohen’s \( d \) for differences between conditions.

4.3 Results

Of the nine participants, all completed the rest condition and seven completed the exercise condition, with all seven successfully finishing the HIIE protocol. Average participant heart rate percentages and RPE scores for the exercise trial and average resting heart rate percentages are shown in Table 4-2.

Table 4-2 - Mean participant RPE scores and percentage heart rates for high and low intensity exercise and mean resting heart rate percentages. All heart rates are displayed as a percentage of predetermined HR\(_{\text{max}}\). Results are displayed as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Exercise RPE</th>
<th>Exercise HR (% of HR(_{\text{max}}))</th>
<th>Resting HR (% HR(_{\text{max}}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Intensity</td>
<td>16 ± 2</td>
<td>91 ± 3</td>
<td></td>
</tr>
<tr>
<td>Low Intensity</td>
<td>11 ± 3</td>
<td>72 ± 4</td>
<td>33 ± 7</td>
</tr>
</tbody>
</table>

4.3.1 Blood markers of oxidative stress (PC, LOOH and total ROS).

To determine if the exercise protocol induced oxidative stress in blood, and to assess the impact of the biopsy procedure upon oxidative stress in blood, PC, LOOH and total ROS were assessed. To assess the impact on antioxidant capacity in blood, SOD and UA were analysed.
4.3.1.1 Plasma PC

4.3.1.1.1 Resting conditions

Resting conditions plasma PC appear to decline slightly over the duration of the testing protocol (Table 4-3) and there were no significant differences over the time course of the study, a large effect size was found between the Baseline and 120 minutes post-exercise (SE = 1.10) and 180 minutes post-exercise (SE = 1.15). Under resting conditions, LOOH concentration remained relatively stable (Table 4-4), with only a slight increase seen at time point 3, which coincides with the post-exercise draw. Compared with baseline, this was not significant, however there was a medium effect ($p = 0.28$, $SE = 0.65$). Total ROS concentration did not change from resting conditions ($p = 1.00$), however a very large effect size was seen between baseline and 30 min ($SE = 2.08$) and 180 min ($SE = 1.23$). In regards to antioxidant response, under resting conditions, plasma SOD activity gradually declined throughout the testing period until time point 5, 30 min post-exercise equivalent, although this was not significantly different to baseline ($p = 0.07$, $SE = 1.29$; very large) (Table 4-7). Serum UA displayed no change under resting conditions (Table 4-8). These findings suggest that the biopsy procedure had little to no impact on the markers of oxidative stress and antioxidant capacity.

4.3.1.1.2 Exercise conditions

Under exercising conditions, PC concentration appears to reduce at the cessation of exercise and remain reduced over time, however this reduction is not significant and produces only a small effect size at 0 min, 30 min and 120 min post-exercise ($SE = 0.48$, 0.44 and 0.48, respectively) and a medium effect at 60
min and 180 min post-exercise (SE = 0.63 and 0.55, respectively) (Table 4-3). When PC concentrations are compared between the two conditions, there is no significant difference at any time point, although a large effect size is seen at 120 min post-exercise between the exercise and rest conditions, with the PC concentration being higher under exercising conditions.
Table 4-3 Plasma protein carbonyl concentration at baseline, during either exercise or resting conditions and throughout 180 minutes of recovery time. Values are means ± standard deviations. Protein carbonyls are expressed as µg/ mg total protein. Ex 1 = end of first exercise bout and Ex2 = end of second exercise bout or equivalent time under resting conditions. Time is expressed in minutes starting from the cessation of the final exercise bout or equivalent time under resting conditions.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ex 1</th>
<th>Ex2</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise</td>
<td>10.8 ± 9.2</td>
<td>11.4 ± 6.7</td>
<td>10.4 ± 6.8</td>
<td>7.6 ± 2.0</td>
<td>7.8 ± 2.7</td>
<td>5.6 ± 3.4</td>
<td>6.4 ± 3.7</td>
<td>7.0 ± 3.2</td>
</tr>
<tr>
<td>Rest</td>
<td>8.7 ± 3.6</td>
<td>8.9 ± 5.8</td>
<td>8.0 ± 3.9</td>
<td>8.4 ± 3.8</td>
<td>7.4 ± 4.0</td>
<td>6.9 ± 4.3</td>
<td>5.1 ± 2.9</td>
<td>5.3 ± 2.0</td>
</tr>
</tbody>
</table>
4.3.1.2 Serum LOOH

Under exercising conditions there was a non-significant, but slight increase in LOOH at the onset of exercise and values remained slightly elevated until cessation of exercise, where it returned close to baseline levels (Table 4-4). At 120 min and 180 min post-exercise the LOOH concentration began to increase again, this was not significantly higher than baseline, but produced a large and very large effect size, respectively (120 min; $p = 0.19$, SE = 1.04, 180 min; $p = 0.13$, SE = 1.41). All other time points were not significantly different from baseline.

When comparing the two conditions, the exercising condition had a higher concentration of LOOH compared to the rest group across all time points, although this was not significant ($p = 0.90$, SE = 0.45; small). The greatest difference at individual time points between the two conditions was seen after the first HIGH intensity exercise, where the exercising condition had a significantly higher LOOH concentration compared with resting conditions ($p < 0.01$, SE = 2.07; very large). The exercising group also had higher LOOH concentrations compared with the rest group after the second high intensity exercise ($p = 0.06$, SE = 1.12; large) and at 180 min post-exercise ($p = 0.08$, SE = 1.74; very large).

4.3.1.3 Whole blood total ROS

The percentage change from baseline in whole blood total ROS is shown in Table 4-5. Results are highly variable between participants at all time points under both conditions. Under exercising conditions there were no changes seen compared with baseline. No significant difference was found between the two conditions, however there was a large effect size seen between the two conditions at the 30 min time point (SE = 0.82).
Table 4-4 Serum lipid hydroperoxides concentration at baseline, during either exercise or resting conditions and throughout 180 minutes of recovery time. Values are means ± standard deviations. Lipid hydroperoxides are expressed as mmol.L⁻¹. Ex 1 = end of first exercise bout and Ex2 = end of second exercise bout or equivalent time under resting conditions. Time is expressed in minutes starting from the cessation of the final exercise bout or equivalent time under resting conditions. The alpha is accepted at p = 0.05 and displayed as a * if different from baseline and a # when different between conditions.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ex 1</th>
<th>Ex2</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise</td>
<td>37.1 ± 18.7</td>
<td>54.0 ± 23.4#</td>
<td>28.0 ± 21.9</td>
<td>23.6 ± 15.2</td>
<td>24.2 ± 19.8</td>
<td>29.5 ± 16.1</td>
<td>54.6 ± 14.8</td>
<td>59.9 ± 13.1</td>
</tr>
<tr>
<td>Rest</td>
<td>23.0 ± 14.9</td>
<td>17.1 ± 9.4#</td>
<td>10.3 ± 4.3</td>
<td>37.0 ± 26.8</td>
<td>28.8 ± 16.4</td>
<td>32.2 ± 25.6</td>
<td>32.5 ± 35.1</td>
<td>32.1 ± 18.4</td>
</tr>
</tbody>
</table>

Table 4-5 Whole blood total ROS percentage change from baseline under either exercise or resting conditions and throughout 180 minutes of recovery time. Values are means ± standard deviations. Results are expressed as a percentage (%) change from baseline value, which is set to 100%. Ex 1 = end of first exercise bout and Ex2 = end of second exercise bout or equivalent time under resting conditions. Time is expressed in minutes starting from the cessation of the final exercise bout or equivalent time under resting conditions.

<table>
<thead>
<tr>
<th></th>
<th>Ex 1</th>
<th>Ex2</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise</td>
<td>87.7 ± 67.3</td>
<td>150 ± 141</td>
<td>66.9 ± 28.4</td>
<td>132 ± 100</td>
<td>156 ± 160</td>
<td>131 ± 90.5</td>
<td>78.9 ± 47.6</td>
</tr>
<tr>
<td>Rest</td>
<td>145 ± 76.4</td>
<td>115 ± 65.1</td>
<td>102 ± 78.8</td>
<td>67.2 ± 24.5</td>
<td>152 ± 101</td>
<td>106 ± 66.9</td>
<td>73.6 ± 27.1</td>
</tr>
</tbody>
</table>
4.3.2 Muscle markers of oxidative stress (PC and LOOH)

4.3.2.1 Muscle PC

Muscle PC content is shown in Figure 4-2. Under resting conditions, PC concentration in skeletal muscle appeared to increase slightly at the 180 min post-exercise time point compared with baseline, however this was not significant ($p = 0.63$, SE = 0.24; small, 110% of baseline measurement). Under exercising conditions, muscle PC concentrations were lower at baseline compared with rest (Exercise baseline was 69% of rest baseline) and showed a similar upward trend towards the 180 min post-exercise time point, however, this also was not significant ($p = 0.25$, SE = 0.65; medium, 137% of baseline measurement). When the two conditions were compared, the exercise group was lower across all time points ($p = 0.04$, SE = 0.65; medium). The greatest difference between the two conditions was immediately post-exercise, however this only approached significance ($p = 0.07$, SE = 0.98; large).
Figure 4-2 Muscle protein carbonyl concentration at baseline, immediately post exercise and 3 hours post exercise or equivalent time under resting conditions. Values are means ± standard deviations. Protein carbonyls are expressed µg/ mg total protein. Note; Ex = Exercise.
4.3.2.2 Muscle LOOH

Muscle LOOH concentration is presented in Figure 4-3. The data show a slight increase in LOOH concentrations immediately after and 3 hours into recovery in the exercise condition, although this increase is not significant ($p = >0.05$, SE = 0.01 and 0.55, respectively). Concentrations remained similar throughout the resting condition. When the two conditions are compared there are no significant changes between the conditions at any time point, however, there is a large effect size between the exercise and rest muscle LOOH concentration at the 180 min time point ($p = 0.12$, SE = 1.02).
Figure 4-3 Muscle lipid hydroperoxides concentration at baseline, immediately post-exercise and three hours post exercise, or the equivalent times under resting conditions. Values are means ± standard deviations. Lipid hydroperoxides are expressed as mmol.L⁻¹.
In summary, across multiple oxidative stress markers blood assays showed a high level of variation between individuals and time points, which makes interpretation of the results very challenging. Muscle markers were a little more consistent, however they still provided variability between individuals. The overall trend at each time point between the resting and exercise conditions are shown for blood and muscle in Table 4-6. Exercise increased LOOH in both blood and skeletal muscle, with a 44% increase above baseline at 180 minutes in muscle and 47% above baseline at 120 min and 61% above baseline at 180 minutes in blood.

Table 4-6 - Summary of oxidative stress response in blood and muscle between resting and exercise conditions. Where -- represents no change, ↑ represents an increase from resting and ↓ represents a decrease from resting conditions. B = Baseline, Ex1 = after the first high intensity exercise bout, Ex 2 = after the second high intensity exercise bout, 0 – 180 represent minutes into recovery.

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>Ex1</th>
<th>Ex2</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
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<tbody>
<tr>
<td>Plasma PC</td>
<td>--</td>
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<td>Serum LOOH</td>
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<tr>
<td>Whole blood ROS</td>
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<td>↑</td>
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</tr>
<tr>
<td>Muscle PC</td>
<td>↓</td>
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<td>Muscle LOOH</td>
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</tbody>
</table>

4.3.3 Antioxidants (SOD, UA)

4.3.3.1 Plasma SOD

Under both exercise and rest conditions there was great variability in SOD results (Table 4-7). During the exercise protocol SOD activity appeared to increase, then it dropped below baseline at 30 min post exercise ($p = 0.04, \text{SE} = 3.61$; very large) and remained slightly reduced for the remainder of the protocol. When exercise and resting conditions were compared there were no significant differences between the two conditions mean across all time points. However, the exercise condition showed greater fluctuation in SOD concentration across the protocol, whereas under resting conditions SOD concentration remained relatively
unchanged. At time point 3, after the second HIGH intensity bout of exercise, there was a significant difference seen in SOD activity between the two conditions ($p = 0.04$, $SE = 1.60$; very large) with the exercise condition being higher. No other significant differences were found at any other time points.

Unfortunately, due to the size of sample collected from the skeletal muscle biopsies, we were unable to complete SOD analysis in muscle.

4.3.3.2 Serum UA

Under exercising conditions UA concentration significantly increased at 30 min post-exercise ($p = 0.03$, $SE = 1.52$, very large) compared with baseline, and remained elevated for the remainder of the testing period compared with baseline; at 60 min ($p = 0.19$, $SE = 0.84$; large), 120 min ($p = 0.06$, $SE = 1.27$, very large) and at 180 min post-exercise ($p = 0.24$, $SE = 0.72$, medium), however these changes were not significant (Table 4-8). When comparing the two conditions across all time points there was no significant difference. However, at 30 min post exercise the exercise group had significantly higher UA concentration when compared to the resting group ($p = 0.04$, $SE = 1.51$; very large), the exercise group also had higher UA concentration at the 120 min post exercise time point, however this only approached significance ($p = 0.07$, $SE = 1.50$; very large).
Table 4-7 Plasma SOD activity at baseline, during either exercise or resting conditions and throughout 180 minutes of recovery time. Values are means ± standard deviations. SOD activity is expressed as U/µl. Ex 1 = end of first exercise bout and Ex2 = end of second exercise bout or equivalent time under resting conditions. Time is expressed in minutes starting from the cessation of the final exercise bout or equivalent time under resting conditions.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ex 1</th>
<th>Ex2</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise</td>
<td>1.8 ± 0.4</td>
<td>1.1 ± 0.6</td>
<td>3.3 ± 2.0</td>
<td>2.0 ± 1.3</td>
<td>0.9 ± 0.1</td>
<td>1.2 ± 0.6</td>
<td>1.0 ± 0.5</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>Rest</td>
<td>1.7 ± 0.9</td>
<td>1.5 ± 0.3</td>
<td>1.0 ± 0.4</td>
<td>0.9 ± 0.2</td>
<td>0.8 ± 0.4</td>
<td>1.7 ± 1.1</td>
<td>1.7 ± 0.5</td>
<td>1.3 ± 0.4</td>
</tr>
</tbody>
</table>

Table 4-8 Serum uric acid concentration at baseline, during either exercise or resting conditions and throughout 180 minutes of recovery time. Values are means ± standard deviations. UA concentration is expressed as µmol/L. Ex 1 = end of first exercise bout and Ex2 = end of second exercise bout or equivalent time under resting conditions. Time is expressed in minutes starting from the cessation of the final exercise bout or equivalent time under resting conditions. The alpha is accepted at p = 0.05 and displayed as a * if different from baseline and a # when different between conditions.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ex 1</th>
<th>Ex2</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise</td>
<td>295 ± 57.2</td>
<td>300 ± 59.0</td>
<td>299 ± 68.9</td>
<td>312 ± 65.9</td>
<td>377 ± 50.8*#</td>
<td>353 ± 78.4</td>
<td>380 ± 75.1</td>
<td>350 ± 90.5</td>
</tr>
<tr>
<td>Rest</td>
<td>304 ± 66.2</td>
<td>323 ± 58.2</td>
<td>333 ± 68.6</td>
<td>328 ± 52.8</td>
<td>306 ± 42.4#</td>
<td>288 ± 42.7</td>
<td>289 ± 42.9</td>
<td>288 ± 35.0</td>
</tr>
</tbody>
</table>
4.3.4 Creatine Kinase (CK)

Serum samples were analysed for CK concentrations and are displayed in Table 4-9. CK is widely used as a biochemical marker of muscle damage (Brancaccio, Lippi, & Maffulli, 2010). Under resting conditions CK remained relatively stable with only a slight increase at time point four in the blood draw immediately post-exercise. This increase was not statistically significant, but had a very large effect ($p = 0.06, \ SE = 1.47$). Under exercise conditions CK was more variable. Initially CK concentrations gradually increased over the duration of the exercise protocol, then began to decline post-exercise below baseline. There were no significant changes from baseline at any time points under exercising conditions, however a large effect was seen at 60 min post exercise compared with baseline ($p = 0.20, \ SE = 0.87$).

When the two conditions were compared there was a significant difference between the two conditions across all time points, with the exercising group showing higher CK concentrations ($p = <0.01, \ SE = 0.99; \text{large}$). The greatest difference was seen at time point 3 for the blood drawn after the second HIGH intensity period, although this was not significant, there was a very large effect size ($SE = 1.24$). Large effect sizes were found between the conditions at baseline ($SE = 1.11$), after the first HIGH intensity bout ($SE = 1.11$), and at 0 min, 30 min and 180 min post-exercise ($SE = 0.85, \ 1.18 \text{ and } 0.83$, respectively).
Table 4-9 Serum creatine kinase concentration at baseline, during either exercise or resting conditions and throughout 180 minutes of recovery time. Values are means ± standard deviations. CK concentration is expressed as U/L. Ex 1 = end of first exercise bout and Ex2 = end of second exercise bout or equivalent time under resting conditions. Time is expressed in minutes starting from the cessation of the final exercise bout or equivalent time under resting conditions.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ex 1</th>
<th>Ex2</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise</td>
<td>111 ± 54.7</td>
<td>120 ± 66.5</td>
<td>137 ± 72.7</td>
<td>138 ± 80.0</td>
<td>118 ± 57.1</td>
<td>75.3 ± 17.2</td>
<td>102 ± 49.7</td>
<td>97.1 ± 46.8</td>
</tr>
<tr>
<td>Rest</td>
<td>67.1 ± 9.2</td>
<td>67.5 ± 11.1</td>
<td>73.3 ± 9.2</td>
<td>88.6 ± 18.6</td>
<td>68.5 ± 13.5</td>
<td>69.4 ± 1.5</td>
<td>66.8 ± 8.9</td>
<td>68.5 ± 14.0</td>
</tr>
</tbody>
</table>
In summary, it was found that antioxidant capacity in blood increased in response to the HIIE protocol (Table 4-10). It was also found that muscle damage, represented by CK concentration, was elevated in the exercise condition compared with the resting condition, which is as expected based on previous findings (Wiewelhove et al., 2016).

Table 4-10 - Summary of antioxidant response and muscle damage in blood and muscle under exercising conditions, compared with resting conditions. Where -- represents no change, ↑ represents an increase from resting and ↓ represents a decrease from resting conditions. B = Baseline, Ex1 = after the first high intensity exercise bout, Ex 2 = after the second high intensity exercise bout, 0 – 180 represent minutes into recovery. The alpha is accepted at p = 0.05 and displayed as a * if different from baseline and a # when different between conditions.

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>Ex1</th>
<th>Ex2</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
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<tbody>
<tr>
<td>Plasma SOD</td>
<td>--</td>
<td>--</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>Serum UA</td>
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<tr>
<td>CK</td>
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4.4 Discussion

The main aims of this chapter were to assess the impact of the skeletal muscle biopsy procedure on markers of oxidative stress and antioxidant capacity in blood and muscle, and then to assess the relationship between the two tissue types within this analysis. Additionally, the aim was to show that the HIIE protocol induced oxidative stress changes. Firstly, it was found that the biopsy procedures had little, to no impact on the measured outcomes of oxidative damage (plasma PC; Table 4-3, whole blood total ROS; Table 4-5, Serum LOOH; Table 4-4), antioxidant defence (SOD; Table 4-7 and UA; Table 4-8) and muscle damage (CK; Table 4-9). This suggests that the procedure is a suitable method for tissue collection for research into oxidative stress and antioxidant capacity, even when multiple procedures are completed. Secondly, the results show that the HIIE protocol was of a sufficient intensity to cause some oxidative stress, a change in antioxidant capacity and a potential increase in skeletal muscle damage in young,
healthy males. This provides a suitable exercise protocol for use in future studies within this field. Additionally, it was found that blood and muscle tissues did not always provide correlations between muscle observations and blood. Therefore, the use of skeletal muscle biopsies alongside blood sampling, is vital for obtaining insight into the response of this tissue to exercise-induced oxidative stress.

4.4.1 Skeletal biopsy impact

To understand the impact of the biopsy procedure itself markers of redox status and muscle damage were measured following repeated muscle biopsies under resting and exercise conditions. In resting conditions there appeared to be little or no immediate impact of the muscle biopsy on either muscle or plasma PC. Plasma PC tended to decline over the duration of the resting condition testing period, although this was not statistically significant. There also appeared to be a large effect over time when the baseline measurements were compared with the 120 min and 180 min samples. It may be hypothesised that this may be due to the participants reporting to the lab after a 12 hour fast and remaining fasted for the duration of the testing protocol. This is supported by the data of Asadi (Asadi et al., 2015) who found that serum PC concentration significantly reduced following two days of Ramadan fasting in CVD patients. A reduced PC content in blood may, therefore, be representative of a protective effect of acute fasting on the oxidative damage of proteins. No difference was found across any time points in muscle PC concentrations.

Serum and muscle LOOH concentrations both showed high inter-individual variability under resting conditions, with a general trend to stay close to baseline levels following the biopsy procedures. This suggests that the biopsy had a limited impact upon LOOH in the muscle tissue and in systemic blood samples.
No difference was detected from baseline at any time-point under resting conditions in serum UA and CK concentrations.

The biopsy had little impact on the variables measured, suggesting that the assessment of oxidative stress and antioxidant status following exercise is representative of exercise-related changes, rather than an artefact caused by the biopsy procedure.

**4.4.2 HIIE protocol suitability**

The modified HIIE protocol elicited an increase in some oxidative stress markers and alterations in antioxidant protection when compared to the rested state. It is well established that exercise of sufficient duration or high enough intensity can cause a disruption in the balance between oxidative stress and antioxidant capacity (Bailey et al., 2004; Bloomer, 2008; Fogarty et al., 2011). These findings are in support of preliminary research in the lab using C2C12 skeletal muscle cells (see Chapter 3), where an intermittent protocol elicited the greatest increase in ROS.

High intensity intermittent exercise (HIIE) has received interest as a mode of exercise to elicit a change in oxidative stress and antioxidant status in a research setting due to its time-efficient nature and the ability for participants to maintain an overall higher work output (Milanović, Sporiš, & Weston, 2015b). It was found that the protocol of four minutes high intensity (90-95% VO_{2\text{max}}) followed by three minutes of lower intensity (70% VO_{2\text{max}}) repeated three times produced a change in some markers of oxidative stress (Table 4-6) and antioxidant capacity (Table 4-10).
Plasma LOOH increased at the onset of exercise and remained elevated for the duration of the testing protocol when compared with resting conditions, with a significantly higher concentration 3 hours post-exercise. Muscle LOOH concentrations also tended to be slightly elevated under exercising conditions compared with resting conditions, which was most prominent at 3 h post-exercise. However, this was not statistically significant. Fogarty (Fogarty et al., 2013), reported a similar, but significant, increase in serum LOOH following an exhaustive, acute exercise protocol. Significance may have been reached due to different exercise protocols and a younger cohort with higher VO_{2\text{max}} results or possibly due to differences in analytical methods and equipment. It is known that strenuous exercise causes an inflammatory response, which may lead to an increase in macrophage reactions within the tissues, possibly contributing to a rise in LOOH. This may help to explain why LOOH levels increased closer to the end of the testing period, rather than immediately post-exercise. A number of other studies have found that LOOH levels rise between 48 h and 96 h post-exercise (Close, Ashton, Cable, Doran, & MacLaren, 2004; Close, Ashton, McArdle, & MacLaren, 2005; Sacheck, Milbury, Cannon, Roubenoff, & Blumberg, 2003). The mechanism responsible for a delayed increase in plasma LOOH could be damage to cellular mechanisms following strenuous exercise. For example, cell membranes are rich in polyunsaturated fatty acids (Nikolaidis & Mougios, 2004) which can be released into the blood as a result of exercise-induced muscle damage. Unsaturated fatty acids are more prone to lipid peroxidation (Hulbert, 2005) and therefore could raise LOOH concentrations in the days following exercise. Additionally, exercise in the fasted state has been shown an elevated fatty acid contribution to energy provision (Long et al., 2008).
This can increase the concentration of fatty acids in the blood stream and can subsequently lead to increased exposure of fatty acids to peroxidation.

CK concentrations were compared across all time points between the two conditions to assess for skeletal muscle damage. The exercise group displayed significantly higher overall CK concentration, suggesting that HIIE caused an increase in muscle damage. This finding is supported by Wiewelhove et al., (2016), who found that CK levels were significantly higher 24 hours after a variety of HIIE running protocols when compared to baseline (Wiewelhove et al., 2016). It is thought that an increase in CK is also linked with an increase in plasma LOOH concentrations, due to increased lipid peroxidation resulting in a rise in membrane permeability (Sjödin et al., 1990). This may help explain the delayed release of CK into the bloodstream (Newham, Jones, & Edwards, 1986). However, other research has suggested that HIIE using a cycle ergometer, rather than running, may cause little or no muscle damage as represented by fluctuations in CK concentrations (Bogdanis et al., 2013). This is important to consider when selecting a modality of HIIE for testing or exercise prescription, especially in those populations who may be vulnerable to the effects of skeletal muscle damage, such as aged individuals.

Plasma PC content appeared to be relatively unaffected by the exercise protocol, with only a large effect size, but no significant difference, seen at 120 min post-exercise compared with baseline and no difference between the two conditions at any time point. This is in agreement with Bloomer (Bloomer, Goldfarb, & McKenzie, 2006) who also found no increase in plasma PC concentrations following traditional HIIE. A limited change in PC content following high intensity exercise is supported by Fogarty (Fogarty et al., 2011), who found that following
three bouts of incremental exercise there was no significant change in blood PC content. However, the present results are in contrast to recent research completed by Bogdanis (Bogdanis et al., 2013), who found that plasma PC concentrations significantly increased 30 min post-exercise, and peaked at 24 hours post-exercise, before returning close to baseline at 48 hours. Their findings are suggestive that a peak in plasma PC following HIIE may occur later than measured in the current study, and it is advisable that future research should complete blood analysis the day following HIIE to ensure the full impact of HIIE is measured.

In the current study, skeletal muscle protein PC was significantly lower in the exercise condition across the full duration of the study, the greatest difference being seen immediately post exercise. However, there were no significant differences at any of the individual time points between the two conditions. This is supported by Devries (Devries et al., 2008) who found no change in PC content of skeletal muscle biopsy samples in healthy, obese females following exercise training. It is possible that prolonged duration of exercise may be a more potent stimulus for muscle PC alteration than exercise intensity (Alessio et al., 2000; Bloomer et al., 2005) or that the high intensity exercise only causes a short-term impact on muscle PC concentration.

Further research is needed, and results taken after 3-hour time point to identify when the post-exercise LOOH and PC levels peak and then return to baseline after exercising. Providing participants with a meal during the testing protocol may also eliminate the impact of fasting upon markers of oxidative stress and antioxidant capacity. It is important to note that the content of such meal would need to be carefully controlled and individualised.
Antioxidant capacity was seen to increase during the exercise session with both SOD activity and UA concentrations rising above baseline. However, at 30 min post-exercise UA concentrations continued to rise to a significantly higher concentration than both baseline and resting protocol, whereas SOD activity was significantly reduced at the same time point. These findings would indicate that antioxidant capacity increases during exercise, probably caused by the mobilisation of antioxidants into the blood to counteract the rise in ROS activity. Other researchers have found similar, transient increases in antioxidant capacity during or immediately after HIIE (Fisher et al., 2011) whereas others have found that antioxidant levels peak 24 – 48 hr post exercise (Baker, Bailey, Hullin, Young, & Davies, 2004; Bogdanis et al., 2013; Chatzinikolaou et al., 2010).

4.4.3 Blood and skeletal muscle relationship

In the current study muscle PC content tended to increase over time in all conditions, whereas plasma PC was unaffected. This finding suggests that plasma samples may not be representative of muscle tissue PC response. A recent study by Parker (Parker et al., 2018) found that plasma redox status was not representative of redox-sensitive protein signalling in skeletal muscle, specifically c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor kappa B (NF-κB).

However, in the current study blood and muscle gave similar trends in LOOH concentration in response to exercise. This highlights the importance of measuring a range of oxidative stress markers and antioxidant capacity in both tissue types.
4.5 Conclusions

From the research in this chapter, it may be concluded that the muscle biopsy procedure has limited impact upon markers of oxidative stress and antioxidant capacity in blood or skeletal muscle tissues. However, there is a distinct difference in some of the results for blood and muscle samples, which support the need for both skeletal muscle sampling and blood collection and analysis across a range of redox markers in future research. It may also be concluded that the modified HIIE protocol used in the current study, provides sufficient stimulus to generate a change in oxidative stress and antioxidant capacity in young, healthy men compared with resting conditions.
Chapter 5 - The effect of acute CoQ supplementation on exercise-induced redox changes in young and aged men.
Chapter 5 – Study 4 – The effect of acute CoQ supplementation on exercise-induced redox changes in young and aged men.

5.1 Introduction

As previously discussed in Chapters 3 and 4, high intensity intermittent exercise (HIIE) has been shown, within our laboratory and others, to cause an oxidative stress response in humans (Parker, Mcguckin, & Leicht, 2014; Parker et al., 2016; Wadley, Chen, et al., 2016) and in cell culture (see Chapter 3). It was therefore proposed to apply the exercise stress model used in Chapter 4 to help identify the impact of a dietary antioxidant supplement on markers of oxidative stress and antioxidant capacity in skeletal muscle in young and aged males. In the current chapter, the focus is specifically on the use of CoQ as the dietary supplement.

Skeletal muscle health and function is a key component of maintaining overall health and independence as we age. It is thought that a deterioration in muscle health is caused by a combination of increased oxidative stress (Cobley et al., 2019; Fulle et al., 2004; Ji, 2001; Liochev, 2013; Sohal & Forster, 2007), physical inactivity, dysfunction in metabolism (Fulle et al., 2004; Guescini et al., 2017; Ji, 2015; Marzetti et al., 2008) and a progressive loss of protective mechanisms (Pansarasa et al., 1999; Pellegrino et al., 2011). A combination of exercise and improved nutrition is often suggested to limit the progression of age-associated muscle function decline (Calvani et al., 2013; Guescini et al., 2017). It has been suggested that antioxidant supplements may help to elevate protective mechanisms and help reduce the increases in oxidative stress seen with age (Landi et al., 2017; Ryan et al., 2010; Xu et al., 2010). By contrast, the beneficial effects of regular exercise with advancing age are well established (Fulle et al., 2004; Lee et al., 2012; Lenk et al., 2010; Viña et al., 2016).
CoQ is a lipid soluble, vitamin like substance that is largely located within the inner mitochondrial membrane (Bhagavan & Chopra, 2006), it acts as a potent antioxidant, primarily preventing the initiation and propagation phases of lipid peroxidation (Lars Ernster & Dallner, 1995; Irani, 2007). CoQ also acts indirectly by regenerating α-tocopherol, another potent lipid antioxidant (Quinn, Fabisiak, & Kagan, 1999). Additionally, the location of CoQ close to mitochondrial sources of ROS is ideal to reduce oxidative damage to nearby cellular structures.

CoQ is fundamental in the provision of ATP by acting as an electron carrier between complexes I and II to complex III of the ETC (López-Lluch et al., 2010). Supplementation may help to limit the age-associated dysregulation of metabolism (Fulle et al., 2004; Ji, 2015). CoQ has also been shown to limit permeability transition pore (PTP) opening and subsequently reduce cell apoptosis (Guescini et al., 2017). It is well understood that as we age there is an increase in muscle cell apoptosis and subsequent loss of skeletal muscle fibres (McIntire & Hoffman, 2011). There is a greater loss of type II fibres, leading to a higher proportion of type I fibres in aged skeletal muscle (Faulkner et al., 1995; Lexell, 1995; Örlander et al., 1978; Verdijk et al., 2007). Type I fibres have a higher mitochondrial density, and therefore a higher concentration of CoQ, due to CoQ being primarily located within the inner mitochondrial membrane.

Humans synthesise CoQ endogenously, however it is thought that the capacity for production reduces with age (Niklowitz et al., 2016; Sohal & Forster, 2007). Supplementation in an aged population may, therefore, help to restore lost CoQ and help limit the age-associated increases in oxidative stress, metabolic dysfunction, loss of muscle fibres and loss of protective antioxidant mechanisms within skeletal muscle.
Studies have shown promising results in increasing blood CoQ concentration following supplementation in a young population (Bhagavan & Chopra, 2006; Bloomer, Canale, McCarthy, & Farney, 2012; Tomasetti, Littarru, Stocker, & Alleva, 1999). There is, however, limited research on CoQ supplementation uptake into skeletal muscle in young participants (Cooke et al., 2008) and no recent research into the impact of CoQ supplementation on skeletal muscle in an aged population. In a young cohort, Svensson (Svensson et al., 1999) found that supplementation with 120 mg CoQ daily had no impact on skeletal muscle CoQ concentrations after 1, 11, 15 or 20 days of supplementation, despite an increase in serum CoQ concentrations. This research was later supported by Zhou (Zhou et al., 2005), who found similar results in serum and skeletal muscle concentrations following supplementation with 150 mg CoQ for four weeks in a young cohort.

Cooke (Cooke et al., 2008) supplemented healthy young adults with 200 mg of CoQ or placebo for a period of 14 days. An initial dose of 200 mg supplement or placebo was administered on the first day, 60 min after the first consumption a battery of exercise tests were completed followed by blood and skeletal muscle sample collection, allowing for acute analysis. The exercises and collections were repeated after 14 days to assess extended supplementation. Analysis in skeletal muscle showed that there was a slight increase in CoQ content after acute supplementation however significance was not reached. No increase was seen after 14 days of CoQ consumption. No other markers were measured in the skeletal muscle, making it difficult to determine the impact of a slightly elevated CoQ concentration on oxidative stress. To date, there is no known investigation with CoQ supplementation in an aged population, where skeletal muscle levels may already be compromised.
An acute supplementation strategy has therefore been adopted, based on promising results from Cooke (Cooke et al., 2008). It may also be possible that the beneficial effects of increased CoQ concentration within the skeletal muscle may only be short lived following supplementation. The impact of CoQ in a young, healthy population has shown a limited effect on exercise-induced oxidative stress and antioxidant capacity (Bloomer et al., 2012; Kon et al., 2008; Östman, Sjödin, Michaëlsson, & Byberg, 2012), however, there may be an impact in an aged population.

5.1.1 Objectives

The objectives of the current study are:

- To assess the impact of HIIE on oxidative stress in an aged population.
- To assess the effect of supplementation on blood and muscle CoQ levels in young and aged males.
- To understand the effect of CoQ supplementation on aged skeletal muscle markers of exercise-induced redox alterations.
- To identify differences in exercise-induced oxidative stress and antioxidant capacity following CoQ supplementation between a young and an aged population.

5.2 Materials and methods
5.2.1 Human subjects and experimental design

Four young and 5 aged males volunteered to participate in the cross-over study. All subjects completed a medical history questionnaire and consent forms (see appendices A and B) prior to participation. Participants were recreationally active (participating in a minimum of 3 recreational sessions per week), non-smokers
and not taking any dietary supplements or medication. Ethical approval was granted as described in section 2.2.

5.2.2 Preliminary testing

Prior to experimental testing, anthropometric data was collected (Table 4-1) and participants performed an incremental exercise test to exhaustion to elicit VO$_{2\text{max}}$, as described in section 2.2.3. Participants returned to the laboratory for a familiarisation session to assess the suitability of the HIIE protocol and the level of resistance was adjusted to ensure the correct range of heart rate would be achieved.

Table 5-1 Anthropometric data for YOUNG ($n = 4$) and AGED ($n = 5$) participants. All values are means ± standard deviation.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>YOUNG</th>
<th>AGED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>26 ± 3</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>Body Mass (kg)</td>
<td>106 ± 24</td>
<td>71 ± 10</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>183 ± 8</td>
<td>175 ± 6</td>
</tr>
<tr>
<td>BMI (kg/cm$^2$)</td>
<td>32 ± 8</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>VO$_{2\text{max}}$ (ml.kg/min)</td>
<td>35 ± 14</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>Heart rate max (bpm)</td>
<td>179 ± 8</td>
<td>161 ± 7</td>
</tr>
</tbody>
</table>

5.2.3 Supplementation with CoQ

Participants were provided with three capsules of either 100 mg CoQ supplement (300 mg/day total) or placebo (Pharma Nord, Vejle, Denmark) and asked to consume the capsules at breakfast, lunch and dinner the day before the main trial as CoQ absorption is enhanced when taken with food (Ochiai et al., 2007). The placebo capsules were identical to the CoQ supplement and manufactured at the same laboratory (soyabean oil, gelatin, hydrogenated soyabean oil, glycerol (E 442), iron oxide (E172)), with only the removal of the active ingredient. All capsules were a kind gift from Pharma Nord, Denmark.
5.2.4 Experimental testing

To assess the impact of a dietary intervention on an elderly population, an exercise protocol was utilized to provide an adequate level of oxidative stress to participants. Research shown in Chapter 4 is in agreement with previous research, that a healthy population is not under oxidative stress or reduced antioxidant capacity under resting conditions. Therefore the recruitment of healthy subjects demanded additional stress to be applied to exacerbate this increased oxidative stress and reduced antioxidant defence experienced in age-related disease states (Doria et al., 2012; Jackson, 2016; Mecocci et al., 1999). High-intensity, intermittent exercise (HIIE) was deemed most appropriate as it has been well established that an increased oxidative stress response occurs following exercise of short duration at a high intensity, or long duration at a moderate intensity (Bloomer, 2008; Wiewelhove et al., 2016). This method of exercise stress has also been proven successful in increasing oxidative stress in our previous research in both cell culture, described in Chapter 3, and in young, healthy males, described in Chapter 4.

Participants were instructed to refrain from strenuous exercise and not to consume alcohol or caffeine for 48 hours prior to testing. They reported to the laboratory after an overnight fast, although water could be consumed *ad libitum*. Biometric data was collected, and a cannula fitted as described in section 2.3.1. Baseline blood and muscle samples were collected and processed as described in sections 2.3 and 2.4. Participants completed a five-minute incremental warm-up starting at 50W, which was followed by four minutes of high intensity exercise at 90 – 95% heart rate max (HR_{max}), predetermined at their VO_{2max} test and familiarisation session. This was followed by 3 minutes of lower-intensity,
recovery exercise at 70% of HR\textsubscript{max}, each set being repeated for an additional two times. The structure of the HIIE protocol was identical to the protocol used in Chapter 4 and based on previous research within our laboratory, which showed a highest increase in ROS production following a 3 x 5 min intermittent protocol in C2C12 muscle cells.

Following the exercise protocol, participants were asked to remain within the laboratory for a further three hours, during which they could move freely and consume water \textit{ad libitum}. Participants returned for the cross-over condition a minimum of 6 weeks later to allow for a wash out period from the exercise and for skeletal muscle to recover.

\textbf{5.2.5 Haematology & skeletal muscle samples}

Blood was drawn from the cannula at baseline, after each bout of high intensity exercise then at 30, 60, 120 and 150 minutes post-exercise. Blood samples were collected into serum separation tubes (SST) for serum analysis, and EDTA vacutainers for plasma analysis, inverted three times and stored on ice for further analysis.

Skeletal muscle biopsies were collected at baseline, immediately post-exercise and at 180 minutes post-exercise, using the protocol described in section 2.4.

\textbf{5.2.6 Biochemical analysis}

\textit{5.2.6.1 Protein quantification}

The Bradford Assay (Bradford, 1976) was used to determine protein concentration to allow for normalisation of data (see section 2.6.1).
5.2.6.2 Total reactive oxygen species (ROS)

Whole blood total ROS production was detected by fluorescence of DHE using the method of Magwenzi (Kearney et al., 2015) (see section 2.6.2).

5.2.6.3 Lipid hydroperoxides (LOOH)

Serum and skeletal muscle homogenate LOOH were measured spectrophotometrically using the Ferrous Oxidation of Xylenol orange (FOX) assay (see section 2.6.4).

5.2.6.4 Protein carbonyls (PC)

Plasma and skeletal muscle homogenate PC were quantified spectrophotometrically using the method described by Mesquita (Mesquita et al., 2014)(see sections 2.6.6 and 2.7.4).

5.2.6.5 Superoxide dismutase (SOD)

Total plasma SOD activity was quantified using a xanthine oxidase as a ROS-generating system and NBT as a ROS detector to assess SOD activity against a standard curve of bovine SOD (see section 2.6.5).

5.2.6.6 Coenzyme Q10 quantification

Skeletal muscle CoQ concentrations were determined at Liverpool John Moores University using a reversed-phase HPLC with ultraviolet (UV) detection according to the method described by Boitier (Boitier et al., 1998). CoQ quantification required a large muscle sample size for analysis.
5.2.6.7 Additional biochemical analysis

The ABX Pentra 400 auto-analyser (Horiba, Montpellier, France) was used to analyse serum creatine kinase (CK) and uric acid (UA). Calibration and quality controls were completed prior to analysis in accordance with the manufacturer’s guidelines.

5.2.7 Statistical analysis

Data were assessed using SPSS as described in section 2.11. The alpha was accepted at \( P = < 0.05 \) and indicated by a \(^*\); all descriptive data sets are expressed as a mean value \( \pm \) standard deviations. Standardised effect size (SE) was calculated using \( \eta^2 \) for the ANOVA analysis and Cohen’s \( d \) for differences between conditions.

5.3 Results

The following section presents the main experimental findings following CoQ supplementation and placebo in young and aged participants.

All participants completed the exercise protocol. Participant heart rates averaged 90-95\% \( HR_{\text{max}} \) over the duration of the high intensity sessions and 70-75\% for the lower intensity sessions. Intensity was modified as required to keep participants heart rates within these ranges. Participant average RPE values are shown in Table 5-2 for each high and low intensity period under placebo and CoQ sessions.
Table 5-2 Mean participant RPE scores and percentage heart rate for HIGH and LOW periods of exercise for young and aged participant groups following placebo and CoQ supplementation. All results are expressed as a mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Placebo RPE</th>
<th>CoQ RPE</th>
<th>Placebo HR (%)</th>
<th>CoQ HR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young HIGH</td>
<td>15 ± 2</td>
<td>14 ± 2</td>
<td>90 ± 3</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>Young LOW</td>
<td>10 ± 2</td>
<td>10 ± 2</td>
<td>72 ± 4</td>
<td>72 ± 1</td>
</tr>
<tr>
<td>Aged HIGH</td>
<td>16 ± 2</td>
<td>15 ± 2</td>
<td>91 ± 2</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>Aged LOW</td>
<td>11 ± 1</td>
<td>11 ± 1</td>
<td>72 ± 4</td>
<td>72 ± 2</td>
</tr>
</tbody>
</table>

Due to a large amount of haemolysation of blood samples caused by a defective cannulation in the YOUNG groups, we had limited sample volumes and complete superoxide dismutase (SOD) analysis in these groups was not possible. SOD analysis in the AGED groups were also not completed. Unfortunately, due to the small amount of muscle sample collected, LOOH analysis in skeletal muscle could not be completed. However, in Chapter 4 serum LOOH was representative of muscle LOOH values and therefore serum LOOH should be representative of muscle LOOH in this study. For future research, it may be beneficial to increase the number of samples collected at each time point, or increase the needle gauge to increase the size of muscle sample captured.

5.3.1 CoQ uptake

5.3.1.1 Serum CoQ concentration

Serum CoQ concentrations were measured at baseline to assess the uptake of CoQ into the blood after 24 hour of supplementation with either CoQ or placebo in both the YOUNG and AGED groups (Table 5-3). Individual data for YOUNG participants are shown in Table 5-4 and for AGED participants in Table 5-5.
Supplementation increased serum CoQ concentration in both YOUNG and AGED groups. YOUNG CoQ group concentrations were significantly higher than YOUNG placebo ($p = 0.02$, SE $= 2.13$; very large). AGED CoQ were also significantly higher than AGED placebo ($p < 0.01$, SE $= 2.62$; very large). Both of these findings suggest that supplementation with 300 mg CoQ for 24 hours was successful in increasing serum CoQ concentration in both YOUNG and AGED populations.

There was no significant difference between YOUNG placebo and AGED placebo, although the AGED group tended to have slightly lower concentrations with a medium effect size ($p = 0.42$, SE $= 0.55$). Following CoQ supplementation, no difference was found between the YOUNG and AGED groups. Individual data shown in Tables 5 & 6 clearly show an increase in CoQ for each participant following supplementation; they also show the wide variation in individual response to the same CoQ dose and in non-supplemented individuals.

5.3.1.2 Muscle CoQ concentration

The normal range of skeletal muscle CoQ concentrations is 140 – 580 pmol/mg protein (Duncan et al., 2005) however, some of the participants had concentrations outside of this range. Mean muscle CoQ concentrations are shown in Table 5-5 and individual data are shown in Table 5-7 for the YOUNG group and Table 5-8 for the AGED group. The mean results show that CoQ supplementation increased the muscle CoQ content slightly in both age groups at baseline compared with placebo, however this was more prominent in the AGED CoQ group ($p = 0.32$, SE $= 0.63$; medium). The individual YOUNG data in Table 5-7 suggest that this is not always representative as not all participants displayed this trend. Participant YOUNG 2 showed a decline in muscle CoQ
content at baseline following CoQ supplementation compared with placebo. A similar result was found in the AGED group where participants 3 and 5 both showed a reduction in muscle CoQ content following supplementation compared with placebo. The YOUNG placebo group has a lower baseline CoQ muscle concentration compared with the AGED placebo ($p = 0.27, \text{SE} = 0.78; \text{medium}$) and AGED CoQ groups ($p = 0.06, \text{SE} = 1.36; \text{very large}$) which was unexpected. Previous research has indicated that CoQ content of metabolically active tissues, such as skeletal muscle, deteriorates as we age (Turunen et al., 2004).

Over the duration of the study all muscle CoQ concentrations fell below baseline, under all conditions by 180 min post-exercise. The most significant reduction from baseline is seen in the AGED CoQ group, CoQ concentrations reduce significantly, immediately post-exercise ($p = 0.05, \text{SE} = 1.32; \text{very large}$) and at 180 min post-exercise ($p = 0.03, \text{SE} = 1.65; \text{very large}$). However, despite this decline, the concentration at 180 min post-exercise was still higher than the AGED placebo group ($p = 0.31, \text{SE} = 0.70; \text{medium}$) and both the YOUNG CoQ ($p = 0.09, \text{SE} = 1.29; \text{very large}$) and significantly higher than the YOUNG placebo group ($p = 0.04, \text{SE} = 1.73; \text{very large}$). The AGED placebo group also declined over the duration of the study compared to baseline, however this did not reach significance at 180 min post-exercise ($p = 0.18, \text{SE} = 0.99; \text{large}$). The only group to show a slight increase in CoQ concentration immediately post-exercise was the YOUNG placebo group, however this did not reach significance ($p = 0.50, \text{SE} = 0.51; \text{medium}$), at 180 min post-exercise these values had returned close to baseline. This suggests HIIE causes a reduction in muscular CoQ concentrations for up to 3 hours post-exercise, however, CoQ intake can limit this response in an aged population.
Table 5-3 - Serum CoQ at baseline following 24 hours supplementation with either 300 mg CoQ or placebo in YOUNG and AGED groups. Values are means ± standard deviations. CoQ concentrations are expressed per pmol/mg protein.

<table>
<thead>
<tr>
<th></th>
<th>Baseline Placebo</th>
<th>Baseline CoQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>YOUNG</td>
<td>1033 ± 510</td>
<td>2309 ± 679</td>
</tr>
<tr>
<td>AGED</td>
<td>799 ± 318</td>
<td>2043 ± 592</td>
</tr>
</tbody>
</table>

Table 5-4 - Individual results for YOUNG serum total CoQ at baseline following 24 hours supplementation with either 300 mg CoQ or placebo. CoQ concentrations are expressed per pmol/mg protein.

<table>
<thead>
<tr>
<th></th>
<th>Baseline Placebo</th>
<th>Baseline CoQ</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young 1</td>
<td>1761</td>
<td>2535</td>
<td>44</td>
</tr>
<tr>
<td>Young 2</td>
<td>621</td>
<td>1761</td>
<td>184</td>
</tr>
<tr>
<td>Young 3</td>
<td>994</td>
<td>3169</td>
<td>219</td>
</tr>
<tr>
<td>Young 4</td>
<td>756</td>
<td>1769</td>
<td>134</td>
</tr>
</tbody>
</table>
Table 5-5 - Individual results for AGED serum total CoQ at baseline following 24 hours supplementation with either 300 mg CoQ or placebo. CoQ concentrations are expressed per pmol/mg protein.

<table>
<thead>
<tr>
<th></th>
<th>Baseline Placebo</th>
<th>Baseline CoQ</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aged 1</td>
<td>759</td>
<td>2670</td>
<td>252</td>
</tr>
<tr>
<td>Aged 2</td>
<td>654</td>
<td>1467</td>
<td>124</td>
</tr>
<tr>
<td>Aged 3</td>
<td>493</td>
<td>1945</td>
<td>295</td>
</tr>
<tr>
<td>Aged 4</td>
<td>757</td>
<td>2625</td>
<td>247</td>
</tr>
<tr>
<td>Aged 5</td>
<td>1333</td>
<td>2294</td>
<td>72</td>
</tr>
</tbody>
</table>

Table 5-6 - Muscle total CoQ content at baseline, immediately post exercise and 3 hours post exercise. Values are means ± standard deviations. CoQ concentrations are expressed per pmol/mg protein. The alpha is accepted at p = 0.05 and displayed as a * if different from baseline and a # when different between conditions.

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>0 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo Young</td>
<td>171 ± 98.3</td>
<td>197 ± 72.0</td>
<td>186 ± 48.7</td>
</tr>
<tr>
<td>CoQ Young</td>
<td>296 ± 184</td>
<td>209 ± 74.0</td>
<td>197 ± 65.7</td>
</tr>
<tr>
<td>Placebo Aged</td>
<td>320 ± 87.7</td>
<td>354 ± 228</td>
<td>216 ± 84.6</td>
</tr>
<tr>
<td>CoQ Aged</td>
<td>364 ± 74.1</td>
<td>252 ± 94.8</td>
<td>266 ± 38.1</td>
</tr>
</tbody>
</table>
Table 5-7 Individual results for YOUNG muscle total CoQ content at baseline, immediately post exercise and 3 hours post exercise following placebo or CoQ supplementation. CoQ concentrations are expressed per pmol/mg protein.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>0 min</th>
<th>180 min</th>
<th></th>
<th>Baseline</th>
<th>0 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Placebo</td>
<td>Placebo</td>
<td></td>
<td>CoQ</td>
<td>CoQ</td>
<td>CoQ</td>
</tr>
<tr>
<td>Young 1</td>
<td>283</td>
<td>195</td>
<td>229</td>
<td></td>
<td>557</td>
<td>303</td>
<td>252</td>
</tr>
<tr>
<td>Young 2</td>
<td>372</td>
<td>728</td>
<td>223</td>
<td></td>
<td>290</td>
<td>232</td>
<td>255</td>
</tr>
<tr>
<td>Young 3</td>
<td>131</td>
<td>126</td>
<td>133</td>
<td></td>
<td>151</td>
<td>150</td>
<td>145</td>
</tr>
<tr>
<td>Young 4</td>
<td>99</td>
<td>270</td>
<td>195</td>
<td></td>
<td>187</td>
<td>149</td>
<td>135</td>
</tr>
</tbody>
</table>

Table 5-8 Individual results for AGED muscle total CoQ content at baseline, immediately post exercise and 3 hours post exercise following placebo or CoQ supplementation. CoQ concentrations are expressed per pmol/mg protein.

<table>
<thead>
<tr>
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<td>Placebo</td>
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<tr>
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<td>149</td>
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</table>
5.3.2 Blood markers of oxidative stress

5.3.2.1 Plasma protein carbonyls (PC)

Plasma PC concentrations are shown in Table 5-9, with individual data shown in Table 5-9-1 for AGED. The results suggest an increased PC content in young participants following 24 hours CoQ supplementation in comparison to the placebo trial, throughout the duration of the testing period. This difference between the two conditions did not reach significance at any time point, however a very large effect size was suggested at 60 min post exercise ($p = 0.15, \text{SE} = 2.25$) and a large effect was seen at baseline ($p = 0.22, \text{SE} = 1.18$), immediately post-exercise ($p = 0.31, \text{SE} = 0.95$) and at 180 min post-exercise ($p = 0.49, \text{SE} = 0.80$). Also, AGED participants did not show any significant difference in plasma PC concentrations between the placebo and CoQ supplementation trials at any time point.

All groups presented with a slight decline in PC over the duration of the testing period; both the YOUNG CoQ group and YOUNG placebo group showed a very large effect between the baseline and 180 min post-exercise time point ($p = 0.20, \text{SE} = 1.71$ and $p = 0.18, \text{SE} = 1.32$, respectively). The YOUNG CoQ group had significantly higher plasma PC compared with all other groups’ mean concentration across all time points; YOUNG placebo ($p = <0.01$), AGED CoQ ($p = <0.01$) and AGED placebo ($p = <0.01$), however no differences were found between the other group means across all time points.
Table 5-9 Plasma protein carbonyl concentration at baseline, during exercise and throughout 180 minutes of recovery time. Values are means ± standard deviations. Protein carbonyls are expressed as µg/mg total protein. Ex 1 = end of first exercise bout and Ex2 = end of second exercise bout. Time is expressed in minutes starting from the cessation of the final exercise bout. The alpha is accepted at p = 0.05 and displayed as a * if different from baseline and a # when different between conditions.

<table>
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<tr>
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<th>60 min</th>
<th>120 min</th>
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</tr>
<tr>
<td>Placebo Young</td>
<td>5.0 ± 3.4</td>
<td>3.5 ± 3.6</td>
<td>3.8 ± 5.1</td>
<td>5.3 ± 3.7</td>
<td>4.4 ± 6.2</td>
<td>4.0 ± 3.9</td>
<td>2.8 ± 3.8</td>
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<tr>
<td>CoQ Young</td>
<td>7.0 ± 3.9</td>
<td>8.5 ± 1.1</td>
<td>7.5 ± 1.6</td>
<td>9.9 ± 4.0</td>
<td>7.5 ± 1.3</td>
<td>15.5 ± 5.3</td>
<td>6.6 ± 4.7</td>
<td>5.8 ± 0.7</td>
</tr>
<tr>
<td><strong>Placebo Aged</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo Aged</td>
<td>3.5 ± 4.0</td>
<td>4.0 ± 3.7</td>
<td>5.6 ± 5.0</td>
<td>4.5 ± 4.1</td>
<td>6.8 ± 9.2</td>
<td>3.5 ± 4.7</td>
<td>2.6 ± 3.8</td>
<td>2.5 ± 2.4</td>
</tr>
<tr>
<td>CoQ Aged</td>
<td>4.2 ± 4.4</td>
<td>5.5 ± 4.0</td>
<td>3.6 ± 3.2</td>
<td>4.3 ± 3.6</td>
<td>4.3 ± 3.7</td>
<td>3.0 ± 3.1</td>
<td>3.4 ± 3.8</td>
<td>3.5 ± 3.3</td>
</tr>
</tbody>
</table>
Table 5-9-1 Individual plasma protein carbonyl concentration for AGED group at baseline, during exercise and throughout 180 minutes of recovery time. Protein carbonyls are expressed as µg/mg total protein. Ex 1 = end of first exercise bout and Ex2 = end of second exercise bout. Time is expressed in minutes starting from the cessation of the final exercise bout. The alpha is accepted at p = 0.05 and displayed as a * if different from baseline and a # when different between conditions.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ex1</th>
<th>Ex2</th>
<th>0 min</th>
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<th>60 min</th>
<th>120 min</th>
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<tr>
<td>Placebo Aged 1</td>
<td>8.18</td>
<td>5.75</td>
<td>8.90</td>
<td>6.29</td>
<td>6.41</td>
<td>10.8</td>
<td>8.17</td>
<td>5.57</td>
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<tr>
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<td>4.87</td>
<td>3.58</td>
<td>5.93</td>
<td>7.20</td>
<td>6.51</td>
<td>6.38</td>
<td>6.50</td>
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<tr>
<td>Placebo Aged 2</td>
<td>5.45</td>
<td>6.33</td>
<td>9.50</td>
<td>8.70</td>
<td>4.61</td>
<td>0.67</td>
<td>2.06</td>
<td>3.66</td>
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<tr>
<td>CoQ Aged 2</td>
<td>2.80</td>
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<td>4.32</td>
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<tr>
<td>Placebo Aged 3</td>
<td>7.93</td>
<td>9.51</td>
<td>7.27</td>
<td>22.6</td>
<td>5.88</td>
<td></td>
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<td>3.26</td>
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<tr>
<td>CoQ Aged 3</td>
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<td>9.03</td>
<td>5.91</td>
<td>7.09</td>
<td>4.64</td>
<td>2.74</td>
<td>0.22</td>
<td>6.88</td>
</tr>
<tr>
<td>Placebo Aged 4</td>
<td>0.18</td>
<td>0.11</td>
<td>0.08</td>
<td>0.09</td>
<td>0.17</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>CoQ Aged 4</td>
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<td>0.04</td>
<td>0.08</td>
<td>0.10</td>
<td>0.17</td>
<td>0.12</td>
<td>0.11</td>
<td>0.10</td>
</tr>
<tr>
<td>Placebo Aged 5</td>
<td>0.10</td>
<td>0.07</td>
<td>0.24</td>
<td>0.15</td>
<td>0.11</td>
<td>0.08</td>
<td>0.11</td>
<td>0.09</td>
</tr>
<tr>
<td>CoQ Aged 5</td>
<td>0.25</td>
<td>0.33</td>
<td>0.30</td>
<td>0.20</td>
<td>0.37</td>
<td></td>
<td></td>
<td>0.12</td>
</tr>
</tbody>
</table>
Serum lipid hydroperoxide data is presented in Table 5-10, with individual data presented in table 5-10-1. There were no significant differences in the average concentration across all time points between any groups. At baseline, CoQ supplementation appeared to increase LOOH concentration in both the YOUNG ($p = 0.16$, SE = 1.43; very large) and AGED ($p = 0.19$, SE = 1.24; very large). However, the YOUNG CoQ group LOOH concentration tended to decrease over the duration of the experimental protocol in contrast to the placebo groups which tended to increase. The YOUNG CoQ group had a significant reduction in LOOH concentration, when compared to baseline, during exercise ($p = <0.01$, SE = 6.89; very large) and immediately post exercise ($p = 0.01$, SE = 3.39; very large).

The AGED CoQ group provided more variable results, showing a maintenance of LOOH concentration during exercise, followed by a large increase immediately post-exercise ($p = 0.19$, SE = 0.93: large). However, concentrations returned close to baseline at 30 min post exercise ($p = 0.05$, SE = 1.61; very large) and finally falling well below baseline at the 180 min post-exercise time point ($p =0.11$, SE 1.11; large) when compared to baseline. The placebo AGED group show an increase from baseline in lipid hydroperoxide concentrations during exercise ($p = 0.15$, SE = 1.40; very large), immediately post-exercise ($p = 0.30$, SE = 0.96; large), a drop at 60 min post-exercise ($p = 0.19$, SE = 1.30; very large), and then an increase again at 120 min post-exercise ($p = 0.21$, SE = 1.23; very large) with peaks at 180 min post-exercise ($p =0.11$, SE = 1.62: very large). There is a significant difference between the AGED CoQ and placebo groups at 60 min post-exercise ($p =0.05$, SE = 3.13; very large) with the supplemented group showing increased LOOH
compared with the placebo group. However, at 180 min post-exercise the AGED CoQ group is significantly lower than the AGED placebo group ($p = 0.04$, SE = 1.61: very large). No difference is found between the two YOUNG groups at each time point.

The increase in LOOH concentrations in both groups at baseline is unexpected due to the antioxidant properties of CoQ. However, CoQ appears to alter the profile of LOOH concentration during recovery in the AGED population, causing a shift in the LOOH peak post-exercise to earlier in recovery, allowing LOOH concentration to return below baseline by 3 hour post-exercise. This may suggest a faster clearance of LOOH post-exercise due to a higher concentration of CoQ in both the blood and skeletal muscle, as seen by the CoQ concentration data.

5.3.2.3 Whole blood total ROS

The percentage change from baseline in whole blood total ROS is shown in Table 5-11. There was a great level of variability between participants in all groups. The highest total ROS values across all time points were seen in the YOUNG placebo group, whereas the lowest levels were seen in the AGED placebo group, however there was no significant differences found between the means across time points for all groups.

The YOUNG CoQ and AGED placebo groups displayed a significant reduction in total ROS after the second bout of exercise compared to baseline measures ($p = 0.02$, SE = 2.50; very large, $p = <0.01$, SE =2.83; very large, respectively). The YOUNG CoQ group total ROS concentration fluctuated post-exercise with a significant reduction compared to baseline seen at 30 min post-exercise ($p = 0.04$, SE = 1.87: very large) however concentrations appeared to return close to
baseline by 180 min post-exercise. The AGED placebo group also displayed fluctuations post-exercise, with a significant reduction immediately after the second exercise bout (p < 0.01, SE = 2.83; very large) which increased slightly before falling again at 120 min post-exercise (p = 0.08, SE = 1.26; very large) and 180 min-post-exercise (p = 0.06, SE; 1.40; very large).

The YOUNG placebo group displayed a significant increase in total ROS following the first exercise bout (p = 0.04, SE = 1.80; very large) which remained elevated following the second exercise bout (p = 0.14, SE = 1.12; large), and increased above baseline again at 30 min post-exercise (p = 0.06, SE = 1.46; very large), before gradually returning to baseline at 180 min post-exercise.

Supplementation in the AGED group appeared to dampen and delay the variations of the total ROS concentrations, the AGED CoQ group total ROS reduced immediately post-exercise (p = 0.07, SE = 1.18; large) then raised slightly above baseline until the 180 min post-exercise time point where it fell significantly lower than baseline concentrations (p = 0.01, SE = 1.87; very large). When comparing the CoQ AGED group to the placebo AGED group there were no significant differences at any time point, however a large effect size was seen between the two conditions after the second bout of exercise (p = 0.18, SE = 0.83) with the CoQ group showing higher concentrations than the placebo group.

CoQ in the YOUNG group appeared to dampen the fluctuation of total ROS concentrations over the duration of the study when compared to the YOUNG placebo group. Although differences did not reach significance, a very large effect was seen between the placebo and CoQ YOUNG groups after both the first (p = 0.28, SE = 1.47) and second (p = 0.20, SE = 1.85) exercise bouts and at 30 min post-exercise (p = 0.11, SE = 1.73), with the CoQ YOUNG group displaying...
lower concentrations of total ROS. A large effect size was seen immediately post-exercise ($p = 0.20$, $SE = 1.01$) between the two conditions with the placebo YOUNG group displaying lower concentrations compared with the CoQ YOUNG group.
Table 5-10 - Serum lipid hydroperoxides concentration at baseline, during exercise and throughout 180 minutes of recovery time. Values are means ± standard deviations. Lipid hydroperoxides are expressed as mmol.L⁻¹. Ex 1 = end of first exercise bout. Time is expressed in minutes starting from the cessation of the final exercise bout. The alpha is accepted at \( p = 0.05 \) and displayed as a * if different from baseline and a # when different between conditions.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ex 1</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
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<tbody>
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<td>Placebo Young</td>
<td>42.5 ± 14.5</td>
<td>36.6</td>
<td>31.6 ± 13.9</td>
<td>45.3 ± 0</td>
<td>55.3 ± 36.6</td>
<td>47.3 ± 19.6</td>
<td>51.5 ± 7</td>
</tr>
<tr>
<td>CoQ Young</td>
<td>58.3 ± 5.9</td>
<td>11.2 ± 7.7*</td>
<td>26.5 ± 11.9*</td>
<td>51.3 ± 67.6</td>
<td>44.6 ± 37.1</td>
<td>53.8 ± 64.7</td>
<td>30.3</td>
</tr>
<tr>
<td>Placebo Aged</td>
<td>24.8 ± 4.0</td>
<td>42.4 ± 17.3</td>
<td>41.8 ± 24.7</td>
<td>52.3 ± 53.0</td>
<td>32.9 ± 7.7#</td>
<td>56.6 ± 36.3</td>
<td>149.0 ± 108.6#</td>
</tr>
<tr>
<td>CoQ Aged</td>
<td>45.7 ± 23.4</td>
<td>46.0 ± 35.2</td>
<td>78.6 ± 44.3</td>
<td>41.3 ± 28.4</td>
<td>51.4 ± 3.3#</td>
<td>76.2 ± 13.2*</td>
<td>24.9 ± 10.0#</td>
</tr>
</tbody>
</table>
Table 5-10-1 Individual serum lipid hydroperoxides concentration at baseline, during exercise and throughout 180 minutes of recovery time in the AGED group. Lipid hydroperoxides are expressed as mmol.L⁻¹. Ex 1 = end of first exercise bout. Time is expressed in minutes starting from the cessation of the final exercise bout. The alpha is accepted at p = 0.05 and displayed as a * if different from baseline and a # when different between conditions.

<table>
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<tr>
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<th>Baseline</th>
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<th>60 min</th>
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<td>Placebo Aged 1</td>
<td>21.7</td>
<td>34.5</td>
<td>28.3</td>
<td>24.4</td>
<td>40.3</td>
<td>42</td>
<td>36.4</td>
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</tr>
<tr>
<td>CoQ Aged 1</td>
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<td>67</td>
<td>51.6</td>
<td>23.2</td>
<td>73.2</td>
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<td>20.2</td>
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<td>58.2</td>
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<tr>
<td>CoQ Aged 2</td>
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<td>15.6</td>
<td>59.2</td>
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<td>83.1</td>
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<tr>
<td>Placebo Aged 4</td>
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<td>67.0</td>
<td>14.5</td>
<td>24.9</td>
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<tr>
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<td>97.2</td>
<td>85.8</td>
<td>89.5</td>
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<td>33.4</td>
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<tr>
<td>CoQ Aged 5</td>
<td>23.1</td>
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<td>29.7</td>
<td>49.1</td>
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</tbody>
</table>
Table 5-11 - Whole blood total ROS percentage change from baseline under exercise conditions and throughout 180 minutes of recovery time. Values are means ± standard deviations. Results are expressed as a percentage (%) change from baseline value which is set to 100%. Ex 1 = end of first exercise bout and Ex2 = end of second exercise bout. Time is expressed in minutes starting from the cessation of the final exercise bout. The alpha is accepted at p = 0.05 and displayed as a * if different from baseline and a # when different between conditions.

<table>
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<tr>
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<th>Ex 1</th>
<th>Ex2</th>
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<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
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</thead>
<tbody>
<tr>
<td>Placebo Young</td>
<td>157 ± 44.9</td>
<td>152 ± 65.6*</td>
<td>61.5 ± 64.2</td>
<td>215 ± 111*</td>
<td>186 ± 192</td>
<td>189 ± 200</td>
<td>96.5 ± 40.0</td>
</tr>
<tr>
<td>CoQ Young</td>
<td>110 ± 9.7</td>
<td>61.4 ± 21.8*</td>
<td>124 ± 59.0</td>
<td>77.4 ± 17.2</td>
<td>194 ± 274</td>
<td>114 ± 89.9</td>
<td>69.5 ± 54.2</td>
</tr>
<tr>
<td>Placebo Aged</td>
<td>79.0 ± 40.0</td>
<td>55.3 ± 22.3*</td>
<td>83.7 ± 55.4</td>
<td>117 ± 65.7</td>
<td>83.8 ± 66.5</td>
<td>68.1 ± 35.9</td>
<td>61.0 ± 39.4</td>
</tr>
<tr>
<td>CoQ Aged</td>
<td>99.5 ± 57.2</td>
<td>90.2 ± 55.1</td>
<td>62.2 ± 45.5</td>
<td>81.9 ± 38.5</td>
<td>103 ± 68.1</td>
<td>109 ± 162</td>
<td>63.9 ± 27.3*</td>
</tr>
</tbody>
</table>
5.3.3 Muscle markers of oxidative stress

5.3.3.1 Muscle protein carbonyls (PC)

Mean muscle PC concentrations are shown in Table 5-12 with individual participant data presented in Table 5-13 for the YOUNG participants and Table 5-14 for the AGED group. Participants supplemented with CoQ in the AGED group had the highest baseline PC concentration compared with all other groups, however this difference only approached significance when compared with the AGED placebo group ($p = 0.06$, SE $= 1.53$; very large). The AGED CoQ group maintained a higher mean concentration of PC throughout the duration of the testing period compared with all other groups, however this did not reach significance. The AGED placebo group had the lowest baseline concentrations compared to all other groups, but significantly increased after the completion of exercise ($p = 0.04$, SE $= 1.58$; very large) and continued to increase at 180 min post exercise ($p = 0.12$, SE $= 1.11$; large) when compared with baseline. CoQ appeared to have no impact on PC concentration in plasma of AGED participants. However, despite a higher baseline concentration, CoQ appeared to attenuate the post-exercise increase in PC seen in the AGED placebo skeletal muscle and maintained a constant level of protein carbonyls throughout the testing period in the AGED CoQ group.

YOUNG participants displayed lower PC concentrations in skeletal muscle across all time points under both conditions compared with the AGED groups, however no significant differences were found. CoQ supplementation appeared to limit the decline in muscle PC over time that was seen in the YOUNG placebo group, despite similar baseline concentrations. The greatest difference was seen at 180 min post-exercise, but this did not reach significance. However a very
large effect size was found ($\rho = 0.11$, SE = 1.74). Again, this was unexpected due to the proposed antioxidant protection from CoQ. Individualised data sets show great variability in response between participants, which could contribute to the mixed results.
Table 5-12 - Muscle protein carbonyl concentration at baseline, immediately post exercise and 3 hours post exercise. Values are means ± standard deviations. Protein carbonyls are expressed as µg/mg total protein. The alpha is accepted at p = 0.05 and displayed as a * if different from baseline and a # when different between conditions.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>0 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo Young</td>
<td>0.81 ± 0.46</td>
<td>0.89 ± 0.66</td>
<td>0.28 ± 0.10</td>
</tr>
<tr>
<td>CoQ Young</td>
<td>0.79 ± 0.42</td>
<td>0.60 ± 0.19</td>
<td>0.64 ± 0.27</td>
</tr>
<tr>
<td>Placebo Aged</td>
<td>0.64 ± 0.22</td>
<td>1.05 ± 0.29*</td>
<td>1.20 ± 0.68</td>
</tr>
<tr>
<td>CoQ Aged</td>
<td>0.99 ± 0.23</td>
<td>1.06 ± 0.45</td>
<td>0.97 ± 0.29</td>
</tr>
</tbody>
</table>

Table 5-13 - Individual results for YOUNG participant muscle protein carbonyl concentration at baseline, immediately post exercise and 3 hours post exercise following placebo of CoQ supplementation. Protein carbonyls are expressed as µg/mg total protein.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>0 min</th>
<th>180 min</th>
<th>Baseline</th>
<th>0 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Placebo</td>
<td>Placebo</td>
<td>CoQ</td>
<td>CoQ</td>
<td>CoQ</td>
</tr>
<tr>
<td>Young 1</td>
<td>0.82</td>
<td>1.82</td>
<td>0.23</td>
<td>1.35</td>
<td>0.74</td>
<td>0.83</td>
</tr>
<tr>
<td>Young 2</td>
<td>1.26</td>
<td>0.88</td>
<td>0.21</td>
<td>0.69</td>
<td>0.33</td>
<td>--</td>
</tr>
<tr>
<td>Young 3</td>
<td>0.35</td>
<td>0.45</td>
<td>0.39</td>
<td>0.76</td>
<td>0.71</td>
<td>--</td>
</tr>
<tr>
<td>Young 4</td>
<td>--</td>
<td>0.39</td>
<td>--</td>
<td>0.34</td>
<td>0.63</td>
<td>0.44</td>
</tr>
</tbody>
</table>
Table 5-14 - Individual results for AGED participant muscle protein carbonyl concentration at baseline, immediately post exercise and 3 hours post exercise following placebo or CoQ supplementation. Protein carbonyls are expressed as µg/mg total protein.

<table>
<thead>
<tr>
<th></th>
<th>Baseline 0 min</th>
<th>Baseline 180 min</th>
<th>Baseline 0 min</th>
<th>Baseline 180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Placebo</td>
<td>CoQ</td>
<td>CoQ</td>
</tr>
<tr>
<td>Aged 1</td>
<td>0.82</td>
<td>1.32</td>
<td>0.86</td>
<td>0.74</td>
</tr>
<tr>
<td>Aged 2</td>
<td>0.66</td>
<td>1.11</td>
<td>0.73</td>
<td>1.50</td>
</tr>
<tr>
<td>Aged 3</td>
<td>0.88</td>
<td>1.11</td>
<td>1.25</td>
<td>1.07</td>
</tr>
<tr>
<td>Aged 4</td>
<td>0.37</td>
<td>1.15</td>
<td>--</td>
<td>0.58</td>
</tr>
<tr>
<td>Aged 5</td>
<td>0.46</td>
<td>0.55</td>
<td>--</td>
<td>1.70</td>
</tr>
</tbody>
</table>
A summary of CoQ effect on markers of oxidative stress in blood and muscle of YOUNG and AGED groups is shown in Table 5-15. CoQ supplementation appeared to increase PC concentration in the YOUNG, but not in the AGED. However, supplementation appeared to increase serum LOOH in the AGED group initially, but reduce LOOH later in the protocol, whereas the YOUNG LOOH was reduced following supplementation. Whole blood total ROS concentrations gave very mixed results in both YOUNG and AGED groups, although fluctuations in results appeared to be reduced with CoQ compared to placebo. YOUNG skeletal muscle PC concentrations increased slightly 3 hours post-exercise following supplementation, whereas the AGED CoQ group displayed a higher baseline PC compared to placebo, but no impact later in the protocol.

Table 5-15 Impact of CoQ supplementation on markers of oxidative stress in YOUNG and AGED groups compared with placebo across all time points.

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>Ex1</th>
<th>Ex2</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>YOUNG Plasma PC</td>
<td>↑</td>
<td>--</td>
<td>--</td>
<td>↑</td>
<td>--</td>
<td>↑</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>YOUNG Serum LOOH</td>
<td>↑</td>
<td>↓</td>
<td>--</td>
<td>↓</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>↓</td>
</tr>
<tr>
<td>YOUNG Whole blood ROS</td>
<td>--</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>YOUNG Muscle PC</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>↑</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>AGED Plasma PC</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>AGED Serum LOOH</td>
<td>↑</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>↑</td>
<td>--</td>
<td>--</td>
<td>↓</td>
</tr>
<tr>
<td>AGED Whole blood ROS</td>
<td>--</td>
<td>--</td>
<td>↑</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>AGED Muscle PC</td>
<td>↑</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

5.3.4 Antioxidant capacity

5.3.4.1 Serum uric acid (UA)

Serum uric acid (UA) concentrations are displayed in Table 5-16. The mean concentrations across all time points show a significant difference between the YOUNG CoQ group and both the AGED placebo ($p = <0.01$) and the AGED CoQ groups ($p = 0.02$), however no difference is found when compared with the YOUNG placebo group ($p = 1.00$).
Both YOUNG groups displayed a relatively stable UA concentration throughout the duration of the study with no significant differences at any time point compared with baseline concentrations. However, the YOUNG CoQ group did show slight increases in UA between baseline, and concentrations after the first exercise bout ($p = 0.30$, SE $= 0.98$; large), the second exercise bout ($p = 0.28$, SE $= 1.03$; large), 30 min post-exercise ($p = 0.20$, SE $= 1.11$; large), and at 60 min post-exercise ($p = 0.33$, SE $= 0.80$; large). Both AGED groups displayed a slight incline in UA concentrations over the duration of the study when compared to baseline concentrations however this did not reach significance.

The YOUNG CoQ group had higher UA concentrations compared to the AGED CoQ group after the first ($p = 0.24$, SE $= 1.25$; very large) and second ($p = 0.22$, SE $= 1.30$; very large) exercise bout. The YOUNG CoQ group also had higher UA concentrations compared to the AGED placebo group at baseline ($p = 0.21$, SE $= 0.98$; large), after both exercise bouts (First $p = 0.16$, SE $= 1.62$; very large, Second $p = 0.28$, SE $= 1.28$; very large), immediately post-exercise ($p = 0.18$, SE $= 1.07$; large), and 30 min post-exercise ($p = 0.34$, SE $= 0.85$; large).

The YOUNG placebo group had higher UA concentrations compared to the AGED placebo group at baseline ($p = 0.17$, SE $= 1.11$; large), after the first ($p = 0.29$, SE $= 1.26$; very large) and second ($p = 0.42$, SE $= 0.96$; large) exercise bouts and immediately post exercise ($p = 0.15$, SE $= 1.16$; large). The YOUNG placebo group also had higher UA concentrations compared to the AGED CoQ group after the first ($p = 0.45$, SE $= 0.85$; large) and second ($p = 0.39$, SE $= 0.99$; large) and immediately post-exercise ($p = 0.27$, SE $= 0.83$; large). No differences were seen between the two YOUNG groups at any time points or the two AGED groups at any time points.
Table 5-16 - Serum uric acid concentration at baseline, during exercise and throughout 180 minutes of recovery time. Values are means ± standard deviations. UA concentration is expressed as µmol/L. Ex 1 = end of first exercise bout and Ex2 = end of second exercise bout. Time is expressed in minutes starting from the cessation of the final exercise bout. The alpha is accepted at p = 0.05 and displayed as a * if different from baseline and a # when different between conditions.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ex 1</th>
<th>Ex 2</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo Young</td>
<td>370 ± 24</td>
<td>379 ± 20.5</td>
<td>387 ± 17</td>
<td>386 ± 41.9</td>
<td>389 ± 54.8</td>
<td>361 ± 11</td>
<td>390 ± 22.6</td>
<td>393 ± 45.8</td>
</tr>
<tr>
<td>CoQ Young</td>
<td>367 ± 45.6</td>
<td>416 ± 53</td>
<td>420 ± 56.9</td>
<td>387 ± 58.3</td>
<td>421 ± 51.3</td>
<td>409 ± 58.5</td>
<td>390 ± 28.3</td>
<td>378 ± 38.2</td>
</tr>
<tr>
<td>Placebo Aged</td>
<td>298 ± 87.9</td>
<td>305 ± 80.6</td>
<td>330 ± 82.2</td>
<td>314 ± 77.5</td>
<td>350 ± 106</td>
<td>357 ± 101</td>
<td>371 ± 99</td>
<td>362 ± 93.8</td>
</tr>
<tr>
<td>CoQ Aged</td>
<td>326 ± 77.5</td>
<td>325 ± 86.8</td>
<td>329 ± 81.2</td>
<td>332 ± 82.2</td>
<td>358 ± 71.1</td>
<td>355 ± 77.8</td>
<td>351 ± 90.7</td>
<td>354 ± 69.3</td>
</tr>
</tbody>
</table>
5.3.5 Muscle damage (CK)

Skeletal muscle damage was assessed by measuring serum CK concentration (Table 5-17). Mean concentration across all time points was significantly different between the YOUNG CoQ group and both the AGED CoQ \( (p < 0.01) \) and AGED placebo \( (p < 0.01) \). No significant differences were found between the other groups for mean concentration across all time points.

The YOUNG placebo group had higher CK concentration compared with the AGED placebo group at baseline \( (p = 0.12, \ SE = 1.25; \ \text{very large}) \), after the first \( (p = 0.29, \ SE = 0.85; \ \text{large}) \) and the second exercise bout \( (p = 0.25, \ SE = 1.12; \ \text{large}) \), immediately post-exercise \( (p = 0.07, \ SE = 1.55; \ \text{very large}) \), at 60 min post-exercise \( (p = 0.28, \ SE = 0.91; \ \text{large}) \), 120 min post-exercise \( (p = 0.36, \ SE = 0.89; \ \text{large}) \) and at 180 min post-exercise \( (p = 0.16, \ SE = 1.32; \ \text{very large}) \).

The YOUNG CoQ group had higher CK concentrations compared with the AGED CoQ group at baseline \( (p = 0.18, \ SE = 0.94; \ \text{large}) \), immediately post-exercise \( (p = 0.11, \ SE = 1.15; \ \text{large}) \), 30 min post-exercise \( (p = 0.20, \ SE = 0.92; \ \text{large}) \) and 60 min post-exercise \( (p = 0.27, \ SE = 0.84; \ \text{large}) \).

Both YOUNG groups displayed a similar profile for CK concentration over the duration of the study, suggesting a limited impact of CoQ. The AGED groups also displayed similar profiles across the study, however these values were generally lower than the YOUNG groups.

5.3.6 Total Cholesterol

Serum total cholesterol levels were measured throughout the study and are presented in Table 5-18. There was a significant difference in the group means
across all time points between the YOUNG CoQ and the AGED placebo groups ($p = 0.04$). All other group means were similar across all time points.

All groups displayed a similar profile over the duration of the study, total cholesterol levels increased during exercise and then slowly returned to baseline levels. The YOUNG CoQ group had the highest values, which increased from baseline after the second exercise bout ($p = 0.11$, SE = 1.42: very large) and remained elevated immediately post-exercise ($p = 0.14$, SE = 1.22: very large). Similar changes were seen in the AGED CoQ group after the second exercise bout ($p = 0.07$, SE = 1.27: very large) and immediately post-exercise ($p = 0.12$, SE = 1.07: large) and in the AGED placebo group after the first ($p = 0.28$, SE = 0.84: large) and second ($p = 0.30$, SE = 0.95: large) exercise bouts and immediately post exercise ($p = 0.19$, SE = 1.04: large).

Total cholesterol levels were higher in the YOUNG CoQ group compared with the AGED CoQ group after the second exercise bout ($p = 0.22$, SE = 0.80; large), 30 min post-exercise ($p = 0.21$, SE = 0.84: large) and 60 min post exercise ($p = 0.12$, SE = 1.15: large). The YOUNG placebo group had higher total cholesterol levels compared with the AGED placebo group at baseline ($p = 0.08$, SE = 1.48: very large), however no great differences were found across any other time points between the two placebo groups.

In summary, CoQ appeared to have little impact on UA, CK and total cholesterol levels in either the YOUNG or AGED groups. However, there appears to be a difference between the two ages under both conditions. The YOUNG placebo displayed higher UA, CK and total cholesterol than the AGED placebo, and the YOUNG CoQ displayed higher UA, CK and total cholesterol than the AGED CoQ.
This highlights that there are distinct differences in response to exercise across age when assessing antioxidant capacity, muscle damage and cholesterol levels.
Table 5-17 - Serum creatine kinase concentration at baseline, during exercise conditions and throughout 180 minutes of recovery time. Values are means ± standard deviations. CK concentration is expressed as U/L. Ex 1 = end of first exercise bout and Ex2 = end of second exercise bout. Time is expressed in minutes starting from the cessation of the final exercise bout.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ex 1</th>
<th>Ex 2</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo Young</td>
<td>186 ± 52.7</td>
<td>185 ± 88.7</td>
<td>194 ± 92.3</td>
<td>212 ± 62.5</td>
<td>161 ± 81</td>
<td>160 ± 55.9</td>
<td>152 ± 48.4</td>
<td>175 ± 41.5</td>
</tr>
<tr>
<td>CoQ Young</td>
<td>154 ± 42.6</td>
<td>165 ± 82.4</td>
<td>179 ± 89.4</td>
<td>226 ± 102</td>
<td>173 ± 62.1</td>
<td>160 ± 58.8</td>
<td>137 ± 41</td>
<td>133 ± 33.9</td>
</tr>
<tr>
<td>Placebo Aged</td>
<td>122 ± 47.3</td>
<td>127 ± 37.8</td>
<td>117 ± 29.3</td>
<td>135 ± 33</td>
<td>141 ± 58.6</td>
<td>119 ± 29.3</td>
<td>118 ± 26.1</td>
<td>129 ± 26.2</td>
</tr>
<tr>
<td>CoQ Aged</td>
<td>115 ± 39.4</td>
<td>129 ± 44.6</td>
<td>134 ± 43.1</td>
<td>135 ± 44.5</td>
<td>124 ± 41.4</td>
<td>117 ± 43.5</td>
<td>121 ± 47.5</td>
<td>118 ± 36</td>
</tr>
</tbody>
</table>

Table 5-18 - Serum total cholesterol at baseline, during exercise conditions and throughout 180 minutes of recovery time. Values are means ± standard deviations. Total cholesterol concentration is expressed as mmol/L. Ex 1 = end of first exercise bout and Ex2 = end of second exercise bout. Time is expressed in minutes starting from the cessation of the final exercise bout.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ex 1</th>
<th>Ex 2</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo Young</td>
<td>5.91 ± 0.46</td>
<td>5.83 ± 0.34</td>
<td>6.08 ± 0.36</td>
<td>6.11 ± 0.39</td>
<td>5.42 ± 0.87</td>
<td>5.54 ± 0.36</td>
<td>5.20 ± 0.12</td>
<td>5.75 ± 0.47</td>
</tr>
<tr>
<td>CoQ Young</td>
<td>5.55 ± 0.38</td>
<td>6.41 ± 1.16</td>
<td>6.64 ± 1.02</td>
<td>6.27 ± 0.74</td>
<td>5.86 ± 0.96</td>
<td>5.75 ± 0.68</td>
<td>5.46 ± 0.29</td>
<td>5.27 ± 0.32</td>
</tr>
<tr>
<td>Placebo Aged</td>
<td>5.15 ± 0.57</td>
<td>5.70 ± 0.72</td>
<td>5.78 ± 0.88</td>
<td>5.80 ± 0.67</td>
<td>5.43 ± 0.75</td>
<td>5.02 ± 0.66</td>
<td>5.16 ± 0.75</td>
<td>5.15 ± 0.82</td>
</tr>
<tr>
<td>CoQ Aged</td>
<td>5.36 ± 0.72</td>
<td>5.89 ± 0.24</td>
<td>6.05 ± 0.26</td>
<td>5.95 ± 0.31</td>
<td>5.22 ± 0.51</td>
<td>5.17 ± 0.22</td>
<td>5.32 ± 0.30</td>
<td>5.16 ± 0.62</td>
</tr>
</tbody>
</table>
The results from the YOUNG placebo group in the current study showed very similar trends to the results from the exercising group from Chapter 4. Although, the values were not always directly comparable between the two groups, this is most likely due to a smaller YOUNG placebo group compared with the exercise condition. Results displayed the same trends in the YOUNG placebo and exercise condition from Chapter 4 for serum LOOH, plasma PC, and UA. However, CK values present the same trend over the duration of the testing protocol, the YOUNG placebo group from the current study displayed higher values across all time points compared with the exercise condition from Chapter 4. Muscle PC values were significantly different between the two groups at baseline ($p = 0.01$, SE = 1.55; very large) and immediately post-exercise ($p = 0.01$, SE = 1.20; very large), however no difference was found at 180 minutes post exercise. The high level of variability across whole blood total ROS concentrations produced similar profiles across the majority of the testing protocol for both groups.

The results from the AGED placebo group in the current study and the exercise group from Chapter 4 show similar differences between them as the YOUNG and AGED placebo groups. However, the AGED placebo show closer values to the exercise group from Chapter 4 for CK concentration.

This demonstrates that the HIIE protocol is appropriate for increasing oxidative stress in both young and aged participants. It also further supports the HIIE protocol as a functional tool for future research into oxidative stress.
5.4 Discussion

The main findings of this chapter were that an acute, 24 hr CoQ supplementation showed a trend towards increased mean muscle CoQ content (Table 5-6) and a significant increase in serum CoQ concentration (Table 5-3) in both age groups at baseline. This would suggest that a dietary CoQ intake of 300 mg/day may be taken up by the tissues after supplementing for only 24 hours. These findings are in agreement with those of Cooke (Cooke et al., 2008) who also found a trend for increased muscle CoQ concentrations following an acute supplementation phase in young healthy participants using a dose of 200 mg/day. However, the present results are in contrast to those from Svensson (Svensson et al., 1999) and Laaksonen (Laaksonen et al., 1995) who found an increase in plasma CoQ concentrations, but no change in skeletal muscle CoQ after supplementation. Both of these studies used a lower dose of CoQ than the current study and over a longer duration which may have impacted the uptake of CoQ into the tissues.

The HIIE protocol appeared to have little impact on muscle CoQ concentration immediately post-exercise, compared to baseline, however concentrations fell by 180 min post-exercise (Table 5-6) in both YOUNG and AGED groups. CoQ intake caused a reduction in skeletal muscle CoQ concentration immediately after exercise, compared to baseline, and remained reduced at 180 min post-exercise (Table 5-6). This reduction immediately post-exercise brought the CoQ concentrations in line with baseline measures of the placebo group. From these novel findings we hypothesise that the reduction in CoQ concentration post-exercise may be caused by the mobilisation of CoQ from muscle and their use in the skeletal muscle to protect against the increase in oxidative stress brought about by the HIIE. In the current study total CoQ was measured and it would be
interesting in future research to determine the ratio between ubiquinol and ubiquinone to give a better understanding on any changes in the redox state of CoQ. CoQ is also fundamental to ATP production (Crane, 2001, 2007) and a higher rate of metabolism in the exercising muscle could cause a reduction in total CoQ concentration within the skeletal muscle itself. For future research, it would be beneficial to analyse serum CoQ content post-exercise to determine if the reduction in skeletal muscle CoQ is aligned with an increase in serum CoQ concentration. If there is a mobilisation of CoQ from skeletal muscle, it may be a protective mechanism to increase antioxidant activity in the blood.

It was unexpected to find that the AGED group had a higher skeletal muscle CoQ concentration pre-supplement compared with the younger participants (320.5 ± 87.7 pmol/mg protein and 171.0 ± 98.3 pmol/mg protein, respectively). These results are in contrast to the widely accepted theory that CoQ concentration in blood and other tissues declines with age (Ernster & Forsmark-Andrée, 1993; Tang et al., 2001). It may be hypothesised that this could be due to a shift in skeletal muscle composition with age to a higher percentage of type I skeletal muscle fibres (McIntire & Hoffman, 2011). Due to the oxidative nature of type I fibres, they have a higher concentration of mitochondria, which is where the majority of CoQ is located (Bhagavan & Chopra, 2006). Therefore, a higher percentage of type I fibres in aged skeletal muscle would most likely increase the amount of type I fibres taken in these biopsy sample and could lead to an increased CoQ content. It is also possible that the body adapts to an increase in oxidative damage with age by increasing the concentration of endogenous antioxidants, such as CoQ. The present findings may also reflect the selection process of using healthy, recreationally active aged participants to ensure
completion of the HIIE protocol. This could limit the application of the results to an active aged population and not to a sedentary one.

Serum LOOH levels were not influenced by CoQ in YOUNG participants. However, the AGED population displayed elevated serum LOOH under placebo conditions after exercise, peaking at 180 min post-exercise, in accordance with previous research (Marzani, Felzani, Bellomo, Vecchiet, & Marzatico, 2005). Interestingly, CoQ ameliorated this response and lipid hydroperoxide concentrations remained close to baseline levels in the AGED CoQ group, making them comparable to the YOUNG groups. This finding supports previous research that showed a reduction in lipid peroxidation products following CoQ intake (Del Pozo-Cruz et al., 2014; Hernández-Ojeda et al., 2012; Sarmiento, Diaz-Castro, Pulido-Moran, Kajarabille, et al., 2016; Sarmiento, Diaz-Castro, Pulido-Moran, Moreno-Fernandez, et al., 2016). The mechanism responsible for increased LOOH in an aged population is not yet fully understood. However, it could, in part, be as a result of increased lipid content of aged skeletal muscle fibres, which could increase the amount of lipid exposed to ROS produced within skeletal muscle during exercise. When CoQ concentration is increased within the skeletal muscle following supplementation this may provide protection of these lipids and help to return LOOH concentrations closer to those of a young population. Additionally, it is understood that lipid peroxidation increases with age (Laaksonen et al., 1995), due to a reduced efficiency of metabolism and antioxidant protection. However, with an increased CoQ concentration within the blood and skeletal muscle, there is more available CoQ to scavenge free radicals (Del Pozo-Cruz et al., 2014; Turunen et al., 2004) and more rapidly regenerate $\alpha$-tocopherol, another potent lipid antioxidant.
5.5 Conclusions

From the current study it may be concluded that supplementation with 300 mg/day CoQ for 24 hours shows a trend towards increased CoQ concentration in skeletal muscle and significant increases in blood CoQ concentrations in both a young and aged population. Unexpectedly, it was found that aged individuals showed a tendency for increased CoQ concentration in skeletal muscle without supplementation. The results also suggest that CoQ intake can reduce the exercise-induced increase in LOOH seen in an aged population, shifting them towards those observed in a young population.
Chapter 6 - The effect of CoQ on redox balance of young and aged C2C12 skeletal muscle cells following biochemically simulated exercise.
CHAPTER 6 – Study 4 – The effect of CoQ on redox balance of young and aged C2C12 skeletal muscle cells following biochemically simulated exercise.

6.1 Introduction

As previously discussed in Chapter 5, research from our laboratory and others has suggested discrepancies between blood and muscle tissue responses to exercise-induced redox balance in terms of certain markers (Child et al., 1999; Meydani et al., 2017; Parker et al., 2018). It was concluded that collecting of both skeletal muscle biopsies and blood is required for accurate analysis. However, the muscle biopsy procedure is invasive and has the potential to impact negatively on short-term muscle performance. In vulnerable groups, such as an aged population, healing may also not occur as efficiently (Dice, 1993; Fulle et al., 2004). These are just some of the reasons why it can be difficult to recruit participants. Therefore, alternative methods of analysis need to be developed to provide less invasive methods of preliminary testing to determine if human research is of value, to allow for optimisation of protocols and to assess multiple compounds.

Cell culture models have successfully been used to optimise research protocols, allowing pilot testing in cells prior to human based research. This valuable tool limits the need for time consuming, expensive studies for which recruitment can be difficult. A biochemical exercise protocol in Chapter 3 was therefore investigated, in which an intermittent, 3 x 5 min exposure elicited the greatest increase in ROS. This was then applied to human based research in Chapters 4 and 5.

Research into CoQ as a supplement for general health has increased in recent years, with an interest in its potential to improve muscle health and function, and delay the onset of age-related decline (del Pozo-Cruz et al., 2014; Laaksonen et
al., 1995; Svensson et al., 1999; Wada et al., 2007). The results from Chapter 5 suggested that CoQ was taken up into the skeletal muscle to some extent, in both young and aged participants. CoQ was also able to limit the increase in exercise-induced LOOH in an aged population. However, this research is invasive and there is a need for a safe, effective screening tool to be developed.

An ageing model in cell culture allows for the assessment of pharmacological compounds, across a range of aged cell types to provide an alternative view of ageing skeletal muscle. Additionally, a physiologically relevant dose of CoQ has not yet been identified for use in cell culture. Extremely high doses that can be applied in cell culture are not representative of what is feasible in human skeletal muscle-based studies, and the identification of a physiologically relevant dose needs to be made for a more applicable cell culture model. The development of these aspects of a cell culture model in conjunction with the biochemical exercise model used in Chapter 3 could provide a toolkit for assessing multiple compounds at young and aged states under rest and exercise stressed conditions. Further comparisons can be made between skeletal muscle biopsy data and the cell culture model following the analysis of human skeletal muscle samples in Chapters 4 and 5.

6.1.1 Objectives:

- Identify an appropriate, physiologically relevant dose of CoQ for use in cell culture.
- Develop a model of skeletal muscle ageing in cell culture.
- Assess the impact of CoQ treatment on measures of biochemical exercise-induced oxidative stress in ‘young’ and ‘old’ cell cultures.
6.2 Materials and methods

Cells were cultured under aseptic conditions as described in section 2.8.

6.3 Experimental Flasks

For experimentation, cells were seeded at $20 \times 10^4$ with 8 ml growing medium. As described in section 2.8.7, growing medium was replaced with 8 ml differentiation medium at 70 - 80% confluence and used for experimentation once myotubes had been formed.

6.4 C2C12 experimental procedures

6.4.1 C2C12 Coenzyme Q10 Treatment

Coenzyme Q10 (Cambridge Biosciences, Cambridge, UK) was suspended in 100% dimethylformamide (DMF) to form a stock solution which was frozen in 50 µl aliquots. For experimentation, coenzyme Q10 stock was diluted with growing medium, to reduce the concentration of the residual solvent to 0.01% v/v DMF, and applied to the cells. Fresh treatment was made for each experimental day. During experimentation the control solution was prepared using 2% differentiation medium and 0.01% v/v DMF to eliminate solvent interaction.

6.4.2 C2C12 Biochemical Exercise Mimic

A biochemical exercise mimic treatment was applied to the cells, as previously described in section 3.2.1.

6.5 C2C12 biochemical analysis

6.5.1 Protein quantification

Cell samples that had been experimented on in flasks and stored at -80°C in cryovials were treated as described previously in blood samples (section 2.6.1).
6.5.2 Lipid hydroperoxides (LOOH)

Lipid hydroperoxides were measured spectrophotometrically using the Ferrous Oxidation of Xylenol orange (FOX) assay, as previously described in blood (section 2.6.4). A standard curve of H₂O₂ concentrations between 0 – 5 µl was measured for comparison, 20 µl of H₂O₂ concentrations were added to 180 µl of FOX assay reagent.

The FOX assay reagent was prepared using aqueous solution containing 250 µM ammonium ferrous sulphate, 100 µM Xylenol orange, 100 µM sorbitol and 25 mM of sulphuric acid (H₂SO₄) (all from Sigma-Aldrich Poole, UK). Deproteinised cell samples in 100% methanol were defrosted on ice in the dark. Once thawed, 20 µl of cell sample was added to 108 µl FOX reagent. The samples were incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 550 nm against the standard curve.

6.5.3 Protein carbonyls (PC)

Carbonyl groups of oxidized proteins were quantified using a modified spectrophotometric method as previously described (section 2.6.6). Cell samples in PBS were defrosted on ice, in the dark. Once thawed, up to 1 mg protein sample was added to 100 µl of deionised water and 10 µl 10 mM DNPH solution and incubated in the dark at room temperature for 10 minutes. After incubation, 50 µl of sodium hydroxide was added to each well and incubated for a further 10 minutes. Absorbance was measured at 450 nm against a blank (Magellan, Tecan, Infinite 2000 Pro, Zurich, Switzerland).

6.5.4 Superoxide dismutase (SOD)

Total SOD activity was quantified using a modified SOD Assay Kit based on Cayman Chemicals standard kit as previously described for blood (section 2.6.5).
Cell samples in PBS were defrosted in the dark on ice. Once thawed, samples were inverted and then centrifuged at 4°C (3000 x g for 5 minutes). Cell samples were diluted 1:1000 in 500 mM Tris-HCl and 10 µl of the diluted sample was added to 20 µl NBT and 170 µl of sample assay buffer. Absorbance was measured at 560 nm against the standard curve.

6.5.5 Ageing process
Skeletal muscle myoblasts were passaged until a minimum of P30, but a maximum of P45. Previous research (Pronsato et al., 2013) demonstrated signs of senescence in C2C12 cells with high passage numbers. Cell cultures of passage ≥30 were classified as ‘old’. To ensure a distinct difference in cell age, ‘young’ cells were classified between P16 – P22.

6.5.6 CoQ dose response
C2C12 myotube cultures were treated with a range of physiologically-relevant CoQ concentrations (1-1000 nM) for 24 h and analysed for total ROS by fluorescence to identify the most appropriate dose for use in the remaining research.

6.6 Statistical analysis
Data were assessed using SPSS as described in 2.11. The alpha was accepted at P = < 0.05 and indicated by a *, all descriptive data sets are expressed as a mean value ± standard deviations. Standardised effect size (SE) was calculated using η² for the ANOVA analysis and Cohen’s d for differences between conditions.
6.7 Results

6.7.1 Optimisation of CoQ dose

CoQ treatment reduced total ROS production in young non-stressed cells at concentrations between 1 nM and 500 nM (16.3% - 24.5% differences in means compared to control) are shown in Table 6-1. The 24.5% decrease in mean ROS production, induced by 500 nM CoQ was statistically significant ($p = 0.02$, SE =1.48; very large). Concentrations above 500 nM did not alter ROS production in non-stressed cells. For the purpose of the remaining research in this chapter, the 500 nM dose was used.
Table 6-1 – ROS production as a percentage of control following 24 hours incubation with a range of physiologically relevant CoQ concentrations (nM). Values are means ± standard deviations. Control is set at 100%. The alpha was accepted at \( p = < 0.05 \) and indicated by a *.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>250</th>
<th>500</th>
<th>750</th>
<th>1000</th>
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</thead>
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<tr>
<td>Percentage change from control (%)</td>
<td>84 ± 13</td>
<td>78 ± 5</td>
<td>83 ± 6</td>
<td>86 ± 4</td>
<td>76 ± 13*</td>
<td>93 ± 11</td>
<td>95 ± 14</td>
</tr>
</tbody>
</table>
6.7.2 Impact of CoQ on oxidative stress with age

Following the optimisation of the exercise mimic protocol (Chapter 3), the original concentration of the biochemical exercise-mimic, represented in the data as 100% concentration, was used. This was applied to the cells using the intermittent 3 x 5-minute protocol. Following optimisation of the CoQ dose, cells were exposed to 24-hour incubation with 500 nM concentrations of CoQ or a control lacking CoQ. Cells were categorised using the abbreviations below in Table 6-2.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Abbreviation used</th>
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<tbody>
<tr>
<td>YOUNG</td>
<td>Y</td>
</tr>
<tr>
<td>OLD</td>
<td>O</td>
</tr>
<tr>
<td>CoQ supplemented</td>
<td>Q</td>
</tr>
<tr>
<td>Non-supplemented</td>
<td>-</td>
</tr>
<tr>
<td>Exercise stressed</td>
<td>Ex</td>
</tr>
<tr>
<td>Non-exercise stressed</td>
<td>Cont</td>
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</tbody>
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6.7.2.1 Lipid Hydroperoxide (LOOH) response

There was a significant difference between all groups for LOOH as determined by one-way ANOVA ($F(7, 36) = 2.34$, $p = 0.045$, $\eta^2 = 0.31$; small). A Bonferroni post-hoc test was completed to distinguish where significance was found.

The YOUNG, supplemented exercise-stressed group (YQEx) displayed significantly higher concentrations of LOOH compared with the YOUNG, supplemented control group (YQCont), ($p = 0.05$, $SE = 2.54$; very large), however this effect was not seen in the OLD population, or when placebo treatment was administered.
Figure 6-1 - Lipid hydroperoxide concentration (LOOH) in YOUNG and OLD cells following 24 hour incubation with CoQ or Placebo treatment under exercise or control conditions. Values are means ± standard deviations. Lipid hydroperoxides are expressed as mmol.L⁻¹. The alpha was accepted at \( P = < 0.05 \) and indicated by a *.

6.7.2.2 Protein carbonyls (PC)

There was a significant difference between all groups for PC as determined by one-way ANOVA (\( F (7, 33) = 7.82, p = <0.001, \eta^2 = 0.62; \text{medium} \)). A Bonferroni post-hoc test was completed to distinguish where significance was found.

The main difference in PC concentration appeared between the different age groups (Figure 6-2). All YOUNG groups displayed a lower concentration of PC compared with all OLD groups. The YQEx group showed a significantly lower PC concentration when compared with OQEx (\( p = 0.014, \text{SE} = 2.00; \text{very large} \)), OQCont (\( p = 0.012, \text{SE} = 3.32; \text{very large} \)), OEx (\( p = 0.009, \text{SE} = 3.26; \text{very large} \)), and OCont (\( p = 0.001, \text{SE} = 3.93; \text{very large} \)). The YQCont group showed a significantly lower PC concentration when compared with OQEx (\( p = 0.027, \text{SE} = 1.93; \text{very large} \)), OQCont (\( p = 0.023, \text{SE} = 3.31; \text{very large} \)), OEx (\( p = 0.018, \text{SE} = 3.30; \text{very large} \)), and OCont (\( p = 0.003, \text{SE} = 4.06; \text{very large} \)). A significant
difference was also found between the non-supplemented YEx group and all OLD groups; OQEx ($p = 0.015$, SE = 2.24; very large), OQCont, ($p = 0.013$, SE = 3.89; very large), OEx ($p = 0.010$, SE = 3.79; very large), and OCont ($p = 0.002$, SE = 4.57; very large).

The only YOUNG group that did not reach a significant reduction compared with the OLD groups was the YCont group ($p = 1.0$ for all comparisons). The YCont group was also the only group that displayed a difference within the same age category. The YCont group had higher PC concentrations than YQEx ($p = 0.70$, SE = 2.15; very large), YQCont ($p = 1.0$, SE = 2.09; very large), and YEx ($p = 0.53$, SE = 2.49; very large). Comparisons within the OLD groups showed no difference in PC concentrations.
Figure 6-2 - Protein carbonyl (PC) concentration in YOUNG and OLD cells following 24 hour incubation with CoQ or Placebo treatment under exercise or control conditions. Values are means ± standard deviations. PC concentration is expressed as µg/mg total protein. The alpha was accepted at $P = < 0.05$. Significance is indicated by a * for a difference compared with YQEx, indicated by a # for a difference compared with YQCont and a + for a difference compared with YEx.

6.7.3 Impact of CoQ on antioxidant capacity with age
6.7.3.1 Superoxide Dismutase (SOD) response

There was a significant difference between all groups for SOD as determined by one-way ANOVA ($F(7, 29) = 4.36$, $p = 0.002$, $\eta^2 = 0.51$; medium). A Bonferroni post-hoc test was completed to distinguish where significance was found.

The OLD, non-supplemented control group (OCont) displayed a higher concentration of SOD activity compared with all other groups. This increase reached significance when compared with the OLD, supplemented exercise (OQEx) and control (OQCont) groups ($p = 0.001$, SE = 3.94; very large, and $p = 0.037$, SE = 2.39; very large, respectively) and also compared with the YOUNG,
non-supplemented exercise group (YCont) \( (p = 0.025, \ SE = 3.5; \text{very large}) \) as shown in (Figure 6-3). Although significance was not reached, the OCont group also displayed a large to very large standardised effect size when SOD activity was compared to all other groups; OEx \( (p = 0.18, \ SE = 1.86; \text{very large}) \), YCont \( (p = <1.0, \ SE = 0.83; \text{large}) \), YQCont \( (p = 0.74, \ SE = 1.58; \text{very large}) \), and YQEx \( (p = 0.22, \ SE = 2.42; \text{very large}) \).

The OLD, supplemented exercise stressed group (OQEx) appeared to have a reduced SOD activity level compared with the other groups. This reduction only reached a significant \( p \) value in comparison to the OCont group, however there also appeared to be a very large standardised effect size between OQEx and OQCont \( (p = 1.0, \ SE = 1.13; \text{very large}) \), YCont \( (p = 0.61, \ SE = 1.05; \text{very large}) \) and OEx \( (p = 1.0, \ SE = 1.77; \text{very large}) \).

![Figure 6-3 - Superoxide dismutase (SOD) activity in YOUNG and OLD cells following 24 hour incubation with CoQ or Placebo treatment under exercise or control conditions. Values are](image-url)
6.8 Discussion

The main aims of this chapter were; to investigate an ageing and biochemical exercise protocol for use in cell culture, to identify a physiologically relevant CoQ dose for treatment in cell culture and, finally, to examine the impact of CoQ supplementation on biochemical exercise-induced oxidative stress and antioxidant capacity in young and aged models of skeletal muscle.

In order to provide a difference in ‘age’ for the cells, Pronsato (Pronsato et al., 2013) found that high population doublings, and therefore a higher passage number, of the cells caused a senescence phenotype. The present research supports this finding, with distinct differences in response between the YOUNG and OLD cells. Specifically, there was a significant difference between PC concentrations in the two age groups. The older cells, regardless of the exercise or supplement conditions, displayed elevated PC production. This suggests that as cells are passaged repeatedly their response changes, as would be expected with an ageing muscle cell. However, this finding is in contrast to the outcome in Chapter 5 in human subjects, where PC concentrations did not differ between the two age groups in plasma or skeletal muscle measurements.

The model of biochemical exercise investigated in Chapter 3, was used to examine the impact of the dietary supplement CoQ on oxidative. To ensure a suitable dose of CoQ preliminary research was carried out using a variety of physiologically relevant doses of CoQ (Duncan et al., 2005). It was found that a dose of 500 nM reduced total ROS production to the greatest extent, whereas doses of a higher concentration appeared to have little or no impact on ROS
production. Interestingly, this would suggest that for in vivo research, the exposure of tissues to a higher dose of a CoQ may not provide additional protection when compared to a moderate dose. It is possible that there is a maximum CoQ uptake for skeletal muscle cells in culture and the mitochondria may become saturated (Estornell et al., 1992; Littarru & Tiano, 2007).

The application of CoQ to the cells provided alterations in oxidative stress markers in cell culture, however, they were highly varied. The greatest change was seen in antioxidant capacity in the form of SOD activity levels of the OLD cells. Supplementation reduced SOD activity in both the exercise stressed and control group when compared with the non-stressed placebo old group.

The cell culture model developed in this chapter provides a useful tool for optimising protocols and pre-screening pharmaceutical and dietary compounds, however it is important to note its limitations when researching the impact of markers of oxidative stress and antioxidant capacity and applying these findings to human-based research. Cell culture research can provide an insight into the mechanisms behind CoQ supplementation with age and exercise. While exposure of tissues to CoQ in human based research is dependent upon successful CoQ digestion, uptake and utilisation, alongside the interplay of many other physiological interactions. In cell culture, the cells are suspended in an unnatural environment during the cell culture process (Halliwell, 2014). The O₂ tension in vivo usually falls around 1-10 mmHg, whereas in cell culture O₂ tension is significantly higher, around 150 mmHg, this increased presence of O₂ can cause an increase in ROS production (de Groot & Littauer, 1989; Yusa, Freeman, & Crapo, 1984). Cell culture medium is usually deficient in many antioxidants present in the human body and may lead to an over interpretation of the effect of
an applied antioxidant (Halliwell & Gutteridge, 2007.; Leist, Raab, Maurer, Rösick, & Brigelius-Flohé, 1996; Smith, Visioli, & Hagen, 2002). Due to the isolation of the cells in culture there is also a loss of cell communication from different neighbouring cell types and the cell matrix is lost (Halliwell, 2014). It is, therefore, important to consider these limitations to cell culture research when applying it to, or combining it with, human based research of a similar nature. However, preliminary research in cell culture remains invaluable as an initial optimisation of methodology and a screen for future dietary interventions, prior to use in human subjects.

6.9 Conclusions

The current study investigated a cell culture model of ageing. This protocol provides a structured toolkit through which to investigate the impact of dietary and pharmacological compounds on skeletal muscle, without the need for skeletal muscle biopsies. However, a physiological dose of CoQ was unable to limit the impact of these stressors within the cell model, which was in contrast to our human based research (Chapter 5).
Chapter 7 – General Discussion
7.1 Summary of findings

This thesis has advanced the existing knowledge of ageing in oxidative stress in human skeletal muscle by directly comparing oxidative stress markers and antioxidant capacity in muscle biopsies and blood and begun the development of an in vitro screening tool. It provides intriguing potential mechanisms for future study of muscle oxidative stress in biopsy-based trials, including basic controls, such as the impact of muscle biopsies, which tend not to be included in previous studies. This thesis has demonstrated that with the use of a HIIE protocol, the impact of the biopsy procedure is limited and therefore the observed oxidative stress is due to the exercise intervention. This is in alignment with the main aims of the thesis, which were to assess the impact of multiple biopsies on markers of post-exercise redox status, to compare redox responses in young and aged populations to HIIE and CoQ supplementation and to develop a cell culture toolkit for less invasive preliminary assessment of dietary compounds and optimisation of protocols.

The novel findings of this thesis are;

- Multiple biopsies had little to no impact on redox markers in both blood and skeletal muscle samples of a young, healthy male population.
- HIIE elicited greater increases in LOOH concentrations in aged compared with young males without supplementation.
- Aged males had higher basal skeletal muscle CoQ concentrations compared with young males.
- Acute supplementation with 300 mg CoQ reduced exercise-induced increases in LOOH in aged males.
• The successful development of a cell culture model reflecting skeletal muscle ageing and biochemical exercise to allow the testing of multiple compounds at young and aged states under rest and exercise stressed conditions.

• Repeated exposure of C2C12 muscle cells to a biochemical exercise stimulus on consecutive days resulted in a reduction in total ROS.

7.1.1 The effect of skeletal muscle biopsies on redox status in blood and skeletal muscle.

From the results discussed in Chapter 4, it was possible to conclude that multiple biopsy procedures had little effect on markers of oxidative stress, antioxidant protection and muscle damage. This suggests that the results of previous research using skeletal muscle biopsies are representative of the impact of the exercise protocol on oxidative stress and antioxidant status, rather than an impact of the skeletal muscle biopsy procedure itself (Fogarty et al., 2013).

Within this same chapter, evidence was provided in support of previous research that blood analysis gave an unreliable representation of skeletal muscle redox status for selected markers (Parker et al., 2018). These findings support the need for skeletal muscle biopsy procedures alongside blood sample collection when investigating oxidative stress and antioxidant status within the skeletal muscle tissue.

7.1.2 The effect of acute CoQ supplementation on exercise-induced changes in skeletal muscle redox status in young and aged males.

In Chapter 5, the HIIE protocol from Chapter 4 was used to assess the impact of exercise and acute supplementation with CoQ on healthy young and aged males. An acute 300 mg CoQ supplementation was identified which caused a significant increase in plasma CoQ concentration and a trend towards increased skeletal
muscle CoQ concentration in both the YOUNG and AGED groups. In addition, typically it is accepted that CoQ concentrations decline with age (Ernster & Forsmark-Andrée, 1993; Sohal & Forster, 2007), although this was not the case in our study. The unexpectedly higher baseline CoQ concentration found in muscle of the AGED group, compared with the YOUNG group, is similar to data in a collection of studies that found higher blood CoQ content in older participants (Del Pozo-Cruz et al., 2014; Laaksonen et al., 1995; Wada et al., 2007). However, it appears that no studies have found higher muscle CoQ in an aged population. The mechanism behind the elevated CoQ levels in an aged population is currently unknown. It may be speculated that this could be as a result of fibre type composition change with age. As we age, skeletal muscle mass is lost, with type IIb muscle fibres being lost at a higher rate than type I (Faulkner et al., 1995; Lexell, 1995; Pansarasa et al., 2002) and type I fibre proportion can also increase with age (Lexell, 1995; Örlander et al., 1978). CoQ concentration is higher in type I due to the oxidative nature of these fibres and the higher number of mitochondria present (Leary et al., 2003). A greater loss in type IIb fibres would therefore, increase the proportion of type I fibres, possibly causing an incidental increase in the amount of CoQ present. However, fibre type composition of the skeletal muscle samples was not analysed and therefore this is only speculative.

Increased muscle CoQ concentration in an aged population could also be an adaptation to higher levels of oxidative stress with age to help protect against oxidative damage. CoQ is considered an important factor in many age-related diseases (Hernández-Ojeda et al., 2012) and further understanding of this mechanism would be of great value.

The results from Chapter 5 suggested that CoQ supplementation provided some protection against the exercise-induced increase in LOOH in the AGED group,
but not in the YOUNG group. As we age the rate of lipid peroxidation increases with exercise (Laaksonen et al., 1995; Marzani et al., 2005); thought to be due to increased ROS production and a decline in protective mechanisms. It is possible that by increasing the CoQ concentration within skeletal muscle, the protection of lipids is increased due to a higher concentration of this lipid specific antioxidant and the regeneration of \( \alpha \)-tocopherol, another potent lipid antioxidant.

### 7.1.3 The effect of CoQ on redox balance of young and aged C2C12 skeletal muscle cells following biochemically simulated exercise.

It was demonstrated in Chapter 4 that skeletal muscle biopsies are fundamental for investigation into skeletal muscle redox status. However, they are invasive and often unappealing to participants. To reduce the need for skeletal muscle biopsy procedures during the preliminary phases of skeletal muscle redox research, a cell culture toolkit was developed to provide an adequate method for pre-screening dietary compounds. This was used to investigate a biochemically stimulated exercise model (Chapter 3) and develop an ageing model (Chapter 6) allowing for early testing across both young and aged cells. Although the results may not be directly comparable to those of human skeletal muscle biopsies due to limitations of using cell culture in redox research (Halliwell, 2014; Halliwell & Whiteman, 2004), they provide a valuable early screening tool. This could reduce the need for skeletal muscle biopsies, especially in vulnerable populations, such as the aged. As discussed in Chapter 6, the cell model to assess CoQ application on young and aged cells was used to demonstrate the application of the cell culture toolkit.
7.2 Practical implications and recommendations for future research

The research in this thesis strengthens the justification for using skeletal muscle biopsies alongside blood collection in research on oxidative stress in skeletal muscle, and provides a robust cell culture model for future research. However, further research is required to understand the mechanisms by which ROS and antioxidants are impacted by HIIE, dietary supplementation and the ageing process.

7.2.1 Blood and biopsy sample timings and protein signalling measurements for future research

Chapters 4 and 5 identified that HIIE altered redox status in blood and showed a trend towards changes in the skeletal muscle during exercise and through 3 hours recovery. Previous findings (Bloomer et al., 2006, 2005; Close et al., 2005) have shown that some markers, especially those in skeletal muscle, may take longer to display the true impact of exercise on redox status. For example it has been found that LOOH (Bloomer et al., 2005; Close et al., 2004, 2005) and PC (Bloomer et al., 2005; Lee et al., 2002) often have a biphasic response to exercise, with the greatest peak seen at 24-48 hours post-exercise. In taking this research forward, this suggests that future research should take additional biopsies and blood samples at 24- and 48-hours post-exercise. These samples would also help to identify when ROS levels return to baseline. However, it is important to note that the impact of the biopsy procedure, as analysed in Chapter 4, would need to be repeated at these later time points to ensure that there is no interference from the biopsy procedure itself.

Changes in blood and skeletal muscle ROS, antioxidants and muscle damage in response to exercise and CoQ have been identified. However, these only provide
a snapshot of what is happening in the whole body. To allow for better understanding of the mechanisms involved in these changes and the impact on adaptation, especially in an aged population, more specific redox-sensitive protein signalling measurements could be used, as suggested by others (Henríquez-Olguín et al., 2016; Margaritelis et al., 2016; Parker et al., 2018).

Downstream markers of redox-sensitive protein signalling have been linked to exercise adaptations in skeletal muscle (Parker et al., 2016, 2018); for example c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which are some of the key redox-sensitive protein signalling pathways for adaptation to exercise (Kang et al., 2009; Kramer & Goodyear, 2007; Parker et al., 2018). Parker et al., (2018), found that plasma status did not reflect skeletal muscle phosphorylation of these signalling pathways, supporting the need for muscle biopsies in this line of research. One of the key downstream proteins is the master transcriptional regulator of mitochondrial biogenesis, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Cobley et al., 2012; Hood, 2009; Pilegaard, Saltin, & Neufer, 2003), the transcriptional response of which subsequent mitochondrial adaptation has been shown to remain intact with advanced ageing (Cobley et al., 2012; Iversen et al., 2011). However, it is important to note that these pathway networks are complex and adaptations to these pathways may occur independent of exercise-induced ROS. Therefore, measurement of these signalling pathways in future research could help identify mechanisms behind the variations in redox status response to HIIE and age.
7.2.2 Individualisation

The data in Chapters 4 and 5 show that individuals respond very differently to exercise stress and supplementation. Similar to previous findings, some individuals could be classed as ‘responders’ and other as ‘non-responders’ to supplementation with CoQ (Alf, Schmidt, & Siebrecht, 2013; Bloomer et al., 2012; Miles, 2007; Niklowitz, Sonnenschein, Janetzky, Andler, & Menke, 2007; Svensson et al., 1999; Zhou et al., 2005) and to exercise (Fisher-Wellman & Bloomer, 2009; Kerksick, Kreider, & Willoughby, 2010). To try and account for individualisation, in both of the human studies in this thesis (Chapters 4 and 5), a cross-over study design was implemented with each participant acting as their own control. Additionally, the exercise protocols were individually tailored and adjusted on the day of exercise to ensure all participants were exercising at the same relative intensity. Despite using these measures, the data still showed great variability between participants.

The data suggest that some participants were ‘responders’ to the CoQ supplementation in Chapter 5 displaying a larger increase in baseline muscle CoQ when taking the supplement compared to the placebo (Table 5-14 and Table 5-15, YOUNG and AGED individual data, respectively), whereas others displayed no change, or even a decrease in CoQ content in skeletal muscle. It was also interesting that each individual’s muscle CoQ concentration presented a different response to exercise and throughout recovery. Those who exhibited limited or no increase in CoQ content prior to exercise, often displayed higher CoQ after exercise and into recovery than after taking placebo (Table 5-15; AGED 1 and AGED 2). This may pose an important factor to consider when assessing the impact of a dietary supplement on oxidative stress; those who are responders
may display an amplified change in redox status, whereas those who are not may show limited change. It is also important to note that CoQ supplementation may not lead to an initial response in the skeletal muscle, however increased serum CoQ content may alter muscle CoQ concentrations following exercise. It may be hypothesised that this is due to an increase in blood flow to the exercising muscles and therefore an increased availability of serum CoQ. In Chapter 5, some individuals had lower baseline CoQ concentrations than others. Although all individuals in both groups showed an increase in serum CoQ concentration after supplementation, the individual with the lowest baseline values showed the greatest percentage increase in serum CoQ following supplementation in the AGED group (AGED 3; 492.8 pmol/mg protein baseline incurred a 294.6% increase), however this was not the case in the YOUNG group. Both YOUNG and AGED participants with the highest baseline serum CoQ values had the lowest percentage increases (44.0% and 72% increases in YOUNG and AGED, respectively). It is therefore difficult to determine if these individuals were ‘responders’ or if they were deficient in CoQ at baseline and benefitted more from the supplementation.

From individual data in Chapter 4, it was apparent that despite the exercise being individually tailored, some individuals had a more pronounced increase in exercise-induced ROS. This is in agreement with previous research (Margaritelis et al., 2014; Margaritelis, Paschalis, Theodorou, Kyparos, & Nikolaidis, 2018; Paschalis, Theodorou, Margaritelis, Kyparos, & Nikolaidis, 2018) in which it was found that individual responses can vary greatly both at rest and after exercise. This may be due to differences in previous exposure to this exercise type, training status and overall health of the participant. It is also important to acknowledge that although participants were asked to refrain from strenuous exercise, caffeine
and alcohol and report to the lab after an overnight fast, no other dietary controls or recordings were conducted in either of the human based studies (Chapters 4 and 5). For future research, dietary control in the weeks prior to testing may be beneficial. Since the findings highlight the importance of tailoring supplementation to the individual, based on their personal responses to exercise and supplementation.

7.2.3 Young and aged blood and skeletal muscle redox markers respond differently to exercise and dietary supplementation

As shown in Chapter 5, acute supplementation with 300 mg CoQ attenuated the exercise-induced increase in LOOH concentrations in the AGED group. This reduction lowered the AGED group LOOH concentrations in line with the YOUNG group results.

The LOOH process can be terminated when two free radicals interact to form a non-radical. Antioxidants, vitamin C and E and CoQ can produce stable radicals which can also terminate LOOH production (Matsuo & Kaneko, 2000). CoQ has been shown to inhibit both initiation and propagation phases of lipid peroxidation (Bentinger, Brismar, & Dallner, 2007; Kaikkonen, Tuomainen, Nyyssonen, & Salonen, 2002; Zhou et al., 2005). It is thought that its location within mitochondrial cell membranes, in close proximity to unsaturated fatty acids, promotes its effectiveness in preventing lipid peroxidation (Turunen et al., 2004). However, it is hypothesised that LOOH may also play an important role in cell signalling and the beneficial adaptations that exercise promotes. It is currently unclear if there is an optimal LOOH concentration to promote adaptations, but limit damage.
Research has shown distinct differences in the oxidative and antioxidant response of old and young skeletal muscle to exercise (Doria et al., 2012; Pansarasa et al., 1999; Vasilaki et al., 2006), which data in this thesis supports (Chapter 5). It is generally accepted that aged muscle is slower to respond and less resistant to exercise stress than that of their younger counterparts (Hunter, Pereira, & Keenan, 2016; Layec et al., 2018). The skeletal muscle of old rats and mice (Vasilaki et al., 2003; Vasilaki, McArdle, Iwanejko, & McArdle, 2006; Vasilaki et al., 2006; Vasilaki, Jackson, & McArdle, 2002) were unable to produce the increased levels of ROS after an acute bout of exercise seen in their younger counterparts. This limited the exercise-induced heat shock protein upregulation, which is fundamental to cell protection and adaptation to exercise (Henstridge, Febbraio, & Hargreaves, 2016), and antioxidant protection. However, it is accepted that regular exercise training can protect against age-related decline in redox functioning (Bori et al., 2012; Cobley et al., 2014; Tweedie et al., 2010). Research within the field of ageing muscle has focused on reverting aged muscle responses back to those of younger tissues. The results from Chapter 5 demonstrated that antioxidant supplementation may be effective at achieving this. Since acute supplementation with CoQ was able to reduce exercise-induced increases in LOOH in skeletal muscle and significantly reduce LOOH in serum. This supports previous findings that old and young tissues respond differently to an exercise stimulus and that dietary supplementation can help aged skeletal muscle to respond similarly to young skeletal muscle. It is thought that the higher concentration of LOOH levels in aged skeletal muscle may in part be caused by the age-related loss of type II muscle fibres and therefore an increase in type I, oxidative fibres (Mecocci et al., 1999; Pansarasa et al., 1999; Pansarasa et al., 2002). The impact of CoQ supplementation on LOOH levels is most likely due to
its lipophilic nature, and therefore its close proximity to lipids makes it ideal for protecting such lipids from damage.

7.2.4 Cell culture – an important alternative to skeletal muscle biopsies

Skeletal muscle research in an aged population is important to help understand the ageing process and how to improve quality of life with advancing age. However, it can be difficult to carry out research in this field due to the vulnerable nature of an aged population, and the importance of maintaining muscle mass. Skeletal muscle biopsies are invasive and incur a recovery period, which, although relatively short, may make recovery from a skeletal muscle biopsy difficult in some aged individuals. It is therefore ethically important to find viable alternatives to skeletal muscle biopsies in the aged, diseased or frail populations.

In Chapter 6, we explored a cell culture toolkit which can provide important pre-screening for dietary interventions and allow a more targeted approach for compounds selected for human based research.

It was found that CoQ supplementation had no impact on ROS production and antioxidant protection in C2C12 cells. This may have been due to the dominant CoQ homologue in rodents being CoQ9 and that the cells used were from mouse skeletal muscle. The supplementation applied to the cells was CoQ10, which is more widespread in human tissues. Therefore, it may be possible that supplementation with CoQ9 may have given different results. However, the difference in responses of the cell culture to CoQ 10 and CoQ 9 was not tested, therefore this is only speculation.
7.3 Conclusions

It is well established that exercise in humans increases oxidative stress in skeletal muscle (Khassaf et al., 2003; Petersen et al., 2012), and blood (Bloomer et al., 2006, 2005). With age, the ability to protect against this increase diminishes (Doria et al., 2012; Fulle et al., 2004; Pansarasa et al., 1999; Viña et al., 2016). The work presented in this thesis has contributed to this evidence base underlying these characteristics with the following novel findings:

- Repeated skeletal muscle biopsy procedures had limited impact on skeletal muscle and blood markers of oxidative stress, antioxidant capacity and muscle damage for up to 3 hours post-biopsy.
- HIIE elicited a greater increase in LOOH concentration in aged males compared with their younger counterparts without supplementation.
- Supplementation with 300 mg CoQ per day reduced the exercise-induced increase in LOOH in an older population.
- The successful development of a cell culture toolkit for assessing dietary supplementation, ageing and the impact of a biochemical exercise stimulus.
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