How do different dietary dairy proteins, ingested post-exercise, effect adaptations to endurance training?

A thesis by

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Declaration of Authenticity

I, Karen Hill, declare that the PhD thesis entitled “How do different dietary proteins, ingested post-exercise, effect adaptations to endurance training?”, is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Chapter 5 – Citrate synthase assay and western blot analysis was carried by a fellow research student.

Signature:  Date: 29/6/2017

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Abstract

It is well established that endurance exercise training leads to cardiovascular, skeletal muscle, and metabolic adaptations, with important implications for both athletic performance and health. While many studies have addressed the effects of endurance exercise training on such adaptations, very few have examined the role of post-exercise nutritional supplementation in facilitating or increasing the magnitude of the adaptive response. The beneficial effects of post exercise nutrition, in the form of carbohydrate and protein, following an acute bout of exercise has been the focus of many investigations. The results however remain equivocal due to methodological differences, such as the training status of participants, the treatment groups not being isocaloric, differing CHO contents, different protein sources, exercise modes and protocols, and outcome measurements all being different.

The quality of the protein source used in post exercise supplementation may also affect training adaptations. Animal-source of proteins, such as milk and the constituent proteins of milk, casein and whey, are classified as being of high biological availability and quality. The types of proteins that are best for achieving muscle recovery and adaptations after endurance exercise are not defined.

The significant aim of this PhD candidature was to determine the role of protein supplementation, when included in the training diet over an extended period, on endurance training adaptations. Along with investigating how different proteins affect the signalling pathways that regulate endurance training adaptations. As such the research presented in this thesis examines 8 weeks of supplementation, with and without an endurance training program, with micellar caseins or caseinates, whey protein isolates or a carbohydrate matched and isocaloric group in animal and human models.

The first study demonstrated that after 8 weeks of supplementing with whey protein isolates, this group had lower body fat compared to the carbohydrate group at week 4 ($P < 0.05$) and week 8 ($P < 0.05$). The micellar caseins group had lower body fat compared to carbohydrate group only after 8 weeks ($P < 0.05$).

A key finding in the second study was that despite matching all rodents across groups according to exercise performance, the carbohydrate supplemented animals were unable to perform for as long in the time to exhaustion test compared to both whey protein isolates and micellar caseins groups ($P < 0.05$); 14:19 ± 4 min, 29:81 ± 11 min and 25:51 ± 6 min..

There was no significant difference between protein groups in several measures including; enzyme activity of citrate synthase and β-hydroxyacyl-CoA dehydrogenase (βHAD), mitochondrial respiration or lean mass.

The third study examined post-exercise supplementation with calcium caseinates compared to whey protein isolates in trained cyclists in a double-blind manner. Participants were provided all meals and snacks for the duration of the study. Endurance exercise performance, body composition and mitochondrial respiration, along with proteins involved in mitochondrial biogenesis showed no difference between protein groups after 8 weeks of supplementation and endurance training.

This research has established that micellar caseins and whey protein isolates may have beneficial effects on body composition and mitochondrial function without exercise, however following exercise training these mitochondrial function differences are diminished. Despite this, animals supplemented with the different proteins and undertook an endurance training protocol for 8 weeks performed significantly better in the time to

exhaustion test. Thus, it appears that this improved time to exhaustion is not related to improved mitochondrial function, but by some other, yet to be determined factor.

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Abbreviations

AA	Amino acid
ACC	acetyl-CoA carboxylase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	5' AMP-activated protein kinase
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
BCAA	Branched chain amino acid
BIOPS	biopsy preserving solution;
BM	body mass
cDNA	complementary DNA
CHO	carbohydrate
CI+IIE	maximal electron transport system capacity through CI+II
CI+IIP	maximal oxidative phosphorylation state through CI+CII
CIIE	maximal electron transport system capacity through CII
CIL	leak respiration state through Complex I
CIP	maximal oxidative phosphorylation state through CI
CIVE	maximal noncoupled respiration through CIV
Complex I	NADH-Q reductase or CI
Complex II	succinate-coenzyme Q reductase or CII

Complex III	cytochrome reductase or CIII
Complex IV	cytochrome c oxidase
Complex V	ATP synthase or CV
COX	cytochrome c oxidase
CREB	cAMP response element-binding protein
CS	citrate synthase
DRP1	dynamin-related protein-1
DXA	Dual-energy X-ray absorptiometry
E	maximal ETS capacity
EAA	Essential amino acid
ETS	electron transport system
$FADH_2$	flavin adenine dinucleotide
FAO	fatty acid oxidation
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone;
FSR	fractional protein synthesis rate
GXT	graded exercise test
HIT	high intensity interval training
IMF	intermyofibrillar mitochondria
IMM	inner mitochondrial membrane
IMS	intermembrane space
IMTG	intramuscular triglyceride
L	leak respiration

MHC	myosin heavy chain
MiR05	respiration medium
MPC	milk protein concentrate
mRNA	messenger RNA
mtDNA	mitochondrial DNA
MPS	mitochondrial protein synthesis
NADH	nicotinamide adenine dinucleotide
O_2	oxygen
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
P	maximal oxidative phosphorylation
PGC-1α	PPARγ coactivator 1-alpha
PHF20	plant homeodomain finger-containing protein 20
SD	standard deviation
SEM	standard error of the mean
SIT	sprint interval training
SS	subsarcolemmal mitochondria
SUIT	substrate-uncoupler-inhibitor titration protocol
TCA	tricarboxylic acid cycle
TFAM	mitochondrial transcription factor A
TT	cycling time trial
$\dot{V}O2_{max}$	maximal oxygen uptake

W_{LT}	power at the lactate threshold
W_{Peak}	peak power output
WPI	whey protein isolates
β-HAD	β-hydroxy acyl-CoA dehydrogenase

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Parker, L., C. S. Shaw, L. Banting, I. Levinger, **K. M. Hill**, A. J. McAinch and N. K. Stepto (2016). "Acute low-volume high-intensity interval exercise and continuous moderate-intensity exercise elicit a similar improvement in 24-h glycemic control in overweight and obese adults." Frontiers in physiology **7**.

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Chapter 1: Literature review

1.1 Introduction

The concern for what to eat for good sports performance long predates sports science. Athletes have always been advised about what to eat, but the academic field, now known as sports nutrition, began in the exercise physiology laboratories (Juzwiak 2016). Historians consider the first studies of sport nutrition to be those of carbohydrate and fat metabolism conducted in Sweden in the late 1930s (Dunford 2010). Technology such as the Bergstom needle was developed in the 1960's and 1970's to help scientists measure human response to exercise. Endurance runner's and cyclists were most frequently studied because they were at most risk of depleting muscle glycogen stores and these sports could be easily replicated in a laboratory with stationary bikes or treadmills (Dunford 2010). Some research was conducted on protein, but protein was much more difficult than carbohydrate because protein is found in so many different places in the body. Most of the interest for protein came from bodybuilders wanting to maximise muscle protein synthesis (Applegate and Grivetti 1997).

Sports nutrition has developed from a niche discipline into a very important element of sports performance. As athletes approach their limits with respect to training volume and

intensity, good nutritional practice becomes of greater importance. Particularly for elite athletes who need to maximise training to increase potential for competition. Because full and often rapid recovery is necessary for optimal performance, athletes should practice nutritional strategies that maximise recovery (Burke 2010). Meeting energy and nutrient needs is a priority for athletes to facilitate achievement of peak performance.

The manipulation of macronutrients for the benefit of athletes in optimising recovery and body composition, can also benefit the general population. With increasing prevalence of obesity and metabolic disorders, macronutrients such as dietary protein have been studied for its obesogenic and metabolic effects for supporting weight loss and preventing weight re-gain (Larsen, Dalskov et al. 2010). This literature review section of the thesis aims to provide background information on endurance training adaptations and body composition changes and how post – exercise co-ingestion of carbohydrate and proteins can affect these processes.

1.1.2 Exercise and performance

In the search for novel and better methods of preparation for competition, scientist, coaches and practitioners should constantly seek out the latest information on training, nutrition and psychology in an attempt to gain a competitive edge for athletes (Coffey and Hawley 2006). The quality and quantity of nutritional intake harnesses many benefits for athletic populations. Nutritional strategies implemented prior to and during exercise can profoundly alter exercise performance and prolong endurance capacity (Rodriguez, DiMarco et al. 2009). Further, effective post-exercise nutritional strategies can replenish depleted energy stores, attenuate indices of muscle damage and augment the recovery of endurance capacity and muscle function (Pritchett, Pritchett et al. 2011). In addition to exercise performance and recovery, dietary modifications can profoundly alter the adaptive response to exercise.

In humans endurance performance capacity is characterized by a large degree of inter-individual variation, even among well trained athletes (Myburgh 2003). This capacity can be assessed using physiology, biochemistry and histology techniques. The most frequently used tests include determination of maximal oxygen uptake ($VO_{2\ max}$), lactate threshold, oxidative enzyme activities and the percentage of slow twitch muscle fibres (Myburgh 2003). All these parameters correlate well with endurance performance in events ranging

from 3000 m to various ultramarathon distances (Larsen 2003). An optimal training regime is paramount to improving these parameters that lead to endurance performance.

Different training techniques are used by endurance athletes to improve performance. Training practices of elite endurance athletes has consisted of interval training for several decades now (Hawley, Myburgh et al. 1997). Such training consists of a number of continuous (5 min) high-intensity exercise bouts alternated with short (1 min) rest intervals at a lower intensity (Hawley, Myburgh et al. 1997). This type of training is thought to improve fatigue resistance of muscle fibres and induce metabolic and performance adaptations that resemble traditional endurance training (Little, Safdar et al. 2010).

Although repeated supramaximal high-intensity interval training (HIT) has not been traditionally used in aerobic endurance training programs, recent research has shown that this type of training can improve performance in well-trained cyclists (Stepto, Hawley et al. 1999, Laursen, Shing et al. 2002). Another study demonstrated that six sprint interval training sessions (30 s ‘all out” Wingate tests, 4 min recovery) performed over 2 weeks increased citrate synthase activity and endurance capacity during cycling at 80% $VO_{2\ peak}$

(Burgomaster, Hughes et al. 2005). This type of sprint interval training has also been demonstrated to increase muscle glycolytic enzymes as well as oxidative enzymes and endurance performance over longer duration (7 weeks) in recreationally active males (MacDougall, Hicks et al. 1998).

1.2 Endurance exercise adaptations

For the competitive endurance athlete, the goal of physical training is to increase the ability to sustain the highest average power output or speed of a movement for a given distance or time. This depends on the rate and efficiency at which chemical energy can be converted into mechanical energy for muscle contraction (Smiles, Hawley et al. 2016). Enhancement of endurance performance needs to induce physiological and metabolic adaptations that enable the individual to; increase the rate of energy production from both aerobic and oxygen-independent pathways, maintain tighter metabolic control (match ATP production with hydrolysis), minimize cellular disturbances, increase economy of motion and improve fatigue resistance of working muscles (Hawley 2002).

Endurance training elicits both central and peripheral adaptations, alters neural recruitment patterns, and causes profound changes in muscle bioenergetics and enhanced morphological, metabolic, substrate and acid-base status in skeletal muscle (Hawley 2002). These include; fast-to-slow fibre-type transformation (Zierath and Hawley 2004), glycogen sparing effects due to alterations in substrate metabolism (Holloszy, Rennie et al. 1977), enhanced lactate kinetics and increased mitochondrial density (Coffey and Hawley 2007). Moreover, repeated bouts of endurance exercise alter the expression of a host of gene products that promote adaptation towards a fatigue resistant phenotype (Adhihetty, Irrcher et al. 2003). Mitochondria are the main sub-cellular structures that determine the oxidative capacity and fatigue resistance to prolonged contractile activity in skeletal muscle (Hoppeler and Fluck 2003).

Endurance training has been shown to increase mitochondrial protein content by 50–100% within 6 weeks, but a protein turnover half-life of 1 week means a continuous training stimulus is required to maintain elevated mitochondrial content (Zierath and Hawley 2004). While enhanced oxygen kinetics, substrate transport and buffering capacity all contribute to enhanced endurance capability in skeletal muscle, improved endurance is due primarily to

increased mitochondrial density and enzyme activity termed ‘mitochondrial biogenesis’ (Irrcher, Adhihetty et al. 2003).

1.2.1 Endurance Performance and the role of skeletal muscle

Skeletal muscles must cope with a large range of activities, from supporting the body weight during long periods of upright standing, to performing explosive movements or sustained contractile force for long periods of time. To deal with these divergent activities our muscle contains cells with different metabolic and contractile properties, e.g. different fibre types (Bottinelli and Reggiani 2000).

The classification system for mammalian skeletal muscle is based on myosin heavy chain (MHC) isoforms (Bottinelli and Reggiani 2000). The major fibre types are type I, IIa, IIx and IIb (Westerblad, Bruton et al. 2010). In addition, a minority of fibres express more than one MHC. Rodent muscles express all four types of MHC, whereas type IIb MHC is not expressed in humans (Westerblad, Bruton et al. 2010). The slow twitch fibres (type I), found in rat soleus muscle, exhibit a high blood flow capacity, a high capillary density, and a high mitochondrial content making this fibre type more fatigue resistant (Wells, Selvadurai et al.

2009). Fast twitch type II fibres generally have a lower oxidative capacity, are high energy consuming and depend mainly on anaerobic metabolism, such as the rat gastrocnemius muscle (Bottinelli and Reggiani 2000). In contrast, sprint-trained athletes will generally have a higher proportion of type II fibres in their legs compared to endurance-trained athletes that have a higher proportion of type I fibres (Costill, Daniels et al. 1976, Saltin, Henriksson et al. 1977).

It is not surprising to find a high quantity of type I fibres in endurance trained muscle due to the high capillary density and oxidative capacity of these fibres. Studies using endurance training paradigms have been commonly used to explore the phenotypic plasticity of mitochondria (Koulmann and Bigard 2006). It is well known that endurance training promotes an increase in both the mitochondrial volume density and the concentration of mitochondrial proteins per gram of muscle (Hoppeler, Howald et al. 1985). One interesting finding is that the increased mitochondrial volume density occurred in all three fibre types in humans (Howald, Hoppeler et al. 1985). In addition to these quantitative changes, endurance training is associated with an improvement of coupling and regulation properties of mitochondrial respiration in human skeletal muscle (Zoll, Koulmann et al. 2003). In rat

skeletal muscles, these qualitative changes in muscle respiration rate occur in all fibre types, whatever their initial oxidative capacity (Zoll, Sanchez et al. 2002).

1.2.2 Molecular basis of exercise training

The key components of a training program are the volume, intensity and frequency of exercise sessions (Hawley 2002). Modifications in the muscle cells that persist for extended periods as a consequence of training are termed ‘chronic’ adaptations, whereas cellular alterations that occur in response to a single training session are ‘acute’ responses (Booth and Thomason 1991). In line with this reasoning, the acute signalling response following each distinct exercise bout can be considered as the initial step leading to gradual changes in protein content and enzyme activity that modulate chronic adaptations (Egan and Zierath 2013).

Muscle contraction generates transient increases in the quantity of messenger RNA (mRNA) for a multitude of genes which typically peaks 3-12 hours post exercise, leading to a new steady-state of the same encoded protein (Bickel, Slade et al. 2005). Therefore, frequent bouts of exercise result in acute increases in transcriptional activity and subsequent protein

synthesis and this may be potentiated when appropriate nutrient strategies are implemented post-exercise (Mahoney, Parise et al. 2005, Coffey and Hawley 2007).

1.2.3 Mitochondrial structure and function

Mitochondria are the main subcellular structures that determine oxidative capacity and fatigue resistance to prolonged endurance training in skeletal muscle. Chronic endurance training is known to induce increases in mitochondrial volume and function, allowing greater work capacity and an improved resistance to fatigue (Irrcher, Adhihetty et al. 2003). Endurance training also leads to a shift in skeletal muscle mitochondria toward an increased use of lipids as a substrate source (Hoppeler and Fluck 2003).

From a structural standpoint, mitochondria range generally from 0.5 to 1.0 μm in diameter (Henze and Martin 2003), form an integrated reticulum that is continually remodelled by fusion and fission processes (Picard, Shirihai et al. 2013), and are divided in four main compartments: the outer membrane (OMM), the intermembrane space (IMS), the inner membrane (IMM) and the matrix (Figure 1.1). The OMM consists of a phospholipid bilayer housing protein structures called porins that make it permeable to small molecules (<10,000

Da), including adenosine diphosphate (ADP) and adenosine triphosphate (ATP) (Henze and Martin 2003). Conversely, the IMM is made of a protein-rich, highly-folded complex structure that is only permeable to oxygen, water, and carbon dioxide. The wrinkled shape of the IMM generates several infolds and pockets (cristae) that increase its surface area and maximise its ability to house the four complexes of the electron transport system (ETS) and ATP synthase required for the generation of ATP (Hoppeler and Fluck 2003). The compartment inside the IMM is defined as the matrix and hosts mitochondrial (mt) DNA, ribosomes, and the enzymes of the tricarboxylic acid (TCA) cycle (Ojuka, Andrew et al. 2016). The TCA cycle, which includes the enzyme citrate synthase (CS), is the final common pathway for the oxidation of fuel molecules such as carbohydrates, lipids and amino acids.

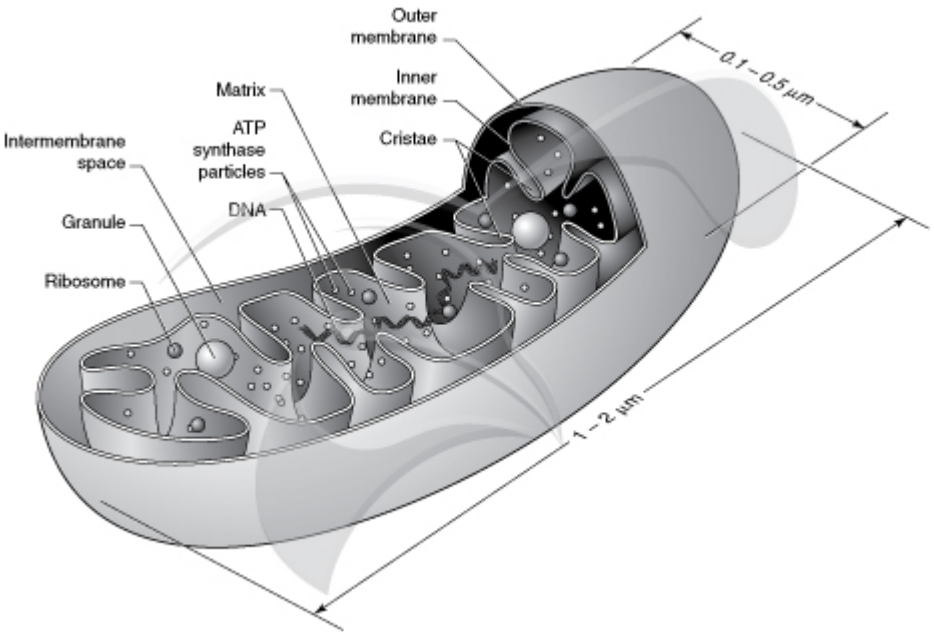


Figure 1.1: *Mitochondria structure (www.quailridgestudio.com)*

During the TCA cycle, a small amount of ATP is generated alongside the reducing equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). These high-energy electron carriers can transfer electrons to coenzyme Q in a reaction catalysed by complex I (NADH-Q reductase or CI) in the case of NADH, or by complex II (succinate-coenzyme Q reductase or CII) in the case of $FADH_2$. From coenzyme Q, the electron pair is transferred to cytochrome c in a reaction catalysed by the enzyme complex III (cytochrome reductase or CIII), and then onto its final acceptor, molecular

oxygen (O_2), in a process catalysed by complex IV (cytochrome c oxidase (COX) or CIV). During electron transfer, protons are pumped from the matrix to the IMS, with the resulting electrochemical gradient representing the proton-motive force that allows generation of ATP by phosphorylation of ADP in a reaction catalysed by complex V (ATP synthase or CV) (Saraste 1999). The combination of these processes taking place along the IMM is described as oxidative phosphorylation (OXPHOS) (Figure 1.2).

Mitochondria are the main site for oxidation of free fatty acids and these fatty acids require transport into the mitochondria by membrane proteins (Houten and Wanders 2010). The degradation of fatty acids occurs via mitochondrial fatty acid β-oxidation (FAO) (Bartlett and Eaton 2004). The β-oxidation pathway is a cyclic process consisting of 4 enzymatic reactions, with each cycle yielding an acyl-CoA shortened by two carbon atoms, an acetyl-CoA, and one NADH and one $FADH_2$ as electron carriers (Ojuka, Andrew et al. 2016). The resulting acyl-CoA enters another cycle of FAO; the acetyl-CoA can enter the TCA cycle and the electron carriers deliver the electrons to the electron transport chain, as shown in Figure 1.2 (Nsiah-Sefaa and McKenzie 2016).

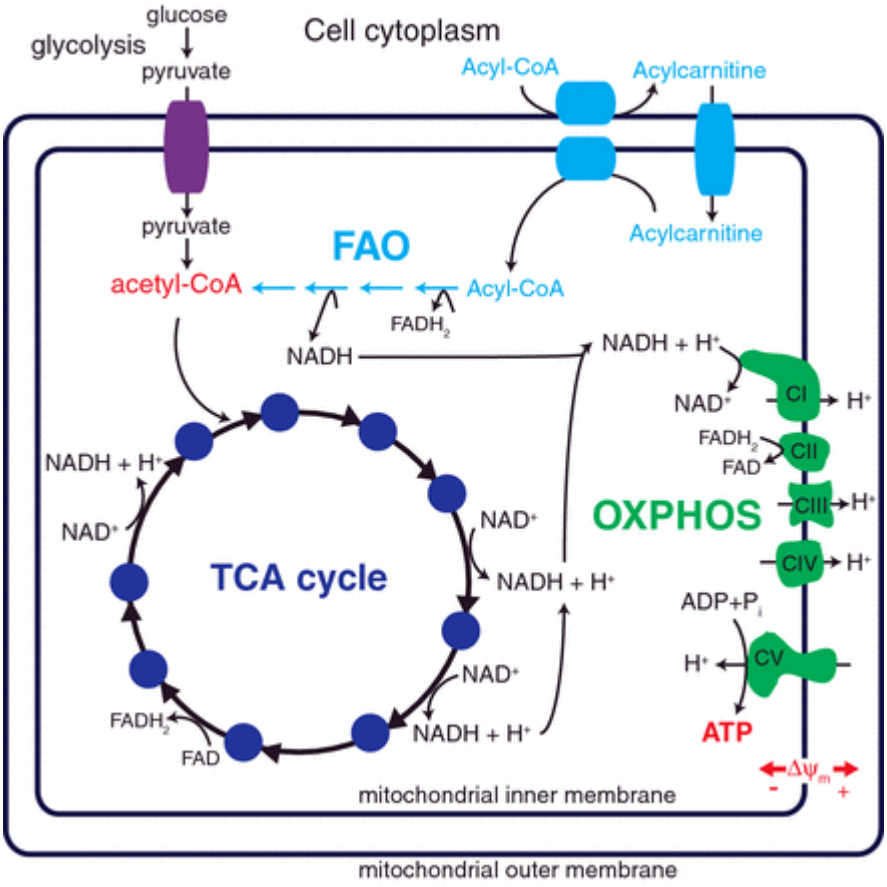


Figure 1.2: *Glucose breakdown through glycolysis and the TCA cycle (dark blue) generates reduced NADH and $FADH_2$. Fatty acid β-oxidation (FAO, light blue) of fatty acyl-CoA esters is performed in four enzymatic reactions that also generates NADH and $FADH_2$, as well as acetyl-CoA. Electrons derived from NADH and $FADH_2$ are utilized by the five OXPHOS complexes (green) to generate ATP. Complex I (CI, NADH: ubiquinone oxidoreductase), CII, complex II (succinate: ubiquinone oxidoreductase), complex III (CIII,*

*ubiquinol: ferricytochrome c oxidoreductase) and complex IV (CIV, cytochrome c oxidase) pump electrons out of the mitochondrial matrix to generate a membrane potential ($\Delta\psi_m$) that drives the synthesis of ATP by complex V (CV) (*Nsiah-Sefaa and McKenzie 2016*).*

1.2.4 Mitochondrial biogenesis

Mitochondrial biogenesis is the process by which an increase in the mitochondrial reticulum takes place within the cell and is governed by transcriptional regulation requiring the integration of two genomes (nuclear and mitochondrial), and a series of processes such as signalling, transcription, translation, post-translational events, and protein import (Irrcher, Adhihetty et al. 2003).

PGC-1α is a transcriptional coactivator that has been shown to regulate mitochondrial content and respiration in human skeletal muscle (Hood 2009). Studies in human skeletal muscle have shown that exercise training leads to an increase in PGC-1α protein (Perry et al 2010) and suggest PGC-1α mRNA is sensitive to training intensity (Egan and Zierath 2013).

The Tumour suppressor p53 is also an important regulator of mitochondrial biogenesis (Saleem, Carter et al. 2011). Studies in cells and mice indicate that deletion of p53 leads to a reduction in mitochondrial respiration and content and endurance performance (Matoba, Kang et al. 2006). p53 is also upregulated after a single bout of exercise in human skeletal muscle (Bartlett and Eaton 2004) and like PGC-1α, has been shown to regulate the mitochondrial transcription machinery via modulation of mitochondrial transcription factor A (TFAM) (Saleem and Hood 2013). Furthermore, by regulation of downstream targets, p53 can control mitochondrial respiration by modulating the balance between glycolytic and oxidative pathways (Matoba, Kang et al. 2006) and has been linked with mitochondrial dynamics and the maintenance of mitochondrial autophagy (Saleem, Carter et al. 2011). Although the signalling events upstream of p53 are not yet fully known, plant homeodomain finger-containing protein 20 (PHF20) has been shown to up-regulate p53 transcription (Park, Kim et al. 2012) and to stabilize p53 protein (Cui, Park et al. 2012), emphasizing the role of PHF20 as a regulator of p53.

The mitochondrial reticulum network is continuously remodelled by fusion and fission events, which add or subtract from it, respectively. These processes act as quality control to preserve mitochondrial DNA (mtDNA) and influence respiratory capacity (Hood, Tryon et

al. 2015). In mammalian cells, mitofusion -2 (MFN2) is one of the mitochondrial membrane proteins regulating the fusion process. Mitochondrial membrane fission is mediated by dynamin-related protein 1 (Drp1) (Hood, Tryon et al. 2015). It has been reported that p53 can transcriptionally upregulate DRP1, which conveys the p53 apoptotic signal and initiates fission (Bergeron, Ren et al. 2001). Furthermore, p53 can also modulate mitochondrial fusion by binding directly to the promoter of MFN2 and MFN2 mRNA and protein content are upregulated in a p53-dependent manner (Twig and Shirihai 2011).

1.3 Endurance exercise performance and the role of body composition

Body composition and body weight are two of the many factors that contribute to optimal exercise performance (Loucks 2004). Taken together, these two factors may affect an athlete's potential for success for a given sport. Body weight can influence an athlete's speed, endurance, and power, whereas body composition can affect an athlete's strength, agility, and appearance (O'Connor and Slater 2011). Most athletes require a high strength-to-weight ratio to achieve optimal athletic performance, and because body fat adds to weight without adding to strength, low body fat percentages are often emphasized within many sports, especially endurance sports (Rodriguez, DiMarco et al. 2009).

Increased access to dual-energy X-ray absorptiometry (DXA) allows this technology to be used in assessment of body composition for monitoring athletes (Nana, Slater et al. 2015). DXA provides information on three compartments of body composition, according to the terminology “fat mass,” “lean mass” or the “fat-free soft tissue” and “bone mineral content” (Nana, Slater et al. 2015).

While the accessibility and use of DXA is increasing among athletes, there are a number of limitations around DXA use. This includes manufacturers’ body composition estimation algorithms are not developed on athletes and comparing results between different DXA machines (need specific regression equations) is not recommended (Nana et al 2014). A number of factors can affect reliability of scans such as if the participant is in a fasted state or not, and body positioning on the bed (Margulies et al 2005). Adopting a standardised protocol for DXA use to measure body composition of athletes can help reduce variability between scans and technical errors.

Optimal body composition is not only important for athletic performance, it is also important for maintaining health in the general population (Bendtsen, Lorenzen et al. 2013). Data is

beginning to accumulate suggesting increased protein content in the diet, may reduce the loss of lean body mass in a hypo energetic state (Mettler, Mitchell et al. 2010, Wycherley, Moran et al. 2012). Greater improvements in body composition have been demonstrated with chocolate milk post exercise in untrained participants over 4.5 weeks (Ferguson-Stegall, McCleave et al. 2011). In contrast, a study supplementing carbohydrate-protein post-endurance exercise for 8 weeks did not demonstrate a positive effect on body composition compared to isocaloric carbohydrate beverage, however, this study did not control dietary intake over the study duration and the protein in the test beverage was whey protein concentrate (Cramer, Housh et al. 2012). Most studies investigating the benefits of protein on body composition with positive results have used whey protein isolate, casein or a combination (milk) (Halton and Hu 2004, Bendtsen, Lorenzen et al. 2013).

Protein has been shown to effect appetite regulation as it is more satiating than fat and carbohydrate (Astrup 2005), along with increasing gastrointestinal hormones that regulate satiety (Halton and Hu 2004). Furthermore, diet induced thermogenesis is greater for protein (20-35% of ingested energy) than carbohydrate (5-15% of ingested energy) and fat (0-3% of ingested energy) (Westerterp, Wilson et al. 1999). Less evidence exists regarding the

effects different protein types have on maintenance of lean mass and fat mass when an endurance athlete is in energy balance.

1.4 Influence of nutrition on endurance training adaptations

Exercise physiology and nutrition are intimately linked. The adaptation to exercise training can be amplified or reduced by nutritional intake (Juekendrup 2017). Exercise stimulus is essential to drive adaptation, but nutritional substrates can optimize this response (Stellingworth 2014).Nutritional modifications including carbohydrate, fat and protein, to augment training adaptations and performance have been investigated for many years (Maughan 2003, Hawley, Burke et al. 2011). A single bout of exercise increases protein synthesis and, to a lesser extent, muscle protein breakdown rates. However, post exercise protein balance will remain negative in the absence of food intake (van Loon, Saris et al. 2000).

1.4.1 Overview of the role of fats

Fat oxidised during an exercise session has not been thought to limit performance due to the adipose stores in the body; Even the leanest athletes having relatively large adipose stores

(Burke, Kiens et al. 2004). However, intramuscular triacylglycerol (IMTG) stores provide an important energy source for contracting muscle (Watt, Heigenhauser et al. 2002). Increased fat intake has been suggested to spare muscle glycogen and therefore delay the onset of fatigue during sub-maximal endurance exercise (Goedecke, Christie et al. 1999). Studies in rats and humans that have a high fat diet for more than 7 days increases fat oxidation and decreases muscle glycogen utilisation during exercise (Lambert, Speechly et al. 1994, Lapachet, Miller et al. 1996).

Studies in which trained individuals have been exposed to a high fat diet (> 60-65% of dietary energy) for 5-28 days show markedly higher rates of fat oxidation and reduced rates of muscle glycogen use during submaximal exercise compared with consumption of an isoenergetic high carbohydrate diet (Lambert, Speechly et al. 1994, Goedecke, Christie et al. 1999). However, examination of the performance outcomes from these studies show a lack of a performance benefit. This seems to be the consensus amongst other studies that have not found high fat diets to benefit performance (Angus, Hargreaves et al. 2000, Horowitz, Mora-Rodriguez et al. 2000). These results are also supported by a 3 week study carried out by Burke *et al*. (2017). This study found adaptation to a ketogenic low carbohydrate, high fat (LCHF) diet increased rates of whole-body fat oxidation during

exercise in race walkers. However, the increased rates of fat oxidation resulted in reduced economy (increased oxygen demand for a given speed). In contrast to training with diets providing chronic or periodised high carbohydrate availability, adaptation to a LCHF diet impaired performance despite a significant improvement in peak aerobic capacity (Burke et al. 2016).

Dietary fat is not only a source of energy for athletes but also a source of essential fatty acids and fat soluble vitamins (McArdle, Katch et al. 2009). The recommended levels of dietary fat intake for athletes are around 20-25% of total energy intake (Broad and Cox 2008), primarily based on prioritising carbohydrate and protein intake within total energy requirements.

1.4.2 Overview of the role of carbohydrates

It has been known for a long time that carbohydrate feeding can improve endurance cycling performance and performance of other endurance sports (Kerksick, Harvey et al. 2008). Performance in events lasting longer than 2 hours is usually improved when carbohydrate is ingested before and during exercise (Jeukendrup 2008). It is well documented that

depletion of muscle glycogen stores can limit performance during prolonged endurance events and inhibit subsequent training sessions (Halson, Lancaster et al. 2004).

Dietary carbohydrates play a significant role in sports performance as a key fuel source for the brain and central nervous system and as a substrate for muscular activity at differing intensities. Given its limited storage capacity in the body, guidelines for carbohydrate intake for athletes are designed to meet daily fuel and recovery needs (Rodriguez, DiMarco et al. 2009). Studies investigating ways to spare, replenish and maximise muscle glycogen stores have been at the forefront of research for several years. Carbohydrate loading before competition is well practiced to maximise glycogen stores (Kerksick, Harvey et al. 2008). The use of carbohydrate and electrolyte sports beverages during competition and training, have been well studied to prevent a decline in performance during prolonged endurance events and aid recovery (Jeukendrup, Jentjens et al. 2005).

Daily carbohydrate guidelines for athletes are given based on the athlete's body size ($g \cdot kg^{-1}$ body mass) and training load, along with the frequency, duration and intensity of the activity (Thomas, Erdman et al. 2016). These guidelines are recognised and used by the

Australian Institute of Sport and widely referenced in the literature (Burke et al. 2011; Juekendrup 2008). Since activity levels change from day to day, carbohydrate intake should fluctuate to reflect this. On larger or more intense training days, carbohydrate intake should be increased to match training load and reduced on recovery or rest days (Burke, Loucks et al. 2006). The carbohydrate guidelines (Table 1) are intended to provide high carbohydrate availability to meet the carbohydrate needs of the muscle and central nervous system (Thomas, Erdman et al. 2016).

Table 1.1: *General guidelines for the suggested carbohydrate intakes (*Thomas, Erdman et al. 2016*).*

Situation	**Carbohydrate targets**	**carbohydrate intake**
Daily needs for fuel and recovery		
Light	Low intensity or skill-based activities	3-5 g/kg/d
Moderate	Moderate exercise program (eg, ~1 h/d)	5-7 g/kg/d
High	Endurance program (eg, 1-3 h/d moderate to high-intensity exercise)	6-10 g/kg/d
Very high	Extreme commitment (eg, >4-5 h/d moderate to high-intensity exercise)	8-12 g/kg/d

1.4.3 Overview of the role of proteins

The word protein is derived from the Greek word *proteos*, means primary or 'most important' (Dunford 2010). In the sporting environment, protein nutrition was initially favoured by bodybuilders, but it now a common consideration for the majority of athletes (Tipton 2008). However, the effectiveness of protein nutrition for endurance athletes is yet to be fully elucidated.

Proteins are nitrogen- containing substances that are formed by amino acids. They serve as the major structural component of muscle and other tissues in the body, in addition to a number of other components within the body including hormones, enzymes and haemoglobin (Tipton 2008). Proteins can also be used as energy; however, they are not the primary choice (Hoffman and Falvo 2004).

The primary role of dietary proteins in sports nutrition is to support metabolic adaptation, repair, remodelling, and for protein turnover (Thomas, Erdman et al. 2016). Thus, many athletes and coaches are under the belief that high intensity training creates a greater protein

requirement (Thomas, Erdman et al. 2016). This stems from the notion that if more protein and amino acids were available to exercising muscle it would enhance protein synthesis (Hoffman and Falvo 2004).

Unlike strength athletes, the goal for endurance athletes is not necessarily to maximize size and strength, but to prevent loss of lean tissue which can have a significant detrimental effect on endurance performance (Hoffman and Falvo 2004). Therefore, these athletes need to maintain muscle mass to ensure adequate performance and mitochondrial adaptations. Current data suggest that dietary protein intake necessary to support metabolic adaptation, repair, remodelling, and for protein turnover generally ranges from 1.2 to 2.0 g/kg/day (Thomas, Erdman et al. 2016).

Studies show that muscle protein synthesis is optimised in response to exercise by the consumption of high biological value protein, providing ~10 g essential amino acids in the early recovery phase (0 to 2 hours after exercise) (Beelen, Burke et al. 2010, Phillips 2012). This translates to a recommended protein intake of 0.25 to 0.3 g/kg BW or 15 to 25 g protein across the typical range of athlete body sizes (Moore, Robinson et al. 2009). More recently,

a study found 40g of protein to increase muscle protein synthesis more than 20g (Macnaughton et al. 2016). However, this was consumed after a whole body resistance training. The optimal amount of protein to be consumed depends on the type of exercise performed. This study also found there was great individual variation once higher (40g) protein amounts were consumed (Macnaughton et al. 2016). The exercise-enhancement of muscle protein synthesis, determined by the timing and pattern of protein intake, responds to further intake of protein within the 24-hour period after exercise, and may ultimately translate into chronic muscle protein accretion and functional change (Areta, Burke et al. 2013).

Traditional protein intake guidelines focused on total protein intake over the day (grams per kilogram), newer recommendations now highlight that the muscle adaptation to training can be maximised by ingesting high quality protein after key exercise sessions and every 3 to 5 hours over multiple meals (Moore, Robinson et al. 2009, Thomas, Erdman et al. 2016).

1.5 Different types of protein

1.5.1 Overview of different types of protein

The notion that endurance athletes require a greater protein consumption than the general population is generally accepted. This increase in protein intake above the recommended dietary intake of 0.8 $g \cdot kg^{-1} \cdot day$, is to aid in an elevated level of functioning and possibly adaptation to the exercise stimulus (Phillips and Van Loon 2011). Despite the prevalence of high protein diets in athletic and sedentary populations, information available concerning the type of protein (animal vs vegetable) to consume is limited, with dairy proteins and vegetable proteins such as soy being the major sources used in sports nutrition.

Protein quality describes characteristics of a protein in relation to its ability to achieve defined metabolic actions (Millward, Layman et al. 2008). Traditionally, this has been in the context of a food proteins ability to provide specific patterns of amino acids to satisfy the demands for synthesis of protein and other metabolites (Millward, Layman et al. 2008). The quality of a protein is vital when considering the nutritional benefits, it can provide. The composition of various proteins may be so unique that their influence on physiological function in the human body could be quite different. Determining the quality of a protein is determined by assessing its essential amino acid composition, digestibility and

bioavailability of amino acids (FAO/WHO 1990). Quality refers to the availability of amino acids that it supplies, and digestibility considers how the protein is best utilized (Tarnopolsky 2004). Animal protein sources are considered complete proteins as they contain all the essential amino acids. Proteins from vegetable sources are considered incomplete as they are lacking one or more essential amino acids (Phillips 2012).

An area of expanding research in recent years has been the comparative effects of different protein sources; the advantages and disadvantages of animal versus vegetable and their implications on athletic performance (Haub, Wells et al. 2002, Brown, DiSilvestro et al. 2004). Chronic training studies have shown that the consumption of milk-based protein after resistance exercise is effective in increasing muscle strength and favourable changes in body composition (Hartman, Tang et al. 2007, Josse, Tang et al. 2010). To date, dairy proteins seem to be superior to other tested proteins such as soy and proteins of vegetable origin, largely due to leucine content and the digestion and absorptive kinetics of branched-chain amino acids in fluid-based dairy foods (Pennings, Boirie et al. 2011). It has been postulated that dairy affects weight control in adults however, findings from randomised clinical trials (RCT) have been conflicting. Several studies have suggested dairy consumption has beneficial effects on weight loss (Zemel et al. 2004). Increased dairy consumption has

positive effects on body composition such as loss of fat mass and maintaining lean mass, when energy intake is restricted. These RCT's were not completed in athlete's and the pattern of weight loss in terms of body composition is important to monitor. Preserving lean mass is important for an athletic (and non-athletic) population for performance goals (Phillips and Zemel 2011).

Dairy protein is made up of 2 classes of proteins: casein (80%) and whey proteins (20%). Whey protein and casein are both classified as high quality proteins based on human amino acid (AA) requirements, digestibility and their bioavailability (Wolfe, Rutherfurd et al. 2016). Nevertheless, differences in their physiological effects have been attributed to differences in their AA composition (Dunford and Doyle 2011). Whey protein contains a higher proportion of the branched chain amino acids (BCAA) leucine, isoleucine and valine compared to casein (Paul 2009). Casein contains higher proportions of some of the other essential AA including histidine, methionine and phenylalanine than whey protein (Paul 2009). In addition, casein also contains a higher proportion of several non- EAAs including arginine, glutamic acid, proline, serine and tyrosine (Dunford and Doyle 2011).

Table 1.2: Amino acid profile of whey protein isolate and calcium caseinates, provided by Fonterra. Micellar casein was provided by Murray Goulburn however this analysis is an average based on Bulk Nutrients products. Average per 100g.

	Whey Protein	Calcium caseinates	*Micellar casein
Isoleucine	6.4	5.4	4.4
Leucine	14.6	9.8	8.2
Lysine	11.4	8.6	7.1
Methionine	2.4	2.9	2.2
Phenylalanine	3.9	5.0	4.1
Threonine	5.4	4.6	3.8
Tryptophan	2.4	1.4	4
Valine	5.7	6.5	5.3
Histidine	2.0	2.8	2.4

Alanine	5.8	3.4	2.8
Arginine	3.1	3.8	2.9
Aspartate	12.8	8.0	6.5
Cysteine	4.1	1.2	0.7
Glutamate	18.0	22.0	18.4
Glycine	1.8	1.9	1.6
Proline	4.6	10.0	8.1
Serine	4.6	5.7	4.9
Tyrosine	4.3	5.4	4

Caseins are phosphoproteins that precipitate from raw milk by acidification. The phosphoproteins are dispersed in milk in the form of micelles that are stabilised by κ-caseins. The casein micelles are maintained as a colloidal suspension in milk. In contrast, whey proteins are the proteins that remain soluble after precipitation of casein and consist of β-lactoglobulin, α-lactalbumin, albumin and lactoferrin and lactoperoxidase making up

the rest (Farrell, Jimenez-Flores et al. 2004, Luhovyy, Akhavan et al. 2007). Milk is commonly separated into different protein fractions for different food applications (Smithers 2008). Milk protein concentrate (MPC), produced by ultrafiltration of skimmed milk, contains both casein and whey in similar proportions to whole milk. Micellar caseins can be extracted from MPC by further ultrafiltration. Casein is produced from skim milk by acid precipitation or enzymatic coagulation, washing and drying. Caseinates are produced by treatment of acidified coagulate casein curd with an alkali such as sodium hydroxide or calcium hydroxide, which forms sodium or calcium caseinates respectively; caseinates contain approximately 90% protein (Gaucheron 2005).

Whey protein concentrate is produced by coagulation of milk with enzyme rennet or acid and further ultrafiltration and drying produces whey protein concentrates containing approximately 25-80% protein. Additional processing can produce whey protein isolates containing 90% or greater protein with low amounts of lactose and lipids (Smithers 2008). For this thesis, whey protein isolates, micellar caseins and calcium caseinates will be the focus.

The speed of absorption of dietary amino acids by the gut varies according to the type of ingested dietary protein (Bos, Metges et al. 2003). Amino acids are potent modulators of protein synthesis, breakdown and oxidation, so such different patterns of post prandial aminoacidemia might well result in different post prandial protein kinetics and gains (Boirie, Dangin et al. 1997). After whey protein ingestion, the plasma appearance of dietary amino acids is fast, high and transient (Boirie, Dangin et al. 1997). This amino acid pattern is associated with an increased protein synthesis and oxidation and no change in protein breakdown (Pennings, Boirie et al. 2011). In contrast, the plasma appearance of dietary amino acids after a casein meal is slower, lower and prolonged with a different whole body metabolic response; protein synthesis slightly increases, oxidation is moderately stimulated, but protein breakdown is markedly inhibited. The latter metabolic profile results in a better leucine balance (Boirie, Dangin et al. 1997). Even with a positive leucine balance being achieved with casein protein, much of the research has focused on whey protein post exercise, with more of a focus on resistance training than endurance training.

Table 1.3: The differences and similarities between whey protein isolates, calcium caseinates and micellar caseins.

Whey Protein Isolates	**Calcium Caseinates**	**Micellar Casein**
Fast digestion	Fast digestion	Slow digestion
High quality	High quality	High quality
High BCCA levels	High non-essential AA levels	High non-essential AA levels
Increases protein synthesis	?	Reduces protein break down
90% protein	90% protein	83% protein

1.5.2 Whey protein isolates

Whey protein supplements contain differing amounts of particular proteins due to variation in production and include beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, lactoferrin, immunoglobulins, lactoperoxidase enzymes, and glycomacropeptides (Marshall 2004). Separation technologies such as membrane-based separation technologies include

ultrafiltration to concentrate proteins, or diafiltration (DF) to remove most lactose, minerals and low molecular weight components (Ha and Zemel 2003). Whey protein isolates (WPI) are produced with 90% protein concentration and are of high quality and purity (Marshall 2004). WPC and WPI are commonly used in the sports industry.

Whey proteins compared with other protein sources have been shown to be more effective at promoting protein synthesis due to the high concentration of essential amino acids (Cribb 2005). Studies have shown only essential amino acids are required for protein synthesis (Ha and Zemel 2003, Cribb 2005). Whey proteins however are more effective than individual amino acids due to the prolonged elevated blood levels after consumption (Tipton, Elliot et al. 2004).

Protein synthesis is reduced during endurance exercise due to the increased oxidation of skeletal muscle branched chain amino acids and the use of gluconeogenic amino acids as an energy source (Ha and Zemel 2003). Extracellular amino acid concentration act as signals to promote protein synthesis. A reduction in this concentration decreases protein synthesis, subsequently influencing protein balance (Wolfe and Miller 2002). Maintaining net protein

balance is as important for endurance athletes as it is for strength athletes (Rodriguez 2009). Adaptive responses in the muscle occur as a result of exercise stressing the muscle (Tipton and Wolfe 2004). The adaptive response from the muscle differs depending on the type of exercise, for example endurance training induces mitochondrial biogenesis and increases muscle oxidative capacity, but not muscle growth and in contrast, resistance training mainly stimulates muscle hypertrophy through stimulating protein synthesis (Atherton, Babraj et al. 2005). mTOR is a signalling pathway involved in protein synthesis and is possibly linked to the beneficial effects of whey protein through integrating signals from nutrients and exercise (Blomstrand, Eliasson et al. 2006, Mascher, Andersson et al. 2007, Morrison, Hara et al. 2008)

1.5.3 Caseins

The casein in cow's milk comprises alpha-s1, alpha-s2, beta and kappa-casein. Micellar casein can be extracted from milk protein concentrate by further ultrafiltration (Fox and Brodkorb 2008). Micellar caseins are produced from skim milk by acid precipitation or enzymatic coagulation, washing and drying. Caseinates are produced by treatment of acidified or coagulated casein curd with alkali such as sodium hydroxide or calcium

hydroxide, which forms sodium or calcium caseinates respectively; caseinates contain approximately 90% protein (Fox and Brodkorb 2008).

An attractive property of the micellar casein is its ability to form a clot or gel in the stomach (Gaucheron 2005). This clot is able to provide a sustained slow release of amino acids into the blood stream (Boirie, Dangin et al. 1997). Micellar casein has limited uses in the food industry due to its insolubility, making it expensive to produce (Paul 2009).

Calcium caseinate, as distinguished from micellar casein, is soluble (and is thus used in numerous food processes), and so digestion rates of this form of caseins are not overtly different from those of whey (Reitelseder, Agergaard et al. 2011). The aminoacidemia reported in this investigation by Reitelseder et al. (2011) reflects not differential digestion kinetics but merely slightly differing amino acid contents.

Micellar caseins and caseinates typically contain higher levels of the conditionally essential amino acids glutamine and arginine (variations among manufactures) (Paul 2009). All forms of stress, including exercise, significantly deplete glutamine levels and arginine becomes

conditionally essential under a variety of catabolic conditions (Di Pasquale 2007, Gleeson 2008),therefore, providing potential benefit of using micellar caseins or caseinates.

1.6 Influence of post-exercise protein ingestion on endurance exercise performance and adaptations

For competitive endurance athletes, the desired outcome of an endurance training regimen is to increase the ability to sustain a desired power output or speed of movement over a given time (Smiles, Hawley et al. 2016). Effective post-exercise nutritional strategies can replenish depleted energy stores, attenuate indices of muscle damage and augment the recovery of endurance capacity and muscle function (Breen, Philp et al. 2011). In addition to exercise performance and recovery, dietary modifications can profoundly alter the adaptation process.

The effectiveness of protein nutrition for endurance athletes is yet to be fully elucidated. Thus, improving our understanding of how nutritional strategies can optimize the adaptive response to endurance exercise could provide the knowledge necessary to allow athletes to successfully modify their training and dietary habits and achieve their competitive goals.

1.6.1 Carbohydrate-protein co-ingestion for endurance exercise performance

It has been suggested that ingesting a carbohydrate and protein post-exercise may be more beneficial than carbohydrate alone for aiding muscle recovery and performance (Antonio and Stout 2002). In support of this hypothesis, several studies have shown that carbohydrate plus protein ingestion can extend endurance time-to-exhaustion (Saunders, Kane et al. 2004, Saunders 2007) and result in significantly greater glycogen restoration than carbohydrate alone (Williams, Raven et al. 2003, Cramer, Housh et al. 2012). However, these studies investigated acute recovery after a glycogen depleting bout of exercise. Little is known about the long-term performance benefits of carbohydrate and protein co-ingestion post-exercise.

According to short-term studies, the addition of protein to carbohydrate specifically improved late-exercise cycling time-trial performance (Saunders, Moore et al. 2009). Time to exhaustion has also been improved in running when carbohydrate and protein, in the form of milk, was ingested post exercise compared to carbohydrate alone (Lunn, Pasiakos et al. 2012). The mechanism behind the improved running performance was unclear (Lunn, Pasiakos et al. 2012). Several methodological differences between studies make it difficult

to discern whether any benefits observed for carbohydrate plus protein are the result of a protein-mediated effects. Studies examining carbohydrate – protein co-ingestion post exercise as part of a training diet and subsequent performance are lacking.

Cepero et al. (2010) compared casein and whey protein on performance and recovery, however failed to find any difference between groups. Another study found no difference on performance when comparing a carbohydrate and protein to isocaloric CHO supplements on time-to-exhaustion (Richardson, Coburn et al. 2012). A large amount of research exists on whey proteins effects on adaptations to endurance training, however these studies are all short term and do not completely control the diet by matching carbohydrate and total energy content. The types of proteins that are best for achieving muscle recovery and adaptations after endurance exercise are also not defined.

One potential mechanism several studies have shown with co-ingestion of whey protein with carbohydrate to benefit recovery and subsequent performance from endurance exercise, is by promoting muscle glycogen synthesis (Ivy, Goforth et al. 2002, Williams, Raven et al. 2003, Luden, Saunders et al. 2007, Morifuji, Kanda et al. 2010). In contrast,

other studies have found addition of whey protein to have no effect on muscle glycogen synthesis (Carrithers, Williamson et al. 2000, Green, Corona et al. 2008, Howarth, Moreau et al. 2009). Furthermore, protein supplementation may only be of benefit when carbohydrate intake is suboptimal (Kerksick, Harvey et al. 2008, Howarth, Moreau et al. 2009). When post-exercise beverages contain similar energy content but different proportions of carbohydrate and protein, they have a similar effect on muscle recovery and subsequent exercise performance in well-trained cyclists (Goh, Boop et al. 2012).

1.6.2 Carbohydrate-protein co-ingestion for signalling pathways involved with endurance exercise adaptations

Dietary protein intake post-exercise may play an important role in promoting aspects of skeletal muscle adaptation to endurance training (Hawley, Tipton et al. 2006, D'Antona, Ragni et al. 2010). It is well-established that BCAA, in particular leucine and carbohydrate (via insulin mediated pathways) regulate translation initiation via mammalian targets of rapamycin complex I (mTORC1) (Kimball 2006). Furthermore, mTORC1 is thought to be involved in activation of mitochondrial biogenesis (Cunningham, Rodgers et al. 2007), and long-term feeding of BCAA to mice led to mTORC1 activation, improved mitochondrial oxidative capacity and exercise endurance (Kimball 2006). These findings suggest that the

consumption of protein in the early post-exercise period may potentiate exercise-induced adaptive signalling in humans. In addition, amino acids have been shown to stimulate muscle protein synthesis via insulin independent pathways (Blomstrand, Eliasson et al. 2006). Essential amino acids directly phosphorylate downstream signalling proteins such as mTOR and the regulatory proteins (s6k1, eIF4E or eIF-2B complex) (Blomstrand, Eliasson et al. 2006).

In a longer-term study, Hill et al. (2013) demonstrated that 2 weeks of protein-carbohydrate supplementation with endurance training increased PGC-1α mRNA compared with carbohydrate only intervention. An acute study investigating protein co-ingestion also supported the upregulation of gene expression of mitochondrial adaptation with an upregulation of fatty-acid transport and electron transport chain components however, long term studies are lacking (Rowlands, Thomson et al. 2011).

1.6.3 Carbohydrate-protein co-ingestion on mitochondrial protein synthesis

Research on the nutritional requirements of endurance athletes has predominantly focused on carbohydrate ingestion protocols designed to replace glycogen stores and rehydration

(Burke, Loucks et al. 2006). However, muscle damage and muscle protein breakdown also result from endurance exercise (Smiles, Hawley et al. 2016). More recently, the co-ingestion of protein and carbohydrate has been investigated for enhancing muscle protein repair and regeneration as well stimulating myofibrillar and mitochondrial protein synthesis (Breen, Philp et al. 2011).

The co-ingestion of whey proteins and carbohydrates has been investigated to determine how post-exercise ingestion affects both myofibrillar and mitochondrial protein synthesis (Breen, Philp et al. 2011). Breen et al (2011) found myofibrillar protein synthesis increased with co-ingestion of protein after endurance exercise but not mitochondrial protein synthesis. A long term study conducted in mice found that feeding a high protein diet from the age of 8 weeks significantly decreased the amount of muscle mitochondria, mitochondrial activity, and running distance at age 50 weeks, compared to standard chow (Mitsuishi, Miyashita et al. 2013). However, muscle mass and grip power was increased. The effects of co-ingestion of carbohydrate and protein during recovery on mitochondrial protein synthesis pathways is unclear.

1.7 Summary

As outlined in this review of the literature, sports nutrition has evolved and become an integral part of an athletes' diet to ensure optimal athletic performance.

Long term studies investigating post-exercise co-ingestion of protein and carbohydrate on endurance training adaptations are lacking. In addition, longer-term studies with dietary protocols that replicate what an athlete would do in their training and competition regime and how this effects endurance adaptations are needed.

1.8 Aims and hypothesis

1.8.1 Study 1

The aim of the present study was to compare the effects of long term (8 weeks) supplementation with a fast (whey protein isolates) and slow (micellar caseins) digesting protein compared to control group (carbohydrate) on mitochondrial function, enzyme activity and body composition in an animal model. It was hypothesized that protein supplementation will have a greater effect on body composition by reducing fat mass and maintain and/or increasing lean mass and mitochondrial function compared to the carbohydrate group.

1.8.2 Study 2

The aim of the present study was to measure the effect of feeding different proteins (micellar caseins and whey protein isolates) during an endurance training program on endurance performance in an animal model. In addition, this study aimed to measure the effect of feeding different proteins (micellar caseins and whey protein isolates) during an endurance training program on mitochondrial function and body composition in an animal model. It was hypothesized that protein supplementation would have a greater effect on performance,

body composition by reducing fat mass and maintain and/or increasing lean mass and mitochondrial function compared to the carbohydrate group.

1.8.3 Study 3

The aims of this study were to understand the effect of post-exercise supplementation, in the form of carbohydrate and protein, on endurance exercise performance, skeletal muscle adaptations associated with endurance training such as mitochondrial respiration and mitochondrial biogenesis, along with body composition changes following 8 weeks of supplementation in trained individuals. This will be achieved while participants replicate carbohydrate and protein timing and quantities ingested in line with current Australian sports nutrition recommendations, that replicate what an athlete does in 'real world' training and racing. It was hypothesized that whey protein isolates would have a greater improvement in performance, body composition and adaptations, compared to calcium casienates.

Chapter 2: General methods section.

2.1 Experimental design

2.1.1 Animal model

Two animal studies were designed to investigate the effects of feeding different proteins (micellar caseins and whey protein isolates) for 8 weeks on body composition and mitochondrial adaptations in rodents (study 1), and body composition, mitochondrial adaptations, and endurance performance in rodents following an 8-week endurance training program (study two).

Experimental procedures were approved by Howard Florey Animal Ethics Committee (AEC 11-046). Seven-week-old male Sprague Dawley rats were individually housed in an environmentally-controlled laboratory (ambient temperature 22-24°C) with a 12-hour light/dark cycle (7:00 - 63 19:00). A total of 77 male Sprague Dawley rats were utilised across two studies. Evidence from past studies using Sprague Dawley rats has shown that endurance performance on a running treadmill can be explored in this rat species (Morifuji et al. 2010).

Rats were randomly assigned to receive either a Control diet consisting of standard chow (65% carbohydrate, 22% protein & 13% fat total energy), Standard chow + whey protein isolate (65% carbohydrate, 30% protein & 5% fat total energy) or Standard chow + calcium casienate (65% carbohydrate, 30% protein & 5% fat total energy). The nutritional content of the diet is explained in more detail in section 2.2.1. Ad libitum access to food and water was maintained throughout the duration of the study, with supplements provided each day, and body weight and food consumption recorded daily.

Exercise training was carried out 5 times per week with performance tests in the final week. Body composition was measured mid-way and in the final week of the experimental period. Both protocols will be outlined in more detail in the following sections. Following eight weeks of dietary intervention and/or exercise training, rats were then deeply anaesthetised with sodium pentabarbitone (100 mg/kg; Virbac, Peakhurst, Australia) and then euthanised via cardiac puncture. Soleus and gastrocnemius muscles were removed and one portion (10-20 mg) was immediately immersed in ~3 mL of ice-cold BIOPS (a biopsy preserving solution) for in-situ measurements of mitochondrial respiration; the remaining portion was immediately frozen in liquid nitrogen and stored at -80°C for subsequent analyses.

2.1.2 Human model

The aim of this study was to provide a detailed understanding of how skeletal muscles respond to different post-training carbohydrate and protein supplements during an 8-week training program in athletes. Experimental procedures were approved by Victoria University Human Research Ethics Committee (HRETH 12-330). Seventeen (nine in WPI+CHO and eight CAS+CHO) cyclists were recruited from local cycling clubs.

This study consisted of 8 weeks of an endurance-training program (cycling) with supplementation after each exercise session. Participants were randomly assigned to one of two groups; whey protein isolates and carbohydrate (WPI+CHO) or calcium caseinates and carbohydrate (CAS+CHO). Familiarisation testing was carried out two days prior to baseline testing. Baseline testing consisted of a DXA scan to assess body composition, a graded exercise test (GXT) for determination of lactate threshold and $VO_{2\ max}$ and a 2-hour steady state cycle followed by 20-km time trial as a measurement of endurance performance (these procedures are explained in more detail below). Post-exercise testing followed the exercise and nutrition intervention (Figure 2.1) was performed in the same order as pre-training. Blood and muscle samples were taken at rest pre and post the exercise training and nutrition intervention.

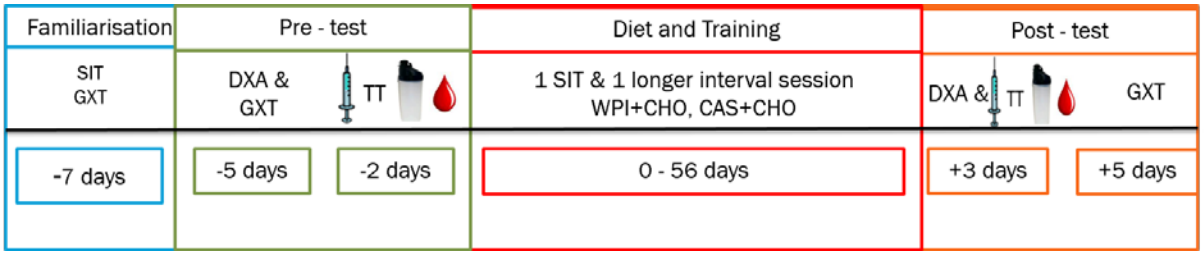


Figure 2.1: *Study design: Familiarisation sessions and pre-testing was carried out 1 week prior to the dietary and exercise intervention. Pre-testing provided baseline data for body composition (DXA), GXT and aerobic capacity as well as muscle and plasma insulin response. Diet (WPI+CHO or CAS+CHO) and training interventions was continued for 8 weeks with the last training session being a GXT, followed by post-test data.*

SIT – Sprint interval training; GXT – graded exercise test (lactate and VO_2 max); DXA – Dual Energy X Ray Absorptiometry; *Biopsy; TT – time trial; Blood samples; nutrition; WPI+CHO – whey protein isolates and carbohydrates; CAS+CHO – calcium caseinates and carbohydrates.*

2.2 Dietary intervention

2.2.1 Animal studies

The Sprague Dawley rats were randomly allocated to either a carbohydrate (CHO), CHO+ whey protein isolate (WPI+CHO) or CHO+ micellar casein (CAS+CHO) for the duration of the 8-week study. The animals had ad libitum access to standard chow, purchased from Specialty Feeds (Glen Forrest, WA, Australia). The soy free option was chosen to keep the protein source standardised between groups. See Table 2.1 and 2.2 for nutritional composition and ingredient list, respectively.

Table 2.1: *Soya free rat and mouse chow from Specialty Feeds.*

Calculated Nutritional Parameters	
Protein	18.5%
Total fat	5.00%
Crude fibre	3.4%
Digestible energy	14.3 MJ/Kg
% Total calculated digestible energy from protein	23.4%
% Total calculated digestible energy from lipids	15%

Table 2.2: *Ingredients list for Specialty feeds Soya free rat and mouse chow*.

Ingredients
Wheat, Mill mix, Fish meal, Refined Canola oil, Dicalcium phosphate, Lime sand, Salt, Methionine Vitamin and trace mineral premix.

The animals were provided individual supplements daily that were isocaloric and matched for carbohydrate content. According to previous research conducted at Howard Florey, Sprague Dawley rats consumed 20-30 g of standard chow per day. In addition to this the WPI+CHO diet supplement consisted of 2.5 g WPI and 0.68 g CHO, the CAS+CHO diet

consisted of 3 g micellar caseins and 0.5 g CHO and the CHO diet consisted of 0.8 g CHO and 0.2 g fat. Supplements were mixed into a paste with water and served to the animals in a petri dish; I observed to ensure supplements were eaten and not tipped over.

The WPI and CAS supplements were provided by Murray Goulburn. Due to micellar caseins supplement containing more moisture, fat, and CHO, 3.1 g of Micellar casein was given compared to 2.5 g of WPI, to ensure all rats were received the same amount of target protein (20 g). Micellar caseins had a higher carbohydrate content therefore, less carbohydrate in the form of sucrose was added. Canola oil was the fat source added to the CHO group to match total energy content of supplements. This was chosen as it was the fat source used the standard chow. See Table 2.3 for nutritional composition of protein supplements.

Table 2.3: *Breakdown of micellar casein and whey protein isolate supplements.*

	Micellar caseins	Whey protein isolates
moisture g/100g	5.3	3.4
fat g/100g	1.9	0.9
protein g/100g	76.3	90.7
cho g/100g	8.3	0.4
ash g/100g	8.2	4.3

2.2.2 Human study

The study consisted of two dietary groups to investigate the benefits of post-exercise carbohydrate and protein supplements on endurance training adaptations. A well-controlled dietary intervention was employed to investigate adaptations such as mitochondrial respiration and mitochondrial proteins.

The two dietary groups were isocaloric and matched for carbohydrate content; whey protein isolates (WPI+CHO) and calcium caseinates and carbohydrate (CAS+CHO). The carbohydrate content of the diets was matched given the crucial role that fuel plays in performance along with them being isocaloric.

Participants were provided all meals, snacks, and supplements for the duration of the intervention to aid with compliance. Mission Foods Australia (GRUMA, Asia & Oceania, LLC) provided several food items that provided the basis of the main meals. The fruit and vegetable items were ordered weekly from the University's fruit and vegetable provider. Supermarket items were ordered from Coles online weekly and delivered to Victoria University Footscray Park campus. Food boxes tailored to the participant's individual requirements were packed weekly and delivered to their house.

The diets provided 9 g per kilogram body mass ($g \cdot kg^{-1} \cdot bm^{-1}$) of carbohydrate per day, 1.8 $g \cdot kg^{-1} \cdot bm^{-1} \cdot day$ of protein and 1 $g \cdot kg^{-1} \cdot bm^{-1} \cdot day$ of fat. The carbohydrate guidelines for fuelling and recovery for endurance athletes were based off the Australian Institute of Sport guidelines (AIS Sports Nutrition 2014). Diets were created using Food Works nutrition data

base (Xyris Software Australia) to ensure all macro and micronutrient requirements were met. Participants were provided with weekly menus to follow, and asked to tick off meals and snacks once consumed or to add anything extra that was not on the list.

Participants were instructed to follow acute fuelling strategies (Table 2.4) for training rides and races outside of the study training sessions and this information was included in their weekly menus. This fuelling strategy was used through the 2-hour, steady-state cycle followed by 20 km time trial.

Table 2.4: *Acute fuelling strategies (*Jeukendrup 2008*)*

Carbohydrate guidelines for training and racing	
Less than30 min	No CHO
30-75 min	little CHO (depending on intensity)
1-2 h	up to 30 g per hour
2-3 h	up to 60 g per hour
>2.5 h	up to 90 g per hour

Protein supplements were provided by Fonterra Co-operative Group Limited (Table 2.5 and 2.6). Calcium casienate and whey protein isolates supplements provided 20 g of protein and were mixed with 27.5 g of sugar.. Calcium caseinates were utilised due to the expense and difficulty of obtaining sufficient micellar caseins from the manufacturer to be able to complete this study. Participants mixed these in water and drank directly after testing and each training session.

Table 2.5: *Breakdown of protein supplements utilised in the human study.*

	Calcium casienates	Whey protein isolates
Moisture g/100 g	3.9	4.7
Fat g/100 g	1	0.3
Protein g/100 g	92.6	93.9
CHO g/100 g	0.1	0.4
Ash g/100 g	3.9	1.5

Table 2.6: *The grams of (free) amino acids per 100grams of crude protein.*

	Whey Protein isolates	Calcium caseinates protein
Isoleucine	6.4	5.4
Leucine	14.6	9.8
Lysine	11.4	8.6
Methionine	2.4	2.9
Phenylalanine	3.9	5.0
Threonine	5.4	4.6
Tryptophan	2.4	1.4
Valine	5.7	6.5
Histidine	2.0	2.8
Alanine	5.8	3.4
Arginine	3.1	3.8
Aspartate	12.8	8.0
Cysteine	4.1	1.2
Glutamate	18.0	22.0
Glycine	1.8	1.9
Proline	4.6	10.0

Serine	4.6	5.7
Tyrosine	4.3	5.4

2.3 Exercise intervention

2.3.1 Animal study

All animals were familiarised with the motor-driven treadmill (Columbus Instruments, Columbus, OH, USA) for 3–4 days, 5 min/day, on a 10° incline. The running speed was 15 m/min initially, and gradually increase to 25 m/min. Following the familiarization, all animals performed an incremental test to exhaustion (exhaustion will be defined as the inability of the rats to be able to run on the treadmill and the inability to upright themselves when placed on their backs) on a treadmill inclined to 15°, starting at 10 m/min with increments of 3 m/min every 2 min. At least 72 h later, the rats performed a time trial to exhaustion at the individually determined maximal velocity reached during the incremental test. Exhaustion was determined when the rat stopped running and had to be replaced onto the treadmill 3 successive times despite encouragement using a tail brush, tail pinching or compressed air on the tail (Betik, Baker et al. 2008, Betik, Aguila et al. 2016).

The rats performed an endurance training program for eight weeks in conjunction with the feeding regime. Supplementation occurred directly after each training session. The rats began eating their supplement as soon as it was put in their cage and were monitored to ensure it was not tipped over and all the supplement consumed. The training program consisted of seven (1^{st} wk) to twelve (8^{th} wk) 2-min intervals (interspersed with 1 min of rest) performed five times/wk for 8 weeks. The intensity of the intervals was initially set at 80% of the peak speed reached for each rat during the incremental test and was increased by 5% each week (Bishop, Thomas et al. 2010). In week 8 of the training program, the same incremental test to exhaustion and time trial were performed as the beginning of the study.

2.3.2 Human study

The training program consisted of one sprint interval training session and one longer interval training session per week for 8 weeks carried out at Victoria University, Footscray Park campus. These two sessions were standardised due to the results from previous studies to improve fatigue resistance (Hawley, Myburgh et al. 1997) and oxidative capacity (MacDougall, Hicks et al. 1998). Participants continued their normal training program outside of these training sessions. Participants training program, while no data was

collected, anecdotally consisted of a criterium race and longer aerobic ride on Saturday and Sunday, respectively. Many participants also commuted on their bikes.

The sprint interval training session consisted of 30-second maximum effort intervals on an electronically-braked cycle ergometer (Velotron, RacerMate, Seattle, WA, USA). A similar 7-week training protocol to Macdougall (1998) was used. In this study, he showed that relatively brief but intense sprint training can result in an increase in both glycolytic and oxidative enzyme activity. The program (Table 2.7) began with four intervals with 4 minutes of recovery between intervals, and increased one interval each week except week 4 and reached nine intervals in week 7. Week 8 provided a taper week before final testing in week 9.

The recovery interval was fixed at 4 min and athletes cycled at a low cadence (50 revolutions per minute) against a light resistance (30 W) to reduce venous pooling in lower limbs and minimize feelings of light-headedness or nausea. All training sessions were supervised by myself and/or a research student who adjusted resistance, timed the recovery intervals, and provided verbal encouragement during the exercise bouts.

The participant's bike set up was closely matched to their personal bike set up to ensure optimal comfort and pedalling efficiency. Participants brought in their own bike seats and cycling shoes.

Table 2.7: *Weekly schedule for sprint interval training sessions.*

Week	**Number of sprint intervals**
1	four
2	five
3	six
4	six
5	seven
6	eight
7	nine
8	six

On the same cycle ergometer described above, participants completed one Longer interval session per week. The objective of this type of training is to expose the physiological power systems to sustained exercise at an intensity corresponding to the athlete's highest current steady state pace. This is helping to improve the fatigue resistance of the muscle and to improve lactate kinetics (Hawley, Myburgh et al. 1997).

After baseline testing, each training session consisted of six to eleven 5-minute cycling bouts at 80% of the subject's peak power output (Table 2.8) Between each interval participants rested for 1 minute or cycled against a low resistance. As with the sprint interval training schedule, week 8 was taper week before testing in week 9.

Table 2.8: *Weekly schedule for longer interval training sessions.*

Week	**Number of longer intervals**
1	Six
2	Seven
3	Eight
4	Nine
5	Nine
6	Ten
7	Eleven
8	Six

2.4 Body composition

2.4.1 Animal study - Echo Magnetic Resonance Imaging (Echo MRI)

Body composition of the rats was analysed using an EchoMRI ™ Whole Body Composition Analyzer (EchoMRI, Houston TX). The machine was calibrated according to the manufacturer's instructions using canola oil as calibration medium. Live, conscious rats were restrained within a clear plastic cylinder which limited their ability to turn around, but did not restrict breathing. The restrained rats were then placed inside the EchoMRI™ Analyzer for a two-minute scan, which determined fat and lean tissue as well as free and total body water content of the animals. Each animal was scanned in duplicate, to ensure accuracy of measurements. Animals were restrained for no longer than 10 minutes and were returned to cages following the scans. Echo MRI is a validated method to obtain body composition in rats and mice (Taicher, Tinsley et al. 2003).

2.4.2 Human study – Dual Energy X Ray Absorptiometry (DXA)

Participants presented to Western Centre for Health and Research (WCHRE), St Albans, Australia in the morning, where possible for DXA scans to be performed after an overnight

fast. This time was not suitable for two participants, therefore a food diary was kept and this was mimicked for the follow up scan.

A phantom scan was performed prior to each scan to calibrate the machine. A phantom scan serves as an external standard for the analysis of different tissue components (Santos et al. 2010). Participants were instructed to wear light exercise attire or wear a hospital gown (provided) with no metal objects or piercings that could not be removed. They were lined up within the markings of DXA bed with a band around their feet to maintain position for the entire length of the scan. Participants were instructed to stay as still as possible and refrain from talking or moving for the duration of the scan.

DXA gives detailed images of body composition including bone mineral mass, lean body mass and body fat mass of participants. There is relatively low radiation exposure with this procedure (Nana, Slater et al. 2015). DXA accurately estimates total body composition and the composition of weight loss in athletic participants and can be used for nutritional monitoring and evaluation during dietetic and exercise interventions (Nana, Slater et al. 2015). The procedure may take up to 10 minutes, where the participant is asked to lie still face up on the DXA bed with arms and legs tucked closely to the body. I completed the

Clinical densitometry training course prior to my study and completed the DXA scans associated with this study.

2.5 Performance tests – human study

2.5.1 Graded Exercise Test (GXT)

A discontinuous graded exercise test was performed on an electronically-braked cycle ergometer (Lode Excalibur, v2.0, Groningen, The Netherlands) for determination of peak oxygen uptake (VO_{2peak}), lactate threshold (W_{LT}) using the modified D_{Max} method (Bishop, Jenkins et al. 2000) and peak power output (W_{Peak}), and determination of the training intensities used during the training intervention.

The test consisted of 4-min incremental steps with 30 seconds of rest between each step. The GXT commenced at 90 W and increased 30 W every 4 min until volitional exhaustion. During the 30-s rest capillary blood samples were taken from the fingertip. Participants were instructed to keep a cadence of 90-100 rpm and were given verbal encouragement throughout the test. Participants had access to elapsed time and cadence values, whilst every other variable was concealed from their view. The test was terminated when a subject reached volitional exhaustion or when his cadence dropped below 60 rpm. The maximal

power output (W_{Peak}) was determined as the power of the last completed stage when participants stopped at the end of a stage. If a participant stopped during a stage, the W_{Peak} was determined as the power of the last completed stage plus a fraction of the difference in power between the last completed stage and the incomplete stage equivalent to each completed minute of the non-completed stage. The lactate threshold was identified as the power at which capillary blood lactate increased1 mM above baseline (Coyle, Martin et al. 1983), and was calculated using Lactate-E software (Newell, Higgins et al. 2007). Wpeak was calculated as previously reported (Kuipers, Verstappen et al. 1985, Hawley and Noakes 1992):

$$W_{peak} = W_{final} + \left(\frac{t}{240} \bullet 30 \right)$$

where W_{final} was the power output of the last completed stage and t was the time in seconds of any final uncompleted stage.

Capillary blood sampling during the GXT

Blood samples (~50 µl) were taken from the fingertips of each participant at rest, after completion of the warm up during the 30 s of recovery between stages and immediately after the termination of the test.

2.5.2 Peak Oxygen Uptake Test

After the GXT, the participants performed 5 min of active recovery at 20 W on the cycle ergometer, followed by a square-wave $VO_{2\ peak}$ test. This comprised a steady-state cycle to volitional fatigue at a supramaximal power output, equating to 105% of W_{peak} achieved during the GXT. A similar protocol has previously been reported to elicit $\dot{V}O_{2peak}$ values no different to those determined during either a ramp incremental test performed 5 min previously (Rossiter, Kowalchuk et al. 2006), or a GXT performed 3 min previously (Sedgeman, Dalleck et al. 2013). Participants were advised to accelerate to 90–100 rpm at the commencement of a 5-s countdown, and to maintain a high but not fixed cadence until volitional fatigue. Consistent verbal encouragement was provided throughout. Expired gases were analysed every 15-s using a custom-made metabolic cart. A two-point calibration of the gas analysers (S-3lA/II and CD-3A analysers, Ametek, PA, USA) was performed before each test using one certified gravimetric gas (16.1% O_2, 4.17% CO_2; BOC Gases, Chatswood, Australia) and ambient air. Ventilation was recorded every 15 s. The ventilometer (KL Engineering, Sunnyvale, CA, USA) was calibrated at the start of each day using a 3-L syringe (MedGraphics, St. Paul, MN). Peak $\dot{V}O_2$ was calculated as the mean of the two highest consecutive 15-s values.

2.5.3 2-hour, steady state followed by 20 km cycling Time Trial

The cycling time trials were performed on an electronically-braked cycle ergometer (Velotron, RacerMate, Seattle, WA, USA). This trial was performed at baseline and following the 8-week training and nutrition intervention.

In line with endurance athlete nutrition guidelines (AIS Sports Nutrition 2014) for events longer than 90 minutes, a carbohydrate loading regime was undertaken 2 days prior to this performance test by each athlete. During the 2-hour cycle participants ingested carbohydrate at a rate of 70 $g \cdot h^{-1}$and *ad libitum* water intake. The carbohydrate was provided in a sports drink made with a combination of fructose and glucose, and participants were reminded to drink on a regular basis to ensure carbohydrate content was consumed.

Prior to beginning the time trial, participants completed a warm-up consisting of 10 min of cycling at a high cadence at a self-determined power output. The 2-hour constant-load ride was set at 95 % onset of the blood lactate accumulation (OBLA). During this time heart rate, capillary blood lactate (as per GXT procedure described below) and rate of perceived effort

(RPE) was measured every 15 minutes. Participants could listen to music or watch a movie on a laptop screen. This was recorded and provided during the post test.

On completion of 2 hours of cycling there was a 2-min rest period before participants began the 20-km cycling time trial test. The participants were instructed to complete the 20-km time trial as quickly as possible. Gearing and cadence was self-selected. The gearing on the velotron bike was set up the same as the participants own bike. Participants were aware of the distance covered but received no verbal stimuli, times, or other information.

2.6 Blood collection - Human study

Following the 20-km time trial, a post-exercise beverage was consumed (either WPI+CHO or CAS+CHO), and venous blood samples were taken every 20 min for 4 h. Blood was collected via venous cannulation. These techniques are performed regularly in the exercise performance laboratory and I completed the necessary training to collect blood samples or another member of the research team that was licensed in venepuncture and venous cannulation techniques.

2.7 Muscle collection

2.7.1 Animal study

At the end of the experimental period, rats were deeply anaesthetised with sodium pentabarbitone (100 mg/kg; Virbac, Peakhurst, Australia), soleus and gastrocnemius muscles were removed and then rats euthanised via cardiac puncture. The soleus and gastrocnemius muscles were dissected out, cleaned of excess blood, fat and connective tissue, and split in two portions. A small portion of each muscle sample (10-20 mg) was immediately immersed in an eppendorf containing 1mL of BIOPS, a muscle preserving solution placed on ice. This portion was immediately used for in-situ measurements of mitochondrial respiration using the permeabilised fibre technique (described in detail below). The second portion immediately frozen in liquid N_2, and subsequently stored at -80°C for subsequent analysis.

2.7.2 Human study

Each participant received a total of two muscle biopsies; one prior to the dietary and exercise intervention and one following the 8 weeks of the intervention. The muscle biopsy procedure was performed by a qualified and experienced medical doctor, using aseptic techniques. In this procedure needle biopsies were taken from the *vastus lateralis* muscle

with suction. There are no large blood vessels or nerves in the region that was biopsied. Prior to the biopsy, the skin around the area of investigation was cleaned with surgical spirit, and the skin and subcutaneous tissue was anaesthetised with 1% xylocaine. Using aseptic techniques, the skin and muscle fascia was punctured with a sterile scalpel blade. The tip of the biopsy needle with the inner cylinder was introduced into the muscle to a depth of 2-5 cm. The inner cylinder was then drawn back a few cm without moving the needle, and pushed back to cut a small piece of muscle bulging into the cylinder because of suction applied to the needle. This can be repeated several times to increase the mass of muscle taken. The procedure took on average 5-10 seconds, and the weight of tissue ranged from 100-400 mg.

Once obtained, muscle samples were immediately processed, cleaned of excess blood, fat and connective tissue, and split in two portions. A small portion of the biopsy samples (10-20 mg) was immediately immersed in an eppendorf containing 1mL of BIOPS, a muscle preserving solution and placed on ice. This portion was immediately used for in-situ measurements of mitochondrial respiration using the permeabilised fibre technique (described in detail below). The second portion (80-390 mg) was weighed, immediately

frozen in liquid N_2, and subsequently stored at -80°C for subsequent analysis of markers of mitochondrial biogenesis such as gene and protein expression and enzymatic activity.

2.8 Muscle analysis

2.8.1 Mitochondrial respiration – Human and rat study

Muscle samples were obtained from the *vastus lateralis* muscle in humans and soleus and white portion of gastrocnemius muscles in rats. Mitochondrial respiration utilising glycolytic protocol was investigated in all three muscle samples. A Fatty acid protocol was also investigated in the rat soleus muscle (protocol is described in the next section).

Fibre preparation and high-resolution respirometry.

Muscle fibres were mechanically separated using pointed forceps in ice-cold BIOPS, a biopsy preserving solution containing (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2$, 20 taurine, 50 MES, 15 Na_2phosphocreatine, 20 imidazole and 0.5 Dithiothreitol adjusted to pH 7.1 (Pesta and Gnaiger 2012). The plasma membrane was subsequently permeabilised by gentle agitation for 30 min at 4°C in BIOPS containing 50 µg/mL of saponin, and was followed by 3 washes in MiR05, a respiration medium

containing (in mM, unless specified) 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose and 1 g/L BSA essentially fatty acid-free, pH 7.1 (Pesta and Gnaiger 2012). Mitochondrial respiration was measured in duplicate (from 3-4 mg wet weight of muscle fibres) in MiR05 at 37°C using the high-resolution Oxygraph-2k (Oroboros, Austria). Oxygen concentration (nmol mL^{-1}) and flux (pmol s^{-1} mg^{-1}) were recorded using DatLab software. Re-oxygenation by direct syringe injection of O_2 was necessary to maintain O_2 levels between 275 and 450 nmol mL^{-1} and to avoid potential oxygen diffusion limitation.

Mitochondrial respiration protocol – Glycolytic protocol

Mitochondrial respiration measurements were taken using a substrate uncoupler-inhibitor titration (SUIT) protocol (Pesta and Gnaiger 2012). Firstly, the substrates pyruvate (final chamber concentration; 2 mM) and malate (5 mM) were added in the absence of adenylates for measurement of leak respiration (L) with electron entry through Complex I (CI) (CIL). Next, adenosine diphosphate (ADP) was added (5 mM) for measurement of maximal oxidative phosphorylation (OXPHOS) capacity (P) with electron input through CI (CIP), followed by addition of succinate (10 mM) for measurement of P with electron supply through CI and Complex II (CII) combined (CI+IIP). This state provides convergent

electron input to the Q-junction through CI (NADH provided by malate and pyruvate) and CII ($FADH_2$ provided by succinate) and supports maximal mitochondrial respiration by reconstruction of the citric acid cycle function. Cytochrome c (10 μM) was next added to assess outer mitochondrial membrane integrity – increases in O_2 flux > 6% indicated compromised membrane integrity, in which data was excluded. A series of stepwise carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone titrations (FCCP, 0.75-1.5 μM) were performed, to determine maximal uncoupled respiration and electron transport system (ETS) capacity (E), with convergent electron input through CI+II (CI+IIE). Rotenone (0.5 μM), an inhibitor of CI, was added next to determine E with electron input through CII alone (CI+IIE). Antimycin A (2.5 μM), an inhibitor of Complex III (CIII), was then added to measure residual oxygen consumption capacity (ROX); this was used to correct all respiration values. Mitochondrial respiration measures were expressed in both absolute oxygen flux (mass-specific mitochondrial respiration), and corrected to citrate synthase activity (mitochondrial-specific respiration).

2.8.2 Mitochondrial respiration –Animal study only

Mitochondrial respiration protocol – Fatty acid protocol

Mitochondrial respiration measurements were also taken using a fatty acid SUIT protocol in the soleus muscle (Pesta and Gnaiger 2012). The SUIT sequence, with final chamber concentration in brackets, was as follows: Malate (5 mM) and octanoylcarnitine (0.2 mM) in the absence of adenylates were added for measurement of LEAK respiration (Leak). ADP (5 mM) was then added for measurement of maximal OXPHOS capacity (P). Cytochrome c (10 μM) was then added to test for outer mitochondrial membrane integrity; an exclusion criterion was set such that if a chamber showed an increase in O_2 flux > 6% after addition of cytochrome c, it was discarded. This was followed by a series of stepwise carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone titrations (FCCP, 0.75-1.5 μM) for measurement of ETS capacity (E). This was followed by addition of antimycin A (2.5 μM), an inhibitor of Complex III (CIII), to obtain a measurement of ROX. ROX was subtracted from all other measurements to account for oxidative side reactions. Due to time constraints, the measurement of electron transport through CII only by addition of Rotenone to inhibition CI was not conducted.

2.8.3 Citrate Synthase (CS) enzyme assay - Human and animal study

Sample preparation

Approximately 10-20 mg of frozen muscle was homogenised 4 times for 5 seconds with a hand-held mini-homogenizer (Kontes Pellet Pestle Cordless Motor, Kimble Chase, NJ, USA.) on ice (1:20 w/v) in an ice-cold lysis buffer containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM benzamidine, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF), adjusted to pH 7.4. Muscle homogenates were rotated end over end at 4 °C for 60 min, after which they were centrifuged for two cycles of 10 min at 15 000 g at 4 °C separated by a similar re-homogenization step to the initial one. After the second centrifugation, the supernatant was taken as the whole muscle lysates for enzyme activity assay.

CS activity was determined in triplicate on a microtiter plate by adding: 5 μL of a 2 mg/mL muscle homogenate, 40 μL of 3 mM acetyl CoA in Tris buffer and 25 μL of 1 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in Tris buffer to 165 μL of 100 mM Tris buffer (pH

8.3) kept at 30°C. At this point 15 µL of 10 mM oxaloacetic acid were added to the cocktail and the plate was immediately placed in a spectrophotometer kept at 30°C (xMark Microplate Spectrophotometer, Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia). Following 30 s of linear agitation, absorbance at 412 nm was recorded every 15 s for 3 min (Srere 1969).

2.9 Statistics

Chapter 3 - All data are expressed as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was performed to assess total accumulated food and energy intake, muscle enzyme activities, and mitochondrial function. Changes in body composition were evaluated by using a repeated-measures ANOVA design. Differences were considered statistically significant if the P value was less than 0.05. All statistical calculations were conducted using GraphPad Prism 7 software (GraphPad Software Inc, La Jolla, CA, USA).

Chapter 4 - All data are expressed as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was performed to assess total accumulated weekly food and energy intake, muscle enzyme activities and mitochondrial respiration. Changes in body composition were evaluated by using a repeated-measures ANOVA design. Tukeys post hoc test was utilised and differences were considered statistically significant if the P value

was less than 0.05. All statistical calculations were conducted using GraphPad Prism 7 software (GraphPad Software Inc, La Jolla, CA, USA).

Chapter 5 - All statistical analysis was completed using Graph Pad Prism Software 7.0. Normality of data sets was analysed using Sharipo-Wilk normality test. The change was calculated from pre to post for each group and an independent t-test performed

Chapter 3: Effects of 8 weeks of protein supplementation in rats, on body composition and mitochondrial function.

3.1 Abstract

3.1.1 Background

Protein intakes above the recommended dietary intakes may help to promote healthy aging, appetite regulation, weight management, and athletic performance. Body mass is an important issue for both the general population and athletes, with obesity and its related health concerns a major problem for the general population. Furthermore, mitochondria play a key role in energy metabolism in many tissues, including skeletal muscle. Therefore, investigating how different types of proteins affect body composition and mitochondrial function in healthy animals may help in providing a better framework for these outcomes in humans.

3.1.2 Materials and methods

Sprague Dawley rats were randomly assigned to one of the following three experimental groups for 8-weeks; carbohydrate (CHO), carbohydrate plus whey protein isolate (CHO+WPI), or carbohydrate plus micellar casein (CHO+CAS). The control diet consisting of standard chow (65% carbohydrate, 22% protein & 13% fat) and the protein groups consisted of standard chow + whey protein isolate (65% carbohydrate, 30% protein & 5%

fat) or Standard chow + calcium casienates (65% carbohydrate, 30% protein & 5% fat). Body mass and food consumption was weighed and recorded daily and body composition was determined at 4 and 8-weeks via an Echo MRI^{TM} system. Mitochondrial respiration was assessed in soleus and white portion of gastrocnemius muscle.

3.1.3 Results, discussion and conclusion

This study demonstrated 8 weeks of supplementing with whey protein isolates or micellar caseins decreased body fat in Sprague Dawley rats compared to carbohydrate matched control group. In addition, the micellar caseins group showed a significant increase in oxidative phosphorylation compared to whey protein isolates when investigating fatty acid substrate entry in rat soleus muscle. This research has established that micellar caseins and whey protein isolates may have beneficial effects on body composition and mitochondrial function without the stimulus of exercise.

3.2 Introduction

Body mass is an important issue for both the general population and athletes. Obesity and its related health concerns are a major problem for the general population, with estimates that 63.4% of Australians are overweight or obese (ABS 2015). Consequently, there is a large percentage of the Australian population for which strategies to promote weight loss is an important concern. Mitochondria play a key role in energy metabolism and reduced mitochondria function has been associated with obesity and its health concerns (Johannsen and Ravussin 2009). Many athletes also restrict energy intake to achieve a certain body mass category, for aesthetic reasons, or to attain better force to mass ratio to improve performance (Thomas, Erdman et al. 2016).

Research is now emerging for a positive role of protein ingestion at intakes above the recommended dietary intake (RDI) to assist in promoting positive changes in body composition and managing body mass (Phillips, Chevalier et al. 2016). In particular, there is increasing interest in the roles of different types of protein on lean mass retention during ageing, weight loss and weight control (Millward, Layman et al. 2008). Investigating how different types of proteins affect body composition in healthy animals may help to provide a better understanding into how to improve body composition in humans.

The nutritional properties of milk proteins have been studied in both animals (Rutherfurd and Moughan 1998) and humans (Bos, Gaudichon et al. 2000), due to their excellent nutritional properties, digestibility and effects on postprandial protein utilisation (Lacroix, Bos et al. 2006). Dairy protein is made up of 2 classes of proteins: casein (80%) and whey proteins (20%). Casein and whey are both complete proteins containing all essential amino acids, but they differ in the way they are digested and absorbed. Casein coagulates in the acidic environment of the stomach, which delays gastric emptying and slows the postprandial increase in plasma amino acids; this gives micellar casein the label as a 'slow' protein (Boirie, Dangin et al. 1997). Whey induces a fast, high, and transient increase in plasma amino acids, which has seen it labelled as a 'fast' protein (Boirie, Dangin et al. 1997). These properties make these two milk proteins attractive supplements to aid muscle growth and repair for both resistance-based (Areta, Burke et al. 2013) and endurance-based exercise (Howarth, Moreau et al. 2009). However, whether postprandial induced increases in protein synthesis by milk proteins is sufficient to result in net skeletal muscle mass accretion over time without exercise and reduce fat mass to aid in body mass management, is yet to be established.

Milk proteins provide a potent anabolic stimulus due to their amino acid composition and insulinotropic effects, although whether whey proteins or caseins have a greater differential effect on muscle mass and/or body composition is not yet well understood (McGregor and Poppitt 2013). An earlier study by Boirie et al. (1997) found that postprandial protein synthesis was increased 68% after ingestion of whey protein but only 31% after ingestion of casein. In the same study casein also inhibited whole body protein breakdown and so despite the more rapid appearance of whey derived amino acids, net protein balance was higher following casein ingestion (Boirie, Dangin et al. 1997). Although casein and whey differ in their effect on protein balance, it is unknown how each protein effects body composition changes.

Increased satiety and diet-induced thermogenesis observed with high-protein diets may also translate into beneficial effects on body mass and composition over time. Whey proteins that are more rapidly digested appear to have greater influence on satiety than casein, which induces a slower hyper aminoacidemia (Hall, Millward et al. 2003). Whey has also shown greater suppression of appetite in the next hour of feeding than egg-albumin, soy protein and casein (Hall, Millward et al. 2003, Anderson, Tecimer et al. 2004, Bowen, Noakes et al. 2006). These studies indicate that whey protein may have unique properties in maintaining

a healthy body mass through its greater satiating effects compared to other protein sources. In addition to these satiating effects of protein, it is well documented that diet induced thermogenesis is greater for protein (20-35% of ingested energy) than carbohydrate (5-10% of ingested energy) and fat (0-3% of ingested energy) (Westerterp, Wilson et al. 1999, Mikkelsen, Toubro et al. 2000, Halton and Hu 2004).

Mitochondrial adaptations, such as increased density and function are usually associated with endurance exercise training (Arany 2008). Studies have investigated the benefit of protein and exercise on markers of mitochondrial biogenesis such as peroxisome proliferator-activated receptor gamma coactivator (PGC-1α) (Hill, Stathis et al. 2013). However, studies investigating the anabolic nature of dietary protein and its ability to either improve mitochondrial function without exercise stimulus are lacking. Protein may play a role on satiety, thermo-regulation and muscle anabolism and mitochondria play an important role in energy production, therefore the need to investigate the relationship between protein intake and mitochondrial function.

The aim of the present study was to therefore compare the effects of 8 weeks of supplementation with a fast (whey protein isolates) or slow (micellar caseins) digesting protein, compared to control diet (carbohydrate), on body composition and mitochondrial function and enzyme activity in an animal model. It was hypothesized that protein supplementation will have a greater effect on body composition and mitochondrial function compared to the carbohydrate group.

3.3 Methods

3.3.1 Animals

Twenty-four male Sprague Dawley rats were individually housed in an environmentally controlled laboratory (ambient temperature 22-24°C) with a 12-hour light/dark cycle (7:00 - 19:00). Animals were obtained at 7 weeks (~150 g) and acclimatised for 5 days before being separated out into individual cages. Experimental procedures were approved by the Howard Florey Animal Ethics Committee (AEC 11-046).

3.3.2 Experimental design

Animals were randomly assigned to one of the following three experimental groups (eight in each group); carbohydrate (CHO), carbohydrate plus whey protein isolate (CHO+WPI), or carbohydrate plus micellar casein (CHO+CAS). Body mass and food consumption was weighed and recorded daily and body composition was determined at 4 and 8 weeks via an Echo MRI^{TM} system (Echo-MRITM 900, Houston, TX, United States of America) according to manufacturer's instructions and as previously validated (Taicher, Tinsley et al. 2003).

At the end of the 8-week experimentation animals were euthanised by lethal injection and blood collected via cardiac puncture. One soleus and white gastrocnemius muscle was

quickly removed and placed in ice-cold preservation buffer (BIOPS) for transportation (approximately 30 minutes) to Victoria University, Footscray Park campus for the assessment of mitochondrial respiration. The same muscles were removed from the contralateral hind limb, frozen in liquid nitrogen, and stored at -80°C until subsequent biochemical analysis.

3.3.3 Diets

Rats were fed a standard Soya-free rat and mouse chow (Specialty feeds), ad libitum. Every day rats were supplemented with either CHO+WPI, CHO+CAS, or CHO. These supplements were isocaloric and matched for carbohydrate content and mixed into a paste with water and fed in a petri dish; animals were observed to ensure supplements were eaten and not tipped over. The control diet consisted of standard chow (65% carbohydrate, 22% protein & 13% fat), the CHO+WPI diet consisted of standard chow + whey protein isolate (65% carbohydrate, 30% protein & 5% fat), and the CHO+CAS diet consisted of standard chow + micellar casein (65% carbohydrate, 30% protein & 5% fat).

The CHO+WPI diet consisted of daily supplementation with 2.5 g WPI and 0.68 g CHO, the CHO+CAS diet of 3.1 g micellar casein and 0.5 g CHO, and the CHO diet of 0.8 g CHO and 0.2 g fat. CHO+CAS was provided 3.1 g of supplement compared to CHO+WPI 2.5 g

to ensure all rats were receiving the same amount of target protein. Micellar casein has a higher carbohydrate content; therefore, less carbohydrate in the form of sucrose was added. Canola oil was the fat source added to the CHO group to match total energy content of supplements. This was chosen as it was the fat source used in the standard chow fed. The WPI and micellar casein supplements were provided by Murray Goulburn Nutritionals, Victoria, Australia. Total energy intake includes standard chow plus protein supplement for the 8-week dietary intervention.

3.3.4 Enzyme assays

Frozen muscle was pulverised in liquid nitrogen, weighed, homogenised and then assessed for the activity of citrate synthase (CS) and beta-hydroxyacyl-CoA (β-HAD), adapting previously described methods (Srere 1969) for use in a plate reader.

3.3.5 Mitochondrial respiration

Muscle fibres were separated and permeabilised as previously described by Pesta and Gnaiger (2012). Mitochondrial respiration was measured in duplicate (from 3–4 mg wet weight of muscle fibres) in MiR05 at 37°C by using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria). Mitochondrial respiration measurements were taken using

two different substrate uncoupler-inhibitor titration (SUIT) protocols to assess a glycolytic protocol in both soleus and gastrocnemius and a fatty acid protocol in the soleus muscle. These were conducted according to previously described methods (Pesta and Gnaiger 2012).

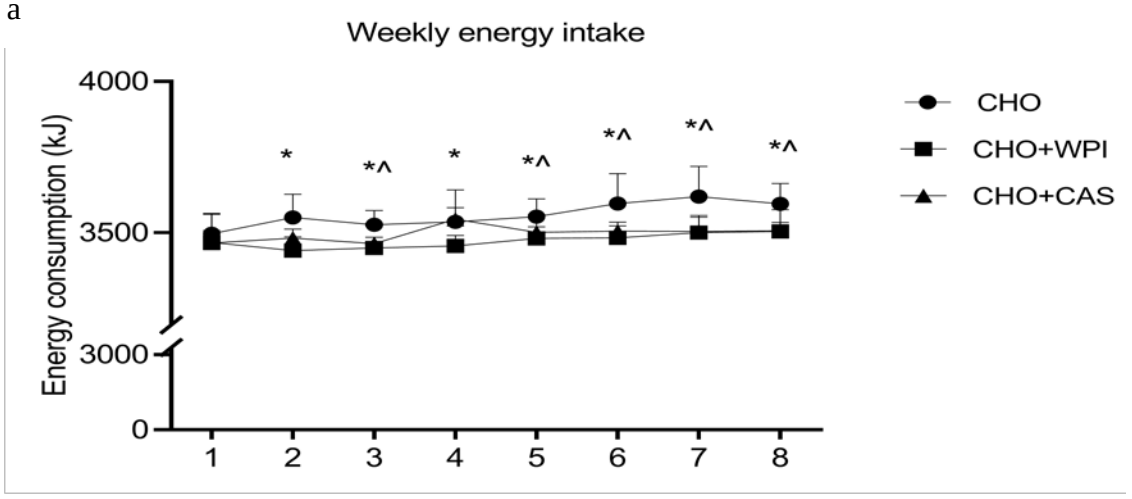
3.3.6 Statistics

All data are expressed as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was performed to assess total accumulated food and energy intake, muscle enzyme activities, and mitochondrial function. Changes in body composition were evaluated by using a repeated-measures ANOVA design. Differences were considered statistically significant if the P value was less than 0.05. All statistical calculations were conducted using GraphPad Prism 7 software (GraphPad Software Inc, La Jolla, CA, USA).

3.4 Results

3.4.1 Energy intake and body composition

a



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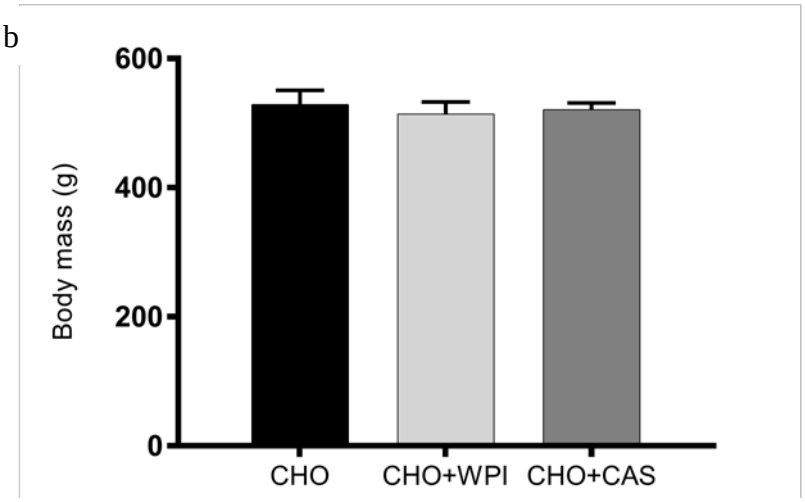

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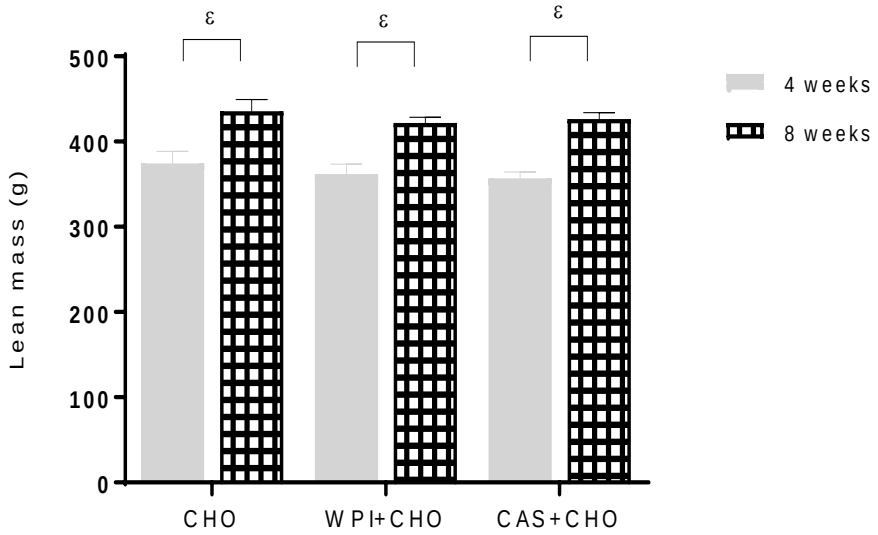
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ε
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400
300
200
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Lean mass (g)
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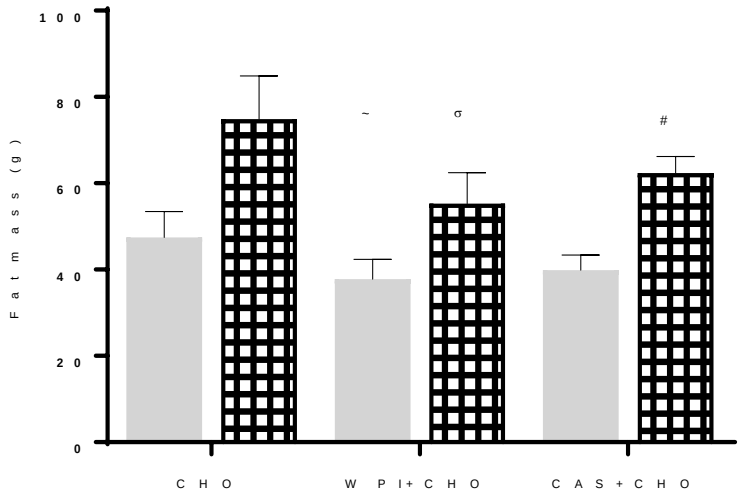
WPI+CHO
CAS+CHO
4 weeks
8 weeks

d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Fat mass (g)
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CAS+CHO

Figure 3.4.1: *Weekly energy intake and body composition for the carbohydrate (CHO), carbohydrate and whey protein isolate (CHO+WPI) and carbohydrate and micellar casein (CHO+CAS) supplemented groups during the 8-week dietary intervention. Total energy intake over 8 weeks (a). Body mass (b) of animals in each group. Lean mass (c) at 4 weeks and 8 weeks for each group with a main effect of time (ε). Fat mass (d) at 4 and 8 weeks for each group. * Significantly different (P < 0.05) vs. CHO, ^ Significantly different (P < 0.05) vs. CHO, # vs. CHO+WPI ~Significantly different (P < 0.05) vs. CHO at 4 weeks and vs.* $^{\sigma}$ *CHO at 8 weeks. n = 8 in each group. Values are mean ± SEM.*

The weekly mean energy intake is shown in Figure 3.1a. CHO group had significantly higher energy intake then CHO+WPI in week 2 and 4 and CHO group was significantly higher than both protein groups and in week 3, 5, 6, 7 and 8 ($P<0.05$; Figure 3.1a). The total body mass was not significantly altered between groups (Fig 3.1b) and body mass increased in all groups over the 8 week intervention period; however, in terms of body composition the CHO+WPI group had lower body fat (Fig 3.1d) compared to the CHO group at week 4 ($P < 0.05$) and week 8 ($P < 0.05$). The CHO+CAS group however had a lower body fat (Fig 3.1d) compared to CHO group only after 8 weeks ($P < 0.05$). Lean mass showed a main

effect of time; however, there was no significant difference between supplement groups at 4 or 8 weeks (Fig 3.1c).

3.4.2 Enzyme activity

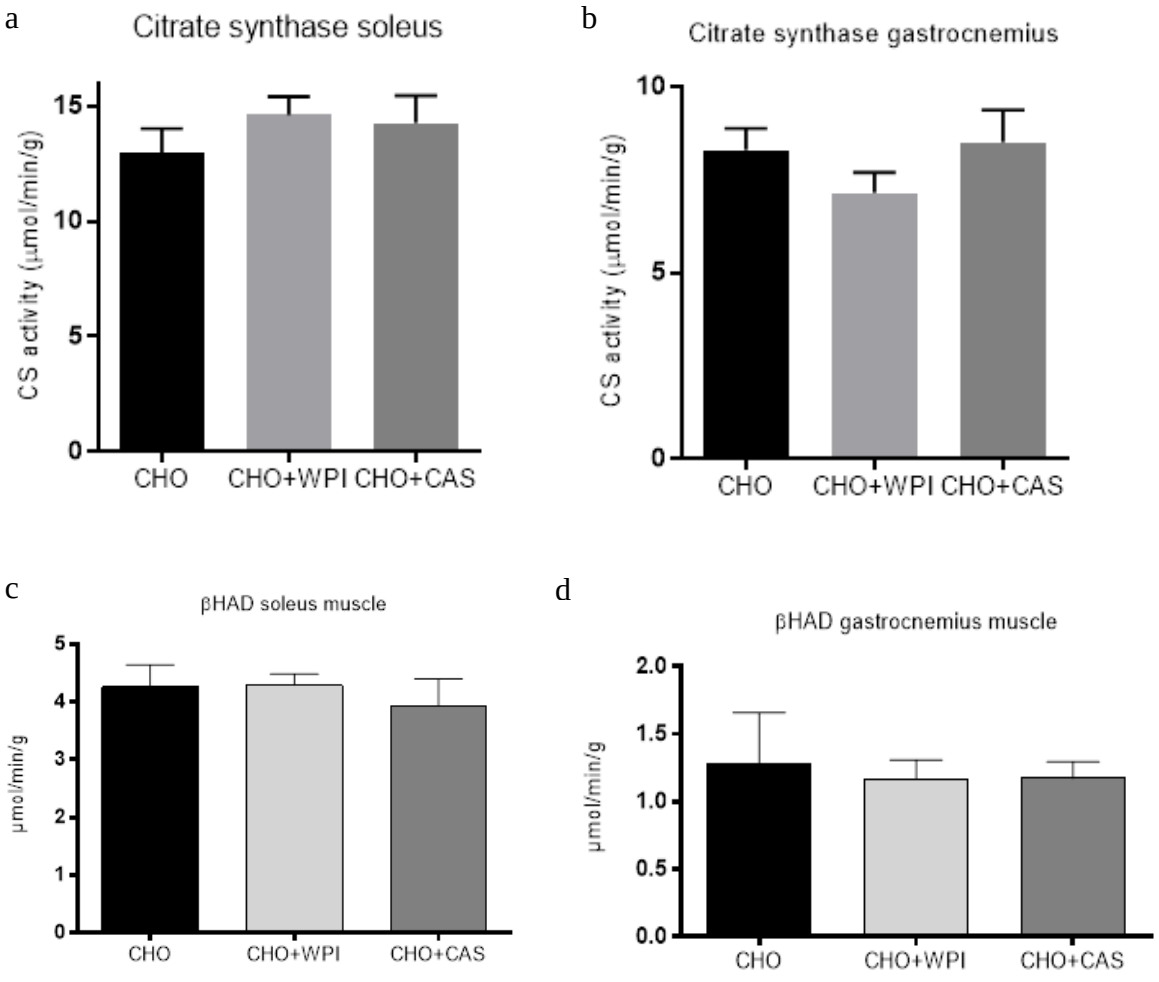


Figure 3.4.2: *Citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (βHAD) are shown in soleus and white gastrocnemius muscles for carbohydrate (CHO), carbohydrate and whey protein isolate (CHP+WPI) and carbohydrate and micellar casein (CHO+CAS)*

after 8 weeks of supplementation. Comparison of CS activity in soleus muscle (Fig 2a) with n = 7 in each group and white gastrocnemius muscle (Fig 2b) with n=8 in CHO+WPI, n=7 in CHO and n = 6 CHO+CAS. Comparison of βHAD activity in soleus muscle (Fig 2c) with n =8 CHO+WPI and CHO+CAS and n = 6 CHO and white gastrocnemius muscle (Fig 2d) with n= 8 CHO+WPI and CHO+CAS and n = 6 CHO. Values are mean ± SEM.

After 8 weeks of supplementation with either CHO, CHO+WPI, or CHO+CAS, both CS and βHAD activity was not significantly different between groups in either the soleus or white gastrocnemius muscles.

3.4.3 Mitochondrial respiration

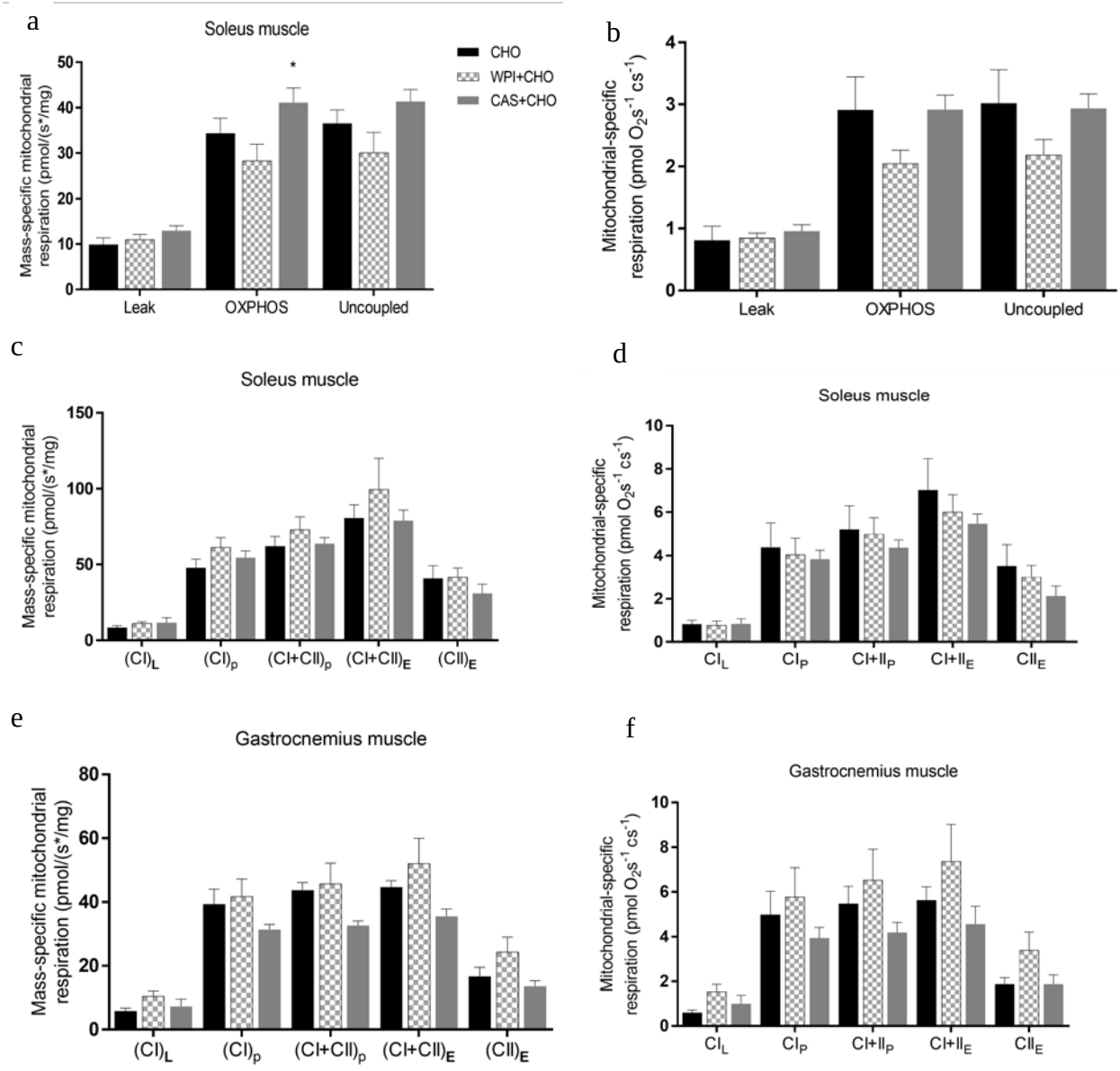


Figure 3.4.3: *Mitochondrial respiration after 8 weeks of dietary intervention; carbohydrate (CHO), carbohydrate and whey protein isolate (CHO+WPI) or carbohydrate and micellar casein (CHO+CAS). a, c and e are mass-specific mitochondrial respiration (pmol O2 s^{-1} mg^{-1}) and b, d and f are mitochondrial specific respiration (obtained by normalising mass-specific mitochondrial respiration values by citrate synthase activity expressed per g of tissue) in both soleus and white gastrocnemius muscle. A fatty acid protocol was used in soleus muscle (a and b) and glycolytic protocol in soleus and white gastrocnemius (c, d, e and f). Respiration values are: CI_L: Leak respiration state (L) in the absence of adenylates and limitation of flux by electron input through Complex I (CI); CI_P: maximal oxidative phosphorylation state (P) with saturating levels of ADP and limitation of flux by electron input through CI; $CI+II_P$: P with saturating levels of ADP and limitation of flux by convergent electron input through CI + Complex II (CII); $CI+II_E$: maximal electron transport system (ETS) capacity (E) with saturating levels of ADP and limitation of flux by convergent electron input through CI+II; CII_E: E with saturating levels of ADP and limitation of flux by electron input through CII. *Significantly different ($P < 0.05$) vs. CHO+WPI (a). Fig 3a n = 8 in each group, Fig 3b n = 7 in each group, Fig 3c n = 6 in each group, Fig 3d n = 5 CHO, n = 6 in CHO+WPI and CHO+CAS, Fig 3e n = 6 in each group and Fig 3d n = 6 CHO and CHO+WPI and n = 5 CHO+CAS. Values are mean ± SEM.*

The CHO+CAS group had significantly higher oxidative phosphorylation (OXPHOS) compared to the CHO+WPI group. When normalised to citrate synthase (activity expressed per gram of tissue) no differences were detected. There were also no differences between groups for mass-specific or mitochondrial-specific (normalised to CS activity) respiration in the soleus muscle. Once normalised to CS activity no differences between groups existed. There was no effect of the addition of cytochrome *c* as a control for outer mitochondrial membrane integrity.

3.5 Discussion

Body mass and mitochondrial function have important health and athletic implications therefore, investigating how different types of proteins affect body composition and mitochondrial function in healthy animals may help in providing a better framework for these outcomes in humans. The present study showed that 8-weeks of supplementing with whey protein isolates or micellar caseins improved body composition by lowering fat mass, in Sprague Dawley rats compared to a carbohydrate matched control group. Furthermore, The CHO+CAS group had significantly higher OXPHOS compared to the CHO+WPI group in mass-specific mitochondrial respiration in the soleus muscle.

The CHO group had a significantly higher total energy intake over 8-weeks of the intervention, compared to CHO+WPI and CHO+CAS groups. This is in accordance with the findings of other studies completed in humans that have demonstrated protein to be more satiating than fat and carbohydrate (Astrup 2005, Johnstone, Horgan et al. 2008). Furthermore, CHO+CAS had significantly lower total energy intake than CHO+WPI group. Currently, no clear evidence exists that indicates one protein source, when comparing whey and caseins, is more satiating than another (Bendtsen, Lorenzen et al. 2013).

Despite statistical significant differences in total energy intake, the body mass of rats in the current study were not significantly different. The current study didn't show a difference in body weight at the end of the 8-week intervention however, body fat was significantly lower in the CHO+CAS and CHO+WPI groups compared to CHO. The studies comparing the effects of different protein sources on body weight are inconclusive (Gilbert, Bendsen et al. 2011). However, Nakazato et al. (2008), demonstrated a higher protein group (35% of total energy), using casein supplementation, to have lower body weight compared to the lower protein consumption (15% total energy) group. The lower protein intake (15%) in the study by Nakazato et al. (2008) was lower than the CHO control group (22% of total energy intake) in the current study. Higher protein diets may increase diet induced thermogenesis contributing to higher energy expenditure and use of fat as an energy source (Johnston, Day et al. 2002, Westerterp 2004). Therefore, total protein content may be more important than protein source when a high-quality protein is used (animal source compared to plant) (Gilbert, Bendsen et al. 2011).

Fat mass was significantly lower in the CHO+WPI and CHO+CAS compared to CHO group after 8 weeks of supplementation. This finding is supported by other studies that have reported high protein diets (50% total energy) to reduce adiposity while maintaining lean

mass in animals (Lacroix, Gaudichon et al. 2004, Pichon, Huneau et al. 2006, Blouet, Ono et al. 2008). However, fat mass reduction occurred in the current study with lower protein content; 30% total energy in the protein supplemented groups. Reductions in fat mass with moderate protein intakes (38% total energy) have been reported in another long term (10 weeks) study by Zhou et al. (2011). Positive changes in body composition seem to occur with moderate intakes of protein therefore higher intakes may not be necessary to see beneficial changes (Zhou, Keenan et al. 2011).

Higher protein diets do not only support reduction in adiposity but are usually associated with increases or maintenance of lean mass (Bendtsen, Lorenzen et al. 2013). Although the current study did not find a change in lean mass between groups, other studies do support this finding. For example, when iso-energetic diets have shown no statistically significant difference between body mass loss, these studies showed an improved body composition (an increase in fat free mass/fat mass ratio) and metabolic profile with a relatively high protein diets (Layman, Boileau et al. 2003, Johnston, Tjonn et al. 2004). The relatively high protein diets all consist of 25-30% of energy from protein, which is like that utilised in the current investigation. Maintaining lean mass is essential for healthy ageing and has been

shown to improve metabolic syndrome conditions and glucose metabolism (Phillips, Chevalier et al. 2016).

All three groups in the current study increased lean mass from 4weeks to 8weeks without a difference between groups. Insulin response may play a role in maintaining lean mass. Although not measured in this study, I have shown in a previous study in humans (Hill, Stathis et al. 2013) the insulinotrophic effect of whey protein isolates. Bos et al. (2003) found a very similar hormonal response between milk and soy protein and Van Loon et al. (2000) showed a similar insulin response between pea protein and casein. While another study found whey increased insulin concentration more than casein in healthy human subjects (Veldhorst, Nieuwenhuizen et al. 2009).

To my knowledge no other studies have assessed the effect of protein supplementation without exercise on mitochondrial function using high-resolution respirometry. Studies have investigated an acute protein feed on muscle protein synthesis (Paddon-Jones, Westman et al. 2008) or assessed mitochondrial content via citrate synthase or succinate dehydrogenase activity (SDH). Nakazato and Song (2008) observed increased PGC-1α protein content of the gastrocnemius muscle in rats fed on a high protein diet. A high protein (casein) diet (35% of total energy) significantly induced SDH activity and PGC-1α protein content in the

gastrocnemius muscle, supporting a shift to more oxidative properties. However, there was no difference in the soleus muscle (Nakazato and Song 2008). The current study observed the CHO+CAS supplemented group to have a significant increase in oxidative phosphorylation compared to the CHO+WPI group, when a fatty acid oxidation protocol was used in soleus muscle. Indicating, casein may have a greater effect on mitochondria than WPI. Although, specific increases in OXPHOS flux were not apparent after normalization to CS, indicating that gross changes mainly resulted from increased mitochondrial mass (Vincent et al. 2015).

In conclusion, although there were no differences in lean mass between groups, supplementing with whey protein isolates or micellar caseins for 8 weeks had positive effects on body composition by reducing fat mass compared to a CHO control group. Furthermore, micellar caseins improved oxidative phosphorylation compared to whey protein isolates in soleus muscle when investigating fatty acid oxidation through high resolution respirometry. Whether these changes are enhanced with endurance exercise training is unclear.

Chapter 4: The effects of 8 weeks of treadmill running plus protein supplementation on body composition and mitochondrial respiration in rats.

4.1 Abstract

4.1.1 Background

The quality and quantity of nutritional intake harnesses many benefits for athletic populations. Nutritional strategies implemented prior to and during exercise can profoundly alter exercise performance and prolong endurance capacity. Further, effective post-exercise nutritional strategies can significantly alter the adaptive response to exercise and potentially improve performance. Few studies have examined the influence of long term post-exercise protein ingestion on mitochondrial function and endurance performance.

4.1.2 Methods and materials

Sprague Dawley rats were randomly assigned to one of the following three experimental groups for 8-weeks; carbohydrate, carbohydrate plus whey protein isolate, or carbohydrate plus micellar casein, as per chapter 3. All animals were familiarized with a motor-driven treadmill and performed an incremental test to exhaustion (TTE) that determined running speed for the training program. The program consisted of 5 sessions per week for 7 weeks,

with a TTE completed 72 hours after the last training session. Body mass and food consumption was weighed and recorded daily and body composition was determined at 4 and 8-weeks via an Echo MRI^{TM} system. Mitochondrial respiration was assessed in soleus and white portion of the gastrocnemius muscle.

4.1.3 Results, discussion and conclusion

This study demonstrated that despite matching all rodents across groups according to exercise performance prior to supplementation, following the 8-week supplementation period the carbohydrate supplemented animals were exhausted quicker in the time to exhaustion test compared to both micellar caseins and whey protein isolates groups ($P < 0.05$); 14:19 ± 4 min, 29:81 ± 11 min and 25:51 ± 6 min. The CHO+WPI group had a greater increase in lean mass from 4 to 8-weeks compared to CHO group. There was no significant difference between protein groups in several measures including; enzyme activity of citrate synthase and β-hydroxyacyl-CoA dehydrogenase (βHAD), mitochondrial respiration or lean mass.

4.2 Introduction

Endurance training stimulates positive adaptations throughout the entire cardiovascular system, making it an excellent intervention for improving health and performance and for preventing and treating various diseases, particularly obesity and type 2 diabetes (Bonen, Dohm et al. 2006, Thomas, Elliott et al. 2006). The response is achieved primarily by application of an appropriate training stimulus, but can be modulated by the nutrient, metabolic and hormonal environment (Maughan 2003). Food intake before, during and/or after training can therefore modify the adaptations seen with endurance training. As shown in chapter 3, 8 weeks of supplementing with whey protein isolates or micellar caseins decreased body fat in Sprague Dawley rats compared to carbohydrate matched control group. In addition, the micellar caseins group showed a significant increase in oxidative phosphorylation compared to whey protein isolates when investigating fatty acid substrate entry in rat soleus muscle. This chapter will investigate the same dietary approach with the addition of an endurance training program.

Endurance training elicits a variety of metabolic and morphological changes, including mitochondrial biogenesis, fast to slow muscle fibre type transformation and alterations in substrate metabolism, along with improvements in body composition (Smiles, Hawley et al.

2016). It is well accepted that protein supplementation and the timing of supplementation is critical for resistance training adaptations (Tang, Moore et al. 2009). Recent research is also emerging that post-exercise protein supplementation is just as important for endurance athletes (Rodriguez 2009). However, endurance athletes do not generally prioritise post-exercise protein consumption due to beliefs it will increase muscle mass (Ferguson-Stegall, McCleave et al. 2011). To add to the uncertainty for endurance athletes the types of protein that have the best effect when included as part of a long-term training regime is also unclear.

Milk proteins are attractive sports supplements to aid muscle growth and repair for resistance based exercise (Areta, Burke et al. 2013) and endurance based exercise (Howarth, Moreau et al. 2009). Casein and whey are both complete proteins containing all essential amino acids, but they differ in the way they are digested and absorbed (Have, Engelen et al. 2007). Micellar caseins coagulates in the acidic environment of the stomach, which delays gastric emptying and thus slows the postprandial increase in plasma amino acids, giving micellar casein the name ‘slow’ protein (Boirie, Dangin et al. 1997). Whey proteins do not coagulate like micellar caseins in the stomach and thus induce a fast, high and transient increase in plasma amino acids and as a result are called ‘fast’ proteins (Boirie, Dangin et al. 1997).

Milk proteins provide a potent anabolic stimulus due to their amino acid composition and insulinotropic effects, although whether whey protein or casein have greater differential effects on muscle mass and/or function is not yet well understood (McGregor and Poppitt 2013). An earlier study by Boirie et al. (1997) found that postprandial protein synthesis was increased by 68% after ingestion of whey protein but only 31% after ingestion of casein. In the same study casein also inhibited whole body protein breakdown and so despite the more rapid appearance of whey derived amino acids, net protein balance was higher following casein ingestion (Boirie, Dangin et al. 1997). Despite casein ingestion resulting in a higher net protein balance, no study to my knowledge, has investigated if inclusion of micellar casein results in improvements in endurance adaptations compared to whey protein isolates or carbohydrate diet.

Endurance training has been shown to improve substrate utilization in favour of a switch to using fatty acids as a fuel source to spare muscle glycogen and prolong muscular fatigue (Myburgh 2003). Pesta et al. (2011) showed oxidative OXPHOS capacity increased two-fold in human muscle following 10 weeks of endurance training, using substrate combination of octanolycartine and malate. To my knowledge, no study has looked at the

addition of protein post-exercise over a longer period to determine its effect of fatty acid oxidation.

As casein and whey differ in their effect on protein balance, it is unknown how each protein effects body composition changes, which is an important consideration for endurance athletes. Proteins are found to induce greater weight and fat mass loss than carbohydrates (Krieger, Sitren et al. 2006). These results are often observed when energy is restricted or in obese animal models, however how casein or whey protein supplementation effects body composition when consumed in conjunction with undertaking an endurance training program is unclear.

Aims

The aim of the present study was to measure the effect of feeding different proteins (micellar caseins and whey protein isolates) during an endurance training program on endurance performance in an animal model. In addition, this study aimed to measure the effect of feeding different proteins (micellar caseins and whey protein isolates) during an endurance training program on mitochondrial function and body composition in an animal model.

It is hypothesised that both protein groups will have a greater effect on performance.

4.3 Methods

4.3.1 Experimental groups

Twenty-four male Sprague Dawley rats were individually housed in an environmentally controlled laboratory (ambient temperature 22-24°C) with a 12-hour light/dark cycle (7:00 - 19:00). Animals were obtained at 7 weeks (~150 g) and acclimatised for 5 days before being separated out into individual cages. Experimental procedures were approved by the Howard Florey Animal Ethics Committee (AEC 11-046).

Animals were randomly assigned to one of the following three experimental groups and underwent an endurance training program for 8 weeks; carbohydrate (CHO), carbohydrate plus whey protein isolate (CHO+WPI), or carbohydrate plus micellar casein (CHO+CAS). Body weight and food consumption was weighed and recorded daily and body composition was determined at 4 weeks and 8 weeks via Echo MRI^{TM} system (Echo-MRITM 900, Houston, TX, United States of America) according to manufactures instructions and as previously validated (Taicher, Tinsley et al. 2003).

4.3.2 Exercise training

All animals were familiarized with a motor-driven treadmill (Columbus Instruments, Columbus, OH, USA) for 3-4 days, 5 min/day, on a 10-degree incline. The running speed was initially 15 m/min and was gradually increased to 25 m/min within 2 days. Following the familiarization, all animals performed an incremental test to exhaustion (TTE) on the treadmill inclined to 15°, starting at 10 m/min with increments of 3 m/min every 2 min (Henderson, Wagner et al. 2002). Exhaustion was determined when the rat stopped running and had to be replaced onto the treadmill 3 successive times despite encouragement using a tail brush, tail pinching or compressed air on the tail (Betik, Baker et al. 2008, Betik, Aguila et al. 2016).

The training program was adapted from Bishop et al. (2010) and consisted of seven (1st wk) to 14 (7th wk) 2-min intervals (interspersed with 1 min of rest) performed five times/wk for 7 wk. The intensity of the intervals was initially set at 80% of the peak speed reached for each rat during the incremental test and was increased by 5% each week. Rats were matched, based on the peak speed reached during the incremental test, with a rat from alternate training group, and rats trained concurrently on the treadmill during each training session to ensure that all pairs performed identical amounts of training. Only two rats could be trained

together at one time, therefore rats (matched on peak speed) alternated training partners. In the 8th wks., at least 72 h after the last training session, rats performed a TTE test at the pre-training maximal velocity reached during the incremental test.

4.3.3 Diets

The dietary intervention used in this study is the same as that detailed in Chapter 3 of this thesis. In short, rats were fed standard chow from Specialty feeds- Soya free rat and mouse diet, ad libitum. The three experimental diets were the control diet which consisted of standard chow (65% carbohydrate, 22% protein & 13% fat total energy), standard chow + whey protein isolate (65% carbohydrate, 30% protein & 5% fat total energy) or standard chow + micellar casein (65% carbohydrate, 30% protein & 5% fat total energy). Rats were supplemented with one of CHO+WPI, CHO+CAS or CHO. These supplements were isocaloric and matched for carbohydrate content and mixed into a paste with water and fed in a petri dish. Supplements were given directly post-training session on training days to ensure optimal timing. On non-training days' supplements were given after food and body weight records were taken. Total energy intake was measured to include standard chow plus the supplements for the 8-week dietary intervention.

4.3.4 Enzyme assays

Frozen muscle was pulverised in liquid nitrogen, weighed, and homogenised and the activity of citrate synthase (CS) and beta-hydroxyacyl-CoA (βHAD) was measured adapting previously described methods (Srere 1969, Czok and Bergmeyer 1974) for use in a plate reader.

4.3.5 Mitochondrial respiration

On completion of endurance performance test rats were then deeply anaesthetised with sodium pentabarbitone (100 mg/kg; Virbac, Peakhurst, Australia), soleus and gastrocnemius muscles were removed and then rats euthanised via cardiac puncture. One portion (10-20 mg) of each muscle was immediately immersed in ~3 mL of ice-cold BIOPS for in-situ measurements of mitochondrial respiration. Muscle fibres were separated and permeabilised as previously described by Pesta and Gnaiger (2012). Mitochondrial respiration was measured in duplicate (from 3–4 mg wet weight of muscle fibers) in MiR05 at 37 °C by using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria). Mitochondrial respiration measurements were taken using two different substrate uncoupler-inhibitor tritration (SUIT) protocols to assess a glycolytic protocol in both soleus

and gastrocnemius and a fatty acid protocol in the soleus muscle. These were conducted according to previously described methods (Pesta and Gnaiger 2012).

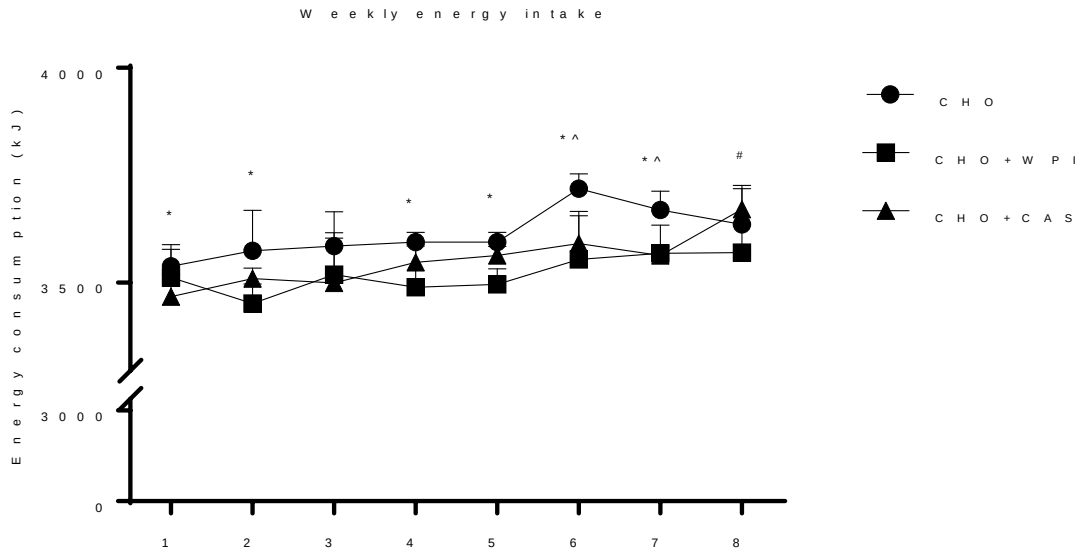
4.3.6 Statistics

All data are expressed as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was performed to assess total accumulated weekly food and energy intake, muscle enzyme activities, mitochondrial respiration and exercise performance data.. Changes in body composition were evaluated by using a repeated-measures ANOVA design. Tukeys post hoc test was utilised and differences were considered statistically significant if the P value was less than 0.05. All statistical calculations were conducted using GraphPad Prism 7 software (GraphPad Software Inc, La Jolla, CA, USA).

4.4 Results

4.4.1 Energy intake and body composition

a



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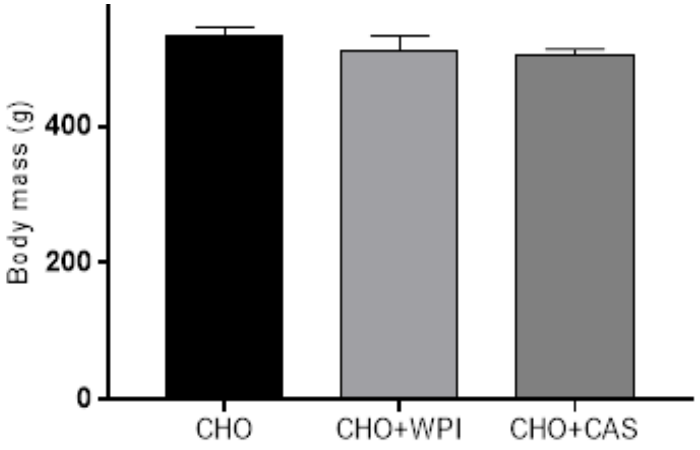

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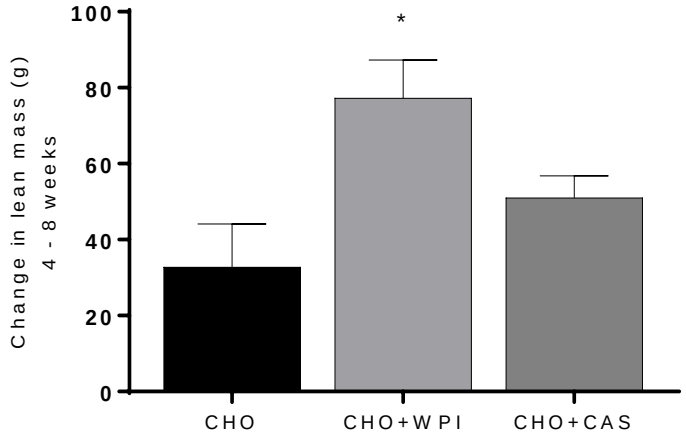

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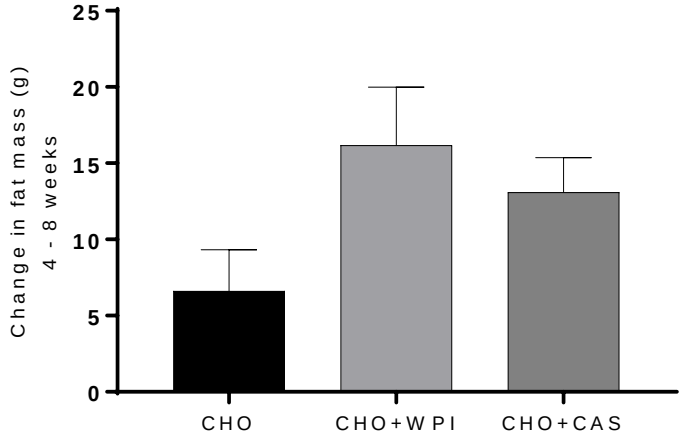


Figure 4.4.1: *Weekly energy intake and body composition for the carbohydrate (CHO), carbohydrate and whey protein isolate (CHO+WPI) and carbohydrate and micellar casein*

*(CHO+CAS) supplemented groups during the 8-week diet and exercise intervention. Mean weekly energy intake (a) and body mass (b) of animals in each group. The change in lean mass (c) and fat mass (d) from 4 to 8 weeks. *Significantly different (P < 0.05) vs. CHO, ^ CAS+CHO Significantly different (P < 0.05) vs. CHO, # Significantly different (P < 0.05) vs. CHO+WPI. n = 8 in each group. Values are mean ± SEM.*

The weekly mean energy intake is shown in Figure 4.1a. CHO group had significantly higher energy intake then CHO+CAS in week 1, week 2 CHO group was significantly higher than CHO+WPI and again in week 4, week 5, 6 and 7. CHO group was significantly higher than both protein groups and in week 8 and CHO+CAS group was significantly higher than CHO+WPI (P<0.05; Figure 4.1a). Body mass was recorded daily and total body mass was not significantly altered between groups (Fig 4.1b); 533 ± 34 g, 511 ± 61 g and 504 ± 22g for CHO, CHO+WPI and CHO+CAS respectively.. However, in terms of body composition the CHO+WPI supplemented rats had a greater increase in lean mass from 4 to 8 weeks compared to CHO supplemented rats.

4.4.2 Time trial to exhaustion

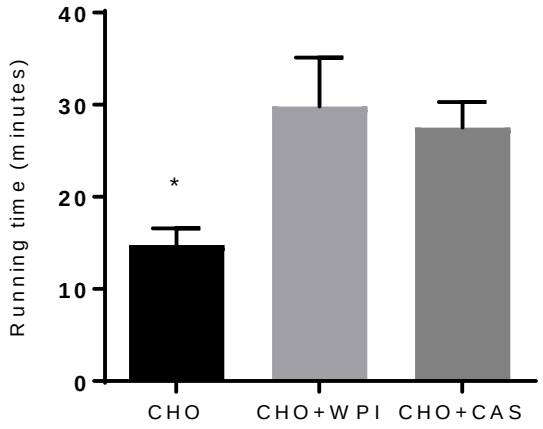


Figure 4.4.2: *Maximal running time completed during time trial to exhaustion (TTE) completed at the end of 8 weeks of supplementation with either carbohydrate (CHO), carbohydrate and whey protein isolate (CHO+WPI) or carbohydrate and micellar casein (CHO+CAS) along with endurance training program. *Significantly different (P < 0.05) vs. CHO+WPI and CHO+CAS. n = 6 in CHO and n = 5 in CHO+WPI and CHO+CAS. Values are mean ± SEM.*

Rats completed the time trial to exhaustion 72 hours after the last training session. The CHO supplemented group was unable to perform for as long as the CHO+WPI and CHO+CAS supplemented groups ($P < 0.05$); 14:19 ± 4 min, 29:81 ± 11 min and 25:51 ± 6 min. The

number of rats was reduced in each of the groups because of rats refusing to run to exhaustion despite encouragement or toenails breaking and the feet bleeding before the rat reached exhaustion.

4.4.3 Enzyme assays

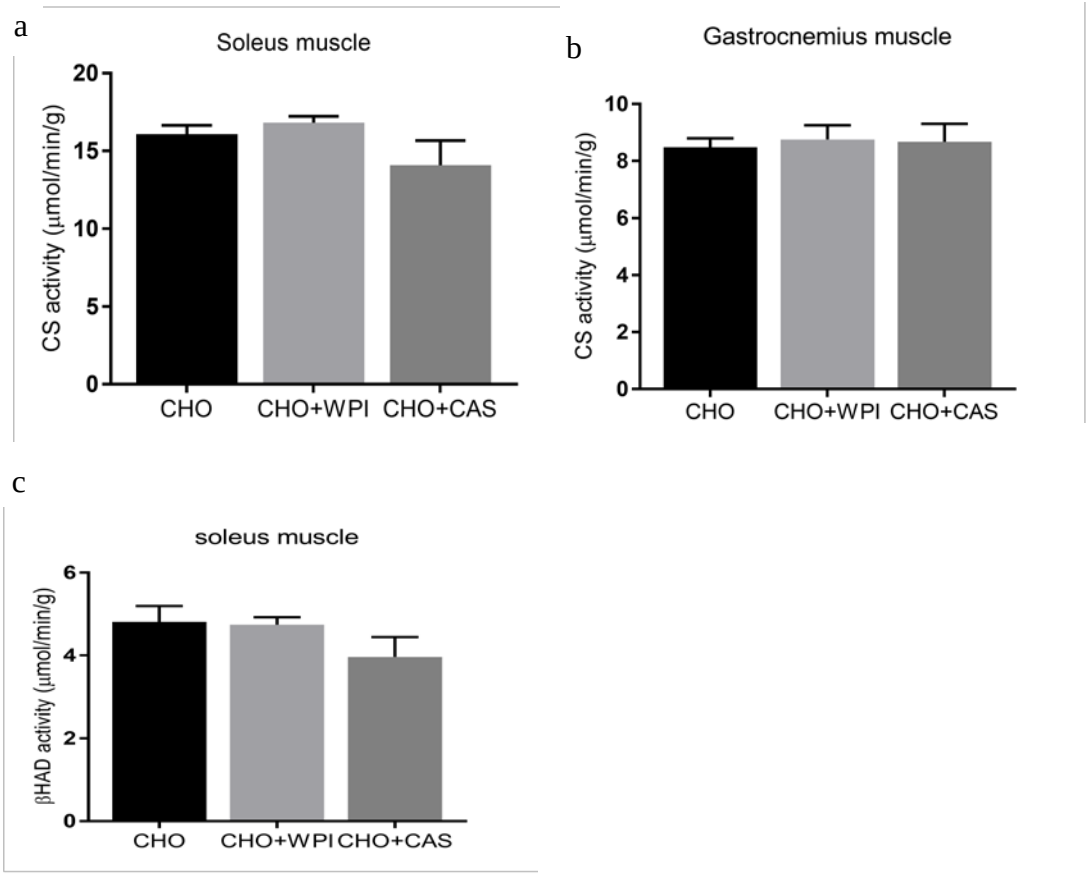


Figure 4.4.3: *Citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (βHAD) activity are shown in soleus and white gastrocnemius muscles for carbohydrate (CHO), carbohydrate and whey protein isolate (CHO+WPI) and carbohydrate and micellar casein*

(CHO+CAS) after 8 weeks of diet and exercise training. Comparison of CS activity in soleus muscle (Fig 4.3a) with n=8 in CHO+WPI and CHO+CAS and n = 7 in CHO. White gastrocnemius muscle (Fig 4.3b) with n=7 in CHO+WPI and CHO+CAS and n=7 in CHO. Comparison of βHAD activity in soleus muscle (Fig 4.3c) with n =7 CHO+WPI and n = 6 CHO and CHO+CAS. Values are mean ± SEM.

Enzyme activity of citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (βHAD) measured in soleus and white gastrocnemius muscle are shown in Figure 4.3a-c. After 8 weeks of supplementation with either CHO, CHO+WPI or CHO+CAS along with endurance exercise training, CS or βHAD activity wasn't significantly altered between groups in either soleus or white gastrocnemius muscles.

4.4.4 Mitochondrial respiration

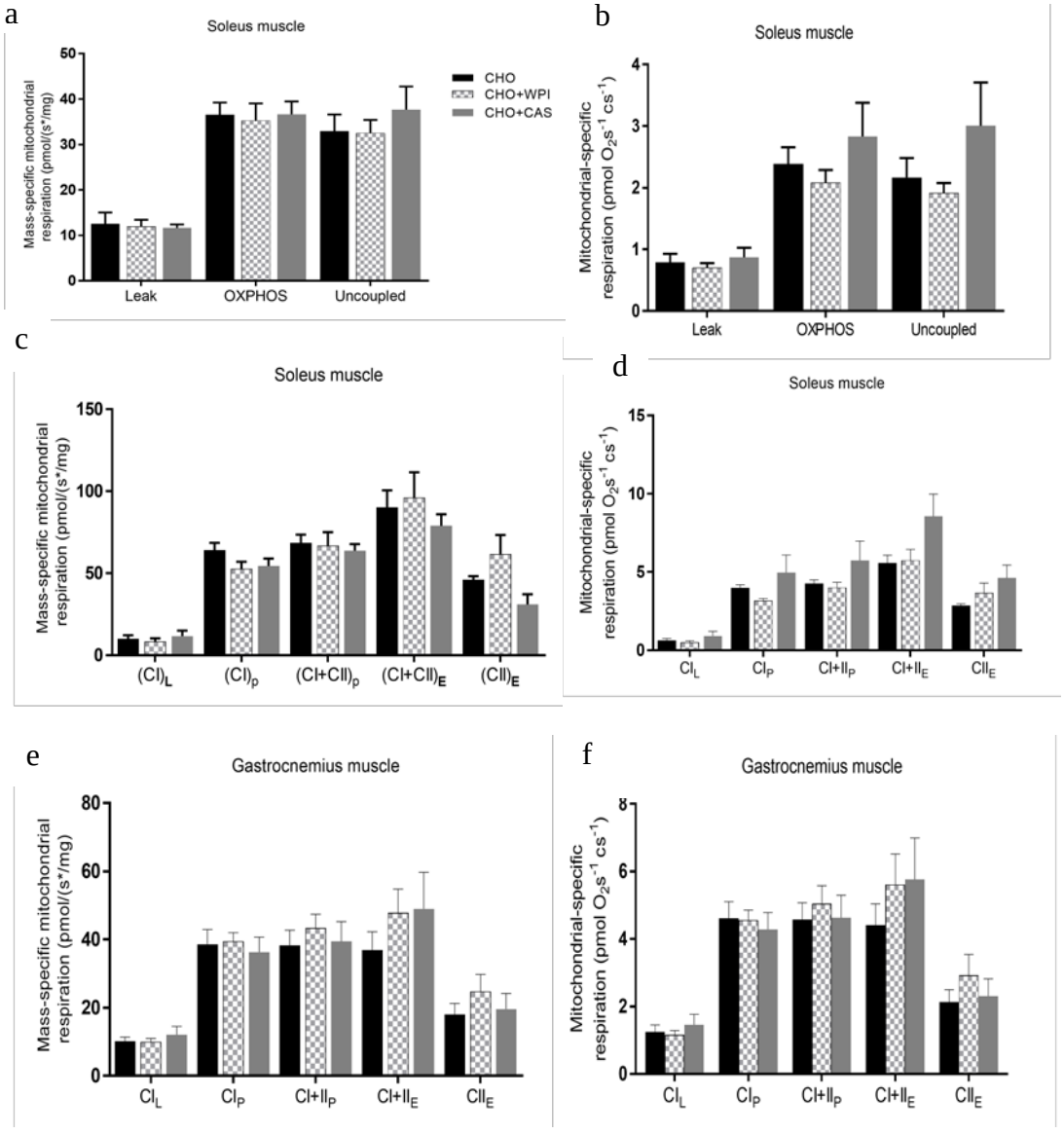


Figure 4.4.4: *Mitochondrial respiration after 8 weeks of diet and exercise intervention; carbohydrate (CHO), carbohydrate and whey protein isolate (CHO+WPI) or carbohydrate and micellar casein (CHO+CAS). a, c and e are mass-specific mitochondrial respiration (pmol O2 s^{-1} mg^{-1}) and b, d and f are mitochondrial specific respiration (obtained by normalising mass-specific mitochondrial respiration values by citrate synthase activity expressed per gram of tissue) in both soleus and white gastrocnemius muscle. A fatty acid protocol was used in soleus muscle (a and b) and glycolytic protocol in soleus and white gastrocnemius (c, d, e and f). Respiration values are: CI_L: Leak respiration state (L) in the absence of adenylates and limitation of flux by electron input through Complex I (CI); CI_P: maximal oxidative phosphorylation state (P) with saturating levels of ADP and limitation of flux by electron input through CI; $CI+II_P$: P with saturating levels of ADP and limitation of flux by convergent electron input through CI + Complex II (CII); $CI+II_E$: maximal electron transport system (ETS) capacity (E) with saturating levels of ADP and limitation of flux by convergent electron input through CI+II; CII_E: E with saturating levels of ADP and limitation of flux by electron input through CII. Fig 4.4a and b n = 7 in each group, Fig 4.3c and d n = 7 in CHO and CHO+CAS and n = 4 CHO+WPI, Fig 4.3e and f n = 7 in each group. Values are mean ± SEM.*

Mass-specific mitochondrial respiration (expressed as pmol O_2 s^{-1} mg^{-1}) results are shown in Figures 4.4a, c and e. A fatty acid protocol was carried out in rat soleus muscle (Figures 4.4a and b) after 8 weeks of endurance exercise training and supplementation with CHO, CHO+WPI or CHO+CAS. This protocol was completed in soleus muscle only due to the oxidative capacity of this muscle type and due to equipment availability, which meant too long a delay for the gastrocnemius muscle protocol to be tested. There was no difference between supplemented groups before or after normalising to citrate synthase (activity expressed per gram of tissue). A glycolytic protocol was run in the soleus and white gastrocnemius muscle. There were no differences between the supplemented group with either mass-specific or mitochondrial-specific respiration in white gastrocnemius muscle. The data presented in this study showed no effect of the addition of cytochrome *c* as a control for outer mitochondrial membrane integrity.

4.5 Discussion

This study determined the effects of 8 weeks of post-exercise supplementation with either whey protein isolates or micellar caseins compared to an isocaloric, carbohydrate matched carbohydrate group on time to exhaustion, body composition, and mitochondrial function in Sprague Dawley rats. The CHO+WPI group showed a greater increase in lean mass from 4 to 8 weeks compared to CHO group, despite energy consumption being significantly lower, in CHO+WPI and CHO+CAS compared to CHO group. Despite the higher energy intake in the CHO group during the 8 weeks of supplementation, this group was also unable to perform as long in the time trial to exhaustion compare to CHO+WPI and CHO+CAS group.

A key finding in the current study was that despite matching all rodents across groups according to exercise performance and then these animals completing identical training programs (all matched animals completed training sessions together, and thus training load was identical) the CHO supplemented group was exhausted quicker than CHO+WPI and CHO+CAS in the time trial to exhaustion (TTE). There was however no difference in TTE between protein groups. This indicates that the supplementation with either of these dairy proteins had a superior effect on endurance performance following endurance training compared to carbohydrate supplementation alone. Studies investigating performance results

for carbohydrate and protein co-ingestion post-exercise remain equivocal, with the clear majority of studies examining the acute effects of post-exercise recovery on exercise performance. Recent studies comparing different protein types on endurance performance have observed no difference in 20-km cycling time trial performance when supplementing with carbohydrate plus whey protein hydrolysate, carbohydrate plus casein hydrolysate or isoenergetic carbohydrate drink consumed before a 20-km cycling time trial (Cepero González, Padial et al. 2010). Casein hydrolysate digests much like whey hydrolysate in terms of digestion time, with amino acid composition being the main the difference (Lacroix, Bos et al. 2006). This contrasts with the current study that found regardless of the type of protein consumed, time trial to exhaustion was extended compared to CHO. The difference in timing of ingestion may play a role in the performance benefit seen in the current study compared to Cepero et al. (2010).

Niles, Lachowetz et al. (2001) found an ergogenic effect on subsequent run times-to-exhaustion with an isocaloric carbohydrate and milk and whey protein isolate combination drink compared with carbohydrate alone when it was provided acutely during 2 hour of recovery (Niles, Lachowetz et al. 2001). Plasma insulin levels were also higher during the recovery period with the carbohydrate and milk and whey protein isolate combination drink,

which may be a factor for greater rates of glycogen repletion (Niles, Lachowetz et al. 2001), which has been shown in other studies (Hill, Stathis et al. 2013). Furthermore, Saunders, Kane et al. (2004) found cyclist performed 40% longer following the consumption of a carbohydrate-protein beverage (Saunders, Kane et al. 2004). However, it should be noted the beverages were not isocaloric (CHO-PRO 581 kcals; CHO 391 kcals) so it is hard to comment if the improvement in time to exhaustion was a result of the added protein or the extra energy. Practically it would be more beneficial to examine the effects of these nutritional strategies over a longer duration similar to a training regime, as utilized in the current study. In support of the current study, it has been found that endurance training and a diet with whey protein hydrolysate for 4 weeks in mice increased time to exhaustion during running (Aoi, Takanami et al. 2011).

The increase in time to fatigue in CHO+WPI and CHO+CAS supplemented groups compared to CHO was not associated with improvements in mitochondrial respiration or citrate synthase content; a measure of mitochondrial function and content respectively. To my knowledge, this is the first study to compare WPI and micellar caseins to a CHO content matched control group over a longer (8-weeks) period on mitochondrial function using high

resolution respirometry. However, in contrast to the current study, improvements in citrate synthase and succinate dehydrogenase have been observed following 31 days of cycle training in healthy male volunteers without dietary control (Phillips, Green et al. 1996).

Mitochondrial respiration has previously been shown to increase with endurance training alone (Holloszy 1974). However, the current study showed no training effect, when compared to the mitochondrial respiration data obtained following 8 weeks of feeding (Refer to chapter 3 Figure 3.3 for these results). The training protocol was adapted from Bishop et al. (2010) and these animals showed a large increase in oxidative capacity in both slow (soleus) and fast-twitch (EDL) muscles over 5 weeks with a 10% increase in interval speed each week. In the current study, the lack of increase in oxidative capacity in either muscle type may be due to the 5% increase in speed of intervals each week over 7 weeks which may have been insufficient to bring about training adaptation in mitochondrial respiration. The current study was also not able to use electric shock on the motorised treadmill and this may have contributed to a lack of training intensity in some sessions and making it harder to keep rats running. However, despite these limitations and lack of change in mitochondrial respiration, TTE was significantly greater in the CHO+WPI and CHO+CAS groups compared to the CHO group, indicating that there were alterations in

metabolic or other adaptations between groups with training, but just not in the measurements undertaken. An alternative mechanism not measured in this study that may have resulted in improved TTE, may have been improved buffering capacity of the muscle or mental functioning of the rats. Some research indicates BCAA's play an important role in central fatigue.

In trained humans, one of the most consistent adaptations is an increase in muscle mitochondria, which is reflected in elevations in the maximal activities of representative enzymes of the tricarboxylic cycle (TCA) and β-oxidative pathway (Holloszy and Coyle 1984, Green, Jones et al. 1991). The current study in rats did not result in any difference between protein and carbohydrate supplemented groups when a mitochondrial respiration protocol examined fatty acid oxidation through specific substrate protocols. A study by Mitsuishi et al. (2013) examined the long-term effects of a high-protein diet on changes in muscle properties and physical performance, focusing especially on muscle mitochondria. Mice were assigned to protein-adjusted diets with various ratios of protein (10, 20, 30, and 50%) and fat (10 and 45%) from the age of 8 weeks, with measurements taken at 20 and 50 weeks. The results of this study by Mitsuishi et al. (2013) showed that high protein and high fat/high protein increased muscle mass and muscle strength however, they suppressed

muscle mitochondrial activity and decreased acute endurance performance (Mitsuishi, Miyashita et al. 2013). The current study, observed greater time to exhaustion with both protein groups compared to CHO and no difference in mitochondrial function or enzyme activity.

The CHO+WPI group showed a significant increase in the lean mass from 4 weeks to 8 weeks compared to CHO supplemented group but no difference to the CHO+CAS group. This result is in line with a study done in collegiate female athletes that found no difference between whey protein and casein protein supplemented pre and post training sessions for 8 weeks (Wilborn, Taylor et al. 2013). Although this study is not very specific on the exact protein composition of the whole diet as only casein and whey protein is mentioned. Body weight of animals fed a mixture of soy and whey protein isolate were lower than trained animals fed each protein diet separately (Elia, Stadler et al. 2006). This contrasts with the current study which found no difference in body weight at the end of 8 weeks of supplementing CHO+WPI, CHO+CAS or CHO with endurance training, despite small albeit significant differences in total energy consumption over the 8 weeks. However, CHO+WPI group had a greater increase in lean mass from 4 to 8 weeks compared to CHO.

In summary, the present study revealed that long term carbohydrate and protein co-ingestion post-exercise, extended time to exhaustion in Sprague Dawley rats compared to carbohydrate supplementation alone. There is no clear mechanism by which the extended TTE observed in the present study after consumption of either proteins can be explained. In addition, lean mass accretion from 4 to 8 weeks was greater in CHO+WPI group compared to the CHO. This improvement in performance was not associated with improvements in mitochondrial function, thus it appears that this improved time to exhaustion is not related to improved mitochondrial function, but by some other, yet to be determined factor.

Chapter 5: Effects of post – training supplementation with different proteins on muscle and performance adaptations in trained male cyclists

5.1 Abstract

5.1.1 Background

Post-exercise nutrition is critical to facilitate endurance exercise adaptations and performance. The beneficial effects of post exercise nutrition, following an acute bout of exercise has been the focus of many investigations. Few studies however, have investigated long term post-exercise nutrition, in the form of carbohydrate and protein on potential endurance adaptations in skeletal muscle, such as mitochondrial function, content and proteins involved in mitochondria biogenesis.

5.1.2 Materials and methods

Trained, male cyclists were recruited from local cycling clubs and randomly assigned in a double-blind manner to one of two dietary groups; carbohydrate and whey protein isolates or carbohydrate and calcium caseinates. Participants completed 2 training sessions per week at Victoria University, in addition to their own training and these included one sprint interval

session and one longer interval session. Participants were provided all meals, snacks and supplements for the 8-week duration of the study. Baseline and post-exercise testing followed the dietary and exercise intervention consisted of a DXA scan to assess body composition, graded exercise test (GXT) for determination of lactate threshold and $VO_{2\ peak}$ and 2-hour steady state cycle followed by 20-km time trial as a measurement of endurance performance. Blood and muscle samples were taken pre and post the exercise training and nutrition intervention.

5.1.3 Results, discussion and conclusion

This study demonstrated that when comparing two high quality proteins post-exercise for 8-weeks, carbohydrate and calcium caseinates increased CS activity however, neither supplement altered mitochondrial function or protein expression. Endurance exercise performance and body composition was comparable between proteins. These results indicate that when two high quality protein are ingested post-exercise for 8-weeks, time-trial performance, body composition and mitochondrial function are similar for trained cyclists.

5.2 Introduction

There is an increasing recognition that nutrition has a key role in promoting endurance adaptations that take place in the muscle and other tissues in response to each training session (Broad and Cox 2008). For example, it is well recognised that carbohydrate supplementation before, during and after prolonged exercise has beneficial effects on physical performance, sustained maximal voluntary muscle contraction, and perceived exertion, mainly because it maintains glycaemia (Nybo 2003, Utter, Kang et al. 2004, Fulco, Kambis et al. 2005). Furthermore, it is well recognised protein supplementation following resistance exercise enhances the effect of exercise by promoting a positive net protein balance in skeletal muscle (Phillips, Tipton et al. 1997, Moore, Robinson et al. 2009). Less is known about the stimulatory effect on specific endurance training adaptations such as mitochondrial function. Chapter 4 demonstrated that long term carbohydrate and protein co-ingestion post-exercise, extended time to exhaustion in Sprague Dawley rats compared to carbohydrate supplementation alone. In addition, lean mass accretion from 4 to 8 weeks was greater in CHO+WPI group compared to the CHO.

Studies investigating post-exercise protein supplementation on endurance training adaptations have established carbohydrate and protein co-ingestion post exercise increases

mixed muscle protein synthesis (Levenhagen, Carr et al. 2002, Howarth, Moreau et al. 2009), increases PGC-1α messenger RNA content and increases plasma insulin (Hill, Stathis et al. 2013) and improves body composition (Longland, Oikawa et al. 2016), compared to carbohydrate alone. However, few studies have investigated the type of protein that is most beneficial to bring about these adaptations. Animal-source proteins, such as milk and the constituent proteins of milk, casein and whey, are classified as being of high biological availability and quality (Schaafsma 2005, Phillips, Tang et al. 2009). Casein and caseinates are used to a lesser extent than whey protein isolates, mostly because of the formulation issues and higher cost structure (Paul 2009). Whey proteins originate from the soluble whey fraction removed primarily during cheese manufacturing, whereas casein comes from the solid fraction of skim milk after exposure to low pH (Paul 2009).

The digestion rates of whey protein isolates and calcium caseinates are very similar (Reitelseder, Agergaard et al. 2011). Few studies have compared whey protein isolates and calcium caseinates effect on endurance training adaptations and body composition. Although, studies using casein often do not define if they use micellar casein or if it is a caseinate, making interpretation difficult. Pennings et al. (2011) investigated if the different digestion rates or amino acid profiles were responsible for the increase in protein accretion

in the post absorptive state in older men when comparing whey protein, casein and casein hydrolysate. The casein hydrolysate mimicked the digestion rate of whey protein isolate, however this study showed whey protein to be superior at increasing protein accretion (Pennings, Boirie et al. 2011).

The beneficial effects of post exercise nutrition, following an acute bout of exercise has been the focus of many investigations (Williams, Raven et al. 2003, Berardi, Price et al. 2006, Ivy, Ding et al. 2008). Increases in rates of myofibrillar but not mitochondrial protein synthesis have been shown when protein was ingested post-exercise following sprint cycling (Coffey, Moore et al. 2011). Few studies however have investigated post exercise nutrition, in the form of carbohydrate and protein on potential endurance adaptations in skeletal muscle, such as mitochondrial function, content and proteins involved in mitochondria biogenesis.

Thus, the aims of this project are to understand the effect post-exercise protein supplementation has on skeletal muscle adaptations associated with endurance training such as mitochondrial respiration and mitochondrial biogenesis, along with body composition

changes following 8 weeks of supplementation in trained individuals. This will be achieved while participants replicate carbohydrate and protein timing and quantities ingested in line with current Australian sports nutrition recommendations, that replicate what an athlete does in ‘real world’ training and racing. It was hypothesised that whey protein isolates would have a greater improvement in performance, body composition and adaptations, compared to calcium casienates.

5.3 Methods

5.3.1 Participants

Participants.

Seventeen healthy, competitive cyclists with at least 2 years of cycling were recruited from local cycling clubs and volunteered to take part in this study

. All participants completed the trial, however if one of the testing procedures was not completed due to illness this is indicated in the figure legend.

Ethical approval. Participants were informed of the study requirements, benefits and risks before giving written informed consent. Approval for the studies procedures, which conformed to the standards set by the latest revision of the Declaration of Helsinki, was granted by the Victoria University Human Research Ethics Committee (HRETH 12-330).

5.3.2 Study design

The experimental protocol (Figure 5.1) began with a familiarisation session, and baseline testing that consisted of, in the following order, a Dual Energy X-ray Absorptiometry (DXA) scan, a graded exercise test (GXT) and $VO_{2\ peak}$ test, a 2-h constant load cycle followed by a 20-km cycling time trial (20k-TT), and a resting muscle biopsy; all tests were separated

by a minimum of 48 h. After initial screening and testing, participants were matched by the power attained at the lactate threshold (WLT) and randomly assigned in a double-blind manner to one of the two groups; carbohydrate and whey protein isolates (CHO+WPI) or carbohydrate and calcium casienates (CHO+CAS) group. Following the baseline testing participants undertook 8 week of training attending two training sessions, in addition to other training sessions, at Victoria University, Footscray Park campus and were provided with all meals, snacks and supplements. After completion of the 8 weeks of training all baseline tests were repeated seventy-two hours following the last training session.

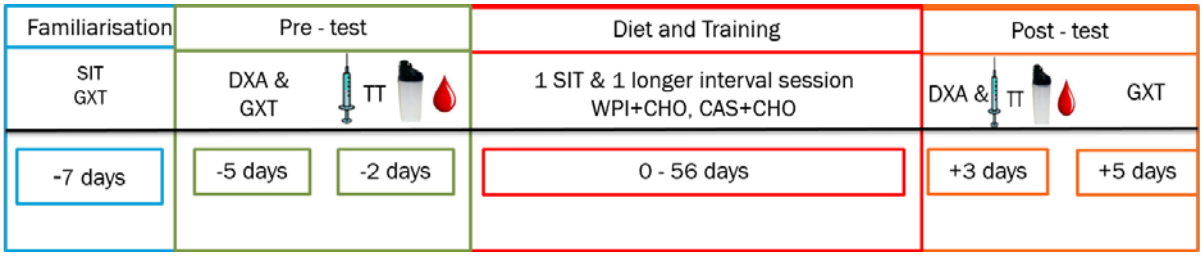


Figure 5.3.1: *Study design: Familiarisation sessions and pre-testing was carried out 1 week prior to the dietary and exercise intervention. Pre-testing provided baseline data for body composition (DXA), GXT and aerobic capacity as well as muscle and plasma insulin response. Diet (WPI+CHO or CAS+CHO) and training interventions was continued for 8 weeks with the last training session being a GXT, followed by post-test data. SIT – Sprint interval training; GXT – graded exercise test (lactate and VO_2 max); DXA* – Dual Energy

X Ray Absorptiometry;  *Biopsy; TT – time trial;*  *Blood samples;*  *nutrition; WPI+CHO – whey protein isolates and carbohydrates; CAS+CHO – calcium caseinates and carbohydrates.*

5.3.3 Testing procedures

5.3.3.1 Body composition analysis

Where possible, participants presented to Western Centre for Health and Research (WCHRE), St Albans, Australia in the morning, after an overnight fast for a DXA scan to determine body composition. If this time was not suitable for the participants, a food diary was kept and this was repeated prior to post-training scan.

See chapter 2 for a detailed description of the procedure.

5.3.3.2 Graded exercise test

A discontinuous graded exercise test was performed on an electronically-braked cycle ergometer (Lode Excalibur, v2.0, Groningen, The Netherlands) for determination of peak oxygen uptake ($VO_{2\ peak}$) (Rossiter, Kowalchuk et al. 2006), the lactate threshold (W_{LT}) using the modified D_{Max} method (Bishop et al. 2000), and peak power output (W_{Peak}), the

results of this test were also used to determine the training intensities used during the training intervention (Hawley, Myburgh et al. 1997).

Blood samples (~50 µL) were taken from the fingertips of each participant at rest, after completion of the warm up, during the 30 s of recovery between stages, and immediately after the termination of the test. Glucose and lactate concentrations were determined using an automated YSI 2300 STAT plus glucose and lactate analyser (YSI incorporated, USA).

5.3.3.3 Maximal oxygen uptake (VO_2 max)

After the GXT, the participants performed 5 min of active recovery at 20 W on the cycle ergometer, followed by a square-wave $VO_{2\,max}$ test at 105% W_{peak} achieved during the GXT, as previously described (Rossiter, Kowalchuk et al. 2006).

5.3.3.4 Muscle biopsies

Each participant received a total of two muscle biopsies; one prior to the dietary and exercise intervention and one following the 8-week intervention. The muscle biopsy procedure was

performed by a qualified and experienced medical doctor, using aseptic techniques as previously described (Evans, Phinney et al. 1982).

Once obtained, muscle samples were immediately processed, cleaned of excess blood, fat and connective tissue, and split into two portions. A small portion of the biopsy samples (10-20 mg) was immediately immersed in an eppendorf containing 1 mL of BIOPS, a muscle preserving solution and placed on ice. This portion was immediately used for in-situ measurements of mitochondrial respiration using the permeabilised fibre technique (described in detail below). The second portion (80-350 mg) was weighed, immediately frozen in liquid N_2, and subsequently stored at -80°C for subsequent analysis of markers of mitochondrial biogenesis (gene and protein content and enzyme activity).

5.3.3.5 Endurance performance test

The endurance performance test was performed on an electronically-braked cycle ergometer (Velotron, RacerMate, Seattle, WA, USA). Participants completed a 2-hour constant load cycle followed by 20 km time trial (Smith, Pascoe et al. 2013). Participants first completed a warm-up consisting of 10 min of cycling at a high cadence at a self-determined power output. They then completed a 2-hour constant-load cycle at 95% of the intensity associated with the onset of blood lactate accumulation (OBLA). Every 15 minutes heart rate and rate

of perceived exertion (RPE) were recorded and capillary blood samples were taken for the analysis of plasma glucose and lactate concentrations using an automated YSI 2300 STAT plus glucose and lactate analyser (YSI incorporated, USA). Participants could listen to music or watch movies on laptop screen during the 2-hour constant-load cycle. This was recorded and provided during the post test. On completion of 2 hours of cycling there was a 2-min rest period before participants began the 20-km cycling time trial. The participants were instructed to complete the 20-km time trial as quickly as possible. Gearing and cadence were self-selected. The gearing on the velotron bike was set up the same as the participants own bike. Participants were aware of the distance covered, but received no verbal stimuli, times, or other information.

In line with endurance athlete nutrition guidelines (Thomas, Erdman et al. 2016) that recommend carbohydrate loading to be beneficial for events longer than 90 minutes, this practice was undertaken 2 days prior to this performance test by each athlete. During the 2-hour cycle participants received verbal reminders to drink 200 ml of sports drink every 15 minutes and at random time points through the 20-km TT, to ensure a total of 140g of carbohydrate was consumed. This was made up in two 750 mL water bottles and they had

ad libitum access to water. The sports drink was made with combination of fructose and glucose.

5.3.4 Training intervention

The training program consisted of one sprint interval training session and one longer interval training session per week for 8 weeks carried out at Victoria University, Footscray Park campus. Participants continued their racing and training program outside of these training sessions. In addition to the two supervised exercise sessions included in the study, all participants raced club level criteriums on weekends, along with another long ride. The participant's bike set up was closely matched to their personal bike set up to ensure optimal comfort and pedalling efficiency. Participant's brought in their own bike seats and cycling shoes.

The sprint interval training session consisted of 30-s maximum effort intervals on an electronically-braked cycle ergometer (Velotron, RacerMate, Seattle, WA, USA). The 7-week training protocol was adapted from Macdougall et al., (1998). The program began with four intervals with 4 minutes of recovery between intervals and increased one interval

each week (except week 4) and reached nine intervals in week 7. Week 8 provided a taper week before final testing in week 9.

Table 5.1: *Weekly schedule for sprint interval training sessions.*

Week	**Number of sprint intervals**
1	four
2	five
3	six
4	six
5	seven
6	eight
7	nine
8	six

The longer interval training session per week was adapted from Hawley et al. (1997), and consisted of six to eleven 5-min cycling bouts at 80% of the subject's W_{peak}. Between each interval, participants rested for 1 minute or cycled against a low resistance. As with the sprint interval training schedule, week 8 was a taper week before testing in week 9.

Table 5.2: *Weekly schedule for longer interval training sessions.*

Week	**Number transition training sessions**
1	Six
2	Seven
3	Eight
4	Nine
5	Nine
6	Ten
7	Eleven
8	Six

5.3.5 Dietary intervention

The dietary intervention was carried out in a double-blind manner, with protein supplements being packaged and labelled in brown lunch bags, by a fellow researcher. The two dietary groups were isocaloric and matched for carbohydrate content; whey protein isolates (CHO+WPI) and calcium caseinates and carbohydrate (CHO+CAS). Participants were provided all meals, snacks and supplements for the duration of the intervention to aid with compliance. The fruit and vegetable items were ordered weekly from the University's fruit and vegetable provider. Supermarket items were ordered online weekly and delivered to Victoria University Footscray Park campus. Mission Foods Australia (GRUMA, Asia & Oceania, LLC) also generously provided a number of food items. Food boxes for participants were packed weekly and delivered to their house.

The diets provided 9 g per kilogram body mass ($g \cdot kg^{-1} \cdot bm^{-1}$) of carbohydrate per day, 1.8 $g \cdot kg^{-1} \cdot bm^{-1}$ of protein and 1 $g \cdot kg^{-1} \cdot bm^{-1}$ of fat per day. The carbohydrate guidelines for fuelling and recovery for endurance athletes were based off the Australian Institute of Sport guidelines (AIS Sports Nutrition 2014). Diets were created using Food Works nutrition data base (Xyris Software Australia) to ensure all macro and micronutrient requirements were

met. Participants were provided with weekly menus to follow, and asked to tick off meals and snacks once consumed or to add anything extra that was not on the list.

Participants were instructed to follow acute fuelling strategies (Table 5.3) for training rides and races outside of the study training sessions (Jeukendrup 2008). Carbohydrate loading involved participants increasing CHO intake to 10 g · kg^{-1} ·bm^{-1} · day, and was used prior to events lasting longer than 90 minutes (AIS Sports Nutrition 2014). Both fuelling strategies (CHO loading and acute fuelling) were used prior to and during the 2-h steady state cycle and 20 km time trial.

Table 5.3: *Acute fuelling strategies* (Jeukendrup 2008)

Carbohydrate guidelines for training and racing	
Less than 30 min	No CHO
30-75 min	little CHO (depending on intensity)
1-2 h	up to 30 g per hour
2-3 h	up to 60 g per hour
>2.5 h	up to 90 g per hour (combination of glucose and fructose)

Protein supplements were provided by Fonterra Co-operative Group Limited and the amino acid profile is shown in the table below. Calcium casienates and whey protein isolates were mixed with 27.5 g of sugar. Participants mixed these in water and drank directly after testing and each training session conducted at Victoria University and sessions completed outside of Victoria University.

Table 5.4: *The grams of (free) amino acids per 100 grams of crude protein*

	Whey Protein	Caseinate protein
Isoleucine	6.4	5.4
Leucine	14.6	9.8
Lysine	11.4	8.6
Methionine	2.4	2.9
Phenylalanine	3.9	5.0
Threonine	5.4	4.6
Tryptophan	2.4	1.4
Valine	5.7	6.5
Histidine	2.0	2.8
Alanine	5.8	3.4
Arginine	3.1	3.8
Aspartate	12.8	8.0
Cysteine	4.1	1.2
Glutamate	18.0	22.0
Glycine	1.8	1.9
Proline	4.6	10.0
Serine	4.6	5.7
Tyrosine	4.3	5.4

5.3.6 Muscle analysis

5.3.6.1 Citrate synthase activity assay

Citrate Synthase (CS) activity was determined in triplicate on a microtiter plate by adding: 5 µL of a 2 mg/mL muscle homogenate, 40 µL of 3mM acetyl CoA in Tris buffer and 25 µL of 1mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in Tris buffer to 165 µL of 100 mM Tris buffer (pH 8.3) kept at 30°C. At this point 15 µL of 10 mM oxaloacetic acid were added to the cocktail and the plate was immediately placed in a spectrophotometer kept at 30°C (xMark Microplate Spectrophotometer, Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia). Following 30 s of linear agitation, absorbance at 412 nm was recorded every 15 s for 3 min. CS activity was calculated and is reported as mol kg of protein^{-1} h^{-1}.

5.3.6.2 Mitochondrial respiration

Fibre preparation and high-resolution respirometry.

Muscle fibres were separated and permeabilised as previously described by Pesta and Gnaiger (2012). Mitochondrial respiration was measured in duplicate (from 3–4 mg wet weight of muscle fibers) in MiR05 at 37°C by using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria). Oxygen concentration (in nanomoles per millilitre) and flux (in picomoles per second per milligram) were recorded with DataLab software (Oroboros).

Reoxygenation by direct syringe injection of oxygen was necessary to maintain oxygen levels between 275 and 450 nmol/mL and to avoid potential oxygen diffusion limitation.

Mitochondrial respiration protocol.

Mitochondrial respiration measurements were taken using a substrate uncoupler-inhibitor titration (SUIT) protocol to assess glycolytic pathways. These were conducted according to the methods of Pesta and Gnaiger (2012).

5.3.6.3 Western blot analysis

Muscle lysates (10–25 μg) were separated by electrophoresis on SDS polyacrylamide gels (8–12%) and transferred to PVDF membranes, before being blocked for 1 h in 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST). Membranes were incubated overnight at 4°C with the following primary antibodies: apoptosis-inducing factor (AIF), dynamin-related protein 1 (DRP1), mitofusin 2, p53, and PHF20 (all from CST); PGC-1α (EMS-Millipore, Billerica, MA, USA); synthesis of cytochrome *c* oxidase 2 (SCO2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and TFAM and total oxphos (Abcam,

Cambridge, UK). The membranes were then incubated at room temperature with the appropriate host species–specific secondary antibody for 90 min, before being exposed to a chemiluminescence solution. Three TBST washes were performed between each step. Protein bands were visualized with a Bio-Rad Versa-Doc imaging system, and bands were quantified with Bio-Rad Quantity One software (Bio-Rad). An internal standard was loaded on each gel, and each lane was normalized to that value, to reduce gel-to-gel variability. Coomassie blue staining was used to qualitatively verify correct loading and equal transfer between lanes (Welinder and Ekblad 2011). Statistical analysis was performed on the raw density data.

5.4 Statistics

All statistical analysis was completed using Graph Pad Prism Software 7.0. Normality of data sets was analysed using Sharipo-Wilk normality test. The change was calculated from pre to post for each group and an independent t-test performed.

5.5 Results

5.4.1 Participant parameters

Table 5.5: *Baseline characteristics for the participants.*

*All values are mean ± SD. *Significantly different (P < 0.05) vs. WPI+CHO. n=9 WPI+CHO and n=8 CAS+CHO for all measures except maximal oxygen uptake, n = 6 in both groups (due to experimental error).*

Measurement	**WPI + CHO (n=9)**	**CAS + CHO (n=8)**
Age (y)	30 ± 6	29 ± 2
Height (cm)	183 ± 6	177 ± 7
Mass (kg)	81 ± 5	72 ± 7*
Lactate threshold (watts)	247±30	243±55
Maximal oxygen uptake ($ml \cdot kg^{-1} \cdot min^{-1}$)	57 ± 6	64 ± 5
Baseline time trial data (minutes)	35.07 ± 1.3	35.73 ± 3.98

Whey protein isolates and carbohydrates (WPI+CHO); Calcium caseinates and carbohydrates (CAS+CHO).

Participants baseline characteristics are shown in Table 5.5 Body mass was significantly different between protein groups at baseline ($P < 0.05$).

5.4.3 Dietary intake

Table 5.6: Macronutrient and total energy content of 8 weeks of dietary intervention. Values are means ± SD. n=9 WPI+CHO and n=8 CAS+CHO.

	WPI + CHO		**CAS + CHO**	
	g · day^{-1}	**g · kg^{-1} · bm^{-1}·day**	**g · day^{-1}**	**g · kg^{-1} ·bm^{-1}·day**
Carbohydrate (g)	573 ± 53	7.4 ± 0.7	557 ± 82	7.9 ± 0.6
Fat (g)	73 ± 6	0.9 ± 0.5	69 ± 7	1 ± 0.07
Protein (g)	136 ± 8	1.7 ± 0.2	134 ± 9	1.9 ± 0.07
Total kJ	14732 ± 855		14178 ± 1497	

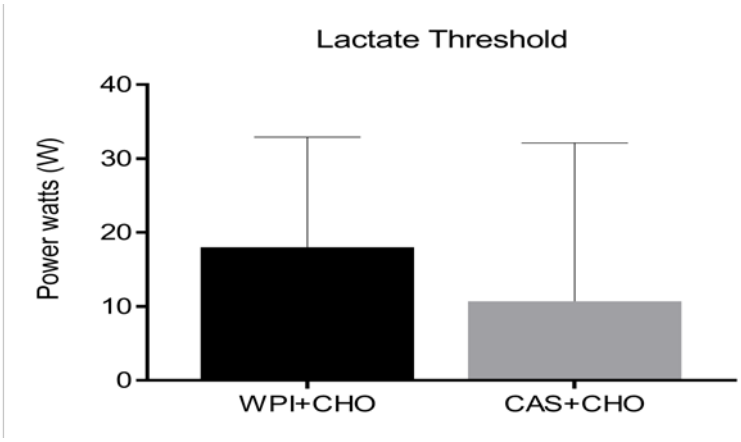
Table 5.7: *Macronutrient and total energy content of 2-day carbohydrate loading phase. Values are means ± SD. *Significantly different (P < 0.05) vs. WPI+CHO. n=9 WPI+CHO and n=8 CAS+CHO.*

	WPI + CHO		**CAS + CHO**	
	g · day^{-1}	**g · kg^{-1} ·bm^{-1}·day**	**g · day^{-1}**	**g · kg^{-1} ·bm^{-1}·day**
Carbohydrate (g)	764 ± 46	9.8 ± 0.3	689 ± 72*	9.9 ± 0.3
Fat (g)	52 ± 5	0.7 ± 0.02	52 ± 9	0.7 ± 0.1
Protein (g)	131 ± 15	1.6 ± 0.1	119 ± 14	1.7 ± 0.1
Total kJ	17469 ± 1246		15513 ± 1769	

There were no differences between carbohydrate and whey protein isolates (WPI+CHO) group and carbohydrate and calcium caseinates (CAS+CHO) group for macronutrient and total energy content of 8 weeks of dietary intervention (Table 5.6). Despite providing all food for participants each group did not reach the target intake of carbohydrate (9 $g \cdot kg^{-1} \cdot bm^{-1} \cdot$ day) during the 8-week training phase. During the 2-day carbohydrate load CAS+CHO had significantly lower total carbohydrate intake in grams compared to WPI+CHO group (Table 5.7 $P < 0.05$), however there were no differences in the $g \cdot kg^{-1} \cdot bm^{-1} \cdot$ day intake during this period or in other macronutrients consumed.

5.4.4 Performance measurements

a



b

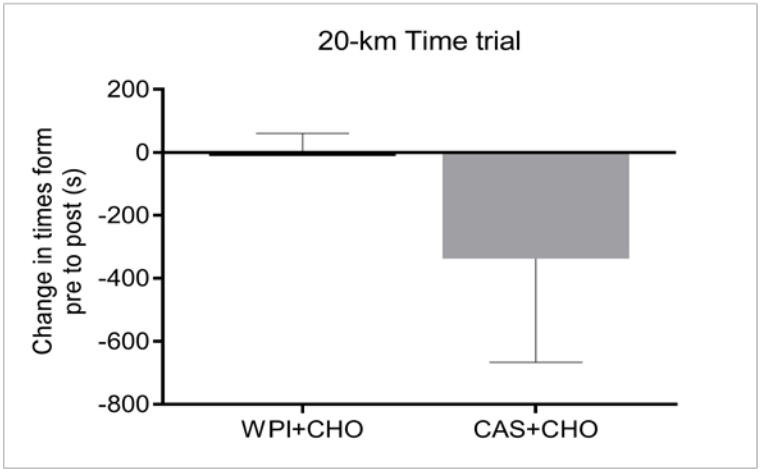


Figure 5.2: *The change in Lactate threshold (a) and 20 km cycling time trial (b) for whey protein isolates and carbohydrate (WPI+CHO) and calcium caseinates and carbohydrates*

(CAS+CHO) groups from pre to post 8 weeks of dietary and endurance training. Lactate threshold WPI+CHO pre n = 9 post n = 7. CAS+CHO pre n = 8 post n = 7; 20 km time trial WPI+CHO pre n =7 and post n = 8, CAS+CHO n = 7 pre and post. All values are mean ± SEM.

Lactate threshold was measured during graded exercise test at baseline and post intervention (Figure 5.2a). 48 hours following this, participants completed a 2 hours of steady state cycling at 95% of OBLA before completing 20-km time trial (Figure 5.2b). There was no significant change in lactate threshold or time to complete 20-km between protein groups. One participants results may have caused the large shift in the group mean due to being the only one to complete the time trial nearly 4 minutes quicker than his pre-time. Participants completed all the allocated training sessions throughout the study period.

5.4.5 Body Composition

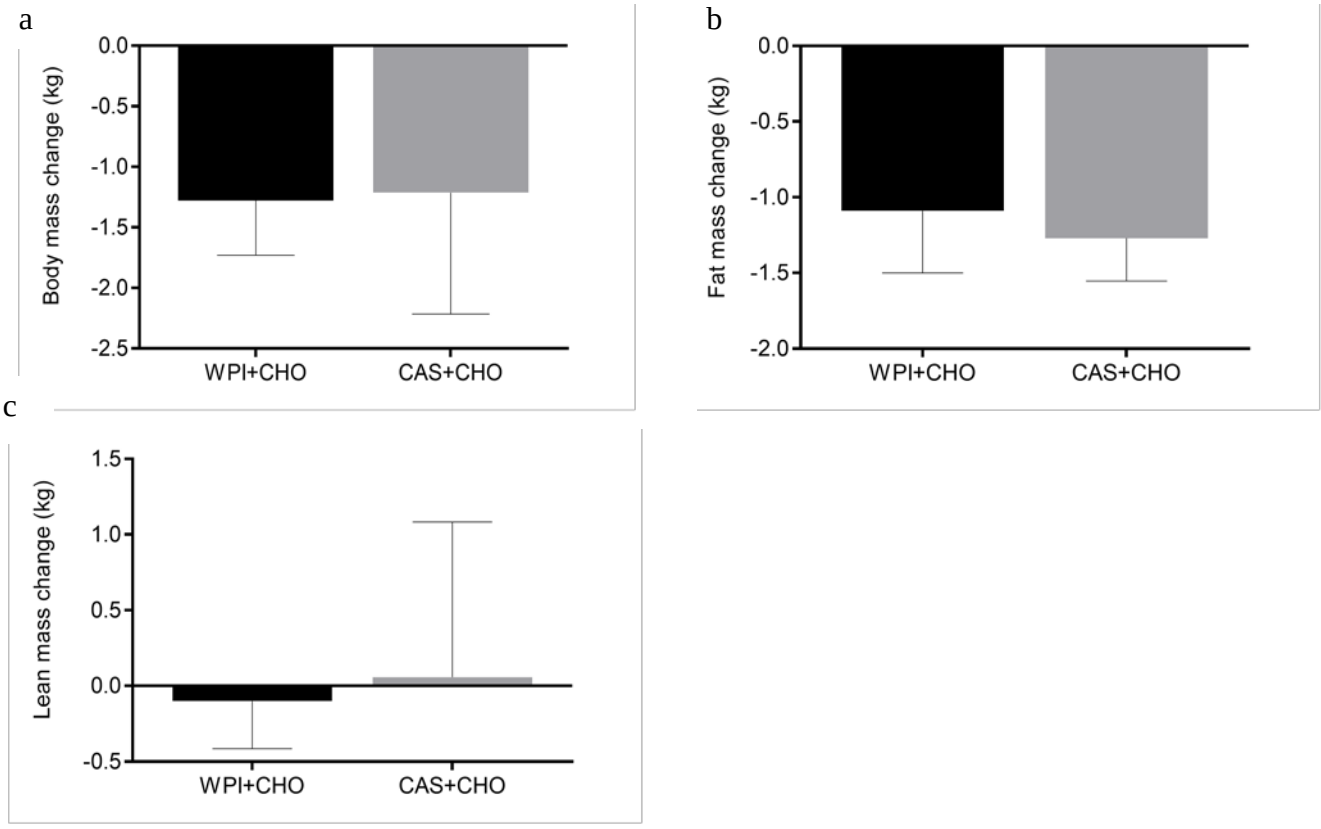


Figure 5.4.3 a-c: *The change in body composition from pre to post 8 weeks dietary and training intervention between protein groups; whey protein isolates and carbohydrate (WPI+CHO) and calcium caseinates and carbohydrate (CAS+CHO). The change in body mass (a), lean mass (b), and fat mass (c) are shown. n = 9 WPI+CHO and n = 8 CAS+CHO. All values are mean ± SEM.*

The change in body composition was measured between WPI+CHO and CAS+CHO, using DXA at baseline (pre) and after 8 weeks of dietary and exercise training (Fig 5.3a-c). No significant changes in body mass (Fig 5.3a), Lean mass or fat mass (Fig 5.3c) was shown between protein groups.

5.4.5 Citrate synthase and mitochondrial respiration

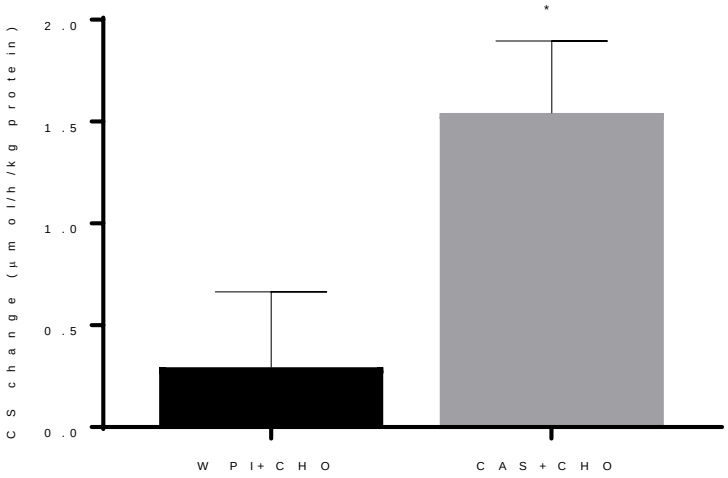
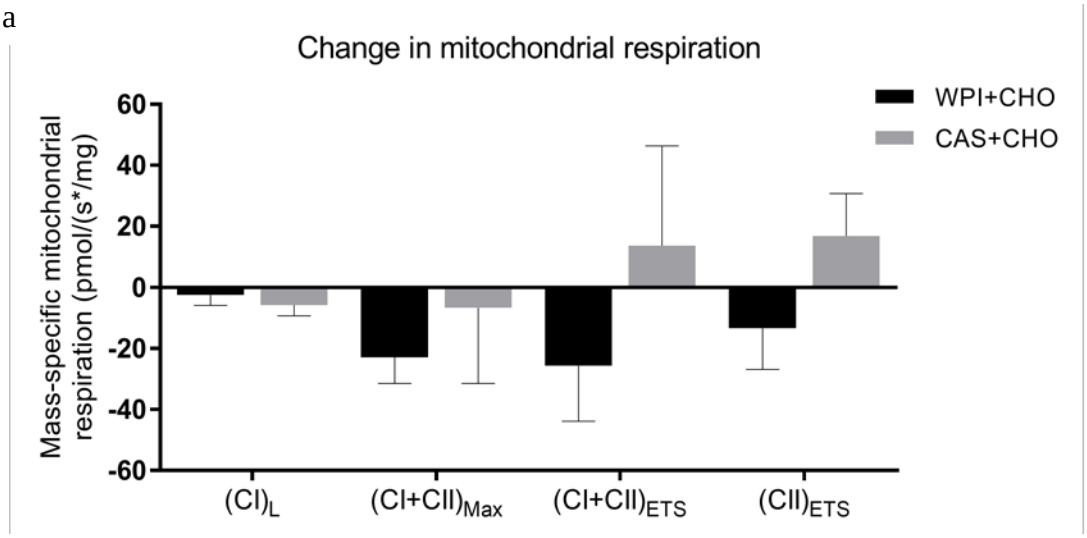


Figure 5.4.4: *The change in maximal activity (mmol kg protein^{-1} h^{-1} w.w.) of citrate synthase (CS) measured in whole muscle homogenates prepared from muscle biopsy samples (v. lateralis) obtained pre and post 8 weeks of dietary and exercise training. Protein groups were whey protein isolates and carbohydrate (WPI+CHO) or calcium caseinates and carbohydrate (CAS+CHO). *Significantly different (P < 0.05) vs. WPI+CHO. n = 8 WPI+CHO and n = 7 CAS+CHO. All values are mean ± SEM.*

CS activity had a significantly greater change from pre to post intervention in the CAS+CHO group compared to pre-treatment ($P < 0.05$).

a


Change in mitochondrial respiration
WPI+CHO
CAS+CHO
Mass-specific mitochondrial respiration (pmol/(s*/mg)
60
40
20
0
-20
-40
-60
(CI)L
(CI+CII)Max
(CI+CII)ETS
(CII)ETS

b

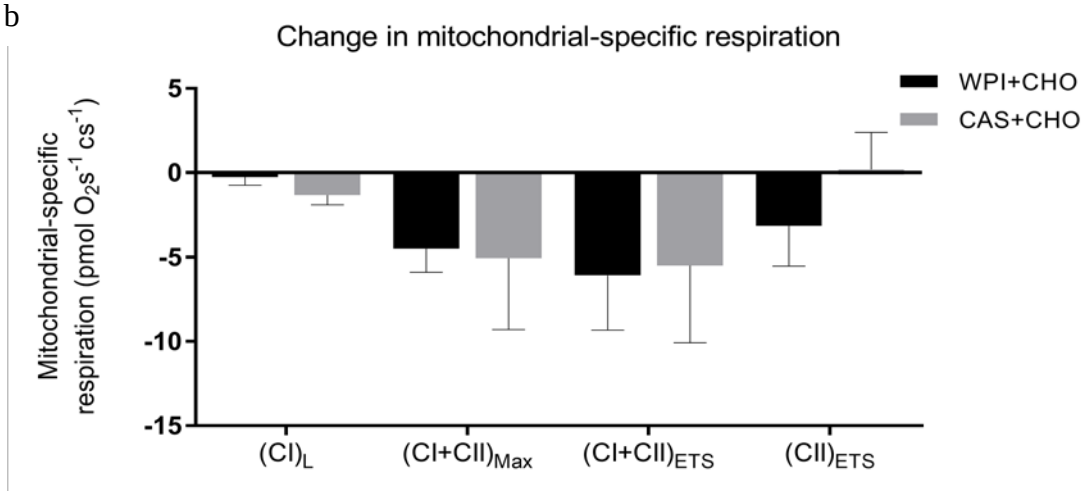

Change in mitochondrial-specific respiration
WPI+CHO
CAS+CHO
Mitochondrial-specific respiration (pmol O2s-1 cs-1)
5
0
-5
-10
-15
(CI)L
(CI+CII)Max
(CI+CII)ETS
(CII)ETS

***Figure 5.4.5 a-b**: Mitochondrial respiration changes from pre to post 8- week dietary and exercise intervention, between whey protein isolates and carbohydrate (WPI+CHO) and calcium caseinates and carbohydrate (CAS+CHO) protein groups. Respiration was measured in permeabilised fibres prepared from muscle biopsy samples (vastus lateralis) obtained pre and post 8 weeks of dietary and exercise intervention. Respiration values are: $(CI)_L$: Leak respiration state (L) in the absence of adenylates and limitation of flux by electron input through Complex I (CI); CI_P: maximal oxidative phosphorylation state (P) with saturating levels of ADP and limitation of flux by electron input through CI; $CI+II_P$: P with saturating levels of ADP and limitation of flux by convergent electron input through CI + Complex II (CII); $CI+II_E$: maximal electron transport system capacity (E) with saturating levels of ADP and limitation of flux by convergent electron input through CI+II; CII_E: E with saturating levels of ADP and limitation of flux by electron input through CII. n = 6 for both groups. All values are mean ± SEM.*

Mass-specific mitochondrial respiration changes are shown in Figure 5.5a and when normalised to CS to activity mitochondrial-specific respiration is shown in Figure 5.5b. No significant changes between protein groups were shown in either mass-specific or

mitochondrial-specific respiration. The data presented in this study showed no effect of the addition of cytochrome *c* as a control for outer mitochondrial membrane integrity.

5.4.6 Protein content of subunits from the five ETS complexes

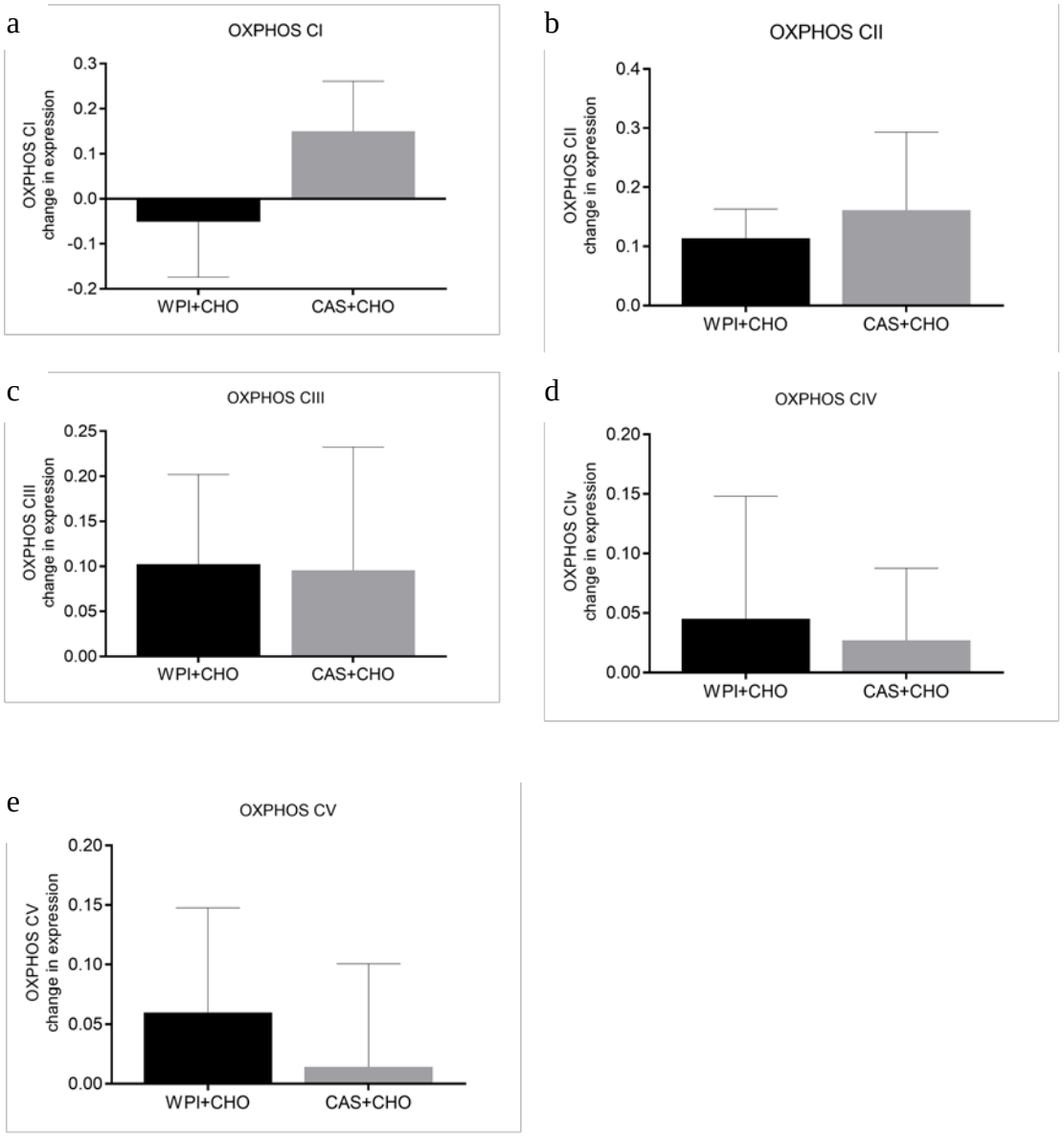


***Figure 5.6a-e**: The change in protein content of subunits from the five complexes of the electron transport system (ETS), complex I to V (CI to CV), in whole muscle lysates prepared from skeletal muscle biopsy samples (vastus lateralis). Biopsies were obtained pre and post 8-weeks of dietary intervention with whey protein isolates and carbohydrate (WPI+CHO) or calcium caseinates and carbohdyrates (CAS+CHO) and training intervention. n = 8 in WPI+CHO and n =7 in CAS+CHO. All values are mean ± SEM.*

The was no significant changes in protein content of the subunits from the five ETS complexes from pre to post 8-weeks of dietary and exercise intervention, between protein groups (Fig 5.6).

5.4.7 Protein content of transcription factors

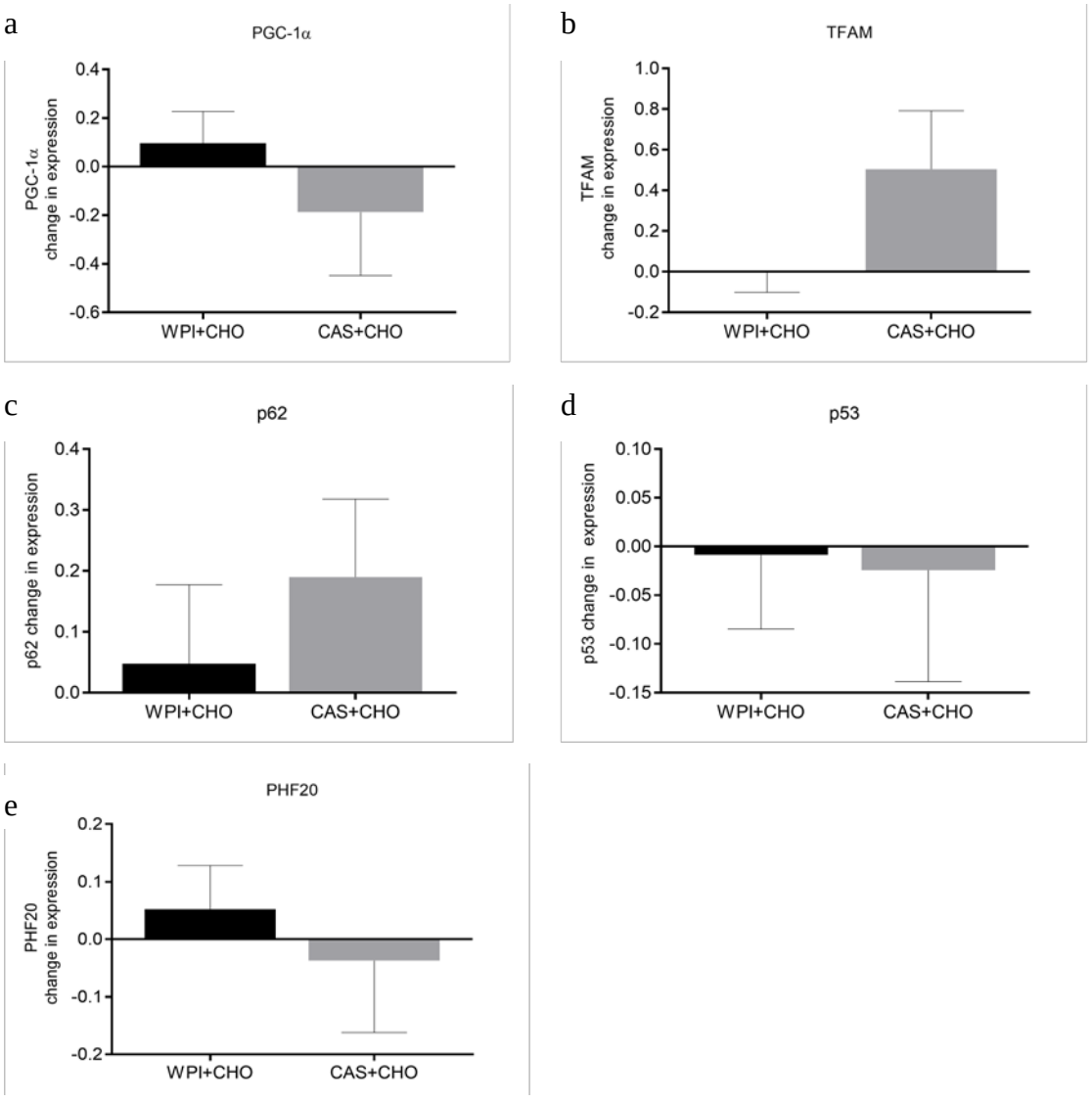


Figure 5.7 a-e: *The change in protein content of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), (a), mitochondrial transcription factor A (TFAM) (b), P62 (c), p53 (d) and plant homeodomain finger-containing protein 20 (PHF20) (e) in whole muscle lysates prepared from skeletal muscle biopsy samples (vastus lateralis). Biopsies were obtained pre and post 8 weeks of dietary and training intervention with whey protein isolates and carbohydrates (WPI+CHO) and calcium caseinates and carbohydrate (CAS+CHO). n = 8 in WPI+CHO and n = 7 in CAS+CHO. All values are mean ± SEM.*

5.4.8 Protein content of DRP1 and MFN2

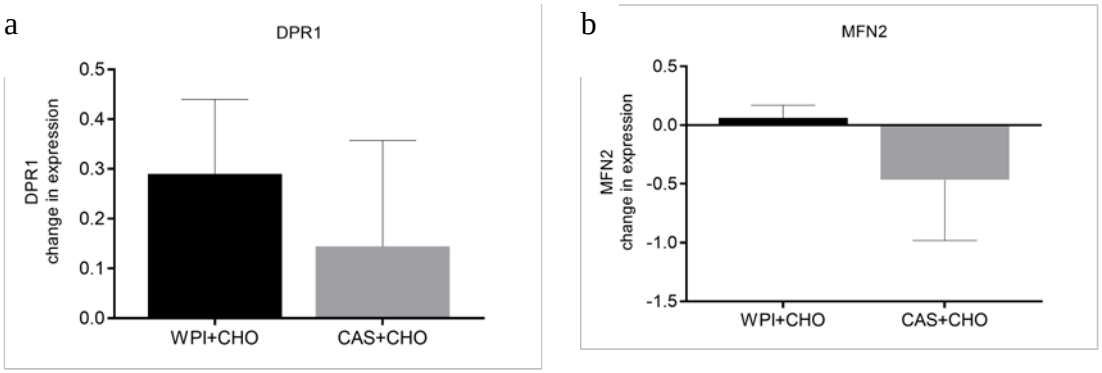


Figure 5.8 a-b*: The change in protein content of Dynamin related protein 1 (DRP1), (a) and Mitofussion 2 (MFN 2) (b) in whole muscle lysates prepared from skeletal muscle biopsy samples (vastus lateralis). Biopsies were obtained pre and post 8 weeks of dietary and training intervention with whey protein isolates and carbohydrates (WPI+CHO) and calcium caseinates and carbohydrate (CAS+CHO). n = 8 in WPI+CHO and n = 7 in CAS+CHO. All values are mean ± SEM*

The protein content of the transcription factors (Fig 5.7 a-e) peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), mitochondrial transcription factor A (TFAM), P62, p53 and plant homeodomain finger-containing protein 20 (PHF20) showed no change between WPI+CHO and CAS+CHO groups from pre to post 8 weeks of dietary and exercise training. There was also no change in protein content of DRP1 and MFN2 between groups (Fig 5.8).

5.6 Discussion

The results from the present study demonstrate that long term (8-week) post-exercise supplementation with WPI does not have added benefits compared to calcium caseinates. In fact, calcium caseinates group had increased change in CS activity compared to WPI group, while other measures of body composition and markers of mitochondrial adaptations showed no difference in change between protein groups. This is important as WPI is marketed as having superior benefits compared to other proteins (Ha and Zemel 2003) and is a popular supplement amongst athletes.

There was no difference in macronutrient content or total energy intake for the 8-week dietary intervention between the WPI and CAS groups. The 2-day carbohydrate (CHO) loading phases implemented prior to the performance tests showed CAS+CHO to have significantly less CHO compared to WPI. However, as CHO provided was based on $g \cdot kg^{-1} \cdot bw^{-1}$ (Thomas, Erdman et al. 2016) when normalised to body mass there was no difference in CHO consumed between both groups. Despite there being no differences between the groups regarding macronutrient content or total energy intake during the 8-week intervention, neither group consumed the quantity of CHO the study was aiming ($9 g \cdot kg^{-1} \cdot bw^{-1}$) for; $7.4 g \cdot kg^{-1} \cdot bw^{-1}$ and $7.9 g \cdot kg^{-1} \cdot bw^{-1}$ for the WPI and CAS groups respectively. Even though participants were provided with all meals, snacks and supplements for the duration of the study they did not consume all snacks provided each day or consume the quantity of carbohydrates through all of their training sessions.

Studies investigating the diets of athlete's show they under consume compared to their requirements (Masson and Lamarche 2016, Heikura, Stellingwerff et al. 2017). Conflicting and evolving nutrition research, food product advertising, the complexity of behaviours needed to achieve specific sports nutrition intake and strong media coverage of nutrition issues make it challenging for clear nutrition messages to be translated to the athletic population (Spendlove, Heaney et al. 2012). While participants consumed more than what they normally would (anecdotal evidence), they still fell short of the aims of the study and current guidelines (AIS Sports Nutrition 2014). Despite the dietary guidelines for endurance athletes being based on carbohydrate-rich, low fat dietary approach, recent media attention surrounding high-protein or high-fat and low-carbohydrate diets may contribute to lack of carbohydrate intake and uncertainty about consumption of carbohydrate-rich foods (Hendrie, Coveney et al. 2008, Doering, Reaburn et al. 2016, Masson and Lamarche 2016).

Body mass was higher in the WPI+CHO group at the beginning of the trial compared to CAS+CHO group however, there was no significant change between protein groups in body mass, lean mass and fat mass from pre-to post intervention. While there was no significant difference between groups, the WPI+CHO group lost 1.27 ± 0.45 kg and CAS+CHO lost 1.21 ±1 kg of BM over the 8 weeks and 1.08 ± 0.41 kg and 1.27 ± 0.28 kg of fat mass for WPI+CHO and CAS+CHO, respectively. Studies of shorter duration have found WPI to stimulate post prandial protein accretion more effectively than casein and casein hyrolysate and attributed this finding to a combination of WPI's faster digestion and absorption kinetics and higher leucine content (Pennings, Boirie et al. 2011). However, calcium caseinates has a similar digestion rate to WPI and as demonstrated in the current study when included in the diet post- exercise over a longer duration, has similar effects to WPI. Chapter 3 of this thesis showed WPI and micellar

casein reduced adiposity compared to CHO. Indicating, fast or slow digesting proteins seem to be superior compared to CHO at improving body composition. Whether the casein is in slow (micelle form) or faster (caseinates) digestion form doesn't seem to make a difference on body composition when comparing to WPI.

The change in time to complete the 20-km time trial (TT) from pre to post testing, between the WPI+CHO and CAS+CHO groups was not significantly different. However, when elite endurance cycling performance can come down to seconds, the non-significantly quicker times of the CAS+CHO group (- 338 ± 328 s) compared to WPI+CHO group (+ 3.77 ± 57.87 s) may be important. Further investigation is warranted to compare different protein types on cycling endurance performance when included in an athlete' 'real world' training and racing schedule. Meanwhile, several studies have shown that carbohydrate plus protein ingestion can extend endurance time-to-exhaustion compared to CHO (Saunders, Kane et al. 2004, Saunders 2007). However, this result hasn't been replicated in acute feeding studies comparing different protein (casein and hydrolysed whey) drinks on 20-km cycling TT results (Cepero González, Padial et al. 2010). In addition, when comparing milk-based proteins on subsequent cycling 20-km TT performance in trained male cyclists, there was no difference between high carbohydrate/low protein, low carbohydrate/high protein or isocaloric carbohydrate. Potentially, the type of protein is less important when two high quality proteins are compared.

The CAS+CHO group showed a significantly greater increase in CS from pre to post 8-week diet and exercise intervention compared to WPI+CHO; CS is commonly used as a marker of mitochondrial quantity (Lundby and Jacobs 2016). Even though CS activity increased there

were no functional changes in mitochondrial respiration. Mitochondrial respiration was not different between WPI+CHO and CAS+CHO groups and did not show an improvement within groups after the training intervention, including when normalised to CS.

Previous studies in humans have shown that acute and/or chronic endurance exercise increases mixed muscle protein synthesis independent of protein ingestion (Pikosky, Gaine et al. 2006, Harber, Konopka et al. 2010, Mascher, Ekblom et al. 2011). While it would be assumed these increases in mixed muscle protein synthesis would be related to mitochondrial proteins and enzymes, recent studies have instead shown increases in myofibrillar protein synthesis with endurance based exercise (Breen, Philp et al. 2011, Coffey, Moore et al. 2011, Rowlands, Nelson et al. 2015). While myofibrillar proteins and signalling pathways weren't measured in the current study, mitochondrial proteins of the electron transport system, complexes I – V and involved in mitochondrial biogenesis showed no difference between WPI+CHO and CAS+CHO groups before or after 8 weeks of dietary and exercise training. An acute study investigating two different training modes and supplemented a non-nutritive control or carbohydrate + essential amino acid beverage pot-exercise, found p53 and COXIV expression upregulated with the non-nutritive control beverage (Margolis, Murphy et al. 2017). Future investigations should focus on long-term post-exercise protein ingestion and its impact on mitochondrial biogenesis and adaptations to training.

In summary, a number of factors contribute to success in endurance sport and nutrition is a key component. WPI has been touted as a superior protein due to its high leucine content and faster digestion rate however, the current study demonstrated that when two high quality proteins are

ingested post-exercise for 8-weeks results are comparable. In fact, calcium caseinates showed a greater increase in CS activity. Further studies are needed to investigate long term supplementation in trained individuals that replicates what an athlete does in ‘real world’ training and racing.

Chapter 6: Overall discussion and future directions

6.1 General discussion

In the search for novel and better methods of preparation for competition, scientists, coaches, athletes, and practitioners, constantly seek out the latest information on training and nutrition to gain a competitive edge for athletes (Coffey and Hawley 2006). It is well established that endurance exercise training leads to cardiovascular, skeletal muscle, and metabolic adaptations, with important implications for both athletic performance and health. The quality and quantity of nutritional intake harnesses many benefits for athletic populations. These benefits not only impact athletes, but can also have many benefits for improving the overall health of the general population.

While many studies have addressed the effects of endurance exercise training on adaptations, very few have examined the role of post-exercise nutritional supplementation in facilitating or increasing the magnitude of the adaptive response. The beneficial effects of post exercise nutrition, in the form of carbohydrate and protein, following an acute bout of exercise has been the focus of many investigations (Williams, Raven et al. 2003, Berardi, Price et al. 2006, Ivy, Ding et al. 2008). The results however remain equivocal due to methodological differences, such as the training status of participants, the treatment groups not being isocaloric, differing CHO contents, different protein sources, exercise modes and protocols, and outcome measurements all being different.

Research is now emerging for a positive role of protein ingestion at intakes above the recommended dietary intake (RDI) to assist in promoting positive changes in body

composition and managing body mass (Phillips, Chevalier et al. 2016) for both the general population and athletes in achieving health and athletic performance. In particular, there is increasing interest in the roles of different types of protein on lean mass retention during weight loss, weight control and ageing (Millward, Layman et al. 2008). Investigating how different types of proteins affect body composition in healthy animals may help in providing a better framework to improve body composition in humans.

The quality of the protein source used in post exercise supplementation may also affect training adaptations. Animal-source proteins, such as milk and the constituent proteins of milk, casein and whey, are classified as being of high biological availability and quality (Schaafsma 2005, Phillips, Tang et al. 2009). I have previously demonstrated that WPI consumption in the presence of adequate CHO causes an enhanced plasma insulin response and increased PGC-1α mRNA expression in trained individuals following exercise (Hill, Stathis et al. 2013). These results raise the question as to whether habitual consumption of these proteins offers greater advantage for muscle function particularly via enhanced mitochondrial function, along with improving body composition.

The significant aim of this PhD candidature was to determine the role of protein supplementation, when included in the training diet over an extended period, on endurance training adaptations. Along with investigating how different proteins affect the signalling pathways that regulate endurance training adaptations. As such the research presented in this thesis examined 8 weeks of supplementation, with and without an endurance training program,

with micellar caseins or caseinates, whey protein isolates or a carbohydrate matched and isocaloric group in animal and human models.

6.1.1 The effects of protein on body composition

The role protein plays in optimising body composition was investigated in chapters three, four and five, in an animal model, with and without endurance training, and in a human model. Body mass is an important issue for both the general population and athletes. Obesity and its related health concerns are a major problem for the general population (Johannsen and Ravussin 2009). Consequently, there is a large percentage of the Australian population for which strategies to promote weight loss is an important concern. Many athletes also restrict energy intake to achieve a certain body mass category, for aesthetic reasons, or to attain better power to mass ratio to improve performance (Thomas, Erdman et al. 2016).

The findings from chapter three demonstrate 8 weeks of supplementing with WPI or micellar caseins reduced fat mass in Sprague Dawley rats compared to a carbohydrate matched control group. This finding is supported by other studies using moderate (35% total energy) and higher (50% total energy) protein intakes (Lacroix, Bos et al. 2006, Zhou, Keenan et al. 2011). This indicates, reductions in adiposity can occur with moderate amounts of dietary protein, when high quality proteins are ingested. These changes in fat mass were shown without an increase in lean mass when there is no exercise stimulus.

The findings from chapter four established the WPI supplemented rats had a greater increase in lean mass from 4 to 8 weeks compared to CHO supplemented rats, when an endurance training program was implemented. A key finding from chapter five demonstrated that when two high quality proteins are supplemented in humans while undertaking an endurance training program the change in body composition is comparable. The results from this thesis demonstrate protein ingestion is beneficial for body composition changes compared to CHO and high quality proteins have comparable effects.

6.1.2 The role post-exercise protein ingestion has on exercise performance

The role post-exercise protein ingestion has on time to exhaustion performance tests was investigated in chapters four and five, in an animal and human model, respectively. A key finding in chapter four was that despite matching all rodents across groups for exercise performance prior to the intervention, the carbohydrate supplemented animals were unable to perform for as long in the TTE test compared to both micellar caseins and whey protein isolates groups, with no difference between protein groups. This improvement in performance was not associated with improvements in mitochondrial function, thus it appears that this improved time to exhaustion is not related to improved mitochondrial function, but by some other, yet to be determined factor.

Chapter five demonstrated a similar effect when WPI and calcium caseinates were supplemented post-exercise in humans. 20-km cycling TT, following 2-hour constant load cycle, was not significantly different between protein groups following 8-weeks of nutrition and endurance training in humans. Calcium caseinate was used instead of a slow

digesting micellar casein due to its greater accessibility, and although the digestive properties are quicker than micellar caseins it demonstrated to have the similar performance benefits to WPI. WPI is commonly used amongst athletes due to its strong influence in the sports nutrition market based on the high levels of BCAA's and positive effects on muscle protein synthesis (Ha and Zemel 2003) however, the current study has demonstrated no added benefit compared to calcium caseinate.

6.1.3 The effects of protein on mitochondrial adaptation

The effect protein has on mitochondrial function and content was investigated in chapters three, four and five, in an animal model, with and without endurance training, and in a human model. Mitochondria play a key role in energy metabolism in many tissues, including skeletal muscle (Johannsen and Ravussin 2009). Mitochondrial dysfunction has been associated with obesity, type 2 diabetes mellitus and ageing (Johannsen and Ravussin 2009). Thus, making these organelles an important focus of research. Endurance type exercise has been shown to be a potent stimulator for improving mitochondrial function and content (Granata, Oliveira et al. 2016).

In chapter three, the micellar caseins group showed a significant increase in oxidative phosphorylation compared to whey protein isolates when investigating fatty acid substrate entry in rat soleus muscle. Increasing the use of fat as a fuel source is a well-known adaptation to endurance exercise (Holloszy 1974) therefore, other potential mechanisms need investigating to identify the pathways of protein supplementation increasing fatty acid oxidation. In chapter four, when an endurance training program was implemented in an animal

model this improvement in oxidative phosphorylation was not observed. The same amount of protein supplementation was used in the non-training and training studies, indicating the protein quantity may not have been sufficient to see changes in mitochondrial function, despite improvements in performance. Chapter five demonstrated CAS+CHO supplementation to result in a greater increase in CS compared to the WPI+CHO group. However, this increase in a marker of mitochondrial quantity did not translate into greater quality or functioning. In summary, this thesis demonstrated micellar casein supplementation to increase OXHPOS without an endurance training program in an animal model, along with calcium caseinate increasing CS compared to WPI. Thus, micelle caseins and calcium casienates may provide greater mitochondrial adaptations when included in an athlete's diet over a longer period of time.

6.2 Overall summary

The results from the studies undertaken for this thesis provide novel information regarding the 8-week supplementation of WPI compared to micellar caseins or calcium caseinates and carbohydrate group, with and without exercise, in both animal and human models. WPI and micellar caseins reduced fat mass compared to a CHO control group without exercise, while WPI also had a greater increase in lean mass compared to the CHO group in combination with an endurance exercise program in an animal model. In addition, WPI and calcium caseinates showed no difference on body composition in trained athletes. Endurance time to exhaustion was extended with WPI or micellar caseins supplementation compared to CHO control group in an animal model. When endurance performance was tested in trained cyclists both had comparable effects. Furthermore, micellar caseins improved oxidative phosphorylation compared to WPI in rat soleus muscle when investigating fatty acid oxidation through high

resolution respirometry without exercise and calcium caseinates showed a greater increase in CS compared to WPI in trained cyclists. Overall, these three studies have demonstrated protein supplementation provides beneficial body composition and performance improvements compared to CHO. Furthermore, the quality of protein may be important as calcium caseinates and WPI has comparable outcomes. Further investigations are needed to elucidate the mechanisms behind the benefits of these milk-based proteins.

6.3 Recommendations for future research

The results from this thesis pose further questions that warrant future investigation especially surrounding the potential health and athletic benefits of whey protein isolates and micellar caseins for improving body composition and mitochondrial function, when included in the diet longer term.

6.3.1 Body composition and fat oxidative capacity

Chapter three has provided preliminary information regarding the benefits of WPI and micellar caseins on body composition and mitochondrial function. Based on tissues already collected, further analysis to investigate if the reduction in fat mass and increased oxidative phosphorylation via a fatty acid mitochondria protocol are related. Protein-induced lipid oxidation has been investigated by a limited number of studies. Lorenzen, Frederiksen et al. (2012) observed a small increase in lipid oxidation after casein compared to whey, but in contrast Acheson, Blondel-Lubrano et al. (2011) observed no difference between the proteins. The suggested mechanism for the increased lipid oxidation after consumption of casein compared to whey, may be due to differences in post prandial insulin response (Bendtsen,

Lorenzen et al. 2013). Insulin is known to suppress lipid oxidation; therefore, the current study could be improved by investigating the differences between postprandial insulin response between micellar caseins, WPI and CHO groups.

Uncoupling proteins (UCP), in particular UCP1 and UCP3 have been suggested as therapeutic targets for treating obesity and its related diseases, due to their role in mitochondrial uncoupling and energy metabolism (Busiello, Savarese et al. 2015). In future studies, taking brown adipose tissue to measure UCP1 and in the current investigation examining UCP3 content in skeletal muscle is warranted. UCP3 is thought to play a role in skeletal muscle mitochondrial fatty acid oxidation (Cioffi, Senese et al. 2009) therefore, this may potentially help explain the increased OXPHOS with the fatty acid protocol in the current study (Chapter 3).

Furthermore, investigating the gastrointestinal hormone response of micellar caseins compare to WPI and CHO may give a wider view of how these proteins regulate energy intake and their effect on satiety. The current study (Chapter 3) showed WPI to have a lower energy intake compared to CHO group but no difference compared to micellar caseins group. The mechanism behind this is not clear. Some studies but not all have shown whey to stimulate incretin hormones to a greater extent than other protein sources such as casein and milk (Hall, Millward et al. 2003, Veldhorst, Nieuwenhuizen et al. 2009). Investigating satiety hormones such as CCK, glucagon-like polypeptide, peptide YY and the incretin hormones would provide a more complete picture of the satiating effect of different proteins. In addition, investigating muscle and liver glycogen levels and intramuscular triglyceride levels to assess if different protein sources affect storage of these nutrients is warranted.

In addition to investigating isolated proteins on body composition and fat metabolism, future studies may examine the potential added benefit of whole dairy products such as milk or yoghurt. These whole foods may provide additional benefits on body composition, appetite and satiety and muscle function compared to isolated proteins. These dairy products contain other vitamins and minerals such as calcium, which has been shown to reduce adiposity (Zemel, Shi et al. 2000) and the probiotics in yoghurt have beneficial effects on gut health, which flows onto additional whole body benefits, including muscle adaptations (Hemarajata and Versalovic 2013).

6.3.2 Muscular adaptation

The WPI+CHO group had a significantly greater increase in lean mass from 4 to 8-weeks compared to CHO group. Due to the lack of change between groups with regards to mitochondrial function, investigating myofibrillar protein synthesis pathways such as mTORC1 and its downstream regulators is warranted. Furthermore, it would be worthwhile to investigate if mTOR pathways influence PGC-1α, even though there was not an increase in mitochondrial function. In addition, studies have shown myofibrillar and not mitochondrial protein synthesis pathways to be upregulated in acute studies (Breen, Philp et al. 2011). Moreover, it should be examined if the endurance training plus post-exercise protein supplementation induced fibre type transformation.

The current study used the same protein dose in the training study (Chapter 4) as the non-training study (Chapter 3) and this may have been insufficient to see any mitochondrial training adaptations. Along with investigating the level of stress hormones such as cortisol (Hill, Zack

et al. 2008) in animals because of involuntary treadmill running and examining if this effects training adaptations would be worthwhile.

Furthermore, Chapter 5 provided interesting results showing that both WPI and calcium caseinates have a similar effect on mitochondrial adaptations. However, more long-term studies are needed in athlete's comparing the effects of different proteins on mitochondrial adaptations. Future research is warranted to investigate the effect of whole foods compare to isolated proteins. While acute studies provide an important look at what happens in the short term, whether this transfers into longer term muscular adaptation is unclear.

In addition to muscle protein synthesis pathways, future investigations should focus on the role of different pathways for muscle protein breakdown in adaptation to endurance exercise and the impact of post-exercise protein ingestion. In acute studies, micellar casein has been demonstrated to reduce muscle protein breakdown compared to WPI, resulting in more positive protein balance (Boirie, Dangin et al. 1997). It is clear that nutrition may influence the degradative side of the adaptation to exercise training (Tipton 2008), but very little information exists on the role of protein, particularly in long-term human studies.

6.3.3 Endurance exercise performance

Chapter 4 demonstrated WPI and micellar caseins extended time to exhaustion in rats compared to CHO group. This result occurred without any change in mitochondrial function, as measured by mitochondrial respiration. Further analysis of skeletal muscle to investigate muscle

glycogen stores and intramuscular triglyceride stores, to identify if protein supplementation had a greater effect on energy storage would be beneficial. This has been identified as one method for protein improving performance when carbohydrate intake is suboptimal (Berardi, Price et al. 2006). Not that carbohydrate was necessarily suboptimal in the current study as rats had *ad libitum* access to standard chow. In addition, measuring markers of muscle damage to identify if the training program had a greater effect on muscle damage in CHO and this may be a mechanism for the reduced time to exhaustion.

In addition to muscular fatigue playing a role in endurance exercise performance, central fatigue may be another mechanism. Central fatigue is largely unexplored, but there is increasing evidence that exercise-induced increases in the plasma free tryptophan/BCAA ratio are associated with increased brain serotonin and the onset of fatigue during prolonged exercise (Davis 1995). There are also strong theoretical grounds for a beneficial role of nutrition in delaying central fatigue with dietary supplementation with BCAA's by reducing tryptophan uptake and also brain serotonin synthesis and release, thereby delaying fatigue (Newsholme and Blomstrand 2006). However, this theory needs further investigation to determine if BCAA's in dairy protein would have the same effect as supplemental BCCA's.

A potential limitation of the study detailed in Chapter 5, that may have effected performance, was controlling only two training sessions and not collecting data from participants training sessions outside of the study. These two session may not have been sufficient for trained cyclist to obtain further adaptation. On the other side, participants may have been over training and this may have resulted in the lack of adaptation. The total energy, CHO and protein intake in

the current study would not have been sufficient to support a period of overtraining. Training periodization is the principle of cyclical training design within an athletes training program, that contains meso (weeks) and macro (months) cycles that make up the yearly plan (Issurin 2010). For future long-term studies, it may be beneficial to design an 8-week training block for cyclist, leading in to a major race to encourage participation and adherence or investigate a 12-week base training phase. This training program would be accompanied by the above mentioned dietary control strategies to investigate post-exercise protein supplementation. Controlling a one full macro cycle of a training plan would potentially ensure training adaptation occurs.

6.3.4 Dietary control

Long-term well controlled dietary studies can be expensive and this may be a barrier to them being carried out. The current study showed that when all meals and snacks are provided for the duration of the study, participants fall short of CHO intake. Another, more cost-effective way, may be to investigate individual participant nutrition knowledge via questionnaire, provide meal plans based on participant's budget, time constraints and likes/dislikes that meets athletic guidelines and have them record intake in an online app. This dietary information would be provided by a sports dietitian/nutritionist familiar with the endurance type training and guidelines. This type of support and education may provide a better framework for participants to understand the importance of reaching macro and micronutrient goals.

6.3.5 Additional factors for future study's

Future studies may benefit from incorporating the 'whole' athlete in measurements due to a number of factors that affect performance and adaptation in an athlete's 'real world' training and competition schedule. While many participants are juggling work and/or study, family commitments, training and potentially a social life, assessing the impact of sleep on long- term studies with the use of accelerometers would also be worthwhile. Sleep can impact the quality of training and or testing throughout a study due to the implications on recovery (Marshall and Turner 2016). Along with monitoring sleep, it would be important to assess immune function and stress hormones and how these impact adaptations to long-term training studies and whether protein supplementation has beneficial effects compared to CHO. This may allow an athlete to train at a greater intensity for a longer period of time.

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