

**SKELETAL MUSCLE CARNITINE METABOLISM
DURING INTENSE EXERCISE IN HUMAN
VOLUNTEERS**

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ABSTRACT

Carnitine plays a key role in the regulation of skeletal muscle metabolism during intense exercise and increasing muscle carnitine content enhances PDC flux during 30 minutes of continuous intense exercise at 80% W_{\max} , reducing reliance on non-mitochondrial ATP production and improving work output. The studies in this thesis evaluated a carnitine feeding strategy that did not rely on the high carbohydrate load previously used to increase muscle carnitine; then further investigated the role of carnitine during intense exercise in healthy volunteers and whether increasing skeletal muscle carnitine content could augment the adaptations to a chronic period of submaximal high-intensity intermittent training (HIT).

Following acute $^2\text{H}_3$ -L-carnitine feeding, the rate of carnitine uptake into skeletal muscle was directly quantified for the first time *in vivo* in humans and shown to be increased up to 5-fold by the ingestion of an 80 g carbohydrate formulation. This promoted a positive forearm carnitine balance, an effect that was entirely blunted when the carbohydrate load was supplemented with 40 g of whey protein, suggesting a novel antagonisation of insulin-stimulated muscle carnitine transport by amino acids.

Skeletal muscle biopsy sampling found acetylcarnitine accumulation and non-mitochondrial ATP production during single-leg knee extension at 85% W_{\max} to be minimal, suggesting that PDC flux is probably not limiting to oxidative ATP production under these exercise conditions. Conversely, PDC flux appeared to decline over repeated bouts of intense cycling exercise at 100% W_{\max} , as evidenced by greater non-mitochondrial ATP production in the face of similar acetylcarnitine

accumulation. This identified submaximal HIT as an exercise paradigm during which muscle carnitine availability would be expected to influence oxidative ATP delivery and exercise performance.

Manipulation of muscle carnitine content by daily carnitine and carbohydrate feeding resulted in the elevation of free carnitine availability and maintenance of PDC flux during repeated bouts of intense exercise. However, profound improvements in oxidative ATP delivery in response to submaximal HIT during the second bout eclipsed any effect of this carnitine-mediated increase in PDC flux on non-mitochondrial ATP production and indeed, carnitine supplementation did not potentiate any of the increases in maximal workload, oxygen consumption or exercise capacity above submaximal HIT alone.

Collectively, these novel data further advance our understanding of skeletal muscle carnitine transport and the interplay between carnitine metabolism, PDC flux and non-mitochondrial ATP production during intense exercise. These findings have important implications for the development of nutritional and exercise prescription strategies to enhance human performance and health in both athletic and clinical populations.

PUBLICATIONS

Conference Abstracts

CE Shannon, PL Greenhaff & FB Stephens. Optimising an oral insulinotropic formulation for L-carnitine feeding in humans. Presented at the *European Congress of Sports Sciences*, Brugges (2012).

CE Shannon, AV Nixon, PL Greenhaff & FB Stephens. Protein ingestion impairs insulin-stimulated net muscle carnitine uptake in healthy young men. Presented at *Experimental Biology Conference*, San Diego (2014).

CE Shannon, R Ghasemi, PL Greenhaff & FB Stephens. Increasing muscle total carnitine content maintains PDC flux during repeated bouts of very intense exercise. Presented at the *European Congress of Sports Sciences*, Amsterdam (2014).

Manuscripts

FB Stephens, C Chee, BT Wall, AJ Murton, CE Shannon, LJC van Loon, K Tsintzas (2014). Lipid induced insulin resistance is associated with an impaired skeletal muscle protein synthetic response to amino acid ingestion in healthy young men. *Diabetes*, In Press.

FB Stephens, B Mendis, CE Shannon, S Cooper, CA Ortori, DA Barrett, P Mansell, K Tsintzas (2014). Fish oil omega-3 fatty acids partially prevent lipid induced insulin resistance in human skeletal muscle without limiting acylcarnitine accumulation. *Clin Sci*, 127, 315-22.

FB Stephens, BT Wall, K Marimuthu, CE Shannon, D Constantin-Teodosiu, IA MacDonald, PL Greenhaff (2013). Skeletal muscle carnitine loading increases energy expenditure, modulates fuel metabolism gene networks and prevents body fat accumulation in humans. *J Physiol.*, 591, 4655-66.

S Awad, FB Stephens, CE Shannon, DN Lobo (2012). Perioperative perturbations in carnitine metabolism are attenuated by preoperative carbohydrate treatment: Another mechanism by which preoperative feeding may attenuate development of postoperative insulin resistance. *Clin Nutr.*, 31, 717-20.

DECLARATION

All of the work presented in this thesis was the result of procedures conducted by myself, including all *in vivo* human exercise tests, blood and skeletal muscle preparation, expired gas analysis and biochemical tissue analysis, with the following exceptions:

In Chapter 3, cannulations for study 1A were performed by Ian Bennett and cannulations and measurements of forearm blood flow in study 1B were performed by Aline Nixon. In Chapter 4, all human experiments for study 2A were completed by Bente Stallknecht and colleagues at the University of Copenhagen. Muscle biopsies for Chapter 4 (study 2B) and Chapter 5 were carried out by Reza Ghasemi.

I hereby declare that the present thesis has been composed by myself and that it is a record of the work performed by myself, except where assistance has been acknowledged. No part of this thesis has been submitted in any other application for a higher degree and all sources of information have been appropriately referenced.

Signed: _____

Chris Shannon

Date: _____

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LIST OF ABBREVIATIONS

ACS	acyl-CoA synthetase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATB ^{0,+}	neutral amino acid transport (
ATP	adenosine triphosphate
Ca ²⁺	calcium ion
CACT	carnitine acylcarnitine translocase
CAT	carnitine acetyltransferase
CoASH	free coenzyme A
CPT1	carnitine palmitoyltransferase 1
CPT2	carnitine palmitoyltransferase 2
Cr	free creatine
dw	dry muscle weight
DCA	dichloroacetate
FFA	free fatty acid
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
H ⁺	hydrogen ion
HK	hexokinase
IMCL	intramyocellular lipid
IMP	inosine monophosphate
K ⁺	potassium ion
kcal	kilocalories
kJ	kilojoules

K_m	Michaelis-Menten constant
LCFA	long-chain fatty acyl-CoA
LDH	lactate dehydrogenase
μM	micromolar
μmol	micromoles
mM	millimolar
mmol	millimoles
mU	standard international milli units
Na^+	sodium ion
NAD^+	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
OCTN2	novel organic cation transporter 2
PCr	phosphocreatine
Pi	inorganic phosphate
PDC	pyruvate dehydrogenase complex
PDCa	activation status of the PDC
PDK	pyruvate dehydrogenase kinase
PFK	phosphofructokinase
TAG	triacylglycerol
TCA	tricarboxylic acid cycle
$\text{VO}_{2\text{max}}$	maximal rate of oxygen uptake
ww	wet muscle weight
W_{max}	maximal workload during an incremental exercise test

CHAPTER 1: GENERAL INTRODUCTION

1.1 Regulation of fuel metabolism during exercise

1.1.1 Overview

History

Skeletal muscle contractile activity is a prerequisite for all locomotive tasks and relies exclusively on the chemical energy derivable from the hydrolysis of adenosine triphosphate (ATP). At the start of the 20th century and prior to the discovery of ATP, it was widely believed that muscle contraction was supported solely by the direct combustion of oxygen. Following the identification of ATP in 1950, Cain and Davies (1962) were able to demonstrate that the energy required to perform a single muscle contraction was identical to that lost through the decrease in ATP content when resynthesis was inhibited using fluoro-dinitrobenzene, thus proving that ATP was explicitly responsible for the energy necessary to fuel muscle contraction. Experiments in amphibian muscle by Fletcher and Hopkins (1907) established that lactate accumulated during contraction in the absence of oxygen and it was acknowledged at this time that this could be associated with contraction-induced fatigue. Work from the pioneering exercise physiologist AV Hill led to the view that “lacticacidogen” (now known as fructose-1-6-diphosphate), an intermediate in the conversion of glycogen to lactate, was responsible for the energy derivable from anaerobic glycolysis. The discovery of phosphocreatine in skeletal muscle (Eggleton and Eggleton, 1927), together with a series of experiments from Hartree and Hill (1928) and Lundsgaard (1938) reconciled the existence of two distinct oxygen-independent pathways of energy production. In 1933, Margaria and colleagues investigated the relationship between oxygen consumption and blood lactate concentrations during exercise and recovery in human volunteers. They proposed that the difference between the oxygen consumption and the total energy demand of

the work done (the so-called oxygen deficit) must indeed be dependent on both a lactic and an “alactic” component, but were unable to measure any indication of intramuscular phosphocreatine breakdown at this time. The advent of muscle biopsy sampling in human volunteers, largely facilitated by the introduction of Bergstrom’s percutaneous needle technique (Bergstrom & Hultman, 1966), allowed for the direct measurement of skeletal muscle metabolism during exercise. Muscle glycogen availability was identified as a crucial and manipulable determinant of exercise capacity and fatigue development during submaximal exercise (Bergström *et al.*, 1967; Bergström & Hultman, 1967). During more intense exercise, studies from Karlsson and Saltin (Karlsson *et al.*, 1970; Karlsson & Saltin, 1970) assessed the role of intramuscular ATP, PCr and lactate concentrations in the development of fatigue. They also related the magnitude of change in these metabolites to the oxygen deficit, i.e. the shortfall between total ATP demand and mitochondrial ATP production.

Routes of ATP Resynthesis

The primary ATP dependent processes in contracting skeletal muscle involve the myosin, Ca^{2+} and Na^+/K^+ ATPase enzymes, which are responsible for approximately 70, 25 and <5%, respectively, of total ATP consumption. During high-intensity exercise, ATP demand can increase over 100-fold above resting turnover rates and as such, requires a dynamic and highly regulated matching from ATP resynthesis pathways to defend the limited intramuscular ATP store (20-25 $\text{mmol}\cdot\text{kg dw}^{-1}$). Illustrated in **Figure 1.1**, the principle routes for the resynthesis of ATP within skeletal muscle are the hydrolysis of phosphocreatine, the glycolytic conversion of glucose-6-phosphate to pyruvate and the mitochondrial oxidation of acetyl-CoA. Glucose-6-phosphate is derivable from the phosphorylation of either blood-borne

glucose or intramuscular glycogen, whilst mitochondrial acetyl-CoA is generated mainly from the decarboxylation of pyruvate and from the β -oxidation of fatty acids. The generation of acetyl-CoA from both carbohydrate and lipid precursors relies on the availability of skeletal muscle carnitine which, through the carnitine acyltransferase class of enzymes is able to reversibly bind coenzyme-A esters of various chain lengths. This reversible binding facilitates the translocation of long-chain fatty acyl groups into the mitochondrial matrix for subsequent β -oxidation and independently promotes the conversion of pyruvate to acetyl-CoA by buffering intramitochondrial acetyl-group production. The importance of these roles during exercise will be discussed further in **section 1.2**.

The main substrates that fuel skeletal muscle contraction are listed in **Table 1.1**. Mitochondrial ATP resynthesis is by far the most efficient pathway for the maintenance of intramuscular ATP concentrations, yielding 38 or 39 mmols of ATP for every one mmol of glucose or glycogen phosphorylated, respectively. Lipid substrates provide an even greater energy yield, with 106 mmols ATP obtained from the mitochondrial oxidation of one mmol palmitate. Adipose tissue triacylglycerides also represent the most abundant substrate for ATP resynthesis in the body, with the average lean male having approximately 10 kg adipose tissue, compared to whole body carbohydrate stores (mainly muscle and liver glycogen) of only 0.5 kg (Maughan, 1997). Given the greater ATP mole yield associated with fatty acid oxidation, this equates to an almost 50-fold larger energy store of lipids than carbohydrates. In contrast to mitochondrial oxidation, glycolysis provides only three mmol ATP per mmol glucose, whilst the degradation of PCr resynthesizes ATP with a 1:1 ratio.

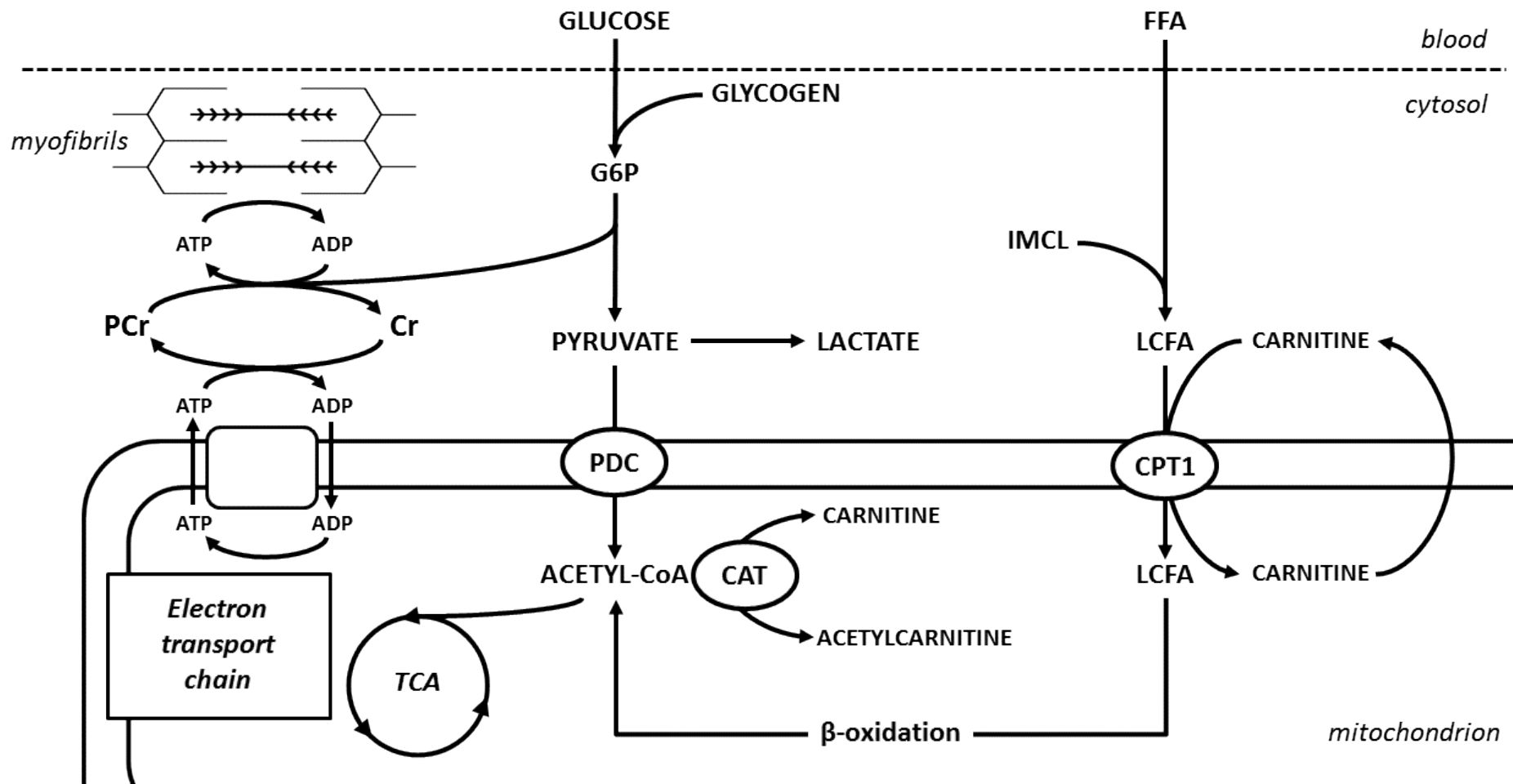


Figure 1.1 Schematic showing pathways of ATP resynthesis in skeletal muscle.

Reciprocally, the maximal rate of mitochondrial ATP production is about half that which can be achieved from glycolysis and approximately one sixth that which can be obtained from the combined non-mitochondrial reactions (glycolysis plus PCr; see **Table 1.1**). These differences in the total energy yield and rate of ATP delivery from a given substrate mean that fuel selection within skeletal muscle is largely dictated by the intensity and duration of muscle activation. It is also important to consider that different sub-populations of muscle fibres exist within skeletal muscle, which can broadly be classified as type I (slow) or type II (fast). These two fibre types possess distinct biochemical characteristics (see **Table 1.2**) and their pattern of recruitment, again largely determined by the exercise intensity, will influence the metabolic responses to an exercise challenge. Under most exercise scenarios, mitochondrial oxidation predominates as the major route of ATP resynthesis. However, when the rate of ATP hydrolysis exceeds the capacity of mitochondrial ATP delivery, as can occur during high-intensity exercise, reliance on non-mitochondrial ATP production must increase. In the next sections, the governance of fuel selection by exercise intensity is described in more detail, with reference given to how this determines the likely limitations to exercise performance. Although theoretically represented as a continuum, exercise intensities will be categorised into prolonged submaximal exercise, high-intensity submaximal exercise and maximal (i.e. requiring maximal effort) exercise, including repeated bouts.

Table 1.1 Maximal rates and capacity for ATP resynthesis from different substrates

Pathway	Maximal Rate (mmol·kg dw⁻¹·s⁻¹)	Capacity for ATP delivery (mmol·kg dw⁻¹)
PCr hydrolysis	9	80
Glycolysis	5.3	300
Glycogen oxidation	2.5	10000
Lipid oxidation	1	>500,000

Values are expressed in mmol of ATP per kg dry muscle mass and are based on Bangsbo *et al.* (1990), Hultman *et al.* (1991) and Maughan (1997).

Table 1.2 Biochemical characteristics of type I and type II muscle fibres

Fibre type characteristic	Type I	Type II
Glycogen content ^a	400	480
PCr content ^a	80	90
PFK activity ^b	7.5	15.6
Glycogen phosphorylase activity ^b	2.8	7.3
Citrate synthase activity ^b	10.8	7.6

a [mmol·kg dw⁻¹] from Soderlund *et al.* (1992); b [mmol·min⁻¹·kg ww⁻¹] from (Maughan and Gleeson, 2004), values of type II fibres represent the average of values for type IIa and type IIx.

1.1.2 Prolonged submaximal exercise

Submaximal exercise intensities refer to workloads where the rate of ATP resynthesis required to sustain muscle contraction is lower than the maximal rate that can be supported by mitochondrial ATP delivery alone. Strictly speaking, this equates to any workload that requires steady-state oxygen consumption rates below the maximal obtainable rate (100% $\text{VO}_{2\text{max}}$). However, as will be discussed, beyond exercise intensities of ~85% $\text{VO}_{2\text{max}}$ (or even lower for untrained persons), a steady-state oxygen consumption rate is rarely attained (Hill *et al.*, 2002) and exercise time is often restricted by factors different to those which limit exercise performance at lower intensities. Therefore, prolonged submaximal exercise generally refers to steady-state exercise intensities below 85% $\text{VO}_{2\text{max}}$. As mentioned above, mitochondrial ATP resynthesis is sustained utilising acetyl-CoA derived primarily from lipid or carbohydrate substrates. The relative proportions of these substrates contributing to total ATP delivery is principally an acute function of exercise intensity, but is also altered by other factors including exercise duration (Edwards *et al.*, 1934), diet (Roepstorff *et al.*, 2005), gender (Venables *et al.*, 2005) and training status (Bergman & Brooks, 1999).

Lipid availability to skeletal muscle is dependent upon the appropriate lipolysis of adipose tissue TAG stores, release of these liberated fatty acids into the circulation and effective uptake of the fatty acids across the sarcolemma. In addition, intramyocellular lipid droplets (IMCL) provide a viable pool of fatty acids to the mitochondria and represent an important substrate during exercise (van Loon *et al.*, 2003). As will be discussed, under most exercise conditions the rate of ATP resynthesis attributable to lipid oxidation is regulated at the level of the

mitochondrion and limited by the entry of long-chain fatty acids into the organelle matrix. In line with the lower maximal rate of ATP resynthesis that can be supported by lipids compared to carbohydrate, it has long been known from respiratory exchange measurements made on expired gases that muscle contraction relies more heavily on lipid oxidation during low-intensity exercise than during high-intensity exercise (Krogh & Lindhard, 1920). More recent studies investigating whole-body fat oxidation rates over a number of exercise increments suggest that the absolute rate of lipid oxidation during low to moderate intensity exercise increases linearly with ATP demand, with maximal rates of lipid oxidation occurring at an exercise intensity between 50 – 65% $\text{VO}_{2\text{max}}$ (Achten *et al.*, 2002; Achten & Jeukendrup, 2003; Achten *et al.*, 2003; Achten & Jeukendrup, 2004). When the exercise intensity is increased beyond that which elicits maximal fatty acid oxidation rates, reliance on lipid substrates drops sharply and, when measured at the whole-body level, can be almost negligible beyond approximately 80% $\text{VO}_{2\text{max}}$ in untrained volunteers.

The development of stable isotope tracer methodology has enabled a more detailed evaluation of the effects of exercise intensity on skeletal muscle substrate selection. For example, based on the plasma disappearance of [6,6- $^2\text{H}_2$]glucose and [$^2\text{H}_2$]palmitate, combined with indirect calorimetry measurements, Romijn and colleagues (1993) determined total fat and carbohydrate oxidation rates in volunteers exercising for 30 minutes each at 25, 65 and 85% $\text{VO}_{2\text{max}}$. Whilst estimated rates of carbohydrate oxidation increased linearly with exercise intensity, lipid oxidation only increased between the initial two workloads, from 27 to 43 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, before declining at 85% $\text{VO}_{2\text{max}}$ to 30 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$. A subsequent study from van Loon *et al.* (2001) further illustrated that at rest and during low (40% $\text{VO}_{2\text{max}}$) to

moderate (57% $\text{VO}_{2\text{max}}$) intensity exercise, the relative contribution of lipid oxidation to total energy expenditure was reasonably consistent at approximately 50%. However, when the exercise intensity was increased to 72% $\text{VO}_{2\text{max}}$, [U- ^{13}C]palmitate oxidation declined, reflecting a reduction in both the absolute and relative rate of total fat oxidation. The additional employment of muscle biopsy sampling in this latter study confirmed that the reduction in whole-body lipid oxidation coincided with an increased reliance on muscle glycogen stores, such that the glycogenolytic rate increased from 0.9 $\text{mmol}\cdot\text{kg dw}^{-1}\cdot\text{min}^{-1}$ at 57% $\text{VO}_{2\text{max}}$ to 6.2 $\text{mmol}\cdot\text{kg dw}^{-1}\cdot\text{min}^{-1}$ at 72% $\text{VO}_{2\text{max}}$. Moreover, lactate accumulation at the highest workload (29.5 $\text{mmol}\cdot\text{kg dw}^{-1}$) was also double that of the moderate intensity workload (15.6 $\text{mmol}\cdot\text{kg dw}^{-1}$). This accelerated glycolytic rate is likely responsible for the restriction of lipid oxidation at higher exercise intensities, although the mechanisms behind this are not fully understood. The authors of the aforementioned study proposed that, under these conditions, free carnitine availability could become limiting to mitochondrial fatty acid oxidation (van Loon *et al.*, 2001), a discussion point that will be developed in **section 1.2**.

The greater rate of muscle glycogenolysis seen with increasing exercise intensities is likely related, at least in part, to the progressive recruitment of type II muscle fibres during more intense exercise. Indeed, single fibre staining for glycogen revealed significantly more glycogen remaining in type II fibres compared to type I, which were mostly glycogen depleted, following three hours of cycling exercise at 31% $\text{VO}_{2\text{max}}$ (Gollnick *et al.*, 1974). In the same study, the proportion of both fibre types staining negative for glycogen increased progressively during exercise at 64% (two hours) and 83% $\text{VO}_{2\text{max}}$ (one hour), which was consistent with the greater rates of

whole-muscle glycogenolysis as intensity increased (0.7, 2.2 and 5.0 mmol·kg dw⁻¹·min⁻¹, respectively). Biochemical measurement of fibre type-specific glycogenolysis confirms these findings, with over twice the glycogen utilisation seen in type I (195 mmol·kg dw⁻¹) compared to type II fibres (90 mmol·kg dw⁻¹) over the first 15 minutes of one-legged exercise at 61% VO_{2max} (Ball-Burnett *et al.*, 1991). In both fibre types, the rate of muscle glycogen utilisation was greater over the first 15 minutes of exercise compared to the remainder of the bout (~two hours in total). Similarly, muscle lactate increased from a resting value of 9.3 mmol·kg dw⁻¹, to 50.4 mmol·kg dw⁻¹ after 15 minutes of exercise before declining to 25.5 mmol·kg dw⁻¹ after one hour. This increased reliance on anaerobic glycolysis during the initial several minutes of exercise likely reflects the delay in mitochondrial carbohydrate oxidation in reaching the required rate of ATP resynthesis. This point will be considered further in the **section 1.1.3**. Above exercise intensities of ~65% VO_{2max}, where muscle glycogen becomes the predominant substrate, it is intuitive that muscle glycogen reserves might become the limiting factor in exercise capacity. This was classically demonstrated by a series of studies from Bergstrom and Hultman in the 1960s. By manipulating the pre-exercise muscle glycogen levels they were able to demonstrate a close relationship between resting glycogen availability and the time to exhaustion during exercise at 75% VO_{2max}, which coincided with the near complete depletion of muscle glycogen stores (Bergström & Hultman, 1967). They further observed that consuming a carbohydrate-rich diet following glycogen depleting one-legged cycling exercise increased resting muscle glycogen of the exercised leg (normally about 300-400 mmol·kg dw⁻¹) to approximately 900 mmol·kg dw⁻¹, with no change in the non-exercised leg (Bergstrom & Hultman,

1966). This illustrates one of the most robust adaptations to exercise training in skeletal muscle, that is, an increase in substrate availability.

1.1.3 High-intensity submaximal exercise

High-intensity submaximal exercise refers to exercise workloads above approximately 85% $\text{VO}_{2\text{max}}$, which generally represent non-steady state conditions whereby oxygen consumption will trend towards the maximal attainable rate (Poole *et al.*, 1988; Hill *et al.*, 2002), but can be sustained at a fixed workload for more than a few seconds. Theoretically, exercise at 100% $\text{VO}_{2\text{max}}$ could be sustained by the maximal capacity of mitochondrial respiration using muscle glycogen as the sole fuel. However, glycogen oxidation takes several minutes to reach its maximal rate and thus during this “lag” period, non-mitochondrial sources of ATP resynthesis must cover the energy deficit. High-intensity exercise is thus characterised not only by the eventual requirement for a near-maximal mitochondrial respiration, but also the immediate mismatch in mitochondrial ATP delivery and total ATP demand. The intramuscular ATP store ($25 \text{ mmol}\cdot\text{kg dw}^{-1}$), in isolation, could sustain intense muscle contraction for less than one second and thus alternative pathways for ATP resynthesis must be rapidly activated.

One of the earliest studies to investigate the time-course of non-mitochondrial ATP delivery during high-intensity exercise was that by Karlsson and Saltin in 1970. They demonstrated that during exhaustive constant load exercise bouts at 90 - 125% $\text{VO}_{2\text{max}}$, skeletal muscle PCr was degraded to $\sim 20 \text{ mmol}\cdot\text{kg dw}^{-1}$, approximately 25% of resting values, within the first two minutes of exercise for each workload. Lactate also accumulated most rapidly over this initial period, reaching values of ~ 80

mmol·kg dw⁻¹ within two minutes and continuing to increase until exhaustion. Furthermore, the ATP resynthesis from these two pathways was closely related to the oxygen deficit during exercise, particularly at the highest workloads. The products of ATP hydrolysis (ADP and AMP) are powerful allosteric activators of creatine kinase and phosphofructokinase, the rate-limiting enzymes in PCr degradation and glycolysis, respectively, and signal to accelerate the rate of ATP delivery to match ATP demand. During a ten minute bout of cycling at 90% VO_{2max}, AMP and ADP concentrations in skeletal muscle were found to increase approximately 10-fold and 3-fold, respectively, over the first minute of exercise (Howlett *et al.*, 1998). Together with the rapid degradation of phosphocreatine (PCr was less than 50% of resting values within one minute of exercise) and the reciprocal elevation of inorganic phosphate, accumulation of these metabolites was associated with the activation of glycolytic and mitochondrial ATP production. Despite the latter, substantial lactate accumulation occurred throughout the exercise bout, reaching values in excess of 100 mmol·kg dw⁻¹ by 10 minutes. This reflects an imbalance between pyruvate production from glycolysis and pyruvate oxidation, particularly during the initial minute of exercise, when the rate of lactate accumulation was greatest (31 mmol·kg dw⁻¹·min⁻¹). As will be discussed below, the metabolic events occurring during the early seconds/minutes of high-intensity exercise may be crucial in the development of fatigue later in the bout.

Unlike prolonged, submaximal exercise, fatigue during high-intensity exercise is less easily explained by substrate availability. Indeed, in the study by Karlson and Saltin (1970), muscle glycogen content at exhaustion was above 100 mmol·kg dw⁻¹ and thus, presumably not limiting, for all workloads. Furthermore, the constancy of PCr

concentrations ($\sim 20 \text{ mmol}\cdot\text{kg dw}^{-1}$) from two minutes until exhaustion at 90% and 100% $\text{VO}_{2\text{max}}$ (approximately 16 minutes and 6 minutes, respectively) would suggest that the continued rate of PCr hydrolysis equalled its resynthesis from mitochondrial ADP phosphorylation (Perry *et al.*, 2012) for the remainder of the exercise. Thus, neither muscle glycogen nor PCr depletion could be considered as limiting to exercise performance at these workloads. Instead, fatigue is commonly cited as resulting from substantial lactate production, the accompanying accumulation of hydrogen ions and the reciprocal decline in muscle pH that occur during high-intensity exercise. Allosteric inhibition of the key glycolytic enzymes glycogen phosphorylase (Chasiotis *et al.*, 1983) and phosphofructokinase (Trivedi & Danforth, 1966) by muscle acidosis has been proposed to limit ATP turnover during high-intensity exercise. However, Bangsbo *et al.* (1996) have reported that following the elevation of systemic lactate concentrations by prior intense arm exercise, glycogenolysis ($\sim 35 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{min}^{-1}$) was unchanged during a subsequent bout of exhaustive leg-extension exercise, despite a lower muscle pH and a 25% reduction in exercise time. Thus it would appear that the acidosis-induced inhibition of glycolytic enzymes can be overcome in contracting human muscle. This of course does not rule out any negative effects on hydrogen ion accumulation or acidosis on other aspects of the contractile cascade (Gladden, 2004), some of which will be discussed in the subsequent section (see **section 1.14**). In support of this, interventions to increase the hydrogen ion buffering capacity of skeletal muscle by β -alanine supplementation (Hill *et al.*, 2007), sodium bicarbonate (Bird *et al.*, 1995) or sodium citrate (McNaughton & Cedaro, 1992) ingestion have all been shown to increase high-intensity exercise performance. Consistent with the likely role of accelerated lactate production in the development of fatigue, the oxidation of

pyruvate, or its reciprocal conversion to lactate, represents a key branch-point in skeletal muscle metabolism. Moreover, the rapid activation of non-mitochondrial pathways of ATP delivery at the onset of high-intensity and maximal exercise is vital to compensate for the comparatively slow activation of mitochondrial ATP resynthesis. The reason that carbohydrate oxidation is more slowly activated than non-mitochondrial ATP resynthesis may be related to the proximity of the phosphocreatine stores to the site of ATP utilisation or the greater number of enzymatic reactions involved. In particular, substrate flux through the pyruvate dehydrogenase complex (PDC), the rate-limiting enzyme in the conversion of pyruvate to acetyl-CoA, has been studied with regard to its regulatory role in mitochondrial ATP production.

The pyruvate dehydrogenase complex

Carbohydrate oxidation initiates with the glycolysis of skeletal muscle glycogen or glucose to pyruvate in the cytosol. Depending on the redox state of the cell, pyruvate can either be reduced to lactate via the lactate dehydrogenase reaction or converted to acetyl-CoA by the PDC. Pyruvate can also enter a number of other pathways, including the anaplerotic/gluconeogenic reactions catalysed by alanine aminotransferase and pyruvate carboxykinase, but these are quantitatively less important in energy metabolism. The PDC is a mitochondrial membrane-bound multi-enzyme complex that catalyses the irreversible decarboxylation of pyruvate to acetyl-CoA and is considered one of the rate-controlling reactions in carbohydrate oxidation. The catalytic activity of the PDC is regulated by covalent transformation between its active and inactive forms (Linn *et al.*, 1969), as well as by end-product inhibition. Transformation of the PDC to its active form (PDCa) is determined by the

competing activities of the PDC phosphatases (activators) and kinases (deactivators), whilst in-vitro observations suggest end-product inhibition to be principally regulated by the ratios of acetyl-CoA to CoASH and NADH to NAD⁺ (Pettit *et al.*, 1975). However, measurements of PDCa in human skeletal muscle biopsies would suggest that exercise is able to override the inhibition by acetyl-CoA accumulation on PDC transformation to its active form, possibly via calcium-stimulated phosphatase activation (Constantin-Teodosiu *et al.*, 2004). Flux through the PDC ultimately determines the fate of pyruvate and is thus considered a branch-point between mitochondrial and non-mitochondrial ATP resynthesis.

The Acetyl-group Deficit

As previously discussed, the deficit between total ATP demand and mitochondrial ATP delivery is most expansive under non-steady state conditions. These occur at the onset of exercise or during the transition to a higher exercise increment and were classically thought to result from a delay in the oxygen transport pathway (Margaria *et al.*, 1933). However, a series of studies from Timmons and colleagues indicated that the delay in mitochondrial ATP delivery could be due to a lag in the rate of PDC activation at the onset of contraction (Timmons *et al.*, 1996; Timmons *et al.*, 1997; Timmons *et al.*, 1998a; Timmons *et al.*, 1998b). Using a perfused hind-limb contraction model in canine gracilis muscle, with which blood-flow and oxygen delivery can be kept constant, it was demonstrated that maximal activation of the PDC (using the pharmacological PDK antagonist dichloroacetate; [DCA]) reduced reliance on non-mitochondrial ATP production during 20 minutes of subsequent contraction (Timmons *et al.*, 1996). Consistent with the suspected role of inorganic phosphate and hydrogen ion accumulation in the impairment of contractile function, DCA administration also attenuated the decline in force production by 40%. A

parallel study using the same model demonstrated that this reduction in PCr hydrolysis and lactate accumulation was most prominent over the first minute of contraction (Timmons *et al.*, 1997), findings that were later reproduced in humans performing eight minutes of single leg extension exercise (Timmons *et al.*, 1998a; Timmons *et al.*, 1998b).

One apparent paradox of the studies from Timmons and colleagues is the observation that acetylcarnitine accumulation, an indication of excessive PDC flux relative to the TCA cycle, is greatest during the initial minutes of exercise (**Figure 1.5**), when PDC flux is proposed to be limiting mitochondrial ATP production. However, using the same canine contraction model as previously employed, Roberts *et al.* (2002, 2005) sampled multiple muscle biopsies over the first minute of contraction, affording a much greater temporal resolution than previous studies. This approach demonstrated that during the first 20 seconds of contraction, acetylcarnitine and acetyl-CoA concentrations tended to decrease from baseline values, providing unequivocal evidence of the existence of a mitochondrial inertia at the level of the PDC. Moreover, DCA administration prior to the start of muscle contraction reduced PCr hydrolysis and lactate accumulation, with the majority of this effect attributable to the first 20 seconds of contraction (Roberts *et al.*, 2002). Similar results were obtained when acetyl-group availability was increased independently of changes in PDC activation, by the infusion of sodium acetate (Roberts *et al.*, 2005). The authors concluded that the pre-contraction accumulation of acetylcarnitine and acetyl-CoA facilitated a greater acetyl-group flux into the TCA cycle, thus increasing the contribution from mitochondrial respiration to total ATP resynthesis. In this respect, any intervention that can accelerate the delivery of acetyl-CoA to the TCA cycle has potential to reduce reliance on non-mitochondrial ATP production. It is of note that

manipulation of the muscle carnitine pool has been demonstrated to influence PDC flux and acetyl-group availability to the TCA cycle (Wall *et al.*, 2011), a facet that will be explored further in **section 1.2**.

Not all studies have demonstrated an effect of DCA administration on mitochondrial ATP delivery. For example, (Savasi *et al.*, 2002) reported that whilst maximal activation of the PDC with DCA prior to a 90 second exercise bout at 90% $\text{VO}_{2\text{max}}$ was sufficient to elevate acetyl-group availability (increasing acetyl-CoA 4-fold and acetylcarnitine 5-fold compared to a control condition), no differences were observed in PCr degradation or lactate accumulation at either 30 seconds or 90 seconds of exercise. It has been argued that increasing acetyl-group availability is unlikely to enhance mitochondrial ATP delivery when the initial rate of PCr degradation is greater than $\sim 1 \text{ mmol}\cdot\text{kg}^{-1}\cdot\text{s}^{-1}$ (Timmons *et al.*, 2004), as was the rate in the study by Savasi and colleagues. It is also important to consider that under certain conditions PDC activation may be uncoupled from PDC flux (Putman *et al.*, 1993) and, theoretically, the pharmacologically-induced stockpiling of acetyl-groups at rest may actually serve to dampen further increases in PDC flux during subsequent exercise.

1.1.4 Maximal exercise

Maximal exercise efforts, i.e. not constant load, place the greatest demand possible on ATP resynthesis pathways and ATP delivery declines in parallel with work output. By means of example, the average rate of skeletal muscle PCr degradation during the first six seconds of a 30 second maximal cycle sprint was found to approximately $4.9 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{s}^{-1}$ (Boobis, 1983). Over the subsequent 24 s the average rate of PCr degradation had fallen to approximately $1.1 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{s}^{-1}$.

During the same exercise bout, ATP production derived from muscle lactate accumulation averaged $4.8 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{s}^{-1}$ over the initial six seconds and was $3.8 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{s}^{-1}$ over the subsequent 24 seconds. The progressively lower rates of PCr hydrolysis and lactate accumulation over the 30 seconds was associated with a corresponding decline in work output, such that the average power during the first six seconds was 847 watts, compared to 649 watts when averaged over the entire 30 second bout. Similar rates of PCr degradation and glycolytic ATP delivery have been observed over comparable time periods of electrical stimulation, which can be refined to elicit near-maximal intensity involuntary contractions (Hultman *et al.*, 1991). Using this model in combination with single muscle fibre analysis, Soderlund *et al.* (1992) demonstrated that the rate of total ATP turnover was substantially greater in type II fibres ($13.4 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{s}^{-1}$) compared to type I fibres ($4.9 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{s}^{-1}$), over 20 seconds of intermittent (1.6:1.6 seconds; 50Hz) stimulation. This was attributable to a greater rate of PCr degradation in type II fibres, particularly during the first 10 seconds of contraction (5.3 vs $3.3 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{s}^{-1}$), as well as a high glycogenolytic rate in these fibres ($6.3 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{s}^{-1}$) compared to a negligible rate in type I fibres ($0.6 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{s}^{-1}$). Using similar electrical stimulation protocols, epinephrine infusion (Greenhaff *et al.*, 1991) and blood flow occlusion (Greenhaff *et al.*, 1993) have independently been shown to accelerate the glycogenolytic rate in type I fibres (6 and 11-fold, respectively) but not type II fibres over 64 seconds of contraction. These data suggest that glycolytic ATP delivery from type II fibres is near-maximal under these conditions and are consistent with the maximal reported activity of glycogen phosphorylase in each fibre type (**Table 1.2**)

During maximal exercise, glycolytic rates begin to decline within 10-20 seconds of contraction, whilst the rate of PCr hydrolysis declines almost immediately following the onset of contraction (Hultman *et al.*, 1991). Fatigue during maximal effort exercise ultimately results from this inability to maintain the extremely high rates of ATP delivery required at these workloads. Furthermore, the reduction in the overall rate of non-mitochondrial ATP delivery would appear to be primarily related to the capacity of the type II muscle fibres. For example, the rate of PCr degradation in type II fibres declined by more than half between 10 and 20 seconds of maximal contraction, whilst it remained relatively constant in type I fibres (Soderlund *et al.*, 1992). Similarly, the fall in the glycolytic rate in mixed-muscle from 20-30 seconds could be almost entirely accounted for by a 45% reduction in type II fibre glycogenolysis. The authors proposed that the high oxidative capacity of the type I muscle fibres enables continued resynthesis of ATP at a rate sufficient to match PCr hydrolysis in these fibres. Conversely, the low oxidative capacity of the type II muscle fibres prohibits the matching of mitochondrial ATP resynthesis to the high rate of PCr hydrolysis, resulting in a rapid decline in type II fibre PCr availability. Coupled with the concomitant decline in type II fibre glycolysis, this precludes the further production of non-mitochondrial ATP delivery at the rate necessary to sustain maximal muscle contraction. Aside from the possible direct effects of myofibrillar PCr depletion, the impairment of contractile function during high-intensity or maximal exercise may also be related to the disruption of sarcoplasmic reticulum calcium cycling, secondary to local reductions in phosphocreatine availability (Duke & Steele, 2001). Furthermore, the reciprocal accumulation of inorganic phosphate that accompanies PCr hydrolysis has also been implicated in the development of fatigue under these conditions. For example, it was shown that force production in

intact skeletal muscle was enhanced in the presence of pyruvate compared to glucose or lactate, an effect apparently mediated by a concurrent lowering of myocellular inorganic phosphate concentrations (Phillips *et al.*, 1993). A number of specific detrimental effects of inorganic phosphate accumulation on contractile function have been proposed, including the direct or indirect (reduced calcium sensitivity) interference with cross-bridge formation and the possible impairment of sarcoplasmic reticulum calcium release or uptake (Westerblad *et al.*, 2002). Finally, it has been speculated that muscle potassium efflux during high-intensity exercise may result in the accumulation of extracellular potassium and subsequently contribute to the development of fatigue (Bangsbo *et al.*, 1996). This is supported by the finding that the enhancement of fatigue-resistance gained following exercise training was associated with lower interstitial potassium accumulation during high-intensity one-legged exercise (Nielsen *et al.*, 2004).

Repeated bouts of exercise

Exercise paradigms that involve repeated bouts of high-intensity exercise are a useful tool for the study of metabolic regulation during skeletal muscle contraction, demanding substantial ATP delivery from both mitochondrial and non-mitochondrial pathways. For example, Gaitanos *et al.* (1993) demonstrated that across a series of six-second maximal bicycle sprints separated by 10 seconds of recovery, the rate of glycolytic ATP production fell markedly from $6.6 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{s}^{-1}$ during the first sprint, to $0.9 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{s}^{-1}$ during the tenth bout, whilst PCr degradation also declined, from 7.4 to $4.2 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{s}^{-1}$. Similarly, the reported contributions from anaerobic glycolysis, PCr degradation and mitochondrial oxidation to total ATP production during an initial 30 second bout of maximal sprint exercise (300-

400% VO_2max) range between 50-55, 23-28 and 16-28%, respectively (Bogdanis *et al.*, 1996; Trump *et al.*, 1996; Parolin *et al.*, 1999). However, during a third bout, following four minutes recovery between bouts, the absolute rate of non-mitochondrial ATP production declines such that anaerobic glycolysis and PCr degradation each provide at most 15% of the total ATP resynthesis, with the relative contribution from mitochondrial ATP delivery increasing to approximately 70%. Given the lower rates of ATP production that can be sustained by mitochondrial oxidation compared to anaerobic glycolysis and PCr degradation, this shift in substrate metabolism is associated with a reduction in the overall rate of ATP production. Moreover, when repeated bouts of maximal effort exercise are performed, work output declines in parallel with total ATP production over successive bouts (McCartney *et al.*, 1986; Spriet *et al.*, 1989; Trump *et al.*, 1996; Parolin *et al.*, 1999).

Given the near-equilibrium rate constant of the creatine kinase reaction during recovery from high-intensity exercise (Sahlin *et al.*, 1979), the reduction in PCr degradation over repeated bouts of exercise is likely related to an incomplete resynthesis of PCr between bouts. Trump *et al.* (1996) investigated the influence of PCr recovery on skeletal muscle metabolism during three 30 second maximal bicycle sprints by occluding blood flow to one leg between bouts two and three. Occlusion prevented PCr resynthesis such that prior to the start of bout three, the PCr concentration in the occluded leg ($21 \text{ mmol}\cdot\text{kg dw}^{-1}$) was one third that of the non-occluded leg ($63 \text{ mmol}\cdot\text{kg dw}^{-1}$). The blunted ability to resynthesize ATP from PCr hydrolysis in the following bout was associated with a 15% reduction in work output compared to the non-occluded leg (5.8 vs 6.8 kJ, respectively). Positive correlations

have been reported between the extent of PCr resynthesis during recovery and the work output attained in a subsequent bout, whilst the recovery of muscle pH does not seem to influence subsequent power output (Bogdanis *et al.*, 1995; Bogdanis *et al.*, 1996). This supports the notion that PCr availability *per se*, rather than muscle acidosis, is responsible for the progressive decline in ATP production over repeated bouts of maximal exercise. In particular, and consistent with the lower oxidative capacity of type II muscle fibres (**Table 1.2**), it has been suggested that lower rates of PCr resynthesis in these muscle fibres during recovery may be a primary determinant of the decline in work output during repeated bouts of high-intensity exercise (Casey *et al.*, 1996).

The mechanisms that govern the reduction in glycolytic rate with successive high-intensity exercise bouts are less clear though have been proposed to be related to the inhibitory effect of hydrogen ion accumulation on glycogen phosphorylase activity (Spriet *et al.*, 1989). However, given the accumulation of other key glycolytic activators during high-intensity exercise (ADP, AMP, Pi, IMP), it would seem unlikely that allosteric modulation of phosphorylase can wholly explain the reduction in lactate accumulation during repeated bouts. Putman *et al.* (1995) calculated that the ATP delivered from PDC flux increased progressively over three maximal 30 second cycle sprints, such that it equalled 220 mmol in bout 1, 255 mmol in bout 2 and 357 mmol in bout 3. This was attributed to the maintenance of a sequentially greater (2.2 and 2.8-fold) pre-bout PDC activation above resting levels ($0.45 \text{ mmol}\cdot\text{kg ww}^{-1}\cdot\text{min}^{-1}$), as PDCa was maximal ($\sim 3.0 \text{ mmol}\cdot\text{kg ww}^{-1}\cdot\text{min}^{-1}$) by the completion of each bout. As the overall work output decreased with each sprint (19.3, 16.3 and 14.2 kJ, respectively), PDC flux accounted for a progressively

greater proportion of total ATP production (29, 33 and 63%, respectively) and thus may partially explain the lower lactate accumulation during latter bouts. Relatively little is known about the metabolic responses to repeated bouts of high-intensity non-maximal exercise, where the workload can be fixed and thus total ATP demand maintained constant over successive bouts. Manipulation of PDC flux by carnitine under these conditions, where PDC is likely to be activated more slowly than during maximal exercise, may represent a possible strategy to enhance mitochondrial ATP delivery and improve exercise performance.

1.2 Carnitine metabolism during exercise

The increase in ATP turnover during exercise requires the integration of greatly accelerated rates of fat and carbohydrate oxidation. As mentioned previously, both these pathways depend upon adequate carnitine availability and changes in the free carnitine pool during exercise are closely linked with changes in substrate utilisation in skeletal muscle. Carnitine (3-hydroxy-4-*N*-trimethylaminobutyric acid) is a naturally occurring quaternary amine compound, synthesised from the amino acids lysine and methionine. Being a zwitterionic molecule, carnitine contains a positively charged amine and a negatively charged carboxyl group, as well as a centrally positioned hydroxyl group (**Figure 1.2**). This hydroxyl group functions as a binding site for acyl residues and is fundamental to the two primary biochemical roles of carnitine in skeletal muscle, where around 95% of whole body carnitine stores are sequestered (Brass, 1995).

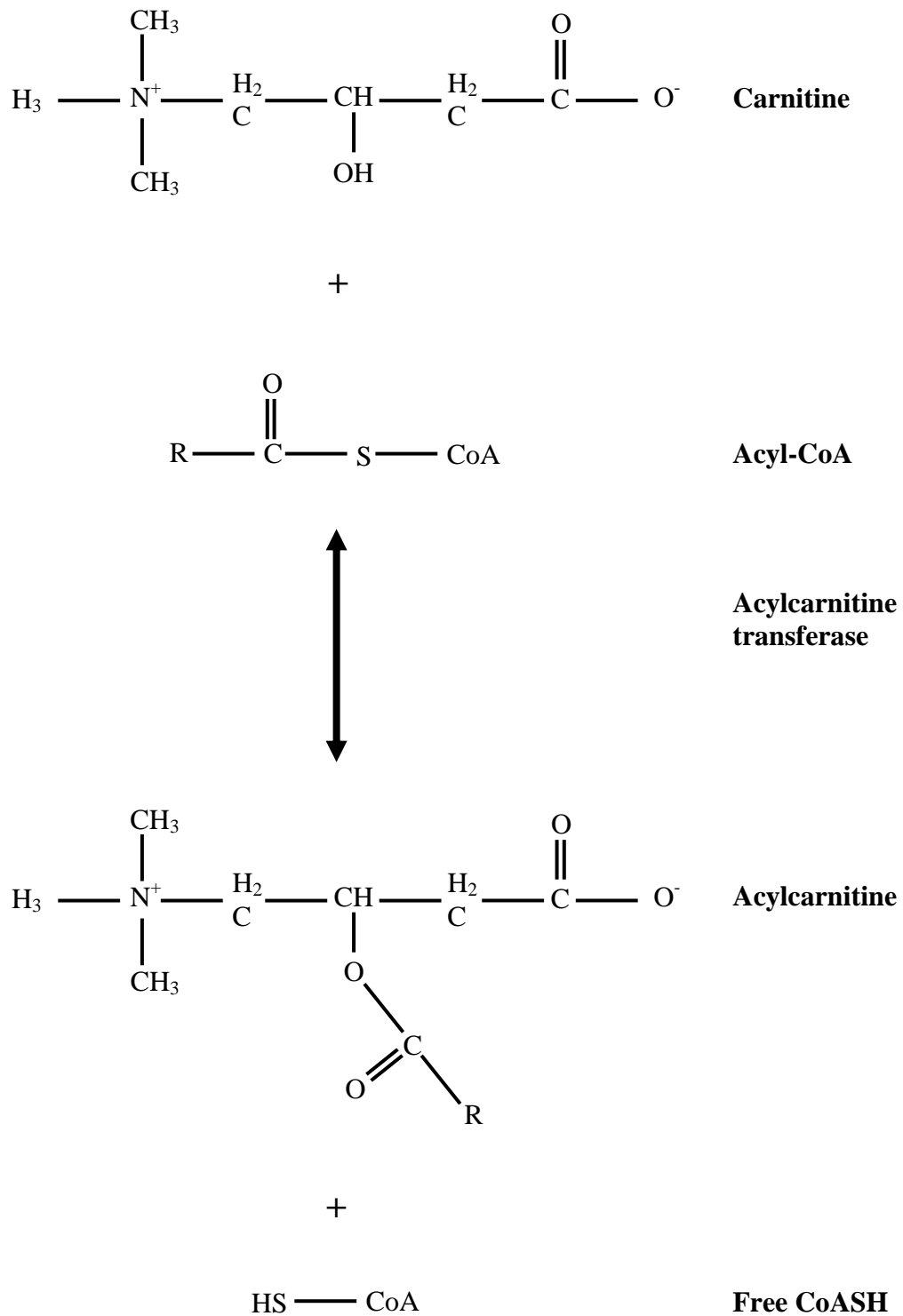


Figure 1.2 Structure of carnitine and the acyltransferase reaction

1.2.1 Carnitine regulates fat oxidation during exercise

Translocation of long chain fatty acids

Mitochondrial combustion of lipids is one of the most vital pathways for energy production in humans. In skeletal muscle, the primary substrates for mitochondrial lipid oxidation are cytosolic long-chain fatty acids, derived either from plasma-borne free fatty acids, or the lipolysis of plasma lipoprotein- or intramuscular-triglyceride depots. Once activated to their respective CoA esters via acyl-CoA synthetase, long-chain fatty acyl-CoAs must be converted to acylcarnitine esters before they are able to permeate the mitochondrial membranes and access the matrix enzymes of the β -oxidation pathway (Fritz & Yue, 1963). This reversible trans-esterification is facilitated by carnitine palmitoyltransferase 1 (CPT1) and is illustrated schematically in **Figure 1.3**. Owing to the close association between ACS and CPT1 within the contact sites of the outer mitochondrial membrane (Hoppel *et al.*, 1998; Hoppel *et al.*, 2002), activated long-chain acyl-CoAs would appear to be preferentially trafficked towards the mitochondrial matrix for subsequent oxidation. Once formed, acylcarnitines are translocated from CPT1, on its cytosolic-facing site of the outer mitochondrial membrane (Murthy & Pande, 1987; Fraser *et al.*, 1997), to CPT2 on the inner mitochondrial membrane (Woeltje *et al.*, 1987). This process necessitates a stoichiometric 1:1 exchange of the cytosolic acylcarnitine with intra-mitochondrial free carnitine by the enzyme carnitine acylcarnitine translocase (CACT). Upon exposure to CPT2 acylcarnitine is trans-esterified back to free carnitine and LCFA-CoA, which can then be sequentially cleaved of two-carbon acetyl-CoA units by the enzymes of the β -oxidation cycle. The free carnitine product of CPT2 is subsequently available to participate in the CACT reaction and as such, under

normal resting conditions mitochondrial fatty acid flux can be sustained with minimal disturbance to the free carnitine pool.

Carnitine deficiencies impair fat metabolism

The paramount involvement of carnitine and its associated enzymes in fat oxidation is highlighted by multiple pathophysiological scenarios in humans. For example, systemic carnitine deficiency (Engel & Angelini, 1973); chronic haemodialysis, which results in secondary carnitine deficiency (Calvani *et al.*, 2004; Evans *et al.*, 2004); or genetic mutations in CPT2 (Orngreen *et al.*, 2005) all result in marked suppression of whole-body lipid oxidation, increased intramuscular lipid storage and impairments in exercise performance. Furthermore, pharmacological partial blockade of CPT1 via Etomoxir administration acutely recapitulates these perturbations of lipid metabolism in healthy volunteers (Hinderling *et al.*, 2002). The absence of reports concerning human genetic defects in CPT1 would suggest that these are incompatible with life and indeed homozygous knockout of the skeletal muscle isoform of CPT1 is embryonically lethal in mice (Ji *et al.*, 2008). Interestingly, genetic variants in the skeletal muscle CTP1 gene, particularly the E531K variant, have recently been linked with lower rates of post-exercise fat oxidation (Gomez-Gomez *et al.*, 2014) and an increased prevalence of obesity and the metabolic syndrome in humans (Robitaille *et al.*, 2007; Auinger *et al.*, 2013).

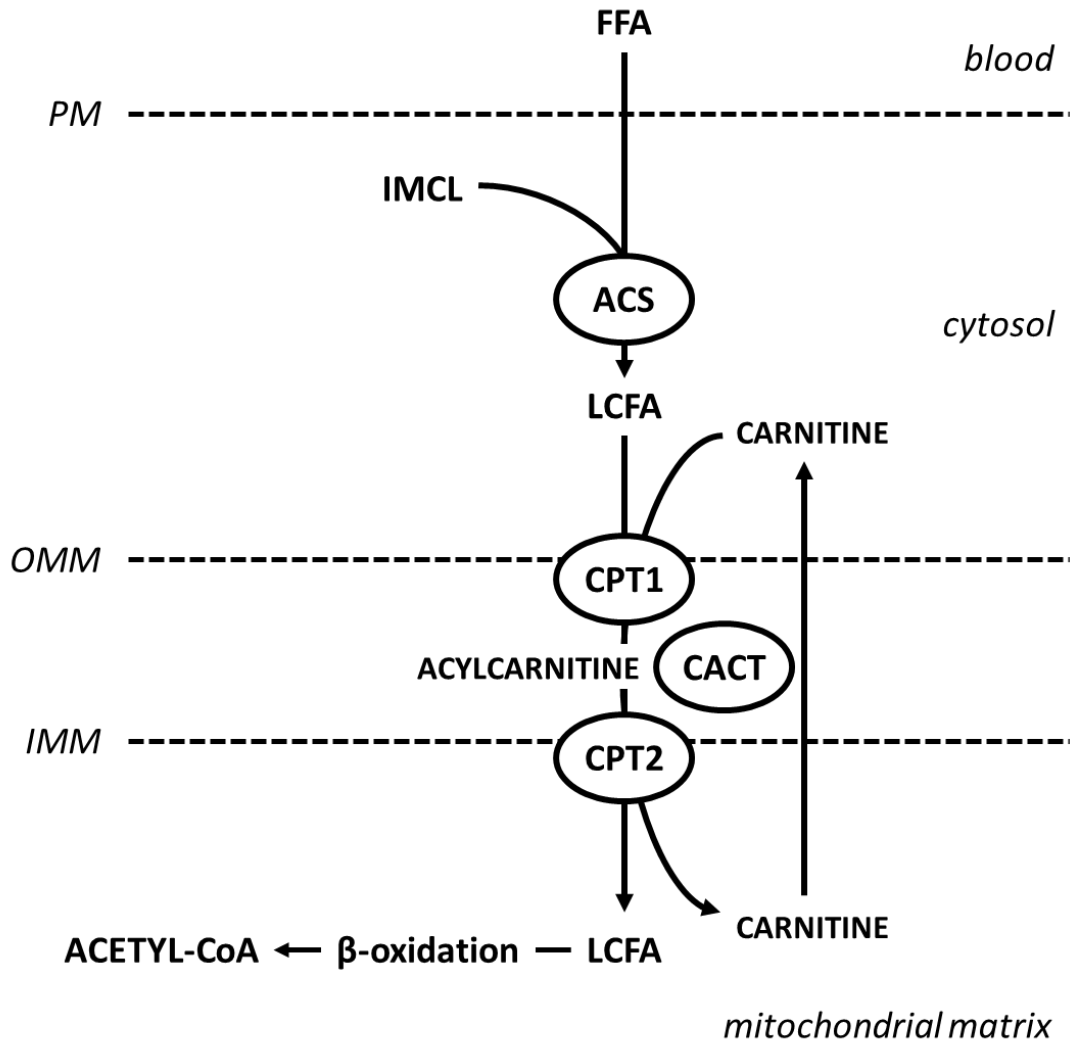


Figure 1.3 Schematic illustrating the role carnitine in fatty acid oxidation. PM plasma membrane, OMM outer mitochondrial membrane, IMM inner mitochondrial membrane.

Carnitine acetylation limits CPT1 flux

As previously discussed, above exercise intensities of approximately 65% $\text{VO}_{2\text{max}}$, there is a sharp decline in the absolute rate of whole body fat oxidation. Concurrent with an acceleration of glycolytic rate, PDC flux is also increased at higher exercise intensities and is associated with the formation of acetylcarnitine (see Acetyl Group Buffering). Studies in human volunteers confirm that the increase in acetylcarnitine concentrations within skeletal muscle during exercise of increasing intensity is paralleled by a reciprocal decline in free carnitine content (Constantin-Teodosiu *et al.*, 1991). The first evidence that this acetylation of the free carnitine pool was mechanistically responsible for the decline in lipid oxidation was provided by van Loon *et al.* (2001), who observed a 35% reduction in [^{13}C]-palmitate oxidation during cycling exercise at 72% $\text{VO}_{2\text{max}}$ compared to 57% $\text{VO}_{2\text{max}}$. This coincided with a lowering of intramuscular free carnitine concentrations to $5.6 \text{ mmol}\cdot\text{kg dw}^{-1}$, or approximately 30% of the total carnitine pool (excluding long-chain acylcarnitine), which was argued could possibly limit CPT1 flux and led to the hypothesis that increasing skeletal muscle free carnitine availability could alleviate this inhibition of fat oxidation. It has since been demonstrated that increasing the skeletal muscle carnitine pool via a chronic nutritional strategy, such that the free carnitine concentration during a 30 minute bout of cycling exercise at 80% $\text{VO}_{2\text{max}}$ was $8.8 \text{ mmol}\cdot\text{kg dw}^{-1}$, was unable to prevent the decline in whole-body lipid oxidation (Wall *et al.*, 2011). Although free carnitine availability in this study was likely increased above the intracellular concentration expected to limit CPT1 flux (0.5 mM; McGarry *et al.*, 1989) it still only represented ~30% of the total carnitine pool. Thus, the marked decline in lipid oxidation during exercise at intensities above ~65% $\text{VO}_{2\text{max}}$ is paralleled by a rapid acetylation of the free carnitine pool, whereas

acetylcarnitine accumulation is far more moderate at lower exercise intensities, when lipid oxidation is still increasing (see **Figure 1.5**). As a consequence of these (and other) findings, it has recently been proposed that the absolute rate of lipid oxidation will decline when carnitine acetylation reaches 50% of the total carnitine pool (Stephens & Galloway, 2013). This hypothesis predicts that at such exercise intensities, the intra-mitochondrial free carnitine concentration will become limiting to the CACT reaction flux, which will in turn restrict the delivery of free carnitine to the cytosolic site of CPT1 and inhibit fatty acid entry into the mitochondria. This acknowledges that an elevation of the total muscle carnitine pool will not only increase free carnitine availability to CPT1, but also to the CAT reaction. As such, this intervention might only be expected to increase the rate of lipid oxidation under conditions of low glycolytic flux.

An additional implication of this working model is that free carnitine availability will be limiting to CPT1 flux at any exercise intensity, even when lipid oxidation is increasing. This is consistent with the observation that acetylcarnitine concentrations generally decline with progressive exercise durations at these intensities (**Figure 1.5**). For example, Watt *et al.* (2002) found that the increase in plasma fatty acid oxidation with prolonged (four hours), moderate intensity (57% $\text{VO}_{2\text{max}}$) exercise coincided with a reduction in PDC activation and a return in acetylcarnitine concentrations to resting values. This also aligns with the finding that increasing muscle total carnitine content by 21%, such that free carnitine availability was increased from 14.5 to 19.6 $\text{mmol}\cdot\text{kg dw}^{-1}$, resulted in a 31% suppression of PDCa during 30 minutes of exercise at 50% $\text{VO}_{2\text{max}}$ (Wall *et al.*, 2011). This was associated with a near-halving of glycogen utilisation (from 49.9 to 26.9 $\text{mmol}\cdot\text{kg}$

dw⁻¹) and has subsequently been shown to concomitantly increase whole body fat oxidation rates by about 10%, from 0.61 to 0.67 mg min⁻¹·kg⁻¹ lean mass (Stephens *et al.*, 2013). Interestingly, the latter study found that this carnitine-mediated increase in fat oxidation was able to prevent the 1.8 kg gain in fat mass observed in the control group (attributed to chronic carbohydrate overfeeding), and was reflected in the modulation of related metabolic gene networks. These findings were consistent with earlier work from this group, demonstrating that acute elevation of the muscle carnitine compartment was associated with a blunting of resting PDCa and glycogen utilisation (Stephens *et al.*, 2006a). The authors concluded that the increase in free carnitine availability had promoted a greater CPT1-mediated oxidation of lipids, as supported by increased long-chain acyl-CoA content, leading to an elevation of mitochondrial acetyl-CoA availability and thus driving the suppression of PDC and glycogenolytic fluxes (Garland & Randle, 1964).

1.2.2 Carnitine maintains PDC flux during exercise

Acetyl group buffering

As previously discussed, at rest and during steady-state low intensity exercise (<50% VO_{2max}) the contribution from fat oxidation to total energy expenditure is 55-80%, with the remaining portion fulfilled almost entirely by carbohydrate oxidation (Romijn *et al.*, 1993; van Loon *et al.*, 2001). Reciprocally, non-mitochondrial ATP generation (and thus glycolytic flux) proceeds at a relatively low rate. Specifically, pyruvate-derived acetyl group production through the PDC is well matched to acetyl-CoA flux through the tricarboxylic acid cycle. However, during times of accelerated glycolytic flux, as occurs at higher exercise intensities (>70% VO_{2max}), acetyl-CoA production from the decarboxylation of pyruvate would appear to exceed

its rate of condensation with oxaloacetate. The resting muscle CoASH pool is approximately $15 \mu\text{mol}\cdot\text{kg} \text{wm}^{-1}$ which, in isolation, would be sufficient to support maximal PDC flux of $20\text{-}25 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{kg} \text{wm}^{-1}$ (Constantin-Teodosiu *et al.*, 1991) for less than one second of intense muscular contraction. If acetyl-group delivery was supported solely by the formation of acetyl-CoA, this rapid acetylation of the mitochondrial CoASH pool would prevent succinyl-CoA formation via the α -ketoglutarate dehydrogenase reaction, resulting in the complete inhibition of TCA cycle flux. By reversibly binding to mitochondrial acetyl-groups, carnitine prevents this depletion of the CoA pool, facilitating an increase in the catalytic activity of the PDC (**Figure 1.4**). Experiments on blowfly flight muscle by Childress and Sacktor (1966) demonstrated that the tissue had a carnitine content comparable to human skeletal muscle ($\sim 4 \text{mmol}\cdot\text{kg} \text{ww}$), despite lacking the capacity to oxidise fatty acids. The findings that carnitine enhanced the conversion of pyruvate to acetyl-CoA and that acetylcarnitine accumulated during flight (4-fold increase) led to the hypothesis that free carnitine buffers excess acetyl-CoA. The formation of acetylcarnitine from carnitine and acetyl-CoA is catalysed by the mitochondrial matrix enzyme carnitine acetyltransferase (CAT). Compared to relatively small pool of CoASH in skeletal muscle ($\sim 90 \mu\text{mol}\cdot\text{kg} \text{dw}^{-1}$), free carnitine ($\sim 20 \text{mmol}\cdot\text{kg} \text{dw}^{-1}$) provides a far greater capacity for acetyl-group transfer during conditions of accelerated mitochondrial acetyl-group delivery. Indeed, the extent of acetylcarnitine accumulation in muscle during exercise can be an order of magnitude higher than any increase in acetyl-CoA concentration (Constantin-Teodosiu *et al.*, 1992). The observations of Childress and colleagues were subsequently confirmed in contracting frog (Alkonyi *et al.*, 1975), rat (Carter *et al.*, 1981), and human (Constantin-Teodosiu *et al.*, 1991; Constantin-Teodosiu *et al.*, 1992, 1993) skeletal muscle and it was proposed that this acetyl-

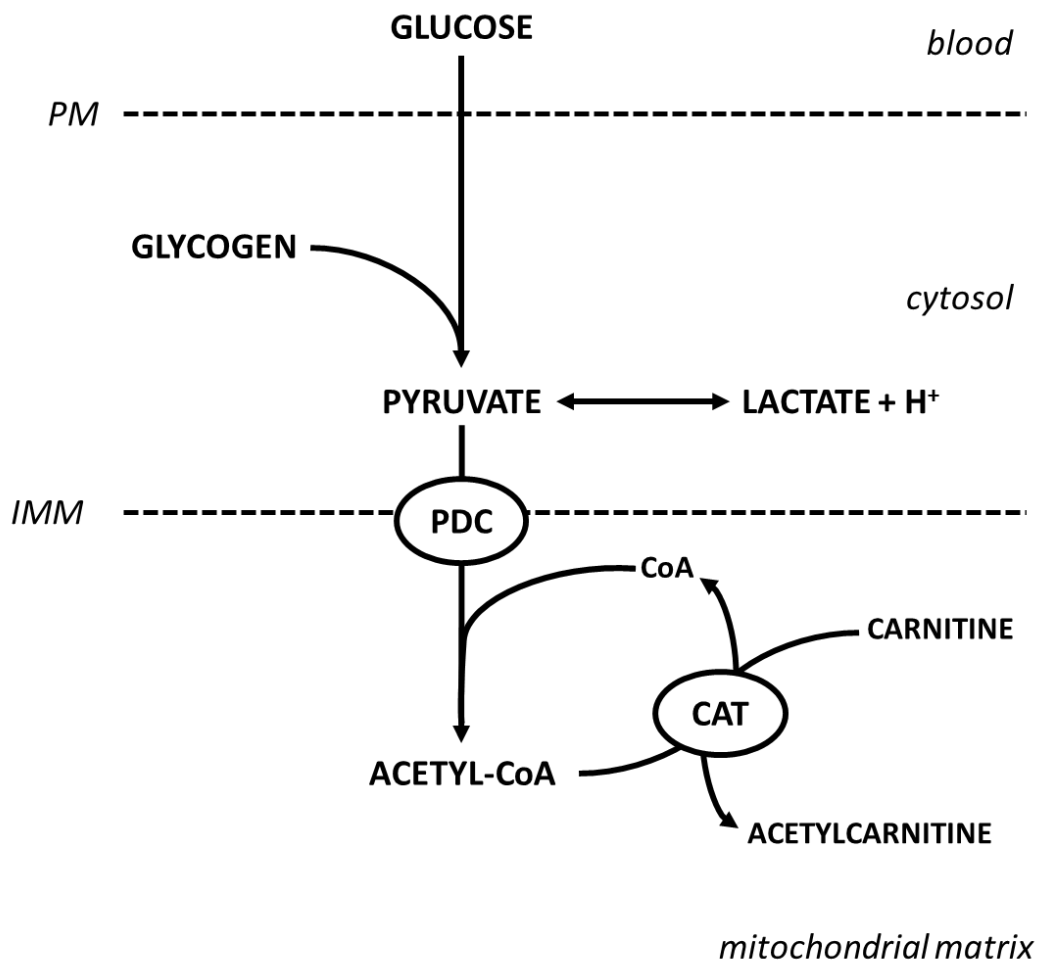


Figure 1.4 Schematic showing role of carnitine in acetyl-group buffering. PM plasma membrane, IMM inner mitochondrial membrane.

buffering role of carnitine and the CAT reaction was tightly coupled to the activity of the PDC.

Acetylcarnitine formation during exercise

The formation of acetylcarnitine by CAT is crucial to the maintenance of the high rates of carbohydrate oxidation evoked by high-intensity exercise. A comparison of 25 published studies that have measured acetylcarnitine formation during exercise of various intensities and durations demonstrates that acetylcarnitine accumulates most rapidly during the initial minutes of exercise, suggesting that the CAT reaction is particularly important over this period (**Figure 1.5**). Consistent with the greater dependence on non-mitochondrial ATP delivery during the first few minutes of exercise, acetylcarnitine accumulation would also appear to suggest that PDC flux is accelerated more rapidly than TCA cycle flux over this period. During exercise at 75% $\text{VO}_{2\text{max}}$ and above, most studies report that acetylcarnitine remains elevated or continues to accumulate, albeit at a lesser rate, for the remainder of exercise (Sahlin, 1990; Constantin-Teodosiu *et al.*, 1992; Leblanc *et al.*, 2004). Thus, once the initial mitochondrial inertia has been overcome (see Acetyl-group Deficit) and oxidative ATP delivery begins to approach the total ATP demand, PDC flux and hence acetyl-group availability would appear to become better matched to the demands of the TCA cycle. However, estimates of in vivo TCA cycle flux have challenged this concept.

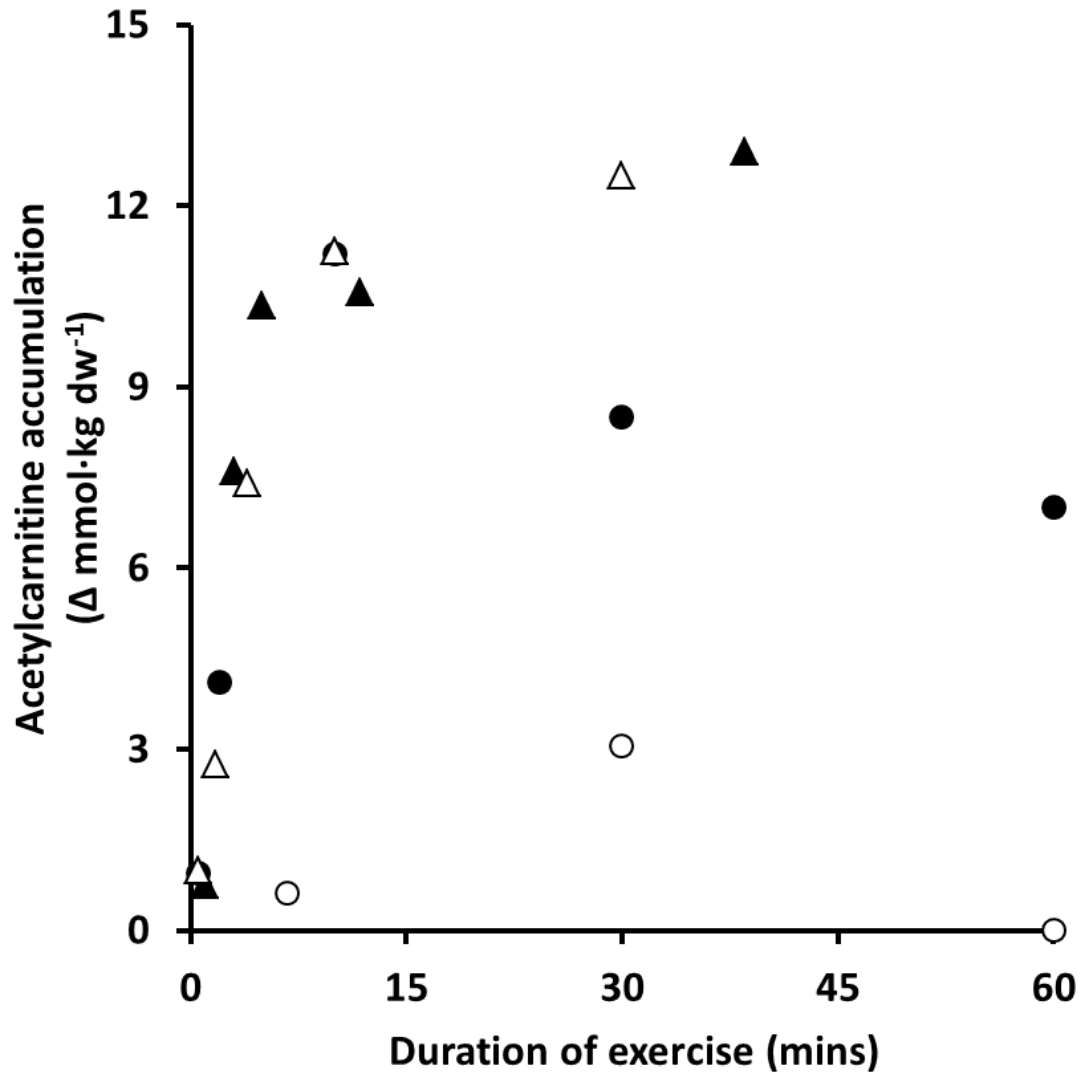


Figure 1.5 Acetylcarnitine formation at different exercise intensities.

Open circles <50% VO_{2max}, closed circles 65% VO_{2max}, closed triangles 75% VO_{2max}, open triangles 90% VO_{2max}. Values are averaged from studies that have determined acetylcarnitine content in skeletal muscle before and after voluntary exercise of various intensities and durations (Harris *et al.*, 1987; Hiatt *et al.*, 1989; Sahlin, 1990; Constantin-Teodosiu *et al.*, 1991; Constantin-Teodosiu *et al.*, 1992; Spencer *et al.*, 1992; Putman *et al.*, 1993; Putman *et al.*, 1995; Howlett *et al.*, 1998; Howlett *et al.*, 1999a; Parolin *et al.*, 2000; St Amand *et al.*, 2000; Tsintzas *et al.*, 2000; van Loon *et al.*, 2001; Campbell-O'Sullivan *et al.*, 2002; Gibala *et al.*, 2002a; Gibala *et al.*, 2002b; Savasi *et al.*, 2002; Leblanc *et al.*, 2004; Roepstorff *et al.*, 2005; Sahlin *et al.*, 2005; Vollaard *et al.*, 2009; Marwood *et al.*, 2010; Wall *et al.*, 2011).

Gibala *et al.* (1998) found that during five minutes of leg extension exercise at 60% maximal exercise capacity, in-vitro determination of PDCa accounted for only 77% of the estimated TCA cycle flux (calculated from leg O₂ uptake). In this exercise model it is quite likely that lipid oxidation provides a substantial portion of the acetyl-groups entering the TCA cycle (Helge *et al.*, 2007), even during more intense workloads. Moreover, the relatively low muscle mass recruited during single-leg knee extension lessens the restrictions imposed by circulating catecholamine concentrations and local vasoconstriction on the active muscle oxygen uptake, compared to two-legged cycling exercise (Helge *et al.*, 2007; Boushel & Saltin, 2013). With these considerations, it might be expected that total TCA cycle flux be greater than flux through the PDC under the exercise conditions employed by Gibala and colleagues. Indeed, the maximal activity of citrate synthase, the enzyme regulating acetyl-group entry into the TCA cycle, is approximately 10-fold greater than that of the PDC (Rasmussen *et al.*, 2001). It is also interesting to note that the reported K_m of the CAT reaction for acetyl-CoA (40 μM; Childress & Sacktor, 1966) is substantially lower than that of the condensation of acetyl-CoA with oxaloacetate (500 μM; Newsholme & Leech, 1983), raising the possibility that under certain conditions acetyl-CoA could be preferentially converted to acetylcarnitine over entry to the TCA cycle. Consistent with the finding that acetylcarnitine concentrations tend to decline during 20 seconds of electrical stimulation (Roberts *et al.*, 2002), acetylcarnitine accumulation is negligible during a single bout of maximal cycling exercise, lasting either ten (Howlett *et al.*, 1999b) or 30 seconds (Parolin *et al.*, 1999). Under these conditions, mitochondrial (oxidative) ATP delivery cannot approach the required rate of ATP hydrolysis and the duration of exercise is probably insufficient for an acetyl-group excess to develop. Indeed, whole body

oxygen uptake reaches at most 60% and 85% of $\text{VO}_{2\text{max}}$ within 15 and 30 seconds of maximal cycle sprinting (Putman *et al.*, 1995), reflecting a submaximal mitochondrial respiration.

Carnitine matches glycolytic and TCA cycle fluxes

As discussed, acetylation of the free carnitine pool indicates that acetyl-group delivery from the PDC exceeds acetyl-CoA entry into the TCA cycle. Similarly, lactate accumulation during exercise largely reflects an imbalance between pyruvate formation (i.e. glycolytic rate) and disposal through the PDC. Thus during high-intensity exercise, the simultaneous accumulation of both lactate and acetylcarnitine can be considered to reflect an overall imbalance between glycolytic and TCA cycle fluxes. It has been proposed that the availability of free carnitine, if limiting to PDC flux, could in part regulate the matching of substrate fluxes in skeletal muscle during high-intensity exercise (Siliprandi *et al.*, 1990). However, the study by Siliprandi and colleagues made no attempt to measure skeletal muscle metabolism and was unlikely to have successfully manipulated muscle carnitine availability (Stephens *et al.*, 2006b). To date, only one study has addressed the impact of manipulating skeletal muscle carnitine content on fuel metabolism during high-intensity exercise (Wall *et al.*, 2011). Elevating muscle total carnitine content by 21% was associated with a 50% increase in PDCa following a 30 minute exercise bout at 80% $\text{VO}_{2\text{max}}$. Acetylcarnitine accumulation also appeared to be greater, suggesting that flux through the PDC had indeed been augmented during the exercise bout. Moreover, the post-exercise PCr/ATP ratio was higher following carnitine loading, implying reduced reliance on PCr degradation during exercise. Glycolytic flux was accelerated following the intervention period, probably as a result of the daily carbohydrate load

needed to increase muscle carnitine uptake, but lactate accumulation was significantly lower in the carnitine fed group than in the control group (carbohydrate only). Altogether, the findings from Wall and colleagues demonstrated that increasing free carnitine availability during high-intensity exercise enabled a greater flux through the PDC, facilitating a better matching of glycolytic and TCA cycle fluxes and thus reducing reliance on non-mitochondrial ATP resynthesis. Importantly, the metabolic impact of muscle carnitine loading translated into an 11% improvement in work output during a subsequent 30 minute performance trial.

Disease states under which skeletal muscle mitochondrial ATP production is impaired are often reflected in perturbed carnitine metabolism. For example, patients suffering from unilateral peripheral arterial disease have reduced resting muscle free carnitine content compared to their non-symptomatic leg (Hiatt *et al.*, 1992). These patients commonly experience exercise-induced claudication which severely limits exercise tolerance and thus any physical task can represent an intense exercise challenge. Hiatt and colleagues also reported that those patients who were able to exercise for longer accumulated greater amounts of acetylcarnitine, whilst resting acetylcarnitine content was a negative predictor of exercise tolerance. Although derangements in carnitine metabolism are perhaps secondary to other features of this disease (e.g. tissue perfusion, mitochondrial volume), these findings demonstrate the importance of acetyl-group buffering in reflecting the metabolic state of skeletal muscle. Indeed, evidence suggests that mitochondrial oxidative capacity is impaired in peripheral arterial disease patients (Hands *et al.*, 1986; Bauer *et al.*, 1999). Furthermore, targeting of the PDC to reduce reliance on non-mitochondrial ATP production may represent a means to increase the efficacy of exercise prescription in

this population (Calvert *et al.*, 2008), or indeed in any clinical population in which mitochondrial ATP production appears impaired (e.g. chronic obstructive pulmonary disease, mitochondrial myopathy).

1.2.3 Novel areas of carnitine metabolism during exercise

The data from the study by Wall *et al.* (2011), described above, suggest that increasing skeletal muscle carnitine content could facilitate the enhancement of oxidative ATP provision during continuous high-intensity exercise, as reflected by the lower rate of PCr degradation and diversion of glycogenolysis away from lactate production. These findings could potentially have a wide applicability, not only as an ergogenic tool to improve athletic performance, but also with respect to the efficacy of exercise prescription in pathologies where mitochondrial ATP production and hence, exercise capacity, may be impaired. However, whether the metabolic alterations observed during 30 minutes of exercise at 80% $\text{VO}_{2\text{max}}$ (Wall *et al.*, 2011) are transferable to other exercise paradigms is unclear and requires further investigation. In order to fully exploit the utility of skeletal muscle carnitine loading, further clarification is needed as to the roles of carnitine metabolism and PDC flux in the facilitation of oxidative ATP production under various exercise conditions.

Carnitine metabolism during single leg exercise

As already discussed, during the initial few minutes of high-intensity exercise, non-mitochondrial ATP-producing pathways are rapidly activated to compensate for the deficit in ATP turnover associated with the more gradual rise in oxidative ATP delivery towards the required ATP demand. Similarly, the formation of acetylcarnitine in skeletal muscle during the first 5-10 minutes of high-intensity

exercise is generally considered to reflect a rate of PDC flux that is in excess of acetyl-group entry to the TCA cycle (**Figure 1.5**). Wall *et al.* (2011) reported that acetylcarnitine accumulation during 30 minutes two-legged cycling exercise at 80% VO_{2max} was more than double that observed during an equivalent bout at 50% VO_{2max} . This demonstrates how the proposed imbalance between PDC and TCA cycle fluxes becomes more exaggerated as the exercise intensity, and thus the demand for non-mitochondrial ATP production, is increased. However, a study from Gibala and co-workers (1998) has challenged this concept, estimating TCA cycle flux to be 10-30% greater than PDC flux over five minute periods of single-legged knee extension exercise. Should acetylcarnitine formation indeed reflect the balance between PDC and TCA cycle fluxes, under these conditions acetylcarnitine accumulation might be considered somewhat paradoxical. As acetylcarnitine metabolism was not determined in the study by Gibala and colleagues, definitive conclusions cannot be drawn on the relevance of acetylcarnitine accumulation during the single-leg knee extension model, when oxidative ATP delivery may not be impeded by the same limitations as during two-legged cycling (Boushel & Saltin, 2013).

Repeated bouts of high-intensity exercise

The progressive increase in the relative contribution from mitochondrial ATP resynthesis to total ATP production is well characterised during repeated sprint (maximal effort) exercise, with a concurrent decline in non-mitochondrial ATP delivery. A confounding factor concerning the investigation of skeletal muscle metabolism during repeated sprints is the inherent reduction in work output over successive bouts. The implementation of a slightly lower exercise intensity (e.g.

100% $\text{VO}_{2\text{max}}$), where the workload can be fixed and sustained over repeated bouts provides an exercise paradigm that relies on a rapid and near-maximal activation of mitochondrial ATP production and yet still demands a substantial non-mitochondrial component. Using this type of paradigm, Bangsbo *et al.* (2001) reported that oxidative ATP delivery increased in a second three minute bout of single-leg knee extension exercise at 100% W_{max} , whilst reliance on non-mitochondrial ATP production declined, relative to the first bout. In line with the aforementioned relationship between PDC flux, oxidative ATP production and acetylcarnitine accumulation, it might be expected that under these conditions, where TCA cycle flux is apparently accelerated during a second exercise bout at a fixed workload, acetylcarnitine accumulation would decline. Acetylcarnitine was not determined in the study by Bangsbo and colleagues and indeed, relatively little is known about skeletal muscle carnitine metabolism during repeated-bout exercise, or during the recovery between exercise bouts and how this might influence subsequent bout performance.

Effects of training on carnitine metabolism during exercise

A limited number of studies have reported data on skeletal muscle carnitine metabolism during exercise in response to training. For example, Leblanc *et al.* (2004) observed a training-induced reduction in PDC activation and acetylcarnitine accumulation during 15 minutes of constant-load cycling exercise at 80% $\text{VO}_{2\text{max}}$. Taken together with the calculation that absolute rates of pyruvate oxidation were lower following training, this would indicate that flux through the PDC was suppressed post-training. Whilst this might initially appear counterintuitive to a training adaptation, the relative proportion of pyruvate production that was diverted

towards oxidation actually increased and was accompanied by lesser reliance on non-mitochondrial ATP delivery, as evidenced by lower PCr degradation, lactate accumulation and glycogenolytic flux. The latter were probably related to a better preservation of resting ADP and AMP concentrations, a finding consistent with the reported increase in maximal citrate synthase activity (an enzymatic marker of mitochondrial content). Finally, a reduction in pyruvate oxidation with a concomitant decrease in non-mitochondrial ATP production would imply that the contribution from lipid oxidation to energy provision was increased. This is certainly plausible given the increased availability of free carnitine, in the face of a lower glycolytic flux. In agreement with this, a second study that observed comparable training adaptations with respect to acetylcarnitine accumulation and non-mitochondrial ATP production, also reported a lower post-training respiratory exchange ratio during exercise (70% pre-training $\text{VO}_{2\text{max}}$), suggesting that fat oxidation was indeed enhanced by training at this workload (Vollaard *et al.*, 2009). If alterations in carnitine metabolism during exercise are an important aspect of the metabolic responses to training in skeletal muscle, it would be pertinent to investigate whether increasing muscle carnitine during training could influence these adaptations. As will be discussed below, the current strategy to increase muscle carnitine availability is not without limitations and could benefit from further empirical optimisation.

1.3 Increasing skeletal muscle carnitine content

1.3.1 Carnitine homeostasis

Although the regulation of muscle carnitine stores is poorly understood, muscle carnitine uptake would appear to be primarily driven by the sodium-dependent organic cation transporter OCTN2 (Tamai *et al.*, 1998) and relies on a tightly-

controlled plasma carnitine availability. Plasma carnitine is in turn regulated by intestinal dietary absorption, renal reabsorption and endogenous biosynthesis (see **Chapter 3**). The difficulties in increasing muscle carnitine content are multifaceted and are addressed in greater detail in **Chapter 3**. Briefly, the bioavailability of orally ingested boluses of L-carnitine is less than 20% and declines further as dose size increases (Harper *et al.*, 1988). Secondly, the renal reabsorption of carnitine is near-saturated at normal plasma carnitine concentrations, meaning that any elevation of plasma carnitine concentration is matched by rapid urinary carnitine elimination (Brass *et al.*, 1994). Consequently, a sustainable increase in systemic carnitine availability would require constant oral dosing. Finally, the active transport of carnitine into muscle by OCTN2 must occur against a considerable (>100-fold) concentration gradient and would appear to be saturated by normal plasma carnitine concentrations under basal conditions (Stephens *et al.*, 2006b). These features of carnitine homeostasis are reflected in the results from multiple studies demonstrating the failure of L-carnitine administration to measurably impact the muscle carnitine stores. For example, Vukovich and colleagues (1994) found no difference in muscle carnitine following two weeks of oral L-carnitine supplementation (6 g per day) despite maintaining elevated serum carnitine concentrations throughout the intervention period. Similar results were found following three months of supplementation (2 x 2g per day; Wachter *et al.*, 2002). Moreover, Brass *et al.* (1994) found no impact of acute intravenous administration of carnitine upon muscle carnitine content. A further common finding from the three studies described above is the absence of any effect of carnitine supplementation on muscle metabolism or performance during exercise. Indeed, these findings cast doubt on a number of

studies that report the ergogenic effects of acute oral dosing of L-carnitine, as it is difficult to understand how such a strategy could alter skeletal muscle metabolism.

Insulin stimulates muscle carnitine accretion

The coupling of OCTN2 carnitine transport with the Na^+ gradient makes it an electrogenic process, and reduction of the membrane potential via vanilomycin-induced potassium diffusion has been shown to transiently increase OCTN2 activity (Tamai *et al.*, 1998). This feature was the basis for the hypothesis that manipulating the sarcolemma membrane potential via non-specific targeting of the Na^+/K^+ -ATPase pumps with insulin could enhance OCTN2-mediated skeletal muscle carnitine transport. A series of novel studies from Stephens and colleagues (2006a, b, 2007a; 2007b) were able to demonstrate that insulin can indeed augment muscle carnitine transport and have led to the development of a dietary regime to successfully and sustainably increase muscle carnitine content by a physiologically relevant amount (Wall *et al.*, 2011). The mechanism by which insulin stimulates Na^+/K^+ ATPase activity is not entirely clear, but is likely tissue specific and multifaceted (Sweeney & Klip, 1998). Insulin-signalled translocation of $\alpha 2$ and $\beta 1$ subunits from an intracellular storage depot, to the plasma membrane, has been demonstrated in skeletal rat muscle (Hundal *et al.*, 1992). Other potential mechanisms include an insulin-induced increase in intracellular K^+ concentrations, or in ATPase sensitivity to Na^+ (Ewart & Klip, 1995).

Initially it was shown that maintaining serum insulin at a supraphysiological level ($\sim 150 \text{ mU}\cdot\text{L}^{-1}$) during a five hour carnitine infusion resulted in a significant 13% increase in skeletal muscle carnitine content (Stephens *et al.*, 2006b). This was

associated with the induction of a 2.3-fold increase in skeletal muscle OCTN2 mRNA expression. Importantly, no change in muscle carnitine content or OCTN2 expression was observed when carnitine was infused with circulating insulin clamped at fasting levels. This is likely due to the considerable concentration gradient that carnitine must be transported against, from plasma into muscle (~40 μM and 4 mM in plasma and muscle, respectively). The reported muscle specific K_m of OCTN2 for carnitine is around 3.6 μM (Tamai *et al.*, 1998), suggesting that basal muscle carnitine uptake is saturated at normal plasma concentrations. Subsequent studies clarified that this insulin-mediated increase in muscle carnitine content required a threshold serum insulin concentration of somewhere between 50 and 75 $\text{mU}\cdot\text{L}^{-1}$ (Stephens *et al.*, 2007a) and necessitates an elevation of plasma carnitine (Stephens *et al.*, 2006a) most likely because the basal plasma carnitine compartment was insufficient to measurably increase muscle stores. In an attempt to make the manipulation of muscle carnitine more practically appealing, a subsequent study investigated whether carbohydrate feeding (to stimulate insulin secretion) with an oral bolus of L-carnitine could acutely increase muscle carnitine uptake (Stephens *et al.*, 2007b). The anticipated effect of short-term L-carnitine feeding on muscle carnitine content is so low as to be undetectable in muscle biopsy samples, so urinary carnitine excretion was used as a surrogate marker of whole body carnitine retention. Over a two week period of daily L-carnitine feeding, the average 24 hour urinary carnitine excretion was reduced by 29% with carbohydrate co-ingestion, compared to L-carnitine alone (188 vs 252 mg per day, respectively). Furthermore, plasma carnitine concentrations following a single oral bolus of L-carnitine were lower with carbohydrate ingestion and were negatively related to the seven hour area under the serum insulin x time curve (Stephens *et al.*, 2007b), supporting the notion of insulin-

stimulated plasma carnitine clearance into skeletal muscle. Utilising a similar feeding strategy, Wall *et al.* (2011) subsequently demonstrated that chronic (24 weeks) twice-daily L-carnitine feeding (3 g per day) in a carbohydrate beverage resulted in a 21% increase in total muscle carnitine stores. This was the first study to confirm the efficacy of carbohydrate-L-carnitine feeding to measurably increase muscle carnitine and provided a critical tool for investigating the impact of manipulation of the muscle carnitine pool on exercise metabolism.

1.3.2 Current limitations

Chronic carbohydrate intake

The feeding strategy used to increase muscle carnitine content by Wall and colleagues (160g daily carbohydrate load; ~650 kcal) is perhaps not appropriate for long-term, mass implementation. The control group, who consumed the same amount of carbohydrate in absence of L-carnitine, gained an average of 3% body weight ($P < 0.01$) over the first 12 weeks of the study. It is interesting to note that L-carnitine appeared to protect against this weight gain, through a coordinated up-regulation of fat oxidation at both a physiological and genomic level (Stephens *et al.*, 2013). Nevertheless, there is concern that high carbohydrate diets adversely affect triacylglycerol and high-density lipoprotein (HDL) concentrations (Petersen *et al.*, 2007). Indeed, increased dietary carbohydrate intake is associated with reduced clearance of very low density lipoproteins (Parks *et al.*, 1999), particles which have been implicated in the progression of atherogenesis (Zilversmit, 1995). It is also possible that some of the negative effects of carbohydrate feeding observed in the control group could have suppressed the adaptations to skeletal muscle carnitine loading, thus masking the true magnitude of the response. A supplemental protocol

that could have dyslipidemic properties would be unsuitable for chronic use, particularly in populations who might already be at risk of metabolic syndrome. It is therefore necessary to devise oral nutritional formulations which produce a physiologically high insulinaemic response, but with a relatively low caloric and carbohydrate load. It is well known that other macronutrients, namely proteins and amino acids, can stimulate insulin secretion *in vivo*, and are therefore potential candidates to achieve this.

Urinary carnitine as a measure of muscle carnitine retention

The most decisive way to determine the effectiveness of a feeding strategy in elevating muscle carnitine stores is to obtain a muscle biopsy sample following a chronic period (at least 12 weeks) of the feeding intervention. Clearly, this is a very time-consuming and invasive approach and is the reason less direct, more acute measures have been performed previously (Stephens *et al.*, 2007b). The main limitation with previous investigations into the manipulation of whole body carnitine stores is the reliance on urinary measures of carnitine retention. The lower plasma and urinary carnitine concentrations following carbohydrate ingestion during the acute carnitine feeding study by Stephens and colleagues could equally have been attributed to a lower intestinal absorption. Given that a negative correlation was found between the area under the time curves for plasma carnitine and serum insulin concentrations, it seems highly likely that the suppression of plasma carnitine was indeed due to increased muscle uptake. Nevertheless, a more direct acute measure of muscle carnitine accretion would be a valuable tool in the investigation of novel ways to improve the efficacy of muscle carnitine loading.

1.4 Aims and hypotheses

The general aim of these studies was to investigate whether skeletal muscle carnitine loading could alter the metabolic adaptations to high-intensity exercise training. Initially this involved the generation of experimental data to ascertain the most appropriate feeding strategy to elevate muscle carnitine. Secondly, an exercise paradigm was identified under which the manipulation of muscle carnitine stores could be most beneficial to the expected metabolic adaptations. Finally, the identified feeding strategy and exercise paradigm were implemented into a chronic exercise training study.

Study one

Given the disadvantages of a high carbohydrate intake, the principle aim of study 1 (**Chapter 3**) was to investigate whether the carbohydrate load used to increase whole body L-carnitine retention in previous studies could be reduced using a carbohydrate and whey protein combination. Initially, the impact of this oral formulation upon whole-body carnitine retention was assessed using urinary and plasma carnitine measurements (part A). A second aim of study one was to obtain a more direct estimate of muscle carnitine uptake and accretion rates, and to determine the efficacy of the chosen oral formulations in acutely enhancing these rates (part B). The latter approach utilised the forearm arterio-venous balance model of net substrate uptake and circumvented any confounding influence of the possible inconsistencies in intestinal absorption. It was hypothesised that following acute L-carnitine feeding, an orally administered carbohydrate and protein (PRO) solution would reduce urinary carnitine retention, relative to a flavoured water control (CON) and to the same extent as a carbohydrate only beverage (CHO). These were secondary to the

hypothesis that PRO and CHO would stimulate similar physiologically high insulin responses. In part B it was subsequently hypothesised that PRO and CHO would promote similar rates of insulin-stimulated net muscle carnitine balance, above that observed with CON.

Study two

In **Chapter 4**, two studies were conducted to investigate the concept that skeletal muscle acetylcarnitine accumulation can reflect the balance between PDC flux and TCA cycle flux, thus further informing on the balance between oxidative and non-mitochondrial ATP production, during different exercise paradigms. In study 2A, carnitine acetylation and non-mitochondrial ATP production were determined in a single-leg knee extension exercise model, using comparable workloads to those previously characterised for two-legged cycling (Wall *et al.*, 2011). It was hypothesised that, consistent with the apparent excess of TCA cycle flux relative to PDC flux during this modality of exercise (Gibala *et al.*, 1998), acetylcarnitine accumulation and non-mitochondrial ATP production would be negligible during work at both 55% and 85% W_{max} . In study 2B, we investigated acetylcarnitine accumulation, PDC activation and non-mitochondrial ATP delivery during two successive bouts of high-intensity bicycle exercise, where the ATP demand could be fixed and sustained across both bouts, but oxidative ATP provision is apparently increased in the second bout (Bangsbo *et al.*, 2001). This also provided the opportunity to study the recovery of these parameters during a five minute passive rest period between the two exercise bouts. It was rationalised that a five minute recovery period would allow PCr to return to basal values between bouts and thus

hypothesised that acetylcarnitine accumulation and non-mitochondrial ATP production would decline during the second bout relative to the first.

Study three

Study three (**Chapter 5**) investigated the impact of increasing skeletal muscle carnitine content on the metabolic adaptations to high-intensity bicycle exercise training. Based on previous data on the responses at 80% $\text{VO}_{2\text{max}}$ to muscle carnitine loading, and on data from the previous studies of this thesis, it was proposed that increasing muscle carnitine would maintain PDC flux during a second bout of exercise and thus alter the training adaptations to this type of exercise. Specifically, it was hypothesised that carnitine loading would reduce PCr degradation and glycolytic flux during repeated bouts of exercise, secondary to increasing activation and flux through the PDC and compared to a control group. It was further hypothesised that this could alter the functional adaptations to the training intervention, including divergent gains in $\text{VO}_{2\text{max}}$ and work output.

CHAPTER 2: GENERAL METHODS

2.1 Human Volunteers

Volunteer recruitment

For all of the studies described in this thesis, with the exception of Chapter 4 Study A, young (18-35 years), healthy, male volunteers were recruited from the University of Nottingham and surrounding area through responses to posters, social media and local newspaper advertisements. All recruitment strategies were approved by the University of Nottingham Ethics Committee. For Chapter 4 Study A, young healthy volunteers were recruited in accordance with the Ethics Committee for Medical Research in Copenhagen (Stallknecht *et al.*, 2007).

Volunteer Screening

Prior to study participation, all volunteers attended a preliminary visit to give informed consent and complete a general health screening. Volunteers received a study information sheet in advance of the screening and were asked to read it thoroughly. During the preliminary visit the study timeline, protocol and procedures were explained explicitly before written consent was obtained. Volunteers were informed that they were free to withdraw from the study at any point. The subsequent general health screening consisted of a health questionnaire (see **Appendix 1.2**), measurements of height, weight and blood pressure and a 12-lead electrocardiogram (Schiller, Altgasse, Switzerland) and small (<10 ml) blood sample from an antecubital vein. The blood sample was screened for blood cell count and serum indices of hepatic and renal function by the Departments of Haematology and Clinical Chemistry, Queen's Medical Centre, Nottingham, UK. Screening results were overseen by the study medic before volunteers were recruited onto the study.

Determination of maximal oxygen consumption

For Chapters 4 and 5, volunteers completed a continuous incremental exercise test to exhaustion to determine their individual maximal oxygen consumption (VO_{2max}) and the workload that would elicit this under these exercise conditions (W_{max}), on an electronically-braked cycle ergometer (Lode Excalibur; Lode, Groningen, The Netherlands). Following a five minute warm-up period cycling against a workload of 50-100 watts ($\sim 1W/kg$), the first stage commenced at the same workload and was increased by a 30W increment every three minutes in a step-wise fashion until volitional exhaustion. Heart rate (3-lead electrocardiogram; GE Medical Systems Technology Inc. Buckinghamshire, UK), oxygen consumption and carbon dioxide production (mixing chamber mode, V_{max} ; SensorMedics corporation, California, USA) were measured continuously throughout the test. In addition, the rating of perceived exertion (RPE) was determined on the Borg Scale (Borg, 1970) in the last 30 seconds of each incremental stage. Upon cessation of exercise and following a short rest, volunteers cycled again at a workload 20-30 watts greater than the maximal increment achieved in the previous test. This confirmation procedure was also repeated on a separate occasion, within one week of the initial test. Oxygen consumption values were averaged over 20 second intervals and VO_{2max} was taken as the highest value obtained across all three occasions. W_{max} was calculated as:

$$W_{max} = W_F + 30 \cdot \left(\frac{T_F}{180} \right)$$

where W_F is the workload of the final *complete* stage and T_F is the time attained in the final stage.

Calculation of mechanical efficiency

Mechanical cycling gross efficiency, herein referred to as mechanical efficiency, is a whole-body measure of the fraction of total energy expenditure that can be ascribed to the actual work done relevant to the exercise task. It is calculated by the equation:

$$GE\% = \frac{W}{VO_2 \times c \times 0.06} \times 100$$

where GE is the gross mechanical efficiency, W is the power output in watts, VO₂ is the oxygen consumption at that power output in ml·min⁻¹ and c is the energy equivalent of one litre of oxygen in kJ. When the whole-body respiratory exchange ratio exceeds 1.0, as was always the case for the calculation of mechanical efficiency at W_{max}, the value used for c is 21.13 kJ·L⁻¹.

Determination of body composition

For **Chapter 5**, volunteers received a dual energy X-ray absorptiometry (DEXA) scan (Lunar Prodigy DEXA; GE Medical Systems, Bedford, UK) at three time-points throughout the study to determine fat mass and fat free mass. A questionnaire was completed prior to each scan to confirm volunteer suitability for the scan (see **Appendix 1.3**) and any metallic accessories that could interfere with the image were removed. Volunteers lay supine on the scanning platform, remaining motionless for the duration of the scan (approximately seven minutes), which involves two low-dose X-ray beams (40 – 70 keV) passing through the body incurring a total radiation dose of less than 1 µSv. Estimations of fat and fat-free tissue masses are subsequently automated from the attenuation ratio of the two X-ray beams and

separated into leg, trunk and arm specific masses using the standardised regions specified in the software package (enCORE 2005 version 9.1; GE Medical Systems, U.K). All scans were analysed during a single session by an experienced operator.

Blood Sampling

For **Chapter 3**, volunteers were cannulated to enable regular (up to every ten minutes) arterialised-venous blood sampling. A sterile 12G cannula was placed retrograde into a dorsal vein on the back of the hand. Lidocaine (1%) was injected (approximately 1 ml) subcutaneously prior to cannula insertion to minimise discomfort. The cannula was kept patent by a slow running 0.9% saline infusion. To arterialise the blood, this hand was then placed in an air-warming unit set to heat the hand to 55°C and kept there for the remainder of the visit. This method has been demonstrated to produce venous concentrations of the molecules of interest that are close to the arterial concentrations, without altering core body temperature (Gallen & Macdonald, 1990). For Chapter 3, it was necessary to sample venous blood draining directly (or as close as possible) from the forearm tissue. An ultrasound probe was used to locate the deep-lying branch of an antecubital vein of the contralateral forearm and a sterile cannula was inserted as above. Accurate placement of the cannula was verified using ultrasound.

Muscle biopsy sampling

Muscle biopsies were obtained from the vastus lateralis muscle using the percutaneous needle method as described by Bergstrom (1975). Briefly, a small area of the thigh was shaved and cleaned using iodine solution. Following local anaesthetic of the skin and proximal tissue (Lidocaine; 2 %) a small incision (<1cm)

was made through the fascia using a sterile scalpel blade. A five millimetre Bergstrom needle (Bignell Surgical Instruments Ltd, West Sussex, UK) was used (with suction) to obtain 100 – 200 mg (wet weight) muscle tissue which was rapidly frozen in liquid nitrogen-cooled isopentane. Post-exercise muscle biopsies were obtained and frozen within 15 seconds of the cessation of exercise. For Study 2A (**Chapter 4**), two muscle biopsies were obtained from a single incision, in opposite directions (distal-proximal) to one another, from each leg.

2.2 Plasma and serum analysis

Serum Insulin

For the studies in Chapter 3, two millilitres of freshly drawn blood was allowed to clot and following centrifugation (3,000 G for ten minutes), the serum was removed and stored at -80°C for analysis using a radioenzymatic kit (Coat-A-Count RIA; Seimens Healthcare, Erlangen, Germany). This assay involves a 24 hour incubation period during which endogenous insulin from the sample competes with [¹²⁵I]-insulin for binding sites on an insulin antibody lining the test tube. The tubes were then decanted, blotted thoroughly and counted in a gamma radiation scintillation counter (COBRA II Auto Gamma; Canberra-Packard, Melbourne, Australia). Counts per minute were used to interpolate serum insulin values from a [\log_{conc}] standard curve of known insulin concentrations, normalised to the blank which represented 100% [¹²⁵I]-insulin binding (**Figure 2.1**). The insulin assay kit had an intra-assay coefficient of variation (CV) of 2.8% and an inter-assay CV of 7.1%.

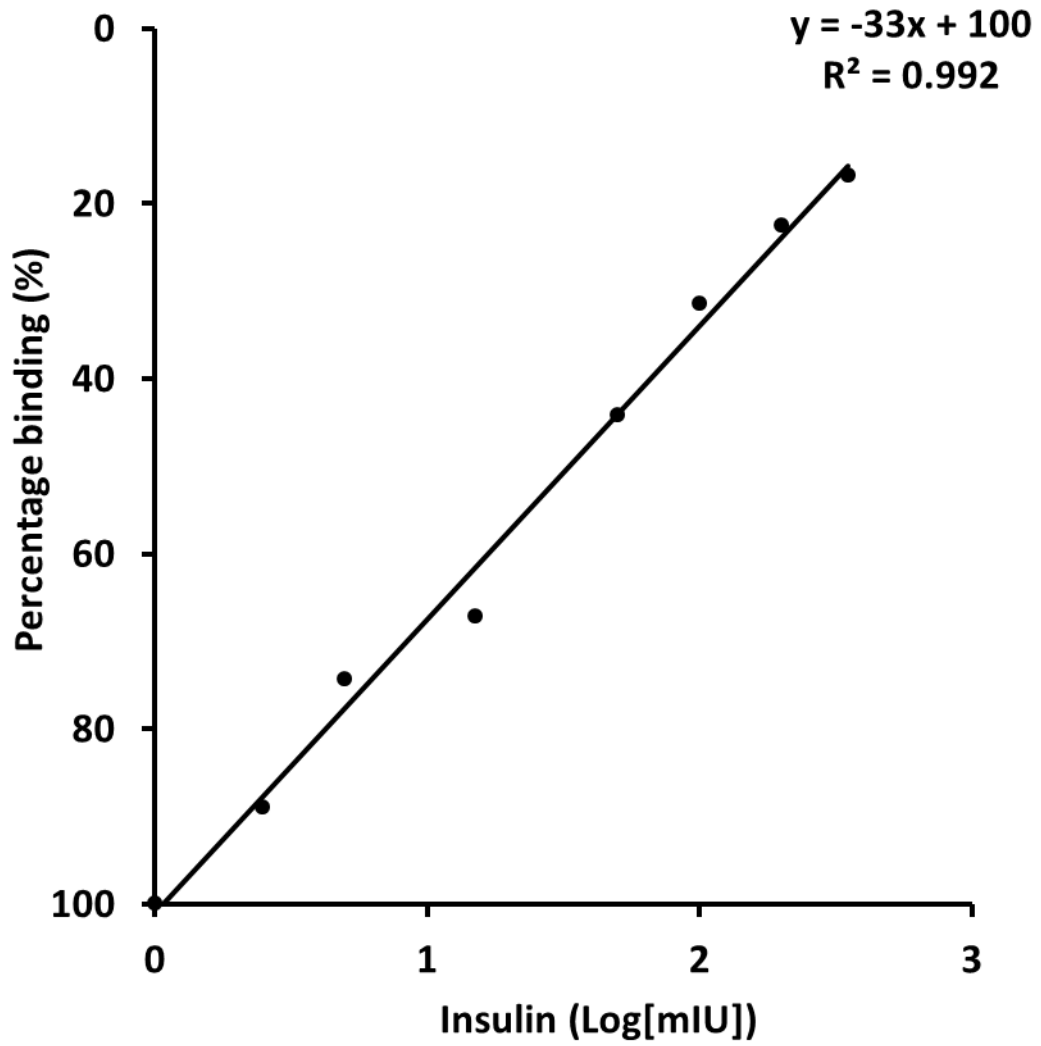
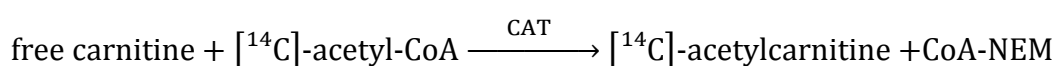


Figure 2.1 Serum insulin assay standard curve. Representative example of the response of the serum insulin assay to increasing concentrations (0-350 mU·L⁻¹) of exogenous insulin standard.

Plasma and urine total, free and acylcarnitine

Plasma was separated from two millilitres of freshly drawn blood (lithium heparinized vacutainer), centrifuged (3,000G for 10 min) and immediately frozen at -80°C for the subsequent analysis of carnitine based on the original method of Cederblad and Lindstedt (1972). Thirty microlitres of plasma or urine (1:9 dilution) was deproteinized in one millilitre chloroform:methanol (3:2) and vigorously vortexed for two minutes. After centrifugation (7,000G for 10 min) the supernatant was decanted into glass test tubes and dried to residue under oxygen-free nitrogen. The residue was resuspended in 120 µl of distilled water and assayed directly for free carnitine. One hundred microliters of potassium phosphate buffer (pH 6.2; 250 mM) containing both [¹⁴C]-acetyl-CoA (1.6 µM) and unlabelled acetyl-CoA (75 µM) was added to each sample. The acetylation reaction was initiated by the addition of 20 µl CAT (0.4 U) and allowed to proceed for 20 minutes at room temperature (final reaction volume was 240 µl). NEM was included in the reaction mixture (4 mM) to remove any liberated coenzyme A and quench the reaction as follows:



The positively charged [¹⁴C]-acetylcarnitine product was separated from the unreacted [¹⁴C]-acetyl-CoA (negatively charged) by passing the sample through a positive ion exchange column (DOWEX chloride counter ion resin, 100-200 mesh) and then washing the columns with H₂O. The flow-through was collected in scintillation vials to which 10 ml scintillation fluid was added, vortexed vigorously and subsequently counted for [¹⁴C] β-radiation using a scintillation counter (TriCarb, 2100TR, Canberra-Packard, Melbourne, Australia). Counts per minute were used to

interpolate carnitine values from a linear standard curve of known carnitine concentrations (0-3600 pmol) with a typical $R^2 > 0.999$ (**Figure 2.2**). The intra-assay coefficient of variation (CV) was less than 2%, whilst the inter-assay CV was less than 5%. For the determination of total carnitine, the dried residue was incubated (2 hours @ 50°C) in 100 µl potassium hydroxide (KOH; 0.1 M) to ensure complete hydrolysis of the acylcarnitine ester bonds. Total carnitine was then assayed as above in the neutralised (20 µl 0.5 M HCl) samples. The difference between total (hydrolysed) carnitine and free carnitine was assumed to equal plasma (or urine) acylcarnitine concentration.

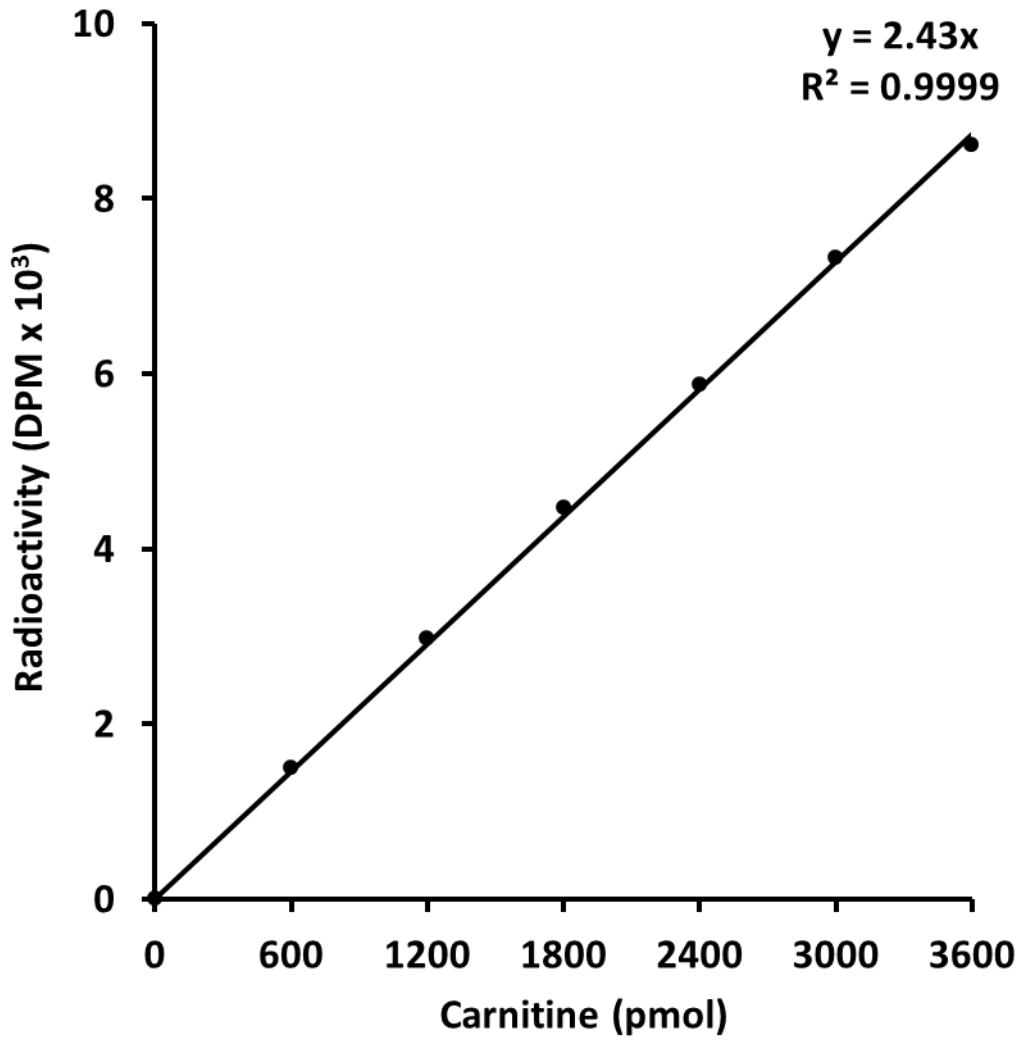


Figure 2.2 Free carnitine assay standard curve. Representative example of the linear response of the carnitine assay to increasing amounts (0-3600 pmols) of exogenous L-carnitine standard.

Plasma [methyl-²H₃]-L-carnitine enrichment

Solid phase extraction

The molar percent excess (MPE) tracer enrichment, used to calculate the rate of plasma carnitine disappearance in **Chapter 3**, was determined by the parallel quantification of L-carnitine (m/z 162) and ²H₃-L-carnitine (m/z 165) in each sample using high performance liquid chromatography mass spectrometry. Plasma samples were prepared for LCMS with prior solid phase extraction using strong cation exchange. Two hundred microliters of plasma was mixed with an acetyl-lysine internal standard (20 μM) and acidified in 800 μl formic acid (2%), vortexed briefly and centrifuged at 13,000G for two minutes. Strong cation exchange columns (30 mg Oasis MCX 33 μm, 80Å; Waters) were prepared with 1 ml methanol (70%) and equilibrated with 1 ml formic acid (2%). Samples were then loaded and a flow rate of approximately 1 ml/min was set. Columns were subsequently washed, sequentially, with 1 ml formic acid (2%) and 1 ml methanol (70%), allowed to vacuum dry for 1-2 minutes and the flow-through discarded. Columns were eluted with 1 ml ammonium hydroxide (35%) in methanol (50%) and the eluate retained in low-bind microtubes. Sample eluates were dried to residue overnight in a vacuum centrifuge, resuspended in formic acid (0.1%) and centrifuged at 13,000G for two minutes. Two hundred microliters of the supernatant was decanted into an auto-sampler vial for subsequent LCMS quantification.

Liquid chromatography mass spectrometry

All chromatographic experiments were performed on a Waters 2700 auto-sampler module using a C18 Brownlee column (2.1 x 300 mm, 5 μm) with a 50 μl injection volume and a single mobile phase of 5% acetonitrile / 0.1% formic acid in HPLC

grade water (isocratic mode). Flow rate throughout separation was 0.3 ml/min. Mass spectrometry experiments were performed on a Micromass Quattro Ultima triple quadrupole mass spectrometer (Waters, MA, USA) in positive ion mode with nitrogen as the collision gas. The collision voltage (30 V), capillary voltage (3.6 kV), cone voltage (100 V), source temperature (96 °C) and desolvation temperature (250 °C) were kept constant for experiments. Molecules of interest were quantified in MSMS ion monitoring mode with transitions of (m/z 162 → 60) for L-carnitine and (m/z 165 → 63) for ²H₃-L-carnitine.

2.3 Muscle metabolite, substrate and enzyme analysis

Muscle metabolite extraction

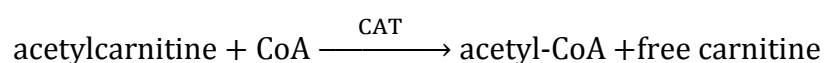
Muscle biopsy samples were cut (10-50 mg ww) and freeze-dried for 24 hours. Visible blood, fat and connective tissue were removed before powdering using sterile forceps and separate portions were weighed out for metabolite extraction (3-10 mg dw) and the determination of glycogen (1-3 mg dw). Eighty volumes of ice-cold perchloric acid (0.5 M with 1 mM EDTA) was added to the muscle powder before gentle vortexing (5 RPM) for ten minutes at 6°C to precipitate acid-soluble metabolites (Harris *et al.*, 1974). After centrifugation (2,000G for 3 mins) the decanted supernatant was neutralised with 0.25 volumes of potassium bicarbonate (2.2 M), re-centrifuged and the neutralised supernatant stored at -80°C for subsequent metabolite analysis. The acid-insoluble muscle pellet from the initial centrifugation was blotted and digested in 500 µl KOH (0.2 M) for two hours at 50°C, neutralised with 20 µl PCA (5M) and the supernatant (12,000G for 3 min) decanted and stored at -80°C for the analysis of long-chain acylcarnitine and the acid-insoluble glycogen fraction.

Muscle free, long-chain acyl and total carnitine

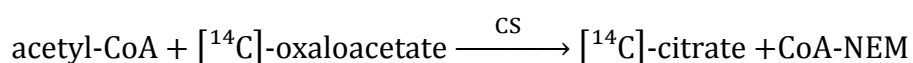
Muscle free carnitine and long-chain acylcarnitine was determined in 10 µl PCA extract (made up to 120 µl with distilled water) or 120 µl long-chain extract, respectively, as described above for plasma and urine free carnitine. Total carnitine was calculated as the sum of free carnitine, long chain acylcarnitine and acetylcarnitine (see below).

Muscle acetylcarnitine

Muscle acetylcarnitine content was determined as described by Pande and Caramancion (1981) and modified by Cooper *et al.* (1988). Fifty microliters of diluted PCA extract (1:49 – 1:99) or standard (0-125 pmol) was incubated in 200 µl HEPES buffer (pH 7.4; 125 mM) containing EDTA (1 mM) and freshly reduced (1 mM DTT) coenzyme A (25 µM). The acetyltransferase reaction was initiated by the addition of 20 µl CAT (0.4 U):



Following a 30 minute incubation at room temperature the reaction was terminated by heating the samples at 95°C for three minutes to denature the CAT and 30 µl NEM (40 mM) was subsequently added to remove any excess CoA. The acetyl-CoA formed, proportional to the initial acetylcarnitine concentration, was condensed with 20 µl [¹⁴C]-oxaloacetate (freshly prepared from [¹⁴C]-aspartate; see **Appendix 2.1**) by the addition of 20 µl citrate synthase (0.4 U):



Following a 20 minute incubation at room temperature, the excess [^{14}C]-oxaloacetate (negatively charged) was removed by transamination back to [^{14}C]-aspartate via the addition of glutamate-oxaloacetate transaminase in the presence of excess potassium glutamate (133 mM). Following a final 20 minute incubation at room temperature the [^{14}C]-citrate (negatively charged), proportional to the initial acetylcarnitine concentration, was separated from the [^{14}C]-aspartate (positively charged) by the addition of one millilitre of negative ion exchange resin (DOWEX, hydrogen counter-ion resin, 50-100 mesh). Samples were vortexed vigorously and allowed to sediment, before 500 μl was decanted and mixed with 5 ml scintillation fluid. After vigorous vortexing, samples were counted for [^{14}C] β -radiation using a scintillation counter (TriCarb, 2100TR, Canberra-Packard, Melbourne, Australia). Counts per minute were used to interpolate acetylcarnitine values from a linear standard curve of known acetylcarnitine concentrations (0-125 pmol) with a typical $R^2 > 0.999$ (**Figure 2.3**).

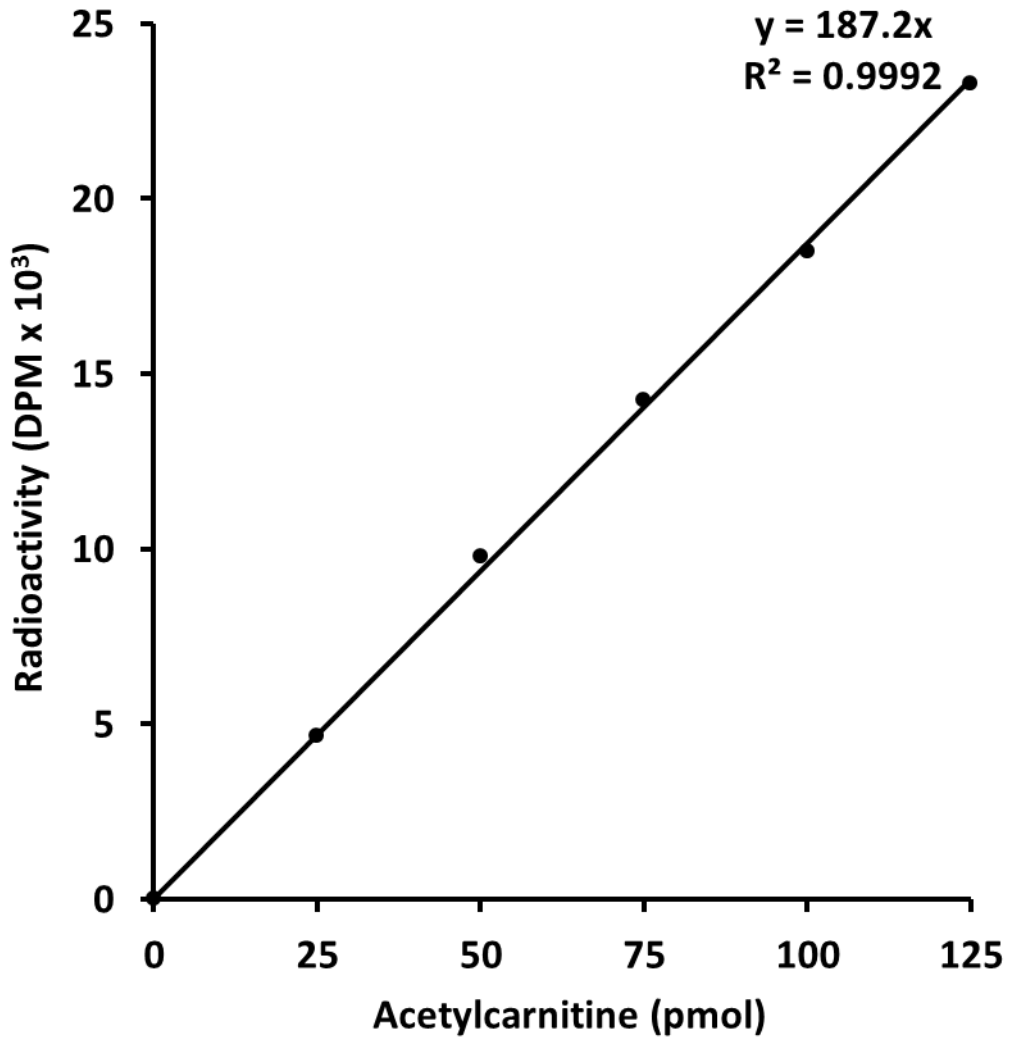
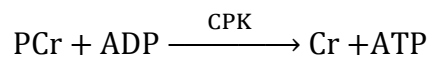
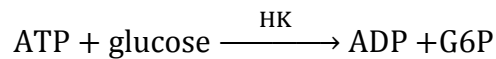
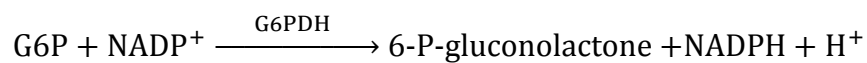


Figure 2.3 Acetylcarnitine assay standard curve. Representative example of the linear response of the acetylcarnitine assay to increasing concentrations (0-125 pmols) of exogenous L-acetylcarnitine standard.

Muscle adenosine triphosphate and phosphocreatine

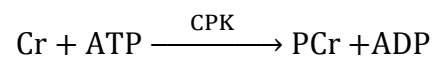
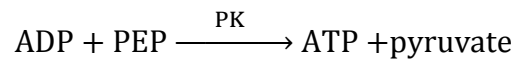
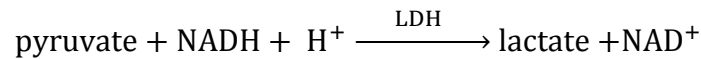
Adenosine triphosphate (ATP) and phosphocreatine (PCr) were assayed sequentially using the method of Harris *et al.* (1974) in which NADPH production is coupled to three reactions and can be followed spectrophotometrically. Twenty microlitres of PCA extract was aliquoted in duplicate into a 96-well plate and mixed with 225 μ l triethanolamine buffer (pH 7.5) containing NADP (1 mM), ADP (0.04 mM), glucose (5 mM) and EDTA (1 mM). Baseline light absorbance was read at a wavelength of 340 nm before the sequential addition of four microlitres (1.5 U) of glucose-6-phosphate dehydrogenase, hexokinase and creatine phosphokinase. After the addition of each enzyme, the plate was mixed and incubated for 20 minutes at 25°C and the absorbance was again recorded. The sequential reactions were as follows:



Muscle creatine

Free creatine was assayed spectrophotometrically (Harris *et al.*, 1974) as the disappearance of NADH. Twenty microlitres of PCA extract was added in duplicate to each well and mixed with 300 μ l glycine buffer (pH 9.0) containing ATP (1.5 mM), phosphoenolpyruvate (1 mM), NADH (0.15 mM) and the coupling enzymes lactate dehydrogenase (LDH) and pyruvate kinase (PK). Baseline light absorbance was read at a wavelength of 340 nm before the addition of four microlitres (1.5 U) of

creatine phosphokinase. After the enzyme was added, the plate was mixed and incubated for 35 minutes at 25°C before again reading absorbance. The coupled reactions were as follows:



Muscle lactate

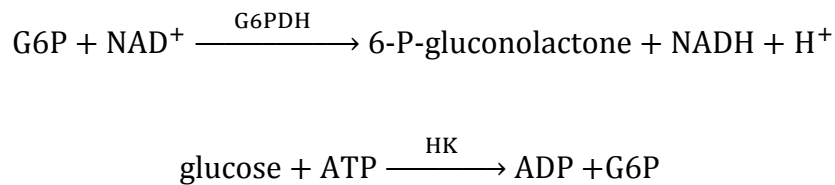
Lactate was assayed spectrophotometrically based on the method of Lundholm *et al.* (1963). Twenty microlitres of PCA extract was aliquoted in duplicate and mixed with 200 µl of glycine buffer (pH 9.2; 100 mM) containing NAD⁺ (3 mM) and hydrazine (260 mM) to quench the reaction in favour of pyruvate production. After a baseline absorbance reading at 340 nm, four microlitres of lactate dehydrogenase (5.5 U) was added. The plate was mixed, incubated for 30 minutes at 25° and read a second time.



Muscle Glycogen

Muscle glycogen was determined by a modified version of the method employed by Harris *et al.* (1974). Glycogen was extracted from 1-3 mg freeze-dried muscle powder by hot alkaline digest (10 minutes at 80°C in 100 mM sodium hydroxide).

Following neutralisation with HCl (100 mM), the glycogen was enzymatically hydrolysed in a sodium phosphate/citrate buffer (200 mM) using amyloglucosidase. Samples were incubated with the enzyme for one hour at room temperature and then centrifuged for three minutes at 20,000 G. The liberated glucose was measured spectrophotometrically; twenty-five microlitres of extract was aliquoted to each well and mixed with 250 μ l TEA buffer (pH 8.2) containing NAD⁺ (1 mM), ATP (0.75 mM), EDTA (1 mM), DTT (1 mM) and glucose-6-phosphate dehydrogenase. After baseline absorbance measurement at 366 nm, 1.5 U hexokinase was added to each well and the plate was incubated at 25°C for 15 minutes. The change in absorbance after 15 minutes was corrected for a distilled-water blank and used to calculate glycosyl-units.



Calculation of metabolite concentrations

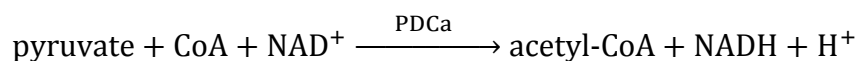
For all spectrophotometric assays, absorbance values were automatically normalised to a one centimetre path-length using pre-programmed path-length correction values for the software (Spectra Max 190, Molecular Devices Ltd; Wokingham, UK) and changes in absorbance were corrected for the background change in absorbance of a distilled-water blank. Metabolite concentrations were then calculated from the following equation:

$$\text{Conc}_{\text{met}} = \frac{\{[\text{Vol}_2 \times (\text{Abs}_2 - \text{Bl}_2)] - [\text{Vol}_1 \times (\text{Abs}_1 - \text{Bl}_1)]\} \cdot \text{EF}}{\text{Vol}_s \times \varepsilon}$$

Where Conc_{met} is the concentration of the metabolite given in millimoles per kilogram of dry muscle mass; Vol is the pre- or post-enzyme well volume or sample volume given by the suffices 1,2 or S, respectively; Abs is the sample absorbance, Bl is the blank absorbance, EF is the PCA extraction factor (~ 100) and ϵ is the extinction coefficient for NADPH at 340 nm ($6.22 \text{ cm}^{-1} \cdot \text{mmol}^{-1}$) or NADH at 366 nm ($3.4 \text{ cm}^{-1} \cdot \text{mmol}^{-1}$). A one or two point standard was always included with each assay to confirm reactions had run to completion.

Pyruvate dehydrogenase activation status

The activation status of the pyruvate dehydrogenase complex (PDCa) is a measurement of the phosphorylation state of the enzyme, and in most situations where blood flow is not restricted, will be an indication of the actual activity or flux of the enzyme (Constantin-Teodosiu *et al.*, 1991). 3-20 mg wet muscle was homogenised (50 seconds at 400 RPM) in 30 volumes ice-cold Tris buffer (pH 7.8; 50 mM; see **Appendix 2.2**), which contained sucrose (200 mM); sodium fluoride (50 mM) and dichloroacetate (5 mM), to inhibit PDC phosphatase and kinase activity, respectively; and the membrane detergent Triton X-100 (0.1%). Thirty microliters of homogenate was then added to 720 μl pre-warmed (37°C) Tris-HCl buffer (100 mM) which contained Coenzyme A (0.5 mM) and cofactors NAD^+ (1 mM) and thiamine pyrophosphate (1 mM). The decarboxylation reaction was initiated by the addition of 30 μl pyruvate (1 mM) as follows:



The reaction was terminated in sequential aliquots after 1, 2 and 3 minutes by adding 210 μ l of the reaction mix to 40 μ l perchloric acid (0.5 M). Aliquots were subsequently neutralised with 10 μ l potassium bicarbonate (2.2 M) and centrifuged for three minutes at 20,000G to pellet muscle cell debris. Supernatants were removed and stored at -80°C for the determination of the reaction product, acetyl-CoA, using a similar radioenzymatic assay as described above for acetylcarnitine. Ten to twenty microliters of neutralised supernatant was made up to a volume of 120 μ l in distilled water and the assay commenced with the addition of NEM (see Acetylcarnitine Assay), with acetyl-CoA concentrations interpolated from linear standard curve of known acetyl-CoA concentrations (0-150 pmols) with a typical $R^2 > 0.999$ (**Figure 2.4**). PDCa was then calculated as the linear ($R^2 > 0.99$) increase in acetyl-CoA concentrations over time (rate of acetyl-CoA production) and corrected for the protein content of the muscle homogenate (see Protein content), expressed as nmol acetyl-CoA per mg protein per minute.

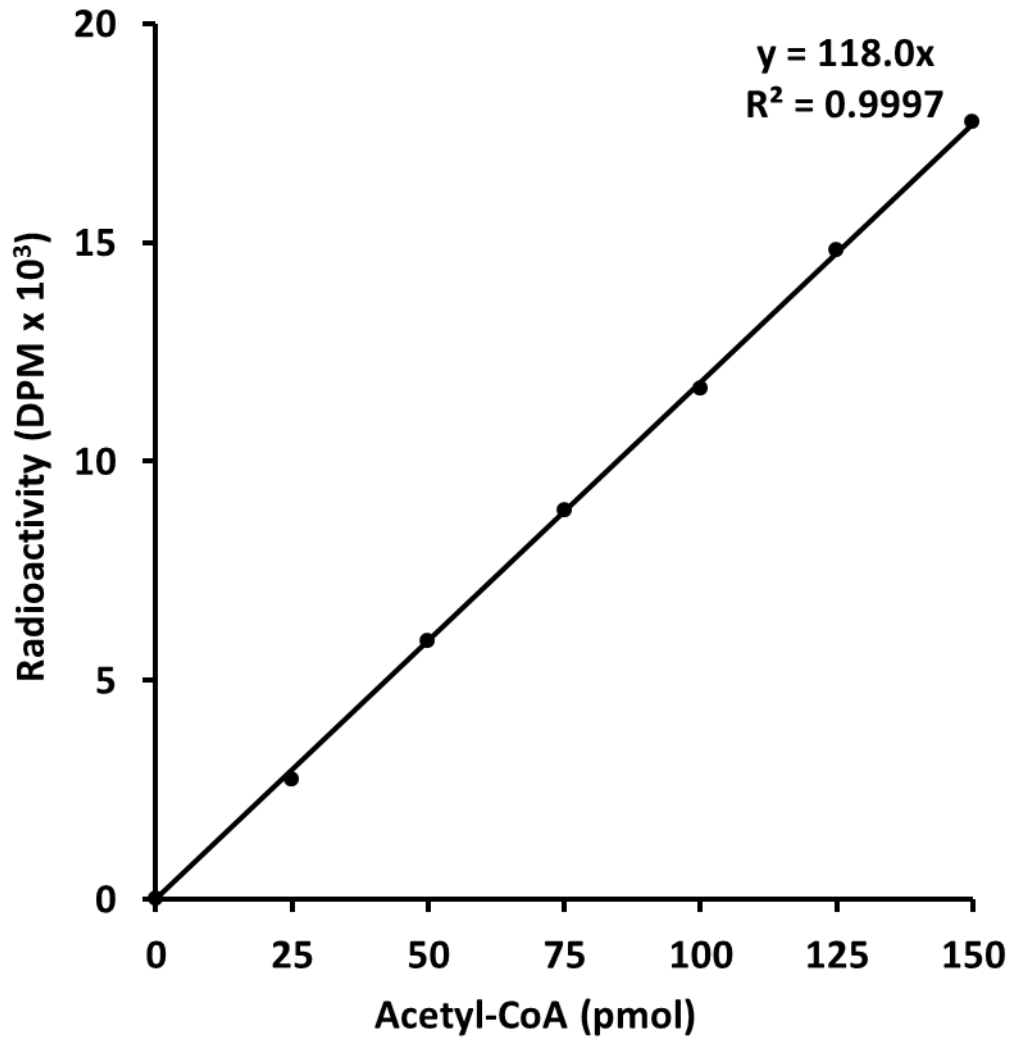


Figure 2.4 Acetyl-CoA assay standard curve. Representative example of the linear response of the acetyl-CoA assay to increasing concentrations (0-150 pmols) of exogenous acetyl-CoA standard.

Protein assay of muscle homogenates

PDCa values measured in skeletal muscle were normalised to the protein content of the homogenate as determined by the Bradford protein assay (Bradford, 1976). Four microliters of muscle homogenate, diluted in 996 μ l distilled water was added to 250 μ l BioRad reagent (Coomassie Brilliant Blue dye G-520; BioRad laboratories) and vortexed well. After a five minute incubation samples were aliquoted (250 μ l) in triplicate into a 96 well plate and the absorbance was read at 595 nm. Protein content was determined from a bovine serum albumin standard curve (0 – 30 mg/ml), also aliquoted in triplicate (**Figure 2.5**).

Calculation of non-mitochondrial ATP production

In **Chapter 4** and **Chapter 5**, non-mitochondrial ATP production during a given exercise bout was estimated based on the theoretical ATP resynthesis from skeletal muscle ATP breakdown, PCr degradation and lactate accumulation, given by the equation:

$$\text{Non-mitochondrial ATP production} = 2 \cdot \Delta[\text{ATP}] + [\text{PCr}] + 1.5 \cdot \Delta[\text{lactate}]$$

It should be noted that this will always represent an underestimate of the ATP delivery from non-mitochondrial sources in non-occluded muscle, as it does not consider the glycolytic ATP delivery accounted for by lactate efflux. It also ignores ATP turnover associated with the accumulation of intermediary metabolites, for example, glycerol-3-phosphate or pyruvate (Spriet *et al.*, 1987). However, this calculation provides a relative index that is reflective of the overall reliance on non-mitochondrial ATP delivery and thus, the matching of oxidative ATP production to total ATP demand.

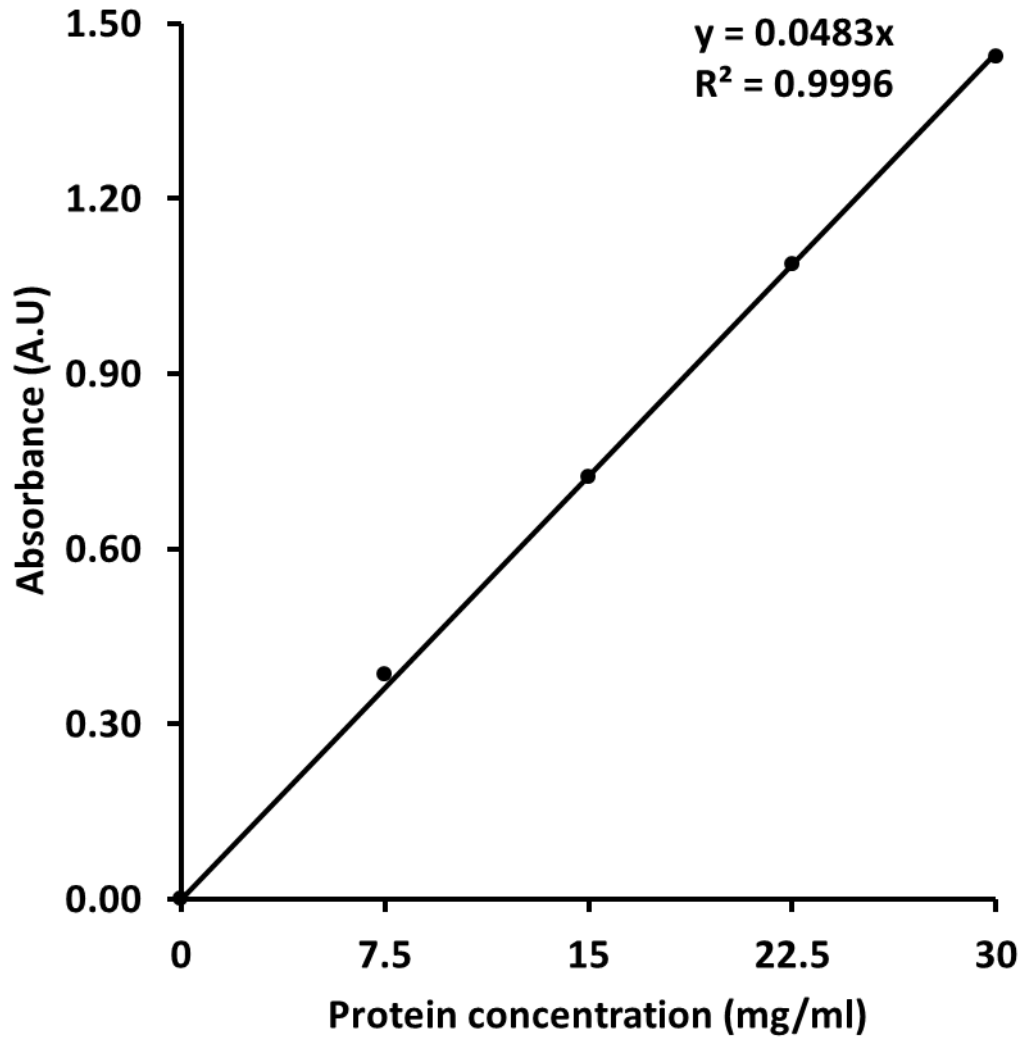


Figure 2.5 Protein assay standard curve. Representative example of the linear increase in absorbance in response to increasing protein concentrations over a range of 0 – 30 mg/ml BSA.

2.4 Statistical analyses

For the studies in this thesis, all time-dependent variables were compared using two-way analysis of variance (ANOVA) to identify main effects of time or treatment. Significant main effects were isolated post-hoc using a student's t-test with a Bonferoni stepwise correction for multiple comparisons. Time-independent variables in Chapter 3, including urinary carnitine excretion and area under the curve values, were compared using one-way ANOVA with Tukey post-hoc testing. Single comparisons, such as delta metabolite values between bouts in Chapter 4, were performed using a paired Student's t-test. Area under the analyte x time curve analysis, performed for Chapter 3, employed curve integration using the trapezium rule. Correlation analysis in Chapter 3 was performed using the Pearson product-moment correlation coefficient. All statistical tests were performed using GraphPad Prism V6 (GraphPad Software Inc; La Jolla, CA, USA) and an alpha level of $P < 0.05$ was deemed accepted as statistically significant. All data in text, tables and figures are presented as mean \pm standard error of the mean (SE).

**CHAPTER 3: ACUTE EFFECTS OF CARBOHYDRATE
AND WHEY PROTEIN INGESTION ON WHOLE
BODY L-CARNITINE RETENTION, ABSORPTION
AND NET MUSCLE BALANCE**

3.1 Introduction

Whole body carnitine stores in healthy, non-vegetarian humans equate to approximately 20g, with the vast majority (~95%) of this located in skeletal muscle (Brass, 1995). As outlined in the **General Introduction**, manipulation of the skeletal muscle carnitine stores may represent an appealing intervention for both athletic and clinical populations, but strategies to sustainably increase muscle carnitine content still require optimisation. Compartmental analysis of [³H]-carnitine kinetics in humans suggests that the skeletal muscle carnitine reserve is a relatively stable pool, with a predicted turnover time of approximately 190 hours (Rebouche and Engel, 1984). As skeletal muscle lacks the requisite γ -butyrobetaine hydroxylase enzyme for the terminal step of endogenous carnitine synthesis (Rebouche & Seim, 1998), muscle carnitine stores are restricted to the extraction of carnitine from the extracellular fluid, an active transport process thought to be facilitated by the sodium-dependent novel organic cation transporter OCTN2 (Tamai *et al.*, 1998). Moreover, muscle carnitine uptake occurs against a considerable (>100-fold) concentration gradient and thus any meaningful increase in the rate of muscle carnitine accretion requires a targeted up-regulation of muscle carnitine transport. Simultaneous elevation of plasma carnitine and serum insulin concentrations has proven to be an effective strategy to increase muscle carnitine uptake in healthy subjects and would appear to be driven by the insulin-mediated up-regulation of OCTN2 (Stephens *et al.*, 2005; see **General Introduction**). However, the limitations of the current carnitine loading regime, in particular the requirement for a large carbohydrate load, warrant the investigation into alternative oral formulations that can stimulate similarly high insulin responses, but utilise lower carbohydrate loads.

The potential for non-glucose substances to stimulate insulin release was initially recognised by Cochrane and colleagues (1956) following studies on cases of pathological hypoglycaemia. Floyd *et al.* (1966) later demonstrated that the intravenous infusion of essential amino acids, either individually or in combination with one another, resulted in a marked stimulation of insulin release. It was subsequently observed that the addition of protein to a carbohydrate meal could have a synergistic effect on insulin secretion (Rabinowitz *et al.*, 1966; Pallotta & Kennedy, 1968). In-vitro studies in pancreatic beta cells have partially elucidated the mechanisms behind the insulinotropic properties of amino acids. For example leucine and phenylalanine, which would appear to be particularly potent insulin secretagogues (van Loon *et al.*, 2000), are thought to stimulate beta cell respiration both through allosteric activation of glutamate dehydrogenase and by acting as a fuel for the enzyme (Sener & Malaisse, 1981). Given the large rise in plasma availability of leucine, phenylalanine and other amino acids following protein feeding (Nilsson *et al.*, 2007), it would seem likely that the insulin response to protein ingestion is secondary to such effects of individual amino acids. A number of studies have focused on manipulating the insulinotropic properties of amino acids and protein to optimise post-exercise glycogen synthesis (Ivy *et al.*, 2002) or protein anabolism (Rasmussen *et al.*, 2000). Of particular interest is a study from Steenge *et al.* (2000), which demonstrated that the combination of 50 g whey protein and 47 g carbohydrate was as effective as 96 g carbohydrate in stimulating an insulin-mediated ~25% increase in whole-body creatine retention. Similarly to carnitine, creatine is co-transported into muscle in a sodium-dependent manner and it is thought that the insulin-mediated increase in muscle uptake of both compounds is related to the stimulation of N^+/K^+ -ATPase driven sodium efflux. Based on the

findings of Steenge and colleagues (2000), whey protein would appear to be an attractive candidate to reduce the amount of carbohydrate needed to stimulate muscle carnitine uptake.

Strategies to optimise insulin-stimulated muscle carnitine uptake are dependent upon adequate L-carnitine absorption in order to maximise muscle carnitine delivery. However, the effects of whey protein ingestion on the intestinal absorption of L-carnitine have not before been tested and it has been speculated that amino acids could actually impair L-carnitine absorption (Taylor, 2001). Whilst the bioavailability of carnitine from a non-vegetarian diet, providing $2\text{-}12\ \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (Rebouche & Engel, 1984; Rebouche, 1992) ranges from 45-85% (Rebouche & Chenard, 1991), oral bolus doses of L-carnitine are absorbed with a much lower efficiency. For example, Harper *et al.* (1988) demonstrated that 16% of a 2 g oral bolus dose and only 5% of a 6 g dose were absorbed in healthy, non-vegetarian volunteers. The finding that L-carnitine bioavailability may be inversely related to dose size probably reflects the intestinal transport kinetics of L-carnitine. Studies in human intestine mucosa have characterised the intestinal absorption of L-carnitine as a predominantly active, sodium-dependent, saturable transport process with a K_m for carnitine of approximately $560\ \mu\text{M}$ (Hamilton *et al.*, 1986). This would imply that postprandial carnitine absorption from the jejunal lumen of healthy humans, where fasting carnitine concentrations ($\sim 26\ \mu\text{M}$) can increase by up to 10-fold following a standard meal containing approximately 50 mg carnitine (Li *et al.*, 1992), is predominantly facilitated by active transport. In contrast, a large component of the absorption of a $50\ \text{mg}\cdot\text{kg}^{-1}$ oral bolus of L-carnitine (approximately 4000 mg), which has been shown to elevate intrajejunal total carnitine concentrations to almost 25,000

μM , can likely be attributed to passive diffusion (Li *et al.*, 1992). The active component of intestinal carnitine transport was initially ascribed to the novel sodium dependant cation transporter OCTN2 (Duran *et al.*, 2002). However, the discovery that the OCTN2-deficient juvenile visceral steatotic mouse still retains approximately 50% of gut carnitine transport capacity would suggest the existence of additional transport mechanisms (Yokogawa *et al.*, 1999). For example, the amino acid transporter $\text{ATB}^{0,+}$, which is strongly expressed in the intestinal tract (Hatanaka *et al.*, 2004) has been shown to transport carnitine in a Na^+ - and Cl^- -dependent manner (Nakanishi *et al.*, 2001). Because $\text{ATB}^{0,+}$ is energised by the transmembrane gradients of both Na^+ and Cl^- , it exhibits a much higher transport capacity for carnitine than OCTN2. As such, intestinal carnitine transport by OCTN2, which has a reported K_m for carnitine of $4.3 \mu\text{M}$ (Tamai *et al.*, 1998), will become saturated at a much lower carnitine concentration than transport by $\text{ATB}^{0,+}$ ($K_m=1000-2000 \mu\text{M}$; Nakanishi *et al.*, 2010). Given these characteristics, it seems likely that the contribution from $\text{ATB}^{0,+}$ to intestinal carnitine transport would be of greater importance following oral bolus L-carnitine ingestion, when intestinal carnitine concentrations are rapidly elevated above normal postprandial levels. However, the study from Nakanishi *et al.* (2001) also demonstrated that transport of L-carnitine by $\text{ATB}^{0,+}$ was strongly inhibited by several amino acids, including leucine and phenylalanine, both of which are highly enriched in whey protein. Given the already low bioavailability of bolus doses of L-carnitine, any feeding strategy that has the potential to further compromise carnitine absorption may not represent an appropriate means of increasing skeletal muscle carnitine content and requires careful investigation.

A further consideration when designing strategies to optimise muscle carnitine uptake is the renal handling of bolus doses of L-carnitine. In healthy persons, the renal reabsorption of carnitine is a very efficient process and around 95% of carnitine is conserved during glomerular filtration when plasma carnitine concentrations are in their normal range (Rebouche & Engel, 1984). The reported K_m values for the renal tubular reabsorption of carnitine, which would also appear to be facilitated by OCTN2 in the brush border membrane (Tamai *et al.*, 1998), range between 20 μ M and 55 μ M (Rebouche & Mack, 1984). This is equivalent to the normal physiological range of plasma carnitine concentrations and implies that any marked elevation of the plasma carnitine pool, as occurs with oral dosing or intravenous administration, results in a rapid saturation of renal reabsorption with renal clearance rates increased to match the filtered load (Stephens *et al.*, 2006b). By means of example, 70-80% of an intravenous bolus dose of L-carnitine is recovered in the urine within 24 hours (Harper *et al.*, 1988). No detectable degradation of intravenously-delivered carnitine occurs prior to its excretion (Rebouche & Engel, 1984) and, as such, the urinary excretion of carnitine has previously been used as an indirect means to estimate whole-body carnitine retention (Stephens *et al.*, 2007b). However, any potential impact of co-ingested macronutrients on L-carnitine absorption could invalidate the use of urinary carnitine excretion, in isolation, as a marker of muscle carnitine uptake. With this in mind, a more direct estimate of acute muscle carnitine accretion may be necessary to evaluate the efficacy of prospective chronic L-carnitine feeding regimens.

To investigate whether, following acute L-carnitine ingestion, an orally administered carbohydrate and protein solution (PRO) could increase muscle carnitine accretion

two separate acute studies were performed. Initially, the impact of PRO on whole-body carnitine retention was investigated by measuring plasma total carnitine and urinary carnitine responses (study A). Based on the results of study A, a protocol was subsequently developed to more directly estimate the rate of muscle carnitine accretion stimulated by PRO, using an arteriovenous forearm balance model with an isotopically labelled L-carnitine tracer (study B). In both studies, the responses to PRO were compared to those of flavoured water and carbohydrate alone. This combination of net muscle carnitine balance and urinary carnitine excretion thus provided an indirect index of carnitine absorption for comparison across trials. In study A it was hypothesised that 40 g whey protein plus 40 g carbohydrate (PRO) would reduce urinary carnitine excretion, relative to a flavoured water control (CON) and to the same extent as an isocaloric carbohydrate solution (CHO). This was secondary to the hypothesis that PRO and CHO would stimulate similar serum insulin responses compared to CON. In study B, it was further hypothesised that PRO would increase muscle carnitine uptake and promote a positive net carnitine balance, comparable to CHO, whilst CON would promote a net zero balance.

3.2 Methods

Volunteers

Fifteen healthy, non-vegetarian male volunteers were recruited to participate in these studies. Eight participated in study A (age 23.2 ± 3.7 years; BMI 24.2 ± 2.4 kg·m⁻²) and seven in study B (24.2 ± 5.0 yrs, 23.3 ± 3.1 kg·m⁻²). Both protocols were approved by the University of Nottingham Medical School Ethics Committee. Prior to the study, each participant attended a routine medical screening (see General Methods) and written consent was obtained.

Experimental Protocol

Study A. Volunteers completed three randomised laboratory visits having fasted from 10.00 pm the previous evening. The experimental protocol for study visits is illustrated schematically in **Figure 3.1**. Upon arrival (approximately 9.00 am) volunteers laid semi-supine on a bed with one hand placed inside an air-warming unit heated to ~55°C. A cannula was inserted into a dorsal vein of the heated hand, allowing arterialized-venous blood sampling (Gallen & Macdonald, 1990). Volunteers ingested a 200 ml beverage of flavoured water containing 4.5 g L-carnitine tartrate (3g L-Carnitine; Lonza Ltd, Basel, Switzerland) at t=0 hours, then were given a 700 ml treatment drink (see beverage composition) at t=1, 2.5 and 4 hours to consume over 5 minutes. After a six hour sample collection period (see sample collection and analysis), the cannula was removed and volunteers were fed a standardised meal.

Study B. Shown in **Figure 3.2**, the protocol for study B was consistent with that for the initial three hours of study A. In addition to the procedures described above, a second cannula was placed into a deep-lying antecubital vein of the contralateral arm, for the sampling of venous blood draining from the forearm tissue (Andres *et al.*, 1954). The 200 ml beverage at t=0 hours additionally contained 1% [methyl-²H₃]-L-carnitine hydrochloride (Cambridge Isotope Laboratories Inc., Andover, MA) and volunteers were given a single 500 ml treatment drink at t=1 hour, with the sampling period lasting three hours. Brachial artery blood flow of the non-heated arm was also determined using Doppler ultrasound (Aplio SSA-770A, Toshiba Medical Systems) with a 12MHz transducer. The brachial artery (10 cm proximal to the antecubital fossa) was imaged in two-dimensions and the arterial luminal

diameter was determined using online video callipers with images synchronised to the cardiac cycle using a three-lead ECG. Blood velocity was determined at the same anatomical location using pulsed-wave Doppler (variable frequency) and the signal was integrated using the online software to give time-averaged velocity values.

Beverage Composition

The composition of the beverages was as follows: flavoured water (<1 g carbohydrate; CON), 80 g carbohydrate polymer (Vitargo orange flavour, Swecarb AB, Stockholm, Sweden; CHO) and 40 g of the carbohydrate polymer plus 40 g whey protein (PRO-10.com, Birmingham, UK; PRO). All drinks were orange flavoured and were mixed thoroughly in water one hour prior to consumption. The selected carbohydrate was a high-molecular weight glucose polymer and was identical to that which had been used to successfully increase muscle carnitine content in previous studies (Wall *et al.*, 2011; Stephens *et al.*, 2013), but therefore differed to the type of carbohydrate used by Stephens *et al.* (2007b) to acutely increase whole-body carnitine retention, which contained only simple sugars (low molecular weight). The calorie content of the PRO drink was matched to this and halved the amount of carbohydrate ingested.

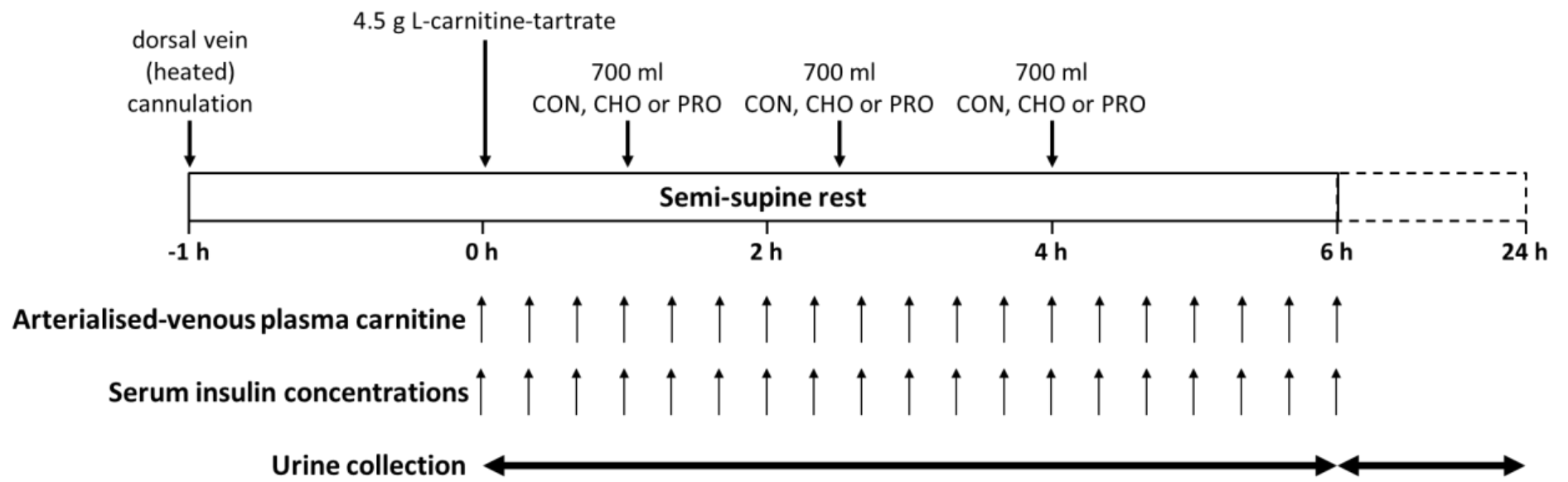


Figure 3.1 Schematic showing the protocol for study 1A.

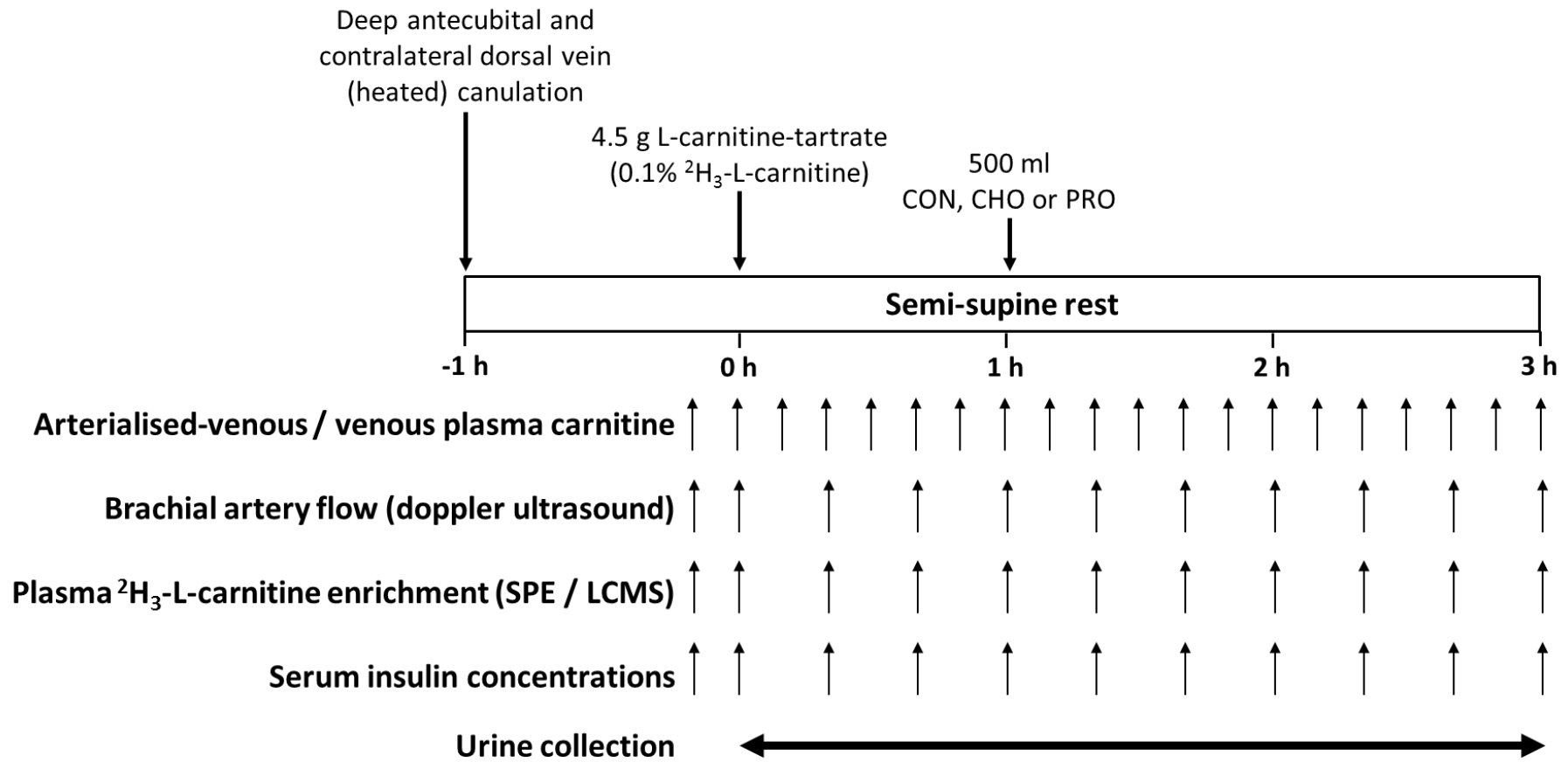


Figure 3.2 Schematic showing the protocol for study 1B.

Sample collection and analysis

Blood samples were drawn every 20 minutes in study A and every 10 minutes in study B for the determination of serum insulin and plasma free and total carnitine concentrations. A total blood volume of 285 ml (95 ml per visit) and 420 ml (140 ml per visit) was sampled for study A and B, respectively. Urine was collected during all study visits and also for the 18 hour post-visit period in study A and analysed for total carnitine content. Following plasma sample purification with strong cation exchange solid phase extraction (SPE), $^2\text{H}_3$ -L-carnitine enrichment in study B was measured using liquid chromatography mass spectrometry (see General Methods).

Calculations

Six hour or 24 hour urinary total carnitine excretion (U_{TC}) in milligrams was calculated as:

$$U_{TC} = \frac{C_U \cdot V_U \cdot M}{1000}$$

where C_U is the average total carnitine concentration in the urine (in $\mu\text{mol}\cdot\text{L}^{-1}$), V_U is the total volume of urine passed over the collection period (in L) and M is the molecular weight of carnitine ($161.2 \text{ g}\cdot\text{mol}^{-1}$). Mean brachial artery luminal diameter (D_M) was calculated as:

$$D_M = \left(\frac{1}{3} \cdot D_{PS}\right) + \left(\frac{2}{3} \cdot D_{ED}\right)$$

where D_{PS} and D_{ED} are the average values from triplicate measures made at peak vascular systole and end diastole, respectively. Mean brachial artery blood flow (BAF) is subsequently given by the equation:

$$BAF = v \cdot \pi \left(D_M / 2 \right)^2$$

where v is the time averaged blood velocity. For the calculation of net carnitine balance and carnitine rate of disappearance, brachial artery blood flow was converted to forearm plasma flow (F) by the equation:

$$F = BAF \cdot (1 - H)$$

where H is the haematocrit fraction in whole blood for each volunteer. Net carnitine balance (NCB) was calculated from the arterialised-venous (A_C) – venous (V_C) difference in plasma carnitine concentration determined biochemically using the Fick principle and is given as:

$$NCB = F \cdot (A_C - V_C)$$

Plasma carnitine fractional extraction (FE_C) was also calculated, to provide a flow-independent marker of muscle carnitine uptake, by the following equation:

$$FE_C = (A_C - V_C) / A_C \times 100$$

The rate of carnitine disappearance (R_d) and, therefore, forearm carnitine uptake was determined from standard equations based on the steady-state tracer enrichment values (TTR) converted to molar percent excess values for arterialised-venous (MPE_A) and venous (MPE_V) plasma carnitine concentrations:

$$MPE = TTR / (1 + TTR)$$

$$R_d = [(MPE_A \times A_C) - (MPE_V \times V_C)] \cdot \frac{F}{MPE_A}$$

Statistics

Time dependent variables (serum insulin, plasma carnitine, plasma flow, net carnitine balance, rate of disappearance and carnitine extraction) were analysed with a two way (*drink* \times *time*) repeated measures analysis of variance (ANOVA) with significant main effects isolated post-hoc using a paired t-test with Bonferoni stepwise correction for multiple comparisons. Areas under the curve above baseline (AUC) were calculated for the same variables using the trapezium rule and were compared, along with urinary carnitine excretion, using a one way ANOVA with Tukey post-hoc for multiple comparisons. Pearson moment correlations were also performed between urinary carnitine excretion, plasma carnitine AUC and serum insulin AUC. In study B, time dependent variables (measured every 10 minutes) are averaged over 20 minute periods.

3.3 Results

Serum Insulin

In study A, serum insulin concentrations were similar between trials at baseline (5 ± 1 , 4 ± 0 and 3 ± 0 $\text{mU}\cdot\text{L}^{-1}$ for CON, CHO and PRO, respectively) and did not change throughout the CON trial (displayed in **Figure 3.3A**). Serum insulin concentration rose rapidly following the ingestion of each beverage during CHO and PRO, peaking at $t=80$, 180 and 260 minutes. Peak insulin concentration following the first drink was no different between CHO (78 ± 19 $\text{mU}\cdot\text{L}^{-1}$) and PRO (78 ± 25 $\text{mU}\cdot\text{L}^{-1}$) but remained elevated during CHO at $t=120$ min compared to PRO ($P<0.0001$). Peak responses during CHO were also greater than PRO following the second (95 ± 16 vs 51 ± 6 $\text{mU}\cdot\text{L}^{-1}$; $P<0.0001$) and third (66 ± 8 vs 39 ± 10 $\text{mU}\cdot\text{L}^{-1}$; $P<0.01$) drinks such that the area under the insulin x time curve AUC above baseline during CHO (12.6 ± 2.0 $\text{U}\cdot\text{L}^{-1}\cdot\text{min}$) was 59% ($P<0.05$) greater than PRO (7.9 ± 1.2 $\text{U}\cdot\text{L}^{-1}\cdot\text{min}$). For study B, serum insulin rose from similar baseline concentrations (7 ± 2 , 5 ± 1 and 6 ± 1 $\text{mU}\cdot\text{L}^{-1}$ for CON, CHO and PRO, respectively) following CHO and PRO ingestion, peaking at $t=100$ minutes (64 ± 10 and 72 ± 10 $\text{mU}\cdot\text{L}^{-1}$, respectively) and remained elevated above CON for the rest of the visit (**Figure 3.3B**). Serum insulin was greater in CHO compared to PRO at $t=140$ minutes (42 ± 13 and 24 ± 4 $\text{mU}\cdot\text{L}^{-1}$, respectively; $P<0.05$) but AUC above baseline was no different between trials (4.5 ± 0.8 and 3.8 ± 0.6 $\text{U}\cdot\text{L}^{-1}\cdot\text{min}$, respectively; $P=0.7$).

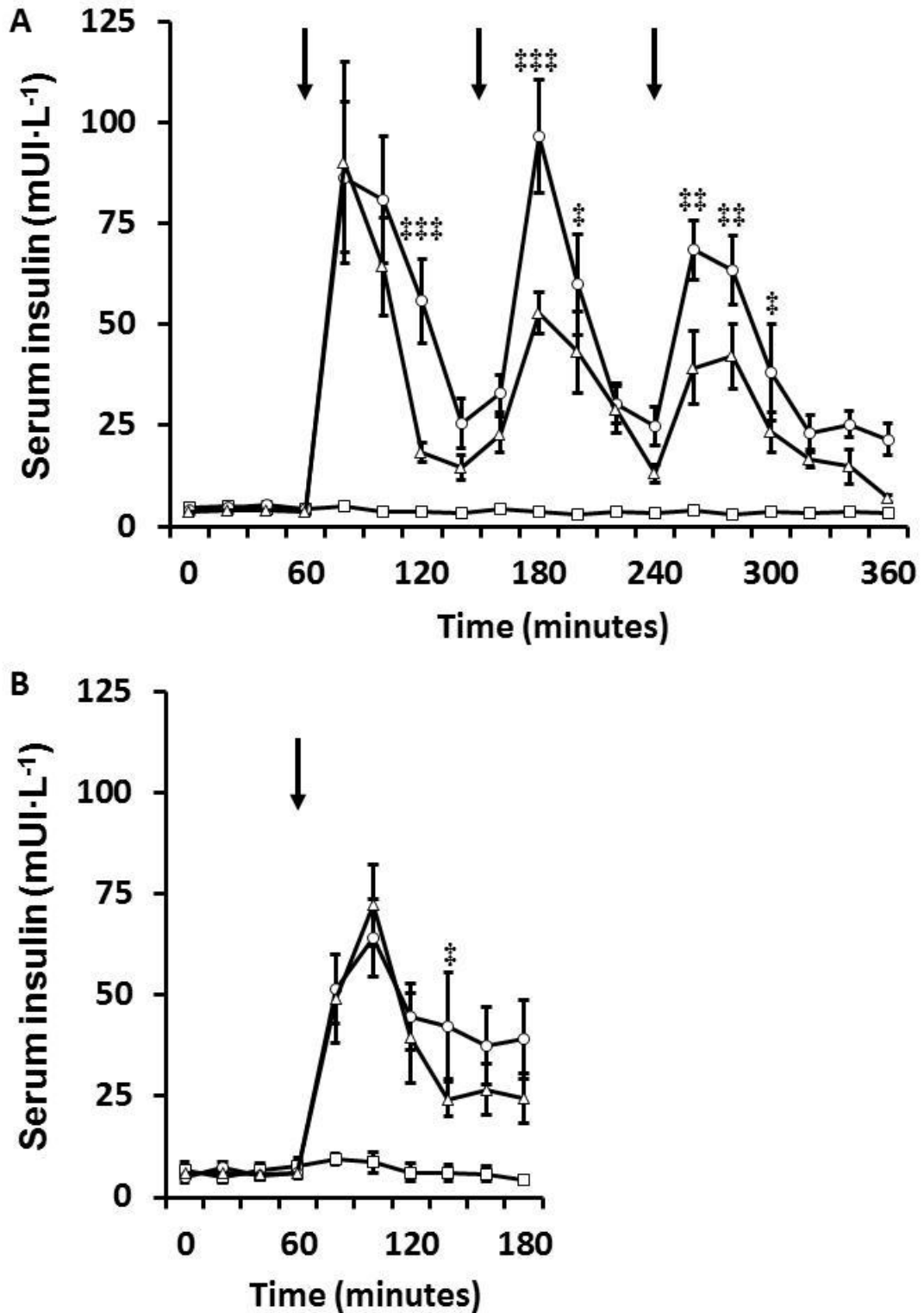


Figure 3.3 Serum insulin responses following ingestion of 3 g L-carnitine (t=0) and 3 x 700 ml (A) or 1 x 500 ml drink (B; arrows) containing flavoured water (CON; squares), 80 g carbohydrate (CHO; circles) or 40 g carbohydrate + 40 g whey protein (PRO; triangles). Values are mean \pm SE, n=8 (A) or 7 (B). † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ for CHO vs PRO.

Plasma carnitine

Plasma total carnitine concentrations during both studies are displayed in **Figure 3.4**. Basal plasma total carnitine (TC) concentrations were similar across visits in study A (41 ± 4 , 42 ± 4 and 44 ± 4) and study B (46 ± 3 , 45 ± 3 and $46 \pm 3 \mu\text{mol}\cdot\text{L}^{-1}$) for CON, CHO and PRO, respectively and were no different over the first hour of either study. In study A, Plasma TC following CON ingestion increased to $65 \pm 3 \mu\text{mol}\cdot\text{L}^{-1}$ at $t=100$ minutes and peaked at $66 \pm 3 \mu\text{mol}\cdot\text{L}^{-1}$ at $t=180$ minutes, before slowly declining throughout the rest of the trial but still remaining elevated above baseline at $t=360$ minutes. The rise in plasma TC in CHO was initially slower, such that CON was greater ($P<0.05$) than CHO from $t=80$ to 120 minutes, but more prolonged, such that plasma TC peaked in CHO ($70 \pm 3 \mu\text{mol}\cdot\text{L}^{-1}$) at a later time of $t=260$ minutes and was elevated above CON ($P<0.05$) from $t=240$ to 360 minutes. Plasma TC in PRO increased more rapidly than CON or CHO and was elevated above CON ($P<0.05$) from $t=100$ to 260 minutes and above CHO ($P<0.05$) from $t=80$ to 200 minutes. After peaking ($82 \pm 5 \mu\text{mol}\cdot\text{L}^{-1}$) at $t=140$ minutes, plasma TC in PRO declined towards CON values and was lower than CHO ($P<0.01$) from $t=320$ to 360 minutes. The six hour plasma TC AUC during PRO ($7.5 \pm 0.9 \text{ mmol}\cdot\text{L}^{-1}\cdot\text{min}$) was 25% ($P<0.01$) and 15% ($P=0.05$) greater than CON ($6.0 \pm 0.8 \text{ mmol}\cdot\text{L}^{-1}\cdot\text{min}$) and CHO ($6.5 \pm 0.7 \text{ mmol}\cdot\text{L}^{-1}\cdot\text{min}$), respectively, which were no different with respect to each other. Study B plasma TC responses during were overall similar to those described for study A, with PRO producing greater increases than CHO or CON ($P<0.001$). Plasma TC during CHO was also suppressed relative to CON at $t=160$ ($P<0.05$). Plasma TC AUC during PRO (3.5 ± 0.5) was 67% and 84% greater than CON (2.1 ± 0.2 ; $P<0.05$) and CHO ($1.9 \pm 0.3 \text{ mmol}\cdot\text{L}^{-1}\cdot\text{min}$; $P<0.01$), respectively.

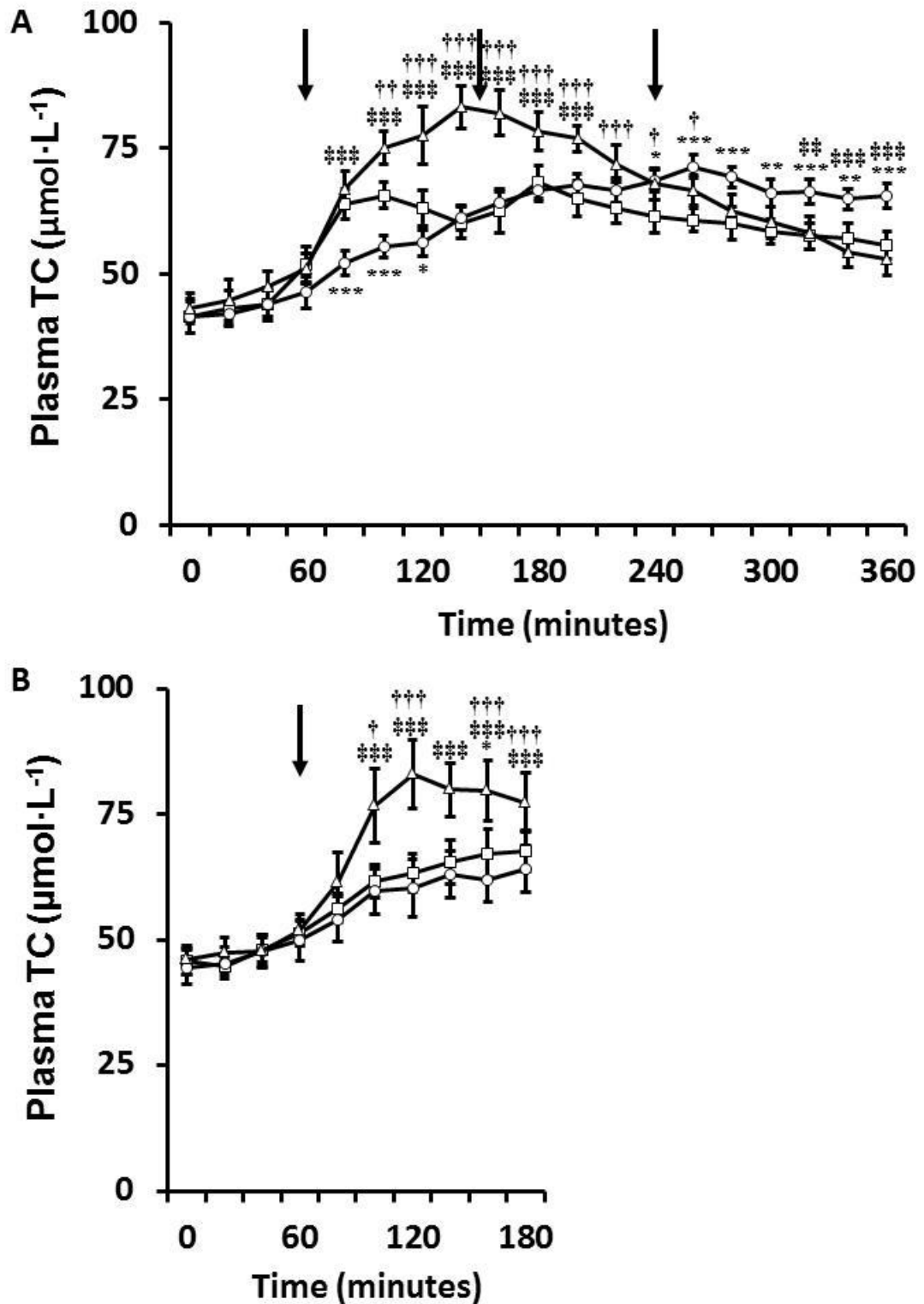


Figure 3.4 Plasma total carnitine responses following ingestion of 3 g L-carnitine ($t=0$) and 3 x 700 ml (A) or 1 x 500 ml drink (B; arrows) of either CON (squares), CHO (circles) or PRO (triangles). Values are mean \pm SE for $n=8$ (A) or 7 (B). * $P<0.05$ for CHO vs CON; † $P<0.05$, ††† $P<0.001$ for PRO vs CON; ‡‡‡ $P<0.001$ for CHO vs PRO.

Urinary carnitine

As shown by **Figure 3.5A**, six hour urinary TC excretion during study A was 71% and 23% greater following PRO (183 ± 14 ; $P < 0.001$) and CHO (149 ± 15 ; $P < 0.05$) than CON (107 ± 10 mg) and tended to be greater in PRO than CHO ($P = 0.06$). After 24 hours, PRO (322 ± 33) remained 34% greater ($P < 0.01$) than CON (240 ± 25) whilst CHO (333 ± 32 mg) was 39% greater, though not significantly ($P = 0.12$ vs CON), and was no different to PRO. In study B, three hour urinary TC excretion in study B tended to be greater in PRO (92 ± 28) than CHO (45 ± 13 ; $P = 0.14$) and CON (46 ± 5 mg; $P = 0.05$), which were no different (**Figure 3.5B**)

Plasma flow

Plasma flow during study B (**Figure 3.6**) was similar at baseline for CON, CHO and PRO (40 ± 7 , 47 ± 10 and 44 ± 9 ml·min⁻¹, respectively) and did not change over the first hour. A main trial effect ($P < 0.05$) was observed such that plasma flow was greater in CHO and PRO than CON from $t = 120$ and $t = 140$ minutes, respectively, for the remainder of the visit. Plasma flow AUC above the averaged pre-drink baseline was greater in CHO (0.5 ± 0.6 ; $P < 0.05$) and tended to be greater in PRO (0.4 ± 0.6 ; $P = 0.05$) than CON (-0.5 ± 0.2 L·min).

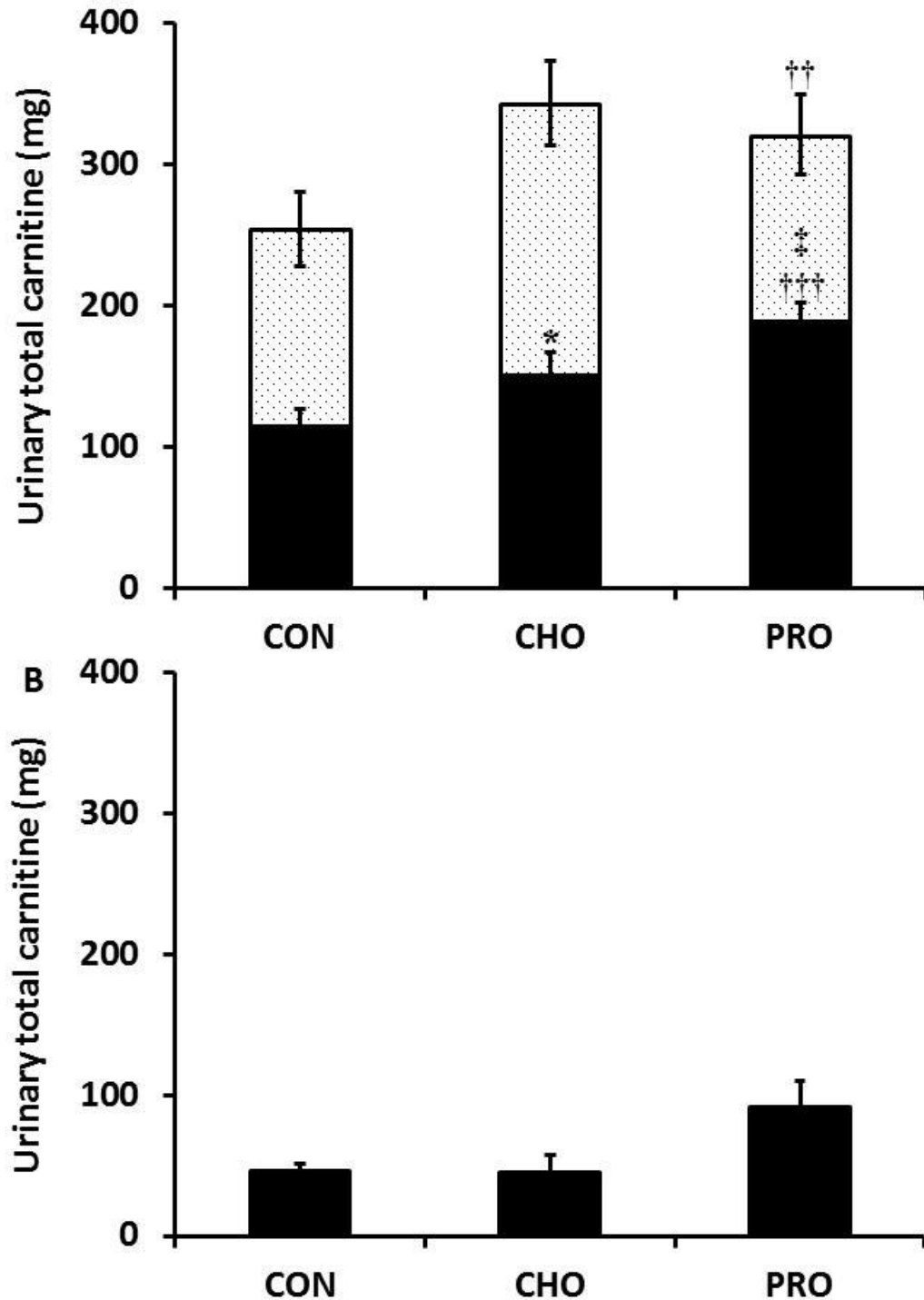


Figure 3.5 (A) Six (solid bars) and 24 (dotted bars) hour urinary total carnitine excretion following ingestion of 3 g L-carnitine and 3 x 700 ml drink of either CON, CHO or PRO. (B) Three hour urinary total carnitine excretion following ingestion of 3 g L-carnitine and 1 x 500 ml of either CON, CHO or PRO. Values are mean \pm SE for n=8 (A) or 7 (B). * $P<0.05$ for CHO vs CON; † $P<0.05$, ††† $P<0.001$ for PRO vs CON. Main effect ($P<0.05$) of trial in study B, PRO vs CON ($P=0.05$) and vs CHO ($P=0.14$).

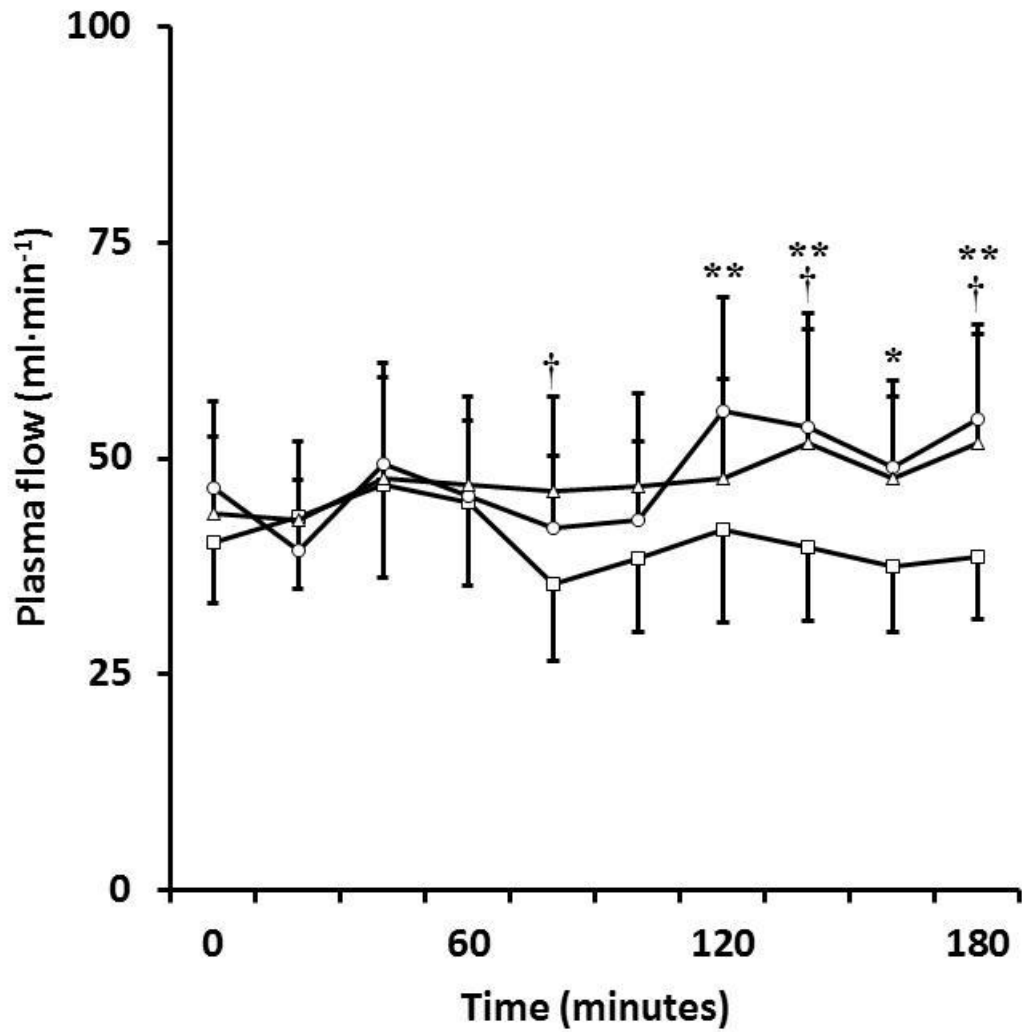


Figure 3.6 Plasma flow responses following ingestion of 3 g L-carnitine (t=0) and 1 x 500 ml drink (arrow) of either CON (squares), CHO (circles) or PRO (triangles). Values are mean \pm SE for n=7. * $P<0.05$, ** $P<0.01$ for CHO vs CON; † $P<0.05$ for CHO vs PRO.

Net forearm carnitine balance and extraction

Net carnitine balance (NCB) across the forearm in study B is shown in **Figure 3.7A** and was unchanged over the one hour following L-carnitine ingestion. However, there was a main effect of time ($P < 0.05$) such that NCB at $t = 120$ minutes was greater ($P < 0.05$) in CHO (170 ± 74) than CON (-18 ± 37) and PRO ($-91 \pm 104 \text{ nmol} \cdot \text{min}^{-1}$). NCB in PRO was also lower than CHO at $t = 100$ ($P < 0.05$) and $t = 180$ ($P < 0.01$) minutes. Net carnitine extraction was increased ($P < 0.05$) in CHO compared to PRO at $t = 100, 120$ and 180 minutes and tended to be increased ($P = 0.09$) above CON at $t = 120$ minutes.

Carnitine MPE and disappearance rate

Arterialised-venous plasma [methyl- $^2\text{H}_3$]-L-carnitine enrichment (MPE) reached a steady state after $t = 80$ minutes in all trials, with a similar enrichment attained in CON and CHO ($\sim 0.14\%$), but a slightly higher enrichment in PRO of $\sim 0.2\%$ (**Figure 3.8**). There was no difference between the arterialised-venous and venous MPE values during the CON or PRO trials, but there was a main effect (MPE_A vs MPE_V , $P < 0.05$) during CHO, with MPE_A tending to be greater ($P = 0.06$) than MPE_V at $t = 100$ mins in CHO. R_d for plasma carnitine was no difference to zero in either CON or PRO, but was significantly greater than zero during CHO at $t = 100$ mins ($P < 0.05$) and tended to be greater than CON (main effect $P = 0.12$). R_d at $t = 100$, which coincided with peak serum insulin concentration in CHO, was 5-fold greater during CHO (520 ± 148) compared to CON ($102 \pm 189 \text{ } \mu\text{mol} \cdot \text{min}^{-1}$), though this did not reach statistical significance (**Figure 3.7B**).

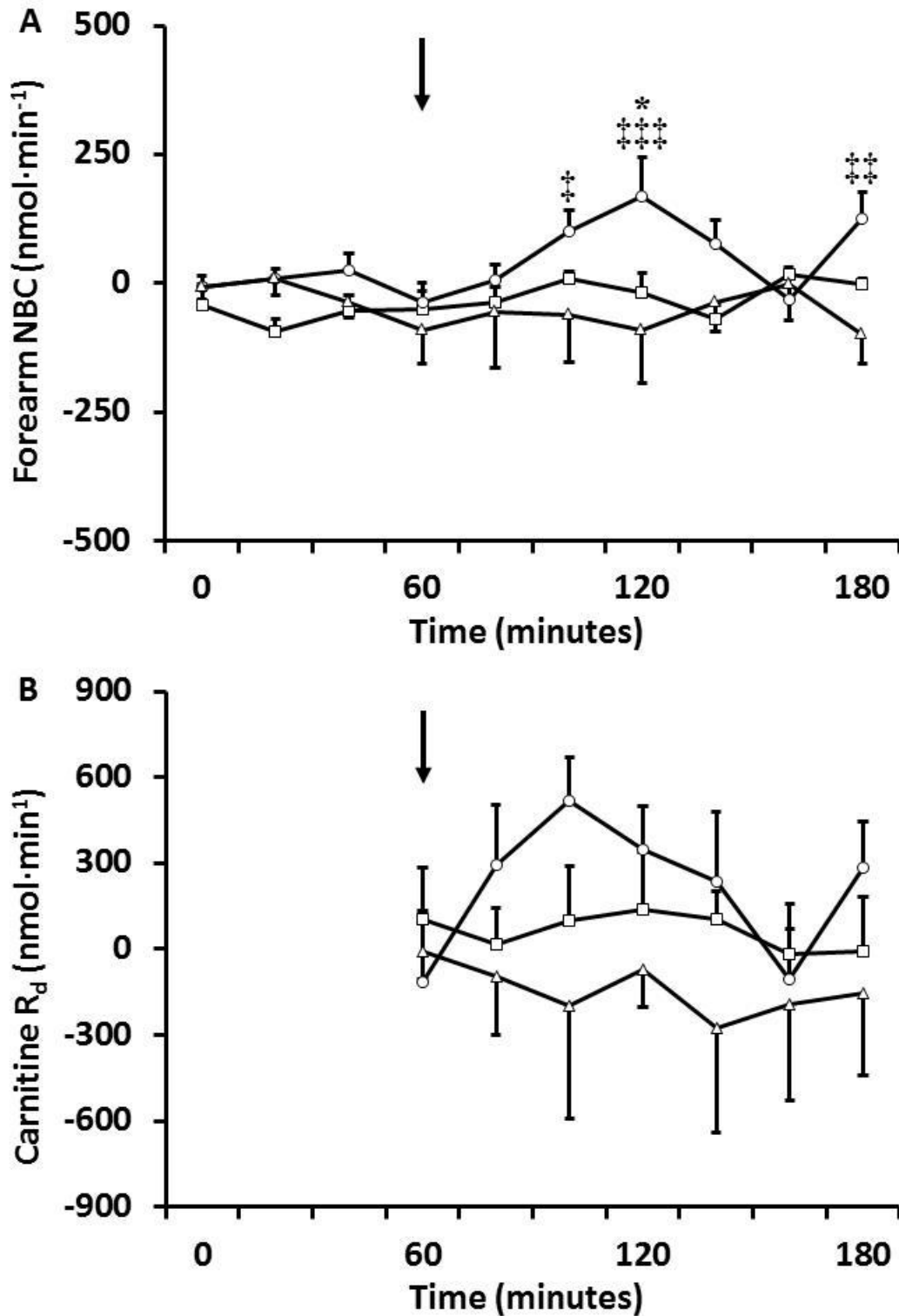


Figure 3.7 Net carnitine balance (A) and rate of carnitine disappearance (B) following ingestion of 3 g L-carnitine (1% ²H₃-L-carnitine) at t=0 and 1 x 500 ml drink (arrow) of either CON (squares), CHO (circles) or PRO (triangles). Values are mean ± SE for n=7 except PRO in B (n=6). * *P*<0.05 for CHO vs CON; ‡ *P*<0.05, †† *P*<0.001, ††† *P*<0.001 for CHO vs PRO.

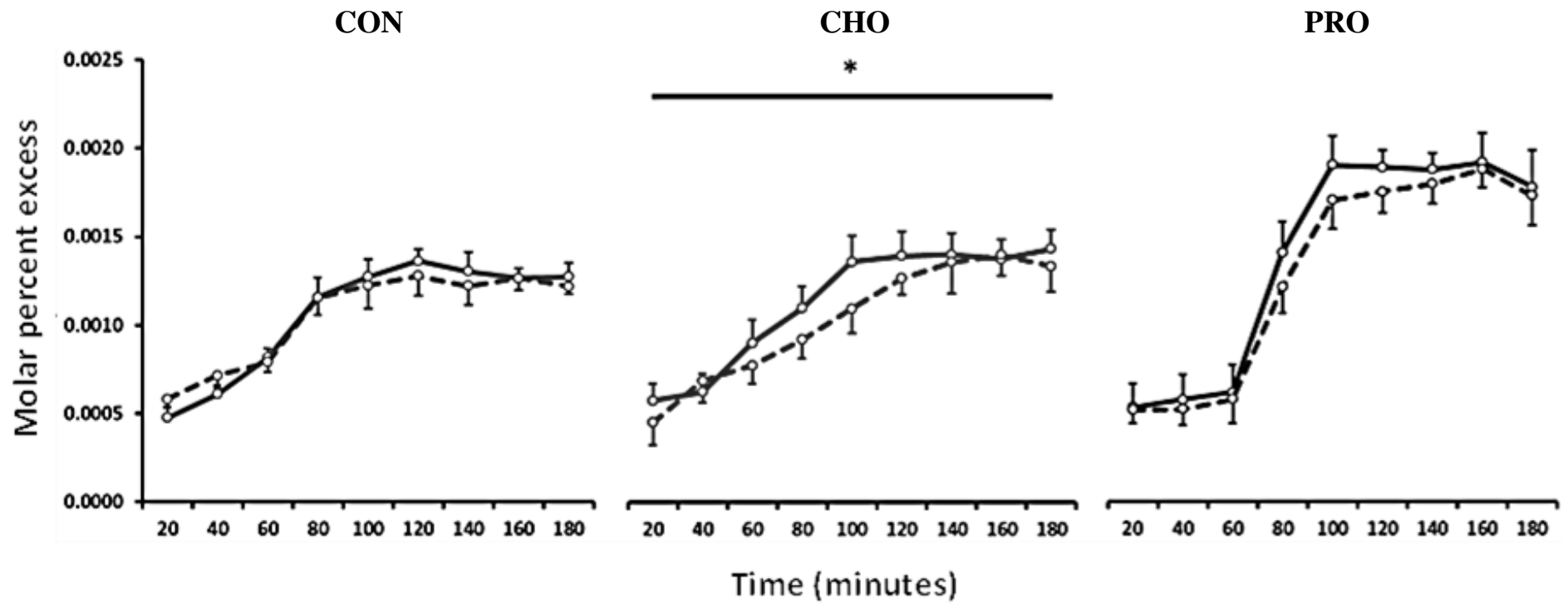


Figure 3.8 [methyl- $^2\text{H}_3$]-L-carnitine enrichment in arterialised-venous (solid line) and venous (dashed line) blood samples (MPE) following ingestion of 3 g L-carnitine (1% $^2\text{H}_3$ -L-carnitine) and 1 x 500 ml drink of either CON, CHO or PRO. * $P < 0.05$ for main effect of AV difference in CHO.

Correlation analyses

For study A there was a strong, positive correlation ($R^2=0.81$; $P<0.01$) between six hour urinary carnitine excretion and plasma TC AUC during CON and a moderate, positive correlation ($R^2=0.56$; $P<0.05$) during PRO. No correlation was found between these variables during CHO. Additionally, there were strong negative correlations between six hour urinary excretion and serum insulin AUC during CHO ($R^2=0.72$; $P<0.05$) and PRO ($R^2=0.74$; $P<0.05$).

3.4 Discussion

It was previously shown that, following acute oral L-carnitine feeding, repeated ingestion of a carbohydrate drink (CHO) resulted in a lower urinary carnitine excretion compared to ingestion of a flavoured water control (Stephens *et al.*, 2006c). Based on the assumption that L-carnitine absorption would be similar and maximal during both visits, these findings were taken to indicate that the carbohydrate facilitated an insulin-mediated increase in muscle carnitine uptake, leading to greater whole-body carnitine retention. In study A of the current investigation, it was thus hypothesised that a protein-carbohydrate (PRO) blend could promote a similar increase in whole-body carnitine retention, secondary to the hypothesis that PRO would stimulate a comparable serum insulin response to CHO. Contrary to this hypothesis, urinary carnitine excretion was increased following CHO and PRO compared to CON, suggesting differential L-carnitine absorption across trials and thus invalidating the use of urinary and plasma carnitine concentrations in the relative determination of whole-body carnitine retention. Study B was subsequently conducted to circumvent the effect of potential alterations in L-carnitine absorption on the inference of muscle carnitine accretion, using parallel

measures of net forearm carnitine balance with plasma carnitine concentrations and urinary carnitine excretion over a tightly controlled three hour period. This demonstrated a clear stimulation of net muscle carnitine uptake following CHO, but not PRO, despite similar serum insulin responses. Thus it can be concluded that 80 g carbohydrate is able to promote a positive net muscle carnitine balance. Furthermore, it would appear that the addition of 40 g protein to 40g carbohydrate antagonises this insulin-mediated muscle carnitine accretion. Collectively, these studies also indicate that CHO and PRO treatment drinks, both containing a high-molecular weight glucose polymer, were able to increase the systemic availability of L-carnitine, presumably due to altered intestinal absorption.

Carbohydrate promotes net muscle carnitine uptake

Studies from this lab have previously shown that acutely elevating plasma carnitine via intravenous infusion has no impact on muscle carnitine content (Stephens *et al.*, 2005). Furthermore, acute (Soop *et al.*, 1988) or chronic (Wachter *et al.*, 2002) oral dosing of L-carnitine does not appear to effect net leg carnitine balance or muscle carnitine accretion, respectively. In agreement with these data and consistent with the high concentration gradient between the plasma and tissues, 3 g L-carnitine had no detectable impact on NCB in the CON trial of study B. In support of this, there was a strong positive correlation between plasma carnitine AUC and urinary carnitine excretion during CON in study A, which most likely reflects the near saturation of renal carnitine reabsorption above basal plasma carnitine concentrations (Harper *et al.*, 1988). In contrast, CHO feeding facilitated a positive NCB across the forearm compared to CON and no correlation with urinary carnitine excretion, providing further support for the ingestion of L-carnitine in a carbohydrate beverage

as a means to promote an insulin-mediated increase in muscle carnitine content (Wall *et al.*, 2011).

In a prior study from Stephens *et al.* (2007b), carbohydrate feeding (4 x 90 g) following L-carnitine ingestion suppressed 24 hour urinary carnitine excretion, both acutely and sustainably over a two week period. Moreover, plasma carnitine was also numerically lower (though not significantly) throughout the CHO trial and was negatively related to the serum insulin response. Consistent with this, plasma carnitine in study A was suppressed following the first CHO drink relative to CON and this coincided with the first peak in CHO serum insulin concentrations. However, despite repeated elevation of serum insulin following the second and third drinks in CHO, plasma carnitine concentrations continued to rise such that they were actually greater than CON during the final two hours of the visit. This posed the question as to whether the suppression of plasma total carnitine following CHO ingestion in the previous trial (Stephens *et al.*, 2007b) and following the first CHO drink of the current study (A) was indeed due to insulin-stimulated muscle carnitine transport or rather, due to a slower L-carnitine absorption from the gut. Indeed, the delayed peak in plasma TC concentration during CHO in the current trial could reflect an impairment of L-carnitine absorption. However, six hour urinary carnitine excretion during CHO exceeded that of CON and appeared to be greater over the entire 24 hour period following L-carnitine ingestion. This would imply that total systemic L-carnitine availability during CHO was at least as high as during CON and argues against a role for CHO in impairing L-carnitine absorption. Moreover, should carbohydrate *per se* have slowed L-carnitine absorption, plasma TC responses during

PRO (40 g CHO) would also be expected to reflect this, which was not the case (discussed later).

If it assumed that the majority of the measured carnitine extraction from the plasma occurred into the forearm musculature (>90% of whole body carnitine stores exist in skeletal muscle) it is possible to estimate whole body rates of insulin-stimulated muscle carnitine accretion in study B. Insulin was elevated from 80 minutes to 180 minutes, over which period the mean area under the NCB x time curve was 7.9 μmol greater in CHO than CON. Assuming a forearm muscle mass of approximately 0.6 kg (Andres *et al.*, 1954; Allwood *et al.*, 1959) and a whole body muscle mass of 30 kg, this equates to a whole body muscle carnitine uptake of 63 mg greater than CON. This is in excellent agreement with the estimate of 60 mg carnitine retention calculated from differences in urinary carnitine excretion in a previous study (Stephens *et al.*, 2007b). Extended to a chronic feeding scenario, this would equate to a daily increase in muscle carnitine content of 57 $\mu\text{mol}\cdot\text{kg dw}^{-1}$, which would be expected to increase muscle total carnitine content ($\sim 22 \text{ mmol}\cdot\text{kg dw}^{-1}$) stores by 22% over 12 weeks. Again, this extrapolation is almost identical to the 21% increase in muscle carnitine content reported by (Stephens *et al.*, 2013) and provides indirect validation for the values of net carnitine balance determined here. In further agreement with our baseline NCB data, one previous study measuring leg carnitine balance found no evidence of net carnitine uptake or efflux at rest (Soop *et al.*, 1988).

Net balance models normally preclude definitive conclusions on whether a positive net balance is due to increased substrate uptake, reduced substrate efflux, or a

combination of the two. The use of a [methyl-²H₃]-L-carnitine tracer in the current study enabled a more direct estimation of muscle carnitine uptake. The average rate of forearm carnitine disappearance throughout the CON trial was ~63 nmol·min⁻¹, which equates to 6.3 μmol·kg·hr⁻¹. In good agreement with this, compartmental modelling of intravenously administered L-[methyl-³H]carnitine kinetics estimated basal rates of muscle carnitine uptake to be 11.6 μmol·kg·hr⁻¹ (Rebouche *et al.*, 1984). In general, R_d data was highly variable between individuals and despite a 5-fold greater R_d observed in CHO over CON, differences did not reach statistical significance when compared across trials. Interestingly however, peak R_d during CHO coincided with peak serum insulin concentrations (t=100 minutes). Furthermore, venous tracer dilution (MPE_{A-V}) was only observed during CHO, consistent with the proposed mechanism of action that skeletal muscle carnitine uptake can be augmented via an insulin-mediated increase in OCTN2 activity. It could also be argued that the increase in NCB during CHO above CON was due to a nutrient-mediated increase in tissue perfusion, as forearm plasma flow was greater following CHO compared to CON ingestion. However, carnitine fractional extraction, which does not depend on plasma flow, was also elevated following CHO. Furthermore, whilst plasma flow was similarly elevated above CON following PRO, no change in NCB or fractional extraction was observed with PRO. Taken together, the increase in NCB observed with CHO cannot be explained, at least not exclusively, by an increased carnitine delivery to the muscle.

Protein antagonises insulin-mediated net muscle carnitine uptake

A principle aim of the current study was to determine whether the insulin response to 40 g carbohydrate plus 40 g protein (PRO) would be sufficient to promote the same

increase in whole-body carnitine retention as that observed with CHO alone. The relationship between serum insulin concentration and muscle carnitine uptake is not unambiguous, but based on previous studies from our group it seems likely that a serum insulin threshold for stimulating muscle carnitine uptake exists somewhere between 50 and 70 $\text{mU}\cdot\text{L}^{-1}$ (Stephens *et al.*, 2007a; Stephens *et al.*, 2007b). Over the six hour study visit, the serum insulin response during PRO was lower than CHO, largely as a result of diminished serum insulin concentrations following the second and third drinks. The blunted insulin response observed after repeated PRO ingestion could perhaps be related to the fact that subjects had been fasting for ~10 hours prior to ingesting the first drink. Although the overall insulin response to PRO was lower than to CHO, peak serum insulin concentrations following the first drink were comparable and hence it seems reasonable to expect that the insulin response to PRO would be sufficient to increase muscle carnitine uptake above CON.

As discussed for CHO, it was impossible to discern from study A whether whole-body carnitine retention following PRO was enhanced over CON. However, study B demonstrated that whilst CHO was able to promote a positive net carnitine balance, PRO appeared to have no effect on muscle NCB. This was surprising as the serum insulin AUC in study B was no different between CHO and PRO. Indeed, the initial insulin response between 60-120 minutes, the time period over which CHO promoted a positive NCB, was almost identical between CHO and PRO. This would suggest that the insulin-mediated increase in NCB promoted by CHO was somehow inhibited by PRO. This is supported by the finding that the rate of carnitine disappearance following PRO was not significantly different to zero. This would appear to be the first study to report evidence of an inhibitory effect of whey

protein or amino acids on muscle carnitine accumulation and the prospective mechanism is unclear. However, it would seem likely that the absence of a positive NCB following PRO be due to an inhibition of OCTN2-mediated carnitine uptake, as no alternative mechanisms appear operative to facilitate skeletal muscle carnitine accumulation. This is supported by the finding that defects within the OCTN2 gene in patients suffering from primary carnitine deficiency can reduce skeletal muscle carnitine concentrations to less than 1% of normal values (Treem *et al.*, 1988). Transport of carnitine by OCTN2 is known to be inhibited by several xenobiotics including steroidal, zwitterionic and organic cationic compounds, as well as carnitine derivatives such as D-carnitine and acylcarnitines of various chain lengths (Ohashi *et al.*, 1999; Wu *et al.*, 1999; Tamai *et al.*, 2000). In contrast, endogenous compounds, including the amino acids leucine, lysine and arginine, do not appear to influence basal OCTN2-mediated carnitine transport (Ohashi *et al.*, 1999; Kobayashi *et al.*, 2005), at least at the concentrations tested (500 μ M). However, this does not necessarily rule out a role for amino acids in antagonising the insulin-stimulated up-regulation of OCTN2 carnitine transport. Similarly to carnitine, the sodium-dependent co-transport of amino acids in skeletal muscle occurs against a steep transport gradient and is augmented by hyperinsulinemia (Shotwell *et al.*, 1983). Moreover, Bonadonna *et al.* (1993) demonstrated that the insulin-mediated increase in forearm amino acid uptake is associated with a reversal in net forearm potassium balance, implicating the stimulation of Na^+/K^+ -ATPase activity as a putative mechanism. Based on values reported for OCTN2 (Tamai *et al.*, 2000) and the Na^+ -dependent amino acid transport systems (Jacquez, 1973; Chen & Russell, 1989), transport of both carnitine and amino acids would appear to share an affinity for sodium in the same order of magnitude (1-5 mM). Whether the extracellular amino

acid concentrations following PRO were sufficient to effectively out-compete OCTN2 for sodium is unknown, but seems plausible given that plasma amino acid availability was likely several-fold higher than that of carnitine.

It cannot be discounted that our measurement of net carnitine balance in PRO was violated by the very rapid rise in plasma carnitine concentrations, providing a non-steady state condition during the period of greatest serum insulin concentrations. However, correcting the venous carnitine concentrations for the expected transit time through the forearm (Cobelli *et al.*, 1989; Tessari *et al.*, 1991) did not influence the relative differences in net carnitine balance or R_d between trials. Indeed, when using an arteriovenous difference model, rapid increases in plasma substrate concentrations between two time points are more likely to lead to an overestimation of substrate uptake over that period.

Effects of macronutrient co-ingestion on L-carnitine absorption

The plasma total carnitine pool following L-carnitine ingestion can be considered as a function of intestinal absorption and carnitine clearance, with the latter influenced by urinary carnitine excretion and tissue carnitine uptake (likely primarily into muscle). The reported K_m for intestinal carnitine transport is approximately $560 \mu\text{mol}\cdot\text{L}^{-1}$ (Hamilton *et al.*, 1986) and it has been demonstrated that the absolute bioavailability of a 6 g oral bolus (~300 mg) of L-carnitine is no greater than that of a 2 g bolus (~320 mg; Harper *et al.*, 1988). Thus it was expected that a 3 g dose of L-carnitine, which would likely increase jejunal carnitine concentrations to approximately $19,000 \mu\text{mol}\cdot\text{L}^{-1}$ (Li *et al.*, 1992), would saturate intestinal active carnitine transport and thus evoke maximal and equivalent carnitine absorption

during all trials. However, the divergent responses in plasma carnitine and urinary carnitine excretion, when considered together with the calculated differences in net forearm carnitine balance indicate that carnitine absorption was not comparable across CON, CHO and PRO.

Plasma carnitine responses in CON were very similar between study A and B, increasing from an average basal value of $43 \mu\text{mol}\cdot\text{L}^{-1}$ to a three hour peak of $67 \mu\text{mol}\cdot\text{L}^{-1}$. Urinary carnitine excretion after three (study B), six and 24 hours in CON represented 1.5, 3.6 and 8.0% of the oral dose, respectively. This is in very good agreement with other studies that have reported 24 hour urinary carnitine excretions of 7-8% from a similar oral bolus (Harper *et al.*, 1988; Stephens *et al.*, 2007b). Consistent with the dose-dependent renal elimination rates of intravenously administered boluses of L-carnitine (Harper *et al.*, 1988), we also observed a strong positive correlation between plasma carnitine AUC and six hour urinary carnitine excretion. This also likely reflects the rapid saturation of tubular reabsorption following elevation of plasma carnitine concentration. Based on an approximate bioavailability of 16% for oral L-carnitine of this dose size (Harper *et al.*, 1988) it can be predicted that around 480 mg of L-carnitine would have been absorbed in the CON trial, with half of this excreted in the urine within 24 hours. Carbohydrate feeding in study A increased 24 hour urinary carnitine excretion to 11% of the ingested L-carnitine dose, such that it was 93 mg greater than in CON. Based on the greater NCB found with CHO in study B, it can be assumed that muscle carnitine uptake would also have been greater in CHO over the six hour study A. Thus, increased urinary carnitine excretion following CHO, in the face of greater muscle carnitine uptake, relative to CON, would imply that intestinal carnitine absorption

was greater with CHO. Our calculation of insulin-stimulated whole-body muscle carnitine retention (63 mg) would suggest that at least 150 mg more carnitine was absorbed during CHO than CON trials. This figure likely represents a conservative estimate, given that CHO was fed more frequently in study A and thus insulin-stimulated muscle carnitine uptake may have been even greater.

Why contrasting results on urinary carnitine excretion were evident in the previous acute carbohydrate-L-carnitine feeding study (Stephens *et al.*, 2007b) may be due to modifications of the treatment drinks consumed. The main difference between this study and the aforementioned study is that the current study utilised Vitargo, a very high-molecular weight carbohydrate (500,000-700,000 g·mol⁻¹) compared to the lower molecular weight glucose-syrup (~500 g mol⁻¹) used previously. Vitargo has previously been shown to increase gastric emptying by 120% (Leiper *et al.*, 2000) in the 10 minute post-ingestion period, as well as both increase and accelerate the post-ingestion rises in blood glucose and serum insulin (Stephens *et al.*, 2008), compared to a low molecular weight solution. Secondly, the Vitargo solution has a very low osmolality (<50 mOsmol·L⁻¹), which could also be expected to increase fluid absorption in the duodenum segment relative to a glucose-syrup formulation (Gisolfi *et al.*, 1998). A greater gastric emptying following both CHO and PRO would be expected to increase L-carnitine delivery to the small intestine which, along with a potentially greater fluid absorption, could be expected to increase L-carnitine absorption by facilitating greater passive diffusion (Li *et al.*, 1992). Thus, the sustained elevation of plasma carnitine concentrations in CHO relative to CON in study A could reflect a progressive increase in L-carnitine absorption following repeated CHO ingestion. It should also be noted that macronutrient availability *per*

se could modulate intestinal transport activity for L-carnitine, by increasing total solute flux over that observed with CON (Gisolfi *et al.*, 1998).

The addition of whey protein appeared to further influence the pharmacokinetics of L-carnitine. Across both studies, the average rate of increase in plasma carnitine concentration over the first hour following PRO ingestion was approximately $0.5 \mu\text{mol}\cdot\text{L}^{-1}\cdot\text{min}^{-1}$, which was around 3-fold faster than following CON or CHO ingestion ($\sim 0.17 \mu\text{mol}\cdot\text{L}^{-1}\cdot\text{min}^{-1}$ for both). Peak plasma carnitine concentration during PRO was also 20-30% greater than CON or CHO trials, and occurred consistently earlier. As discussed above, it is possible that the inclusion of a high-molecular weight carbohydrate in PRO promoted an increased rate of gastric emptying, which could potentially explain differences between CON and PRO plasma TC concentrations. The question remains as to whether the differences in plasma and urinary TC concentrations between PRO and CHO can be explained purely by the additional differences in tissue uptake. Based on an average plasma volume of 3.25 L, the plasma carnitine compartment at three hours was increased by 79 and 32 μmol (13 and 5 mg) above baseline in CHO and PRO trials, respectively. Urinary carnitine excretion was 45 mg and 92 mg, respectively. Thus it follows that the estimated difference in whole body muscle carnitine uptake ($\sim 92 \text{ mg}$) more than compensates for the difference in plasma and urinary carnitine values. It should be noted that measurements of net carnitine balance were particularly variable during the PRO trial and, as such, these extrapolations should be viewed with caution. Indeed, if the differences in plasma carnitine concentrations between CHO and PRO were purely due to a greater muscle carnitine uptake in CHO, one might expect the arteriovenous difference in CHO to be far greater than what was observed here.

Independent effects of whey protein ingestion without carbohydrate on L-carnitine absorption cannot therefore be ruled out, and would be plausible given the primary sources of dietary carnitine (red meat, fish, dairy products) are foodstuffs rich in amino acids. Why then, the uptake of carnitine by skeletal muscle would be inhibited by compounds it is habitually co-ingested with is somewhat perplexing. Perhaps this may serve to preserve plasma carnitine concentrations during postprandial periods, when serum insulin concentrations would be elevated, which would in fact be entirely consistent with the stability and slow turnover of the muscle carnitine pool.

Conclusions

The novel application of an arteriovenous forearm balance model with oral $^2\text{H}_3$ -L-carnitine tracer methodology in this study confirmed the efficacy of a carbohydrate beverage in promoting muscle carnitine accretion when co-ingested with an L-carnitine bolus. A relatively large bolus of whey protein (40 g), with 40 g carbohydrate, appeared to blunt this stimulation of net muscle carnitine uptake, despite stimulating comparable insulin secretion and intestinal carnitine absorption to 80 g carbohydrate alone. These studies also suggest, for the first time, that macronutrients co-ingested with L-carnitine may alter its pharmacokinetic properties which should facilitate further developments of strategies used to increase skeletal muscle carnitine content.

**CHAPTER 4: SKELETAL MUSCLE CARNITINE
METABOLISM, PDC FLUX AND NON-
MITOCHONDRIAL ATP PRODUCTION DURING
SINGLE-LEG KNEE EXTENSION AND REPEATED-
BOUT CYCLING EXERCISE**

4.1 Introduction

Skeletal muscle acetylcarnitine concentrations reflect the balance between pyruvate-derived acetyl-group production from the PDC and condensation with oxaloacetate by citrate synthase. Therefore, the accumulation of acetylcarnitine during exercise can be considered as being indicative of an imbalance between PDC flux and TCA cycle flux. As discussed in the **General Introduction**, acetylcarnitine accumulation is readily observable during the first 5 - 10 minutes of submaximal exercise above a relative intensity of approximately 50% VO_{2max} , with acetylcarnitine concentrations reportedly stabilising as the exercise continues (Sahlin *et al.*, 1990; Constantin-Teodosiu *et al.*, 1992). This plateau in acetylcarnitine concentrations would suggest that a steady-state is reached, at which point PDC and TCA cycle fluxes can be assumed to be well matched. In a previous study from our lab, it was demonstrated that increasing skeletal muscle total carnitine content over a 24 week period was associated with a suppression of PDC flux during 30 minutes of cycling exercise at 50% VO_{2max} , as evidenced by a lower rate of glycogen utilisation, PDCa and acetylcarnitine accumulation (Wall *et al.*, 2011). These findings were consistent with the role of carnitine in CPT1-mediated fat oxidation under conditions when PDC flux is relatively moderate and not limiting to TCA cycle flux. Intriguingly, when the exercise was continued for 30 minutes at 80% VO_{2max} , the increase in muscle carnitine availability facilitated a greater PDC flux, as reflected by an increase in PDCa and acetylcarnitine accumulation. Moreover, this carnitine-mediated increase in PDC flux was associated with a reduced reliance on non-mitochondrial ATP production, resulting from a lower rate of PCr degradation and a diversion of glycogenolysis away from lactate accumulation. These findings would suggest that carnitine availability is somehow limiting to PDC flux during continuous high

intensity exercise. Moreover, a sparing of PCr and lower muscle lactate accumulation at the same absolute workload implies that the increase in PDC flux was able to facilitate a greater contribution from oxidative ATP production, which also suggests that PDC flux could be limiting to TCA cycle flux under these exercise conditions.

In contrast to the proposition that PDC flux may limit TCA cycle flux during exercise, Gibala and colleagues (1998) have reported that estimations of TCA cycle flux actually exceed PDC flux. Based on values of leg oxygen uptake, it was determined that TCA cycle flux was approximately 30% greater than PDCa at a workload of 60% W_{\max} and remained 10% greater at 100% W_{\max} . Thus, if acetylcarnitine does reflect the imbalance between PDC and TCA flux during 5-10 min of exercise, then under these conditions one might expect its accumulation to be negligible. However, neither acetylcarnitine nor glycolytic flux were determined in this study and hence inferences about intermediary skeletal muscle metabolism upstream of the TCA cycle cannot be made. Moreover, this study utilised a single-leg knee extension model where, unlike two-legged cycling, mitochondrial ATP production would not appear to be limited by muscle oxygen delivery (Andersen & Saltin, 1985; Boushel *et al.*, 2011). Previously, Jorn Helge and colleagues from the University of Copenhagen have performed a study to interrogate leg substrate metabolism during single-leg knee extension at 55% and 85% W_{\max} (Stallknecht *et al.*, 2007). They kindly allowed us to perform further analysis on the muscle samples obtained which provided an opportunity to further investigate skeletal muscle acetylcarnitine metabolism as an indication of the integration between PDC and TCA cycle fluxes, in a single-leg model using comparable relative workloads to

those previously employed by Wall *et al.* (2011) for two-legged cycling (i.e. 50% and 80% W_{\max}).

Another scenario that can provide valuable insight into the matching of PDC and TCA cycle fluxes is during two repeated bouts of high-intensity, submaximal exercise, where the workload can be maintained constant across both bouts. Under these conditions, it is proposed that oxidative ATP delivery and thus, TCA cycle flux, will be greater in the second bout of exercise (Bangsbo *et al.*, 2001), possibly as a result of a greater PDC flux during this second bout (Putman *et al.*, 1995). Indeed, a study from Bangsbo and colleagues (2001) found that leg VO_2 , an indication of TCA cycle flux, during two 3 minute bouts of single-leg knee extension exercise at 100% W_{\max} (~66 watts) was greater during the second bout, whilst reciprocally, the average rate of non-mitochondrial ATP production was approximately 33% lower. During a single high-intensity, three minute exercise bout, it is unlikely that acetylcarnitine concentrations would reach a steady-state and thus the imbalance between PDC flux and TCA cycle flux would be exemplified (Timmons *et al.*, 1998b). Furthermore, if reliance on non-mitochondrial ATP production is indeed lessened during a subsequent exercise bout, one would expect acetylcarnitine accumulation to also be lower compared to the initial exercise bout. Indeed, although acetylcarnitine accumulation or PDCa were not determined in the study by Bangsbo *et al.* (2001), PDCa has been demonstrated to be maximal after 3 min of contraction in this model (Bangsbo *et al.*, 2002). However, as discussed above, it is possible that the restrictions to oxidative ATP production differ between the single-leg knee extension and two-legged cycling exercise models and no study has previously

determined acetylcarnitine metabolism and non-mitochondrial ATP delivery during repeated bouts of two-legged cycling exercise at this fixed workload (100% W_{\max}).

The overall aim of the current studies was to assess acetylcarnitine accumulation and non-mitochondrial ATP delivery during exercise paradigms where TCA cycle flux is reportedly higher than PDC flux, thus further characterising the balance between PDC flux and oxidative ATP production. This is important if L-carnitine supplementation is to be used to enhance athletic performance or improve the efficacy of exercise prescription in populations where oxidative ATP production may be impaired (see **General Introduction**). In study A it was hypothesised that, in line with the reported excess of TCA flux relative to PDC flux (Gibala *et al.*, 1998) and in contrast to two-legged cycling (Wall *et al.*, 2011), acetylcarnitine accumulation and non-mitochondrial ATP production during single-legged knee extension would be negligible at both 55% and 85% W_{\max} . In study B, it was proposed that acetylcarnitine accumulation and non-mitochondrial ATP production would both decline during a second bout of high-intensity two-legged cycling exercise, when TCA cycle flux is purportedly accelerated (Bangsbo *et al.*, 2001) relative to a first bout at the same fixed workload (100% W_{\max}).

4.2 Methods

4.2.1 Study A

Volunteers

Following a routine screening and written consent (see **General Methods**), ten healthy male volunteers (age 26 ± 2 years, BMI 24.5 ± 0.8 kg·m⁻², VO_{2max} 50 ± 2 ml·kg⁻¹·min⁻¹) completed the exercise protocol approved by the Ethics Committee for Medical Research in Copenhagen (Stallknecht *et al.*, 2007). A complete data set of muscle biopsies was available for a subset of eight of these volunteers.

Experimental protocol

Volunteers completed the single experimental visit following a 12 hour (overnight) fast and having abstained from vigorous physical activity, caffeine or alcohol for 2 days prior to the visit. Following a 30 minute rest period, volunteers completed two 30 minute single-leg knee extension exercise bouts at 55 and 85% of their previously determined maximal kicking power (W_{\max} ; Anderson and Saltin, 1985), separated by a 30 minute rest interval (**Figure 4.1**). The second exercise bout was performed on the contralateral leg to the first bout, with the order of the legs determined by randomisation. In accordance with the objectives of the larger trial that this study formed a part of, a third exercise bout was completed at 25% W_{\max} prior to the exercise at 55% W_{\max} , using the same leg as was recruited for the exercise at 85% W_{\max} . Furthermore, the exercise at 55% W_{\max} was continued for an extra 90 minutes following the initial 30 minute bout. This meant that following the initial exercise bout at 25% W_{\max} , there was a three hour recovery period before the commencement

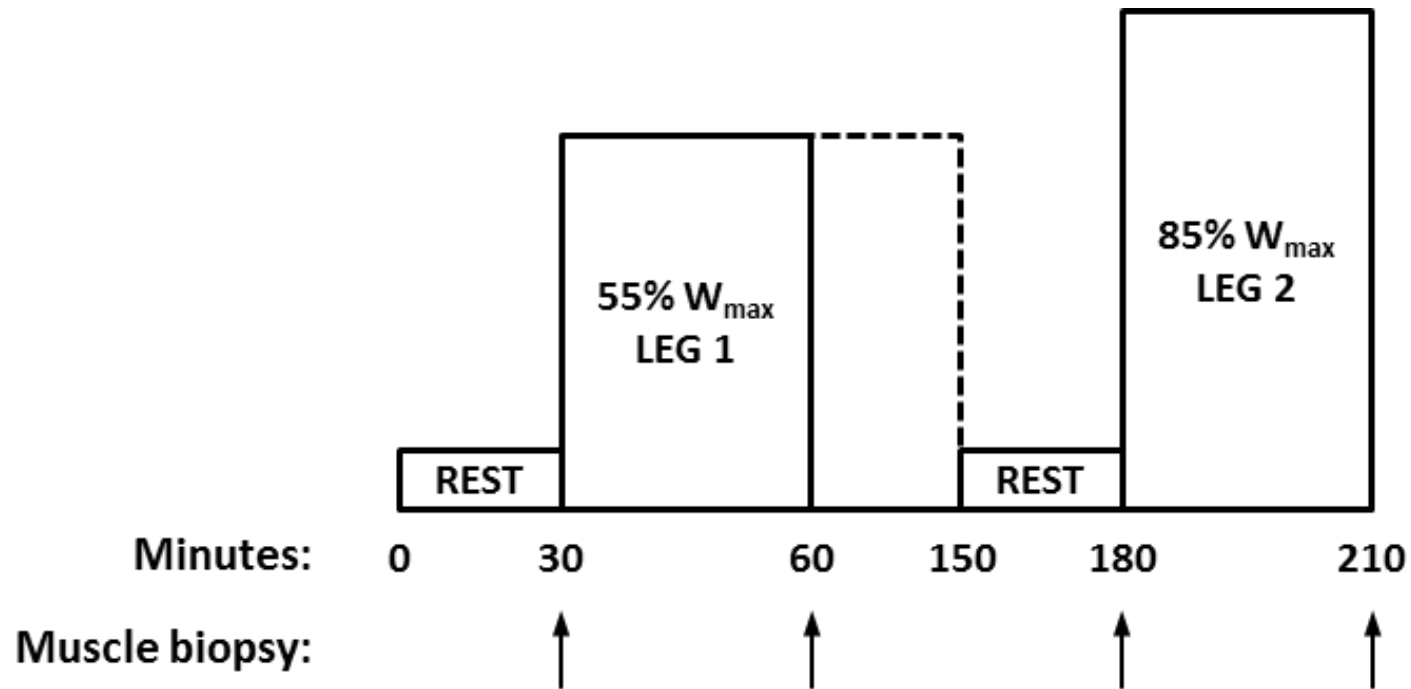


Figure 4.1 Schematic outlining the protocol for Study 2A. Volunteers completed two single-leg knee extension exercise bouts at 55 and 85% W_{max} , respectively, separated by a 30 minute recovery period. Muscle biopsies (arrows) were obtained after 30 minutes of each exercise bout. Dotted lines indicate extension of the 55% W_{max} exercise bout (see text for details).

of the final exercise bout at 85% W_{\max} with the ipsilateral leg and thus it was assumed that any influence of the initial low-intensity exercise bout on skeletal muscle metabolism during this latter bout would be minimal.

Sample collection and analysis

Muscle biopsies were obtained immediately before and after each exercise bout and frozen rapidly (10-15 seconds) in liquid nitrogen for the determination of ATP, phosphocreatine, lactate, glycogen, carnitine and acetylcarnitine concentrations (see **General Methods**). Data collection for study A was completed at the University of Copenhagen (Stallknecht *et al.*, 2007) and metabolite analysis was carried out at the University of Nottingham.

4.2.2 Study B

Volunteers

Seven healthy, non-vegetarian male volunteers (mean \pm SD age 23 ± 4 years; BMI 23.6 ± 2.0 $\text{kg}\cdot\text{m}^{-2}$; $\text{VO}_{2\max}$ 48.0 ± 6.0 $\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) were recruited to participate in study B, which was approved by the University of Nottingham Medical School Ethics Committee. Prior to the study, each participant attended a routine medical screening (see **General Methods**).

Experimental protocol

Volunteers attended the lab 2-3 times prior to the main experimental visit to determine the exercise workload (W_{\max}) corresponding to the attainment of maximal oxygen uptake (see **General Methods**) and to ensure that they were fully

familiarised with the exercise protocol. On the day of the main experimental visit, volunteers arrived at the laboratory at approximately 8.30 am following an overnight fast and having abstained from alcohol, caffeine or strenuous exercise for 48 hours. Following a 30 minute period of supine rest, a muscle biopsy was obtained from one leg and volunteers completed a brief warm-up period (3 minutes at 25% W_{\max}) on the cycle ergometer. It has previously been shown that neither acetyl-CoA nor acetylcarnitine concentrations are altered by either one minute of cycling at 35% $VO_{2\max}$ (Howlett *et al.*, 1998) or three minutes at 30% $VO_{2\max}$ (Constantin-Teodosiu *et al.*, 1991). Volunteers subsequently cycled for three minutes at their pre-determined 100% W_{\max} workload and, upon cessation of exercise, a second biopsy was immediately obtained from the same leg as the first. Volunteers then rested for five minutes before completing a second three minute bout of exercise at the same absolute workload, with further biopsies obtained from the contralateral leg immediately before and after the second bout (**Figure 4.2**). The order of the biopsies was randomised between legs, but the biopsied leg was kept consistent within bouts.

Sampling and analysis

Muscle biopsies were rapidly (~10 seconds) frozen in liquid nitrogen-cooled isopentane and a portion (approximately 50 mg ww) was freeze-dried for the determination of metabolites. A second portion (10-20 mg ww) was used for the determination of PDC activation status.

Statistics

Muscle metabolite concentrations for both studies were compared using a two-way ANOVA (*intensity or bout x time*) and any significant main effects were isolated

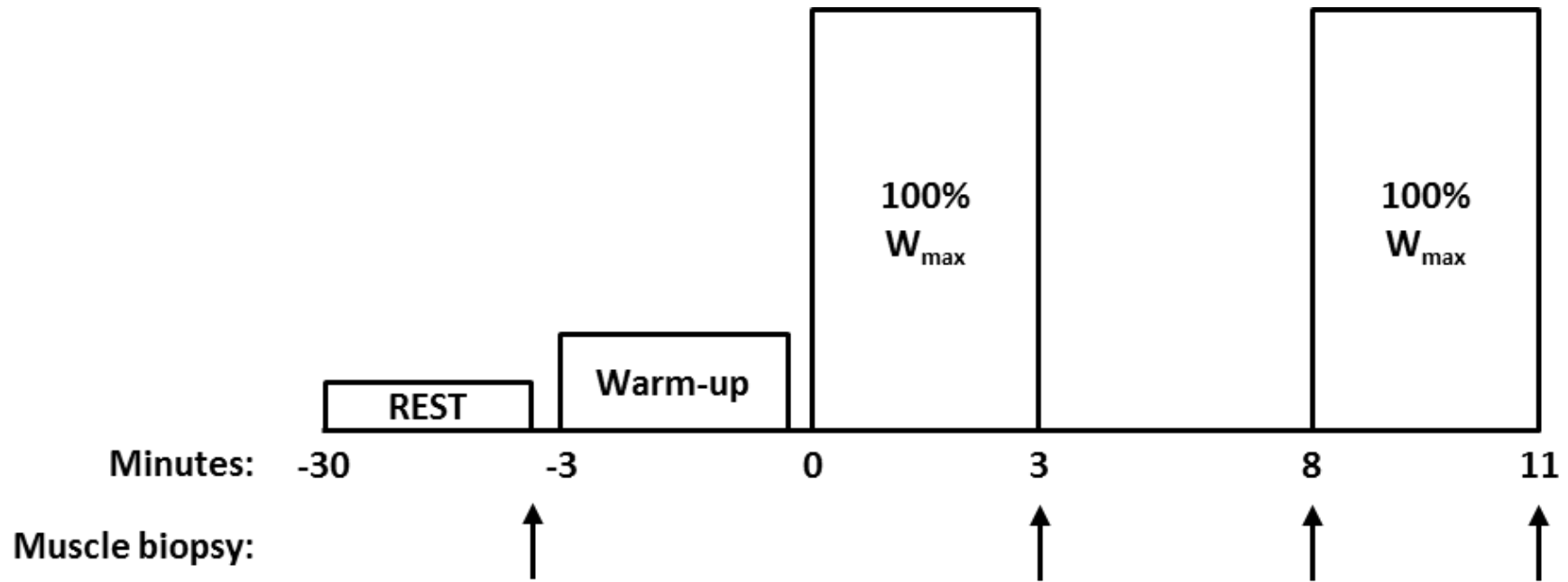


Figure 4.2 Schematic outlining the protocol for study 2B. Volunteers completed two 3 minute bouts of cycle ergometer exercise at 100% VO_{2max} , separated by a five minute passive recovery period. Muscle biopsies (arrows) were obtained before and after each exercise bout.

post-hoc using paired t-tests with Bonferoni stepwise correction for multiple comparisons. Delta values for metabolites (PCr degradation, lactate accumulation, glycogen utilisation, acetylcarnitine accumulation or non-mitochondrial ATP production) were compared between exercise intensities using paired t-tests. Data are presented as mean \pm standard error.

4.3 Results

4.3.1 Study A

Skeletal muscle ATP, PCr, lactate and glycogen

The concentrations of muscle metabolites before and after each exercise bout are shown in **Table 4.1**. Resting levels of ATP and PCr were no different between legs and remained unchanged throughout exercise. Lactate was also unchanged during exercise at 55% W_{\max} and although it increased slightly during exercise at 85% W_{\max} ($P < 0.05$), lactate accumulation was no different between the two exercise workloads (2.6 ± 0.9 and 6.2 ± 2.2 mmol·kg dw⁻¹ for 55 and 85%, respectively). Muscle glycogen utilisation was not significant during exercise at 55% W_{\max} and as such, the pre-exercise glycogen content for each leg was similar. As shown in **Figure 4.3** however, muscle glycogenolysis at 85% W_{\max} was 3-fold greater than at 55% W_{\max} (116.7 ± 41.2 vs 39.3 ± 13.9 mmol·kg dw⁻¹, respectively; $P < 0.01$). The total non-mitochondrial ATP production during each exercise bout is shown in **Figure 4.4** and was similar between exercise at 55% and 85% W_{\max} (0.9 ± 5.9 and 15.0 ± 5.0 mmol·kg dw⁻¹, respectively; $P = 0.13$).

Table 4.1 Skeletal muscle metabolite concentrations before and after two 30 minute bouts of single-leg knee extension exercise at 55 or 85% W_{max} . Data are presented as mean \pm SE for 8 subjects in $mmol \cdot kg \text{ dw}^{-1}$. ^a Main effect of time, ^b main effect of intensity, ^c interaction effect for 2-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs PRE of same bout; † $P < 0.05$ vs POST 55% W_{max} .

	55% W_{max}		85% W_{max}	
	PRE	POST	PRE	POST
ATP	26.3 \pm 0.8	26.6 \pm 1.0	26.1 \pm 0.6	26.3 \pm 0.7
PCr	83.7 \pm 2.3	86.1 \pm 2.1	85.7 \pm 2.4	79.5 \pm 3.9
Lactate ^a	4.1 \pm 0.4	6.7 \pm 1.5	4.2 \pm 0.6	10.4 \pm 1.8*
Glycogen ^{a,c}	403 \pm 34	363 \pm 37	423 \pm 39	308 \pm 29***
Carnitine ^{a,b}	18.9 \pm 1.0	14.6 \pm 1.2*	17.3 \pm 1.0	10.6 \pm 1.0**†
Acetylcarnitine ^a	0.8 \pm 0.2	6.1 \pm 1.9*	2.3 \pm 0.8	8.1 \pm 1.8**

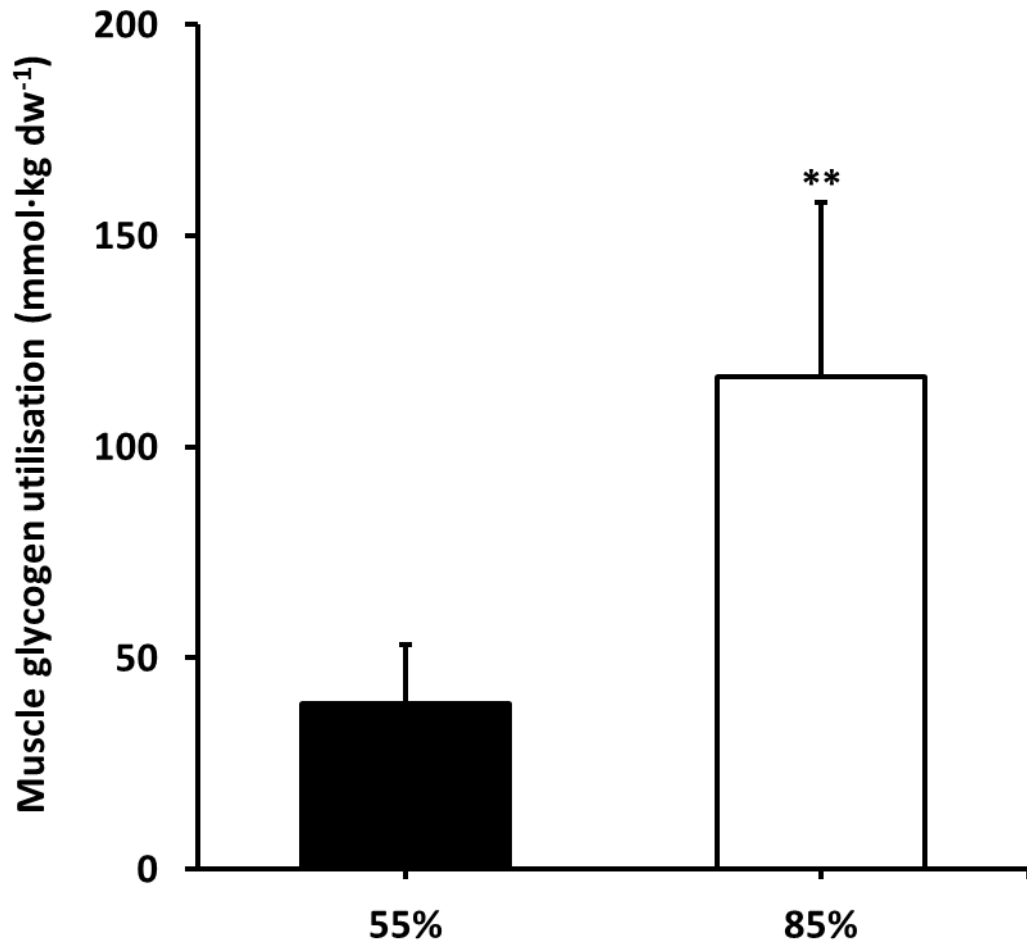


Figure 4.3 Skeletal muscle glycogen utilisation (Δ glycogen) during 30 minutes of single-leg knee extension exercise at 55% (black bar) or 85% (white bar) W_{\max} . Data are presented as mean \pm SE for 8 subjects in $\text{mmol}\cdot\text{kg dw}^{-1}$. $**P < 0.01$ vs 55% W_{\max} for paired t-test analysis.

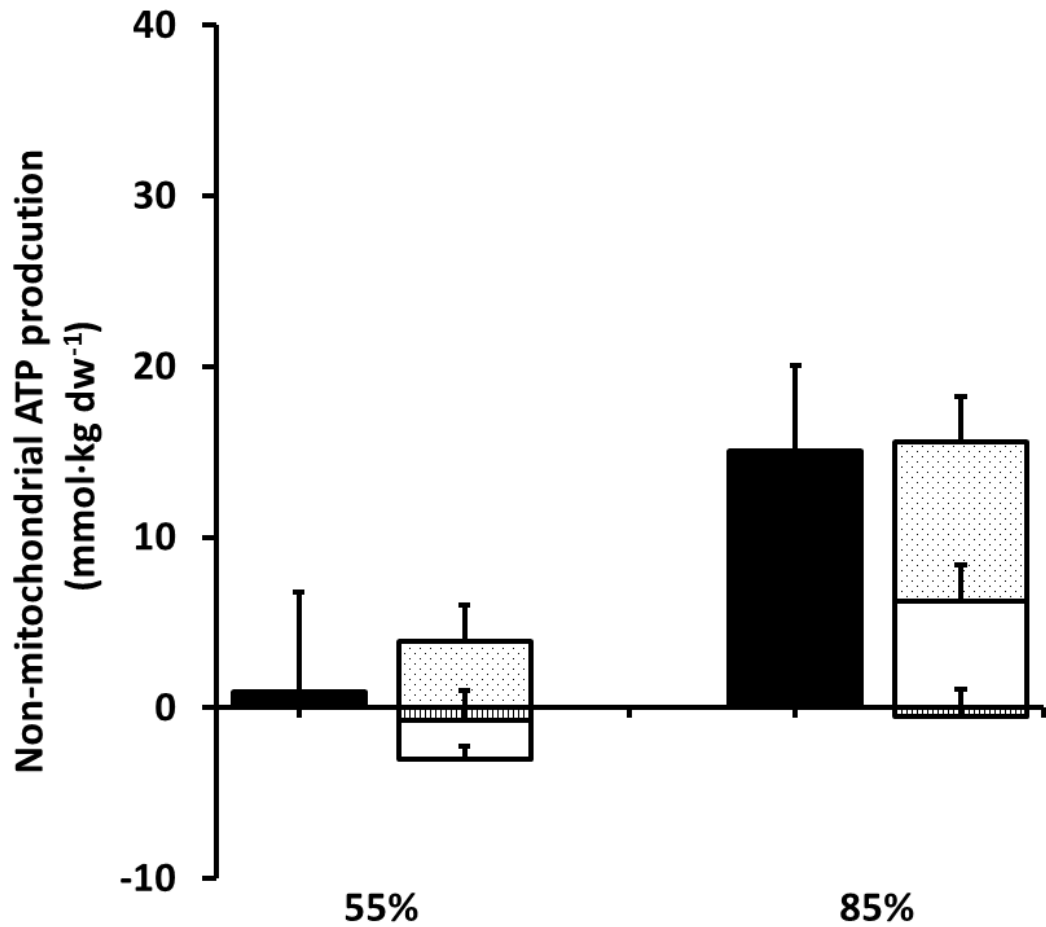


Figure 4.4 Net skeletal muscle ATP production from non-mitochondrial sources (black bars), comprising ATP (striped bars), PCr (white bars) and lactate (dotted bars) during 30 minutes of single-leg knee extension exercise at 55% or 85% W_{max} . Data are presented as mean \pm SE for 8 subjects in $\text{mmol}\cdot\text{kg dw}^{-1}$. Total non-mitochondrial ATP production was no different between workloads ($P=0.13$; paired t-test). Negative values imply net ATP or PCr resynthesis from oxidative metabolism.

Skeletal muscle carnitine metabolism

Free carnitine concentrations declined from similar resting values by approximately 23% ($P<0.05$) and 39% ($P<0.01$) during exercise at 55% and 85% W_{\max} , respectively. Although post-exercise free carnitine availability was lower ($P<0.05$) following exercise at 85% W_{\max} compared to 55% W_{\max} (**Table 4.1**), the absolute decline in free carnitine availability during exercise was no different between workloads (4.3 ± 1.5 and 6.8 ± 2.4 mmol·kg dw⁻¹ at 55 and 85%, respectively; $P=0.23$). Reciprocally, acetylcarnitine concentrations increased during both exercise workloads, with acetylcarnitine accumulation equivocal between exercise at 55% and 85% W_{\max} (5.3 ± 1.9 and 5.8 ± 2.0 mmol·kg dw⁻¹, respectively; $P=0.80$; **Figure 4.5**).

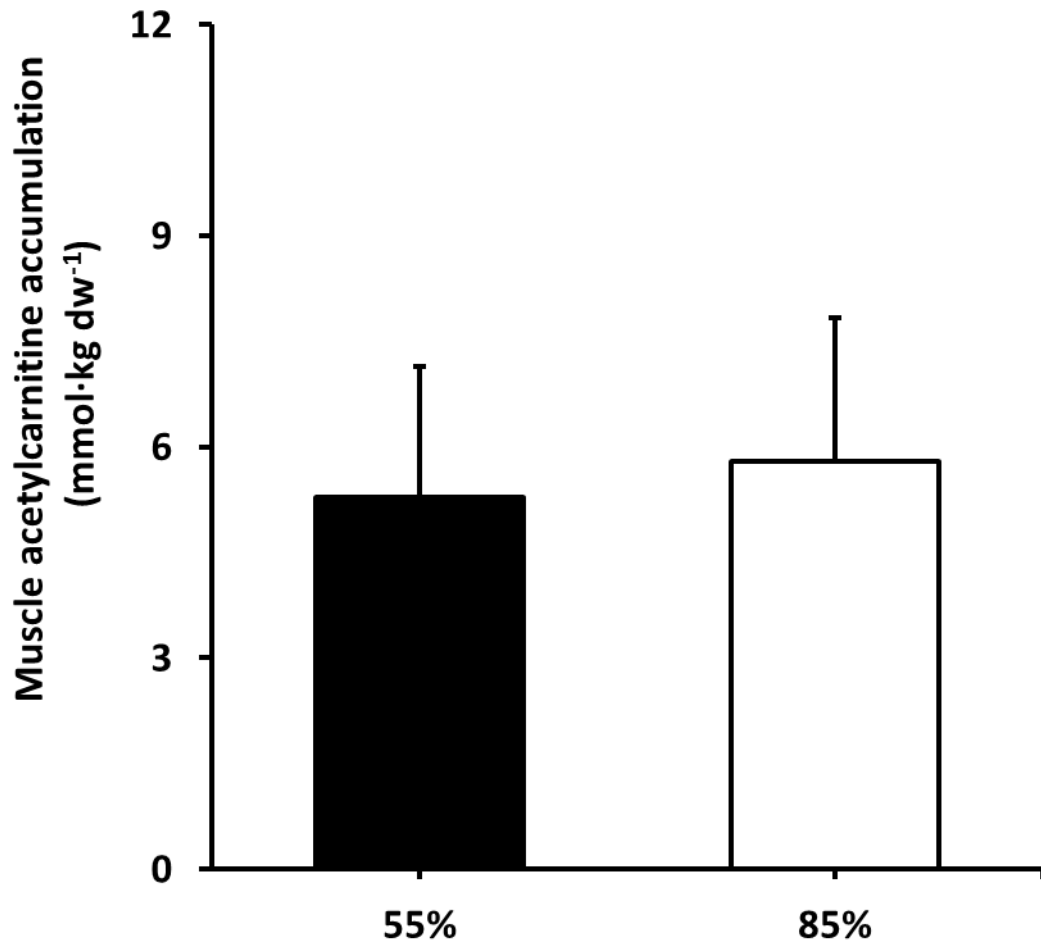


Figure 4.5 Skeletal muscle acetylcarnitine accumulation (Δ acetylcarnitine) during 30 minutes of single-leg knee extension exercise at 55% (black bar) or 85% (white bar) W_{\max} . Data are presented as mean \pm SE for 8 subjects in $\text{mmol}\cdot\text{kg dw}^{-1}$. Acetylcarnitine accumulation was no different between workloads ($P=0.80$; paired t-test).

4.3.2 Study B

Skeletal muscle ATP and PCr

Skeletal muscle metabolite concentrations before and after each exercise bout are shown in **Table 4.2**. ATP concentrations tended to decrease 11% ($P=0.09$) during the first bout of exercise and declined ($P<0.05$) by a similar amount (16%) in the second bout of exercise. During bout one, PCr decreased to 64% of its resting value and was completely resynthesized over the recovery period such that the pre-exercise values were identical for each bout. During bout two, PCr declined ($P<0.001$) to 26% of the pre-bout value with PCr degradation being 2-fold greater ($P<0.05$) during bout two ($60.9 \pm 7.7 \text{ mmol}\cdot\text{kg dw}^{-1}$) compared to bout one ($29.3 \pm 9.0 \text{ mmol}\cdot\text{kg dw}^{-1}$; **Figure 4.6**).

Lactate accumulation and glycogenolysis

There were main effects of exercise ($P<0.01$) and bout ($P<0.001$) on muscle lactate concentration, with lactate increasing significantly ($P<0.05$) during bout one and tending ($P=0.05$) to remain elevated at the start of bout 2. Lactate increased ($P<0.05$) by a similar amount during bout two such that there was no difference in lactate accumulation between bouts (50.3 ± 19.8 and $59.3 \pm 4.9 \text{ mmol}\cdot\text{kg dw}^{-1}$ for bouts 1 and 2, respectively; **Figure 4.7**). Glycogen content declined during both exercise bouts (main effect of exercise $P<0.001$), although glycogenolysis tended ($P=0.11$) to be 2.1-fold greater during bout two ($99.4 \pm 17.9 \text{ mmol}\cdot\text{kg dw}^{-1}$) compared to bout one ($54.4 \pm 13.7 \text{ mmol}\cdot\text{kg dw}^{-1}$; **Figure 4.8**).

Table 4.2 Skeletal muscle metabolite concentrations before and after two 3 minute bouts of cycling exercise at 100% W_{max} separated by 5 minutes of passive recovery. Data are presented as mean \pm SE for 7 subjects in mmol·kg dw⁻¹. ^a Main effect of time, ^b main effect of bout, ^c interaction effect for 2-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs PRE of same bout; † $P < 0.05$, †† $P < 0.01$ vs POST bout one.

	Bout 1		Bout 2	
	PRE	POST	PRE	POST
ATP ^a	27.9 \pm 1.2	24.8 \pm 1.4	26.3 \pm 1.5	22.2 \pm 1.7*
PCr ^{a,b,c}	82.4 \pm 3.6	53.1 \pm 9.5*	82.1 \pm 2.9†	21.1 \pm 6.2***††
Lactate ^{a,b}	6.5 \pm 1.5	56.8 \pm 18.9*	44.3 \pm 12.3	103.6 \pm 13.1*†
Glycogen ^{a,b}	403.8 \pm 43.3	351.9 \pm 48.2	373.2 \pm 55.8	265.0 \pm 54.9***†

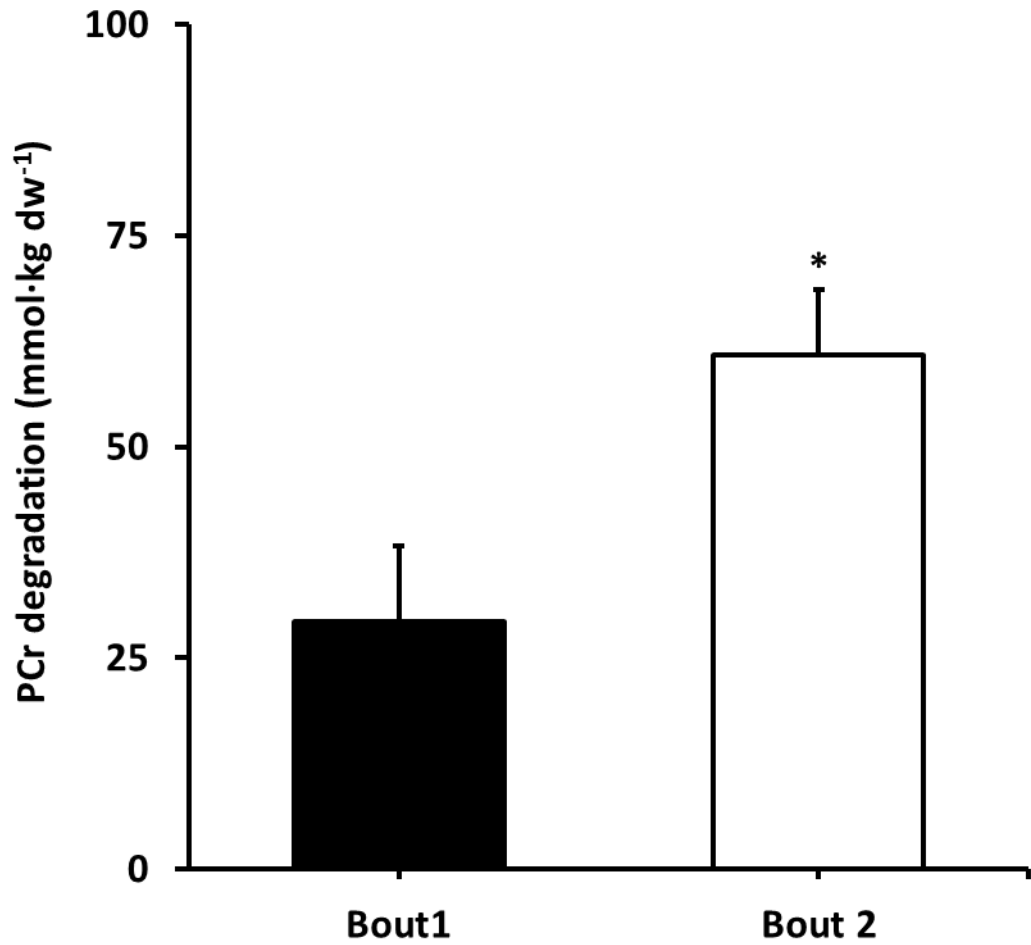


Figure 4.6 Skeletal muscle phosphocreatine degradation (Δ PCr) during two 3 minute bouts of cycling exercise at 100% W_{\max} separated by 5 minutes of passive recovery. Data are presented as mean \pm SE for 7 subjects in $\text{mmol}\cdot\text{kg dw}^{-1}$. * $P<0.05$ versus bout one (paired t-test).

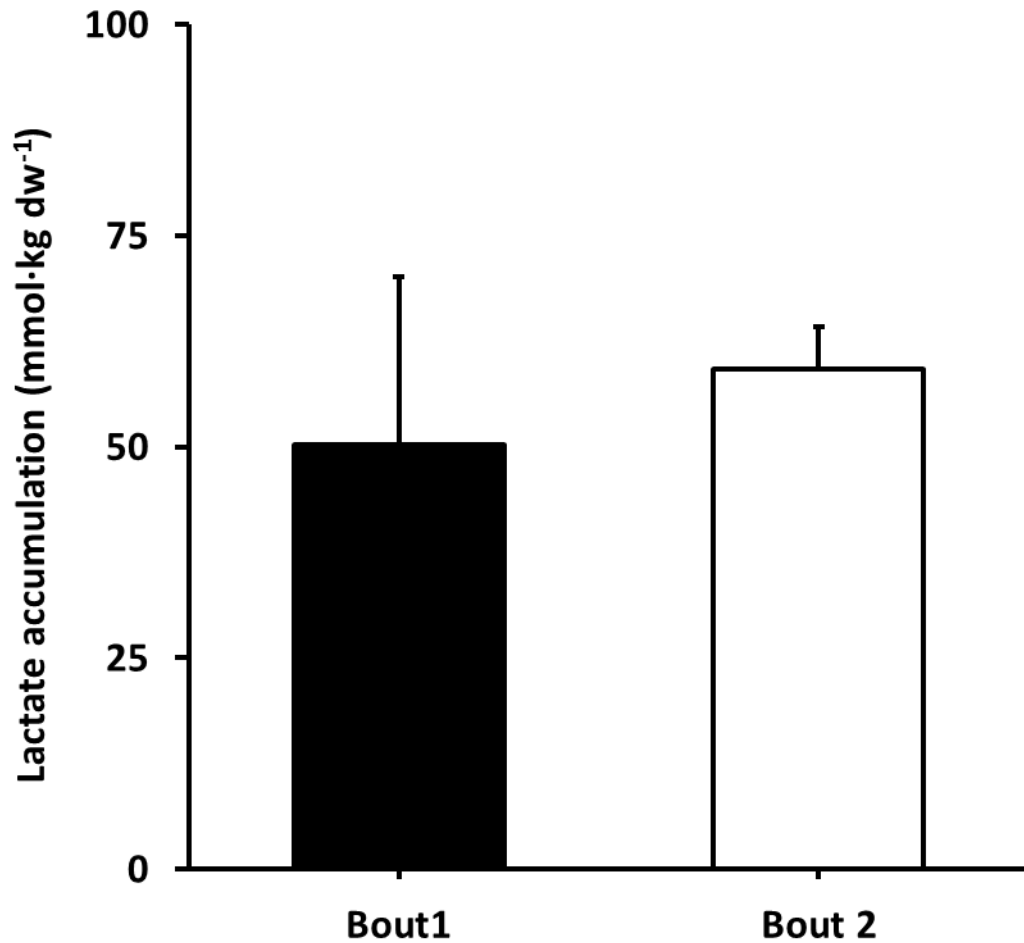


Figure 4.7 Skeletal muscle lactate accumulation (Δ lactate) during two 3 minute bouts of cycling exercise at 100% W_{\max} separated by 5 minutes of passive recovery. Data are presented as mean \pm SE for 7 subjects in $\text{mmol}\cdot\text{kg dw}^{-1}$. Lactate accumulation degradation was not significantly different between the two exercise bouts ($P=0.68$ for paired t-test).

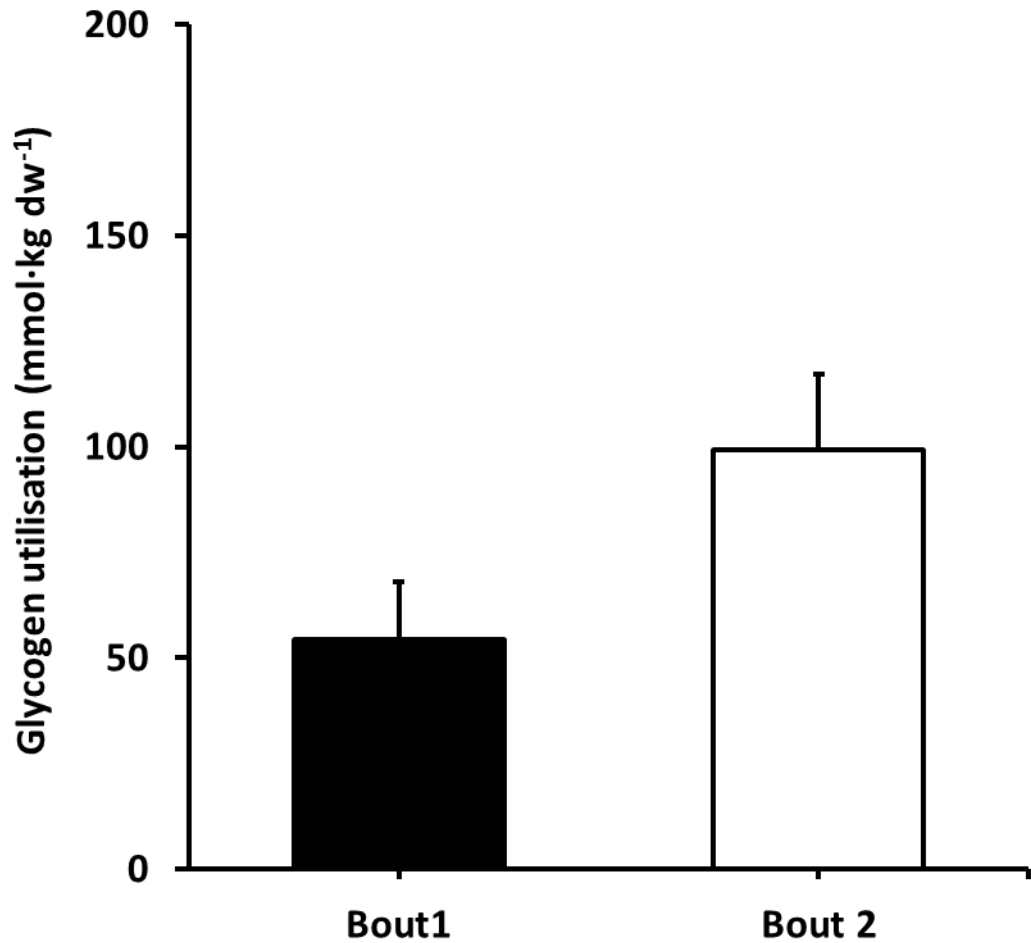


Figure 4.8 Skeletal muscle glycogen utilisation (Δ glycogen) during two 3 minute bouts of cycling exercise at 100% W_{\max} separated by 5 minutes of passive recovery. Data are presented as mean \pm SE for 7 subjects in $\text{mmol}\cdot\text{kg dw}^{-1}$. Glycogen degradation was not significantly different between the two exercise bouts ($P=0.11$ for paired t-test).

Skeletal muscle carnitine metabolism

Resting acetylcarnitine concentration was $4.0 \pm 1.4 \text{ mmol}\cdot\text{kg dw}^{-1}$, increasing to 9.3 ± 2.0 and $12.5 \pm 1.0 \text{ mmol}\cdot\text{kg dw}^{-1}$ following bout one and bout two, respectively (main effect of exercise; $P < 0.001$; **Figure 4.9**). Acetylcarnitine was unchanged during the recovery period such that concentrations before the start of bout two ($8.3 \pm 2.1 \text{ mmol}\cdot\text{kg dw}^{-1}$) tended to be 2.1-fold greater than at rest ($P = 0.10$) whilst the absolute accumulation was similar between bout one and bout two (5.3 ± 2.1 vs $4.2 \pm 1.5 \text{ mmol}\cdot\text{kg dw}^{-1}$). Free carnitine concentration was $15.9 \pm 1.1 \text{ mmol}\cdot\text{kg dw}^{-1}$ at rest and mirrored the changes in acetylcarnitine, with free carnitine tending to decline during bout one (33%; $P = 0.12$) and bout two (41%; $P = 0.13$). Free carnitine remained unchanged during the recovery period (**Figure 4.9**).

Pyruvate dehydrogenase complex activation status

Shown in **Figure 4.10**, PDCa tended to increase 3.2-fold ($P = 0.06$) during bout one, from a resting value of 3.6 ± 0.9 to $11.5 \pm 2.6 \text{ nmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$, before returning to basal values prior to the start of bout two ($4.4 \pm 1.0 \text{ nmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$). During bout two, PDCa increased 5-fold ($P < 0.05$) to a value of $15.5 \pm 3.1 \text{ nmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$.

Non-mitochondrial ATP production

The calculated non-mitochondrial ATP production was not significantly different ($P = 0.29$) between bouts, despite a 42% increase in the second bout (110.9 ± 40.3 vs $158.0 \pm 14.4 \text{ mmol ATP}\cdot\text{kg dw}^{-1}$ for bouts one and two, respectively; **Figure 4.11**).

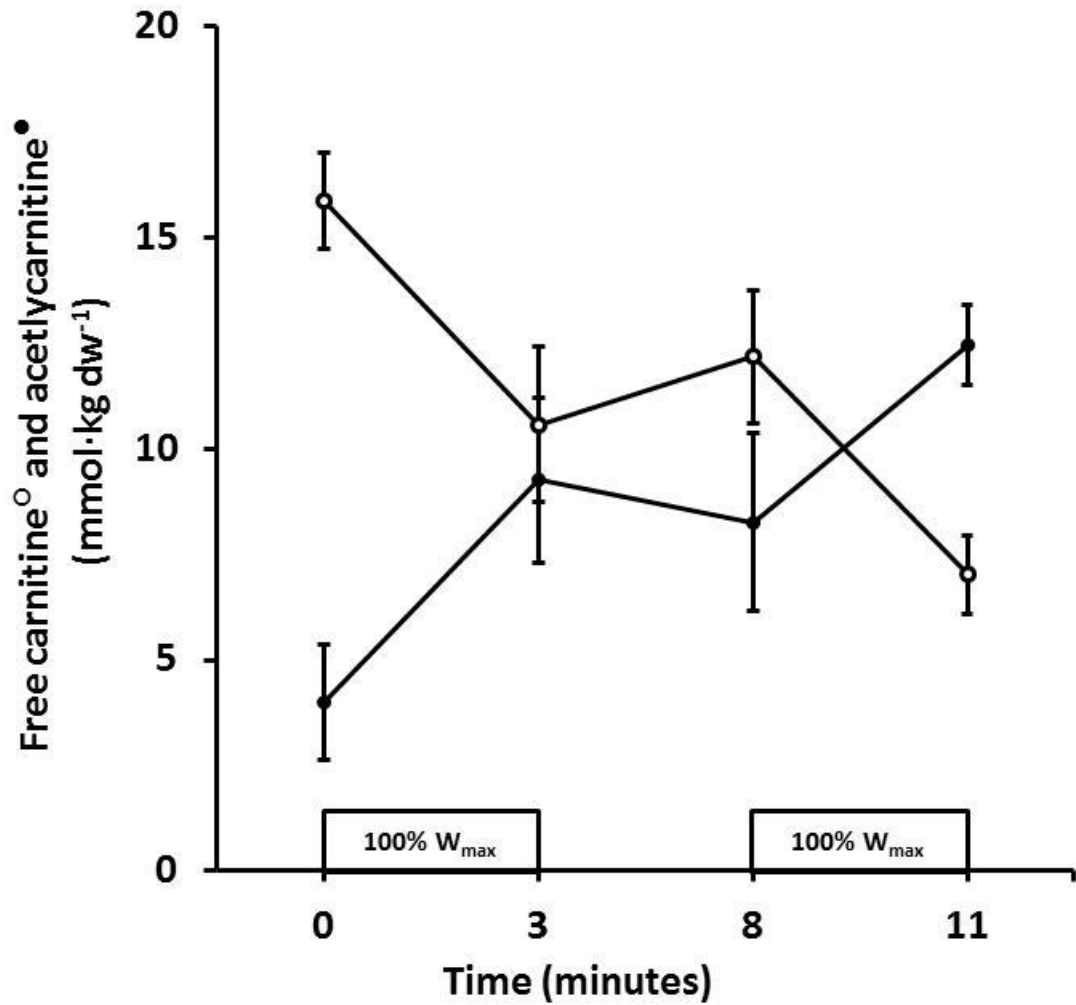


Figure 4.9 Skeletal muscle free carnitine (white circles) and acetylcarnitine (black circles) concentrations before and after two 3 minute bouts of cycling exercise at 100% W_{max} separated by 5 minutes of passive recovery. Data are presented as mean \pm SE for 7 subjects in $\text{mmol}\cdot\text{kg dw}^{-1}$. Main effects of exercise ($P<0.001$) and bout ($P<0.01$) for two-way ANOVA were observed for both free carnitine and acetylcarnitine concentrations.

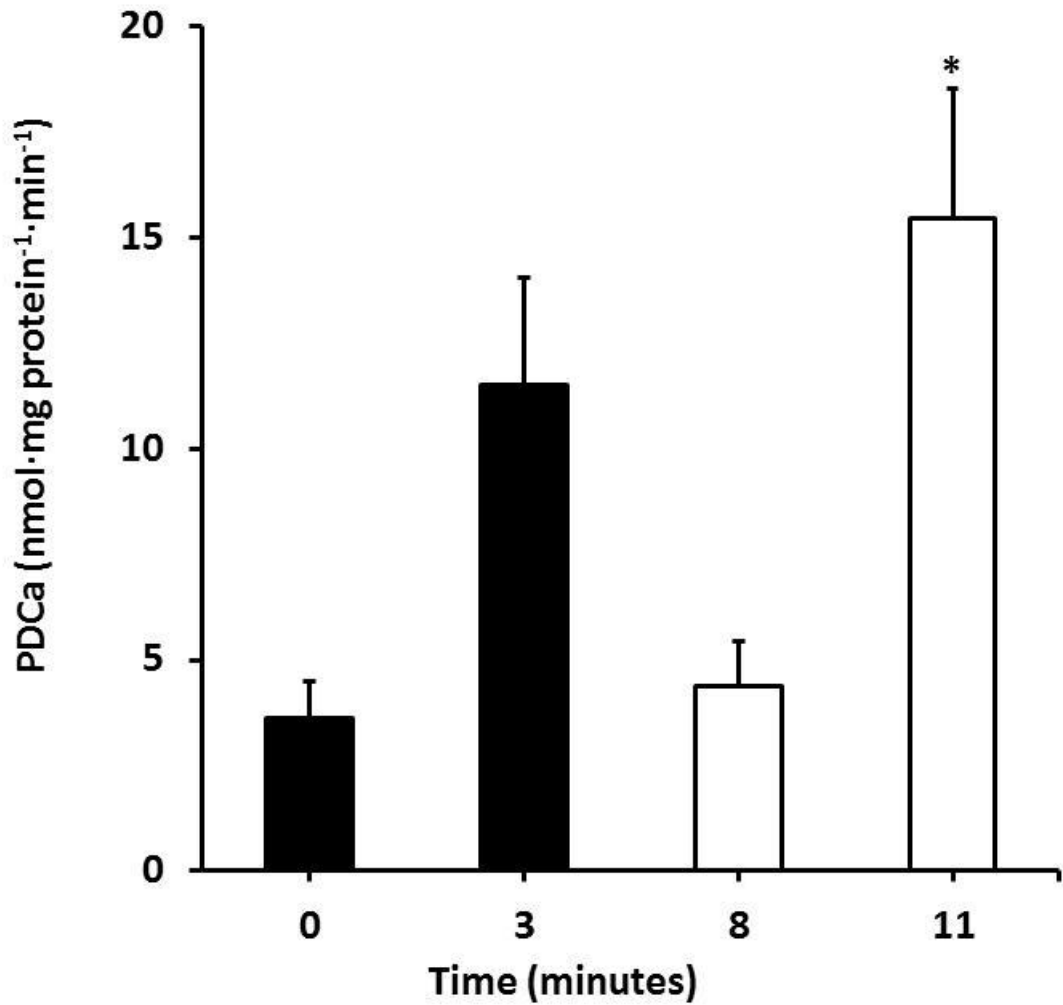


Figure 4.10 Activation status of the pyruvate dehydrogenase complex in skeletal muscle before and after two 3 minute bouts of cycling exercise at 100% W_{max} separated by 5 minutes of passive recovery. Black bars show bout 1 response; white bars show bout 2 response. Data are presented as mean \pm SE for 7 subjects in nmol acetyl-CoA·mg protein⁻¹·min⁻¹. Main effects of exercise ($P < 0.001$) for two-way ANOVA. * $P < 0.05$ vs pre-bout value.

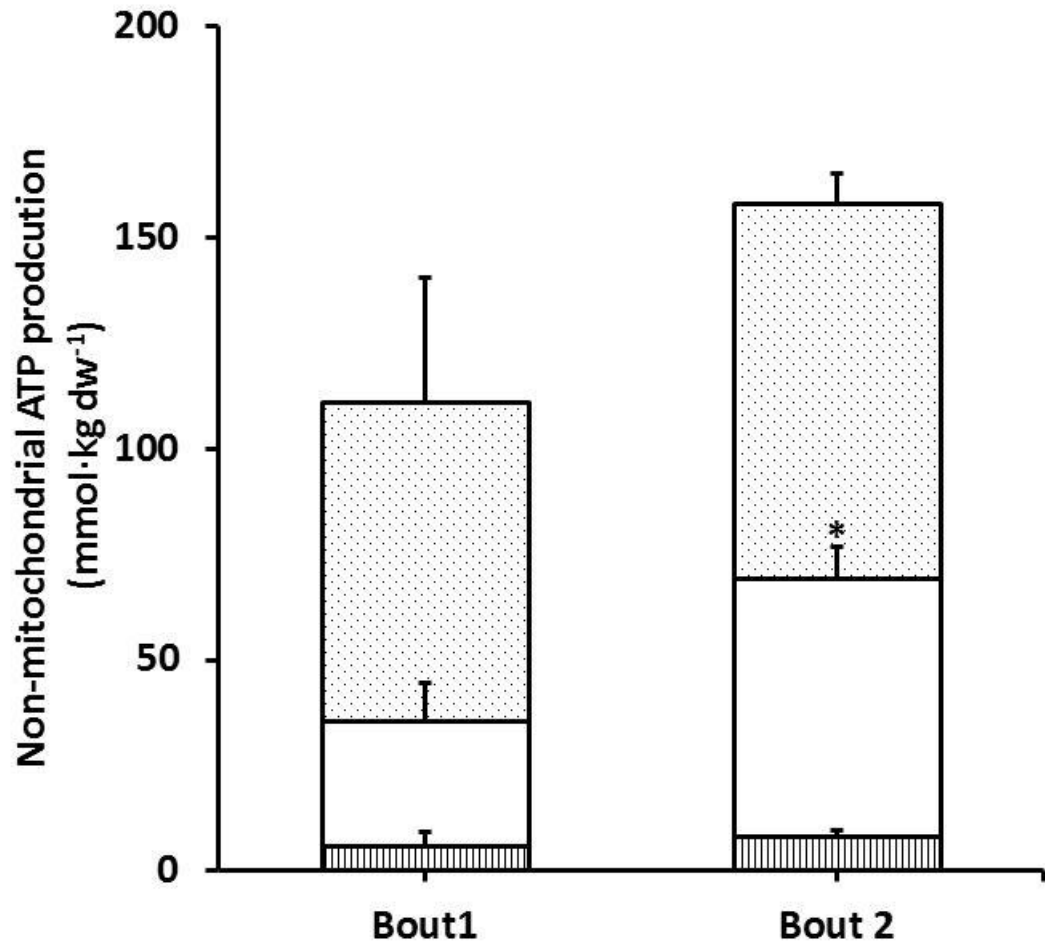


Figure 4.11 Skeletal muscle ATP production from non-mitochondrial sources, comprising ATP (striped bars), PCr (white bars) and lactate (dotted bars) during two 3 minute bouts of cycling exercise at 100% W_{max} , separated by 5 minutes of passive recovery. Data are mean \pm SE for 7 subjects. * $P < 0.05$ vs bout 1 for PCr. Total non-mitochondrial ATP production was not significantly different between the two exercise bouts ($P = 0.29$ for paired t-test).

4.4 Discussion

The current studies were performed to provide further insight into the interplay between PDC and TCA cycle fluxes by determining acetylcarnitine accumulation and non-mitochondrial ATP production under different exercise conditions, where the demand and capacity for oxidative ATP production may vary. The results demonstrate that acetylcarnitine accumulation and non-mitochondrial ATP production during single-leg knee extension exercise are considerably lower than the equivalent values reported for two-legged cycling exercise at comparable relative workloads. Secondly, non-mitochondrial ATP production is increased during a second bout of high-intensity cycling exercise in the face of similar acetylcarnitine accumulation which, in contrast to repeated bouts of single-leg knee extension exercise, would suggest that both PDC and TCA cycle flux decline during a second bout when the workload is fixed. The rates of acetylcarnitine accumulation and non-mitochondrial ATP production across these various exercise paradigms are illustrative of the relative matching between glycolytic, PDC and TCA cycle fluxes and, as will be discussed, are consistent with the proposal that free carnitine availability can dictate PDC flux and hence influence oxidative ATP provision during high-intensity exercise.

Single-leg knee extension at 55 and 85% W_{max}

During two-legged cycling, Wall *et al.* (2011) reported a near doubling of acetylcarnitine accumulation when the exercise intensity was increased from 50% to 80% VO_{2max} . As a consequence of this elevation in skeletal muscle acetylcarnitine content ($\sim 17 \text{ mmol}\cdot\text{kg dw}^{-1}$ at 80% VO_{2max}), free carnitine availability was reduced to an extent that it likely became limiting to further increases in PDC flux, as

evidenced by the near doubling of non-mitochondrial ATP production at 80% compared to 50% $\text{VO}_{2\text{max}}$. In accordance with this, increasing skeletal muscle carnitine availability enhances PDC flux and reduces reliance on non-mitochondrial ATP production during exercise at 80% $\text{VO}_{2\text{max}}$ (Wall *et al.*, 2011). In contrast, in study A, acetylcarnitine accumulation was similar at 55% and 85% W_{max} and thus acetylcarnitine content following the latter bout was only $8.1 \text{ mmol}\cdot\text{kg dw}^{-1}$, less than half that measured by Wall and colleagues (2011). Although glycogen utilisation was 3-fold greater at 85% W_{max} compared to 55% W_{max} , lactate accumulation remained relatively low (only $6.2 \text{ mmol}\cdot\text{kg dw}^{-1}$) and PCr degradation was similarly negligible. As a result, reliance on non-mitochondrial ATP production at 85% W_{max} was substantially lower than the values reported by Wall *et al.* (2011) at 80% $\text{VO}_{2\text{max}}$, suggesting a better matching of glycolytic, PDC and TCA cycle fluxes during single-leg knee extension exercise compared to two-legged cycling. These findings are consistent with the greater oxygen uptake per unit of muscle mass recruited during single-leg knee extension exercise (Boushel & Saltin, 2013) as well perhaps, as the lower catecholamine response and hence lower adrenergic stimulation of vasoconstriction and glycolysis (Greenhaff *et al.*, 1991), compared to two-legged cycling. Indeed, it has previously been argued that TCA cycle flux exceeds PDC flux during moderate to intense knee extension exercise (Gibala *et al.*, 1998) and thus, acetylcarnitine accumulation under these conditions would be somewhat paradoxical.

Although the calculation of TCA cycle flux by Gibala and colleagues assumed that mitochondrial ATP production was supported entirely by carbohydrate oxidation, the discrepancy between PDC and TCA cycle fluxes would actually suggest additional

acetyl-group provision independent from the PDC, likely from non-carbohydrate substrates. Indeed, the absolute rates of leg fat oxidation observed during study A were substantial and actually increased from 55% to 85% W_{\max} (see **Appendix 3.2**). In this respect, another consequence of extensive acetylation of the total carnitine pool during high-intensity exercise (70% in the study by Wall et al., 2011) is the expected restriction on CPT-mediated fatty acid oxidation (Stephens *et al.*, 2013). Thus, the lower acetylcarnitine accumulation in study A is a likely mechanism to explain why fat oxidation is preserved at higher relative exercise intensities during single-leg knee extension (Helge *et al.*, 2007) compared to two-legged cycling. Nevertheless, it is difficult to relate acetylcarnitine accumulation to an increase in PDC flux when the rate of β -oxidation is increasing in parallel. However, it is known that carbohydrate oxidation is more rapidly activated than fat oxidation at the onset of exercise and thus PDC flux likely delivers the majority of mitochondrial acetyl-groups during the initial minutes of exercise. As such, the 5-6 mmol·kg dw⁻¹ acetylcarnitine accumulation that occurred in both bouts of study B could still reflect an imbalance between PDC flux and TCA cycle flux during this initial period, while leg oxygen uptake and TCA cycle flux were still rising. If it is assumed that measurements made after 30 minutes of exercise represent the steady-state acetylcarnitine concentration, under these conditions it would seem equally appropriate to view acetylcarnitine accumulation as simply the expansion of the mitochondrial acetyl-group pool associated with the moderate increase in PDC flux. Moreover, when a significant proportion of acetyl-groups entering the TCA cycle are provided from the β -oxidation of fatty acids, TCA cycle flux is unlikely to be limited by PDC flux. On the contrary, the refinement of PDC flux to acetyl-group demand when glycolytic flux is relatively low is illustrated by the finding that the

augmentation of fat oxidation in response to skeletal muscle carnitine loading (Stephens *et al.*, 2013) was previously associated with an approximately 30% reduction in both PDCa and acetylcarnitine accumulation (Wall *et al.*, 2011).

Repeated bouts of high-intensity cycling exercise

The rate of acetylcarnitine accumulation of $1.8 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{min}^{-1}$ during bout one of study B was similar to that generally reported over the initial minutes of cycling exercise at intensities between 75-90% $\text{VO}_{2\text{max}}$ ($\sim 2.2 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{min}^{-1}$; see **figure 1.5 in General Introduction**) and coincided with a 3.2-fold increase in the activation of the PDC. During the five minute passive recovery period, acetylcarnitine concentrations remained relatively stable whilst PDCa returned to resting values. Based on the findings of Bangsbo and colleagues (2001), it was hypothesised that acetylcarnitine accumulation would be lower during a second bout of exercise. However, acetylcarnitine concentrations in the subsequent bout actually increased by a comparable amount (from an elevated pre-bout value), such that the post-exercise free carnitine concentration was approximately 34% lower compared to the first bout. Therefore, given the apparently lower rate of mitochondrial ATP provision in the face of similar acetylcarnitine accumulation, PDC flux was presumably lower during the second bout of exercise. Thus it can be proposed that, despite a similar PDC activation, the continued decline of free carnitine availability during the second bout of exercise restricted PDC flux and consequently limited the contribution from the TCA cycle to total ATP production.

Previous studies have suggested that during repeated bouts of high-intensity exercise, non-mitochondrial ATP delivery is reduced (Putman *et al.*, 1995; Bangsbo

et al., 2001) and VO_2 kinetics are accelerated in the second bout relative to the first (Putman *et al.*, 1995; Bangsbo *et al.*, 2001; Campbell-O'Sullivan *et al.*, 2002). For example, during two 3 minute bouts of single-leg knee extension exercise at 100% thigh $\text{VO}_{2\text{peak}}$, Bangsbo and colleagues (2001) reported that the combined ATP production from PCr degradation and lactate accumulation decreased from 175 $\text{mmol ATP}\cdot\text{kg dw}^{-1}$ during the first bout, to 135 $\text{mmol ATP}\cdot\text{kg dw}^{-1}$ in the second bout. In contrast, in study B non-mitochondrial ATP production was no lower during the second bout of exercise and, if anything, averaged 42% higher values. The latter was primarily accounted for by an approximately 2-fold greater PCr degradation during the second bout of exercise relative to the first. PCr resynthesis relies heavily on oxidative ATP provision via the mitochondrial creatine kinase shuttle (Perry *et al.*, 2012) and thus the extent of PCr degradation, which has been shown to reach a steady-state within two minutes of high-intensity exercise (Karlsson & Saltin, 1970), can be considered a very sensitive marker of mitochondrial ATP delivery. In addition to the increase in PCr degradation, muscle glycogen utilisation tended to be 2.1-fold greater during the second exercise bout. If it is assumed firstly, that the total ATP demand of contraction was similar for each bout (the external workload was fixed) and secondly, that the intensity and duration of the exercise precluded any significant contribution from fat oxidation (see **General Introduction**), this increase in glycogen utilisation would suggest a greater reliance on anaerobic glycolysis. Whilst muscle lactate accumulation was no greater during the second bout, the rate of muscle lactate efflux was not determined and so it is plausible that total lactate production increased in parallel with glycogen utilisation. Thus although the difference in non-mitochondrial ATP production between bouts was not significant in study B, the increased PCr degradation and glycogen utilisation strongly suggest

that mitochondrial ATP provision was compromised during the second exercise bout.

The opposing results obtained from study B and the study from Bangsbo and colleagues (2001), which both used a protocol involving two 3 minute exercise bouts at 100% W_{max} separated by a similar recovery period, are probably attributable to the previously discussed differences between single-leg knee extension and two-legged cycling exercise. In particular, the restricted blood flow per unit muscle mass during exercise involving a large muscle mass, such as cycling, is likely to limit oxidative ATP delivery to a greater extent than during exercise involving a smaller mass (Boushel *et al.*, 2011). Therefore pulmonary oxygen kinetics, which are known to be accelerated during a second exercise bout (Campbell-O'Sullivan *et al.*, 2002), may be a more primary determinant of mitochondrial ATP production during single-leg knee extension exercise. Nevertheless, previous studies using two-legged cycling protocols have reported a reduced reliance on non-mitochondrial ATP production during a second exercise bout. For example, Putman *et al.* (1995) provided evidence to suggest that PDC flux and mitochondrial ATP production increased progressively over three 30 second maximal effort cycle sprints. However, the total work performed (and thus contraction intensity) declined by 17% and 24% in the second and third bout, respectively, relative to the first. Moreover, the protocol employed by Putman and colleagues (1995) did not enable complete PCr resynthesis between exercise bouts resulting in a 12% and 19% reduction in pre-bout PCr availability before the second and third bout, respectively. Thus the demand and capacity for non-mitochondrial ATP production would have been reduced progressively over

each sprint, whilst it was maintained constant by the fixed workload and complete PCr resynthesis in the current study B.

It should be noted that most previous protocols investigating skeletal muscle metabolism during repeated bout exercise do not include a warm-up period. In the current study we chose to include a warm-up period to better reflect the nature of a practical exercise session. It has previously been demonstrated that a 10 minute exercise bout at 55% VO_{2max} accelerates oxygen uptake kinetics and reduces non-mitochondrial ATP production by 89% over the first minute of a subsequent bout at 75% VO_{2max} , compared to when no prior low-intensity exercise is performed (Campbell-O'Sullivan *et al.*, 2002). Thus it is possible that previous reports of increased oxidative ATP delivery during repeated exercise bouts are attributable to the “warm-up” effect of the first exercise bout. Conversely, the current protocol removed this effect by ensuring that subjects were already warmed-up prior to the start of the first bout and thus enabled us to investigate the true differences between first and second bouts. Interestingly, in the study by Campbell-O'Sullivan *et al.* (2002) non-mitochondrial ATP production for the remaining nine minutes of the bout at 75% VO_{2max} was actually 96% greater in the prior-exercise condition such that, if anything, it was 11% greater over the entire 10 minute bout. Thus, whilst prior exercise appeared to accelerate PDC and TCA cycle flux at the start of a second bout, it did not ultimately favourably impact upon the total oxidative ATP production over the entire bout.

Conclusions

In conclusion, these studies principally emphasise the differences between the metabolic responses to single-leg knee extension exercise and two-legged cycling exercise, but also demonstrate the consistency in the relationship between acetylcarnitine metabolism and oxidative ATP provision across both exercise modalities. During single-leg knee extension, the ATP demand of contraction can be met almost entirely by mitochondrial ATP delivery up to a workload of at least 85% W_{\max} and thus muscle PCr degradation and lactate accumulation are much lower than during two-legged cycling at comparable relative intensities. Similarly, the relatively low levels of acetylcarnitine accumulation likely reflect the moderate increase in PDC flux during the initial minutes of exercise and the subsequently mild expansion of the mitochondrial acetyl-group pool. In comparison, during a single three minute bout of high-intensity cycling exercise, which elicits a substantially higher ATP demand, non-mitochondrial ATP production provides a much greater proportion of total ATP production. In this situation, acetylcarnitine accumulation reflects an immediate imbalance of PDC and TCA cycle flux, as it would appear no other pathways can significantly contribute to the mitochondrial acetyl-group pool. This situation is exacerbated further in a second bout of exercise, where acetylcarnitine accumulation continues to the extent that free carnitine availability would appear to become limiting to PDC flux and reliance on non-mitochondrial ATP production must increase. In this respect, increasing free carnitine availability to enhance PDC flux may represent a targetable approach to improve exercise performance during repeated bouts of high-intensity submaximal cycling.

**CHAPTER 5: MANIPULATING SKELETAL MUSCLE
CARNITINE CONTENT: METABOLIC ADAPTATIONS
TO SUBMAXIMAL HIGH-INTENSITY
INTERMITTENT EXERCISE TRAINING AND
CONSEQUENCES FOR EXERCISE PERFORMANCE**

5.1 Introduction

The transfer of pyruvate-derived acetyl-groups to free carnitine prevents excessive acetyl-CoA accumulation and thus maintains a viable mitochondrial pool of CoASH in the face of accelerated flux through PDC, for example during high-intensity cycling exercise. The utility of this acetylation of the free carnitine pool during exercise is exemplified by the study of Wall *et al.* (2011), in which an increase in muscle total carnitine content was associated with increased PDCa and acetylcarnitine accumulation during continuous exercise at 80% $\text{VO}_{2\text{max}}$ and a subsequent decrease in PCr degradation and lactate accumulation. As highlighted by the findings in **Chapter 4**, this carnitine-mediated enhancement of PDC flux and subsequent reduction in non-mitochondrial ATP production could become particularly pertinent during a second bout of exercise, where PDC flux would appear to decline in concert with free carnitine availability. Assuming that, under these conditions, a similar alteration of metabolic flux is obtainable following skeletal muscle carnitine loading, it is plausible that this could influence the adaptations to a chronic period of exercise training. For example, Wall *et al.* (2011) found that in addition to the metabolic alterations noted above, subjects were able to complete more work in an all-out work performance test in the carnitine-loaded state. If reproduced over multiple training sessions, this enhanced work output could reasonably be expected to provide a greater stimulus for metabolic and/or functional adaptations to the exercise.

Exercise training is known to elicit improvements in exercise performance and capacity, which at the whole body level is reflected by increases in maximal power output, $\text{VO}_{2\text{max}}$ and exercise endurance (Holloszy *et al.*, 1977). The metabolic

adaptations that underpin these functional improvements are also well characterised, such as an increased reliance on oxidative ATP delivery and reciprocal reductions in phosphocreatine degradation, lactate accumulation and glycogen utilisation (Vollaard *et al.*, 2009). A lower requirement for non-mitochondrial ATP production would suggest a better matching of mitochondrial, PDC and glycolytic fluxes during exercise. In agreement with this, Vollaard *et al.* (2009) found that the accumulation of both lactate and acetylcarnitine within skeletal muscle during a 10 minute exercise bout at 70% VO_{2max} was reduced following their six week endurance training protocol. In another study, PDC activation and acetylcarnitine accumulation during 15 minutes of exercise at ~80% VO_{2max} were reduced following seven weeks of exercise training (Leblanc *et al.*, 2004). It seems intuitive to expect that similar adaptations to training are manifest during more intense exercise, where there is a greater mismatch between glycolytic and mitochondrial substrate fluxes. Indeed, the recently popularised HIT (high-intensity interval training) type exercise paradigm has been shown to elicit many adaptations classically thought to be primarily associated with endurance-type training (ET) paradigms. For example, Burgomaster *et al.* (2008) demonstrated that both HIT and ET facilitated comparable reductions in PCr degradation and glycogen utilisation during exercise following training, despite marked differences in the overall training volume completed. Although studies utilising HIT have typically employed maximal sprint efforts, it has also been shown that training sessions consisting of 8-12 one minute bouts of cycling exercise at 100% W_{max} (submaximal) are sufficient to induce robust increases in markers of skeletal muscle oxidative capacity over a two week period, as well as enhance exercise performance (50 and 750 kJ time trial) and resting muscle glycogen content (Little *et al.*, 2010). Hence this type of submaximal HIT paradigm has been

proposed as a viable time-efficient strategy to improve health and fitness (Gillen & Gibala, 2013) in an increasingly sedentary population, when a “lack of time” is frequently cited as one of the major perceived barriers to physical activity (Trost *et al.*, 2002).

The overall premise of the current study is that the previously reported effects of skeletal muscle carnitine loading will alter the metabolic and functional adaptations to a 24 week period of submaximal HIT. At present, daily L-carnitine and carbohydrate feeding is the only known efficacious dietary strategy to increase the muscle carnitine stores over a 12-24 week period (Wall *et al.*, 2011). As discussed in the **General Introduction**, the carbohydrate load associated with the current L-carnitine supplementation protocol may have a negative impact upon body composition (Wall *et al.*, 2011), hence the efforts to reduce the carbohydrate requirement of L-carnitine supplementation in **Chapter 3** of this thesis. However, it would appear that replacement of some of this carbohydrate with whey protein may interfere with the insulin-mediated stimulation of muscle carnitine accretion (see **Chapter 3**) and thus, the previously validated use of a twice-daily L-carnitine and carbohydrate formulation likely still represents the most appropriate approach to increase muscle carnitine content in healthy volunteers. It is therefore also important to assess whether the differential treatment effects on body fat mass observed by Wall *et al.* (2011) are abated in the current intervention, as periods of structured exercise training are known to impact positively upon body composition (Nordby *et al.*, 2012). It was hypothesised that manipulation of muscle carnitine content with daily L-carnitine and carbohydrate feeding would alter metabolic flux during repeated bouts of exercise at 100% W_{max} (reduced PCr degradation and lactate

accumulation), particularly during a second bout, when PDC flux would appear to decline. Secondly, it was proposed that any carnitine-mediated reduction in non-mitochondrial ATP production during exercise would enable a greater amount of work to be completed over progressive training sessions and thus augment the training-induced gains in exercise capacity ($\text{VO}_{2\text{max}}$, Watt_{max} and cycling efficiency), performance (work output) and body composition.

5.2 Methods

Volunteers

Fourteen healthy, non-vegetarian male volunteers (mean \pm SE age 23.2 ± 1.1 years; BMI $24.4 \pm 0.9 \text{ kg}\cdot\text{m}^{-2}$; $\text{VO}_{2\text{max}}$ $41.6 \pm 2.1 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) were recruited to participate in this study, which was approved by the University of Nottingham Medical School Ethics Committee. Prior to the study, each participant gave informed consent to take part and attended a routine medical screening (see **General Methods**).

Experimental protocol

The overall study protocol is depicted in **Figure 5.1**. Prior to the baseline study visit, volunteers completed a $\text{VO}_{2\text{max}}$ assessment, confirmation visit and were familiarised with the exercise protocol (see **General Methods**). On the study day (**Figure 5.2**), volunteers arrived at approximately 8.30 am following an overnight fast and underwent a full body DEXA scan to determine total body, fat and lean mass and a blood sample was taken for the determination of plasma total carnitine concentration. A resting muscle biopsy was obtained and the proceeding acute exercise protocol was identical to that described in study 2B (See **Chapter 4**). This study day was

repeated following 24 weeks of exercise training and supplementation (see below), with $VO_{2\max}$ and W_{\max} re-assessed after 4, 8, 12, 18 and 24 weeks to monitor improvements in fitness. Mechanical cycling efficiency at W_{\max} was also determined at 0, 12 and 24 weeks (see **General Methods**). Plasma carnitine was measured every six weeks to assess systemic carnitine availability and confirm compliance to the supplementation protocol. In addition, a resting biopsy and DEXA scan were obtained at 12 weeks to monitor any progressive impact of the intervention on total muscle carnitine and body composition, respectively.

Training protocol

Volunteers trained three times per week (normally Monday, Wednesday and Friday) over a 24 week period and were required to complete >85% of all training sessions for inclusion in the final data set. All training sessions were supervised and involved a three minute warm-up at 25% W_{\max} followed by 3 x 3 minute exercise bouts at 100% W_{\max} and a fourth bout to exhaustion, each separated by five minute passive recovery periods. During the final bout of each training session, volunteers received standardised verbal encouragement to exercise as long as they could (typically less than six minutes). The absolute training workload was adjusted, in line with the reassessment of W_{\max} at regular intervals (see **Figure 5.1**), to maintain a constant relative intensity of 100% W_{\max} . Additionally, when volunteers were able to exercise for six minutes in the final bout during more than two consecutive training sessions, the workload was increased by a further 1% for the subsequent training session. Thus the training paradigm was designed to maximise work output, in every progressive training session.

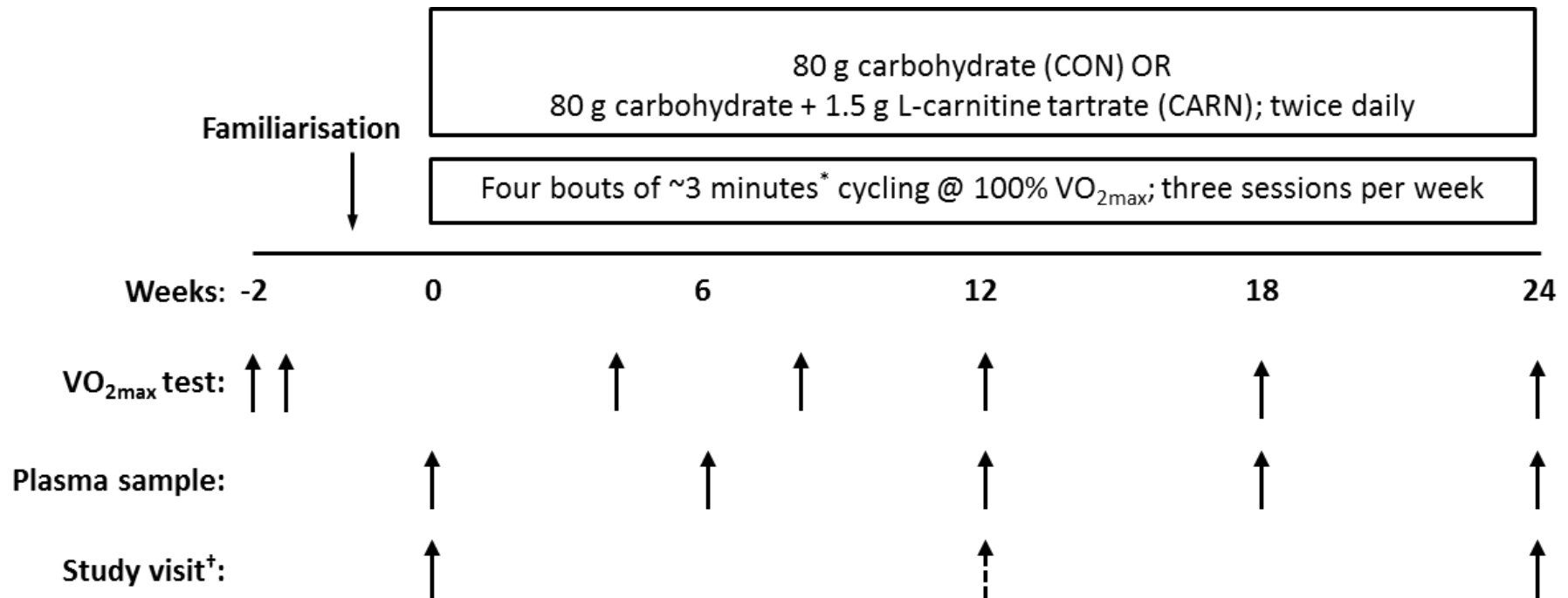


Figure 5.1 Schematic showing the experimental study protocol. Fourteen healthy, male volunteers were randomised into either CON (n=7) or CARN (n=7). *The first three exercise bouts lasted three minutes, the fourth bout was open-ended to allow exercise until exhaustion but generally lasted 0-6 minutes. † The dotted arrow indicates that only a DEXA scan and resting biopsy was obtained at 12 weeks.

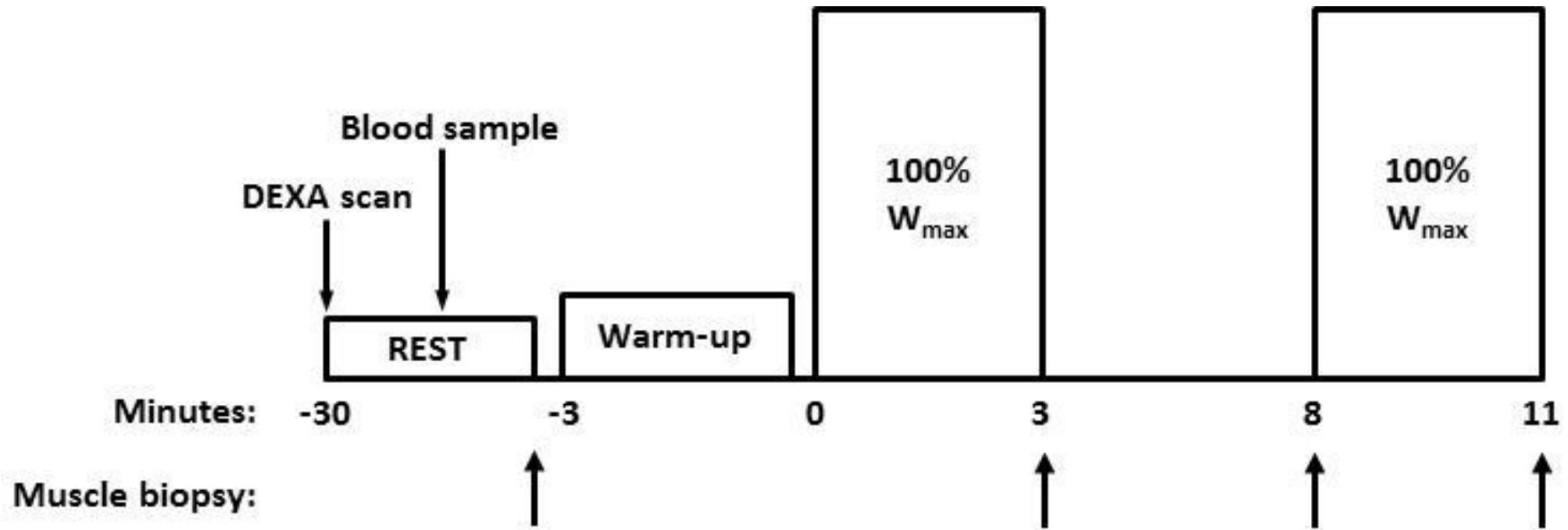


Figure 5.2 Schematic showing the protocol for study visits, completed at 0 and 24 weeks.

Supplementation protocol

Volunteers were assigned to receive twice-daily beverages of either carbohydrate alone (80 g maltodextrin; Maldex 180, SYRAL Belgium) or 80 g carbohydrate plus 3 g L-carnitine tartrate (1.5 g L-carnitine; Nutramet, UK) in a randomised, double-blinded fashion. The exact formulation utilised by Wall *et al.* (2011) was not available for use in the current study, but the quantities of L-carnitine and carbohydrate provided by Nutramet were identical to those used previously. Drinks were made up in 500 ml cold water from sachets of powder and were matched for flavour, appearance and carbohydrate type. Volunteers were instructed to consume one beverage first thing in the morning, and the second beverage four hours later to maximise the time period over which plasma carnitine and serum insulin concentrations were simultaneously elevated (Wall *et al.*, 2011 and **Chapter 3**).

Sampling and analysis

Plasma from samples taken at 0, 6, 12, 18 and 24 weeks were analysed for total carnitine. Skeletal muscle free, acetyl, acyl and total carnitine, as well as ATP, PCr, lactate, glycogen and PDCa were determined in biopsy samples at 0 and 24 weeks. Skeletal muscle total carnitine was additionally determined at 12 weeks (see **General Methods**).

Statistics

Data were compared using two-way ANOVA for the effect of training (exercise x time) for each group and treatment (group x time) for each biopsy condition with Bonferoni-corrected t-test post-hoc analysis (see **General Methods**). Data are presented as mean \pm SE.

5.3 Results

5.3.1 Plasma and muscle total carnitine

Plasma total carnitine concentrations at baseline were similar between CON and CHO (42.8 ± 2.4 and $40.8 \pm 1.4 \mu\text{mol}\cdot\text{L}^{-1}$) and remained stable in CON throughout the 24 week intervention period. However, following six weeks, plasma carnitine concentrations in CARN were elevated above both CON and baseline, remaining approximately 24% greater than CON for the duration of the intervention (**Figure 5.3**). Skeletal muscle total carnitine content, shown in **Figure 5.4**, was no different between groups at baseline but an interaction effect (group x time; $P < 0.05$) occurred, such that total carnitine was decreased after 12 weeks ($P < 0.05$ vs 0 weeks) in CON, but not CARN. After 24 weeks, total carnitine content in CON was no different from 12 weeks or 0 weeks, whilst the change from baseline in muscle carnitine was significantly greater in CARN compared to CON (1.5 ± 0.7 vs $-0.9 \pm 0.6 \text{ mmol}\cdot\text{kg dw}^{-1}$; $P < 0.05$).

5.3.2 Skeletal muscle metabolites

Carnitine metabolism and PDCa

The effect of training on skeletal muscle free carnitine concentrations during exercise is shown in **Figure 5.5** for both CON and CARN. In the CON group, training appeared to attenuate the exercise-induced decline in free carnitine, but only during bout two ($\Delta\text{FC} = 4.5 \pm 1.4$ and $1.4 \pm 1.3 \text{ mmol}\cdot\text{kg dw}^{-1}$ at 0 and 24 weeks, respectively; $P = 0.09$). As such, CON free carnitine at the end of bout two tended to be 40% higher at 24 weeks compared to baseline ($P = 0.07$).

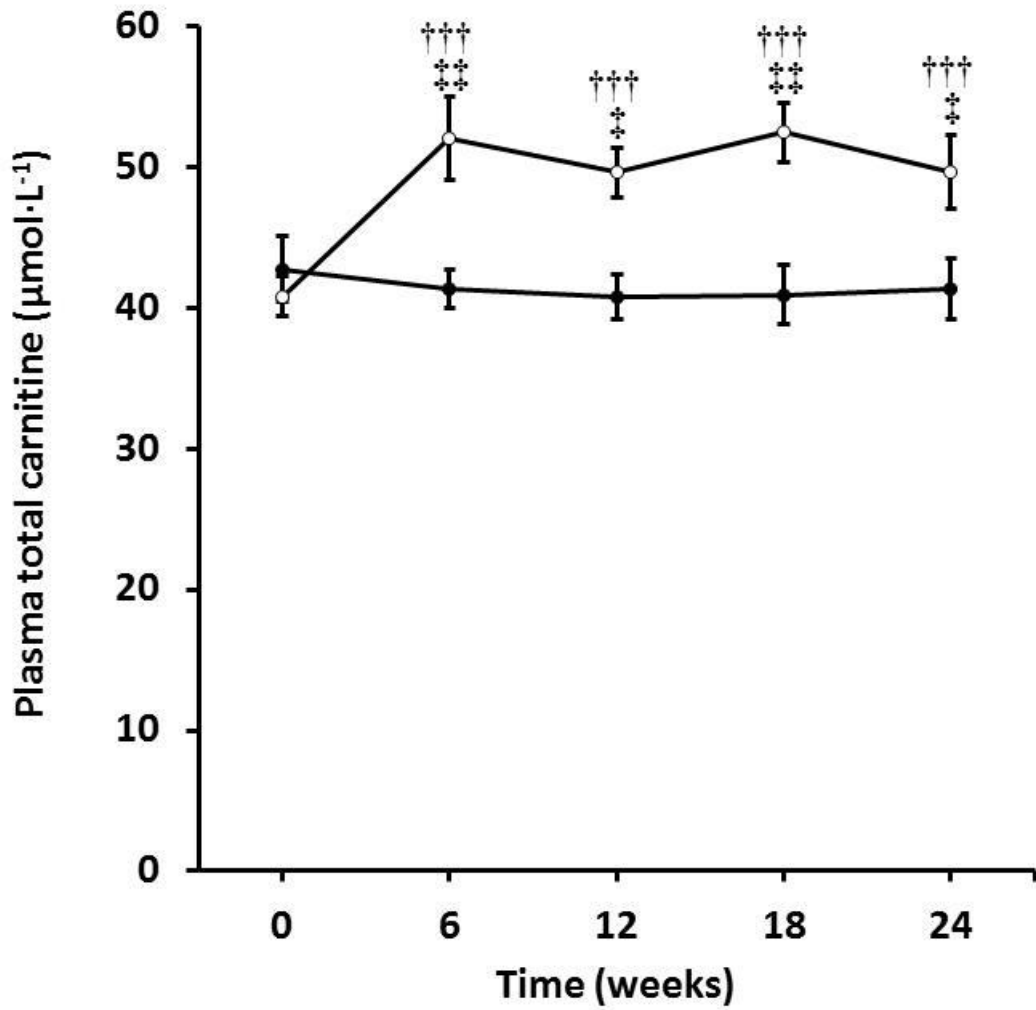


Figure 5.3 Plasma total carnitine concentrations at 0, 6, 12, 18 and 24 weeks of exercise training, receiving twice daily drinks of either 80 g carbohydrate (CON; black circles) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white circles). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*group x time*): †† P <0.01, ††† P <0.001 vs 0 weeks; † P <0.05, †† P <0.01 vs CON.

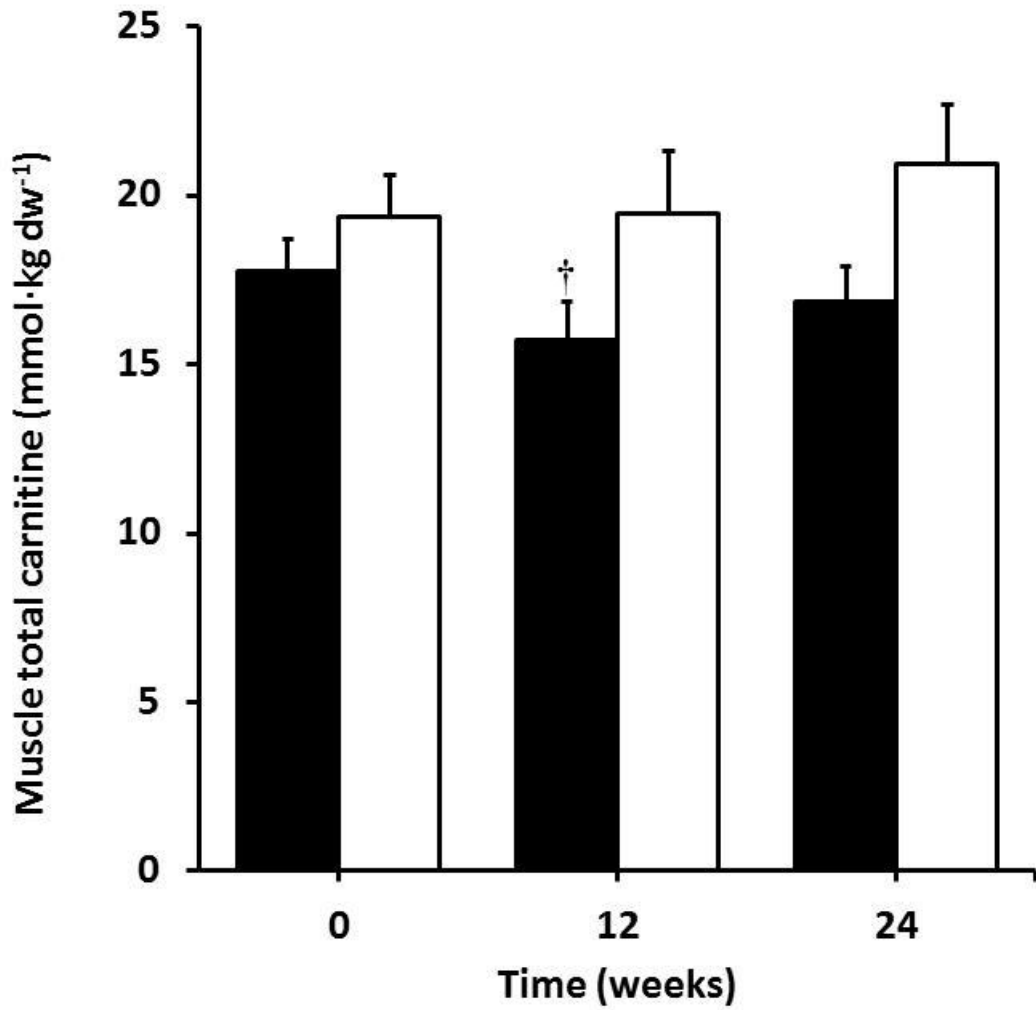


Figure 5.4 Skeletal muscle total carnitine content at 0, 12 and 24 weeks of exercise training, receiving twice daily drinks of either 80 g carbohydrate (CON; black bars) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white bars). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*group* \times *time*): † $P < 0.05$ vs 0 weeks.

In the CARN group, resting free carnitine availability was 31% greater than CON ($P<0.05$) after 24 weeks and, in contrast to CON, the decline in free carnitine during bout one was reduced with training ($\Delta FC = 5.2 \pm 1.4$ and 1.9 ± 1.1 mmol·kg dw⁻¹ at 0 and 24 weeks, respectively for CARN; $P<0.05$). This resulted in a 39% greater free carnitine content at the end of bout one in CARN compared to CON ($P<0.05$), which also represented a 35% increase from baseline ($P<0.05$). Free carnitine availability prior to the onset of the second bout remained 45% greater in CARN than CON ($P<0.05$) at 24 weeks, but was no different at the end of the second bout. Although not significant, the bout two decline in free carnitine at 24 weeks was 3.9-fold greater in CARN than CON ($P=0.12$). The changes in acetylcarnitine accumulation with training in CON mirrored the preservation of free carnitine observed in bout two, with acetylcarnitine concentrations tending to be 36% lower ($P=0.06$) at the end of bout two compared to baseline (see **Table 5.1**). Although no significant differences were observed between CON and CARN acetylcarnitine concentrations during exercise at either time-point, bout one acetylcarnitine accumulation was quantitatively 2.9-fold lower after training in CARN, compared to baseline ($\Delta AC = 4.0 \pm 1.8$ and 1.2 ± 0.6 mmol·kg dw⁻¹ at 0 and 24 weeks, respectively; $P=0.18$). Conversely, in bout two the percentage of the carnitine pool that was acetylated only increased in CARN ($P<0.05$) and not CON ($P=0.9$) at 24 weeks. There was no difference in PDCa between CON and CARN at either time-point and PDCa was not influenced by training in either group (**Figure 5.6**).

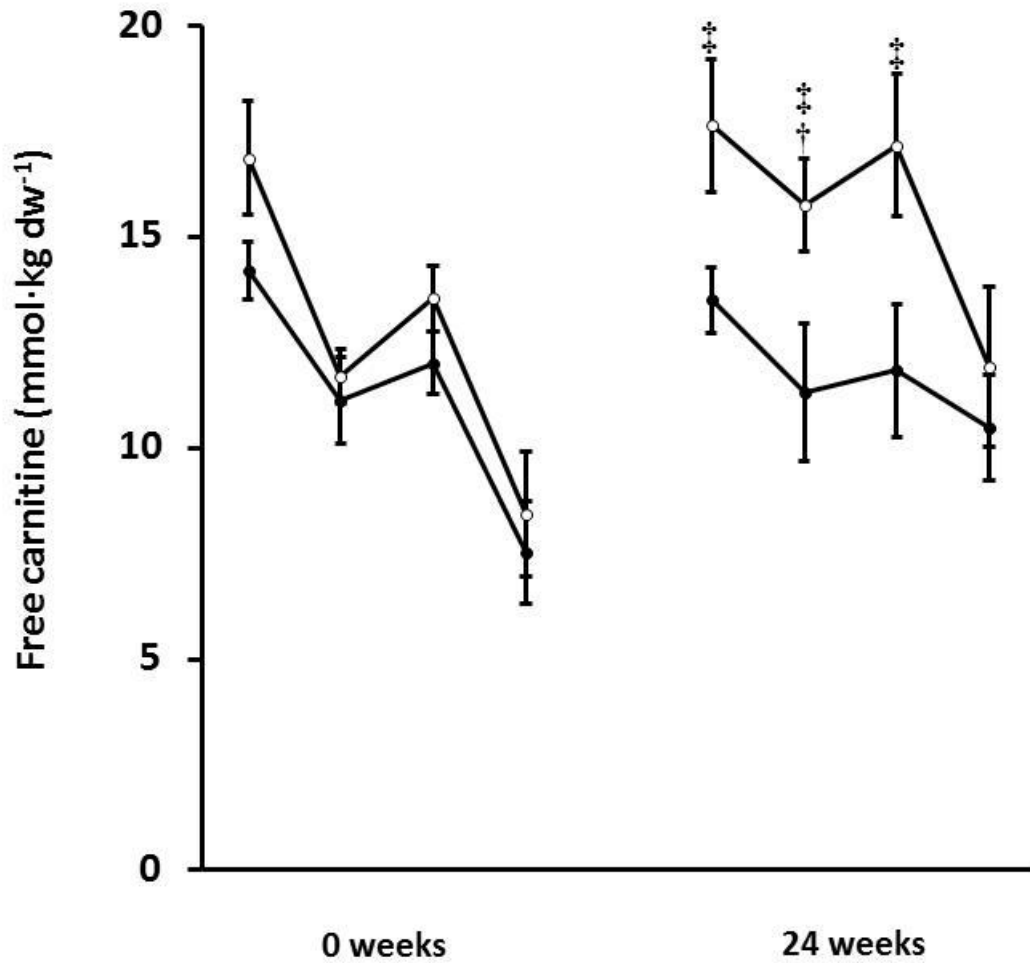


Figure 5.5 Skeletal muscle concentrations of free carnitine before and after two 3 minute bouts of exercise at 100% W_{max} , at baseline (left) and following 24 weeks of exercise training (right) receiving twice daily drinks of either 80 g carbohydrate (CON; black circles) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white circles). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*exercise x time*): † $P < 0.05$ vs 0 weeks; (*time x group*): ‡ $P < 0.05$ vs CON.

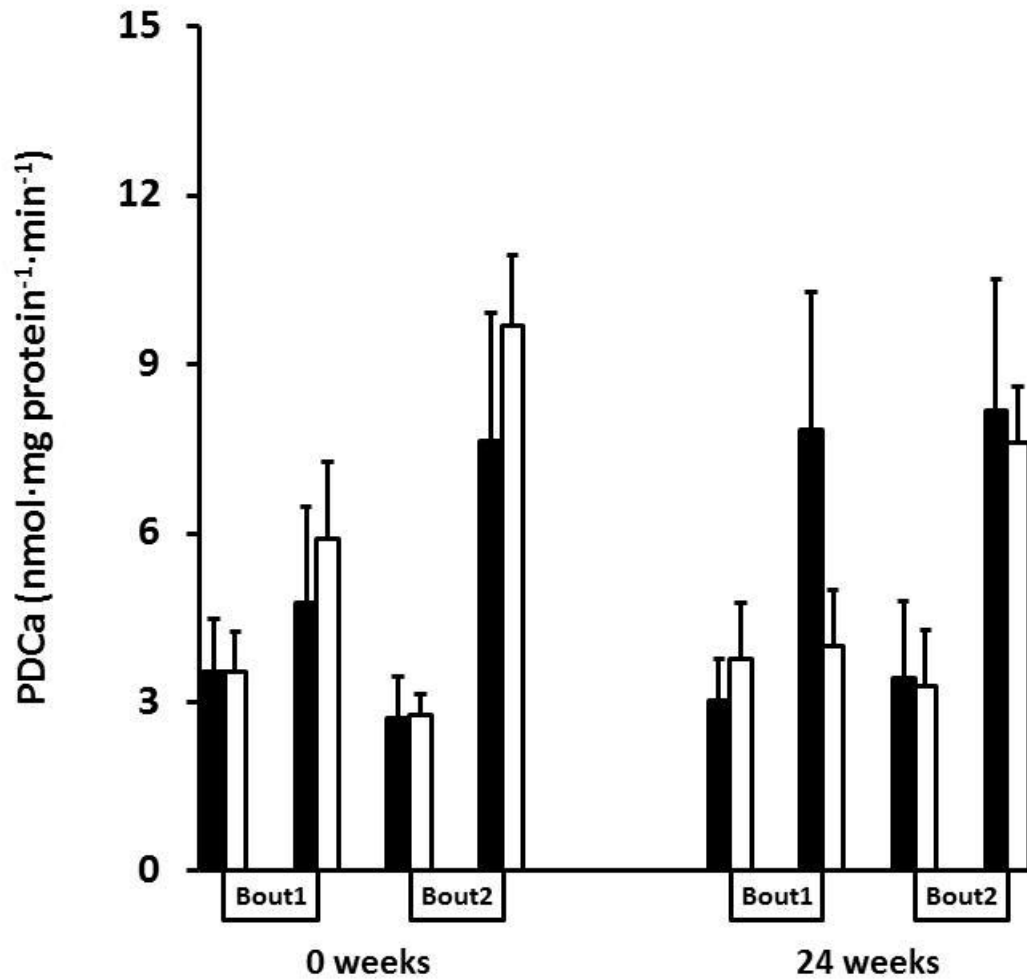


Figure 5.6 Activation status of the pyruvate dehydrogenase complex before and after two 3 minute bouts of exercise at 100% W_{max} , at baseline (left) and following 24 weeks of exercise training (right) receiving twice daily drinks of either 80 g carbohydrate (CON; black circles) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white circles). Values are mean \pm SE for 7 subjects expressed as a rate of acetyl-CoA production in $\text{nmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$.

Skeletal muscle ATP and PCr

ATP concentrations during bout one were not influenced by training or supplementation, although the decline in ATP tended to be less after training across both groups (main effect of time $P=0.08$). ATP content at the end of bout two was no different after training in CON ($P=0.16$) but was 20% greater in CARN ($P<0.05$) compared to baseline. Resting skeletal muscle PCr concentrations were similar before and after training in both CON and CARN (**Table 5.1**). PCr degradation during bout one tended ($P=0.07$) to be reduced by training across both groups (**Figure 5.7**) but the post-bout PCr concentration was only increased in CARN ($P<0.05$), not CON ($P=0.37$), at 24 weeks compared to baseline. In bout two, PCr degradation tended to be lower at 24 weeks in both CON ($P=0.07$) and CARN ($P=0.09$) compared to baseline such that the post-bout PCr concentrations were similarly increased above baseline in both groups ($P<0.05$). Once corrected for workload, bout one PCr degradation tended to be reduced at 24 weeks in CARN ($P=0.08$) but not CON ($P=0.60$), whilst bout two PCr degradation was lower in both groups ($P<0.05$), compared to 0 weeks (see **Appendix 3.3**).

Table 5.1 Skeletal muscle metabolites before and following 2 x 3 min bouts of exercise at 100% W_{max} , before and after 24 weeks of exercise training receiving twice daily drinks of either 80 g carbohydrate (CON) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*exercise x time*): * P <0.05, ** P <0.01, *** P <0.001 vs PRE value of same bout; † P <0.05 vs 0 weeks.

	BOUT ONE				BOUT TWO			
	PRE		POST		PRE		POST	
	CON	CARN	CON	CARN	CON	CARN	CON	CARN
ATP	25.6 \pm 1.3	24.9 \pm 0.9	23.5 \pm 1.1	22.1 \pm 1.7	24.2 \pm 2.0	21.7 \pm 1.4	20.0 \pm 1.8	19.7 \pm 1.2
PCr	72.3 \pm 4.0	74.7 \pm 3.4	55.4 \pm 7.2*	50.1 \pm 7.7*	74.1 \pm 3.9	73.4 \pm 5.1	25.8 \pm 7.3***	23.4 \pm 3.6***
0 Lactate	4.5 \pm 0.6	7.2 \pm 1.7	39.0 \pm 10.9*	51.2 \pm 20.7**	15.8 \pm 2.1	19.6 \pm 7.3	91.3 \pm 15.4***	83.9 \pm 8.2***
Glycogen	378 \pm 28	413 \pm 20	318 \pm 21	347 \pm 15	399 \pm 23	375 \pm 31	243 \pm 13**	264 \pm 24**
Acetylcarnitine	2.8 \pm 0.7	2.3 \pm 0.9	5.7 \pm 1.2	6.4 \pm 1.6	5.0 \pm 1.0	5.3 \pm 2.0	9.0 \pm 0.6*	9.8 \pm 1.1*
ATP	23.6 \pm 1.5	24.0 \pm 1.0	25.0 \pm 1.1	24.2 \pm 1.6	23.6 \pm 1.1	23.8 \pm 1.1	22.8 \pm 1.1	23.6 \pm 1.6 [†]
PCr	73.9 \pm 3.8	78.1 \pm 3.2	65.5 \pm 5.7	70.8 \pm 5.3 [†]	77.6 \pm 1.9	78.5 \pm 4.1	49.1 \pm 7.2*** [†]	48.9 \pm 9.2*** [†]
24 Lactate	8.0 \pm 2.4	6.7 \pm 1.5	23.0 \pm 7.3	16.7 \pm 6.1 [†]	23.0 \pm 3.1	20.0 \pm 4.5	50.2 \pm 9.7* [†]	53.6 \pm 11.7* [†]
Glycogen	567 \pm 33 ^{†††}	570 \pm 46 ^{†††}	501 \pm 32 ^{†††}	536 \pm 40 ^{†††}	516 \pm 48 [†]	532 \pm 44 ^{†††}	462 \pm 55 ^{†††}	479 \pm 55 ^{†††}
Acetylcarnitine	2.9 \pm 0.7	2.8 \pm 0.7	4.9 \pm 1.2	4.0 \pm 0.7	4.2 \pm 0.6	3.9 \pm 0.9	5.7 \pm 0.9	7.7 \pm 1.9

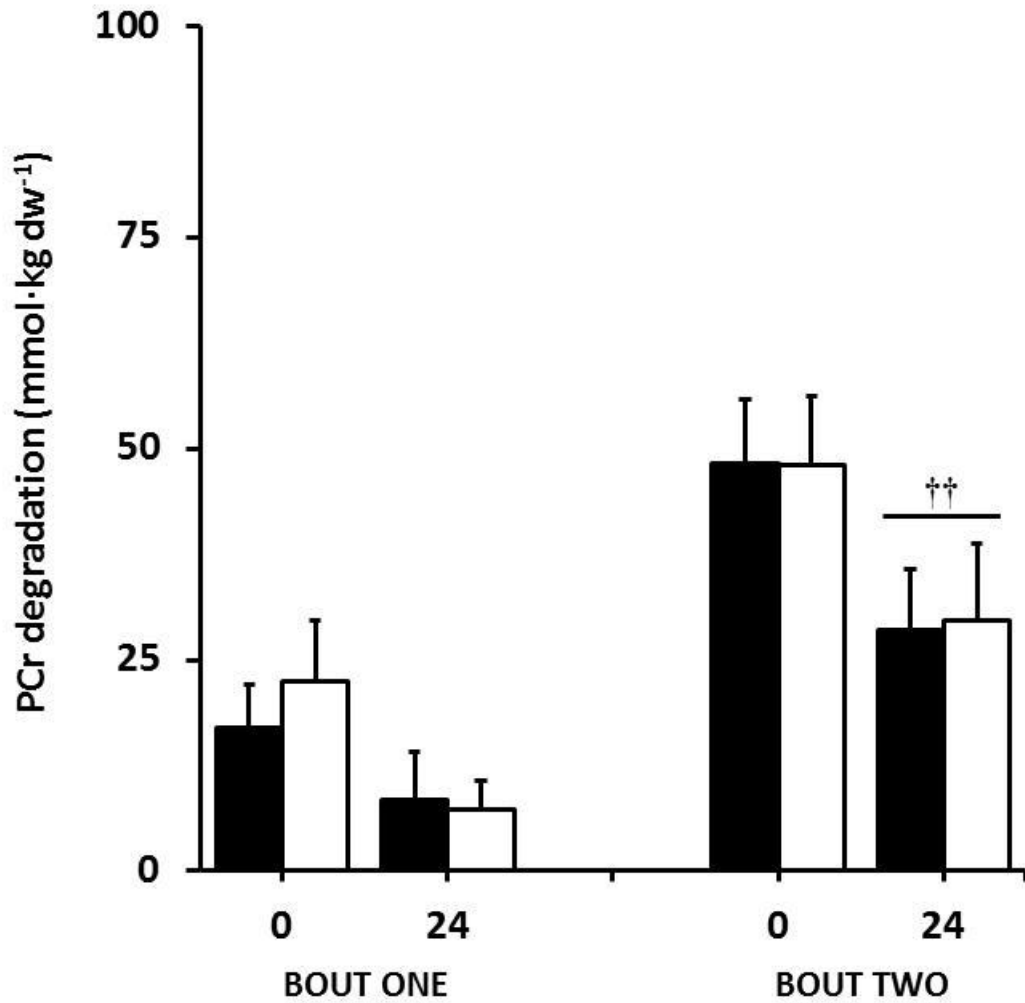


Figure 5.7 Muscle phosphocreatine degradation during two 3 minute bouts of exercise at 100% W_{max} , before and after 24 weeks of exercise training receiving twice daily drinks of either 80 g carbohydrate (CON; black bars) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white bars). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*time x group*): †† $P < 0.01$ vs 0 weeks.

Muscle lactate accumulation and glycogen utilisation

Muscle lactate concentrations were no different between CON and CARN at any time-point (**Table 5.1**). However, muscle lactate accumulation (**Figure 5.8**) during bout one tended to be 77% lower at 24 weeks in CARN ($P=0.08$) compared to 0 weeks, but showed no tendency to change in CON ($P=0.39$). These differences remained after correcting for workload, with bout one lactate accumulation tending to be lower in CARN ($P=0.05$) but not CON ($P=0.35$) after training. As such, the post-bout lactate tended to be lower in CARN ($P=0.06$) but not CON ($P=0.57$), compared to baseline. Conversely, lactate accumulation during bout two was 64% lower after training in CON ($P<0.05$), but not significantly different in CARN, despite a 48% reduction ($P=0.14$), although the latter tended towards significance after correcting for workload ($P=0.08$ for CARN, 24 vs 0 weeks; see **Appendix 3.4**).

The resting muscle glycogen content was increased at 24 weeks by 50% ($P<0.01$) and 38% ($P<0.05$) in CON and CARN, respectively, compared to 0 weeks. Glycogen utilisation during bout one was not influenced by training in either group (**Figure 5.9**) and accordingly, the post-bout muscle glycogen content was greater at 24 weeks in both groups ($P<0.01$) compared to baseline. During bout two, muscle glycogen utilisation at 24 weeks was 65 and 53% lower in CON and CARN, respectively, though this difference was only significant for CON ($P<0.05$). Nevertheless, the muscle glycogen remaining after bout two was 90 and 81% greater in CON and CARN, respectively, at 24 weeks compared to baseline ($P<0.01$). Workload correction had no impact on the pattern of glycogen utilisation in either group.

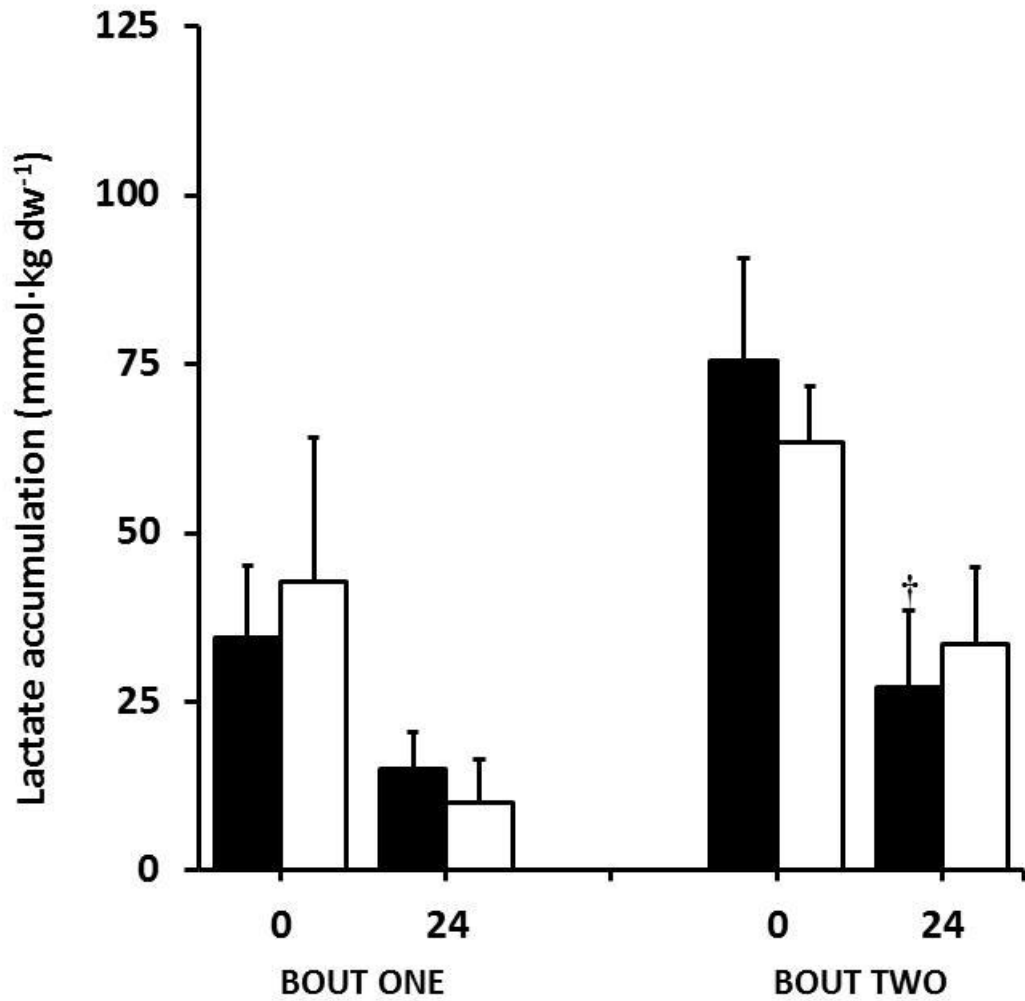


Figure 5.8 Muscle lactate accumulation during two 3 minute bouts of exercise at 100% W_{max} , before and after 24 weeks of exercise training receiving twice daily drinks of either 80 g carbohydrate (CON; black bars) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white bars). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*time* x *group*): † $P < 0.05$ vs 0 weeks.

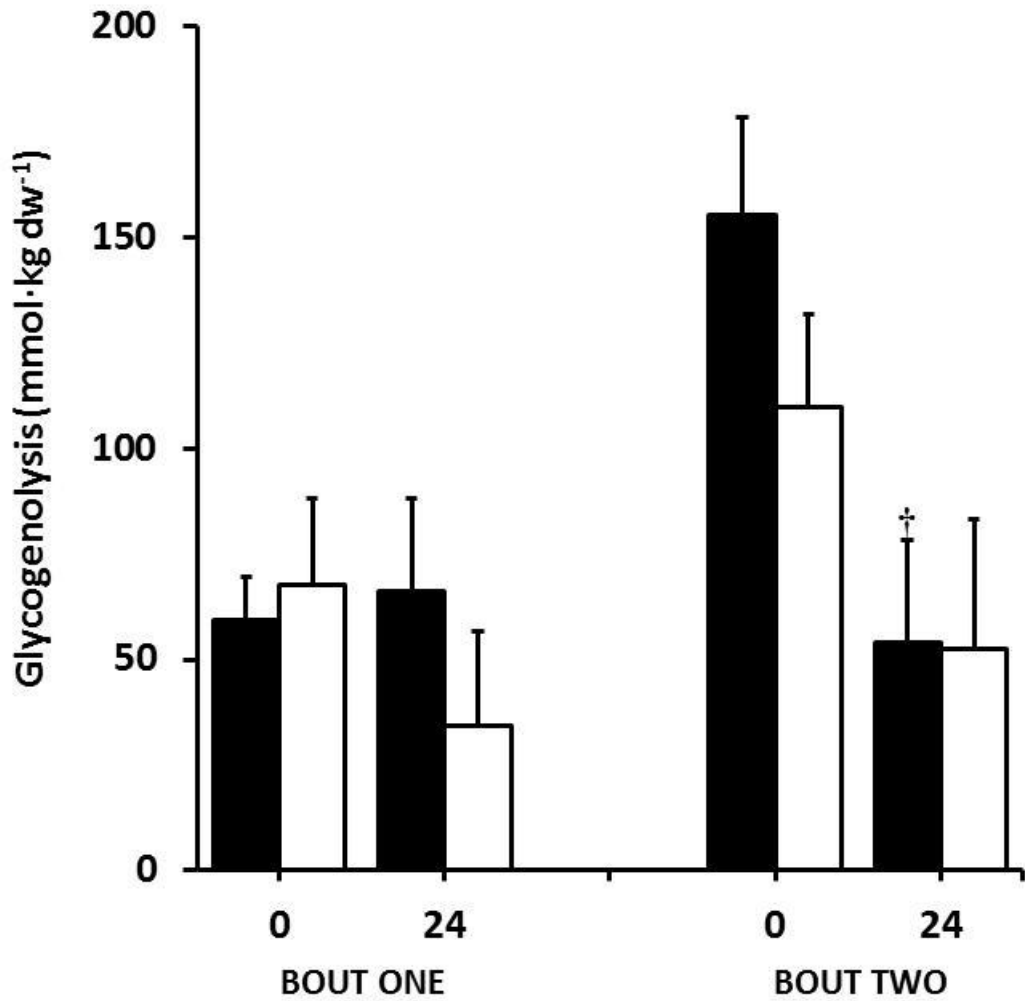


Figure 5.9 Muscle glycogen degradation during two 3 minute bouts of exercise at 100% W_{max} , before and after 24 weeks of exercise training receiving twice daily drinks of either 80 g carbohydrate (CON; black bars) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white bars). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*time x group*): * $P < 0.05$ vs 0 weeks.

Non-mitochondrial ATP production

The calculated ATP production from non-mitochondrial sources was similar at baseline between CON and CARN in bout one (72.9 ± 23.1 and 96.2 ± 38.8 mmol·kg dw⁻¹, respectively) and bout two (169.9 ± 30.9 and 150.3 ± 15.2 mmol·kg dw⁻¹, respectively). Following 24 weeks of exercise training, non-mitochondrial ATP production during bout one had fallen by 61 and 77% in CON and CARN, respectively (**Figure 5.10A**), though this reduction only tended to be significant in CARN ($P=0.07$) and not CON ($P=0.32$). In bout two, training reduced non-mitochondrial ATP production in both CON ($P<0.01$) and CARN ($P<0.05$) by a similar amount (**Figure 5.10B**). All differences in non-mitochondrial ATP production persisted after correction for workload.

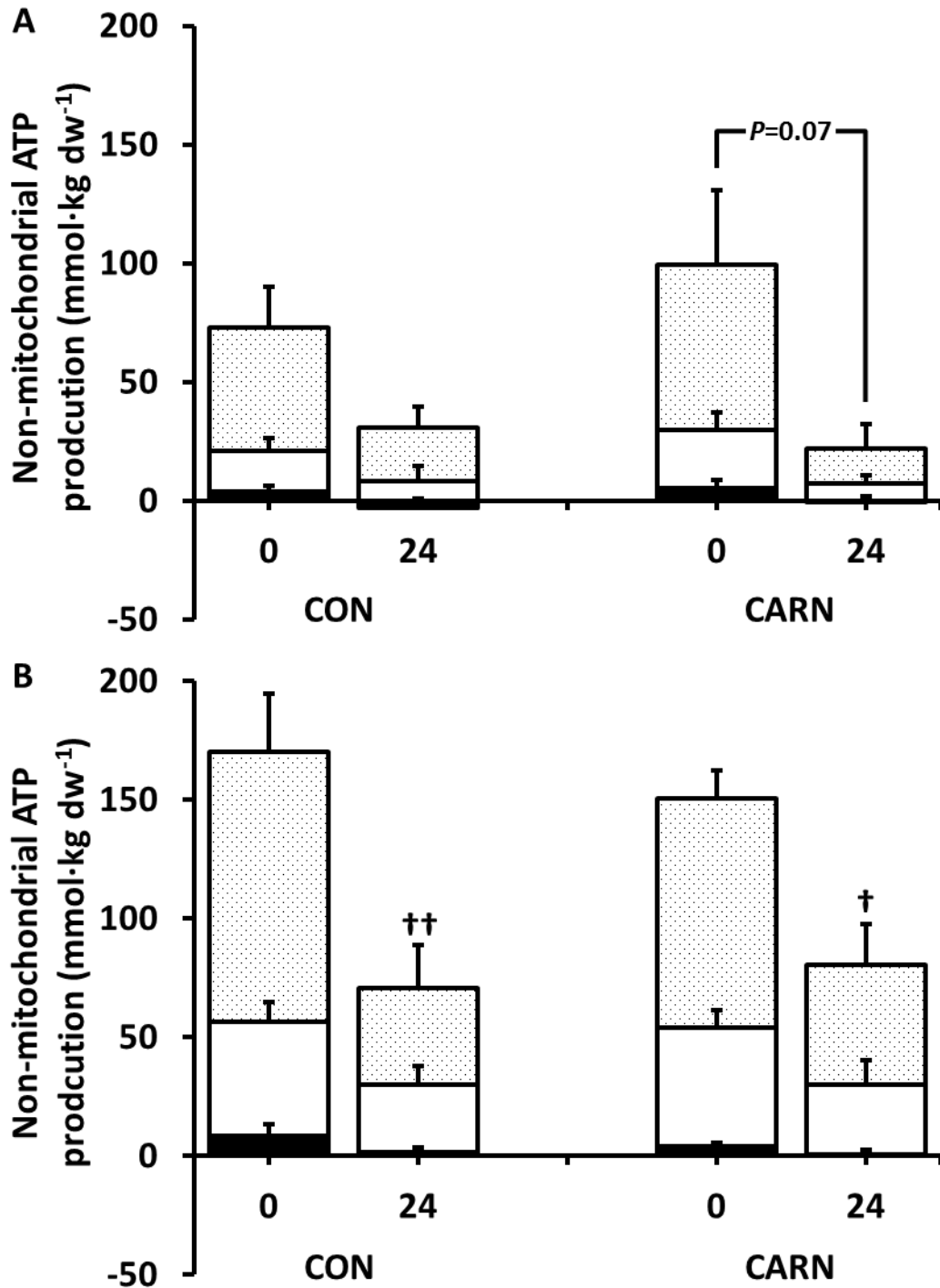


Figure 5.10 Non-mitochondrial ATP production determined from the change in muscle ATP (black bars), PCr (white bars) and lactate (spotted bars) during a first (A) and second (B) 3 minute bout of exercise at 100% W_{max} , before and after 24 weeks of exercise training receiving twice daily drinks of either 80 g carbohydrate (CON; black bars) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white bars). Values are mean \pm SE for 7 subjects. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ vs 0 weeks for total non-mitochondrial ATP production.

5.3.3 Whole body adaptations

VO_{2max} and Watt_{max}

Baseline VO_{2max} was 3.12 ± 0.29 and 3.16 ± 0.24 L·min⁻¹ in CON and CARN, respectively and was increased in both groups ($P < 0.01$) after 8 weeks of training, to 3.36 ± 0.34 and 3.39 ± 0.20 L·min⁻¹, respectively. No further increases in VO_{2max} occurred after 8 weeks, although values remained elevated above baseline in both CON (3.34 ± 0.31 , 3.41 ± 0.32 and 3.47 ± 0.30 L·min⁻¹, $P < 0.01$) and CARN (3.40 ± 0.20 , 3.34 ± 0.18 and 3.40 ± 0.16 , $P < 0.001$) at 12, 18 and 24 weeks, respectively. When normalised to body weight, the improvement in VO_{2max} did not reach significance until 24 weeks, increasing from 41.3 ± 3.1 to 45.2 ± 3.2 ml·kg⁻¹·min⁻¹ in CON and from 42.0 ± 2.9 to 44.5 ± 2.1 ml·kg⁻¹·min⁻¹ in CARN ($P < 0.05$). The percentage change from baseline in absolute VO_{2max} was no different between CON and CARN at any time point over the 24 week training period (**Figure 5.11**). W_{max} was similar at baseline in CON and CARN (206 ± 24 and 208 ± 24 watts, respectively) and was increased by a similar amount in both groups at 8 weeks (233 ± 25 and 235 ± 20 watts, respectively), with no further increases occurring at 12 (232 ± 24 and 239 ± 20), 18 (239 ± 26 and 238 ± 18) or 24 weeks (232 ± 23 and 243 ± 18 watts, respectively). Normalising W_{max} to body weight or lean mass did not influence statistical differences with respect to either training or group.

Mechanical efficiency

Mechanical efficiency at W_{max} was no different between groups at baseline (19.2 ± 0.7 and $19.3 \pm 0.9\%$ in CON and CARN, respectively) and was unchanged over 24 weeks in CON (**Figure 5.12**). However, mechanical efficiency in CARN increased to $20.8 \pm 0.8\%$ after 12 weeks ($P < 0.05$) and remained elevated after 24 weeks (21.2

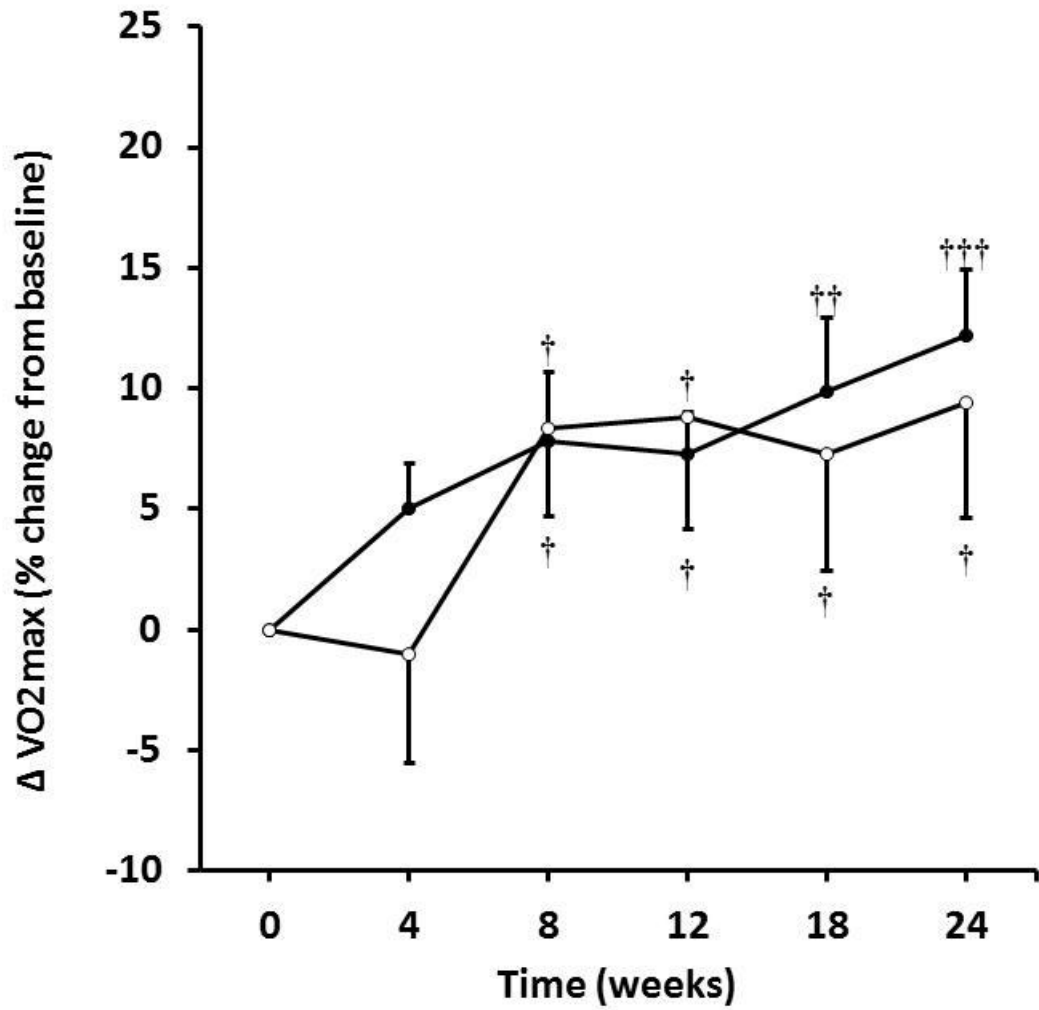


Figure 5.11 Percentage change in maximal oxygen uptake (VO_{2max}) at baseline and at 4, 8, 12, 18 and 24 weeks exercise training, receiving twice daily drinks of either 80 g carbohydrate (CON; black circles) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white circles). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*time x group*): † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ vs 0 weeks.

$\pm 0.6\%$; $P<0.01$), such that the change from baseline at 24 weeks was greater in CARN than CON (1.9 ± 0.8 and $-0.2 \pm 0.7\%$, respectively; $P<0.05$).

Work output

The work completed during the training sessions increased from baseline values of 153 ± 24 and 166 ± 18 kJ to 8 week values of 189 ± 24 and 196 ± 20 kJ in CON ($P<0.01$) and CARN ($P<0.05$), respectively. Work output remained elevated in CON (187 ± 27 , 191 ± 24 and 195 ± 25 kJ) and CARN (202 ± 19 , 192 ± 18 and 196 ± 19 kJ) at 12, 18 and 24 weeks, respectively but no further increases occurred after 8 weeks in either group. The percentage improvement from baseline is shown in **Figure 5.13** and was no different between CON and CARN at any time point.

Body composition

Total body mass was no different between CON and CARN at baseline (75.2 ± 3.6 and 76.3 ± 5.9 kg, respectively), 12 weeks (75.9 ± 3.3 and 77.8 ± 6.1 kg, respectively) or 24 weeks (76.7 ± 3.4 and 77.6 ± 5.9 kg, respectively). Lean body mass was similar in CON and CARN at baseline (55.3 ± 3.6 and 55.8 ± 3.4 kg, respectively) and increased by a small amount in CON at 12 (56.0 ± 3.8 kg; $P=0.09$) and 24 (56.5 ± 3.4 kg; $P<0.01$) weeks. The 1.2 kg increase in lean body mass over 24 weeks in CON was primarily due to a 0.9 kg increase ($P<0.05$) in lean trunk mass. Lean body mass in CARN did not change significantly after either 12 (56.0 ± 3.4 kg; $P=0.34$) or 24 (56.5 ± 3.4 kg; $P=0.12$) weeks, although the overall change in lean mass at 24 weeks was no different between groups (Δ lean mass = 1.2 ± 0.3 vs 0.7 ± 0.2 kg in CON and CARN, respectively; $P=0.15$). Fat mass was similar

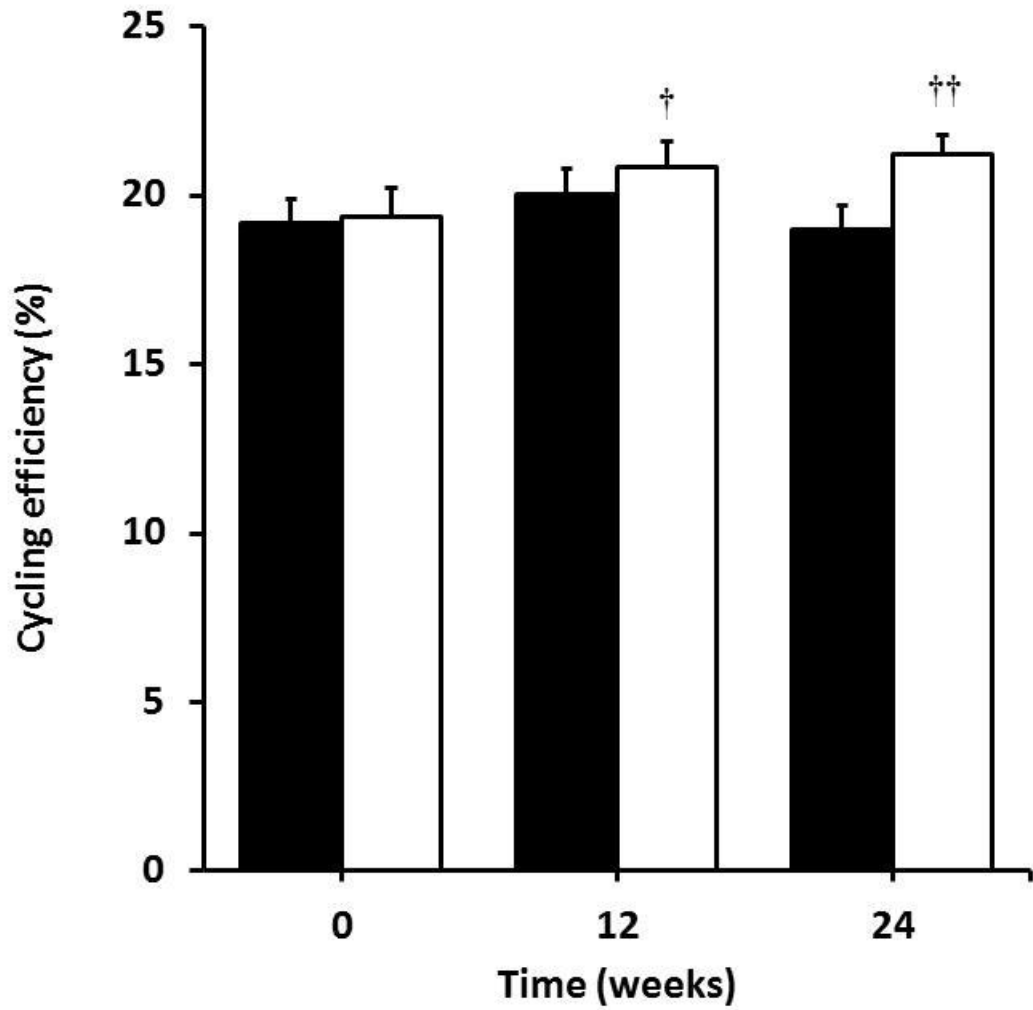


Figure 5.12 Mechanical efficiency at W_{\max} , determined as the percentage of VO_2 accounted for by mechanical work (see **General Methods**), at baseline and at 12, and 24 weeks exercise training, receiving twice daily drinks of either 80 g carbohydrate (CON; black bars) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white bars). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*time x group*): † $P < 0.05$, †† $P < 0.01$, vs 0 weeks.

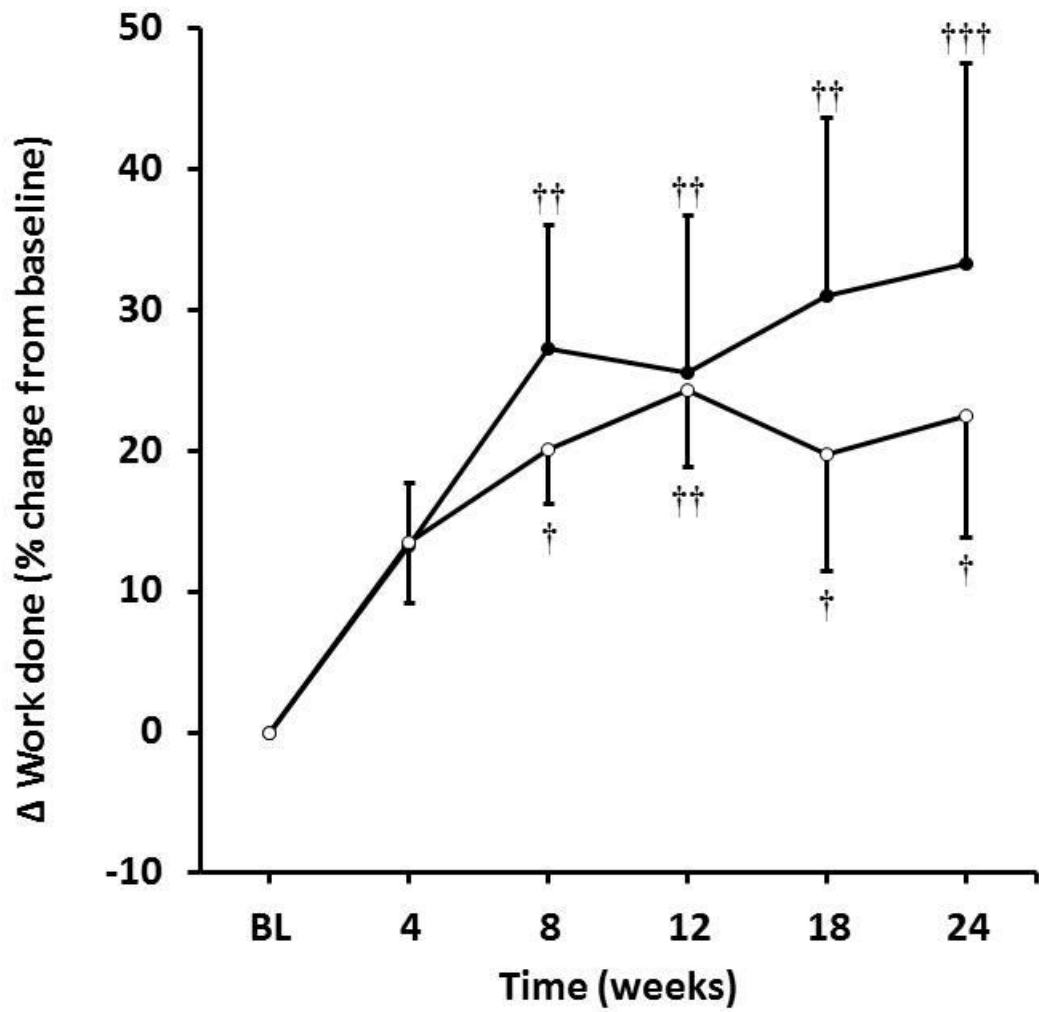


Figure 5.13 Work completed during training sessions at baseline and at 4, 8, 12, 18 and 24 weeks exercise training, receiving twice daily drinks of either 80 g carbohydrate (CON; black circles) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white circles). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*time x group*): † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ vs 0 weeks.

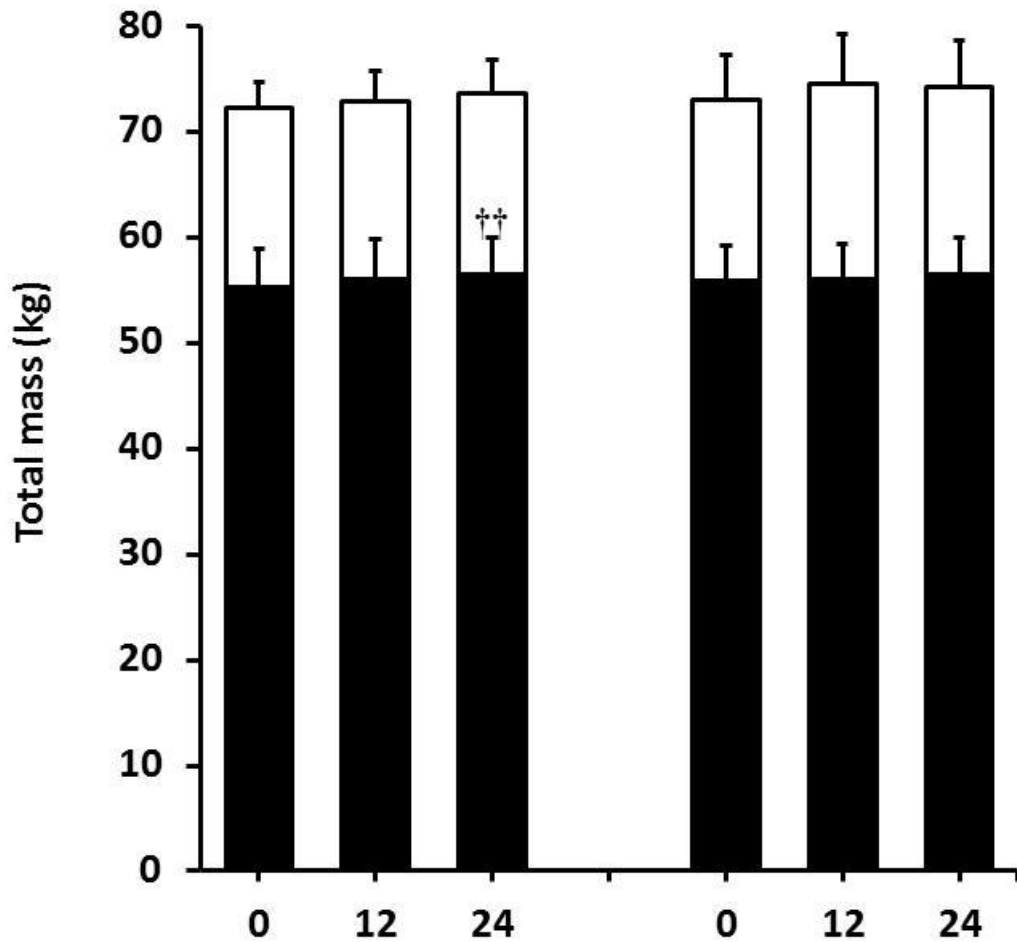


Figure 5.14 Total lean (black bars) and fat (white bars) mass at 0, 12 and 24 weeks of exercise training, receiving twice daily drinks of either 80 g carbohydrate (CON) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*time x group*): †† $P < 0.01$ vs 0 weeks.

between groups and did not change over 12 or 24 weeks in either CON or CARN (Figure 5.14).

5.4 Discussion

The present study investigated the influence of a proven carnitine loading strategy on skeletal muscle carnitine stores during a chronic period of intense exercise training and evaluated its impact upon the metabolic and functional adaptations to this type of training paradigm. These data clearly demonstrate that daily L-carnitine and carbohydrate feeding completely prevented the 12% decline in skeletal muscle carnitine content associated with a 12 week period of submaximal HIT in healthy, untrained volunteers. This preservation of total muscle carnitine content enabled the better maintenance of free carnitine availability during repeated bouts of high-intensity exercise, following 24 weeks of submaximal HIT. Interestingly, a trend towards a training-induced reduction in non-mitochondrial ATP production during an initial three minute bout of exercise at 100% W_{max} was evident in CARN but not CON, which is consistent with carnitines role in the matching of glycolytic and mitochondrial fluxes. However, neither PCr degradation nor lactate accumulation was different between groups in the first or second exercise bout following submaximal HIT. Moreover, in line with this apparent inability of carnitine supplementation to influence substrate metabolism during repeated bouts of exercise beyond submaximal HIT alone, the gains in VO_{2max} , W_{max} and work performance were similar between groups. Taken together this would suggest that high-intensity repeated-bout exercise training *per se*, results in a better matching of PDC and TCA cycle flux during multiple bouts of exercise such that carnitine availability is not limiting to oxidative ATP production. Nevertheless, mechanical efficiency during

exercise at 100% W_{\max} was enhanced in the CARN group over 12 and 24 weeks of training, but not in CON.

Muscle total carnitine content

Limited data exist on the plasticity of the muscle carnitine stores during chronic periods of exercise training. Endurance-trained individuals purportedly have augmented levels of muscle total carnitine (Arenas *et al.*, 1991), although the same study reported a somewhat paradoxical 6% reduction in muscle total carnitine in the same group of athletes following 120 days of structured endurance training. Two other studies have previously determined skeletal muscle carnitine concentrations following shorter controlled periods of structured exercise training. Following six weeks endurance training (45 minutes cycling at 70% $VO_{2\max}$, 4 times per week), the sum of the free and acetylcarnitine values reported by Vollaard *et al.* (2009) was 11% lower than at baseline. The equivalent values reported by Leblanc *et al.* (2004) declined by approximately 19% over a seven week training period (60 minutes cycling at 75% $VO_{2\max}$, 5 times per week). Although direct statistical comparisons were not made in either study, these data would suggest that several weeks of exercise training could facilitate a decline in muscle carnitine content. In agreement with this, the current data demonstrate that in previously untrained volunteers, intense repeated-bout exercise training is associated with a 12% reduction in muscle total carnitine content over a 12 week period. However, this decline appeared to be transient, as muscle carnitine content was somewhat restored following 24 weeks of continued training. One possible explanation for this measured decline could relate to changes in whole-body lean mass, which increased linearly in the CON group over the duration of the 24 week intervention. An increase in lean mass, comprising

presumably of mainly skeletal muscle, would require an increase in whole-body carnitine retention (or endogenous synthesis) and muscle carnitine uptake to maintain skeletal muscle carnitine concentrations at the same level. Thus the reduction in muscle carnitine concentrations at 12 weeks may simply reflect the relatively slow transport of carnitine into skeletal muscle (Rebouche & Engel, 1984) in the face of a greater whole-body skeletal muscle mass. However, the gains in lean mass in the CON group were relatively modest (~1.3%) and are therefore perhaps insufficient to entirely explain the 12% reduction in muscle carnitine content. The decline in muscle carnitine content could also be related to alterations in fibre-type composition, as the stimulus provided from our exercise training intervention could conceivably result in a shift towards a more oxidative muscle phenotype in these previously untrained volunteers (Buller *et al.*, 1960; Yan *et al.*, 2011). Indeed, studies in rats have suggested that the total muscle carnitine content of the oxidative soleus muscle is somewhat lower than that of gastrocnemius muscle (mixed fibre type) or the primarily glycolytic muscles, extensor digitorum longus and tibialis anterior (Porter, 2012). Although human data would not appear to support the existence of fibre-type differences in total carnitine content (Constantin-Teodosiu *et al.*, 1996), the influence of training on this relationship has not previously been tested. Finally, given the improved matching of PDC and TCA cycle fluxes following training, evidenced by lower acetylcarnitine accumulation at the same absolute workload (Vollaard *et al.*, 2009), it could be argued that this type of training reduces the dependence on free carnitine availability during high-intensity exercise. Thus the decline in skeletal muscle carnitine content from 0 -12 weeks in the CON group could reflect an adaptation to a lower carnitine requirement over the period when gains in exercise performance (and thus presumably metabolic adaptations)

were greatest. Importantly, and consistent with the increase in muscle total carnitine content observed following 12 (Stephens *et al.*, 2013) and 24 (Wall *et al.*, 2011) weeks of L-carnitine and carbohydrate feeding, this same feeding strategy was able to prevent the decline in muscle carnitine content in the current study. As a result, the change in muscle carnitine from baseline to 24 weeks was greater in the CARN group than in the CON group, thus providing the opportunity to assess whether the manipulation of muscle carnitine availability could have influenced the adaptations to the repeated bout exercise training paradigm.

Metabolic adaptations to training and carnitine

Consistent with the previously reported impact of high-intensity repeated bout exercise upon mitochondrial oxidative capacity (Little *et al.*, 2010), reliance on non-mitochondrial ATP delivery was reduced at 24 weeks in the current study, regardless of group. This training effect is particularly notable when it is considered that both groups were exercising at an approximately 15% greater absolute workload during the post-training biopsy visit. Moreover, the reductions in bout one non-mitochondrial ATP delivery at 24 weeks were only significant in the CARN treated group. Although not statistically different to CON values, bout one post-exercise PCr and lactate concentrations were only significantly elevated and suppressed, respectively, at 24 weeks in the CARN group. That carnitine may have been able to accentuate the sparing of non-mitochondrial ATP delivery following 24 weeks is consistent with the 35% reduction in non-mitochondrial ATP production following 24 weeks of skeletal muscle carnitine loading demonstrated by Wall *et al.* (2011) and implies that oxidative ATP provision during the first exercise bout was increased to a greater extent following training in CARN versus CON. Interestingly, the carnitine-

mediated reduction in non-mitochondrial ATP delivery in the study by Wall *et al.* (2011) was paralleled by increases in PDCa and acetylcarnitine accumulation, whereas we observed no difference, or even lower PDCa and carnitine acetylation following training in the CARN group. Thus it could be speculated that during a single bout of high-intensity exercise, the combination of training and increased muscle carnitine availability facilitated an appropriately greater delivery of mitochondrial acetyl-groups, thus better matching glycolytic and TCA cycle fluxes whilst at the same time improving the matching between the PDC and TCA cycle fluxes.

It was originally hypothesised that the impact of increasing skeletal muscle carnitine might be greater during a second bout of exercise, when PDC flux would appear to decline. The reliance on non-mitochondrial ATP production during bout two was robustly reduced with training in both groups, although it remained substantially greater than during bout one. In the CON group, acetylcarnitine accumulation at 24 weeks was similar across both bouts which, as discussed in **Chapter 4**, would suggest PDC flux still declined during the second bout. In contrast, bout two carnitine acetylation was significantly greater than bout one in the CARN group after 24 weeks, which would be indicative of a better maintenance of PDC flux and was consistent with our hypothesis that increasing free carnitine availability at the onset of a second bout could avert the decline in PDC flux during repeated bouts. Unlike in bout one however, this apparent acceleration of acetyl-group delivery did not seem to translate into any further enhancement of oxidative ATP provision beyond training alone, as the decrement in non-mitochondrial ATP production at 24 weeks was equal between groups. The potency of the training response in both groups is

underlined by the observation that bout two non-mitochondrial ATP production at 24 weeks was very similar to that measured during bout one at baseline. Nevertheless, the finding that oxidative ATP production still declines in a second bout of exercise in the face of an enhanced PDC flux would perhaps suggest that acetyl-group delivery is limiting to oxidative ATP production during a first, but not a second bout of high-intensity exercise.

Impact on whole-body adaptations

The mean increase in VO_{2max} at 24 weeks was 0.36 and 0.24 $L \cdot min^{-1}$ for the CON and CARN groups, respectively, whilst the change in W_{max} was 26 and 30 watts, respectively, which represented a 15 and 20% increase from baseline. However, neither the relative nor absolute improvements in VO_{2max} or W_{max} appeared to be influenced by CARN treatment. It was originally hypothesised that, consistent with the findings from Wall *et al.* (2011), CARN would enable a greater amount of work to be completed during training sessions and thus potentially augment the training stimulus to increase VO_{2max} and W_{max} . In this respect, given that no effect of CARN treatment was apparent on metabolism during the second bout of exercise, or work output during training sessions, it is perhaps unsurprising that no differences between groups were observed in the gains in VO_{2max} or W_{max} , particularly as skeletal muscle carnitine loading *per se* would not appear to influence VO_{2max} (Wall *et al.*, 2011). Interestingly however, a significant interaction effect was observed for mechanical efficiency, suggesting that the relative oxygen cost of work at W_{max} was reduced over time in CARN, but not CON. Data from another study recently completed in our lab demonstrate that this same L-carnitine and carbohydrate feeding protocol, in the absence of exercise training, does not appear to impact upon

mechanical efficiency during exercise at W_{\max} (see **Appendix 3.5**). This would suggest that the increase in mechanical efficiency at W_{\max} observed in the current study is a result of manipulating free carnitine availability during training, rather than an acute effect of skeletal muscle carnitine loading *per se*. Moreover, given that the exercise training was performed at the same workload as that for which mechanical efficiency was determined, albeit under different exercise conditions (repeated bout vs continuous incremental protocol), it is quite possible that this effect would have been similarly manifest during training sessions. Taken together with the findings reported above, it therefore seems highly likely that daily L-carnitine and carbohydrate feeding would have altered metabolic flux during the training sessions, which raises the question as to why no apparent impact was observed upon work output and the associated markers of exercise performance.

As we did not investigate skeletal muscle metabolism during exercise after 12 weeks of the intervention, it is impossible to ascertain over what time period the differential effects discussed above were occurring. However, given that skeletal muscle carnitine content was reduced after 12 weeks in CON, and differences in mechanical efficiency were already manifest at this point, it seems likely that at least some effects of L-carnitine feeding would have been present for the latter 12 weeks of the intervention. Given that no impact of CARN on non-oxidative ATP delivery was observed during the second exercise bout, it would also seem unlikely that any further impact would be manifest during subsequent exercise bouts, particularly as free carnitine concentrations were no different between groups following the second bout at 24 weeks. In this regard, it seems likely that the metabolic effect of manipulating free carnitine availability would not have been of sufficient magnitude

to influence work output during the fourth exercise bout in training sessions. Lastly, it should also be acknowledged that as work output remained equivalent between groups, the apparent enhancement of oxidative ATP production with CARN could possibly be expected to dampen the training stimulus, as reductions in both the myocellular oxygen tension and PCr/Cr ratio have been identified as metabolic events that may regulate various signalling processes leading to skeletal muscle adaptation in response to exercise (Egan & Zierath, 2013).

Conclusions

In conclusion, this study demonstrates that daily L-carnitine and carbohydrate feeding can be used to manipulate the skeletal muscle carnitine stores during a chronic period of intense exercise training, when total muscle carnitine content would otherwise seem to decline. However, the consequent elevation of muscle free carnitine availability during repeated-bout exercise training does not ultimately appear to influence the training-induced increase in oxidative ATP provision across repeated bouts of high-intensity cycling exercise, despite the potential preservation of PDC flux during a second bout of exercise. Finally, and likely as a result of this latter finding, neither work output, VO_{2max} or body fat were differentially altered by carnitine treatment. Nevertheless, these data provide further evidence for the acetyl-group buffering role of carnitine in the facilitation of PDC flux in human skeletal muscle and suggest that oxidative ATP production during single high-intensity exercise efforts can be enhanced with L-carnitine and carbohydrate feeding over a chronic period of submaximal HIT.

CHAPTER 6: GENERAL DISCUSSION

6.1 Overview of findings

The overall aim of this thesis was to further investigate the role of carnitine during high-intensity submaximal exercise and to test the hypothesis that increasing skeletal muscle carnitine content in healthy human volunteers could augment the adaptations to a chronic period of submaximal high-intensity intermittent training (HIT). Initially this attempted to identify an L-carnitine feeding strategy that did not rely on the high carbohydrate load previously used by our group to increase carnitine content. Thereafter, it identified an exercise paradigm during which muscle carnitine availability might influence PDC flux and non-mitochondrial ATP production and that when implemented in a training program would potentially induce greater gains than classical endurance exercise training at an intensity where we also know carnitine availability to be limiting (e.g. 30 min at 80% $\text{VO}_{2\text{max}}$; Wall et al., 2011).

Through the novel application of a $^2\text{H}_3$ -carnitine tracer with a forearm carnitine balance model and parallel estimations of whole-body carnitine retention, a carbohydrate formulation was demonstrated to have greater potential for increasing muscle carnitine accretion compared to a whey protein-carbohydrate formulation, which appeared to antagonise the insulin-mediated stimulation of muscle carnitine uptake (**Chapter 3**). The potential inhibitory action of amino acids on muscle carnitine transport is a novel finding and may have important implications with respect to our understanding of the chronic regulation of skeletal muscle carnitine content.

The acute exercise studies in **Chapter 4** demonstrated that acetylcarnitine accumulation and the reliance on non-mitochondrial ATP production during

moderate and high-intensity single-leg knee extension is minimal compared to previously reported values during two-legged cycling, suggesting that PDC flux is probably not limiting to oxidative ATP production under these exercise conditions. Conversely, non-mitochondrial ATP production during repeated bouts of high-intensity, fixed-workload cycling was found to increase in a second bout in the face of similar acetylcarnitine accumulation, suggesting that PDC flux declines during this second bout. This was in contrast to previous studies of maximal sprint efforts, or single-leg knee extension, where PDC flux is thought to increase with progressive bouts.

Drawing on these initial findings, the final study of this thesis (**Chapter 5**) assessed whether increasing skeletal muscle carnitine content by daily L-carnitine and carbohydrate feeding could prevent this decline in PDC flux during repeated bouts of exercise and thus increase work output and concomitantly enhance the adaptations to a 24 week period of submaximal high-intensity interval training (HIT). L-carnitine supplementation was successful in manipulating skeletal muscle carnitine availability and appeared to preserve PDC flux across repeated bouts of exercise, but did not ultimately influence non-mitochondrial ATP production following submaximal HIT. In line with this latter observation, L-carnitine did not potentiate any of the functional adaptations induced by submaximal HIT alone. Collectively, these data further underline the importance of the acetyl-group buffering role of carnitine in skeletal muscle PDC flux during exercise and should aid the development of nutritional strategies to improve exercise performance in both athletic and clinical populations.

6.2 Implications for skeletal muscle carnitine accumulation

Whey protein inhibits insulin-stimulated muscle carnitine transport

As mentioned in the **General Introduction** and **Chapter 3**, the factors that regulate skeletal muscle carnitine content are poorly understood, but would appear to be highly dependent on the functionality of OCTN2 (Treem *et al.*, 1988). Insulin is known to increase muscle carnitine transport by OCTN2 (Stephens *et al.*, 2006b) and the finding in **Chapter 3** that carbohydrate ingestion is able to promote a positive forearm carnitine balance is consistent with this. Moreover, these data provide the most direct estimate to date of insulin-stimulated muscle carnitine uptake *in vivo* and confirm the expected rates of muscle carnitine accumulation based on previous acute (Stephens *et al.*, 2007b) and chronic (Stephens *et al.*, 2013) feeding studies from this lab. A key question to arise from **Chapter 3** is why a large oral bolus of whey protein might inhibit insulin-stimulated muscle carnitine transport, given that dietary L-carnitine is typically sourced from amino acid-rich foods (Mitchell, 1978). In view of the fact that plasma carnitine availability is unaltered by a standard mixed meal (containing approximately 350 μmols carnitine; Li *et al.*, 1992), it is questionable as to whether carnitine transport into muscle would be appreciably different between basal and postprandial periods and indeed, plasma carnitine concentrations would appear to be far more tightly regulated than the reportedly slow turnover of the muscle carnitine stores (Rebouche & Engel, 1984). Nevertheless, following whey-protein and carbohydrate co-ingestion, plasma carnitine availability and serum insulin concentrations were sufficiently elevated to levels that were able to promote muscle carnitine uptake following carbohydrate ingestion alone. Whether these findings infer a direct influence of amino acid availability on OCTN2 activity or an indirect effect mediated, for example, by sodium-amino acid co-transport requires

further investigation. One further possible mechanism could involve the inhibition of OCTN2 by acylcarnitines (Ohashi *et al.*, 1999), as total plasma acylcarnitine concentrations during the PRO trial were raised throughout the period when serum insulin was elevated (see **Appendix 3.1**). The reason for the greater plasma acylcarnitines during PRO is unclear, although acylcarnitines are known to accumulate in response to excessive amino acid oxidation, either as direct by-products of amino acid catabolism (e.g. propionylcarnitine, isovalerylcarnitine) or as a result of incomplete β -oxidation (Stephens *et al.*, 2014). Cell-based studies to investigate the rate of intracellular carnitine accumulation in the presence of variable insulin, amino acid and acylcarnitine concentrations would be a useful approach to address this mechanism.

Regulation of muscle carnitine content: Diet and exercise

Another novel finding from this thesis relating to muscle carnitine transport was the observation that total muscle carnitine content declined over 12 weeks of submaximal HIT and daily carbohydrate feeding in the control group. Although it has previously been speculated that high-intensity exercise training could lead to a reduction in the muscle carnitine stores (Arenas *et al.*, 1991 and **Chapter 5**), it cannot be excluded that the increase in daily carbohydrate load, rather than the submaximal HIT, was responsible for the decline in muscle total carnitine content. Interestingly, in two previous prolonged studies from our lab, total muscle carnitine content was 6-10% lower (though not statistically significant) following 12 weeks of daily carbohydrate feeding in control subjects (Wall *et al.*, 2011; Stephens *et al.*, 2013). As well, vegetarians, who will typically obtain a relatively large proportion of their energy intake from carbohydrates, have lower levels of muscle and plasma

carnitine compared to their non-vegetarian counterparts (Stephens *et al.*, 2011). Whilst the latter is undoubtedly related to a habitually lower carnitine intake (Rebouche, 1992), L-carnitine infusion studies from our lab have also suggested that the stimulatory effect of insulin on muscle carnitine accumulation and OCTN2 expression is diminished or absent in vegetarian subjects (Stephens *et al.*, 2011). Reciprocally a non-vegetarian diet, which is typically associated with an increased fat intake, could conceivably promote muscle carnitine accumulation given the emerging role of the PPAR nuclear receptors (of which fatty acids are ligands) in the regulation of OCTN2 expression and carnitine transport activity (Ringseis *et al.*, 2007; Maeda *et al.*, 2008; Zhou *et al.*, 2014a; Zhou *et al.*, 2014b). Thus aside from the influence of habitual diet on plasma carnitine availability, macronutrient availability may also be involved in the chronic (i.e. transcriptional) regulation of muscle carnitine transport and accretion.

With respect to the role of submaximal HIT in muscle carnitine content, studies in rats have indicated that OCTN2 may be translocated to the sarcolemma membrane in response to muscle contraction (Furuichi *et al.*, 2012) and thus it has been speculated that regular muscle contraction could actually promote muscle carnitine accretion. However, human data does not support a role for exercise in acutely stimulating muscle carnitine uptake, with or without oral L-carnitine supplementation to increase plasma carnitine availability (Soop *et al.*, 1988). On the contrary, it has been suggested that high-intensity exercise could acutely increase muscle carnitine efflux, potentially via acetylcarnitine export (Brass, 2004). However, the majority of studies that have determined muscle carnitine moieties before and after exercise have been unable to detect differences in total carnitine content, suggesting minimal net

loss/gain from the skeletal muscle compartment during exercise (Carlin *et al.*, 1986; Harris *et al.*, 1987; Constantin-Teodosiu *et al.*, 1992; van Loon *et al.*, 2001; Wall *et al.*, 2011). Thus it would perhaps seem more likely that the decline in muscle total carnitine content in the control group of **Chapter 5** is perhaps related to something other than the accumulative effect of repeated acute bouts of intense exercise on muscle carnitine efflux *per se*. Finally, this decline in muscle carnitine content with training and/or carbohydrate feeding could also explain why the observed increase in muscle carnitine content in the L-carnitine supplemented group was somewhat less than projections based on the results from **Chapter 3** and on previous research (Stephens *et al.*, 2007b; Wall *et al.*, 2011).

Skeletal muscle carnitine loading in clinical populations

In **Chapter 5**, the previously reported increase in fat mass in response to 12 weeks carbohydrate feeding (Control group; Stephens *et al.*, 2013) appeared to be offset by submaximal HIT, consistent with the observed effects of an increase in physical activity levels on body composition (Nordby *et al.*, 2012). However, the development of an L-carnitine feeding approach that does not rely on such a large carbohydrate load (160 g per day) is still desirable if the emerging metabolic benefits of skeletal muscle carnitine loading in young, healthy volunteers are to be translated to other populations, such as overweight or obese, diabetic, or elderly individuals. Despite the finding that 40 g whey protein plus 40 g carbohydrate was unable to promote a positive forearm carnitine balance in **Chapter 3**, the potential for other reduced-carbohydrate formulations to increase muscle carnitine accretion cannot be disregarded. Indeed, our lab has recently demonstrated that daily L-carnitine feeding in a formulation containing 44 g carbohydrate, 14 g protein and 11 g fat was able to

increase muscle carnitine content over 24 weeks in older (65-75 years old) adults (Chee *et al.*, 2014). Thus the use of a smaller protein load could be efficacious in lowering the carbohydrate requirement of carnitine supplementation without inhibiting insulin-mediated muscle carnitine transport. Further chronic studies are currently in progress in our lab which will address the efficacy of similar feeding strategies in overweight and diabetic subjects.

6.3 Oxidative ATP provision declines during high-intensity repeated-bout cycling exercise

An interesting and novel observation in **Chapter 4** was the increase in PCr degradation during a second bout of cycling exercise at 100% W_{max} , data that were reproduced and thus validated using the same exercise paradigm in **Chapter 5**. Combining the datasets from **Chapter 4** with the baseline (pre-training) data from **Chapter 5** emphasises this finding and clearly demonstrates that reliance on non-mitochondrial ATP production is significantly greater, by approximately 70% across these 21 subjects, during the second bout of exercise (**Figure 6.1**). This was related to both a 121% increase in PCr degradation and a 54% greater lactate accumulation. Given that the mechanical work performed during each bout would have been identical, an increased reliance on non-mitochondrial routes of ATP production must indicate either a reduction in oxidative ATP production or a lower metabolic efficiency of muscle contraction, i.e. a greater ATP demand per unit work. The latter could feasibly result from a lowering of muscle pH at the onset of contraction through altered Ca^{2+} -ATPase kinetics (Wolosker *et al.*, 1997), as muscle lactate was greater prior to the start of the second bout (**Table 6.1**). Alternatively, it is possible that muscle temperature, which has been shown to influence whole-body oxygen

uptake during cycling exercise (Ferguson *et al.*, 2001), was greater during the second exercise bout. However, the impact of either muscle pH or temperature on metabolic efficiency would appear to be relatively small during dynamic human skeletal muscle contraction (Bangsbo *et al.*, 2001) and probably insufficient to explain the 70% greater non-mitochondrial ATP production during bout two. Thus it seems likely that the increased reliance on PCr degradation and anaerobic glycolysis was a direct compensation for the inability to sustain oxidative ATP production in the second bout. This is in contrast to single-leg knee extension exercise, as it was shown in **Chapter 4** that oxidative ATP provision is well matched to the total ATP demand during continuous exercise at 55% or 85% W_{\max} , whilst it has previously been shown that oxidative ATP provision is better matched to total ATP demand during a second exercise bout at 100% W_{\max} (Bangsbo *et al.*, 2001). Our findings also differ from maximal two-legged cycling conditions, where a progressive decline in work output parallels the decline in non-mitochondrial ATP production during each bout (Putman *et al.*, 1995).

The progressively greater reliance on non-mitochondrial ATP production is consistent with exercise performance during this type of repeated bout paradigm, which we found elicited exhaustion in our untrained subjects within 3-4 bouts (9-12 minutes). If, as previous studies have argued, oxidative ATP provision was indeed able to provide an increasingly higher proportion of total ATP production during each bout, then reliance on anaerobic glycolysis would reciprocally decrease with each bout and exercise performance would be expected to continue until muscle glycogen stores were depleted.

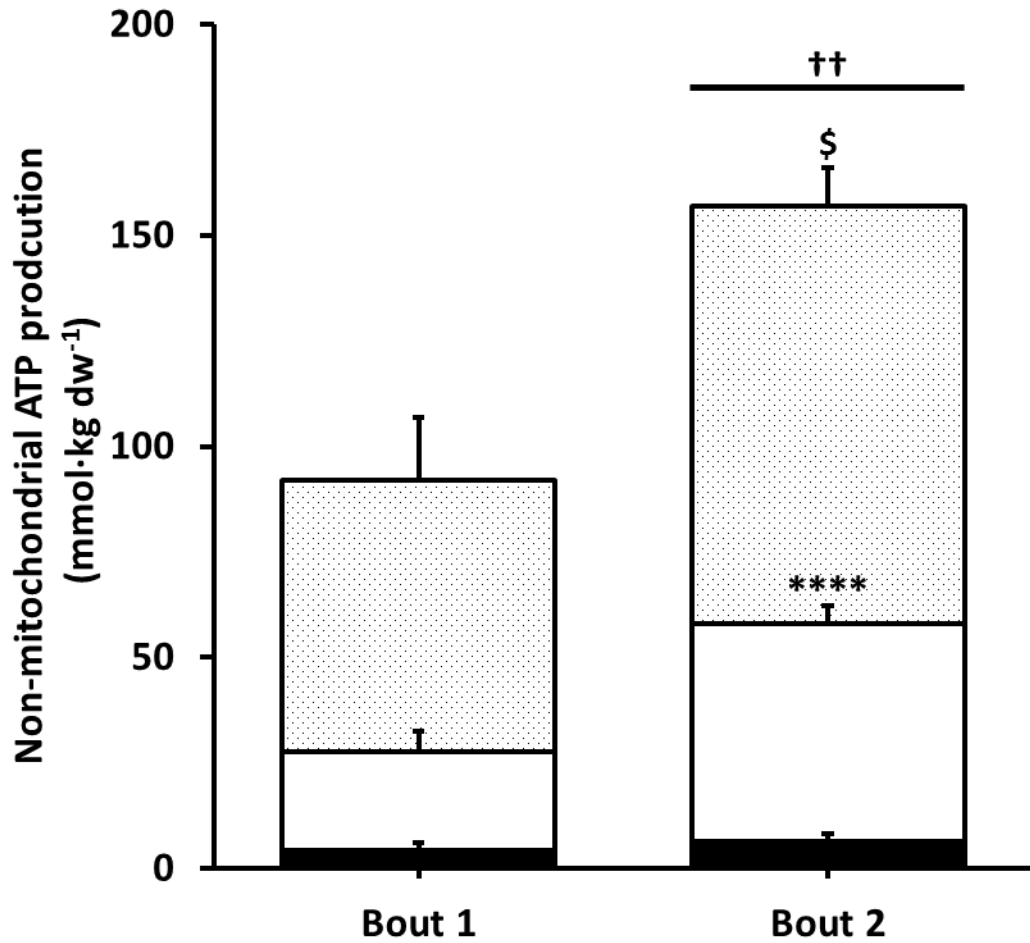


Figure 6.1 Skeletal muscle ATP production from non-mitochondrial sources, comprising ATP (black bars), PCr (white bars) and lactate (dotted bars) during two 3 minute bouts of cycling exercise at 100% W_{max} , separated by 5 minutes of passive recovery. Data are mean \pm SE for 21 subjects. **** P <0.0001 for PCr; \$ P =0.07 for lactate; †† P <0.01 for total non-mitochondrial ATP, vs bout one.

Table 6.1 Skeletal muscle metabolite concentrations before and after two 3 minute bouts of cycling exercise at 100% W_{\max} separated by 5 minutes of passive recovery. Data are presented as mean \pm SE for 21 subjects in $\text{mmol}\cdot\text{kg dw}^{-1}$ except PDCa ($\text{nmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$). Values with different letters are significantly different ($P<0.05$) from each other following one-way ANOVA with bonferoni post-hoc comparison.

	Bout 1		Bout 2	
	PRE	POST	PRE	POST
ATP	25.8 \pm 0.8 ^a	23.6 \pm 0.8 ^a	24.0 \pm 1.0 ^a	20.8 \pm 0.9 ^b
PCr	76.4 \pm 2.2 ^a	53.1 \pm 4.8 ^b	75.4 \pm 2.6 ^a	23.9 \pm 3.2 ^c
Lactate	6.1 \pm 0.8 ^a	48.9 \pm 9.6 ^b	26.9 \pm 5.2 ^c	92.9 \pm 7.2 ^d
Glycogen	399.3 \pm 17.5 ^a	337.8 \pm 17.2 ^b	382.4 \pm 21.2 ^a	258.1 \pm 19.2 ^c
Free Carnitine	15.4 \pm 0.6 ^a	11.1 \pm 0.8 ^b	12.5 \pm 0.8 ^b	7.9 \pm 0.8 ^c
Acetylcarnitine	3.1 \pm 0.6 ^a	7.2 \pm 1.0 ^b	6.1 \pm 1.1 ^b	10.4 \pm 0.6 ^c
PDCa	3.7 \pm 0.5 ^a	6.5 \pm 1.5 ^{ab}	3.9 \pm 0.8 ^a	10.6 \pm 1.4 ^b

Indeed, it is generally accepted that muscle glycogen availability is unlikely to become limiting to exercise performance during continuous exercise at such intensities (see **General Introduction** and Karlsson and Saltin, 1970). However, with our exercise paradigm muscle glycogen utilisation increased by 2-fold in the second exercise bout (**Table 6.1**) to a rate of over 40 mmol·kg dw⁻¹·min⁻¹, which could quite conceivably elicit fatigue within a further two bouts. Such a marked increase in glycogenolysis would perhaps suggest an increased recruitment of type II muscle fibres in this second exercise bout (Greenhaff *et al.*, 1991; Soderlund *et al.*, 1992) and would appear to be in contrast with the progressive reduction in muscle glycogen utilisation during continuous exercise performance (Karlsson & Saltin, 1970) or maximal sprint performance (Spriet *et al.*, 1989).

Based on the arguments discussed above and the combined data set from **Chapter 4** and **Chapter 5**, which clearly demonstrate that non-mitochondrial ATP production and glycolytic flux are greater during a second bout of exercise in the face of similar acetylcarnitine accumulation, it seems reasonable to conclude that despite a greater activation of PDC, oxidative ATP production and thus PDC flux were lower during a second exercise bout. In other words, the ratio of acetylcarnitine accumulation to glycolytic flux may be considered an informative index of PDC flux in human skeletal muscle during high-intensity exercise. Reasons for the discrepancy in these findings with those of different exercise models were discussed in **Chapter 4**, though it remains unclear as to what mechanisms are restricting oxidative ATP provision during a second bout of high-intensity cycling exercise. Whole body oxygen consumption during this type of exercise paradigm would not appear to decline during a second bout (see **Appendix 3.6**) and thus would seem unlikely to be

limiting to TCA cycle flux, although this may not necessarily reflect the oxygen uptake of the working muscle mass. In **Chapter 4** it was postulated that this suppression of oxidative ATP production could be related to an inhibition of PDC flux, secondary to a reduction in free carnitine availability. **Chapter 5** subsequently tested the hypothesis that increasing muscle carnitine availability could abate this decline in oxidative ATP production, the implications of which will be discussed below.

6.4 Adaptations to chronic submaximal HIT and carnitine supplementation

Submaximal HIT improves matching between glycolytic, PDC and TCA cycle fluxes

Given the current popularity of the HIT exercise paradigm to increase fitness and health in various sedentary populations (Gillen & Gibala, 2013), our submaximal HIT intervention was a pertinent and timely approach to investigate carnitine metabolism during exercise training. A particularly novel aspect of our study is its 24 week duration, as most previous HIT studies have only investigated relatively short-term responses (Sloth *et al.*, 2013). Prior studies have demonstrated the ability of 2-6 weeks HIT to reduce glycogen utilisation and lactate accumulation with a concomitant increase in lipid oxidation during constant load exercise at intensities of 60 - 90% $\text{VO}_{2\text{max}}$ (Burgomaster *et al.*, 2006; Burgomaster *et al.*, 2008). Markers of oxidative capacity in resting skeletal muscle also reportedly increase in response to short-term submaximal HIT (Gibala *et al.*, 2006; Little *et al.*, 2010; Cochran *et al.*, 2014). However, this is the first study to demonstrate the profound impact of long-term submaximal HIT upon oxidative ATP production during this type of exercise protocol, where the demand for mitochondrial ATP production is stressed near-maximally. Data from a combined group of 18 subjects to have completed our

training intervention demonstrate the marked effect of submaximal HIT on skeletal muscle metabolism during two bouts of exercise at 100% W_{\max} , resulting in reductions in non-mitochondrial ATP production of 61 and 52% during the first and second bout, respectively (**Figure 6.2**). Moreover, and consistent with prior reports on short-term HIT (Burgomaster *et al.*, 2006), resting muscle glycogen content was increased approximately 50% after 24 weeks of submaximal HIT in these 18 volunteers, whilst net muscle glycogen utilisation throughout both exercise bouts was reduced by approximately 40%. In line with the discussion above (see section **6.3**), these training-induced alterations in skeletal muscle substrate availability and utilisation are reflective of a better matching between glycolytic and mitochondrial fluxes and more than likely facilitated the ~30% increase in work output at 100% W_{\max} . We also noted an ~10% improvement in $VO_{2\max}$, which is in agreement with the range (4-13.5%) reported in a recent meta-analysis on the adaptations to HIT intervention (Sloth *et al.*, 2013). All together it seems probable that these adaptations are indicative of an increased capacity for oxidative ATP production in skeletal muscle, particularly given the strong correlation between training-induced improvements in whole-body $VO_{2\max}$ and skeletal muscle citrate synthase activity (Vigelso *et al.*, 2014), a reliable marker of mitochondrial content (Larsen *et al.*, 2012). Interestingly, most of the measured improvements in W_{\max} , $VO_{2\max}$ and exercise performance occurred during the first 8-12 weeks of our intervention and therefore it would be also salient to address whether this apparent plateau in functional adaptation is related to a “ceiling” in the metabolic adaptations observed after 24 weeks of training. Indeed, given that most prior HIT studies have been of a

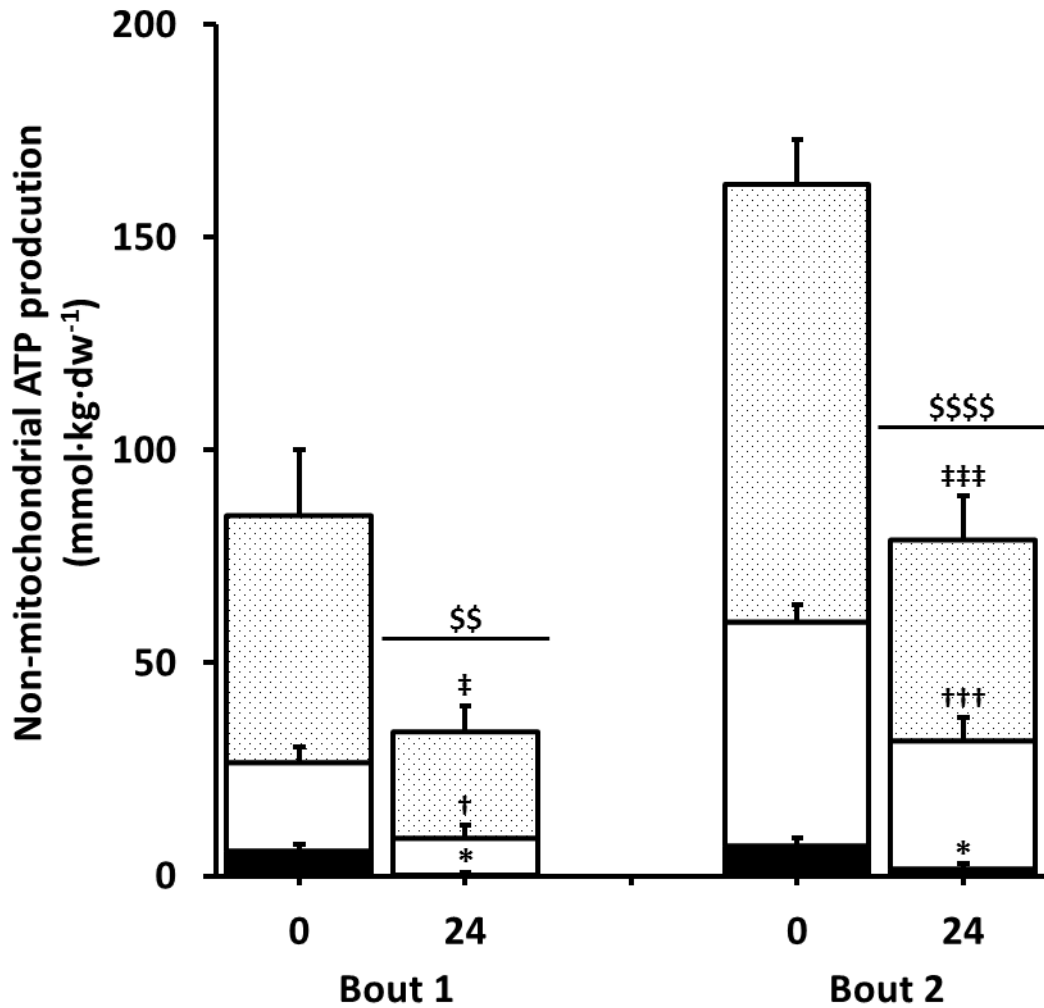


Figure 6.2 Skeletal muscle ATP production from non-mitochondrial sources, comprising ATP (black bars), PCr (white bars) and lactate (dotted bars) during two 3 minute bouts of cycling exercise at 100% W_{max} , separated by 5 minutes of passive recovery before and after 24 weeks of submaximal HIT. Data are mean \pm SE for 18 subjects. * $P < 0.05$ for ATP; †† $P < 0.01$, ††† $P < 0.001$ for PCr; ‡ $P < 0.05$, †††† $P < 0.0001$ for lactate; \$\$\$ $P < 0.0001$ for total non-mitochondrial ATP vs 0 weeks.

shorter duration, these findings now question whether continued meaningful improvements in exercise capacity are possible with a HIT model, beyond an 8-12 week period. Ongoing work will investigate the genomic adaptations that occurred in resting muscle biopsy samples over 12 and 24 weeks of intervention.

Manipulating muscle carnitine availability during submaximal HIT

Despite a robust training-induced reduction in non-mitochondrial ATP production across both bouts of our exercise protocol (discussed above), an apparent decline in PDC flux during the second exercise bout still persisted following 24 weeks of submaximal HIT (see **Figure 6.2**). Drawing on the findings from **Chapter 4** it was rationalised that increasing free carnitine availability could maintain PDC flux during this second bout of exercise and thus improve work capacity during submaximal HIT, leading to greater functional adaptations. Consistent with this hypothesis, manipulation of the skeletal muscle carnitine pool with L-carnitine and carbohydrate feeding resulted in an increased free carnitine availability during both bouts of cycling exercise at 100% W_{max} and appeared to preserve PDC flux during the second bout. Thus a novel finding to arise from this study was that, despite the aforementioned carnitine-mediated preservation of PDC flux, the training-induced reduction in PCr utilisation and lactate accumulation – and thus non-mitochondrial ATP production - was no different between groups across either exercise bout.

Given the impressive improvement in oxidative ATP delivery in response to submaximal HIT discussed above, it is possible that the training-induced improvement in the balance of glycolytic, PDC and mitochondrial fluxes during the second bout was greater than that which could be expected from manipulating free

carnitine availability. Indeed, this tighter matching of metabolic fluxes could lessen the dependence on free carnitine availability as demonstrated by the training-induced blunting of about two acetylcarnitine accumulation in the Control group (see **Table 5.1**). In this respect, muscle carnitine loading should not be discounted as a potential strategy to enhance the efficacy of exercise prescription in patients suffering from impairments in oxidative ATP delivery (e.g. peripheral vascular disease), as reliance on non-mitochondrial ATP production in these individuals is likely to be even more exaggerated (Hands *et al.*, 1986; Bauer *et al.*, 1999).

The reciprocal relationship between carnitine-mediated PDC flux and non-mitochondrial ATP production demonstrated by Wall *et al.* (2011) implied that PDC flux was limiting TCA cycle flux and oxidative ATP delivery during continuous exercise at 80% $\text{VO}_{2\text{max}}$. In contrast, here we demonstrate that increasing PDC flux in a second bout of high-intensity exercise is not able to reduce the reliance on non-mitochondrial ATP production. If it is assumed that acetylcarnitine accumulation is in equilibrium with acetyl-CoA production (Constantin-Teodosiu *et al.*, 1993), this could imply that under these conditions, oxidative ATP production is restricted by factors other than acetyl-group delivery, possibly by sites distal to the TCA cycle. For example a limitation at the level of the electron transport chain would restrict NAD^+ production, an important cofactor for several enzymes of the TCA cycle, which is consistent with the high redox potential that would have been needed to drive the greater rate of lactate production during the second exercise bout. Moreover, oxygen uptake by the working muscle mass is known to be compromised during high-intensity two-legged cycling (Boushel & Saltin, 2013), secondary to the high demand on cardiac output and blood flow distribution (Calbet *et al.*, 2007), in

addition perhaps, to a sympathetic vasoconstriction-mediated decline in muscle peak perfusion (Savard *et al.*, 1989). Whether these limitations are more marked and could restrict oxygen supply to the electron transport chain during a second bout of exercise is a fascinating supposition but requires further examination. Alternatively, if submaximal HIT is considered as a chronic stimulus to improve the matching of glycolytic and mitochondrial fluxes and thus lower the requirement for carnitine availability, it might be speculated that increasing free carnitine availability under these conditions could alter the equilibrium of the CAT reaction. Thus further studies attempting to manipulate the skeletal muscle carnitine pool should also determine skeletal muscle acetyl-CoA and CoASH content. Finally, as previously discussed it is difficult to delineate the independent effects of skeletal muscle carnitine availability and submaximal HIT from the chronic interaction of the two. Therefore, the impact of skeletal muscle carnitine loading on the acute responses to repeated bout exercise in untrained volunteers warrants future investigation.

6.5 Concluding remarks

Over the past two decades, the skeletal muscle carnitine pool has received attention as a dynamic, manipulable component of human skeletal muscle metabolism, being one of the most important buffers of the integration of fat and carbohydrate metabolism, and glycolytic and oxidative ATP production. Recent advances have demonstrated the efficacy of increasing muscle carnitine availability and its utility in elucidating the determinants of exercise performance and metabolism, the implications of which are under current investigation in our lab with respect to ageing, weight loss and type II diabetes. Novel data from the studies in this thesis provide valuable information relating to skeletal muscle carnitine transport that can

assist in the development of more efficacious strategies to accelerate muscle carnitine accumulation and in these investigations. Moreover, these data further advance our knowledge of the interplay between carnitine metabolism, PDC flux and non-mitochondrial ATP production during high-intensity exercise and challenge the current understanding of oxidative ATP delivery during high-intensity repeated bout exercise.

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APPENDIX 1: HUMAN VOLUNTEER DOCUMENTS

Appendix 1.1 Example of a healthy volunteers consent form

***Metabolic Physiology Group,
School of Life Sciences,
University of Nottingham Medical School,
Queen's Medical Centre Nottingham***



The University of
Nottingham

Title of Project: **Effects of six months of L-carnitine and carbohydrate feeding on the adaptations to high-intensity exercise training**

Name of Investigators: **Chris Shannon, Dr Francis Stephens, Professor Paul Greenhaff**

Healthy Volunteer's Consent Form

Please read this form and sign it once the above named or their designated representative, has explained fully the aims and procedures of the study to you

- I voluntarily agree to take part in this study.
- I confirm that I have been given a full explanation by the above named and that I have read and understand the information sheet given to me which is attached.
- I have been given the opportunity to ask questions and discuss the study with one of the above investigators or their deputies on all aspects of the study and have understood the advice and information given as a result.
- I agree to the above investigators contacting my general practitioner [and teaching or university authority if appropriate] to make known my participation in the study where relevant.
- I agree to comply with the reasonable instructions of the supervising investigator and will notify him immediately of any unexpected unusual symptoms or deterioration of health.
- I authorise the investigators to disclose the results of my participation in the study but not my name.
- I understand that information about me recorded during the study will be kept in a secure database. If data is transferred to others it will be made anonymous. Data will be kept for 7 years after the results of this study have been published.
- I authorise the investigators to disclose to me any abnormal test results. (delete this if not applicable)
- I understand that I can ask for further instructions or explanations at any time.
- I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing.
- I confirm that I have disclosed relevant medical information before the study.

- I shall receive an inconvenience allowance of £X per study visit (involving biopsies), plus an additional £X upon completion of the study. If I withdraw from the study for medical reasons not associated with the study a payment will be made to me proportional to the length of the period of participation. If I withdraw for any other reason or fail to complete any part of the study, the payment to be made, if any, shall be at the discretion of the supervising investigator.
- I have not been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance; having an invasive procedure (eg venepuncture >50ml, endoscopy) or exposure to ionising radiation.
- I understand that I should not take part in any other research study during my participation in this study, or within three months of completing this study.

Name:

Address:

Telephone number:

Signature: **Date:**

I confirm that I have fully explained the purpose of the study and what is involved to:

.....

I have given the above named a copy of this form together with the information sheet.

Investigators Signature: **Name:**

Study Volunteer Number:

Appendix 1.2 Example of a healthy volunteer's health questionnaire

**Metabolic Physiology Group,
School of Life Sciences,
University of Nottingham Medical School,
Queen's Medical Centre Nottingham**



Title of Project: **Effects of six months of L-carnitine and carbohydrate feeding on the adaptations to high-intensity exercise training**

Name of Investigators: **Chris Shannon, Dr Francis Stephens, Professor Paul Greenhaff**

Healthy Volunteer's Questionnaire

Please remember, all information will be treated in the strictest of confidence.

NAME _____

HEIGHT _____

DATE OF BIRTH _____

WEIGHT _____

ADDRESS _____

BMI _____

HR LYING _____

PHONE NO _____

HR STANDING _____

EMAIL _____

BP LYING _____

BP STANDING _____

Are you a smoker? **YES / NO**

Are you taking ANY medication? **YES / NO**

If yes, please give details:

Do you or have you ever suffered from any of the following?

Cardiovascular Disease	YES / NO
Metabolic Diseases e.g. Diabetes	YES / NO
Epilepsy	YES / NO
Nervous Disorder	YES / NO
Any other CHORNIC medical condition	YES / NO

If yes to any of the above, please give details:

Have you ever fainted? *E.g. on standing, in warm room, following fasting* **YES / NO**

Roughly, how many units of alcohol do you consume per week? _____ **units**

Roughly, how many cups of coffee, tea, or cola do you drink a day? _____ **units**

Are you, or have you ever been a Vegetarian or Vegan? **YES / NO**

If yes, please give details:

If you are not a Vegetarian or Vegan, how often do you eat meat or fish? ___ **times/week**

Do you eat red meat? *E.g. beef, lamb etc.* **YES / NO**

If yes, how often? _____ **times/week**

Have you ever taken any sports nutritional supplements? **YES / NO**

E.g. creatine, amino acids

If yes, please give details:

How would you assess your present fitness level? **POOR / AVERAGE / HIGH**

How regularly do you take part in physical activity? Please state the type of activity and how often.

Have you taken part, or intend to take part in any other trial within three months

prior to and following this particular study? *E.g. other studies, blood donor* **YES / NO**

If yes, please give details e.g. dates:

Appendix 1.3 Healthy volunteer's DEXA suitability questionnaire

DEXA Questionnaire

Please complete the following questions to the best of your ability. If you have any questions, please ask the DEXA operator for assistance.

- Have you had any investigations involving x-rays within the last week? **YES/NO**
 - If 'YES', please give details.....
.....

- Have you had any investigations involving x-rays within the last month? **YES/NO**
 - If 'YES', please give details.....
.....

- Have you had any investigations involving x-rays within the last year? **YES/NO**
 - If 'YES', please give details.....
.....

- Do you have any of the following medical devices in your body? Please tick any which apply & describe the device in the adjacent space provided.
 - Ostomy devices (e.g. colostomy bag).....
 - Prosthetic devices (e.g. hip or knee replacement).....
 - Surgical devices (e.g. metal clips, pins, plates).....
 - Pacemaker or pacemaker leads.....
 - Radioactive implants (i.e. for treatment of cancer).....
 - Catheters or tubes.....

- Are you exposed to ionising radiation as part of your job? **YES/NO**

- Do your clothes contain any metal such as buttons, zips or clips? **YES/NO**

Any clothes containing metal will need to be removed before you have the DEXA scan. Tops and trousers are available to change into, and lockers are provided.

- Are you wearing any jewellery? **YES/NO**

Any jewellery, including watches and piercings will need to be removed before you have the DEXA scan. If you are unable to remove any items, please inform the DEXA operator.

- Do you have any foreign objects in your body? e.g. shrapnel, buckshot **YES/NO**

I certify that the information given above is, to the best of my knowledge, true and correct.

Name:.....

Signed:
...../...../.....

Date:

Witnessed:

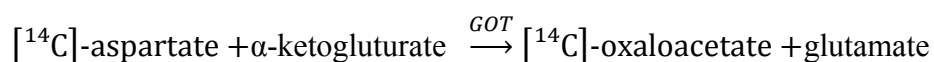
Name:.....

Signed: Date:/...../.....

APPENDIX 2: ANALYTICAL BUFFERS

Appendix 2.1 Preparation of [¹⁴C]-oxaloacetate

Assays of acetylcarnitine and acetyl-CoA (for PDCa) required the fresh synthesis of [¹⁴C]-oxaloacetate by the transamination of [¹⁴C]-aspartic acid (40 μM) with α-ketoglutarate (30 mM) in a 0.5 M HEPES buffer (pH 7.4) containing 11 mM EDTA (Cooper *et al.*, 1986).

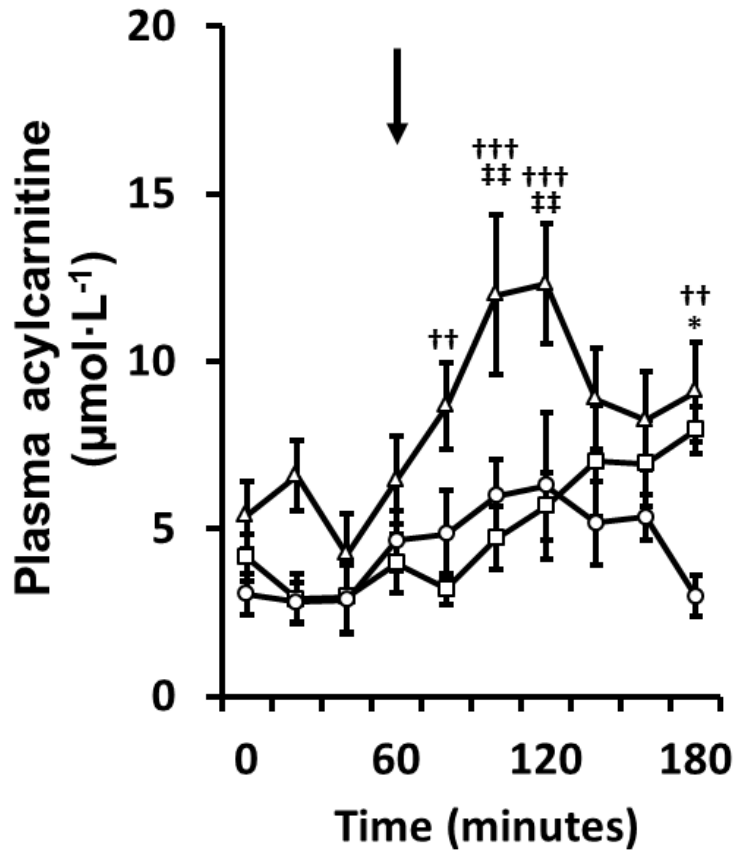


The reaction was initiated by the addition of glutamate-oxaloacetate transaminase and allowed to proceed for 10 minutes at room temperature. The transamination was terminated with 1M PCA, neutralised with 0.6 M KOH and diluted 2.5 times in EDTA (11 mM). This solution was prepared immediately before the citrate synthase condensation reaction step of the acetyl-CoA assay (as **General Methods**).

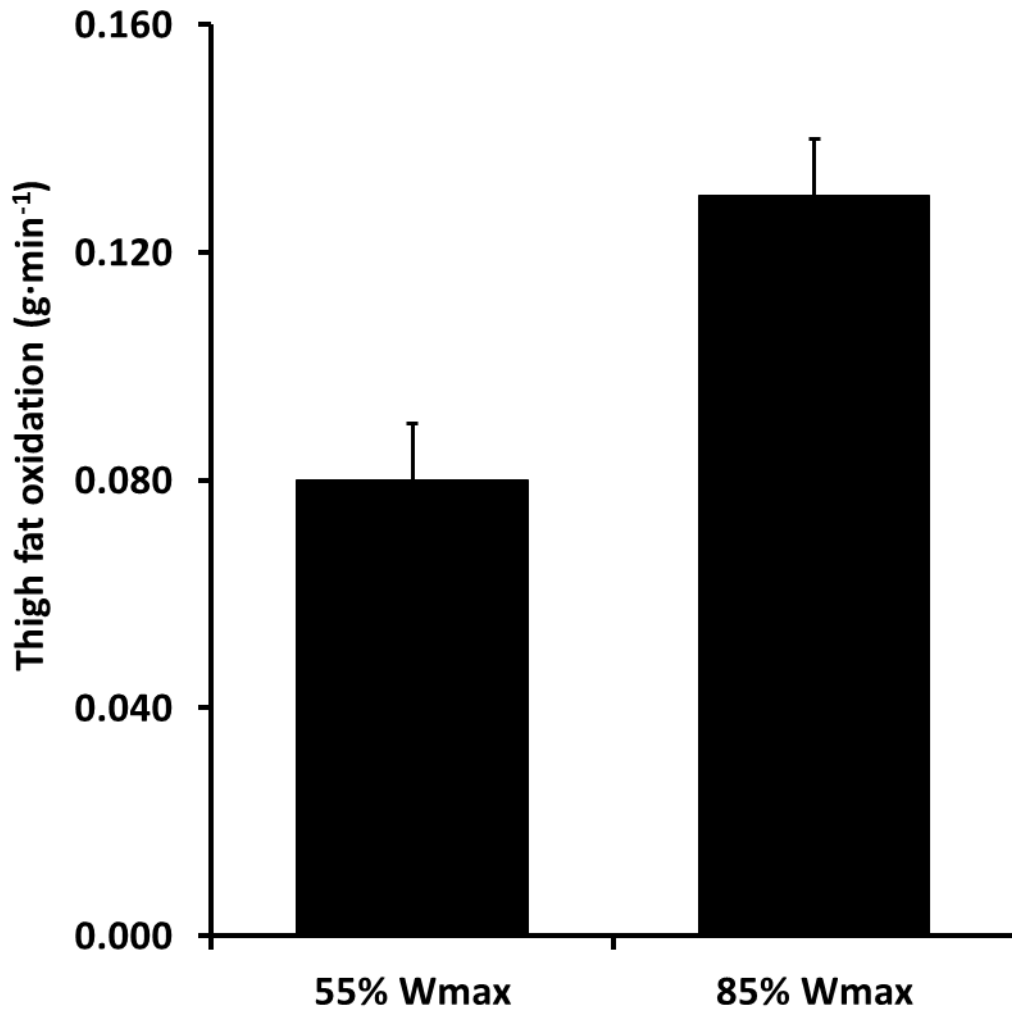
Appendix 2.2 Homogenisation buffer for PDCa extraction

Reagent	Concentration (mM)
Sucrose	200
Potassium chloride	50
Magnesium chloride hexohydrate	5
EGTA	5
Tris	50
Sodium fluoride	50
Dichloroacetate	5
Triton X-100	0.1%

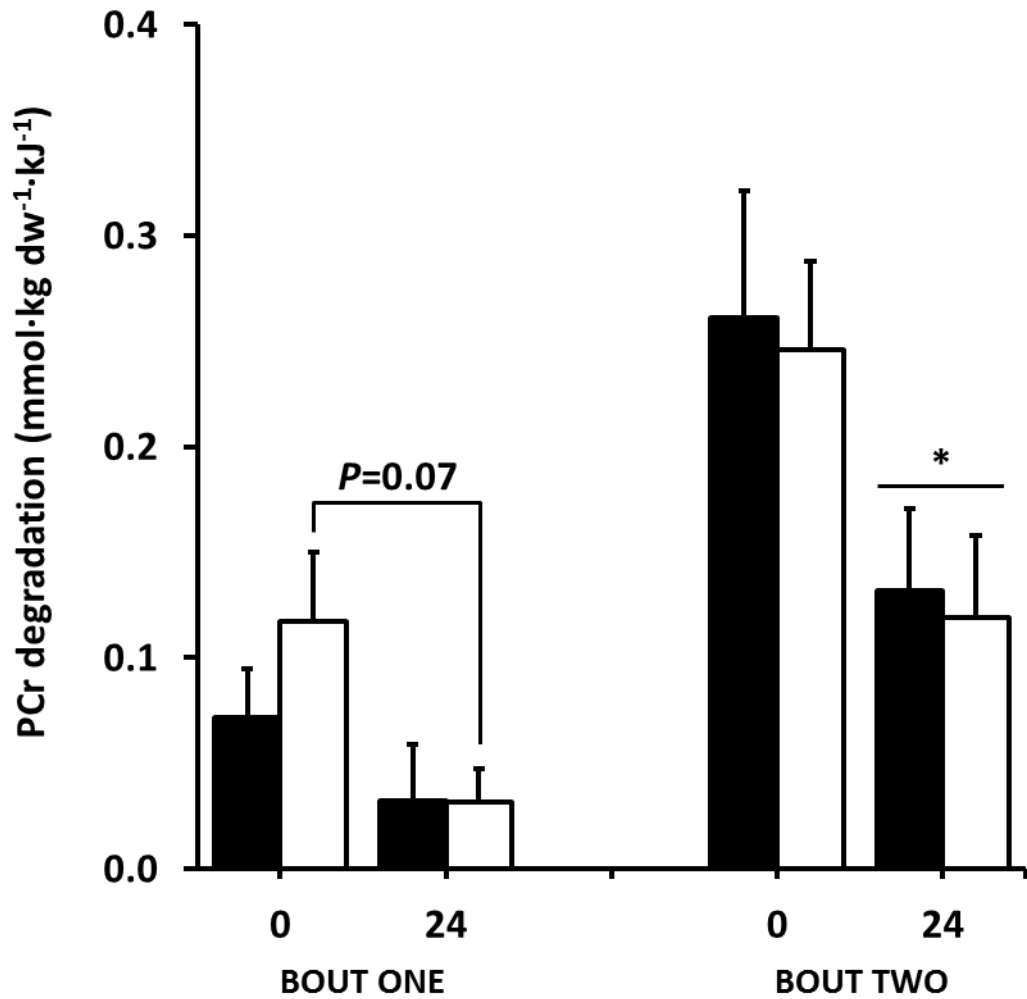
APPENDIX 3: SUPPLEMENTARY FIGURES



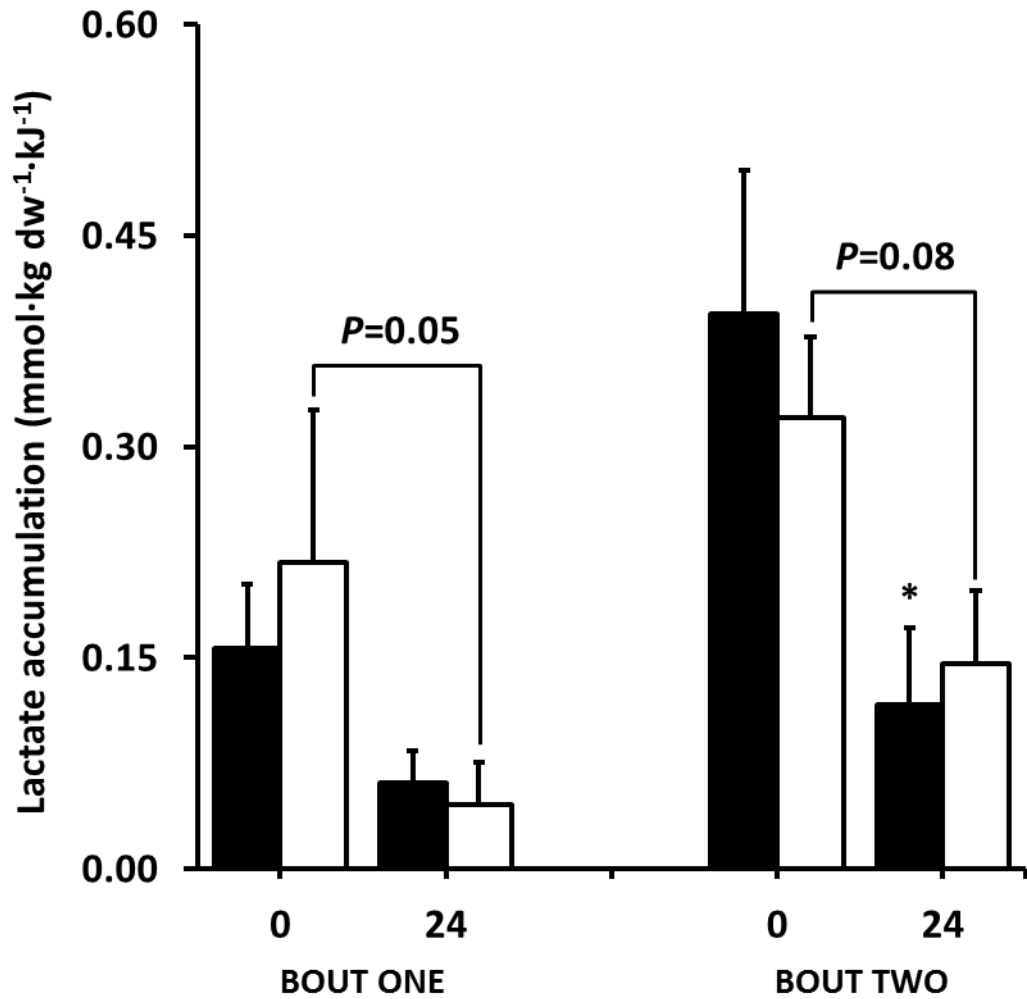
Appendix 3.1 Plasma acylcarnitine concentrations following ingestion of 3 g L-carnitine (t=0) and 1 x 500 ml drink (arrow) of either CON (squares), CHO (circles) or PRO (triangles). Values are mean \pm SE for n= 7. * $P<0.05$ for CHO vs CON; †† $P<0.01$, ††† $P<0.001$ for PRO vs CON; ††† $P<0.01$ for CHO vs PRO. Supplementary data from **Chapter 3**; Study 1B.



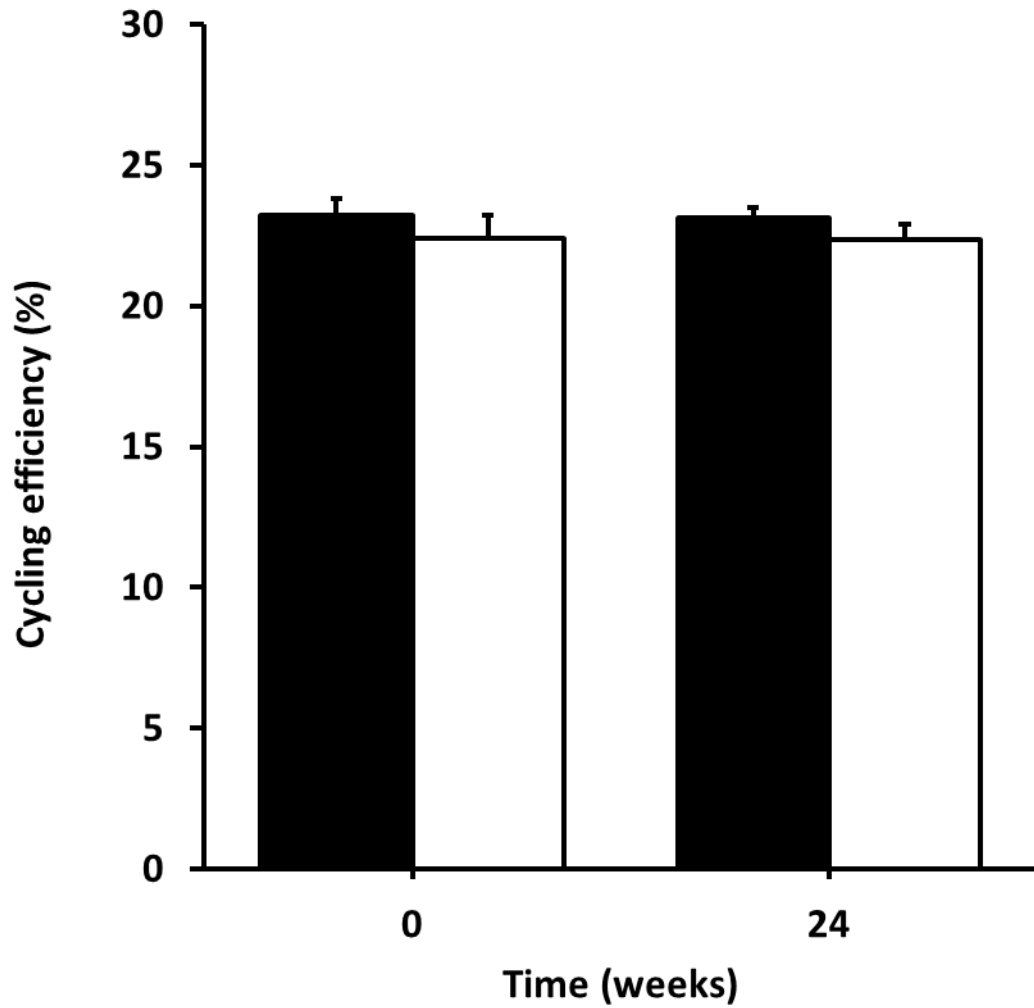
Appendix 3.2 Leg thigh fat oxidation during 30 minutes of single-leg knee extension exercise at 55 and 85% W_{\max} . Supplementary data from **Chapter 4**; Study 2A.



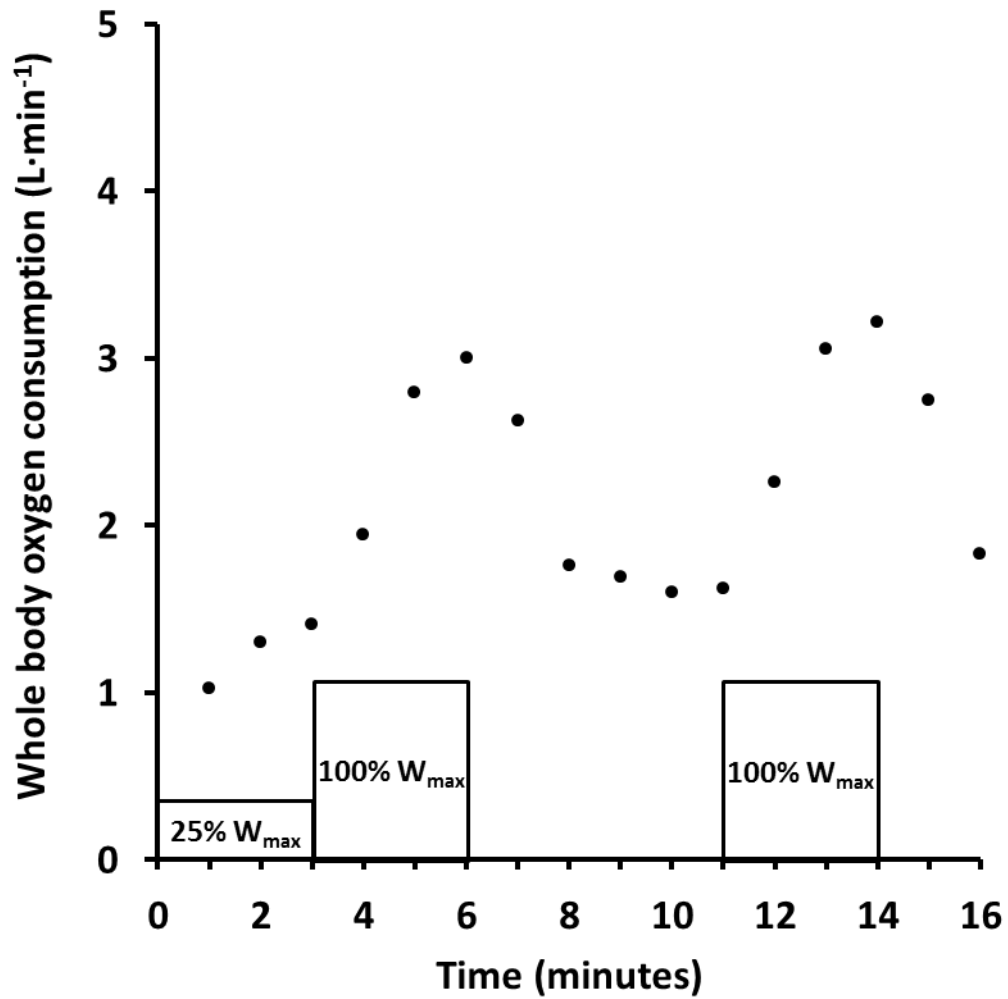
Appendix 3.3 Muscle PCr degradation, corrected for work output, during two 3 minute bouts of exercise at 100% W_{max} , before and after 24 weeks of exercise training receiving twice daily drinks of either 80 g carbohydrate (CON; black bars) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white bars). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*time* x *group*): * P <0.05 vs 0 weeks. Supplementary data from **Chapter 5**; Study 3.



Appendix 3.4 Muscle lactate accumulation, corrected for work output, during two 3 minute bouts of exercise at 100% W_{\max} , before and after 24 weeks of exercise training receiving twice daily drinks of either 80 g carbohydrate (CON; black bars) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white bars). Values are mean \pm SE for 7 subjects. 2-way ANOVA (*time* x *group*): * $P < 0.05$ vs 0 weeks. Supplementary data from **Chapter 5**; Study 3.



Appendix 3.5 Cycling efficiency at W_{\max} , determined as the percentage of VO_2 accounted for by mechanical work (see **General Methods**), at baseline and 24 weeks of twice-daily 80 g carbohydrate (CON; black bars) or 80 g carbohydrate plus 1.5 g L-carnitine tartrate (CARN; white bars) supplementation. Values are mean \pm SE for 7 subjects. Supplementary data from **Chapter 5**; Study 3.



Appendix 3.6 Whole body oxygen uptake during two 3 minute exercise bouts at 100% W_{\max} separated by 5 minutes of passive recovery and preceded by a 3 minute warm-up period at 25% W_{\max} . Boxes indicate timings of exercise periods. Data are minute-averaged values from a single trial. Supplementary data from **Chapter 5; Study 3**.