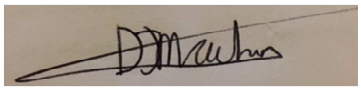
INVESTIGATING THE OPTIMISATION OF THE ERGOGENIC EFFECTS OF L-CARNITINE SUPPLEMENTATION IN HUMANS

Submitted by David James Machin to the University of Exeter as a thesis for the degree of Masters by Research in Sports and Health Sciences

In September 2019

I certify that all the material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

Signature: 

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Abstract

L-carnitine, when consumed alongside high dose oral carbohydrates or infused under insulin clamp conditions increases muscle total carnitine. This is likely via increased insulin augmented Na^+/K^+ -ATPase pump activity via Na^+ dependent OCTN2 carnitine transport. Increased muscle total carnitine is associated with numerous physiological effects including increased fatty acid metabolism and improved exercise time trial performance. However, significant practical and health issues including weight gain exist with the current mechanism of carbohydrate/ insulin augmented carnitine uptake. The purpose of this thesis therefore was to investigate an alternative methodology that could stimulate increased muscle carnitine uptake in humans without the calorific load required via oral carbohydrates. This was investigated by using caffeine to stimulate Na^+/K^+ -ATPase pump activity similarly to that of the previously identified action of insulin. The effects of caffeine ingestion during hypercarnitinemia on Na^+, K^+ and plasma carnitine amongst other measures were investigated. Experimental group participants consumed 9mg/kg/bw caffeine over a period of 5 hours intravenous infusion of L-carnitine (C&C), with carnitine only (CARN) and caffeine only (CAFF) placebo groups also investigated. Combined hypercarnitinemia and caffeine decreased steady state plasma carnitine by 10.2% (~30 μmol.L-1) compared to carnitine infusion alone. Rate of carnitine clearance from plasma increased by 9.2% (C&C 205.1 μmol.L-1 vs 187.9 μmol.L-1 CARN) and rate of tissue uptake also increased proportionately (C&C 36.9 μmol.L-1vs 33.7 μmol.L-1CARN). Caffeine ingestion increased steady state whole blood Na+ (C&C 138.1 mmol/L, CAFF 138.2 mmol/L vs CARN 137.6mmol/L) whilst simultaneously decreasing K+ (C&C 4.1mmol/L, CAFF 4.1mmol/L vs CARN 4.3 mmol/L). Consequently, the changes in carnitine

clearance were likely stimulated by caffeine's actions influencing Na^+/K^+ kinetics, due to increased Na^+/K^+ -ATPase pump activity. Further pilot data appears to indicate that caffeine ingestion acetylated the muscle carnitine pool with free carnitine decreasing between baseline and post infusion (-1.8mmol/kg CAFF vs -0.5mmol/kg CARN) with the caffeine mediated decrease being largely attenuated when caffeine was consumed in a state of hypercarnitinemia (-0.9mmol/kg C&C). After ~14 hours post infusion CAFF continued to acetylate the carnitine pool, whilst CARN was unchanged and C&C returned towards baseline (CAFF -2.3mmol/kg, CARN +0.6mmol/kg, C&C -0.3mmol/kg) with pre-exercise muscle free carnitine obtained the absolute highest value in the C&C group (CARN 10.7mmol/kg, CAFF 10.7mmol/kg vs C&C 12.5mmol/kg). Neither carnitine, caffeine nor a combination of the two appeared to significantly alter any markers of metabolism or exercise performance in the pilot data (n=2) regardless of condition. Collectively these novel findings indicate that it is likely that caffeine is able to augment human skeletal muscle carnitine uptake but may lead to increased acetylation of the muscle carnitine pool. The direct effects of these findings on muscle total carnitine, metabolism and exercise performance are yet to be identified. However, a novel mechanism for increasing plasma carnitine clearance and thus likely increased skeletal muscle carnitine uptake appears to have been discovered.

Key Words: L-carnitine, Plasma carnitine clearance, Caffeine, Na^+/k^+ -ATPase pump, OCTN2

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Contained Within Chapter Three: Experimental Results

Declaration

The material contained within this thesis is original work conducted and written by the author.

Key Thesis Abbreviations

ATP - Adenosine tri phosphate

CAFF - Caffeine only experimental condition

CARN - Carnitine only experimental condition

C&C - Caffeine and carnitine experimental condition

EXE - Exercise

FC - Free carnitine

K^+ - Potassium

Na^+ - Sodium

RER - Respiratory exchange ratio

RMR - Resting metabolic rate

RPM - Revolutions per minute

TC - Total carnitine

$V0^2$ - Oxygen uptake

$V0^2$ Max - Maximum oxygen uptake

Acknowledgements

The completion of this thesis has only been made possible with the assistance, guidance and support of a number of people.

Firstly, I would like to thank my supervisor team, Professor Francis Stephens and Dr Benjamin Wall. Your dedication and passion is inspiring, and without your help, guidance and faith in me throughout my programme this thesis would not have been possible. I would also like to thank Dr Marlou Dirks for her time and assistance throughout my research.

Secondly, I would like to acknowledge the hard work and contributions made by Alistair Monteyne, Mariana Coelho, Andrew Davenport, George Pavis, Sean Kilroe and Tom Jameson whose time and efforts towards this research have been invaluable.

I am extremely thankful to the technical teams whose advice, assistance and support enabled the completion of my research. A special thanks to Jamie Blackwell, Sam Bailey, Luke Connolly and Garry Massey.

I would also like to thank my fellow postgraduate research students for creating a fantastic working environment and providing support, encouragement and opportunities to broaden my expertise.

Finally, I would like to thank my family for believing in me throughout the process and for all the support you have provided for me throughout my education.

Chapter One: Introduction and Literature Review

1.0 Introduction to Literature Review

Since its discovery in 1905 the importance of carnitine in a number of key metabolic functions including translocation of long chain fatty acids into the mitochondrial matrix for β- oxidation, and in the regulation of the mitochondrial acetyl- CoA/CoASH ratio has been established. A mechanism to enable manipulation of the human skeletal muscle carnitine pool has since been discovered whereby using carbohydrates the carnitine pool can be increased, leading to improvements in exercise performance and metabolic changes. This mechanism, however, is not only impractical but due to the large volume of required carbohydrates is not without long term risks including weight gain and blunted insulin sensitivity. This thesis seeks to explore an alternative methodology that will enable safe and convenient increases in skeletal muscle carnitine. This introductory chapter briefly explores the history and metabolic roles of carnitine, the mechanisms behind muscle carnitine transport before finally exploring a number of potential avenues to augment skeletal muscle carnitine accumulation including our working hypothesis for this thesis.

1.1 L-carnitine: History and Structure

Carnitine is a water-soluble quaternary amine (Tomita and Sendju, 1927) classified as 3-hydroxy-4-N,N,N-trimethylaminobutyric acid. Carnitine was first discovered over 100 years ago in meat extract (Gulewitsch and Krimberg, 1905) during the investigation of constituents of certain meats. Despite its early discovery the physiological importance of carnitine only began to surface during the late 1940's when Fraenkel and Blewett (1947) noted that the Tenebrio Molitor (meal worm beetle larvae) required a charcoal filtrate for survival. The

charcoal filtrate, which was generated from liver or yeast extract, was named vitamin B_T. Following on from this work, Carter et al (1952) discovered that vitamin B_T was identical to the previously discovered carnitine amine and identified it as a vital growth factor for the Tenebrio Molitor beetles. Although in these early works it was initially identified as a vitamin, due to the conditionally essential nature of carnitine it has since been reclassified as a vitamin- like nutrient (Zurbriggen, 2000) similar to that of choline and taurine. Further research has characterised specific roles of carnitine in energy metabolism where it is both an essential cofactor for mitochondrial fatty acid translocation and a cellular buffer for maintenance of the mitochondrial acetyl coenzyme A to coenzyme A ratio (the latter function is particularly pertinent high intensity during exercise). Both functions will be discussed in more detail in later sub-chapters.

In humans, carnitine is synthesised from the amino acids lysine and methionine. A peptide-bound lysine is converted into e-N-trimethyllysine via donation of S-adenosylmethionine. E-N-trimethyllysine is then hydroxylated into Beta-hydroxy-e-N-trimethyllysine via the aldolase enzyme which is then converted into y trimethylaminobutyraldehyde where it loses a glycine molecule passively (Bremer, 1983). A final dehydrogenation process into Gamma Butyrobetaine leads to a hydroxylation process forming L-carnitine. This L-carnitine can then be acetylated to form acetyl- L-carnitine (Bach, 1982). This multistage process relies upon the availability of vitamins C, B6 and riboflavin to function (Borum, 1983). Whilst this process predominantly takes place within the liver, the brain and kidneys are also responsible for a small portion of synthesis. As both carnitines' key functions relate to muscle metabolism, it is pertinent that 95% of the body's carnitine is stored within the skeletal muscles (Brass, 1995),

meaning it is available to act directly within the skeletal muscle system. Carnitine homeostasis is maintained via endogenous carnitine synthesise in combination with an adequate dietary carnitine intake. Adults on average obtain 75% of carnitine through dietary intake and only 25% from the aforementioned endogenous biosynthesis (Vaz and Wanders, 2002). The average carnitine requirements for adults are estimated at 5μmol/day/kg of body weight (0.8mg/day/kg) (Vaz and Wanders, 2002) and the oral bioavailability of carnitine is in the range of 57-84%. Interestingly the bioavailability of an oral carnitine supplement is significantly lower, ranging from 14-18% (Rebouche, 2004). Consequently, whilst this initially may indicate that smaller dietary dosages are more efficiently absorbed it is likely that bioavailability of large doses is lower due to saturation of absorption at far lower doses than supplemented.

L-carnitine predominantly occurs naturally in animal tissue and milk however small amounts can be found in vegetables, grains and fruits (Steiber, 2004). As carnitine is almost exclusively obtained via meat and dairy products, vegan and vegetarian diets generally tend to be low in carnitine. Plasma and urinary carnitine in an adult population is significantly lower in non-meat eaters than meat eaters (Lombard et al, 1989) and vegetarians have also been shown to have a reduced skeletal muscle carnitine transport capacity (Stephens et al, 2011). Non-meat eaters, individuals with reduced OCTN2 levels and those with a specific carnitine deficiency may all benefit from increased carnitine supplementation for health and body functionality reasons as it appears carnitine supplementation alone may increase muscle carnitine in individuals with physiologically low muscle total carnitine (Magoulas and Hattab, 2012).

1.1.1 The role of carnitine within the human body

Fatty acids can be defined as a family of lipid macronutrient class molecules consisting of essential or non-essential fatty acids (Kaur et al, 2014) and formulated with a carboxylic acid and long aliphatic chain. Fatty acid metabolism is crucial in many aspects of homeostasis including the critical maintenance of body functionality. This is especially relevant in cardiac muscle which due to a very high energy demand obtains 50-70% of its ATP from fatty acid β-oxidation (Lopaschuk et al, 2010). Fatty acids also provide an additional source of energy to cover the energy deficiency created by the increased demand for ATP during exercise. As the role fatty acid metabolism plays in exercise performance is widely known, the potential mechanisms to optimise the usage of fatty acids for energy during exercise have been researched in great depth. This includes research into the hypothetical low intensity 'fat burning zone' style exercise where fatty acid metabolism is the dominating source of energy, particularly during exercise lasting 90 minutes or longer (Holloszy et al, 1998). During low intensity exercise glycogen breakdown is not overly stimulated, thus fatty acid metabolism provides the dominating energy source (Wolfe, 1998) preserving glucose for increased energy demand. The remaining energy deficit is therefore filled via glucose metabolism (Van Hall et al, 2002) with a constant state of flux influencing the selection ratio between substrates, which alters depending on exercise intensity amongst other factors. Carnitine has multiple functional roles within cellular metabolism with the primary role being the translocation of fatty acyl- CoAs into the mitochondrial matrix. This is of significant importance due to the impermeable nature of the mitochondrial membrane to direct fatty acyl- CoA transport (Houten and Wanders, 2010). This means fatty acyl- CoAs require conversion into acyl-

carnitine before being able to enter the mitochondrial matrix. Without carnitine the body is unable to sufficiently metabolise fat as is witnessed in individuals with severe carnitine deficiency (Engel and Angelini, 1973).

In its most basic, form reactions between carnitine and acyl CoA are catalysed by the outer mitochondrial enzyme CPT 1 (an 88kDa enzyme) (Murthy and Pande, 1987) forming acyl carnitine which is then translocated across the mitochondrial membrane (reaction 1). An initial reversible esterification between Acyl- CoA and free carnitine forms acylcarnitine and CoASH within the cytosol (Stephens, 2007). This long chain acylcarnitine is then transported via the 32 kDa enzyme CACT (located within the mitochondrial membrane) (Pande, 1975) into the mitochondria at a 1:1 exchange ratio with mitochondrial free carnitine. Once within the mitochondrial matrix the long chain acylcarnitine is then trans-esterified into acyl- CoA and free carnitine (a reversal of the initial reaction) via CPT2 (71kDa) (Woeltje et al, 1991) (reaction 2). The free carnitine produced from this reaction becomes available for CACT mediated transport back through the mitochondrial membrane into the cytosol, where it is then available for the original reaction. The long chain acyl- CoA within the mitochondria enters the beta oxidation pathway forming acetyl-CoA and entering the TCA cycle for fatty acid metabolism (Fernie et al, 2004).

CPT1

(1) Carnitine + Acyl-CoA $\longleftrightarrow$ Acylcarnitine + CoASH

CPT2

(2) Acylcarnitine + CoASH $\longrightarrow$ Acyl- CoA + Carnitine

A second crucial role of carnitine is as a buffer for excess acetyl groups. As previously stated, fatty acids and glucose compete for production of ATP (whilst fatty acids have a greater capacity to produce ATP, the process requires more oxygen thus is rated less efficient (Fernie et al, 2004)). This relationship relies on the Randle cycle (Randle et al, 1963) whereby fatty acid oxidation inhibits glucose oxidation by regulation of the TCA cycle from acetyl Co A production via fatty acid beta-oxidation. Furthermore, PDC is inhibited directly via increases in the acetyl CoA/ CoA ratio. By providing a buffer for the increases in acetyl groups carnitine may prolong the efficacy of this reaction.

This role of carnitine was first identified in blowfly muscle (Childress and Sacktor, 1966) where during the first stage of flight acetyl- CoA was produced above that of its utilisation within the TCA cycle at a proportionate increased rate to that of acetylcarnitine, generating the hypothesis that through CAT acetylcarnitine was formed which maintained a viable supply of mitochondrial CoASH. Further studies looking at carnitine within rats noted that the acetylation state of carnitine and CoA within rat hearts perfused with palmitate suggested the CAT reactants remained near equilibrium despite large steady state variations (unlike during the citrate synthase reaction). It was therefore hypothesised that the CAT ‘system’ acted as a buffer of acetyl- CoA against the rapid changes that were occurring (Pearson and Tubbs, 1967). Final confirmation of this theory is demonstrated in the relationship identified between tissue metabolic activity and CAT activity (Snoswell and Koundakjian, 1972). Consequently, it is now universally accepted that CAT plays a key role in modulating the mitochondrial acetyl CoA/ CoA ratio.

Changes in the ratio of free carnitine (decrease) to acetylcarnitine (increase) during intense exercise (Harris and Foster, 1990) further demonstrates the

capacity for carnitine to function as an acetyl acceptor during exercise. Carlin et al (1990) demonstrated these ratio changes took place during high intensity exercise in horses. Whilst the exercise did not alter total carnitine, it resulted in a significant change in carnitine ratios with a decrease in free carnitine post exercise mirrored with an equivalent rise in acetylcarnitine, providing a 'real world' example of the role of carnitine in regulating the acetyl- CoA/ CoA ratio during exercise. This mechanism of action delays the inhibition of the PDC and TCA cycles by maintaining a readily available supply of CoASH. Constantin-Teodosiu et al (1991) calculated that during vigorous exercise, assuming maximal PDC activity the body's stored supply of CoASH would become acetylated within one second. The effectiveness and rapidity of the CAT reaction for the purpose of maintaining PDC activity prevents this occurring.

Whilst this thesis predominantly focuses on the role of carnitine within muscle tissue it is likely that further roles of carnitine exist within the body. The enhanced effectiveness of which are likely linked directly to carnitine supplementation as opposed to standard biochemical mechanisms utilising the body's stored carnitine pool and appear to augment markers of health in individuals. There is potential that these benefits may be enhanced during an optimal carnitine supplementation strategy therefore the benefits of discovering a model to increase carnitine uptake within the body may also be pertinent for health and wellbeing. Whether these roles are directly or indirectly related to carnitines muscular function is debated, however it is not unreasonable to suggest that increasing the body's stored carnitine pool may well be beneficial. L-carnitine supplementation appears to improve recovery and survival after a myocardial infarction (Corbucci and Loche 1993) and although this is likely linked to the buffering effects discussed above, alternative protective roles of

carnitine may exist. Alongside other cardiac benefits, carnitine supplementation also improves metabolic profiles within an ischemic heart and improves exercise tolerance and ventricular function in angina patients (Canale et al, 1988; Kamikawa et al, 1984). There may also be a role for carnitine within the field of insulin resistance. Obese patients with insulin resistance appear to have reduced CACT expression (Peluso et al, 2002) which supports the theory that decreased mitochondrial uptake results in lipotoxicity (thought to be one primary process leading to insulin resistance (Morino et al, 2006)). Leading on from this, Kilici et al (2010) demonstrated diminished plasma carnitine levels in diabetic patients with Tamamogullari et al (1999) showing approximately 25% lower carnitine concentrations in type 2 diabetes patients compared to control. This lower carnitine may be due to reduced mRNA of carnitine biosynthetic enzymes (Noland et al, 2009) amongst other reasons. Therefore, carnitine deficiency alongside decreased related enzymatic activity may lead to a negative spiralling effect strongly linked to causality or deterioration of these conditions. By finding an efficient mechanism to increase the body's stored carnitine we can hypothesis that this could lead to improvements in health and wellbeing in a number of conditions.

Due to the roles of carnitine discussed in previous sub chapters we can conclude that carnitine plays an important role in both health and exercise and therefore strategies to optimise the ergogenic effects of carnitine in both infirm and athletic populations warrant further research.

1.2 Skeletal Muscle Carnitine Transport

Due to the critical role carnitine plays in both fatty acid translocation and acetyl group buffering, a significant amount of research has investigated carnitine

supplementation. Multiple studies have investigated low and high dosages (up to 6g per day) over differing time periods (up to 3 months) with the vast majority failing to show improvements in exercise performance or cause any changes to metabolic state (Broad et al, 2005; Barnett et al, 1994; Wachter et al, 2002). It was hypothesised that carnitine failed to alter metabolism as a result of unchanged skeletal muscular carnitine availability, rather than the ineffectiveness of the carnitine supplement per se. Vukovich et al (1994) supplemented 4g/day for 14 days finding no improvements in performance or markers of lactate accumulation and was the first crucial study to note that muscle total carnitine did not increase throughout the supplementation period, concluding that as there was no increased carnitine availability no effects were seen. It therefore stands to reason that simply increasing plasma carnitine via carnitine supplementation alone is not sufficient to increase skeletal muscle carnitine which appears to be necessary for any significant metabolic effects to occur. This finding, combined with the assumption that free carnitine availability is rate limiting to skeletal muscle fat oxidation during exercise, has led to significant research searching for an effective mechanism that could increase skeletal muscle carnitine accumulation to improve exercise performance.

Carnitine is actively transported into skeletal muscle through the plasma membrane (Rebouche, 1977) via the Na+ dependent skeletal muscle carnitine transporter OCTN2 against a concentration gradient of greater than 100-fold (Wachter et al, 2002), with skeletal muscle carnitine concentration significantly higher than in the plasma (Geoges et al, 2000). This process is saturable, with fasted plasma carnitine concentration approximately 50 μmol.1-1 when compared to the in vitro Km of OCTN2 of 4.3 μmol.1-1 (Rebouche et al, 1977). As discussed previously, this reaffirms that carnitine availability itself is not a

rate-limiting step to carnitine transport. Transport of carnitine via the OCTN2 shuttle is thought to be the predominant, if not exclusive, method of skeletal muscle carnitine uptake. This theory is supported by previous OCTN2 transport inhibition studies which have blocked OCTN2 via mildronate (3-92,2,2-trimethylhydrazinium-propionate), a gamma-butyrobetaine hydroxylase inhibitor known to reduce Na^+/K^+ -ATPase activity. Blocking Na^+/K^+ -ATPase pump activity reduced the functionality of Na^+ dependent OCTN2 carnitine transporter and consequently inhibited skeletal muscle carnitine uptake (Georges et al, 2000). Ouabain (another Na^+/K^+ -ATPase pump inhibitor) has also been demonstrated to inhibit carnitine transport in vitro further reinforcing this hypothesis (Rebouche, 1977). Despite this it is worth noting that carnitine may be able to passively diffuse down a concentration gradient (Stanley et al, 1987), however this has only been noted in cases where skeletal muscle carnitine levels appear significantly lower than plasma carnitine (Treem et al, 1988). Therefore, this process appears redundant in individuals with 'normal' levels of carnitine and whilst some suggestion of the existence of a low affinity antiport system exist this appears to only become relevant in patients with extreme carnitine deficiency and for all intents and purposes we believe OCTN2 transport remains the primary mechanism behind skeletal muscle carnitine uptake.

1.2.1 Current mechanism of augmenting carnitine uptake

Stephens et al (2006) proposed that a state of hyperinsulinemia combined with hypercarnitinemia may augment skeletal muscle carnitine uptake via insulins potential action in increasing Na^+/ K^+ -ATPase pump activity (Clauson, 2003). Insulin can augment sensitivity of the Na^+/ K^+ -ATPase pump to Na^+ thus may augment Na^+ coupled carnitine transport secondary to stimulating intracellular

Na^+ flux. Insulin purportedly increases Na^+/ K^+ -ATPase pump activity via increased translocation of α2 and β1 pump subunits from intracellular storage into the plasma membrane (Sweeny and Klip, 1998). This theoretical mechanism is supported by previous work which has shown Na^+ dependent skeletal muscle uptake of amino acids and other nutrients are augmented via insulin (Zorzano et al, 2000) implying augmentation of Na^+ dependent transport of carnitine may similarly be increased. Carnitine- Na^+ cotransport via OCTN2 is at a 1:1 ratio (Taamai et al, 2001) therefore lowering intracellular Na^+ concentration would increase the electrochemical gradient for Na^+ thus further driving carnitine transport. Muscle carnitine can be measured in three ways; firstly, measuring muscle total carnitine via biopsy techniques gives a direct measure of muscle carnitine content, despite being the gold standard this mechanism is invasive. A second reliable measure of muscle carnitine is examining plasma carnitine concentration as a marker of carnitine change within muscles. A decreased plasma carnitine concentration during hypercarnitinemia indicates increased muscle carnitine uptake, which can be supported by calculating the rate of plasma carnitine clearance and muscle tissue uptake. Finally, magnetic resonance imaging (MRI) can also be used to provide an indication of muscle carnitine levels and its effects on muscle metabolism (Taylor et al, 2004). This is the least used methodology within literature to date.

Whilst previous research showed states of high plasma hypercarnitinemia (~550 μmol.L) alone was unable to increase muscle total carnitine (supporting the previously discussed theory that exclusively increasing plasma carnitine alone is insufficient stimulus to increase muscle carnitine). When combined with hyperinsulinemia (~150μmol.L) this led to significantly increased muscle total

carnitine from 22.0 mmol/Kg to 24.7 mmol/Kg (a 13% increase) and demonstrated a 2.3-fold increase in OCTN2 expression (Stephens et al, 2006a: Stephens et al, 2006b). Plasma total carnitine concentration was also significantly lower during hyperinsulinemia, this is a significant finding as it indicates that a state of hyperinsulinemia had increased plasma carnitine clearance and therefore carnitine uptake rate into the muscle tissues. This combined with an unchanged urinary total carnitine and the 13% increase in muscle carnitine data, resulted in Stephens et al concluding that insulin significantly stimulates skeletal muscle total carnitine accumulation during hypercarnitinemia.

After this ground-breaking work, further studies quickly followed exploring numerous avenues related to insulin augmented carnitine uptake (Stephens et al, 2007a, Stephens et al, 2007b, Stephens et al, 2007c). Stephens et al examined varying concentrations of hyperinsulinemia (5, 30, 55 and 105mIU.m^{-2}.min^{-1}) under hypercarnitinemia conditions whereby a significant effect of insulin on plasma TC was only identified in the 55 and 105 μm01.min-1 insulin groups confirming that a threshold existed for the stimulatory effect of insulin and that only a high circulating serum insulin (>55mU.L^{-1}) is capable of stimulating skeletal muscle carnitine accumulation. This was supported by an increased calculated plasma carnitine clearance (170ml.min^{-1}) and tissue uptake rate (33μmol.min^{-1}) which further demonstrated that high circulating insulin increased carnitine clearance from the plasma. This increase above that of the renal clearance (100 ml.min^{-1}) provides an estimated skeletal muscle total carnitine increase of 10% which is directly comparable to the 13% increase demonstrated by measuring skeletal muscle total carnitine via biopsy. These robust proof of concept research papers evidenced that skeletal muscle

carnitine can be increased by approximately 15% and enabled further work (Stephens et al, 2007b) to examine whether this increase in skeletal muscle carnitine altered fat and carbohydrate oxidation. A 30% decrease in muscle PDC activity and a 40% decrease in muscle lactate were noted at rest implying that glycolytic flux and carbohydrate oxidation were inhibited, most likely due to carnitine mediated increases in fat oxidation. Whilst all studies had taken place under carnitine infusion and insulin clamp conditions, a third study in 2007 by Stephens et al investigated whether oral doses of 3g/day L-carnitine combined with 4*94g carbohydrate/day over a single day would be sufficient to augment carnitine uptake. As in hypercarnitinemia infusion studies, plasma TC concentration was lower than control in the carbohydrate group and when extended to 14 days (3g/day L-Carnitine combined with 2 *94g carbohydrate/day) urinary TC during the 14 day visits remained lower than a carnitine only control group suggesting in both cases that oral carbohydrates augment skeletal muscle carnitine uptake (decreased plasma carnitine combined with decreased urinary carnitine indicates the ‘missing’ carnitine has been accumulated within skeletal muscle tissue). The calculated effect size of carnitine accumulation from this study appeared to be a daily increase of ~0.1%. Subsequently, assuming a cumulative increase, to obtain similar increases to that obtained under insulin clamp conditions a 150-day protocol of oral carbohydrates combined with carnitine would be required.

With this knowledge, Wall et al (2011) undertook the most significant oral carnitine study to date, a chronic oral L-carnitine feeding study (4g carnitine & 160g carbohydrates a day) for 168 days. This demonstrated a 21% increase in muscle total carnitine supporting the previous work from Stephens et al and was the first study to conclusively show muscle carnitine can be increased

chronically and substantially by dietary means. This study also examined the effects of increased muscle carnitine on exercise performance and metabolism. At workloads eliciting 50% $V0^2$ max the increased muscle carnitine group used 55% less muscle glycogen and had a 31% lower PDC activation whereas at 80% $V0^2$ max muscle PDC activation was 38% higher and acetyl carnitine content was 16% greater and participants impressively demonstrated an 11% increase in workload compared to baseline during a performance trial. It was hypothesised that increased muscle carnitine leads to glycogen sparing during low intensity, which is then available in later stages of exercise and provides further evidence of the buffering capacity of L-carnitine as discussed in the previous sub-chapter (1.1.2). These findings were demonstrated after a chronic feeding study and it is not known if these findings would apply in an acute environment or during periods of fasting as opposed to carbohydrate loaded. It is also as yet unknown if the effectiveness during exercise was due to any interaction effects between the carnitine and carbohydrates or whether increased carnitine, augmented via a different mechanism, also would elucidate these changes.

Whilst it has now been demonstrated that muscle total carnitine can be increased through oral supplementation and that, once increased, it can significantly alter metabolism and improve exercise performance, the mechanisms required (either insulin clamps or 160g carbohydrates for 168 days) are either impractical or bring their own significant health risks, particularly if applied to an infirm population (including patients with diabetes and those at risk of obesity or in groups with already blunted insulin sensitivity). As a result of this options to increase muscle carnitine that reduce the calorific load have been considered.

One popular theory is that utilising a carbohydrate/ protein blend may provide a suitably insulinogenic oral formulation which would be sufficient to stimulate muscle carnitine uptake (whey protein and carbohydrate combination has been previously used to increase insulin mediated muscle creatine uptake (Steenge et al, 2000)) so it stands to reason similar uptake will be demonstrated in carnitine. Shannon et al (2016) compared serum insulin, forearm carnitine balance and carnitine disappearance acutely between a 40g protein/ carbohydrate blend and 80g carbohydrates alone (both groups supplementing with 3g oral L-carnitine). This study found that the carbohydrate/ protein beverage acutely blunted the insulin mediated increase in forearm carnitine balance thus suggesting this would not be a suitable solution. This may be due to excessive amino acid oxidation and incomplete β oxidation increasing plasma acyl carnitines (Stephens et al, 2014) which in turn may cause limited inhibition of OCTN2 thus reducing carnitine transport. A second possible mechanism for the lack of effect may be related to the large protein bolus impairing insulin stimulated mechanisms. Glucose disposal has been shown to be blunted with a large protein bolus (Smith et al, 2014) thus a similar effect on inhibiting insulin mediated muscle carnitine uptake may have taken place. Furthermore, high Na^+ dependent amino acid flux could potentially limit muscle carnitine transport (Chen and Russell, 1989; White et al, 1982). Despite this, there may still be some promise in this mechanistic design, as the majority of dietary carnitine is obtained through consumption of meat, it is unlikely to be protein per se that is attenuating carnitine uptake. This study used a higher dose than would be expected for a normal meal, therefore altering the carbohydrate/ protein ratio may still lead to some positive effects on skeletal muscle carnitine accumulation with a slightly reduced calorific load than carbohydrate alone.

Other options to safely and effectively increase skeletal muscle carnitine accumulation may lie in finding an alternative methodology to stimulate Na^+ dependent transport. Na^+/ K^+ -ATPase pump activity (thus Na^+ flux) is increased (potentially maximally in working muscles) via exercise (Clausen et al, 2013) therefore optimally timed carnitine ingestion around exercise bouts may be sufficient (especially when combined with carbohydrates) to stimulate increased carnitine transport. Broderick et al (2017) noted that in mice acute exercise stimulated carnitine biosynthesis and increased OCTN2 expression adding further weight to this theory. However, this mechanism would only be relevant in athletes and we are seeking to identify a strategy that may also be useful for both the general and infirm population. Another potential mechanism to increase Na^+/ K^+ -ATPase activity appears to be increasing certain AMPK activators (Benziane et al, 2012), one such activator being caffeine. This may act via a similar mechanism to insulin however without the large calorific load required by carbohydrate ingestion. Although the consensus of this mechanism is currently divided with certain AMPK factors appearing to actually blunt carnitine uptake in C2C12 skeletal muscle myotubes (Shaw et al, 2017). This interesting study found a wide range of AMPK factors actually inhibited L-carnitine uptake. Inhibition of carnitine was not dantrolene sensitive (dantrolene inhibited caffeine augmented calcium release but did not alter carnitine inhibition status) and caffeine appeared not to competitively inhibit carnitine transport. Carnitine uptake was partially restored via compound C (an AMPK inhibitor). This suggests that an AMPK or PKA related factor may be responsible for inhibiting L-carnitine uptake. Whilst this raises doubts on our theory, until fully investigated in vivo we cannot confirm this hypothesis as there may be significant differences between in vitro and in vivo results. We will

examine the mechanistic probability of success in vivo below. This thesis will seek to find an alternative methodology to increase plasma carnitine clearance via utilisation of the AMPK activators pathway. We will specifically focus on caffeine, a widely used, safe drug.

1.3 Caffeine

1.3.1 Structure of caffeine

Caffeine is widely consumed globally with 92-97% of North Americans estimated to consume some form of caffeine (Heishman and Henningfield, 1992) whilst in the UK a relatively large daily dose of 359-621mg appears to be the norm (Bruce and Lader, 1986). Due to the difference in caffeine quantity between various sources, there is of course wide variability in daily consumption, however it is clear that caffeine usage is extremely prevalent and generally considered safe.

Caffeine (1,3,7-trimethylxanthine) is a xanthine compound with methyl (CH3) groups at 1,3 and 7 carbons (Gummadi et al, 2012). The loss of a single methyl group results in conversion to paraxanthine (3rd carbon demethylation), theobromine (1st carbon) and theophylline (7th carbon) with full demethylation resulting in xanthine (Gummadi et al, 2011). 84% of caffeine is directly metabolised into paraxanthine (Cappelletti et al, 2015) via the 450 enzyme system (CYP1 A1/2), 10% can then be further metabolised into 1 methylxanthine with the remaining 90% converted to 1,7-dimethylurate via CYP2A6 (Caubet et al, 2002). There is also limited direct metabolisation into theobromine and theophylline (Miners and Birkett, 1986). Metabolisation via CYP1A appears to be the rate limiting step of plasma clearance potentially

accounting for 40% of individual variance in caffeine kinetics (Kalow and Tang, 1991).

The oral bioavailability of caffeine is exceptionally high with intestinal uptake around 99% (Blanchard and Sawers, 1983) with peak absorption time appearing to be approximately 45 minutes post ingestion (Magkos and Kavouras, 2005). This translates to peak plasma caffeine values within 30-60 minutes which appear to vary widely based on individual physiology and method of caffeine consumption (Bonati et al, 1982) with the half-life of caffeine also widely variable between individuals suggested to be between 2.7- 9.9 hours.

Caffeine is known to have many physiological and neurological effects on the body with noted improvements in anaerobic running capacity (Bishop and fletcher, 2010), power output (Astirino et al, 2008) and aerobic exercise (Desbriw et al, 2012) to name but a few. Caffeine also appears to have a significant influence on plasma electrolytes (Geethavani et al, 2014) amongst other mechanistic actions.

Caffeine is generally identified as a safe drug with a high toxic level around the range of serum 200uM (Fredholm, 1985). Assuming 1mg/kg equates to 5-10uM then a toxic dose would be around 30mg/kg (Carrilo and Benitez, 2000). Single bolus dosages between 3-9mg/kg are generally seen to be safe and are recommended for optimal sporting performance (Pickering and Kiely, 2018) although certain side effects (including nausea and headaches) can begin to appear from 6mg/kg depending on individual differences and tolerance.

1.3.2 The potential role of caffeine in stimulating skeletal muscle carnitine accumulation

Summarising previous work from Stephens et al (2006, 2007) and Wall et al (2011) as discussed in previous sub- chapters we can conclude that it has been robustly shown that skeletal muscle carnitine can be increased orally and that once increased it can alter metabolism and improve endurance exercise performance.

Caffeine appears to actively stimulate the Na^+/ K^+ -ATPase pump thus may mediate increased Na^+ flux hence leading to increased carnitine transport via similar mechanisms to insulin. Caffeine is thought to stimulate the Na^+/ K^+ -ATPase pump via a number of pathways; firstly, via a direct stimulating effect of caffeine on Na^+/ K^+ -ATPase pump activity. Rat extensor digitorum longus muscle when exposed in vitro to 1mM caffeine demonstrated a stimulatory effect to Na^+ /K^+ transport (Rogus et al, 1977). Further supporting this, in isolated rat muscle, theophylline stimulated K^+ influx which was negated by ouabain (Clausen and Hansen, 1977). This mechanism is thought to work via caffeine (or its metabolites) directly influencing pump activity via adrenergic β2 receptors. A second potential mechanism relies on caffeine inhibiting the activity of phosphodiesterase (Clausen and Everts, 1988) which leads to increased intracellular cAMP. This increase in cAMP activates Na^+/ K^+ -ATPase pump activity which is stimulated via caffeine binding to adenosine receptors (Ammon, 1991). Caffeine (and its metabolites) are able to cross the plasma membrane and have been shown to impair breakdown of cAMP (via inhibition of phosphodiesterase) leading to elevated cytosolic cAMP thus stimulated pump activity (Rall, 1985).

Caffeine ingestion may also stimulate Na^+/ K^+ -ATPase pump activity indirectly via increasing plasma epinephrine and to a lesser extent norepinephrine (Lindinger et al, 1993). Epinephrine and norepinephrine stimulate the β2 adrenoceptor linked Na^+/K^+ -ATPase pumps (Brown et al, 1983). Furthermore in vitro rat studies have shown increased pump activity with both catecholamine's and β 2 adrenoceptor agonists (salbutamol) which increased electrolyte activity (Juel, 1988). Despite this promising in vitro mechanism, due to large individual variances in epinephrine response to caffeine ingestion (Lindinger et al, 1993) it does appear unlikely that this is the primary mechanism of action. It has also been theorised however that a combined direct and indirect effect may exist. The stimulatory effect of epinephrine appears to be mediated by cAMP activated protein kinase (Everts et al, 1988) therefore increased epinephrine may further stimulate Na^+/K^+ activity in combination with the direct effects of caffeine and its metabolites. Clausen and Flatmen (1987) noted that the metabolite of caffeine theophylline directly potentiated the stimulatory effect of epinephrine on K^+ kinetics in rats, suggesting that it may be a combined rather than an isolated mechanism driving Na^+/ K^+ -ATPase pump activation via caffeine ingestion.

These theorised effects have been positively supported by a number of in vivo publications. Firstly, caffeine has been shown to alleviate exercise induced increases in plasma K^+ (Lindinger et al, 1993) likely due to the increased activity of Na^+/ K^+ -ATPase pump activity. Lindinger et al noted that 6-9mg caffeine significantly attenuated plasma K^+ accumulation (4.88meq/l caffeine vs 5.37meq/l placebo group). Plasma epinephrine also saw a 1.4 to 2-fold increase in the caffeine group further supporting the indirect stimulation hypothesis. Interestingly as Na^+/ K^+ -ATPase pump activity is thought to be maximal during

exercise (Clausen, 1988) the increased activation must therefore have occurred in non-contracting tissues suggesting that caffeine may also activate pump activity during rest, this is positively confirmed by Cordingley et al (2016) who recently demonstrated that with 5mg/kg of caffeine blood K^+ was significantly attenuated 80 minutes following caffeine consumption during resting conditions. Epinephrine and norepinephrine were also significantly elevated when compared to a placebo condition. A dosage response relationship between caffeine and serum and urinary electrolytes has also been identified with no significant changes in 45mg, 90mg and 180mg dosages however with an increased dose of up to 400mg significant acute increases in urinary sodium alongside reduced serum K^+ were identified (Geethavani et al, 2014). This supports the hypothesis of caffeine's direct and indirect action upon the Na^+/ K^+ -ATPase pump which has led to increased electrolyte flux and indicates the existence of a dose response relationship between caffeine and electrolytes.

Based on the current literature it therefore appears plausible that ingesting caffeine will stimulate the Na^+/ K^+ -ATPase pump either indirectly (via epinephrine) and/or directly (via increasing cAMP) leading to increased Na^+ flux hence increased OCTN2 activity. This will then create similar intracellular conditions to the high circulating insulin conditions previously demonstrated to augment carnitine transport thus caffeine should theoretically augment skeletal muscle carnitine accumulation.

1.4 General Introduction

As discussed throughout the literature review skeletal muscle carnitine (L-3-hydroxy-4-N,N,N-trimethylaminobutytic acid) plays a critical role in a number of key metabolic functions within the human body. Its primary role is as an

essential cofactor for the translocation of long chain fatty acids through the impermeable cell membrane into the mitochondrial matric for β- oxidation (Houten and Wanders, 2010). Additionally, carnitine provides essential regulation of the acetyl CoA/ CoASH ratio (Constanton-Teodosiu et al, 1991) thus enabling prolonged PDC and TCA functionality (Carlin et al, 1990) via the formation of acetylcarnitine during exercise. As more than 95% of carnitine is stored within skeletal muscle the ability to manipulate the skeletal muscle carnitine pool can result in direct altered metabolic functionality within muscle cells (Brass, 1995). Despite this the efficacy of carnitine supplementation alone has been largely disproved (Vukovich et al, 1994) whereby increasing plasma carnitine via carnitine alone fails to alter muscle carnitine levels.

Stephens et al (2006) demonstrated that the muscle carnitine pool can be manipulated during a state of hypercarnitinemia by either elevating serum insulin using an insulin clamp or via large dose carbohydrate ingestion. This was extended into a chronic oral study whereby it was confirmed that oral carnitine combined with oral carbohydrates can increase muscle carnitine and results in improved time trial exercise performance (Wall et al, 2011). Despite the mechanistic success of carbohydrate augmented carnitine uptake, it remains a relatively impractical and unhealthy long-term strategy (potentially increased risk of weight gain and blunted insulin sensitivity) therefore this thesis will focus on identifying a safe and effective alternative.

Carnitine is actively transported into skeletal muscle through the plasma membrane via the Na^+ dependent skeletal muscle carnitine transporter OCTN2 against a concentration gradient of >100 fold (Wachter et al, 2002). Insulin augmented transport likely works due to increased sensitivity of the Na^+/K^+ -ATPase pump to available Na^+ whilst under high levels of circulating insulin

thus resulting in increased stimulated Na^+ coupled carnitine transport via OCTN2 secondary to insulins actions of stimulating intracellular Na^+ flux (Stephens et al, 2007). A two-fold increase in OCTN2 activation whilst muscle cells are exposed to hyperinsulinemia (Wall et al, 2011) strongly supports this hypothesis. Finding an alternative mechanism to maximise Na^+/K^+ -ATPase pump activity (thus intracellular Na^+ flux and Na^+ dependent skeletal muscle carnitine transport) may be crucial to manipulating carnitine uptake independent of insulin.

Caffeine has been shown to stimulate Na^+/ K^+ -ATPase pump actively similarly to that of the action of insulin (Lindinger et al, 1993) thus is likely to increase Na^+ flux, therefore augmenting increased carnitine transport via Na^+ dependent OCTN2. Based on the previously discussed evidence we hypothesise that caffeine will increase Na^+/K^+ -ATPase pump activity thus leading to increased Na^+ dependent OCTN2 activity. This will stimulate increased skeletal muscle carnitine transport which we will indirectly examine via plasma carnitine concentration and plasma carnitine clearance rates, a robust indicator of changes within muscle carnitine as demonstrated by Stephens et al (2006, 2007). Stephens work demonstrated the efficacy of measuring changes in the muscle carnitine pool using muscle biopsy samples, plasma carnitine kinetics and measures of plasma carnitine clearance. All of which methodologies provided robust and reliable mechanisms of indicating any changes to the muscle carnitine pool.

Wall et al (2011) demonstrated that with an increased muscle carnitine content, muscle glycogen utilisation is halved during low intensity exercise, muscle lactate accumulation was substantially reduced. These physiological changes appeared alongside a well maintained PCr/ATP ratio during high intensity

exercise. In addition, a 35% improvement in work output as assessed via a 30-minute time trial was demonstrated, hypothesised to result directly from the carnitine induced changes in muscle fuel metabolism ratios. This study demonstrated significant benefit to exercise performance therefore if the benefits of carbohydrate augmented skeletal muscle carnitine uptake translate directly to that of a potential caffeine augmented carnitine uptake this would be a significant finding for athletes looking to improve performance.

1.4.1 Hypothesis to be investigated in this research

The principle aim of this thesis is to investigate whether caffeine would decrease steady state plasma carnitine concentration and increase plasma carnitine clearance. As demonstrated in previous research plasma carnitine is a robust marker of change in muscle total carnitine thus this would indirectly demonstrate increased skeletal muscle carnitine accumulation within healthy human volunteers similarly to that of the action of insulin. This thesis further aims to enhance existing knowledge regarding the mechanism behind skeletal muscle carnitine transport as well as the influence of caffeine on the Na^+/K^+ -ATPase pump and blood metabolites including glucose and lactate. In addition, the purported ergogenic effects of carnitine supplementation on substrate utilisation and exercise performance will be examined.

The following hypothesis will be tested:

1) We hypothesise that consuming caffeine whilst in a state of hypercarnitinemia will stimulate Na^+/K^+ -ATPase pump activity therefore increasing Na^+ dependent OCTN2 carnitine transport thus increasing plasma carnitine clearance into skeletal muscle and decreasing plasma carnitine concentration compared to hypercarnitinemia alone.

2) We further hypothesise that any increase in muscle carnitine content with caffeine ingestion alongside hypercarnitinemia would lead to improved time trial performance and increased fat metabolism during exercise.

Our null hypothesis is that there is no effect of caffeine ingestion on carnitine uptake.

Chapter Two: Methodology

2.0 Introduction to Methodology

In order to fully investigate our hypothesises an in-depth experimental trial needs to be performed in healthy human participants. This study needs to be robustly designed and rigorously controlled to ensure any effects, however small are true effects of the investigated condition. This chapter explains the methodology and equipment that will be utilised. The methodology and equipment have been described in sufficient detail so that one could replicate the study contained within this thesis using the procedures explained throughout this chapter. The study was a proof of concept infusion-based design in which participants consumed caffeine or placebo supplements whilst undergoing a carnitine or placebo infusion. An exercise protocol was then performed the following day to examine changes in substrate utilisation and exercise performance.

2.1 General experiment procedures and protocols

2.1.1 Ethics and informed consent

Ethical approval was obtained for all studies included in this thesis from the University of Exeter Sports and Health Sciences ethics committee in accordance with the *Declaration of Helsinki (1964)*. Ethical approval was received prior to any testing taking place. Participants were provided with an information sheet and were given a minimum of 48 hours to read and understand the detailed description of the studies purposes, requirements and outcomes. Participants then attended a formal screening and talk through with an individual suitably qualified to take informed consent and any questions were fully answered. Participants then provided verbal and written informed consent.

Participants were made fully aware at all stages of the screening process and throughout the study that they were free to withdraw from the experiment at any time and without reason.

2.1.2 Participants and recruitment

Six healthy, recreationally active male and female participants were recruited for this thesis (Mean ± SD: Age 24 ±5 years, Height 175.8 ± 8.8cm and weight 69.76 ± 12.65 Kg). The inclusion and exclusion criteria for this thesis were as follows: no vegetarians or vegans (non-meat eaters may have either a lower plasma carnitine value or a reduced skeletal muscle carnitine transport capacity both of which could alter investigated outcomes). Smokers were excluded due to the effects of nicotine on metabolism. Individuals taking prescribed medication were routinely excluded both for their safety and to remove any possible interaction effects between medication and investigation outcomes. Participants were required to be recreationally active (taking part in non-elite sport 2-4 times per week) and healthy (as assessed by a medical screening form, an International physical activity questionnaire, a physical activity readiness questionnaire and BMI). Participants were asked to provide a list of supplements they were currently taking and asked to abstain from any supplementation that could interact with carnitine or influence metabolism (as a general rule all supplements were excluded for the duration of the study).

Recruitment was undertaken via posters, emails, flyers, social media and word of mouth and individuals were recruited from the both the University of Exeter student body and the local Exeter community.

Participants completed a three-day nutritional diary prior to undertaking the study and also reported habitual caffeine usage. Participants were asked to

abstain from caffeine, alcohol and vigorous exercise for 48 hours prior to entering the laboratory. Participants were provided with a low calorie, macro-controlled meal for the evening before (average ~370Kcal dependent on bodyweight) and were informed to avoid large quantities of carnitine containing foods the day prior to testing. Subjects were provided with an individually calculated calorie-controlled breakfast (~550Kcal) with calorie content calculated by height and bodyweight and instructed to consume the meal 4 hours prior to entering the laboratory (~07:00). Participants were then provided with a further controlled evening meal (~1300Kcal) in the laboratory to be consumed by 21:00 and instructed to return to the laboratory for exercise the following day after an overnight fast. Each infusion visit was performed at the same time (±1.5 hours between different participants) and (± 30 minutes between individual participants) to avoid any potential for diurnal variation. Participants were provided with a wrist worn accelerometer (GENEactiv) to wear for 3 days each visit (day prior to any testing, infusion day and exercise day), which was used to examine any potential effects of caffeine on sleep duration.

2.1.3 Infusion and supplementation

Studies were run in a randomised controlled repeated measures cross over study design. The order of conditions for each participant were randomly allocated using an online random number generator (Research Randomizer, 1997) with numbers assigned for each participant and for each condition. This was counterbalanced to ensure equal distribution of conditions and ensure no order effects could occur. There were three conditions: L-carnitine and caffeine (C&C), L-carnitine only (CARN) and Caffeine only (CAFF).

2.1.3a Carnitine infusion

Carnitine was purchased from Sigma-Aldrich (Gillingham, UK) in powder form (L-Carnitine inner salt Mw 161.2). This was then prepared into an infusate (60Mm) following the local SOP in a sterile controlled environment by the investigator after training and under supervision. This was prepared no longer than two hours prior to infusion. On placebo visits the carnitine placebo was saline alone. Carnitine dosage for each individual was set at 10mg/kg/hour for a steady state 5 hour infusion with an initial 10 minute primer bolus dose of 15mg/kg.

A cannula (BD Venflon 20G Pink) was inserted into the antecubital vein in the non-dominant forearm and was initially flushed with 2ml of saline to clear the cannula and to keep it patent. The infusion bag (carnitine or placebo) was then attached to the cannula via a three-way port (BD Connecta, Sweden) through a filtered volumat line with a pre-calculated dead space. This ran through an infusion machine (Volumat MC Agilia, Fresnius Kabi, Chesire) with the rate of infusion calculated as follows:

6g carnitine in 500ml saline = 12mg / ml

*Bolus (mg) = bodyweight (kg) * 15mg*

*Total bolus (ml) = bolus mg * mg / ml*

Bolus rate per minute (ml) = bolus ml / 10

*Total steady state infusion (mg) = (weight * 10 (mg / kg / hr)) * 5*

*Total infusion (ml) = total infusion (mg) * 12 (mg / ml)*

Infusion rate per minute (ml) = total infusion (ml) / 300

The overall infusate preparation, the quantity of carnitine weighed, the infusion calculations and pump settings were recorded, checked and signed for by a second qualified individual.

2.1.3b Caffeine supplementation

Fine balance scales were utilised to weigh out 6mg/kg caffeine (Sigma- Aldrich, Gillingham Mw 194.19) and loaded into in a solid white gelatine capsule (Capsule connection 00, Prescot, AZ). From previous research It appears that a minimum dose of 6mg/kg is required to influence Na^+/ K^+ kinetics. Due to the relatively rapid half-life of caffeine (~5 hours (Bonati et al, 1982)) a single bolus caffeine dose may be insufficient to maximally stimulate OCTN2 transport therefore multiple 'top up' doses were administered at set intervals throughout the infusion. This initial 6mg/ kg dose was administered after the 10-minute infusion bolus at onset of steady state carnitine infusion (T=0). A single dose tablet of 1mg/kg caffeine was ingested at 2 hours, 3 hours and 4 hours throughout the infusion for a total of 9mg/kg over the 5 hours. Each dose was provided with a measured 50ml water to consume and all participants were instructed to consume all the provided water alongside the capsule. The caffeine placebo was dextrose (Bulk powders, Essex). Although carbohydrates are known to stimulate carnitine uptake the maximum provided dosage of ~750mg is far lower than the stimulatory threshold and would have no impact on carnitine kinetics. The placebo was weighed out and loaded into solid white gelatine capsules identical in size and appearance to that of the caffeine capsules ensuring participants remained blinded to which condition they were in. Caffeine dosages were calculated on a excel spreadsheet and the weighed caffeine tablets were peer checked for safety and accuracy.

2.1.4 Exercise protocols

Participants undertook a $V0_2$ Max test on an electronically braked cycle ergometer as described below to elucidate an individual's maximum rate of

oxygen consumption. This was followed by confirmation trials, familiarisation trials, and 3 exercise protocols (once under each supplementary condition).

2.1.4a $V0_2$ maximum testing

Participants entered the laboratory after a minimum 4 hour fast to undertake a continuous incremental exhaustive $V0_2$ max protocol on an electronically braked cycle ergometer (Lode Excalibur sport V2 electrically braked cycle ergometer, Lode BV, Groningen, Netherlands). Starting wattage was set at bodyweight ±5 Watts and increased between 20-40 watts every three minutes based on participants individual differences, assessed at screening visit via fitness score. Oxygen consumption was measured using a gas analyser (Cortex Metalyser 3b, Cortex, Leipzig, Germany) and $V0_2$ was accepted when a plateau in oxygen consumption was achieved despite an increasing workload. Other secondary criteria for acceptance were an RER of >1.15, Borg scale rating of perceived exertion (Williams, 2017) of 20 or heart rate 95% of predicted max. Participants who failed to obtain a sustained $V0_2$ plateau repeated testing or undertook a further confirmation trial. Participants were provided with verbal encouragement throughout the test. All participants undertook at least one confirmation trial consisting of a number of 3-minute bouts (initial wattage same as starting wattage for $V0_2$ Max, then increase to a mid-range wattage before increasing to a wattage just above that reached during the previous $V0_2$ Max test). If a $V0_2$ Max plateau (maximum oxygen consumption unchanged despite increased workload) was still not achieved, then a second confirmation trial was undertaken at least three days later. $V0_2$ Max data was then extracted from the cortex system and used to calculate 50% and 75% of an individual's $V0_2$ Max, the wattage required for a participant to obtain these $V0_2$ boundaries was then calculated.

2.1.4b Exercise familiarisation testing

Participants entered the laboratory after an overnight fast. They then followed the exercise protocol on the same lode Excalibur used for $V0_2$ Max testing with 30 minutes at a wattage designed to elicit 50% $V0_2$ max followed by a 5 minute break, then a further 30 minutes at 75% $V0_2$ max followed by a 5 minute break before a 15 minute all out time trial using the alpha formula on lode software, this was calculated from cadence and watt max and was set so ~70% watt max was at 90 rpm for all participants. Subjects wore the cortex gas analyser mask during the first and last 10 minutes at each stage and provided Borg scale readings every 10 minutes. Saddle height and other positional settings on the lode were recorded during the familiarisation visit and kept identical throughout the rest of testing. If participants $V0_2$ was out of the 50% and 75% range during the familiarisation stages, then minor adjustments were made to the exercise protocol wattages.

2.1.4c Exercise testing

Subjects entered the lab at ~06:30 after an overnight fast following a calorie-controlled meal and infusion trial the previous afternoon. Resting respiratory exchange ratio (RER) (the ratio between carbon dioxide produced and oxygen used) was recorded 30 minutes prior to undertaking exercise whilst participants were supine in a darkened room using the cortex gas analyser system. Subjects then completed the protocol identical to that of the familiarisation trial (30 minutes at 50% $V0_2$, 30 minutes at 75% $V0_2$ followed by a 15-minute time trial). Water intake was ad lib on visit one and recorded and kept the same throughout participant's future trials. All trials took place at the same time (±30 minutes) to ensure minimum differences in fasting and sleep differences between trials.

Participants were allowed music and provided encouragement during the 50% and 75% bouts however identical conditions were maintained for each performance time trial (room temperature set to 18°c, no music or talking, no one else in room during this time period) to ensure minimal differences between exercise trials. All participants were informed when they had 10 minutes and 5 minutes remaining however no encouragement was provided. The positional settings of the bike were kept identical for each subjects' trials.

2.2 Experimental Measures

2.2.1 Blood sampling protocol

2.2.1a During infusion

During the infusion visits the non-dominant hand was placed in hot water for ~10 minutes to pre-warm the hand. A cannula (BD Venflon 20G blue) was then inserted retrogradely into a superficial vein on the dorsal surface and secured in place using tegaderm plaster (Tegaderm I.V. transparent dressing 1633). 2ml of blood using a disposable sterile 2ml syringe (Terumo, Leuven, Belgium) was initially drawn to ensure the cannula was flowing before a saline drip was attached, dripping at the minimum permitted steady rate (~500ml/6hours) to keep the cannula patent throughout the experiment. The hand was then placed in a hand warming unit (Medical engineering unit, University of Nottingham) which was air warmed to 55°c (Liu et al, 1992). The hand resided in the box for the duration of the visit. The baseline blood sample was drawn a minimum of 20 minutes after hand was initially introduced into the hotbox. This methodology provided arterialised venous drainage of the hand enabling a more stable arterialised venous blood sample. Blood was drawn at baseline and then every 30 minutes during the infusion. When drawing blood an initial 2ml waste was

drawn to remove any saline from the cannula line before 6ml of blood was removed using a sterile 10ml syringe (Terumo, Leuven, Belgium). 3ml was immediately injected into a lithium heparin coated vacutainer (BD Vacutainer, Bristol, UK) and centrifuged for 10 minutes at 4°c at 10000g (Labcare centrifuge, Buckinghamshire). The plasma was then aliquoted into 4 ~500µl centrifuge tubes (Eppendorf microtubes, Sigma, UK) which were snap frozen in liquid nitrogen before being transferred to a -80°c freezer for storage prior to analysis. A further 2ml of whole blood was aliquoted into a spray coated silica and polymer gel (SST) containing vacutainer (BD vacutainer, Bristol, UK) and left to clot at room temperature for a minimum of 30 minutes before being spun at 10000g for 10 minutes at 4°c and the supernatant then aliquoted into 2 centrifuge tubes and immediately snap frozen in nitrogen and transferred to the -80°c freezer. The remaining 1ml of whole blood was immediately analysed using a blood analyser system for sodium, potassium and haematocrit (NOVA biomedical, Runcorn, UK) and glucose and lactate (YSI 2500 Lactate Analyser, YSI, UK). The order of this process was kept identical for every sample and was as follows: 3ml blood into pre-iced heparin tube and replaced on ice, 2ml of blood into SST tube and left in vacutainer holder to clot, whole blood electrolyte and metabolite measures, then place heparin tube in centrifuge and spin. This whole process took no longer than 1 minute and the blood in the tube was kept continually agitated. After the final blood sample, the cannula was then removed as per the SOP with firm pressure being applied for ~5 minutes to ensure no hematoma.

2.2.1b During exercise

On arrival participants were asked to rest in a supine position on a bed whilst a 20 G cannula (Venflon) was inserted into the antecubital vein of the non-

dominant arm. A three-way tap was then attached to a saline drip which was kept at a continual minimal drip to keep the cannula patent. The cannula was secured using tegaderm and the line was taped to the arm and back using micropore tape to ensure it did not interfere with exercise. Blood samples were taken at baseline and then every 10 minutes during exercise excluding the performance time trial. A baseline blood sample of ~6ml was drawn and treated exactly the same as during infusion visits (4ml plasma, 2ml to clot and 1ml for NOVA and YSI). All blood samples following on from this excluded the NOVA measurements but were otherwise processed identically.

2.2.2 Muscle sampling

Muscle biopsy samples were obtained from the vastus lateralis muscle immediately before and after infusion and immediately prior to exercise during resting supine conditions by a qualified individual using the percutaneous needle biopsy technique as described previously (Bergstrom et al, 1962). Upon removal from the leg the muscle sample was placed on gauze in a petri dish where it was immediately divided into two samples (~60mg and ~30mg) using serrated forceps before being snap frozen in liquid nitrogen within 60 seconds of removal from the leg. The samples were then transferred to 2ml cryogenic vials and stored in a -80°c freezer ready for processing. Samples were taken from the same leg each visit (3 left leg, 3 right then a further 3 left leg.

2.3 Analysis

2.3.1 Plasma analysis for caffeine, paraxanthine, theophylline and theobromine

Plasma concentrations of caffeine and its principal metabolites were determined by reversed phase high performance liquid chromatography (HPLC), following a

method determined by Holland et al (1998). HPLC was performed using a Perkin Elmer Flexar LC system, comprising of a chilled peltier auto sampler used in conjunction with binary pump and photodiode array (PDA) detector (Perkin Elmer, MASS, USA). A single aliquot of plasma for each subject at each measured time point was removed from storage -80°c freezer and defrosted on ice. All time points and visits for each subject were run in the same batch to decrease between run variability. Defrosted samples were immediately vortexed before 250µl of plasma was pipetted into a centrifuge tube. The pipette tip was inserted two thirds into plasma sample providing optimal extraction ensuring no air or particulates were accidently pipetted. This was then deproteinised using 250µl of 0.8M perchloric acid and vortexed immediately. The sample was then centrifuged at 14000g at room temperature for 210 seconds in a centrifuge (MSE Micro Centaur Plus) to remove precipitated proteins. 350µl of the resulting supernatant was pipetted into a glass HPLC vial where it was neutralised with 27µl of 4 M sodium hydroxide before final vortexing. The samples were then added to the HPLC chilled autosampler loading tray in an ascending then descending subject specific order to reduce any ordering effects that may have occurred ready for direct injection onto the HPLC column. An initial blank sample (H20) was run at the start of every subject batch. Deproteinised sample (50 µl) was eluted isocratically [mobile phase - 15mM potassium phosphate (pH 4.9) and methanol (85:15 v/v)] for 30 minutes through a reverse-phase analytical column [C18HD, 5µm, 250 mm x 4.6 mm (Perkin Elmer, MASS, USA) with 10 x 4.6 mm guard cartridge], with constant flow rate of 1.9 ml/min. Resulting peaks were detected by ultraviolet absorbance at 274nm on the PDA.

Peaks were integrated using Chromera software version 4.1.2.6210 (Perkin Elmer, MASS, USA). Calibration curves were determined from aqueous standards of analytes with concentrations of 10, 5, 3.333, 2.222, 1.481, 0.987, 0.439, and 0.195 µg/ml. Retention times: theobromine (5.6min) paraxanthine (9), theophylline (10.2), caffeine (19.0).

Each readout was checked manually to ensure peak compliance.

2.3.2 Plasma carnitine analysis

In an identical manner to caffeine one aliquot of plasma for each subject at each time point was removed from the -80°c freezer. All visits for each subject were run as a single batch to reduce any between run variability. Batches of two subjects were run at a time. Plasma was defrosted on ice, vortexed and immediately replaced on ice. Total carnitine analysis methodology was via the radioenzymatic method (Cederblad et al,1990). 20µl of plasma was added to a pre-chilled centrifuge tube on ice. 1ml of chloroform:methanol (3:2) solution was added to the plasma and centrifuged at 12000g for 10 minutes. The supernatant formed was decanted into a new centrifuge tube and placed under a low power air blower to evaporate in a fume hood, a centrifuge holder was designed and built using a 3D printer to allow all of each participant's condition data to be run together. Once the chloroform:methanol solution had fully evaporated 10µl of ultrapure water (Thermo Scientific) in addition to 100µl of 0.1 M KOH was pipetted into the centrifuge tubes. The centrifuge tubes were then placed in a solid heating block with caps closed and incubated at 50°c for 2 hours. Post incubation samples were neutralised utilising 10µl 1M Hydrochloric acid leaving a final total volume of 120µl which was transferred to a 3ml clear Perspex tube.

A 100µl solution consisting of 25µl phosphate buffer (pH 6.5), 40µl unlabelled acetyl CoA (300µ M), 25µl 14c labelled acetyl- CoA (4µM) and 10µL NEM (40mM) was added to each tube. After vortexing 10µl of carnitine acetyltransferase (5mg/ml H20) was added to each tube every 20 seconds and immediately vortexed. After exactly 30 minutes of room temperature incubation 2mL Dowex resin (1g dowex:1ml distilled H20) was added to each tube every 20 seconds and immediately vortexed. Once the Dowex slurry had settled 500µl of supernatant was pipetted into a scintillation vial and 3ml scintillation fluid was added. The scintillation vial was then added to the scintillation counter (Hidex 300SL) ready for analysis.

A carnitine standard curve was run with every batch of samples. This consisted of duplicated ratios of L-carnitine to H20; 0:120, 15:105, 30:90, 45:75, 60:60, 75:45, 90:30. The scintillation counter was then loaded with one blank vial, the carnitine standards, the plasma samples and then 3 radiation swap test strips and activity was counted for 60 seconds.

This was then applied to a standard curve using excel and the data converted into µmol L-1.

2.3.3 Muscle carnitine analysis

Muscle was removed from the -80°C freezer and weighed. Approximately 50mg muscle tissue was then freeze dried for 48 hours. Freeze dried muscle was powdered using serrated forceps and scalpel in a pestle ensuring all connective tissue was removed. Powdered muscle was reweighed into 2ml centrifuge tubes, which were subsequently burst spun in a centrifuge to ensure powder sank to bottom of tube. A solution of PCA 0.5M/ EDTA 1mM was added to each tube (at a ratio of 1ml/12.5mg powder). Samples were then continually vortexed

for 10 minutes whilst on ice. After 10 minutes samples were spun at 10000g for 3 minutes at 4°c and the supernatant was then decanted. Supernatant was neutralised utilising KHC03 2.2M (~1/4 of decanted PCA/EDTA volume with the exact ratio calculated using pH testing strips to ensure an exact pH of 7). Upon dissipation of produced C02, samples were spun at 10000g for a further 3 minutes at 4°c. Produced supernatant was decanted, labelled as muscle extract and refrozen at -80°c in preparation for subsequent analysis.

Muscle total carnitine was measured from the muscle extract. Extract was removed from -80°c freezer and defrosted on ice. 10µl extract was then pipetted into a new Eppendorf, 100µl of 0.1m KOH was then added and the Eppendorf was incubated at 50°c for 2 hours before being removed from heat and neutralised with 10µl of 1M HCL. The same process for scintillation counter analysis as used for plasma carnitine analysis was then followed including running a new standard curve with each run to calculate muscle total carnitine.

Muscle free carnitine was elucidated by adding 10µl muscle extract to 110µl ultrapure water in a 3ml Perspex tube before running scintillation counter testing as described in previous analysis.

2.3.4 Statistical analysis

All statistical analysis was performed using GraphPad software (GraphPad, Prism, USA). Data sets were screened for normality prior to testing. Significance was accepted at $P<0.05$ and values of $P<0.1$ were assessed as trends. A series of two-way ANOVAs (time and treatment effects) were run and where appropriate Bonferroni post hoc analyses was applied. Students paired t tests were used to compare means. All data is presented as mean ± standard

deviation unless otherwise stated. GPower software (GPower3.0.10, Germany) was used to calculate power of study design and utilised as an estimation of effect size. Due to recruitment and study timing issues muscle and exercise testing was only performed in n=2 therefore statistical analysis was conducted for infusion data only (n=6). Due to mechanical error blood metabolites were not obtained for one subject so K^+, Na^+, glucose and lactate (n=5) and HCT (n=4).

Chapter Three: Experimental Results

3.0 Introduction to Experimental Results

The following chapter describes the results obtained during the experimental study contained within this thesis. A concise description of all important findings for each measure has been provided and where appropriate a graphical representation of data has been presented. All results are discussed and explained in future chapters.

3.1 Infusion Data

3.1.1 Plasma carnitine

Plasma carnitine was unchanged (baseline 55.4±10.8μmol/ml vs 52.7± 4.9μmol/ml steady state (SS)) throughout five hours of placebo saline infusion (CAFF condition).

In the carnitine only infusion (CARN) plasma carnitine increased from baseline after an initial bolus and 30 minutes of SS carnitine infusion from 47.4μmol/ml to 340.1μmol/ml. Plasma carnitine in CARN condition was higher at every time point than both baseline and CAFF.

In the caffeine and carnitine condition (C&C) plasma carnitine increased from baseline after an initial bolus and 30 minutes of SS carnitine infusion from 47.0μmol/ml to 328.6μmol/ml. Plasma carnitine in C&C condition was higher at every time point than both baseline and CAFF. Post hoc Bonferroni analysis of the steady state infusion data comparing C&C vs CARN trended to show a lower plasma carnitine at T=210, 240 and 300 ($P<0.08$) during the C&C condition (Figure 1).

Rate of total carnitine clearance from plasma was calculated from individual plasma carnitine data. During a 5 hour carnitine infusion the rate of total carnitine clearance from plasma was increased (Figure 2) with the ingestion of caffeine (P<0.0001) (C&C 205.1 ±15.19 ml.min-1 vs 187.9 +-13.9 ml.min-1 CARN). This was also reflected in an increased (P<0.0001) calculated rate of tissue uptake when caffeine was ingested during carnitine infusion (figure 3) CARN 33.7 +-5.3μmol.min-1 vs 36.9+-5.3μmol.min-1 C&C.

Figure 1.

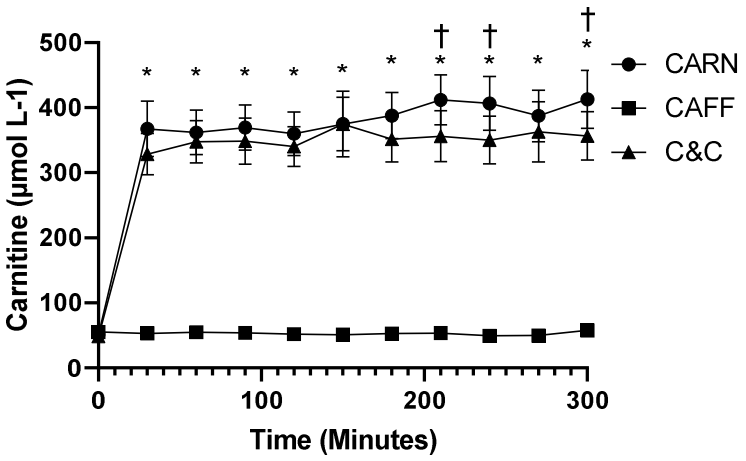

*Fig.1. Plasma TC concentrations over the course of 5 hours of intravenous carnitine or saline infusions combined with dextrose placebo or caffeine. Values expressed as mean ± SEM (n=6). *, (P<0.05) C&C and CARN significantly greater than CAFF (Saline) condition and baseline at every time point. †, (P<0.1) CARN trends to be higher than C&C at T=210, T=240 and T=300. There is no difference in baseline values between conditions.*

Figure 2.

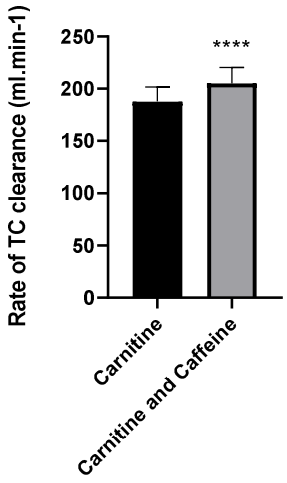

*Fig.2. Rate of TC clearance from plasma during an Intravenous infusion of L-carnitine vs L-carnitine and caffeine capsules. ****, (P<0.0001) TC had a significantly greater rate of clearance from plasma during infusion when combined with caffeine vs carnitine alone.*

Figure 3.

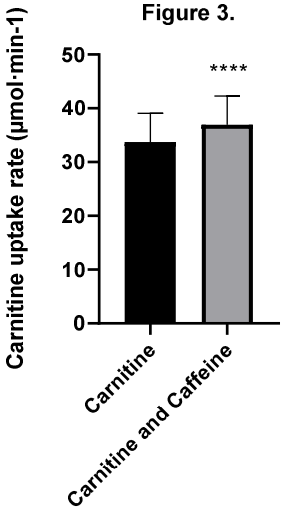

*Fig. 3. TC uptake rate into tissue (calculated as plasma concentration x plasma clearance) during a 5 hour infusion of L-carnitine when combined with caffeine vs dextrose placebo. ****, (P<0.0001) Carnitine had a significantly increased uptake rate into tissue when combined with caffeine vs carnitine alone.*

3.1.2 Plasma caffeine

Plasma caffeine was unchanged (baseline 0.2µg/mL Vs 0.1µg/mL SS) throughout the 5 hours infusion in the caffeine placebo condition (CARN).

In the caffeine only group (CAFF) plasma caffeine increased from baseline after an initial 6mg/kg dose at T=0 from 0.03µg/mL to 6.6µmg/mL after 30 minutes.

Plasma caffeine in the CAFF condition was higher at every time point than both baseline and CARN condition.

In the caffeine and carnitine group (C&C) plasma caffeine increased from baseline after an initial 6mg/kg dose at T=0 from 0.03µg/mL to 8.9µg/mL after 30 minutes. Plasma caffeine in the C&C condition was higher at every time point than both baseline and CARN condition. Plasma caffeine remained elevated throughout the 5 hour infusion and excluding baseline there were no effects of time at any point within the C&C condition demonstrating a constant SS in plasma caffeine (Figure 4). There were no differences between C&C and CAFF conditions at any time (SS CAFF 7.2µg/mL vs 7.7µg/mL C&C).

Figure 4.

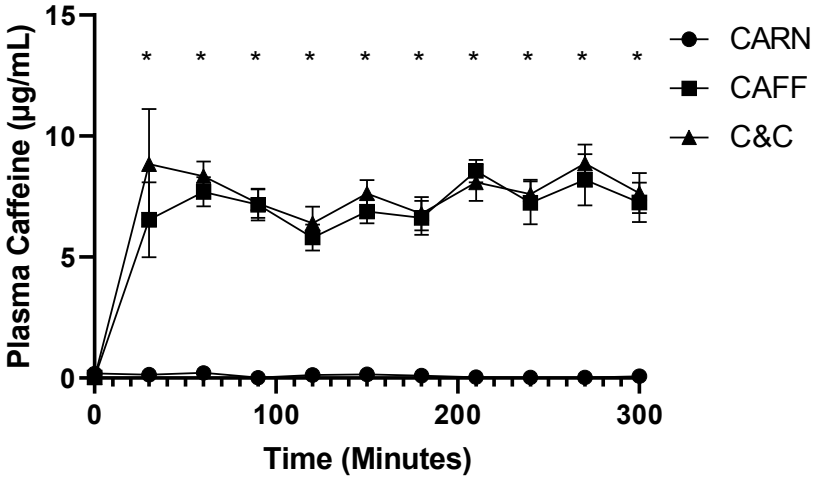

Fig.4. Plasma caffeine concentration during a 5 hour intravenous infusion of L-Carnitine or saline. Caffeine/ dextrose placebo ingested at T=0 6mg/kg, T=120 1mg/kg, T=180 1mg/kg and T=240 1mg/kg. All values expressed as mean ± SEM (n=6)., (P<0.05) CAFF and C&C greater than CARN at all time points excluding T=0. No differences between CAFF and C&C at any time points.*

3.1.3 Caffeine metabolites

3.1.3a Paraxanthine

There was a significant effect of time and condition on plasma paraxanthine ($P<0.0001$) (figure 5).

Paraxanthine was unchanged (baseline 0.3µg/mL Vs 0.2µg/mL SS) in the CARN group (caffeine placebo).

In the caffeine only group (CAFF) plasma paraxanthine increased from baseline after an initial 6mg/kg dose of caffeine at T=0 from 0.1µg/mL to 0.5µg/mL after 30 minutes and continued to increase linearly till 2.3µg/mL after 300 minutes. Plasma paraxanthine in the CAFF condition was higher at every time point than both baseline and CARN condition.

In the caffeine and carnitine group (C&C) plasma paraxanthine increased from baseline after an initial 6mg/kg dose of caffeine at T=0 from 0.05µg/mL to 0.6µg/mL after 30 minutes and continued to increase linearly until the end of infusion reaching 2.2 µg/mL after 300 minutes. Plasma paraxanthine in the C&C condition was higher at every time point than both baseline and CARN condition. There were no differences between C&C and CAFF conditions at any time.

Figure 5.

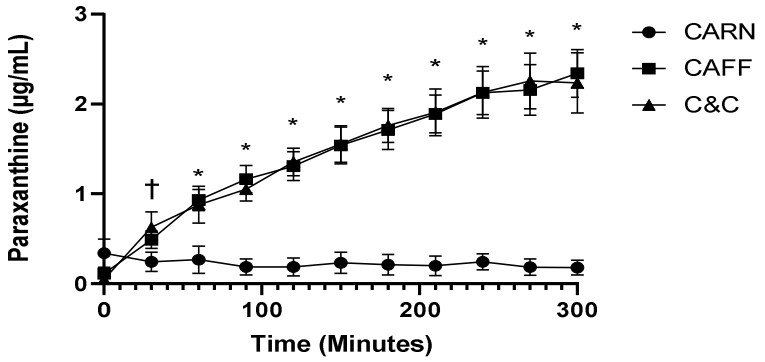

*Fig. 5. Plasma paraxanthine concentration during a 5 hour infusion of L-Carnitine or Saline when combined with Caffeine or Placebo. Caffeine ingested at T=0 6mg/kg, T=120 1mg/kg, T=160 1mg/kg, T=240 1mg/kg in C&C and CAFF conditions. †, (P<0.05) C&C greater than CARN. *, (P<0.0001) C&C and CAFF greater than CARN. No differences at baseline between any conditions or between C&C and CAFF at any time point.*

3.1.3b Theophylline

There was a significant effect of time and condition on plasma theophylline (P<0.0005) (figure 6). Theophylline was unchanged (baseline 0.04µg/mL Vs 0.02µg/mL SS) in the CARN group (caffeine placebo).

In the caffeine only group (CAFF) plasma theophylline increased from baseline after an initial 6mg/kg dose of caffeine at T=0 from 0.01µg/mL to 0.06µg/mL after 30 minutes and continued to increase steadily 0.22µg/mL after 300 minutes. Plasma theophylline in the CAFF condition was higher after 120 minutes than both baseline and CARN condition and remained higher at all time points until the termination of infusion.

In the caffeine and carnitine group (C&C) plasma theophylline increased from baseline after an initial 6mg/kg dose of caffeine at T=0 from 0.02µg/mL to 0.5µg/mL after 30 minutes and continued to increase linearly till the end of infusion reaching 0.13 µg/mL after 300 minutes. Plasma theophylline in the C&C condition was higher after 150 minutes than both baseline and CARN

condition and at all time points thereafter. At T=240 and T=300 CAFF had elevated theophylline compared to C&C, there was no differences it at any other time points.

Figure 6.

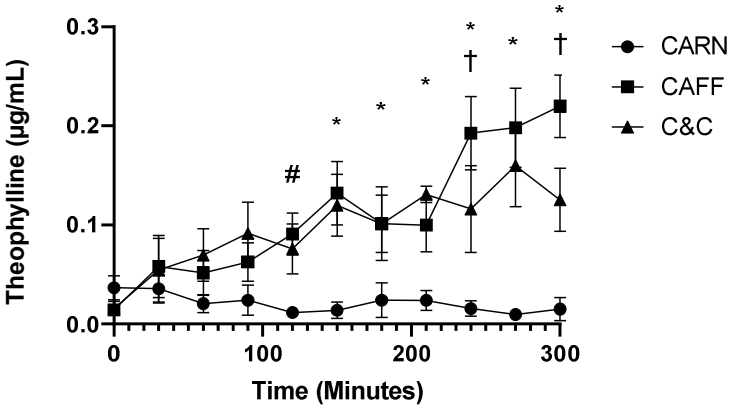

*Fig. 6. Plasma theophylline concentration during a 5 hour infusion of L-Carnitine. #, (P<0.05) CAFF greater than CARN. *, (P<0.01) C&C and CAFF greater than CARN. †, (P<0.05) CAFF greater than C&C.*

3.1.3c Theobromine

There was no effect of time or condition on plasma theobromine throughout the 5 hour infusion (P>0.1) in all conditions. Theobromine was unchanged (baseline 0.9µg/mL vs 0.6µg/mL SS) in the CARN group (caffeine placebo) (Figure 7).

In the caffeine only group (CAFF) theobromine was unchanged (baseline 1.1µg/mL vs 0.9µg/mL SS) and was not different to CARN or C&C conditions

In the caffeine and carnitine group (C&C) theobromine was unchanged (baseline 0.4µg/mL vs 0.5µg/mL SS) and was not different to CARN or CAFF conditions.

Figure 7.

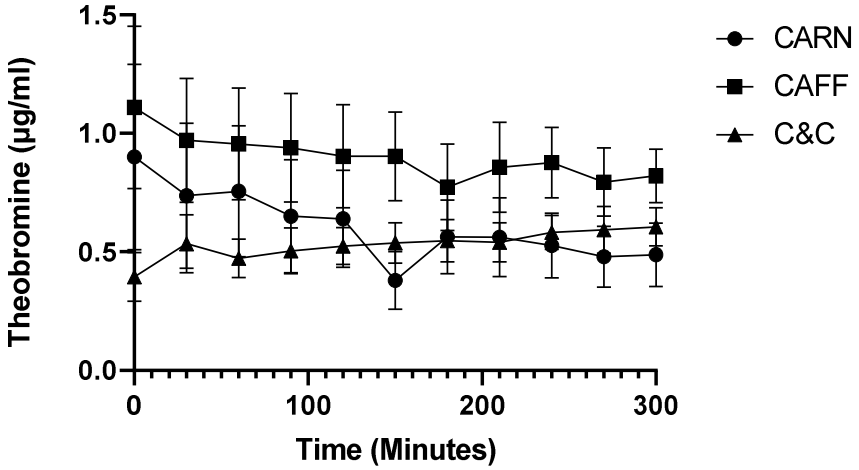

Fig.7. Plasma theobromine levels during a 5 hour infusion of L-carnitine with or without caffeine ingestion. There were no significant effects of time or condition on plasma theobromine levels.

3.1.4 Whole blood data

3.1.4a Sodium (Na^+)

Whole blood Na^+ remained unchanged (baseline 137.5mmol/L vs 137.6mmol/L SS) throughout the 5 hours infusion in the caffeine placebo condition group (CARN).

In the caffeine only group (CAFF) Na^+ increased (baseline 137.6mmol/L vs 138.2mmol/L SS) after 9mg/kg caffeine ingested throughout five hours. Na^+ in CAFF condition was higher ($P<0.05$) than CARN at T=30, 60, 150 minutes and trended ($P<0.1$) to be higher T=240 and 270minutes (Figure 8).

In the caffeine and carnitine group (C&C) Na^+ increased (baseline 137.6mmol/L vs 138.1mmol/L SS) during the 5 hour infusion. Na^+ in C&C vs CARN was higher ($P<0.05$) at T= 60 and 150 minutes and trended ($P<0.1$) to be higher at T=30 and T=300 minutes. There were no differences in Na^+ at any time points between C&C and CAFF.

There was a difference in steady state infusion whole blood Na^+ levels between conditions (P<0.05). CARN (137.6 +-0.9) was lower (P<0.05) than CAFF (138.2+-1.1) and trended (P=0.1) to be lower than C&C (138.1+-0.8). There was no difference between CAFF and C&C conditions (Figure 9).

Figure 8.

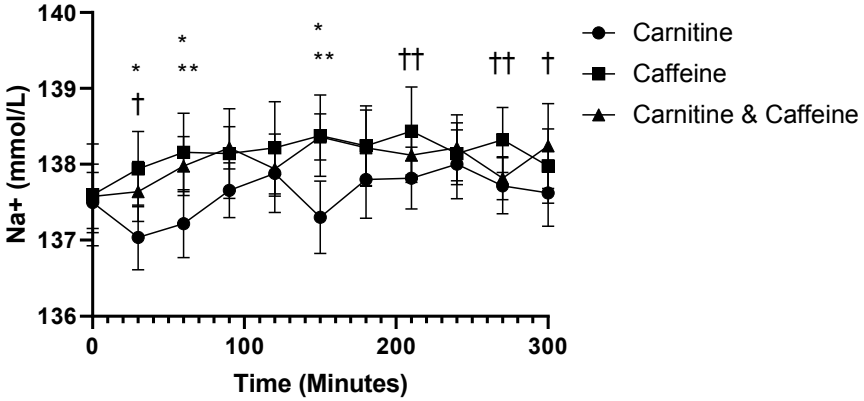

*Fig. 10. Whole blood Na+ concentration during a 5 hour intravenous infusion of L- Carnitine or saline. Caffeine/ Destrose placebo ingested at T=0 6mg/ kg, T=120 1mg/kg, T=180 1mg/kg, T=240 1mg/kg. *, (P<0.05) CAFF greater than CARN. **, (P<0.05) C&C greater than CARN. †, (P<0.1). C&C greater than CARN. ††, (P<0.1) CAFF greater than CARN. No differeces at any time point between CAFF and C&C or at baseline between any conditions.*

Figure 9.

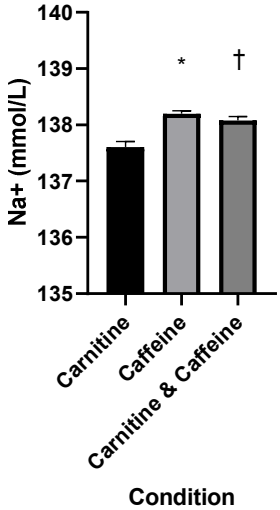

*Fig.11. Whole blood steady state average Na+ concentration during a 5 hour intravenous infusion of L-Carnitine or saline. Combined with total 9mg/kg caffeine in C&C and CAFF conditons. *, (P<0.05) significantly higher than CARN. †, (P=0.1) trended to be higher than CARN. No difference between CAFF and C&C conditions.*

3.1.4b Potassium (K^+)

Whole blood K^+ remained unchanged (baseline 4.3mmol/L vs 4.3mmol/L SS) throughout the 5 hours infusion in the caffeine placebo condition group (CARN).

In the caffeine only group (CAFF) K^+ decreased (baseline 4.3mmol/L vs 4.1mmol/L SS) with the ingested of 9mg/kg caffeine throughout five hours. K^+ was significantly lower than CARN after 300 minutes (Figure 10).

In the caffeine and carnitine group (C&C) K^+ also decreased throughout the 5 hour infusion (baseline 4.3mmol/L vs 4.1mmol/L SS).

There was a difference in steady state infusion whole blood K^+ levels between conditions ($P<0.05$). CARN (4.3+-0.2mmol/L) was higher than both CAFF (4.1+-0.2mmol/L) and C&C (4.1+-0.3mmol/L) (Figure 11). There was no difference between CAFF and C&C conditions.

Figure 10.

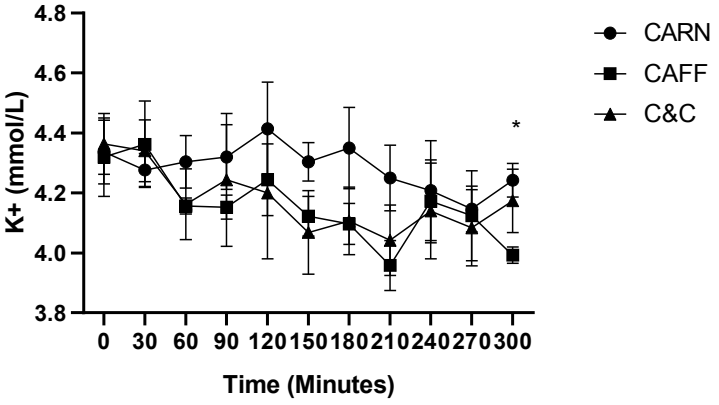

*Fig.8. Whole blood Potassium concentration during a 5 hour intravenous infusion of L-Carnitine or saline. Caffeine/ dextrose placebo ingested at T=0 6mg/kg, T=120 1mg/kg, T=180 1mg/kg and T=240 1mg/kg. *, (P<0.05) CARN greater than CAFF.*

Figure 11.

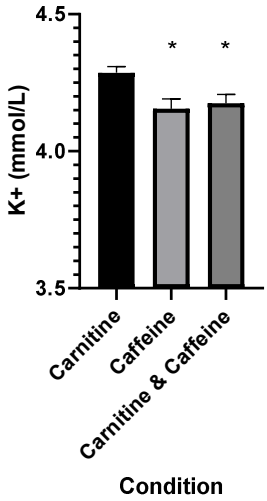

*Fig.9. Whole blood average steady state K+ concentration during a 5 hour intravenous infusion of L-Carnitine or saline combined with total 9mg/kg caffeine in C&C and CAFF conditons. Values expressed as mean ± SEM (n=5). *, (P<0.05) significantly lower than CARN. No difference between CAFF and C&C conditions.*

3.1.4c Glucose

Whole blood glucose decreased with time in all conditions (P=0.05) throughout the 5 hour infusion. There were no differences in glucose at any time points between CAFF, C&C or CARN conditions (Figure 12). In the CARN group (caffeine placebo) glucose decreased (baseline 4.5 mmol/L vs 4.2mmol/L T=300) gradually throughout the 5 hour infusion.

In the caffeine only group (CAFF) glucose decreased throughout the 5 hour infusion (baseline 4.4mmol/L vs 4.1mmol/L T=300).

In the caffeine and carnitine group (C&C) glucose also decreased (baseline 4.4mmol/L vs 4.1mmol/L T=300) during the 5 hour infusion.

Figure 12.

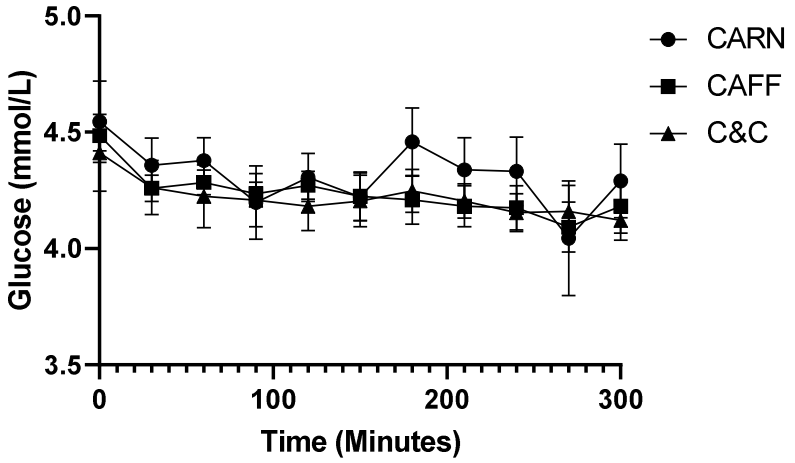

Fig.12. Whole blood glucose during a 5 hour infusion of L-carnitine with or without caffeine capsules. There were no significant effects of time or condition on blood glucose.

3.1.4d Lactate

In the CARN group (caffeine placebo) lactate decreased from baseline to 60 minutes (baseline 0.6 mmol/L vs 0.4mmol/L T=60) and thenceforth remained consistent for the remainder of infusion (0.4mmol/L SS). There was a trend for condition effect (P<0.1) (Figure 13).

In the caffeine only group (CAFF) lactate remained consistently higher than CARN (P<0.1) throughout the 5 hour infusion (CARN SS 0.4mmol/L vs 0.5mmol/L CAFF SS). Lactate was higher (P<0.05) in CAFF vs CARN conditions at T= 90, 150, 180, 210, 270 and 300 and trended to be higher (P <0.1) at T=120.

In the caffeine and carnitine group (C&C) lactate remained consistently higher than CARN (P<0.1) throughout the 5 hour infusion (CARN SS 0.4mmol/L vs 0.5mmol/L CAFF SS). Lactate was higher (P<0.05) in C&C vs CARN conditions at T= 150, 210 and 300 minutes and trended to be higher (P<0.1) at T= 240 and

270 minutes. There were no differences between CAFF & C&C group at any time points.

Figure 13.

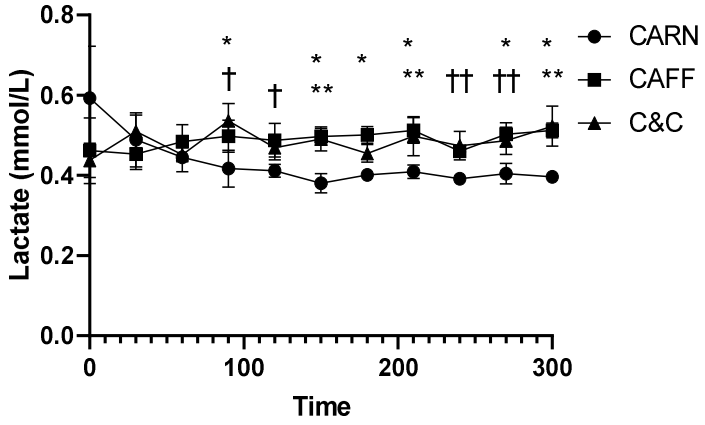

*Fig. 13. Whole blood lactate concentration during a 5 hour intravenous infusion of L- Carnitine or saline. Caffeine/ Dextrose placebo ingested at T=0 6mg/ kg, T=120 1mg/kg, T=180 1mg/kg, T=240 1mg/kg. *, (P<0.05) CAFF greater than CARN. **, (P<0.05) C&C greater than CARN. ††, (P<0.1). C&C greater than CARN. †, (P<0.1) CAFF greater than CARN. There were no differences at any time points between CAFF and C&C.*

3.1.4e Haematocrit (HCT)

In the CARN group (caffeine placebo) HCT decreased marginally from baseline throughout the 5 hour infusion (baseline 39.8% vs 38.5% T=300). There was a trend for condition effect (P<0.1) (Figure 14).

In the caffeine only group (CAFF) HCT remained consistently higher than CARN (P<0.1) throughout the 5 hour infusion (CARN SS 38.8% vs 40.1% CAFF SS). HCT was higher (P<0.05) in CAFF vs CARN conditions at all time points.

In the caffeine and carnitine group (C&C) HCT remained consistently higher than CARN (P<0.1) throughout the 5 hour infusion (CARN SS 38.8% vs 40.5% C&C SS). HCT was higher (P<0.05) in C&C vs CARN condition at all time

points. There were no differences between CAFF & C&C group at any time points.

Figure 14.

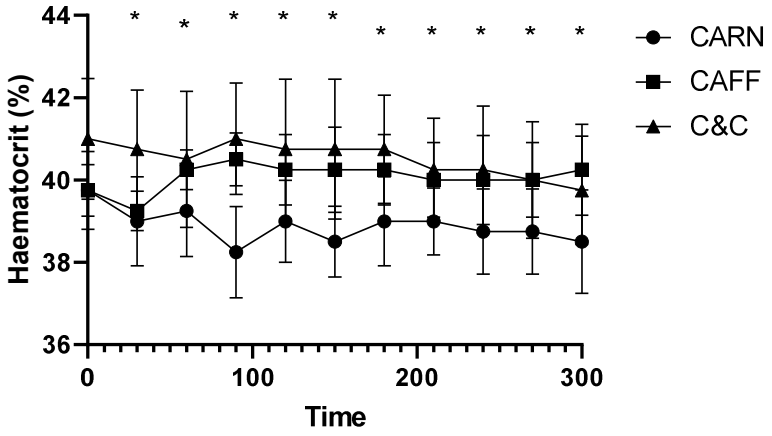

*Fig. 14. Haematocrit concentration during a 5 hour intravenous infusion of L-Carnitine or saline. Caffeine/ Dextrose placebo ingested at T=0 6mg/ kg, T=120 1mg/kg, T=180 1mg/kg, T=240 1mg/kg. *, (P<0.05) CAFF and C&C greater than CARN. No significant difference at baseline or between CAFF and C&C conditions at any time.*

3.1.5 Respiratory exchange ratio (RER) and fat and carbohydrate utilisation

RER refers to the ratio between of carbon dioxide produced in metabolism vs the quantity of oxygen utilised. There were no effects of time on RER during the 5 hour infusion in any conditions (Figure 15). There was a trend for condition effects (P<0.1).

During the caffeine placebo condition (CARN) RER trended (P<0.1) to be lower than CAFF. Average fuel utilisation during the 5 hours in CARN condition was 60% fat and 40% carbohydrate (Figure 16).

During the carnitine placebo condition (CAFF) average fuel utilisation was 53.3% fat and 46.7% Carbohydrate. This trended (P<0.1) to have increased carbohydrate and decreased fat utilisation compared to the CARN condition.

During the C&C condition average fuel utilisation was 56.7% fat and 43.3% carbohydrate. This was not different to either CARN or CAFF conditions.

Figure 15.

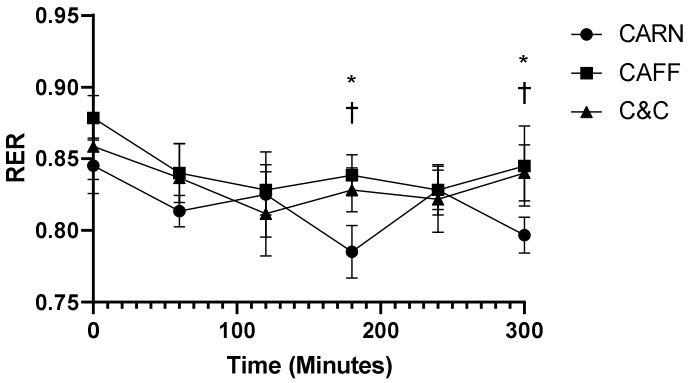

*Fig. 15. RER during a 5 hour infusion of L-Carnitine or Saline. *, (P≤0.05) CAFF greater than CARN. †, (P<0.1) C&C greater than CARN. No differences between CAFF and C&C at any time and no differences between groups at baseline.*

Figure 16.

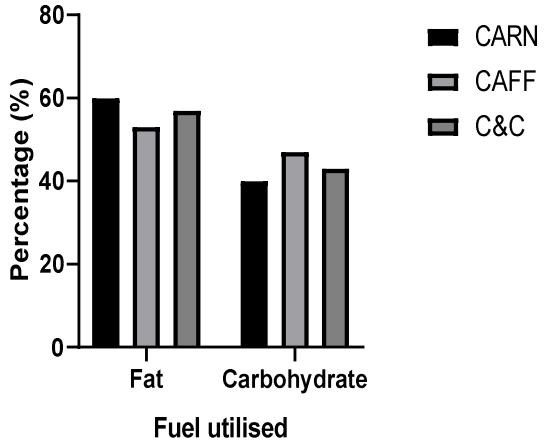

Fig. 16. Average fuel usage in percentage during a 5 hour infusion of L-carnitine with or without caffeine capsules. There was no significant difference in fuel usage between conditions.

3.2 Muscle and Exercise data (n=2)

3.2.1 Muscle carnitine

Total carnitine data was measured but excluded due to high variability in duplicates during analysis. Muscle free carnitine data was therefore examined instead. Stats were not run as data is n=2.

Caffeine appears to reduce muscle free carnitine (Figure 18) and this reduction appears to be blunted by the carnitine infusion. Total carnitine and acetyl carnitine data would be required to examine whether muscle total carnitine has been increased via caffeine augmented carnitine uptake. Free carnitine was reduced in all groups, with minor decreases in the no caffeine condition and a large decrease with the presence of caffeine alone (-0.5mmol/kg CARN vs -1.8mmol/kg CAFF). However, this decrease was largely attenuated by a state of hypercarnitinemia (-0.9mmol/kg C&C). After ~14 hours post infusion caffeine alone appeared to continue to acetyl the free carnitine pool, whilst carnitine only remained unchanged and C&C returned towards baseline (CAFF -2.3mmol/kg vs CARN +0.6mmol/kg vs C&C -0.3mmol/kg). The highest absolute pre-exercise muscle free carnitine (~14 hours post infusion) value was obtained in the C&C group (CARN 10.7mmol/kg, CAFF 10.7mmol/kg vs C&C 12.5mmol/kg).

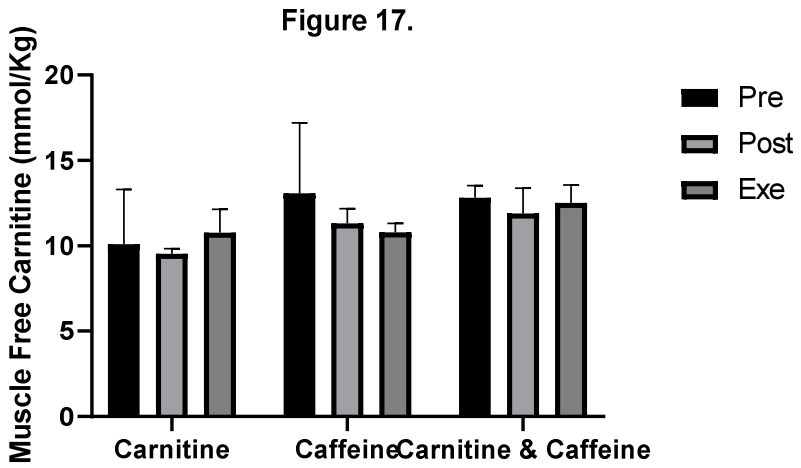

Fig. 17. Muscle free carnitine immediately pre and post infusion and ~14 hours post infusion proir to exercise. Caffeine appears to reduce muscle free carnitine post infusion and continues to reduce free carnitine ~14 hours later. This decline appears to be attenuated in the caffeine and carnitine group.

3.2.2 Pre-exercise plasma carnitine

Plasma carnitine was measured ~14 hours post infusion pre-exercise (Figure 18).

Carnitine placebo (CAFF) was unchanged from end of infusion T=300 to pre-exercise (T=300 59.9 µmol L-1 vs 52.8 µmol L-1 pre-exe) as expected.

Caffeine placebo (CARN) decreased from end of infusion value but remained elevated compared to CAFF condition (T=300 391.1µmol L-1 vs 94.5µmol L-1 pre-exe).

In the experimental condition (C&C) plasma carnitine decreased from end of infusion but remained elevated compared to start of infusion baseline and CAFF condition (T=300 356.5µmol L-1 vs 68.1µmol L-1 pre-exe).

Plasma carnitine in CAFF remained at baseline values, in the CARN condition it was still largely elevated after 14 hours whilst C&C remained elevated above that of CAFF but had decreased further than that of CARN.

Figure 18.

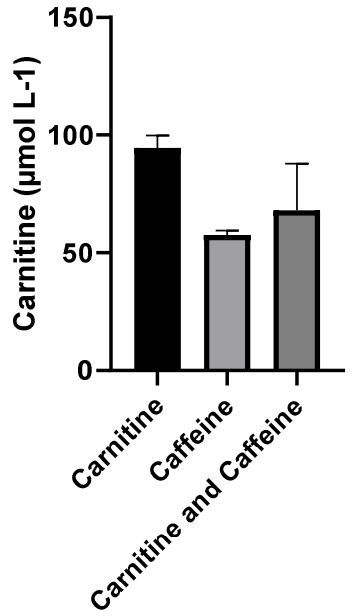

Fig.18. TC concentration in plasma ~14 hours post carnitine infusion and caffeine capsules in each condition. CARN is elevated vs CAFF and C&C. The rise in C&C above CAFF is attenuated vs CARN.

3.2.3 Whole blood data

3.2.3a Exercising glucose

There was no difference in baseline glucose levels between conditions (~4.19±0.22mmol/ L).

During 50% exercise intensity there were no differences in glucose levels between conditions however glucose had slightly increased from baseline (~4.37±0.22mmol/L).

During 75% exercise intensity there were no large differences in glucose levels between all conditions however glucose had increased slightly further from baseline above and beyond that of 50% (~4.57 ± 0.53).

Although the differences were very minor, glucose in C&C condition appeared marginally higher during 75% when compared to the CARN and CAFF conditions (Figure 19).

Figure 19.

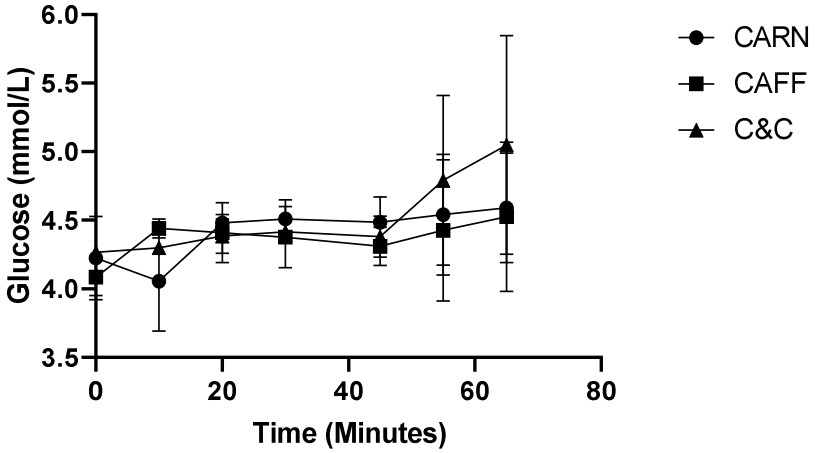

Fig. 20. Whole blood glucose levels during exercise protocol. There were no significant effects of time or condition on blood glucose levels.

3.2.3b Exercising lactate

There was no difference in baseline lactate levels between conditions (~0.61±0.05mmol/ L).

During 50% exercise intensity there were again no differences in lactate levels between conditions however lactate had increased from baseline (~1.03±0.12mmol/L).

During 75% exercise intensity there were no differences in lactate levels between all conditions however lactate had increased further from baseline above and beyond that of 50% (~3.57 ± 1.13).

It is worth noting that, similarly to glucose, although the differences were very minor, lactate in C&C condition appeared marginally higher at all time points (including baseline) when compared to CARN and CAFF conditions.

Figure 20.

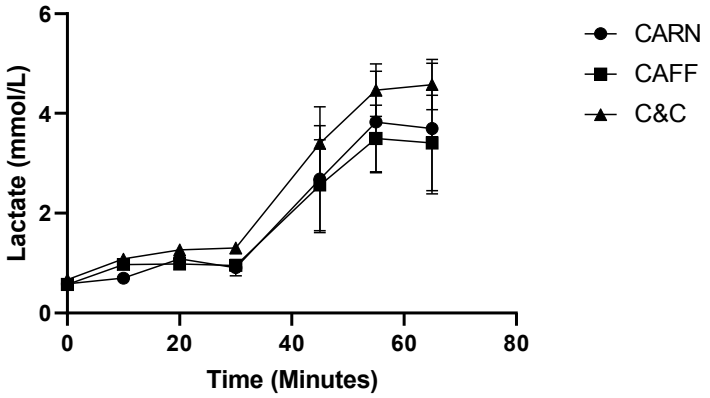

Fig.19. Whole blood lactate during 30 minutes exercise at 50% V02 Max and 30 minutes at 75% V02 Max. Lactate was higher during 75% compared to 50% in all groups. There were no differences between groups however C&C condition appeared slightly higher than both groups at all time points.

3.2.4 Heart rate

Baseline heart rate was not recorded in any groups.

During 50% exercise intensity there were no major differences in heart rate between conditions (~118 bpm).

During 75% exercise intensity there were no large differences in heart rate between all conditions however heart rate had increased above that at 50% (~169bpm).

Heart rate in the C&C condition appeared marginally higher during both 50% (121bpm) and 75% (172bpm) when compared to average and CARN and CAFF conditions (Figure 21).

Figure 21.

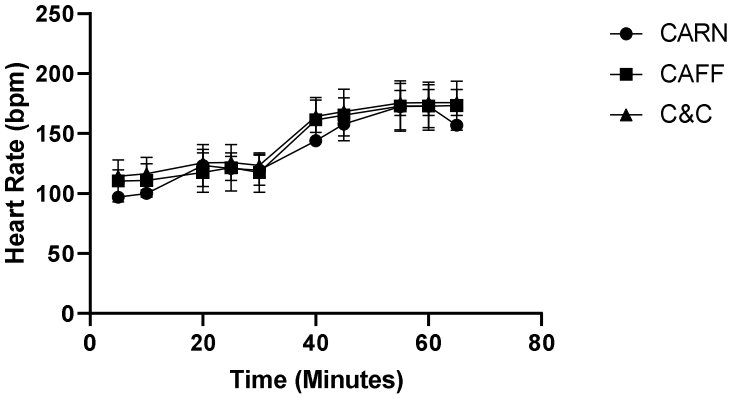

Fig. 21. Heart rate (bpm) during exercise protocol. No clear differences between groups in this small population subset. Heart rate was elevated at 75% compared to 50% in all conditions.

3.2.5 Respiratory exchange ratio (RER) and fat and carbohydrate utilisation

Baseline RER was similar between CAFF and C&C conditions (~0.8) whilst CARN had a slightly elevated baseline RER (0.85) (Figure 22). This indicated a 50:50 fat:carbs fuel utilisation in CARN condition whilst both CAFF and C&C utilised ~60:40 fat:carbs.

During 50% exercise intensity combined RER increased to 0.86 (baseline 0.82). This indicated an increased shift towards carbohydrate metabolism. All conditions demonstrated increased carbohydrate usage at 50% (CARN + 7%, CAFF +6%, C&C +23%.

During 75% exercise intensity combined RER between all groups increased further to 0.93, indicating a further shift towards carbohydrate metabolism. All conditions demonstrated increased carbohydrate usage compared to 50% (CARN +24%, CAFF+ 26%, C&C+24%).

RER in the C&C condition appeared higher during both 50% (carbohydrate usage 56.7%) and 75% (carbohydrate usage 80%) when compared to average and CARN and CAFF conditions (Figure 23).

Figure 22.

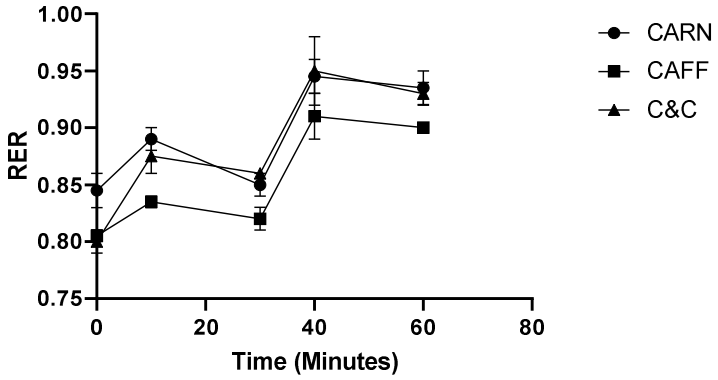

Fig.22. Respiratory exchange ratio (RER) during exercise protocol. A distinct increase in RER from exercise at 50% V02 compared to exercise at 75% V02 occurs. CAFF appears to have a lower RER at all time points.

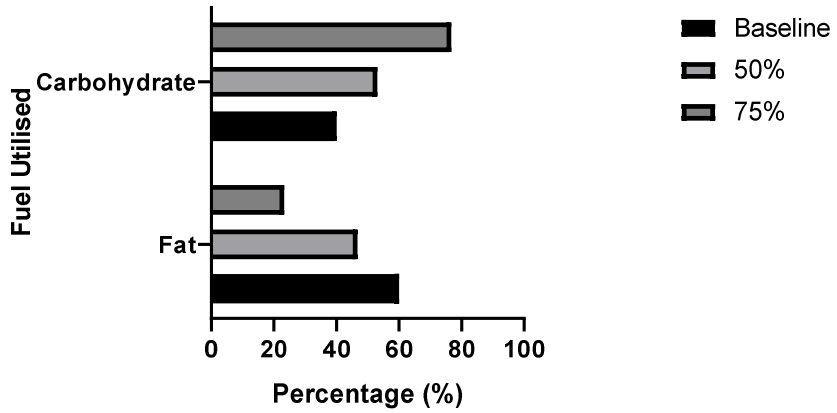

Fig. 23. Average fuel utilised (%) during each exercise stage with all conditions combined. A distinct shift from fat to carbohydrates takes place as exercise intensity increases.

3.2.6 Rating of perceived exertion (RPE)

Baseline RPE was identical between all conditions (RPE=7).

During 50% exercise intensity RPE increased in all groups (CARN=8.7, CAFF=9.2, C&C = 9.5).

During 75% exercise intensity RPE continued to increase in all groups (CARN=14.3, CAFF =14.6, C&C= 14.9).

Whilst all conditions were similar (±1). RPE in the C&C condition was marginally higher during both 50% and 75% (Figure 24).

Figure 24.

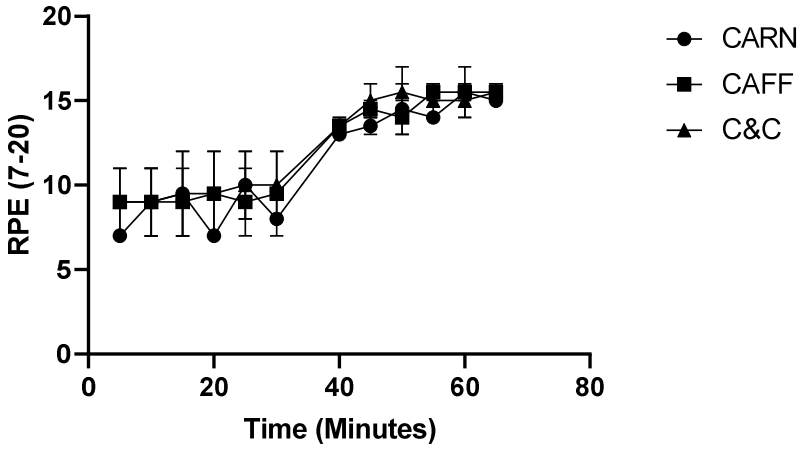

Fig.24. Rating of percieved exertion as measured using the BORG scale. Little noticible effects of condition on RPE were present. RPE appeared to increase significantly from 50% V0^2 exercise compared to 75%.

3.2.7 Power output (Kj)

In the carnitine placebo condition (CAFF) the average power output was 147.5Kj. This was almost identical to the caffeine placebo condition (CARN) 147.8Kj. The C&C condition was markedly lower (138.1Kj) (Figure 25). This was mainly due to a decrease in subject 2 power output (CARN 172.5, CAFF 171.7 vs C&C 159.9) however subject 1 also demonstrated a more reserved decline in the C&C condition (CARN 123.2, CAFF 123.4 vs C&C 116.3).

Figure 25.

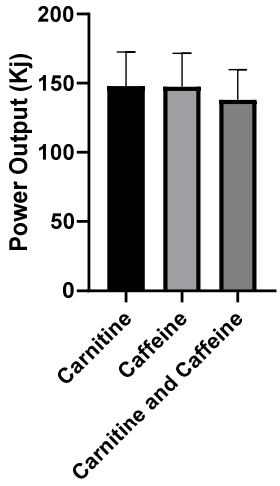

Fig. 25. Power Output (Kj) during a 15 minute TT. C&C condition appears to have a slightly lower Power output compared to both CARN and CAFF conditions.

3.2.8 Sleep

Caffeine did not affect sleep time significantly (Figure 26) however it is worth noting sleep quality was not assessed. CARN had the shortest sleep time (418 minutes) compared to both CAFF (448 minutes) and C&C (459 minutes). It is therefore unlikely that caffeine reduced sleep time per se which may potentially have directly influenced exercise performance.

Figure 26.

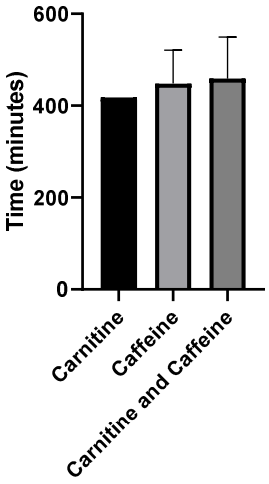

Fig.26. Sleep data from GeneActiv accelerometer. No large differences in sleep time between any groups. CARN had a slightly lower sleep time vs CAFF and C&C. Caffeine did not therefore reduce sleep time after an infusion test day.

Chapter Four: Discussion, conclusions and suggestions for future work

4.0 Introduction to Discussion

This chapter provides an overview of the major experimental findings of this thesis. This chapter links the findings to current and previous research and explains the importance of any major findings. This chapter also provides an insight into the rationale behind many of the effects (or lack of) seen. We finally discuss the relevance of our findings and provide suggestions about how future research could be directed to further elucidate the effects of caffeine on carnitine accumulation. The final section of this thesis draws together all our experimental work, explains the major findings and provides a comprehensive conclusion to our work.

4.1 Summary of Purpose of Research

The purpose of this thesis was to investigate optimising the ergogenic effects of L-carnitine with our primary aim being to seek a safe mechanism of increasing skeletal muscle carnitine accumulation as an alternative to that of the current effective but inconvenient and potentially unhealthy carbohydrate/ insulin methodology currently utilised. We theorised that caffeine ingestion would activate similar mechanistic pathways to that of insulin thus we proposed caffeine would augment skeletal muscle carnitine uptake via a similar mechanism. To investigate this theory, an initial proof of concept study was undertaken to provide a detailed look at plasma carnitine kinetics and obtained some pilot data to provide a brief insight into muscle and exercise data. The proof of concept study examined ingesting high dose caffeine whilst in a state of hypercarnitinemia and its effects on steady state plasma total carnitine levels and plasma carnitine kinetics, it further explored the mechanistic effects of

caffeine supplementation on certain electrolyte activity. The additional pilot data looked directly at a number of measures of skeletal muscle carnitine content and further examined any potential effects on exercise performance and metabolism. This thesis addressed the following research questions:

1) Does caffeine supplementation augment indirect markers of human skeletal muscle carnitine accumulation under hypercarnitinemia?
2) Does caffeine stimulate Na^+/K^+ flux thus likely lead to increased Na^+/K^+ -ATPase pump activity?
3) Do any changes in plasma carnitine relate directly to muscle data and does this appear to have an impact on exercise performance (albeit in pilot data)

4.2 Discussion of Findings: Part 1 – Infusion and Major Findings

This section summarises the major findings in the proof of concept study and pilot data including an explanation for the changes in plasma carnitine, plasma caffeine and measured whole blood readings including Na^+ and K^+. The effects of caffeine and carnitine on muscle free carnitine and exercise performance and markers of metabolism will also be examined in a small population sub set. This novel study is the first to demonstrate that oral caffeine ingestion can alter plasma carnitine kinetics. Oral caffeine ingestion under hypercarnitinemia increased the rate of total carnitine clearance from the plasma by 9.2% and appeared to decrease steady state plasma carnitine concentration by 10.2%. We have also demonstrated that caffeine appears to increase Na^+/K^+ -ATPase activity as witnessed via proportionately decreased K^+ and increased Na^+ in caffeine supplemented conditions. This is the first study to our knowledge that

has demonstrated significant changes in plasma carnitine in vivo during resting conditions without utilising insulin clamp or carbohydrate protocols.

4.2.1 Plasma carnitine kinetics

4.2.1a Steady state plasma carnitine

As would be anticipated regardless of caffeine status a carnitine infusion significantly increased plasma carnitine compared to baseline and the caffeine only control.

Whilst steady state plasma carnitine was 10.2% lower throughout the infusion when carnitine was combined with caffeine these values did not reach overall significance. However, the difference between CARN and C&C in the time points towards the end of infusion trended towards significant ($P<0.08$). A failure to achieve overall significance can be a result of a number of factors. Firstly, that there was genuinely no effect. Secondly, that the effect size was so small that it would never reach significance at any level or thirdly that the population sample size (n=6) was too small for the effect size obtained to be statistically significant despite a real difference being identified. We propose that the third scenario is the most likely. The failure to achieve significance despite a relatively large difference in values combined with small SEM potentially resulted from the slightly underpowered nature of the study as opposed to the lack of effect size from caffeine. Our initial GPower calculations for study size were based on data from Stephens et al (2006) (where insulin significantly reduced steady state plasma carnitine) which produced an effect size dz of 0.843 with a suggested sample size of 14 participants required for significance. However due to unforeseen recruitment and timing issues we were only able to examine 6 participants in the study timeframe, likely creating a slightly

underpowered study design. To investigate this hypothesis, a further GPower calculation power assessment was performed, utilising our plasma carnitine data this returned an effect size dz of 0.958 and advised that steady state plasma carnitine would reach statistical significance with a sample group of 11 compared to the 6 we analysed. Assuming a similar trend is replicated and can be extrapolated in additional participants we can tentatively conclude that there is a trend for a lower steady state plasma carnitine in groups consuming caffeine and with a marginally larger sample size caffeine would show significant decreases in steady state plasma carnitine concentration under hypercarnitinemia (potentially to a greater degree than that of insulin due to the larger dz).

4.2.1b Rate of total carnitine clearance and tissue uptake

Rate of total carnitine clearance from plasma was calculated as the rate of elimination divided by the steady state plasma concentration (Stephens et al, 2006). The plasma total carnitine concentrations during caffeine ingestion was at a steady state throughout the infusion at 351.9 μmol.min-1 and the rate of elimination was ~ 72μmol.min-1. Rate of elimination was calculated for each participant individually as ((bodyweight*carnitine infusion mg/kg/hr)/MW of carnitine/60)*1000. Rate of clearance was then calculated as: Rate of elimination/Steady state concentration*1000. As the rate of carnitine infusion was equal to its rate of elimination thus clearance was calculated as 205ml.min-1 (C&C) compared to 187.9ml.min-1 (CARN). This demonstrated a significantly increased rate of carnitine clearance from the plasma (9.2%) in the caffeine and carnitine group.

Rate of tissue uptake was further calculated assuming renal clearance calculated to be ~100ml.min-1 (Pace et al, 2000) giving a difference of

105ml.min-1 in C&C condition. Rate of tissue uptake equals plasma concentration x plasma clearance therefore tissue uptake rate was calculated using the following equation: (Steady state concentration* (rate of carnitine clearance from plasma-100))/1000). Tissue uptake rate was calculated as ~33 µmol.min-1 CARN condition and was significantly greater in the C&C condition at ~37 µmol.min-1. This can be calculated as a hypothetical skeletal muscular carnitine increase of ~1.7g during the 5 hour infusion (discounting bolus dose) when carnitine was combined with caffeine. With skeletal muscle carnitine content estimated to be ~20g (Shannon et al, 2016) we can estimate that hypercarnitinemia when combined with oral caffeine ingestion has increased the body's total carnitine by approximately 8.5% in 5 hours. Due to the mechanistic action of caffeine we can reasonably assume that plasma carnitine clearance is likely to remain elevated for a period post infusion. Stephens et al (2006) calculated from plasma carnitine data that during hyperinsulinemia there was a 10% increase in muscle carnitine, which was supported directly by a 13% increase in muscle total carnitine. Our data appear to directly correlate to these findings published by Stephens et al thus indicating the rates of uptake we see are augmented above that of normal rates and likely will have led to increases in muscle carnitine. Whilst urinary carnitine clearance was not examined in this study, previous work utilising a similar protocol identified no differences in urinary total carnitine clearance during a 5 hour carnitine infusion (Stephens et al, 2006), this suggests that any changes identified in plasma total carnitine concentration throughout the infusion are unlikely to be due to increased urinary carnitine excretion. Furthermore, when plasma carnitine exceeds normal range of ~60umolL the high capacity renal clearance of plasma appears to become saturated. Stephens et al (2006) hypothesised that during a 5 hour infusion of

carnitine plasma carnitine filtration is at maximum and renal carnitine reabsorption is saturated therefore explaining why changes in urinary carnitine excretion between conditions would be expected if muscle carnitine was not increased. As 95% of the body's carnitine is stored within muscle it stands to reason that due to the saturation of renal carnitine reabsorption and the probability of unchanged urinary carnitine excretion therefore the changes in plasma carnitine are likely to also be mirrored directly proportionately within the muscle.

4.2.1c Plasma carnitine pre-exercise

Plasma carnitine was also examined pre-exercise ~14 hours post infusion (CARN 94.5± 5.2 µmol L-1, CAFF 57.5± 2.8 µmol L-1and C&C 68.1 ± 19.8 µmol L-1). Whilst these were marginal changes in a small sample group (n=2) we can theorise potentially interesting findings. Firstly, as expected caffeine only was almost identical to previous day's measures. Secondly, whilst carnitine alone decreased significantly from its previous steady state peak it did appeared to remain elevated above baseline. This implies that carnitine in the plasma had not been fully cleared by the following day, suggesting saturated renal clearance throughout the 14 hours. The caffeine and carnitine group (which we calculated to have a significantly higher rate of plasma carnitine clearance) had a lower plasma carnitine pre-exercise than carnitine only (which would support this hypothesis). The caffeine and carnitine plasma carnitine value is also almost identical to that Stephens et al (2006) obtained 24 hours after an infusion of carnitine under hyperinsulinemia conditions which was shown to increase muscle carnitine (68.1 µmol L-1vs 72.9 µmol L-1 respectively). The higher carnitine only value compared to carnitine and caffeine suggests that the plasma carnitine clearance rate has remained elevated post carnitine infusion/

caffeine ingestion and therefore may indicate that skeletal muscle carnitine uptake is also increased for a number of hours post infusion thus muscle carnitine may be still be elevated the following morning This data indicates that renal clearance has been at maximum for the previous 12 hours therefore the lower value indicated in the C&C suggests increased plasma carnitine transport continues for a number of hours post infusion which we can hypothesise has likely been transported into muscle tissue.

Chronic oral carnitine supplementation was demonstrated to significantly increase circulating plasma carnitine alongside increased muscle carnitine content after 12 weeks and 24 weeks (Wall et al, 2011) compared to a placebo, our data is in line, acutely, with these findings.

4.2.2 Caffeine data

4.2.2a Plasma caffeine

Whilst research has previously looked at caffeine, its effects on plasma and its washout period there is limited research examining the ability to maintain 'steady state' plasma caffeine over a number of hours via oral supplementation. To the best of our knowledge this is the first study to have maintained an elevated steady state plasma caffeine for 5 hours (~7.7 ± 0.3 µg/mL) C&C and (7.2 ± 0.2 µg/mL) CAFF. This was a critical requirement of our study as a failure to raise plasma caffeine levels sufficiently would be unlikely to optimally stimulate any potential effects of caffeine on Na^+/K^+ -ATPase pump activity. We were able to confirm that an elevated caffeine plasma concentration was maintained for 5 hours thus created an optimal environment for the hypothesised impact on carnitine uptake (it was previously identified that 6mg/kg dosages were required to alter K^+/ Na^+ activity and we were

successfully able to maintain plasma caffeine at a level obtained during 6mg/kg caffeine ingestion throughout the entire 5 hours with the use of 'top up' maintenance doses). There were no significant differences in plasma caffeine between the caffeine and caffeine and carnitine groups at any time point indicating a reliable and repeatable methodology to stimulate steady state plasma caffeine. There was also no effect of time (from T=30 to T=300) showing that we were able to successfully instigate a steady state of elevated caffeine plasma for 5 hours via one initial 6mg/kg dose with top up 1mg/kg doses at T=120, T=180 and T=240. The methodological timing of caffeine ingestion we used prevented plasma caffeine falling too low (despite its rapid rate of clearance) yet avoided a cumulative build-up. The majority of previous research that investigates caffeine's ability to alter Na^+/ K^+ levels haven't directly measured plasma caffeine, however it appears a single dose of around 400mg or 6mg/kg is required for short term enhanced pump activation (Lindinger et al, 1993). Doses of 6mg/kg appear to peak in the plasma at ~7.7ug/mL (40 umol/m) (Spreit et al, 2014; Graham and Spreit, 1995) and our average steady state caffeine of 7.7ug/mL (C&C) follows exactly the dosage response curve expected from 6mg/kg. Since we successfully maintain plasma caffeine at this value for 5 hours (never decreasing significantly) we propose that assuming reactions and effect size occur linearly, any effects on Na^+/K^+ activity that a single 6mg/kg dose may have will be prolonged for the duration of this time period.

Caffeine appeared slightly but not significantly elevated from baseline ~14 hours post infusion (C&C and CAFF ~ 0.49ug/ml vs 0.01ug/ml CARN) n=2. These values appear lower than that required to stimulate performance

enhancements (Spriet, 2014) so any changes in exercise performance during study two are unlikely to be the result of direct caffeine mediated pathways.

4.2.2b Caffeine metabolites

It has been proposed that whilst the activation of the Na^+ /K^+ -ATPase pump may occur directly via increased plasma caffeine, the metabolites of caffeine may also play a role in this process. Therefore it was also crucial that alongside increased plasma caffeine we were further able to elevate the key metabolites of caffeine. Plasma paraxanthine was increased by 2281% (CAFF) and 4300% (C&C) linearly in both caffeine conditions at T=300. The greater percentage increase in C&C condition is likely due to the lower baseline values as peak values at T=300 were near identical between the two conditions. Paraxanthine remained significantly elevated ~14 hours post infusion. Plasma theophylline also increased by 2100% (CAFF) and by 1200% (C&C) at T=300 and continued to increase after 14 hours. Both paraxanthine and theophylline peaked towards the end of the infusion, which is also when the greatest differentiation in plasma carnitine values were identified, this may indicate an interesting mechanistic possibility that caffeine metabolites may be, at least in part, a key determinate of caffeine augmented skeletal muscle carnitine uptake. Caffeine metabolites remained elevated ~14 hours post exercise and could potentially have caused alterations in exercise performance, however if caffeine metabolites do alter Na^+/K^+ -ATPase pump activity then it stands to reason pump activation may have been elevated for a prolonged time post infusion thus leading to an even greater skeletal muscle carnitine accumulation than would be initially anticipated.

Theobromine appears to be the slowest major metabolite of caffeine and was unchanged throughout the 5 hours infusion in all conditions. We can therefore

conclude that the effects of theobromine on Na^+/K^+ -ATPase pump activity, intracellular Na^+ flux and carnitine transport are likely to be negligible as changes in plasma carnitine and whole blood electrolytes occurred long before any increases on plasma theobromine. This thesis further increases the understanding of caffeine metabolites and their distinct roles in Na^+/ K^+ activity.

4.2.3 Whole blood data

Whilst previous measures were examined from arterialised venous blood that had been centrifuged and separated into plasma the following measures were examining instantly in the whole blood (no centrifugation) using NOVA and YSI devices.

4.2.3a Sodium (Na^+)

Whilst previous data has generally focused on the effects of caffeine on plasma K^+ (Lindinger et al,1993) which has led to the hypothesis that any changes seen were due to increased Na^+/K^+ -ATPase pump activity there remains limited data looking directly at Na^+. We were able to identify a significant effect of time and condition on whole blood Na^+ over the 5 hour period in which 9mg/kg caffeine was consumed. CARN Na^+ was visibly lower at every time point (excluding baseline) than both caffeine conditions. Whole blood Na^+ concentration over the 5 hour infusion was significantly higher in the caffeine conditions (C&C 138.1 mmol/L ($P<0.1$), CAFF 138.2 mmol/L ($P<0.05$) vs CARN 137.6 mmol/L). These differences indicate that caffeine likely stimulates Na^+ flux leading to increased presence of whole blood Na^+. This is one of the first papers to demonstrate caffeine ingestion alone can directly influence blood Na^+ during resting conditions in vivo. Combined with other previous works that have identified directly opposing effects of decreased K^+ during caffeine ingestion alongside in

vitro work that has noted similar Na^+ findings (Lindinger et al, 1996) we can hypothesise that the increased whole blood Na^+ is due to caffeine (or its metabolites) causing increased activation of Na^+/K^+ -ATPase pump activity. It is interesting to note that whilst the caffeine only group was significantly greater than carnitine alone, Na^+ in the caffeine and carnitine group appeared to have a slightly attenuated increase in whole blood Na^+ which could suggest an increased Na^+ dependent transport of carnitine due to the hypercarnitinemia conditions thus leading to less Na^+ in whole blood, the changes are very small however and this is purely a theoretical observation.

4.2.3b Potassium (K^+)

Caffeine significantly lowered whole blood K^+ values throughout the 5 hours (C&C and CAFF 4.1mmol/L vs 4.3 CARNmmol/L). This finding is directly in line with previous research both in vivo (Lindinger et al, 1993) and in vitro (Hawke et al, 1999) that have shown caffeine or its metabolites either directly reduce K^+ or attenuates the exercise induced rises in K^+. Our findings, when combined with previous research and the combined effects on Na^+ during infusion, present a strong case for us to hypothesis that high dose caffeine ingestion increases Na^+/K^+ -ATPase pump activity thus increasing electrolyte flux.

We have demonstrated that caffeine both significantly increases Na^+ concentration whilst simultaneously decreasing K^+ concentration identical to the function of the Na^+/K^+ -ATPase pump (Castillo et al, 2015) and demonstrates the likely scenario that would occur if pump activity was increased (as demonstrated by the exercise model whereby exercise augmented pump activity leads to greater Na^+:K^+ ratios than at rest (Lindinger et al,1993).

4.2.3c Glucose during infusion

There were no significant effects of any condition on whole blood glucose. Whilst C&C and CAFF conditions had a slightly lower steady state concentration compared to CARN this minor change wasn't significant and with a limited group size we can't draw any conclusions from this finding. We may have expected glucose to increase in caffeine only groups signifying a shift towards glucose metabolism with an inverse shift in the carnitine and caffeine group if, as hypothesised a shift towards fat metabolism occurred (Stephens et al, 2006). It has been demonstrated that habitual high caffeine intake can increase fasting insulin concentrations but has no influence on resting glucose levels (Van Dam et al, 2004). Caffeine when ingested alongside carbohydrates tend to decrease glucose disposal (Greer et al, 2001), this increase in blood glucose is suggested to be due to an acute interference of caffeine on insulin action (Pizziol, 1998) and may be variable depending on caffeine tolerance and fitness state. Due to the previously discussed research showing insulin augments skeletal muscle carnitine uptake one concern was that caffeine's potential negative impact on insulin action may reduce the current steady state rate of carnitine uptake, however in our experiment the lack of significant changes in glucose levels indicates that the acute influence of caffeine ingestion on glucose and therefore most likely insulin was minor.

4.2.3d Lactate during infusion

Previous literature suggests that caffeine appears to slightly accelerate the onset of blood lactate accumulation during incremental exercise (Gaesser and Rich, 1985) although there is limited research examining caffeine's influence on resting blood lactate values. Our research tentatively supported increased lactate levels with caffeine ingestion even at rest with whole blood lactate in

both caffeine groups trending to be higher than carnitine alone. This may be due to caffeine causing minor increased availability of glycogen (Laurent et al, 2000) thus slightly increasing lactic acid, potentially through stimulation of the cori cycle where lactate is produced by anaerobic glycolysis (Rogatzki et al, 2015). If lactate did significantly alter metabolism this may interfere or mask any potential metabolic effects that carnitine may produce however the changes in lactate throughout our infusions appear minor and we hypothesis would be unlikely to significantly alter metabolism.

4.2.3e Haematocrit (HCT)

Haematocrit is defined as the percentage of red blood cells in the blood. Therefore, decreased hydration status theoretically is indicated by increased haematocrit and visa/versa. All participants consumed the same amount of water orally throughout the lab visit (200ml) and received ~500ml saline to ensure the hand cannula remained patent with an additional average total carnitine/placebo infusion of ~380ml including bolus for a total of ~1080ml over a ~6hour period. Haematocrit was only recorded in 4 participants, however there was a trend effect of condition (P=0.1). During infusion the carnitine only condition had a significantly lower HCT at every time point compared to both caffeine containing groups. Although in a limited group size these results strongly indicate an effect of caffeine on whole blood haematocrit. As all conditions had similar volume of fluids this implies caffeine may have possibly increased dehydration status. This is supported by previous findings which have identified a minor diuretic effect of caffeine (Armstrong, 2002). This diuretic effect potentially led to increased urination in both the caffeine conditions thus decreased plasma volume and therefore increased haematocrit percentage. Whilst previous research shows that the mild diuretic effect of caffeinated drinks

is counterbalanced by the fluid it is consumed in (e.g coffee) due to the method of ingestion (capsules) and limited fluid intake throughout the day this could reasonably account for the changes in HCT. This could potentially have impacted the Na^+/K^+ -ATPase (our proposed methodology of carnitine transport) as dehydration is shown to cause electrolyte imbalance (El Sharkaway et al, 2013) which could affect pump activity. However as HCT never went outside normal range values (52% for men and 48% for women) and the difference in condition only appeared to trend rather than achieve significance, in conjunction with an increased whole blood K^+ we can assume that it is unlikely that under these conditions a sufficient state of dehydration to influence the Na^+:K^+ balance had taken place.

4.2.4 RER during infusion

Respiratory exchange ratio (RER) can be used to indicate and therefore calculate the proportion of fuel utilised in which a lower RER is indicative of fat metabolism, whilst a higher RER shows increased carbohydrate metabolism. An RER OF 0.7 suggests exclusively fat metabolism whilst 1 and above indicates carbohydrates are being used as the primary energy source. Caffeine trended to increase RER compared to carnitine over the 5 hour period (0.82± 0 vs 0.84±0) but this shift towards carbohydrate metabolism appeared to be blunted in the caffeine and carnitine group (0.83± 0). Whilst these were only minor changes if extended across a larger sample size it would support the theory that increased skeletal muscle carnitine shifts towards increases fatty acid metabolism and attenuates the caffeine stimulated increase in RER caused by increased glycolysis, this is tentatively supported by the small changes in lactate data we observed. There is mixed discussion on whether caffeine per se directly alters RER and whilst our results propose an interesting theoretical

observation, due to the insensitivity of indirect calorimetry, the current literature debate on caffeine's effects on RER and the small effect size means no firm conclusions on this can be drawn. However, it certainly doesn't exclude the theory that caffeine has augmented skeletal muscle carnitine uptake which in turn has increased fat metabolism thus blunting the shift towards carbohydrate metabolism stimulated via caffeine intake.

4.3 Discussion of Findings: Part 2 – Exercise and Muscle Data

Pilot exercise data was obtained to give an indication whether caffeine had augmented skeletal muscle uptake as observed directly within muscle tissue and then to further investigate if it had remained increased by the following morning and therefore had any effects on exercise performance and muscle metabolism. As all values are N=2 there is of course a possibility that any results are anomalies, errors or not indicative of a large data set therefore results from all data are supportive of that of the proof of concept infusion results and act as pilot testing for future studies as opposed to providing conclusive data.

4.3.1 Muscle carnitine

Muscle total carnitine and muscle free carnitine were measured from muscle samples taken by biopsy from the vastus lateralise muscle. All samples were run as repeats to ensure reliability of measures. Unfortunately, there was a number of discrepancies in repeat measures for muscle total carnitine, potentially due to the K0H stage of the reaction not fully freeing the carnitine within the cells. All total muscle carnitine data was therefore deemed unreliable and excluded from the study. Due to the issues described in obtaining muscle total carnitine data, muscle free carnitine (the amount of 'available' carnitine)

was therefore measured. The free carnitine duplicates were able to be replicated accurately and reliably. Free carnitine data interestingly appears to show that caffeine may be acetylating the carnitine pool thus reducing the free carnitine. This effect however appears to be blunted by the carnitine infusion. The first and obvious observation from this is that C&C does appear to influence muscle carnitine kinetics and whilst from this data we can't categorically say it is due to increased muscle TC we can see that plasma carnitine is decreased leading to increased plasma carnitine uptake followed by a significant difference in muscle free carnitine in the C&C group compared to both CARN and CAFF. Caffeine is hypothesised to activate cAMP and it is also thought that under conditions of high PDC flux muscle free carnitine is generally decreased (Shannon et al, 2016) therefore caffeine may be increasing the acetylation of carnitine via increasing PDC flux. Increased plasma carnitine clearance may lead to increased total carnitine, therefore the difference between C&C and CAFF may be explained by a larger baseline pool of free carnitine. There is also a possibility that although total carnitine is likely to have increased (based on our plasma carnitine findings and that the decrease in free carnitine are significantly blunted in the C&C group compared to CAFF), due to the reductions in free carnitine, this may potential alter the baseline muscle carnitine ratio which may have an impact on exercise performance and metabolism thus may be different to that of insulin augmented carnitine uptake due to the different ratios of free and acyl carnitine. Spriet et al (1992) identified no significant changes in acetyl carnitine levels despite participants consuming 9mg/kg caffeine which appears in contradiction to our findings. However, looking at the raw resting data from this study acetylcarnitine (mmol/kg) increases non-significantly with caffeine ingestion (Placebo 3.2 vs 4.9 CAFF)

and free carnitine decreases (Placebo 20.3 vs 18.4 CAFF) in a similar nature to our study. Whilst these changes were also small (potentially not significant as 'masked' by the far larger changes that occurred during exercise) it is interesting to note the similarities between our findings. This potentially increased acetylation of carnitine is a novel finding and worth investigating, especially as caffeine has become so widely used as an ergogenic aid in sports performance. During high intensity exercise the primary role of carnitine is in acetyl group buffering via the formation of acetyl carnitine and maintaining free CoASH levels which are critical for mitochondrial flux (Harris and Foster, 1990). During exercise there is significant depletion of free carnitine pool therefore the improved buffering capacity may rely on increased total carnitine levels also having a proportionate increase in muscle free carnitine (Wall et al, 2011) and if, as possibly indicated, that caffeine's mechanism of action increases acetylation of the free carnitine pool which therefore may reduce the buffering capacity thus potentially decreasing the effectiveness of this mechanism to alter high intensity exercise performance.

4.3.2 Whole blood data

4.3.2a Exercising glucose

Whole blood venous glucose demonstrated minor increases from baseline to 50% and further small increases at 75%. These increases likely indicate glucose being released into the blood stream to support the exercising muscles. Blood glucose in all conditions at 50% were similar ~4.4mmol/L suggesting no differences (or at least nothing that was sensitive enough to be picked up by whole blood glucose). At 75% glucose was maintained at 4.4mmol/L in the caffeine only group but increased slightly to 4.5 in carnitine only and further increased to 4.7mmol/L in the C&C condition. This implies slightly more glucose

was being used during exercise at 75% in this condition. It is however a small change in a limited group thus the effects are likely to be negligible. Wall et al (2011) noted that with increased muscle carnitine, participants had a 55% reduction in muscle glycogen utilisation at 50% $V0_2$max compared to a control condition, likely due to augmented skeletal muscle lipid oxidation (thus blunted PDCa and glycolytic flux). An inhibited PDCa via a carnitine mediated increase in acetyl- CoA would likely reduce muscle carbohydrate kinetics as directed by the Randle cycle (Randle et al, 1963). Whilst we have no access to muscle glycogen data therefore are conclusions are limited it appears unlikely that similar findings were noted in our pilot data.

4.3.2b Exercising lactate

Lactate was recorded every 10 minutes from venous blood. As would be expected lactate increased with exercise intensity and overtime (combined data showed lactate increased from 0.6 at rest to 1.0 at 50% followed by 3.6 at 75%). Subject one had a consistent lactate profile ± 0.5bpm between intervention conditions, suggesting no differences between conditions. Subject 2 had a consistent lactate profile between CAFF and CARN conditions ± 0.05bmp however (similarly to heart rate data) lactate was comparatively high during the C&C condition (+0.8). We would anticipate that lactate may actually be lower in the C&C condition if muscle carnitine had been increased. Wall et al (2011) demonstrated that muscle lactate content is 44% lower during 80% exercise in groups with higher muscle carnitine and resulted in a reduction in muscle lactate accumulation during exercise. This however did not appear to be the case as observed indirectly in our pilot data.

4.3.3 Heart rate

Heart rate was recorded every 5 minutes during exercise and the increased heart rate from that at 50% to 75% $V0_2$ were in line with previous studies that have shown similar heart rates at these range of exercise intensities (Potteiger et al, 2000). CARN and CAFF both had a heart rate of 115 during 50% exercise however C&C was slightly higher (121). This is a small difference in a pilot data set therefore any conclusions remain tentative. The slightly higher heart rate may indicate participants are finding the same volume of work slightly harder compared to other conditions.

At 75% heart rate was the same ± 8bpm between groups. Similar to 50% data it is difficult to draw conclusions with minor changes in a small data set. When looking at each individual's data subject one had a very stable heart rate at 75% between conditions (±5bpm). The majority of differences identified were via subject's two heart rate results during the C&C condition. Exercise heart rate remained consistent 128 CARN and 124 CAFF however was elevated to 136 in the C&C condition at 75% $V0_2$. This reinforces the need to a larger sample group to draw conclusions from this data set.

4.3.4 RER during exercise

Combined exercise data shows RER during 30 minutes exercise at 50% $V0_2$ was at 0.86 compared to 0.93 at 75% $V0_2$. Whilst this difference was not perhaps as pronounced as expected (0.86 being a relatively high RER at 50% $V0_2$ considering an overnight fast), there is certainly a distinct shift between fat utilisation at 50% exercise switching to increased carbohydrate usage at 75%. Interestingly both carnitine groups demonstrated a higher RER than the caffeine only group. Our hypothesis that increased skeletal muscle carnitine would

increase fat metabolism during exercise and carbohydrate sparing (in line with Wall et al 2011) is not directly supported by this initial pilot data, however the lack of participants and the potentiality for variability in RER measurements mean further research would be required to confirm this finding.

4.3.5 Rating of perceived exertion

Rating of perceived exertion was assessed by a 7-20 borg scale (Day et al, 2004). Values were obtained every 5 minutes throughout exercise. No moderate or larger changes between conditions were noted. We would expect that perceived exertion may have decreased slightly in the C&C condition if muscle carnitine uptake was both increased and available. Wall et al (2011) demonstrated a decreased perceived exertion compared to baseline and control in a group with increased skeletal muscle carnitine. The lower perceived exertion is possibly due to the increased buffering effects of carnitine at high intensity exercise, and it has furthermore been shown that a carnitine loaded rat soleus muscle had a 25% reduction in fatigue compared to a control during electrically stimulated contractions (Brass et al, 1993). This did not appear to occur in our small sample set and may either be masked by lack of participants or potentially be linked to an interaction with caffeine metabolites that had remained elevated post infusion, this is unlikely however as caffeine is commonly associated with a decreased RPE (Doherty and Smith, 2005). Further investigation is needed to identify if RPE was genuinely unchanged during exercise after caffeine augmented carnitine uptake.

4.3.6 Power output

Power output (Kj) was assessed using a 15 minute time trial following 60 minutes exercise as described in methods section (chapter 2). Power output in

CARN and CAFF was 147Kj with both subjects obtaining the same power output ± 0.6Kj. However, power output declined by 6% in the C&C group dropping to 138.1Kj. This resulted from a slightly lower value in subject one (116 vs 123 vs 123) and a large difference in subject two (159 vs 172 vs 173). This is in keeping with all other exercise data that suggests negative exercise performance variables from subject two in the C&C condition (Higher HR, RER and Lactate compared to other conditions). It is interesting that both participants had reduced power output in the C&C condition despite being extremely consistent in the other conditions. This is against our hypothesis that C&C would improve exercise performance via increased muscle carnitine and is opposed to previous research in which an almost identical exercise protocol was followed but showed increases of (11%) in time trial performance (Wall et al, 2011). Time trial performance can be influenced significantly by minor changes and these decreases could potentially be explained by factors such as participant feeling unwell, a painful pre-exercise biopsy or other variables other than a specific effect of carnitine. The changes witnessed here would be amplified due to the population size examined. A larger scale investigation would be necessary to determine the true changes.

4.3.7 Effects on sleep

Sleep was assessed using a wrist worn accelerometer and overall sleep time calculated using GeneActiv software. Sleep pre-exercise was assessed and compared. The primary reason was to see if caffeine significantly reduced sleep time which may have accounted for performances differences between conditions if caffeine groups had performed significantly worse the following morning. Caffeine did not reduce sleep quantity and in fact caffeine groups had a longer sleep time than carnitine alone: carnitine 418mins, caff 448mins and

C&C 459 mins. Evidently, this doesn't take into account quality and stage duration of sleep which may have been impaired or altered by caffeine ingestion (Landolt et al, 1995), however we can conclude that caffeine hasn't affected sleep time. Therefore, sleep time alone is unlikely to be a key determinant in time trial performance variations.

4.4 Summary of all data presented within this thesis

4.4.1 Habitual caffeine intake, caffeine tolerance and blunted responses

Caffeine tolerance in individuals is widely reported (Shi et al, 1993) with caffeine appearing to have reduced effects in individuals who regularly consume high dose caffeine. For this reason, a subsidiary aim of this study was to see whether there was any impact of habitual caffeine intake/ tolerance on effectiveness of caffeine at stimulating Na^+/K^+ -ATPase pump activity thus potentially reducing skeletal muscle carnitine uptake. Three subjects reported habitual caffeine intake as low which was confirmed by nutrition diary (daily intake averaged at 4.4mg over three days). One subject was calculated as a medium user (135.0mg/day) and the final two participants were reported as high users (366.7mg/day). Whilst these group sizes are both small and imbalanced (3:1:2) data means have been compared to see if any pilot trends appear.

Interestingly the low caffeine intake group had a higher baseline caffeine concentration during carnitine infusion (0.17mmol/kg vs 0.02mmol/kg in both medium and high groups). This could potentially indicate increased sensitivity to caffeine in these individuals, however all caffeine containing food and drink had been avoided for 48 hours prior to testing so it is likely these differences may be due to individual variation. During both caffeine ingestion conditions (CAFF and C&C) average steady state plasma caffeine concentration was higher in both

the low (7.86mmol/kg) and medium (8.52mmol/kg) groups compared to high habitual users (6.06mmol/kg). High caffeine user's peak plasma caffeine also demonstrated a delayed response, appearing at 60 minutes as opposed to a 30-minute peak in the low and medium users. This appeared to directly or indirectly reduce the effectiveness of the Na^+/K^+ -ATPase pump with a smaller increase in Na^+ in the high caffeine intake group compared to low users (0.49mmol/L vs 0.55mmol/L respectively). This was combined with an apparent attenuation of decreased K^+ appearing in high caffeine users compared to low caffeine users (-0.07mmol/L vs- 0.17mmol/L). When observed together these changes appear to indicate reduced pump activity thus lower Na^+ flux and potentially reduced carnitine uptake. This appears to have reduced plasma carnitine transport and has translated directly into a much higher steady state plasma carnitine concentration in the C&C condition in high caffeine users compared to low caffeine (364µmol.L-1 vs 315µmol.L-1). Suggesting reduced plasma carnitine clearance thus reduced muscle carnitine uptake. Although these results are from a small data set it raises an interesting question as to whether caffeine tolerance may blunt the apparent positive effects of caffeine on carnitine uptake thus warrants future research comparing habitual caffeine users.

4.4.2 Effects on exercise

Plasma carnitine remained lower in C&C vs CARN pre-exercise possibly suggesting that transport of carnitine into muscle remained elevated 14 hours post infusion. Whilst caffeine had returned to near baseline the metabolites of caffeine all remained significantly elevated and may have been available to act on metabolism or other exercise factors. Whether the effects seen during exercise are true physiological effects as a result of supplementation are

therefore questionable. The changes are all relatively small and may are only be highlighted due to the small sample size. Secondly one visit by subject two appears to have skewed all exercise data (and although this could be a true effect of the C&C condition, potentially related to the differing ratio of free to acetyl carnitine due to the caffeine supplementation, we would have expected this to also occur during the caffeine only group). It could equally be due to an external factor affecting subject two on that specific visit, especially considering the consistency displayed in other conditions and the differences between subject one and subject two. We can therefore draw no real conclusions on the effectiveness of acute caffeine augmented carnitine supplementation on exercise performance from this study.

4.5 Limitations and Directions for Future Research

The most significant limitation of this thesis was the limited sample size examined. Due to unforeseen issues with recruitment only 6 participants completed the proof of concept infusion study with only 2 participants completing the ‘pilot’ data. Whilst 7 participants were enough to see significant differences under a similarly designed study which used insulin rather than caffeine (Stephens et al, 2006) and we did uncover significant changes in rate of clearance and uptake amongst other factors. An increased sample size would have increased the accuracy and reliability of our findings and decreased our margins of uncertainty. Variables with a smaller effect size (electrolyte/lactate data for example) or those with larger variation (carnitine steady state concentration) may have failed to reach significance due to this limited sample size despite obvious changes apparently occurring when looking at raw data sets. In the pilot data, if the results from one individual were

anomalies (as may have possibly happened in one visit of subject two) it leaves us unable to draw many meaningful conclusions from this data set.

A second limitation was the failure for any muscle total carnitine results to be obtained due to chemical/ mechanical error. It was believed that during either the metabolic extraction phase or incubation with KOH the muscle extract failed to totally elucidate the amount of total carnitine present thus large variations between repeat measures meant the data had to be excluded. This was of course a key measure for our pilot data and was extremely unfortunate not have access to the results for observations.

From the results of our proof of concept study, a strong case can be made that caffeine augments carnitine clearance from plasma under a state of hypercarnitinemia via increased activation of Na^+/K^+ -ATPase activity and increased Na^+ flux and Na^+ coupled carnitine transport. We hypothesis that this will directly lead to increased skeletal muscle carnitine and whilst not demonstrated in our pilot data (potentially due to issues discussed previously) this could lead to improved exercise performance. A number of key avenues for future research based on this study are worth examination: firstly, and most importantly, a study directly looking at muscle carnitine levels under similar conditions would confirm that the differences in plasma carnitine we discovered have in fact translated directly to increased muscle carnitine (rather than into some other tissue or increased excretion). Secondly, for full investigation, a similar study to our pilot data collection should be run which would allow both muscle carnitine to be examined directly as well as any effects on exercise that this may have had. Depending on results from these studies important future research identifying any tolerance / caffeine status effects on Na^+/K^+ -ATPase pump activity and muscle carnitine differences should be examined, as small

pilot data from our study indicated a possible tolerance effect which would be extremely relevant when applying this to athletes. Another key study should examine if there is a caffeine threshold and the optimal supplementation strategy to gain the most ergogenic benefit. A supplementation protocol of 9mg/kg, whilst safe acutely, possibly is unwise to be consumed long term therefore examining different dosages of caffeine to see if a lower dose is equally effective may be critical to the usefulness of caffeine as a co supplement for carnitine augmentation. Finally, a chronic study examining if oral carnitine can be increased via oral caffeine would demonstrate the most 'real life' and applicable benefits of caffeine on carnitine. It would also be interesting to examine the effects of a mixed carbohydrate / caffeine supplement to see if they work synergistically or in fact inhibit one another, if a synergistic blend is demonstrated this may allow a safe and realistic level of daily caffeine and carbohydrate co-supplementation which increases skeletal muscle carnitine.

4.6 Applications for the Study

Due to the proof of concept nature of the study the absolute current real world applications for this study are small however have the future potential to be great. We appear to be the first study to have indirectly demonstrated an alternative mechanism to that of carbohydrate to increase skeletal muscle carnitine which was the primary aim of this study.

We could potentially tentatively advise athletes that consuming caffeine alongside carnitine may augment skeletal muscle carnitine uptake which based on previous research may improve exercise performance. However, since we are unable to suggest an accurate dose (or even that caffeine is able to increase carnitine via oral ingestion) this advice would be purely theoretical.

However, it may provide an alternative strategy to carbohydrates to theoretically optimise any potential ergogenic effects of carnitine and may be the best suggestion for individuals who are required to avoid carbohydrates until further research can confirm or deny this hypothesis.

4.7 Conclusions

We have demonstrated that 9mg/kg caffeine when taken orally over 5 hours increases certain electrolyte activity, simultaneously increasing sodium and decreasing potassium which we hypothesise indicates increased Na^+/K^+ -ATPase pump activity. We further hypothesise that this increased pump activity leads to increased transport of plasma carnitine thus results in a witnessed decreased plasma carnitine concentration and a significantly increased rate of carnitine muscle tissue uptake. A small sample size of muscle data indicates that caffeine may lead to increased acetylation of the carnitine pool leading to an increased acetyl carnitine: free carnitine ratio.

We hypothesise that this lower plasma TC concentration during caffeine ingestion was a result of caffeine mediated increase in skeletal muscle carnitine uptake as opposed to increased renal clearance (or transport into alternative organs). In future these results warrant further investigation examining the effects of caffeine on carnitine directly within the muscle and any impact on exercise this may have.

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Appendicies

Participant Information Sheet

Version B3 08 October 2018

Participant Information Sheet

Study title

Does caffeine stimulate L-carnitine accumulation within skeletal human muscle?

Invitation and brief summary

We would like to invite you to take part in a research study investigating the effects of caffeine on carnitine transport in muscles. Taking part in the study is entirely up to you so before you decide, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information and to discuss it with other people to decide whether you wish to take part or not.

Thank you for taking the time to read this information.

What's involved?

The study will involve a total of 4 lab visits. The visits will be as follows: one introductory and pre-screening visit and three infusion lab visits (explained in more detail below). The estimated time commitment for this study in total is 21 hours. We are doing this experiment due to the effect that carnitine has on muscle metabolism and exercise performance. If the amount of carnitine within our muscles can be increased the way we 'burn' fat and carbohydrate in our muscles becomes more efficient and endurance performance is improved. However muscle carnitine has so far only been increased orally by 6 months of supplementation along with large quantities of sugar which inevitably could lead to negative health consequences. If we can find a non-calorie alternative to increase carnitine transport this could make carnitine a viable supplement in the health and exercise fields.

What would taking part involve?

We have invited you to take part because we are looking for healthy recreationally active male and female participants aged 18-30. You must not be vegetarian or vegan as non-meat eaters can have a different response to

carnitine intake. Furthermore if you smoke or take any form of prescription medication you will also be unable to participate in this study.

If you agree to take part you will be asked to attend a laboratory at the University of Exeter's St. Luke's campus on seven separate occasions.

Preliminary testing visit (visit 1) ~1 hour

This visit will provide you with an opportunity to discuss the study with the investigators and ask all the questions you may have regarding any aspects of the study's objectives, procedures or potential results. You will then be asked to sign a consent form stating that you are happy to take part. You will be given a food diary and asked to record your food and drink intake for 3 days. This will enable us to work out your habitual L-carnitine and caffeine intakes.

Test days 1, 2 and 3 (~7 hours each)

You will be asked to follow as closely much as possible your normal diet the day prior to testing. However, you will be asked to avoid anything high in caffeine (coffee, energy drinks etc) and foods high in carnitine (Red meat); this will be explained in detail during the screening visit. An evening meal will be provided for the night before testing (this can be eaten at home and won't require a laboratory visit). Breakfast on the test day will also be provided and you will be asked to consume this at 07:00 (again this will be provided in advance and can be eaten at home not requiring a lab visit).

You will then be asked to attend the laboratory for 11:00 having not eaten or drunk anything apart from water and the provided breakfast. Nothing further will then be consumed until completion of the infusion.

A cannula will be placed in your hand which will then be placed in a warming box which improve our ability to sample blood. A second cannula will also be placed in your forearm vein for infusion. Blood samples will be taken from the cannula in the hand every 30 minutes for the duration of the visit for a total of 11 5ml samples (55ml blood total).

Carnitine or saline will then be intravenously infused for a total of 5 hours through the forearm cannula. You will also be asked to take tablets containing either 6mg/kg caffeine or flour (as a placebo) at the start of infusion followed by tablets containing 1mg/kg caffeine or flour after 2,3 and 4 hours for a total of 9mg/kg.

You will be asked to remain at rest (i.e. on the bed) for the duration of the infusion and are free to bring in books/ laptops or watch dvds that can be provided for entertainment.

You will be asked to wear a face mask for 10 minutes every hour during the infusion that will let us measure the percentage of fat or carbohydrates you are using as energy.

There will be 1 week between infusion visits and you will be required to follow the above protocol on 3 occasions to complete all 3 experimental conditions (i.e. caffeine only, carnitine only or caffeine and carnitine).

What are the possible benefits of taking part?

The main benefits of the proposed research are educational and there will be limited personal benefit to you. However you will be paid an inconvenience allowance of £75 for your time and have a number of meals provided.

What are the possible disadvantages and risks of taking part?

There are minimal risks involved with this study.

Blood sampling via cannulation may cause some temporary discomfort however it is a safe procedure performed regularly only by qualified individuals.

Further supporting information

Do I have to take part?

Please remember that participation in this study is entirely voluntary. It is up to you to decide whether you would like to take part or not and if you decide to take part you are free to leave the study at any time without giving a reason as to why you wish to do so. If you do decide to participate in this study you will be asked to sign a consent form before you start. You will be given a copy of the consent form and this information sheet for your own records.

Are my results confidential?

If you consent to take part in this study you have a right to privacy. Your name will be linked to an ID number on a password protected database and only these IDs will be used as labels during blood, muscle and data analyses.

What will happen to my blood and/or tissue samples?

Your blood or tissue samples will be stored in a secure storage area while they are waiting for analysis. It is possible that there may be some samples left over after analysis. You will have the opportunity on the consent form for this study to specify whether we may keep these samples to use for other studies. If you agree to this you may, if you wish, say whether there are some types of research that you do not want your samples used for. Any other study wishing to use your surplus samples must first obtain approval from our ethics committee.

What will happen to the results of this study?

The results will increase our understanding of the mechanisms behind L-carnitine accumulation and the relationship between caffeine and L-carnitine. We aim to publish the findings in research journals and to present them at conferences in the UK or abroad. Your data will always remain anonymous and your name will not appear on any results. If you wish a summary of results will be emailed to you at the end of the study

Who has reviewed this study?

All research activity at the University of Exeter is examined and approved by an ethics committee to protect your interests. This study has been approved by the Ethics Committee of Sport and Health Sciences, College of Life and Environmental Sciences, University of Exeter.

Who is funding/sponsoring this study?

This study is funded by the University of Exeter

Contacts for further information

If you would like more information or if you have any further questions about the study please contact the investigators using the details below:

David Machin	Dr Benjamin Wall
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Exeter University	Exeter University
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Informed Consent

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Study: Does caffeine stimulate L-carnitine accumulation within skeletal human muscle?

Researcher: Mr David Machin

Organisation: The University of Exeter

Version: B3. 08/10/2018

Participant Identification Number: ID no.

Informed Consent form for participants

Please initial box

I confirm that I have read and understand the information sheet version B3 dated 08.10.2018 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason. ☐

I understand that any information given by me may be used in future reports, articles or presentations by the research team. ☐

I understand that my name will not appear in any reports, articles or presentations. ☐

I will be asked to provide 33 venous blood samples of 5ml over the course of the study and I understand the blood sampling and cannulation procedures. ☐

I understand that my blood samples may be transported to another Instution in the UK or overseas for analysis. ☐

I give consent for my blood to be used in future studies ☐

I give consent for my surplus blood samples to be used for other studies subject to additional ethical approval (initial neither or one only of the boxes):

With no restrictions ☐

Or,
With the following restrictions: _____

_____ ☐

☐

I have reported any allergies or illnesses to the research team. Particularly food allergies to ensure that the controlled diets are safe for my consumption ☐

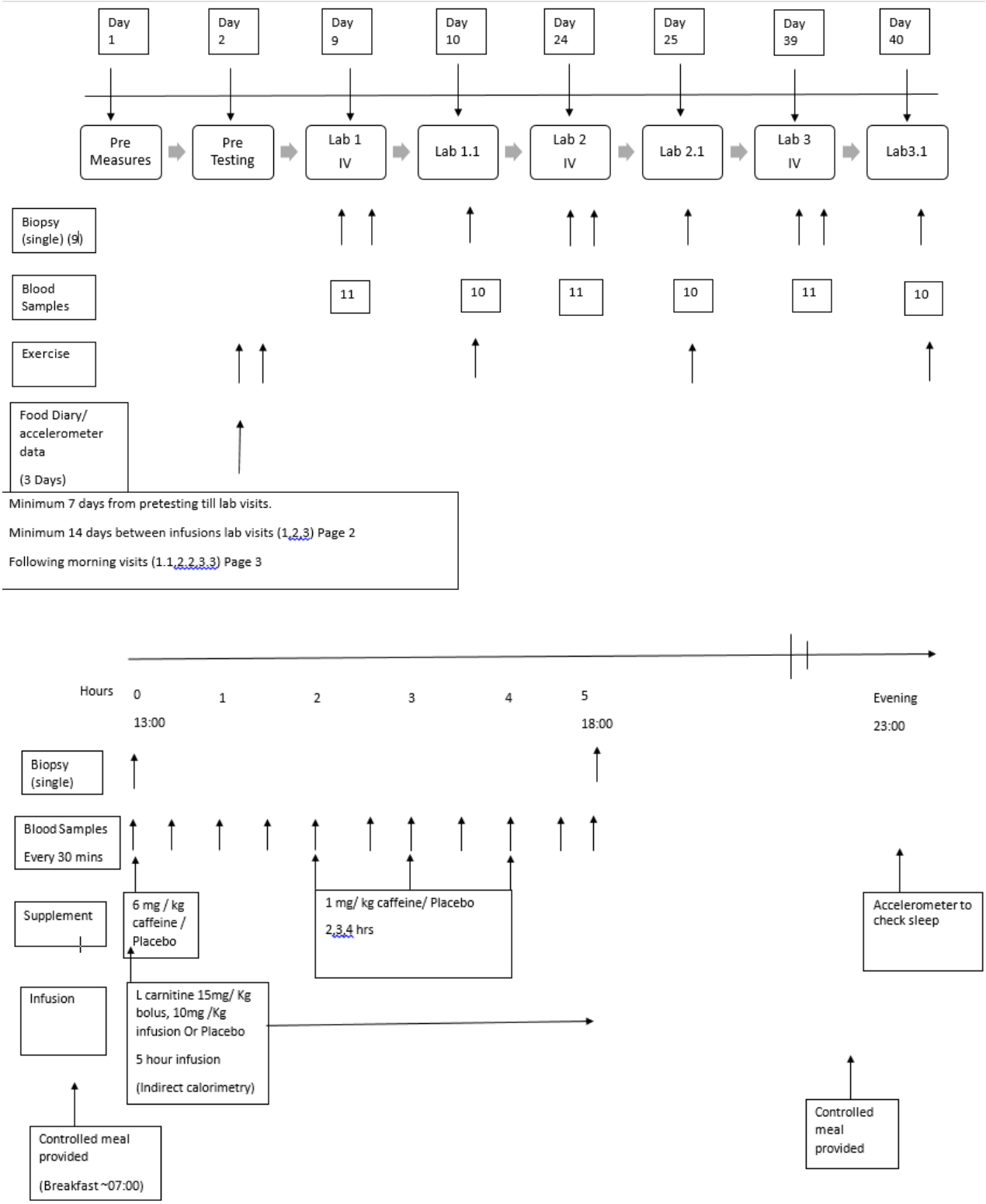
I agree to take part in the above study. ☐

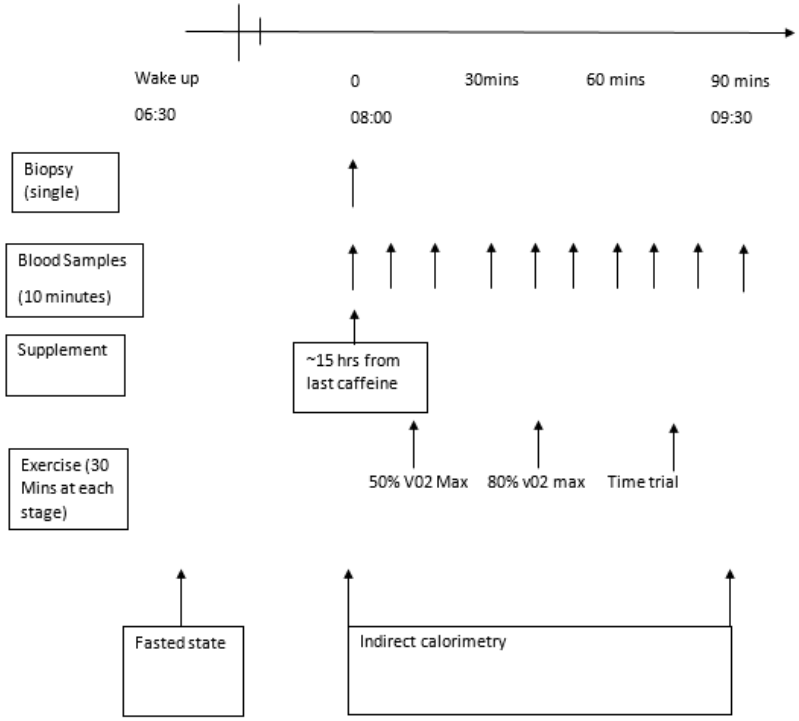
I understand that I will receive a £75 inconvenience allowance if I complete all aspects of the study but only a proportion of the allowance will be paid if visits/measurements are missed.

Name of Participant	Date	Signature
_____	_____	_____

Name of Researcher	Date	Signature
_____	_____	_____

Protocol Schematic




Wake up
06:30
0
08:00
30mins
60 mins
90 mins
09:30
Biopsy (single)
Blood Samples (10 minutes)
Supplement
~15 hrs from last caffeine
Exercise (30 Mins at each stage)
50% V02 Max
80% v02 max
Time trial
Fasted state
Indirect calorimetry

Test Day Sheets

COLA Study

Participant		Date	

MUSCLE	A		B	
	Stopwatch	Clock	Stopwatch	Clock
RESTING				

Infusion Start Time:

	CARN	HIS	CARN	HIS
RESTING				
Estimated Weight				

Tablet 1 Time:	Water consumed:

Notes:

Tablet 2 time:	Water consumed:

Blood

Tablet 3 time:	Water consumed:

	0	1	2	3	4	5	6	7	8	9	10	Infusate
Plasma												
Serum												

Tablet 4 time:	Water consumed:

Notes:

Lidocane	Biopsy 1	Biopsy 2	Biopsy 3

RMR

	0	1	2	3	4	5						
RMR												

Bolus RPM		Infusion RPM	
Total Bolus		Total Infusion	
Bolus Ml/Hr		Infusion Ml/ Hr	

Notes:

COLA Study

Participant		Date	

MUSCLE	A	
	Stopwatch	Clock
RESTING		

	Predicted V02	WATTS
50%		
75%		
TT		

	CARN	HIS
RESTING		
Estimated Weight		

50% Time:	Water consumed:

Notes:

75% time:	Water consumed:

Blood

Time trial time:	Water consumed:

	0	1	2	3	4	5	6					
Plasma												
Serum												

Notes:

Lidocane	Biopsy 1	Biopsy 2	Biopsy 3

RMR

	0	0-10	20-30	30-40	50-60							
RMR												

Notes:

Infusion													
Visit:		Subject Code:			Date:								

Time point	RMR	Blood	Na+	K+	Lactate	Glucose	Ser A	Ser B	Pla A	Pla B	Pla C	Pla D	Muscle
Baseline (0)													
30 (1)													
60 (2)													
90 (3)													
120 (4)													
150 (5)													
180 (6)													
210 (7)													
240 (8)													
270 (9)													
300 (10)													

Exercise															
Subject code:				Visit :		Date:									

Time point	RMR	HR	RPE	Blood	Na+	K+	Lactate	Glucose	Ser A	Ser B	Pla A	Pla B	Pla C	Pla D	Muscle
Baseline (0)															
5															
10 (1)															
15															
20 (2)															
25															
30 (3)															
40															
45 (4)															
50															
55(5)															
60															
65 (6)															