The significance of CYP1A2 genotype on caffeine metabolism and exercise performance

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Abstract

Objective: The objective of this study was to investigate whether a single nucleotide polymorphism (C to A transversion at position -163 downstream of the first transcribed nucleotide) in the enzyme that metabolizes caffeine (CYP1A2), would explain the variability seen in caffeine related responses in endurance exercise performance. In a double blind crossover trial, well trained male endurance athletes (n=11, mean VO_2 max 69±4 $mL.kg^{-1}.min^{-1}$) ingested either caffeine (5 $mg.kg^{-1}$) or a placebo 60 minutes prior to performing a lab based experimental protocol involving a two hour steady state cycle (70% VO_2 max) followed by a 30 minute time trial to measure performance. The rate of caffeine metabolism over seven hours (inclusive of exercise period) was also determined by the HPLC analysis of plasma caffeine and its major metabolites, paraxanthine, theophylline and theobromine. Caffeine metabolism at rest over a similar seven hour period was also determined in the same manner.

Results: Caffeine improved endurance performance by 7.1% (p=0.037) compared to a placebo. Caffeine also significantly elevated heart rate during the time trial (p=0.003); and RPE (p=0.010) and VO_2 (p=0.047) during steady state exercise. There was no correlation between caffeine or paraxanthine concentrations at the start of the time trial and subsequent performance and the rate of caffeine metabolism was not significantly different between resting or exercising trials. Furthermore there was no significant interaction between caffeine treatment and CYP1A2 genotype on performance or any other

variables measured. However there was a trend for carriers of the C allele showing faster metabolism than those homozygous A/A (p=0.097).

Conclusions: Caffeine is ergogenic during endurance exercise, however individual responses were variable. In this study this variability could not be explained by CYP1A2 genotype. However the small sample size in this study especially when subjects were divided into genotype groups, makes drawing conclusions difficult.

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Approval for the research was obtained from Massey University Human Ethics Committee (HEC: Southern A Application 11/35) for the experiments described in this thesis.

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Abbreviations

137X	1,3,7-trimethylxanthine (caffeine)
17U	1,7-dimethyluric acid
17X	1,7-dimethylxanthine (paraxanthine)
1U	1-methyluracil
1X	1-methylxanthine
AFMU	5-acetylamino-6-formylamino-3-methyluracil
AUC	Area Under the Curve
BW	Body Weight
Caffeine-Ex	Exercise trial with Caffeine
Caffeine-Rest	Resting trial with Caffeine
cAMP	cyclic adenosine monophosphate
CNS	Central Nervous System
CYP1A2	Cytochrome P450 1A2
DNA	Deoxyribonucleic Acid
EDL	Extensor digitorum longus muscle
EDTA	Ethylenediaminetetraacetic acid
FFA	Free Fatty Acids
HIT	High Intensity Training
HPLC	High Performance Liquid Chromatography
HR	Heart Rate
NMR	Nuclear Magnetic Resonance
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase Chain Reaction
Placebo- Ex	Exercise trial with Placebo
PX	Paraxanthine
RER	Respiratory Exchange Ratio
RPE	Rating of Perceived Exertion
SNP	Single Nucleotide Polymorphism
TB	Theobromine
TFA	Trifluroacetic Acid
TP	Theophylline

USDA	United States Department of Agriculture
VO_2	Volume of Oxygen
VO_2 max	Maximal Oxygen Uptake
WADA	World Anti-Doping Agency

Chapter 1 Introduction

1.1. Caffeine use in sport

Prior to 2004, caffeine intake that produced urinary caffeine concentrations greater than 12 $\mu g.mL^{-1}$ was banned in competitive sport. However; in 2004 caffeine was removed from the World Anti-Doping Agency's (WADA) prohibited list. The reasons for removal from the WADA prohibited list are not public, but possibly include the acknowledged widespread social use of caffeinated beverages and the difficulties in differentiating this from intentional use to improve athletic performance. Additionally, caffeine is positively associated with an ergogenic effect at doses between 3-6 $mg.kg^{-1}$ (Cox et al., 2002; Desbrow, Biddulph, et al., 2012) caffeine ingested at these doses is well below that required to produce urinary caffeine concentrations of 12 $ug.mL^{-1}$(Cox et al., 2002). Nevertheless, since 2004, caffeine remains part of the Agency's monitoring program in order to detect any patterns of misuse ("World Anti-Doping Agency (WADA) Monitoring Program," 2015).

Investigation into caffeine consumption since its removal from the prohibited list has indicated that caffeine use has not substantially changed since 2004. In the period from 2004 to 2008, 74% of 20,000 international competitive athletes competing in a range of sports were shown to have consumed caffeine (measurable urinary caffeine concentrations). Similarly, prior to 2004, 67% of athletes were shown to use caffeine (calculated from the data presented by Van Thuyne and Delbeke (2006)). Furthermore, urinary caffeine concentration indicated that in sports involving cycling, athletes had

significantly higher urinary caffeine concentrations than non-endurance sports such as soccer, swimming, and basketball (Del Coso, Munoz, & Munoz-Guerra, 2011). However; this trend is not different to before caffeine removal from the banned list. Urinary caffeine concentration in cyclists was previously significantly higher than most other sports except for bodybuilding and volleyball (Van Thuyne, Roels, & Delbeke, 2005).

As illustrated by Del Coso et al. (2011), caffeine is commonly ingested by both cyclists and triathletes during competition. For example, a high prevalence of planned caffeine use was observed in well-trained elite and age group competitors from 16 countries competing in the 2005 Hawaiian Ironman Triathlon World Championships (Desbrow & Leveritt, 2006). Of 140 randomly selected athletes participating in this event, 89% reported they planned to use cola beverages, caffeinated gels, pre-race coffee, energy drinks or NoDoz® tablets during the competition. Fifty of these athletes were tested following completion of the race; with plasma caffeine detected in all samples. The presence of plasma caffeine following an eleven hour race suggested that this group of athletes were probably ingesting caffeine for its ergogenic effect. As urinary caffeine concentration was not measured, these authors were unable to assess whether urinary values were above the former 12 $\mu g.mL^{-1}$ limit. However, the plasma caffeine concentrations recorded, indicate that the caffeine dose consumed by these athletes was unlikely to reach the former cut-off limit.

Thus there is very good evidence that caffeine was and still is commonly consumed by endurance athletes for its ergogenic effects.

Caffeine and caffeine-containing foodstuffs have interested exercise scientists for many years because of its ergogenic potential. Much research has shown improved performance, especially during endurance exercise (Burke, 2008; Graham, 2001a). However, not all studies have shown caffeine to have this effect (Hunter, Gibson, Collins, Lambert, & Noakes, 2002; Jacobson, Febbraio, Arkinstall, & Hawley, 2001). This inconsistency may be as a result of differing study designs, caffeine dosages or exercise intensity and duration. On the other hand, the individual response may have a genetic basis. The activity of cytochrome P450 1A2 (CYP1A2), the main enzyme responsible for caffeine metabolism in the liver, has been shown to be widely variable between individuals (Sachse et al., 2003). Thus; some of the variability in exercise performance may be explained by levels of caffeine and/or caffeine metabolites, paraxanthine and theophylline in relation to the timing of physical work.

1.2. Aims and objectives

The aim of this research was to assess whether a genetic polymorphism in the primary enzyme responsible for caffeine metabolism (CYP1A2) alters the rate of caffeine metabolism and performance during endurance exercise.

The objectives of this research were to:

- Determine whether the CYP1A2 single nucleotide polymorphism (at position -163 downstream of the first transcribed nucleotide) has different

rates of caffeine metabolism in carriers of the C allele compared to individuals homozygous for variant A at rest and during exercise.

- Determine whether different activity of CYP1A2 in C allele individuals affects performance benefits of caffeine on endurance cycling relative to individuals homozygous for variant A.

Chapter 2 Literature Review

Caffeine is a widely consumed psychoactive substance found in many beverages and foods such as coffee, tea and soft drinks (Fredholm, Battig, Holmen, Nehlig, and Zvartau, 1999). It is a methylxanthine that is well absorbed and easily crosses the blood brain barrier resulting in its well-known stimulant properties. This review will assess the current literature pertaining to the caffeine content of coffee and other beverages and foods; factors affecting caffeine absorption and metabolism and the most likely mechanisms producing performance enhancing effects. In particular; the focus of this review is the effect of caffeine on endurance exercise performance and how polymorphisms of CYP1A2 affects caffeine metabolism.

2.1. Caffeine content of various dietary sources

2.1.1. Café coffee

The caffeine content of various foods and beverages can vary widely. Rudolph et al. (2012) analysed 121 caffeine-containing food and beverages available on the Austrian market. These authors found that coffee, although highly variable depending on the roasting and type of preparation, had the highest caffeine content of all the samples tested (36-804 $mg.L^{-1}$). Within the coffee preparations; espresso coffee had the highest caffeine content, whereas the lowest was seen in a beverage from a vending machine

(coffee/cocoa based) (Rudolph et al., 2012). In this study a variety of food and beverages such as coffee, tea, colas, energy drinks and chocolate contributed to the Austrian population's caffeine intake. However the most variable were coffee (9-200 mg of caffeine in typical cup) and coffee based beverages, whereas colas and energy drinks had more consistent caffeine concentrations.

The burgeoning number of cafés in both New Zealand and Australia shows that coffee drinking is becoming a large part of culture within these countries. Furthermore, many cyclists consume pre-race coffee (Desbrow & Leveritt, 2006). However the caffeine content from a "single shot" of ground coffee in an espresso/short black (the basis of many of the commonly consumed drinks such as cappuccinos, lattes, flat whites etc.) varies widely between different establishments. For example, a single shot espresso collected from different retail vendors with espresso machines in five retail shopping centres on the Gold Coast, QLD, Australia, had caffeine content ranging between 25-214 mg (Average 106 mg; Desbrow, Hughes, Leveritt, & Sheelings, 2007). Coffee samples collected in Sydney, Brisbane and Melbourne were also similarly variable, ranging from 54-189 mg per serve (Desbrow, Henry, & Scheelings, 2012).

The variability of the caffeine content in coffee can arise from many factors such as the variety of coffee bean, where it is grown or the way it is prepared. The Arabica coffee bean is now widely used but has a lower concentration of caffeine than the Robusta coffee bean (Knight, Knight, Mitchell, & Zepp, 2004; Rodrigues et al., 2007). Many coffee roasters will use a blend of Arabica and Robusta coffee beans from different origins (regions) as each

bean gives coffee a distinct taste but the latter variety is significantly cheaper and easier to grow (Ludwig et al., 2014; Machado, Breder, Ximenes, Simoes, & Vigo, 2009; Rodrigues et al., 2007). Crozier, Stalmach, Lean, and Crozier (2012) investigated the effect washing (unwashed Arabica beans from Ethiopia and washed Arabica beans from Colombia) and roasting temperature (high temperature (350°C) short time or low temperature (270°C) for long time) had on caffeine concentration. Washing made no difference to caffeine content; however roasting reduced the caffeine concentration by approximately 80% irrespective of which temperature was used. In contrast, a recent study by the laboratory of Ludwig et al. (2014) observed that roasting had no effect on the caffeine content, though in this study much lower temperatures were used (≤ 219°C).

This variability can also be found across retail chains. The caffeine content of Starbucks® 'breakfast blend' coffee brewed at one establishment ranged from 259-564 mg per large cup (approximately 500 mL) (McCusker, Goldberger, & Cone, 2003). Desbrow, Henry, et al. (2012) saw similar variability with espresso coffees (n= 4 to 7) purchased from the same retailer across different locations. Nine retailers including McDonalds, Muffin Break and Coffee Club had espresso coffee caffeine content ranging from 49-214 mg (7.6-38.1%CV within establishment). The greatest variability was seen at Donut King (82-214 mg).

2.1.2. Other caffeine-containing beverages

Manufacturers of pre-packaged coffee flavoured milks purchased in Australia were shown to underestimate the caffeine content of each drink (Desbrow,

Biddulph, et al., 2012)(Desbrow, Biddulph, et al., 2012). These drinks often had guarana added (naturally occurring caffeine containing compound) and ranged between 33-197 mg caffeine/serve. Thus they have the potential to provide unknowing consumers with a high caffeine intake.

Energy drinks such as Red Bull® and Monster Energy Drink®, have become very popular with young people and athletes alike (Rosenbloom, 2014). Extensive sponsorship and marketing of these drinks and their smaller volume counterpart, energy shots, has resulted in these drinks being associated with extreme sports such as snowboarding, surfing and BMX racing (Rosenbloom, 2014). Most energy drinks are high in sugar and caffeine, for example, a serving (240g) of either Red Bull® or Monster Energy Drink® will give the consumer either 77 or 86 mg of caffeine respectively (USDA National Nutrient Database for Standard Reference, 2015). These drinks also contain an array of components such as taurine, B vitamins, and glucuronolactone which manufacturers purport to aid in mental and physical performance. However, in an extensive review, McLellan and Lieberman (2012) concluded that there was a lack of evidence for any of these other ingredients having an effect on physical or cognitive performance. Leaving caffeine (whether from caffeine or other caffeine containing constituents e.g. guarana or kola nut) to be primarily responsible for the physiological effects users experience.

As with coffee preparations, both black and green teas have variable caffeine content depending on how it is brewed. As part of a large study investigating caffeine exposure in pregnant women, Bracken et al. (2002), analysed a range of tea samples (n=82) that were either purchased or prepared in the

home. Tea brewed for longer than three minutes had caffeine content ranging from 24-359 $\mu g.mL^{-1}$ whereas tea brewed three minutes or less ranged from 13-228 µg of caffeine per mL. Rudolph et al. (2012) saw approximately 20% higher caffeine content in teas brewed for six minutes compared to three minutes but did not see any tendency for black tea to contain more caffeine than green tea.

Figure 2.1 gives an indication of the average caffeine content in some commonly consumed food and beverages. As mentioned, many of the coffee and tea preparations will be highly variable; however, it does give the reader an overview of the approximate caffeine concentration across different types of food and beverages.

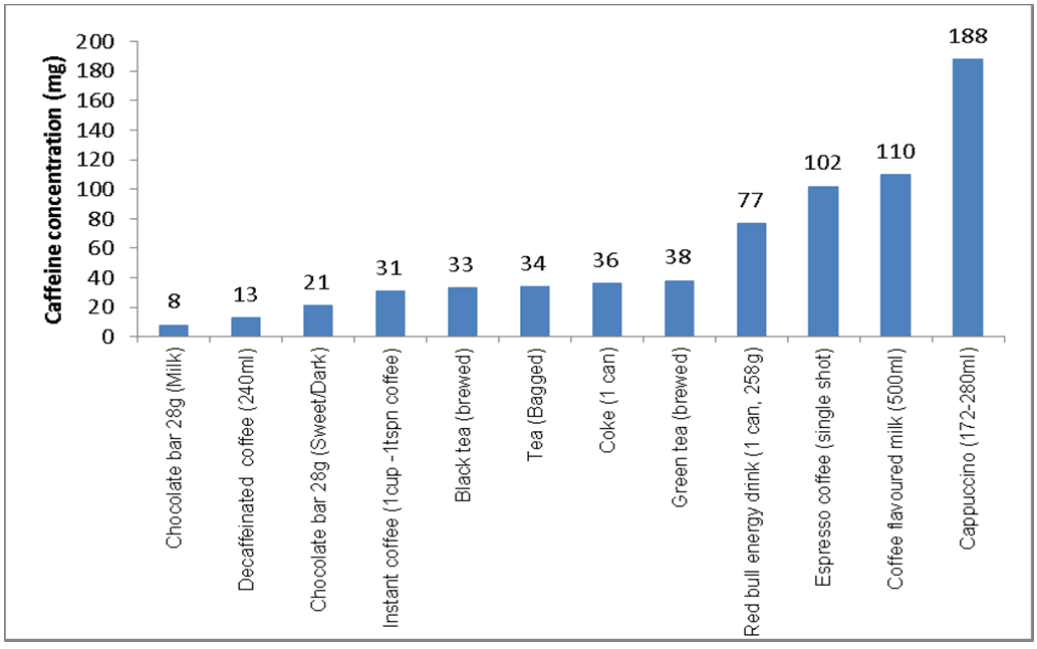

Figure 2.1. Representation of caffeine content (mg) found in a usual serving size of some common caffeine containing food and drinks. Data taken from Desbrow et al. (2012); Fredholm et al. (1999); Ludwig et al. (2014); Mandel (2002); Rudolph et al. (2012) and USDA National Nutrient Database for Standard Reference (2015)

2.1.3. Caffeine absorption

Oral caffeine is completely absorbed from the stomach and small intestine and is 100% bioavailable (Blanchard & Sawers, 1983). Thus the rise in plasma caffeine concentration corresponds to the oral dose given (Magkos & Kavouras, 2005). Being hydrophobic and circulating primarily unbound (Fredholm et al., 1999), caffeine is readily able to pass through all biological membranes including blood cells and the intestinal wall. However, caffeine may be absorbed differentially depending on how it is administered. For instance, Mumford and colleagues (1996) administered oral caffeine-containing foodstuffs (chocolate, cola, and caffeine in capsules) to subjects in a fasted state. Peak caffeine concentration was reached approximately 30 minutes after ingestion of the caffeine containing capsules. However, caffeine absorption was delayed (1.5 - 2h) and a lower maximum caffeine concentration was seen when caffeine was consumed as part of cola or chocolate ingestion; despite all three products possessing an equivalent amount of caffeine (72 mg). Caffeine absorption is also significantly faster from a gum containing 200 mg caffeine than an equivalent dose in capsule formulation (Kamimori et al., 2002).

2.1.4. Effect of carbohydrate and breakfast on absorption

Coffee and other caffeine-containing substances are often consumed with meals, particularly breakfast. Furthermore, many caffeine-containing beverages also contain significant quantities of carbohydrate. Both matrices are likely to have significant effects on the rate of caffeine absorption. Whilst many studies have shown absorption of pure caffeine to be complete within

approximately one hour (Blanchard & Sawers, 1983; Liguori, Hughes, & Grass, 1997; Mumford et al., 1996), Skinner et al. (2013) showed that consumption of a high carbohydrate meal prior to caffeine ingestion, significantly slowed the appearance of caffeine in the blood. To be applicable to sports people ingesting caffeine both before and during events, many caffeine and exercise studies simulate a typical race day scenario (i.e. give a high carbohydrate breakfast prior to caffeine consumption). In many of these studies it can be seen that peak plasma caffeine concentration was reduced and delayed (two hours or more) compared to those who consumed caffeine in a fasted state (Table 2.1). Therefore any research designed to study the effect of caffeine on physiological parameters including performance should control concurrent food intake, otherwise caffeine pharmacokinetics and subsequent effects will vary.

Table 2.1. The effect breakfast/carbohydrate has on caffeine absorption.

	Caffeine dose	Breakfast	Plasma caffeine concentration at 60 minutes ($\mu mol.L^{-1}$)	Caffeine peak (minutes)	Exercise time (cycling)
Skinner, Jenkins, Folling, et al. (2013)	6 $mg.kg^{-1}$	Fasted	40	60	Rested
	9 $mg.kg^{-1}$	Fasted	65	60	
	6 $mg.kg^{-1}$	**2 g CHO/kg**	**20**	**120**	
	9 $mg.kg^{-1}$	**2 g CHO/kg**	**25**	**180** *(just above 120 min)*	
Desbrow, Barrett, Minahan, Grant, and Leveritt (2009)	**1.5 $mg.kg^{-1}$**	**2 g CHO/kg** *Caffeine ingested 60 minutes after breakfast*	**~9**	**120**	120 minutes (70% VO_2 peak) + 7$kJ.kg^{-1}$ TT
	3 $mg.kg^{-1}$	**2 g CHO/kg** *Caffeine ingested 60 minutes after breakfast*	**~11**	**180** *(not measured after this)*	
Hulston and Jeukendrup (2008)	5.3 $mg.kg^{-1}$	Fasted (but glucose infusion)	~20	105 min?	105 minutes (62%VO_2 max) + TT
Cureton et al. (2007)	1.2 $mg.kg^{-1}$ before exercise + 0.6mg/kg every 15 min	Fasted – o/n (except 4 subjects had a small meal or CHO drink 3h or more before	~14	Not measured past 2h 15 min *(still increasing*	120 minutes (60-75% VO_2 max) +15 min TT
Cox et al. (2002)	**6 $mg.kg^{-1}$ 1h before cycling**	**2 g CHO/kg** *Caffeine ingested 60 minutes after breakfast*	**~40**	**~2h after dose**	120 minutes (70% VO_2 peak) + 7$kJ.kg^{-1}$ TT
Conway, Orr, and Stannard (2003)	6 $mg.kg^{-1}$	No breakfast (fasted)	~38	~1h after dose	90 minutes (70% VO_2 peak) + 30 min TT
Kovacs, Stegen, and Brouns (1998)	150 $mg.L^{-1}$ caffeine 225 $mg.L^{-1}$ caffeine 320 $mg.L^{-1}$ caffeine	Subjects arrived fasted. Consumed standardised breakfast. Caffeine 30 min after breakfast	Plasma caffeine after 75 minutes: ~ 4 ~ 5 ~ 9	Not measured past 2h 15 min *(still increasing)*	~ 1 hour TT

Note: Plasma caffeine concentration at 60 minutes post ingestion and the time taken to reach a peak is shown along with the exercise time (where relevant), the caffeine dose used and whether the results are from a fasted or fed state. Where values are approximate they have been converted from µg.mL-1 or taken from a graph. TT denotes time trial. The trial of Skinner, Jenkins, Folling, et al. (2013) specifically investigated the effect carbohydrate has on caffeine absorption at rest however other data is collated from various protocols used for measuring performance during endurance exercise.

2.1.5. Effect of habitual intake

The effect of habitual caffeine intake on caffeine pharmacokinetics has provided variable results. Collomp et al. (1991) illustrated slowed caffeine elimination in heavy coffee drinkers (4-5 cups/day) compared to light coffee drinkers (maximum of one cup per day). However in a study by Skinner et al. (2014) habitual caffeine intake was not associated with any changes in the rate of caffeine metabolism. Furthermore habituation made no difference to the rate of caffeine absorption or maximum caffeine concentrations. Whether or not this then means habituation can affect caffeine's ergogenic potential is a point of conjecture. In this context Tarnopolsky and Cupido (2000) showed that habituation had no effect on the response of acute caffeine (6 $mg.kg^{-1}$) ingestion to electrical stimulation (two minute tetanic stimulation of the common peroneal nerve). In this research low frequency stimulation following acute caffeine ingestion resulted in potentiation of the contraction force irrespective of whether the subject was a habitual or non-habitual caffeine user. This indicates that there is no effect of habituation on the peripheral ergogenic effects of caffeine at least.

2.1.6. Effect of exercise on caffeine absorption and elimination

Exercise of a submaximal intensity has been shown to have little effect on the rate of caffeine absorption when compared to rest (Collomp et al., 1991; McLean & Graham, 2002). This was seen both when subjects exercised (60 minutes, 30% VO_2 max) immediately following caffeine ingestion (Collomp et al., 1991) and when exercise (90 minutes, 65% VO_2 max) commenced one hour after caffeine ingestion (McLean & Graham, 2002).

In contrast, exercise was found to effect peak caffeine concentration, however results generated are inconsistent. When subjects performed light exercise immediately following caffeine ingestion, peak caffeine concentration was elevated (Collomp et al., 1991). However, when moderate endurance exercise was commenced one hour following caffeine consumption, exercise had no effect on peak caffeine concentration (McLean & Graham, 2002). It is likely that the different rates of caffeine metabolism seen during these studies, was due to the timing of exercise following caffeine ingestion. Hepatic blood flow is compromised when exercise intensity increases. As caffeine breakdown occurs in the liver, the reduced blood flow in response to exercise may result in slowed caffeine breakdown and subsequently, caffeine concentration remains elevated.

Variable results were also seen in caffeine elimination; with either no effect (McLean & Graham, 2002) or accelerated (Collomp et al., 1991) caffeine elimination with exercise. Similarly to the peak caffeine concentration, the different caffeine ingestion and exercise protocols may have resulted in differences in the rate of caffeine metabolism occurring in the liver and subsequent caffeine elimination.

The protocol used by McLean and Graham (2002) is likely of more relevance to athletes. Caffeine is often consumed an hour prior to exercise along with a pre-exercise meal, and higher exercise intensities are commonly used. Additionally, this study was well controlled with respect to physical fitness, chronic caffeine consumption of subjects and abstention of foods known to alter caffeine metabolism.

2.1.7. Effect of gender, age, body composition and training status on caffeine pharmacokinetics

Peak serum caffeine concentration and the time taken to reach a peak following caffeine ingestion are not affected by age or gender (McLean & Graham, 2002; Skinner et al., 2014). However having greater fat mass has been associated with increased rate of caffeine absorption and slower caffeine elimination. Kamimori, Somani, Knowlton, and Perkins (1987) demonstrated that at rest, obese individuals (n=3) had increased rate of caffeine absorption but slower elimination. Skinner et al. (2014) also found that higher fat mass was significantly associated with a slower rate of caffeine elimination.

Of more relevance to the athlete, training status may also modify caffeine pharmacokinetics. Skinner et al. (2014) saw a trend towards lower peak caffeine concentrations in trained cyclist/triathletes compared to active individuals. Vigorous training programs have also been shown to increase the activity of CYP1A2 (Boel, Andersen, Rasmussen, Hansen, & Dossing, 1984; Vistisen, Poulsen, & Loft, 1992) which leads to increased caffeine breakdown.

2.2. Caffeine metabolism

The main enzyme responsible for caffeine (1,3,7–trimethylxanthine) metabolism is cytochrome P450 1A2 (CYP1A2) (Sachse, Brochmoller, Bauer, & Roots, 1999). This enzyme metabolises caffeine in the liver via N3, N1 and N7 demethylation (Fig. 2.2) to form paraxanthine (PX; 80%), theobromine (TB; 11%) and theophylline (TP; 5%) respectively (Magkos &

Kavouras, 2005). Additional hepatic demethylation and oxidation of these metabolites forms multiple urates and acetylated uracil derivatives which can be recovered in the urine, less than 3% of which is caffeine (Mandel, 2002). With higher caffeine doses or repeated caffeine consumption, paraxanthine accumulates in the plasma due to saturation of paraxanthine metabolism resulting in reduced paraxanthine clearance (Mandel, 2002). Paraxanthine has also been shown to be pharmacologically active at high concentrations. Thus, heavy caffeine users may accumulate paraxanthine to a level that provides pharmacological activity (Benowitz, Jacob, Mayan, & Denaro, 1995). Besides caffeine, CYP1A2 is important to the metabolism of many drugs such as paracetamol and antipsychotic (clozapine) and anti-depressant (imipramine) medications (Sachse et al., 1999).

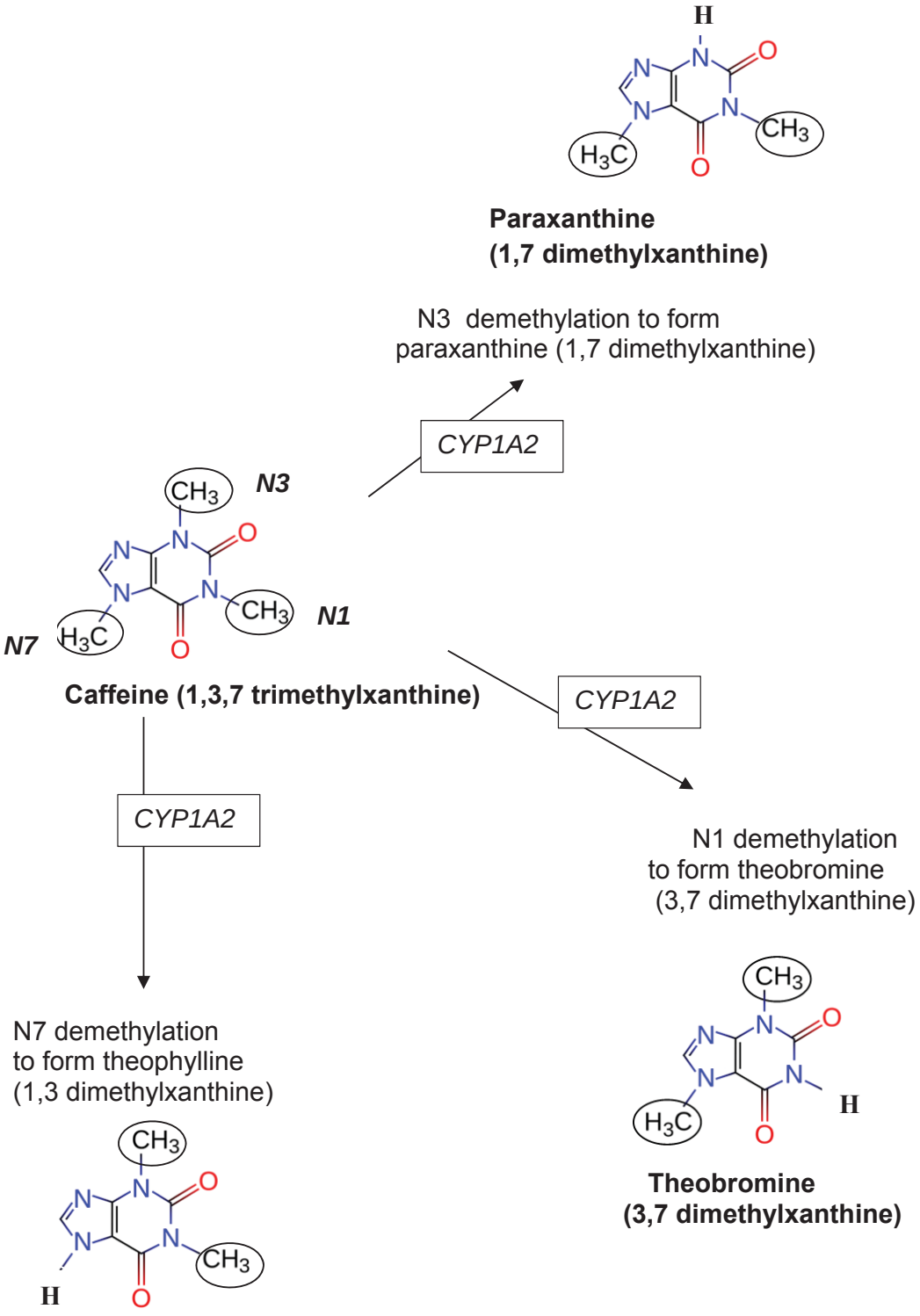

Figure 2.2. **CYP1A2 enzymatic metabolism of caffeine in the liver to produce the three major caffeine metabolites, paraxanthine, theophylline and theobromine. (Caffeine image, 2015) Modifications of the metabolite structures have been made.**

2.3. CYP1A2 genotype

It has been shown that there is a wide variation in individual CYP1A2 activity, with polymorphisms of the CYP1A2 gene being associated with changes in enzymatic activity (Sachse et al., 1999).

2.3.1. CYP1A2 (-163C>A)

A single nucleotide polymorphism (SNP) of CYP1A2 involves either a C or A nucleotide at position -163 downstream of the first transcribed nucleotide. However, there appears to be some confusion about the nomenclature of this enzyme. According to the current nomenclature downloaded from CYP1A2 @ Human Cytochrome P450 (CYP) Allele Nomenclature Committee (2015) the C variant at position -163 is considered to be the wild-type allele and is denoted CYP1A2*1A. Early sequencing by Sachse et al. (1999) of 236 Caucasian volunteers genotyped for the CYP1A2 C to A transversion at position 734 (-163 downstream of the first transcribed nucleotide), reported 46% were homozygous A/A, 43% heterozygous A/C and 10% were homozygous C/C genotype. These authors suggest that as the A variant is more frequent it should be termed CYP1A2*1A and the C variant CYP1A2*1B. In later work by these authors (Sachse et al., 2003) they continue to use CYP1A2*1A to denote the A variant and change the name of the C variant to CYP1A2*1F, citing this as current CYP1A2 allele nomenclature as of 2 July 2002.

Cornelis, El-Sohemy, and Campos (2004) Cornelis, El-Sohemy, Kabagambe, and Campos (2006) Cornelis, El-Sohemy, and Campos (2007) and Palatini et al. (2009) also all refer to the C variant as CYP1A2*1F. However as

mentioned, current CYP1A2 allele nomenclature (2015) has changed, so that the wild type allele is termed CYP1A2*1A (carriers of the C variant) and a C to A transversion at this position (-163C>A), is termed the CYP1A2*1F allele (i.e. the A variant). Regardless of the nomenclature used, however, most authors refer to the A variant as being responsible for rapid metabolism or higher inducibility and carriers of the C allele having reduced enzyme inducibility or slow metabolism.

2.3.2. Differences in the rate of caffeine metabolism in relation to genotype

Initial findings by Sachse et al (1999), indicated that those that were homozygous for the A allele (A/A) had faster caffeine metabolism than those who were carriers of the C allele (both heterozygous A/C and homozygous C/C). This was based on the plasma paraxanthine (PX) to caffeine ratio five hours following caffeine ingestion. This effect was only seen in smokers. However, as urinary PX/Caffeine ratios rather than plasma were used for assessing non-smokers, a lack of sensitivity in the urine assay, may have resulted in the absence of an effect.

Urinary caffeine metabolite ratios were again used to investigate CYP1A2 activity (Sachse et al., 2003). However this study used the ratio of AFMU +1U+1X/17U 8 hours after instant coffee was consumed (4g). Where AFMU denoted 5-acetylamino-6-formylamino-3-methyluracil; 1U, 1-methyluracil; 1X, 1-methylxanthine; 17U, 1,7-dimethyluric acid; 17X, 1,7-dimethylxanthine, (paraxanthine), and 137X, 1,3,7-trimethylxanthine (caffeine). As with this group's previous study (Sachse et al., 1999), the A/A genotype had

significantly higher activity but it was only seen in smokers. Non-smokers (n=100) had no differences in CYP1A2 activity. Other researchers have also shown that CYP1A2 activity is significantly higher in smokers. However, there were no differences in enzyme activity between genotypes (Aklillu et al., 2003; Nakajima et al., 1999).

Sachse et al. (2003) also report that six common CYP1A2 polymorphisms (-3858G>A, -2464T>delT, -740T>G, -164A>C, 63C>G, 1545T>C) in British Caucasians are in linkage disequilibrium such that only changes in -164 A>C and 1545T>C will enable the other polymorphisms to be inferred. The most frequent linkages were:

-3858G/-2464T/-740T/**-164A**/63C/**1545T** (61.8%),

-3858G/-2464T/-740T/**-164C**/63C/**1545C** (33.3 %) and

-3858G/**-2464delT**/-740T/**-164A**/63C/**1545C** (3.5%)

Aklillu et al. (2003) evaluated inter-individual CYP1A2 activity in Ethiopians to ascertain whether there was any environmental influences involved in CYP1A2 activity. They investigated both Ethiopians living in Ethiopia (n=100) and Ethiopians living in Sweden (n=73). However they found that environmental factors had no influence on CYP1A2 activity between the two groups, lending support to a study in twins (Rasmussen, Brix, Kyvik, & Brosen, 2002) that CYP1A2 activity is mainly governed by genetic factors. During the course of their investigation, Aklillu et al. (2003) identified four haplotypes present in Ethiopians **CYP1A2*1A** (-164C), **CYP1A2*1F** (-164A),

CYP1A2*1J (-164A; -740T>G), **CYP1A2*K** (-164C; -740T>G; -730C>T). Both the CYP1A2*1J and CYP1A2*1K are novel haplotypes.

CYP1A2 polymorphisms at position -2964 G>A and 734 (-164A>C) on enzyme activity were investigated in 3 localities in Mexico (Castorena-Torres et al., 2005). Eight hour urinary caffeine metabolite ratios were significantly increased in those that had the intron I mutation A/A at position -164 compared to heterozygous subjects (C/A) irrespective of smoking status. Other environmental factors such as obesity, or consumption of alcohol, food containing low amounts of PAH (Polycyclic aromatic hydrocarbons) or caffeine containing food and beverages did not affect the enzyme activity. The activity of the CYP1A2 enzyme was not affected by polymorphism at position -2964.

Activity of CYP1A2 enzyme was significantly increased by an intense exercise program consisting of 8-11 hours per day for 30 days (Vistisen, Loft, & Poulsen, 1991). In this study 23 healthy males showed a 70% increase in urinary metabolite ratio from baseline after undertaking the exercise program. This effect was additive to the effect of smoking. Other CYP1A2 substrates (antipyrine, andaminopyrine) have also been shown to increase the activity of CYP1A2 with increased physical fitness (Boel, 1984).

Various population studies have shown that both the A and C variants at position -164 are similar in frequency. The A variant (A/A genotype) was seen in 54%, 46% and 45% of Mexican, Caucasian and Costa Rican study populations respectively. Frequency of those heterozygous for C/A genotype in the same study populations was 46%, 43% and 44%

respectively (Castorena-Torres et al., 2005; Cornelis, El-Sohemy, et al., 2007; Sachse et al., 1999)

Thus a study investigating the activity of this enzyme in relation to exercise is warranted. In addition, a relatively even distribution of subjects within the two groupings i.e. those who are homozygous A/A and those who are carriers of the C allele is likely.

2.4. Caffeine mechanisms of action on exercise performance

Scientists have been researching caffeine and its ergogenic potential for many years. The actions of caffeine throughout the body are widespread. Thus the (ergogenic) mechanisms of action may be different at different times and during different types of exercise. Therefore, determining the mechanism by which caffeine exerts its ergogenic effects has provided much speculation and debate (Fredholm et al., 1999; Graham, 2001a; Tarnopolsky, 2008). It is beyond the scope of this review to discuss in detail all relevant published research; however, the more pertinent theories such as the original hypothesis of glycogen sparing and subsequent investigations into peripheral and central actions of caffeine will be summarized below.

2.4.1. Glycogen sparing

Early caffeine and performance research revealed lowered respiratory exchange ratios (RER) and increased plasma free fatty acids during exercise

following caffeine ingestion. This led to the hypothesis that increased fat oxidation and subsequent glycogen sparing were the mechanisms by which caffeine produced its ergogenic effects (Costill, Dalsky, & Fink, 1978; Ivy, Costill, Fink, & Lower, 1979). This theory was established from experiments where cyclists, after consuming caffeine were either able to ride longer to exhaustion or were able to increase work production during an isokinetic ride (2 hour). However, this early research relied on respiratory measurements and fatty acid concentrations in blood to assess substrate metabolism rather than more direct measures of glycogen stores such as muscle biopsies.

Other research also found decreased muscle glycogen utilization when caffeine was consumed prior to cycling 65-70% VO_2 max (Erickson, Schwarzkopf, & McKenzie, 1987). However, blood free fatty acids (FFA) and glucose were not significantly different compared to the control. Spriet et al. (1992) also observed increased cycling time to exhaustion with caffeine ingestion but these authors only saw a glycogen sparing effect within the initial 15 minutes of cycling. Although both these studies used muscle biopsies to directly measure glycogen use, they both had small sample sizes (n=5 and 8 respectively).

In the late nineties it began to emerge that glycogen sparing may not be caffeine's main mechanism of action. Using more specific measures of muscle metabolism such as muscle biopsies or Nuclear Magnetic Resonance (NMR) spectroscopy, Jackman et al. (1996) and Laurent et al. (2000) observed no differences in muscle glycogen concentration following exhausting exercise with prior caffeine consumption. Laurent et al. (2000) used NMR spectroscopy to non-invasively examine muscle glycogen during

an exercise protocol, that had glycogen super compensated trained athletes perform 120 minutes at 65% VO_2 peak followed by alternating one minute sprints with one minute rest until exhaustion. These authors found that compared to a placebo, caffeine failed to exert any glycogen sparing effects in glycogen super compensated athletes, concluding that caffeine's effect is negated when glycogen stores are saturated.

Graham et al. (2000) also showed that caffeine ingestion (5 $mg.kg^{-1}$) did not alter leg glucose uptake (muscle biopsy) during a 60 minute exercise protocol at 70% VO_2 max indicating that caffeine ingestion was not increasing fat oxidation or sparing glycogen. Additionally these authors did not find any significant differences in pulmonary RER with caffeine ingestion and furthermore, the RER results showed that carbohydrate oxidation was the primary substrate during this exercise. However these authors did see an increase in arterial fatty acid mobilization and circulating adrenaline.

The discrepancy in many of the studies investigating glycogen sparing mystified many scientists. Indeed some could not explain why their own group had different findings (Graham, 2001b). Due to muscle glycogen biopsies being highly variable and the possibility of type II statistical error (small sample sizes); significant differences in muscle glycogen content may not have been detected. Thus, Graham (2008) pooled the data from some of the smaller studies; (Chesley, Howlett, Heigenhauser, Hultman, & Spriet, 1998; Graham et al., 2008; Greer, Friars, & Graham, 2000; Spriet et al., 1992; Thong et al., 2002), to provide a much larger data set. However, although glycogen levels did trend towards a sparing effect it was not significant even with the much larger sample size (n= 37).

Acute caffeine ingestion results in increases in epinephrine and norepinephrine secretion by the adrenal medulla, leading to multiple effects throughout the body such as increased lipolysis. Epinephrine was increased during exercise in both male and female trained cyclists but was significantly higher after consumption of an energy drink containing caffeine (160mg, approximately 2.35mg.kg^{-1}) during and immediately following one hour cycling at 70% W Max compared to placebo (Ivy et al., 2009). Epinephrine significantly increased with caffeine consumption (4.45mg.kg^{-1}) when caffeine was administered as either capsules, coffee or decaffeinated coffee plus caffeine) during a one hour rest period and during endurance exercise (treadmill running to exhaustion) in trained endurance runners compared to placebo and decaffeinated coffee (Graham, Hibbert, & Sathasivam, 1998). Van Soeren and Graham (1998) also saw significantly increased plasma epinephrine concentration even when a two or four day caffeine withdrawal period was imposed.

To date, glycogen sparing as a mechanism of action has been discounted as the main mechanism responsible for caffeine's ergogenic effect and the focus has shifted to many other mechanisms relating to direct action by caffeine on muscle and those via the central nervous system.

2.4.2. Peripheral actions of caffeine

In-vitro studies have demonstrated that caffeine can both promote the direct release of calcium from the sarcoplasmic reticulum promoting muscle contraction and also inhibit cyclic nucleotide phosphodiesterase, which in

turn increases cyclic adenosine monophosphate (cAMP) leading to increased lipolysis (J. K. Davis & Green, 2009). As such these effects may contribute to the biochemical actions of caffeine. However doses of caffeine at a concentration 100 times and 20 times higher than that reached by normal caffeine consumption are required for caffeine to mobilize release of calcium or inhibit phosphodiesterase and subsequent cyclic nucleotide breakdown respectively. Caffeine ingestion at these concentrations is toxic and is therefore unlikely to be reached during human caffeine consumption, ruling out these as mechanisms contributing to caffeine's effects (Fredholm et al., 1999).

However, Tarnopolsky (2008) stated increased calcium release from the sarcoplasmic reticulum following caffeine administration is likely to be involved in increasing contractile force during submaximal intensities and as such may play a part in the multifactorial effects of caffeine on endurance exercise performance. In this review, the author based this conclusion on three studies (Lopes, Aubier, Jardim, Aranda, & Macklem, 1983; Mohr, Van Soeren, Graham, & Kjaer, 1998; Tarnopolsky & Cupido, 2000) that have shown attenuated fatigue during electrically evoked contraction in humans following caffeine administration and some publications (Block, Barry, & Faulkner, 1992; Connett, Ugol, Hammack, & Hays, 1983; James, Kohlsdorf, Cox, & Navas, 2005) that have shown caffeine in the physiological ($\mu mol.L^{-1}$) range can enhance force output in isolated muscle preparations. In these human studies it appears that low frequency of stimulation following caffeine administration enhanced contraction force. Although variable, Lopes et al (1983) saw increased tension developed with low frequency of stimulation

following a low dose (50mg) of caffeine compared to a placebo. Tarnopolsky & Cupido (2000) using a caffeine dose that has been found to be ergogenic in endurance exercise (6mg.kg^{-1}) also found that low (20Hz) but not high (40Hz) frequency stimulation of the common peroneal nerve resulted in potentiation of the contraction force compared to a placebo. These results suggest that the ergogenic effect of caffeine may be partially mediated by a direct effect on skeletal muscle.

In-vitro studies using a variety of species (rat, frog, and mouse) and muscle types (soleus, extensor digitorum longus (EDL)) have shown increases in muscle contractions with physiologically relevant doses of methylxanthines. A small but significant increase in muscle force and power was seen in non-fatigued muscle with prior caffeine (70 μmol.L^{-1}) incubation, however this level of caffeine was not able to prevent fatigue (James et al., 2005). Other studies have also shown increases in isometric force however this effect was short lived (Reading, Murrant, & Barclay, 2004). Taken together, it demonstrates that caffeine may enhance power over short time periods, but may not enhance endurance activities.

2.4.3. Sympathetic nervous system stimulation

Many studies (Ivy et al., 2009; Laurent et al., 2000; Spriet et al., 1992; Van Soeren & Graham, 1998) have shown that the sympathetic nervous system is stimulated with caffeine ingestion by significantly increased adrenaline concentration at rest and during exercise compared to a placebo. It may be possible that some of the responses to caffeine are secondary to increased

sympathetic stimulation rather than being responsible for the ergogenic effect of caffeine on exercise performance (Graham et al., 2008). For example, the inotropic (altered force of contraction) effect of caffeine on heart rate are well described (Davis et al., 2003) , and probably simply reflect direct sympathetic stimulation, a function of caffeine-induced adrenaline release rather than being related to any ergogenic effect.

Studies using tetraplegic individuals (Mohr et al., 1998), where the spinal cord has to function independently from the brain have shown that caffeine may have a direct effect on various peripheral tissues. In these studies, caffeine ingestion did not cause a rise in circulating adrenaline, but it did increase exercise time indicating that caffeine may have a direct action, independent of the brain and circulating adrenaline. However many of caffeine's effects are via the central nervous system (CNS) and it is generally considered that caffeine's main mechanism of action is by blocking adenosine receptors and subsequent effects.

2.4.4. Adenosine and blocking of adenosine receptors

Caffeine is structurally similar to adenosine (Figure 2.3), thus it is able to bind to adenosine receptors but not activate them. Adenosine is ubiquitously produced and binds to four known G- protein coupled receptors A_1, A_{2A}, A_{2B} and A_3 (Fisone, Borgkvist, & Usiello, 2004). These receptors occur in muscle, liver, the heart, adipose tissue, vascular endothelium and the nervous system (Graham et al., 2008). The actions of adenosine are varied depending on tissue, location and affinity of each receptor subtype to adenosine.

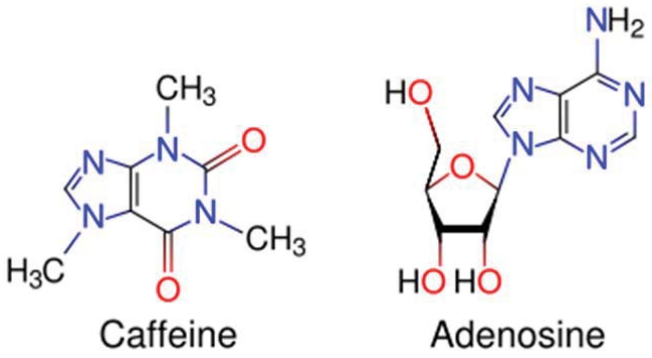

Figure 2.3. Structure of caffeine and adenosine (2015)

By binding to adenosine receptors but not activating them, caffeine may have a central mechanism via neurotransmitters. The central actions of adenosine are the release of dopamine and norepinephrine which leads to decreased wakefulness and lowered motor activity. It has been shown that caffeine can block the effects of adenosine on A_{2A} receptors in the CNS of rodents (Davis et al., 2003) and to a lesser extent, A_1 receptors at low concentration i.e. equivalent to a single cup of coffee in humans (Davis et al., 2003; Fredholm et al., 1999). This has led to the idea that the blocking of adenosine receptors in the brain is the main mechanism of action of caffeine at physiological concentrations (Fredholm et al., 1999). Although most people think of caffeine as having stimulatory actions, in reality, it is suppressive effects on adenosine action that induces central nervous system stimulation. Without the suppressive actions of adenosine, caffeine promotes increased alertness and motor activity (Fisone et al., 2004). This would potentially reduce the sensation of exercise-induced 'central' fatigue (Tarnopolsky., 2008).

Thus the actions of caffeine via inhibition of adenosine will depend on the tissue and receptor isoform it blocks (Graham et al., 2008). In the brain A_{2B} and A_3 receptors have low affinity to adenosine whereas A_1 and A_{2A} receptors demonstrate high affinity to adenosine. Hence A_1 and A_{2A} are believed to be important in caffeine binding and inactivating adenosine receptors whereas A_{2B} and A_3 receptors are thought to be of no consequence to caffeine inhibition of adenosine receptors in the brain. The A_1 receptor has widespread distribution with the majority being located on presynaptic nerve terminals, where binding of adenosine inhibits the release of neurotransmitters. Binding of caffeine to A_1 receptors will lead to the blocking of adenosine inhibition of neurotransmitters with the subsequent neurotransmitter release (e.g. glutamate, dopamine and acetylcholine) which are believed to be responsible for caffeine's positive effect on arousal, vigilance and attention (Fisone et al., 2004; Jones, 2008).

The group of Davis et al. (2003) showed that CNS caffeine administration delayed fatigue in rodents, predominantly by centrally mediated adenosine receptor antagonism. In this investigation, rat treadmill running time to fatigue was increased after CNS caffeine administration ($0.6mg.kg^{-1}$) compared to control. However with CNS administration of an A1 and A2 adenosine receptor agonist (NECA), rat treadmill run time to fatigue was decreased compared to a control (vehicle solution). In contrast, caffeine administered peripherally made no difference to rat treadmill run time to exhaustion. In addition, when caffeine was administered to the CNS five minutes prior to the NECA, the reduction in run time seen with NECA alone was blocked (i.e. caffeine plus NECA promoted a longer run time to fatigue

than NECA alone), indicating that the ergogenic effects of caffeine results from the CNS blocking adenosine receptors.

2.4.5. Perceived exertion (RPE)

Another potential centrally mediated caffeine mechanism is its effect on ratings of perceived exertion (RPE). It has often been shown that caffeine reduces people's perception of effort (Cole et al., 1996; Costill et al., 1978; Cox et al., 2002). Cole et al. (1996) found that caffeine ingestion increased work output over a range of perceived exertion. As this was short duration exercise, it was unlikely that metabolic factors significantly affected performance, and the authors speculate that the caffeine induced a rise in catecholamine's altered neuronal excitability. Thus masking a person's perception of fatigue may provide a mechanism for caffeine's ergogenic effect.

In a meta-analysis, Doherty and Smith (2005) found that RPE was lower following caffeine ingestion during constant load exercise but was not different at the end of exhausting exercise. These authors also found that caffeine dose, withdrawal period and the time between caffeine ingestion and exercise did not appear to influence caffeine's effect on RPE. However trained individuals (high VO_2 max) showed the greatest reduction in RPE.

Most studies use the Borg scale to assess RPE. The research group of Jenkins and colleagues (2008) used the D-RPE scale which incorporates three components of effort sense (CNS factors, local factors and cardio respiratory factors) to clarify the perceptual responses of caffeine during

exercise. In this work, RPE-O monitored motivation, drive and task aversion to give an overall perception of effort. The amount of strain, force sensation and fatigue in the working muscles measured perception of effort in the legs (RPE-L) and heart rate and breathing rate and depth were indicators of chest perceived exertion (RPE-C). These authors found that low doses of caffeine (1, 2 and 3 $mg.kg^{-1}$) did not affect perceptual response during a 15 minute cycle at 80% VO_2 peak even though the 2 and 3 $mg.kg^{-1}$ dose increased subsequent cycling performance. Similarly Ivy et al. (2009) found that an energy drink containing approximately 2.35 $mg.kg^{-1}$ caffeine did not change RPE (Borg scale) in both female and male trained cyclists compared to placebo during one hour cycling at 70% W Max.

As discussed, caffeine is metabolised in the liver to form paraxanthine and theophylline. Both paraxanthine and theophylline also have pharmacological activity (Greer et al., 2000). Thus this activity also needs to be considered when looking at the biological actions of caffeine (Fredholm et al., 1999). However, administration of caffeine (oral or intravenously) to caffeine-naive individuals does not result in significant accumulation of these dimethylxanthines and as such they are not thought to contribute to the observed caffeine physiological effects (Magkos & Kavouras, 2005). Gradual accumulation of paraxanthine occurs when caffeine is chronically consumed however its significance had not been evaluated at the time of writing (Magkos & Kavouras, 2005).

There have been years of research on the effects of caffeine within the body. However, not all of these effects may be relevant to exercise performance. It has been shown that caffeine stimulates the central nervous system (CNS)

and as many people consuming a cup of coffee can attest, caffeine can produce feelings of increased arousal and there may also be reduced feeling of pain and perceived exertion during exercise. Much research has also illustrated that caffeine mobilises free fatty acids (FFA) probably via adrenaline stimulated lipolysis. It is believed that many of the actions of caffeine are centrally mediated and as a result of blocking the suppressive effects of adenosine (i.e. inhibiting the neurotransmitters dopamine and norepinephrine release leading to subsequent decreased arousal and sleep promotion). However, whether the ergogenic effects are due to the direct action of caffeine on skeletal muscle or due to CNS control remains to be clarified. In all likelihood it is probable that caffeine's ergogenic effect is in response to multiple factors rather than just a single mechanism.

2.5. Effects of caffeine on endurance performance

Various exercise protocols such as time to fatigue, time trial, or different caffeine ingestion protocols such as provision of caffeine with carbohydrate, differing caffeine dose before or repeated dose during exercise have all been utilised to investigate the effect caffeine has on exercise performance. However, much of the research has yielded conflicting results.

Many earlier studies (Costill et al., 1978; Spriet et al., 1992) measured the time taken to reach exhaustion. This is a measure of endurance capacity rather than endurance performance (Kovacs et al., 1998). However when a time trial is used to measure performance, the cyclist performs the greatest amount of work in the time frame given. This better replicates the

competitive situation and is thus a more appropriate measure of exercise performance (Jenkins et al., 2008). Time trials appear to be very reproducible (Astorino, Cottrell, Lozano, Aburto-Pratt, & Duhon, 2012; Jeukendrup, Saris, Brouns, & Kester, 1996) thus this section of the review will only focus on those studies that have used a time trial as a measure of performance.

2.5.1. Effect of caffeine co-ingested with carbohydrate

Consumption of carbohydrate has been shown to be beneficial to exercise performance especially during endurance events (Burke, Kiens, & Ivy, 2004). As carbohydrate is commonly consumed prior to and during events, investigation into whether caffeine would improve performance above that of carbohydrate alone has been assessed. Researchers found that providing caffeine, above that of carbohydrate alone, resulted in either no additional effect (Hunter et al., 2002; Jacobson et al., 2001) or, enhanced performance (Cureton et al., 2007; Hulston & Jeukendrup, 2008; Kovacs et al., 1998) above carbohydrate alone was demonstrated.

Using a randomised placebo controlled, crossover trial, (Kovacs et al., 1998) investigated the effect of adding various doses (0, 150, 225 and 320 $mg.L^{-1}$) of caffeine to a carbohydrate electrolyte drink (7% CHO). This drink was consumed during a warm up protocol and provided a mean caffeine intake of 2.1, 3.2 and 4.5$mg.kg^{-1}$ BW respectively. Approximately 35 minutes later the well trained subjects performed a set amount of work as quickly as possible (approximately 1 hour). The results showed that cycling performance was improved above carbohydrate alone in a dose related manner for the 150

and 225 mg.L^{-1} caffeine but there was no further improvement with the 320 mg.L^{-1} dose. A commercial caffeinated (5.3 mg.kg^{-1} caffeine over entire trial) sports drink that also provided carbohydrate, electrolytes, taurine, carnitine, vitamins B3, B6 and B12 and sucralose was also shown to enhance time trial performance (15 min after two hours at 60-75% VO_2 max) of highly trained cyclists (n=16) above that of a placebo and a non-caffeinated carbohydrate electrolyte sports drink (Cureton et al., 2007).

Hulston and Jeukendrup (2008) investigating the effect of caffeine co-ingested with carbohydrate on substrate metabolism during exercise found enhanced performance with caffeine plus carbohydrate above that of carbohydrate alone. However exogenous carbohydrate oxidation was not increased by caffeine ingestion. This is in contrast to a previous investigation (Yeo, Jentjens, Wallis, & Jeukendrup, 2005) where caffeine enhanced carbohydrate oxidation during exercise. Both these studies had well trained subjects exercising at 65% VO_2 max for two hours; however, Hulston and Jeukendrup (2008) used smaller caffeine (5.3 vs 10 mg.kg^{-1}) and carbohydrate (0.71 vs 0.80 g.min^{-1}) doses compared to Yeo et al. (2005) to be more representative of what athletes use during competition.

However not all studies have shown additional benefits of caffeine above that of carbohydrate ingestion. When highly trained, non-habituated caffeine users were provided caffeine as a 6 mg.kg^{-1} body weight (BW) dose, there was no additive effect of caffeine over carbohydrate alone on time trial performance following 120 minutes steady state cycling (Jacobson et al., 2001). Hunter et al. (2002) also did not see a difference in time to complete a 100 km time trial or average power in highly trained cyclists consuming

carbohydrate (7%), caffeine (6 $mg.kg^{-1}$ + 0.33 $mg.kg^{-1}$ every 15 minutes), a mixture of both carbohydrate and caffeine or placebo.

It can be seen, that although these studies all used highly trained athletes, they employed variable study designs. Thus differences in exercise intensity and duration used in individual experiments may have contributed to the contrasting results seen in studies investigating caffeine performance above that of carbohydrate alone. It has been shown that caffeine breakdown is slowed in heavy coffee drinkers (Collomp et al., 1991). Therefore the use of non-habitual caffeine users as study subjects in the work of Jacobsen et al (2001) may also have contributed to the lack of effect seen. The studies showing enhanced performance above carbohydrate alone (Cureton et al., 2007; Hulston & Jeukendrup, 2008; Kovacs et al., 1998) all used subjects who were identified as caffeine users.

2.5.2. Effect of low doses of caffeine

Drinks such as coke and red bull that provide low doses of caffeine (2 and 2.35 $mg.kg^{-1}$ respectively) along with carbohydrate have been investigated for their potential to enhance exercise performance. Enhanced performance was seen with coca cola ingestion instead of a sports drink late in an exercise protocol (Cox et al., 2002). This was repeated in a more rigorous placebo controlled experiment, where cyclists were given various cola beverages (5 $ml.kg^{-1}$ at 80 and 120 minutes steady state followed by 5 $ml.kg^{-1}$ during a 7 $kJ.kg^{-1}$ time trial). Coke enhanced performance (3.3%) compared to a decaffeinated cola drink (6% carbohydrate). When the cola drink was

provided containing the same concentration of caffeine (13 mg.100mL^{-1}) or carbohydrate (11%) as coke, the enhanced performance was only 2% and 1% respectively. Ivy et al. (2009) found that an energy drink (red bull) given as a dose of approximately 2.35 mg.kg^{-1} caffeine ingested 40 minutes prior to a time trial (one hour cycling at 70% W Max) improved performance (4.7%) in both female and male trained cyclists compared to a placebo.

The lowest dosage of caffeine required to produce an ergogenic effect has also been investigated. In a caffeine dose response study, low and moderate doses (1.5 and 3 mg.kg^{-1}) of caffeine with readily available carbohydrate did not elicit improved time trial performance following 120 minutes of steady state cycling (Desbrow et al., 2009). However, when these same authors used a time trial only (1 hour) both a 3 mg.kg^{-1} and 6 mg.kg^{-1} caffeine dose was found to be ergogenic (Desbrow, Biddulph, et al., 2012). Jenkins et al. (2008) also found an ergogenic effect of 2 and 3 mg.kg^{-1} doses. However this study only used 15 minutes cycling before the time trial.

2.5.3. Effect of divided doses of caffeine

Cox et al. (2002) found that ingesting 6 mg.kg^{-1} BW as either a single dose or divided into a series of doses throughout the exercise protocol enhanced time trial performance following 120 minutes of steady state cycling regardless of when the caffeine was ingested. Conway et al., (2003) also investigated the effect of divided doses of caffeine using the same dose as Cox et al. (2002). In contrast these authors found that when caffeine was ingested as either a single or divided dose, cycling time trial performance,

after 90 minutes steady state cycling was not significantly enhanced. However there was a strong trend towards an ergogenic effect, but the researchers have concluded that low subject numbers (n=8) may have contributed to the lack of significance in these results (Conway et al., 2003). Nonetheless it does appear that the subjects in this trial arrived at the lab after an overnight fast and then completed the time trial (30 minutes) after 90 minutes of steady state exercise (70% VO_2 max) without any breakfast or carbohydrate during the ride. Reduced glycogen levels may have contributed to a lack of significant effect. Subjects completing high intensity cycling intervals (HIT 8 x 5 min) with normal glycogen levels showed significantly higher % peak power output than when they had low glycogen levels (Lane et al., 2013). Further, increases in self-selected % peak power output above that of the normal glycogen state were seen with caffeine ingestion (3 $mg.kg^{-1}$ BW 60 minutes prior to exercise). However, when caffeine was ingested with low glycogen stores the caffeine was unable to restore the self-selected intensity to normal. Additionally, other authors also found caffeine provided no beneficial effect (200 mg) without carbohydrate on a 20 km time trial following two hours cycling by subjects in negative energy balance (Slivka, Hailes, Cuddy, & Ruby, 2008).

2.5.4. The effect of coffee vs. anhydrous caffeine on endurance performance

As discussed in the previous section, coffee can contain considerable amounts of caffeine (25-300 mg). Thus many athletes use coffee as an ergogenic aid before events (Desbrow & Leveritt, 2006). Graham et al.

(1998) investigating the effects of caffeine or coffee on endurance exercise found that there were no differences in serum caffeine or paraxanthine concentrations between caffeine capsules, coffee or decaffeinated coffee with added caffeine. All treatments were given a total of 4.45 mg caffeine.kg^{-1} in a fasted state. However, endurance performance (treadmill running to exhaustion) was only increased when caffeine was consumed independently of coffee. These authors attribute this to caffeine antagonism of other compounds in the coffee interfering with the ergogenic effects.

Although it is often stated that coffee is inferior to anhydrous caffeine in producing an ergogenic effect, this may not necessarily be the case. Many authors cite the work of Graham et al. (1998), however in contrast to this work, Hodgson et al. (2013) more recently found that a 5 $mg.kg^{-1}$ dose of either caffeine or coffee 60 minutes prior to exercise significantly improved time trial finishing times compared to both decaf (4.7%) and placebo (4.3%). In this study subjects cycled for 30 minutes at 55% VO_2 max before completing a target amount of work (approximately 45 minute time trial). As time trials provide more reproducible results than time to exhaustion (Jeukendrup, 1996), this study may be more relevant than the work of Graham et al. (1998) which used treadmill running time to exhaustion to measure performance. However as noted above the concentration of caffeine in purchased coffee is highly variable and whether lower doses of caffeine in coffee have the same effect is yet to be determined.

2.5.5. The effect of caffeine withdrawal on endurance performance

Most research uses a caffeine withdrawal period before conducting trials. However whether this is necessary or not has been investigated. Six male recreational athletes showed significantly increased cycling time to exhaustion with caffeine ingestion (6 $mg.kg^{-1}$) compared to a placebo, regardless of whether two or four day withdrawal period was imposed (Van Soeren & Graham, 1998). In a better controlled experiment, Irwin et al. (2011) used a time trial to measure performance, and also found that a four day withdrawal period had no impact on the ergogenic effect of a 3 $mg.kg^{-1}$ caffeine dose (n=12 well trained cyclists). In this experiment caffeine ingestion for the non-withdrawal trial was controlled i.e. all subjects refrained from caffeine containing food and beverages during a four day withdrawal period, however during this time subjects were either given daily placebo or caffeine capsules (1.5 $mg.kg^{-1}.day^{-1}$) to represent a withdrawal trial and non-withdrawal trial respectively. Thus, over the course of the experiment subjects completed a placebo and caffeine time trial for both withdrawal and non-withdrawal trials in a double blind crossover manner. Withdrawal from caffeine in those habituated to it can result in headaches, loss of focus, fatigue, and lack of motivation (Fredholm et al., 1999; Irwin et al., 2011; Van Soeren & Graham, 1998). Thus these results suggest that imposing a withdrawal period on an athlete may not provide any additional benefit and cause unnecessary discomfort.

2.5.6. The effect of caffeine on endurance performance in relation to CYP1A2 genotype

As can be seen, moderate and high doses of caffeine have produced conflicting time trial performance results (Conway et al., 2003; Cox et al., 2002; Jacobson et al., 2001; Jenkins et al., 2008). This inconsistency may be as a result of differing study designs, caffeine dosages or exercise intensity and duration, but it may also have a genetic basis. Womack et al. (2012) investigated whether a specific CYP1A2 polymorphism (a C/A polymorphism at intron 1 of the cytochrome P450 gene) had any influence on the ergogenic effect of caffeine supplementation (6 $mg.kg^{-1}$) in trained (n=35) cyclists performing a time trial. These authors saw significantly faster times to complete a 40 km time trial with caffeine compared to placebo in A/A homozygotes that was not seen in those that were carriers of the C allele. It was noted that the A/A homozygote group had slower times during the time trial with placebo ingestion than the carriers of the C allele raising the concern that the two groups may have been different in their exercise capabilities. However, when four subjects with extremely slow placebo times were removed from the dataset to give similar placebo times with both groups there was still a significant effect of caffeine in the A/A homozygotes compared to the C allele carriers. These authors speculated that as A/A homozygotes have been shown to have faster metabolism of caffeine (Sachse et al., 1999) it will result in increased paraxanthine and theophylline. These metabolites have higher adenosine receptor binding affinities than caffeine (Daly, Buttslamb, & Padgett, 1983) thus they may be contributing to the ergogenic effect seen. However; as these metabolites along with plasma

caffeine concentration were not measured it remains to be seen if this is the case.

2.6. Summary

It is now commonly accepted that caffeine has a role in increasing endurance exercise performance. Although much research has gone into understanding the mechanisms by which caffeine exerts its ergogenic effects, the exact mechanisms still remain to be clarified. Caffeine exerts effects on a variety of tissues throughout the body making it difficult to determine what is producing the ergogenic effect. However, it is generally considered that the main effects are due to blocking of adenosine receptors and subsequent stimulation of the central nervous system.

As discussed, individual performance results can be quite variable. A single nucleotide polymorphism in the main enzyme responsible for caffeine breakdown in the liver (CYP1A2 C>A transversion at position -163) has been shown to result in differences in the rate of caffeine breakdown. To date, little research has investigated whether this polymorphism is responsible for the individual variability seen in endurance exercise performance. Thus investigation into any differences in the rate of caffeine metabolism in response to different genotypes and if genotype has an effect on endurance exercise performance is warranted.

Chapter 3 Materials and Methods

3.1. General design

The study was a randomised placebo controlled double blind trial. Subjects were tested on three different occasions consisting of two exercise trials, where the subject ingested gelatine capsules containing either caffeine (5 $mg.kg^{-1}$) or placebo (white flour), and a third session where the subject was given gelatine capsules containing caffeine (5 $mg.kg^{-1}$) at rest. Variable results (Collomp et al, 1991; McLean & Graham, 2002) have been seen in the rate of caffeine metabolism with exercise. Thus a resting trial was deemed necessary to rule out exercise influencing the rate of caffeine metabolism.

Trials were designated Caffeine-Ex (exercise trial with caffeine), Caffeine-Rest (resting trial with caffeine) and Placebo-Ex (exercise trial with placebo). An overview of a trial day can be seen in Figure 3.1.

The study was conducted at the Human Performance Laboratory within the School of Sport and Exercise, and the Human Nutrition Laboratory, Massey University, Palmerston North, New Zealand. The study was approved by the Human Ethics Committee of Massey University (HEC: Southern A Application 11/35).

The exercise protocol used for this research was based on the work of Cox et al. (2002). A 5 $mg.kg^{-1}$ caffeine dose was chosen as variable results have

been seen with lower doses (1-3mg) but generally an ergogenic effect has been found when using doses between 5-6mg (Cureton et al., 2007; Cox et al., 2002; Desbrow et al., 2009; Desbrow et al., 2012; Jenkins et al., 2008) Although it has been shown that a caffeine withdrawal period may not be necessary for endurance exercise performance (Irwin et al.,2011; Van Soeren & Graham, 1998) it was decided to include a 48 hour caffeine abstinence to be comparable to other studies and reduce the possible influence of caffeine metabolites affecting pharmacokinetic parameters.

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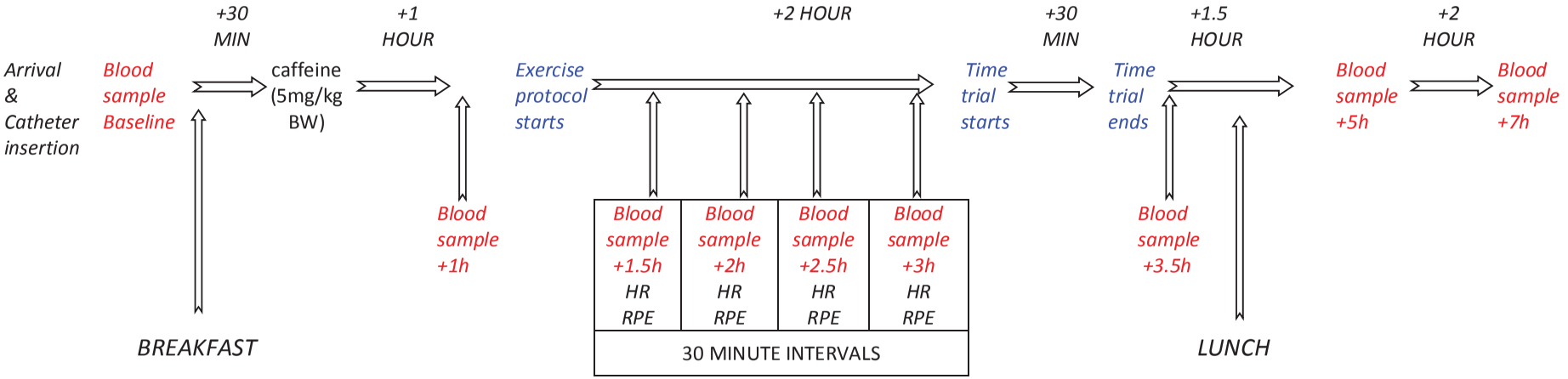

Figure 3.1 **Diagrammatic representation of exercise trial days. Blood samples are timed from caffeine ingestion. Caffeine-Rest followed the same protocol without the exercise component**

3.2. Recruitment process

Initial recruitment was by flyers (Appendix 1) distributed around the university, local cycle shops and clubs. Subsequently, business cards (smaller version of flyers) were also handed out prior to local cycling club rides.

3.3. Subject characteristics

Eleven healthy well trained male cyclists and triathletes volunteered to participate in the study. All subjects were non-smokers with a mean age of 31±3 years, weight 77±4 kg, height 181±2cm relative VO_2 max of 69±4 $mL.kg^{-1}.min^{-1}$ and peak power 433±17 W. Subjects trained an average of 10.6±4.3 hours of aerobic activity per week of which 7.0±3.5 hours was specifically cycling activity. The average workload performed by the subjects during steady state exercise (70%VO_2 max) was 223±17 W.

Subjects were informed of the procedures and the possible risks before obtaining their written consent (Appendix 2).

Usual weekly caffeine intake for subjects is shown in Table 3.1, with 27% of subjects being classified as low (<7/week), 45% moderate (7-14/week) and 27% high (>14/week) habitual caffeine users.

Table 3.1. Number of caffeinated coffee or energy drinks consumed by the subjects per week

Caffeinated coffee or energy drinks consumed/week	# of subjects (n=11)	% of subjects
<7/week	3	27
7-14/week	5	45
14-21/week	2	18
>21/week	1	9

3.4. Inclusion criteria

Inclusion criteria required regular activity as a cyclist or triathlete and age between 18 and 50 years old. Participants were required to be aerobically fit (VO_2 max at least 55 $mL.kg^{-1}.min^{-1}$) and capable of cycling at 70% VO_2 max for two hours followed by a 30 minute self-paced time trial. If they had participated in regular cycling training (road cycling, mountain biking, multisport, triathlon, etc.) over the previous six months, along with high-intensity training or competed in races in the past year they were deemed to be capable of completing the trial.

3.5. Exclusion criteria

Subjects were asked to complete a health questionnaire (Appendix 3) before participating. Those aged over 40 were considered to be at a higher risk of cardiovascular incidents; thus they were asked to provide a doctors certificate before participating. Subjects were excluded from the trial if they reported a history of heart disease, diabetes, kidney trouble, uncontrolled asthma, or were naïve to caffeine.

3.6. Diet and activity before the testing

All subjects were asked to refrain from ingesting caffeine containing foods and beverages for 48 hours prior to each test day. Each subject was given a list of common caffeine containing foods and drinks to avoid as well as non-prescription cold/flu or headache medication that contain caffeine (Appendix 4). In addition they were asked to refrain from brassica vegetables such as broccoli, due to their interference with the functioning of P450 enzymes (Vistisen et al., 1992).

During this time, a two day diet record (Appendix 5) was completed by each participant to monitor caffeine abstention compliance and to standardise pre-trial diet i.e. the same diet was repeated prior to each performance test.

Each participant was also instructed to refrain from heavy exercise for 24 hours prior to each test day. During this time they kept a physical activity log (Appendix 6). The same physical activity was repeated on subsequent test days.

3.7. Preliminary testing

As part of the selection criteria and to calculate the workload required for the steady state cycling (70% of VO_2 max), each potential participant completed a VO_2 max test. To ensure this test had no influence on the subsequent exercise trial, it was a requirement that it was performed at least 48 hours

prior to the exercise trial. Just prior to the VO_2 max test the subject was weighed, and this weight was then used for calculating the amount of caffeine required (5 $mg.kg^{-1}$ body weight (BW), and carbohydrate content in the breakfast meal (1.5 g $CHO.kg^{-1}BW$).

3.8. VO_2 max

Subjects performed a stepwise sub maximal test in which they cycled at a constant self-selected cadence for four minutes at each of four workloads (100W, 150W, 200W, 250W) on an electronically-braked cycle ergometer (Lode Excalibur, Netherlands). Expired respiratory gases were collected by Douglas bag during the final minute of each workload. After completion of the last workload, subjects were given a short (five minutes) rest. Then, they began a ramp test (30W increment/minute) to exhaustion. Exhaustion was defined as when a cadence of at least 60 rpm was unable to be maintained, volitional fatigue or a heart rate within 10 beats per minute (bpm) of calculated heart rate max (220-age). When it was judged that the subject was nearing their respiratory compensation point, the subject was asked to attach the nose clip, insert the two-way, rebreathing mouthpiece and start breathing into the apparatus. Between two and four consecutive Douglas bags of expired air (approximately 30 seconds worth) were collected from the subject in the last stages of the test. This was done to ensure that a representative sample of expired air during the final stages of the test would be available for analysis. The Douglas bag volume and relative proportions of O_2 and CO_2 were measured using a dry gas meter (Harvard, United Kingdom) and gas analysers (Ametek, USA) respectively. VO_2 and VCO_2

were calculated using the Haldane transformation (Wilmore & Costill, 1973). A respiratory exchange ratio (RER) of greater than 1.1 indicated that VO_2 max had been reached and calculated results were able to be used for subject inclusion in the trial and subsequent calculation of work rate. Appendix 7 shows the VO_2 max test form used.

3.9. Caffeine

Anhydrous caffeine was sourced from Blackburn Distributions (Nelson, United Kingdom). See Appendix 8 for Certificate of Analysis. Gelatine capsules were filled with a measured dose of caffeine or placebo and then given to an independent researcher not involved with the trial, to facilitate double blinding and order randomisation. Exercise trial capsules were returned labelled as either A or B.

3.10. Sample collection and analyses

3.10.1. Experimental exercise protocol

The same experimental protocol and conditions were followed for each of the experimental exercise trial days (placebo and caffeine). Appendix 9 shows the form used to record all data during the exercise trial.

Subjects were asked to arrive early morning at the lab in a euhydrated state after an overnight fast. The start time was replicated as close as possible on subsequent trial days and was not greater than 1 h difference. A cannula was inserted by a trained phlebotomist via Teflon catheter (Becton Dickinson,

Sandy, Utah, USA) placed in antecubital vein. Whilst the cannula was inserted it was flushed every 30-60 minutes to keep it patent.

As subjects would not normally race on an empty stomach, a standard breakfast prior to the supplementation was provided to mimic normal physiological conditions for these athletes. The breakfast consisted of a breakfast drink (Sanitarium Up & Go (vanilla flavour), New Zealand) a banana and a cereal bar (WeightWatchers, Tasti products, Auckland, New Zealand). Current recommendations are to consume 1-4 g carbohydrate per kg BW, 1-4 hour pre exercise (Burke, Cox, Cummings, & Desbrow, 2001; Burke, Hawley, Wong, & Jeukendrup, 2011). The weight of the Up & Go and banana was calculated to give the subject a breakfast that was high in carbohydrate (1.5 $g.kg^{-1}$ BW) and low in fibre, protein and fat (see Appendix 10 for macronutrient composition of the breakfast). Exactly 30 minutes after breakfast the gelatine capsule containing either caffeine or placebo was given with 250 mL of water. The subject then sat quietly for a further hour before starting exercise. Fifteen minutes prior to starting the exercise protocol, the subject was given a high carbohydrate cereal bar (containing 22.3g carbohydrate).

A 4 mL blood sample was collected through an indwelling cannula into a lithium heparin coated blood tube prior to caffeine/placebo supplementation (baseline), and a 4 mL sample collected again at 60 minutes post ingestion for caffeine and metabolite analysis. The 60 minute post ingestion sample was collected whilst the subject commenced the warm-up on the bike. The blood sample was placed immediately in a centrifuge (Eppendorf 5804R, Germany) where it was spun at 3000rpm (1620xg) for 10 minutes at 4 °C.

Plasma was removed and aliquots were frozen initially at -20°C and then later at -80°C. All subsequent blood samples were treated in this manner.

The exercise trial consisted of a 5 min warm-up at a workload half that of the workload calculated to elicit 70% VO_2 max. Subjects then cycled for 115 min at the workload calculated to elicit 70% VO_2 max. During this time the ergometer was set in hyperbolic mode so that cadence and work rate were independent of each other.

Every thirty minutes during the steady state exercise, respiratory gas was collected and measured for assessing the current workload as a percentage of their VO_2 max and for calculating rates of substrate metabolism. Ratings of perceived exertion (RPE) and heart rate (POLAR, RS8000CX GPS) were recorded at the same time. Appendix 11 shows RPE scale. A further blood sample (lithium heparin) was also taken every 30 minutes and treated as above.

The recommendation for fluid intake during exercise is 0.4 - 0.8 L per hour (Sawka et al., 2007); the exact amount dependent upon environmental conditions. Thus due to this being a laboratory based experiment conducted in a temperature controlled room with a fairly constant relative humidity it was decided that the lower level of this recommendation (0.4 $L.hour^{-1}$) should be sufficient to maintain adequate hydration. Recommended carbohydrate intake during endurance exercise is 30-60 g carbohydrate per hour (Burke et al., 2001; Burke et al., 2011). Thus to also maintain circulating blood glucose levels during the exercise, fluid was given as a carbohydrate based drink

(Powerade®). The subject was asked to consume 400 mL of this drink each hour during the steady state exercise.

Immediately following 115 min of steady state cycling, the subjects were instructed to ride as hard as possible for timed thirty minutes. The amount of work completed on this ride was used as a measure of the cyclist's performance. During this time the ergometer was set to linear mode where a linear factor was calculated so that workload was proportional to cadence using the following formula:

$$W = L.(rpm)^2 \qquad \text{(Conway et al., 2003)}$$

Where W was a work rate eliciting 80% VO2 max, L was the linear factor and rpm was the average pedalling rate in the final stages of the VO_2 max test.

No verbal encouragement was provided to participants during the time trial, however, subjects were given a verbal countdown consisting of halfway, 10, 5, 3, 2 and 1 minute to go. In the final minute they were told 30 seconds, 20 seconds to go and then given a final countdown from 10 seconds. Performance during the time trial was measured by the amount of energy expended (kJ) that the subject accumulated during the 30 minute period.

An additional drink of Powerade® was made available for the subject during the time trial. During this time the subject chose their own hydration protocol. This same self-selected hydration protocol was then replicated during the subsequent performance test.

Immediately upon completing the time trial a further blood sample was taken. After a 10 min cool down period subjects got off the bike and showered and changed. On completion of the exercise on each of the trial days, the subjects were asked to comment on whether they thought they had ingested caffeine or placebo. Participants were given a standardised lunch from the Subway® menu. They were allowed to choose what they liked as long as it didn't contain caffeine and the choice was replicated on subsequent trial days.

A further blood sample was taken at five and seven hours after caffeine ingestion. Subjects were required to refrain from caffeine containing food and drinks up until this time. On completion of the exercise part of the trial subjects were free to go from the laboratory and return at specified times for their five and seven hour samples. Subjects were provided all food during the seven hour caffeine testing period which was kept consistent during each trial day. However water could be consumed as required. Subjects were also asked to not exercise again until the last blood sample had been taken.

Subjects were required to have a minimum washout period of seven days before repeating the experimental protocol (caffeine or placebo). To minimise any changes in fitness levels a maximum of two weeks between test days was allowed.

3.10.2. Caffeine intervention at rest

The same experimental protocol was followed as the exercise trials without the subject performing the exercise. Blood samples were taken at the same time points as performed during the exercise trial.

3.10.3. HPLC quantification of plasma caffeine and metabolite (theobromine, paraxanthine and theophylline) concentrations

Plasma caffeine and metabolite (paraxanthine, theophylline and theobromine), concentrations were determined using a Shimadzu Prominence High Performance Liquid Chromatography (HPLC) system (Shimadzu, Kyoto, Japan) consisting of a quaternary solvent delivery module (LC-20AT), Autosampler (SIL-20A) and a CT-20A column oven with a Phenomenex Luna 5µ C18 (2) 100A 250 x 4.60mm column with C18 guard column attached. Detection was by a UV/Vis photodiode array detector. The mobile phase and gradient used were adapted from the method of Ali, O'Donnell, Starck, and Rutherfurd-Markwick (2015). Briefly the HPLC was conditioned with 98% mobile phase A (0.1% trifluroacetic acid; TFA) and 2% mobile phase B (30% acetonitrile in TFA (0.1%) at 1 $mL.min^{-1}$. The following gradient for each injection was used over 42 minutes; 2% B to 30% B (20 min), 30% B to 100% B (8 min), 100% B (2 min), 100% B to 2% B (2 min) and 2% B for 10 min. Shimadzu LC solution software was used to quantify peaks at 274nm based on standard retention times and concentrations ranging from 1-10 $\mu g.mL^{-1}$. The method was determined to be linear in the range of 1-100 $\mu g.mL^{-1}$ and the limit of detection 0.1$\mu g.mL^{-1}$.

Plasma samples were prepared according to the method described by Conway et al (2002). Briefly, 300 µL of serum was deproteinised by an equal volume of 0.8 mol.L^{-1} perchloric acid. The samples were mixed by vortex and then centrifuged for 4 min at 12000rpm. 400 µL of the resulting supernatant was neutralised with 60 µL of 0.4 mol.L^{-1} sodium hydroxide of which 20 µL was injected onto the HPLC column.

Caffeine, paraxanthine (1,7-dimethylxanthine), theobromine and theophylline standards along with perchloric and trifluroacetic acids were purchased from Sigma-Aldrich (St Louis, USA). Acetonitrile (gradient grade for liquid chromatography LiChrosolv®) was purchased from Merck Millipore (Billerica, USA).

CYP1A2 activity was determined by calculation of the paraxanthine to caffeine ratio at 5 and 7 hours after caffeine administration (Fuhr & Rost, 1994)Figure 3.2 shows a typical HPLC chromatogram of measured caffeine and the main metabolites paraxanthine, theophylline and theobromine in the plasma of a subject following caffeine ingestion (5 h post ingestion).

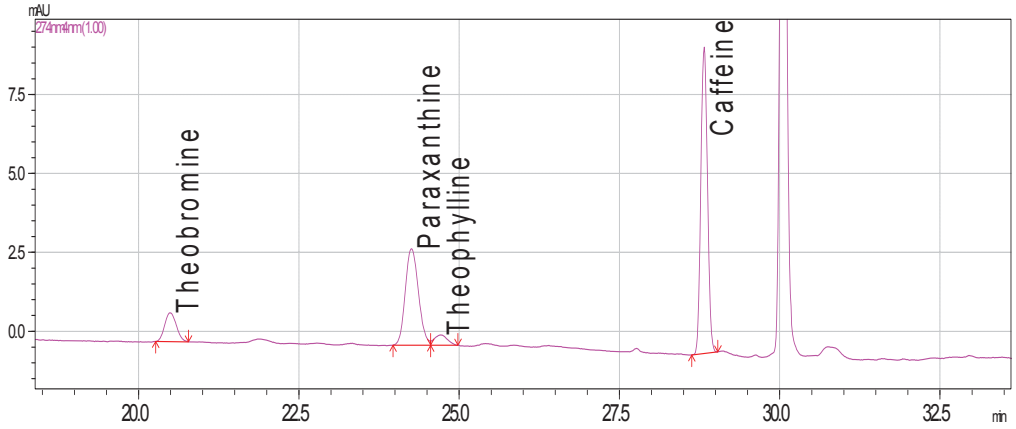

Figure 3.2. Typical HPLC chromatogram of deproteinised plasma showing caffeine and main metabolites

Caffeine pharmacokinetic parameters were calculated based on the method described in Ali et al. (2015).

3.10.4. Genotyping

Whole blood was collected into Ethylenediaminetetraacetic acid (EDTA) vacutainers and the DNA extracted using an E.Z.N.A Blood DNA kit (OMEGA bio-tek D3392). See Appendix 12 for a diagrammatic representation of the protocol.

DNA concentration of the extracted DNA was measured on a nanophotometer (Implen, Germany). The amount of DNA required (approximately 50 ng) was calculated by dividing the DNA concentration into 50 (50/DNA concentration). A260/A280 was measured on all DNA samples

to ensure purity. All samples had a ratio of approximately 1.8 therefore they were considered pure and thus were used for subsequent genotyping.

The calculated volume of DNA was then mixed with PCR grade water to give a total volume of 15.4 µL. Sufficient volume was prepared for duplicate analysis and kept on ice whilst preparation of reaction mix occurred.

Genotyping was performed according to manufacturer instructions (TIB MOLBIOL LightSNiP, rs762551 CYP1A2*1F; Appendix 13). Briefly, in a reaction tube on ice, enough reaction mix for all the samples was prepared by mixing reagent mix (1 µL per sample, light cyclerFast start Master HybProbe), fast start DNA master mix (2 µL per sample) and $MgCl_2$ (1.6 µL per sample). 4.6 µL of this reaction mix was pipetted into the bottom of each well (96well plate) and then 15.4 µL of the diluted sample prepared above was then added in duplicate. A blank and controls (one of each genotype previously measured) were also added in duplicate. Samples were then analysed using real time PCR (Roche LightCycler 480, Rotkreuz, Switzerland). CYP1A2 genotype was assigned based on melting temperature. Figure 3.3 shows typical melting peaks for each of the three genotypes. A single melting peak represents carriers of the homozygous A/A (54±4°C) or A/C (64±4°C) allele whereas a double melting peak (56°C and 68°C) represents a heterozygous (A/C) individual.

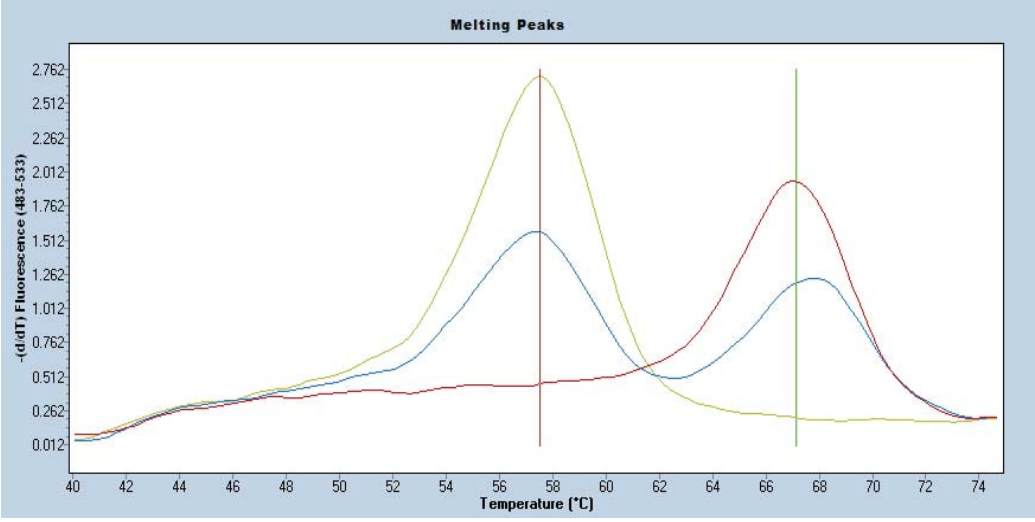

Figure 3.3. Melting peaks for CYP1A2 genotype (rs76251)

3.10.5. Calculating rate of substrate oxidation

Substrate oxidation rates were calculated according to the method described in Jeukendrup and Wallis (2005).

3.10.6. Statistical analyses

All data was entered into software for statistical analysis (IBM SPSS Statistics 22). Prior to statistical analysis all data was checked for normal distribution using Kolmogorov-Smirnov p values. A p–value greater than 0.05 indicated that the data was not significantly different from normal thus was able to be used for statistical analysis. Some data (RPE, VO_2, and RER) at various time points were not normally distributed, in these instances, except RPE, the data was log transformed to normal distribution before statistical analysis.

Analysis of variance (ANOVA), taking into account trial order and genotype was used to investigate the effect of caffeine on performance data (work done during the time trial) compared to a placebo. Heart rate during the time trial was also analysed in the same manner. Other dependent variables which were taken in time series (blood, expired respiratory gases, HR) were compared between experimental trials using a three-way, repeated measures ANOVA. To include the known genotype and whether it affected the dependent variables, a two-way ANOVA was used for the performance measure and a three way repeated measures ANOVA for all other dependent variables. The confidence to accept the null hypothesis (that genotype would not alter the response to caffeine) was set at a level of 95%.

Chapter 4 Results

4.1. CYP1A2 genotype (rs762551)

Of the eleven subjects, six were homozygous A/A, four were heterozygous A/C and one was homozygous C/C. Fig.4.1 shows a visual representation of the individual melting peaks showing the distinct groupings. Those with a single peak at 56°C were designated homozygous A (A/A). Those with a single peak at 68°C were designated homozygous C (C/C) and those with a peak at 56°C and 68°C were designated heterozygous (A/C). For the purposes of analysis, in accord with other published research (Aklillu et al., 2003; Sachse et al., 1999) subjects were grouped as either homozygous A (A/A), or carriers of the C allele (A/C and C/C). Although a small sample size; this study shows similar distribution to previously reported investigations (Castorena-Torres et al., 2005; Cornelis, Ei-Sohemy, & Campos, 2007; Sachse et al., 1999).

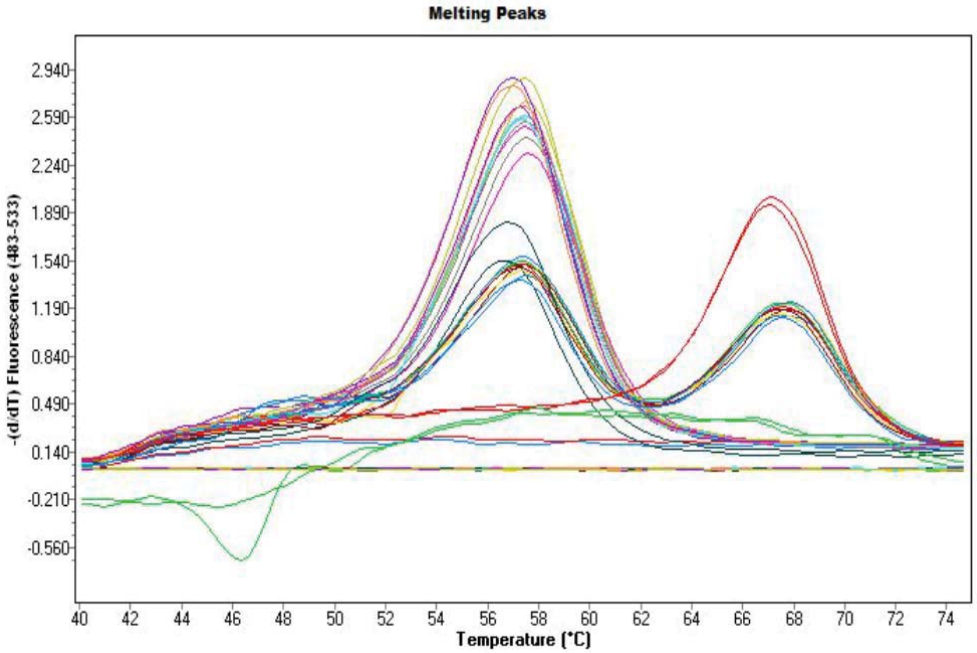

Figure 4.1. A visual representation of the different melting peaks showing distinct groupings.

There was no significant differences in the two genotype groups in relation to age, relative VO_2 max, weight, or habitual caffeine intake, indicated by one way ANOVA.

Review of individual exercise and diet diaries indicated that the subjects complied with the exercise and diet restrictions required (Appendix 4) prior to participating in one of the trial days. Minimal circulating caffeine concentration ($<0.1 \mu g.mL^{-1}$) at the start of each trial confirmed there was good compliance by all subjects in avoiding caffeine during the 48 hours prior to testing.

4.2. Performance data

4.2.1. Performance during time trial

The average accumulated energy expenditure (kJ) on completion of the 30 minute time trial after ingesting either placebo or caffeine can be seen in Figure 4.2. This represents an average time trial difference of 7.1% between caffeine ingestion and placebo.

Time trial performance with caffeine or placebo, and accounting for trial order and genotype both as dichotomous variables, were compared. A repeated measures ANOVA revealed a main effect of caffeine treatment ($p=0.037$) indicating that caffeine, regardless of genotype or trial order, improves self-paced cycling performance. However there was no significant interaction between treatment and genotype ($p=0.343$) i.e. possessing the CYP1A2 gene variant did not further change the ergogenic effect of caffeine. Nor was there a significant interaction ($p=0.147$) with caffeine intervention and order of the trial thus indicating that the ergogenic effect of caffeine was not modulated by trial order.

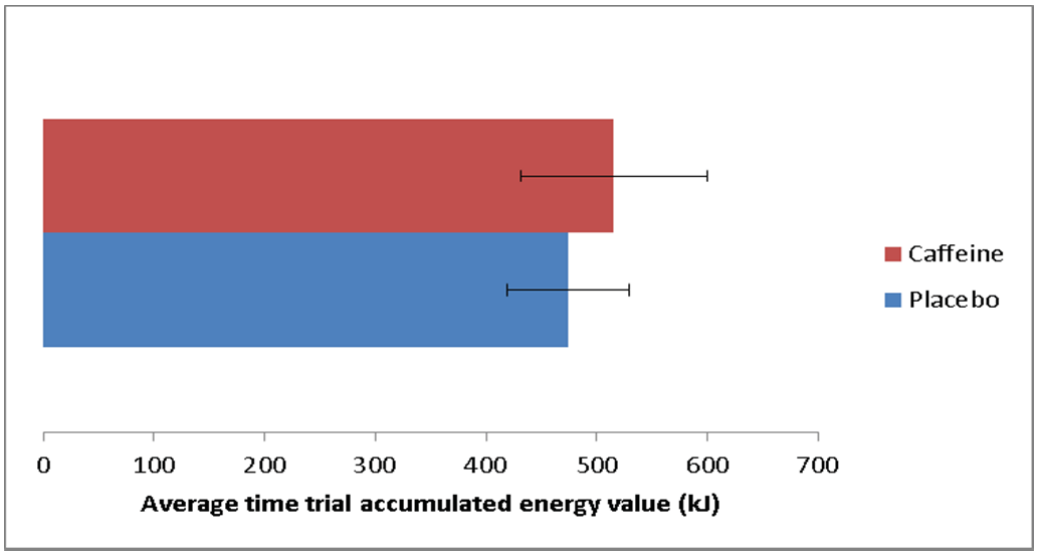

Figure 4.2. Average accumulated energy value (kJ) ± SD on completion of time trial after ingesting either placebo or caffeine.

4.2.2. Heart rate during time trial

Heart rate was significantly higher (p=0.003, main effect) with caffeine ingestion during the time trial (Fig. 4.3). However this response was not different between the genotypes (p=0.118, genotype x treatment interaction), nor was this modulated by trial order (p=0.595, trial order x treatment). Neither was there a three way interaction between genotype, trial order and treatment (p= 0.958).

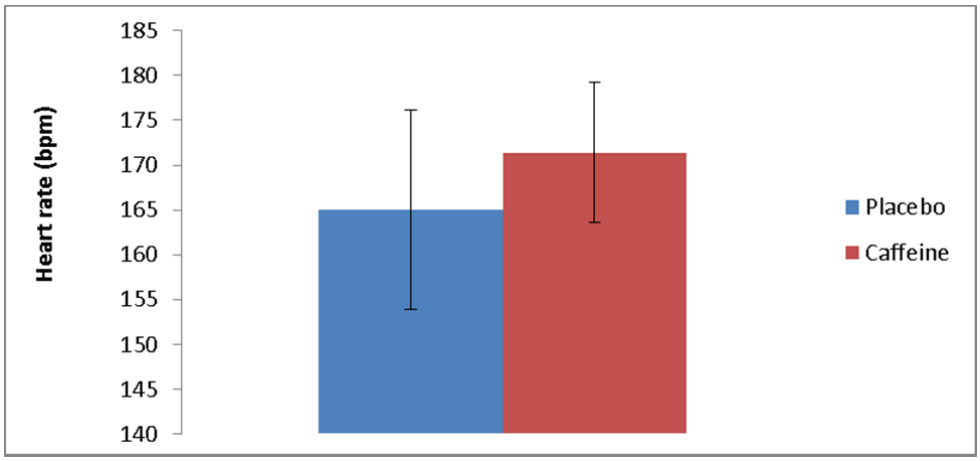

Figure 4.3. Comparing the effect of caffeine on heart rate during the time trial. Values are mean ±SD.

4.3. Data during steady state exercise

4.3.1. Rating of perceived exertion (RPE)

The RPE significantly increased over time (main effect, p=0.020) and was significantly higher with placebo than caffeine (main effect, p=0.010) (Fig.4.4). There was no significant interaction between treatment (caffeine) and genotype (p=0.484).

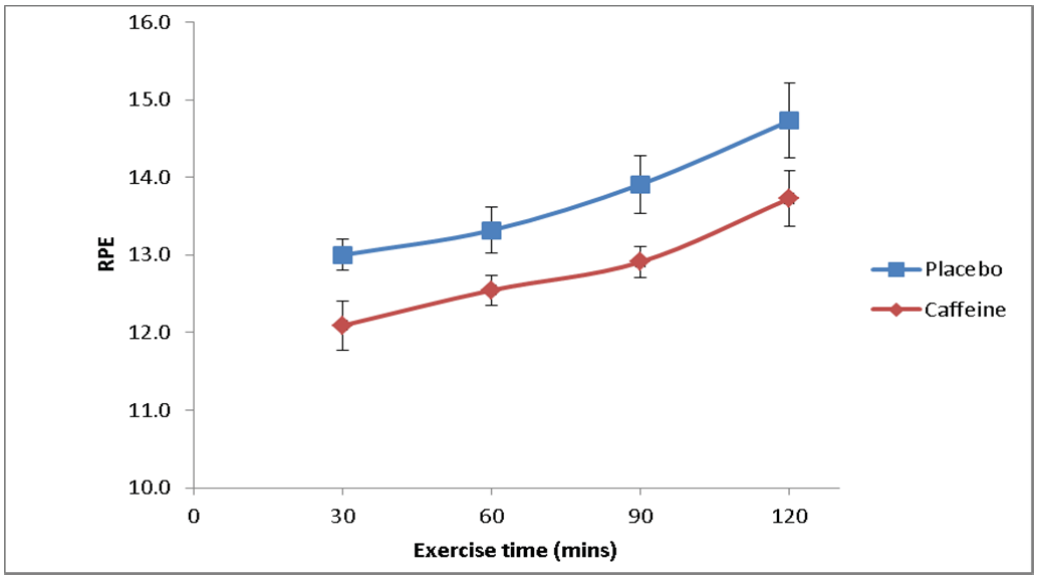

Figure 4.4. RPE during 120 minutes steady state exercise comparing RPE vs. exercise time for placebo and caffeine dosed subjects. Values are mean ±SD.

4.3.2. Heart rate (HR)

Heart rate during steady state exercise significantly increased over time (main effect, p=0.013) (Fig 4.5) but was not significantly different with caffeine or genotype. However the interaction between treatment and time approached significance (p=0.079).

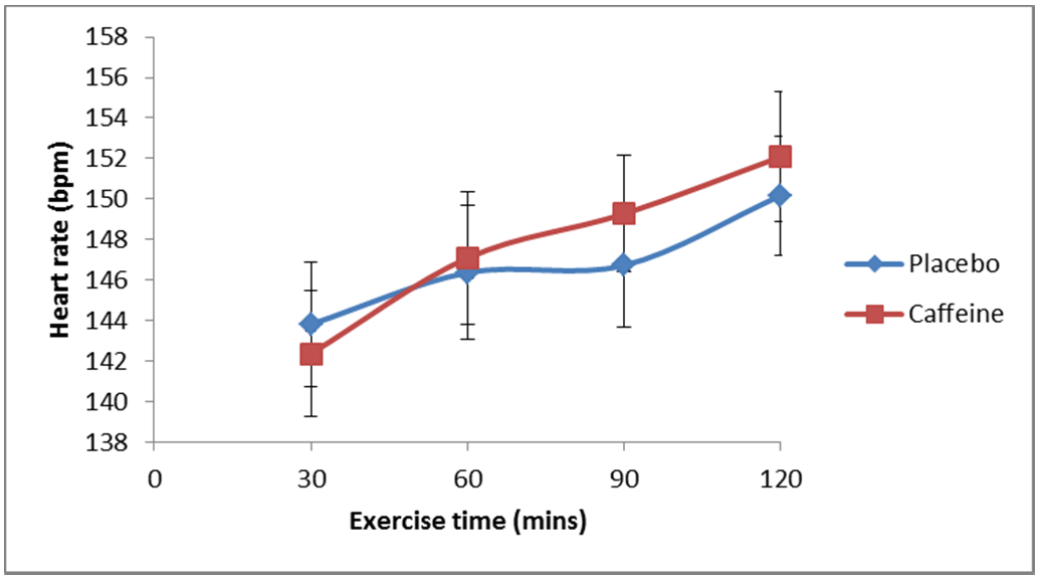

Figure 4.5. Heart rate during 120 minutes steady state exercise comparing heart rate vs. exercise time for placebo and caffeine dosed subjects. Values are mean ±SD.

4.3.3. Rate of oxygen consumption (VO_2)

Oxygen uptake during steady state significantly changed over time (main effect, p=0.007) and was significantly higher with caffeine compared to placebo (main effect, p=0.047) (Fig.4.6). However there was no significant interaction between treatment and genotype (p=0.757).

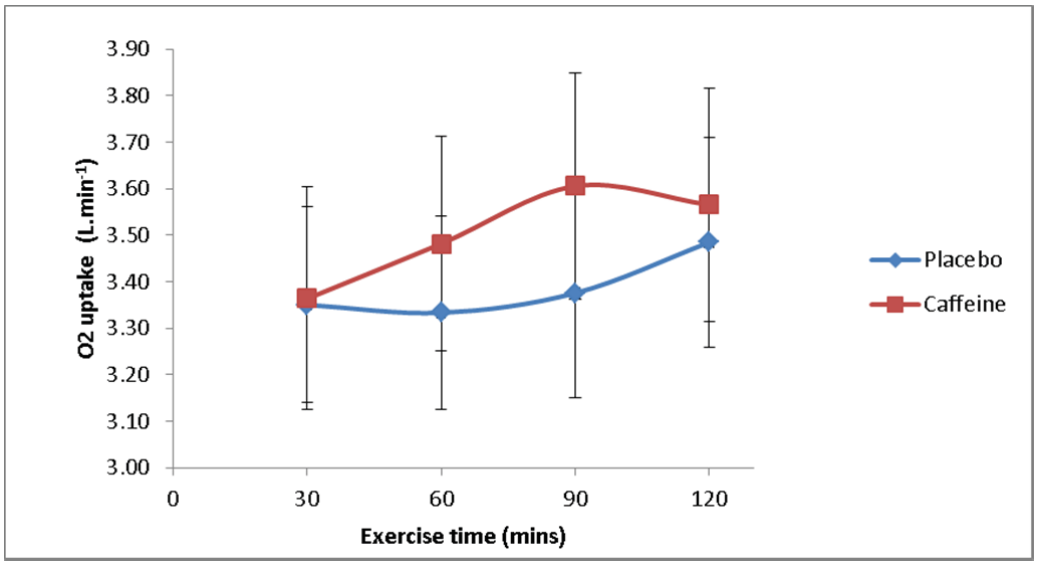

Figure 4.6. **VO_2 during 120 minutes steady state exercise comparing O_2 uptake vs. exercise time for placebo and caffeine dosed subjects. Values are mean ±SD.**

Oxygen consumption/heart beat (oxygen pulse) during steady state was not significantly different over time (p=0.491) but was approaching significance with caffeine treatment (main effect, p=0.081) (Fig.4.7). There was no significant interaction between treatment and genotype (p=0.657).

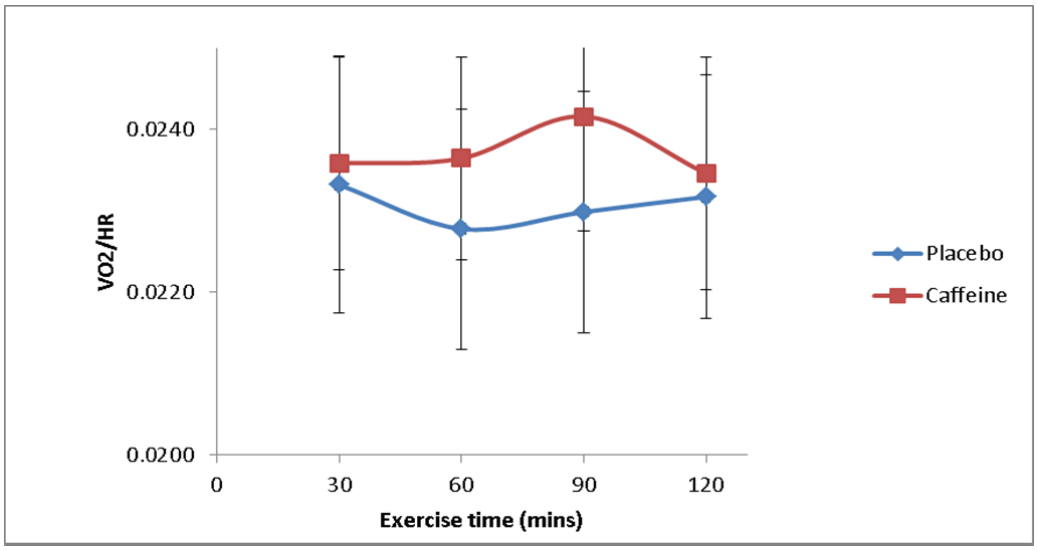

Figure 4.7. **Oxygen consumption/heart beat during 120 minutes steady state exercise comparing VO_2/HR vs. exercise time for placebo and caffeine dosed subjects. Values are mean ±SD.**

4.3.4. Respiratory exchange ratio (RER)

Respiratory exchange ratio (Fig.4.8) decreased over the course of the 120 minutes exercise (p=0.009) and was approaching significance (p=0.08) with caffeine treatment compared to placebo. But there was no significant interaction between treatment and genotype (p=0.709).

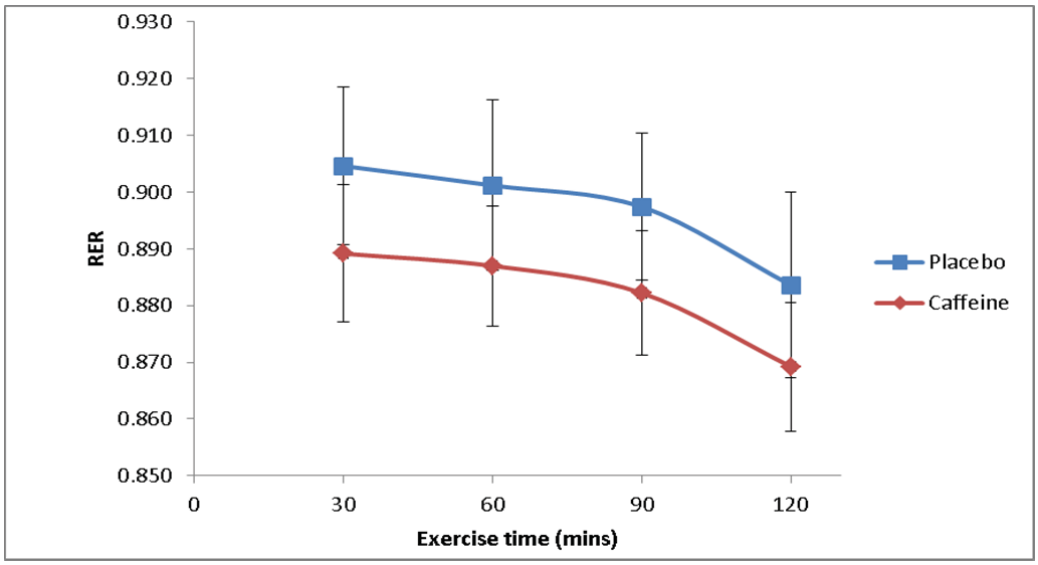

Figure 4.8. Respiratory exchange ratio during 120 minutes steady state exercise comparing RER vs. exercise time for placebo and caffeine dosed subjects. Values are mean ±SD.

4.3.5. Calculated rates of substrate metabolism

Fat oxidation significantly increased over time (p=0.01) however there was no interaction between treatment (caffeine; p=0.078), or genotype (p=0.575) (Fig. 4.9). In contrast, carbohydrate oxidation did not significantly change over time (p=0.887) or with treatment (p=0.754) or genotype (p=0.578).

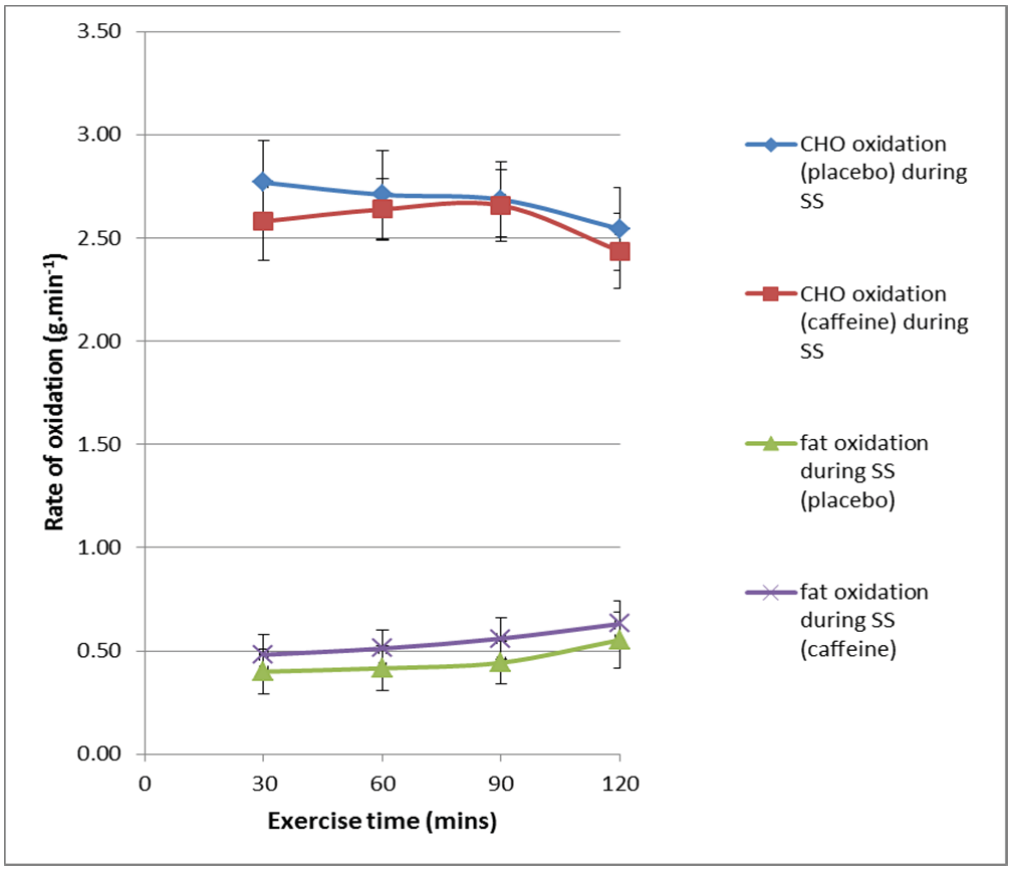

Figure 4.9. Carbohydrate and fat oxidation during 120 minutes steady state exercise comparing both carbohydrate and fat oxidation rates vs. exercise time for placebo and caffeine dosed subjects. Values are mean ±SD.

4.3.6. Subject perception of caffeine ingestion

Five subjects correctly guessed they had ingested caffeine. Two were completely wrong and four subjects were unsure as to whether they were on caffeine or placebo. One subject with a large increase in time trial performance with caffeine above placebo (20%) was unsure until the seven hour time point when they experienced heart palpitations. This subject had already completed both exercise trials but was withdrawn from the caffeine resting trial. There appeared to be no differences in perception of caffeine ingestion based on genotype.

4.4. Caffeine and metabolites

4.4.1. Plasma caffeine and caffeine metabolite concentrations

Plasma caffeine was not detected during the Placebo-Ex trial. Caffeine and its major metabolite concentrations during Caffeine-Ex trial at one, three, five and seven hours following caffeine ingestion can be seen in Table 4.1. The relative proportion of Paraxanthine (PX), Theobromine (TB) and Theophylline (TP) formed by CYP1A2 breakdown of caffeine three hours after ingestion were approximately 78%, 15% and 6% respectively.

As expected there was a significant effect of time on caffeine concentration during Caffeine-Ex and Caffeine–Rest trials (p=0.000). Peak caffeine concentration (31.2±4.0 $\mu mol.L^{-1}$ (exercise trial) and 29.5±4.2 $\mu mol.L^{-1}$ (resting trial) varied between individuals but was reached between one and three hours following caffeine ingestion at which point a steady decline in caffeine concentration was seen (Fig 4.10). Peak caffeine concentration was not significantly different between resting and exercise trials (p=0.264). There was no interaction at any time point between caffeine concentration and genotype during Caffeine–Ex (p=0.408) or Caffeine-Rest (p=0.765) trials.

Table 4.1. **Average concentration (μmol.L^{-1}) during the exercise trial of caffeine and main metabolites paraxanthine (PX), theobromine (TB) and theophylline (TP) at baseline and 1, 3, 5 and 7 hours after caffeine ingestion. The percentage of each metabolite at a given time point is also shown.**

		caffeine	PX	TB	TP	%PX	%TB	%TP
Baseline	μmol.L^{-1}	0.08	0.02	0.83	ND			
	SD	0.20	0.06	1.52	0.00			
Concentration (1h)	μmol.L^{-1}	14.99	1.57	0.97	0.13	58.2	37.2	4.6
	SD	12.95	1.44	1.34	0.10	28.9	29.7	3.7
Concentration (3h)	μmol.L^{-1}	27.53	6.41	1.40	0.52	77.9	16.0	6.1
	SD	5.41	1.38	1.15	0.17	9.7	9.3	0.9
Concentration (5h)	μmol.L^{-1}	21.24	8.00	1.61	0.64	78.5	15.3	6.2
	SD	5.14	1.49	1.08	0.15	7.2	7.5	0.5
Concentration (7h)	μmol.L^{-1}	15.76	8.89	1.70	0.75	78.8	14.6	6.6
	SD	4.86	1.23	0.95	0.17	5.8	6.0	0.8

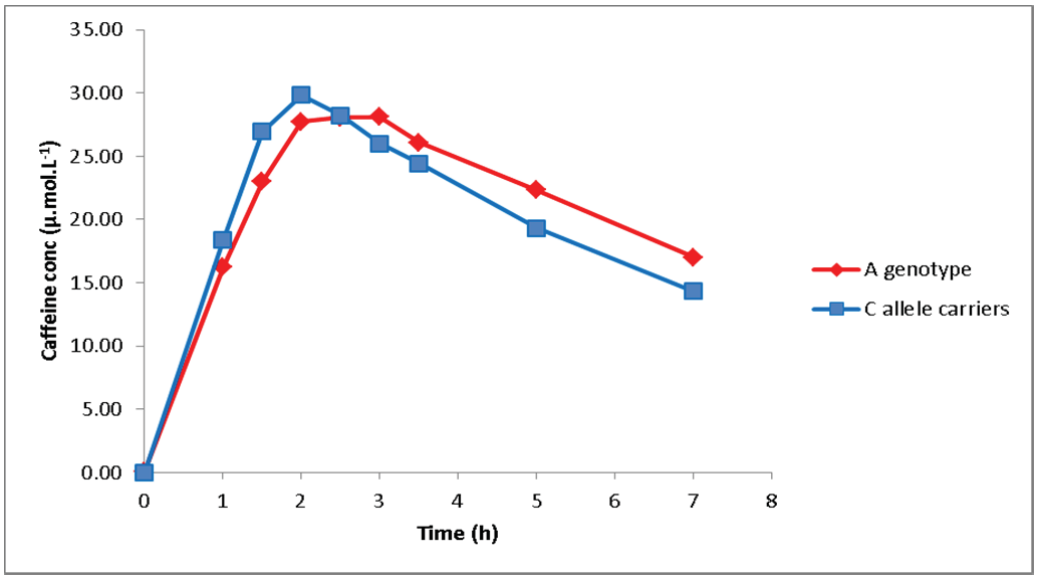

Figure 4.10. **The effect of genotype on caffeine concentration over time. Data is from both resting and exercise trials for all subjects. Values are mean ±SD.**

There was no correlation between caffeine or paraxanthine concentration at the start of the time trial and subsequent performance (p=0.843 and 0.409 respectively). Plasma concentrations of PX and TP increased steadily from baseline and were still rising at the last sampling time point (seven hours post-caffeine ingestion). Many subjects had detectable theobromine at baseline however as seen with the other metabolites it increased steadily over the course of the experiment and was still rising at the seven hour time point.

4.4.2. Estimated rates of caffeine metabolism

Metabolite ratios (Paraxanthine/Caffeine)

Carriers of the C allele appeared to have higher PX/caffeine ratios than A/A genotype however it was not significant (p=0.097) possibly due to large individual variability and small sample size (Fig. 4.11). There was no difference in PX/caffeine ratios between exercise and rest. When both resting and exercise data was combined, PX/caffeine ratios were still not significantly different between genotypes at either five, or seven hours post caffeine ingestion

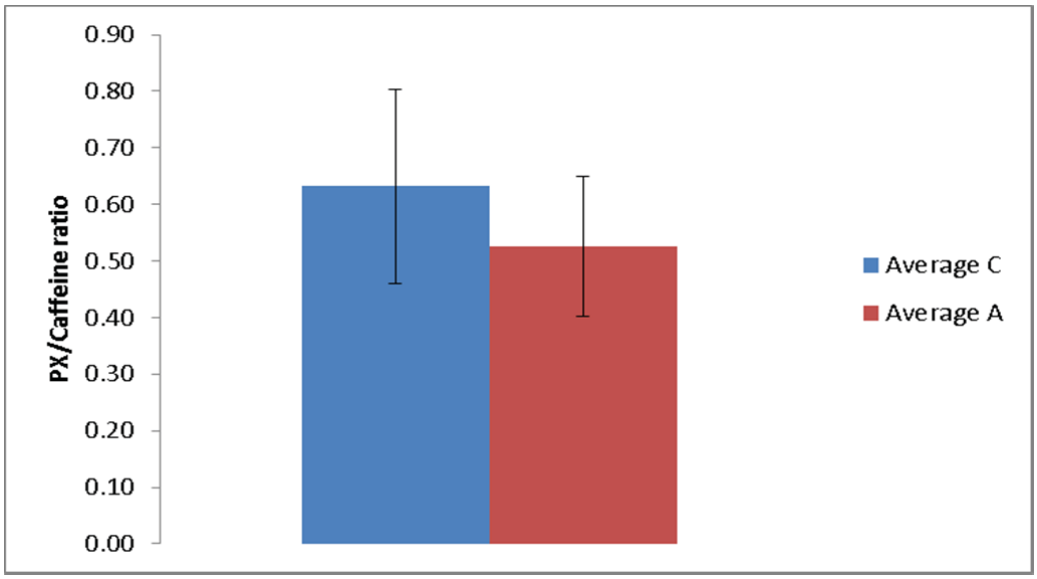

Figure 4.11. PX/Caffeine ratio at 7 hours. Data is from both resting and exercise trials for all subjects. Values are mean ±SD.

Combined metabolites (TP+PX+TB) to caffeine ratio at seven hours was also not significantly different between groups (p=0.117 resting trial, p=0.547 exercise trial).

Pharmacokinetic parameters

The pharmacokinetic parameters for Caffeine-Rest and Caffeine-Ex are shown in Table 4.2 and 4.3 respectively. There were no significant differences seen between genotype groups and any of the parameters measured. Nor were there any significant differences between resting and exercise trials in plasma clearance (p=0.568), elimination half-life (p=0.462), apparent volume of distribution (p=0.207) or peak plasma concentration (p=0.264).

Table 4.2. Pharmacokinetic parameters for Caffeine–Rest trial.

	Plasma Clearance (mL.min^{-1}.kg^{-1})	Elimination half-life (h)	Apparent volume of distribution (L.kg^{-1})	Peak plasma conc (µmol.L^{-1})
Mean	2.80	5.98	1.45	26.8
SD	1.13	1.13	0.31	9.75
Mean (A/A)	2.69	6.44	1.50	29.67
SD	0.17	1.20	0.31	2.58
Mean (C allele carriers)	2.95	5.29	1.36	23.34
SD	0.49	0.60	0.32	14.21

Table 4.3. Pharmacokinetic parameters for Caffeine–Ex trial

	Plasma Clearance (mL.min^{-1}.kg^{-1})	Elimination half-life (h)	Apparent volume of distribution (L.kg^{-1})	Peak plasma conc (µmol.L^{-1})
Mean	2.82	5.27	1.24	31.2
SD	0.53	1.57	0.22	3.96
Mean (A/A)	2.79	5.8	1.33	31.16
SD	0.69	1.8	0.23	5.25
Mean (C allele carriers)	2.87	4.7	1.13	31.25
SD	0.33	1.2	0.15	2.19

Chapter 5 Discussion

5.1. Purpose of the study

The present study was designed to examine whether caffeine (5 $mg.kg^{-1}$ BW) affected exercise performance differentially based on a single nucleotide polymorphism (SNP) within the enzyme (CYP1A2) involved in caffeine metabolism. It was hypothesised that different genotypes at position -163 would result in changes in the rate of caffeine elimination during exercise and thus the different plasma caffeine concentration (or paraxanthine) would account for the variability often seen in the ergogenic effects of caffeine on exercise performance. In addition, the pharmacokinetics of caffeine metabolism based on CYP1A2 genotype during rest compared to exercise was examined.

5.2. Main findings

The main finding was that caffeine improved endurance performance compared to a placebo; however CYP1A2 genotype did not differentially affect this ergogenic effect. CYP1A2 genotype also had no effect on heart rate (steady state and time trial), VO_2, RER or carbohydrate or fat oxidation.

5.2.3. The effect of caffeine on endurance performance

Consistent with other investigations (Cureton et al., 2007; Hodgson et al., 2013; Hulston & Jeukendrup, 2008) the present study showed that a 5 $mg.kg^{-}$

[1] dose of caffeine one hour prior to exercise, significantly improved (7.1%) endurance performance compared to a placebo. Nevertheless, individual performance data varied widely. All subjects but one had improved performance with caffeine. For this one subject, performance was better during the placebo trial than the caffeine trial. The lack of a positive response to caffeine in this one subject could be due to a learned effect, since this subject received placebo in the second trial. This subject was also the least experienced in participating in laboratory-based exercise trials. Although subjects were randomised as to trial order and there were no trial order effects, no pre familiarisation trial was undertaken. This was not deemed necessary as Jeukendrup et al. (1996) had shown when validating endurance performance testing that there was no learning effect. The variability and negative response in endurance performance seen in one subject is consistent with Astorino et al. (2012). These authors showed that time trial performance was repeatable but there was large variation between subjects with one out of the nine subjects having negative performance.

Heart rate was significantly elevated with caffeine (171 bpm) compared to placebo (165 bpm) during the time trial. This is a similar response to that seen by Womack et al. (2012), Kovacs et al. (1998) and Hulston and Jeukendrup (2008) where heart rate was elevated during a time trial. This, together with the significant effect of caffeine on time trial performance simply confirms that subjects were working harder during the caffeine time trial, probably because of the central effects of caffeine on perceived effort.

5.2.4. The effect of CYP1A2 polymorphism on exercise performance

To our knowledge only one study, Womack et al. (2012), has previously examined the effect of CYP1A2 polymorphism on exercise performance. Whilst the findings of the present study showed an overall ergogenic effect of caffeine, there was no significant effect of genotype on exercise performance. This is in contrast to Womack et al. (2012) who found that following a 6 $mg.kg^{-1}$ caffeine dose, individuals homozygous for the A allele completed a simulated 40 km cycling time trial significantly faster than carriers of the C allele. However, the study designs were very different. Though both studies had subjects ingest caffeine one hour prior to exercise, in the current study the time trial was preceded by a two hour endurance ride. Thus the time trial was performed three hours after caffeine ingestion compared to one hour in the trial of Womack and colleagues (2012). In the current study a two hour steady state ride prior to the time trial (time trial starting three hours post caffeine ingestion) was chosen to ensure that caffeine would be well absorbed but differences in the rate of metabolism as a result of the CYP1A2 enzyme were more likely to be seen. Why Womack et al. (2012) were able to resolve a difference in performance relating to CYP1A2 activity with exercise one hour following caffeine ingestion is curious and we could speculate that it is unlikely to be directly related to plasma caffeine concentrations. However, as CYP1A2 metabolises caffeine in the liver and hepatic blood flow is reduced during exercise, it could be that with the longer exercise in our study, any differences in CYP1A2 activity due to genotype, may have less of an effect and other (unknown) factors may have a larger influence creating the variability seen.

The Womack group (2012) hypothesised that the slower metabolism due to the C allele would be more advantageous to ergogenic potential of caffeine due to caffeine being in the system longer. However in contrast to their original hypothesis they saw an ergogenic potential with the A allele; which they speculated was possibly due to the quicker production of paraxanthine and or theophylline providing increased ergogenic benefit. However, as these researchers did not measure caffeine or any metabolites it is difficult to draw any firm conclusions. In contrast, in the present study plasma caffeine and its three main metabolites paraxanthine, theobromine and theophylline were also measured over a seven hour period.

5.2.5. Ergogenic potential of caffeine metabolites

Paraxanthine and theophylline have been shown to have ergogenic properties and similar sympathiometric actions to caffeine when given in comparable doses (Benowitz et al., 1995; Greer et al., 2000). Greer et al. (2000) saw an ergogenic effect of a 4.45 $mg.kg^{-1}$ dose of theophylline which corresponded to 30 $\mu mol.L^{-1}$ theophylline in plasma (15 minutes into exercise session). In the current trial low concentrations of paraxanthine and theophylline (6.4, and 0.5 $\mu mol.L^{-1}$ respectively) were seen in the plasma at the start of time trial (three hours post caffeine ingestion) making it unlikely that they are responsible for an ergogenic effect. However, it is possible that paraxanthine may be influencing Ca^{2+} concentrations in skeletal muscle; as low physiological concentrations (10 $\mu mol.L^{-1}$) of paraxanthine have been shown to increase [Ca^{2+}] transiently to subcontracture levels in resting skeletal muscle (Hawke, Allen, & Lindinger, 2000). Additionally, low doses of

caffeine eliciting between 5-16 μmol.L^{-1} plasma caffeine (Cox et al., 2002; Desbrow, Biddulph, et al., 2012) during exercise have been found to be ergogenic. Therefore it is conceivable that the low doses of paraxanthine from caffeine metabolism may also be contributing to the ergogenic effects. However, from a thorough literature search, it does not appear that the dose response relationship of paraxanthine has been specifically investigated in relation to effects on exercise performance.

In the current study caffeine and its metabolites showed similar concentrations and trends in the plasma compared to other published research using a similar dose of caffeine (McLean & Graham, 2002; Skinner, Jenkins, Folling, et al., 2013). More specifically, three hours after ingestion (start of the time trial), caffeine concentration (taking into account any seen at baseline) was 28 μmol.L^{-1} (5 mg.kg^{-1} dose) compared to 33 (McLean & Graham, 2002) and 36 μmol.L^{-1} (Skinner, Jenkins, Folling, et al., 2013) at the same time point following a 6 mg.kg^{-1} dose. Similarly paraxanthine concentrations of the current study and that of McLean and Graham (2002) and Skinner, Jenkins, Folling, et al. (2013) at three hours were 6.4, 7.8 and 9 μmol.L^{-1} respectively. However in the current study we did not observe any correlation between caffeine (or paraxanthine) concentration at the start of the time trial and subsequent performance. This is consistent with the observations of (Skinner, Jenkins, Taaffe, Leveritt, & Coombes, 2013) who showed that coinciding the peak serum caffeine concentration with the start of a 40 km time trial did not improve endurance performance above that of a placebo. However when caffeine was given one hour before exercise (usual practice) there was a significant improvement in time-trial performance.

Theophylline (0.5 μmol.L^{-1}) and Theobromine (0.6 μmol.L^{-1}) at three hours were also consistent with that of (McLean & Graham, 2002) (0.7 μmol.L^{-1} and 1.0 μmol.L^{-1} respectively). Data from the current study also showed subjects with higher theobromine at baseline (0.8 μmol.L^{-1}) in agreement with these authors.

5.2.6. The effect of CYP1A2 polymorphism on rate of caffeine metabolism

Many studies investigating CYP1A2 enzyme activity report that the C>A tranversion at position -163 downstream of the first transcribed nucleotide is responsible for changes in the rate of caffeine metabolism (Castorena-Torres et al., 2005; Sachse et al., 2003; Sachse et al., 1999). The rate of caffeine elimination can be determined by calculation of the area under the concentration-time curve (AUC) or, by a method that closely resembles systemic caffeine clearance, the plasma paraxanthine/caffeine (PX/Caff) ratio taken at a single time point (5-7 hours) after caffeine administration (Fuhr & Rost, 1994) The initial major metabolic reaction in caffeine metabolism is the 3-N demethylation of caffeine to paraxanthine. This reaction is almost exclusively (85%) mediated by CYP1A2 (Kot & Daniel, 2008). Thus the PX/Caff ratio can infer the rate of CYP1A2 enzyme activity.

The PX/Caff ratio at seven hours post caffeine ingestion was not significantly different ($p=0.097$) between those that were homozygous A/A or carriers of the C allele. This is in contrast to many authors who state that those homozygous A/A show faster metabolism. However many of these authors (Cornelis, Bae, Kim, & El-Sohemy, 2010; Cornelis et al., 2004; Cornelis et al.,

2006; Palatini et al., 2009) have not phenotyped their subjects but cite the work of Sachse et al. (1999), Han et al. (2001), Ghotbi et al. (2007) and Castorena-Torres et al. (2005). Although these authors using a variety of metabolite ratios in plasma and urine have found higher inducibility with the A/A genotype, it has only consistently been seen in smokers and with only one study (Castorena-Torres et al., 2005) showing that non-smokers had differences in the rate of caffeine metabolism. As would be expected of most trained athletes the subjects in the current trial were all non-smokers thus the lack of significant differences between the genotypes is not unexpected. However it can't be conclusively determined that there is no effect of genotype on the rate of metabolism due to the very small sample size of the present study, and the fact that significance was approached.

Indeed, although it was not significant, the PX/Caff ratio did show a trend that carriers of the C allele appeared to have slightly faster metabolism than the A/A genotype. This was not expected as it conflicts with others who have shown that those who are homozygous A/A may be faster metabolisers of caffeine than carriers of the C allele. Why this is the case is unknown; however, as mentioned, very little data in non-smokers shows faster metabolism in the A/A group compared to carriers of the C allele. Again due to the very small sample size in the present study, no definitive conclusions can be drawn. However, the impact of the CYP1A2 gene on rates of caffeine metabolism does warrant further rigorous investigation. Specific genotype grouping was based on the specific melting temperature provided with the kit used and if time and funding permitted, genotyping would be confirmed by sequencing.

Caffeine clearance (2.8 $mL.min^{-1}.kg^{-1}$) and half-life (5.6h) were highly variable and thus not significantly different between genotypes. However they were in the same direction as the PX/caffeine ratio; that is, the C group had higher clearance rates and faster half-life than the A group. These results are consistent with a review by (Magkos & Kavouras, 2005) where half-life for caffeine (dose <10 $mg.kg^{-1}$) ranges from 2.5-10 hours and plasma clearance rates of approximately 1-3 $mL.kg^{-1}.min^{-1}$. A more recent study by Perera, Gross, and McLachlan (2010) also showed a similar half-life of 5.5±1.9h. In addition an intense exercise program has been shown to increase the activity of CYP1A2 enzyme (Vistisen et al., 1991). The subjects in the current study spend many hours training (10.6 ±4.3h of aerobic activity per week) thus it would be expected to see faster caffeine clearance than those not training so vigorously. This is reflected in the higher caffeine clearance seen in the current study compared to a range of 1.3-1.6 $mL.min^{-1}.kg^{-1}$ seen by others (Collomp et al., 1991; McLean & Graham, 2002; Perera et al., 2010).

There were no differences seen between resting and exercise trials with caffeine clearance (2.8 $mL.min^{-1}.kg^{-1}$ for both trials) and elimination half-life (6.0 and 5.3 hours respectively). These results are similar to McLean and Graham (2002) who observed elimination half-life of 5.7 hours (resting) and 6.3 hours (exercising) and no differences in caffeine clearance rates. Ali et al. (2015) had substantially longer elimination half-life (17.6h) and lower plasma clearance (0.95$mL.min^{-1}.kg^{-1}$) in exercising subjects than the present study. However this study involved females who were also taking oral contraceptives and it has been shown that CYP1A2 is more active in males

than females, and also inhibited by oral contraceptives (Rasmussen et al., 2002).

Studies in monozygotic and dizygotic twins have shown that CYP1A2 activity is mainly governed by genetic factors (72.5%) and the remainder of the variability is due to unique environmental factors (Rasmussen et al., 2002) such as smoking, oral contraceptives, ingestion of cruciferous vegetables and physical fitness. In the present study the subjects were all male, highly trained, non-smokers and were specifically told not to eat cruciferous vegetables in the 48 hours preceding the trials. Therefore it is unlikely that any of these factors would have influenced the results seen in this study.

5.3. Habitual caffeine intake

There was no correlation between habitual caffeine intake and CYP1A2 activity. However, it is hard to make an accurate prediction of how much caffeine was consumed by the subjects as they were only asked whether they consumed coffee or other caffeinated beverages <7 per week, 7-14 per week, 14-21 per week or >21 per week.

5.4. Effect of caffeine on other physiological parameters

As expected, heart rate, VO_2 and RPE increased over the duration of the steady state ride; explained by the 'slow component' of oxygen uptake and associated cardiovascular drift. However, heart rate during the steady state was not affected by caffeine ingestion (mean 152 bpm) compared to placebo (150 bpm).

As would be expected with subjects working at approximately 70% VO_2 max, carbohydrate was their primary energy source (RER approximately 0.91). Interestingly, VO_2 was higher with caffeine during steady state exercise however no interaction between genotype was seen. This is difficult to explain, but could be a result of a higher cadence in the caffeine trial because perceived exertion was less. A higher cycling cadence for the same submaximal cycling workload has previously been associated with increased rate of oxygen consumption (Doherty & Smith, 2005). Accordingly, RPE during steady state exercise was significantly affected by caffeine compared to placebo. Additionally, if lipid oxidation is greater during submaximal exercise, the rate of oxygen utilization should theoretically be greater. Caffeine, as a stimulant, encourages lipolysis, and increases fatty acid availability to the working muscles (Graham, 2001a). Whether this may have occurred in the present trial, we cannot confidently ascertain, since our estimates of substrate oxidation rely upon VO_2 for their calculation. Nevertheless, RER significantly decreased over the course of the 120 minutes steady state exercise and was approaching significance ($p=0.08$) with caffeine treatment, suggesting that caffeine consumption had an effect on fuel selection towards increased fat oxidation. However although many other studies have seen increased fat oxidation with caffeine it is now generally considered that increased fat oxidation does not result in “glycogen sparing” and other factors such as adenosine receptor antagonism provide the mechanism for increased performance.

5.5. Limitations and future work

There are several limitations to the present study. Due to the relatively invasive nature (blood sampling) and exercise capabilities required of the subjects, the sample size was relatively small particularly when the subjects were divided into genotype groups (n=5 and n=6). Based on the results of previous studies (Cox et al., 2002; Hulston & Jeukendrup., 2008; Jenkins et al., 2008) following similar protocols to the present research a larger sample size would be necessary to provide statistical power to any conclusions drawn from the present results. These previous studies saw significant differences in pre- and post- caffeine supplementation endurance outcomes following the administration of between 2 to 6 mg of caffeine to between 10 to 13 well-trained endurance athletes.

For this study only one SNP was investigated (i.e. CYP1A2 A>C transversion at position -163 of the first transcribe nucleotide). It appears that different haplotypes may be more important than individual SNPs when looking at rate of caffeine metabolism (Ghotbi et al., 2007). Thus other SNPs could also be investigated. However this would need an even larger sample size. Variations in caffeine responses have also been seen with adenosine receptor A_{2A} polymorphism (Cornelis, El-Sohemy, et al., 2007). Thus investigation into polymorphisms of the A_{2A} receptor may also provide answers.

Habitual caffeine intake was assessed on the basis of a simple frequency questionnaire. The subjects were only questioned about how many coffee or energy drinks they consumed per week. Future studies should also ask

about other sources of caffeine such as tea, soft drinks and chocolate to get a better indication of total habitual caffeine concentration.

The lack of a large sample size was a big factor in this research. Thus any modifications that make the study less onerous on the subjects should be considered. The use of saliva has been validated as an appropriate sample matrix for the analysis of paraxanthine/caffeine ratio and thus assessment of in vivo CYP1A2 activity (Perera et al., 2010). These authors found that saliva PX/caff ratio at 4 hours following oral administration of a 100 mg caffeine tablet accurately measured CYP1A2 activity. Saliva sampling would be less invasive and a lot more pleasant for subjects thus would recommend any future work assessing CYP1A2 activity that sampling saliva rather than plasma would be an appropriate option.

Further investigation into the rate of caffeine metabolism in relation to CYP1A2 polymorphism is warranted. A large study investigating caffeine metabolism at rest could be easily undertaken. The group used for this study were all highly trained athletes thus it is conceivable that they may react differently than the general population. Alternatively an investigation comparing trained with sedentary individuals, or low compared to high habitual caffeine consumers may be beneficial.

This research has looked at CYP1A2 and its role in caffeine metabolism and its significance to exercise. However caffeine metabolism is only a small part of the role CYP1A2 plays in the body. It is a major player in the metabolism of many drugs and in the removal of unwanted substances such as polycyclic aromatic hydrocarbons (PAHs) which have been associated with various

cancers (Castorena-Torres et al., 2005). Much of the current research uses caffeine as a probe for CYP1A2 activity not for any interest in caffeine itself but for research investigating therapeutic drug dosages (Perera et al., 2010).

5.6. Conclusions

Caffeine is ergogenic during endurance exercise, however individual responses were variable. In this study this variability could not be explained by CYP1A2 genotype. This was a very well-controlled study that used a placebo based, double blind, randomised design. All subjects had similar fitness levels and variables such as exercise and food intake, especially abstinence from cruciferous vegetables and caffeine containing food and beverages, prior to, and during the trial were kept consistent to minimise any influence on exercise performance and caffeine pharmacokinetics. However, the small sample size in this study especially when subjects were divided into genotype groups made drawing any conclusions relating exercise performance to genotype and subsequent caffeine pharmacokinetics difficult.

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Caffeine image. (2015). Retrieved from http://commons.wikimedia.org/wiki/File:Caffeine.svg

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Appendix 1 Recruitment Flyer

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Are you interested in....

Having your fitness assessed by a VO_2 max test?

Finding out if a caffeine supplement affects your exercise performance?

WE ARE LOOKING FOR MALE VOLUNTEERS....

- Cyclists, triathletes, mountain bikers
- 18 – 50 years of age
- Consume some coffee, energy drinks or caffeine supplements

TO TAKE PART IN A TRIAL...

Investigating whether a caffeine supplement affects performance differently in individuals who have different variations of a gene involved in breaking down caffeine

For more information please contact:

Michelle McGrath or Dr Jasmine Thomson,
Institute of Food, Nutrition and Human Health Massey University
Tel: 063569099 ext 5995 (Michelle) or 84559 (Jasmine)
Email: M.McGrath@massey.ac.nz or J.A.Thomson@massey.ac.nz

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 11/35. If you have any concerns about the conduct of this research, please contact A/Prof Hugh Morton, Chair, Massey University Human Ethics Committee: Southern A telephone 06 350 5799 x 4265, email humanethicssouthA@massey.ac.nz

Te Kunenga ki Pūrehuroa

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Appendix 2 Participant Consent Form

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Caffeine Supplementation and Genotype Study

PARTICIPANT CONSENT FORM - INDIVIDUAL

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree to participate in this study under the conditions set out in the Information Sheet.

Signature: _____ **Date:** _____

Full Name - printed _____

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Appendix 3 Participant Health Questionnaire

MASSEY UNIVERSITY
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TE KURA HAUORA TANGATA

Caffeine Supplementation and Genotype study

HEALTH QUESTIONNAIRE

NAME				DATE	
ADDRESS					
PHONE		(HM)			(MOB)
Occupation					
Current GP				Phone	
Address					
Person to contact in case of emergency				Phone	

Please answer the following questions. For most people physical activity should not pose a hazard. This questionnaire has been designed to identify the small number of persons (18-50 years of age) for whom physical activity might be inappropriate and to provide the researchers with descriptive information about the participant. If you have any doubts or difficulty answering the questions, please ask the investigator for guidance. These questions help us to determine whether the proposed physical activity requirement of this study is appropriate for you. Your answers will be kept strictly confidential.

HEALTH HISTORY

Has your GP advised you not to perform vigorous exercise?	Yes ☐	No ☐
Have you been diagnosed with a heart condition?	Yes ☐	No ☐
Have you been diagnosed with high blood pressure?	Yes ☐	No ☐
Have you been diagnosed with a heart condition?	Yes ☐	No ☐
If yes please detail below:		
Have you been diagnosed with high cholesterol?	Yes ☐	No ☐
Have you been diagnosed with diabetes?	Yes ☐	No ☐
Do you feel chest pain while doing physical activity?	Yes ☐	No ☐
In the past month, have you had chest pain while not doing physical activity?	Yes ☐	No ☐
Do you lose balance due to dizziness or loss of consciousness?	Yes ☐	No ☐
Do you have a back or joint pain that may be made worse with vigorous exercise?	Yes ☐	No ☐
Do you suffer from asthma?	Yes ☐	No ☐
If yes, do you control it with medication?	Yes ☐	No ☐
Do you suffer from a blood borne contagious disease?	Yes ☐	No ☐

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Do you suffer from any other condition which we need to be aware of before you undertake this study?			Yes ☐	No ☐
If yes, please detail below:				
Do you currently smoke?			Yes ☐	No ☐
If yes, please indicate average packs per day?	<0.5 ☐	0.5-1 ☐	1.5-2 ☐	>2 ☐
Are you currently taking medication?			Yes ☐	No ☐
If yes, please detail below:				

PHYSICAL ACTIVITY & TRAINING HISTORY

Have you previously done a maximal exercise test?	Yes ☐	No ☐
Over the last 6 months, have you been performing vigorous exercise regularly?	Yes ☐	No ☐
How long have you purposely trained in a sport involving cycling?		years
Which sports?		
Over the past 6 months, what was your average volume of aerobic exercise?		h/wk
Average volume of cycling training over this period?		h/wk

DIET RELATED

Over the last month, has your diet been stable?			Yes ☐	No ☐
Do you have any food allergies?			Yes ☐	No ☐
If yes, to which foods?				
Do you have any food intolerances/dislikes?			Yes ☐	No ☐
If yes, which foods don't you eat?				
Are you Vegetarian?			Yes ☐	No ☐
Do you regularly consume caffeinated coffee or energy drinks?			Yes ☐	No ☐
If yes, please indicate approx. how many per week?	<7 ☐	7-14 ☐	14-21 ☐	>21 ☐
Do you take caffeine supplements?			Yes ☐	No ☐
If yes, please indicate approx. how often	<1/week ☐	weekly ☐		>1/week ☐

I have completed the questionnaire to the best of my knowledge, and any doubts or difficulty I had answering questions were addressed by the researcher to my satisfaction.

Participant Signature _____ DATE _____

Appendix 4 List of Foods and Drinks to Avoid

48 Hours Prior to Trial

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Caffeine Supplementation and Genotype study

List of foods to avoid 48 hours (2 days) prior to caffeine intervention study:

Caffeinated drinks including:

- Coffee
- Green or black tea
- Energy / sports drinks
- Fizzy drinks
- Milo
- Hot chocolate
- Chocolate milkshakes
- Alcohol

Foods:

- Chocolate and products containing chocolate
- Foods containing coffee
- Brassica vegetables such as:
  - Broccoli
  - Cauliflower
  - Brussel sprouts
  - Cabbage

Medication:

- Non prescription cold/flu or headache medication that contain caffeine

N.B. In addition to the avoidance of these foods for 48 hours please abstain from any form of vigorous physical activity for 24 hours prior to the study days.

Thank you for your cooperation.

Te Kunenga ki Pūrehuroa
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Appendix 5 Two Day Diet Record

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

GENOTYPE AND CAFFEINE METABOLISM STUDY

Two Day Diet Record and Nutrient Analysis

Two day diet record

- Record all that you eat and drink for two (2) days prior to each of the three days when you come in to participate in the study.

On the diet record sheet include:

- The time of day the food was eaten
- Where it was eaten
- Precise description of the food (brand, preparation, condiments)
- Amount eaten

Note:

- Choose from foods not excluded on the food list
- Eat your normal quantities of food
- Weigh or measure portion sizes if you are unsure of how to record them. Use standard measure such as "cups"
- When describing amount of pasta, rice etc., make sure you record whether portion sizes are dry or cooked portions
- Remember to include all drinks, including water. If you drink from a sipper bottle throughout the day, record it as such. I.e. you don't need to record every sip.
- Record cuts and types of meat, type of milk, type of bread, fruit varieties, brand names, cooking methods used etc.
- If you eat packaged foods please attach the food labels
- If you eat mixed dishes (i.e. a beef curry) please attach a recipe

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Name…… Date…… Day……

Time food was eaten	**Place eaten**	**Complete description of food** (Preparation, variety, brand. If possible attach the recipe or nutrition label)	**Amount consumed** (units, measures, weight)

Appendix 6 24 Hour Physical Activity Record

Date ..... Name .....

In order to complete your physical activity diary you need to record all activities (including being at rest or sleeping) for 24 hours prior to the supplementation. Using the chart below estimate what your Physical Activity Rate (PAR) for each of the activities.

PAR 1.2 (1.0 to 1.4)	
Lying at rest	Reading
Sitting at rest	Watching TV; reading; writing; calculating; playing cards; listening to radio; eating
Standing at rest	
PAR 1.6 (1.5 to 1.8)	
Sitting	Sewing; knitting; playing piano; driving
Standing	Preparing vegetables; washing dishes; ironing; general office and laboratory work
PAR 2.1 (1.9 to 2.4)	
Standing	Mixed household chores (dusting and cleaning); washing small clothes; cooking activities; hairdressing; playing snooker; bowling
PAR 2.8 (2.5 to 3.3)	
Standing	Dressing and undressing; showering; 'hoovering'; making beds
Walking	3-4km/h; playing cricket
Industrial	Tailoring; shoemaking; electrical; machine tool; painting and decorating
PAR 3.7 (3.4 to 4.4)	
Standing	Mopping floor; gardening; cleaning windows; playing table tennis; sailing
Walking	4-6km/h; golf
Industrial	Motor vehicle repairs; carpentry; chemical; joinery; bricklaying
PAR 4.8 (4.5 to 5.9)	
Standing	Polishing furniture; chopping wood; heavy gardening; volleyball
Walking	6-7km/h
Exercise	Dancing; moderate swimming; gentle cycling; slow jogging
Occupational	Labouring; hoeing; road construction; digging and shovelling; felling trees
PAR 6.9 (6.0 to 7.9)	
Walking	Uphill with load or cross country; climbing stairs
Exercise	Average jogging; cycling
Sports	Football; more energetic swimming; tennis; skiing

Time	Activity	Physical activity Rate (PAR)
08.30		
08.45		
09.00		
09.15		
09.30		
09.45		
10.00		
10.15		
10.30		
10.45		
11.00		
11.15		
11.30		
11.45		
12.00		
12.15		
12.30		
12.45		
13.00		
13.15		
13.30		
13.45		
14.00		
14.15		
14.30		
14.45		
15.00		
15.15		
15.30		
15.45		
16.00		
16.15		

Time	Activity	Physical activity Rate (PAR)
16.30		
16.45		
17.00		
17.15		
17.30		
17.45		
18.00		
18.15		
18.30		
18.45		
19.00		
19.15		
19.30		
19.45		
20.00		
20.15		
20.30		
20.45		
21.00		
21.15		
21.30		
21.45		
22.00		
22.15		
22.30		
22.45		
23.00		
23.15		
23.30		
23.45		
24.00		
00.15		

Time	Activity	Physical activity Rate (PAR)
00.30		
00.45		
01.00		
01.15		
01.30		
01.45		
02.00		
02.15		
02.30		
02.45		
03.00		
03.15		
03.30		
03.45		
04.00		
04.15		
04.30		
04.45		
05.00		
05.15		
05.30		
05.45		
06.00		
06.15		
06.30		
06.45		
07.00		
07.15		
07.30		
07.45		
08.00		
08.15		

Appendix 7 VO_2 Max Test Form

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TE KURA HAUORA TANGATA

CAFFEINE AND GENOTYPE VO_2MAX

Subject ID:	***Date:***	***Time:***
Age:	***BW (kg):***	***Ht (cm):***
Barometric Pressure (mmHg):		***$mmHg_x$ = InHg*25.4***
Humidity (%):		
Temperature (°C):		
Loader protocol:	*Submax:*	*Ramp test:*
HR_{max} (bpm)	*220-age=*	

CYCLE ERGOMETER DIMENSIONS

Cycle _____ Handlebar Extn (cm) _____ Handlebar Ht (cm) _____

Seat Extn (cm) _____ Seat Ht (cm) _____ Seat-Pedal (cm) _____

Crank Length (cm) _____

Warm up at ~100W or load comfortable for cyclist.
Maintain cadence as high as possible/comfortable (>80-90rpm)

GAS ANALYSIS

Work rate	Nose clip & mouth piece in (mins)	Collection start time (mins)	Bag #	HR (bpm)	collection time (secs) *Convert to minutes for spreadsheet*	Expired O2	Expired CO2	Sample pump time (mins)	Sample pump flow rate $L.min^{-1}$	Temp exp air (°C)	Volume in bag after sample removed (L)
100	**2.5**	**3.0**									
150	**6.5**	**7.0**									
200	**10.5**	**11.0**									
250	**14.5**	**15.0**									
		Max									

STOP TIME _____ _____W HR @ STOP_____

STOP WHEN EXHAUSTED:
A CADENCE >60RPM UNABLE TO BE MAINTAINED
VOLITIONAL FATIGUE
HR WITHIN 10BPM OF HR_{MAX}

VO2 MAX (rel) =
VO2 MAX (abs)=
RER=

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Appendix 8 Caffeine Certificate of Analysis

Highest Quality
Nutritional Ingredients

BLACKBURN DISTRIBUTIONS LTD

Blackburn Distributions Ltd
Unit 4B Pendle Industrial Estate
Wenning St off Southfield St
Nelson
Lancashire
BB9 0LE

www.BlackburnDistributions.com
sales@blackburndistributions.com

Tel: 01282 878254
Fax: 01282 699237

BLACKBURN DISTRIBUTIONS LTD

Caffeine Anhydrous Powder
BP2012, EP7.0, USP35, FCC8

Original specification: 992418V02
Batch: 1031305542
Manufacturing date: 01/05/2013
Expiry date: 01/04/2017

Certificate of Analysis

Caffeine Anhydrous Powder
BP2012, EP7.0, USP35, FCC8

Original specification: 992418V02
Batch: 1031305542
Manufacturing date: 01/05/2013
Expiry date: 01/04/2017

TEST	SPECIFICATION	RESULTS
Appearance	A white crystalline powder or silky white crystals	White crystalline powder
Colour and Appearance	clear and colourless BP/EP	Pass
Assay	98.5 to 101.0% EP/USP	99.5%
Identification	Chemical reaction, Passes test IR, Passes test	Pass Pass
Acidity	NMT 0.2ml of 0.01 M Sodium Hydroxide is required to change the colour of the indicator to blue BP/EP	< 0.2 ml
Heavy metals	Max 10 ppm	< 10 ppm
Alkalinity	no precipitate is formed USP	Pass
Related Substances	Unspecified impurites: 0.10% max BP/EP Total: 0.1% max BP/EP disregard limit: 0.05% max BP/EP	< 0.1% < 0.1% < 0.1% < 0.1% < 0.1%
Loss on drying	Max 0.5 % EP	0.11%
Melting Range	235 to 237.5°C	235.9 to 236.7°C
Lead	Max 1 mg/kg	< 1 mg/kg
Residue on ignition	Max 0.1 %	0.04%
Readily Carbureted Substance	Passes test	Pass
Sulphate	Max 500 ppm	< 500 ppm

Appendix 9 Exercise Trial Day Data Form

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Caffeine Supplementation and Genotype Study

Date:
Subject ID:
Subject name:
START TIME: Breakfast: Caffeine: HR monitor:
Session:
Intervention (A or B):
Breakfast + fluid given:
Loader protocol:
Data saved as:
Warm up: Steady state: Time trial linear factor
Barometric Pressure (mmHg): ***$mmHg_x = InHg*25.4$***
Humidity (%):
Temperature (°C):

CYCLE ERGOMETER DIMENSIONS

Handlebar Extn (cm) _____
Handlebar Ht (cm) _____
Seat Extn (cm) _____
Seat Ht (cm) _____

Notes

_____

_____

_____

_____

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Initial weight: _____

Weight after cycling: _____

Urine sample collection 1 _____

Urine sample collection 2 _____

Blood Sampling:

Tube label	Sample	Time due	Actual time	Caffeine timer	Comments
1	Baseline				
2	1hour				
3	1.5 hour				
4	2 hour				
5	2.5 hour				
6	3 hour				
7	3.5 hour				
8	5 hour				
9	7 hour				

ANALYSIS

Time	HR (bpm)	Bag #	RPE	Collection time (sec) *Convert to minutes for spreadsheet*	Expired O2	Expired CO2	Sample pump time (min)	Sample pump flow rate $L.min^{-1}$	Temp exp air (°C)	Volume in bag after sample removed (L)

2 hour steady state: accumulated energy value _____ kJ

Total energy value (2hr +TT) _____ kJ

Time trial: accumulated energy value _____ kJ

Data saved as_____

Lunch given _____

Subject guess (caffeine/placebo) _____

Appendix 10 Macronutrient Content of the Standardised Breakfast

	Banana	Up & Go	Cereal Bar
	Per 100g	Per 100g	Per serving (40g)
Weight (g)	100	100	40
Energy (kJ)	431	330	508
Carbohydrate (g)	24	12	22.3
Protein (g)	1	3.4	1.8
Total fat (g)	0	1.5	0.4

Appendix 11 RPE Scale

HEART RATE: _____ bpm

During the exercise bout, we want you to pay close attention to how hard you feel the exercise work rate is. This feeling should reflect your total amount of exertion and fatigue, combining all sensations and feelings of physical stress, effort, and fatigue. Don't concern yourself with any one factor, such as leg pain, shortness of breath or exercise intensity, but try to concentrate on your total, inner feeling of exertion. Try not to underestimate or overestimate your feeling of exertion; be as accurate as you can.

6	
7	Very, very light
8	
9	Very light
10	
11	Fairly light
12	
13	Somewhat hard
14	
15	Hard
16	
17	Very hard
18	
19	Very, very hard
20	

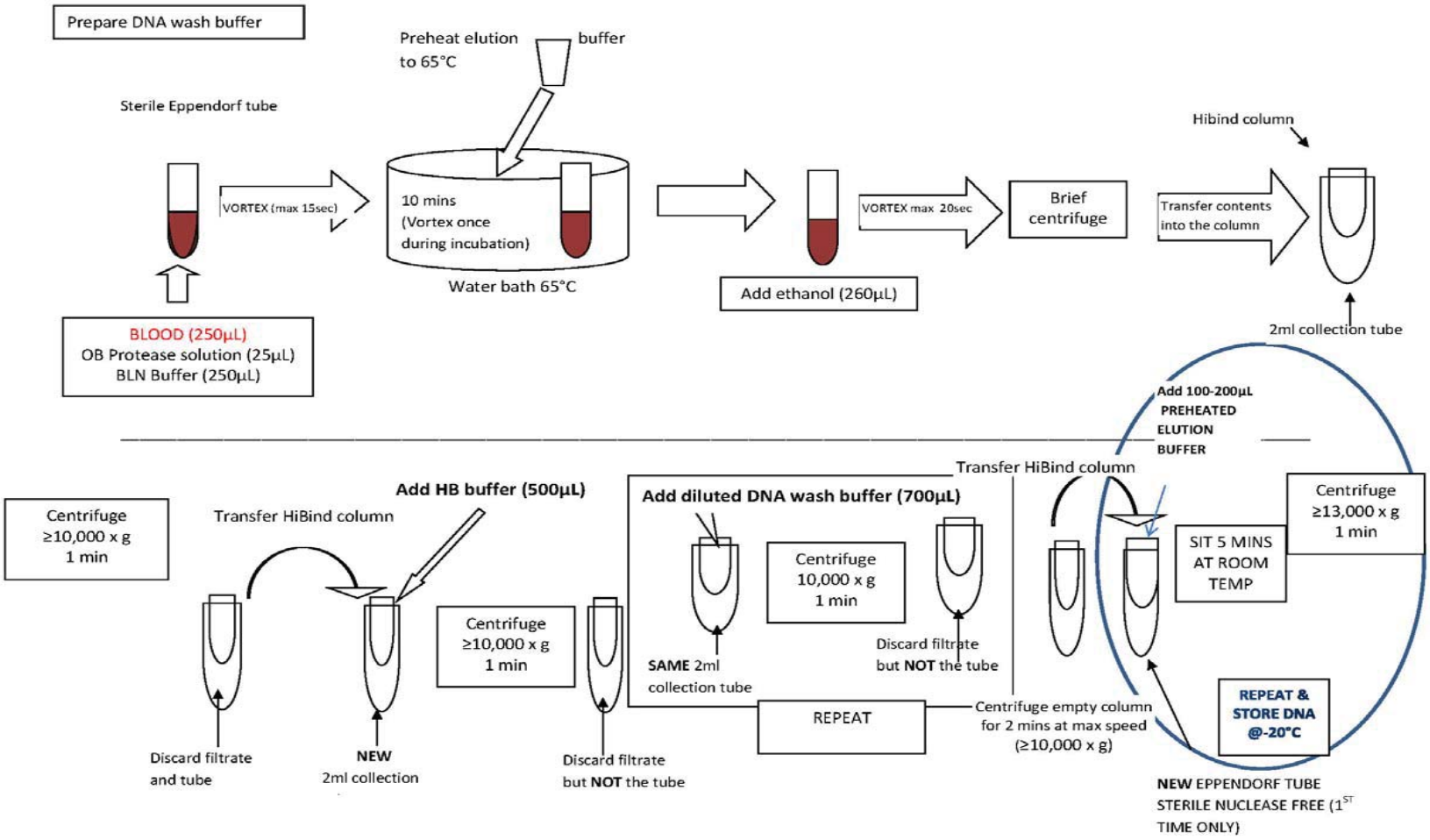
While participating in exercise, it is common to experience changes in mood. Some individuals find exercise pleasurable, whereas others find it to be unpleasurable. Additionally, feeling may fluctuate across time. That is, one might feel good and bad a number of times during exercise. Scientists have developed this scale to measure such responses.

+5	Very good
+4	
+3	Good
+2	
+1	Fairly good
0	Neutral
-1	Fairly bad
-2	
-3	Bad
-4	
-5	Very bad

Estimate here how aroused you actually feel. Do this by circling the appropriate number. By "arousal" here is meant how "worked-up" you feel. You might experience high arousal in one of a variety of ways, for example as excitement or anxiety or anger. Low arousal might also be experienced by you in one of a number of different ways, for example as relaxation or boredom or calmness.

1	Low arousal
2	
3	
4	
5	
6	High arousal

Appendix 12 DNA Extraction Method


Prepare DNA wash buffer
Preheat elution buffer to 65°C
Sterile Eppendorf tube
VORTEX (max 15sec)
10 mins (Vortex once during incubation)
Water bath 65°C
Add ethanol (260µL)
VORTEX max 20sec
Brief centrifuge
Hibind column
Transfer contents into the column
2ml collection tube
BLOOD (250µL)
OB Protease solution (25µL)
BLN Buffer (250µL)
Add 100-200µL PREHEATED ELUTION BUFFER
Transfer HiBind column
Centrifuge ≥13,000 x g 1 min
SIT 5 MINS AT ROOM TEMP
REPEAT & STORE DNA @-20°C
NEW EPPENDORF TUBE STERILE NUCLEASE FREE (1ST TIME ONLY)
Centrifuge empty column for 2 mins at max speed (≥10,000 x g)
Add diluted DNA wash buffer (700µL)
Centrifuge 10,000 x g 1 min
SAME 2ml collection tube
Discard filtrate but NOT the tube
REPEAT
Centrifuge ≥10,000 x g 1 min
Discard filtrate but NOT the tube
Add HB buffer (500µL)
Transfer HiBind column
Centrifuge ≥10,000 x g 1 min
Discard filtrate and tube
NEW 2ml collection

Appendix 13 Rs762551 CYP1A2 Genotyping Method

(Supplied by TIB MOLBIOL)

LightSNiP

rs762551 CYP1A2*1F

Preparation of parameter-specific reagents (96 reactions):

One reagent vial contains all primers and probes to run 96 LightCycler® reactions.

Spin vial before opening to ensure the yellow pellet is located at the base of the reaction tube.

Add 100 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

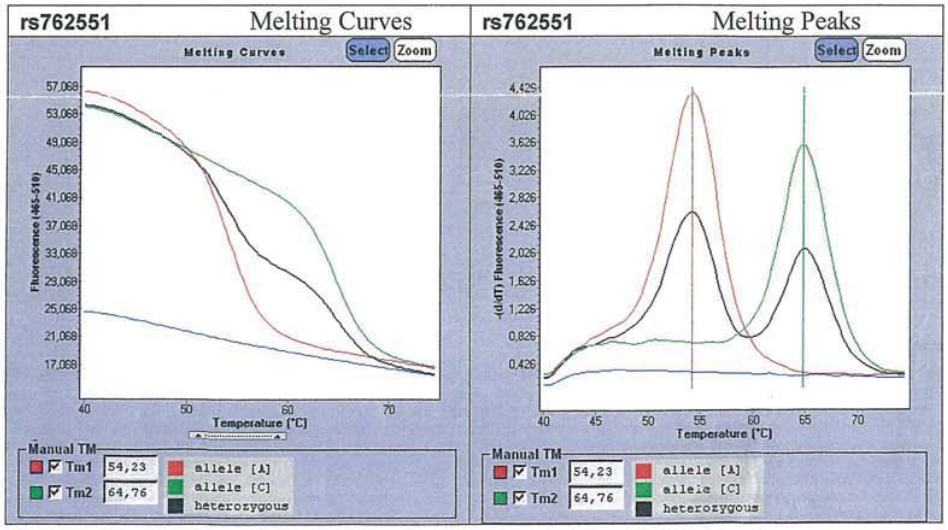
►Use 1 µl **Reagent Mix** for a 20 µl PCR reaction.

Preparation of the reaction mix:		**Settings:**
20 µl reaction mixture		**LightCycler® 480 Instrument**
H_2O	14.4 – 10.4 µl	**Block Type:** 384 or 96
Reagent Mix	1.0 µl	**Detection Format:** Simple Probe
FastStart DNA Master[(1)]	2.0 µl	LightCycler® 480 Instrument I: 483-533
$MgCl_2$ (25 mM)	1.6 µl	LightCycler® 480 Instrument II: 465-510
DNA	1.0 – 5.0 µl (~ 50 ng)	
Final $MgCl_2$ conc.:	3.0 mM	

[(1)]LightCycler® FastStart DNA Master HybProbe (Roche Diagnostics)

Programming LightCycler® 480 Instrument:

Program:	*Denaturation*	*Cycling*			*Melting*			*Cooling*
Parameter								
Analysis Mode	*None*	*Quantification*			*Melting Curves*			*None*
Cycles	*1*	*45*			*1*			*1*
Segment	*1*	*1*	*2*	*3*	*1*	*2*	*3*	*1*
Target [°C]	95	95	60	72	95	40	75	40
Hold [hh:mm:ss]	00:10:00	00:00:10	00:00:10	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
*Ramp Rate [°C/s] **384***	*4.6*	*4.6*	*2.4*	*4.6*	*4.6*	*2.0*	-	*2.0*
*Ramp Rate [°C/s] **96***	*4.4*	*4.4*	*2.2*	*4.4*	*4.4*	*1.5*	-	*1.5*
Acquisition Mode	None	None	Single	None	None	None	Continu.	None
Acquisitions [per °C]							3	



Reference: ATGCTCAAAGGGTGAGCTCTGTGGGC [A/C] CAGGACGCATGGTAGATGGAGCTTA

http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=762551

Preparation of the reaction mix:		Settings:
20 µl reaction mixture		**LightCycler® 1.x / 2.0 Instruments**
H_2O	14.4 – 10.4 µl	LightCycler® 1.x Instrument: channel F1
Reagent Mix	1.0 µl	LightCycler® 2.0 Instrument: channel 530
FastStart DNA Master[1]	2.0 µl	
$MgCl_2$ (25 mM)	1.6 µl	
DNA	1.0 – 5.0 µl (~ 50 ng)	
Final $MgCl_2$ conc.:	3.0 mM	

[1]LightCycler® FastStart DNA Master HybProbe (Roche Diagnostics)

Programming LightCycler® 1.x / 2.0 Instruments:

Program:	*Denaturation*	*Cycling*			*Melting*			*Cooling*
Parameter								
Analysis Mode	*None*	*Quantification*			*Melting Curves*			*None*
Cycles	*1*	*45*			*1*			*1*
Segment	*1*	*1*	*2*	*3*	*1*	*2*	*3*	*1*
Target [°C]	95	95	60	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:10	00:00:10	00:00:15	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [°C/s]	*20.0*	*20.0*	*20.0*	*20.0*	*20.0*	*20.0*	*0.2*	*20.0*
Acquisition Mode	None	None	Single	None	None	None	Continu.	None

Important Notes:

Temperatures reported in the manual are obtained with a LightCycler® 480 instrument version II. The Tm values may differ by up to 4°C when run on other instruments while the ΔTm values are relative constant.

LightSNiP assays are developed based on synthetic targets and verified using a few genomic DNA samples only, representing at least one of two possible genotypes but have not been validated on a larger number of samples. The amplified region is checked for other published polymorphisms (NCBI) at time of the design to avoid the interference due to other SNPs covered by primers or the probe, however, results may be influenced by other SNPs in the region.

The product is intended for research use only and must be validated on samples with a known genotype.