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The influence of the CYP1A2 polymorphism on the effect of caffeine ingestion and mouth rinsing on 3km cycling performance

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Approved and recommended for acceptance as a thesis in partial fulfillment of the requirements for the degree of Master of Science.

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The Influence Of The CYP1A2 Polymorphism On The Effect Of Caffeine
Ingestion And Mouth Rinsing On 3km Cycling Performance

Mark W. Pataky

A thesis submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

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Abstract

PURPOSE: The objectives were to determine the effects of caffeine ingestion and mouth rinsing on cycling performance, and to assess whether the CYP1A2 gene is related to the size of the performance effects. **METHODS:** Thirty-eight recreational cyclists completed four simulated 3-kilometer time trials (TT). Subjects ingested either 6mg/kgBW of caffeine or placebo one hour prior to each TT. Additionally, 25 ml of 1.14% caffeine, or placebo solution was mouth rinsed before each TT. Treatments were: placebo ingestion+placebo rinse (*Placebo*), placebo ingestion+caffeine rinse (*Rinse*), caffeine ingestion+caffeine rinse (*Ingestion+Rinse*), caffeine ingestion+placebo rinse (*Ingestion*). Subjects were genotyped and classified as AA homozygotes or C allele carriers for the rs762551 polymorphism in CYP1A2. Magnitude based inferences were used to evaluate treatment differences. **RESULTS:** Both *Ingestion+Rinse* ('likely') and *Ingestion* ('possibly') improved performance compared to *Placebo*. Performance differences between *Ingestion+Rinse* and *Ingestion* and between *Rinse* and *Placebo* were 'likely trivial.' C allele carriers experienced greater gains ('likely') with *Ingestion* compared to AA homozygotes. Additional analyses revealed that caffeine intake conferred larger benefits for subjects that performed trials prior to 10am. **CONCLUSIONS:** Caffeine ingestion, but not mouth rinse, improved cycling performance. C allele carriers experienced the largest improvement in performance with caffeine ingestion, especially prior to 10am.

Chapter One

Introduction

Consumption of caffeinated beverages began no later than 750-1000AD (19). Caffeine was first isolated by Friedrich Ferdinand Runge in 1819 with the intention of treating diseases associated with aging (39). Today, caffeine is the most consumed psychoactive drug in the world, with an estimated worldwide daily intake of ~75mg/person (18). Fredholm et al. reported that Scandinavian countries consume >400mg caffeine/person/day, whereas countries such as Nigeria and Angola consume as little as 5mg caffeine/person/day (18). The widespread use of caffeine in humans is primarily attributed to the stimulatory effect of caffeine on the central nervous system, enhancing alertness. Caffeine has also proliferated into the world of athletics and the last four decades have yielded sufficient scientific evidence for the efficacy of caffeine ingestion on sprinting and endurance exercise performance (14). In cyclists, caffeine can enhance 1km and 4km sprinting performance (42, 49). Likewise, caffeine provision can improve short duration swimming performance (8), rowing (5), running (20), and performance in variable intensity activities such as tennis and team sports (29, 43). In 2004, a meta-analysis of 40 studies revealed that caffeine intake markedly influences endurance performance, confirming the effectiveness of caffeine use in events greater than 60 minutes (14).

The mechanism by which caffeine improves performance has drawn considerable interest. Early thinking was that caffeine spared muscle glycogen and enhanced fat oxidation during endurance exercise (11, 15, 27, 45) but this notion has largely been discarded (21, 22, 23, 31). Caffeine is now believed to deliver its

effects through the central nervous system (17). Adenosine decreases neuron firing and the release of most neurotransmitters including acetylcholine, dopamine, and noradrenaline (44). Caffeine is an adenosine antagonist that competitively binds to adenosine receptors, thereby promoting continued neuron activity. Likely through this mechanism, caffeine provision can suppress ratings of perceived exertion and pain (11, 37), and increase neural excitability (47) thereby enhancing athletic performance.

Though it is evident that caffeine ingestion can improve performance in events of short and long durations, not all subjects experience the same benefits. There is considerable individual variability with respect to caffeine responsiveness, with some subjects exhibiting no effect at all (6). Genetics have consequently been implicated as an explanation for the individualized ergogenic response to caffeine ingestion (9). In an attempt to address whether or not genetics contribute to the individual ergogenic effect of caffeine, our laboratory recently determined that individuals homozygous for a single nucleotide (A) polymorphism at intron 1 of the cytochrome P450 (CYP1A2) gene appeared to experience a larger ergogenic effect following caffeine ingestion (50). Therefore, there may be a genetic predisposition for responsiveness to caffeine ingestion that is greater in some people than in others. Caffeine is metabolized in the liver by the cytochrome P450-1A2 enzyme, which is coded for by the CYP1A2 gene (51). Being homozygous for the A allele increases the inducibility of the P450-1A2 enzyme (41). If the expression of the P450-1A2 enzyme is increased, as it is in individuals with the A/A genotype, then the rate of caffeine metabolism may also increase. Quicker metabolism of caffeine in

the liver may lead to a higher concentration of caffeine metabolites in the blood that in turn would act on the central nervous system, consequently improving performance.

Beaven and colleagues recently reported that without ingesting, but by rinsing caffeine in the mouth, repeated sprinting performance is enhanced in cyclists (1). It is logical to speculate that there are unidentified caffeine receptors in the oral cavity that respond to the presence of caffeine by transmitting signals to the areas of the brain involved in reward and motor activity, much like what has been shown with carbohydrates (6). Interestingly, in the same study by Beaven, it was also revealed that a caffeine-carbohydrate mouth rinse improved performance beyond that of only a carbohydrate mouth rinse, providing more support for an oral receptor in the mouth for caffeine (1). More research is necessary to profile the efficacy of a caffeine mouth rinse on performance, particularly in real-world time trial scenarios. The idea that mouth rinsing with caffeine can enhance performance through a mechanism not influenced by liver caffeine metabolism invites the possibility that genetic non-responders to caffeine ingestion may be able to derive performance benefits from a caffeine mouth rinse. Additionally, because of the presumed mechanisms through which caffeine consumption and mouth rinsing elicits performance gains, one might expect there to be a further improvement in performance when mouth rinsing in combination to ingesting caffeine than when only ingesting caffeine.

In contrast to Beaven's work, others have shown that a caffeine mouth rinse given throughout a 60 minute cycling time trial does not seem to improve

performance (12), bringing into question the efficacy of a caffeine mouth rinse for sustained power output. Worth noting is that in a subgroup of subjects that did not report regular caffeine consumption there was an observed ergogenic effect to the caffeine mouth rinse even though the mouth rinse in this study contained a relatively low concentration of caffeine that has not been shown to improve performance during caffeine ingestion (28). Some studies have demonstrated that “non-caffeine users” (<50mg caffeine/day) experience a heightened performance benefit during caffeine ingestion (2) and that performance gains are attenuated in habitual caffeine users (16).

This project was designed to address the questions outlined above. Specifically, we aim to determine the effectiveness of a caffeinated mouth rinse on sustained performance. As previously stated the presumed mechanisms of action of caffeine mouth rinsing and ingestion are on the oral receptors and brain, respectively. Differing enhancement in performance between genetic polymorphisms, or between habituation levels of subjects will allow for the assumption that the physiological effect of these factors occurs at either the liver or CNS. In those that do not respond to caffeine ingestion it is possible that a caffeine mouth rinse will elicit a performance enhancing effect that was otherwise not observed by caffeine ingestion. If there is a separate mechanism of caffeine mouth rinsing on the CNS then those who do not respond to caffeine ingestion potentially could experience a performance enhancing effect of a caffeine mouth rinse.

Aims and Hypotheses

Aim 1: To determine if a pre-exercise caffeine mouth rinse improves 3km cycling performance compared to placebo.

Hypothesis 1: A pre-exercise caffeine mouth rinse will improve 3km cycling performance compared to a placebo.

Aim 2: To determine if a caffeine mouth rinse influences 3km cycling performance in C allele carriers of the CYP1A2 polymorphism.

Hypothesis 2: Genetic non-responders (C allele carriers) will not exhibit as great of a performance improvement in response to a pre-exercise caffeine mouth rinse when compared to placebo as genetic responders (A/A homozygous) individuals.

Aim 3: To determine if a pre-exercise caffeine mouth rinse improves 3km cycling performance among “caffeine-users” to a greater degree than “caffeine non-users.”

Hypothesis 3: “Caffeine non-users” will exhibit a greater performance benefit in response to a pre-exercise caffeine mouth rinse than “caffeine-users.”

Aim 4: To determine if a pre-exercise caffeine mouth rinse in addition to caffeine ingestion improves 3km cycling performance compared to caffeine ingestion alone.

Aim 4: A pre-exercise caffeine mouth rinse in addition to caffeine ingestion will improve 3km cycling performance more than caffeine ingestion alone.

Significance

The current study will be the first to examine the performance enhancing effect of a caffeinated mouth rinse on prolonged cycling, providing further evidence of the actions of caffeine in the oral cavity. The effectiveness of caffeine ingestion in combination with a caffeine mouth rinse at enhancing performance will be examined to determine the potential additive effects of these two methods of caffeine administration. Additionally, this study will yield further insight to the factors that can effect performance improvement in regards to caffeine ingestion including genetic responsiveness and previous caffeine intake habits. These results may influence the use of caffeine in athletes competing in events of similar duration.

Chapter Two

Methods

Subjects

All subjects will be recruited from James Madison University and will be required to have performed, at minimum, “occasional” cycling in their weekly exercise routine. Approximately 40 recreationally trained male and female subjects will provide written informed consent to participate in the study approved by the James Madison University Institutional Review Board.

Testing Procedures

Cardiovascular Fitness Test - VO_{2max}

Following height and body weight measurements, subjects will perform an incremental exercise test on a Velotron cycle ergometer (Seattle, WA, USA) until volitional exhaustion. Subjects will begin with a 5-minute warm-up at a self-selected pace. The subjects will then be prompted to select an intensity that “they can maintain for 60-minutes” to begin the test. The workload will then be increased every minute in 15 Watt (W) increments until the subject reaches volitional fatigue. Breath samples will be collected throughout the test and oxygen consumption (VO_{2max}) will be measured via Moxus Modular Metabolic System (AEI Technologies, Bastrop, TX, USA). Heart rate will be measured via Polar heart rate monitor (Lake Success, New York, USA). A fan set on the “medium” setting will be placed 2 meters in front of the Velotron for cooling purposes. All subjects will receive strong verbal encouragement throughout the cardiovascular fitness test.

Familiarization and Experimental Trials

Each subject will perform six exercise trials on a Velotron cycle ergometer, with 3-7 days between each trial. The first two trials will serve as familiarization trials with the subsequent four trials serving as experimental trials in a double blind, randomized, counter-balanced, placebo controlled fashion. The subjects will then be asked to perform a 5-minute warm-up at a self-selected intensity. Two mouth rinses will be provided during the warm-up; one given immediately at the onset of the warm-up, and one 30 seconds before completion of the warm-up. Subjects will be instructed to swish the mouth rinse for 5 seconds, during which the researcher will count down from 5. Following the warm-up subjects will perform a 3km time trial. Subjects will be instructed to treat each trial as if they are in a race. Subjects will not receive verbal feedback or encouragement from the investigators and no visual feedback from the time trial will be provided with the exception of elapsed distance.

Supplementation Protocol

No supplementation will be given during the familiarization trials other than two practice mouth rinses (water only). During the experimental trials subjects will be given a 25ml mouth rinse solution at room temperature containing either: A) 300mg caffeine, 1g saccharine, and 25ml of water (rinse containing 1.14% caffeine) or B) a flavor-matched placebo containing 6g saccharine, and 25ml of water. Additionally, a 6mg/kg of body weight capsule containing either anhydrous caffeine or all-purpose flour (placebo) will be ingested 1 hour prior to each treatment trial.

The following are the four treatment conditions that will be administered: 1) placebo capsule + placebo mouth rinse. 2) placebo capsule + caffeine mouth rinse 3) caffeine capsule + caffeine mouth rinse. 4) caffeine capsule + placebo mouth rinse.

Dietary and Exercise Control

Diet

Subjects will be provided with instructions for recording food intake so that subsequent dietary intake can be replicated. All subjects will record food intake for 24 hours prior to all experimental trials and will be asked to repeat food intake for each treatment trial so that caloric intake is identical between trials. Subjects will be given a copy of their previous food log so that they can easily repeat their diet for the subsequent trial. Subjects will abstain from any alcohol, caffeine, and food, for 24, 12, and 2 hours prior to each experimental trial respectively. Dietary records will be obtained prior to each experimental trial.

Exercise

All subjects will record daily physical activity for 48 hours prior to experimental trials and will be asked to repeat daily physical activity for each experimental trial. Subjects will be given a copy of their previous exercise log so that they can easily repeat their physical activity for the subsequent trial. Subjects will be asked to abstain from any heavy exercise 48 hours prior to each experimental trial. Exercise records will be obtained prior to each experimental trial.

DNA Sampling

Blood Sampling

Approximately 3 ml of blood will be obtained from an antecubital vein after the final time trial. Blood will then be stored in a -80 degree F freezer until DNA extraction takes place. DNA will be extracted via Illustria Blood GenomicPrep mini spin kit (GE Healthcare, Buckinghamshire, UK). Genotyping will then be performed using restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR). Following blood genotyping, subjects will be grouped as A/A homozygous and C allele carriers.

Treatment Blinding Efficacy

Upon conclusion of the study, subjects will be asked to identify which treatment they received for each of the four treatment trials to confirm effectiveness of the treatment blinding procedures.

Statistical Analysis

Univariate Analysis of Variance (ANOVA) will be applied to determine treatment differences for all variables. Simple contrasts between treatment conditions will be used to generate *P* values for subsequent analysis as described below. Statistical analyses will be performed using IBM Statistical Package for Social Sciences (SPSS) 21 for Macintosh (SPSS Inc., Chicago, IL, USA).

Magnitude-based inferences about the data will be derived using methods described by Hopkins and colleagues (Hopkins et al., 2009). A previously

established 'smallest worthwhile change' in performance will be used as the threshold value for a substantial treatment effect (separate treatment conditions vs. placebo) (Hopkins, 2004). The smallest worthwhile change in performance will be defined as $0.3 \times$ the within subject variability across repeated time trials (Hopkins, 2004). The coefficients of variability for the performance parameters will be derived from the familiarization trials of the current investigation.

A published spreadsheet (Hopkins, 2007) will then be used to determine the likelihoods of the true treatment effect (of the population) reaching the substantial change threshold ($0.3 \times CV$); these will be classified as <1% almost certainly no chance, 1-5% = very unlikely, 5-25% = unlikely, 25-75% = possible, 75-95% = likely, 95-99% = very likely, and >99% = almost certain. If the percent chance of the effect reaching the substantial change threshold is <25% and the effect is clear, it will be classified as a 'trivial' effect. If 90% confidence intervals included values that exceeded the substantial change threshold for both a positive and negative effect, effects will be classified as unclear (>5% chance of reaching the substantial threshold for both a positive and negative effect). For ease of interpretation data will be displayed as raw means \pm SD.

Chapter Three

Manuscript

**Caffeine and 3km Cycling Performance;
Effects of Mouth Rinsing, Genotype, and Time-of-Day**

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Abstract

PURPOSE: The objectives were to determine the effects of caffeine ingestion and mouth rinsing on cycling performance, and to assess whether the CYP1A2 gene is related to the size of the performance effects. **METHODS:** Thirty-eight recreational cyclists completed four simulated 3-kilometer time trials (TT). Subjects ingested either 6mg/kgBW of caffeine or placebo one hour prior to each TT. Additionally, 25 ml of 1.14% caffeine, or placebo solution was mouth rinsed before each TT. Treatments were: placebo ingestion+placebo rinse (*Placebo*), placebo ingestion+caffeine rinse (*Rinse*), caffeine ingestion+caffeine rinse (*Ingestion+Rinse*), caffeine ingestion+placebo rinse (*Ingestion*). Subjects were genotyped and classified as AA homozygotes or C allele carriers for the rs762551 polymorphism in CYP1A2. Magnitude based inferences were used to evaluate treatment differences. **RESULTS:** Both *Ingestion+Rinse* ('likely') and *Ingestion* ('possibly') improved performance compared to *Placebo*. Performance differences between *Ingestion+Rinse* and *Ingestion* and between *Rinse* and *Placebo* were 'likely trivial.' C allele carriers experienced greater gains ('likely') with *Ingestion* compared to AA homozygotes. Additional analyses revealed that caffeine intake conferred larger benefits for subjects that performed trials prior to 10am. **CONCLUSIONS:** Caffeine ingestion, but not mouth rinse, improved cycling performance. C allele carriers experienced the largest improvement in performance with caffeine ingestion, especially prior to 10am.

Keywords: CYP1A2, genetics, circadian rhythm, mouthwash

Introduction

Caffeine intake can improve high intensity performance across a continuum of activities, including swimming (Collomp et al., 1992), rowing (Bruce et al., 2000), running (Wiles et al., 1992; Glaister et al., 2008), cycling (Wiles et al., 2006; Santos et al., 2013), and variable intensity activities (Schneiker et al., 2006; Klein et al., 2012). Though unsettled, caffeine is believed to deliver its performance enhancing effects through the central nervous system by blocking adenosine receptors (Ribeiro & Sebastião, 2010) and increasing neural excitability (Walton et al., 2003). This can enhance motor output (Black et al., 2014) and suppress ratings of perceived exertion and pain (Doherty et al., 2005; Motl et al., 2003).

Considerable individual variability associated with the performance benefits of caffeine has long been recognized (Doherty et al., 2002; Meyers & Cafarelli, 2005; Robertson et al., 1978). Genetics have been offered as a possible explanation and viable gene candidates have been identified. For instance, there are genetic variations of the primary enzyme responsible for caffeine metabolism – CYP1A2 (Yang et al., 2010). Homozygosity for the A allele at intron 1 of the CYP1A2 gene increases the inducibility of the enzyme (Sachse et al., 2009). This presumably influences the rate of caffeine metabolism and resultant concentrations of caffeine and caffeine metabolites in the blood. Based on this concept our laboratory recently investigated whether CYP1A2 genotype impacts the performance benefits of caffeine. We reported that individuals homozygous for the A allele experienced a larger ergogenic effect (Womack et al., 2012). This may partially explain the variable performance benefits of caffeine intake.

Interestingly, some investigators have recently observed that rinsing caffeine in the mouth, without ingestion, enhances repeated sprint (Beaven et al., 2013) and 30-minute (Bottoms et al., 2014) cycling performance. It is possible that unidentified receptors in the oral cavity provide afferent feedback in a manner that enhances physical performance, much like what has been shown with carbohydrate rinsing (Chambers et al., 2009). However, recent data refuting the benefits of a caffeine rinse for 60-min cycling performance (Doering et al., 2013) calls this into question and necessitates more work in this area. If mouth rinsing with caffeine ultimately proves beneficial, it could be a practical strategy to avoid the genetic disadvantages associated with alterations in caffeine metabolism, as the ergogenic effect of mouth rinsing would not be affected by hepatic clearance.

Therefore, this study was designed to assess the efficacy of caffeine mouth rinsing on 3-km cycling time trial performance and to determine if caffeine mouth rinsing affects the magnitude of performance gains influenced by the CYP1A2 polymorphism. Because subjects self-selected the time of day at which they performed their laboratory trials, we also had the opportunity to preliminarily examine the possibility that time of day alters the effects of caffeine on cycling performance

Materials and Methods

Subjects

Thirty-eight recreationally trained male and female subjects from James Madison University participated in this study (demographics reported in Table 2). Subjects were required to have performed, at minimum, “occasional” cycling (one day per week) in their typical weekly exercise routine. Subjects were informed of the experimental procedures and risks prior to giving written consent. The study was approved by the James Madison University Institutional Review Board.

Cardiorespiratory Fitness

Following height and body weight measurements, subjects performed an incremental exercise test to exhaustion on a bicycle ergometer (Velotron, Racermate, Inc., Seattle, WA, USA) to determine VO_{2max} . Subjects began with a 5-minute warm-up at a self-selected intensity. Subjects then were prompted to select an intensity that could be maintained for 60-minutes to begin the test. Workload was increased in 15 W increments every minute until volitional fatigue. Metabolic measurements were assessed via Moxus Modular Metabolic System (AEI Technologies, Pittsburgh, PA, USA) throughout the test and VO_{2max} was determined by the highest 30 s mean oxygen uptake value. A fan set on the medium setting was placed 2 meters in front of the ergometer to cool subjects.

Supplementation

A randomly counterbalanced, double blind, placebo controlled design was implemented to compare the effects of the four treatment conditions. During the experimental trials subjects were given two 25ml mouth rinse solutions at room temperature containing either: A) 300mg caffeine (NutraBio Labs, Inc., Middlesex, NJ), 1g saccharine (Sweet'N Low, Brooklyn, NY), and 25ml of water (rinse containing 1.14% caffeine) or B) a flavor-matched placebo containing 6g saccharine, and 25ml of water. Additionally, a 6mg/kg body weight capsule containing either anhydrous caffeine or all-purpose flour (placebo) was ingested 1 hour prior to each treatment trial. The four treatments consisted of a 2 x 2 factorial of capsule and mouth rinse treatments: 1. placebo capsule + placebo mouth rinse (*Placebo*) 2. placebo capsule + caffeine mouth rinse (*Rinse*) 3. caffeine capsule + caffeine mouth rinse (*Ingestion+Rinse*) 4. caffeine capsule + placebo mouth rinse (*Ingestion*).

Performance Trials

Each subject performed six exercise trials (two familiarization trials followed by four experimental trials) on the aforementioned cycle ergometer, with 3-7 days between each trial. Trials were performed at the same time of day to eliminate any possible within-subject circadian effects on performance. The subjects performed a 5-minute warm-up at a self-selected intensity. Two mouth rinses were provided during the warm-up; one immediately at the onset of the warm-up, and another 30 seconds before completion of the warm-up. Subjects swirled the mouth rinse for five seconds and then expectorated the rinse. Following the warm-up subjects

performed a 3km time trial. Familiarization trials were identical to the experimental trials, with the exception that subjects rinsed with water. Subjects were instructed to treat each trial as a competition. Subjects did not receive verbal feedback or encouragement from the investigators and no visual feedback from the time trial were provided, with the exception of elapsed distance.

Average power output during the first, second, and third kilometers were also analyzed to determine if any pacing differences were present between treatments.

Time of Day

Subjects were divided into two separate groups based on the time of day that they performed laboratory trials. 10:00am was selected as the threshold as it emerged as a natural breakpoint and created a group of 15 subjects that performed *Early* trials and 23 subjects that performed *Late* trials. To evaluate the effect of caffeine ingestion on change in performance the *Ingestion* and *Ingestion+Rinse* treatments were averaged for each subject and then compared to placebo.

Dietary and Exercise Control

Subjects recorded food intake for 24 hours prior to the initial experimental trial and were instructed to replicate food intake for the 24-hour period prior to each experimental trial. Subjects were instructed to abstain from any alcohol (24 hrs), caffeine (12 hrs), and food intake (2 hrs) prior to each experimental trial. All subjects recorded daily physical activity for 48 hours prior to experimental trials.

Subjects were instructed to maintain consistent exercise habits between trials and to abstain from any heavy and/or unaccustomed exercise 48 hours prior to each experimental trial.

DNA Sampling

Blood samples [~3ml sample – 5ml BD Vacutainer tube (Becton Dickinson & Company, Franklin Lakes, NJ, USA)] were obtained from an antecubital vein after the final time trial. Blood was then stored at -80 °C until DNA extraction. DNA was extracted via Illustria Blood GenomicPrep mini spin kit (GE Healthcare, Buckinghamshire, UK). Genotyping was then performed using restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR). Following genotyping, subjects were grouped as AA homozygous (i.e. 'fast' metabolizers) and C allele carriers (i.e. 'slow' metabolizers) of the rs762551 single nucleotide polymorphism in the CYP1A2 gene.

Statistical Analysis

Univariate Analysis of Variance (ANOVA) was applied to determine treatment differences. Simple contrasts between treatment conditions were used to generate *P* values for analyses described below. Independent t-tests were used for between group comparisons of genotype, and time of day. All data were log transformed to diminish the effects of nonuniformity. Statistical analyses were performed using IBM Statistical Package for Social Sciences (SPSS) 21 for Macintosh (SPSS Inc., Chicago, IL, USA).

Magnitude-based inferences about the data were derived using methods described by Hopkins and colleagues (Hopkins et al., 2009). A previously established 'smallest worthwhile change' in performance was used as the threshold value for a substantial treatment effect (separate treatment conditions vs. placebo) (Hopkins, 2004). The smallest worthwhile change in performance was defined as 0.3 x the within subject variability (CV=2.3%; calculated from familiarization trials) across repeated time trials, which translated to a 2.1 s difference in performance (Hopkins, 2004).

A published spreadsheet (Hopkins, 2007) was then used to determine the likelihood of the true treatment effect (of the population) reaching the substantial change threshold (0.3 x CV); these were classified as <1% almost certainly no chance, 1-5% = very unlikely, 5-25% = unlikely, 25-75% = possible, 75-95% = likely, 95-99% = very likely, and >99% = almost certain. If the percent chance of the effect reaching the substantial change threshold was <25% and the effect was clear, it was classified as a 'trivial' effect. If 90% confidence intervals included values that exceeded the substantial change threshold for both a positive and negative effect, effects were classified as unclear (>5% chance of reaching the substantial threshold for both a positive and negative effect). For ease of interpretation data is displayed as raw means \pm SD.

Results

Performance

Ingestion+Rinse 'likely' improved performance by $1.4\% \pm 3.3\%$ when compared with *Placebo*, whereas *Ingestion* 'possibly' enhanced performance by $1.1\% \pm 4.1\%$. *Rinse* had a 'likely trivial' effect on time trial performance.

Performance differences between *Ingestion+Rinse* and *Ingestion* and between *Rinse* and *Placebo* were 'likely trivial'. Mean performance times are displayed in Table 1 and mean difference of treatment effects (treatment times – placebo times) with 90 percent confidence intervals are displayed in Figure 1.

Genetics

Twenty-one (55%) subjects were homozygous for the A allele and seventeen (45%) were C allele carriers. Descriptive characteristics of the two genotype groups are shown in Table 2. Among C allele carriers, performance was 'likely' improved by $2.0\% \pm 4.0\%$ and by $1.8\% \pm 3.4\%$ with both *Ingestion* and *Ingestion+Rinse* respectively, whereas AA homozygotes 'possibly' benefited by $1.0\% \pm 3.2\%$ with *Ingestion+Rinse*. All other treatment effects were 'unclear'. Note that there 'likely' was a difference in performance improvement with *Ingestion* between the AA and AC genotypes. Mean treatment effects are displayed in Figure 2.

Time of Day

Fifteen (39%) subjects performed trials prior to 10am (*Early*), of whom seven were C allele carriers and eight were AA homozygotes. Twenty-three (61%)

subjects performed trials later than 10am (*Late*), of whom ten were C allele carriers and thirteen were AA homozygotes. Performance was 'almost certainly' improved with caffeine ingestion in *Early* subjects by $2.0 \pm 2.5\%$, and 'possibly' improved in *Late* subjects by $0.7 \pm 4.3\%$. Among C allele carriers, caffeine ingestion 'almost certainly' and 'possibly' improved performance by $3.5 \pm 2.1\%$ and $0.6 \pm 2.1\%$ in *Early* and *Late* subjects respectively. Mean treatment effects are displayed in Figure 3.

Discussion

The primary goal of this project was to determine if caffeine mouth rinsing and ingestion, alone and in combination, benefit 3km cycling performance, and if effects occurred regardless of CYP1A2 genotype. Although caffeine ingestion improved performance compared to placebo, mouth rinsing had only a trivial effect, regardless of genotype. Also, in contrast to a recent investigation from our laboratory (Womack et al., 2014), C allele carriers experienced greater performance gains with caffeine ingestion than AA homozygotes. More data need to be gathered to provide a sound explanation for this observation. Finally, the *post-hoc* time of day analyses revealed that caffeine imparted marked benefits when trials were performed *Early* compared to *Late*. To our knowledge, this is the first evidence that time of day may impact the magnitude of the effect that caffeine has on cycling performance.

The current data conflict with previous reports that caffeine mouth rinsing can augment 30-minute cycling performance (Bottoms et al., 2014) and power output during repeated sprints (Beaven et al., 2013). However, in line with the current results, Doering's work indicates that caffeine rinsing does not affect ~60-min cycling performance (Doering et al., 2013). We were not able to identify experimental commonalities or differences that would explain why these protocols yielded different results. So, when our data are combined with Doering, and considered with the fact that there are no known oropharangeal receptors for caffeine, we question the utility of caffeine rinsing for performance. However, there

is enough support (Bottoms et al., 2014; Beaven et al. 2013) to warrant follow-up work before unequivocal conclusions are made.

Despite the failure of a caffeine mouth rinse to improve TT performance, caffeine ingestion did enhance performance, as has been demonstrated previously during cycling performances of similar duration (Wiles et al., 2006; Santos et al., 2013). The observation that caffeine intake improved performance supports the efficacy of the current study design and strengthens the interpretation of the mouth rinse findings. In light of the overall mouth rinse results, caffeine mouth rinsing did not benefit the genetic 'non-responders', nor did it further benefit performance when provided along with caffeine ingestion.

We recently reported that AA homozygotes exhibited larger performance benefits with caffeine ingestion, compared to their C allele counterparts (Womack et al. 2014). Thus we expected that the AA homozygotes again would experience a more marked performance enhancement. Surprisingly, the C allele carriers experienced greater gains in performance with caffeine ingestion than did the AA homozygotes. Because of the size and individual consistency of the effect across projects, we do not believe that either finding is coincidental. Yet, there is no clear explanation for the opposing results. One difference between studies is training status. The previous sample population consisted of competitive cyclists, whereas subjects in the current study were recreational cyclists who reported cycling only ~2 days per week. CYP1A2 expression is induced following acute exercise (Kochanska-Dziurowicz et al., 2014) and exercise training (Vistisen et al., 1992). Additionally, some have shown that adenosine receptor (the target of caffeine and

caffeine metabolites) density in skeletal and cardiac muscle is greater in endurance-trained than untrained men (Mizuno et al. 2005). Therefore, it may be that training history and/or current fitness level influences the substrate-receptor interaction between caffeine/metabolites and adenosine receptors, thereby altering the genotype x caffeine effect. Prospective studies should consider evaluating differences in caffeine metabolites across genotype and training status.

Though not *a priori*, the time of day analysis revealed that the benefits of caffeine may be exaggerated *Early* (<10:00am) in the day. Caffeine ingestion improved performance to a greater extent in the *Early* trials compared to the *Late* trials. This is in line with recent findings that caffeine ingestion has a larger effect on neuromuscular performance in the morning than in the afternoon. It seems that the improvement in performance seen in the morning simply elevates performance to what is seen in the afternoon during placebo conditions, with only minimal improvement due to caffeine ingestion in the afternoon (Mora-Rodriguez et al., 2012; Mora-Rodriguez et al., 2014). Additionally, we found that the C allele carriers seemed to be driving the effect in the *Early* trials. All seven C allele carriers who performed *Early* trials performed better with caffeine ingestion, whereas this was true in only five of the eight *Early* AA carriers. Interestingly, CYP1A2 activity is elevated in the morning compared to the evening (Perera et al., 2013), which may contribute to the ergogenic effect of caffeine; consequently adding yet another variable to consider when interpreting the physiological effects of caffeine intake.

It is important to note that all but one of the *Early* subjects were fasted (over night) whereas twenty of the twenty-three *Late* subjects had eaten during the days

of their performance trials, raising the possibility that feeding status factored into the time of day results. However, improvements in performance due to caffeine ingestion have been demonstrated following 6-12 hour fasting (MacIntosh & Wright, 1995; Bruce et al., 2000), and when feedings have taken place approximately 2 hours before testing (Collomp et al., 1992; Schneiker et al., 2006). Moreover, the previously mentioned time-of-day study by Mora-Rodriguez et al. gave subjects a 624kcal bolus one hour prior to all testing, suggesting that fasting does not play a role in the ergogenic effect of caffeine (2014). To our knowledge no studies have directly examined the effect of feeding status on performance following caffeine ingestion. Future investigations should determine whether the time of day and/or fasting influence the performance response to caffeine ingestion.

The present study demonstrates that caffeine mouth rinsing does not affect 3km cycling performance in recreational cyclists. As expected there was an improvement in 3km performance with caffeine ingestion, but there was no further improvement with caffeine ingestion and mouth rinse in combination. Surprisingly, there was a greater performance enhancement in C allele carriers compared to AA homozygotes following caffeine ingestion, thus necessitating follow-up work to explain the dissimilar results compared to our previous work. Finally a greater performance enhancement with caffeine ingestion was observed in *Early* trials compared to *Late* trials.

Perspectives

The present study provides additional evidence of genetic (genotype) and circadian (time of day) factors that affect the ergogenic value of caffeine intake and may allow for prescription of more personalized caffeine intake strategies to maximize performance. For example, perhaps genetics contribute to caffeine metabolism in a manner that would affect the optimal timing and dosage for a particular subject. Further, the time at which caffeine is consumed may strongly influence the magnitude of the desired effect. Future research is critical for the development of personalized caffeine intake guidelines for athletes.

Acknowledgements

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Figure Legends

Figure 1

Effects of Caffeine Mouth Rinsing and Ingestion on 3km Time Trial Performance:

Circles indicate mean treatment differences from placebo and bars depict a \pm 90% confidence interval of *Rinse*, *Ingestion*, and *Ingestion+Rinse* vs. *Placebo*. Dashed lines signify threshold value for a meaningful effect ($0.3 \times CV$). * Signifies a 'likely' improvement in performance. † Signifies a 'possible' improvement in performance. *Placebo* = placebo ingestion+placebo rinse, *Rinse* = placebo ingestion+caffeine rinse, *Ingestion+Rinse* = caffeine ingestion+caffeine rinse, *Ingestion* = caffeine ingestion+placebo rinse.

Figure 2

Effect of Genotype on the Treatment Effect of Caffeine Mouth Rinse and Ingestion

During 3km Time Trial Performance: Bars indicate mean treatment differences from placebo and bars depict a \pm 90% confidence interval of *Rinse*, *Ingestion*, and *Ingestion+Rinse* vs. *Placebo*. * Signifies a 'likely' improvement in performance. † Signifies a 'possible' improvement in performance. ‡ Signifies a 'likely' difference in performance improvement between the AA and AC Genotypes. *Placebo* = placebo ingestion+placebo rinse, *Rinse* = placebo ingestion+caffeine rinse, *Ingestion+Rinse* = caffeine ingestion+caffeine rinse, *Ingestion* = caffeine ingestion+placebo rinse.

Figure 3

Influence of Time of Day on the Treatment Effect of Caffeine Ingestion During 3km Time Trial Performance: Bars depict the treatment effects (\pm 90% CI) of Overall, C allele carrier, and AA homozygote performance with caffeine ingestion. * Signifies an 'almost certain' improvement in performance. † Signifies a 'possible' improvement in performance.

Table 1. 3-km Time Trial Performance

	Placebo	Rinse	Ingestion	Ing+Rinse
Mean Time \pm SD (seconds)	313.7 \pm 30.9	314.1 \pm 29.1	310.2 \pm 30.9†	309.0 \pm 27.4*

† Signifies a 'possible' improvement in performance compared to Placebo. * Signifies a 'likely' improvement in performance compared to Placebo. Placebo = placebo ingestion+placebo rinse, Rinse = placebo ingestion+caffeine rinse, Ingestion+Rinse = caffeine ingestion+caffeine rinse, Ingestion = caffeine ingestion+placebo rinse.

Table 2. Descriptive data of subjects for AA homozygotes and C allele carriers

	All Subjects (n = 38)	AA (n = 21)	C (n = 17)
Height (cm)	171.0 ± 13.7	167.2 ± 16.9	175.7 ± 6.2
Weight (kg)	70.9 ± 11.9	68.0 ± 13.8	74.5 ± 8.0
Age (years)	21.6 ± 1.5	20.8 ± 1.4	21.6 ± 1.5
VO ₂ max (ml/kg/min)	51.4 ± 6.8	51.1 ± 7.0	51.9 ± 6.2

Figure 1. Time Trial Performance

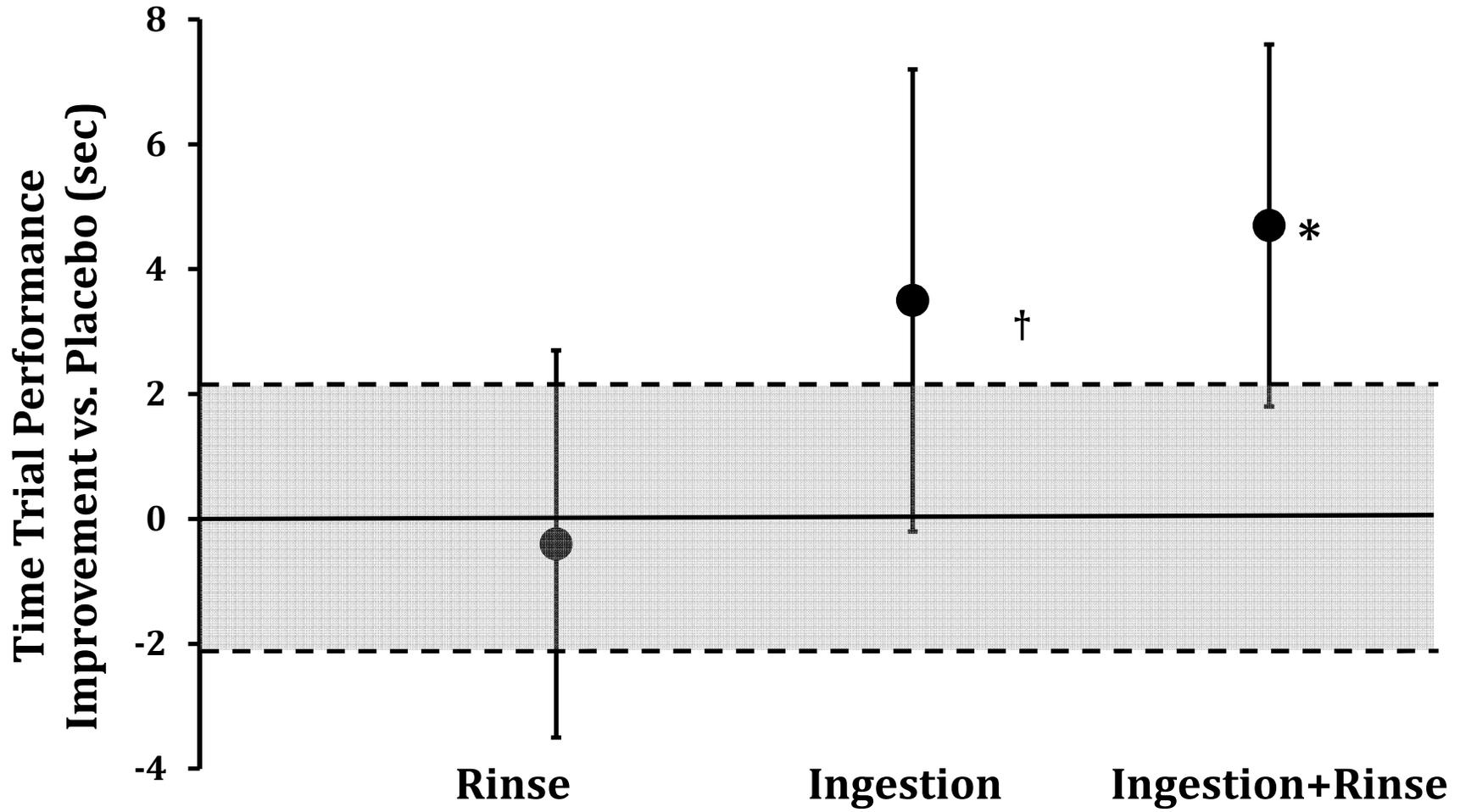


Figure 2. Genotype and Time Trial Performance

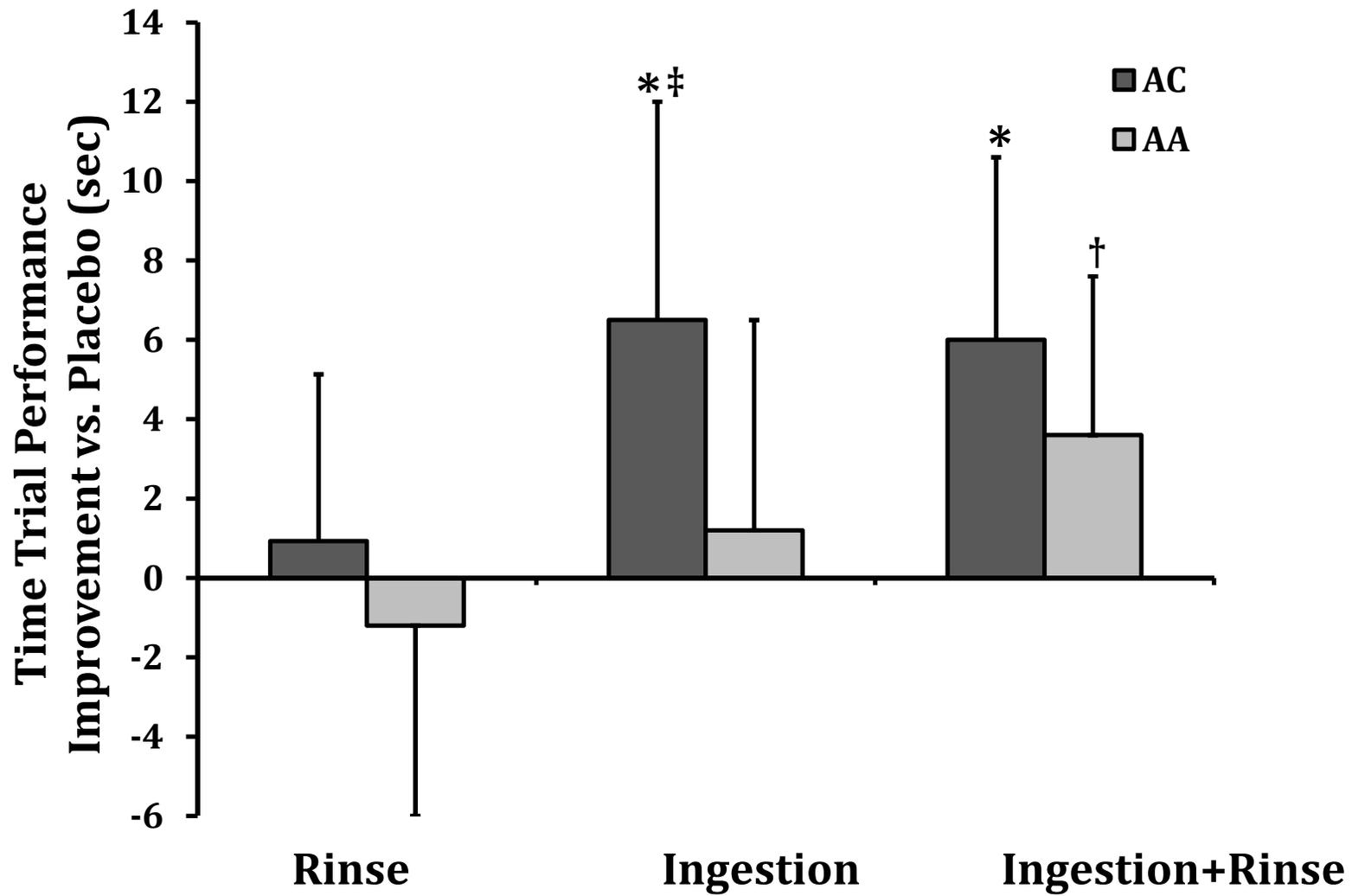
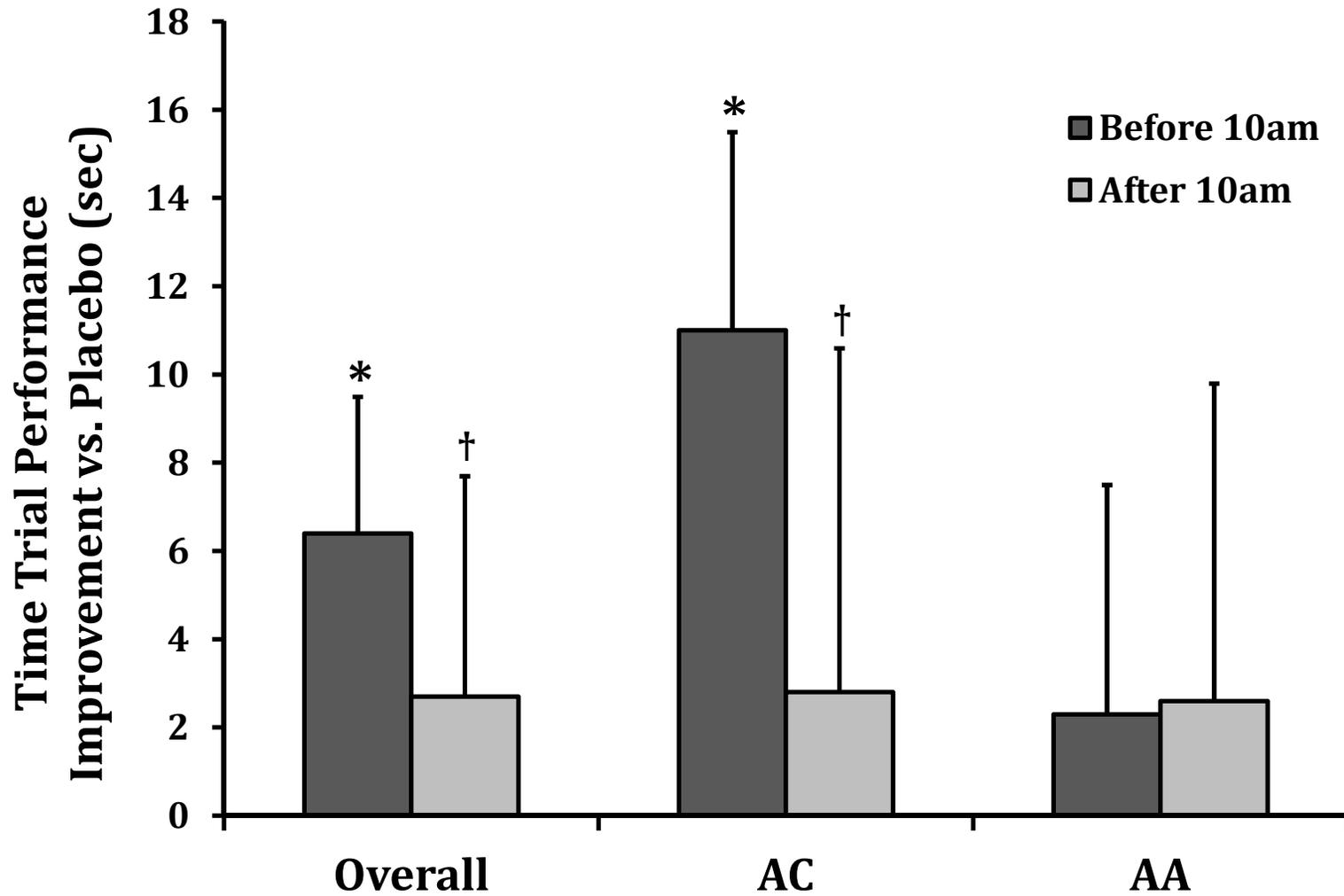


Figure 3. Time-of-Day and Time Trial Performance



James Madison University
Department of Kinesiology
Consent for Investigative Procedure

I, _____, hereby agree on _____ (date) to participate in the research project conducted by Christopher J. Womack, Ph.D., Nicholas D. Luden, Ph.D., Mike Saunders, Ph.D., Mark Pataky, and Jenna Goffe from James Madison University titled *The Influence of a CYP1A2 Polymorphism on the Ergogenic Effects of a Caffeine Mouth Rinse*.

The purpose of this study is to determine whether or not mouth rinsing with caffeine enhances high intensity cycling performance in individuals that do not benefit from caffeine intake due to their genetic predisposition.

Subject Responsibility

I understand that I will undergo the following testing in the study:

This study consists of seven separate exercise tests performed on a stationary cycle (cardiovascular fitness test, two familiarization tests, and 4 3-km time trial tests). All testing will occur in Godwin Hall, room 209, on the campus of James Madison University. You will also be asked about lifestyle behavior such as smoking and physical activity and complete dietary and physical activity records. The total time commitment is estimated to be less than four hours.

Cardiovascular Fitness Test:

If you meet the criteria for the study, the researchers will measure your height and body weight and you will then be asked to perform a cardiovascular fitness test on a bicycle ergometer to determine your peak oxygen consumption (VO_{2max}). You will be asked to ride stationary bicycle ergometer at an initial workload that is 'fairly easy'. The workload will be increased every two minutes during the test. You will be encouraged to continue to cycle until you request to stop due to fatigue or are unable to continue at a cadence of >50 revolutions per minute. To assess oxygen consumption throughout the test, you will need to breathe through a mouthpiece/breathing apparatus that collects your expired breath throughout the test (10-15 minutes).

Familiarization and Time Trial Sessions:

During the familiarization and time trials, you will be asked to complete a 5-min warm up at a self selected intensity followed by 3-km time trial (TT) on a cycle ergometer that will last 3-5 minutes. Each time trial is to be approached with a competition mentality. Between test preparation and completion of the exercise test, each of these tests should take approximately 15-20 minutes.

Supplementation Protocol:

No supplementation will be provided during the familiarization trials. However, you will be asked to practice with the mouth rinse prior to these trials. For the mouth rinse, Dixie cups filled with 25 ml of solution with or without caffeine will be provided 5 minutes, and 30 seconds prior to the TT. You will be instructed to swish the solution in your mouth for 10 seconds, upon which you will spit it into a bucket. The remaining four visits will be experimental trials whereby separate treatments will be provided. The treatments will be: 1. Caffeine mouth rinse; mouth rinse solution administered immediately prior to- and during exercise where you will be instructed to only rinse the caffeine solution in your mouth and then spit it out (without swallowing). A placebo pill will also be administered 1 hour prior to the trial. 2. Caffeine pill; you will ingest the pill whole 1 hour prior to the TT. A placebo mouth rinse solution will also be administered. 3. Caffeine pill + caffeine mouth rinse; a caffeine pill and caffeine mouth rinse will be administered. 4. Placebo; a placebo pill and placebo mouth rinse will be administered.

Dietary, Exercise, and Time of Day Controls:

You will record food intake 24 hours prior to your first experimental trial. You will then be provided with a copy of your initial dietary log, which you will be asked to use to replicate your food intake for 24 hrs preceding each subsequent experimental trial. Additionally, you will abstain from any alcohol and caffeine for 24 hrs and 12 hrs prior to the experimental trials, respectively. Finally, you will avoid food intake for 2 hours preceding each experimental trial.

You will avoid heavy exercise for 48 hrs prior to each experimental trial, record all physical activity performed during this time frame, and maintain consistent exercise habits between trials

All experimental trials will be separated by 3-7 days and performed at the same time of day, with all trials starting within a 2-hour range.

DNA Sampling:

We will extract a sample of your DNA from your blood sample. The DNA will be stored in our laboratory freezer for up to 3 years, but the sample will be coded so that nobody except the primary investigators can detect which sample is yours. The DNA testing will involve determining sequences of DNA for specific genes that are related to caffeine metabolism. We will not use this DNA for any other purpose. The results of this genetic testing will only be available to the primary investigator and you. These results will not be made public and will be stored in a locked file cabinet.

Potential Risks

According to the American College of Sports Medicine's Guidelines for Exercise Testing and Prescription, the risk associated with vigorous exercise in individuals categorized as "low risk" is very minimal, and physician supervision is not necessary. The exercise testing conditions are likely safer than the typical exercise environments of the participants. Subjects that do not meet ACSM criteria for "low risk" will not be allowed to participate in the study. A physician will be available by pager if the need for medical attention arises throughout the study period. In the unlikely event of cardiac or other complications during exercise, an emergency plan is in place. This includes immediate access to a phone to call emergency personnel. In addition, at least one of the listed investigators will be present during all laboratory testing sessions, and all are CPR certified.

The risks of blood sampling using venipuncture include possible mild bruising, and the risk of transfer of blood-borne pathogens, as well as possible risks of infection or skin irritation. These risks are considered to be minimal, and all safety precautions for handling blood samples will be followed according to Occupational Safety and Health Administration (OSHA) protocols, including: investigators will wear latex gloves at all times during blood sampling and testing. A sharps container lined with a biohazard bag will be used for all sharp objects involved in the blood sampling; all other materials (i.e. gloves, gauze pads, etc.) used during the sampling will be put in a separate waste disposal unit lined with a biohazard bag. All trash bags will be taken to Rockingham Memorial Hospital for disposal. All investigators who will be involved in blood draws (and handling of blood) have been trained in these phlebotomy techniques, and completed JMU blood-borne pathogen training.

If you have a wheat allergy you should not participate in this study.

Potential Benefits

There are no known direct benefits. However, volunteering for this project will help determine the impact of a caffeine mouth rinse on cycling performance. *Performance Incentive* – This monetary benefit will only apply to two males and two females. The top 5 female performers (fastest finishing time under the placebo conditions) will be entered into a drawing to win a single \$150 WellsOne Prepaid Visa Card whereas performers 6-10 will be entered into a drawing to win a single \$75 Card. An identical approach will be applied to male subjects (1 \$150 Card and 1 \$75 Card).

Confidentiality

The results of this research will be presented at conferences and published in exercise science journals. The results of this project will be coded in such a way that your identity will not be attached to the final form of this study. The researcher retains the right to use and publish non-identifiable data. However, you can ask that your data be removed from the study at any point prior to presentation and

publication. While individual responses are confidential, aggregate data will be presented representing averages or generalizations about the responses as a whole. All data will be stored in a secure location accessible only to the researcher. Final aggregate results will be made available to you upon request.

Participation & Withdrawal

Your participation is entirely voluntary. You are free to choose not to participate. Should you choose to participate, you can withdraw at any time without consequences of any kind. Your right to withdraw includes the right to request that your DNA and blood samples be discarded at any time. You should be aware that the DNA sample is subject to court subpoena.

Questions

You may have questions or concerns during the time of your participation in this study, or after its completion. If you have any questions about the study, contact Nicholas D. Luden, Ph.D. at ludennd@jmu.edu or by phone at 540-568-4068.

Giving of Consent

I have read this consent form and I understand what is being requested of me as a participant in this study. I freely consent to participate. I have been given satisfactory answers to my questions. The investigator provided me with a copy of this form. I certify that I am at least 18 years of age.

Name of Participant (Printed)

Name of Researcher(s) (Printed)

Name of Participant (Signed)

Name of Researcher(s) (Signed)

Date

Date

For questions about your rights as a research subject, you may contact the chair of JMU's Institutional Review Board (IRB). Dr. David Cockley, (540) 568-2834, cocklede@jmu.edu.

James Madison University
Department of Kinesiology
Health Status Questionnaire

Instructions: Complete each question accurately. All information provided is **confidential**.

Part I: General Information

1. Sex: Male Female
2. Participant Number:
3. Date of Birth (Month/ Day/ Year)

Part II: Medical History

4. Circle any that died of heart attack before age 65: Father Mother Brother
Sister Grandparent

5. Date of last medical exam: _____ Last physical fitness test:

6. Circle operations you have had: Back Heart Kidney Eyes Joint Neck
Ears Hernia

Lung Other _____

7. Please circle any of the following for which you have been diagnosed or treated by a physician or health professional:

Alcoholism	Diabetes	Kidney Problems
Anemia (sickle cell)	Emphysema	Mental Illness
Anemia (other)	Epilepsy	Muscular Injury
Asthma	Eye Problems	Neck Strain
Back Strain	Gout	Obesity
Bleeding trait	Hearing Loss	Orthopedic Injuries
Bronchitis, chronic	Heart Problem	Phlebitis
Cancer	High Blood Pressure	Rheumatoid arthritis
Cirrhosis, liver	Hypoglycemia	Stroke
Concussion	Hyperglycemia	Thyroid problem
Congenital defect	Infectious Mononucleosis	Ulcer
Other _____		

8. Circle all medications taken in the last six months:

Blood thinner	Epilepsy medication	Nitroglycerin
Diabetic pill	Heart-rhythm medication	Other
<hr/>		
Digitalis	High-blood pressure medication	
Diuretic	Insulin	

9. Any of these health symptoms that occur frequently is the basis for medical attention. Circle the number indicating how often you have each of the following:

5 = Very often 4 = Fairly often 3 = Sometimes 2 = Infrequently 1 = Practically never

a. cough up blood
1 2 3 4 5

f. chest pain
1 2 3 4 5

b. abdominal pain
1 2 3 4 5

g. swollen joints
1 2 3 4 5

c. low back pain
1 2 3 4 5

h. feel faint
1 2 3 4 5

d. leg pain
1 2 3 4 5

i. dizziness
1 2 3 4 5

e. arm or shoulder pain
1 2 3 4 5

j. breathless on slight exertion
1 2 3 4 5

Part III: Health Related Behavior

10. Do you smoke? Yes No

11. If you are a smoker, indicate the number of smoked per day:

Cigarettes:

40 or more 20-39 10-19 1-9

Cigars or pipes only:

5 or more or any inhaled less than 5, none inhaled

12. Do you exercise regularly? Yes No

13. How many times in a week do you spend at least 30 minutes in moderate to strenuous/vigorous exercise?

1 2 3 4 5 6 7 days per week

Average Exercise Habits over the Past 2 Months:

Avg. # days of exercise per week _____

Avg. # of days of aerobic exercise per week _____

Avg. # of days of cycling per week _____

14. Can you walk 4 miles briskly without fatigue? Yes No

15. Can you jog 3 miles continuously at a moderate pace without discomfort?
Yes No

16. Weight now: _____ lb. One year ago: _____ lb Age 21:
_____ lb

Caffeine Habits Questionnaire

Please list your approximate WEEKLY intake of the following

Cups of coffee:

Cups of tea:

Cans (12oz) of caffeinated soda:

Servings of chocolate:

Doses of caffeinated pills (No-Doz, Vivarin, etc.):

Other caffeinated beverages not listed (please list specific drink and weekly intake):

INSTRUCTIONS FOR KEEPING YOUR 24-HOUR FOOD RECORD

Keep your record for three days per trial. You will include the day before, the day of, and the day after each trial. Include all meals, snacks, nibbling, and beverages including water and cocktails

1. Fill out the date and day of the week at the top of food record sheet
2. Record the time you consumed your food and/or drink. To be most accurate, fill out the food record as soon as you finish eating.
3. List the first food and/or drink you consumed when you began your day and continue to record until you consume your last food and/or drink of your day (usually before bedtime)
4. List each food and/or drink on a separate line
*Example: cereal with milk, cereal and milk should each be on separate lines
spaghetti, noodles and sauce should each be on separate lines*

Combination foods:

List parts of food on separate lines

Include preparation method, quantity, and brand name of each food

Example: Sandwich (4 oz healthy choice turkey, 2 slices Sara Lee wheat bread, 1 tbsp Hellman's light mayo, 2 oz Kraft American cheese, 1 slice of red fresh tomato)

5. Record the method of preparation
*Example: fried, baked, grilled
salt, oil (olive, canola, corn, other) butter or margarine, spices, etc.*
6. Record quantity consumed
Do not record any food not eaten
Example: two cups of vegetables but ate half, so record one cup

Quantity of food and/or drink
*Example: cups, ounces, liters, grams, each, or other unit of measure
Example: 1 cup of vegetables, 4 ounces of meat, one medium apple*
7. Record brand name
*Example: fast food chain name and/or package name
Example: Wendy's, Betty Crocker, Lean Cuisine, Gatorade*
8. Place any helpful food labels in manila envelope that is attached to folder

USE THE FOLLOWING TO HELP DETERMINE PORTION SIZES AND TYPES OF FOODS

PLEASE SPECIFY	
Beverages	Sugar or creamer? Regular or sugar-free? Alcohol content? Name of drink and ingredients (if mixed drink)
Breads	Butter or margarine added?
Cereal/Milk	Milk, sugar, or fruit added? The type of milk? (skim, 1%, 2%, whole) Cereal: dry or cooked measure?
Dairy	Is yogurt fruited or plain? % fat of milk or yogurt? Indicate brand name of cheese substitute and/or nondairy creamer.
Desserts	Whipped topping added? Frosting? Fat modified (i.e., reduced)? Sugar-free?
Eggs	Preparation method (scrambled, hard-boiled, etc)? Fat used in cooking?
Fast Food	What restaurant? If not a national fast food chain, describe food in detail Size order of fries? Super-size? Extra toppings on sandwich?
Fats/Oils	Regular or salt-free? Stick, tub, or liquid margarine? Reduced calorie or diet product?

Fish	<p>Water or oil packed (fresh or canned)?</p> <p>Baked or fried (With batter or without)?</p> <p>Type of fat added?</p> <p>Raw or cooked weight?</p>
Fruit	<p>Sweetened or unsweetened?</p> <p>Fresh, canned, or frozen?</p> <p>With or without skin?</p>
Meats	<p>Visible fat removed?</p> <p>Light or dark meat? Raw or cooked?</p>
Sugars and Sweets	<p>Regular or reduced-calorie?</p> <p>Don't forget hard candy as well as chocolate.</p>
Vegetables	<p>Raw or cooked?</p> <p>Fresh, frozen, or canned?</p> <p>Low-sodium or regular?</p> <p>Added fat or sauce?</p>

Helpful Hints with Portion Sizes

- 1 teaspoon (5 ml)
 - about the size of the top half / tip of your thumb
- 1oz (28g)
 - approximately inch cube of cheese
 - volume of four stacked dice
 - slice of cheese is about the size of a 3 1/2 inch computer disk
 - chunk of cheese is about as thick as 2 dominoes
 - 1 handful (palm) of nuts
- 2 ounces (57 g)
 - 1 small chicken leg or thigh
 - 1/2 cup of cottage cheese or tuna
- 3 ounces (85 g)
 - serving of meat is about the size of a deck of playing cards (3 exchanges)
 - the size of the palm of your hand
 - 1/2 of whole chicken breast
 - 1 medium pork chop
 - 1 small hamburger
 - unbreaded fish fillet
- 1/2 cup (118 ml)
 - fruit or vegetables can fit in the palm of your hand
 - about the volume of a tennis ball
- 1 cup (236 ml)
 - about the size of a woman's fist
 - breakfast cereal goes halfway up the side of a standard cereal bowl
 - broccoli is about the size of a light bulb
- 1 medium apple = A tennis ball

Treatment Recall

Subject Number: _____

Date: _____

	Trial 1		Trial 2		Trial 3		Trial 4	
Capsule:	PLA	CAF	PLA	CAF	PLA	CAF	PLA	CAF
Confidence:	_____		_____		_____		_____	

	Trial 1		Trial 2		Trial 3		Trial 4	
Rinse:	PLA	CAF	PLA	CAF	PLA	CAF	PLA	CAF
Confidence:	_____		_____		_____		_____	

**Confidence rated on a scale from 1-10. 10 being 100% confident and 1 being not confident at all.

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