Caffeine, exercise physiology, and time-trial performance: no effect of *ADORA2A* or *CYP1A2* genotypes

MARK GLAISTER, KIRAN CHOPRA, ANA LUÍS PEREIRA DE SENA, CASSIE STERNBACH, LIRIDON MORINA, YIANNIS MAVROMMATIS

Faculty of Sport, Health, and Applied Sciences, St Mary's University, Strawberry Hill, Twickenham, UK.

Corresponding Author:

Dr Mark Glaister, Faculty of Sport, Health, and Applied Sciences, St. Mary's University, Waldegrave Road, Strawberry Hill, Twickenham, UK, TW1 4SX.

Tel: (+44)208 240 4012

Fax: (+44)208 240 4212

E-mail addresses

Mark Glaister: mark.glaister@stmarys.ac.uk

Kiran Chopra: kiranchopra95@gmail.com

Ana L Pereira De Sena: senagame@gmail.com

Cassie Sternbach: cassiesternbach@yahoo.com

Liridon Morina: 161528@live.stmarys.ac.uk

Yiannis Mavrommatis: yiannis.mavrommatis@stmarys.ac.uk

ABSTRACT

The aim of this study was to investigate the influence of *ADORA2A* and *CYP1A2* genotypes on the physiological and ergogenic effects of caffeine. Sixty-six male cyclists were screened for *ADORA2A* and *CYP1A2* genotypes; with 40 taking part subsequently in a randomised, double-blind, placebo-controlled study. Trial 1 was used to establish the $\dot{V}O_2$-power output relationship and $\dot{V}O_{2max}$. In trials 2 and 3, participants ingested 5 $mg \cdot kg^{-1}$ of caffeine or placebo one hour before completing a submaximal incremental cycling test, followed by a time-trial (~ 30 mins). Relative to placebo, caffeine led to a significant reduction in time to complete the time-trial (caffeine: 29.7 ± 1.8 mins; placebo: 30.8 ± 2.3 mins); but there was no effect of genotype. During submaximal exercise, caffeine reduced mean heart rate by 2.9 ± 3.7 $b \cdot min^{-1}$, with effects dissipating as exercise intensity increased. Caffeine also significantly reduced perceived exertion by 0.5 ± 0.8, and increased blood lactate by 0.29 ± 0.42 $mmol \cdot L^{-1}$, respiratory exchange ratio by 0.013 ± 0.032, and minute ventilation by 3.1 ± 6.8 $L \cdot min^{-1}$. Nonetheless, there were no supplement × genotype interactions. In conclusion, caffeine influences physiological responses to submaximal exercise and improves time-trial performance irrespective of *ADORA2A* or *CYP1A2* genotypes.

Novelty

- Caffeine affects physiological responses at rest and during submaximal exercise independent of *ADORA2A* or *CYP1A2* genotypes.
- Variability in the effect of caffeine on time-trial performance is not explained by *ADORA2A* or *CYP1A2* genotypes.

Key words: Genetics; coffee; ergogenic; endurance exercise, cycling; cardiorespiratory.

Introduction

Caffeine (1,3,7-trimethylxanthine) is a psychoactive stimulant consumed by most adults (usually in the form of coffee, tea, cola, or energy drinks) as a means of elevating mood, enhancing concentration, increasing wakefulness, improving alertness, and reducing fatigue (Fredholm et al. 1999; Burke 2008). There is evidence also that around 74% of athletes consume caffeine prior to exercise as a means of enhancing performance, with the greatest prevalence in athletes engaged in events requiring sustained bouts of moderate to high-intensity aerobic exercise (Del Coso et al. 2011). Indeed, a dose of 3 – 6 $mg \cdot kg^{-1}$, consumed 30 – 90 minutes prior to closed-loop time-trials lasting ≥ 5 minutes, has been shown to improve performance by approximately 4.5% (Glaister and Moir, 2019).

The mechanism by which caffeine enhances aerobic exercise performance appears to be due, most likely, to the ability of caffeine to act as an adenosine receptor antagonist (Kalmar 2005). Adenosine is a ubiquitous extracellular signalling molecule, the concentration of which increases during exercise due to the breakdown of adenosine triphosphate in energy metabolism (Layland et al. 2014). Adenosine exerts its effects on human physiology by interacting with four G protein-coupled receptors labelled A_1, A_{2A}, A_{2B}, and A_3. For a full review of the physiological effects of each of the receptor subtypes, see Fredholm et al. (2011). However, at present, differential effects of the various adenosine receptor subtypes, coupled with individual variability in receptor density and sensitivity, means that the precise mechanism(s) by which caffeine influences exercise performance remains elusive.

Despite the significant effect of caffeine on aerobic exercise performance, there are reports of considerable individual variability in the magnitude of the response, with some individuals experiencing an ergolytic effect (Guest et al. 2018; Pickering and Kiely 2018; Southward et al. 2018). Potential reasons to explain between-subject variability in the response to caffeine include habituation, method of caffeine ingestion, dosage, training status, and timing of caffeine ingestion (Southward et al. 2018).

Evidence in support of each of these confounding factors is conflicting and, as such, care should be taken by researchers to avoid confirmatory bias. Indeed, the absence of a clear effect of any of the above has led researchers to consider whether between-subject variability in the performance response to caffeine is more likely to be explained by a genetic component, with the most probable candidates being the *ADORA2A* and *CYP1A2* genes (Fulton et al. 2018; Southward et al. 2018).

The *ADORA2A* gene encodes for the A_{2A} adenosine receptors with approximately 45% of individuals having the CT genotype, while the CC and TT genotypes have a fairly equal distribution (20 – 30%) (Southward et al. 2018). In a recent systematic review, Fulton et al. (2018) reported that individuals with the TT genotype were associated with lower habitual caffeine intakes and a greater anxiogenic response to caffeine. In terms of aerobic exercise performance, in the only studies to date, Carswell et al. (2020) reported no influence of *ADORA2A* genotype on the ergogenic effect of caffeine on 15 min cycling time-trial performance; whereas Loy et al. (2015) (pilot study conducted on twelve women with high self-reported caffeine sensitivity and low daily caffeine consumption) reported that caffeine improved 10-minute cycling time-trial performance for TT genotypes (n = 6), but was ergolytic for all but one of the C-allele carriers (CC and CT genotypes).

In contrast to the above, the potential role of the *CYP1A2* gene in the ergogenic response to caffeine appears to lie in the ability of the gene to influence the rate of caffeine metabolism. Caffeine metabolism occurs primarily in the cytochrome P450 system in the liver and mostly (> 90%) by the *CYP1A2* enzyme (Nehlig 2018). Individuals possessing the wildtype (AA) variant of the gene (~40% of the population) are classified as 'fast metabolisers', whereas, those with the CC genotype (~10% of the population) are classified as 'slow metabolisers' (Southward et al. 2018). Despite this apparent clear distinction, the effect of variations in *CYP1A2* genotype on the rate of caffeine metabolism (typical half-life of 4 – 6 hours) has not been resolved (Southward et al. 2018). Moreover, given that caffeine exerts its effects in advance of its metabolism and that ergogenic effects have been observed often in relatively

short (< 1 hour) time-trials (Glaister and Moir 2019), it is difficult to reconcile the mechanism by which the *CYP1A2* gene would influence the response. Nevertheless, there are some discrepant findings (Fulton et al. 2018; Carswell et al. 2020; Spineli et al. 2020), and in the largest studies to date, Spineli et al. (2020) reported significant effects of caffeine (6 $mg \cdot kg^{-1}$) on endurance performance in adolescents (Yo-Yo Intermittent Recovery Test) independent of *CYP1A2* genotype; whereas Guest et al. (2018) reported differential effects of caffeine (4 $mg \cdot kg^{-1}$) on 10 km cycling time-trial performance based on *CYP1A2* genotype, with a significant positive effect in individuals with the AA genotype and a significant negative effect in those with the CC genotype.

In addition to its effects on aerobic exercise performance, caffeine has been found to influence physiological responses to exercise independent of any performance-associated responses (Glaister and Gissane 2018). During fixed intensity submaximal (60 – 85% $\dot{V}O_{2max}$) exercise, using the same dosing strategy highlighted above, caffeine has been found to suppress ratings of perceived exertion (RPE) and increase minute ventilation ($\dot{V}_E$), blood lactate ([BLa]), and blood glucose ([BGl]) (Glaister and Gissane 2018). If the effect of caffeine on exercise performance is influenced by *ADORA2A* and *CYP1A2* genotypes, then those same genes are likely also to influence physiological responses to caffeine. The aim of the present study was therefore to investigate the influence of *ADORA2A* and *CYP1A2* genotypes on physiological and performance responses to caffeine supplementation.

Materials and methods

Participants

Sixty-six male cyclists capable of maintaining a pace of at least 30 $km \cdot h^{-1}$ for 20 km (arbitrary inclusion criterion) volunteered to participate in the genotype screening phase (Phase I) of this study. Prior to screening, participants received written and verbal instructions regarding the nature of the investigation and completed a short questionnaire, which indicated that all had been actively involved

in cycling for approximately 10.3 ± 7.9 years and that 38 had, at some time, used caffeine as a means of enhancing their cycling performance. Prior to commencement, all participants provided written informed consent. Means ± standard deviation for age, height, and body mass of the participants were: 41.9 ± 8.6 years, 1.79 ± 0.08 m, and 76.3 ± 8.9 kg, respectively. Following genotype screening, 40 participants were then selected to participate in the experimental phase (Phase II) of the study. For the *ADORA2A* gene, participants were separated into the three genotype groups (CC, CT, and TT). In contrast, for the *CYP1A2* gene, due to the low frequency of CC genotypes, participants were separated into AA [wildtype] versus C-allele (AC or CC) carriers. Means ± standard deviation for age, height, body mass, $\dot{V}O_{2max}$, and habitual caffeine consumption of the participants in Phase II are presented in Table 1. Participants in Phase II were instructed to avoid food and drink in the hour before each trial, and to refrain from strenuous exercise for 24 hours before each trial. Participants were provided with a list of dietary sources of caffeine and instructed to refrain from consuming these for 24 hours prior to each trial. Prior to the start of Trial 1, participants completed a health screening questionnaire. Participants also completed a 24 hr dietary recall and were instructed to follow that same diet prior to trials 2 and 3. Questionnaires were used to establish: 1) normal daily caffeine intake; and 2) if used for performance enhancement, the extent to which participants perceived that caffeine benefited them (choices were: not at all, a little bit, moderately, and a lot). Ethical approval for the study was granted by St Mary's University Ethics Committee.

Procedures

Phase I

In Phase I of the study, participants provided 2 mL of saliva in a collection vial (SalivaGene collection module II, STRATEC Molecular, Berlin, Germany). A stabiliser, provided by the manufacturer, was then added to the saliva prior to the sample being stored at -20°C until DNA extraction. Genomic DNA was extracted using a commercial kit (PSP® SalivaGene 17 DNA Kit 1011; STRATEC Molecular, Berlin, Germany) in accordance with the manufacturer protocol. Quality and

quantity were assessed using a spectrophotometer (Nanodrop, ThermoFisher, Waltham, MA, USA). Genotyping was performed using TaqMan® single nucleotide polymorphism (SNP) genotyping assays with primers and probes pre-designed (Applied Biosystems, CA, USA) for rs5751876 (*ADORA2A*, assay code: C___2446672_50) and rs762551 (*CYP1A2*, assay code: C___8881221_40), and using a thermocycler (StepOnePlus; Applied Biosystems, CA, USA), with two technical replicates for each sample. The polymerase chain reaction amplification was performed under the conditions specified by the manufacturer. SNPs were accepted when the quality threshold was above 98%.

Phase II

All Phase II trials were completed at approximately the same time of day in a laboratory maintained at a temperature of 18°C. In Trial 1, on arrival at the laboratory, a 600 µL ear lobe capillary blood sample was collected in a lithium-heparin tube (Multivette; Starstedt AG & Co., Nümbrecht, Germany). Blood samples were centrifuged at 2000 g for 5 mins, with subsequently decanted plasma samples (~200 µL) frozen at -80°C until analysed for caffeine content. Participants then completed a submaximal incremental test on an electromagnetically-braked cycle ergometer (Lode Excalibur Sport; Groningen, Holland). The cycle ergometer was fitted with clipless pedals and participants cycled using their own cycling shoes. Prior to Trial 1, the ergometer was adjusted (saddle height and handlebar position) for each participant and the settings were noted for replication in trials 2 and 3. The starting intensity and increment size for each participant in Trial 1 was estimated, based upon feedback from participants regarding their typical race pace, to achieve 5-8 stages beginning at a [BLa] of around 1 $mmol \cdot L^{-1}$ and ending with a [BLa] ≥ 4 $mmol \cdot L^{-1}$. The duration of each increment was 3 minutes, and a 30 s break was provided at the end of each stage to enable a 20 µL blood sample to be obtained from the ear lobe via capillary puncture for the evaluation of [BLa] via an automated analyser (Biosen C-Line; EKF Diagnostic, Ebendorfer Chaussee, Barleben, Germany). The same analyser was used to also evaluate [BGl] in trials 2 and 3.

To limit any effect of cadence on the [BLa] response to exercise (Vercruyssen et al. 2005), participants were given 30 s during the first stage of Trial 1 to achieve a comfortable cadence and were instructed to maintain this throughout all incremental tests (Trials 1-3). Heart rate was monitored at 5 s intervals throughout all trials using a heart rate monitor (Polar s610i; Polar Electro Oy, Kempele, Finland), and ratings of perceived exertion were recorded 15 s from the end of each incremental stage using a 15-point scale (Borg 1970). After five minutes of passive rest, participants completed a second incremental test (Trial 1 only), to determine $\dot{V}O_{2max}$. The test used the same starting intensity and increment size as before; however, the duration of each increment was reduced to 1 minute. The test was terminated when participants reached volitional exhaustion, at which time a final [BLa] measurement was obtained.

Oxygen uptake ($\dot{V}O_2$) was monitored (breath-by-breath) throughout all trials using an on-line gas analyser (Oxycon Pro; Jaeger, Hoechberg, Germany). The analyser was calibrated before each trial using oxygen and carbon dioxide gases of known concentrations (Cryoservice; Worcester, UK) and the flowmeter was calibrated using a 3-litre syringe (Viasys Healthcare GmbH; Hoechberg, Germany). During all trials, participants breathed room air through a facemask (Hans Rudolph; Kansas City, MO, USA) that was secured in place by a head-cap assembly (Hans Rudolph; Kansas City, MO, USA). $\dot{V}O_2$ data were filtered to remove errant breaths caused by coughing, swallowing or sighing. A breath was considered to be errant if the value was outside four standard deviations of the local mean (the two breaths preceding and following the breath of interest). In Trial 1, $\dot{V}O_2$ data were subsequently averaged over the final 30 s of each submaximal stage, with linear regression used to establish the $\dot{V}O_2$-power output relationship for each participant. $\dot{V}O_{2max}$ was determined as the highest 30 s average $\dot{V}O_2$ recorded during the second incremental test of Trial 1 provided that at least two of the following criteria had been met: 1) A plateau in $\dot{V}O_2$; as determined by an increase of less than 2 $ml \cdot kg^{-1} \cdot min^{-1}$ over the previous stage; 2) A respiratory exchange ratio (RER) ≥ 1.15; 3) A heart rate within 10 $b \cdot min^{-1}$ of age predicted maximum; 4) A [BLa] ≥ 8 $mmol \cdot L^{-1}$. The power output at the end of the $\dot{V}O_{2max}$ test (W_{max}) was used in the settings for the following time-trial. If participants terminated the $\dot{V}O_{2max}$ test partway

through a stage, the fraction of time spent in that stage was multiplied by the size of the increment and added to the power output of the previous stage to determine W_{max} (Saunders et al. 2017).

Ten minutes after completion of the $\dot{V}O_{2max}$ test, participants completed a time-trial (for familiarisation purposes) in which they were required to complete a target amount of work equal to 25 mins at 85% of W_{max} (0.85 × W_{max} × 1500). The protocol was the same as that used by Saunders et al. (2017), and was based on a modified version of the protocol outlined by Jeukendrup et al. (2008). The test has been reported to have a test-retest coefficient of variation of 3.0 ± 2.3% (Saunders et al. 2017). For the time-trial, the cycle ergometer was set to the cadence-dependent 'linear mode' with an 'α-factor' equal to (0.85 × W_{max})/(95 × 95). In effect, the 'α-factor' was set so that participants were working at 85% W_{max} when cycling at a cadence of 95 rpm. The same time-trial protocol was repeated in trials 2 and 3. In all instances, the only feedback visible to the participants was how much of the target energy demand remained.

Trials 2-3 followed a balanced, randomised, double-blind, placebo-controlled design in which participants completed a four-stage submaximal incremental test followed by a time-trial under caffeine and placebo supplemented conditions. On arrival at the laboratory, three blood pressure readings were obtained using an automated digital blood pressure monitor (i-C10, OMRON Healthcare Europe B.V. Hoofddorp, Netherlands). Participants then ingested a gelatine capsule containing either 5 $mg \cdot kg^{-1}$ of caffeine (Blackburn Distributions Ltd., Burnley, UK), or placebo (maltodextrin: My Protein; Manchester, UK). After supplementation, participants rested for 40 minutes before the same blood pressure procedures were repeated, along with the collection of a 20 µL ear lobe capillary blood sample for the evaluation of [BLa] and [BGl], and a 600 µL capillary blood sample for the evaluation of plasma caffeine content. 55 minutes after supplementation, participants completed a four-stage (4-minute stages; 1-minute recovery between stages) submaximal incremental test at 40, 55, 70, and 85% of the power output required to elicit $\dot{V}O_{2max}$ (determined from the $\dot{V}O_2$-power output relationship and $\dot{V}O_{2max}$

established in Trial 1). Prior to the start of each incremental test, participants sat passively on the cycle ergometer for 3 minutes to enable resting data to be recorded. Upon completion of each incremental test, participants rested passively for 10 minutes before performing the same time-trial outlined in Trial 1. [BLa] and [BGl] were evaluated at the end of each incremental stage and also at the end of the time-trial. $\dot{V}O_2$, RER, $\dot{V}_E$, breathing frequency (BF), heart rate, and RPE were monitored throughout all incremental tests; with all, apart from RPE, also monitored during the time trials. Respiratory data in the final minute of each incremental stage were used to investigate the effects of supplementation on $\dot{V}O_2$, RER, $\dot{V}_E$, and BF. On completion of Trial 3, participants were asked if they felt they could identify the caffeine trial; if the answer was yes, participants were asked which trial they thought it was.

Statistical Analysis

All statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS for Windows; IBM Corporation, Armonk, New York, USA). Measures of centrality and spread are presented as means ± standard deviation. Pearson correlation was used to investigate the relationship between participant self-reported habitual caffeine consumption and the change in time-trial performance, relative to placebo, following caffeine supplementation. For those participants who reported that they had used caffeine to improve their cycling performance, Spearman's correlation was used to investigate the relationship between participant perceptions of caffeine effectiveness and the change in time-trial performance relative to placebo. The effect of genotype (for each gene) on habitual caffeine consumption was evaluated using one-way analysis of variance (ANOVA). The effects of caffeine supplementation and genotype (for each gene) on resting measures of blood pressure were evaluated using three-way (supplement × genotype × time) mixed ANOVA; with effects on the other physiological measures, at rest, determined using two-way (supplement × genotype) mixed ANOVAs. The effects of caffeine and genotype on physiological responses during the incremental tests of trials 2 and 3 were also evaluated using three-way (supplement × exercise intensity × genotype) mixed ANOVAs. The effects caffeine and genotype on time-trial performance and associated physiological

responses during trials 2 and 3 were evaluated using two-way (supplement × genotype) mixed ANOVAs. The effects of genotype on time-trial performance were also evaluated using one-way analysis of covariance (ANCOVA), with change scores in mean power output included as the dependent variable, genotype as the independent variable, and placebo responses as the covariate. α was set at 0.05 for all analyses. Violations to assumptions of sphericity were adjusted using the Greenhouse-Geisser correction factor. Significant interactions were investigated using *post hoc* tests with Bonferroni adjustments for multiple comparisons. The above analyses provided 95% confidence limits (CL_{95}) for all estimates.

Results

In Phase I of the study, for the *ADORA2A* gene, 11 participants had the CC genotype, 31 had the CT genotype, and 24 had the TT genotype. For the *CYP1A2* gene, 41 participants had the AA genotype, 19 had the AC genotype, and six had the CC genotype. The sample was within the Hardy Weinberg equilibrium for both genes, ($X^2 = 4.09$; $p = 0.130$ for *ADORA2A and* $X^2 = 2.16$; $p = 0.339$ for *CYP1A2*). In Phase II, there were no significant differences between the genotype groups for each gene in baseline measures of age, height, body mass, $\dot{V}O_{2max}$, or habitual caffeine consumption (Table 1). Subject compliance with caffeine restriction prior to each trial was confirmed by the fact that in all non-caffeine supplemented conditions, plasma caffeine concentrations were low (3.2 ± 2.5 μmol·L^{-1}), whereas values were high (43.3 ± 8.8 μmol·L^{-1}) following caffeine. There was no significant relationship between the change in time-trial completion time (placebo time – caffeine time) and the level of habitual caffeine consumption ($r = -0.09$; [CL_{95}: −0.39 – 0.23]). At the end of Trial 3, 22 participants (19 positive time-trial responders to caffeine) correctly identified the caffeine supplement, with nine (six positive responders) being unsure, and nine (eight positive responders) being incorrect. Thirty-eight participants (58%) reported that they had used caffeine previously as a means of enhancing their cycling performance, with five believing that it helped ‘a lot’, 20 feeling that it had a ‘moderate’ effect, 11 feeling that it helped ‘a little bit’, and two believing that it had ‘no effect’. There was no

relationship between participant perceptions of the effectiveness of caffeine and the corresponding change in time-trial performance (ρ = −0.12; [CL_{95}: −0.48 – 0.28]).

Physiological responses at rest

The effects of caffeine supplementation on diastolic and systolic blood pressure are presented in Table 2. In both instances, there was a significant supplement × time interaction effect, with *post hoc* tests revealing that, relative to placebo, caffeine increased diastolic blood pressure by 5.5 ± 6.0 mmHg (CL_{95}: 3.6 – 7.4 mmHg) and systolic blood pressure by 7.1 ± 7.0 mmHg (CL_{95}: 4.8 – 9.3 mmHg). There was no supplement × genotype interaction for either diastolic (*ADORA2A*: p = 0.15; *CYP1A2*: p = 0.78) or systolic (*ADORA2A*: p = 0.21; *CYP1A2*: p = 0.68) blood pressure.

There were no significant effects of caffeine supplementation on resting measures of breathing frequency, [BGl], [BLa], or $\dot{V}O_2$ (Table 2). In contrast, caffeine reduced resting heart rate by 3.2 ± 5.8 b·min^{-1} (CL_{95}: 1.4 – 5.1 b·min^{-1}), and increased resting RER and $\dot{V}_E$ by 0.04 ± 0.09 (CL_{95}: 0.01 – 0.07) and 1.4 ± 3.1 L·min^{-1} (CL_{95}: 0.4 – 2.5 L·min^{-1}) respectively (Table 2). There was no significant supplement × genotype interaction for either gene for any of the responses. p-values for all interaction effects at rest, during submaximal exercise, and during the time-trials are presented in Table 3.

Physiological responses during submaximal exercise

The effects of caffeine supplementation on breathing frequency, [BGl], [BLa], heart rate, RER, RPE, $\dot{V}_E$, and $\dot{V}O_2$ during the submaximal exercise bouts are presented in Figure 1. There were significant effects of exercise intensity on all responses (p < 0.001), but there were no significant effects of supplementation, genotype (for either gene), or any associated interactions on measures of breathing frequency, [BGl], or $\dot{V}O_2$. In contrast, there was a significant effect of supplementation on heart rate (p

< 0.001), and a significant supplementation × exercise intensity interaction ($p < 0.001$). *Post hoc* tests revealed that caffeine significantly reduced heart rate at each of the three lowest exercise intensities, but that the effect dissipated as exercise intensity increased. There was also a significant effect of supplementation on [BLa] ($p < 0.001$), RER ($p = 0.016$), RPE ($p < 0.001$), and $\dot{V}_E$ ($p = 0.008$), but, with the exception of RER, there were no significant effects of genotype, or any associated interactions. *Post hoc* tests revealed that caffeine reduced RPE during submaximal exercise by 0.5 (CL_{95}: 0.2 – 0.8), and increased [BLa] by 0.29 mmol·L^{-1} (CL_{95}: 0.16 – 0.43 mmol·L^{-1}), RER by 0.013 (CL_{95}: 0.003 – 0.023), and $\dot{V}_E$ by 3.1 L·min^{-1} (CL_{95}: 0.9 – 5.3 L·min^{-1}). The only effect of genotype on any of the submaximal exercise responses was with RER, where there was a significant supplement × exercise intensity × *CYP1A2* genotype interaction ($p = 0.002$). When considered by *CYP1A2* genotype, there was a significant supplement × exercise intensity interaction effect on RER for the AC/CC genotype ($p = 0.004$), but not for the AA genotype ($p = 0.628$) (Figure 2). However, *post hoc* tests on the former were unable to locate significant between-supplement differences at any exercise intensity.

Physiological and performance responses during the time-trial

The effects of caffeine supplementation on time-trial performance are presented in Figure 3, with associated physiological responses presented in Table 4. There was a significant effect of supplementation on time-trial completion time ($p < 0.001$) and mean power output ($p < 0.001$). Completion time for the caffeine trial (29.65 ± 1.80 mins) was 1.14 ± 1.24 mins (CL_{95}: 0.74 – 1.54 mins) quicker than placebo (30.78 ± 2.26 mins). Correspondingly, mean power output for the caffeine trial (261 ± 38 W) was 9 ± 10 W (CL_{95}: 6 – 12 W) higher than placebo (252 ± 41 W). There was no significant effect of genotype and no supplement × genotype interaction effect, for either gene, on measures of time-trial performance. ANCOVA confirmed that the effect of caffeine on time-trial performance was not influenced by genotype for either *ADORA2A* ($p = 0.752$) or *CYP1A2* ($p = 0.286$).

There was a significant effect of supplementation on all physiological measures during and immediately following the time-trial, apart from $\dot{V}O_2$ ($F_{(1,34)}$ = 1.9; p = 0.172). *Post hoc* tests revealed that caffeine supplementation increased mean breathing frequency by 1.6 ± 3.6 breaths·min^{-1} (CL_{95}: 0.5 – 2.7 breaths·min^{-1}), end-test [BGl] by 1.00 ± 0.94 mmol·L^{-1} (CL_{95}: 0.69 – 1.31 mmol·L^{-1}), end test [BLa] by 1.83 ± 1.73 mmol·L^{-1} (CL_{95}: 1.27 – 2.39 mmol·L^{-1}), mean heart rate by 3.5 ± 4.5 b·min^{-1} (CL_{95}: 2.0 – 4.9 b·min^{-1}), mean RER by 0.02 ± 0.03 (CL_{95}: 0.01 – 0.03), and mean $\dot{V}_E$ by 9.9 ± 14.6 L·min^{-1} (CL_{95}: 5.2 – 14.5 L·min^{-1}). There was no effect of genotype and no supplement × genotype interactions, for either gene, for any of the time-trial physiological responses.

Discussion

The aim of the present study was to investigate the influence of *ADORA2A* and *CYP1A2* genotypes on physiological and performance responses to caffeine supplementation. The main findings were that during submaximal exercise, caffeine significantly reduced heart rate and perceived exertion, and increased [BLa], RER, and $\dot{V}_E$. Moreover, caffeine significantly improved time-trial performance irrespective of habitual caffeine consumption or perceived knowledge of the supplement, and, apart from $\dot{V}O_2$, increased all associated physiological responses. However, with the possible exception of an effect of *CYP1A2* genotype on RER during submaximal exercise, the effects of caffeine on exercise physiology and performance were not influenced by *ADORA2A* or *CYP1A2* genotypes.

The positive effect of caffeine on time-trial performance is in-line with previous findings (Glaister and Moir 2019) and is most likely due to enhanced central nervous system (CNS) activation resulting from the antagonism of A_1 and A_{2A} adenosine receptors (McLellan et al. 2016). However, while enhanced CNS activation provides the most likely mechanism by which caffeine exerts its ergogenic effect, differential effects of the various adenosine receptor subtypes, coupled with individual

variability in adenosine receptor density and sensitivity, means that more research is necessary to confirm.

Although, relative to placebo, the present study found a significant effect of caffeine on time-trial performance, there was considerable individual variability in the magnitude of the response, with some individuals experiencing an ergolytic effect. The absence of a positive effect of caffeine on time-trial performance in a few individuals is a common finding (Southward et al. 2018); though, as highlighted by Pickering and Kiely (2018), without repeated trials on those same individuals, it is difficult to determine if those individuals are truly 'non-responders'. Nevertheless, the variation in performance was not explained by *ADORA2A* or *CYP1A2* genotypes. The absence of any influence of *ADORA2A* genotype on time-trial performance is in-line with recent findings by Carswell et al. (2020), but contrasts with the findings of Loy et al. (2015) which reported ergolytic effects of caffeine in *ADORA2A* C-allele carriers. Although Loy et al. (2015) used a much smaller sample size with a different sex, training status, and level of caffeine habituation than participants in the present study, it is difficult to explain why those differences would account for the contrasting results. Indeed, the effects of caffeine on time-trial performance show no evidence of between-study heterogeneity despite between-study differences in each of those characteristics (Glaister and Moir 2019). Moreover, there is no apparent mechanistic basis for why *ADORA2A* C allele carriers would experience an ergolytic effect on time-trial performance following caffeine supplementation. In support of the results of the present study and those of Carswell et al. (2020), Grgic et al. (2020) recently reported ergogenic effects of caffeine in *ADORA2A* C allele carriers; albeit in a battery of strength and power tests.

As with *ADORA2A* genotype, previous research into the ability of the *CYP1A2* gene to explain individual variability in the ergogenic effect of caffeine on aerobic exercise performance has shown some contrasting results with some finding the greatest benefit in AA genotypes (Womack et al. 2012; Guest et al. 2018), others in AC genotypes (Pataky et al. 2016), and some finding no effect of genotype

(Algrain et al. 2016; Giersch et al. 2018; Carswell et al. 2020; Spineli et al. 2020). As highlighted earlier, considering that the half-life of caffeine is ~ 4 – 6 hrs it is difficult to rationalise a method by which *CYP1A2* genotype would influence the ergogenic effect of caffeine; particularly since ergogenic effects are reported in time-trials as short as 5 mins (Glaister and Moir 2019). Indeed, as suggested by Southward et al. (2018), individuals with the slower (CC) genotype would probably be expected to derive the greatest benefit of caffeine on endurance performance due to a longer exposure time. In contrast, Guest et al. (2018) reported a significant negative effect of caffeine (4 $mg \cdot kg^{-1}$) on time-trial performance in CC genotypes, whereas in the present study, all the CC genotypes showed a positive response. While it is difficult to explain the contrasting results between the present study and those of Loy et al. (2015) and Guest et al. (2018), it is important to note that the absence of any influence of *ADORA2A* and *CYP1A2* genotypes on time-trial performance in the present study corresponded with the absence of any influence of those same genotypes on resting and exercise physiology also.

The caffeine-induced increase in resting blood pressure observed in the present study is in-line with previous reports (De Giuseppe et al. 2019), as is the absence of any corresponding influence of *CYP1A2* genotype (Renda et al. 2012). Nevertheless, the mechanisms responsible, and the short- and long-term associated health risks are, at present, unclear (De Giuseppe et al. 2019). Similarly, the effects of caffeine on heart rate agree with those previously reported (Fredholm et al. 1999; Glaister et al. 2016), in that caffeine typically reduces resting heart rate by ~ 3 $b \cdot min^{-1}$, with the effect dissipating as exercise intensity increases (Glaister et al. 2016). Moreover, caffeine typically increases heart rate during time-trials due to the corresponding caffeine-induced increase in time-trial intensity (Glaister and Moir 2019). The mechanism to explain the suppressive effect of caffeine on heart rate at rest and during low-intensity exercise is difficult to elucidate. Although adenosine has been shown to increase heart rate due to differential effects on parasympathetic and sympathetic nervous system tone (Rongen et al. 1999), it is difficult to explain why caffeine would do more than just counteract that response.

Although the effect of caffeine on heart rate reduction was influenced by exercise intensity, the reduction in RPE was consistent throughout the incremental test. The ability of caffeine to reduce perceived exertion during submaximal exercise is a consistent finding (Glaister and Gissane 2018), which has been suggested to possibly explain ~ 29% of the corresponding improvement in time-trial performance (Doherty and Smith 2005). While the mechanism(s) to explain the remainder of the improvement, and indeed to explain why caffeine suppresses RPE, remain elusive, it seems unlikely that the effect of caffeine on time-trial performance was influenced by either habituation or a placebo response. Regarding a possible habituation effect, the absence of any significant difference in habitual caffeine consumption between *ADORA2A* genotypes conflicts with reports of lower levels of consumption in TT genotypes (Cornelis et al. 2007). Nevertheless, the lack of any significant relationship between habitual caffeine consumption and the corresponding change in time-trial performance supports previous research showing no effect of caffeine habituation (Gonçalves et al. 2017); though not all studies agree (Pickering and Grgic 2019). Similarly, while there is evidence of a small placebo effect from caffeine (Saunders et al. 2017), the recent suggestion that the true effect could be much larger (De Salles Painelli et al. 2020) is difficult to accept given that there was no apparent link between participant ability to identify the caffeine trial and whether or not caffeine improved performance, along with the fact that caffeine influenced exercise physiology as well as performance.

In most instances, the effects of caffeine on respiratory responses in the present study are the same as those observed previously (Glaister and Gissane 2018; Glaister and Moir 2019), the majority of which can be explained by adenosine receptor antagonism (Glaister and Gissane 2018). The increase in $\dot{V}_E$ following caffeine supplementation, in the absence of any effect on breathing frequency, is most likely explained be either adenosine receptor antagonism or phosphodiesterase inhibition (Howell and Landrum 1997). While the increase in $\dot{V}_E$ could be explained also by the buffering response associated with the disruption of acid-base balance, as indicated by the caffeine-induced increase in [BLa] during submaximal exercise, the increase in $\dot{V}_E$ at rest in the absence of any significant change in [BLa] mitigates against this. Still, the caffeine-induced increase in [BLa] during exercise confirms previous

reports (Glaister and Gissane 2018), and although the mechanism(s) to explain the effect remain unresolved (Glaister and Gissane 2018), the fact that both $\dot{V}_E$ and [BLa] increased more in the time-trial than during submaximal exercise suggests that the increase observed for both in the time-trial is due partly to a direct stimulatory effect of caffeine.

The absence of an effect of caffeine on $\dot{V}O_2$ during the time trial is strange given that caffeine improved performance. Although differences in mean $\dot{V}O_2$ between caffeine and placebo in the present study were reflective of the typical response in time trials (Glaister and Moir 2019), it is difficult to explain why no significant difference was observed (possibly a Type II error). In contrast, the absence of any significant effect of caffeine on $\dot{V}O_2$ at rest and during submaximal exercise is in line with previous findings (Glaister et al. 2016; Glaister and Gissane 2018), but conflicts with the suggestion that caffeine can increase metabolic rate. Then again, as suggested by Fredholm et al. (1999), as a way to explain a caffeine-induced increase in cerebral energy metabolism in the absence of any change in $\dot{V}O_2$, it is possible that caffeine increases metabolic rate in the absence of a change in $\dot{V}O_2$ via an increase in anaerobic glycolysis, as supported by the aforementioned increase in [BLa] (during exercise at least).

Although most of the physiological responses to caffeine measured in the present study were the same as reported previously, there were some anomalous findings. The effect of caffeine on RER is particularly confusing in that while the increase at rest fits with previous reports (Glaister et al. 2016), the submaximal and time-trial responses do not. In a recent meta-analysis, Glaister and Gissane (2018) found no effect of caffeine on RER during submaximal exercise at 60 – 85% $\dot{V}O_{2max;}$ indeed, the tendency was for RER to be reduced with caffeine, though there was considerable between-study heterogeneity. Similarly, research investigating the effects of caffeine on RER during incremental exercise shows a supplement × intensity interaction, with RER either reducing significantly (Stadheim et al. 2013) or tending to be reduced (Glaister et al. 2016) as exercise intensity increases. While the

pattern of the submaximal RER response in the present study was suggestive of a convergence between caffeine and placebo trials as exercise intensity increased, particularly in the *CYP1A2* AC/CC genotypes, there was no suggestion of any reduction in RER with increasing intensity. Likewise, caffeine has been found typically to have no effect on RER during time-trials (Glaister and Moir 2019), though the present study found a small, but significant, increase.

Discrepant findings are evident also in the effects of caffeine on [BGl], with the increase in [BGl] during the time-trial confirming previous findings (Glaister and Moir 2019), but not so for submaximal exercise (Glaister and Gissane 2018). Then again, while there is a lot of evidence to support that caffeine increases [BGl] during submaximal exercise (Glaister and Gissane 2018), there is again considerable between-study heterogeneity, with the effect of exercise intensity reflecting the pattern observed in the present study. Either way, despite the relatively small discrepant findings in some measures of RER and [BGl], most of the physiological responses to caffeine in the present study agree with previous reports (Glaister and Gissane 2018; Glaister and Moir 2019) and do not appear to be influenced by *ADORA2A* or *CYP1A2* genotypes.

At this point it is worth highlighting a few potential limitations with the present study. First, the number of participants with the CC genotype of the *CYP1A2* gene was very small and, as in other studies (Womack et al. 2012; Algrain et al. 2016; Giersch et al. 2018; Carswell et al. 2020), required those participants to be grouped with other C-allele carriers to allow for appropriate statistical analysis. Given the low frequency of CC genotypes in the population, this is an issue which is difficult to address, especially with trained individuals. Indeed, previous research with sample sizes of 101 (Guest et al. 2018) and 100 (Spineli et al. 2020) has only returned eight and nine CC genotypes respectively. Secondly, although a 24-hour period of caffeine abstinence was required prior to all Phase II trials, it is possible that this may have been insufficient to prevent withdrawal symptoms in some participants (McLellan et al. 2016). Then again, while the effect of withdrawal symptoms on the ergogenicity of

caffeine is an area worthy of further investigation (particularly with repeated bouts of abstinence), the likelihood of caffeine withdrawal being a major limitation is mitigated by the fact that: 1) there was no relationship between participant habitual caffeine consumption and the change in time-trial performance; and 2) most of the participants experienced an ergogenic response to caffeine despite many either incorrectly identifying or being unable to identify the caffeine trial.

In conclusion, the results of this study confirm previous reports regarding the effects of caffeine on exercise physiology and time-trial performance. Although the effects of caffeine on blood glucose and RER during submaximal incremental exercise require further research to tease out the precise responses, it seems likely that the effects of caffeine on time-trial performance are influenced by more than just a change in perceived exertion or a placebo response. While all the effects of caffeine may be explained by adenosine receptor antagonism, the differential effects of the various receptor subtypes make is difficult to establish how caffeine is influencing exercise and performance responses. Finally, although the effects of caffeine on exercise physiology and time-trial performance show some individual variability, that variability is not explained by differences in *ADORA2A* or *CYP1A2* genotypes.

Conflict of interest

Dr Mavrommatis is a shareholder and the head of nutrigenetics with the genotyping company Nell Health. He received no financial incentives for the preparation of this manuscript. All other authors declare no conflict of interest.

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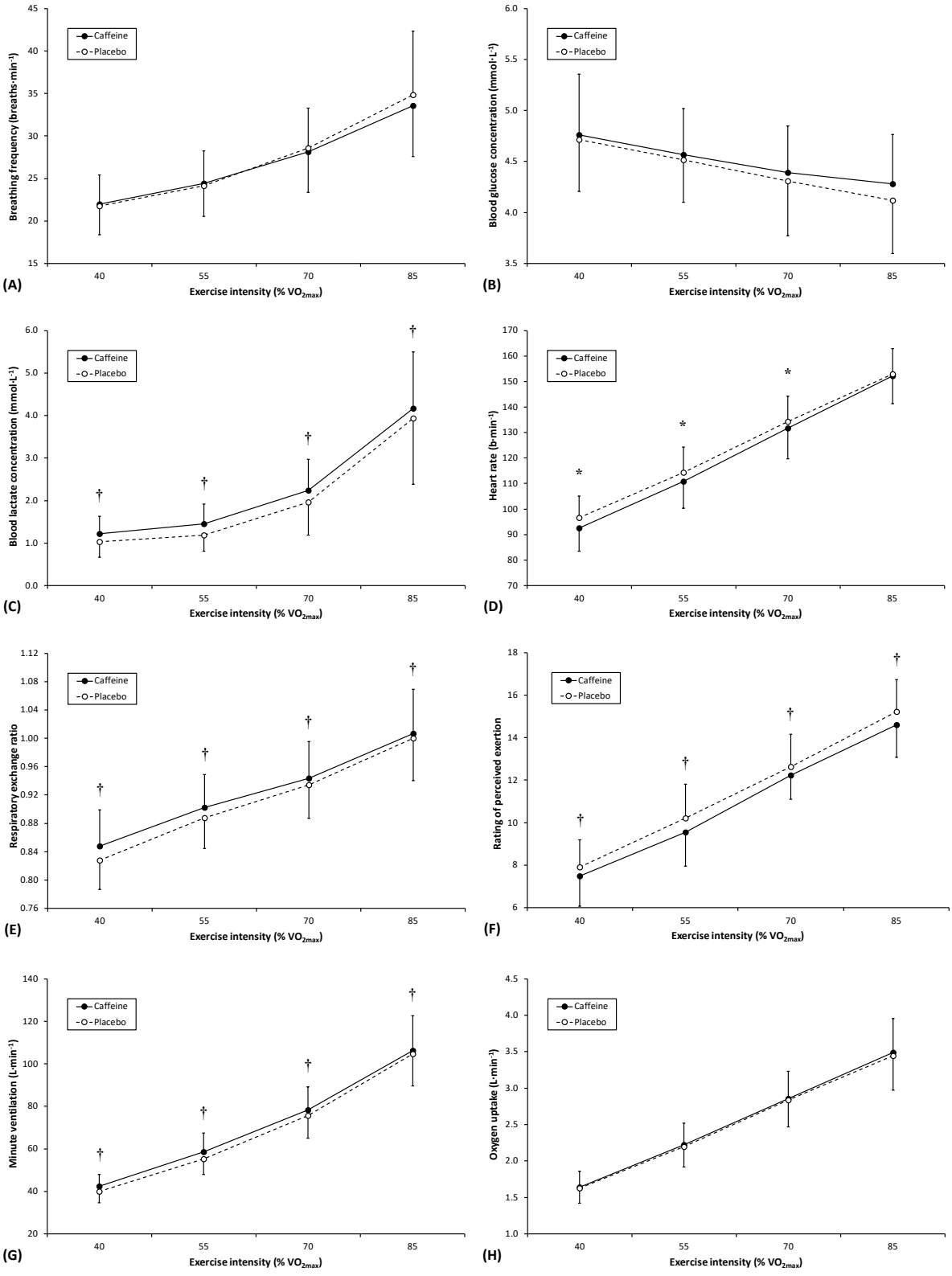

Figure 1. The effects of caffeine supplementation (5 mg·kg^{-1}) on measures of breathing frequency (A), blood glucose (B), blood lactate (C), heart rate (D), respiratory exchange ratio (E), ratings of perceived exertion (F), minute ventilation (G), and oxygen uptake (H), during an incremental test on a cycle ergometer. Values are means, bars are standard deviations. * $p < 0.05$; † main effect ($p < 0.05$) of supplement.

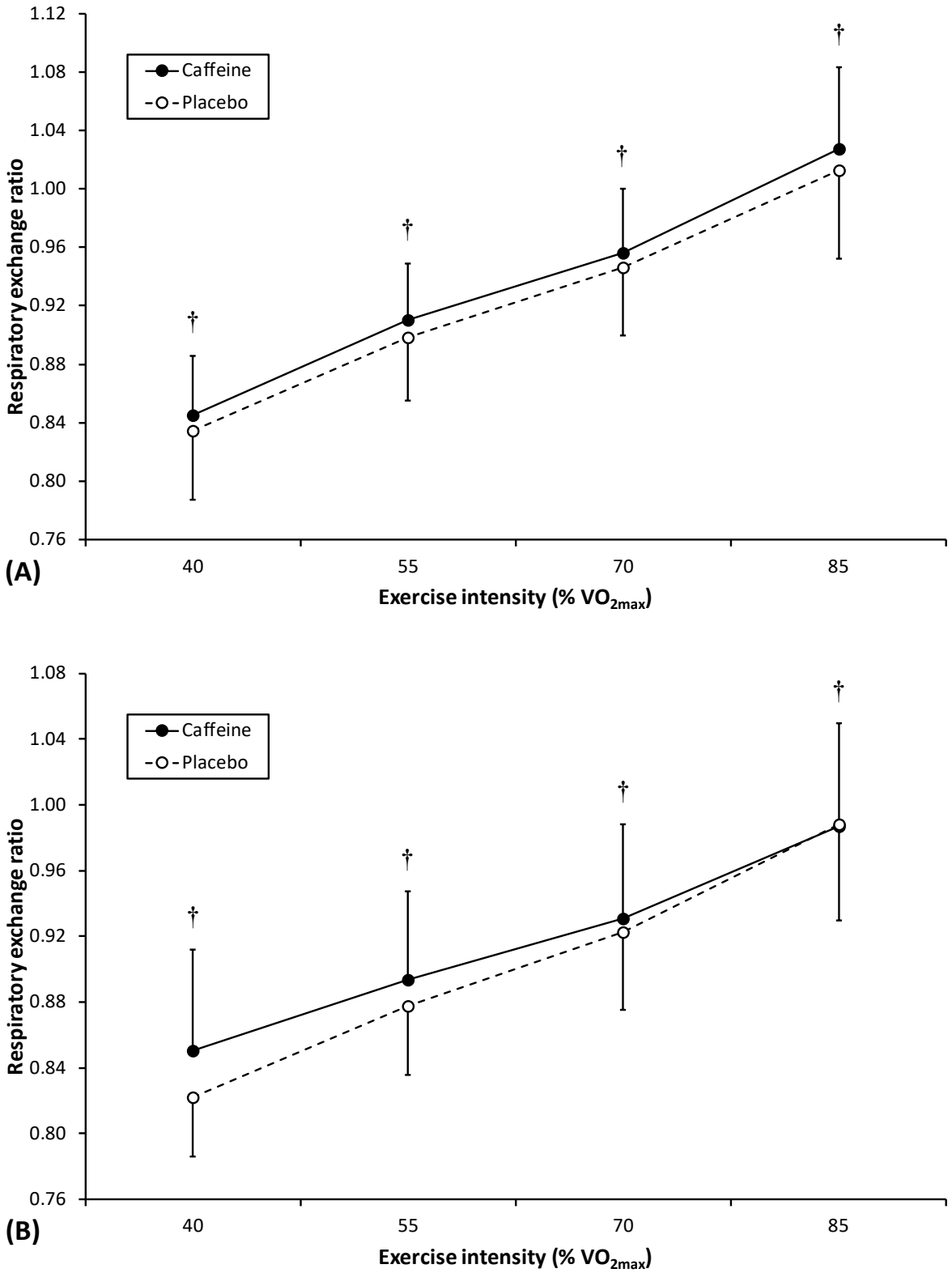

Figure 2. The effects of caffeine supplementation (5 mg·kg^{-1}) on measures of respiratory exchange ratio during an incremental test on a cycle ergometer; differences between *CYP1A2* AA genotype (A) and AC/CC genotypes (B). Values are means, bars are standard deviations. † main effect ($p < 0.05$) of supplement.

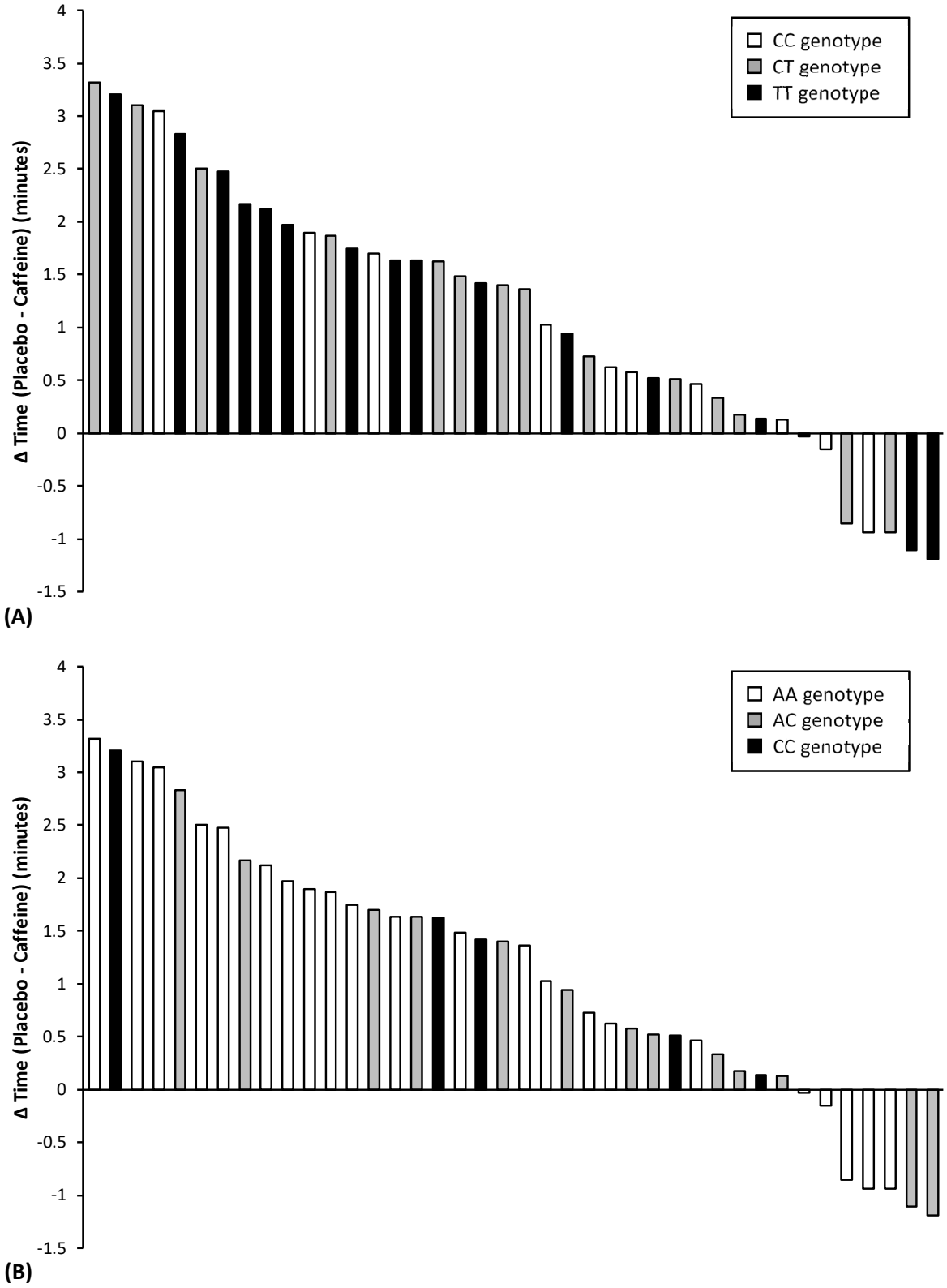

Figure 3. The effects of caffeine supplementation (5 mg·kg^{-1}) and *ADORA2A* (A) and *CYP1A2* (B) genotypes on cycling time-trial performance (~ 30 mins) ranked in order of individual change relative to placebo. Note: a positive value indicates an improvement in performance relative to placebo. Values are individual responses.

Table 1. Participant characteristics for the experimental phase (Phase II) of the study. Values are means ± standard deviation.

Gene	Genotype	n	Age (yrs)	Height (m)	Body mass (kg)	$\dot{V}O_{2max}$ ($L \cdot min^{-1}$)	Caffeine ($mg \cdot d^{-1}$)
ADORA2A	CC	10	43.3 ± 8.5	1.82 ± 0.07	79.5 ± 12.1	4.33 ± 0.37	326 ± 100
	CT	14	41.8 ± 7.6	1.79 ± 0.07	75.1 ± 10.6	4.07 ± 0.46	337 ± 158
	TT	16	44.9 ± 9.3	1.83 ± 0.07	77.2 ± 6.9	4.05 ± 0.45	359 ± 108
CYP1A2	AA	22	43.7 ± 8.5	1.84 ± 0.07	77.7 ± 9.4	4.20 ± 0.43	340 ± 136
	AC/CC	18	43.1 ± 8.6	1.79 ± 0.06	76.1 ± 10.1	4.03 ± 0.44	346 ± 110

Note: Caffeine = estimated habitual caffeine consumption.

Table 2. The effects of caffeine supplementation ($5\ mg \cdot kg^{-1}$) on resting physiological responses. Values are means ± standard deviations.

Supplement	BF ($breaths \cdot min^{-1}$)	[BGl] ($mmol \cdot L^{-1}$)	[BLa] ($mmol \cdot L^{-1}$)	$BP_{(dia)}$ (mmHg)	$BP_{(sys)}$ (mmHg)	Heart rate ($b \cdot min^{-1}$)	RER	V_E ($L \cdot min^{-1}$)	VO_2 ($L \cdot min^{-1}$)
Caffeine	13.1 ± 3.3	4.83 ± 0.57	0.93 ± 0.29	88.6 ± 8.3*	135.9 ± 11.9*	55.4 ± 9.9*	0.86 ± 0.11*	12.3 ± 3.1*	0.35 ± 0.08
Placebo	13.3 ± 3.2	4.90 ± 0.62	0.89 ± 0.30	83.2 ± 8.4	128.8 ± 10.6	58.7 ± 8.7	0.82 ± 0.07	10.8 ± 2.8	0.34 ± 0.09

Note: BF = breathing frequency, [BGl] = blood glucose concentration; [BLa] = blood lactate concentration; $BP_{(dia)}$ = diastolic blood pressure; $BP_{(sys)}$ = systolic blood pressure; RER = Respiratory exchange ratio; V_E = minute ventilation; VO_2 = rate of oxygen uptake. *significant effect ($p < 0.05$) of supplement.

Table 3. Probability values for statistical tests investigating the influence of *ADORA2A* and *CYP1A2* genotypes on the effects of caffeine supplementation ($5\ mg \cdot kg^{-1}$) on physiological responses at rest and during incremental exercise and cycling time-trial performance.

		MAIN EFFECTS AND INTERACTIONS										
		Sup	Int	*ADORA2A*	*CYP1A2*	Sup × Int	Sup × *ADORA2A*	Sup × *CYP1A2*	Int × *ADORA2A*	Int × *CYP1A2*	Sup × Int × *ADORA2A*	Sup × Int × *CYP1A2*
REST	BF	0.726	-	0.510	0.111	-	0.305	0.914	-	-	-	-
	[BGl]	0.535	-	0.300	0.186	-	0.494	0.339	-	-	-	-
	[BLa]	0.248	-	0.730	0.206	-	0.874	0.127	-	-	-	-
	$BP_{(dia)}$	<0.001	-	0.762	0.948	-	0.149	0.783	-	-	-	-
	$BP_{(sys)}$	<0.001	-	0.974	0.684	-	0.213	0.678	-	-	-	-
	Heart rate	0.001	-	0.644	0.878	-	0.969	0.401	-	-	-	-
	RER	0.010	-	0.139	0.845	-	0.140	0.410	-	-	-	-
	V_E	0.005	-	0.759	0.745	-	0.335	0.153	-	-	-	-
	VO_2	0.354	-	0.115	0.327	-	0.903	0.076	-	-	-	-
INCREMENTAL EXERCISE	BF	0.664	<0.001	0.382	0.552	0.091	0.600	0.739	0.480	0.925	0.081	0.303
	[BGl]	0.218	<0.001	0.479	0.572	0.309	0.994	0.873	0.122	0.500	0.995	0.635
	[BLa]	<0.001	<0.001	0.399	0.165	0.444	0.379	0.972	0.484	0.077	0.368	0.746
	Heart rate	<0.001	<0.001	0.631	0.386	<0.001	0.630	0.902	0.865	0.630	0.171	0.725
	RER	0.016	<0.001	0.580	0.220	0.196	0.301	0.952	0.835	0.089	0.323	0.002
	RPE	<0.001	<0.001	0.804	0.340	0.095	0.995	0.778	0.427	0.395	0.957	0.091
	V_E	0.008	<0.001	0.715	0.087	0.530	0.063	0.405	0.947	0.202	0.062	0.253
	VO_2	0.556	<0.001	0.138	0.109	0.525	0.059	0.112	0.213	0.882	0.134	0.672
TIME-TRIAL	MPO	<0.001	-	0.574	0.693	-	0.514	0.386	-	-	-	-
	BF	0.009	-	0.228	0.655	-	0.608	0.849	-	-	-	-
	[BGl]	<0.001	-	0.325	0.968	-	0.111	0.590	-	-	-	-
	[BLa]	<0.001	-	0.210	0.721	-	0.098	0.971	-	-	-	-
	Heart rate	<0.001	-	0.596	0.632	-	0.073	0.206	-	-	-	-
	RER	<0.001	-	0.313	0.673	-	0.058	0.687	-	-	-	-
	V_E	<0.001	-	0.933	0.710	-	0.911	0.080	-	-	-	-
	VO_2	0.172	-	0.415	0.725	-	0.441	0.275	-	-	-	-

Note: BF = breathing frequency, [BGl] = blood glucose concentration; [BLa] = blood lactate concentration; $BP_{(dia)}$ = diastolic blood pressure; $BP_{(sys)}$ = systolic blood pressure; Int = intensity; MPO = mean power output; RER = respiratory exchange ratio; RPE = rating of perceived exertion; Sup = supplement; V_E = minute ventilation; VO_2 = rate of oxygen uptake.

Table 4. The effects of caffeine supplementation (5 $mg \cdot kg^{-1}$) on heart rate and respiratory responses during a cycling time-trial (~ 30 mins), along with blood lactate and glucose responses following that same time-trial. Values are means ± standard deviations.

Supplement	BF ($breaths \cdot min^{-1}$)	[BGl] ($mmol \cdot L^{-1}$)	[BLa] ($mmol \cdot L^{-1}$)	Heart rate ($b \cdot min^{-1}$)	RER	$\dot{V}_E$ ($L \cdot min^{-1}$)	$\dot{V}O_2$ ($L \cdot min^{-1}$)
Caffeine	44.1 ± 8.9*	5.97 ± 1.35*	9.21 ± 2.82*	168.1 ± 11.3*	0.96 ± 0.03*	127.2 ± 24.0*	3.54 ± 0.48
Placebo	42.5 ± 9.4	4.97 ± 1.20	7.38 ± 2.67	164.7 ± 11.6	0.94 ± 0.04	117.3 ± 25.6	3.44 ± 0.48

Note: BF = breathing frequency, [BGl] = blood glucose concentration; [BLa] = blood lactate concentration; RER = respiratory exchange ratio; $\dot{V}_E$ = minute ventilation; $\dot{V}O_2$ = rate of oxygen uptake. *significant effect ($p < 0.05$) of supplement.