Articles in PresS. J Appl Physiol (May 11, 2017). doi:10.1152/japplphysiol.00260.2017

Original article

Article Title: Dispelling the myth that habitual caffeine consumption influences the performance response to acute caffeine supplementation

Submission type: Original Investigation

Running head: Habitual caffeine intake and performance

Authors:

Lívia de Souza Gonçalves[1,2], Vitor de Salles Painelli[1], Guilherme Yamaguchi[1], Luana Farias de Oliveira[1], Bryan Saunders[1], Rafael Pires da Silva[1], Erika Maciel[2], Guilherme Giannini Artioli [1,2], Hamilton Roschel[1,2], Bruno Gualano[1, 2]

Affiliations:

[1] Applied Physiology & Nutrition Research Group, University of São Paulo, Brazil.

[2] Rheumatology Division, School of Medicine, University of São Paulo, Brazil.

Corresponding author:

Bruno Gualano

Av. Mello de Moraes 65

Butanta, 05508-030

Sao Paulo, SP, Brazil.

Phone: +55 11 26481337

Fax: +55 11 3813-5921

E-mail: gualano@usp.br

ABSTRACT

Objective: To investigate the influence of habitual caffeine intake on aerobic exercise performance responses to acute caffeine supplementation. **Methods:** A double-blind, crossover, counterbalanced study was performed. Forty male endurance-trained cyclists were allocated into tertiles according to their daily caffeine intake: low (58 ± 29 $mg \cdot d^{-1}$), moderate (143 ± 25 $mg \cdot d^{-1}$), and high consumers (351 ± 139 $mg \cdot d^{-1}$). Participants completed three trials in which they performed simulated cycling time-trials in the fastest time possible following ingestion of: caffeine (CAF: 6 $mg \cdot kg^{-1}$ BM), placebo (PLA), and no supplement (CON). **Results:** Mixed-model analysis revealed time-trial performance was significantly improved in CAF compared to PLA and CON (29.92±2.18 min vs 30.81±2.67 and 31.14±2.71 min; P = <0.0002). ANCOVA revealed no influence of habitual caffeine intake as a covariate on exercise performance (P=0.47). Time-trial performance was not significantly different between tertiles (P=0.75). No correlation was observed between habitual caffeine intake and absolute changes (CAF – CON) in time-trial performance with caffeine (P=0.524). Individual analysis showed that eight, seven and five individuals improved above the variation of the test in CAF in the low, moderate and high tertiles, respectively. A Fisher's Exact Test did not show any significant differences in the number of individuals who improved in CAF between the tertiles (P>0.05). Blood lactate and ratings of perceived exertion were not different between trials and tertiles (P>0.05). **Conclusion:** Performance effects of acute caffeine supplementation during a ~30 min cycling TT performance were not influenced by the level of habitual caffeine consumption.

Keywords: supplement; daily consumption; endurance; time-trial.

New & Noteworthy

- ✓ There has been a long-standing paradigm that habitual caffeine intake may influence the ergogenicity of caffeine supplementation.
- ✓ Low, moderate and high caffeine consumers showed similar absolute and relative improvements in cycling time-trial performance following acute supplementation of 6 $mg \cdot kg^{-1}$ BM caffeine.
- ✓ Performance effects of acute caffeine were not influenced by the level of habitual caffeine consumption, suggesting that high habitual caffeine intake does not negate the benefits of acute caffeine supplementation.

INTRODUCTION

The most consumed psychoactive substance worldwide, caffeine, is a xanthine-derived alkaloid (1,3,7-trimethilxanthine) naturally found in common dietary products, such as coffee, green-tea extracts, chocolate, and soda.(8) Ingestion of 5-6 $mg \cdot kg^{-1}$BM caffeine results in peak plasma caffeine concentrations of 30-49 $\mu mol \cdot L^{-1}$ within 40 to 60 minutes,(17) and its half-life may vary from 3 to 6 hours.(5) Caffeine use in sports is highly prevalent and supported by meta-analytic data, which has been shown that a median dose of 6 $mg \cdot kg^{-1}$BM 60 min before exercise h improves performance (+1.9%; 95% CI: 0.01% to 3.8%) during both endurance and high-intensity exercise protocols.(11)

The exact mechanisms by which caffeine exerts its ergogenic effects are still under debate, with suggested mechanisms including fatty acid mobilization and oxidation, endogenous glycogen content sparing, calcium ion release from the sarcoplasmic reticulum and potassium ion attenuation in the interstitium (for reviews see. (16, 24) However, its ergogenic effects are most likely due to its ability to act as an adenosine A_1 and A_{2A} receptor antagonist, ultimately resulting in increased dopamine and noradrenaline release, thereby promoting feelings of wakefulness and alertness, and decreasing the rate of perceived exertion and pain during exercise.(27) Regular consumption of caffeine has been associated with an upregulation of the number of adenosine receptors in vascular and neural tissues of the brain.(12, 13) Based on these observations, it could be speculated that habitual and non-habitual caffeine consumers would respond differently to caffeine supplementation during exercise. Indeed, Beaumont, et al.(4) recently demonstrated that 4 weeks of caffeine ingestion (1.5 – 3.0 $mg \cdot kg^{-1} \cdot day^{-1}$) resulted in an increased tolerance to acute caffeine supplementation in previously low habitual caffeine consumers, with the ergogenic effect of acute caffeine supplementation no longer apparent. Based upon these data, it appears reasonable to assume that individuals with different levels of long-term habitual caffeine consumption (i.e., low, moderate or high) may also show differences in the ergogenic response to acute caffeine supplementation.

Studies directly addressing the relationship between habitual intake of caffeine and the effect of caffeine supplementation on exercise performance are inconsistent. Dodd, et al.(10) showed no differences in the effects of caffeine on exercise performance between caffeine naïve individuals and habitual consumers. Conversely, Bell & McLellan(5) demonstrated that non-habitual caffeine consumers cycled longer than habitual consumers during a cycling-to-exhaustion protocol at 80% VO_{2max} following acute caffeine ingestion. These studies are

inconclusive due to several factors including their binary stratification of subjects as habitual (> 300 mg·day^{-1}) or non-habitual consumers (< 50 mg·day^{-1}), disregarding intermediary intakes. Moreover, these studies enrolled a relatively low total number of subjects (n < 21), hampering definitive conclusions. Finally, to assess performance, time-to-exhaustion tests were employed, which have been subjected to criticisms due to poor external validity and large variability.(9, 20) Notwithstanding the lack of solid evidence, there is a belief that individuals who have a low caffeine consumption may have greater performance improvements following acute caffeine supplementation than those who have a high caffeine consumption, which has led to practical recommendations such as removing/reducing caffeine from the diet before supplementing with caffeine for sports competition.(31)

In light of these contrasting data, this study aimed to investigate whether the long-standing notion holds true that habitual intake of caffeine (i.e., low, moderate, and high) influences the effects of acute caffeine supplementation on exercise performance using a large sample of volunteers and a reliable endurance exercise protocol. We hypothesized that habitual caffeine intake would influence the ergogenic effects of caffeine supplementation, with greater aerobic exercise performance gains in individuals with lower regular consumption.

MATERIALS AND METHODS

Participants

Forty-two recreationally-trained male cyclists, out of 125 screened, participated in this study, although only forty were included in the analysis since one individual did not complete the habitual intake form and another did not complete all exercise trials for reasons unrelated to the study. All of them were competitive cyclists at either amateur or professional level, trained at least 4 times/week, and cycled over 150 km/week. The exclusion criteria included the use of any dietary supplement (except carbohydrate and proteins) for at least 6 months prior to the study, and any prior use of anabolic steroids. Participants were fully informed of the nature and possible risks of the experimental procedures before their written consent was obtained. The study was approved by the University of São Paulo's Ethics Review Committee as part of a larger thematic project, the remaining data of which are reported elsewhere (Saunders et al., in press).(30)

Experimental design

This was a double-blind, counterbalanced, crossover study. Participants underwent three experimental trials: caffeine supplementation (CAF), placebo supplementation (PLA) and no supplement (CON). The experimental sessions were performed on different days at least 7 days apart. Participants were randomly assigned to the experimental trials using a Latin Square model.(25)

Participants visited our laboratory on six separate occasions. On the first day, height and body mass were measured, and individuals responded to a validated Food Frequency Questionnaire (FFQ) to assess habitual caffeine intake. Thereafter, a maximal incremental test was performed to determine VO_{2max}. On days 2 and 3, familiarizations to the main exercise test were performed. On days 4, 5, and 6, participants engaged in the main experimental trials, which consisted of a simulated cycling time-trial (TT) performed on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). All exercise tests were individually standardized to be carried out at the same time of day following a 6-h fasting period and 60-min after supplement ingestion.

To tightly control this study, participants were instructed to abstain from training, alcohol and caffeine-containing substances within the 24-h period prior to the experimental sessions. Participants were also asked to record a 24-h food recall before each test, from which carbohydrate, protein, fat and caloric intake were calculated. To assist the participants in refraining from dietary caffeine, they were provided with a comprehensive list of the main products containing caffeine. Additionally, the participants were given a book with instructions and illustrative examples on how to fill out the dietary recall. The food recalls were analyzed before each experimental session by a member of our team to ensure that participants did not consume any food or beverage containing caffeine.

To test the influence of habitual caffeine intake on the exercise responses to acute caffeine supplementation, participants were allocated into tertiles according to their dietary intake of caffeine using statistical software (STATISTICA v.10; StatSoft, Tulsa, USA). This resulted in the following sub-groups: low consumers (58 ± 29 mg·day^{-1}; range = 2 to 101 mg·day^{-1}; 95% CI: 44.2 – 71.7; *n* = 14), moderate consumers (143 ± 25 mg·day^{-1}; range = 104 to 183 mg·day^{-1}; 95% CI: 130.1 – 155.9; *n* = 12), and high consumers (351 ± 139 mg·day^{-1}; range = 190 to 583 mg·day^{-1}; 95% CI: 285.2 – 416.8; *n* = 14) (Table 1).

Maximum oxygen consumption and workload capacity

Participants performed a graded exercise test on a cycle ergometer (Lode Excalibur, Groningen, The Netherlands) to determine VO_{2max}. Individual seat and handlebar position were recorded and replicated for each subsequent visit. Participants began pedaling at a power output of 75 W, with 50 W increments every 4 min for four submaximal stages. Thereafter, work rate was increased by 30 W every minute until volitional exhaustion. The last completed stage plus the fraction of time spent in the final non-completed stage multiplied by 30 W was defined as an individual's power max (W_{max}). Heart rate (HR) was monitored every minute using a transmitter/telemetry unit (Polar System, Finland). Pulmonary gas exchange was determined breath by breath for O_2 and CO_2 concentrations and minute ventilation by use of a portable gas analysis system (K4 b^2, Cosmed, Rome, Italy). The gas analyzer was calibrated immediately before and verified after each test by using a certified gravimetric gas mixture (BOC Gases, Chatswood, Australia). The ventilometer was calibrated pre-

exercise and verified post-exercise using a 3-liter syringe in accordance with the manufacturer's instructions. There were no significant differences between pre- and post-test calibrations for any test.

Simulated cycling time-trial

Participants reported to the laboratory at individually standardized times after a 6-h fasting period. Prior to the main exercise, participants underwent a 5-min warm-up at 125 W, immediately followed by the simulated cycling km TT, which has been shown to be reliable for recreationally-trained cyclists following a familiarization session (CV = 2.9%, intraclass correlation = 0.87 [0.67 – 0.95], smallest meaningful change = 4.8 W; Oliveira et al., in press).(28) Participants were required to perform a set amount of work (mean: 420 ± 69 kJ) in the shortest time possible. Individual total work to be performed was calculated according to the equation of Jeukendrup, Saris, Brouns, and Kester, 1996:(22)

Total amount of work = 0.85 x W_{max} x 1800

Where W_{max} is the maximal workload capacity determined at day 1 and 1800 is duration in seconds (equivalent to 30-min). The ergometer was set in linear mode so that 85% W_{max} was obtained when the participants cycled at 95 rpm. The participants were kept unaware of performance-related information (exercise time and cadence) during the tests. The only information the participants received during the test was the percentage of work performed relative to the preset task, namely following 25, 50, 75, 90 and 100% completion of the total work done (%TWD). At these set intervals during the trial, participants were asked to rate their perceived exertion (RPE) using the 6- to 20-point Borg scale(6) and a fingertip blood sample was collected for the analysis of lactate. A small aliquot (20 μL) of blood was taken and homogenized in a microtube containing the same volume of an ice-cold 2% NaF solution. Samples were centrifuged at 2000 g for 5 min at 4°C to separate plasma from erythrocytes. Plasma was removed and stored at -80 °C until analysis. Plasma lactate was determined spectrophotometrically using an enzymatic-colorimetric method as supplied by a commercially available kit (Katal, Interteck, Sao Paulo, Brazil).No encouragement was provided during the tests. Finally, all persons not involved in the study were excluded from the laboratory to prevent any external disruption.

Supplementation protocol

The participants received a dose of anhydrous caffeine (6 mg·kg^{-1}BM) or placebo (dextrose) provided in gelatin capsules identical in color, size and appearance. No substance was administered in CON. The capsules were ingested with *ad libitum* water intake 60-min before the exercise tests. Participants rested quietly for 60-min before starting the test in all session. Supplements for each participant were prepared and separated by a non-affiliated researcher to ensure double-blinding.

Caffeine and food intake assessments

Habitual caffeine intake was assessed by a specific FFQ adapted from a previously validated questionnaire(7) under the supervision of a qualified nutritionist. The questionnaire was employed to assess the habitual consumption of dietary products rich in caffeine. Portions, in household measures, were used to assess the amount of food consumed according to the following frequency of consumption: a) more than three times a day, b) two to three times a day, c) once a day, d) five to six times a week, e) two to four times per week, f) once a week, g) three times per month, h) rarely or never. The list was composed of 10 dietary products with high caffeine content, according to the Food and Drug Administration(32), namely: a) espresso and espresso drinks, b) brewed coffee, c) instant coffee, d) green tea, e) black tea, f) energetic drinks, g) regular and diet colas drinks, h) dark and milk chocolates, i) sweet cocoa powder, and j) caffeine supplements. Where possible, exact brands of products consumed were identified and subsequent caffeine content of these products obtained. Previously published nutritional tables were used for database construction.(8, 32) Food intake was assessed by means of six 24-h dietary recalls recorded before the exercise sessions (one VO_{2max} test, two familiarization sessions and three main trials). Energy and macronutrient intake were analyzed by the software Avanutri online (Avanutri, Rio de Janeiro, Brazil).

Statistics

Data are presented as mean ± standard deviation (with 95% confidence intervals - CI) and were analysed using SAS statistical software (v. 9.3). Statistical significance was accepted at $P \leq 0.05$. Mixed-model analyses with repeated measures were used to compare the overall effect of caffeine on time-trial performance (TT performance) as well as to compare the food consumption between experimental trials; Trial (CON, PLA and CAF) was used as a fixed factor and participants as a random factor. A mixed-model was used to analyse the overall effect of caffeine on RPE and lactate, where Trial (CON, PLA and CAF) and %TWD (0, 25, 50, 75, 90 and 100%) were considered fixed factors.

The absolute changes in TT performance were also analysed using a mixed-model analysis of covariance (ANCOVA) assuming habitual caffeine intake as a covariate, Trial (CAF and PLA) as a fixed factor and participants as a random factor. A further mixed model was employed to examine the influence of habitual caffeine intake (i.e., Consumption) on the absolute changes in TT performance (i.e., CAF – CON and PLA – CON) in response to supplementation, where Consumption (low, moderate and high) and Trial (PLA and CAF) were fixed factors. A Kenward-Roger correction was used to account for this unbalanced design and make approximate inferences about these fixed effects in a mixed linear model. Tukey-Kramer adjustments for multiple comparisons were performed whenever a significant F-value was obtained. The abovementioned inferential analyses were also performed having the subjects divided into tertiles according to their habitual caffeine intake relative to body weight. However, we opted for not reporting these data as i) results were virtually the same despite the correction for body weight; and ii) literature commonly presents habitual caffeine intake as absolute values,(5, 10) thus facilitating the contrast of findings.

Pearson's correlation was performed between habitual caffeine intake and the absolute change in TT performance. Magnitude based inferences (MBIs) were used to determine the practical significance of caffeine on TT performance using a spreadsheet to establish the likelihood of a meaningful effect on exercise capacity.(3) The smallest worthwhile improvement in time to completion was 36 s, equivalent to half the unbiased typical error associated with the measurement based upon the reliability data from 50 individuals. Qualitative descriptors were assigned to the positive percentile scores as follows: <1%, *almost certainly not beneficial*; 1-5%, *very unlikely beneficial*; 5-25%, *unlikely beneficial*; 25-75%, *possibly beneficial*; 75-95%, *likely beneficial*; 95-99%, *very likely beneficial*; > 99%, *almost certainly beneficial*.(19) A Fischer Exact test

was used to determine any differences in i) supplement identification between trials, and ii) the proportion of individuals who improved in CAF between tertiles.

RESULTS

Overall effect of caffeine on TT performance, RPE and plasma lactate concentrations

Three outliers were visually identified using boxplot analysis; each of these individuals had a performance change in excess of 16% in CAF (two improved, one worsened). Thus, we performed all analyses both with, and without, these three participants. Since the main outcomes remained the same, we decided to only report the analyses with all individuals.

No order effect was shown (F = 0.781; p = 0.461). There was a main effect of Trial on TT performance (F = 9.472; P = <0.01) (Figure 1A), with *post-hoc* tests revealing that performance in CAF was significantly improved compared to PLA (+2.4 ± 5.5%; P = 0.01; 95% CI = -93.3 to -3.3 s; 78% *likely beneficial*) and CON (+3.3 ± 6.4%; P = <0.008; 95% CI = -111.4 to -21.3 s; 92% *likely beneficial*). No significant difference in performance was shown between PLA and CON (+0.8 ± 6.3%; P = 0.62; 95% CI = -63.1 to 26.9 s; 18% *unlikely beneficial*).

There was no main effect of Trial (F = 0.22; P = 0.805) on RPE, but there was a significant main effect of %TWD (F = 118.08; P < 0.0001); RPE increased throughout the exercise test although there were no differences at any time point between CAF, PLA and CON (Figure 1B).

No main effect of Trial (F = 1.44; P = 0.240) on plasma lactate concentrations was observed, although there was a significant main effect of %TWD (F = 69.42; P < 0.0001); plasma lactate significantly increased with exercise compared to rest and post-ingestion for CAF, PLA and CON, although no differences at any time point were observed between the trials. Nevertheless, a significant within-group increase was observed for CAF at 100% compared to the previous time points (i.e., 0, 25, 50, 75, and 90%; all P < 0.05) (Figure 1C).

Influence of habitual caffeine intake on TT performance, RPE and plasma lactate concentrations in response to acute caffeine supplementation

ANCOVA analysis showed no significant effect of habitual caffeine intake as a covariate (F= 0.54, P = 0.47). There was no Consumption x Trial interaction effect on absolute TT performance (F = 0.47; P = 0.756) (Figure 2A), or on TT performance when CAF and PLA data were treated as absolute change compared to CON (F = 1.64; P = 0.207). The absolute difference between CAF and CON was not significantly different between tertiles (low vs moderate: P = 1.000; low vs high: P = 0.996; moderate vs high: P = 0.986) (Figure 2B), nor was the difference between CAF and PLA (data not shown).

MBIs suggested *possible* to *likely* improvements in CAF in the tertiles (Table 2). Individual analysis showed that eight, seven and five individuals improved above the variation of the test with CAF compared to CON in low, moderate and high, respectively (Figure 3), and a Fisher's Exact Test did not show any significant differences in the number of individuals who improved between the tertiles (P > 0.05). There was no correlation between habitual caffeine intake and absolute difference (i.e., CAF – CON) in performance (Figure 4).

No main effect of Consumption (F = 0.63; P = 0.538) or interaction effects (Consumption x Trial x %TWD - F = 0.58; P = 0.945) were observed for RPE between tertiles (data not shown). In addition, no main effect of Consumption (F = 0.77; P = 0.470) or interaction effects (Consumption x Trial x %TWD - F = 1.02; P = 0.437) were detected for plasma lactate concentrations (data not shown).

Food consumption analysis

As expected, tertiles differed significantly in their habitual caffeine intake (P = 0.0001) (Table 1). No significant differences for protein, carbohydrate, fat or caloric intake were observed between low, moderate and high caffeine consumers before CAF, PLA and CON (Table 3).

Side effects and blinding efficacy

Tachycardia was the most frequently reported side effect in CAF (n = 9), followed by feelings of increased wakefulness and attention (n = 7). Despite this, only 17 participants correctly guessed the supplement ingested during both CAF and PLA. Moreover, 13 and 17 participants did not know what supplement they had

ingested during CAF and PLA. In addition, 12 and 8 participants incorrectly guessed the supplement ingested during CAF and PLA. Fisher's Exact Test did not show any significant differences between trials for the proportion of supplement identification (P = 0.57). Fisher's Exact Test did not demonstrate any significant differences between low, moderate and high consumers for the reported side effects (P = 0.77).

DISCUSSION

There has been a long-standing paradigm that habitual caffeine intake may influence the ergogenicity of caffeine supplementation. In contrast to this belief, this study showed that the level of habitual caffeine ingestion was not associated with the magnitude of improvement in cycling TT performance following acute caffeine supplementation.

Caffeine supplementation improved exercise performance by 3.3% compared to CON and 2.4% compared to PLA. These data are in accordance with previous research, with meta-analytic data showing a mean overall improvement of 1.9% with caffeine.(11) However, individual responses to caffeine supplementation exist;(1) in our study, approximately half of the individuals improved above the variation of the test with caffeine. Habitual caffeine intake has been suggested as a potential factor underlying heterogeneous responses to this supplement.(5)

It is known that caffeine acts on the central nervous system antagonizing the adenosine A_1 and A_2 receptors, increasing circulating dopamine and noradrenaline.(27) Experimental data suggest that habitual intake of caffeine is associated with an increased regulation of the adenosine receptors.(12) In support of this notion, clinical studies have demonstrated a differential response to caffeine supplementation regarding mood and cognitive performance in low and high caffeine consumers.(2, 18) This suggests that habitual caffeine intake could influence exercise performance following caffeine supplementation, though current evidence is contrasting.(5, 10) Recently, Beaumont, et al.(4) showed that 4 weeks of caffeine supplementation induced a tolerance to the performance benefits of acute caffeine supplementation in low habitual caffeine consumers (< 75 $mg \cdot day^{-1}$). However, the lack of a post-supplementation placebo trial, to provide a direct comparison with the post-supplementation caffeine trial does not allow conclusive interpretation of the results of this study. However, the relatively acute supplementation period (28 days) may have resulted in several acute adaptations

which do not represent chronic or habitual consumption. In the current study, there was no association between habitual caffeine intake and exercise improvements with caffeine supplementation. Furthermore, low, moderate and high consumers showed similar absolute and relative improvements in exercise performance. This concurs with previous findings suggesting no difference between high and low consumers concerning caffeine's ability to reduce muscle pain during cycling exercise, in which perceptions of pain and effort have been recognized as part of pacing strategies.(14)

Divergent results seen in the literature could be partially explained by methodological differences. Firstly, previous studies employed capacity tests which are known to have limited external validity and high variability (>10%).(9, 20) We employed an exercise performance test with low variability (2.9%) in similarly trained participants.(28) Additionally, we allocated participants into tertiles according to their habitual caffeine intake as assessed by a validated FFQ method, resulting in distinct sub-groups with heterogeneous caffeine intake (i.e., low: 2 to 101; moderate: 104 to 183; high: 190 to 583 $mg \cdot day^{-1}$), allowing the investigation of a broad spectrum of habitual caffeine intake on exercise performance in response to caffeine supplementation. The calculated tertiles in this study are in agreement with the estimated average consumption of caffeine among the healthy population (mean: 165 $mg \cdot day^{-1}$; 90th percentile: 380 $mg \cdot day^{-1}$).(26) In contrast, other studies defined participants as caffeine consumers and naïve or non-consumers, without describing a method to support this approach of categorizing individuals on opposite ends of the spectrum (i.e., <50 or >300 $mg \cdot day^{-1}$).(5, 10) The widely differing range used to characterize habitual caffeine intake may have contributed to previous contrasting results. It also important to note that such a comprehensive comparison performed in the current study was only possible due to the large sample size (n = 40), which is higher than previous studies (17 – 21 participants).(5, 10) In fact, this large sample allowed us to further explore our hypothesis *a posteriori* by allocating participants into quartiles and quintiles, with still no significant differences between them in relation to performance (data not shown).

Athletes are commonly encouraged to refrain from caffeinated products for up to 4 days before supplementing with caffeine to enhance the efficacy of acute supplementation.(31) Despite this, Irwin, et al.(21) showed similar improvements in exercise with caffeine in habitual consumers regardless of a 4 day withdrawal period. Similarly, Van Soeren, et al.(35) showed equal exercise improvements with acute caffeine supplementation in habituated consumers following no, 2-days and 4-days of caffeine withdrawal. In the

current study, to control the protocol, individuals were asked to refrain from caffeine only in the 24-h prior to exercise. It could be suggested that this period of withdrawal may have created an artefact by "normalising" the recent caffeine history of the participants. However, sudden caffeine cessation is unlikely to lead to withdrawal symptoms, any of which are likely only to be moderate,(27) while the previously mentioned studies showed that up to 4 days of caffeine withdrawal do not influence the exercise responses to an acute dose of caffeine supplementation in habitual caffeine consumers.(21, 35) Therefore, we are satisfied that our methodology was sufficient to test our initial hypothesis, although future research investigating the effects of habitual consumption should perhaps record, and not prohibit, caffeine consumption in the 24 h preceding exercise.

At doses of 3-9 $mg \cdot kg^{-1}$BM, caffeine is known to induce specific side effects, such as tachycardia, anxiety, gastrointestinal discomfort, tremors, and insomnia.(17) It is believed that habitual caffeine consumers are less susceptible to these side-effects at the same relative doses when compared to non-consumers.(15) However, when participants were asked about the perceived effects 60-min after acute supplementation, they reported the same side effects regardless of habitual caffeine intake. These results reinforce the notion that the perceptual and exercise individual responses to caffeine may be triggered by other factors, such as genetics, rather than habitual caffeine intake *per se*. In fact, genome-wide association studies have suggested that single nucleotide polymorphisms in genes related to caffeine metabolism (aryl-hydrocarbon receptor [*AHR*], cytochrome P450 1A1 and 1A2 [*CYP1A1-CYP1A2,* Prenyl (Decaprenyl) Diphosphate Synthase, Subunit 2 associated with habitual caffeine and coffee consumption.(23, 29) If the grouping of individuals in the current study reflect different polymorphisms of various caffeine-related genes, then our data would challenge the notion that certain genotypes result in carry over effects to performance. However, this is highly speculative since genotypic analysis was beyond the scope of this study though further studies assessing caffeine-related polymorphisms and exercise are warranted.

A limitation of this study is that we were unable to measure blood caffeine concentrations. However, all previous studies employing doses of 3-9 $mg \cdot kg^{-1}$BM 60-min before a given exercise task have reported significant increases in caffeine concentration.(17, 34) Therefore, it can be assumed that our supplementation protocol was effective in increasing blood caffeine levels. Furthermore, these findings must be confined to male participants, since sexual dimorphism seems to exist in response.(33) We ensured tight control of our experimental measures by having individuals attend the laboratory following a 6-h fast and 24-h post-caffeine

(and alcohol) ingestion. We acknowledge, however, that exercising in a fasted state does not represent the recommendations of current sports nutrition guidelines or the "real life" practices of cyclists, nor does a withdrawal period appear necessary to elicit performance improvements following acute caffeine supplementation.(21) Although a 6 $mg \cdot kg^{-1}$BM was employed in this study, we acknowledge that 3 $mg \cdot kg^{-1}$BM is sufficient to induce performance effects.(17)

In conclusion, performance benefits with acute caffeine supplementation during a ~30 min cycling TT were not influenced by the level of habitual caffeine consumption, refuting the long-standing notion that habitual caffeine intake may negatively affect exercise performance in response to caffeine supplementation.

ACNOWLEDGMENTS

We wish to thank the Laboratório de Determinantes Energéticos de Desempenho Esportivo (LADESP) for access to the ergometer used in this study and the volunteers for their efforts. Livia S. Gonçalves and Luana F. de Oliveira have been financially supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Vitor S. Painelli, Bruno Gualano, Bryan Saunders and Rafael Pires da Silva have been financially supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grants number: 2013/04806-0, 2013/14746-4, 2011/19513-2, FAPESP 2012/13026-5). Bryan Saunders (150513/2015-1), Bruno Gualano and Hamilton Roschel have been financially supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORSHIP INFORMATION

Significant manuscript writer – Livia de Souza Gonçalves, Bruno Gualano, Bryan Saunders, Vitor Painelli

Significant manuscript reviewer/reviser –Hamilton Roschel, Guilherme Giannini Artioli

Concept and design – Bruno Gualano, Bryan Saunders, Hamilton Roschel, Guilherme Giannini Artioli

Data acquisition – Bryan Saunders, Luana Farias de Oliveira, Livia Souza Gonçalves, Guilherme Yamaguchi, Vitor de Salles Painelli, Rafael Pires da Silva, Erika Maciel

Data analysis and interpretation – Livia Souza Gonçalves, Bryan Saunders, Luana Farias de Oliveira, Rafael Pires da Silva, Guilherme Yamaguchi, Erika Maciel

Statistical expertise –Hamilton Roschel, Vitor de Salles Painelli

REFERENCES

1. **Alsene K, Deckert J, Sand P, and de Wit H**. Association between A2a receptor gene polymorphisms and caffeine-induced anxiety. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 28: 1694-1702, 2003.
2. **Attwood AS, Higgs S, and Terry P**. Differential responsiveness to caffeine and perceived effects of caffeine in moderate and high regular caffeine consumers. *Psychopharmacology* 190: 469-477, 2007.
3. **Batterham AM, and Hopkins WG**. Making meaningful inferences about magnitudes. *International journal of sports physiology and performance* 1: 50-57, 2006.
4. **Beaumont R, Cordery P, Funnell M, Mears S, James L, and Watson P**. Chronic ingestion of a low dose of caffeine induces tolerance to the performance benefits of caffeine. *Journal of sports sciences* 1-8, 2016.
5. **Bell DG, and McLellan TM**. Exercise endurance 1, 3, and 6 h after caffeine ingestion in caffeine users and nonusers. *Journal of applied physiology* 93: 1227-1234, 2002.
6. **Borg GA**. Psychophysical bases of perceived exertion. *Medicine and science in sports and exercise* 14: 377-381, 1982.
7. **Bühler. E, Lachenmeier. DW, Schlegel. K, and Winkler. G**. Development of a tool to assess the caffeine intake among teenagers and young adults. *Ernährungs Umschau* 61: 58-63, 2013.
8. **Burke LM**. Caffeine and sports performance. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme* 33: 1319-1334, 2008.
9. **Currell K, and Jeukendrup AE**. Validity, reliability and sensitivity of measures of sporting performance. *Sports medicine* 38: 297-316, 2008.
10. **Dodd SL, Brooks E, Powers SK, and Tulley R**. The effects of caffeine on graded exercise performance in caffeine naive versus habituated subjects. *European journal of applied physiology and occupational physiology* 62: 424-429, 1991.
11. **Doherty M, and Smith PM**. Effects of caffeine ingestion on exercise testing: a meta-analysis. *International journal of sport nutrition and exercise metabolism* 14: 626-646, 2004.
12. **Fredholm BB**. Adenosine actions and adenosine receptors after 1 week treatment with caffeine. *Acta physiologica Scandinavica* 115: 283-286, 1982.
13. **Fredholm BB, Battig K, Holmen J, Nehlig A, and Zvartau EE**. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacological reviews* 51: 83-133, 1999.
14. **Gliottoni RC, Meyers JR, Arngrimsson SA, Broglio SP, and Motl RW**. Effect of caffeine on quadriceps muscle pain during acute cycling exercise in low versus high caffeine consumers. *International journal of sport nutrition and exercise metabolism* 19: 150-161, 2009.
15. **Goldstein ER, Ziegenfuss T, Kalman D, Kreider R, Campbell B, Wilborn C, Taylor L, Willoughby D, Stout J, Graves BS, Wildman R, Ivy JL, Spano M, Smith AE, and Antonio J**. International society of sports nutrition position stand: caffeine and performance. *Journal of the International Society of Sports Nutrition* 7: 5, 2010.
16. **Graham TE**. Caffeine and exercise: metabolism, endurance and performance. *Sports medicine* 31: 785-807, 2001.
17. **Graham TE, and Spriet LL**. Metabolic, catecholamine, and exercise performance responses to various doses of caffeine. *Journal of applied physiology* 78: 867-874, 1995.
18. **Haskell CF, Kennedy DO, Wesnes KA, and Scholey AB**. Cognitive and mood improvements of caffeine in habitual consumers and habitual non-consumers of caffeine. *Psychopharmacology* 179: 813-825, 2005.
19. **Hopikns W**. Probabilities of clinical or practical significance: Sportscience 6. *http://wwwsportsciorg/jour/0201/wghprobhtm* 2002.
20. **Impellizzeri FM, and Marcora SM**. Test validation in sport physiology: lessons learned from clinimetrics. *International journal of sports physiology and performance* 4: 269-277, 2009.
21. **Irwin C, Desbrow B, Ellis A, O'Keeffe B, Grant G, and Leveritt M**. Caffeine withdrawal and high-intensity endurance cycling performance. *Journal of sports sciences* 29: 509-515, 2011.
22. **Jeukendrup A, Saris WH, Brouns F, and Kester AD**. A new validated endurance performance test. *Medicine and science in sports and exercise* 28: 266-270, 1996.
23. **Josse AR, Da Costa LA, Campos H, and El-Sohemy A**. Associations between polymorphisms in the AHR and CYP1A1-CYP1A2 gene regions and habitual caffeine consumption. *The American journal of clinical nutrition* 96: 665-671, 2012.

24. **Magkos F, and Kavouras SA**. Caffeine use in sports, pharmacokinetics in man, and cellular mechanisms of action. *Critical reviews in food science and nutrition* 45: 535-562, 2005.
25. **Mason RL, Gunst RF, and JL H**. Statistical Design and Analysis of Experiments: With Applications to Engineering and Science, Second Edition. *John Wiley & Sons, Inc, Hoboken, New Jersey* 2003.
26. **Mitchell DC, Knight CA, Hockenberry J, Teplansky R, and Hartman TJ**. Beverage caffeine intakes in the U.S. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 63: 136-142, 2014.
27. **Nehlig A**. Are we dependent upon coffee and caffeine? A review on human and animal data. *Neuroscience and biobehavioral reviews* 23: 563-576, 1999.
28. **Oliveira LF, Yamaguchi G, Painelli VS, Silva RP, Gonçalves L, Gualano G, and Saunders B**. Comprehensive reliability analysis of a work-based (~420 kJ) cycling time-trial in recreationally-trained individuals. *Journal of Science and Cycling* In press.
29. **Pirastu N, Kooyman M, Robino A, van der Spek A, Navarini L, Amin N, Karssen LC, Van Duijn CM, and Gasparini P**. Non-additive genome-wide association scan reveals a new gene associated with habitual coffee consumption. *Scientific reports* 6: 31590, 2016.
30. **Saunders B, de Oliveira LF, da Silva RP, de Salles Painelli V, Goncalves LS, Yamaguchi G, Mutti T, Maciel E, Roschel H, Artioli GG, and Gualano B**. Placebo in sports nutrition: a proof-of-principle study involving caffeine supplementation. *Scandinavian journal of medicine & science in sports* 2016.
31. **Sokmen B, Armstrong LE, Kraemer WJ, Casa DJ, Dias JC, Judelson DA, and Maresh CM**. Caffeine use in sports: considerations for the athlete. *Journal of strength and conditioning research* 22: 978-986, 2008.
32. **Somogyi L**. Caffeine Intake in the U.S. Population. *http://wwwfdaorg* 2010.
33. **Tantcheva-Poor I, Zaigler M, Rietbrock S, and Fuhr U**. Estimation of cytochrome P-450 CYP1A2 activity in 863 healthy Caucasians using a saliva-based caffeine test. *Pharmacogenetics* 9: 131-144, 1999.
34. **Tarnopolsky MA**. Caffeine and endurance performance. *Sports medicine* 18: 109-125, 1994.
35. **Van Soeren MH, and Graham TE**. Effect of caffeine on metabolism, exercise endurance, and catecholamine responses after withdrawal. *Journal of applied physiology* 85: 1493-1501, 1998.

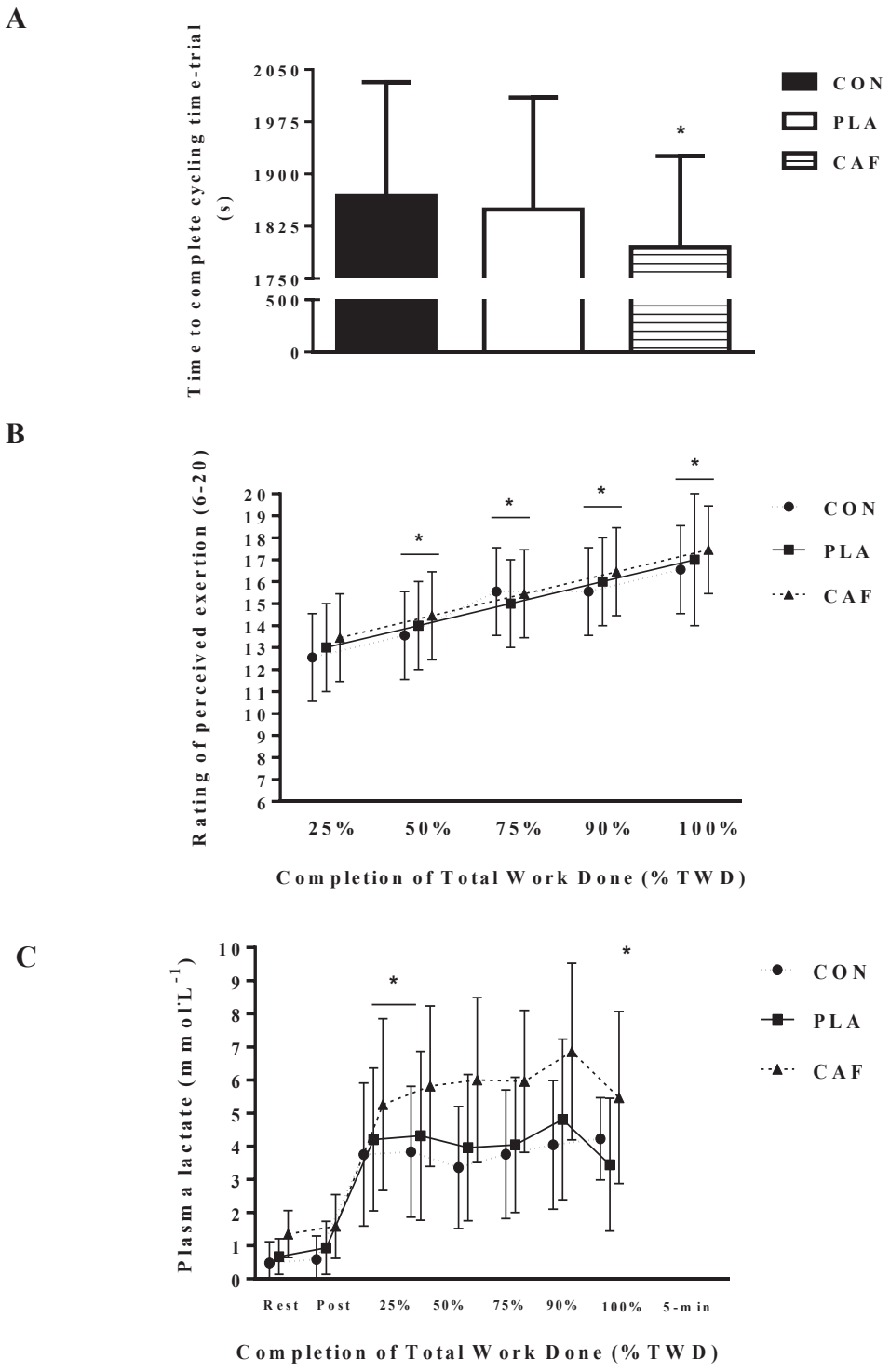
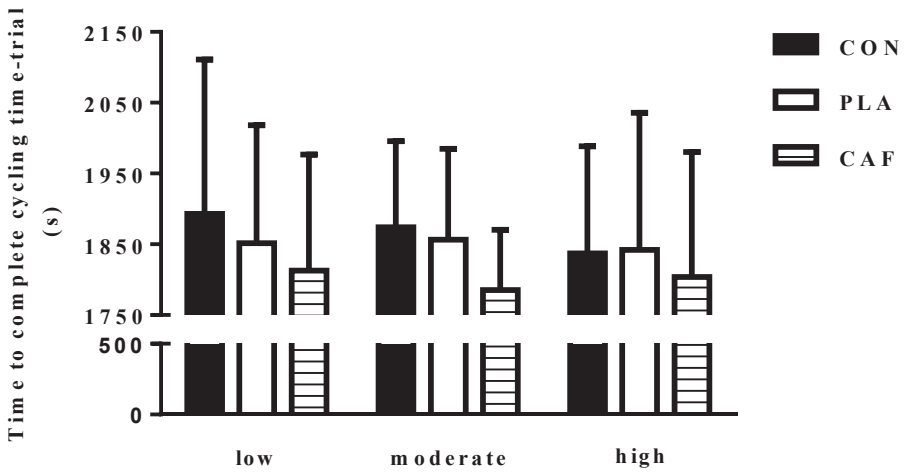


Figure 1: Panel A: Time-trial performance with caffeine (CAF), placebo (PLA) or no supplement (CON) * indicates significant difference ($P < 0.05$) compared to PLA and CON. Panel B: Rating of perceived exertion throughout the simulated time-trial with caffeine (CAF), placebo (PLA) or no supplement (CON). * indicates significant difference ($P < 0.05$) compared to the previous stage. Panel C: Plasma lactate ($mmol \cdot L^{-1}$) throughout exercise with caffeine (CAF), placebo (PLA) or no supplement (CON). * indicates significant difference ($P < 0.05$) compared to the previous stage.

A



B

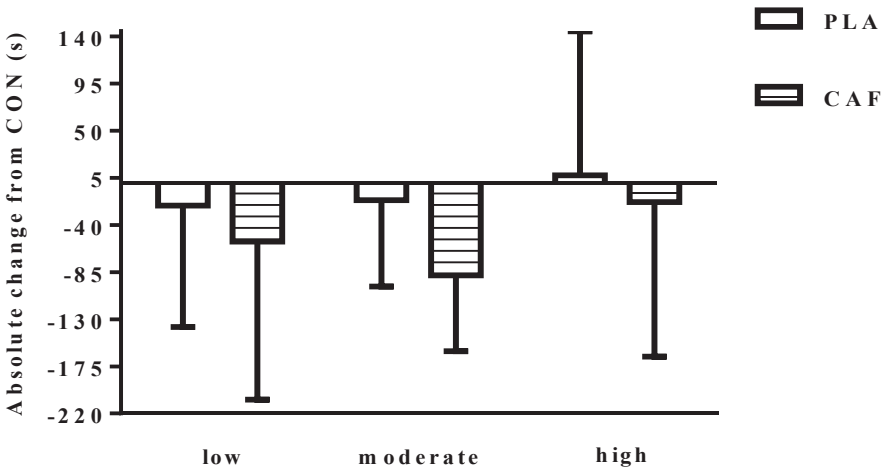


Figure 2: Panel A: Time-trial performance in caffeine (CAF), placebo (PLA) or no supplement (CON) separated according to habitual caffeine intake ($P > 0.05$). Panel B: Absolute change (compared to CON) in time-trial performance after caffeine (CAF) or placebo (PLA) separated according to habitual caffeine intake ($P > 0.05$).

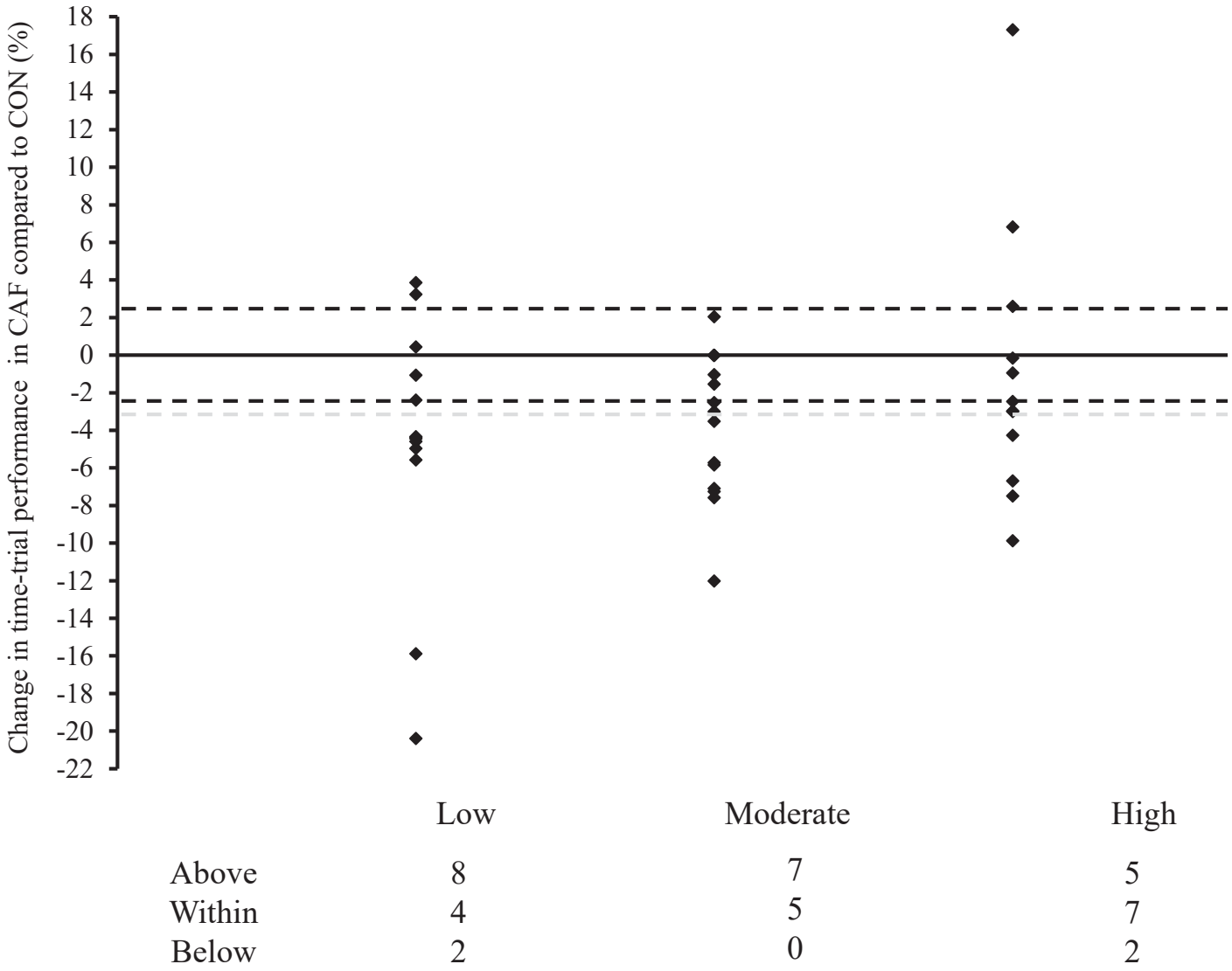


Figure 3: Individual relative change in time-trial performance in CAF vs. CON for low, moderate and high consumers. The black dashed line represents the natural variation of the test (±3.0%); the grey dashed line represents the mean overall improvement with caffeine (+3.3%). The number of individuals who improved above, were within, or worsened beyond the natural variation of the test in each sub-group is displayed below the graph (P>0.05).

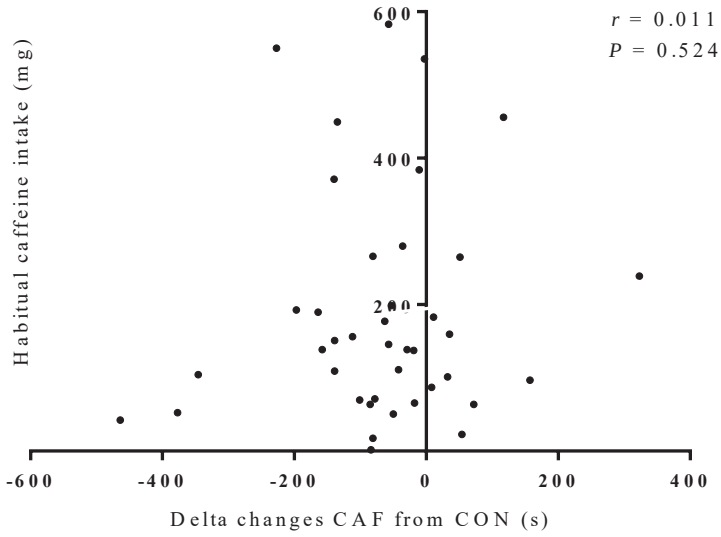


Figure 4: Correlation between habitual caffeine intake and absolute change in exercise performance in CAF vs. CON ($P > 0.05$).

Table 1. Participant characteristics

Mean (SD)	Low (*n*=14)	Moderate (*n*=12)	High (*n*=14)
Age	34 ± 9	37 ± 7	37 ± 8
Height (m)	1.74 ± 0.08	1.77 ± 0.05	1.77 ± 0.03
Weight (kg)	72.08 ± 11.13	74.95 ± 4.92	76.05 ± 8.42
VO_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	50.10 ± 8.45	51.00 ± 6.08	50.90 ± 7.54
Caffeine intake (mg/day)*	58 ± 29	143 ± 25	351 ± 139

Low, moderate and high = habitual caffeine intake
*Significant difference between tertiles ($P < 0.05$)

Table 2. Magnitude-based inferences for time-trial performance across tertiles

	Difference (%)	Chances of treatment	Chances of treatment	Chances of treatment being

		being positive (%)	being trivial (%)	negative (%)
Low				
CAF vs CON	-3.71 ± 7.62	82	17	1
CAF vs. PLA	-1.57 ± 4.25	51	49	0
PLA VS. CON	1.50 ± 15.25	53	44	3
Moderate				
CAF vs CON	-4.57 ± 3.63	99	1	0
CAF vs. PLA	-3.69 ± 4.65	93	6	1
PLA vs. CON	-0.28 ± 9.83	40	53	7
High				
CAF vs. CON	-0.88 ± 7.85	49	46	5
CAF vs. PLA	-1.4 ± 7.68	55	43	2
PLA vs. CON	-1,38 ± 13,77	14	66	20

Legend: CON = control, PLA = placebo; CAF = caffeine
Low, moderate and high = habitual caffeine intake

Table 3: Food consumption data of participants prior to the experimental sessions.

	CON			PLA			CAF		
	Low	Moderate	High	Low	Moderate	High	Low	Moderate	High
Protein (g)	114 ± 42	114 ± 36	123 ± 46	122 ± 61	99 ± 37	105 ± 59	114 ± 59	104 ± 64	128 ± 58
Carbohydrate (g)	360 ± 89	347 ± 105	334 ± 144	354 ± 108	295 ± 76	306 ± 164	357 ± 132	351 ± 151	319 ± 222
Fat (g)	82 ± 21	80 ± 29	77 ± 22	87 ± 36	66 ± 25	80 ± 46	86 ± 30	74 ± 46	84 ± 28
Caloric intake (kcal)	2548 ± 553	2711 ± 1116	2631 ± 1045	2708 ± 716	2338 ± 504	2383 ± 1032	2637 ± 877	2474 ± 1062	2628 ± 1051