Full title: Chronic ingestion of a low-dose of caffeine induces tolerance to the performance benefits of caffeine

Running title: Caffeine tolerance and endurance performance

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Key words: Fatigue, habituation, exercise metabolism, stimulants, supplements

Abstract

This study examined effects of four weeks of caffeine supplementation on endurance performance. Eighteen low-habitual caffeine consumers (<75 mg·day^{-1}) were randomly assigned to ingest caffeine (1.5 – 3.0 mg·kg^{-1}day^{-1}; titrated) or placebo for 28 days. Groups were matched for age, body mass, $\dot{V}O_{2peak}$ and W_{max} (P>0.05). Before supplementation, all participants completed one $\dot{V}O_{2peak}$ test, one practice trial and two experimental trials (acute 3 mg·kg^{-1} caffeine [precaf] and placebo [testpla]). During the supplementation period a second $\dot{V}O_{2peak}$ test was completed on day 21 before a final, acute 3 mg·kg^{-1} caffeine trial (postcaf) on day 29. Trials consisted of 60 min cycle exercise at 60% $\dot{V}O_{2peak}$ followed by a 30 min performance task. All participants produced more external work during the precaf trial than testpla, with increases in the caffeine (383.3 ±75 kJ vs. 344.9 ± 80.3 kJ; Cohen's d effect size [ES] =0.49; P=0.001) and placebo (354.5 ± 55.2 kJ vs. 333.1 ± 56.4 kJ; ES=0.38; P=0.004) supplementation group, respectively. This performance benefit was no longer apparent after four weeks of caffeine supplementation (precaf: 383.3 ± 75.0 kJ vs. postcaf: 358.0 ± 89.8 kJ; ES=0.31; P=0.025), but was retained in the placebo group (precaf: 354.5 ± 55.2 kJ vs. postcaf: 351.8 ± 49.4 kJ; ES=0.05; P>0.05). Circulating caffeine, hormonal concentrations and substrate oxidation did not differ between groups (all P>0.05). Chronic ingestion of a low dose of caffeine develops tolerance in low-caffeine consumers. Therefore, individuals with low-habitual intakes should refrain from chronic caffeine supplementation to maximise performance benefits from acute caffeine ingestion.

Key words: Fatigue, habituation, exercise metabolism, stimulants, supplements

Introduction

Acute caffeine (1,3,7-trimethylxanthine) supplementation approximately one hour before exercise improves endurance performance in laboratory-based studies (Burke, 2008). The same occurs in the field (Berglund & Hemmingsson, 1982), leading to its widespread use by athletes during competition (Desbrow & Leveritt, 2006). To determine optimum conditions by which caffeine improves performance, factors such as dose (Desbrow et al., 2012), source (Hodgson, Randell, & Jeukendrup, 2013), and the timing of intake (Cox et al., 2002) have been investigated. However, habituation to chronic caffeine intake has received less attention (Bell & McLellan, 2002). This is important from a practical standpoint given the high prevalence of daily caffeine intake in the general population (Fitt, Pell, & Cole, 2013) and by athletes during competition (Desbrow & Leveritt, 2006).

Caffeine probably improves exercise performance through its role as a non-selective adenosine receptor antagonist (Fredholm, Bättig, Holmén, Nehlig, & Zvartau, 1999). A prominent role for the adenosine A_1 receptor in mediating the acute performance enhancing effects of caffeine has been demonstrated (Snyder, Katims, Annau, Bruns, & Daly, 1981). However, more recent studies with adenosine A_{2A} receptor knockout mice confirmed that central blockade of this adenosine receptor isoform is largely responsible for the performance enhancing properties of the drug (El Yacoubi et al., 2000). Chronic caffeine intake influences the concentration of A_1 and A_{2A} receptors in several brain regions (Svenningsson, Nomikos, & Fredholm, 1999; Johansson et al., 1993). This includes A_{2A} expression in the striatum (Svenningsson et al., 1999), a sub-cortical region essential for coordinating voluntary actions (Tepper, Wilson, & Koós, 2008). Therefore it is possible that habituation influences performance benefits typical of acute caffeine supplementation. Data from animal studies support this

hypothesis, as chronic exposure to caffeine in the drinking water of rats resulted in tolerance to the performance benefit of a subsequent acute caffeine dose (Karcz-Kubicha et al., 2003). Although these findings have been confirmed in other animal models (Quarta et al., 2004), the doses administered have been large (i.e. 130 $mg \cdot kg \cdot day^{-1}$) and much greater than those typically consumed by the general population (Fitt et al., 2013). Whether the same tolerance develops after habituation to doses typically consumed by the general population is not clear.

The magnitude of performance benefit after an acute 5 $mg \cdot kg^{-1}$ caffeine dose was less pronounced in individuals already habituated to caffeine (>300 $mg \cdot day^{-1}$) than their caffeine-naive counterparts (Bell & McLellan, 2002). Similar metabolic responses have occurred after an acute caffeine dose in comparisons of low-and high-habitual caffeine users (Bangsbo, Jacobsen, Nordberg, Christensen, & Graham, 1992). However, sub-chronic intake (5 days) both of low (3 $mg \cdot kg^{-1}$) and moderate (6 $mg \cdot kg^{-1}$) caffeine doses did not influence thermoregulatory or cardiovascular responses during exercise in the heat (Roti et al., 2006). Furthermore, time-trial performance was similar when individuals received an acute 3 $mg \cdot kg^{-1}$ caffeine dose subsequent to either a four-day habituation (3 $mg \cdot kg^{-1} day^{-1}$) or withdrawal period (Irwin et al., 2011). These data suggest that a greater duration of supplementation is required before the performance benefit of an acute caffeine dose becomes compromised. To date, no study has systematically evaluated a prolonged period of controlled caffeine intake and its influence on endurance performance. Hence, the aim of this study was to examine the effect of a four-week period of controlled caffeine supplementation on endurance performance.

Methods

Participants

Eighteen healthy, recreationally active men (age: 21.2 ± 1.8 y; body mass: 74.1 ± 8.6 kg; stature: 1.75 ± 0.06 m; $\dot{V}O_{2peak}$: 51.4 ± 8.7 ml·kg^{-1}·min^{-1}; W_{max}: 289 ± 46 W) were recruited and completed this study. All participants were free from chronic disease and deemed eligible to participate after the completion of a health screen questionnaire. Habitual caffeine intake was assessed using a modified version of a semi-quantitative food-frequency questionnaire (Addicot, Yang, Peiffer, & Laurienti, 2008) to ensure intake did not exceed 75 mg·day^{-1}. This cut-off point was chosen as it equates to approximately one cup of caffeinated instant coffee (Fitt et al., 2013) and is similar to what has been used previously (Bell & McLellan, 2002). The study was approved by the Ethics Approvals (Human Participants) Sub-Committee at Loughborough University, UK.

Experimental Design

The experimental design is illustrated in Fig 1. All participants attended the laboratory on six occasions. During the initial visit each participant undertook an incremental exercise test to volitional exhaustion on an electronically braked cycle ergometer (Lode Corival, Groningen, the Netherlands) to determine $\dot{V}O_{2peak}$ and peak power output at $\dot{V}O_{2peak}$ (W_{max}) After this visit, each participant completed one practice trial. This was undertaken to ensure that all participants were accustomed to procedures, to minimise order effects from learning or anxiety and ensure attainment of a maximal effort during the performance task.

After these initial tests, each participant completed one acute caffeine trial (precaf) and one placebo trial (testpla), each separated by 5-7 days. Thereafter, participants were randomly assigned to ingest daily doses of caffeine (BDH Ltd, Poole, UK) or starch (250 mg: BHD Ltd, Poole, UK) for 28 days. Both supplementation groups were matched for age, stature, body mass, $\dot{V}O_{2peak}$ and W_{max} (P>0.05). During the first seven days of supplementation, the caffeine group ingested half of the prescribed caffeine dose (1.5 mg·kg^{-1}) in their morning capsule (7-9 am) followed by a placebo capsule (250 mg starch) in the afternoon (1-3 pm). From days 8 to 28, the caffeine group received the full 3 mg·kg^{-1} dose, equally divided between the morning and afternoon capsules. This titrated approach minimised negative influences of caffeine on daily activities in caffeine-naive individuals (e.g. jitteriness, disturbed sleep etc). The placebo group followed the same pattern of intake, but received starch (250 mg) in both capsules. All participants were instructed to ingest the capsules at the same time of day throughout the supplementation period and compliance was verified by telephone contact, email and in person. Both the placebo and caffeine capsules were visually identical and blinded by an external party not involved in any stage of data collection. A second incremental exercise test was completed on the morning of day 21, before the ingestion of any capsules. This followed the same procedure as the initial visit and was undertaken to account for any changes in $\dot{V}O_{2peak}$ before the final single-blind acute 3 mg·kg^{-1} caffeine trial on day 29 (postcaf).

The order of the testpla and precaf trials and assignment to either supplementation group was via a double-blind, randomised design. Participants were instructed to record their dietary intake and physical activity patterns in the 24 hr before their first experimental trial and replicate this on the day before each subsequent experimental

trial. No strenuous exercise, alcohol, or caffeine ingestion was permitted during the 24 hr before any laboratory visit. However, the caffeine provided in the capsules was permitted during the 24 hr before the postcaf trial (caffeine group). No additional dietary caffeine was permitted during the supplementation period in both groups and participants were provided with a list of commonly consumed caffeinated foods and beverage to help achieve this. Participants were also instructed to maintain their usual dietary and exercise patterns throughout the supplementation period. Compliance to these measures was verified at the start of each visit, before any data collection. Finally, all trials were performed at the same time of day to minimise circadian-type variations in performance.

Experimental trials

Participants arrived at the laboratory after an overnight fast (8-10 hr) with the exception of ingesting 500 mL of plain water approximately 90 min before. Upon arrival, post-void nude body mass was recorded to the nearest 10 g (Adam AFW-120K, Milton Keynes, UK) and a heart rate telemetry band (Polar Beat, Kempele, Finland) positioned. After 10 min of supine rest, a 21g cannula was inserted into an antecubital vein to allow repeated blood sampling. The cannula was flushed with a small volume of saline after each sample to ensure patency. A baseline blood sample (7 mL) was collected before participants ingested either 3 $mg \cdot kg^{-1}$ of anhydrous caffeine (precaf and postcaf) or 250 mg of starch (testpla). After 60 min rest, a second 7 mL venous blood sample was drawn before participants cycled for 60 min at an intensity equivalent to 60% $\dot{V}O_{2peak}$. During this period heart rate and rating of perceived exertion (RPE) were recorded every 5 and 10 min, respectively

(Borg, 1982). One-minute expired air samples were collected into Douglas bags every 15 min to determine the rates of fat and carbohydrate oxidation (Peronnet & Massicotte, 1991). Oxygen and carbon dioxide concentrations in each bag were determined with a paramagnetic analyser (Servomex 1400, Sussex, UK) calibrated against gases of known concentration on the morning of each trial. Total volume was quantified (Harvard Dry Gas Meter, Harvard Apparatus, USA) and gas values were expressed as STPD. After each sample was collected, participants were provided with 100 mL of plain water. A third 7 mL blood sample was collected immediately after the fixed-intensity exercise.

After this, there was a 2-3 min delay while the ergometer was set for the performance task. Performance was assessed as the maximum amount of external work (kJ) that could be completed in 30 min. This method is consistent with previous studies (Jenkins, Trilk, Singhal, O'Connor, & Cureton, 2008) and reflected the high ecological validity associated with similar cycle-based performance tests (Jeukendrup, Saris, Brouns, & Kester, 1996). Participants began exercise at 75% $\dot{V}O_{2peak}$, but were free to adjust the intensity of exercise from the outset. During the performance task participants were instructed to maintain a constant cadence. No verbal encouragement was given during this period and contact was limited to the recording of the physiological and perceptual variables. Heart rate was recorded every 5 min and RPE at 10 and 20 min, respectively. A final 7 mL blood sample was collected at completion of exercise, after which the cannula was removed.

Blood collection and analysis

Blood samples (7 mL) were collected directly into dry syringes. A small sample (2 mL) was dispensed into tubes containing K_2EDTA. Duplicate 100 µL sub-samples were rapidly deproteinised in 1 mL of ice-cold 0.3 *M* perchloric acid. These were centrifuged and the resulting supernatant was used to determine blood glucose concentrations (GOD-PAP, Randox Ltd, UK). Haemoglobin was measured in duplicate (cyanmethemoglobin method) and haematocrit in triplicate (microcentrifugation). These values were used to estimate percentage changes in blood and plasma volumes relative to the resting sample (Dill & Costill, 1974). The remaining blood (5 mL) was dispended into tubes containing clotting activator and left at room temperature for at least 60 min before centrifugation at 3000 rpm for 10 min at 4°C. The supernatant was stored at -21°C for the determination of serum prolactin and cortisol in duplicate via ELISA (DRG diagnostics, Germany) and serum caffeine in duplicate with reverse-phase HPLC as previously described (Holland, Godfredsen, Page, & Connor, 1998). The intra-assay coefficient of variation (CV) for serum prolactin, cortisol and caffeine was 4.9%, 5.3% and 2.9%, respectively.

Statistical analysis

All data were analysed using IBM SPSS statistics version 21.0. Normality was assessed with the Shapiro Wilk test. Between-group comparisons of self-reported habitual caffeine intake, stature, body mass, age, $\dot{V}O_{2peak}$ and W_{max} were determined with *t*-tests for independent samples. Repeated measurements of body mass, $\dot{V}O_{2peak}$ and W_{max} were analysed using a two-way (group x time) mixed-design factorial ANOVA. Exercise performance and fasting plasma glucose were analysed

using a two-way (group x trial) mixed-design factorial ANOVA. Variables measured throughout each trial were analysed using a three-way (group x trial x time) mixed-design factorial ANOVA. Where a main effect or interaction occurred, Bonferroni adjusted paired *t*-tests for normally distributed data or Wilcoxon Signed Rank tests for non-normally distributed data were used. Between-group comparisons during the testpla, precaf and postcaf trials were determined with *t*-tests for independent samples. In addition to null-hypothesis testing, magnitude-based inferences were made to examine whether the observed differences in total external work produced were meaningful (Hopkins, 2000). The magnitude of the smallest worthwhile change in performance was set at 3% (~12 kJ), based on the findings of Jenkins et al. (2008) using habituated, recreationally active participants. Cohen's *d* effect size (ES) examined the magnitude of individual differences in total external work produced ([Mean 1 - Mean 2]/pooled SD) and were interpreted as trivial (0-0.19), small (0.2-0.49), medium (0.5-0.79) or large (>0.8) as previously described (Cohen, 1992). Data are presented as means ± SD unless otherwise stated. Statistical significance was accepted at $P<0.05$.

Results

Baseline measures

Self-reported habitual caffeine intake was similar between groups (placebo: 66 ± 6 $mg \cdot day^{-1}$ vs. caffeine: 60 ± 8 $mg \cdot day^{-1}$; $P=0.076$) There were no between-group differences for baseline measures of age (placebo: 21.3 ± 2.2 y; caffeine: 21.0 ± 1.5 y; $P=0.710$), stature (placebo: 1.75 ± 0.06 m; caffeine: 1.76 ± 0.08 m; $P=0.781$), body mass (placebo: 73.3 ± 7.4 kg; caffeine: 74.8 ± 10.1 kg; $P=0.708$), $\dot{V}O_{2peak}$

(placebo: 51.6 ± 9.6 ml·kg^{-1}·min^{-1}; caffeine: 51.2 ± 8.4 ml·kg^{-1}·min^{-}1; *P*=0.860) or W_{max} (placebo: 286 ± 47 w; caffeine: 296 ± 55 w; *P*=0.667). Day 21 body mass (placebo: 73.1 ± 6.8 kg; caffeine: 74.8 ± 10.2 kg), $\dot{V}O_{2peak}$ (placebo: 51.0 ± 9.2 ml·kg^{-1}·min^{-1}; caffeine: 50.6 ± 8.3 ml·kg^{-1}·min^{-1}) and W_{max} (placebo: 282 ± 43 W; caffeine: 289 ± 47 W) was similar to baseline between both supplementation groups (trial x group interactions, *P*>0.646).

Exercise performance

Total external work produced during the testpla trial was similar between the caffeine (344. 9 ± 80.3 kJ) and placebo (333.1 ± 56.4 kJ) group (ES=0.17; *P*=0.723; Fig. 2A). Compared with testpla, total external work produced during the precaf trial increased 12.0 ± 7.4% in the caffeine group (383.3 ± 75 kJ vs. 344. 9 ± 80.3 kJ; ES=0.49; *P*=0.001) and 6.7 ± 4.2% in the placebo group (354.4 ± 55.2 kJ vs. 333.1 ± 56.4 kJ; ES=0.38; *P*=0.004; Fig. 2A). Based on a smallest worthwhile change in performance of 12 kJ, these within-group increases represent an ‘almost certainly beneficial’ (caffeine group) and ‘probably beneficial’ (placebo group) effect on performance, respectively (Table. 1).

Chronic caffeine supplementation resulted in a 7.3 ± 6.3% decrease in total external work produced during the postcaf trial compared with precaf (358 ± 89 kJ vs. 383.3 ± 75 kJ; ES=-0.31; *P*=0.025; Fig. 2A). This diminished response represents a ‘probably harmful’ effect on performance (Table. 1). Total external work produced during the postcaf trial and tetspla was not statistically different (358 ± 89 kJ vs. 344.9 ± 80.3 kJ; ES=0.16; *P*=0.188). However, inferences suggest the difference between these trials represents a ‘possibly beneficial’ effect (Table. 1). Hence, chronic caffeine

supplementation might have not completely eliminated the performance benefit of caffeine (i.e. postcaf vs. testpla; Table. 1).

Participants in the placebo group produced 6.1 ± 2.4% more external work during the postcaf trial than testpla (351.8 ± 49.4 kJ vs. 333.1 ± 56.4; ES=0.33; *P*=0.004; Fig. 2A), with this increase representing a 'probably beneficial' effect on performance (Table. 1). Accordingly, there was no difference between the precaf and postcaf trials (354.4 ± 55.2 kJ vs. 351.8 ± 49.4 kJ; ES=0.05; *P*>0.05).

There were no between-group differences during the precaf (28.7 ± 74.8 kJ; ES=0.44; *P*=0.368) or postcaf (6.2 ± 90.7 kJ; ES=0.09; *P*=0.858) trials (Fig. 2A; Table. 1).

The order of the experimental trials was correctly guessed by two participants in each supplementation group. Furthermore, three participants in each supplementation group correctly guessed whether they received the caffeine or placebo treatment during the habituation period. Therefore, blinding can be considered successful as these odds are less than what could occur purely by chance.

Blood data

Circulating caffeine, cortisol, prolactin and glucose values recorded during exercise are shown in table 2. Acute caffeine supplementation increased serum concentrations during the precaf and postcaf trials, peaking 60 min after ingestion and remaining greater throughout exercise than baseline and testpla (trial x time interaction, *P*<0.05). There were no changes in serum caffeine concentrations during

testpla, with values remaining close to baseline throughout exercise in both groups. The habituation protocol did not influence caffeine metabolism (P=0.605).

Serum cortisol increased progressively throughout exercise (P<0.05), peaking at the end of the performance task in both groups. No influence from trial (P=0.535) or supplementation group (P=0.628) occurred. Similarly, prolactin concentrations increased during exercise (P<0.05), but the rate of increase was similar across trials (P=0.498) and between groups (P=0.649). The greatest concentrations were at the end of the performance task across all trials in both groups (P<0.05). Neither cortisol (P=0.552) or prolactin (P=0.965) were influenced by the habituation protocol.

Fasting plasma glucose was similar across all three trials in both supplementation groups (P=0.465). During exercise, plasma concentrations increased steadily (P<0.05), with similar values across trials (P=0.096) and between groups (P=0.443). Compared with baseline, both blood and plasma volumes were reduced during exercise (P<0.05). No influence of trial (P>0.135) or group (P>0.649) occurred.

Heart rate, substrate oxidation and RPE

Mean heart rate, expired gas and RPE values recorded during exercise are shown in table 3. Exercise caused a progressive increase in heart rate throughout the fixed-intensity exercise (P<0.05). This increase remained similar across trials (P=0.169) and between supplementation groups (P=0.984). Similarly, heart rate increased during the performance task (P<0.05), but this increase was similar across trials (P=0.891) and between groups (P=0.887). Within-group differences in mean heart

263 rate occurred across trials. The greatest values were during the precaf trial in both
264 groups (Table. 3). There were no between-group differences (P>0.274).

265 Rates of carbohydrate oxidation decreased (P=0.026) while rates of fat oxidation
266 increased (P<0.05) during the fixed-intensity exercise. Neither of these were
267 influenced by trial (P>0.784) or group (P>0.328). Furthermore, RER values
268 decreased (P<0.05) while V̇O2 increased (P<0.05) during exercise. No influence
269 from trial (P>0.691) or group (P>0.189) occurred.

270 Exercise induced a steady increase in RPE during the fixed intensity exercise
271 (P<0.05), with similar values across trials (P=0.265) and between groups (P=0.441).
272 Similarly, RPE increased throughout the performance task (P<0.05), but this
273 response was independent of trial (P=0.174) and group (P>0.05).

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275 Discussion:

276 This study examined whether four weeks of controlled caffeine intake influenced
277 endurance performance in a group of recreationally active men with low-habitual
278 caffeine intakes. The results of the present study indicate that chronic
279 supplementation with a titrated low dose of caffeine developed tolerance to the
280 ergogenic effect a subsequent acute caffeine dose. While these results contrast with
281 previous studies that have examined effects of sub-chronic caffeine supplementation
282 (Irwin et al., 2011), this is the first study to examine effects of a prolonged period of
283 controlled caffeine intake typical of the general population (Fitt et al., 2013). This
284 suggests that supplementation protocols in previous studies (Irwin et al., 2011) were
285 too short to influence mechanisms that develop tolerance.

Previous research demonstrated caffeine prolonged time-to-exhaustion because it enhanced fat oxidation late in exercise with a subsequent sparring of muscle glycogen (Costill, Dalsky, & Fink, 1978). The results of the present study are contrary to this as substrate oxidation was not influenced either by acute or chronic caffeine supplementation. Alternatively, chronic caffeine intake could influence caffeine metabolism (Svenningsson et al., 1999). This might lead to an increase in the concentrations of paraxanthine and theophylline, caffeine's primary metabolites (Svenningsson et al., 1999). As these possess a greater affinity for adenosine receptors than caffeine (Fredholm et al., 1999), this could result in enhanced development of tolerance. However, caffeine concentrations were similar between the precaf and postcaf trials in the caffeine group (Table. 2), suggesting the habituation protocol failed to influence caffeine metabolism. Although paraxanthine and theophylline concentrations were not measured, these methylxanthines do not penetrate the blood-brain-barrier with the same efficacy as caffeine (Svenningsson et al., 1999). Therefore, any subtle change in the peripheral concentrations of these metabolites attributable to the chronic supplementation protocol is unlikely to explain the development of tolerance.

Serum cortisol and prolactin were assessed as these are indirect indicators of central noradrenergic (Tsigos & Chrousos, 2002) and dopaminergic (Ben-Jonathan & Hnasko, 2001) activity, respectively. Chronic caffeine supplementation did not influence the circulating concentrations of these hormones (Table. 2), suggesting that neurotransmitter release along these neural pathways does not explain the development of tolerance. Direct analysis of neurotransmitter release with microdialysis (Acquas, Tanda, & Di Chiara, 2002; De Luca, Bassareo, Bauer, & Di Chiara, 2007) and brain imaging techniques (Volkow et al., 2015) also support this

hypothesis. Although high acute caffeine doses increase striatal dopamine release (i.e. 30 $mg \cdot kg^{-1}$; Solinas et al., 2002), lower doses (i.e. 0.25-5 $mg \cdot kg^{-1}$), typically consumed by the general population (Fitt et al., 2013), have not influenced dopamine release both in rat (Acquas et al., 2002; De Luca et al., 2007) and human (Volkow et al., 2015) striatum. Therefore, an alternative mechanism is likely responsible. Chronic caffeine supplementation has been associated with changes in A_{2A} expression across several brain regions (Svenningsson et al., 1999). However, a cross-tolerance to the A_1 receptor probably plays a more important role in mediating the development of tolerance (Karcz-Kubicha et al., 2003). This could involve a functional change in the striatal A_1/A_{2A} heteromer (Ciruela et al., 2006), while others have reported changes in A_1 receptor expression throughout the brain after chronic caffeine supplementation (Johansson et al., 1993). A recent positron emission topography study demonstrated that almost half of *in vivo* cerebral A_1 receptors were occupied by caffeine when participants received an intravenous dose of 4.3 $mg \cdot kg^{-1}$, which corresponded to a plasma concentration of ~8 $\mu g \cdot mL^{-1}$ (Elmenhorst, Meyer, Matusch, Winz, & Bauer, 2012). Participants in the present study were habituated to daily doses of 3 $mg \cdot kg^{-1}$ from days 8 to 28, resulting in serum concentrations of approximately 3.5 $\mu g \cdot mL^{-1}$ (Table. 2). Based on these observations, it could be that the 3 $mg \cdot kg^{-1}$ caffeine dose administered in the present study resulted in the occupation of approximately a quarter of cerebral A_1 receptors. This suggests supplementation with larger daily caffeine doses (i.e. 6-9 $mg \cdot kg^{-1}$), which will ultimately occupy more A_1 receptors, results in accelerated and/or total development of tolerance.

The influence of caffeine habituation in participants is often overlooked in many studies, despite evidence which demonstrates that this influences effects after acute

supplementation (Bell & McLellan, 2002). To minimise this confounder, all participants in the present study were low caffeine consumers before participation. Differences in habitual caffeine consumption are associated with single nucleotide polymorphisms in the ADORA2A gene encoding for the A_{2A} receptor (Cornelis, El-Sohemy, & Campos, 2007). These findings demonstrated individuals with the homozygous recessive (TT) genotype consumed less caffeine than their homozygous dominant (CC) counterparts (Cornelis et al., 2007). Recently, TT carriers performed better during a short performance task (10 min) than CC carriers when supplemented with an acute 5 $mg \cdot kg^{-1}$ caffeine dose (Loy, O'Connor, Lindheimer, & Covert, 2015). Perhaps this could explain the small between-group difference in total external work produced during the precaf trial (28.7 ± 74.8 kJ; ES=0.44), with more TT carriers present in the caffeine group. However, genotype determination was not undertaken in the present study, which limits the extent to which this relationship can be inferred.

Well-trained individuals produce more reliable performance data during cycle-based time-trials than their recreationally active counterparts (Zavorsky et al., 2007). However, recreationally active individuals produced a CV of 1.7% (Zavorsky et al., 2007) and 0.7% (Fleming and James, 2014) during cycle and running-based time-trials, respectively. Furthermore, similar performance tests to that in the present study had a CV of approximately 3% (Jeukendrup et al., 1996). This variability is less than the percentage increase in performance during the precaf trials (caffeine: 12.0 ± 7.4%; placebo: 6.7 ± 4.2%) and the percentage decrease in performance during the postcaf trial compared with precaf in the caffeine group (-7.3 ± 6.3%). Therefore, neither the participant group nor the performance test used in the present study adversely influenced the validity of the performance data.

Ideally, the study design would have incorporated a post-supplementation placebo trial, hence providing a direct comparison with the postcaf trial after the chronic supplementation protocol. It was deemed difficult to implement as timing both trials to occur at the end of the supplementation period was not possible. For example, two randomised trials, undertaken seven days apart, means the supplementation period before the postcaf trial would be twenty-eight days for half the participants and thirty-five days for the remaining participants. Importantly, peak power output and maximal oxygen uptake were similar between the two $\dot{V}O_{2peak}$ tests. Furthermore, heart rate and oxygen uptake during the fixed-intensity exercise was similar during all three trials. This suggests participants maintained similar fitness throughout the study period and exercise intensity was matched before the performance task during each of the experimental trials. Hence, any influence on performance during the postcaf trial in either supplementation group is likely due to participants receiving caffeine or placebo during the chronic supplementation period.

In conclusion, the present findings demonstrate that chronic ingestion of a titrated low dose of caffeine results in the development of tolerance in a group of healthy, recreationally active males with low-habitual caffeine intakes. This occurred despite no changes before and after supplementation in circulating caffeine, hormonal concentrations or substrate oxidation. The influence of chronic caffeine intake should be examined in well-trained individuals with low-habitual caffeine intakes. In addition, futures studies should identify when the tolerance to caffeine occurs and examine whether supplementation with larger daily doses (i.e. 6-9 $mg \cdot kg^{-1}$) influences the rate and extent of the development of tolerance.

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Table 1: Differences in total external work produced (kJ) during the experimental trials within and between supplementation groups

Treatment comparison	Mean ± SD difference and 95% confidence interval (kJ)	ES	Qualitative outcome (beneficial/trivial/harmful)
CAF			Almost certainly beneficial
Precaf-testpla	38.4 ± 19.9 (18.4 to 58.4)	0.49	(100/0/0)
			Possibly beneficial
Postcaf-testpla	13.1 ± 18.2 (-5.2 to 31.3)	0.16	(55/44/1)
			Probably harmful
Postcaf-precaf	-25.3 ± 21.9 (-47.3 to -3.4)	-0.31	(0/9/91)
PLA			Probably beneficial
Precaf-testpla	21.4 ± 13.1 (8.3 to 34.7)	0.38	(94/6/0)
			Probably beneficial
Postcaf-testpla	18.7 ± 11.9 (6.8 to 30.6)	0.33	(91/9/0)
			Unclear
Postcaf-precaf	-2.8 ± 9.8 (-12.7 to 7.1)	-0.05	(50/0/50)
Testpla			Unclear
CAF-PLA	11.8 ± 89.7 (-58.3 to 81.9)	0.17	(50/26/24)
Precaf			Unclear
CAF-PLA	28.7 ± 74.8 (-37.7 to 95.2)	0.44	(70/19/11)
Postcaf			Unclear
CAF-PLA	6.2 ± 90.7 (-68.1 to 80.5)	0.09	(43/26/30)

PLA, Placebo group; CAF, Caffeine group; ES, Cohen's *d* effect size. Qualitative outcome numbers indicate the percentage chance the true value is beneficial, trivial or harmful based on a 12 kJ difference in external work produced during the performance task. An effect was deemed unclear when the percentage chances of benefit and harm were >5%.

Table 2: Circulating caffeine, cortisol, prolactin and glucose concentrations during the experimental trials.

	PLA				CAF			
Variable	-60	0	60	90	-60	0	60	90
Caffeine (μg·mL^{-1})								
Testpla	0.06 ± 0.07	0.06 ± 0.07	0.06 ± 0.07	0.07 ± 0.06	0.13 ± 0.07	0.08 ± 0.10	0.10 ± 0.08	0.05 ± 0.08
Precaf	0.09 ± 0.07	3.54 ± 0.59*$^{\#}$	3.17 ± 0.44*$^{\#}$	2.97 ± 0.23*$^{\#}$	0.28 ± 0.29	3.48 ± 0.57*$^{\#}$	3.40 ± 0.53*$^{\#}$	3.03 ± 0.56*$^{\#}$
Postcaf	0.10 ± 0.09	3.54 ± 0.65*$^{\#}$	3.22 ± 0.44*$^{\#}$	2.97 ± 0.55*$^{\#}$	0.49 ± 0.37	3.69 ± 0.60*$^{\#}$	3.26 ± 0.53*$^{\#}$	3.09 ± 0.66*$^{\#}$
Cortisol (ng·mL^{-1})								
Testpla	131.55 ± 37.22	125.29 ± 59.77	153.22 ± 75.59	211.17 ± 90.96	115.47 ± 14.78	85.30 ± 33.50	163.73 ± 20.75*	236.10 ± 51.18*
Precaf	142.13 ± 26.85	118.00 ± 50.96	177.90 ± 86.66	227.32 ± 90.89	136.25 ± 34.27	104.55 ± 26.11	159.76 ± 46.14	225.63 ± 48.25
Postcaf	146.42 ± 33.79	122.48 ± 36.89	185.70 ± 63.54	249.50 ± 71.88	121.87 ± 42.89	80.30 ± 38.35	168.10 ± 42.36	234.73 ± 38.28*
Prolactin (ng·mL^{-1})								
Testpla	8.13 ± 2.68	7.80 ± 3.16	10.01 ± 2.80	19.65 ± 4.43*	7.83 ± 3.86	7.84 ± 3.02	9.99 ± 2.79	20.53 ± 4.99*
Precaf	7.91 ± 1.78	7.43 ± 1.46	10.39 ± 2.13	19.42 ± 3.18*	7.89 ± 3.65	7.57 ± 3.31	10.23 ± 2.10	20.03 ± 5.22*
Postcaf	7.59 ± 2.50	8.78 ± 3.27	10.37 ± 1.16*	19.25 ± 3.69*	8.33 ± 3.31	7.94 ± 3.66	9.79 ± 3.06	19.68 ± 5.06*
Glucose (mmol·L^{-1})								
Testpla	4.17 ± 0.27	4.18 ± 0.38	4.45 ± 0.51	4.71 ± 0.82	4.26 ± 0.28	4.21 ± 0.35	4.50 ± 0.39	5.03 ± 0.57
Precaf	4.10 ± 0.30	4.10 ± 0.35	4.52 ± 0.51	4.99 ± 1.03	4.19 ± 0.42	4.21 ± 0.35	4.49 ± 0.32	5.35 ± 0.77
Postcaf	4.18 ± 0.22	4.22 ± 0.17	4.70 ± 0.48	5.06 ± 0.75	4.41 ± 0.39	4.25 ± 0.25	4.57 ± 0.37	5.32 ± 0.76

Values are mean ± SD. PLA, Placebo group; CAF, Caffeine group. *denotes a within-trial significant difference (P<0.05) compared with -60. $^{\#}$denotes a significant difference (P<0.05) compared with the corresponding time point in the testpla trial. There were no significant trial x group (P>0.552), time x group (P>0.443) or trial x time x group (P>0.512) interactions for any variable.

Table 3: Mean heart rate, RPE and substrate oxidation during the experimental trials.

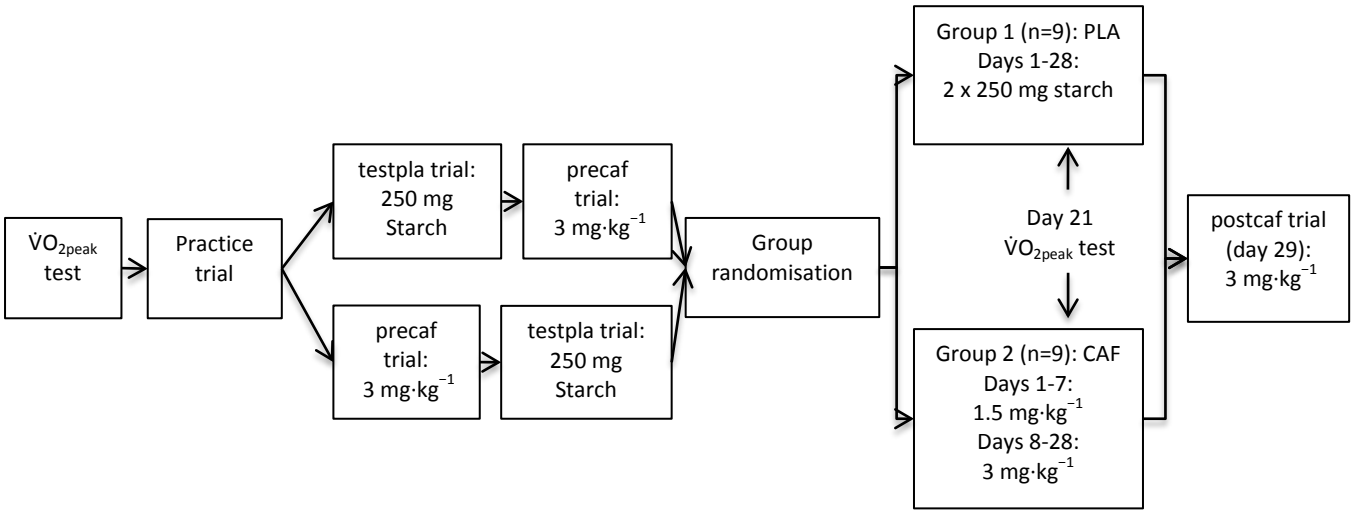
Variable	testpla	precaf	postcaf	*P*
Heart rate (beats·min^{-1}), fixed				
PLA	146 ± 7	145 ± 7	145 ± 8	
CAF	145 ± 6	144 ± 7	146 ± 7	0.312
Heart rate (beats·min^{-1}), PT				
PLA	167 ± 13	172 ± 12*	172 ± 12*	
CAF	169 ± 9	177 ± 5*	171 ± 9†	0.034
RPE, fixed				
PLA	12.7 ± 0.3	12.1 ± 0.8	11.9 ± 1.2	
CAF	12.9 ± 1.2	12.7 ± 1.1	13.0 ± 1.1	0.219
RPE, PT				
PLA	15.8 ± 0.8	15.8 ± 1.0	15.6 ± 1.3	
CAF	16.4 ± 1.0	16.8 ± 1.3	16.6 ± 0.9	0.478
CHO Ox (g·min^{-1})				
PLA	2.02 ± 0.09	2.07 ± 0.05	1.97 ± 0.10	
CAF	2.25 ± 0.09	2.37 ± 0.09	2.16 ± 0.21	0.871
Fat Ox (g·min^{-1})				
PLA	0.40 ± 0.06	0.38 ± 0.04	0.42 ± 0.05	
CAF	0.32 ± 0.05	0.29 ± 0.06	0.37 ± 0.09	0.794
RER				
PLA	0.90 ± 0.01	0.90 ± 0.01	0.89 ± 0.01	
CAF	0.92 ± 0.01	0.92 ± 0.01	0.91 ± 0.02	0.882
$\dot{V}O2$ (L·min^{-1})				
PLA	2.32 ± 0.06	2.30 ± 0.06	2.30 ± 0.09	
CAF	2.31 ± 0.04	2.34 ± 0.06	2.34 ± 0.04	0.472

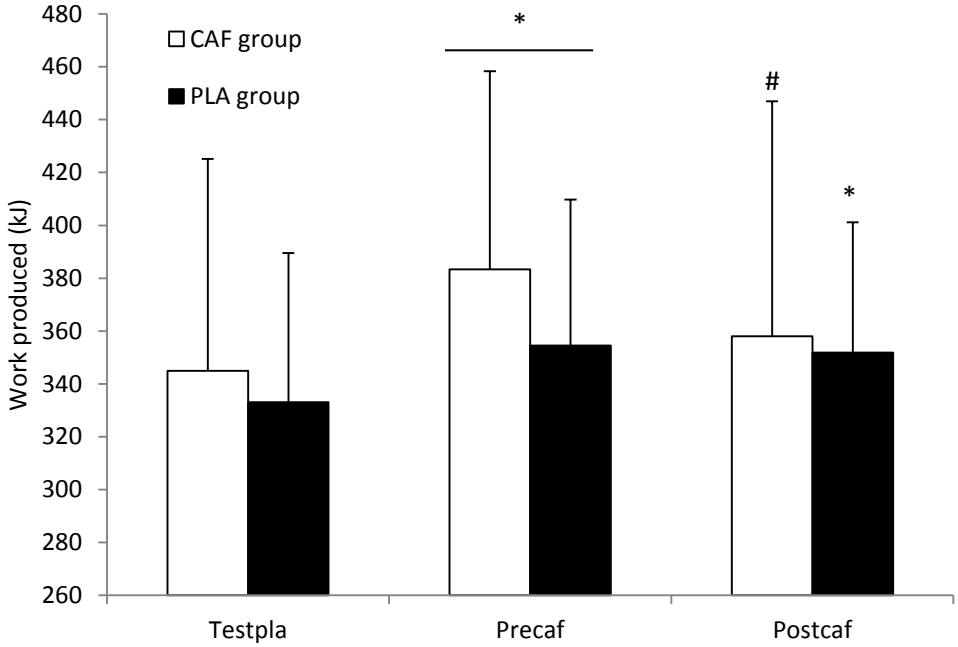
Values are mean ± SD. PLA, Placebo group; CAF, Caffeine group; CHO Ox, carbohydrate oxidation; Fat Ox, fat oxidation; RER, respiratory exchange ratio; VO2, O_2 consumption; RPE, rating of perceived exertion. Fixed, values recorded during the fixed-intensity exercise; PT, values recorded during the performance task. *P* values are derived from trial x group interactions. *denotes a within-group significant difference ($P<0.05$) compared with testpla. †denotes a within-group comparison ($P=0.061$) to precaf.

Figure Captions

Fig. 1: Schematic of the study design

Fig. 2: Total external work produced (kJ) during the experimental trials (A) and individual responses by participants in the placebo (B) and caffeine (C) supplementation group, respectively. A: Trial x group interaction (P=0.017). * and # denote a within-group significant difference (P<0.05) compared with testpla and precaf, respectively.


V̇O2peak test
Practice trial
testpla trial: 250 mg Starch
precaf trial: 3 mg·kg−1
precaf trial: 3 mg·kg−1
testpla trial: 250 mg Starch
Group randomisation
Group 1 (n=9): PLA
Days 1-28:
2 x 250 mg starch
Day 21
V̇O2peak test
Group 2 (n=9): CAF
Days 1-7:
1.5 mg·kg−1
Days 8-28:
3 mg·kg−1
postcaf trial (day 29): 3 mg·kg−1


480
460
440
420
400
380
360
340
320
300
280
260
Work produced (kJ)
CAF group
PLA group
*
#
*
Testpla
Precaf
Postcaf