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1 **A combination of amino acids and caffeine enhances sprint running capacity in a hot,**

2 **hypoxic environment**

3

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24

25 **Abstract**

26 Heat and hypoxia exacerbate central nervous system (CNS) fatigue. We therefore investigated
27 whether essential-amino-acid (EAA) and caffeine ingestion attenuates CNS fatigue in a
28 simulated team-sport specific running protocol in a hot and hypoxic environment.
29 Sub-elite male team-sport athletes (n=8) performed a repeat-sprint running protocol on a non-
30 motorized treadmill in an extreme environment on four separate occasions. Participants ingested
31 one of four supplements: a double placebo, 3 $mg.kg^{-1}$ body mass of caffeine+placebo, 2x7 g
32 EAA (Musashi Create™)+placebo, or caffeine+EAA prior to each exercise session using a
33 randomized, double-blind crossover design. Electromyography (EMG) activity and quadriceps
34 evoked responses to magnetic stimulation were assessed from the dominant leg at pre-, half-time,
35 and post-exercise. Central activation ratio (CAR) was used to quantify completeness of
36 quadriceps activation. Oxygenation of the pre-frontal cortex was measured via near-infrared
37 spectroscopy. Mean sprint work was higher (+174J, 23–24J; mean, 95% confidence interval,
38 $d$=0.30; effect size, likely beneficial) in the caffeine+EAA condition versus EAAs alone, but not
39 caffeine or a placebo. The decline in EMG activity was less (13%, 0–26%; $d$=0.58, likely) in
40 caffeine+EAA versus EAA alone. Similarly, the pre-to-post exercise decrement in CAR was
41 significantly less (-2.7%, 0.4–5.4%; $d$=0.50, likely) when caffeine+EAA were ingested
42 compared to placebo. Cerebral oxygenation was lower (-5.6%, 1.0–10.1%; $d$=0.60, very likely)
43 in the caffeine+EAA condition compared to LNAA alone. Co-ingestion of caffeine and EAA
44 appears to maintain muscle activation and central drive, with a small improvement in running
45 performance relative to EAA alone in hot, hypoxic environments.
46 **Key words** – Repeated sprint exercise, Central fatigue, Hypoxia, Heat, Amino acids, Caffeine

47 **Introduction**

48 Fatigue is characterized by the inability of skeletal muscle to maintain the required power output
49 (Allen et al. 2008), and may occur at a muscular level (peripheral fatigue) or in the central
50 nervous system (CNS, central fatigue). Central fatigue manifests as the inadequacy to voluntarily
51 activate muscles (Gandevia 2001), alterations in cognitive function, perception of effort, and
52 pain (Meeusen et al. 2006). The Central Governor theory highlights the importance of the CNS
53 and the potential role of accumulation and/or depletion of neurotransmitters in fatigue (Noakes
54 2011). Because neurotransmitter synthesis and function can be manipulated using nutritional
55 strategies, it may be possible to use these strategies to reduce central fatigue (Pardridge 1998;
56 Meeusen et al. 2006).
57
58 Caffeine is considered a legitimate nutritional supplement in competitive sport to delay the onset
59 of fatigue. Research on the impact of EAA on fatigue, however, has been equivocal (Davis et al.
60 1999; Watson et al. 2004; Stepto et al. 2011). EAA supplementation did not improve
61 performance in endurance exercise (Madsen et al. 1996; Davis et al. 1999) in normal conditions
62 (thermoneutral, normoxic environment), but may do so in the heat when production of dopamine
63 is augmented. (Mittleman et al. 1998; Tumilty et al. 2011) Reducing dopamine synthesis is
64 associated with a quicker onset of fatigue (Bailey et al. 1993). To our knowledge no studies have
65 examined the effect of EAA supplementation on exercise performance in hypoxia.
66
67 Caffeine may enhance performance in endurance exercise, (Doherty & Smith 2004; Ganio et al.
68 2009), high-intensity running, and sprint activity (Glaister et al. 2008; Astorino & Roberson
69 2010) in normal conditions. Caffeine ingestion prior to exercise can reduce ratings of perceived

70 exertion (RPE) (Doherty & Smith 2005), improve running power (Schneiker et al. 2006), and
71 decrease sprint times (Glaister et al. 2008; Gant et al. 2010). Evidence for an ergogenic effect of
72 caffeine in hypoxia is limited or equivocal when exercise is performed in the heat (Del Coso et
73 al. 2008; Roelands et al. 2011).
74
75 Exercise in the heat and/or at altitude represents a situation of maximal environmental stress that
76 could accelerate the development of central fatigue. Voluntary muscle activation is reduced after
77 exercise in the heat (Skein et al. 2012), possibly related to altered dopamine activity and
78 inhibitory signals from thermoreceptors in the hypothalamus (Nybo 2008). Similarly, voluntary
79 activation is reduced after exercising in hypoxia (Verges et al. 2012; Billaut et al. 2013) as a
80 result of cerebral deoxygenation (Smith & Billaut 2010; Billaut et al. 2013). Therefore,
81 manipulating environmental conditions in combination with nutritional interventions can provide
82 insights into novel supplementation strategies and the mechanisms of CNS fatigue.
83
84 The aim of this study was to investigate whether nutritional supplementation can attenuate
85 central fatigue and reduce performance decrements under conditions of environmental stress
86 (simulated heat and hypoxia). Although these conditions are uncommon, it provides an
87 opportunity to observe the potentially beneficial effects of nutritional manipulations under
88 standardized conditions. Nutritional supplementation, in combination with assessing markers of
89 central fatigue (e.g., muscle recruitment, central drive), will clarify the viability of invoking
90 changes in neurotransmitter concentration in relation to central fatigue. We hypothesized that
91 central fatigue and decrements in intermittent-exercise performance induced by environmental

92 stress would be attenuated by targeted nutritional supplementation strategies utilizing a
93 combination of both caffeine and EAAs.
94
95 **Materials and Methods**
96 **Participants**
97 Ten male, sub-elite football players regularly competing and training (~60 min, 3 times per
98 week) volunteered for the study (Table 1). Two athletes were unable to complete the study, due
99 to issues and/or injuries unrelated to the study. Prior to participation, each subject provided
100 written informed consent for their voluntary participation in the study. The study was approved
101 by the local Human Research Ethics Committee.
102
103 **Participant characterisation/familiarisation**
104 Participants reported to the laboratory 10-14 days prior to the experimental protocols on two
105 occasions. On the first visit, age, height and body mass were recorded, body composition
106 assessed by a whole body light scanner ($[TC]^2$, Cary, U.S.A., (Ryder & Ball 2012)), and
107 participants were familiarized with the exercise protocol. On the second visit, participants
108 performed a 20-m sprint followed, 30 min later, by the Yo-Yo intermittent recovery test (Mohr et
109 al. 2003).
110
111 **Diet and exercise control**
112 Two days prior to each experimental trial participants consumed a moderate-high carbohydrate
113 diet (4 $g.kg^{-1}$ body mass) that provided ~106 $kJ.kg^{-1}$ body mass per day (70% CHO, 15% protein
114 and 15% fat), while controlling protein intake in line with Australian dietary recommendations.

115 Participants were also instructed to abstain from any caffeinated products, alcohol and formal
116 exercise training 24 h prior to testing.
117
118 **Study design**
119 In each session, after voiding, and being weighed, athletes consumed the initial supplement
120 (EAA or Placebo) and remained in the laboratory at normoxic and thermoneutral conditions for
121 the first 2 hours prior to exercise (Figure 1). At the end of this period, one hour before exercise,
122 athletes consumed the second dose of supplements (caffeine, placebo and/or EAA) and rested in
123 the environmental exercise laboratory (15% $O_2$ [~2700m above sea level], 30˚C and 20% relative
124 humidity). Although this combination of conditions is uncommon, in research studies it provides
125 a unique opportunity to invoke central fatigue under standardized conditions and observe the
126 potentially-beneficial effects of nutritional manipulations.
127

**Table 1**: Participant characteristics

| | |
|---|---|
| **Age** | 22 ± 2 |
| **Height (cm)** | 181 ± 6 |
| **Body mass (kg)** | 77 ± 11 |
| **Estimated body fat (%)** | 14.7 ± 4.4 |
| **20m sprint time (sec)** | 3.1 ± 0.1 |
| **Yo-Yo IR1 distance (m)** | 1320 ± 528 |
| **Estimated VO2 max (ml/kg.min$^{-1}$)** | 47.5 ± 4.4 |

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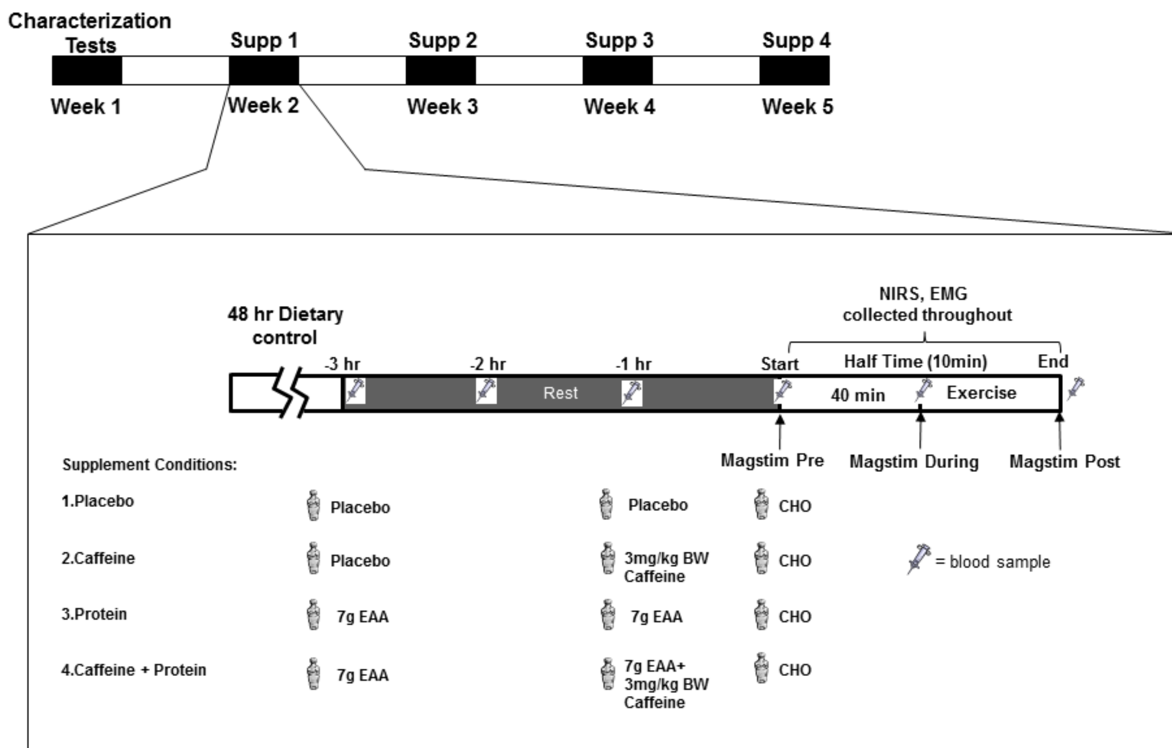


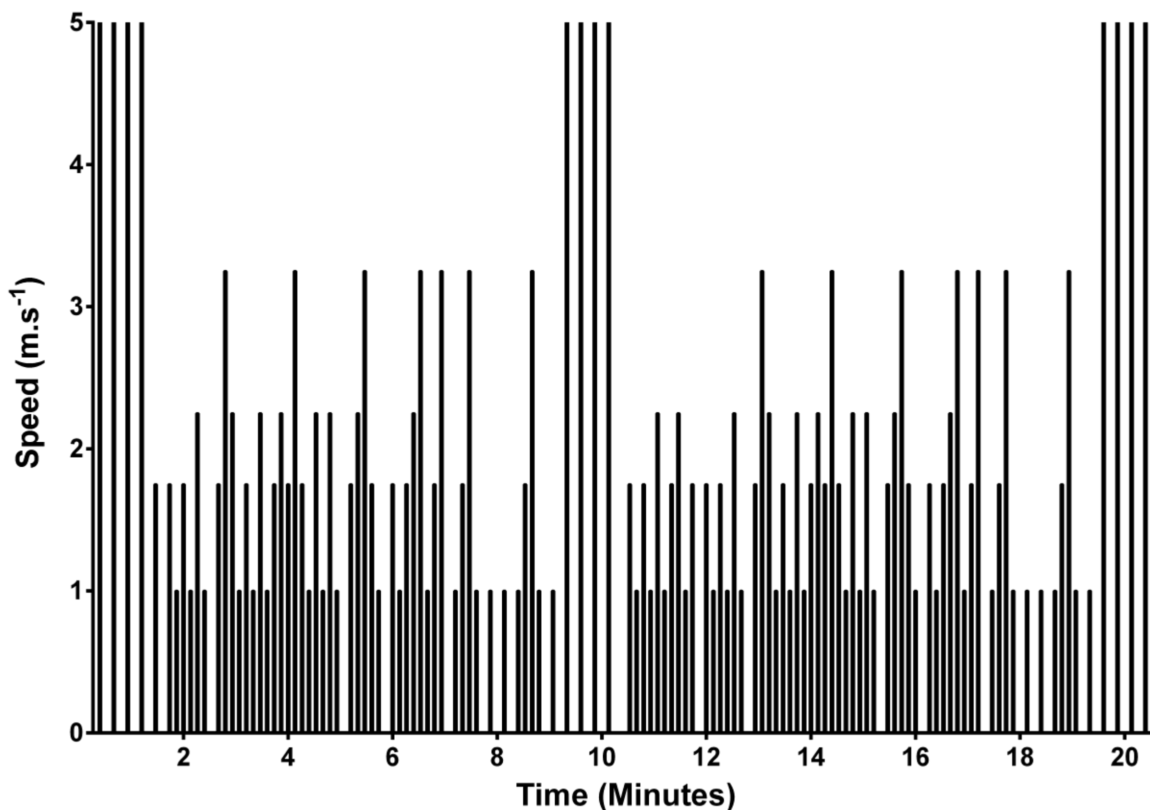

130
131 **Figure 1:** Schematic diagram of the study design. Supp = Supplement, Magstim = Central
132 activation and MVC assessment, BW = Body Weight.

133

134 **Exercise Protocol**

135 The exercise protocol (Figure 2) was designed to mimic the running profile of team-sport
136 athletes (Sirotic & Coutts 2008; Zois et al. 2013). During each session, participants completed a
137 total of 6 sets of 4 s sprints with 8 s of rest between each sprint on a non-motorized treadmill
138 (Woodway Force, Waukesha, U.S.A.). The time between each sprint set was interspersed with
139 standing (22% of total time), walking (27%), jogging (22%), running (12%) and fast running
140 (9%). Mean power, peak power and peak velocities during the exercise protocol were recorded

141 by the treadmill force transducer at 25 Hz. Performance data were stored and analyzed as
142 described previously (Serpiello et al. 2011).




143

144 **Figure 2:** Graphical representation of one half of the exercise protocol, including 3 x 4 x 4
145 second sprints and intermittent activity for participants with a maximal sprint speed of 4.8 – 5.2
146 $m.s^{-1}$. This protocol was completed twice with a 10 minute break in-between to simulate half-
147 time.

148

149 **Supplementation regimens**

150 Four supplement protocols were undertaken in a counterbalanced, randomized and double-blind
151 fashion to provide the participants with a placebo, caffeine only, EAA only, or caffeine + EAA
152 (Figure 1). The EAA was provided in two 7 g doses of Musashi Create™ powder, dissolved in

153 250 ml of water containing; arginine (854 mg), leucine (837 mg), phenylalanine (794 mg), lysine
154 (709 mg), valine (598 mg), histidine (555 mg), isoleucine (512 mg), threonine (418 mg) and
155 methionine (162 mg), to reduce serotonin, and increase dopamine production within the CNS.
156 Musashi also provided an inactive placebo that was indistinguishable in color and taste.
157 Anecdotally, participants were unable to correctly identify any of the treatments. The EAAs or
158 placebos were given to the participants 3 h and 1 h prior to commencing exercise trials. Caffeine
159 (to block the action of adenosine; No-Doz™; 3 $mg.kg^{-1}$ body mass) or placebo ($CaCO_3$) was
160 given to participants as a capsule (blinded) 1 h before exercise. Immediately before the warm-up,
161 participants also consumed a carbohydrate gel (27 g; Powerbar Gel™, Musashi Performance
162 Nutrition, Nestle, Australia) to ensure adequate provision of carbohydrate (Figure 1).
163
164 **Blood sampling**
165 Six 5ml blood samples were taken at regular intervals in each trial (Figure 1). Baseline blood
166 samples (3 h pre-) were taken prior to the ingestion of the initial supplement. Blood samples
167 were analyzed immediately for partial pressure of oxygen ($pO_2$), partial pressure of carbon
168 dioxide ($pCO_2$), oxygen saturation ($sO_2$) (Siemens, Henkestrasse, Germany) and lactate and
169 glucose concentrations (YSI, Yellow Springs, U.S.A).
170
171 **Central fatigue assessment**
172 The knee extension protocol used has been described in detail elsewhere (Billaut et al. 2013).
173 Before, at half-time, and immediately after exercise, participants were instructed to perform a
174 maximum voluntary contraction (MVC) of the quadriceps (dominant leg) against the strain
175 gauge. In this position, a magnetic stimulator (Magstim RAPID; JLM Accutek Healthcare,

176 NSW) and a double 70-mm coil (producing two overlapping circular fields) were used to
177 stimulate each participant's quadriceps during the MVC (Amann et al. 2007; Billaut et al. 2013).
178 The central activation ratio (CAR) was calculated as described previously, where
179 CAR=MVC/(MVC + superimposed twitch) (Gandevia 2001).
180

181 **Surface electromyography (EMG) acquisition and analysis**

182 EMG signals were recorded via skin surface electrodes over the vastus lateralis, rectus femoris,
183 tibialis anterior, biceps femoris, gluteus maximus and lateral gastrocnemius of the dominant
184 lower limb. In each trial, electrodes were secured to the skin with tape and an elastic tubing to
185 prevent cable movements. In the first trial, electrode placement was marked with a permanent
186 marker to ensure that the position of the electrodes was replicated on subsequent visits.
187 EMG signals were collected using the Delsys system (Delsys Inc., Boston, MA) at 2 kHz after
188 being filtered with a 50-Hz line filter. Muscle activity was quantified as the integrated EMG of
189 the signal between the onset and offset of activation of each burst in the last 2 s of every 4 s
190 sprint. EMG signal from all muscles were added together to provide a value representative of
191 overall muscle activation in the lower limb. Data were normalized to the first sprint set to
192 provide a percentage change for total electrical activity to account for a potential
193 disproportionate effect of the first sprint.
194

195 **Near-Infrared Spectroscopy (NIRS) measurement and analysis**

196 Brain oxygenation was measured using NIRS (Artinis Medical Systems, AS Zetten,
197 Netherlands). The protocol allowed the calculation of the tissue saturation index (TSI) for
198 cerebral tissue (Smith & Billaut 2010; Billaut et al. 2013).

199 **Plasma amino acid analysis**

200 Concentrations of the free amino acids histidine, arginine, threonine, lysine, methionine, valine,
201 isoleucine, leucine, phenylalanine, and tryptophanin in plasma were determined using reversed-
202 phase high-performance liquid chromatography (RP-HPLC) as previously reported (Stepto et al.
203 2011). The limit of quantitation for this study was 0.5µmol.$L^{-1}$ with intra- and inter-assay
204 variability of 4.5% and 5.1%, respectively, for tryptophan, and <2.3% and 3.8%, respectively, for
205 the other EAAs.

206

207 **Caffeine analysis**

208 Plasma caffeine concentrations were assessed via RP-HPLC (GBC Scientific HPLC system,
209 GBC Scientific, Victoria, Australia) according to the method of Alvi & Hammami (2011). The
210 internal standard was antipyrine in 30% $HClO_4$ [40 µg/ml]) A 5µm, 4.6x150mm RP C18 column
211 and a 5µm, 4.6x20mm Guard Cartridge insert (Water Atlantis® T3) were used for the separation.
212 The mobile phase consisted of 15 mM potassium phosphate (pH 3.5) and acetonitrile (83:17,
213 v/v), The analysis was performed under isocratic conditions using a flow rate of 1.0 mL.$min^{-1}$.
214 Chromatograms were recorded at 274 nm with a run time of 10 min. The data were collected
215 with a personal computer using EZChrom Elite Chromatography Manager Software and plasma
216 caffeine concentration determined using peak area ratio (caffeine to antipyrine). The assays limit
217 of quantification in plasma was 0.05 mg.$L^{-1}$ with an intra- and inter- assay variability of 2.5%
218 and 5.6% respectively.

219

220

221

222 **Statistical analysis**

223 Data for the 8 participants are expressed as mean ± SD (see Supplementary Tables 1-4). All data
224 were analyzed using a linear mixed model of the estimated true difference between
225 supplementation protocols with Bonferroni *post hoc* adjustments used where appropriate.
226 Estimated mean differences for the fixed effects of time and supplementation strategy, and the
227 interaction of both (time × supplement) were determined for each parameter. Precision of
228 estimation was indicated with 95% confidence intervals (95%CI). Magnitudes of difference
229 between treatments were determined using the standardized mean difference (effect size), the
230 within-subject placebo standard deviation, and are expressed as Cohen's *d*. Chances of the true
231 value of the effect being possibly (≥75%), likely (≥95%), very likely (≥99.5%), or most likely
232 (=100%), were calculated according to established criteria (Hopkins et al. 2009), with clinical
233 chances of benefit or harm considered for running performance variables. An n=8 subjects
234 provided adequate statistical power in a post-only cross-over design assuming a reference
235 difference (between treatments) in work done of 7%, a typical error in repeat sprints of 4.5%,
236 and Type I and II errors of 5% and 20% respectively.

237

238 **Results**

239 **Running performance**: Participants ingesting a combination of caffeine + EAAs completed
240 more mean sprint work (+174 J, 23–324 J; mean difference, 95%CI; *d*=0.30, likely beneficial ;
241 Table 2) and mean sprint power (43 W, 6–81 W; *d*=0.30, likely beneficial), and had higher peak
242 sprint velocity (0.17 $m.s^{-1}$, 0.04–0.31 $m.s^{-1}$; *d*=0.50, very likely beneficial) compared to EAA's
243 alone (Table 2). Mean sprint work, power and velocity were greater in set 1 compared to all
244 other sets (Supplementary Table 1). There was no interaction effect between time and

245 supplement for any running performance variables. Total work performed excluding the sprints

246 showed no significant differences between supplementation protocols.

247

**Table 2**: Comparison of mechanical data between supplementation strategies. Data are mean ± SD

| | Placebo | Caffeine | LNAA | Caffeine + LNAA |
|---|---|---|---|---|
| **Mean Sprint Work (J)** | 2705 ± 581 | 2791 ± 604 | 2678 ± 664 | 2858 ± 763* |
| **Mean Sprint Power (W)** | 676 ± 145 | 698 ± 151 | 669 ± 166 | 715 ± 191* |
| **Peak Sprint Velocity (m.s-$^{1}$)** | 5.78 ± 0.34 | 5.83 ± 0.35 | 5.76 ± 0.44 | 5.94 ± 0.37* |
| **Total Work (Without Sprints) (J)** | 104894 ± 18359 | 108638 ± 15593 | 105047 ± 14985 | 103086 ± 18698 |

***** denotes statistical significant difference from LNAA where p ≤ 0.05

248

249 **Blood parameters**: All supplementation strategies were effective in elevating protein and

250 caffeine concentration (Figure 3, Supplementary table 2).

251

252 **EAA**: In particular, supplementing with EAAs reduced the plasma free tryptophan:LNAA ratio

253 in the caffeine + EAA condition compared to placebo (0.0009, 0.0005–0.0013;*d*=0.76, most

254 likely) and caffeine alone (0.0014, 0.0010–0.0018; *d*=1.21, most likely). There was an effect of

255 time on the plasma free tryptophan:LNAA ratio consistent with ingestion timing.

256

257 **Caffeine**: As expected, plasma caffeine was increased in the caffeine only condition when

258 compared to placebo (4.3 µmol.L$^{-1}$, 3.2–5.4 µmol.L$^{-1}$; *d*=2.65, most likely), and EAA only (4.4

259 µmol.L$^{-1}$, 3.4–5.5 µmol.L$^{-1}$; *d*=2.74, most likely). Plasma caffeine was also higher in the caffeine

260 + EAA condition compared to placebo (4.7 µmol.L$^{-1}$, 3.6–5.7 µmol.L$^{-1}$; *d*=2.88, most likely) and

261 EAA alone (4.8 µmol.$L^{-1}$, 3.7-5.9 µmol.$L^{-1}$; *d*=2.97, most likely; Figure 3B) There was also an
262 effect of time on caffeine concentration, relevant to when caffeine was ingested.
263
264 **$pO_2$ & $SO_2$:** Venous blood $pO_2$ was substantially higher in the caffeine + EAA treatment
265 compared to caffeine alone (7.0 mmHg, 1.7–12.3mmHg; *d*=0.39, likely) and placebo (6.2
266 mmHg, 1.1–11.2 mmHg; 0.35, likely) across the exercise protocol (Supplementary Table 2).
267 Oxygen saturation, as indicated by venous blood $SO_2$, increased from pre- to post-exercise by
268 37% (23-50%; *d*=2.28, most likely) across treatments (Supplementary Table 2).
269
270 **Lactate**: Blood lactate was also substantially higher (0.55 mmol $L^{-1}$, 0.15–0.95; *d*=0.17,
271 possibly; Supplementary Table 2) in the caffeine + EAA group compared to a placebo across all
272 time points.
273
274
275
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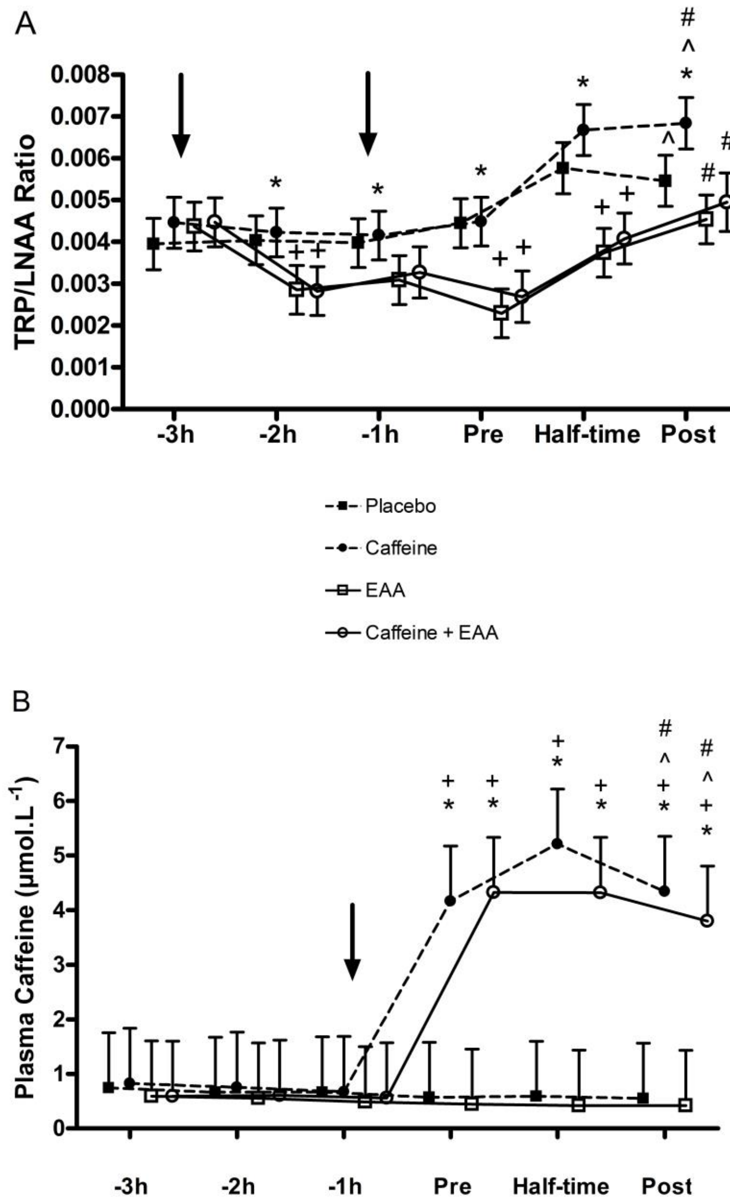



277

278 **Figure 3:** Comparison of Free Tryptophan:LNAA ratio and caffeine concentrations between

279 supplementation strategies. Data are estimated mean ± 95% CI. * denotes significant difference

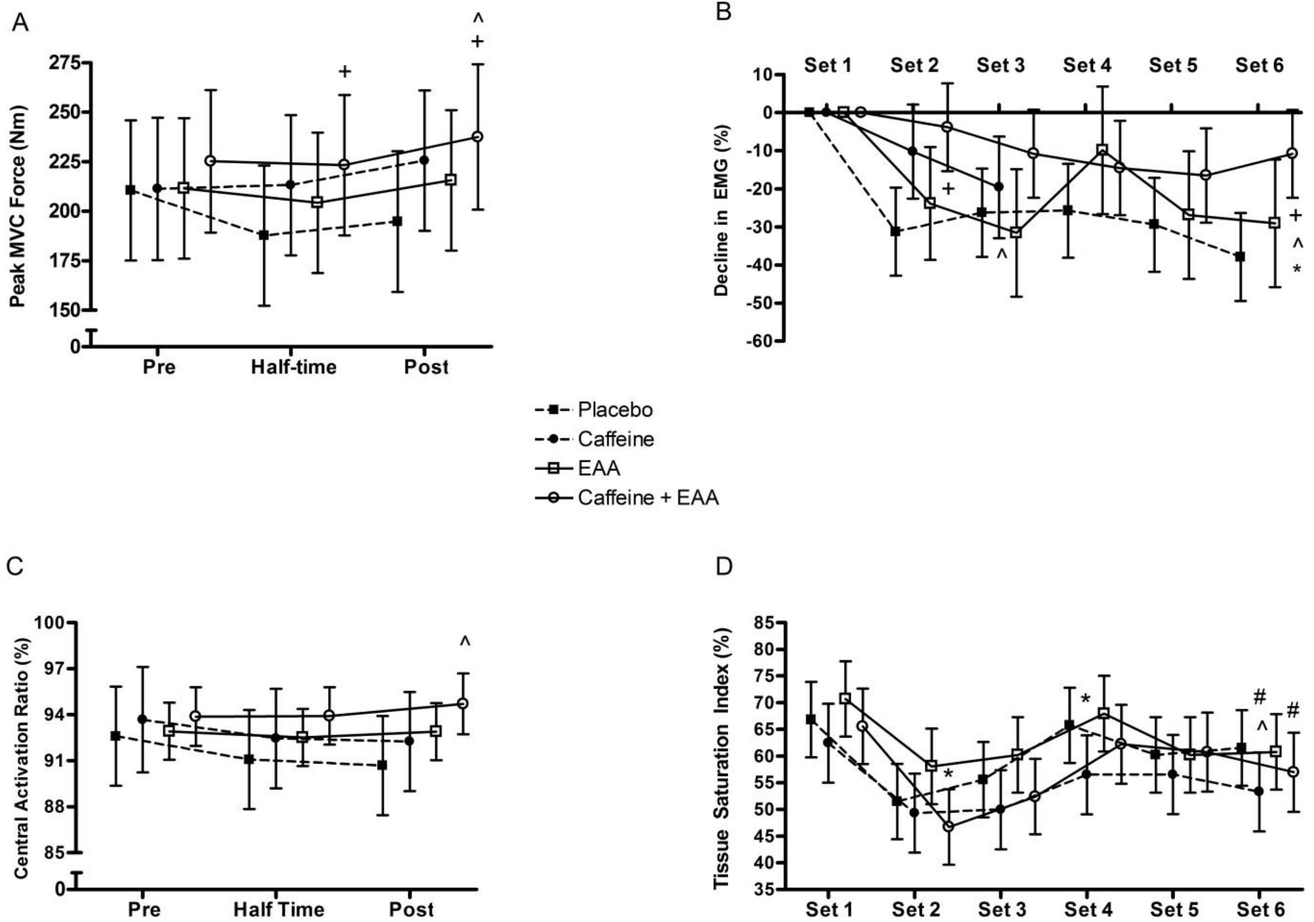
280 from EAA at time point where $p \leq 0.05$. + denotes significant difference from placebo at time

281 point where $p \leq 0.05$. ^ denotes significant difference from EAA overall where $p \leq 0.05$. #

282 denotes significant difference from placebo overall where $p \leq 0.05$. ↓ indicates supplement

283 ingestion time.

284 **MVC**: Overall, MVC force was higher (31nM, 8–54nM; *d*=0.62, likely beneficial) when caffeine
285 + EAA were ingested compared to a placebo (Figure 4A).
286
287 **Peripheral Twitch**: Peripheral twitch force was not different between supplementation protocols
288 across all time points.
289
290 **Central Activation Ratio and EMG**: The decline in normalized EMG activity was attenuated
291 with caffeine + EAA compared with placebo (19%, 9–29%; *d*=0.84, most likely beneficial) and
292 EAA alone (13%, 0–26%; *d*=0.58, likely beneficial). Supplementing with 'caffeine only'
293 resulted in a lower reduction in normalized EMG compared to placebo (15%, 1–29%; *d*=0.67,
294 likely beneficial; Figure 4B). There was no effect of time and no interaction between time and
295 supplement for decline in EMG activity (Supplementary Table 1). Ingesting a combination of
296 caffeine + EAA resulted in a higher overall CAR (2.7%, -5.4 to -0.4%; *d*=0.5, likely beneficial;
297 Figure 4C) compared to a placebo. There was no effect of time, and no interaction between time
298 and supplement.
299
300 **NIRS**: Greater mean declines in TSI across the exercise protocol were observed when caffeine (-
301 8.3%, 3.7–13.0%; *d*=0.9, most likely beneficial) and caffeine + EAA (-5.6%, 1.0–10.1%; *d*=0.6,
302 very likely beneficial) were ingested (the two conditions that produced the highest amount of
303 work), compared with EAAs alone (Figure 4D, Supplementary Table 1).
304
305
306




307

308

309 **Figure 4:** Comparison of central fatigue markers and total work (J) between supplementation

310 strategies. Data are estimated mean ± 95%CI. * denotes significant difference from EAA at time

311 point where $p \leq 0.05$. + denotes significant difference from placebo at time point where $p \leq 0.05$.

312 ^ denotes significant difference from EAA overall where $p \leq 0.05$. # denotes significant

313 difference from placebo overall where $p \leq 0.05$.

314

315

316

317 **Discussion**

318 This study demonstrated, for the first time, that nutritional supplementation with a combination
319 of caffeine and EAAs attenuated the development of central fatigue in response to simulated
320 team-sport exercise in hot and hypoxic environmental conditions. Using EMG activity and CAR
321 as markers of muscle recruitment and central activation, respectively, supplementation with
322 caffeine + EAA delayed the onset of central fatigue in team-sport players, but this effect did not
323 translate into clear performance benefits.

324

325 Our supplementation protocol was designed to target two different neurotransmitter pathways to
326 reduce central fatigue, based on previous literature (Pardridge 1998). Due to the challenges of
327 measuring neurotransmitter concentrations *in vivo* in humans, we can only use a conceptual
328 framework based on prior literature using EAA's containing LNAA's to manipulate
329 neurotransmitters (Pardridge 1998). Our supplementation protocol used EAA's (including
330 LNAA's) to inhibit serotonin synthesis as indicated by a lower free tryptophan:LNAA ratio
331 (Figure 4A; (Pardridge 1998). Additionally, we aimed to promoting dopamine production in
332 the CNS via phenylalanine ingestion (Pardridge 1998). We believe this was achieved, as
333 indicated by the lower ratio of free tryptophan:LNAA and increased phenylalanine
334 concentrations in the plasma when EAAs were ingested with or without caffeine (Figure 4A,
335 Supplementary Table 3). We believe this effectively lowered the serotonin:dopamine ratio,
336 which is considered important in attenuating central fatigue (Fernstrom 2013). Secondly, we
337 aimed to antagonise adenosine receptors via caffeine supplementation (Figure 4, Supplementary
338 Table 3). Isolated EAA and caffeine supplementation are known to effect central drive and motor
339 unit recruitment in thermoneutral (Meeusen et al. 2006) and hot environments (Del Coso et al.

340 2008; Meeusen & Roelands 2010). Antagonizing adenosine or increasing dopamine synthesis
341 has been linked to maintenance of motor unit recruitment and frequency of activation concurrent
342 with improved exercise performance (Del Coso et al. 2008). It is surprising that only caffeine
343 and caffeine + EAA had a clear influence on central fatigue variables. We consider that changes
344 in markers of central drive (CAR and EMG) are likely due to caffeine, and a minor contribution
345 from the EAA's in our combined supplementation protocol.
346
347 Physiological changes in central fatigue variables induced by caffeine and/or EAA, did not
348 translate into a substantial performance benefit. Participants who ingested a combination of
349 caffeine and EAAs performed more work during sprints than with EAA alone, but not
350 significantly more than with a placebo. This is surprising given that supplementation with 3–
351 $6 mg.kg^{-1}$ of caffeine has previously shown to improve sprint times and repeated-sprint
352 performance (Glaister et al. 2008; Gant et al. 2010) compared to a placebo. While EAA
353 supplementation may not improve performance in normal conditions, there is evidence it is
354 beneficial when exercise is performed in the heat (Mittleman et al. 1998; Tumilty et al. 2011).
355 However, the effect of EAA supplementation on high-intensity exercise performance is
356 unknown. Our findings provide new insight into the influence of isolated and combined
357 supplementation protocols on repeat-sprint performance.
358
359 Cerebral oxygenation impairs central drive and motor unit recruitment during repeated-sprint
360 performance in hypoxic conditions (Smith & Billaut 2010; Billaut et al. 2013), and other forms
361 of high-intensity exercise (Rooks et al. 2010). Caffeine ingestion may reduce cerebral blood flow
362 and oxygen availability (Kennedy & Haskell 2011), which could explain the observed reductions

363 in cerebral oxygenation in the final sets of sprints. However, our data appear to dissociate
364 cerebral oxygenation as a mechanism for central fatigue in hot and hypoxic conditions. We
365 consider that reduced cerebral oxygenation observed in the caffeine only and caffeine + EAA
366 trials is a likely consequence of caffeine ingestion and/or the somewhat greater mechanical work
367 produced.
368
369 To our knowledge, this is the first study to measure central fatigue via EMG and magnetic
370 stimulation in conjunction with nutritional supplementation. Although we used a simulated
371 exercise protocol and unique environmental conditions, we believe our data provide novel
372 insights into central fatigue and match performance. This study highlights the potential for
373 nutritional interventions to sustain or enhance exercise performance in challenging
374 environmental conditions. We observed large standard deviations in our performance data, which
375 may be responsible for the lack of significant difference between caffeine + EAA and placebo
376 supplementation protocols. Furthermore, the dose of caffeine used in the study is at the bottom of
377 the recommended range for ergogenic benefit, which may explain why we observed no
378 performance improvements with caffeine supplementation alone. We acknowledge that there
379 may also have been an unaccounted for interaction between pre-exercise carbohydrate feeding
380 and AA supplement on performance. Finally, it is plausible that the environmental conditions
381 chosen for the study induced a level of fatigue that was too great for supplementation alone to
382 overcome, again contributing to the lack of running performance improvements. The observed
383 changes in central fatigue variables may have been too small to enhance performance and these
384 relationships warrant further investigation.
385

386 In conclusion, team-sport athletes who ingest a combination of caffeine and EAA may attenuate
387 central fatigue and perform more work than when ingesting EAA's alone during repeated sprint
388 exercise in hot, hypoxic conditions. The mechanisms explaining this improved performance
389 appear to be associated with central drive and muscle activation, and, possibly, altered
390 neurotransmitter production or function influencing the motivation to exercise. Further work
391 should evaluate similar supplementation and exercise protocols in thermoneutral and normoxic
392 conditions. This line of investigation would help establish the effectiveness of combining
393 supplements for team-sport athletes in more common match and training environments.
394

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**Supplementary Table 1.** Individual time point data for performance outcome variables (6 time points) Data are mean ± SD

| Variable | Condition | Set 1 | Set 2 | Set 3 | Set 4 | Set 5 | Set 6 |
|---|---|---|---|---|---|---|---|
| **Mean sprint work (J)** | **Placebo** | 2860 ± 458 | 2719 ± 466 | 2510 ± 766 | 2819 ± 567 | 2599 ± 638 | 2722 ± 657 |
| | **Caffeine** | 2940 ± 484 | 2775 ± 687 | 2829 ± 664 | 2811 ± 610 | 2686 ± 671 | 2708 ± 660 |
| | **LNAA** | 2911 ± 530 | 2620 ± 798 | 2513 ± 826 | 2820 ± 595 | 2525 ± 680 | 2679 ± 634 |
| | **Caffeine + LNAA** | 2974 ± 677 | 2888 ± 860 | 2837 ± 814 | 2858 ± 781 | 2742 ± 821 | 2867 ± 868 |
| **Mean sprint power (W)** | **Placebo** | 715 ± 115 | 680 ± 117 | 628 ± 191 | 705 ± 142 | 650 ± 159 | 681 ± 164 |
| | **Caffeine** | 735 ± 121 | 694 ± 172 | 707 ± 166 | 703 ± 152 | 672 ± 167 | 677 ± 165 |
| | **LNAA** | 728 ± 132 | 655 ± 199 | 628 ± 206 | 705 ± 149 | 631 ± 170 | 670 ± 158 |
| | **Caffeine + LNAA** | 743 ± 169 | 722 ± 215 | 709 ± 203 | 715 ± 195 | 686 ± 205 | 717 ± 217 |
| **Peak sprint velocity (m.s$^{-1}$)** | **Placebo** | 5.71 ± 0.35 | 5.78 ± 0.29 | 5.79 ± 0.41 | 5.74 ± 0.35 | 5.69 ± 0.40 | 5.97 ± 0.34 |
| | **Caffeine** | 5.81 ± 0.26 | 5.77 ± 0.32 | 6.00 ± 0.30 | 5.64 ± 0.44 | 5.75 ± 0.39 | 6.02 ± 0.29 |
| | **LNAA** | 5.79 ± 0.32 | 5.73 ± 0.47 | 5.79 ± 0.50 | 5.68 ± 0.47 | 5.63 ± 0.50 | 5.92 ± 0.45 |
| | **Caffeine + LNAA** | 5.93 ± 0.29 | 5.91 ± 0.30 | 6.00 ± 0.39 | 5.79 ± 0.30 | 5.87 ± 0.44 | 6.13 ± 0.46 |
| **Mean EMG decline (%)** | **Placebo** | 100 ± 0 | 31.5 ± 31.1 | 26.5 ± 9.2 | 26.7 ± 21.4 | 30.0 ± 17.7 | 38.2 ± 30.2 |
| | **Caffeine** | 100 ± 0 | 9.9 ± 9.7 | 20.9 ± 6.6 | | | |
| | **LNAA** | 100 ± 0 | 25.2 ± 7.1 | 27.7 ± 4.3 | 11.8 ± 9.1 | 28.6 ± 4.0 | 30.2 ± 10.2 |
| | **Caffeine + LNAA** | 100 ± 0 | 4.9 ± 4.9 | 11.8 ± 5.8 | 15.4 ± 10.5 | 17.0 ± 8.5 | 11.9 ± 10.4 |
| **Tissue saturation index (%)** | **Placebo** | 66.8 ± 6.2 | 51.5 ± 4.9 | 55.6 ± 9.2 | 65.8 ± 7.1 | 60.2 ± 9.2 | 61.5 ± 9.8 |
| | **Caffeine** | 62.4 ± 9.3 | 49.1 ± 10.8 | 49.8 ± 11.8 | 56.4 ± 9.7 | 56.5 ± 10.5 | 53.2 ± 14.5 |
| | **LNAA** | 70.7 ± 8.3 | 58.1 ± 10.0 | 60.2 ± 9.3 | 67.9 ± 6.9 | 60.2 ± 9.4 | 60.8 ± 9.6 |
| | **Caffeine + LNAA** | 65.6 ± 9.5 | 46.7 ± 12.7 | 52.4 ± 7.9 | 62.3 ± 11.5 | 60.8 ± 16.9 | 57.1 ± 11.6 |

**Supplementary Table 2.** Venous $pCO_2$, $pO_2$, $sO_2$, lactate, and glucose concentrations between supplementation strategies. Data are mean ± SD

| Variable | Condition | 3hr pre | 2hr pre | 1hr pre | Pre | Half time | Post |
|---|---|---|---|---|---|---|---|
| **$pCO_2$ (mmHg)** | **Placebo** | 57 ± 7 | 59 ± 4 | 62 ± 5 | 54 ± 9 | 33 ± 4 | 30 ± 5 |
| | **Caffeine** | 53 ± 9 | 55 ± 9 | 60 ± 5 | 56 ± 9 | 32 ± 7 | 30 ± 6 |
| | **LNAA** | 56 ± 7 | 57 ± 5 | 59 ± 4 | 50 ± 7 | 31 ± 6 | 31 ± 6 |
| | **Caffeine + LNAA*** | 59 ± 8 | 56 ± 8 | 57 ± 9 | 51 ± 8 | 28 ± 4 | 26 ± 3 |
| **$pO_2$ (mmHg)** | **Placebo** | 31 ± 4 | 24 ± 3 | 20 ± 4 | 27 ± 10 | 53 ± 14 | 63 ± 9 |
| | **Caffeine** | 34 ± 8 | 27 ± 12 | 20 ± 4 | 24 ± 8 | 55 ± 17 | 54 ± 17 |
| | **LNAA** | 33 ± 11 | 24 ± 4 | 20 ± 4 | 33 ± 9 | 64 ± 13 | 60 ± 10 |
| | **Caffeine + LNAA*^** | 33 ± 9 | 29 ± 8 | 24 ± 9 | 30 ± 10 | 65 ± 4 | 71 ± 3 |
| **$sO_2$ (%)** | **Placebo** | 52 ± 11 | 39 ± 10 | 34 ± 12 | 46 ± 23 | 82 ± 12 | 89 ± 8 |
| | **Caffeine** | 65 ± 18 | 45 ± 23 | 31 ± 10 | 43 ± 19 | 80 ± 16 | 79 ± 16 |
| | **LNAA** | 58 ± 20 | 40 ± 12 | 30 ± 12 | 60 ± 19 | 88 ± 10 | 87 ± 8 |
| | **Caffeine + LNAA*^** | 60 ± 13 | 51 ± 17 | 41 ± 20 | 53 ± 21 | 90 ± 1 | 92 ± 1 |
| **Lactate (mmol/L)** | **Placebo** | 0.9 ± 0.2 | 0.8 ± 0.2 | 0.8 ± 0.2 | 0.9 ± 0.1 | 7.1 ± 1.9 | 7.3 ± 1.9 |
| | **Caffeine** | 1.1 ± 0.5 | 0.9 ± 0.3 | 0.8 ± 0.2 | 1.0 ± 0.3 | 7.4 ± 2.0 | 8.5 ± 1.8 |
| | **LNAA** | 1.2 ± 0.7 | 0.9 ± 0.3 | 0.9 ± 0.6 | 1.2 ± 0.5 | 7.5 ± 1.9 | 7.1 ± 1.5 |
| | **Caffeine + LNAA*** | 0.9 ± 0.3 | 0.8 ± 0.1 | 0.9 ± 0.5 | 1.3 ± 0.9 | 8.2 ± 2.4 | 9.4 ± 2.4 |
| **Glucose (mmol/L)** | **Placebo** | 4.0 ± 0.9 | 4.1 ± 0.6 | 4.0 ± 0.4 | 4.7 ± 0.8 | 5.9 ± 0.9 | 5.6 ± 1.7 |
| | **Caffeine** | 4.5 ± 0.5 | 4.5 ± 0.8 | 4.5 ± 1.1 | 4.6 ± 0.7 | 6.1 ± 1.9 | 6.8 ± 1.8 |
| | **LNAA^** | 4.5 ± 1.0 | 3.7 ± 0.3 | 4.4 ± 0.8 | 4.3 ± 0.5 | 6.3 ± 1.5 | 5.7 ± 1.3 |
| | **Caffeine + LNAA** | 3.9 ± 1.0 | 3.7 ± 0.3 | 4.2 ± 1.1 | 4.6 ± 0.9 | 6.8 ± 2.2 | 6.3 ± 1.8 |

* denotes significant difference from placebo treatment over all time points where $p \leq 0.05$

^ denotes significant difference from caffeine treatment over all time points where $p \leq 0.05$

**Supplementary Table 3.** Change in individual LNAA concentrations between supplementation strategies. Data are mean ± SD

| Variable | Condition | 3hr pre | 2hr pre | 1hr pre | Pre | Half time | Post |
|---|---|---|---|---|---|---|---|
| **Histidine (µmol/L)** | **Placebo*^** | 82 ± 6 | 78 ± 6 | 78 ± 5 | 75 ± 8 | 82 ± 10 | 77 ±112 |
| | **Caffeine** | 80 ± 5 | 83 ± 7 | 81 ± 9 | 76 ± 10 | 77 ± 10 | 85 ± 9 |
| | **LNAA#** | 80 ± 9 | 104 ± 11 | 84 ± 9 | 109 ± 21 | 87 ± 9 | 85 ± 7 |
| | **Caffeine + LNAA** | 84 ± 12 | 119 ± 23 | 90 ± 11 | 116 ± 17 | 94 ± 14 | 91 ± 13 |
| **Arginine (µmol/L)** | **Placebo*** | 92 ± 9 | 84 ± 11 | 84 ± 9 | 76 ± 11 | 83 ± 15 | 81 ± 19 |
| | **Caffeine*** | 91 ± 14 | 90 ± 14 | 87 ± 13 | 77 ± 11 | 74 ± 12 | 82 ± 11 |
| | **LNAA** | 90 ± 19 | 124 ± 18 | 105 ± 14 | 139 ± 39 | 103 ± 15 | 96 ± 16 |
| | **Caffeine + LNAA** | 95 ± 16 | 145 ± 24 | 110 ± 11 | 138 ± 23 | 105 ± 9 | 91 ± 6 |
| **Threonine (µmol/L)** | **Placebo*^** | 129 ± 23 | 122 ± 22 | 116 ± 15 | 108 ± 18 | 110 ± 17 | 105 ± 24 |
| | **Caffeine*^** | 132 ± 29 | 131 ± 26 | 126 ± 22 | 116 ± 28 | 105 ± 20 | 115 ± 25 |
| | **LNAA** | 128 ± 32 | 141 ± 23 | 125 ± 19 | 153 ± 37 | 122 ± 18 | 121 ± 15 |
| | **Caffeine + LNAA*** | 136 ± 22 | 163 ± 24 | 137 ± 17 | 157 ± 32 | 129 ± 20 | 122 ± 16 |
| **Lysine (µmol/L)** | **Placebo*^** | 167 ± 26 | 151 ± 21 | 146 ± 17 | 134 ± 22 | 147 ± 22 | 132 ± 27 |
| | **Caffeine*^** | 170 ± 40 | 166 ± 30 | 158 ± 27 | 139 ± 33 | 126 ± 20 | 136 ± 16 |
| | **LNAA*** | 165 ± 51 | 196 ± 36 | 165 ± 31 | 200 ± 52 | 150 ± 28 | 136 ± 24 |
| | **Caffeine + LNAA** | 178 ± 48 | 238 ± 57 | 179 ± 22 | 211 ± 40 | 159 ± 24 | 143 ± 22 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Methionine (µmol/L)** | **Placebo*** | 28 ± 6 | 25 ± 5 | 23 ± 4 | 22 ± 4 | 24 ± 4 | 26 ± 6 |
| | **Caffeine** | 32 ± 6 | 29 ± 4 | 27 ± 4 | 24 ± 6 | 24 ± 5 | 28 ± 4 |
| | **LNAA** | 28 ± 7 | 34 ± 6 | 28 ± 4 | 34 ± 10 | 28 ± 4 | 28 ± 4 |
| | **Caffeine + LNAA** | 32 ± 7 | 40 ± 7 | 28 ± 5 | 34 ± 8 | 28 ± 5 | 27 ± 3 |
| **Valine (µmol/L)** | **Placebo*^** | 255 ± 33 | 249 ± 62 | 241 ± 46 | 237 ± 33 | 240 ± 36 | 237 ± 49 |
| | **Caffeine*^** | 257 ± 25 | 245 ± 28 | 240 ± 44 | 220 ± 21 | 211 ± 26 | 224 ± 43 |
| | **LNAA*** | 250 ± 46 | 320 ± 41 | 284 ± 22 | 372 ± 72 | 305 ± 40 | 285 ± 36 |
| | **Caffeine + LNAA** | 280 ± 45 | 391 ± 67 | 320 ± 50 | 402 ± 47 | 324 ± 38 | 301 ± 47 |
| **Isoleucine (µmol/L)** | **Placebo*^** | 65 ± 15 | 60 ± 25 | 58 ± 17 | 62 ± 11 | 62 ± 14 | 65 ± 24 |
| | **Caffeine*^** | 72 ± 13 | 62 ± 12 | 63 ± 21 | 55 ± 10 | 55 ± 8 | 61 ± 12 |
| | **LNAA** | 65 ± 17 | 100 ± 17 | 82 ± 19 | 122 ± 30 | 87 ± 14 | 77 ± 20 |
| | **Caffeine + LNAA** | 78 ± 15 | 125 ± 32 | 87 ± 24 | 121 ± 24 | 89 ± 21 | 69 ± 20 |
| **Leucine (µmol/L)** | **Placebo*^** | 129 ± 23 | 118 ± 34 | 113 ± 25 | 116 ± 17 | 118 ± 19 | 120 ± 37 |
| | **Caffeine*^** | 140 ± 25 | 125 ± 21 | 123 ± 35 | 108 ± 18 | 106 ± 13 | 116 ± 22 |
| | **LNAA** | 126 ± 29 | 179 ± 25 | 151 ± 25 | 213 ± 50 | 160 ± 23 | 142 ± 29 |
| | **Caffeine + LNAA** | 148 ± 8 | 222 ± 52 | 162 ± 38 | 220 ± 38 | 161 ± 27 | 136 ± 33 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Phenylalanine (µmol/L)** | **Placebo*^** | 57 ± 7 | 52 ± 7 | 51 ± 8 | 52 ± 7 | 55 ± 7 | 57 ± 10 |
| | **Caffeine*^** | 60 ± 10 | 58 ± 9 | 55 ± 10 | 52 ± 7 | 54 ± 6 | 60 ± 9 |
| | **LNAA*** | 55 ± 11 | 81 ± 14 | 73 ± 15 | 102 ± 23 | 88 ± 18 | 86 ± 12 |
| | **Caffeine + LNAA** | 62 ± 13 | 99 ± 24 | 75 ± 4 | 112 ± 22 | 96 ± 18 | 93 ± 14 |
| **Tryptophan (µmol/L)** | **Placebo^+** | 4.0 ± 0.7 | 3.8 ± 0.8 | 3.6 ± 0.8 | 3.9 ± 0.6 | 5.1 ± 1.8 | 5.4 ± 1.0 |
| | **Caffeine^** | 4.6 ± 0.4 | 4.2 ± 0.7 | 4.0 ± 1.0 | 3.9 ± 0.4 | 5.5 ± 1.2 | 6.2 ± 0.8 |
| | **LNAA*** | 4.3 ± 0.7 | 3.6 ± 0.5 | 3.4 ± 0.9 | 3.3 ± 1.1 | 4.2 ± 1.1 | 4.8 ± 1.2 |
| | **Caffeine + LNAA*** | 4.9 ± 0.8 | 4.3 ± 0.8 | 3.9 ± 0.7 | 4.1 ± 1.4 | 4.9 ± 0.8 | 5.2 ± 0.8 |

* denotes significant difference between treatment and caffeine + LNAA treatment over all time points where $p \leq 0.05$
^ denotes significant difference between treatment and LNAA treatment over all time points where $p \leq 0.05$
+ denotes significant difference between treatment and caffeine treatment over all time points where $p \leq 0.05$
# denotes significant difference between treatment and placebo treatment over all time points where $p \leq 0.05$

**Supplementary Table 4.** Individual time point data (3 time points) for main outcome variables. Data are mean ± SD

| Variable | Condition | Pre | Half-Time | Post |
|---|---|---|---|---|
| **Mean MVC force (nM)** | **Placebo** | 211 ± 52 | 188 ± 48 | 195 ± 53 |
| | **Caffeine** | 218 ± 43 | 213 ± 44 | 226 ± 62 |
| | **LNAA** | 212 ± 46 | 204 ± 36 | 216 ± 40 |
| | **Caffeine + LNAA** | 215 ± 35 | 223 ± 41 | 231 ± 33 |
| **Mean twitch force (nM)** | **Placebo** | 68.5 ± 21.6 | 62.7 ± 22.9 | 71.2 ± 24.5 |
| | **Caffeine** | 68.1 ± 19.3 | 65.8 ± 22.9 | 69.5 ± 15.6 |
| | **LNAA** | 68.9 ± 20.9 | 65.0 ± 17.5 | 66.1 ± 14.5 |
| | **Caffeine + LNAA** | 62.8 ± 17.4 | 59.5 ± 12.2 | 71.0 ± 16.3 |
| **Central activation ratio (%)** | **Placebo** | 92.6 ± 2.9 | 91.1 ± 5.3 | 90.7 ± 7.6 |
| | **Caffeine** | 93.5 ± 1.5 | 92.5 ± 2.5 | 92.3 ± 5.5 |
| | **LNAA** | 92.9 ± 4.2 | 92.5 ± 4.7 | 92.9 ± 4.5 |
| | **Caffeine + LNAA** | 94.5 ± 2.0 | 93.9 ± 3.8 | 96.1 ± 2.2 |