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2

3    Effect of a single and repeated dose of caffeine on antigen-stimulated human natural killer

4    cell CD69 expression after high intensity intermittent exercise

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26  **Abstract**

27  Several studies investigating the effect of caffeine on immune function following exercise

28  have used one large bolus dose of caffeine. However, this does not model typical caffeine

29  consumption. Therefore the purpose of this study was to investigate whether small repeated

30  doses of caffeine ingested throughout the day would elicit a similar response as one large

31  bolus dose ingested 1 h prior to exercise on antigen-stimulated NK cell CD69 expression

32  following strenuous intermittent exercise. In a randomized cross-over design, 15 healthy

33  males completed six 15 min blocks of intermittent running consisting of maximal sprinting

34  interspersed with less intense running and walking. Participants had ingested either 0 (PLA),

35  2 $mg \cdot kg^{-1}$ body mass (BM) caffeine on 3 separate occasions during the day (3xCAF) or 1

36  dose of 6 (1xCAF) $mg \cdot kg^{-1}$ BM caffeine, 1 h before exercise. At 1 h post-exercise the

37  number of antigen-stimulated $CD3^{-}CD56^{+}$ cells expressing CD69 was lower on 1xCAF

38  compared with PLA [$P<0.05$; PLA: 42.0 $(34.0)x10^{6}$ $cells \cdot L^{-1}$, 1xCAF: 26.2 $(25.0)x10^{6}$

39  $cells \cdot L^{-1}$], with values on 1xCAF at this time-point remaining close to pre-supplement.

40  1xCAF tended to attenuate the exercise-induced increase in geometric mean fluorescence

41  intensity of CD69 expression on antigen-stimulated $CD3^{-}CD56^{+}$ cells 1 h post-exercise

42  [$P=0.055$; PLA: 141 (28)%, 1xCAF: 119 (20)%]. These findings suggest that although one

43  large bolus dose of caffeine attenuated the exercise-induced increase in antigen-stimulated

44  NK cell CD69 expression 1 h following strenuous intermittent exercise, this attenuation at no

45  point fell below pre-supplement values and as such caffeine does not appear to depress NK

46  cell CD69 expression.

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## Introduction

58 Caffeine is a common element in most people's diets (Heatherley et al. 2006), yet it is not a

59 typical nutrient nor is it essential for health (Graham 2001). Caffeine is legally and socially

60 acceptable and as such is the most widely consumed behaviour-influencing substance in the

61 world (Graham 2001). It is estimated that the mean daily intake of caffeine in the U.K is

62 ~241 ± 173 mg·day$^{-1}$, which is the equivalent to around 2-4 cups of coffee (Heatherley et al.

63 2006). However, daily caffeine intake can vary widely depending upon country. For example

64 the daily caffeine intake in Sweden, Norway and Netherlands is estimated to be

65 approximately 400 mg·day$^{-1}$ (Fredholm et al. 1999).

66

67 Natural killer (NK) cells are a subset of lymphocytes that have innate immune functions,

68 representing approximately 5–20 % of all lymphocytes in peripheral blood (Andoniou et al.

69 2006). NK cells are capable of recognizing and destroying certain tumour cells and virally-

70 infected cells without prior sensitization or specific antigen recognition, and therefore are

71 distinct from other lymphocytes. At rest (unstimulated) NK cells tend to express small

72 amounts of functionally active CD69 (Borrego et al. 1999), however following activation this

73 antigen is rapidly induced (Ziegler 1994). Although the exact role of CD69 has not as yet

74 been elucidated, *in vitro* studies have demonstrated that once expressed on lymphocytes, this

75 antigen appears to act as a co-stimulatory receptor helping to trigger subsequent cell

76 proliferation, cytotoxicity and secretion of cytokines (such as tumour necrosis factor-α and

77 interferon-γ) (Borrego et al. 1999). However, *in vivo* studies have demonstrated that the

78 behaviour of CD69 is more complex, and also appears to act as an immunoregulatory

79 molecule (reviewed in Sancho et al. 2005). Several studies have demonstrated that CD69

80 cross-linking appears to induce the production of the anti-inflammatory cytokine transforming

81  growth factor-β (TGF- β) in lymphocytes, that appears to be able to downregulate the

82  inflammatory response as well as tumour lysis by NK cells (Esplugues et al. 2003; Sancho et

83  al. 2003).

84

85  Acute strenuous exercise causes a temporary perturbation in a range of immune cell functions

86  usually lasting between ~3-24 h.  It is suggested that during this "open window" of altered

87  immunity, viruses and bacteria may gain a foothold, increasing the risk of subsequent

88  infection (Nieman 2000).  Several studies have demonstrated that for several hours following

89  both high intensity continuous and intermittent exercise NK cell number and function are

90  suppressed to below baseline measures (Kappel et al. 1991; McFarlin et al. 2004; Neiman et

91  al. 2007).    NK cells express both adenosine receptors ($A_1$, $A_{2A}$ and $A_{2B}$) and $\beta_2$-

92  adrenoreceptors (Priebe et al. 1990; Shephard 2003). Caffeine is a non-selective adenosine

93  receptor antagonist and also stimulates epinephrine release from the adrenal medulla; as such

94  caffeine has the potential to either increase (via $A_{2A}$ receptor antagonism) or decrease (via $A_1$

95  receptor antagonism and/or increased epinephrine stimulation) NK cell activation depending

96  upon the principal mechanism. Strenuous exercise increases the plasma concentrations of both

97  adenosine and epinephrine (Nieman et al. 2007; Vizi et al. 2002) and it has been proposed that

98  these increases predominately inhibit NK cell function *in vitro* and *in vivo* (Kappel et al.

99  1991; Priebe et al. 1990), via $A_{2A}$ adenosine receptor and $\beta_2$-adrenoreceptor activation of

100  adenylate cyclase and increased intracellular cAMP  (Raskovalova et al. 2006). It is likely that

101  in response to caffeine ingestion and exercise both adenosine and epinephrine mechanisms

102  will be involved.  However, the overall affect on NK cell activation may depend upon which

103  mechanism predominates.

104

105    Caffeine has been shown to increase sympathoadrenal medullary system (SAM) activity and

106    as such plasma epinephrine concentration (reviewed in Graham 2001).  Salivary α-amylase

107    activity is stimulated by increased activity of the sympathetic nervous system (Rohleder et al.

108    2004).  Therefore, it has been suggested that elevations in α-amylase concentration can be

109    considered a good indicator of enhanced sympathetic activity (Anderson et al. 1984; Rohleder

110    et al. 2004).  As such salivary α-amylase has been chosen as a surrogate marker for plasma

111    epinephrine concentration in the present study.

112

113    Despite the reported widespread and frequent use of caffeine within society (Heatherley et al.

114    2006), very few studies have investigated the effect of caffeine on immune cell responses to

115    exercise in healthy individuals.  Of the handful of studies that have been published, all have

116    administered 6 mg·kg$^{-1}$ body mass caffeine in one bolus dose following a 60 h abstinence

117    period (Bishop et al. 2005, 2006; Fletcher and Bishop 2010; Walker et al. 2006, 2007, 2008).

118    However, caffeine is typically consumed in separate doses throughout the day, following an

119    overnight abstention (Fredholm et al. 1999).  To the authors knowledge no study to date has

120    attempted to model natural caffeine consumption in terms of dose and time of consumption

121    when investigating caffeine's effects on immune cell function following exercise.  Recent

122    pilot work from our group has suggested that a small dose of caffeine (2 mg·kg$^{-1}$) ingested

123    prior to exercise increases antigen-stimulated NK cell CD69 expression 1 h following high

124    intensity cycling to the same extent as that of a dose 3 times as large (6 mg·kg$^{-1}$).  Therefore

125    the aim of this study was to investigate whether small repeated doses (2 mg·kg$^{-1}$) of caffeine

126    ingested throughout the day (9:00 h, 12:00 h & 15:00 h) would elicit a similar response as one

127    large (6 mg·kg$^{-1}$) bolus dose ingested 1 h prior to exercise (15:00 h) compared with placebo

128    on antigen-stimulated NK cell CD69 expression following 90 min of high intensity

129    intermittent exercise.  It was hypothesised that both caffeine trials would increase NK cell

130    CD69 expression to a similar extent following high intensity intermittent exercise, and that

131    this increase would be significantly greater when compared with placebo.

132

133    **Methods**

134    *Participants*

135    Fifteen healthy male games (football and rugby) players [mean (SD): age 22 (2) years; body

136    mass 76 (8) kg; $\dot{V}O_{2\,max}$ 54 (3) ml·kg$^{-1}$·min$^{-1}$] volunteered to participate in this study. All

137    participants were fully informed about the rationale for the study and the design of the

138    exercise tests and trial protocol, along with their possible risks and discomforts, before

139    providing written informed consent. The local Ethical Advisory Committee approved the

140    study. Participants did not report taking any medications or experiencing any symptoms of

141    upper respiratory tract infection in the 4 weeks prior to the study. All participants were

142    moderate caffeine users (50 – 250 mg·day$^{-1}$), with an average daily caffeine intake of 159 (61)

143    mg·day$^{-1}$.

144

145    *Preliminary testing*

146    Approximately 2 weeks before the beginning of the main trials, each participant was required

147    to perform a progressive shuttle run test to volitional fatigue to estimate maximal oxygen

148    consumption ($\dot{V}O_{2\,max}$) (Ramsbottom et al., 1988). From this estimate, running speeds

149    corresponding to 55% and 95% $\dot{V}O_{2\,max}$ were calculated from the tables for predicted $\dot{V}O_{2\,max}$

150    values (Ramsbottom et al., 1988). Participants came back into the lab on a separate occasion

151    to undertake a familiarization trial, which required them to perform 3x15 min blocks (45 min)

152    of the LIST. Heart rates (Polar FS1, Polar Electro Oy, Kempele, Finland) were monitored

153    continuously during the familiarization trial.

154

155 *Experimental trial procedures*

156 Participants were instructed to avoid alcohol consumption and strenuous physical activity in

157 the 24 h preceding each experimental trial. Participants were also instructed to stop

158 consuming caffeine from 21:00 h the night before each trial. In an attempt to standardize

159 nutritional status, participants completed a 24 h food diary the day before and the day of the

160 first main trial. Participants were then asked to follow their food diaries for the second and

161 third main trials. Participants performed 3 main exercise trials, each separated by 1 week.

162 Participants were randomly assigned to either the PLA, 1xCAF (1 single dose of 6 mg·kg$^{-1}$

163 caffeine) or 3xCAF (3 repeated doses of 2 mg·kg$^{-1}$ caffeine) trial and acted as their own

164 controls in a repeated-measures, single-blind, cross-over design. Participants arrived at the

165 laboratory at 08:50 h following an overnight fast of 12 h. After 10 min of resting quietly an

166 initial (pre-supplement) blood sample was obtained from an antecubital forearm vein by

167 venepuncture. Following the blood sample, an initial (09:00 h, pre-supplement) saliva sample

168 was obtained. Participants then ingested 0 (PLA) or 2 (3xCAF) mg·kg$^{-1}$ body mass of

169 caffeine powder (BDH Laboratory Supplies, Poole, UK) taken in the form of cellulose

170 capsules (G & G Food Supplies Ltd, West Sussex, UK) with 300 ml plain water. For the 0

171 mg·kg$^{-1}$ dose, participants ingested 2 mg·kg$^{-1}$ body mass of dextrose powder (BDH

172 Laboratory Supplies, Poole, UK). The specific timings of PLA or CAF ingestion along with

173 blood and saliva samples are illustrated in Fig. 1. Participants then rested quietly in the

174 laboratory for 1 h before a further (10:00 h) saliva sample was taken, after which participants

175 were free to leave the laboratory and to start eating. Participants were required to return to the

176 laboratory at 12:00 h to ingest another set of either 0 (PLA) or 2 (3xCAF) mg·kg$^{-1}$ caffeine

177 capsules and to provide a further saliva sample (12:00 h). After the saliva sample participants

178 left the laboratory until 15:00 h, at which time they provided another saliva sample (15:00 h).

179    Participants stopped eating from 15:00 h. Participants then ingested a final set of either 0

180    (PLA), 2 (3xCAF) or 6 (1xCAF) mg·kg$^{-1}$ caffeine capsules immediately after the saliva

181    sample. Participants then rested quietly in the laboratory for 1 h before a further venous blood

182    and saliva sample (16:00 h, pre-exercise) were taken.  Immediately following venous blood

183    and saliva samples pre-exercise body mass (in shorts only) was recorded, after which,

184    participants began six 15 min periods of intermittent shuttle running, which consisted of ~10

185    cycles of walking, maximal sprinting, jogging and cruising (Fig. 2).   Participants were

186    required to run between two lines separated by 20 m at various speeds that related to previous

187    $\dot{V}O_{2\,max}$ values. Each 15 min bout of running was separated by a 3 min rest period.  During

188    this period participants were allowed to consume plain water *ad libitum* and were asked to

189    rate their perceived exertion (RPE) on a Borg 6-20 scale. Heart rates were recorded prior to,

190    during and immediately after each 15 min period of intermittent shuttle running.  A further

191    venous blood and saliva sample (17:45 h, post-exercise) was obtained immediately after

192    cessation of exercise, before post-exercise body mass (in shorts only) was recorded.

193    Participants then consumed 5 ml·kg$^{-1}$ body mass of plain water and rested quietly in the

194    laboratory for a further hour before a final venous blood and saliva sample (18:45 h, 1 h post-

195    exercise) was obtained.  During this time no additional food or fluid was administered to

196    participants.  For all venous blood samples, 20 ml of blood was obtained, with all samples

197    taken from participants in an upright-seated position.  For all saliva samples, participants were

198    asked to swallow to empty the mouth before timed unstimulated whole mixed saliva

199    collections were made into pre-weighed sterile vials (7 ml capacity bijou tubes with screw-

200    top). All collections were made over a 2 min period, unless insufficient volume had been

201    produced, in which case the collection period was increased to 3 min, as necessary.   All

202    collections were made with participants seated; leaning forwards and with their heads tilted

203    downwards. Participants were instructed to allow the saliva to dribble into the tube with

204 minimal orofacial movement. Laboratory conditions throughout the study were 20.5 (0.9) °C

205 and 35.7 (5.4) % relative humidity.

206

207 *Total lymphocyte count and plasma volume*

208 Blood samples were collected into two evacuated monovette tubes (Starstedt, Leicester, UK),

209 one containing $K_3$EDTA (1.6 mg EDTA ml$^{-1}$ blood) and one containing no additive to obtain

210 serum, as well as a sterile 5 ml bijou tube (Sterilin, Staffordshire, UK) containing 15 µl

211 sodium heparin (15 IU heparin ml$^{-1}$ blood). Blood collected into the $K_3$EDTA monovette (7.5

212 ml) was analyzed for total and differential leukocyte counts, hematocrit and hemoglobin

213 contents using a hematology analyzer ($A^C.T^{TM}$ 5diff analyzer, Beckman Coulter, UK).

214 Plasma volume changes were estimated according to Dill and Costill (1974).

215

216 *Lymphocyte culture*

217 Five ml of whole blood transferred into the sterile bijou tube containing heparin was

218 immediately placed on ice and mixed (Thermo Denley, Spiramix) for 20 min before setting

219 up cultures. Due to the large time gap (6 h) between pre-supplement and pre-exercise venous

220 blood samples, 2 lots of cultures were set up: one set was the pre-supplement sample, the

221 other set was the pre-exercise, post-exercise and 1 h post-exercise samples. Cells were

222 cultured in Falcon 12 x 75 mm-polystyrene tubes with caps (Becton Dickinson Biosciences,

223 Oxford, UK) and for all samples stimulated and unstimulated cultures were set up. Tubes

224 were stimulated by a 1:400 or 1:800 Pediacel vaccine (Sanofi Pasteur MSD Ltd, Berkshire,

225 UK), containing diphtheria toxoid, tetanus toxoid, acellular pertussis bacteria, poliovirus and

226 haemophilus influenza type b. Zero or 20 µl of Pediacel working vaccine (either 1:400 or

227 1:800) was added to 200 µl of heparinized whole blood, giving a final stimulant concentration

228    of 1:4000 (optimum) or 1:8000 (sub-optimum) before being incubated for 20 h at 37 °C, 5%

229    $CO_2$. All cell culture work was carried out in a class II laminar flow hood (Esco GB Ltd,

230    Salisbury, UK) by using aspectic techniques.

231

232    *Assessment of lymphocyte subsets and CD69 expression*

233    After incubation, peripheral blood cells were labelled with a cocktail of Pharmingen

234    monoclonal antibodies (Becton Dickinson Biosciences, Oxford, UK) against human

235    lymphocyte cell surface markers, as follows: 5 µl CD3 FITC, 20 µl CD56 PECy5 and 10 µl

236    CD69 PE.  All samples were then vortexed and placed on ice for 20 min, after which

237    erythrocytes were lysed and leukocytes fixed using FACS Lyse (Becton Dickinson, Oxford,

238    UK).  Leukocytes were washed once in 3 ml ice cold phosphate-buffered saline (PBS)

239    containing 0.1% bovine serum albumin (BSA) and 2 mM EDTA (PBS/BSA/EDTA) before

240    being resuspended in 400 µl PBS/BSA/EDTA.  Three-colour flow cytometric analysis was

241    performed using a FACS Calibur flow cytometer with Cell Quest analysis software (Becton

242    Dickinson, Oxford, UK).  Standard gating procedures using side scatter versus forward scatter

243    plots were used to gate on the lymphocyte population.  An unstained unstimulated sample was

244    used to set quadrant boundaries to allow accurate acquisition of stained samples. All samples

245    were set to collect 30,000 lymphocyte events per analysis.

246

247    $CD3^+$ (T cell region) and $CD56^+$ (NK cell region) populations were acquired on quadrant dot

248    plots of FL1 (CD3 FITC) and FL3 (CD56 PE-Cy5), along with quadrant dot plots of FL1

249    (CD3) and FL2 (CD69 PE) and quadrant dot plots of FL3 (CD56) and FL2 (CD69).  This data

250    was then displayed as histogram plots and the percentage of $CD3^-$ cells (total lymphocyte

251    region minus the $CD3^+$ region) expressing $CD56^+$ were derived and from this the percentage

252    of total lymphocytes that were $CD3^-CD56^+$ was determined.  $CD3^-CD56^+$ cells were then

253 gated into a separate region and a CD69$^+$ histogram plot of the cells within this region was

254 used to calculate the percentage expression and geometric mean fluorescence intensity

255 (GMFI) expression of CD69 of CD3$^-$CD56$^+$ cells. Cell counts of CD3$^-$CD56$^+$ were calculated

256 by multiplying the percentage of these cells with the absolute lymphocyte count. The number

257 of CD3$^-$CD56$^+$ cells expressing CD69 were determined by multiplying the percentage of cells

258 expressing CD69 by the total number of CD3$^-$CD56$^+$ cells. To facilitate intersubject

259 comparisons, pre-exercise, post-exercise and 1 h post-exercise CD69 GMFI were expressed

260 as a percentage of the pre-supplement value, as according to Timmons et al. (2006).

261

262 *Saliva flow rate*

263 Saliva samples were stored in their plastic containers at –80 °C prior to analysis. Saliva

264 volume was estimated by weighing the bijou tubes to the nearest mg before and after saliva

265 collection. Saliva density was assumed to be 1.00 $g \cdot ml^{-1}$ (Cole and Eastoe 1988) and from

266 this, the saliva flow rate ($\mu l \cdot min^{-1}$) was determined by dividing the volume of saliva by the

267 collection time.

268

269 Saliva α-amylase

270 Saliva α-amylase activity was measured using a commercially available kit (Infinity$^{TM}$

271 Amylase Liquid Stable Reagent, Thermo Scientific, UK), with proportional reduction of

272 volumes so that the assay could be carried out in a microtitration (96-well) plate. Briefly, 20

273 μl saliva diluted 1:100 with 1.0 mM $CaCl_2$ was mixed with 180 μl of amylase reagent. The

274 plate was incubated for 1 min at 25°C and then the increase in absorbance at 405 nm was

275 recorded every min for a further 4 min period on an automated plate reader. Purified α-

276 amylase from human saliva (A1031, Sigma) was used as a standard. The secretion rate of α-

277 amylase (U·min$^{-1}$) was calculated by multiplying the saliva flow rate by the α-amylase

278 activity.  The intra assay coefficient of variation for α-amylase was 1.1%

279

280 *Serum and saliva caffeine concentration*

281 Serum was obtained from whole blood collected into a serum monovette (5.5 ml), which was

282 left to clot for 1 h before being centrifuged at 1500 g for 10 min in a refrigerated centrifuge at

283 4 ℃.  Saliva samples were transferred into eppendorfs and centrifuged at 12000 rpm for 2 min

284 in a high speed microcentrifuge. Serum and saliva caffeine concentration was determined

285 using a commercially available kit (Emit Caffeine Assay, Dade-Behring, Milton Keynes, UK)

286 on an automatic photometric analyzer (COBAS Miras Plus, Roche Diagnostic Systems,

287 Switzerland).   The intra assay coefficient of variation for serum and saliva caffeine

288 concentration was 2.6% and 1.7%, respectively.

289

290 *Statistical analysis*

291 Data in the text, tables and figures are presented as mean values and their standard deviation

292 ($\pm$ SD).  The data were examined using a two-factor (trial x time) analysis of variance

293 (ANOVA) with repeated measures design.   If the data were not normally distributed,

294 statistical analysis was carried out on the logarithmic transformation of the data. Assumptions

295 of sphericity in the data were checked, and adjustments in the degrees of freedom for the

296 ANOVA were made using the Huynh-Feldt method of correction where appropriate.   Any

297 significant data were assessed using Student's paired *t*-tests with Holm-Bonferroni

298 adjustments for multiple comparisons.  Statistical significance for this study was accepted at

299 $P<0.05$.  The observed powers of the reported main and interaction effects are all >0.8.

300

301  **Results**

302  *Physiological measurements*

303  There were no differences in ratings of perceived exertion between trials during the test [RPE

304  of 13 (2) for all trials].  Likewise, heart rates were similar between trials during exercise

305  [PLA: 171 (12) beat·min$^{-1}$, 1xCAF: 175 (13) beat·min$^{-1}$, 3xCAF: 177 (12) beat·min$^{-1}$; mean

306  of all recordings].

307

308  *Changes in body mass and plasma volume*

309  After exercise, changes in body mass (corrected for fluid intake) were similar for all trials

310  [PLA: -1.8 (0.3) kg, 1xCAF: -1.8 (0.3) kg, 3xCAF: -1.9 (0.4) kg].  There was no significant

311  time x trial interaction effect for changes in plasma volume relative to the initial blood

312  sample.  After exercise plasma volume decreased by 3.3 (4.4) %, 3.4 (6.0) % and 4.8 (6.6) %

313  on the PLA, 1xCAF and 3xCAF trials, respectively.

314

315  *Serum caffeine concentration*

316  A significant time x trial interaction was found for serum caffeine concentration (P<0.01),

317  with higher concentrations at pre (16:00 h), post (17:45 h) and 1 h post-exercise (18:45 h) on

318  both 1xCAF and 3xCAF compared with PLA (P<0.01; Fig. 3).  Serum caffeine concentration

319  was also significantly higher on 3xCAF at pre-exercise compared with 1xCAF (P<0.05).

320  However at post-exercise and 1 h post-exercise serum caffeine concentrations on 1xCAF were

321  significantly higher than 3xCAF (both P<0.01; Fig 3).

322

323  *Saliva caffeine concentration*

324 A significant time x trial interaction was found for saliva caffeine concentration (P<0.01),

325 with higher concentrations at 10:00 h, 12:00 h, 15:00 h and pre (16:00 h), post (17:45 h) and 1

326 h post-exercise (18:45 h) on 3xCAF compared with PLA (P<0.01; Table 1) and at post (17:45

327 h) and 1 h post-exercise (18:45 h) on 1xCAF compared with PLA (P<0.01; Table 1). Saliva

328 caffeine concentrations were also higher on 3xCAF at 10:00 h, 12:00 h, 15:00 h and pre-

329 exercise compared with 1xCAF (P<0.01; Table 1), but lower than 1xCAF at post and 1 h

330 post-exercise (P<0.01; Table 1).

331

332 *Saliva flow rate*

333 Saliva flow rate appeared to increase significantly above pre-supplement (9:00 h) values at

334 10:00 h, 12:00 h, 15:00 h, pre-exercise, post-exercise and 1 h post-exercise (main effect for

335 time: all time points P<0.01, except post-exercise P<0.05; Table 1). However, when

336 compared against pre-exercise values, saliva flow rate fell significantly at post-exercise (main

337 effect for time: P<0.01; Table 1). There were no significant effects of trial/treatment.

338

339 *Saliva α-amylase*

340 A significant time x trial interaction was found for saliva α-amylase activity, which was

341 significantly higher at pre-exercise and 1 h post-exercise on both 1xCAF and 3xCAF

342 compared with PLA (P<0.05; Table 1). A significant time x trial interaction was also found

343 for saliva α-amylase secretion rate, which was higher at 1 h post-exercise on 1xCAF

344 compared with PLA (P<0.05; Table 1). There were no differences between 1xCAF and

345 3xCAF.

346

347 *Circulating lymphocyte counts and numbers of CD3^{-}CD56^{+} cells*

348    There were no time x trial interactions for the number of circulating lymphocytes, but there

349    was a main effect for time (P<0.01) with the number of circulating lymphocytes increasing

350    above pre-supplement values at immediately post-exercise (P<0.01; Table 2).  A significant

351    time x trial interaction was found for the number of CD3$^-$CD56$^+$ cells within the circulating

352    lymphocyte population (P<0.01), with a higher number of CD3$^-$CD56$^+$ cells found at post-

353    exercise on both 1xCAF and 3xCAF compared with PLA (P<0.05; Table 2).  There were no

354    differences between 1xCAF and 3xCAF.

355

356    *Number of unstimulated and antigen-stimulated CD3$^-$CD56$^+$ cells expressing CD69*

357    There were no interaction effects for the number of unstimulated CD3$^-$CD56$^+$ cells expressing

358    CD69.  However there was a main effect for time (P<0.01) with the number of CD3$^-$CD56$^+$

359    cells expressing CD69 increasing significantly above pre-supplement values at pre and post-

360    exercise, only to fall below pre-supplement values at 1 h post-exercise (all P<0.01; Fig. 4a).

361    When cells were stimulated there was a significant time x trial interaction effect, with the

362    number of antigen-stimulated (both doses) CD3$^-$CD56$^+$ cells expressing CD69 lower at 1 h

363    post-exercise on 1xCAF compared with PLA (P<0.05); values on 1xCAF at this time-point

364    remained close to pre-supplement (Fig. 4b & c).  There were no differences between 3xCAF

365    and PLA or 1xCAF and 3xCAF.

366

367    *Geometric Mean Fluorescence Intensity of CD69 expression on unstimulated and antigen-*

368    *stimulated CD3$^-$CD56$^+$ cells*

369    There were no significant interaction effects for the GMFI of CD69 expression on

370    unstimulated CD3$^-$CD56$^+$ cells.  However there was a main effect for time (P<0.01), with the

371    GMFI of CD69 expression on unstimulated CD3$^-$CD56$^+$ cells increasing significantly above

372  pre-supplement values at 1 h post-exercise (P<0.01; Fig. 5a). When cells were stimulated

373  there was a significant time x trial interaction effect, with the GMFI of CD69 expression on

374  antigen-stimulated (1:8000) $CD3^-CD56^+$ cells increasing on all trials above pre-supplement

375  values at 1 h post-exercise (P<0.01; Fig. 5c). However, values tended to be lower on 1xCAF

376  compared with PLA at this time (P=0.055; Fig. 5c). The GMFI of CD69 expression on

377  antigen-stimulated (1:8000) $CD3^-CD56^+$ cells was also significantly lower at immediately

378  post-exercise on 1xCAF compared with both PLA and 3xCAF (P<0.05), with values on

379  1xCAF at this time-point remaining close to pre-supplement values (Fig. 5c).

380

381  **Discussion**

382  The main findings of this study suggest that exercise induced an increase in the number of

383  antigen-stimulated NK cells expressing CD69 as well as in the GMFI of CD69 expression on

384  antigen-stimulated NK cells 1 h following 90 min shuttle running that was attenuated after

385  consuming one large bolus dose (6 mg·kg$^{-1}$) of caffeine 1 h before exercise (15:00 h; 1xCAF).

386  In contrast, ingesting small doses (2 mg·kg$^{-1}$) of caffeine throughout the day to model a more

387  typical pattern of caffeine consumption (09:00 h, 12:00 h & 15:00h; 3xCAF) had little effect

388  on antigen-stimulated NK cell CD69 expression.

389

390  It has been suggested that caffeine's main mechanism of action is predominately via $A_{2A}$

391  adenosine receptor antagonism (Fredholm et al. 1999). As such it was speculated that in the

392  present study both caffeine trials would have increased antigen-stimulated NK cell CD69

393  expression in response to exercise compared with placebo as has previously been found

394  (Fletcher and Bishop 2010). However, this was not the case in this study and as such appears

395  to indicate that in the present study $A_{2A}$ adenosine receptor antagonism was not the

396      predominant mechanism involved. NK cells also possess $A_1$ adenosine receptors (Priebe et

397      al. 1990). As a non-selective adenosine receptor antagonist, caffeine could have antagonized

398      NK cell $A_1$ receptors (Fredholm et al. 1999), which may have restricted the increase in

399      antigen-stimulated NK cell CD69 expression on both caffeine trials in response to high

400      intensity intermittent exercise compared with placebo.

401

402      It could be suggested that the large bolus dose of caffeine attenuated the exercise-induced

403      increase in NK cell CD69 expression following high intensity shuttle running via stimulating

404      the release of epinephrine. Epinephrine stimulates $\beta_2$-adrenoreceptors found on NK cells,

405      leading to an increase in intracellular cAMP levels. An increase in the concentration of

406      cAMP in the cell may have interfered with signals that are necessary for adhesion

407      strengthening (Benschop et al. 1997) and as such could have preferentially mobilized a less

408      active subset of NK cells into the circulation following exercise. Epinephrine has also been

409      shown to inhibit CD69 expression on mitogen-stimulated NK cells (Shimamiya et al. 2003)

410      and as such may have been responsible for the lower number of antigen-stimulated NK cells

411      expressing CD69 and GMFI expression of CD69 on antigen-stimulated NK cells following

412      the large single dose caffeine trial compared with placebo 1 h following high intensity shuttle

413      running.

414

415      Although plasma epinephrine concentrations were not directly determined in this study, saliva

416      α-amylase activity and secretion rate were measured as a surrogate marker, as increases in α-

417      amylase concentration are considered a good indicator of enhanced sympathetic activity

418      (Anderson et al. 1984; Rohleder et al. 2004). Saliva α-amylase activity was significantly

419      higher at 1 h post-exercise on both caffeine trials compared with placebo. However, only the

420   large single dose caffeine trial (1xCAF) showed a significantly higher α-amylase secretion

421   rate at 1 h post-exercise compared with placebo.  As such this may explain why in the present

422   study this was the only caffeine trial to attenuate the exercise-induced increase in both the

423   number of antigen-stimulated NK cells expressing CD69 and their intensity of CD69

424   expression following high intensity shuttle running.  Large (6 and 9 mg·kg$^{-1}$), but not small (3

425   mg·kg$^{-1}$), doses of caffeine have been shown to increase plasma epinephrine concentration in

426   response to high intensity exercise (Graham and Spriet 1995; Walker et al. 2006).  Therefore,

427   it could perhaps be suggested that there may be an epinephrine threshold, above which

428   epinephrine produces inhibitory effects upon NK cell CD69 expression.  However, as plasma

429   epinephrine concentrations were not directly determined in this study, any explanation as to

430   how the large bolus dose of caffeine attenuated the exercise-induced increase in antigen-

431   stimulated NK cell CD69 expression following exercise is only speculation and as such is a

432   limitation of this study.

433

434   The increase in number of antigen-stimulated NK cells expressing CD69 following exercise

435   on PLA could be interpreted in a number of ways due to the multiple complex actions of

436   CD69.   One could suggest that this increase may augment subsequent proliferation,

437   cytotoxicity and pro-inflammatory cytokine release that would appear to favour a pro-

438   inflammatory response via the actions of CD69 as a co-stimulatory receptor (Borrego et al.

439   1999).   However, CD69 has also been shown to act as a negative regulator, downregulating

440   the ability of NK cells to lyse target cells due to the production of TGF-β (Esplugues et al.

441   2003).  As such the increase in the number of NK cells expressing CD69 in response to

442   antigen may act as a regulatory response.  However, given that TGF-β release by CD69$^{+}$ NK

443   cells was not determined it is difficult to ascertain the exact role of CD69 in this study.

444   Future *in vitro* work to determine the nature of the response (e.g. assessment of TGF-β

445     release) would provide greater insight regarding to the physiological nature and implications

446     of these findings.

447

448     Alternatively, it could be argued that as participants were habitual caffeine users, the effects

449     of caffeine on antigen-stimulated NK cell CD69 expression in response to prolonged high

450     intensity exercise in this study may have resulted from participants' tolerance to the effects of

451     caffeine.  It has been demonstrated that tolerance to some of the effects of caffeine can

452     develop quite quickly in habitual caffeine users (reviewed in Fredholm et al. 1999). Studies

453     both in rats and humans suggest that following chronic caffeine intake (~14 days) $A_1$ and $A_{2A}$

454     adenosine receptors are upregulated leading to an increased sensitivity to adenosine

455     (Johansson et al. 1997; Varani et al. 2005). Therefore, as participants were only required to

456     abstain from caffeine overnight, caffeine habituation may have contributed towards the

457     findings that one large bolus dose of caffeine attenuated the exercise-induced increase in

458     antigen-stimulated NK cell CD69 expression 1 h following high intensity shuttle running yet

459     small repeated doses of caffeine had little effect at all.  It should be noted that the mechanisms

460     discussed here are unlikely to occur exclusively; therefore these findings are likely to be the

461     net result of several positive and negative influences.

462

463     The issue of tolerance could also help explain why the findings of the present study contrast

464     with our previous results when caffeine ingestion (2 and 6 mg·kg$^{-1}$) increased antigen

465     stimulated NK cell CD69 expression 1 h following 90 min high intensity exercise compared

466     with placebo (Fletcher and Bishop in press). Tolerance to caffeine dissipates after its

467     discontinuation, with resensitization to caffeine usually occurring within 2-3 days of

468     abstention (Benowitz et al. 1995; Varani et al. 2005). Our previous work employed a 60 h

469     caffeine abstention period and as such likely controlled for any influence of caffeine

470  tolerance. The mode of exercise in the present study was also different to that used in our

471  previous work (intermittent running and continuous cycling, respectively) and may have

472  influenced caffeine metabolism and as such caffeine's effects. However, this seems unlikely

473  as Graham and Spriet (1991) have reported no difference in caffeine metabolism or plasma

474  epinephrine concentration between running and cycling to exhaustion. In addition, serum

475  caffeine concentrations immediately following 90 min continuous cycling (Fletcher and

476  Bishop 2010) and intermittent running (present study) with prior ingestion of 6 mg·kg$^{-1}$ body

477  mass caffeine showed comparable values (continuous cycling, 44 μM; intermittent running,

478  43 μM).

479

480  One limitation of this study was the absence of a resting trial to determine if caffeine acted on

481  antigen-stimulated NK cell CD69 expression without the influence of exercise.  Initial pilot

482  work from our group that looked at NK cell responses to caffeine at rest between 08:30 h and

483  12:00 h suggests that caffeine at doses of 2 or 6 mg·kg$^{-1}$ body mass ingested in one bolus dose

484  at 08:30 h have little effect on antigen-stimulated NK cell CD69 expression up until 12:00 h.

485

486  In conclusion, the findings of the present study suggest that exercise induced an increase in

487  the number of antigen-stimulated NK cells expressing CD69 as well as their intensity of

488  CD69 expression 1 h following 90 min shuttle running that was attenuated by one large bolus

489  dose (6 mg·kg$^{-1}$) of caffeine ingested 1 h prior to exercise.  In contrast, small doses (2 mg·kg$^{-}$

490  $^{1}$) of caffeine ingested throughout the day to model a more typical pattern of caffeine

491  consumption had little effect on antigen-stimulated NK cell CD69 expression.  It should be

492  noted that the attenuations seen with one large bolus dose of caffeine at no point fell below

493  pre-supplement values and as such suggests that caffeine does not depress antigen-stimulated

494  NK cell CD69 expression following 90 min of high intensity shuttle running.

495

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498 Mariani for their help in the data collection.

499

## Ethical Standards

501 The authors declare that the experiments comply with the current laws of the U.K.

502

## Conflict of Interest

504 The authors declare that they have no conflict of interest.

505

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595 **Figure captions**

596 **Fig. 1** Schematic representation of the trial protocol

597 **Fig. 2** Schematic representation of the Loughborough Intermittent Shuttle Running Test
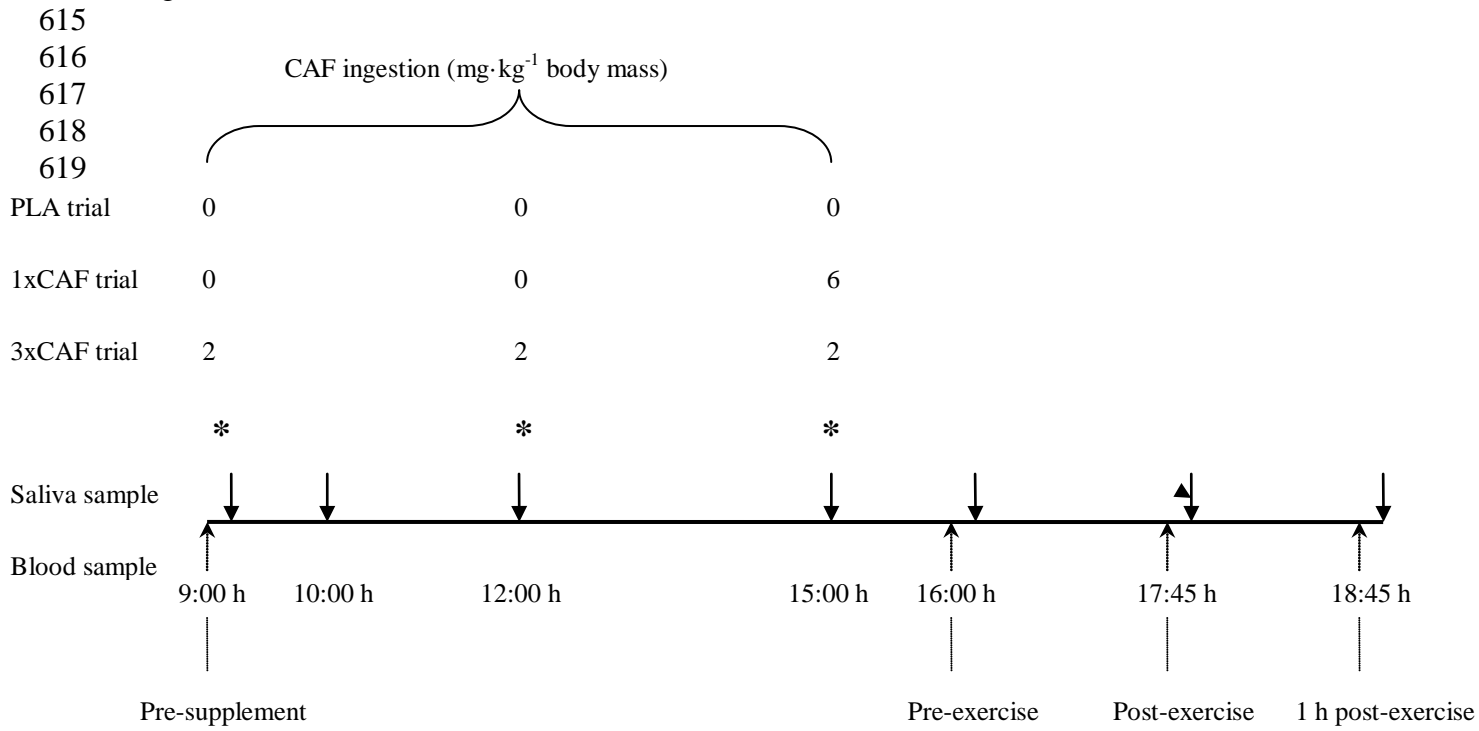
598 (LIST). Adapted from Bishop et al. 2002

599 **Fig. 3** Serum caffeine concentrations during PLA, 3xCAF and 1xCAF trials. * significantly

600 higher than PLA ($P<0.01$). ** significantly higher than PLA and 3xCAF ($P<0.01$).

601 § significantly higher than PLA and 1xCAF ($P<0.01$). $^{\dagger}$ significantly higher than pre-

602 supplement within trial ($P<0.01$). Values are means $\pm$ SD

603 **Fig. 4** Number of (a) unstimulated (b) 1:4000 antigen-stimulated, (c) 1:8000 antigen-

604 stimulated $CD3^{-}CD56^{+}$ NK cells expressing CD69 within the circulating lymphocyte

605 population during PLA, 3xCAF and 1xCAF trials. * significantly lower than PLA ($P<0.05$);

606 † significantly higher than pre-supplement within trial ($P<0.01$). Values are means + SD

607 **Fig. 5** Geometric Mean Fluorescence Intensity of CD69 expression on (a) unstimulated (b)

608 1:4000 antigen-stimulated, (c) 1:8000 antigen-stimulated $CD3^{-}CD56^{+}$ NK cells within the

609 circulating lymphocyte population during PLA, 3xCAF and 1xCAF trials. * significantly

610 lower than PLA and 3xCAF ($P<0.05$); † significantly higher than pre-supplement within trial

611 ($P<0.01$). Values are means + SD and are expressed as a percentage of the pre-supplement

612 value

613

614    Fig. 1
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616
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CAF ingestion (mg·kg$^{-1}$ body mass)

|  | | |
|---|---|---|
| PLA trial | 0 | 0 | 0 |
| 1xCAF trial | 0 | 0 | 6 |
| 3xCAF trial | 2 | 2 | 2 |

Saliva sample

Blood sample

9:00 h    10:00 h    12:00 h    15:00 h    16:00 h    17:45 h    18:45 h

Pre-supplement          Pre-exercise    Post-exercise    1 h post-exercise

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Fig. 2



Heart rates recorded before, during and after each block

15 min intermittent exercise

3 min rest

Exercise time (min)    0    15  15        30  30        45  45        60  60        75  75        90

One cycle of intermittent shuttle running, repeated ~10

Walk

Sprint

Active

Cruise @ 95% $\dot{V}O_{2\,max}$

Jog @ 55% $\dot{V}O_{2\,max}$

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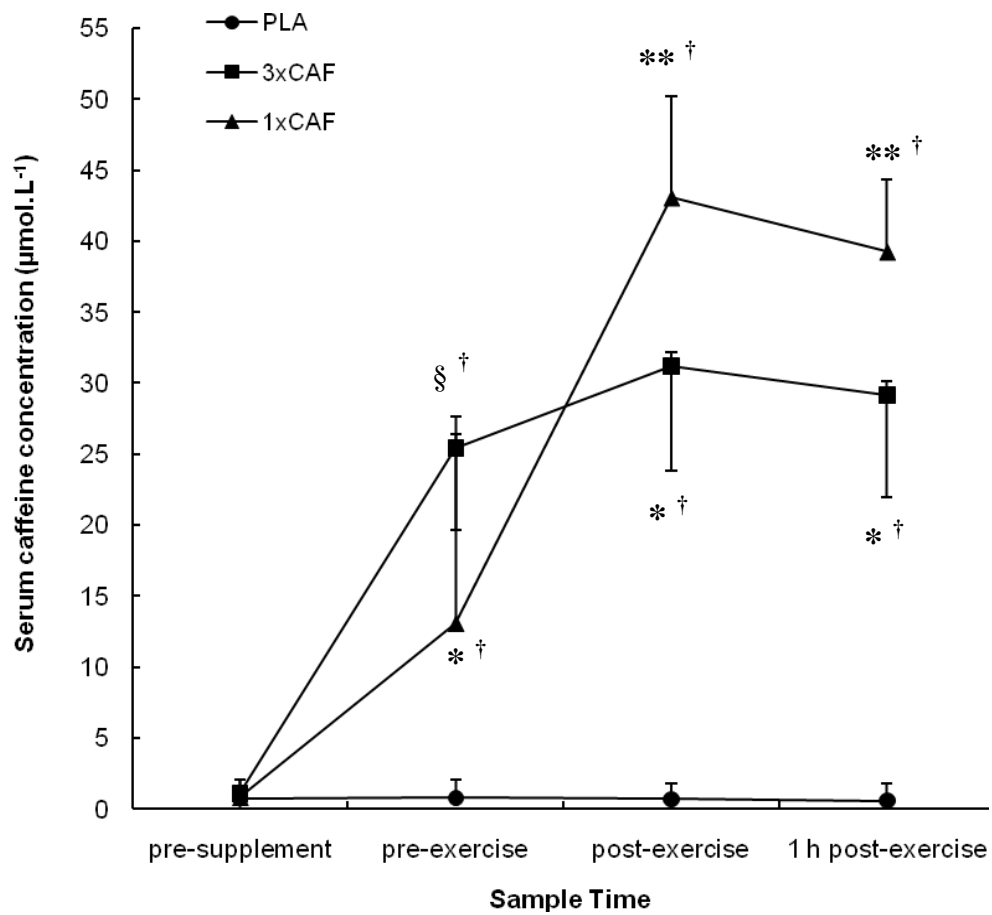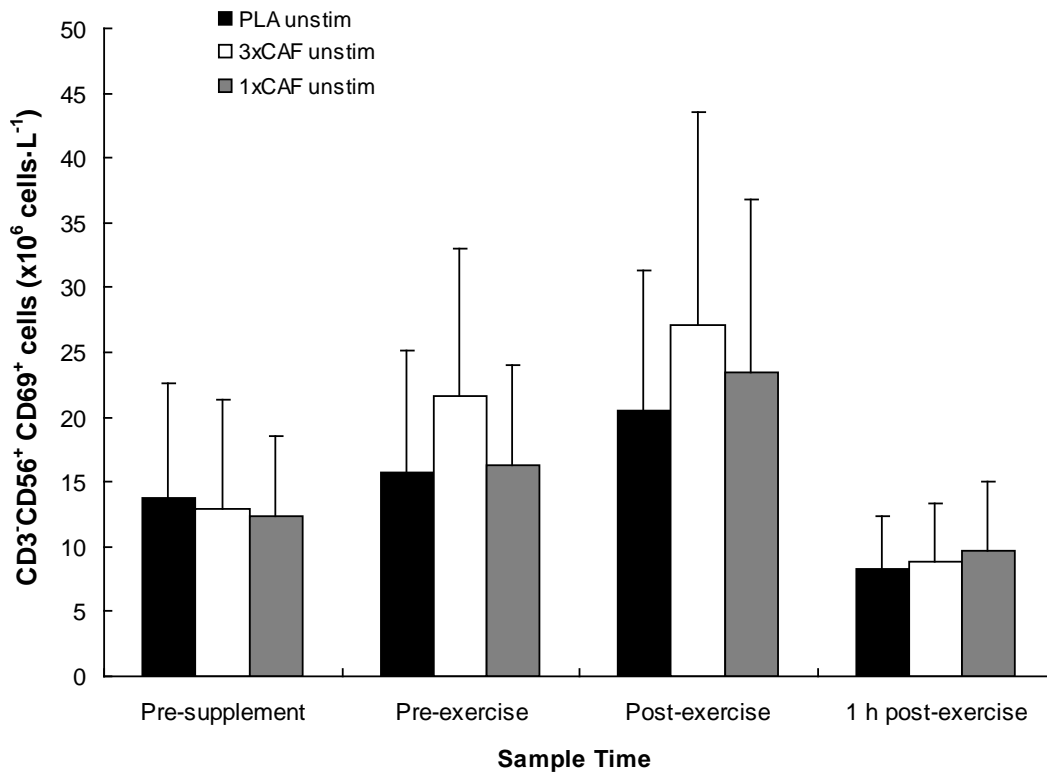641
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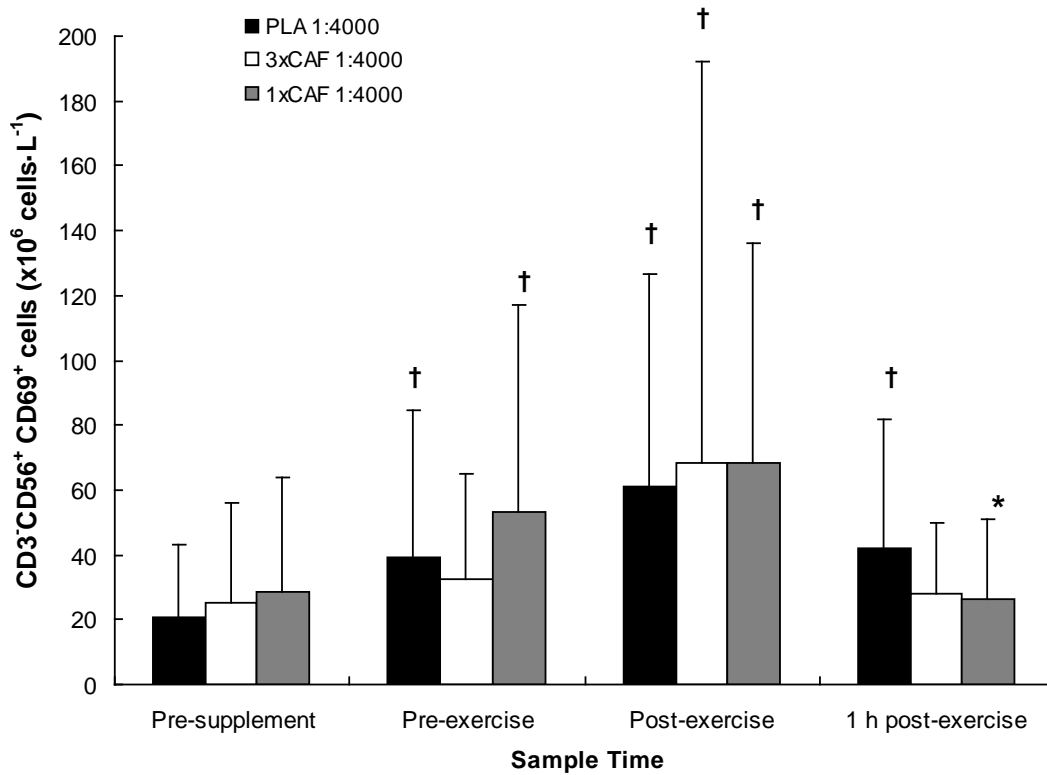644
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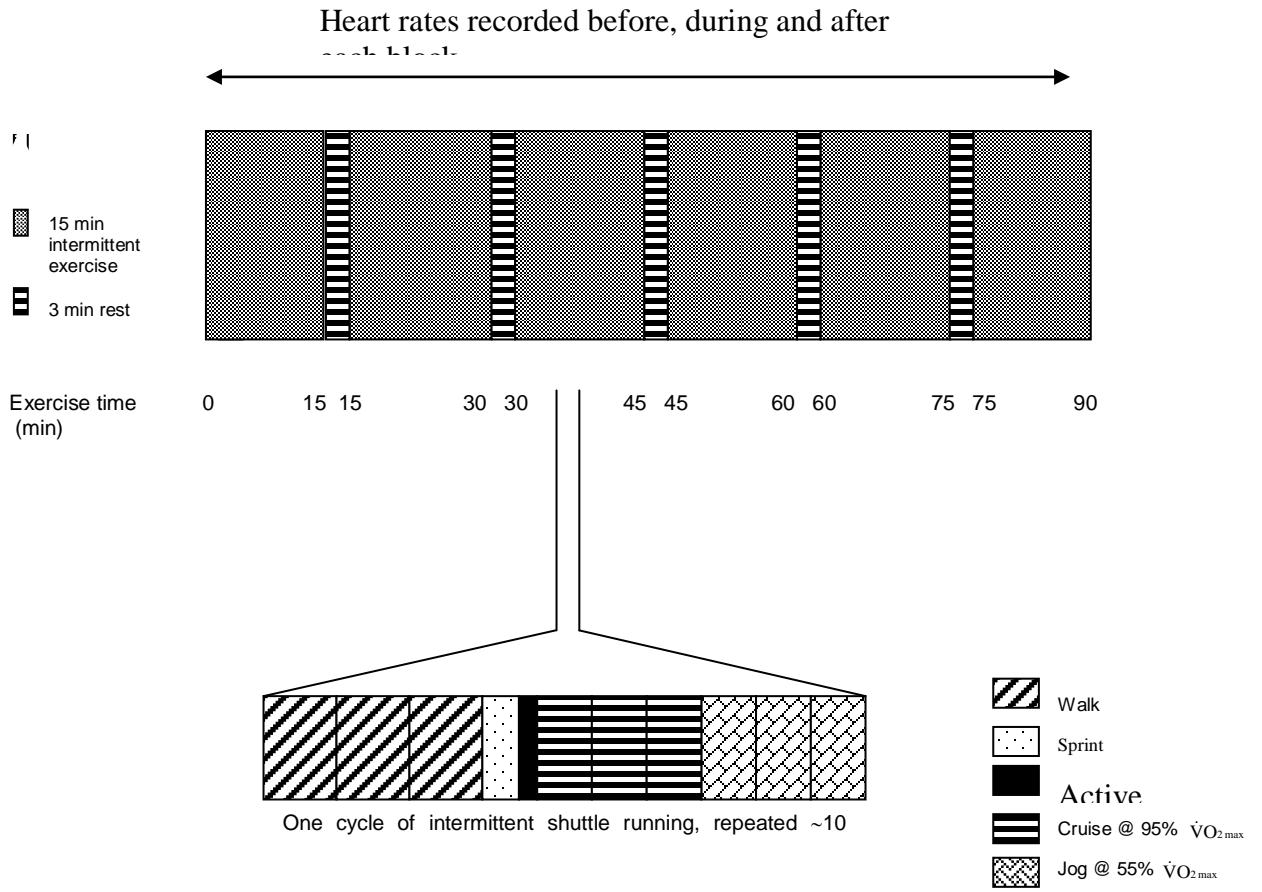
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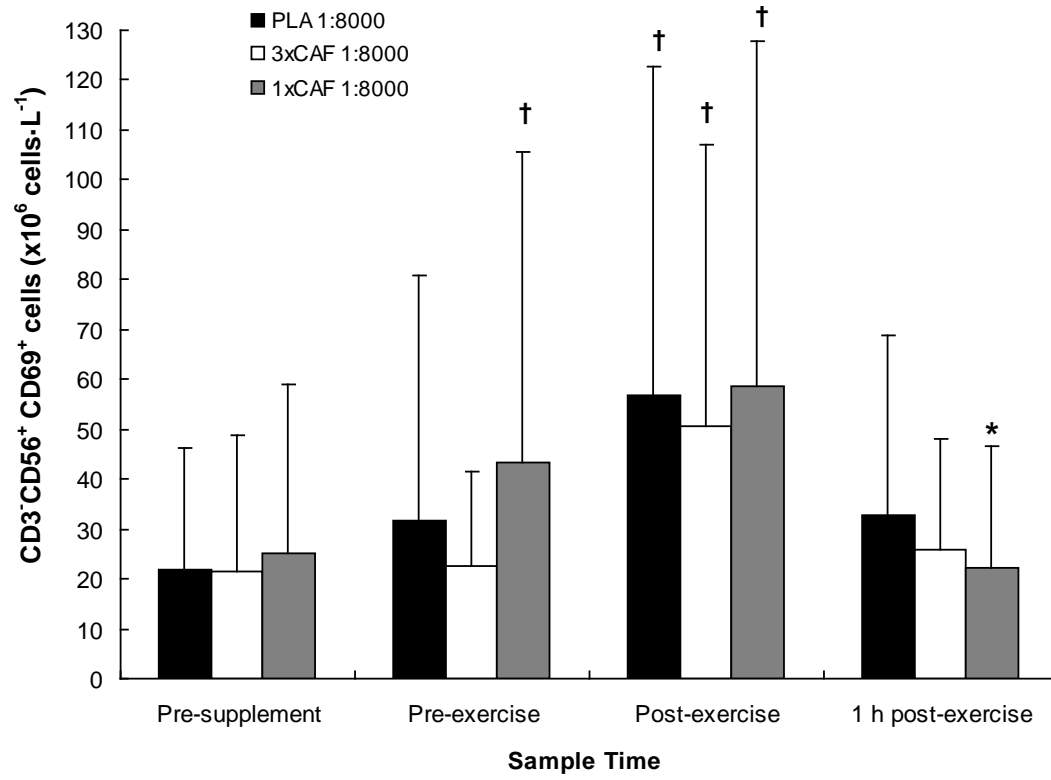
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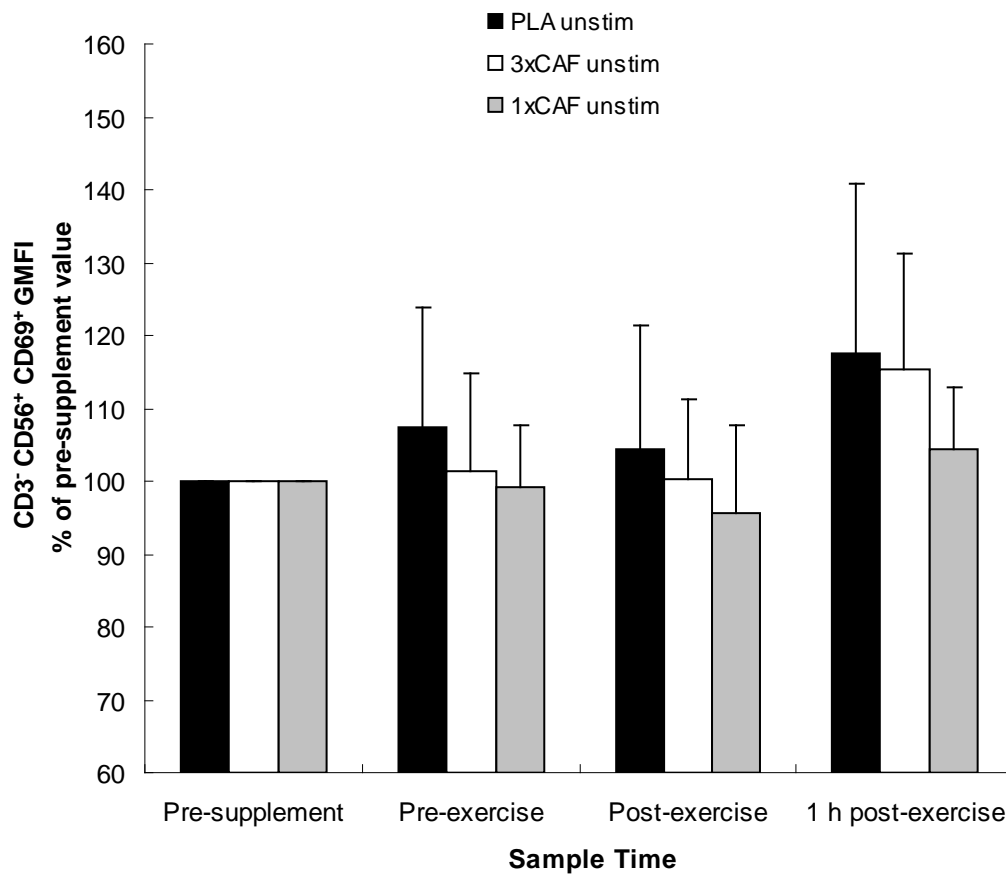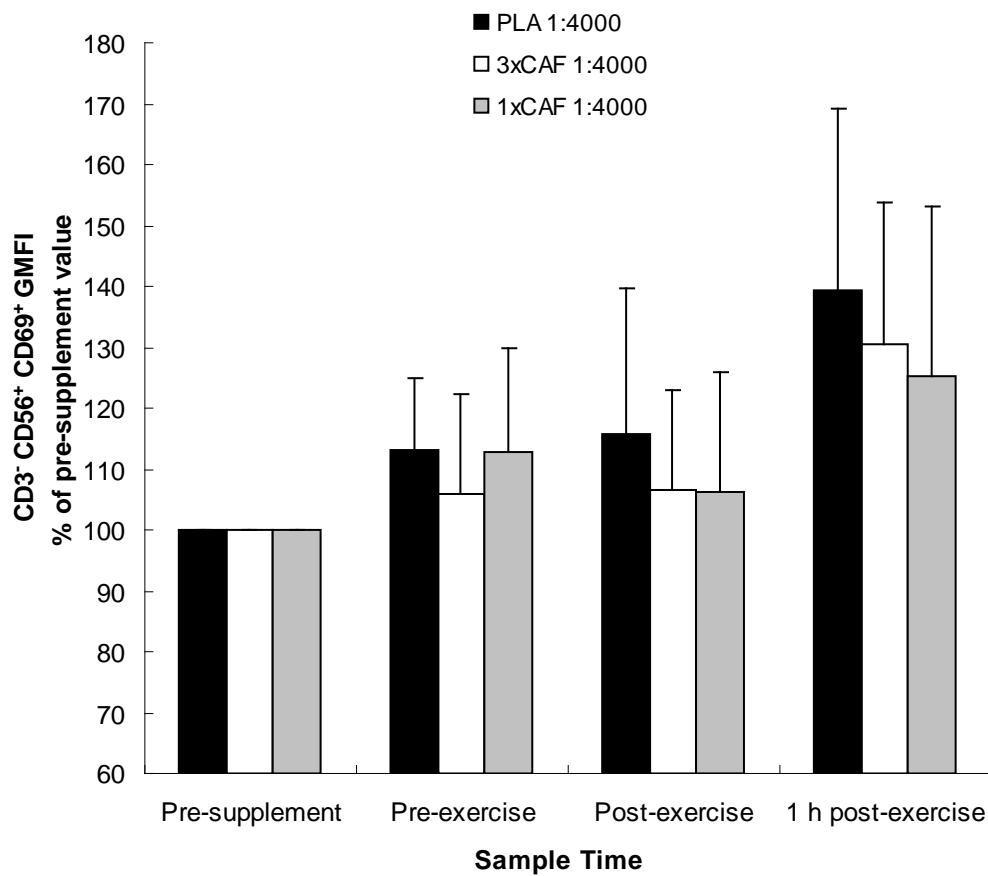660



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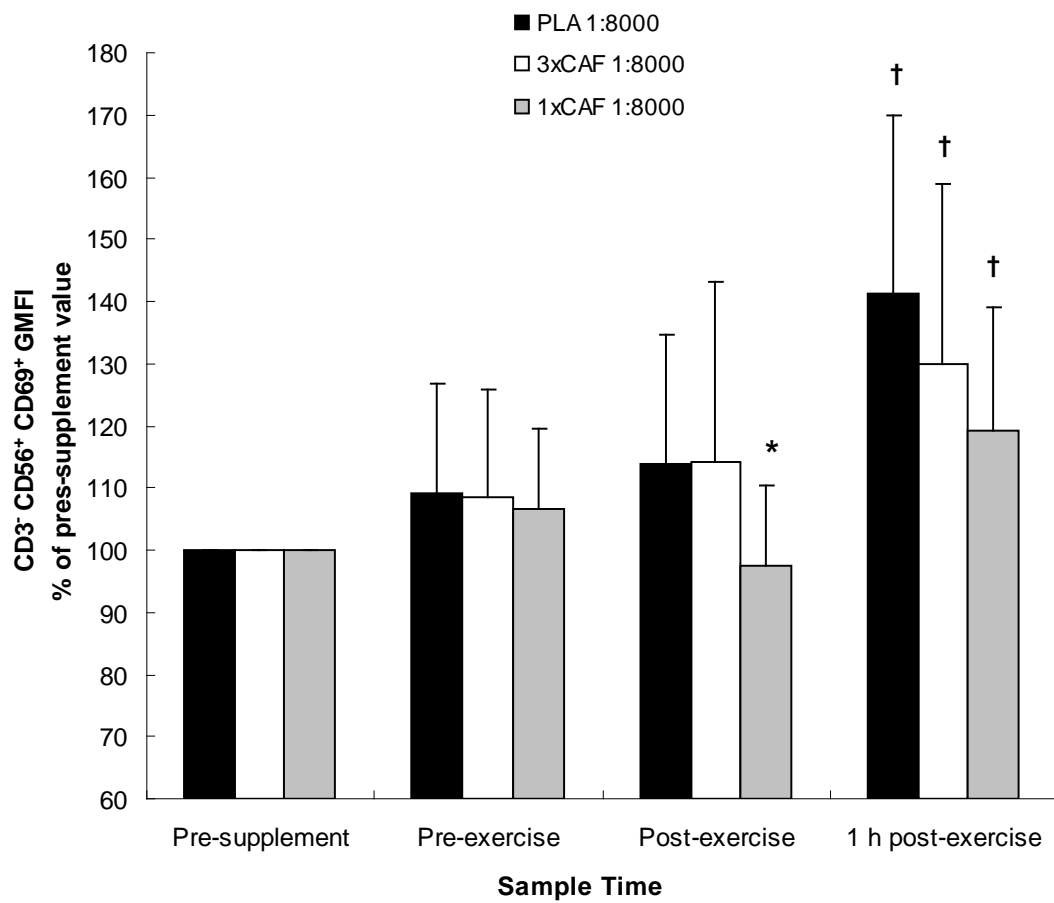
Fig. 4a



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Fig. 5a

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671 Table 1 Saliva caffeine concentration, flow rate, α-amylase activity and secretion rate during PLA, 3xCAF and
672 1xCAF trials

| | 09:00 h (Pre-supplement) | 10:00 h | 12:00 h | 15:00 h | 16:00 h (Pre-exercise) | 17:45 h (Post-exercise) | 18:45 h (1 h post-exercise) |
|---|---|---|---|---|---|---|---|
| **Saliva caffeine concentration ($\mu mol \cdot L^{-1}$)** | | | | | | | |
| PLA | 1 (1) | 1 (1) | 1 (1) | 1 (1) | 0 (0) | 0 (0) | 0 (0) |
| 3xCAF | 1 (1) | 10 (4)*‡ | 8 (2)*‡ | 14 (3)*‡ | 15 (3)*‡ | 20 (5)*‡ | 19 (4)*‡ |
| 1xCAF | 1 (1) | 0 (0) | 0 (0) | 1 (1) | 2 (2) | 30 (4)*§ | 27 (4)*§ |
| **Saliva flow rate ($\mu l \cdot min^{-1}$) [a,b]** | | | | | | | |
| PLA | 441 (268) | 549 (252) | 543 (180) | 567 (203) | 572 (200) | 484 (171) | 683 (248) |
| 3xCAF | 431 (216) | 571 (254) | 586 (185) | 591 (294) | 585 (252) | 467 (209) | 656 (286) |
| 1xCAF | 423 (241) | 575 (240) | 546 (203) | 566 (165) | 642 (223) | 520 (203) | 698 (225) |
| **Saliva α-amylase activity ($U \cdot ml^{-1}$)** | | | | | | | |
| PLA | 392 (257) | 439 (243) | 793 (416) | 967 (373) | 827 (373)† | 1456 (530)† | 867 (494)† |
| 3xCAF | 306 (271) | 608 (346) | 845 (319) | 936 (316) | 997 (313)**† | 1719 (527)† | 1098 (430)**† |
| 1xCAF | 368 (299) | 545 (290) | 905 (442) | 925 (345) | 1023 (369)**† | 1615 (457)† | 1196 (402)**† |
| **Saliva α-amylase secretion rate ($U \cdot min^{-1}$)** | | | | | | | |
| PLA | 174 (167) | 234 (150) | 444 (284) | 569 (433) | 487 (323)† | 695 (343)† | 580 (391)† |
| 3xCAF | 138 (151) | 239 (291) | 526 (353) | 570 (357) | 587 (357)† | 810 (470)† | 742 (452)† |
| 1xCAF | 157 (154) | 314 (192) | 497 (293) | 494 (188) | 660 (327)† | 812 (338)† | 804 (289)**† |

673

674 Values are means (SD)
675 * $P<0.01$, ** $P<0.05$: significantly higher than PLA
676 ‡ significantly higher than 1xCAF, $P<0.01$
677 § significantly higher than 3xCAF, $P<0.01$
678 † $P<0.01$: significantly higher than pre-supplement within trial
679 [a] main effect for time; significantly higher than pre-supplement at all other time-points, all $P<0.01$ except for
680 post-exercise $P<0.05$
681 [b] main effect for time; significantly lower than pre-exercise at post-exercise, $P<0.01$
682

683 Table 2 Number of circulating lymphocytes and CD3$^-$CD56$^+$ cells within the circulating lymphocyte
684 population during PLA, 3xCAF and 1xCAF trials
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| | Pre-supplement | Pre-exercise | Post-exercise | 1 h post-exercise |
|---|---|---|---|---|
| **Lymphocytes (x10$^9$ cells.L$^{-1}$) $^a$** | | | | |
| PLA | 2.10 (0.50) | 2.10 (0.50) | 2.90 (0.80) | 2.10 (0.50) |
| 3xCAF | 2.10 (0.70) | 2.00 (0.50) | 3.10 (1.00) | 1.90 (0.50) |
| 1xCAF | 2.10 (0.40) | 2.10 (0.40) | 3.20 (0.80) | 1.90 (0.60) |
| **CD3$^-$CD56$^+$ cells (x10$^9$ cells.L$^{-1}$)** | | | | |
| PLA | 0.26 (0.08) | 0.30 (0.09) | 0.53 (0.16)$^\dagger$ | 0.25 (0.07) |
| 3xCAF | 0.28 (0.09) | 0.35 (0.10)$^{\dagger\dagger}$ | 0.70 (0.22)**$^\dagger$ | 0.25 (0.07) |
| 1xCAF | 0.29 (0.11) | 0.36 (0.09)$^{\dagger\dagger}$ | 0.80 (0.32)*$^\dagger$ | 0.27 (0.09) |

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704 Values are means (SD)
705 * $P<0.01$; ** $P<0.05$, significantly higher than PLA
706 $^\dagger$ $P<0.01$; $^{\dagger\dagger}$ $P<0.05$, significantly higher than pre-supplement within trial
707 $^a$ main effect for time; significantly higher than pre-supplement at post-exercise, $P<0.01$
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