Citation for published version:
Gonzalez, JT, Green, BP, Campbell, MD, Rumbold, PLS & Stevenson, EJ 2014, 'The influence of calcium supplementation on substrate metabolism during exercise in humans: a randomized controlled trial', *European Journal of Clinical Nutrition*, vol. 68, no. 6, pp. 712-718. https://doi.org/10.1038/ejcn.2014.41

DOI:
10.1038/ejcn.2014.41

Publication date:
2014

Document Version
Peer reviewed version

Link to publication

University of Bath

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 25. Oct. 2020

The influence of calcium supplementation on substrate metabolism during exercise in humans: a randomized controlled trial.

Authors and Affiliations

Javier T. Gonzalez[1,2], Benjamin P. Green[2], Matthew D. Campbell[2], Penny L.S. Rumbold[2], Emma J. Stevenson[1,2].

1 Brain, Performance and Nutrition Research Centre, Faculty of Health and Life Sciences, Northumbria University, Newcastle-upon-Tyne, United Kingdom.

2 Department of Sport, Exercise and Rehabilitation, Faculty of Health and Life Sciences, Northumbria University, Newcastle-upon-Tyne, United Kingdom.

Corresponding author

Javier T. Gonzalez

Faculty of Health and Life Sciences, Northumbria University, Newcastle-upon-Tyne, United Kingdom.

E-mail: j.gonzalez@northumbria.ac.uk

Tel: 0(+44)191 243 4468

Running title: Calcium, exercise and fat oxidation

Potential conflicts of interest: PLRS and EJS have previously received funding from The Dairy Council (UK), which was not related in any way to the work in this article. This study was funded solely by Northumbria University.

29 **Abstract**

30 **Background/Objectives:** High calcium intakes enhance fat
31 loss under restricted energy intake. Mechanisms explaining this
32 may involve reduced dietary fat absorption, enhanced lipid
33 utilization and (or) reductions in appetite. This study aimed to
34 assess the impact of two weeks of calcium supplementation on
35 substrate utilization during exercise and appetite sensations at
36 rest. **Subjects/Methods:** Thirteen physically-active males
37 completed two 14-d supplemental periods, in a double-blind,
38 randomized crossover design separated by a ≥ 4 week washout
39 period. During supplementation, a test-drink was consumed
40 daily containing 400 and 1400 mg of calcium during control
41 (CON) and high-calcium (CAL) periods, respectively. Cycling-
42 based exercise tests were conducted before and after each
43 supplemental period to determine substrate utilization rates and
44 circulating metabolic markers (non-esterified fatty acid,
45 glycerol, glucose and lactate concentrations) across a range of
46 exercise intensities. Visual analogue scales were completed
47 fasted and at rest, to determine subjective appetite sensations.
48 **Results:** No significant differences between supplements were
49 observed in lipid or carbohydrate utilization rates, nor in
50 circulating metabolic markers (both $P > 0.05$). Maximum rates
51 of lipid utilization were 0.47 ± 0.05 and 0.44 ± 0.05 g/min, for
52 CON and CAL respectively, prior to supplementation, and 0.44
53 ± 0.05 and 0.42 ± 0.05 g/min respectively, post-

54 supplementation (main effects of time, supplement, and time x
55 supplement interaction effect all $P > 0.05$). Furthermore, no
56 significant differences were detected in any subjective appetite
57 sensations (all $P > 0.05$). **Conclusions:** Two weeks of calcium
58 supplementation does not influence substrate utilization during
59 exercise in physically-active males.
60
61 **Key words:** appetite, dairy, fat oxidation, lipid utilization, non-
62 esterified fatty acids.
63
64

65 **Introduction**

66 A growing body of research implicates calcium intake in
67 adipose regulation. Epidemiology links high-calcium intake
68 with reduced body fat percentage[1], and interventions
69 demonstrate accelerated loss of adipose tissue[2]. Alongside
70 decreased dietary fat absorption[3], mechanisms possibly
71 contributing to this effect are, elevated lipid utilization[4, 5]
72 and/or reduced appetite[6-8]. Consumption of high-calcium meals
73 have been shown to enhance postprandial lipid utilization by
74 some[8, 9] but not others[6, 7, 10]. Meta-analyses suggest that
75 evidence for chronic (>7 d) calcium intake (increase of ~958
76 mg/d) and lipid utilization, is stronger than acute effects[4]. The
77 reduction in appetite observed with acute calcium intake[6-8],
78 may translate into a chronic reduction in appetite[11-14].

79 The mechanism(s) underlying a change in fat utilization
80 with calcium intake, in humans, is not clear. Most evidence
81 comes from *in vitro* or animal data, and the most prominent
82 theory is currently that of parathyroid hormone (PTH) and/or
83 1,25$(OH)_2$D regulating lipid metabolism[15, 16]. Dietary
84 manipulation of 1,25$(OH)_2$D in humans (oral vitamin D
85 combined with low-calcium diet) however, does not influence
86 whole-body lipid oxidation or lipolytic markers[17]. Similarly,
87 increased lipid oxidation with calcium supplementation has
88 been observed in the presence of similar PTH[18], suggesting
89 other mechanisms may be at play.

90 Increasing the calcium content of a meal by ~900 mg
91 potentiates postprandial circulating glucose-dependent
92 insulinotropic peptide$_{1-42}$ (GIP$_{1-42}$), glucagon-like peptide-1
93 (GLP-1) and insulin concentrations[7]. GLP-1 and insulin
94 suppress appetite[19-21] and emerging evidence suggests that GIP
95 and GLP-1 influence lipid metabolism. GIP stimulates adipose
96 fatty acid re-esterification under hyperinsulinemic
97 hyperglycemia[22]. GLP-1 positively associates with fasting lipid
98 utilization[23], and dipeptidyl peptidase-IV (DPP-IV; an enzyme
99 that degrades the receptor-active form of GIP and GLP-1, but
100 also of other peptides) inhibition, increases lipid utilization[24].
101 The effect size that an elevated calcium intake has on
102 lipid oxidation, tends to be greater when under energy deficit[4].
103 Melanson *et al*. used diet and exercise to produce negative
104 energy balance after 7 d of high-calcium intake[18]. The
105 respiratory quotient was only significantly lower during low-
106 intensity (bench stepping) exercise with high-calcium intake.
107 To our knowledge, this is the only study to examine the effect
108 of a week of elevated calcium intake on lipid oxidation during
109 exercise, and as bench stepping was standardized, it was not
110 possible to discern the effect on maximal lipid utilization.
111 We therefore hypothesized that repeated (daily for 14 d)
112 elevated exposure of GIP$_{1-42}$ and GLP-1 from calcium ingestion
113 (as seen with a similar dose acutely[7]) may influence lipid
114 utilization during exercise. Accordingly, we aimed to

115 investigate the effect of 2 weeks of high-calcium intake
116 (increase of ~ 1000 mg) on maximal lipid utilization during
117 exercise[25]. We also aimed to determine the impact of calcium
118 supplementation on fasting concentrations of plasma GIP_{1-42},
119 GLP-1 and insulin, along with subjective appetite sensations.
120
121 **Methods**
122 **Participants**
123 The sample size was based on the effect size (11%) for lipid
124 utilization obtained by meta-analyses[4] and the typical error of
125 the maximum rate of lipid utilization during exercise of 9%[26].
126 This calculated that 13 participants should provide statistical
127 power above 80% with an alpha level of 0.05.
128 Thirteen physically active males were recruited from
129 the student and staff population of Northumbria University
130 between October 2012 and February 2013. Participants' age,
131 stature, body mass and habitual calcium intakes were (mean ±
132 SD: 26 ± 4 y, 177 ± 6 cm, 78 ± 6 kg, 1040 ± 445 mg/d; for
133 aerobic capacity see Table 2). Participants were self-reportedly
134 physically active (moderate-vigorous activity, ≥30 min, 5
135 times/week [27]), non-smokers, and free of metabolic disease.
136 This study received ethical approval from the Faculty of Health
137 and Life Sciences Ethics Committee at Northumbria
138 University, and participants provided written informed consent

139 prior to the study. This study was registered at
140 ClinicalTrials.gov (NCT01779245).
141
142 **Study design**
143 In a randomized (performed by J.T.G. using an online package:
144 researchrandomizer.org), double-blind (both investigators and
145 participants were blinded, an assistant who was not involved in
146 data collection, prepared and labeled food packages), crossover
147 design, participants underwent a control (CON) and a high-
148 calcium (CAL) supplementation period. Before and after
149 supplemental periods, exercise tests were conducted to
150 determine substrate utilization (Figure 1). Prior to each exercise
151 test participants were asked to replicate their diet for the
152 preceding 24 h, and to abstain from exercise, caffeine and
153 alcohol. Exercise tests began after a 10-14 h overnight fast,
154 during which water was consumed *ad libitum*.
155
156 **Exercise tests**
157 Upon arrival at the laboratory, an intravenous catheter was
158 inserted into an antecubital vein for repeated blood sampling. A
159 5-min resting sample of expired gas was collected in the supine
160 position after an initial 5-min rest period.
161 Following baseline sampling, participants began the
162 exercise test, established by others to assess maximal lipid
163 utilization during exercise[25]. Participants cycled on a

164 mechanically-braked cycle ergometer (Monark Ergomedic
165 874E, Vansbro, Sweden) at an initial power output of 95 W.
166 Power output was increased by 35 W every 3 min until
167 volitional tolerance, with cadence maintained at 70
168 revolutions/min. Expired gas samples were collected for the
169 final min of each of the first 5 stages, and then for the final min
170 of the test, when the participant indicated that they would only
171 be able to continue for a further min. If the participant was
172 unable to complete the full 60 s for the final sample, then the
173 time was recorded. A minimum sample of 30 s was used for
174 analysis. On occasions where the participant was able to
175 continue past this "final" min, an additional sample was
176 collected.
177

178 **Sampling and analysis of expired gases**

179 Expired gas analysis was performed using the Douglas bag
180 technique (Supplementary Information 1) accounting for
181 variance in ambient concentrations of oxygen and carbon
182 dioxide[28].
183

184 **Estimation of carbohydrate and lipid utilization**

185 Substrate oxidation was estimated from oxygen consumption
186 and carbon dioxide values using stoichiometric equations,
187 adjusted for the contribution of glycogen to metabolism[29]:
188

189 LO rate at rest and during exercise =

190 $(1.695 \times \dot{V}O_2)$ - $(1.701 \times \dot{V}CO_2)$

191 CO rate at rest = $(4.585 \times \dot{V}CO_2)$ - $(3.226 \times \dot{V}O_2)$

192 CO rate exercise at 40-50% $\dot{V}O_2$peak = (4.344 ×

193 $\dot{V}CO_2$) - $(3.061 \times \dot{V}O_2)$

194 CO rate exercise at 50-75% $\dot{V}O_2$peak =

195 $(4.210 \times \dot{V}CO_2)$ - $(2.962 \times \dot{V}O_2)$

196

197 LO = lipid oxidation (g/min)

198 CO = carbohydrate oxidation (g/min)

199 $\dot{V}O_2$ and $\dot{V}CO_2$ are L/min

200

201 Carbohydrate utilization rates were only determined up until

202 75% $\dot{V}O_2$peak[29].

203

204 **Supplemental protocol**

205 Participants were provided with pre-packaged bags of

206 milkshake powder to consume once daily. For both

207 supplemental periods, bags contained cocoa powder (Tesco,

208 Dundee, UK) and dried skimmed milk (Tesco, Dundee, UK)

209 providing 987 kJ (235 kcal) energy; 13 g protein; 42 g

210 carbohydrate and 1 g fat. For the CAL supplement, 4 g of a

211 milk-extracted calcium powder (Capolac®, Arla Foods

212 Ingredients amba, Denmark) was added to increase the calcium

213 content. Details of the supplementation, including independent
214 verification are provided in Supplementary Information 2.
215
216 **Anthropometric variables**
217 Body mass was determined to the nearest 0.1 kg using balance
218 scales (Seca, Birmingham, UK) upon arrival at the laboratory,
219 where participants wore only light clothing. Stature was
220 measured to the nearest 0.1 cm using a stadiometer (Seca,
221 Birmingham, UK).
222
223 **Subjective ratings**
224 Subjective appetite ratings were assessed using previously
225 validated, 100 mm VAS[30], upon arrival at the laboratory (in the
226 fasted, resting state). Questions asked included: “how hungry
227 do you feel?”, “how full do you feel?”, “how satisfied do you
228 feel?” and “how much do you think you can eat?”. A
229 combined-appetite score which has been used previously[31] was
230 also employed. Participants rated the palatability of the test
231 drinks following consumption of the first drink using VAS with
232 questions pertaining to visual appeal, smell, taste and overall
233 palatability.
234
235 **Blood sampling and analysis**
236 Blood was sampled at baseline, during the final min of each of
237 the first 5 stages, and immediately after termination of the

238 exercise test, to determine NEFA and glycerol (both kits from
239 RX Daytona, Randox Laboratories Ltd, London, UK) and
240 glucose and lactate (both measured by Biosen C_line, EKF
241 Diagnostics, Magdeberg, Germany) concentrations. Samples
242 were collected into EDTA Vacutainers and immediately
243 centrifuged (10 min, 1509 g, 4°C). At baseline, an additional
244 sample was collected into a non-anticoagulant tube and allowed
245 to stand for 30 min before centrifugation (10 min, 1509 g, 4°C)
246 for PTH (Immunotopics, San Clemente, USA) and insulin (IBL
247 International GmbH, Hamburg, Germany) concentrations. A
248 further EDTA tube with 25 µL aprotinin/mL whole blood was
249 taken at baseline and immediately centrifuged (10 min, 1509 g,
250 4°C) for plasma GIP_{1-42} (Immuno-Biological Laboratories Co.,
251 Ltd, Japan) and total GLP-1 (Epitope Diagnostics, San Diego,
252 CA) concentrations. Samples were stored at -80°C before
253 analysis. Inter-assay CVs for NEFA and glycerol were 1.2 and
254 2.1%, respectively. The intra-assay CV reported by the
255 manufacturers was 4.74 and 1.34% for NEFA and glycerol,
256 respectively.
257
258 **Statistical analysis**
259 Due to difficulties with blood sampling from one participant,
260 data for all blood variables are $n = 12$ apart from when
261 relationships between baseline variables were assessed. As all
262 participants completed all trials, all other data are $n = 13$ and

263 are expressed as mean ± standard deviation (SD) unless stated
264 otherwise. Data were tested for normal distribution using the
265 Anderson-Darling normality test. Data not displaying normal
266 distribution were log-transformed prior to statistical analysis.
267 Insulin sensitivity was assessed by HOMA-IR[32].
268 Two-way (supplement x time) repeated-measures
269 ANOVA were used to detect differences between resting
270 variables, maximal fat oxidation, total fat and carbohydrate
271 oxidation, variables assessed at exhaustion and appetite. Three-
272 way (supplement x time x power output) repeated-measures
273 ANOVA were used to assess differences between circulating
274 variables and substrate utilization during exercise.
275 Following a significant interaction effect, Holm-
276 Bonferroni post-hoc test was used to determine the location of
277 variance and all *P* values reported have been adjusted for
278 multiple-comparisons. Differences between CON and CAL in
279 the change from pre- to post-supplementation were assessed by
280 paired t-tests. Differences were considered significant at $P <$
281 0.05. Relationships between variables were assessed by
282 Pearson product-moment correlation coefficients.
283
284 **Results**
285 **Palatability**
286 Ratings of visual appeal (CON: 76 ± 14, CAL: 79 ± 14), smell
287 (CON: 73 ± 15, CAL: 73 ± 17), taste (CON: 79 ± 13, CAL: 82

288 ± 15) and palatability (CON: 78 ± 15, CAL: 82 ± 13) of the test
289 drinks did not significantly differ (paired t-tests: $P = 0.467$, $P =$
290 0.946, $P = 0.464$, $P = 0.210$, respectively).

291

292 **Resting measures**

293 Resting energy expenditure, lipid and carbohydrate utilization,
294 and circulating metabolites and hormones are displayed in
295 Table 1. A significant interaction effect (supplement x time)
296 was observed for insulin concentration and HOMA-IR, but not
297 for any other resting variables (Table 1). Post-hoc analyses
298 revealed that insulinemia was lower prior to supplementation
299 with CAL vs. CON, but rose during supplementation to a
300 concentration that was higher than both CON post-
301 supplementation and CAL pre-supplementation (Table 1). The
302 pre- to post-supplementation change in insulin concentrations
303 was significantly different between CON and CAL as assessed
304 by paired t-test ($P = 0.006$; Figure 2), as was HOMA-IR ($P =$
305 0.029).

306 No significant main (time or supplement), or interaction
307 (supplement x time) effects were detected for any other resting
308 variable (Table 1; all $P > 0.5$). Similarly, no differences were
309 observed in body mass or subjective appetite (Supplementary
310 Table 1).

311

312 **Exercise measures**

313 $\dot{V}O_2$ peak did not significantly differ between tests (Table 2),
314 nor did any of the metabolic variables measured (Table 2). A
315 main effect of power output was detected for plasma lactate
316 concentration and lipid and carbohydrate utilization (all $P <$
317 0.001). However, no main effects for trial or interaction effects
318 (supplement x time, supplement x power output, time x power,
319 or supplement x time x power output) were observed for any
320 exercise variable (all $P > 0.05$). Figure 3 displays pre- to post-
321 supplementation change in the maximum lipid utilization,
322 which was not significantly different between CON and CAL
323 (paired t-test, $P = 0.619$). NEFA and glycerol concentrations
324 during exercise are displayed in Figure 4, and glucose and
325 lactate concentrations during exercise in Supplementary Figure
326 1.
327
328 **Correlations between variables**
329 No significant relationships were observed between the change
330 in PTH and, resting, maximal or total lipid utilization (r = -
331 0.09, $r = 0.17$ and $r = 0.04$, respectively, all $P > 0.05$). Pre-
332 supplementation (mean of CON and CAL) $\dot{V}O_2$peak however,
333 inversely correlated with pre-supplementation insulinemia
334 (Supplementary Figure 2; $r = -0.65$, $P = 0.016$) and HOMA-IR
335 ($r = -0.72$, $P = 0.006$).
336
337 **Discussion**

338 The main findings indicated by these data are three-fold. 1)
339 two-weeks of calcium supplementation does not influence
340 whole-body lipid metabolism during exercise, in young
341 physically active males; 2) calcium supplementation does not
342 influence circulating concentrations of GIP_{1-42} or total GLP-1,
343 or appetite sensations in the fasted state; 3) calcium
344 supplementation elevated serum insulin concentrations in the
345 fasted state.

346 High-calcium intake for 7 d increases 24-h lipid
347 utilization in overweight individuals, achieved by increasing
348 the consumption of dairy products[18]. The change in substrate
349 metabolism was most apparent during activity. Additionally,
350 meta-analyses indicate that a ~1000 mg increase in calcium
351 intake for ≥7 d increases fat utilization[4]. Therefore, we
352 investigated the effects of a ~1000 mg/d increase in calcium
353 intake for 14 d, on fat utilization during exercise. In the present
354 study, neither maximal fat utilization rates, nor the total amount
355 of fat utilized during exercise were influenced by consumption
356 of additional calcium. Other markers of substrate metabolism
357 support this, as plasma NEFA, glycerol, glucose and lactate
358 concentrations during exercise were unaffected by
359 supplementation, suggesting lipolytic rate[33] and glycolytic
360 flux[34] were unchanged.

361 Participants in the present study and that of Melanson *et*
362 *al.*[18], were both non-obese, healthy and had similar habitual

363 calcium intakes (1040 mg/d vs. ~1100 mg/d, respectively). The
364 only major difference in the participants was the use of
365 physically active individuals in the present study.
366 Consequently, the $\dot{V}O_2peak$ of the participants in the present
367 study was ~ 51 mL/kg/min, compared to ~ 32 mL/kg/min[18]. As
368 training status has a major influence on substrate metabolism
369 during exercise[35, 36], it is conceivable that calcium
370 supplementation is only effective at increasing fat utilization in
371 untrained individuals with limited capacity for fat oxidation.
372 Notwithstanding this, given the higher basal insulin
373 concentrations in response to calcium supplementation seen in
374 the present study, it could be viewed that the absence of
375 suppressed NEFA and glycerol concentrations, or rates of fat
376 utilization somewhat *corroborate* the findings of Melanson *et*
377 *al.*[18] (greater fat utilization under similar insulinemia), as both
378 indicate reduced insulin-suppression of lipid
379 mobilization/utilization.
380 A negative relationship between the change in PTH and
381 the change in fat utilization in response to calcium
382 supplementation was previously reported[10], suggesting reduced
383 PTH was (partially) responsible changes in fat oxidation seen
384 by Gunther *et al.*[37]. PTH was unaffected by calcium
385 supplementation in the present study, although others have
386 shown enhanced fat oxidation with calcium supplementation
387 with no change in PTH[18]. Hence it is presently unclear whether

388 PTH suppression is necessary for calcium supplementation to
389 influence fat oxidation. Both the previous studies used dairy
390 products to increase calcium intake, with potential for
391 confounding from dairy protein, or other bioactive
392 components[38, 39].
393 The present study also indicates that calcium
394 supplementation does not influence appetite sensations, or
395 concentrations of GIP_{1-42} or total GLP-1, when fasted.
396 Although some show high-calcium intake reduces appetite,
397 these responses have, to date, been exclusively studied under
398 energy-restriction[11-14]. The present study provides evidence that
399 calcium supplementation, may not influence appetite sensations
400 (in the fasted state) under *ad libitum* feeding, and therefore,
401 might not result in spontaneous energy restriction. This should
402 however, be confirmed by measures of energy intake under
403 free-living conditions and with postprandial measures of
404 appetite and metabolism, as nutritional status can influence
405 appetite and metabolic responses to exercise[40, 41]. Ingestion of a
406 high-calcium meal transiently enhances postprandial plasma
407 GIP_{1-42} and total GLP-1 concentrations[7]. The present findings
408 indicate that this does not result in upregulation of these
409 peptides when fasted. Supporting this, others have reported that
410 1000 mg of calcium supplementation for 3 weeks does not
411 influence fasting concentrations of total GLP-1 or $GLP\text{-}1_{7-36}$[42],
412 although postprandial concentrations were enhanced,

413 suggesting that the acute response that we previously reported[7]
414 translates into chronic adaptation manifest in the postprandial
415 state.

416 An interesting finding was that fasting insulinemia was
417 elevated by 2 weeks of calcium supplementation. Taken in
418 aggregate with elevated HOMA-IR, this suggests reduced
419 insulin sensitivity. These were not primary outcomes of the
420 study and thus should be interpreted with caution.
421 Notwithstanding this, we previously reported elevated
422 postprandial insulinemia following the consumption of a single
423 high-calcium meal[6, 7]. Physiological hyperinsulinemia induces
424 insulin resistance[43]. Accordingly, it is not inconceivable that
425 this transient elevation, which would likely occur following
426 each CAL test drink ingested (relative to control) over the 14-d
427 supplemental period, underlies the increase in insulinemia and
428 HOMA-IR in the basal state. These findings conflict with some
429 previous work whereby calcium supplementation improves[44, 45]
430 or does not influence[46] insulin sensitivity. These studies
431 however, were performed in individuals at risk of, or diagnosed
432 with, type 2 diabetes.

433 In 314 individuals randomized to calcium plus vitamin
434 D supplementation for 3 y , those with impaired fasting glucose
435 (5.6-6.9 mmol/L; n = 92) improved their insulin sensitivity
436 (assessed by HOMA-IR), whereas those with normal fasting
437 glucose showed no change. Therefore the effects of calcium

438 supplementation may need to be considered in context of the
439 individual's metabolic health. In a metabolically-healthy
440 population, high calcium intake appears to have no benefit (and
441 could possibly be detrimental) for insulin sensitivity. This
442 effect of calcium supplementation on insulin resistance in
443 healthy individuals should be confirmed by a postprandial
444 measure, or a euglycemic hyperinsulinemic clamp. The
445 interpretation of this finding should be taken with caution as
446 pre-intervention insulin and HOMA-IR differed, and this was a
447 secondary outcome. Serum insulin concentrations negatively
448 correlated with $\dot{V}O_2peak$. This is not a novel finding[47], but
449 provides support that the measures of insulin concentration and
450 of oxygen uptake were valid on an inter-individual level.
451 There are some limitations with this study that deserve
452 acknowledgement. The power calculation was based on the
453 primary outcome of whole-body lipid utilization, therefore the
454 blood-based and appetite variables should be interpreted with
455 caution. The method of participant recruitment may have led to
456 a degree of selection bias and, finally we cannot be certain that
457 the CON period resulted in sufficient "calcium deficiency" to
458 influence lipid utilization. Nevertheless, the dietary restrictions
459 imposed (described in Supplementary Information 2), would
460 likely result in a calcium intake approximating ~700 mg/d
461 (including the CON drink). Which, given that physically active
462 individuals likely experience calcium losses during exercise[48],

463 it could be assumed that this produces a similar calcium

464 balance to Melanson *et al.*[18].

465 This is the first double-blind, randomized controlled

466 study to investigate the effects of chronic (> 7 d) high-calcium

467 intake on substrate metabolism during exercise. The blinding

468 was effective, as evidenced by the incapability to correctly

469 identify the test drinks. The supplement was independently

470 verified, and increased calcium intake was not confounded by

471 additional dairy components such as specific amino acids and

472 fatty acids. In conclusion, two-weeks of calcium

473 supplementation (~ 1000 mg/d) in physically active males, does

474 not influence substrate metabolism during exercise or appetite

475 sensations at rest, but does elevate fasting insulin

476 concentrations in serum.

477

478 **Acknowledgements**

479 The authors thank the participants for their commitment and

480 Miss K.L. Luck for assisting with preparation of test-drinks and

481 blinding. The authors extend their gratitude to ARLA Foods

482 Ingredients amba for the donation of the calcium supplement.

483

484 **Funding**

485 This study was funded by Northumbria University.

486

487 **Author contributions**

488 Critically reviewed the paper: JTG, BPG, MDC, PLSR, EJS.

489 Conceived and designed the experiments: JTG, EJS. Performed

490 the experiments: JTG, BPG, MDC. Analyzed the data: JTG,

491 EJS. Wrote the paper: JTG, EJS.

492

493 Supplementary information is available at the *European*

494 *Journal of Clinical Nutrition* website

495 (http://www.nature.com/ejcn)

496

497 **References**

498 1. Tidwell DK, Valliant MW. Higher amounts of body fat
499 are associated with inadequate intakes of calcium and
500 vitamin D in African American women. *Nutr Res* 2011;
501 **31**(7)**:** 527-536. doi: 10.1016/j.nutres.2011.06.005
502
503 2. Onakpoya IJ, Perry R, Zhang J, Ernst E. Efficacy of
504 calcium supplementation for management of
505 overweight and obesity: systematic review of
506 randomized clinical trials. *Nutr Rev* 2011; **69**(6)**:** 335-
507 343.
508
509 3. Christensen R, Lorenzen JK, Svith CR, Bartels EM,
510 Melanson EL, Saris WH *et al.* Effect of calcium from
511 dairy and dietary supplements on faecal fat excretion: a
512 meta-analysis of randomized controlled trials. *Obes Rev*
513 2009; **10**(4)**:** 475-486. doi: 10.1111/j.1467-
514 789X.2009.00599.x
515
516 4. Gonzalez JT, Rumbold PL, Stevenson EJ. Effect of
517 calcium intake on fat oxidation in adults: a meta-
518 analysis of randomized, controlled trials. *Obes Rev*
519 2012; **13**(10)**:** 848-857. doi: 10.1111/j.1467-
520 789X.2012.01013.x
521
522 5. Gonzalez JT, Stevenson EJ. New perspectives on
523 nutritional interventions to augment lipid utilisation
524 during exercise. *Br J Nutr* 2012; **107**(3)**:** 339-349. doi:
525 10.1017/S0007114511006684
526

527 6. Gonzalez JT, Rumbold PLS, Stevenson EJ. Impact of
528 Milk-Calcium on Substrate Metabolism and Appetite
529 Sensations at Rest, During Exercise and Recovery. *Appl*
530 *Physiol Nutr Metab* 2013. doi: 10.1139/apnm-2013-
531 0056
532
533 7. Gonzalez JT, Stevenson EJ. Calcium co-ingestion
534 augments postprandial glucose-dependent insulinotropic
535 peptide1-42, glucagon-like peptide-1 and insulin
536 concentrations in humans. *Eur J Nutr* 2013. doi:
537 10.1007/s00394-013-0532-8
538
539 8. Ping-Delfos WC, Soares M. Diet induced
540 thermogenesis, fat oxidation and food intake following
541 sequential meals: influence of calcium and vitamin D.
542 *Clin Nutr* 2011; **30**(3)**:** 376-383. doi: S0261-
543 5614(10)00210-4 [pii]
544 10.1016/j.clnu.2010.11.006
545
546 9. Cummings NK, James AP, Soares MJ. The acute effects
547 of different sources of dietary calcium on postprandial
548 energy metabolism. *Br J Nutr* 2006; **96**(1)**:** 138-144.
549 doi: S0007114506001887 [pii]
550
551 10. Gunther CW, Lyle RM, Legowski PA, James JM,
552 McCabe LD, McCabe GP *et al.* Fat oxidation and its
553 relation to serum parathyroid hormone in young women
554 enrolled in a 1-y dairy calcium intervention. *Am J Clin*
555 *Nutr* 2005; **82**(6)**:** 1228-1234.
556
557 11. Jones KW, Eller LK, Parnell JA, Doyle-Baker PK,
558 Edwards AL, Reimer RA. Effect of a dairy- and
559 calcium-rich diet on weight loss and appetite during
560 energy restriction in overweight and obese adults: a
561 randomized trial. *Eur J Clin Nutr* 2013; **67**(4)**:** 371-376.
562 doi: 10.1038/ejcn.2013.52
563
564 12. Gilbert JA, Joanisse DR, Chaput JP, Miegueu P,
565 Cianflone K, Almeras N *et al.* Milk supplementation
566 facilitates appetite control in obese women during
567 weight loss: a randomised, single-blind, placebo-
568 controlled trial. *Br J Nutr* 2011; **105**(1)**:** 133-143. doi:
569 10.1017/S0007114510003119
570
571 13. Major GC, Alarie FP, Dore J, Tremblay A. Calcium
572 plus vitamin D supplementation and fat mass loss in
573 female very low-calcium consumers: potential link with
574 a calcium-specific appetite control. *British J Nutr* 2009;
575 **101**(5)**:** 659-663.
576

577 14. Kabrnova-Hlavata K, Hainer V, Gojova M, Hlavaty P,
578 Kopsky V, Nedvidkova J *et al.* Calcium intake and the
579 outcome of short-term weight management. *Physiol Res*
580 2008; **57**(2)**:** 237-245.
581
582 15. Zemel MB. Role of calcium and dairy products in
583 energy partitioning and weight management. *Am J Clin*
584 *Nutr* 2004; **79**(5)**:** 907S-912S.
585
586 16. Soares MJ, Murhadi LL, Kurpad AV, Chan She Ping-
587 Delfos WL, Piers LS. Mechanistic roles for calcium and
588 vitamin D in the regulation of body weight. *Obes Rev*
589 2012. doi: 10.1111/j.1467-789X.2012.00986.x
590
591 17. Boon N, Hul GB, Sicard A, Kole E, Van Den Berg ER,
592 Viguerie N *et al.* The effects of increasing serum
593 calcitriol on energy and fat metabolism and gene
594 expression. *Obesity* 2006; **14**(10)**:** 1739-1746. doi:
595 10.1038/oby.2006.200
596
597 18. Melanson EL, Donahoo WT, Dong F, Ida T, Zemel
598 MB. Effect of low- and high-calcium dairy-based diets
599 on macronutrient oxidation in humans. *Obes Res* 2005;
600 **13**(12)**:** 2102-2112. doi: 10.1038/oby.2005.261
601
602 19. Verdich C, Flint A, Gutzwiller JP, Naslund E,
603 Beglinger C, Hellstrom PM *et al.* A meta-analysis of the
604 effect of glucagon-like peptide-1 (7-36) amide on ad
605 libitum energy intake in humans. *J Clin Endocrin*
606 *Metab* 2001; **86**(9)**:** 4382-4389.
607
608 20. Flint A, Moller BK, Raben A, Sloth B, Pedersen D,
609 Tetens I *et al.* Glycemic and insulinemic responses as
610 determinants of appetite in humans. *Am J Clin Nutr*
611 2006; **84**(6)**:** 1365-1373.
612
613 21. Air EL, Benoit SC, Blake Smith KA, Clegg DJ, Woods
614 SC. Acute third ventricular administration of insulin
615 decreases food intake in two paradigms. *Pharmacol*
616 *Biochem Behav* 2002; **72**(1-2)**:** 423-429.
617
618 22. Asmar M, Simonsen L, Madsbad S, Stallknecht B,
619 Holst JJ, Bulow J. Glucose-dependent insulinotropic
620 polypeptide may enhance fatty acid re-esterification in
621 subcutaneous abdominal adipose tissue in lean humans.
622 *Diabetes* 2010; **59**(9)**:** 2160-2163. doi: 10.2337/db10-
623 0098
624
625 23. Pannacciulli N, Bunt JC, Koska J, Bogardus C, Krakoff
626 J. Higher fasting plasma concentrations of glucagon-

627 like peptide 1 are associated with higher resting energy
628 expenditure and fat oxidation rates in humans. *Am J*
629 *Clin Nutr* 2006; **84**(3)**:** 556-560.
630
631 24. Boschmann M, Engeli S, Dobberstein K, Budziarek P,
632 Strauss A, Boehnke J *et al.* Dipeptidyl-peptidase-IV
633 inhibition augments postprandial lipid mobilization and
634 oxidation in type 2 diabetic patients. *J Clin Endocrin*
635 *Metab* 2009; **94**(3)**:** 846-852. doi: 10.1210/jc.2008-1400
636
637 25. Achten J, Gleeson M, Jeukendrup AE. Determination of
638 the exercise intensity that elicits maximal fat oxidation.
639 *Med Sci Sports Exerc* 2002; **34**(1)**:** 92-97.
640
641 26. Achten J, Jeukendrup AE. Maximal fat oxidation during
642 exercise in trained men. *Int J Sports Med* 2003; **24**(8)**:**
643 603-608. doi: 10.1055/s-2003-43265
644
645 27. Craig CL, Marshall AL, Sjostrom M, Bauman AE,
646 Booth ML, Ainsworth BE *et al.* International physical
647 activity questionnaire: 12-country reliability and
648 validity. *Med Sci Sports Exerc* 2003; **35**(8)**:** 1381-1395.
649 doi: 10.1249/01.MSS.0000078924.61453.FB
650
651 28. Betts JA, Thompson D. Thinking outside the bag (not
652 necessarily outside the lab). *Med Sci Sports Exerc* 2012;
653 **44**(10)**:** 2040; author reply 2041. doi:
654 10.1249/MSS.0b013e318264526f
655
656 29. Jeukendrup AE, Wallis GA. Measurement of substrate
657 oxidation during exercise by means of gas exchange
658 measurements. *Int J Sports Med* 2005; **26 Suppl 1:**
659 S28-37. doi: 10.1055/s-2004-830512
660
661 30. Flint A, Raben A, Blundell JE, Astrup A.
662 Reproducibility, power and validity of visual analogue
663 scales in assessment of appetite sensations in single test
664 meal studies. *Int J Obes Metab Disord* 2000; **24**(1)**:** 38-
665 48.
666
667 31. Anderson GH, Catherine NL, Woodend DM, Wolever
668 TM. Inverse association between the effect of
669 carbohydrates on blood glucose and subsequent short-
670 term food intake in young men. *Am J Clin Nutr* 2002;
671 **76**(5)**:** 1023-1030.
672
673 32. Matthews DR, Hosker JP, Rudenski AS, Naylor BA,
674 Treacher DF, Turner RC. Homeostasis model
675 assessment: insulin resistance and beta-cell function

676 from fasting plasma glucose and insulin concentrations
677 in man. *Diabetologia* 1985; **28**(7)**:** 412-419.
678
679 33. Tunstall RJ, McAinch AJ, Hargreaves M, van Loon LJ,
680 Cameron-Smith D. Reduced plasma free fatty acid
681 availability during exercise: effect on gene expression.
682 *Eur J Appl Physiol* 2007; **99**(5)**:** 485-493. doi:
683 10.1007/s00421-006-0376-5
684
685 34. Blomstrand E, Saltin B. Effect of muscle glycogen on
686 glucose, lactate and amino acid metabolism during
687 exercise and recovery in human subjects. *J Physiol*
688 1999; **514**(1)**:** 293-302.
689
690 35. Sidossis LS, Wolfe RR, Coggan AR. Regulation of fatty
691 acid oxidation in untrained vs. trained men during
692 exercise. *Am J Physiol* 1998; **274**(3 Pt 1)**:** E510-515.
693
694 36. Messonnier LA, Emhoff CA, Fattor JA, Horning MA,
695 Carlson TJ, Brooks GA. Lactate kinetics at the lactate
696 threshold in trained and untrained men. *J Appl Physiol*
697 2013. doi: 10.1152/japplphysiol.00043.2013
698
699 37. Gunther CW, Lyle RM, Legowski PA, James JM,
700 McCabe LD, McCabe GP *et al.* Fat oxidation and its
701 relation to serum parathyroid hormone in young women
702 enrolled in a 1-y dairy calcium intervention. *Am J Clin*
703 *Nutr* 2005; **82**(6)**:** 1228-1234.
704
705 38. Astrup A, Chaput JP, Gilbert JA, Lorenzen JK. Dairy
706 beverages and energy balance. *Physiol Behav* 2010;
707 **100**(1)**:** 67-75. doi: 10.1016/j.physbeh.2010.01.039
708
709 39. Hellgren LI. Phytanic acid--an overlooked bioactive
710 fatty acid in dairy fat? *Ann N Y Acad Sci* 2010; **1190:**
711 42-49. doi: 10.1111/j.1749-6632.2009.05254.x
712
713 40. Gonzalez JT, Stevenson EJ. Assessment of the post-
714 exercise glycemic response to food: considering prior
715 nutritional status. *Nutr* 2013. doi:
716 10.1016/j.nut.2013.05.015
717
718 41. Gonzalez JT, Veasey RC, Rumbold PL, Stevenson EJ.
719 Breakfast and exercise contingently affect postprandial
720 metabolism and energy balance in physically active
721 males. *Br J Nutr* 2013; **110**(4)**:** 721-732. doi:
722 10.1017/S0007114512005582
723
724 42. Trautvetter U, Jahreis G. Effect of supplementary
725 calcium phosphate on plasma gastrointestinal hormones

726 in a double-blind, placebo-controlled, cross-over human
727 study. *Br J Nutr* 2013**:** 1-7. doi:
728 10.1017/S0007114513002341
729
730 43. Iozzo P, Pratipanawatr T, Pijl H, Vogt C, Kumar V,
731 Pipek R *et al.* Physiological hyperinsulinemia impairs
732 insulin-stimulated glycogen synthase activity and
733 glycogen synthesis. *Am J Physiol Endocrinol Metab*
734 2001; **280**(5)**:** E712-719.
735
736 44. Pikilidou MI, Lasaridis AN, Sarafidis PA, Befani CD,
737 Koliakos GG, Tziolas IM *et al.* Insulin sensitivity
738 increase after calcium supplementation and change in
739 intraplatelet calcium and sodium-hydrogen exchange in
740 hypertensive patients with Type 2 diabetes. *Diabetic*
741 *Med* 2009; **26**(3)**:** 211-219. doi: 10.1111/j.1464-
742 5491.2009.02673.x
743
744 45. Sanchez M, de la Sierra A, Coca A, Poch E, Giner V,
745 Urbano-Marquez A. Oral calcium supplementation
746 reduces intraplatelet free calcium concentration and
747 insulin resistance in essential hypertensive patients.
748 *Hypertension* 1997; **29**(1 Pt 2)**:** 531-536.
749
750 46. Mitri J, Dawson-Hughes B, Hu FB, Pittas AG. Effects
751 of vitamin D and calcium supplementation on
752 pancreatic beta cell function, insulin sensitivity, and
753 glycemia in adults at high risk of diabetes: the Calcium
754 and Vitamin D for Diabetes Mellitus (CaDDM)
755 randomized controlled trial. *Am J Clin Nutr* 2011;
756 **94**(2)**:** 486-494. doi: 10.3945/ajcn.111.011684
757
758 47. Bruce CR, Anderson MJ, Carey AL, Newman DG,
759 Bonen A, Kriketos AD *et al.* Muscle oxidative capacity
760 is a better predictor of insulin sensitivity than lipid
761 status. *J Clin Endocrin Metab* 2003; **88**(11)**:** 5444-5451.
762
763 48. Barry DW, Hansen KC, van Pelt RE, Witten M, Wolfe
764 P, Kohrt WM. Acute calcium ingestion attenuates
765 exercise-induced disruption of calcium homeostasis.
766 *Med Sci Sports Exerc* 2011; **43**(4)**:** 617-623. doi:
767 10.1249/MSS.0b013e3181f79fa8
768
769
770

771

772 **Figure legends**
773
774
775 **Figure 1.** Overview of the study design. CAL, high calcium;
776 CON, control.
777
778 **Figure 2.** Change in serum insulin concentration pre- to post-
779 supplementation with CON and CAL. CAL, high-calcium;
780 CON, control. Individual data are presented with group means
781 ± SEM represented by horizontal lines. n = 12. Paired t-test:
782 *Significantly different to CON, P = 0.006.
783
784 **Figure 3.** Change in maximum rates of lipid utilization during
785 exercise pre- to post-supplementation with CON and CAL.
786 CAL, high-calcium; CON, control. Individual data are
787 presented with group means ± SEM represented by horizontal
788 lines. n = 13.
789
790 **Figure 4.** Plasma NEFA and glycerol concentrations during
791 exercise before and after CON and CAL. a, NEFA
792 concentrations with CON supplementation. b, NEFA
793 concentrations with CAL supplementation. C, glycerol
794 concentrations with CON supplementation. d, glycerol
795 concentrations with CAL supplementation. CAL, high-calcium;
796 CON, control; NEFA, non-esterified fatty acid. Data are mean
797 ± SEM. n = 12. Interaction effect (supplement x time x power

798 output), $P = 0.883$ and $P = 0.401$ for NEFA and glycerol,

799 respectively.

800

801
802
803
804
805

806
807