

1 **The Effect of Post-Exercise Carbohydrate and Protein Ingestion on Bone Metabolism**

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21

22 **Abstract**

23 *Purpose*

24 To investigate the effect of feeding carbohydrate and protein (CHO+PRO), immediately or 2
25 h after an exhaustive run, on the bone turnover response in endurance runners.

26 *Methods*

27 10 men (age 28±5 y, height 1.74±0.05 m, body mass 69.7±6.3 kg) performed treadmill
28 running at 75% VO_{2max}, until exhaustion, on three occasions. Blood was collected before and
29 immediately, 1, 2, 3, 4 and 24 h post-exercise, for measurement of β-CTX, P1NP, PTH, PO₄,
30 ACa and Ca²⁺. This was a randomised, counterbalanced, placebo-controlled, single-blinded,
31 cross-over study. The three trials were; i) placebo (PLA), PLA solution was ingested
32 immediately and 2 h post-exercise, ii) immediate feeding (IF), CHO+PRO (1.5 g·kgBM⁻¹
33 dextrose and 0.5 g·kgBM⁻¹ whey) were ingested immediately post-exercise and PLA 2 h post-
34 exercise, and iii) delayed feeding (DF), PLA was ingested immediately post-exercise and
35 CHO+PRO solution 2 h post-exercise. Data were analysed using repeated measures ANOVA
36 and *post-hoc* Tukey's HSD.

37 *Results*

38 At 1 and 2 h post-exercise, β-CTX concentrations were lower in the IF trial than the DF and
39 PLA trials ($P\leq 0.001$). At 3 h post-exercise, β-CTX concentrations were higher in the PLA
40 trial than the IF ($P\leq 0.001$) and DF trials ($P=0.026$). At 4 h post-exercise, β-CTX
41 concentrations were lower in the DF trial than the IF ($P=0.003$) and PLA trials ($P\leq 0.001$). At
42 4 h post-exercise, P1NP was higher in the IF trial than in DF ($P=0.026$) and PLA trials
43 ($P=0.001$). At 3 h post-exercise, PTH was higher in the IF trial than the DF trial ($P\leq 0.001$).

44 *Conclusions*

45 Following exhaustive running, immediate ingestion of CHO+PRO may be beneficial, as it
46 decreases bone resorption marker concentrations and increases bone formation marker
47 concentrations; creating a more positive bone turnover balance.

48

49 **Key words:** Bone resorption, bone formation, post-exercise feeding, endurance athletes

50 **Introduction**

51 Dietary practices can influence both acute bone turnover and long-term bone health (38) and
52 feeding influences the diurnal rhythm of bone turnover markers at rest (31). Feeding of a
53 mixed nutrient meal suppresses all markers of bone turnover (4) and feeding of individual
54 nutrients; glucose, fat, protein and calcium, also suppresses bone resorption at rest (2, 3, 5,
55 14). However, previous studies have only investigated the response in resting, non-athletic
56 participants, who have not performed any prior exercise; it is therefore not known whether
57 there is a similar suppressive effect of nutrient ingestion on bone resorption, after exercise in
58 athletic individuals.

59

60 Prolonged and intense exercise causes increased bone resorption, as shown by increases in C-
61 terminal telopeptide of type 1 collagen (β -CTX) (19, 22, 33), although markers of bone
62 formation, such as N-terminal propeptides of type 1 procollagen (P1NP), are less responsive
63 to acute bouts of exercise (13, 32, 34). Increases in bone resorption, without concomitant
64 increases in bone formation, have been observed for up to four days after a bout of exhaustive
65 running (32). Although not definitive, this suggests that prolonged and intense exercise could
66 lead to an uncoupling or imbalance in bone turnover, favouring increased bone resorption,
67 which may have detrimental effects on bone mass and health (15). This uncoupling has been
68 implicated in the formation of stress fracture injuries (30, 39), which are debilitating injuries
69 for athletes and on average result in 169 days (with a range of 90 to 270 days) of lost training
70 (23, 27). Therefore maintaining coupled bone turnover and anabolic conditions for bone
71 during and after exercise is important for athletes. Given that endurance athletes train
72 multiple times a day preventing bone loss and stress fracture injury will help maximise
73 available training time.

74

75 Pre-exercise feeding has been investigated as a potential means for attenuating the bone
76 resorption response to exercise. Scott *et al.* (34) showed that feeding a mixed nutrient
77 breakfast prior to exercise had no effect on post-exercise β -CTX concentrations compared to
78 fasting and there were no changes in markers of bone formation. This implies that the
79 mechanical loading experienced during exercise over-rides any responses caused by pre-
80 exercise feeding. Scott *et al.* (34) also suggested that the stimulatory effect of PTH on β -CTX
81 may override the effect of pre-exercise feeding, therefore other exercise feeding practices, the
82 subsequent PTH response and related metabolites (calcium and phosphate) require
83 investigation.

84

85 Sale *et al.* (29) showed that carbohydrate (CHO) feeding during exercise attenuated the β -
86 CTX and P1NP responses in the hours following exercise, indicating an acute effect of CHO
87 feeding on bone turnover; however, feeding during intense running is not always well
88 tolerated and is limited by time and practicality. Post-exercise feeding provides a practical
89 opportunity to feed multiple nutrients and in the correct amounts, thus allowing athletes to
90 reach other sports nutrition goals, such as aiding muscle glycogen resynthesis, protein
91 synthesis and maintaining adequate hydration status (16, 36), without the restrictions of
92 gastrointestinal discomfort, which commonly limits nutrient ingestion during exercise. Post-
93 exercise feeding also allows for investigation of the bone turnover response to acute feeding
94 without the confounding effect of mechanical loading. It is not known whether the acute bone
95 turnover response to post-exercise feeding is the same as at rest and whether this varies with
96 different timings of post-exercise nutrient ingestion. The aim of this study was to investigate
97 the effect of feeding carbohydrate and protein (CHO+PRO) immediately or 2 h after a
98 prolonged intense running bout, on the bone turnover response in trained endurance runners.

99 Markers associated with exercise and bone were also measured to explore possible mediating
100 and mechanistic factors.

101 **Methods**

102 *Participants*

103 10 men ([mean \pm 1SD] age 28 ± 6 y, height 1.74 ± 0.05 m, body mass 69.7 ± 6.3 kg, VO_{2max}
104 63.0 ± 5.0 mL·kgBM⁻¹·min⁻¹, weekly running distance 49.9 ± 12.5 km) completed this study
105 that was approved by Nottingham Trent University's Ethical Advisory Committee. All
106 participants were trained endurance runners who had been competing and training
107 consistently for a minimum of 2 years in 10 km, half marathon, marathon or ultra-distance
108 races, without a significant break. Participants had recorded at least one of the following
109 times in the past 2 years; ≤ 35 minutes for 10 km, $\leq 1:25:00$ for half marathon or $\leq 3:00:00$ for
110 marathon. Participants were recruited from local running and triathlon clubs and local races,
111 via posters, flyers and posts on club websites. Consent was obtained by the primary
112 researcher. Participants were non-smokers, had not suffered a fracture in the last 12 months,
113 were free from musculoskeletal injury and did not suffer from any condition known to affect
114 bone metabolism. Compliance with these inclusion criteria was confirmed in the initial visit
115 to the laboratory where health screening was completed and written informed consent was
116 provided.

117

118 *Experimental Design*

119 This was a randomised (Latin Square Design), counterbalanced, placebo-controlled and
120 single-blinded, crossover study. Participants completed a preliminary visit for habituation
121 with trial procedures and measurement of VO_{2max} . Participants then completed three, four-day
122 experimental trials, each separated by 1 week. On days 1 and 2, participants refrained from
123 all exercise and followed a prescribed diet. On day 3, participants performed a bout of
124 treadmill running, at a speed equal to 75% of their previously determined VO_{2max} , until
125 volitional exhaustion. Blood samples (20 mL) were collected before exercise, immediately

126 after exercise and every hour after exercise for four hours. On day 4, participants returned to
127 the laboratory for a fasted follow up blood sample. The three trials consisted of; i) a placebo
128 (PLA) control trial, where the PLA solution was ingested both immediately and 2 h post-
129 exercise, ii) an immediate feeding (IF) trial, where the CHO+PRO solution was ingested
130 immediately post-exercise and the PLA solution 2 h post-exercise, and iii) a delayed feeding
131 (DF) trial where the PLA solution was ingested immediately post-exercise and the
132 CHO+PRO solution 2 h post-exercise. In the PLA trial, the CHO+PRO solution was ingested
133 after the final blood sample to ensure that the energy content and the composition of the diet
134 was identical between trials. This meant that a final PLA solution also needed to be ingested
135 in the IF and DF trials to ensure participant blinding to the trial conditions (Figure 1).

136

137 *Assessment of VO_{2max}*

138 Participants performed an incremental treadmill test to determine lactate threshold, as per
139 Jones and Doust (17), followed by a ramp test to determine VO_{2max} . Level running velocities
140 corresponding to 75% VO_{2max} ($13.0 \pm 0.8 \text{ km}\cdot\text{h}^{-1}$) were calculated based on the regression of
141 VO_2 and velocity.

142

143 *Experimental Dietary Provision*

144 Participants completed a three-day food diary for the measurement of habitual energy intake
145 and macronutrient composition. A diet consisting of 55% CHO, 30% fat and 15% PRO, and
146 isocaloric with habitual diets was designed using dietary analysis software (Nutritics, Dublin,
147 Ireland), for each participant to consume on days 1 and 2 of each trial. Participants provided
148 their own food but were given written and verbal instructions for the preparation of meals,
149 including timings of meals and snacks. Any deviations from prescribed diets were confirmed
150 verbally on day 3 and recorded; there were no significant deviations from prescribed diets.

151

152 ***Experimental Trial Procedure***

153 Participants were asked to maintain their habitual training and record this throughout the
154 study to help maintain consistency across trials. Participants refrained from all exercise on
155 days 1 and 2. Participants arrived at the laboratory on day 3, after fasting from 20:00 the
156 previous evening and consuming 500 mL of water upon awakening. Shortly after arriving,
157 body mass was measured and the first 20 mL blood sample was taken via venepuncture after
158 10 minutes of semi-recumbent rest.

159

160 Participants then ran to volitional exhaustion at 75% $\text{VO}_{2\text{max}}$, which was preceded by a 5-
161 minute warm-up and volitional stretching. At exhaustion a cannula was inserted into a
162 prominent forearm vein, which was kept patent by flushing with saline, a second 20 mL
163 blood sample was taken, with further blood samples taken at 1, 2, 3 and 4 h into recovery.
164 Exact times of exercise commencement, time to exhaustion and blood samples were recorded
165 and were repeated exactly in each trial within-participants to reduce the impact of circadian
166 variation on the results. Due to differences in individual run times to exhaustion between
167 participants, post-exercise blood sample timings vary between participants, but were
168 controlled for within-participants. The baseline blood sample was taken at 08:40 and exercise
169 commenced at 08:50, the blood sample at exhaustion was taken at 10:10 \pm 13 min and blood
170 samples 1 – 4 hours post-exercise were taken at 11:10 \pm 13 min, 12:10 \pm 13 min, 13:10 \pm 13
171 min and 14:10 \pm 13 min.

172

173 Depending on the trial, participants were given either the CHO+PRO or PLA solution to
174 consume immediately after exhaustion. Two and four hours after exhaustion participants
175 were given further solutions to consume. After the final solution was consumed, participants

176 were provided with food and were free to leave the laboratory. Participants consumed a snack
177 at 15:00 and an evening meal at 18:00 and then remained fasted from 20:00 until the next
178 morning. On day 4 participants arrived in the laboratory after consuming 500 mL of water
179 upon awakening and a final 20 mL blood sample was taken.

180

181 ***Recovery Solutions and Evening Meal Composition***

182 The CHO+PRO solution contained 1.5 g·kgBM⁻¹ of CHO (dextrose) and 0.5 g·kgBM⁻¹ of
183 PRO (unflavoured whey isolate) that was made up to a 12.5% CHO solution with water. The
184 whey isolate and dextrose mix was tested for banned substances by LGC Supplement
185 Screening (Cambridgeshire, UK; UKAS Testing Laboratory 1187; Certificate of Analysis
186 91530). Preliminary testing ensured that the PLA solution was taste matched to the
187 CHO+PRO solution using artificial sweetener and flavouring; it consisted of 12 mL·kgBM⁻¹
188 of water, making this the same volume as the CHO+PRO solution. Participants were blinded
189 to the solutions that they were consuming throughout trials. The total volume of fluid
190 consumed in the three recovery solutions was 2,509 ± 227 mL.

191

192 On day 3 the overall diet composition was 2,000 kcal, 55% CHO, 30% fat and 15% PRO.
193 The recovery solution contained approximately 500 kcal depending on individual body mass,
194 therefore the snack and evening meal contained approximately 1,500 kcal. Deviations from
195 prescribed diets were confirmed verbally on day 4 and recorded; there were no significant
196 deviations from prescribed diets. Participants were allowed to ingest plain water on an *ad*
197 *libitum* basis throughout the recovery periods, although none of the participants did this
198 during any of the trials.

199

200 ***Treatment and Storage of Blood Samples***

201 Blood was transferred into precooled tubes and gently inverted 5–8 times; 15 mL of blood
202 was transferred into tubes containing 15%, 0.12 mL of K3E EDTA (Becton Dickinson
203 Vacutainer System, USA) and then centrifuged immediately at $3000 \text{ rev min}^{-1}$, for 10 minutes
204 at 5°C , generating plasma. The remaining 5 mL of blood was transferred into standard serum
205 tubes (Becton Dickinson Vacutainer System, USA), left to clot at room temperature for 60
206 min before being centrifuged at $2000 \text{ rev min}^{-1}$, for 10 minutes at 5°C . Plasma and serum was
207 subsequently stored at -80°C until analysis.

208

209 ***Biochemical Analysis***

210 β -CTX, P1NP and parathyroid hormone (PTH) were measured by electro-chemiluminescence
211 immunoassay on an fully automated COBAS c501 system (Roche Diagnostics, Mannheim,
212 Germany) in blood plasma and were measured in singlicate. The inter-assay CV for β -CTX
213 was $\leq 3\%$ between 0.2 and $1.5 \mu\text{g L}^{-1}$, with sensitivity of $0.01 \mu\text{g L}^{-1}$. The inter-assay CV for
214 P1NP was $\leq 3\%$ between 20 and $600 \mu\text{g L}^{-1}$, with sensitivity of $8 \mu\text{g L}^{-1}$. The inter-assay CV
215 for PTH was $\leq 4\%$ between 1 and 30 pmol L^{-1} , with sensitivity of 0.8 pmol L^{-1} . Phosphate
216 (PO_4), total calcium (Ca) and albumin were measured in serum, using standard colorimetric
217 assays and spectrophotometric methods, performed on an ABX Pentra 400 (Horiba ABX,
218 Montpellier, France). PO_4 was measured using phosphomolybdate, with an inter- and intra-
219 assay CV of $\leq 3.6\%$ between 0.09 and 7.80 mmol L^{-1} . Total Ca was measured using ortho-
220 cresolphthalein complexone, with an inter- and intra-assay CV of $\leq 1.7\%$ between 0.04 and
221 5.00 mmol L^{-1} . Albumin was measured using bromocresol green, with an inter- and intra-
222 assay CV of $\leq 1.9\%$ between 0.02 and 5.99 g dL^{-1} . Because fluctuations in protein,
223 particularly albumin, may cause total Ca levels to change independently of the ionised
224 calcium (Ca^{2+}) concentration, total Ca concentrations were corrected to give an albumin-
225 adjusted Ca (ACa) value: 0.8 mg dL^{-1} was subtracted from the total Ca concentration for

226 every 1.0 g·dL⁻¹ by which the serum albumin concentration was greater than 4 g·dL⁻¹ or 0.8
227 mg·dL⁻¹ was added to the total Ca concentration for every 1.0 mg·dL⁻¹ by which the serum
228 albumin concentration was less than 4 mg·dL⁻¹. *I.e.* $(([\text{Albumin}] - 4) * -0.8) + [\text{Total Ca}]$.
229 Ca²⁺, glucose and lactate were measured in whole blood using a blood gas analyser
230 (Radiometer ABL90 FLEX, Copenhagen, Denmark). Ca²⁺ is estimated directly between pH
231 7.2 and 7.6 with no pH correction applied. The inter- and intra-assay CV for Ca²⁺ was ≤3%
232 between 0.2 and 9.99 mmol·L⁻¹, for glucose was ≤5% between 0 and 60 mmol·L⁻¹ and for
233 lactate was ≤26.7% between 0.1 and 31 mmol·L⁻¹.

234

235 ***Statistical Analysis***

236 The study sample size was calculated to detect changes in β-CTX from pre- to post-
237 exhaustive exercise, with 85% power at an alpha level of $P \leq 0.05$, based on the study by Scott
238 *et al.* (32). Statistical significance was accepted at an alpha level of $P \leq 0.05$. All statistical
239 analyses were performed on raw data. Baseline concentrations were compared using a one-
240 way ANOVA. Parametric assumptions of normality and sphericity were confirmed using the
241 Shapiro-Wilks test and Mauchly's test of Sphericity and where assumptions were violated, a
242 transformation was applied to the data so that the assumptions were satisfied. Normality and
243 homogeneity were achieved following log transformations for ACa and PO₄ data. All data
244 were subsequently analysed using a repeated measures ANOVA, with *Trial* (PLA vs IF vs
245 DF) and *Time* (of sampling) as within participant factors. Tukey's HSD *post-hoc* test was
246 used to compare each time point against baseline and to compare trials at each time point.
247 Effect size for multiple comparisons was calculated using partial (η_p^2) eta-squared (21). *Post-*
248 *hoc* comparisons are reported with Cohen's *d* effect sizes, with $d=0.2$ considered as a small
249 effect, $d=0.5$ considered as a medium effect and $d=0.8$ considered as a large effect (6). These

250 statistical analyses were performed with Statistica (StatSoft, Tulsa, OK) and SPSS (IBM
251 SPSS Statistics 22, Armonk, NY).

252 **Results**

253 ***Exercise variables***

254 The average time to exhaustion (exercise duration) was 01:15:00 ± 00:13:00. There was a
255 significant decrease in body mass from pre-exercise (69.4 ± 6.1 kg) to post-exercise (68.9 ±
256 5.9 kg) ($P=0.001$).

257

258 ***Baseline biochemistry***

259 Baseline concentrations of β -CTX, P1NP, PTH, ACa, Ca^{2+} , PO_4 and albumin were not
260 significantly different between trials (Table 1).

261

262 ***Habitual diet and experimental dietary provision***

263 There were no significant differences between the diets prescribed for days 1 and 2 of each
264 trial and the diets that were actually consumed by participants, for overall energy content or
265 macronutrient composition. Participants' habitual diets were not different from the diet
266 provided on day 3 of trials, for overall energy content, CHO content, fat content and calcium
267 content ($P=0.101$ to 0.523). However, PRO content was significantly higher in the habitual
268 diets compared to the experimental trial diet ($P=0.049$) (Table 2).

269

270 ***Bone turnover markers***

271 ***C-terminal telopeptide of type 1 collagen (β -CTX)***

272 There was a significant main effect of *Trial* ($P\leq 0.001$; $\eta_p^2 = 0.581$) *Time* ($P\leq 0.001$; $\eta_p^2 =$
273 0.744) and a significant *Trial x Time* interaction ($P\leq 0.001$; $\eta_p^2 = 0.630$) for β -CTX. β -CTX
274 concentrations were increased from baseline by the end of exercise in all trials (+8 to +12%).
275 In the PLA trial, β -CTX concentrations remained increased above baseline at 1 h post-
276 exercise (+7%), before decreasing thereafter, being significantly lower than baseline

277 concentrations 3 and 4 h post-exercise (-31 to -42%; $P \leq 0.001$) and 24 h later (-3%). In the IF
278 trial, β -CTX concentrations were significantly lower than baseline at 1 h post-exercise and
279 remained below baseline until the end of the trial (-22 to -61%; $P \leq 0.01$). In the IF trial, β -
280 CTX concentrations were increased above baseline 24 h later (+8%). In the DF trial, β -CTX
281 concentrations were increased above baseline at 1 h post-exercise (+15%), then began to
282 decrease and were significantly lower than baseline concentrations 3 and 4 h post-exercise (-
283 44 to -65%; $P \leq 0.001$). In the DF trial, β -CTX concentrations were increased above baseline
284 24 h later (+8%) (Figure 2A).

285

286 At 1 and 2 h post-exercise, β -CTX concentrations were significantly lower in the IF trial than
287 the DF ($P \leq 0.001$, $d=0.76$) and PLA trials ($P \leq 0.001$, $d=0.84$). At 3 h post-exercise, β -CTX
288 concentrations were significantly higher in the PLA trial than the IF ($P \leq 0.001$, $d=1.13$) and
289 DF trials ($P=0.026$, $d=0.54$). At 4 h post-exercise, β -CTX concentrations were significantly
290 lower in the DF trial than the IF ($P=0.003$, $d=0.82$) and PLA trials ($P \leq 0.001$, $d=1.09$) (Figure
291 2A). All other time points were not significantly different between trials. The overall β -CTX
292 response was significantly lower in the IF trial than the DF trial ($P=0.019$, $d=0.37$) and the
293 PLA trial ($P \leq 0.001$, $d=0.84$).

294

295 *N-terminal propeptides of type I procollagen (PINP)*

296 There was no main effect of *Trial* for PINP, but there was for *Time* ($P \leq 0.001$; $\eta_p^2 = 0.621$)
297 and there was a significant *Trial x Time* interaction ($P \leq 0.001$; $\eta_p^2 = 0.292$). PINP
298 concentrations were significantly increased from baseline by the end of exercise in all trials
299 (+32 to +33%; $P \leq 0.001$) and by 1 h post-exercise PINP had decreased below baseline
300 concentrations in all trials (-3 to -7%). In the PLA trial, PINP concentrations remained below
301 baseline until the end of the trial (-7 to -9%), but were increased above baseline 24 h later

302 (+4%). In the IF trial, P1NP began to increase and reached concentrations above baseline at 3
303 and 4 h post-exercise (+1 to +3%) and 24 h later (+5%). In the DF trial, P1NP concentrations
304 continued to decrease and by 3 and 4 h post-exercise were significantly lower than baseline (-
305 10 to -11%; $P \leq 0.05$), but were increased above baseline 24 h later (+4%) (Figure 2B). At 4 h
306 post-exercise, P1NP was significantly higher in the IF trial than the DF ($P=0.026$, $d=0.20$)
307 and PLA trials ($P=0.001$, $d=0.25$) (Figure 2B). All other time points were not significantly
308 different between trials.

309

310 ***Calcium metabolism***

311 *Parathyroid hormone (PTH)*

312 There was no main effect of *Trial* for PTH, but there was for *Time* ($P \leq 0.001$; $\eta_p^2 = 0.791$) and
313 there was a significant *Trial x Time* interaction ($P \leq 0.001$; $\eta_p^2 = 0.428$). PTH concentrations
314 were significantly increased from baseline by the end of exercise in all trials (+124 to +131%;
315 $P \leq 0.001$) but by 1 h post-exercise had decreased significantly below baseline concentrations
316 in all trials (-17 to -37%; $P \leq 0.05$). In the PLA trial, PTH concentrations remained below
317 baseline until the end of the trial (-3 to -15%) but were increased above baseline 24 h later
318 (+4%). In the IF trial, PTH then began to increase and reached concentrations above baseline
319 3 and 4 h post-exercise (+2 to +7%) and 24 h later (+1%). In the DF trial, PTH continued to
320 decrease and remained below baseline concentrations for the remainder of the trial (-13 to -
321 27%) and 24 h later (-4%) (Figure 3A). At 3 h post-exercise, PTH was significantly higher in
322 the IF trial than the DF trial ($P \leq 0.001$, $d=1.33$) (Figure 3A). All other time points were not
323 significantly different between trials.

324

325 *Albumin-adjusted calcium (ACa)*

326

327 There was no main effect of *Trial* for Aca, but there was for *Time* ($P=0.003$; $\eta_p^2 = 0.290$) and
328 there was a significant *Trial x Time* interaction ($P=0.020$; $\eta_p^2 = 0.191$). Aca concentrations
329 were increased from baseline by the end of exercise in all trials (+2 to +3%). In the PLA trial,
330 Aca concentrations remained above baseline until the end of the trial (+2 to +4%) but had
331 decreased below baseline 24 h later (-1%). In the IF trial, Aca remained above baseline (+2
332 to 3%) until 3 h post-exercise when Aca decreased below baseline (-3%), Aca then
333 increased above baseline 4 h post-exercise (+1%) and remained there 24 h later. In the DF
334 trial, Aca remained above baseline until the end of the trial (+2 to +4%) and returned to
335 baseline 24 h later (Figure 3B). At 3 h post-exercise, Aca was significantly lower in the IF
336 trial than the DF ($P=0.008$, $d=0.79$) and PLA trials ($P=0.001$, $d=0.98$) (Figure 3B). All other
337 time points were not significantly different between trials.

338

339 *Ionised calcium (Ca²⁺)*

340 There was no main effect of *Trial* for Ca²⁺, but there was for *Time* ($P\leq 0.001$; $\eta_p^2 = 0.771$) and
341 a significant *Trial x Time* interaction ($P\leq 0.001$; $\eta_p^2 = 0.321$). Ca²⁺ concentrations were
342 significantly decreased below baseline by the end of exercise in all trials (-5 to -7%;
343 $P\leq 0.001$). In the PLA trial, Ca²⁺ concentrations were still significantly below baseline by 1 h
344 post-exercise (-4%; $P=0.002$) and remained below baseline until the end of the trial and 24 h
345 later (-3%; $P=0.006$). In the IF trial, Ca²⁺ concentrations had returned to baseline by 1 h post-
346 exercise (+1%) and remained at concentrations similar to baseline until the end of the trial
347 and 24 h later (-1%). In the DF trial, Ca²⁺ concentrations had almost returned to baseline by 1
348 h post-exercise (-1%) and remained at concentrations similar to baseline until the end of the
349 trial and 24 h later (-1%) (Figure 3C). At 1 h post-exercise, Ca²⁺ concentrations were
350 significantly lower in the PLA trial than the IF trial ($P=0.010$, $d=1.41$) (Figure 3C). All other
351 time points were not significantly different between trials.

352

353 *Phosphate (PO₄)*

354 There was no main effect of *Trial* for PO₄, but there was for *Time* ($P \leq 0.001$; $\eta_p^2 = 0.581$) and
355 there was a significant *Trial x Time* interaction ($P = 0.007$; $\eta_p^2 = 0.207$). PO₄ concentrations
356 were significantly increased above baseline by the end of exercise in all trials (+21 to +26%;
357 $P \leq 0.001$). By 1 h post-exercise, PO₄ concentrations decreased below baseline in all trials (-5
358 to -13%). In the PLA trial, PO₄ concentrations continued to decrease at 2 h post-exercise (-
359 8%), then increased and returned to baseline 3 h post-exercise. In the PLA trial, PO₄
360 concentrations were increased above baseline at 4 h post-exercise (+14%) and 24 h later
361 (+3%). In the IF trial, PO₄ concentrations started to increase at 2 h post-exercise and
362 increased above baseline 4 h post-exercise (+8%). In the IF trial, PO₄ concentrations were
363 below baseline 24 h later (-2%). In the DF trial, PO₄ concentrations continued to decrease at 2
364 h post-exercise (-8%), concentrations started to increase thereafter, but remained below
365 baseline until the end of the trial and 24 h later (-4%) (Figure 3D). At 1 h post-exercise, PO₄
366 concentrations were significantly lower in the IF trial than the DF trial ($P = 0.049$, $d = 1.03$)
367 (Figure 3D). All other time points were not significantly different between trials.

368

369 *Albumin*

370 There was no main effect of *Trial* for albumin, but there was for *Time* ($P \leq 0.001$; $\eta_p^2 = 0.372$)
371 and there was no *Trial x Time* interaction ($P = 0.054$; $\eta_p^2 = 0.167$). Overall mean albumin
372 concentrations were significantly increased from baseline by the end of exercise (+3 to +4%;
373 $P = 0.011$). There were no other significant changes in albumin concentrations (Figure 4).

374

375 **Discussion**

376 The main findings of the study are that: 1) ingestion of the CHO+PRO solution containing
377 1.5 g·kgBM⁻¹ of CHO and 0.5 g·kgBM⁻¹ of PRO suppressed β-CTX concentrations following
378 an exhaustive run, with a greater overall suppression when the CHO+PRO solution was
379 ingested immediately; 2) immediate ingestion of the CHO+PRO solution resulted in small
380 increases in P1NP concentrations at 3 and 4 h post-exercise; 3) delayed ingestion of the
381 CHO+PRO solution (2 h post-exercise) also resulted in a large suppression of β-CTX
382 concentrations. These findings are novel and have the potential to directly influence an
383 athlete's dietary and/or training practices.

384

385 The response in the PLA trial, showed that the exhaustive running bout caused an immediate
386 increase in bone turnover at the end of exercise, indicated by increased β-CTX and P1NP
387 concentrations above baseline. This was followed by decreased bone turnover during
388 recovery, indicated by decreased β-CTX and P1NP concentrations below baseline. Ingestion
389 of the CHO+PRO solution immediately post-exercise caused a rapid and prolonged (at least 4
390 h) suppression of β-CTX concentrations below baseline levels (-22 to -61%), whereas
391 ingesting the PLA solution immediately post-exercise meant that β-CTX concentrations were
392 increased above baseline by between +7 and +15%. When ingestion of the CHO+PRO was
393 delayed by 2 h, it caused suppression of β-CTX concentrations below baseline (-44 to -65%),
394 which is similar to the suppression caused by immediate ingestion of the CHO+PRO solution
395 and it occurred within the same timeframe, *i.e.*, 1 – 2 h after ingestion.

396

397 This rapid response is important because elite athletes habitually train multiple times a day,
398 meaning that there is often only a few hours in between training sessions and therefore
399 limited time for recovery and food consumption. Although the participants in the present

400 study are not elite athletes, their trained nature means that the results are relevant and may be
401 interpreted and used by elite athletes or practitioners. The results indicate that post-exercise
402 nutrient ingestion or exercise commencement can be timed so that the subsequent training
403 session occurs when bone resorption is at its lowest and bone formation at its highest, *i.e.*, 3 –
404 4 hours after the first exercise bout with immediate ingestion of the CHO+PRO solution. This
405 may maximise the anabolic and minimise the catabolic bone response to the subsequent
406 training session, however further research is needed to investigate whether this intervention
407 does indeed produce a more anabolic environment for bone.

408

409 The significant increase in P1NP concentrations (+32 to +33%) and the larger relative
410 increase in P1NP compared to β -CTX concentrations at the end of exercise is interesting, as
411 markers of bone formation are usually less responsive to acute bouts of exercise than markers
412 of bone resorption (13, 32, 34). Similarly, de Sousa *et al.* (7) reported a 77% increase in
413 P1NP after a high-intensity, interval running session (10 x 800m). In the present study, P1NP
414 concentrations then decreased to below baseline levels at 1 h post-exercise in all trials, but the
415 ingestion of the CHO+PRO solution immediately post-exercise caused P1NP to increase
416 above baseline at 3 and 4 h post-exercise by between +1 to +3%, whereas ingesting the PLA
417 solution immediately post-exercise meant that P1NP remained below baseline concentrations
418 by between -7 and -9%. When the CHO+PRO solution was ingested 2 h post-exercise, P1NP
419 concentrations were suppressed further below baseline concentrations (-10 to -11%). It is
420 possible that P1NP could have increased after the last measurement was taken but was missed
421 by the sampling protocol, therefore it would be useful for future research to examine a longer
422 post-exercise period to investigate the longer term response. The significantly increased
423 P1NP concentrations at 4 h post-exercise in the IF trial compared to the DF and PLA trials is
424 novel, and taken together, these results advocate the feeding of a CHO+PRO solution

425 immediately post-exercise in order to reduce bone resorption marker concentrations and
426 increase bone formation marker concentrations in the short-term recovery from intense
427 exercise.

428

429 The effects of the CHO+PRO solution did not persist to the morning following exercise and
430 β -CTX concentrations were increased in the IF and DF trials (+8%) compared to suppressed
431 β -CTX concentrations in the PLA trial (-3%). P1NP was increased 24 h post-exercise in all
432 trials (+4 to +5%). This increased bone turnover in the IF and DF trials may reflect the bones
433 adapting to a possible hormonal response that is mediated by feeding. It is unlikely that the
434 bones are adapting to the mechanical loading from the running bout alone, as β -CTX
435 concentrations were not increased 24 h post-exercise in the PLA trial. The hormonal
436 mediators of this response are currently unknown; Scott *et al.* (34) and Sale *et al.* (29)
437 recently showed that GLP-2, leptin and ghrelin are unlikely mediators of the effect of CHO or
438 mixed meal feeding on bone turnover. Subsequently, this requires further research including
439 the measurement of other gastro-intestinal hormones.

440

441 Although this increased bone turnover response may be positive in sub-elite populations, elite
442 athletes that train multiple times a day with minimal recovery time and rest days are more
443 likely to suffer from consistently increased bone remodelling, which may have detrimental
444 effects on bone health and enhance the stress fracture risk (25, 26, 28, 30). The trained
445 runners and triathletes in the present study have mean resting bone turnover marker
446 concentrations that are at the upper end of the reference ranges for the non-active, healthy
447 population (7, 11, 12). Further, unpublished data from our laboratory show that elite
448 triathletes have mean resting bone turnover marker concentrations that are higher than the
449 trained runners and triathletes. This is supported by Oosthuysen *et al.* (25) who showed that

450 bone resorption and bone formation markers were significantly elevated each morning after
451 four successive 3 h cycling bouts in well-trained cyclists. Although this is speculative, elite
452 athletes may experience an imbalance between whole-body rates of resorption and formation
453 or, defective coupling (26), meaning that neither bone resorption or bone formation is
454 performed adequately and the quality of the bone may be poorer. Or, athletes may experience
455 accelerated remodelling, which can increase bone microdamage accumulation (30), all of
456 which can increase stress fracture risk (1, 9, 28, 30). Indeed it should be noted that in a
457 normal, healthy basic multicellular unit, the suppression of bone resorption may not always
458 be desired, if the function of bone resorption is to breakdown and remove damaged bone at
459 areas of microdamage accumulation to allow the area to be repaired and strengthened.
460 Therefore, it is crucial for future research to investigate the long term effects of post-exercise
461 suppression of bone resorption on different athletic and non-athletic populations.

462

463 Ingestion of the CHO+PRO solution post-exercise is not sufficient to cause a decrease in
464 bone resorption marker concentrations and/or an increase in bone formation marker
465 concentrations 24 h post-exercise. However, as elite athletes rarely go 24 h without a training
466 session and often have a second session within four hours of finishing the first session, the
467 bone turnover response 24 h post-exercise is less important than the immediate response as it
468 does not reflect real life athlete practice. The more important time point is therefore, 4 h post-
469 exercise, as this may be around the same time that the second training session would start. As
470 we have now investigated the effect of post-exercise feeding after a single acute bout of
471 exercise, future studies should investigate the effect of post-exercise nutrient ingestion on
472 repeated bouts of exercise occurring on the same day.

473

474 The responses of Ca^{2+} and PO_4 to exercise are in line with previous research (37) and the
475 responses are only significantly different between trials at 1 h post-exercise; Ca^{2+}
476 concentrations were lower in the PLA trial compared to the IF trial, suggesting that IF
477 augments the recovery of Ca^{2+} to baseline concentrations, and PO_4 is lower in the IF trial
478 compared to the DF trial. Transient peaks in PTH concentrations, as shown in the present
479 study, are shown to be anabolic for bone (10) and Townsend *et al.* (37) showed that PTH
480 secretion during exercise and recovery is controlled by both Ca^{2+} and PO_4 , therefore these
481 metabolites are likely to be mediating any anabolic effect of increased PTH concentrations.
482 The fact that PTH and P1NP follow the same response could suggest that PTH is mediating
483 an anabolic response in the IF trial, however this response needs to be confirmed.

484

485 At 3 h post-exercise, PTH concentrations were greater in the IF trial than in the DF trial (+7%
486 vs -27%). This response coincides with significantly lower Aca concentrations at 3 h post-
487 exercise in the IF trial compared to the DF and PLA trials (-3% vs +3 to +4%). β -CTX
488 concentrations were at their lowest at 3 h post-exercise in the IF trial. Considering that the
489 action of increased PTH secretion is to increase calcium through mobilisation of the bone
490 reservoir via activation of bone resorption (and also by increasing renal tubular reabsorption
491 and intestinal calcium absorption) (24, 35, 40), this suggests that changes in PTH and calcium
492 metabolism are unlikely to mediate the acute suppression in bone resorption seen with post-
493 exercise CHO+PRO feeding. However, Aca has been shown to be unsuitable when
494 investigating the rapid response of calcium metabolism to exercise (37), which may also be
495 true when investigating CHO+PRO ingestion around exercise. Although Ca^{2+} (non-protein
496 bound calcium) decreased at the end of exercise, because albumin concentrations increased,
497 Aca was normalised and remained fairly unchanged throughout exercise. Changes in
498 albumin could have been effected by the ingestion of dietary protein throughout the recovery

499 period, which has previously been shown to increase circulating albumin concentrations (18,
500 20), however albumin did not change significantly throughout the recovery period. The
501 increase in albumin at the end of exercise could have been to encourage more calcium to be
502 transported around the body, due to the tissues requiring additional Ca^{2+} to keep up with the
503 demand in energy consumption, although the increase in albumin might also just reflect
504 haemoconcentration as a result of the running bout. Transient haemoconcentration can occur
505 rapidly following the onset of acute exercise, possibly even occurring in advance of any
506 significant losses of fluid through sweating or respiration, and it might be argued that
507 significant haemoconcentration would mean that changes in plasma solutes simply reflect
508 shifts in plasma volume. However, one might argue that the level of a plasma solute,
509 irrespective of plasma volume shifts, is more important, since it is this that the body responds
510 to. The data presented herein are uncorrected for plasma volume changes, which could
511 influence the interpretation of the biological data obtained during the recovery period and this
512 should be considered when interpreting results. It is recommended that future studies take this
513 into consideration and correct bone turnover marker data for plasma volume shifts, where
514 appropriate, perhaps even presenting these data both corrected and uncorrected for plasma
515 volume changes.

516

517 In conclusion, following exhaustive running, immediate ingestion of a CHO+PRO recovery
518 solution may be beneficial, as it decreases bone resorption marker concentrations and
519 increases bone formation marker concentrations; creating a more positive bone turnover
520 balance. The mechanisms underlying the acute changes in bone turnover remain unknown,
521 but a change in calcium metabolism is unlikely to fully mediate the response.

522

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526 *of the present study. The results presented herein do not constitute endorsement by the*
527 *American College of Sports Medicine. The results of the study are presented clearly,*
528 *honestly, and without fabrication, falsification, or inappropriate data manipulation.*
529

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628 **Figures**

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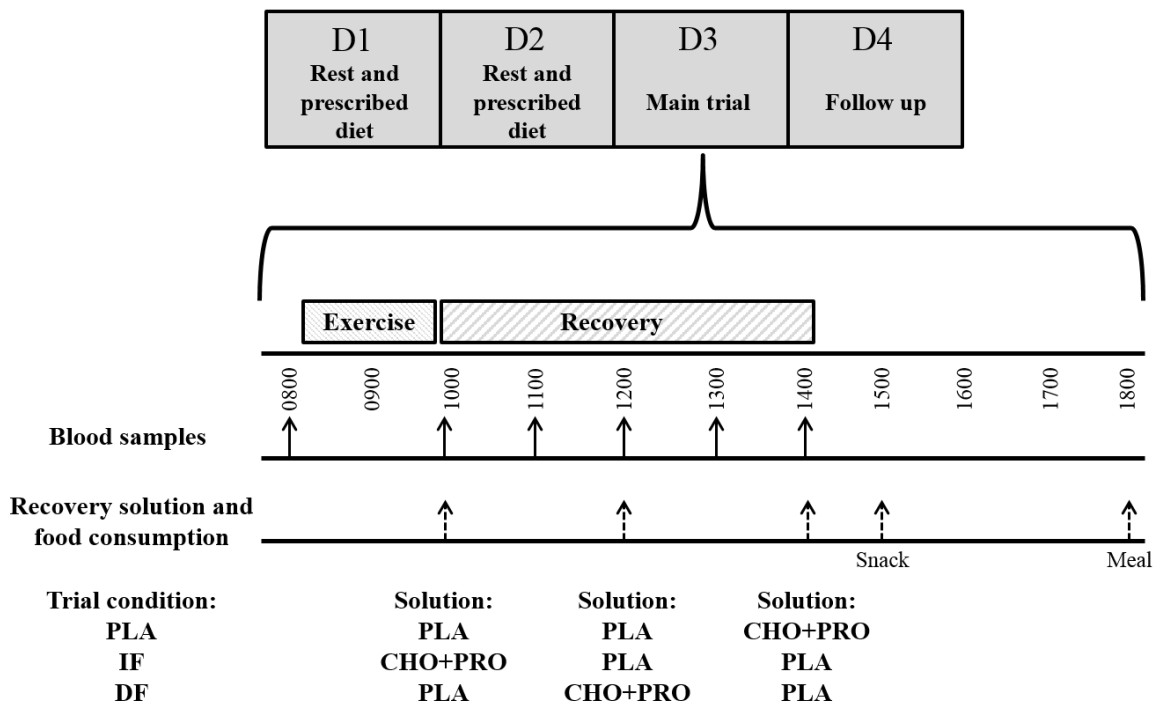
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641 **Figure 1.** Experimental protocol. Exercise was treadmill running at 75%VO_{2max}, followed by

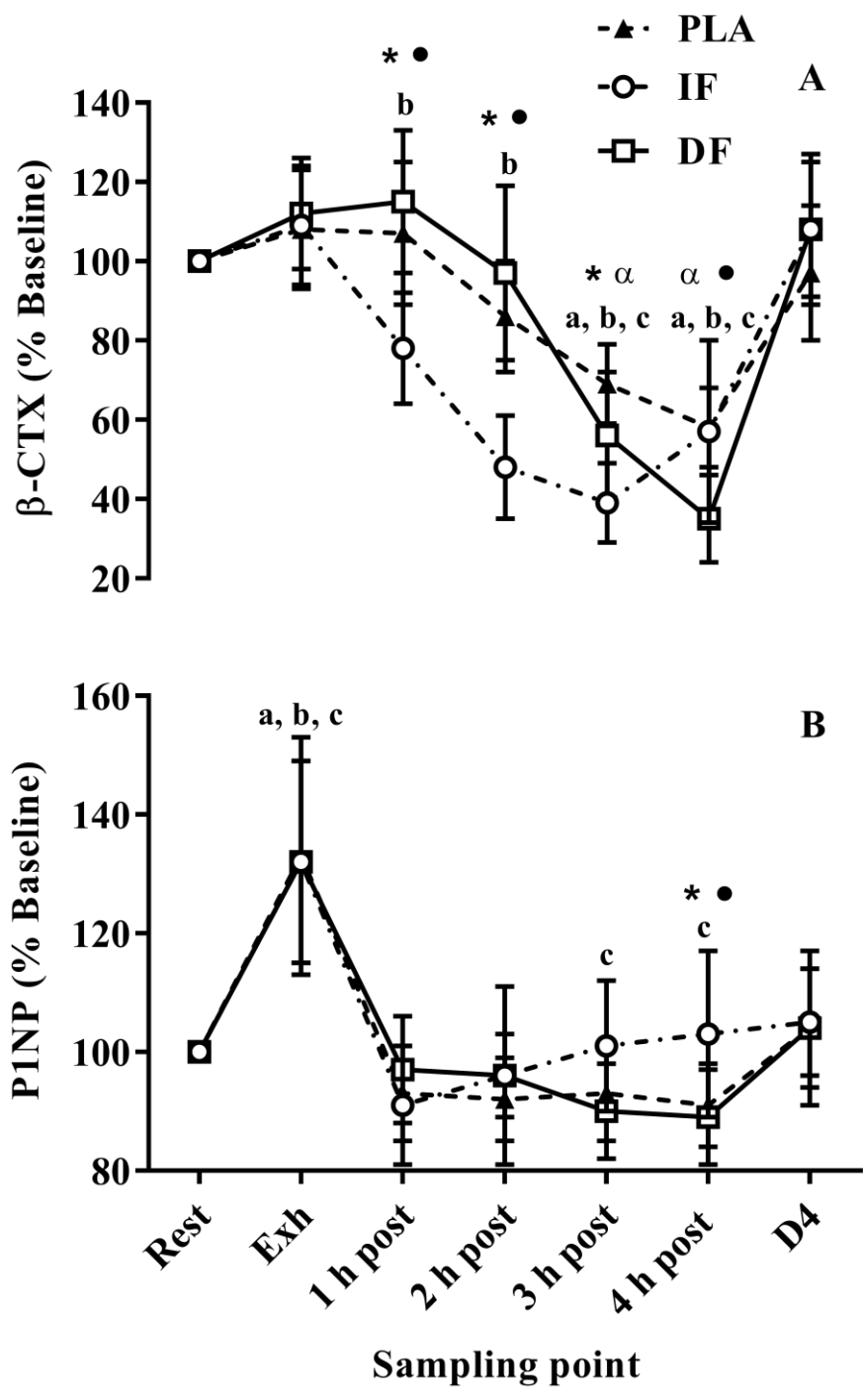
642 4 hours of rested recovery. PLA = Placebo trial, IF = Immediate feeding trial and DF =

643 Delayed feeding trial. Participants departed from the laboratory at the end of the recovery

644 period. Solid vertical arrows denote blood samples. Dashed vertical arrows denote recovery

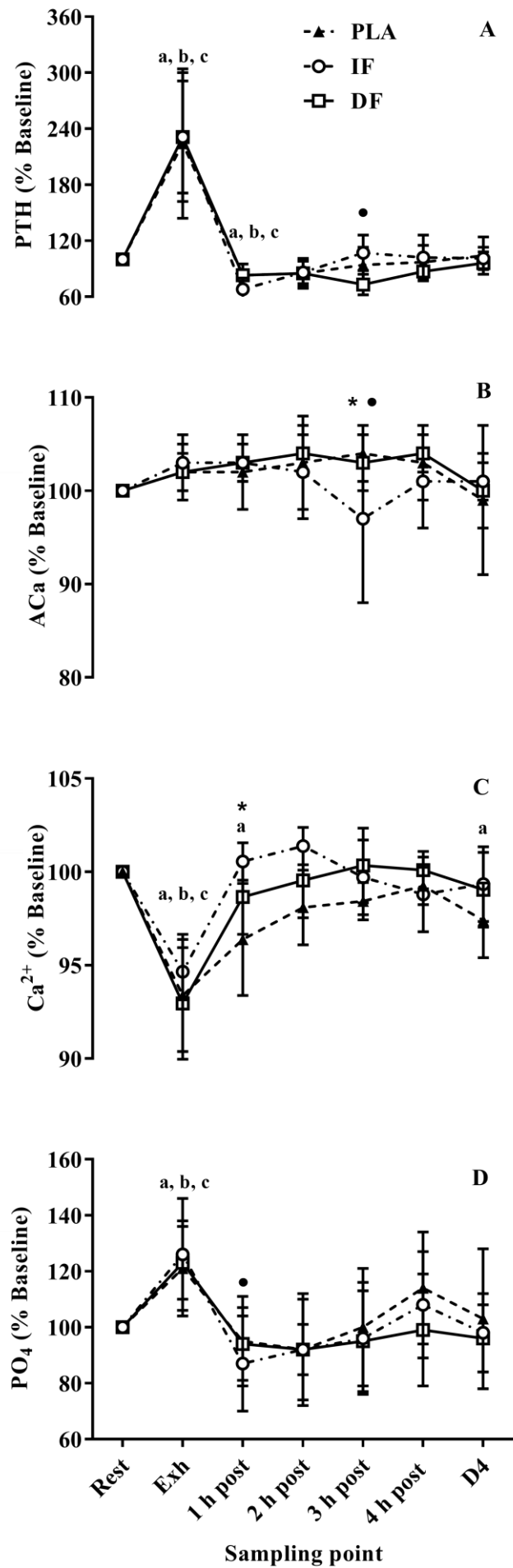
645 solution and food consumption.

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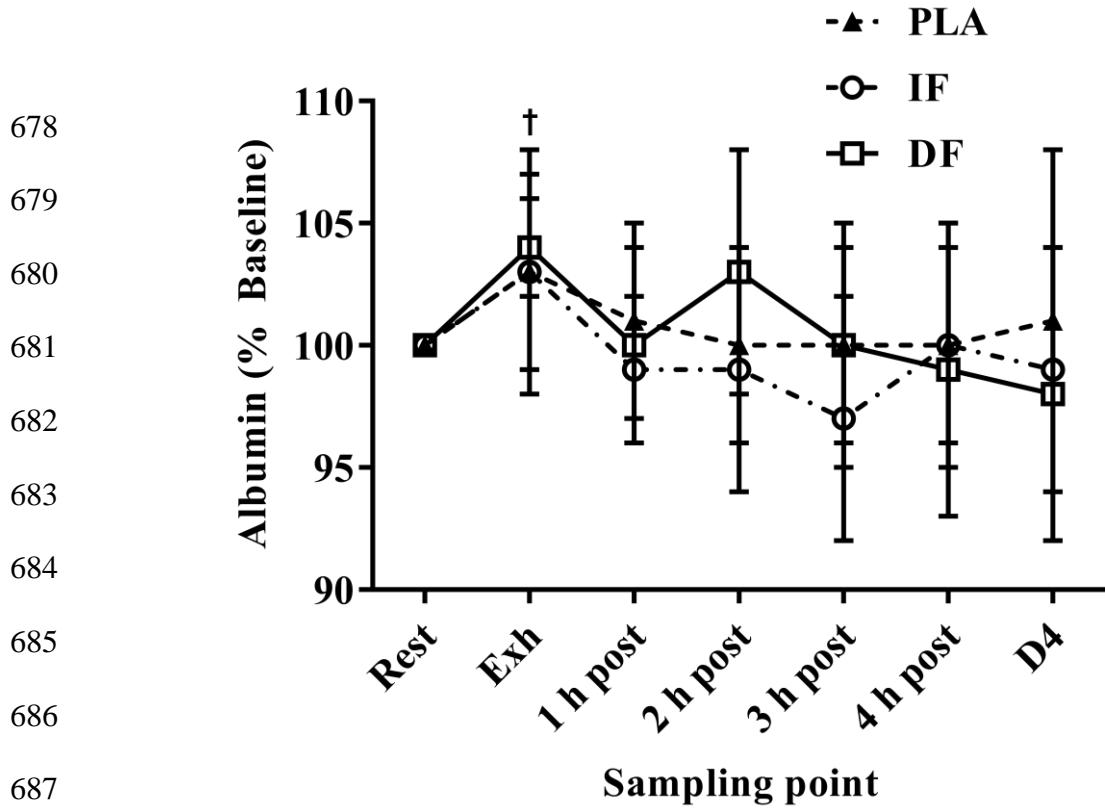


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Figure 2. The percentage change in baseline concentrations of β -CTX (A) and P1NP (B), at Rest (Baseline), Exh (at exhaustion), 1 to 4 hours post-exercise and D4 (follow up sample on day 4), for PLA (filled triangles), IF (open circles) and DF (open squares). Data are mean \pm 1SD. ^a different ($P \leq 0.05$) from baseline (PLA) ^b different ($P \leq 0.05$) from baseline (IF), ^c different ($P \leq 0.05$) from baseline (DF). * IF different ($P \leq 0.05$) from PLA, ^a DF different ($P \leq 0.05$) from PLA, [•] IF different ($P \leq 0.05$) from DF



672 **Figure 3.** The percentage change in baseline concentrations of PTH (A), ACa (B), Ca²⁺ (C)
673 and PO₄ (D) at Rest (Baseline), Exh (at exhaustion), 1 to 4 hours post-exercise and D4
674 (follow up sample on day 4), for PLA (filled triangles), IF (open circles) and DF (open
675 squares). Data are mean ± 1SD. ^a different ($P \leq 0.05$) from baseline (PLA) ^b different ($P \leq 0.05$)
676 from baseline (IF), ^c different ($P \leq 0.05$) from baseline (DF). * IF different ($P \leq 0.05$) from PLA,
677 ^a DF different ($P \leq 0.05$) from PLA, [•] IF different ($P \leq 0.05$) from DF.



688 **Figure 4.** The percentage change in baseline concentrations of albumin at Rest (Baseline),
 689 Exh (at exhaustion), 1 to 4 hours post-exercise and D4 (follow up sample on day 4), for PLA
 690 (filled triangles), IF (open circles) and DF (open squares). Data are mean \pm 1SD. [†]overall
 691 mean concentrations different from baseline ($P \leq 0.05$).