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## **Beetroot Juice Does Not Enhance Altitude Running Performance in Well-Trained Athletes**

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1    **TITLE**

2    Beetroot Juice Does Not Enhance Altitude Running Performance in Well-Trained Athletes

3

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22

23    **RUNNING TITLE**

24    Beetroot juice and performance at altitude.

25 **ABSTRACT**

26 We hypothesized that acute dietary nitrate ( $\text{NO}_3^-$ ) provided as concentrated beetroot juice  
27 supplement would improve endurance running performance of well-trained runners in  
28 normobaric hypoxia. Ten male runners (mean (SD): sea level  $\dot{\text{V}}\text{O}_{2\text{max}}$  66 (7)  $\text{mL}\text{kg}^{-1}\cdot\text{min}^{-1}$ , 10  
29 km personal best 36 (2) min) completed incremental exercise to exhaustion at 4000 m and a 10  
30 km treadmill time trial at 2500 m simulated altitude on separate days, after supplementation  
31 with ~7 mmol  $\text{NO}_3^-$  and a placebo, 2.5 h before exercise. Oxygen cost, arterial oxygen  
32 saturation, heart rate and ratings of perceived exertion (RPE) were determined during the  
33 incremental exercise test. Differences between treatments were determined using means [95%  
34 confidence intervals], paired sample t-tests and a probability of individual response analysis.  
35  $\text{NO}_3^-$  supplementation increased plasma [nitrite] ( $\text{NO}_3^-$ , 473 (226) nM vs. placebo, 61 (37) nM,  
36  $P < 0.001$ ) but did not alter time to exhaustion during the incremental test ( $\text{NO}_3^-$ , 402 (80) s vs.  
37 placebo 393 (62) s,  $P = 0.5$ ) or time to complete the 10 km time trial ( $\text{NO}_3^-$ , 2862 (233) s vs.  
38 placebo, 2874 (265) s,  $P = 0.6$ ). Further, no practically meaningful beneficial effect on time  
39 trial performance was observed as the 11 [-60 to 38] s improvement was less than the *a priori*  
40 determined minimum important difference (51 s), and only three runners experienced a ‘likely,  
41 probable’ performance improvement.  $\text{NO}_3^-$  also did not alter oxygen cost, arterial oxygen  
42 saturation, heart rate or RPE. Acute dietary  $\text{NO}_3^-$  supplementation did not consistently enhance  
43 running performance of well-trained athletes in normobaric hypoxia.

44

45 **KEY WORDS:**

46 Nitrate, nitrite, nitric oxide, exercise, hypoxia.

47 **INTRODUCTION**

48 Exposure to altitude has a profound negative effect on exercise performance because reduced  
49 partial pressure of ambient oxygen causes arterial oxygen desaturation, tissue hypoxia and  
50 disturbed muscle metabolism (Modin et al. 2001). Increasing dietary nitrate via beetroot  
51 supplementation ( $\text{NO}_3^-$ ) is an increasingly popular strategy to improve exercise capacity at sea  
52 level (Hoon et al. 2013). As conjectured by previous publication,  $\text{NO}_3^-$  supplementation may  
53 be particularly effective at altitude due to its ‘oxygen sparing effect’ whereby whole body  
54 oxygen utilisation is reduced during submaximal exercise (Larsen et al. 2007; Bailey et al.  
55 2009; Vanhatalo et al. 2010; Lansley et al. 2011). The mechanism by which  $\text{NO}_3^-$   
56 supplementation has this effect is not completely understood but is likely related to increased  
57 plasma nitrite ( $\text{NO}_2^-$ ) concentration and nitric oxide (NO) production.

58

59 As a physiological signalling molecule, NO plays a key role in the regulation of blood flow,  
60 mitochondrial respiration and biogenesis, muscle contractility, and glucose and calcium  
61 homeostasis (Stamler and Meissner 2001). New evidence also suggests a high NO  
62 bioavailability is characteristic of successful adaptation to altitude (Levett 2011). This notion  
63 is supported by increased concentrations of expired NO and plasma  $\text{NO}_2^-$  observed in Tibetan  
64 highlanders (Beall et al. 2001; Erzurum et al. 2007). Theoretically, compared with sea level,  
65 dietary  $\text{NO}_3^-$  supplementation at altitude may be more beneficial as the reduction process of  
66  $\text{NO}_3^-$  to NO is enhanced under acidic (Modin et al. 2001) and hypoxic conditions (Castello et  
67 al. 2006), whereas the endogenous L-arginine NO synthase (oxygen dependent) pathway is  
68 suppressed.

69

70 Studies investigating the effects of dietary  $\text{NO}_3^-$  supplementation on exercise at high altitude or  
71 normobaric hypoxia are limited, but in general support beneficial effects (Vanhatalo et al. 2011;

72 Masschelein et al. 2012; Muggeridge et al. 2014). Specifically, Masschelein and colleagues  
73 showed partial restoration of oxygen delivery and utilisation in hypoxia, reporting  
74 increased arterial and muscle oxygenation during exercise after  $\text{NO}_3^-$  ingestion compared with  
75 a placebo. Additionally, this and another study (Vanhatalo et al. 2011) demonstrated that dietary  
76  $\text{NO}_3^-$  supplementation improves exercise capacity in hypoxia, as time-to-exhaustion on an  
77 incremental cycling test (Masschelein et al. 2012) and leg extension exercise (Vanhatalo et al.  
78 2011) were longer after  $\text{NO}_3^-$  was ingested. Unfortunately, these studies only demonstrated  
79 statistically significant differences between  $\text{NO}_3^-$  consumption compared with a placebo, of  
80 which the practical performance benefit remained unclear. To address this issue, Muggeridge  
81 and colleagues (2014) investigated the benefit of  $\text{NO}_3^-$  ingestion on trained cyclists during a 16  
82 km time trial at 2500m, and found both a statistically significant and practically meaningful  
83 (2.2%) improvement in performance time. Although these results draw attention to the potential  
84 endurance performance benefits of  $\text{NO}_3^-$  supplementation, there is a requirement for further  
85 studies that investigate acute supplementation protocols, in well-trained athletes, using  
86 practically relevant outcome measures, to determine if  $\text{NO}_3^-$  supplementation can enhance  
87 athletic performance such as endurance running capacity in hypoxia (Hoon et al. 2013; Jones  
88 2013). In addition, anecdotal reports obtained from national level altitude training camps  
89 indicate the possibility of responders and non-responders to ergogenic supplements including  
90  $\text{NO}_3^-$ , but scientific evidence is lacking to support this observation.

91  
92 With an increasing number of athletic camps and competitive running events now held at  
93 altitude each year, well trained runners are increasingly utilising  $\text{NO}_3^-$  supplements despite  
94 minimal evidence of their ergogenic effect. The current investigation therefore aimed to assess  
95 the influence of acute  $\text{NO}_3^-$  ingestion, via beetroot juice, upon endurance running performance  
96 and exercise tolerance at moderate altitude, in a well-trained population. It was hypothesised

97 that compared to a placebo, acute ingestion of a commercially available high-nitrate beetroot  
98 juice shot (~7 mmol NO<sub>3</sub><sup>-</sup>) would statistically (beyond chance) and practically (greater than the  
99 minimum important difference in the majority of participants) enhance exercise performance  
100 in normobaric hypoxia.

101

## 102 METHODS

### 103 *Participants*

104 Ten well-trained competitive male runners (mean (SD): age 37 (13) years, height 1.78 (0.06)  
105 m, body mass 72 (7) kg, sea level  $\dot{V}O_{2\text{max}}$  66 (7) mL kg<sup>-1</sup> min<sup>-1</sup>, 10 km personal best time 36  
106 (2) min) were recruited using opportunistic sampling methods from local running clubs between  
107 January and March 2013. Inclusion criteria detailed: a sub-40 min 10 km run time in the  
108 previous 12 months, non-smoking, and no exposure to altitude greater than 1500 m in the  
109 previous six months. All participants provided written informed consent. Ethical approval was  
110 granted by the Ethics Committee of the School of Sport, Health and Exercise Sciences at  
111 Bangor University (reference ID; MSc03-12/13), and the study was registered on  
112 www.clinicaltrials.gov (reference ID: NCT01795534).

113

### 114 *Design*

115 Participants visited the laboratory on six occasions (**Figure 1**). The first and second visits were  
116 used to familiarise participants with the experimental exercise tests, which involved completion  
117 of a 10 km treadmill time trial at a simulated 2500 m (FiO<sub>2</sub>, 15.4%) and an incremental exercise  
118 test to exhaustion at sea level. This incremental exercise test was also used to determine  
119 maximal oxygen uptake ( $\dot{V}O_{2\text{max}}$ ) at sea level. The study then used a double-blind repeated  
120 measures crossover design where participants received either acute beetroot juice ingestion  
121 (NO<sub>3</sub><sup>-</sup>) or placebo ingestion (PLAC) in a random order. The randomisation was completed by

122 JHM using www.randomization.com. A minimum four-day wash out was used between  
123 supplementations to ensure circulating  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations returned to basal levels  
124 (Wylie et al. 2013). During each supplementation period participants visited the laboratories on  
125 two occasions. The first visit consisted of an incremental exercise test to exhaustion on a  
126 treadmill at a simulated 4000 m ( $\text{FiO}_2$ , 12.8%). This relatively high altitude was chosen to  
127 maximise hypoxemia and thus potentiate any physiological effects of  $\text{NO}_3^-$  supplementation  
128 (enhanced production of NO via exogenous  $\text{NO}_3^-$  reduction occurs in hypoxic conditions  
129 (Castello et al. 2006)). The second visit consisted of a 10 km treadmill time trial at a simulated  
130 2500 m ( $\text{FiO}_2$ , 15.4%), which directly tested moderate altitude endurance performance as  
131 required for events such as the Trans Alps Run, Tour de France, Pikes Peak Marathon and  
132 training camps (Wilber 2004).

133

134 \*PLEASE INSERT FIGURE 1 NEAR HERE\*

135

136 ***Supplementation***

137 Supplementation consisted of either a single 70 mL concentrated shot of beetroot juice (~7  
138 mmol  $\text{NO}_3^-$ , Beet It Sport<sup>TM</sup>, James White Drinks Ltd, Ipswich, UK) or a  $\text{NO}_3^-$  depleted placebo  
139 shot that was identical in appearance, taste and texture (~0.003 mmol  $\text{NO}_3^-$ , James White Drinks  
140 Ltd, Ipswich, UK). Placebo shots were created by passing the  $\text{NO}_3^-$  active beetroot juice through  
141 a Purolite A520E  $\text{NO}_3^-$  selective ion exchange resin before pasteurisation (Lansley et al. 2011).  
142 Supplements were ingested under experimenter supervision 2 h before visits three to six, which  
143 was 2.5 h before each exercise test. Shots were packaged in identical coded containers by James  
144 White Drinks and were distributed by JHM to participants, ensuring blinding of participants  
145 and observers (JTA, TLJ, SJO). To ensure that the placebo had been theoretically effective, a

146 manipulation check was conducted after each visit, asking participants to guess what  
147 intervention ( $\text{NO}_3^-$  or placebo) they had received.

148

149 ***Procedures***

150 One week before testing, participants were fully briefed with regards to the study aims and  
151 design. A list of high  $\text{NO}_3^-$  foodstuffs to avoid throughout the study was presented to each  
152 participant in an attempt to isolate supplemented  $\text{NO}_3^-$  as a cause of any potential effect.  
153 Participants were asked to not increase or decrease training load throughout the study.  
154 Furthermore, twenty four hours before the first familiarisation session, each participant was  
155 asked to produce a diet and activity diary and to repeat these recorded behaviours in the twenty  
156 four hours prior to all trials. Participants were also allocated drinking water equal to  $35 \text{ mL}\cdot\text{kg}^{-1}$   
157 <sup>1</sup> of body mass to be consumed in the 24 hours prior to each visit. Participants were asked to  
158 abstain from the use of any chewing gum or antibacterial mouthwashes as this has previously  
159 shown to lessen the reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  by commensal bacteria within the oral cavity  
160 (Govoni et al. 2008). These actions were then repeated for subsequent visits.

161

162 Each participant completed all exercise tests at the same time of day. At the start of each visit  
163 body mass was measured and urine and capillary blood samples were obtained to ensure runners  
164 were euhydrated (urine specific gravity less than 1.020, refractometer Atago, Japan (Oppliger  
165 et al. 2005)) and had normal hemoglobin (greater than  $13.5 \text{ g}\cdot\text{dL}^{-1}$ , Hemocue Ltd, Derbyshire,  
166 UK). After, a resting venous blood sample was obtained by venepuncture into a lithium-heparin  
167 tube (Monovette Lithium Heparin, Sarstedt, Leicester, UK). This blood sample was placed in  
168 a centrifuge and spun at 4000 rpm at  $4^\circ\text{C}$  for 10 min within 3 min of collection. Immediately  
169 after the centrifugation, plasma was aspirated into eppendorfs and frozen at  $-80^\circ\text{C}$  for a  
170 standardised time period prior to subsequent analysis of NO availability ( $\text{NO}_2^-$  and  $\text{NO}_3^-$ )

171 concentration) as per Wylie et al. (2013). All subsequent data collection was conducted in a  
172 temperature and humidity controlled normobaric hypoxic environmental chamber (Hypoxico  
173 Inc., The Altitude Centre, London, UK, 20.0 (0.1) °C, 40 (3) %).

174

175 ***Incremental exercise test***

176 The chamber was set and maintained at a simulated altitude of 4000 m (ambient oxygen 12.9  
177 (0.1) %). Maximal oxygen uptake was assessed using a continuous incremental exercise test on  
178 a motorised treadmill (h/p/cosmos, Nussdorf, Germany) until volitional exhaustion. The test  
179 started at 10 km·h<sup>-1</sup> with a 0% gradient. Increments were subsequently achieved by increasing  
180 the treadmill speed by 1 km·h<sup>-1</sup> every minute until 16 km·h<sup>-1</sup>. Thereafter the gradient was  
181 increased by 1% every minute until volitional exhaustion. Following a period of active  
182 recovery, where the participant completed light exercise until their heart rate reduced to less  
183 than 100 bpm,  $\dot{V}\text{O}_{2\text{max}}$  was verified by runners returning to the treadmill to complete exercise  
184 at an intensity greater than at exhaustion (i.e. 1% greater gradient). Oxygen consumption was  
185 recorded continuously throughout exercise by a metabolic cart (Metalyser, Cortex, Leipzig,  
186 Germany) with  $\dot{V}\text{O}_{2\text{max}}$  determined as the highest 30 s average at any given time point.  
187 Additionally heart rate by remote transmitter (FT3, Polar, Kempele, Finland), blood oxygen  
188 saturation by fingertip pulse oximeter (7500, Nonin Medical Inc., Minnesota, USA) and overall  
189 rating of perceived exertion (RPE) by Borg CR100 scale (Borg and Borg 2001), were recorded  
190 during the final 15 s of each incremental stage. At exhaustion, blood lactate was also measured  
191 via ear lobe capillary sampling and a portable analyser (Lactate Pro, Ark Ray Inc, Kyoto,  
192 Japan).

193

194

195

196    ***Time Trial***

197    The chamber was set and maintained at a simulated altitude of 2500 m (ambient oxygen 15.4  
198    (0.1) %). After runners had completed a standardised warm up of 3 min at 10 km·h<sup>-1</sup> they  
199    completed a 10 km time trial on a treadmill. Treadmill gradient was set to 1% to better replicate  
200    the physiological demands of outside running (Jones and Doust 1996). Runners were instructed  
201    to complete the distance as quickly as possible. During the time trial runners were blinded to  
202    the elapsed time and speed of the treadmill. Verbal prompts at kilometre intervals were provided  
203    to replicate distance markers during running race competitions. Runners self-selected their  
204    running speed throughout the time trial. Differentiated RPE (legs, chest and overall) was  
205    recorded at the completion of each time trial kilometre to assess trends in pacing. The reliability  
206    of this 10 km time trial protocol at 2500 m simulated altitude was assessed in six similarly  
207    trained runners to be 3.9 (1.0) % (within subjects coefficient of variation), across three time  
208    trials each separated by seven days. The within subjects coefficient of variation of the second  
209    and third time trial alone was assessed to be 2.1 (1.4) %.

210

211    ***Data Analysis***

212    The primary outcome measure was time to complete the 10 km treadmill time trial. All data  
213    extraction was completed whilst experimenters were blinded; only statistical analyses were  
214    completed un-blinded. Data are presented as means (SD) or [95% confidence interval].  
215    Inferential statistical analysis was conducted using the software package SPSS (version 20,  
216    IBM, Portsmouth, UK). Statistical significance was set at  $P \leq 0.05$ . To evaluate the statistical  
217    significance of NO<sub>3</sub><sup>-</sup> supplementation, paired samples *t*-tests were used to assess differences  
218    between NO<sub>3</sub><sup>-</sup> and placebo trials. Magnitude of difference between treatments was calculated  
219    as NO<sub>3</sub><sup>-</sup> minus placebo trial and for the primary outcome measure compared to a minimal  
220    practical important difference determined as 51 s (Cohen's smallest important effect: 0.2 ×

221 between subject SD, confirmed by discussion with expert coaches, and equivalent to 1.8%). A  
222 probability analysis was also undertaken on the primary outcome measure, estimating the  
223 likelihood of a true positive response to  $\text{NO}_3^-$  supplementation (Hopkins 2000). Specifically,  
224 using calculations on precision of change provided by Hopkins (2000), for each runner the  
225 difference between  $\text{NO}_3^-$  and placebo trials was assigned one of the following verbal descriptors  
226 to describe if  $\text{NO}_3^-$  supplementation had a positive effect on their individual time trial  
227 performance: ‘almost certainly not’; ‘very unlikely’; ‘unlikely, probably not’; ‘possibly may’;  
228 ‘likely probable’; ‘very likely’; ‘almost certainly’. Data from the incremental test (i.e.  
229 physiological parameters such as oxygen uptake) were presented and analysed at maximal  
230 exercise capacity (100% altitude specific  $\dot{\text{V}}\text{O}_{2\text{max}}$ ), and at a submaximal workload (45%  
231 altitude specific  $\dot{\text{V}}\text{O}_{2\text{max}}$ ). In order to investigate  $\text{NO}_2^-$  response, baseline plasma  $\text{NO}_2^-$   
232 concentrations and also the difference between  $\text{NO}_3^-$  and placebo trials’ plasma  $\text{NO}_2^-$   
233 concentrations were correlated (Pearson’s  $r$ ) against the difference between  $\text{NO}_3^-$  and placebo  
234 trials for all outcome measures. Finally, *post hoc* independent t-tests were completed to explore  
235 if baseline characteristics (age, body mass,  $\dot{\text{V}}\text{O}_{2\text{max}}$ , haemoglobin), plasma  $\text{NO}_2^-$  responses  
236 (plasma  $\text{NO}_2^-$  concentrations on the placebo trial and difference between  $\text{NO}_3^-$  and placebo  
237 trials’ plasma  $\text{NO}_2^-$  concentrations) or hypoxia responses (average arterial oxygen saturation  
238 on the placebo trial) may explain why some individuals improved time trial performance after  
239  $\text{NO}_3^-$  supplementation.

240

241 For the primary outcome, sample size estimation was completed using both statistical  
242 significance and magnitude based inference methods (Hopkins 2006). Data on expected  
243 reliability of the 10 km time trial between two trials after a familiarisation trial was obtained  
244 from a pilot study on six well trained athletes: the Pearson’s correlation coefficient was 0.98,  
245 the between subject SD was 255 s, and the typical error was 33 s. The minimum practical

246 important difference was therefore set at 51 s. Using the magnitude based inference method  
247 and maximum chances of Type I and Type II clinical errors of 0.5 and 25% respectively, six  
248 participants were estimated as required to detect a difference in means in a post-only  
249 crossover trial. Using the statistical significance method and maximum rates of Type I and  
250 Type II statistical errors of 5 and 20%, respectively, nine participants were required.

251

## 252 **RESULTS**

253 The NO<sub>3</sub><sup>-</sup> and placebo shots effectively altered the independent variable: 2.5 h after NO<sub>3</sub><sup>-</sup>  
254 consumption plasma [NO<sub>3</sub><sup>-</sup>] and [NO<sub>2</sub><sup>-</sup>] were significantly greater than after placebo ([NO<sub>3</sub><sup>-</sup>] in  
255 the NO<sub>3</sub><sup>-</sup> trial, 201.6 (25.9) µM vs. placebo trial, 28.9 (6.4) µM,  $P < 0.001$ ; [NO<sub>2</sub><sup>-</sup>] in the NO<sub>3</sub><sup>-</sup>  
256 trial, 473 (226) nM vs. placebo trial, 61 (37) nM,  $P < 0.001$ ). The runners were considered to  
257 be sufficiently well blinded as to which supplement they received on each visit, as the  
258 manipulation check indicated that only two participants of ten guessed correctly, two guessed  
259 incorrectly, and six were unable to distinguish between the supplements at all.

260

### 261 ***Incremental exercise test***

262 Acute NO<sub>3</sub><sup>-</sup> supplementation did not alter any measured physiological variable or RPE during  
263 maximal or submaximal exercise at 4000 m (**Table 1**). No statistical difference was present in  
264 any parameter obtained at 100% or 45% of  $\dot{V}O_{2\text{max}}$ . There was also no practical performance  
265 difference in time to exhaustion between trials (NO<sub>3</sub><sup>-</sup> – placebo:  $\Delta 1.4\%$ ). No correlations were  
266 observed between baseline plasma [NO<sub>2</sub><sup>-</sup>] or the change in plasma [NO<sub>2</sub><sup>-</sup>] with any maximal  
267 exercise parameter.

268

269 \*PLEASE INSERT TABLE 1 NEAR HERE\*

270

271 **Time Trial**

272 Acute  $\text{NO}_3^-$  supplementation did not improve 10 km running performance at simulated altitude  
273 (2500 m). No statistical difference was observed in time to complete the 10 km time trial ( $\text{NO}_3^-$   
274 , 2862 (233) s vs. placebo, 2874 (265) s,  $P = 0.6$ ). Additionally, compared to the *a priori*  
275 determined minimum practical important difference of -51 s (1.8%) there was also no practical  
276 difference in performance ( $\text{NO}_3^-$  – placebo:  $\Delta$  -11 [-60 to 38] s or  $\Delta$  0.4%: **Figure 2**). Trends in  
277 RPE during the time trial were visually explored but no difference was observed between  $\text{NO}_3^-$   
278 and placebo.

279

280 \*PLEASE INSERT FIGURE 2 NEAR HERE\*

281

282 Results obtained from the probability analysis suggested that three runners experienced a  
283 performance improvement with  $\text{NO}_3^-$  supplementation labelled ‘likely, probable’; one runner  
284 experienced impaired performance labelled ‘likely, probable’; and the remaining six runners  
285 exhibited no strong probability of either improved or impaired performance. Further, no  
286 correlation was observed between baseline  $[\text{NO}_2^-]$  or the change in plasma  $[\text{NO}_2^-]$  with change  
287 in time to complete the 10 km time trial ( $r < 0.48$ ,  $P > 0.1$ ).

288

289 Exploratory *post hoc* analyses suggested that runners who improved time trial performance  
290 responded to hypoxia with greater arterial desaturation, as indicated by lower arterial oxygen  
291 saturation during the placebo time trial (82 (2) vs. 84 (2),  $P = 0.04$ ). There was however no  
292 difference in baseline characteristics (age, body mass,  $\dot{V}\text{O}_{2\text{max}}$ , haemoglobin,  $P > 0.4$ ) or plasma  
293  $\text{NO}_2^-$  responses (plasma  $\text{NO}_2^-$  concentrations on the placebo trial and difference between  $\text{NO}_3^-$   
294 and placebo trials’ plasma  $\text{NO}_2^-$  concentrations,  $P > 0.6$ ) between those runners that did or did  
295 not improve time trial performance after  $\text{NO}_3^-$  supplementation.

296 **DISCUSSION**

297 The current study aimed to assess the influence of  $\text{NO}_3^-$  supplementation upon endurance  
298 running performance at altitude in well-trained runners. The principal finding contradicted the  
299 hypothesis: acute  $\text{NO}_3^-$  supplementation did not enhance endurance running performance in  
300 normobaric hypoxia. Specifically, no statistical or practical difference in 10 km time trial  
301 running performance was observed between  $\text{NO}_3^-$  and placebo trials, whilst probability analysis  
302 of individual responses suggested only three of ten participants had a “likely, probably”  
303 increase in performance. In addition, no significant differences were seen in any measured  
304 physiological or perceptual parameters or time to exhaustion during an incremental treadmill  
305 test in normobaric hypoxia. These findings contrast those of other investigations conducted in  
306 hypoxia that have suggested positive effects of  $\text{NO}_3^-$  supplementation on time to exhaustion  
307 (Vanhatalo et al. 2011; Masschelein et al. 2012) and time trial performance (Muggeridge et al.  
308 2014).

309  
310 It is unlikely that the acute nitrate dose of 7 mmol  $\text{NO}_3^-$  administered in the present study was  
311 simply insufficient to cause an effect. In a previous dose response study completed in normoxia,  
312 time to exhaustion was improved after acute  $\text{NO}_3^-$  supplementation equal to 8 mmol of dietary  
313  $\text{NO}_3^-$  (Wylie et al. 2013). The positive effects in hypoxia on exercise tolerance previously  
314 observed by Vanhatalo et al. (2011) and Masschelein et al. (2012) and on exercise performance  
315 by Muggeridge et al. (2014) were achieved with  $\text{NO}_3^-$  doses that ranged from smaller (5 mmol)  
316 to larger (9 mmol acutely and 5 mmol once daily for six days) doses than used in the present  
317 investigation. Considering that suppression of the endogenous L-arginine NO synthase (oxygen  
318 dependent) pathway occurs in hypoxia (Castello et al. 2006), suggesting a greater reliance on  
319 reduction of  $\text{NO}_3^-$  to NO (potentially reducing the required dose to have a physiological effect),

320 the non-significant finding following dietary supplementation of  $\text{NO}_3^-$  in the present study  
321 remains surprising.

322

323 Theoretically the negative finding of the current investigation may be explained by the well-  
324 trained status of the participants recruited (Hoon et al. 2013). Sea level studies have shown that  
325 the beneficial effects of  $\text{NO}_3^-$  supplementation on exercise performance may be reduced in well-  
326 trained athletes (Wilkerson et al. 2012), and thus well trained athletes may require longer  
327 periods of supplementation to elicit an ergogenic effect (Cermak *et al.*, 2012*a*, 2012*b*). Well-  
328 trained athletes have greater resting plasma  $\text{NO}_3^-$  concentrations (Jungersten et al. 1997),  
329 greater presence of NO synthase (Green et al. 2004), and experience less severe localised  
330 hypoxia and acidosis in the muscle compared to untrained populations (Wilkerson et al. 2012).  
331 Such adaptations allow more NO to be derived from the endogenous NO synthase pathway,  
332 and place less reliance on  $\text{NO}_3^-$  supplementation as a means to maintain adequate NO  
333 concentrations. However we hypothesized that such adaptations in well-trained athletes would  
334 be outweighed by the deleterious effects of hypoxia, allowing a benefit to be observed from  
335 acute nitrate supplementation even in well-trained athletes. Unfortunately the current findings  
336 do not support this hypothesis. As comparison of training status of participants between studies  
337 completed in hypoxia is difficult (Masschelein et al. 2012; Muggeridge et al. 2014; Vanhatalo  
338 et al. 2011), and because completing correlational analyses between baseline fitness or baseline  
339 NO bioavailability and response to supplementation is problematic in homogenous groups such  
340 as recruited herein, an important future direction for research in this area is to investigate the  
341 moderating effect of training status in response to  $\text{NO}_3^-$  supplementation.

342

343 It is also possible that the effects of  $\text{NO}_3^-$  on exercise performance in hypoxia may in part be  
344 dependent upon exercise mode, duration and intensity. Some previous investigations have

345 utilised exercise protocols that are arguably less ecologically valid, over-estimating ergogenic  
346 effects of any intervention (Masschelein et al. 2012; Vanhatalo et al. 2011). In fact even within  
347 sea level studies that have specifically assessed performance through practically relevant time  
348 trial testing, the results of  $\text{NO}_3^-$  supplementation remain mixed (Hoon et al. 2013). Perhaps of  
349 greatest relevance is the study by Muggeridge and colleagues (2014) that utilised a cycling time  
350 trial in hypoxia, which revealed positive effects of  $\text{NO}_3^-$  supplementation. Of interest, the  
351 utilised time trial was noticeably shorter in duration than the test used in the current study (28  
352 vs. 48 min). Possibly the effect size of  $\text{NO}_3^-$  supplementation is reduced in longer duration  
353 activities (Wilkerson et al. 2012). The mechanism remains unknown, but during shorter  
354 duration exercise more type II muscle fibres are recruited, and recent findings suggest the  
355 effects of  $\text{NO}_3^-$  are perhaps preferential to type II fibres (Hernandez et al. 2012; Ferguson et al.  
356 2013).

357

358 Whilst these mechanistic explanations are speculative, detailed analysis within the present  
359 study of individual responses clearly show that the performance benefit of  $\text{NO}_3^-$   
360 supplementation is very variable. A probability analysis addressing the true likelihood of  
361 individual responses to  $\text{NO}_3^-$  supplementation suggested that three participants experienced a  
362 ‘likely/probable’ improvement in performance when supplemented with  $\text{NO}_3^-$ , one participant  
363 experienced a ‘likely/probable’ decrease in performance, whilst the remaining participants had  
364 no strong probability of either enhanced or impaired performance. The reason for the improved  
365 performance in some but not all individuals is of particular interest. A placebo effect can be  
366 excluded as all three participants with improved performance could not differentiate which  
367 supplement they were taking before each time trial. Exploratory *post hoc* analysis suggested  
368 that  $\text{NO}_3^-$  supplementation improved time trial performance in those runners that had the  
369 greatest arterial desaturation in hypoxia. As this exploratory *post hoc* analysis was completed

370 in small numbers, future studies are required to confirm whether individual susceptibility to  
371 hypoxia moderates performance benefits of NO<sub>3</sub><sup>-</sup> supplementation. Future studies are also  
372 required to provide sufficient data for meta-analyses, before NO<sub>3</sub><sup>-</sup> can be accepted as an  
373 ergogenic aid in hypoxia.

374

375 Criticisms of the current work include the use of well-trained athletes. Difficulties surrounding  
376 physiological testing of trained populations include other training and competition  
377 commitments. In order to control for such variables, athletes were encouraged to maintain  
378 consistent training load during the study; however compliance was only confirmed by  
379 inspection of training diaries. Nevertheless the consistency in which these athletes were able to  
380 complete the 10 km time trial, as shown by the acceptable reliability results, suggests that any  
381 effect of other training or competition exercise was minimal on the time trial results of this  
382 study. The acute exposure to hypoxia may be considered another limitation, as the influence of  
383 nitrate supplementation on exercise during longer exposures to hypoxia is unknown. However,  
384 as many athletes do not have adequate time to acclimatize to altitude before training or  
385 competition, the moderate altitude used for the time trial (2500 m) is typical of that experienced  
386 by athletes.

387

### 388 ***Conclusion***

389 This investigation was unable to provide evidence for either a statistically significant or  
390 practically beneficial effect of acute NO<sub>3</sub><sup>-</sup> supplementation on 10km running performance or  
391 exercise tolerance in a maximal incremental test (both completed in normobaric hypoxia).  
392 These results contradict previous studies, most likely due to the inter-individual response to  
393 acute dietary NO<sub>3</sub><sup>-</sup> supplementation that was observed in the present investigation. Further  
394 investigation of the mechanistic reasons for inter-individual responses to supplementation is

395 thus required before  $\text{NO}_3^-$  supplementation can be accepted as an effective ergogenic aid in  
396 hypoxia.

397

398

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402

403   **CONFLICTS OF INTEREST AND SOURCES OF FUNDING**

404   The authors declare they have no conflicts of interests and the study did not receive funding  
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## TABLES

Table 1. Time to exhaustion and other psychophysiological responses at submaximal and maximal exercise intensities during an incremental treadmill exercise test at simulated altitude (4000 m) after acute dietary nitrate and placebo supplementation

	<b>NO<sub>3</sub><sup>-</sup></b>	<b>PLAC</b>	<b>NO<sub>3</sub><sup>-</sup> - PLAC</b>	<b>P</b>
Time to exhaustion (s)	402 ± 80	393 ± 62	9 [-20 to 38]	0.5
<b>45% <math>\dot{V}\text{O}_{2\text{max}}</math></b>				
Speed/Gradient (km·h <sup>-1</sup> / %)	12 (0) / 0 (0)	12 (0) / 0 (0)	-	-
$\dot{V}\text{O}_2$ (mL·kg <sup>-1</sup> ·min <sup>-1</sup> )	26 (2)	26 (2)	0 [-1 to 1]	0.7
SpO <sub>2</sub> (%)	78 (3)	77 (5)	1 [-5 to 3]	0.6
Heart rate (bpm)	136 (13)	134 (12)	1 [-9 to 6]	0.7
Rating of perceived exertion	24 (14)	25 (14)	-1 [-6 to 7]	0.8
<b>100% <math>\dot{V}\text{O}_{2\text{max}}</math></b>				
Speed/Gradient (km·h <sup>-1</sup> / %)	16 (0) / 1 (1)	16 (0) / 1 (1)	-	-
$\dot{V}\text{O}_2$ (mL·kg <sup>-1</sup> ·min <sup>-1</sup> )	48 (4)	48 (5)	0 [-2 to 1]	0.8
SpO <sub>2</sub> (%)	74 (3)	74 (4)	1 [-2 to 3]	0.7
Heart rate (bpm)	155 (12)	158 (26)	-3 [-19 to 12]	0.7
Rating of perceived exertion	79 (27)	79 (30)	0 [-6 to 6]	1.0
[Blood lactate] (mmol·L <sup>-1</sup> )	8.8 (2.0)	8.3 (3.0)	0.5 [-1.2 to 2.1]	0.6

Data are mean (SD) or mean difference [95% confidence interval]; significance determined by paired samples t-test (n = 10); NO<sub>3</sub><sup>-</sup>, 70ml dietary nitrate (beetroot juice) supplementation; PLAC, placebo supplementation;  $\dot{V}\text{O}_{2\text{max}}$ , maximal oxygen uptake at 4000 m; SpO<sub>2</sub>, arterial oxygen saturation; whole-body rating of perceived exertion by Borg CR100 scale.

## FIGURE CAPTIONS

Figure 1: Schematic representation of research design

*n*, number of participants;  $\dot{V}O_{2\text{max}}$ , maximal oxygen uptake incremental exercise test; TT, 10 km time trial.

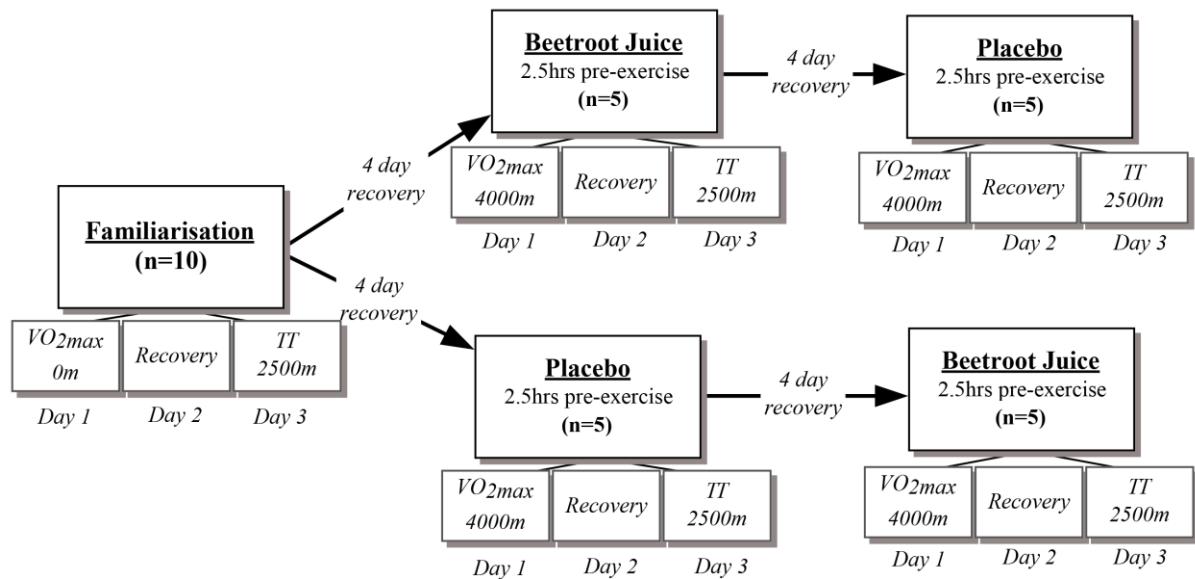


Figure 2: Difference in performance during a simulated altitude (2500 m) 10 km time trial after acute dietary nitrate and placebo supplementation.

$\text{NO}_3^-$ , 70 ml dietary nitrate (beetroot juice) supplementation; PLAC, placebo supplementation; horizontal lines = mean response [95% confidence interval]; dots = individual runner responses. The negative values indicate runners that completed the time trial sooner when supplemented with dietary nitrate than placebo

