

1 **Dietary nitrate supplementation attenuates the reduction in**  
2 **exercise tolerance following blood donation**

3

4 *Original Article*

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13 **Running head:** Nitrate, blood donation and exercise performance

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29 **ABSTRACT**

30 We tested the hypothesis that dietary nitrate-rich beetroot juice (BR) supplementation could  
31 partially offset deteriorations in O<sub>2</sub> transport and utilization, and exercise tolerance, after  
32 blood donation. Twenty-two healthy volunteers performed moderate-intensity and ramp  
33 incremental cycle exercise tests prior to and following the withdrawal of ~450 mL of whole  
34 blood. Before donation, all subjects consumed 7 x 70 mL of nitrate-depleted beetroot juice  
35 shots (PL) in the 48 h preceding the exercise tests. During the 48 h after blood donation,  
36 subjects consumed 7 shots of either BR (each containing 6.2 mmol nitrate; *n*=11) or PL  
37 (*n*=11) before repeating the exercise tests. [Hemoglobin] and hematocrit were reduced by ~8-  
38 9% following blood donation (*P*<0.05), with no difference between the BR and PL groups.  
39 When compared with pre-donation, steady-state  $\dot{V}O_2$  during moderate-intensity exercise was  
40 ~4% lower post-donation in BR (*P*<0.05) but was unchanged in PL. The ramp test peak  
41 power decreased from pre-donation (PL: 341 ± 70 vs. BR: 331 ± 68 W) to post-donation (PL:  
42 324 ± 69 vs. BR: 322 ± 66 W) in both groups (*P*<0.05). However, the decrement in  
43 performance was significantly less in BR (2.7%) compared with PL (5.0%; *P*<0.05). Nitrate  
44 supplementation reduced the O<sub>2</sub> cost of moderate-intensity exercise and attenuated the  
45 decline in ramp incremental exercise performance following blood donation. These results  
46 have implications for improving functional capacity following blood loss.

47

48 **New and Noteworthy:** Dietary nitrate supplementation with beetroot juice lowered the O<sub>2</sub>  
49 cost of moderate-intensity exercise, better preserved muscle oxygenation and attenuated the  
50 decline in incremental exercise test performance following donation of 450 mL whole blood.  
51 These results have implications for improving functional capacity following blood loss.

52

53 **Key words:** blood withdrawal; beetroot juice; O<sub>2</sub> transport; O<sub>2</sub> uptake; exercise performance;  
54 nitric oxide

## 55 INTRODUCTION

56 The peak rate of pulmonary oxygen uptake ( $\dot{V}O_{2\text{peak}}$ ) is an important determinant of exercise  
57 capacity and is influenced by the interaction of several central and peripheral factors (6, 53,  
58 64).  $\dot{V}O_{2\text{peak}}$  and exercise performance can be altered by manipulating the capability of the  
59 cardiovascular system to transport O<sub>2</sub> to contracting skeletal muscles during exercise (5, 11,  
60 18, 51, 57, 67). For example, interventions involving the infusion of erythrocytes (18, 19) or  
61 the stimulation of erythropoiesis (57, 67) to enhance hemoglobin concentration ([Hb]),  
62 increase  $\dot{V}O_{2\text{peak}}$  during maximal exercise. Conversely, limiting O<sub>2</sub> transport to working  
63 muscle by reducing [Hb] via whole blood withdrawal consistently results in a lowered  
64  $\dot{V}O_{2\text{peak}}$  (11, 18, 47, 54). During sub-maximal exercise, however, Panebianco et al. (47)  
65 reported no change in  $\dot{V}O_2$  at two and seven days post 450 mL blood donation, despite  
66 significant reductions in [Hb]. Compensatory adjustments in cardiovascular control, such as  
67 increases in heart rate (HR) and cardiac output ( $\dot{Q}$ ), offset the lower [Hb] and enable muscle  
68 O<sub>2</sub> delivery to be maintained during low-intensity exercise after blood donation (19, 27, 51).

69

70 The gaseous physiological signaling molecule, nitric oxide (NO), plays a key role in the  
71 regulation of vascular tone. NO can be synthesised via the oxidation of L-arginine in a  
72 reaction catalysed by the NO synthases (NOS; 32) or it can be produced via the reduction of  
73 nitrate (NO<sub>3</sub><sup>-</sup>) to nitrite (NO<sub>2</sub><sup>-</sup>) and subsequently NO (8). Recently, dietary NO<sub>3</sub><sup>-</sup>  
74 supplementation has been employed to augment plasma [NO<sub>2</sub><sup>-</sup>] and the potential for O<sub>2</sub>-  
75 independent NO synthesis (4, 38, 65). This NO<sub>3</sub><sup>-</sup>-NO<sub>2</sub><sup>-</sup>-NO pathway may be particularly  
76 important when NOS activity is compromised (20, 42), O<sub>2</sub> availability is limited (14, 25, 34,

77 35) and pH is low (44). Limitations in systemic O<sub>2</sub> transport can result in tissue hypoxia and  
78 greater metabolic perturbation (41, 60), which can contribute to reduced exercise tolerance  
79 (1), as is commonly observed at altitude (2) and in a number of disease states (35, 68). There  
80 is evidence to suggest that NO and NO<sub>2</sub><sup>-</sup> can combat an insufficient muscle O<sub>2</sub> supply by  
81 increasing muscle blood flow via hypoxia-induced vasodilatation (13, 61). Therefore, it is  
82 possible that dietary NO<sub>3</sub><sup>-</sup> supplementation could ameliorate deteriorations in exercise  
83 performance when 'normal' O<sub>2</sub> availability is reduced, during for example, high-intensity  
84 exercise, in hypobaric hypoxia or after blood donation.

85  
86 We and others have reported that, in healthy subjects, dietary NO<sub>3</sub><sup>-</sup> supplementation can  
87 significantly impact the physiological responses to exercise (4, 15, 38, 59). Specifically, a  
88 reduction in the O<sub>2</sub> cost of moderate-intensity exercise has been reported after  
89 supplementation with both sodium NO<sub>3</sub><sup>-</sup> (38, 39, 40) and NO<sub>3</sub><sup>-</sup>-rich beetroot juice (BR; 3, 4,  
90 15, 59, 69). In addition, a significantly increased time to task failure (TTF), indicating  
91 improved exercise tolerance, has been reported following BR ingestion when recreationally-  
92 active, but not highly trained, subjects completed severe-intensity (3, 4, 37) and ramp  
93 incremental exercise (59). These alterations may be due to a NO<sub>2</sub><sup>-</sup> or NO-related reduction in  
94 the ATP cost of muscle contraction (3), greater mitochondrial efficiency (40), changes in  
95 muscle redox status (66), and/or enhanced muscle blood flow, particularly to type II fibres  
96 (21, 22). Such changes could be particularly advantageous after whole blood withdrawal  
97 when [Hb] is reduced and O<sub>2</sub> transport is challenged (11, 18, 54). Indeed, BR  
98 supplementation has been shown to reduce muscle metabolic perturbation during exercise in  
99 normobaric hypoxia and to restore exercise tolerance and oxidative function to the values  
100 observed in normoxia (60, 61). In addition, it has been reported that, when the fraction of  
101 inspired O<sub>2</sub> is lowered to 11-13%, BR supplementation can improve muscle oxygenation

102 status (43), reduce  $\dot{V}O_2$  during sub-maximal exercise (34, 46), and enhance TTF during  
103 incremental exercise (43). BR supplementation has also been reported to increase arterial  $O_2$   
104 saturation following dynamic apnea (i.e., breath-hold diving), which supports an  $O_2$  sparing  
105 effect of  $NO_3^-$  ingestion (48). Collectively, these studies suggest that  $NO_3^-$  ingestion may  
106 enhance the physiological response to exercise when  $O_2$  availability is limited, by sparing  
107 muscle  $O_2$  demand and/or better preserving muscle  $O_2$  supply. However, it is not known  
108 whether the reductions in  $O_2$  carrying capacity and exercise performance subsequent to the  
109 withdrawal of whole blood can be offset by BR supplementation. If so, this may have  
110 important implications for clinical conditions in which [Hb] is lowered, for example in  
111 anemia, following surgery or involuntary blood loss, or in athletes wishing to donate blood  
112 without compromising training.

113

114 The purpose of the present study was to determine whether 48 h of BR supplementation  
115 following 450 mL of whole blood withdrawal alters the physiological responses to sub-  
116 maximal and maximal intensity cycle exercise. It was hypothesized that BR supplementation  
117 would lower the  $O_2$  cost of moderate-intensity exercise, improve muscle oxygenation status,  
118 and attenuate the expected reduction in TTF during ramp incremental exercise following  
119 blood donation.

120

## 121 **METHODS**

### 122 *Subjects*

123 Twenty-two recreationally active and pre-registered National Health Service (NHS) blood  
124 donors (males,  $n = 14$ ; females,  $n = 8$ ) volunteered to participate in this study, which was  
125 approved by the Institutional Research Ethics Committee and conformed to the ethical  
126 principles of the Declaration of Helsinki. None of the subjects were tobacco smokers or

127 habitual users of dietary supplements. All subjects provided written informed consent prior to  
128 the commencement of the study, after the experimental procedures, associated risks and  
129 potential benefits of participation had been explained.

130

131 Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least  
132 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each visit. In addition,  
133 subjects were asked to avoid alcohol consumption, chewing gum and antibacterial  
134 mouthwash throughout each supplementation period and to avoid caffeine intake in the 3 h  
135 preceding each laboratory visit. Each subject recorded habitual diet and exercise undertaken  
136 during the first supplementation period and were asked to replicate these habits during the  
137 second supplementation period. Prior to data collection, subjects were fully familiarized with  
138 the exercise testing procedures. This minimized any possible learning effects during the  
139 study. Exclusion criteria were the presence of known cardiovascular disease, hypertension  
140 and anemia, the use of antihypertensive medication and antibiotics, and having major surgery  
141 or giving blood within 6 months of the study commencing.

142

### 143 *Experimental Overview*

144 Subjects were asked to report to the laboratory on three separate occasions over a ten day  
145 period. The first visit included a 5 min bout of moderate-intensity cycle exercise at 80 W,  
146 followed by a ramp incremental test to task failure with no dietary supplementation. This  
147 served as the pre-intervention familiarization test. Hematocrit (Hct), [Hb], plasma [NO<sub>3</sub><sup>-</sup>] and  
148 [NO<sub>2</sub><sup>-</sup>], pulmonary  $\dot{V}O_2$  dynamics, muscle oxygenation status, HR, blood lactate  
149 concentration ([lactate]), blood glucose concentration ([glucose]) and TTF during ramp  
150 incremental exercise were measured during the first visit and repeated during each visit to the  
151 laboratory. Prior to visit 2, subjects consumed 7 shots of NO<sub>3</sub><sup>-</sup>-depleted beetroot juice (PL)

152 over ~48 h. On the final day of supplementation, subjects completed the same moderate-  
153 intensity exercise bout and ramp incremental test on a cycle ergometer as was performed at  
154 pre-intervention. Two days before the final visit to the lab, subjects attended a National  
155 Health Service (NHS) blood donation clinic. Each subject lay supine on a bed before ~450  
156 mL of whole blood was drawn from an antecubital vein over a 15 min period. The blood  
157 withdrawal was performed by the NHS as part of the national blood donation service.  
158 Following blood donation, each subject was randomly assigned, in a double-blind, placebo  
159 controlled fashion to consume 7 shots of either NO<sub>3</sub><sup>-</sup>-rich beetroot juice (BR;  $n = 11$ ; mean  $\pm$   
160 SD; females,  $n = 4$ : age  $23 \pm 3$  years, body mass  $67 \pm 4$  kg, height  $1.76 \pm 0.05$  m; males,  $n =$   
161  $7$ : age  $26 \pm 5$  years, body mass  $81 \pm 12$  kg, height  $1.80 \pm 0.10$  m) or NO<sub>3</sub><sup>-</sup>-depleted beetroot  
162 juice as a placebo (PL;  $n = 11$ ; mean  $\pm$  SD; females,  $n = 4$ : age  $22 \pm 3$  years, body mass  $77 \pm$   
163  $11$  kg, height  $1.75 \pm 0.10$  m; males,  $n = 7$ : age  $28 \pm 7$  years, body mass  $77 \pm 8$  kg, height  $1.79$   
164  $\pm 0.10$  m) over the next ~48 h. Visit 3 occurred on the final day of supplementation with the  
165 exercise tests conducted 2 h following final supplement ingestion. All tests were performed at  
166 the same time of day ( $\pm 2$  h) to minimise diurnal variation on the physiological variables  
167 under investigation.

168

#### 169 *Exercise tests*

170 During the first visit to the laboratory subjects performed a short bout of low-intensity  
171 exercise at 80 W, followed by a ramp incremental exercise test to task failure on an  
172 electrically-braked cycle ergometer (Lode Excalibur Sport, Gronigen, The Netherlands) for  
173 determination of  $\dot{V}O_{2peak}$  and gas exchange threshold (GET). The protocol began with 3 min  
174 of 'unloaded' baseline cycling at 20 W, followed by 5 min at 80 W and 10 min of passive  
175 rest. Subsequently, 3 min of baseline cycling at 20 W was performed and then the power  
176 output was increased linearly by  $30 \text{ W}\cdot\text{min}^{-1}$  until the subject was unable to continue. The

177 subjects cycled at a self selected cadence (~80 rpm), and this cadence, along with saddle and  
178 handle bar configuration, was recorded and replicated for subsequent tests. Pulmonary gas  
179 exchange was measured breath-by-breath and averaged into 10-s bins.  $\dot{V}O_{2peak}$  was taken as  
180 the highest 30-s mean value attained during the test. The GET was determined as described  
181 previously (59). The work rate that would require 80% of the GET (moderate-intensity  
182 exercise) was calculated, taking into account the mean response time for  $\dot{V}O_2$  during ramp  
183 exercise (59).

184

185 Subjects returned to the laboratory on two further occasions. The second visit was preceded  
186 by PL supplementation ( $n = 22$ ) and the third visit, ~48 h post blood donation, was preceded  
187 by 2 days of either BR ( $n = 11$ ) or PL ( $n = 11$ ) supplementation. The final visit was  
188 conducted 48 h post donation to allow restoration of total blood volume (23) and to minimize  
189 the risk of a syncopal episode occurring during maximal exercise. On each of these two  
190 laboratory visits, subjects completed a single 5-min bout of moderate-intensity exercise (at 80  
191 % of the GET) and a ramp incremental test to task failure, separated by 10 min of passive  
192 rest. The incremental test was terminated when cadence fell more than 10 rpm below the  
193 chosen cadence, despite strong verbal encouragement. TTF was recorded to the nearest  
194 second and the power output achieved at the point of test termination was recorded as the  
195 peak power output (PPO). Feedback on performance was only provided once all  
196 experimentation for the entire study had been completed.

197

### 198 *Measurements*

199 During each visit to the laboratory, a venous blood sample (~4 mL) was drawn from an  
200 antecubital vein into lithium-heparin tubes (Vacutainer, Becton-Dickinson, NJ, USA) and  
201 centrifuged for 10 min at 3000 g and 4°C, within 2 min of collection. Subsequently, the



202 plasma was extracted and frozen at  $-80^{\circ}\text{C}$  for later determination of  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  using a  
203 modified chemiluminescence technique (7) as previously described (69). Blood samples from  
204 a pre-warmed fingertip were collected into four  $30\ \mu\text{l}$  heparinized microhematocrit tubes  
205 (Hawksley and Sons Ltd, Lancing, Sussex, England) which underwent microcentrifugation  
206 for 1 min for the determination of Hct (1560 Micro-haematocrit reader, Hawksley and Sons  
207 Ltd, Lancing, Sussex, England). In addition, blood from the same fingertip was collected into  
208 four microcuvettes for determination of [Hb] (HemoCue AB, Ängelholm, Sweden).

209

210 Pulmonary gas exchange and ventilation were measured breath-by-breath throughout all  
211 exercise tests. Subjects wore a nose clip and breathed through a mouthpiece and impeller  
212 turbine assembly (Jaeger Triple V). The inspired and expired gas volume and gas  
213 concentration signals were sampled continuously at 100 Hz, with the latter using  
214 paramagnetic ( $\text{O}_2$ ) and infrared (carbon dioxide;  $\text{CO}_2$ ) analyzers (Oxycon Pro, Jaeger,  
215 Hoechberg, Germany) via a capillary line connected to the mouthpiece. These analyzers were  
216 calibrated before each test with gases of known concentration, and the turbine volume  
217 transducer was calibrated using a 3-litre syringe (Hans Rudolph, Kansas City, MO, USA).  
218 The volume and concentration signals were time-aligned by accounting for the delay in  
219 capillary gas transit and analyzer rise time relative to the volume signal. Pulmonary  $\text{O}_2$   
220 uptake ( $\dot{V}\text{O}_2$ ),  $\text{CO}_2$  output ( $\dot{V}\text{CO}_2$ ), minute ventilation ( $\dot{V}\text{E}$ ) and respiratory exchange ratio  
221 (RER) were calculated and displayed breath-by-breath. HR was measured at rest and during  
222 all cycle tests using short-range radiotelemetry (Polar S610, Polar Electro Oy, Kempele,  
223 Finland). A fingertip blood sample was collected into a capillary tube over the 20 s preceding  
224 the step transition in work rate to moderate-intensity exercise and the incremental test.  
225 Capillary samples were also collected during the final 20 s of the moderate-intensity exercise  
226 bout and following exhaustion in the ramp test. These samples were analyzed within 60 s of

227 collection to determine blood [lactate] (YSI 2300, Yellow Springs Instruments, Yellow  
228 Springs, OH, USA).

229

230 The oxygenation status of the *m. vastus lateralis* of the right leg was monitored using near-  
231 infrared spectroscopy (NIRS; model NIRO 300, Hamamatsu Photonics KK, Hiugashi-ku,  
232 Japan). Four different wavelength laser diodes provided the light source (776, 826, 845 and  
233 905 nm) and a photomultiplier tube in the spectrometer was used to detect the light returning  
234 from the tissue. The intensity of incident and transmitted light was recorded continuously  
235 throughout exercise at 2 Hz and used to estimate the change in concentration from baseline  
236 for oxygenated, deoxygenated, and total tissue Hb and myoglobin. The NIRS data therefore  
237 represent a relative change based on the optical density measured in the first data point  
238 collected. The deoxyhemoglobin concentration ([HHb]) was assumed to represent the balance  
239 between local O<sub>2</sub> supply and utilization and therefore to provide an estimate of changes in O<sub>2</sub>  
240 extraction within the field of interrogation (28, 36). Prior to the cycling exercise, the right leg  
241 was cleaned and shaved around the belly of the muscle, the probes were placed in the holder  
242 and attached to the skin with an adhesive 20 cm above the fibular head. An elastic bandage  
243 was wrapped around the subject's leg to secure the holder and wires in place and to minimize  
244 the possibility of extraneous light influencing the signal. Pen marks were made around the  
245 probe holder to allow for precise reproduction of the position of the probe in subsequent tests.  
246 The probe gain was set at rest with the subject in a seated position and the leg extended at  
247 down stroke on the cycle ergometer. NIRS data were collected continuously throughout the  
248 moderate-intensity and incremental exercise tests.

249

250 *Supplementation*

251 After completion of the familiarization test, subjects consumed 7 shots of NO<sub>3</sub><sup>-</sup>-depleted  
252 beetroot juice (PL; beetroot juice containing ~0.04 mmol NO<sub>3</sub><sup>-</sup> per 70 mL; Beet It Sport  
253 Stamina Shot, James White Drinks, Ltd., Ipswich, UK) over ~48 h before completing the pre-  
254 donation control trial (PL-Pre and BR-Pre for the PL and BR groups, respectively). This was  
255 done in order to control for the antioxidants and polyphenols that exist in both the NO<sub>3</sub><sup>-</sup>-rich  
256 and NO<sub>3</sub><sup>-</sup>-depleted beverages. The PL was created by passing NO<sub>3</sub><sup>-</sup>-rich BR through a  
257 Purolite A520E ion-exchange resin which selectively removes NO<sub>3</sub><sup>-</sup> (37). After blood  
258 donation, subjects were randomly assigned, in a double-blind, placebo-controlled fashion, to  
259 consume 7 shots of either NO<sub>3</sub><sup>-</sup>-rich (BR; beetroot juice containing ~6.2 mmol NO<sub>3</sub><sup>-</sup> per 70  
260 mL; Beet It Sport Stamina Shot, James White Drinks, Ltd., Ipswich, UK; *n* =11) or NO<sub>3</sub><sup>-</sup>-  
261 depleted beetroot juice (PL; beetroot juice containing ~0.04 mmol NO<sub>3</sub><sup>-</sup> per 70 mL; Beet It,  
262 James White Drinks, Ltd., Ipswich, UK; *n* =11) over ~48 h (PL-Post and BR-Post for the PL  
263 and BR groups, respectively). During both supplementation periods subjects were instructed  
264 to consume 2 x 70 mL of the beverage in the evening (~7 p.m.) two days prior to testing, and  
265 1 x 70 mL in the morning (~10 a.m.) and 1 x 70 mL in the evening (~7 p.m.) one day prior to  
266 testing. On each experimental day, subjects consumed a further 2 x 70 mL, 2 h prior to testing  
267 and 1 x 70 mL on arrival at the laboratory. The supplementation periods were separated by a  
268 mean of 8 days (BR: 7 ± 5 days, PL: 9 ± 5 days).

269

## 270 *Data Analyses*

271 The breath-by-breath  $\dot{V}O_2$  data collected during the exercise tests were initially examined to  
272 exclude errant breaths caused by, for example, coughing, swallowing and sighing, and those  
273 values lying more than four standard deviations (SDs) from the local mean were removed.  
274  $\dot{V}O_{2\text{baseline}}$  was defined as the mean  $\dot{V}O_2$  measured over the last 60 s of baseline cycling and  
275 end-exercise  $\dot{V}O_2$  was defined as the mean  $\dot{V}O_2$  measured over the last 30 s of exercise. The

276 baseline and end-exercise  $\dot{V}CO_2$ , RER,  $\dot{V}E$  and HR values were calculated in the same  
277 manner.

278

279 To provide information on muscle oxygenation, the changes in [HHb] and the tissue  
280 oxygenation index (TOI; calculated as the fraction of oxygenated [Hb] compared to total  
281 [Hb]) during moderate-intensity exercise were assessed at baseline (60 s preceding the  
282 transition to moderate-intensity exercise), in 10 s time bins surrounding 60 s, 120 s, 240 s,  
283 and at end-exercise (mean response over the final 30 s of exercise). During ramp incremental  
284 exercise, the changes in [HHb] and TOI were assessed at baseline, in 10 s time bins  
285 surrounding 120 s, 240 s, 360 s and at task failure.

286

287 Blood lactate accumulation ( $\Delta$  blood [lactate]) was calculated as the difference between  
288 blood [lactate] at end-exercise and blood [lactate] at baseline. Similarly, the change in blood  
289 glucose concentration ( $\Delta$  blood [glucose]) was calculated as the difference between blood  
290 [glucose] at end-exercise and blood [glucose] at baseline.

291

### 292 *Statistical Analyses*

293 Differences in Hct, [Hb], plasma  $[NO_3^-]$  and  $[NO_2^-]$ , pulmonary  $\dot{V}O_2$  dynamics, HR, blood  
294 [lactate], NIRS-derived variables and TTF were assessed using a mixed model ANOVA.  
295 Significant main and interaction effects were further explored using Fisher's LSD.  
296 Independent t-tests were used to assess the relative change between the BR and PL treatment  
297 groups. Pearson's product moment correlation coefficient was used to explore relationships  
298 between changes in [Hb] and Hct and changes in TTF. Statistical analyses were performed  
299 using SPSS version 19.0 (Chicago, IL, USA). Data are presented as mean  $\pm$  SD, unless  
300 otherwise stated. Statistical significance was accepted at  $P < 0.05$ .

301

302 **RESULTS**

303 Subjects' self-reported adherence to the supplementation regimen prior to and post blood  
304 donation was 100%. All subjects reported that their physical activity and dietary patterns  
305 were similar throughout each of the supplementation periods. The ingestion of BR and PL  
306 supplements were well tolerated and no negative side effects were reported. Subjects did,  
307 however, report beeturia (red-stained urine).

308

309 *[Hb] and Hct*

310 The group mean [Hb] and Hct data prior to and following blood donation and BR or PL  
311 ingestion are displayed in Table 1. There was a significant main effect by time for both [Hb]  
312 and Hct ( $P<0.01$ ) but no main effect by group and no interaction effect ( $P>0.05$ ). Prior to  
313 donation, [Hb] and Hct were not different between the BR and PL treatment groups. [Hb] and  
314 Hct were both significantly reduced from pre to post donation ( $P<0.05$ ), with no differences  
315 between PL and BR groups ( $P>0.05$ ).

316

317 *Plasma [NO<sub>3</sub><sup>-</sup>] and [NO<sub>2</sub><sup>-</sup>]*

318 The group mean plasma [NO<sub>3</sub><sup>-</sup>] and [NO<sub>2</sub><sup>-</sup>] pre and post blood donation in the BR and PL  
319 groups are shown in Table 1. There was a significant main effect by time and group and an  
320 interaction effect on plasma [NO<sub>3</sub><sup>-</sup>] and [NO<sub>2</sub><sup>-</sup>] ( $P<0.01$ ). Prior to blood donation, neither  
321 plasma [NO<sub>3</sub><sup>-</sup>] nor [NO<sub>2</sub><sup>-</sup>] were different between groups ( $P>0.05$ ). Following blood  
322 donation, there was a substantial increase in plasma [NO<sub>3</sub><sup>-</sup>] and [NO<sub>2</sub><sup>-</sup>] in the BR group  
323 ( $P<0.05$ ). A small (~11%) rise in plasma [NO<sub>3</sub><sup>-</sup>] ( $P<0.05$ ) was also observed in the PL group  
324 but there was no change in plasma [NO<sub>2</sub><sup>-</sup>] ( $P>0.05$ ).

325

326  $\dot{V}O_2$  response to moderate-intensity and incremental exercise

327 *Moderate-intensity exercise*

328 The pulmonary gas exchange and ventilatory responses to moderate-intensity exercise pre  
329 and post blood donation in PL and BR groups are reported in Table 2 and the group mean  
330  $\dot{V}O_2$  response profiles in BR and PL groups pre and post blood donation are shown in Figure  
331 1. There was a significant main effect by time ( $P<0.01$ ) but no main effect by condition and  
332 no interaction effect ( $P>0.05$ ) for the  $\dot{V}O_2$  measured during the baseline cycling period and at  
333 end-exercise. Prior to donation, there were no differences in baseline or end-exercise  $\dot{V}O_2$   
334 between BR and PL groups ( $P>0.05$ ). Follow-up tests revealed that both baseline  $\dot{V}O_2$   
335 ( $P<0.01$ ) and end-exercise  $\dot{V}O_2$  ( $P<0.05$ ) were reduced in the BR group post-donation  
336 compared with pre-donation.

337

338 The  $\dot{V}CO_2$ ,  $\dot{V}E$ , RER, blood [lactate] and blood [glucose] data during moderate-intensity  
339 exercise are reported in Table 2. Prior to donation, there were no differences in these  
340 variables at baseline or at end-exercise between the BR and PL groups ( $P>0.05$ ) and there  
341 were no significant main effects by condition or time and no interaction effects ( $P>0.05$ ).

342

343 *Ramp incremental exercise*

344 The effects of blood donation and BR and PL supplementation on the ramp incremental test  
345 parameters are reported in Table 3 and illustrated in Figures 2 and 3.

346

347 There was a significant main effect by time on  $\dot{V}O_{2peak}$  ( $P<0.05$ ), but no main effect by  
348 condition or an interaction effect ( $P>0.05$ ). There were no differences between the groups at  
349 baseline ( $P>0.05$ ). Follow-up tests indicated that, from pre to post donation, there was a  
350 significant reduction ( $0.19 \text{ L}\cdot\text{min}^{-1}$ ;  $\sim 5\%$ ) in  $\dot{V}O_{2peak}$  in the PL group ( $P<0.05$ ) but not in the

351 BR group ( $0.12 \text{ L}\cdot\text{min}^{-1}$ ;  $\sim 3\%$ ;  $P > 0.05$ ). There was a significant main effect by time and an  
352 interaction effect ( $P < 0.05$ ) but no main effect by condition ( $P > 0.05$ ) for PPO and TTF. Post  
353 hoc tests revealed a significant reduction in PPO and TTF in both PL and BR groups from pre  
354 to post donation ( $P < 0.01$ ). There were no differences in PPO or TTF between the groups  
355 prior to blood donation ( $P > 0.05$ ). However, the reduction in PPO and TTF following blood  
356 donation was more pronounced in PL compared with BR (5% vs. 3%;  $P < 0.05$ ). The change  
357 in [Hb] and Hct from pre to post donation was correlated with the change in TTF during ramp  
358 incremental exercise in PL ( $r = 0.58$ ;  $P = 0.06$ , and  $r = 0.70$ ;  $P < 0.05$ , respectively) but not BR  
359 ( $r = -0.10$ ;  $P > 0.05$  and  $r = -0.41$ ;  $P > 0.05$ , respectively).

360

361 There was a significant interaction effect, but no main effects by time or group, for peak  
362  $\dot{V}\text{CO}_2$ . Specifically, peak  $\dot{V}\text{CO}_2$  was reduced in the PL group ( $P < 0.05$ ), but was unaffected in  
363 the BR group ( $P > 0.05$ ). There was no main effect by time or condition nor an interaction  
364 effect for peak  $\dot{V}\text{E}$  ( $P > 0.05$ ). There was a significant main effect by time and an interaction  
365 effect for peak RER ( $P < 0.05$ ). Despite no difference at baseline, post hoc tests revealed an  
366 increase in peak RER in the BR group from pre to post donation ( $P < 0.01$ ).

367

### 368 *NIRS measurements*

#### 369 *Moderate-intensity exercise*

370 There were no differences for total Hb (THb) between or within conditions during  
371 the moderate-intensity exercise bout. The [HHb] and TOI values measured during moderate-  
372 intensity exercise are reported in Table 4. There were no main effects by condition or time  
373 and no interaction effect for baseline [HHb] ( $P > 0.05$ ). There was a significant main effect by  
374 time for [HHb] from pre to post donation at 60 s, 120 s, 240 s and end-exercise ( $P < 0.05$ ), but  
375 no main effect by condition or an interaction effect at any time point ( $P > 0.05$ ). Post hoc tests

376 revealed a trend toward an increase in [HHb] in the PL group, but not the BR group, from pre  
377 to post donation at 120 s and 240 s of moderate exercise ( $P<0.10$ ). There were no main  
378 effects by time or interaction effects for TOI at 60 s, 120 s, 240 s and end-exercise ( $P>0.05$ ).  
379 However, there was a trend toward a main effect by condition for all time points ( $P<0.10$ ).  
380 Follow-up tests revealed that blood donation resulted in reductions in TOI in the PL group at  
381 60 s, 120 s and 240 s during moderate exercise, respectively ( $P<0.05$ ; Table 4).

382

### 383 *Ramp incremental exercise*

384 There were no differences for THb between or within conditions during ramp incremental  
385 exercise. The [HHb] and TOI values measured during ramp incremental exercise are reported  
386 in Table 4 and the [HHb] profile is shown in Figure 4. There was a significant main effect by  
387 time ( $P<0.05$ ) but no main effect by condition or an interaction effect ( $P>0.05$ ) for [HHb] at  
388 120 s and 240 s during ramp incremental exercise. Post hoc tests showed that [HHb]  
389 increased from pre to post donation at 240 s in PL ( $P<0.05$ ) but not BR ( $P>0.05$ ; Table 4).  
390 There was a significant main effect by time ( $P<0.05$ ) and a trend for an interaction effect for  
391 [HHb] at 360 s ( $P<0.10$ ) and at end-exercise ( $P<0.05$ ) during the incremental exercise test.  
392 Post hoc tests revealed that [HHb] increased significantly from pre to post donation in the PL  
393 group at both 360 s and end-exercise ( $P<0.05$ ; Table 4). The change in [HHb] from pre to  
394 post donation was higher in PL versus BR at end-exercise ( $P<0.05$ ) and tended to be higher at  
395 360 s ( $P<0.10$ ).

396

## 397 **DISCUSSION**

398 The principal original findings in this study, consistent with our hypotheses, were that  $\text{NO}_3^-$ -  
399 rich beetroot juice ingestion lowered the  $\text{O}_2$  cost of moderate-intensity exercise, better  
400 preserved muscle oxygenation during moderate and ramp incremental exercise and attenuated



401 the reduction in ramp incremental exercise test performance and  $\dot{V}O_{2\text{peak}}$  following blood  
402 donation. These results indicate that dietary  $\text{NO}_3^-$  supplementation can ameliorate decrements  
403 in exercise performance in a situation (i.e. reduction in blood  $\text{O}_2$ -carrying capacity) which  
404 would be expected to compromise physiological function during exercise.

405

#### 406 *Effects of blood donation on [Hb] and Hct*

407 The standard NHS blood bank donation (~450mL) reduced [Hb] and Hct by a similar  
408 magnitude in the PL and BR groups. These results concur with previous studies that have  
409 investigated the influence of whole blood withdrawal on [Hb]. For example, Gordon et al.  
410 (27) and Mora-Rodriguez et al. (45) reported ~8% and ~7% reductions in [Hb], 24 and 48 h  
411 post blood donation, respectively. The ~8% reduction in Hct in the present study is also  
412 similar to the values reported by Burnley et al. (11) and Gordon et al. (27) who reported a ~7-  
413 8% decrease in Hct one day after 450 mL blood donation. The reduction in blood  $\text{O}_2$  carrying  
414 capacity, secondary to the lower [Hb] and Hct, can result in a reduction in muscle  $\text{O}_2$  delivery  
415 and muscle  $\text{O}_2$  diffusing capacity during maximal exercise, with significant implications for  
416 exercise performance (5, 11, 18, 47, 54).

417

#### 418 *Effects of nitrate supplementation on plasma [ $\text{NO}_3^-$ ] and [ $\text{NO}_2^-$ ]*

419 The ingestion of  $\text{NO}_3^-$ -rich BR significantly elevated plasma [ $\text{NO}_3^-$ ] and [ $\text{NO}_2^-$ ] when  
420 compared with baseline values. These findings are in agreement with earlier studies which  
421 also examined the influence of BR supplementation in young, healthy subjects (4, 34, 69).  
422 A small but significant rise in plasma [ $\text{NO}_3^-$ ] was also noted in the PL group post  
423 donation. This may be explained by a slight hemoconcentration or an upregulation in NOS  
424 activity consequent to the reduction in whole body iron concentration after donating blood  
425 (62). Plasma [ $\text{NO}_2^-$ ] rose by ~800% in the BR group from pre to post donation, suggesting

426 appreciably enhanced NO bioavailability. Numerous other studies have also reported  
427 increases in plasma  $[\text{NO}_2^-]$  after BR supplementation, but the percentage increases  
428 attained were approximately half of those reported in this study (56, 69). This finding is  
429 likely a result of the higher dose of  $\text{NO}_3^-$  ingested (~43 mmol over 48 h) when compared  
430 with previous short-term BR supplementation studies. Interestingly, unlike in some earlier  
431 studies (4, 38, 59, 69), BR supplementation did not reduce resting blood pressure (BP)  
432 despite the elevated plasma  $[\text{NO}_2^-]$  (mean arterial pressure, pre- vs. post-donation:  $81 \pm 7$   
433 vs.  $80 \pm 7$  mmHg). Similar BP values pre- vs. post-donation in the PL group indicates that  
434 total blood volume was restored 48 h following blood donation. The lack of effect of BR  
435 on BP in the present study may be related to the relatively low baseline BP values of the  
436 study participants (115/64 mmHg) and the relatively large number of female participants.  
437 It has been reported that females are less sensitive than males to the influence of  $\text{NO}_3^-$   
438 supplementation on BP and that the extent of BP reduction with  $\text{NO}_3^-$  supplementation is  
439 correlated with the baseline BP (33).

440

441 *Effects of blood donation and nitrate supplementation on the physiological responses to*  
442 *moderate-intensity exercise*

443 The  $\dot{V}\text{O}_2$  during both the unloaded baseline period and in the steady state of moderate-  
444 intensity exercise was significantly reduced (by ~4%) in the BR group, but not the PL group,  
445 after blood donation. A similar reduction in the  $\text{O}_2$  cost of moderate-intensity exercise has  
446 been reported by Bailey et al. (4) after six days of non-concentrated  $\text{NO}_3^-$ -rich BR ingestion  
447 and by Larsen et al. (38) after three days of  $\text{NaNO}_3$  supplementation. The present findings are  
448 consistent with those of Kelly et al. (34) who observed that, in hypoxia, BR supplementation  
449 resulted in a decrease in both baseline and steady-state  $\dot{V}\text{O}_2$  when compared with placebo. It  
450 has also been reported that acute (46) and 6 days (43) BR ingestion resulted in significant

451 reductions in  $\dot{V}O_2$  during submaximal cycling exercise in hypoxia (15% and 11%  $O_2$ ,  
452 respectively). Acute BR supplementation has also been reported to better preserve arterial  $O_2$   
453 saturation following dynamic apnea (48).

454

455 The lowering of the  $O_2$  cost of submaximal exercise after  $NO_3^-$  supplementation may be due  
456 to a number of mechanisms, including a reduction in the ATP cost of muscle force production  
457 (4) and/or an improvement in mitochondrial efficiency (40) and/or changes in redox  
458 signalling (66). In addition to changes in muscle contractile or metabolic efficiency, muscle  
459  $O_2$  delivery or its intramuscular distribution may be altered following  $NO_3^-$  supplementation  
460 (21, 22). Exercise, particularly in hypoxia or under conditions that may limit  $O_2$  carrying  
461 capacity, such as blood donation, acts as a potent stimulus for vasodilatation and delivery of  
462  $O_2$  to working muscle (12, 13). Both NO and  $O_2$  compete for the binding site at cytochrome-c  
463 oxidase (COX) in the mitochondrial electron transport chain (9). An elevation in NO  
464 availability via  $NO_3^-$  supplementation, perhaps especially in conditions limiting  $O_2$  delivery,  
465 increases the likelihood of NO binding to COX and therefore inhibiting  $O_2$  consumption at  
466 the mitochondrion (10). As a result, NO may modify the intramuscular distribution of  $O_2$  and  
467 improve the oxygenation status of muscle fibres that are situated further away from the  
468 capillaries (29, 55, 63). Compared to placebo, BR supplementation has been reported to  
469 enable a greater maximal rate of mitochondrial ATP resynthesis ( $Q_{max}$ ) and result in faster  
470 muscle phosphocreatine recovery kinetics following exercise in hypoxia (60, 61), indicating  
471 improved muscle  $O_2$  availability at least in the immediate post-exercise period (61).

472

473 In the present study, TOI was significantly reduced and [HHb] tended to be higher during  
474 moderate-intensity exercise post- compared to pre-donation in the PL group, suggesting that  
475 muscle  $O_2$  availability was lower and a greater muscle fractional  $O_2$  extraction was necessary  
476 to achieve the required  $\dot{V}O_2$  (24, 36). These changes were attenuated in the BR group,

477 consistent with our hypothesis that BR supplementation would better preserve muscle  
478 oxygenation during moderate-intensity exercise when compared with PL. These results are  
479 consistent with Masschelein et al. (43) who reported that BR resulted in a greater muscle TOI  
480 and lower [HHb] during submaximal exercise in normobaric hypoxia. Collectively, these  
481 studies indicate that under conditions which may impair blood O<sub>2</sub> carrying capacity, such as  
482 following blood donation (present study) or in normobaric hypoxia (43), BR ingestion  
483 promotes a better matching between muscle O<sub>2</sub> delivery and O<sub>2</sub> demand, i.e. less O<sub>2</sub> extraction  
484 is required for the same moderate-intensity work rate, perhaps due to the lower exercise  $\dot{V}O_2$   
485 (34) or to preferential alterations in muscle perfusion (21, 22, 61). An increased ratio of O<sub>2</sub>  
486 delivery to O<sub>2</sub> consumption at a given work rate would be expected to retard the rate of  
487 fatigue development and to improve exercise performance.

488

489 *Effects of blood donation and nitrate supplementation on the physiological responses to*  
490 *incremental exercise*

491 As expected, blood donation and the associated reduction in O<sub>2</sub> carrying capacity resulted in a  
492 significant reduction in PPO and TTF during ramp incremental exercise. Panebianco et al.  
493 (47) also reported a significant reduction in PPO during incremental exercise, 2 days post  
494 blood donation. An important original finding in the present study was that ingestion of BR in  
495 the 48 hours post blood donation partly negated the decrement in performance when  
496 compared with PL. Specifically, the reduction in PPO and TTF following blood donation was  
497 significantly more pronounced in the PL group compared with BR. Interestingly, the  
498 reduction in TTF in the PL group was quite well correlated with the reduction in [Hb] ( $r =$   
499  $0.58, P=0.06$ ) and Hct ( $r = 0.70, P<0.05$ ) following blood donation, whereas in the BR group,  
500 the correlations were weaker and non-significant ([Hb]:  $r = -0.10$ ; Hct:  $r = -0.41$ ; both  
501  $P>0.05$ ), implying that BR supplementation compensated for the lower [Hb] and Hct. These

502 findings are consistent with those of Masschelein et al. (43) who reported that, compared to  
503 PL, BR ingestion significantly attenuated the reduction in TTF when incremental exercise  
504 was performed in hypoxia.

505

506  $\dot{V}O_{2\text{peak}}$  was reduced by 5% from pre to 48 h post donation in the PL group. Similarly,  
507 Burnley et al. (11) reported a 4% decrease in  $\dot{V}O_{2\text{peak}}$  during severe-intensity exercise 24 h  
508 following blood donation. This reduction was proportional to the reduced [Hb] and thus the  
509 ability to deliver  $O_2$  to the working skeletal muscle during maximal exercise. In the present  
510 study, the reduced  $\dot{V}O_{2\text{peak}}$  in the PL group following blood donation occurred in conjunction  
511 with an increased muscle [HHb], which may be interpreted as an increase in muscle  
512 fractional  $O_2$  extraction in an (ultimately unsuccessful) attempt to offset the effects of a  
513 reduced [Hb] and lower muscle  $O_2$  delivery (51, 54). In contrast,  $\dot{V}O_{2\text{peak}}$  and [HHb] during  
514 the incremental test were not significantly altered by blood donation in the BR group. These  
515 results may indicate that the  $O_2$  sparing effect of BR ingestion (Figure 2B), coupled perhaps  
516 with altered perfusion distribution (21, 22, 61), enabled muscle oxygenation to be better  
517 preserved during incremental exercise, such that an increased muscle fractional  $O_2$  extraction  
518 was not mandated to achieve a given  $\dot{V}O_{2\text{peak}}$ . Ferguson et al. (21, 22) have reported that, in  
519 rats, BR supplementation can enhance vascular conductance and blood flow to working  
520 muscle and elevate the microvascular partial pressure of  $O_2$  ( $PO_{2\text{mv}}$ ), particularly in type II  
521 fibres. If similar effects occur in humans, this may enhance the blood-myocyte  $O_2$  exchange  
522 gradient during higher intensity exercise, better preserving muscle oxygenation status,  
523 homeostasis and performance. It is also possible that a portion of the preserved ramp  
524 incremental test performance following blood donation with BR compared to PL may be  
525 attributable to effects of  $NO_3^-$  on muscle contractile function (50), perhaps particularly in type  
526 II fibers (31).

527

528 The mechanistic bases for the positive effects of BR ingestion on vascular and metabolic  
529 function in this and other situations warrants further investigation. In particular, while it is  
530 widely believed that the effects may be attributed to greater NO bioavailability or bioactivity,  
531 it is presently unclear precisely how this NO pool is stored and transported. NO is a highly  
532 reactive molecule with a short-half life *in vivo* and its rapid reaction with, for example, O<sub>2</sub> or  
533 heme proteins (30) suggests that the free transport of NO may be limited in plasma and  
534 within cells. It has been proposed that NO<sub>2</sub><sup>-</sup> itself represents a principal means of ‘NO’  
535 storage and transport, with the one electron reduction of NO<sub>2</sub><sup>-</sup> to NO in blood and other  
536 tissues being facilitated, amongst many other factors including xanthine oxidoreductase, by  
537 deoxyhemoglobin and deoxymyoglobin, which will naturally be present in greater abundance  
538 in contracting skeletal muscle (16, 42). However, BR ingestion likely also increases the  
539 production and storage of other reactive nitrogen species. In particular, low molecular weight  
540 thiol groups may react with nitrogen oxides to yield s-nitrosothiol species (SNOs) which can  
541 be transported in the blood as s-nitrosohemoglobin (HbSNO) (17). It has recently been  
542 reported that the reduction in blood pressure following NO<sub>3</sub><sup>-</sup> or NO<sub>2</sub><sup>-</sup> ingestion in a rat model  
543 of hypertension was more closely related to plasma [s-nitrosothiol] than to plasma [NO<sub>2</sub><sup>-</sup>]  
544 (49) and that s-nitrosothiol bioactivity derived through βCys93 may be essential for hypoxic  
545 vasodilation by erythrocytes (70). In contrast, in humans, Gladwin et al. (26) reported a  
546 significant arterial-venous NO<sub>2</sub><sup>-</sup> gradient during forearm exercise and concluded that SNOs  
547 and HbSNO do not play a significant role in the regulation of vascular tone. The role of  
548 SNOs and HbSNO in the physiological effects of nitrate ingestion in humans remains to be  
549 clarified. Equally, the precise mechanisms by which an elevation of tissue [NO<sub>2</sub><sup>-</sup>] following  
550 NO<sub>3</sub><sup>-</sup> ingestion influences metabolic and vascular control at rest and during exercise remains  
551 unclear. While it is possible that NO<sub>2</sub><sup>-</sup> itself is bioactive (58), unresolved questions include

552 the triggers and time course for the possible reduction of  $\text{NO}_2^-$  to NO, and the nature of both  
553 NO transport to, and storage within, biological targets. Resolution of these issues will likely  
554 require synthesis of experimental data deriving from ‘competing’ hypotheses.

555

#### 556 *Perspectives*

557 This study has shown for the first time that despite a significant reduction in [Hb] post blood  
558 withdrawal, BR supplementation lowered the  $\text{O}_2$  cost of moderate-intensity exercise, better  
559 preserved muscle oxygenation during moderate-intensity and ramp incremental exercise, and  
560 attenuated the reduction in  $\dot{V}\text{O}_{2\text{peak}}$  and incremental exercise test performance. These results  
561 may have significant implications for athletes who wish to give blood without significant  
562 detriment to training, individuals with clinical conditions which reduce blood  $\text{O}_2$  carrying  
563 capacity, such as anemia, and in conditions resulting in acute blood loss such as surgery or  
564 military combat. In this context, it is of interest that transfusion of stored blood may impair  
565 vasodilatory capacity, an effect that might be linked to the loss of NO bioavailability that  
566 occurs during blood storage (17, 52). Treating banked blood to better maintain NO stores  
567 might lead to improved functional outcomes following transfusion. In conclusion, BR  
568 supplementation attenuates the decline in functional capacity arising from blood donation.

569

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574

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848

849 **FIGURE LEGENDS**

850 **Figure 1:** Pulmonary oxygen uptake ( $\dot{V}O_2$ ) response following BR and PL supplementation  
851 prior to and following blood donation during a step increment to a moderate-intensity work  
852 rate. Responses prior to blood donation are shown as solid, filled circles, while responses post  
853 blood donation are shown as open, unfilled circles. The dotted vertical line represents the  
854 abrupt imposition of the moderate work rate from a baseline of ‘unloaded’ cycling. *A:* Group  
855 mean  $\dot{V}O_2$  response to moderate-intensity exercise following PL ingestion. *B:* Group mean  
856  $\dot{V}O_2$  response to moderate-intensity exercise following BR ingestion. *C:* Steady state  $\dot{V}O_2$   
857 following PL and BR supplementation relative to pre blood donation baseline. The  $O_2$  cost of  
858 moderate-intensity exercise was reduced following BR supplementation and blood donation  
859 compared with pre donation values, \* $P < 0.05$ .

860

861 **Figure 2:** Group mean pulmonary  $\dot{V}O_2$  response to incremental exercise prior to blood  
862 donation and following BR and PL supplementation after blood donation. Responses prior to  
863 blood donation are shown as solid, filled circles, while responses post blood donation are  
864 shown as open, unfilled circles. The dotted vertical line represents the onset of the ramp  
865 incremental test from a baseline of ‘unloaded’ cycling. The  $\dot{V}O_{2peak}$  was reduced in the PL  
866 group (\*=  $P < 0.05$ ), but not the BR group, after blood donation. TTF was reduced in both  
867 groups post donation ( $\# = P < 0.05$ ), however, the reduction in TTF was greater in the PL  
868 group when compared with the BR group ( $\$ = P < 0.05$ ).

869

870 **Figure 3.** Group mean time to task failure (TTF) in the ramp incremental test prior to and  
871 post blood donation, following BR and PL supplementation. Responses prior to blood  
872 donation are shown as solid, filled bars, while responses post donation are shown as open,

873 unfilled bars. The TTF was reduced in both groups post donation ( $*=P<0.05$ ); however, the  
874 reduction in TTF was greater in the PL group when compared with the BR group ( $^{\#}=P<0.05$ ).

875

876 **Figure 4.** Group mean changes in deoxyhaemoglobin ([HHb]) prior to and post blood  
877 donation, following BR and PL ingestion. Responses prior to blood donation are shown as  
878 solid, filled circles, while responses post blood donation are shown as open, unfilled circles.  
879 The dotted vertical line represents the onset of the ramp incremental test from a baseline of  
880 'unloaded' cycling. [HHb] increased significantly from pre to post donation in the PL group  
881 at 360 s and end-exercise ( $*=P<0.05$ ). [HHb] was not altered from pre to post donation in the  
882 BR group. TTF was reduced in both groups post donation ( $^{\#}=P<0.05$ ), however, the  
883 reduction in TTF was greater in the PL group when compared with the BR group ( $^{\$}=$   
884  $P<0.05$ ).

885

886

**Table 1:** Blood pressure, resting heart rate, plasma nitrate and nitrite concentrations, hemoglobin concentration and hematocrit prior to and following blood donation in the PL and BR groups.

	PL		BR	
	Pre	Post	Pre	Post
<b>Blood pressure (mmHg)</b>				
<i>Systolic</i>	119 ± 7	118 ± 9	115 ± 11	113 ± 11*
<i>Diastolic</i>	69 ± 7	67 ± 7	64 ± 7	63 ± 7
<i>Mean Arterial</i>	86 ± 6	84 ± 8	81 ± 7	80 ± 7
<b>Resting HR (b·min<sup>-1</sup>)</b>	62 ± 9	66 ± 9	66 ± 11	71 ± 10*
<b>Plasma [NO<sub>3</sub><sup>-</sup>] (μM)</b>	45 ± 11	50 ± 14*	47 ± 17	845 ± 350* <sup>§</sup>
<b>Plasma [NO<sub>2</sub><sup>-</sup>] (nM)</b>	73 ± 18	72 ± 21	81 ± 29	619 ± 363* <sup>§</sup>
<b>[Hb] (g·L<sup>-1</sup>)</b>	149 ± 12	132 ± 18*	148 ± 15	137 ± 19*
<b>Hct (%)</b>	45 ± 2	41 ± 4*	45 ± 3	42 ± 5*

Values are mean ± SD. PL, Placebo group; BR, Nitrate group; Pre, pre-donation; Post, post-donation ; HR, heart rate; [NO<sub>2</sub><sup>-</sup>], nitrite concentration; [NO<sub>3</sub><sup>-</sup>], nitrate concentration; [Hb], hemoglobin concentration; Hct, hematocrit. \*Significantly different from pre in the same condition ( $P<0.05$ ). <sup>§</sup>Significantly different from post supplementation value in the PL group ( $P<0.05$ ).

**Table 2:** Ventilatory and gas exchange dynamics, and blood lactate and glucose concentrations during moderate-intensity exercise prior to and following blood donation in the PL and BR groups

	<b>PL</b>		<b>BR</b>	
	<b>Pre</b>	<b>Post</b>	<b>Pre</b>	<b>Post</b>
<b><math>\dot{V}O_2</math> (L·min<sup>-1</sup>)</b>				
<i>Baseline</i>	1.01 ± 0.17	0.97 ± 0.20	0.96 ± 0.20	0.87 ± 0.21 <sup>#</sup>
<i>End exercise</i>	1.72 ± 0.50	1.69 ± 0.53	1.65 ± 0.32	1.59 ± 0.34 <sup>#</sup>
<b><math>\dot{V}CO_2</math> (L·min<sup>-1</sup>)</b>				
<i>Baseline</i>	0.88 ± 0.19	0.86 ± 0.19	0.89 ± 0.19	0.81 ± 0.19 <sup>#</sup>
<i>End exercise</i>	1.60 ± 0.52	1.56 ± 0.50	1.53 ± 0.29	1.54 ± 0.29
<b>RER</b>				
<i>Baseline</i>	0.88 ± 0.08	0.90 ± 0.06	0.89 ± 0.05	0.92 ± 0.09
<i>End exercise</i>	0.94 ± 0.06	0.93 ± 0.06	0.93 ± 0.04	0.96 ± 0.06 <sup>#</sup>
<b><math>\dot{V}E</math> (L·min<sup>-1</sup>)</b>				
<i>Baseline</i>	25 ± 5	24 ± 5	24 ± 5	22 ± 5 <sup>#</sup>
<i>End exercise</i>	42 ± 11	40 ± 11	38 ± 6	38 ± 6
<b>Δ Blood [lactate] (mM)</b>	0.0 ± 0.3	0.1 ± 0.4	0.1 ± 0.3	0.1 ± 0.4
<b>ΔBlood [glucose] (mM)</b>	0.1 ± 0.7	-0.2 ± 0.7	0.00 ± 0.3	0.1 ± 0.5

Values are mean ± SD. PL, Placebo group; BR, Nitrate group; Pre, pre-donation; Post, post-donation; [Bla], blood lactate concentration; [glu], blood glucose concentration; HR, heart rate. <sup>#</sup>Significantly different from pre in the same condition ( $P < 0.05$ ).

**Table 3:** Physiological responses to ramp incremental exercise prior to and following blood donation in the PL and BR groups.

	PL		BR	
	Pre	Post	Pre	Post
$\dot{V}O_{2\text{peak}}$ (L·min <sup>-1</sup> )	3.84 ± 0.91	3.65 ± 0.85*	3.52 ± 0.65	3.40 ± 0.73
$\dot{V}O_{2\text{peak}}$ (mL·kg <sup>-1</sup> ·min <sup>-1</sup> )	49.9 ± 11.0	47.4 ± 10.0*	46.6 ± 6.0	44.9 ± 6.0
Peak power (W)	341 ± 70	324 ± 69*	331 ± 68	322 ± 66*
GET (L·min <sup>-1</sup> )	1.76 ± 0.40	1.68 ± 0.43	1.64 ± 0.44	1.63 ± 0.44
GET (W)	117 ± 29	109 ± 27	116 ± 35	112 ± 24
$\dot{V}CO_{2\text{peak}}$ (L·min <sup>-1</sup> )	4.69 ± 1.12	4.44 ± 0.97*	4.26 ± 0.68	4.36 ± 0.77
RER peak	1.22 ± 0.06	1.22 ± 0.05	1.22 ± 0.06	1.29 ± 0.06*
$\dot{V}E_{\text{peak}}$ (L·min <sup>-1</sup> )	156 ± 44	150 ± 43*	134 ± 28	137 ± 32
HR <sub>peak</sub> (b·min <sup>-1</sup> )	177 ± 16	181 ± 9	178 ± 12	179 ± 10
Δ Blood [lactate] (mM)	6.1 ± 1.4	5.5 ± 1.2	6.1 ± 1.9	6.8 ± 2.5
Δ Blood [glucose] (mM)	-0.2 ± 0.7	0.0 ± 1.1	-0.2 ± 0.4	0.0 ± 1.1

Values are mean ± SD. PL, Placebo group; BR, Nitrate group; Pre, pre-donation; Post, post-donation; GET, Gas exchange threshold; [Bla], blood lactate concentration; [glu], blood glucose concentration; HR, heart rate. \*Significantly different from pre in the same condition ( $P < 0.05$ ).



**Table 4:** Near-infrared spectroscopy-derived [HHb] and TOI dynamics during moderate-intensity and ramp incremental exercise prior to and following blood donation in the PL and BR groups.

	PL		BR	
	Pre	Post	Pre	Post
<i>Moderate-intensity exercise</i>				
<b>[HHb]</b>				
<i>Baseline (AU)</i>	-4.4 ± 3.0	-2.3 ± 3.1	-3.1 ± 3.7	-1.9 ± 2.5
<i>60 s (AU)</i>	-1.2 ± 2.3	2.3 ± 5.0	-0.1 ± 5.0	0.6 ± 3.9
<i>120 s (AU)</i>	-0.9 ± 3.0	3.5 ± 6.2	-0.1 ± 4.9	1.0 ± 3.7
<i>240 s (AU)</i>	-0.7 ± 3.9	2.3 ± 5.2	0.1 ± 4.9	1.1 ± 3.6
<i>End (AU)</i>	0.0 ± 4.4	2.5 ± 4.9	0.0 ± 4.9	1.0 ± 3.4
<b>TOI</b>				
<i>Baseline (%)</i>	65.3 ± 3.4	63.4 ± 3.3*	68.2 ± 4.3	70.1 ± 5.8
<i>60 s (%)</i>	61.9 ± 4.9	57.7 ± 5.0*	64.6 ± 6.5	65.6 ± 8.5
<i>120 s (%)</i>	61.9 ± 4.8	57.1 ± 5.7*	64.8 ± 6.1	65.6 ± 8.8
<i>240 s (%)</i>	60.7 ± 6.6	58.1 ± 4.8*	64.8 ± 6.5	65.8 ± 8.9
<i>End (%)</i>	61.4 ± 6.4	57.8 ± 5.0	65.3 ± 6.3	65.8 ± 8.9
<i>Ramp incremental exercise</i>				
<b>[HHb]</b>				
<i>Baseline (AU)</i>	-6.2 ± 4.1	-3.4 ± 3.6	-5.1 ± 4.1	-2.6 ± 2.5
<i>120 s (AU)</i>	-3.3 ± 5.4	-0.1 ± 5.0	-2.7 ± 5.0	-0.7 ± 3.3
<i>240 s (AU)</i>	-0.8 ± 6.2	3.3 ± 5.8*	-0.6 ± 5.8	1.4 ± 4.4
<i>360 s (AU)</i>	2.0 ± 9.4	7.3 ± 9.1*	1.5 ± 6.6	3.4 ± 5.8
<i>End (AU)</i>	6.2 ± 11.3	12.8 ± 10.1*	3.8 ± 7.6	5.3 ± 7.2
<b>TOI</b>				
<i>Baseline (%)</i>	66.5 ± 3.9	67.3 ± 7.1	71.5 ± 3.9	72.5 ± 4.7
<i>120 s (%)</i>	63.3 ± 5.1	64.6 ± 8.6	68.6 ± 5.5	69.5 ± 6.9
<i>240 s (%)</i>	60.8 ± 6.5	60.7 ± 9.2	65.8 ± 7.5	65.9 ± 9.7
<i>360 s (%)</i>	57.3 ± 11.5	55.4 ± 12.3	61.9 ± 8.6	61.7 ± 11.4
<i>End (%)</i>	49.5 ± 12.6	47.6 ± 14.9	57.1 ± 7.0	57.2 ± 10.9

Values are mean ± SD. PL, Placebo group; BR, Nitrate group; Pre, pre-donation; Post, post-donation; [HHb], deoxygenated haemoglobin concentration; TOI, tissue oxygenation index; AU, arbitrary units. \*Significantly different from pre in the same condition ( $P < 0.05$ ).







