

**TITLE**

Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O<sub>2</sub> uptake dynamics during exercise in hypoxia and normoxia

**AUTHORS**

Kelly, J; Vanhatalo, A; Bailey, SJ; et al.

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1 **Dietary nitrate supplementation: effects on plasma nitrite and**  
2 **pulmonary O<sub>2</sub> uptake dynamics during exercise in hypoxia and**  
3 **normoxia**

4

5 **James Kelly<sup>1</sup>, Anni Vanhatalo<sup>1</sup>, Stephen J Bailey<sup>1</sup>, Lee J Wylie<sup>1</sup>, Christopher Tucker<sup>1</sup>,**  
6 **Stephen List<sup>1</sup>, Paul G Winyard<sup>2</sup>, and Andrew M Jones<sup>1</sup>**

7

8 *<sup>1</sup>Sport and Health Sciences, College of Life and Environmental Sciences, and <sup>2</sup>University of*  
9 *Exeter Medical School, St. Luke's Campus, University of Exeter, Heavitree Road, Exeter,*  
10 *EX1 2LU, United Kingdom.*

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12 **Running Head:** Nitrate supplementation in hypoxia and normoxia

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14 Correspondence to:  
15 Andrew M Jones, PhD  
16 Professor of Applied Physiology  
17 Sport and Health Sciences  
18 St. Luke's Campus  
19 University of Exeter  
20 Exeter EX1 2LU  
21 United Kingdom

22

23

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27

28 **Abstract**

29 We investigated the effects of dietary nitrate ( $\text{NO}_3^-$ ) supplementation on the concentration of  
30 plasma nitrite ( $[\text{NO}_2^-]$ ), oxygen uptake ( $\dot{V}\text{O}_2$ ) kinetics and exercise tolerance in normoxia (N)  
31 and hypoxia (H). In a double-blind, crossover study, twelve healthy subjects completed cycle  
32 exercise tests, twice in N (20.9%  $\text{O}_2$ ) and twice in H (13.1%  $\text{O}_2$ ). Subjects ingested either 140  
33  $\text{ml}\cdot\text{d}^{-1}$  of  $\text{NO}_3^-$ -rich beetroot juice (8.4  $\text{mmol NO}_3$ ; BR) or  $\text{NO}_3^-$ -depleted beetroot juice (PL)  
34 for 3-days prior to moderate-intensity and severe-intensity exercise tests in H and N. Pre-  
35 exercise plasma  $[\text{NO}_2^-]$  was significantly elevated in H-BR and N-BR compared to H-PL ( $P$   
36 = 0.00) and N-PL ( $P = 0.00$ ). The rate of decline in plasma  $[\text{NO}_2^-]$  was greater during severe-  
37 intensity exercise in H-BR ( $-30\pm 22 \text{ nM}\cdot\text{min}^{-1}$ , 95% *CI*; -44, -16) compared to H-PL ( $-7\pm 10$   
38  $\text{nM}\cdot\text{min}^{-1}$ , 95% *CI*; -13, -1;  $P = 0.00$ ) and in N-BR ( $-26\pm 19 \text{ nM}\cdot\text{min}^{-1}$ , 95% *CI*; -38, -14)  
39 compared to N-PL ( $-1\pm 6 \text{ nM}\cdot\text{min}^{-1}$ , 95% *CI*; -5, 2;  $P = 0.00$ ). During moderate-intensity  
40 exercise, steady-state pulmonary  $\dot{V}\text{O}_2$  was lower in H-BR ( $1.91\pm 0.28 \text{ L}\cdot\text{min}^{-1}$ , 95% *CI*; 1.77,  
41 2.13) compared to H-PL ( $2.05\pm 0.25 \text{ L}\cdot\text{min}^{-1}$ , 95% *CI*; 1.93, 2.26,  $P = 0.02$ ) and  $\dot{V}\text{O}_2$  kinetics  
42 was faster in H-BR ( $\tau$ :  $24\pm 13 \text{ s}$ , 95% *CI*; 15, 32) compared to H-PL ( $31\pm 11 \text{ s}$ , 95% *CI*; 23,  
43 38;  $P = 0.04$ ).  $\text{NO}_3^-$  supplementation had no significant effect on  $\dot{V}\text{O}_2$  kinetics during severe-  
44 intensity exercise in hypoxia, or during moderate-intensity or severe-intensity exercise in  
45 normoxia. Tolerance to severe-intensity exercise was improved by  $\text{NO}_3^-$  in hypoxia (H-PL:  
46  $197\pm 28$ ; 95% *CI*; 173, 220 vs. H-BR:  $214\pm 43 \text{ s}$ , 95% *CI*; 177, 249;  $P = 0.04$ ) but not  
47 normoxia. The metabolism of  $\text{NO}_2^-$  during exercise is altered by  $\text{NO}_3^-$  supplementation,  
48 exercise and to a lesser extent, hypoxia. In hypoxia,  $\text{NO}_3^-$  supplementation enhances  $\dot{V}\text{O}_2$   
49 kinetics during moderate-intensity exercise and improves severe-intensity exercise tolerance.  
50 These findings may have important implications for individuals exercising at altitude.

51 **Key Words:** hypoxia; beetroot juice; nitric oxide, efficiency, performance.

52

## 53 **Introduction**

54 Nitric oxide (NO) is a ubiquitous, water soluble, free radical gas which plays a crucial role in  
55 many biological processes. Effective NO production is important in normal physiological  
56 functioning, from the regulation of blood flow, muscle contractility and mitochondrial  
57 respiration, to host defence, neurotransmission and glucose and calcium homeostasis (11, 17,  
58 60). NO production via the oxidation of L-arginine, in a process catalysed by nitric oxide  
59 synthase (NOS), may be blunted in conditions of reduced O<sub>2</sub> availability (52). It is now  
60 widely accepted that NO can also be generated via an alternative pathway, whereby inorganic  
61 nitrate (NO<sub>3</sub><sup>-</sup>) is reduced to nitrite (NO<sub>2</sub><sup>-</sup>) and further to NO. This NOS- and O<sub>2</sub>- independent  
62 NO<sub>3</sub><sup>-</sup> -NO<sub>2</sub><sup>-</sup> -NO pathway represents a complementary system for NO synthesis spanning a  
63 broad range of redox states (49). In addition to being produced endogenously, the body's  
64 NO<sub>3</sub><sup>-</sup> stores can be increased via the diet, with green leafy vegetables and beetroot being  
65 particularly rich in NO<sub>3</sub><sup>-</sup>. Upon ingestion, inorganic NO<sub>3</sub><sup>-</sup> is absorbed from the gut and passes  
66 into the systemic circulation where ~25% of it is concentrated in the saliva (50). Commensal  
67 bacteria in the oral cavity then reduce the NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup> (21). Some salivary NO<sub>2</sub><sup>-</sup> is converted  
68 into NO when swallowed into the acidic environment of the stomach (7), whilst the  
69 remainder is absorbed, increasing circulating plasma NO<sub>2</sub><sup>-</sup> concentration [NO<sub>2</sub><sup>-</sup>]. This NO<sub>2</sub><sup>-</sup>  
70 may be reduced to NO via a number of enzymatic and non-enzymatic pathways (e.g.,  
71 xanthine oxidoreductase and deoxyhemoglobin), which are potentiated in hypoxic  
72 environments, such as may be evident in contracting skeletal muscle (55).

73 NO plays a key role in the physiological response and adaptation to hypoxia. A reduced  
74 fraction of O<sub>2</sub> in inspired air results in reductions in arterial O<sub>2</sub> concentration and intracellular  
75 partial pressure of O<sub>2</sub> (PO<sub>2</sub>). The development of muscle hypoxia leads to increased  
76 metabolic perturbation (46) and reduced functional capacity at altitude (2) and in several  
77 disease conditions (22, 34). In order to restore sufficient O<sub>2</sub> supply, local blood flow is  
78 increased via hypoxia-induced vasodilatation with NO being implicated as a major mediator  
79 of this process (12). NO<sub>2</sub><sup>-</sup> may also promote hypoxic vasodilatation in an NO-independent  
80 manner (16).

81 Dietary NO<sub>3</sub><sup>-</sup> supplementation, in the form of nitrate salts and nitrate-rich beetroot juice (BR),  
82 represents a practical method of increasing circulating plasma [NO<sub>3</sub><sup>-</sup>] (31, 42, 67) and [NO<sub>2</sub><sup>-</sup>]  
83 (4, 33, 62). NO<sub>3</sub><sup>-</sup> supplementation has been shown to reduce resting blood pressure (3, 33, 42)  
84 and oxygen uptake ( $\dot{V}_{O_2}$ ) during submaximal exercise (4, 39, 40, 41, 62, 67), and to improve

85 exercise performance in young, healthy individuals exercising in normoxic conditions (14,  
86 38), but not necessarily in well trained athletes (5-6, 66). These changes may be related to  
87 NO-mediated alterations in mitochondrial efficiency (39), muscle contractile function (3, 28)  
88 and enhanced muscle blood flow, with preferential distribution to type II fibers (23). These  
89 physiological alterations could be particularly beneficial when normal O<sub>2</sub> availability (~21%)  
90 is reduced. Indeed, NO<sub>3</sub><sup>-</sup> supplementation in the form of BR has recently been shown to  
91 reduce muscle metabolic perturbation during exercise in hypoxia and to restore constant-  
92 work-rate exercise tolerance and post-exercise indices of oxidative function to values  
93 observed in normoxia (64). BR supplementation has also been shown to extend incremental  
94 exercise tolerance, improve arterial and skeletal muscle oxygenation (50), and to enhance  
95 cycling economy and time-trial performance (51), in hypoxia. However, while these studies  
96 suggest that BR can improve physiological responses and exercise performance in hypoxia, it  
97 has yet to be determined whether the effects BR are more pronounced in hypoxia relative to  
98 normoxia.

99 The dose-response and pharmacodynamic relationships of BR supplementation have recently  
100 been investigated in normoxia (67) and provides a guide to enable optimal timing and dosing  
101 of BR intake to elicit peak circulating plasma [NO<sub>2</sub><sup>-</sup>] values. However, the kinetics of plasma  
102 [NO<sub>2</sub><sup>-</sup>] during hypoxic exercise and subsequent recovery, and possible changes elicited by  
103 BR supplementation, are presently not known. It was recently reported that during high-  
104 intensity, intermittent running exercise, plasma [NO<sub>2</sub><sup>-</sup>] declined significantly during  
105 exhaustive exercise and showed a tendency to recover back to baseline following 15 min of  
106 passive rest (68). Previous research has reported increases (1, 54) but, more commonly,  
107 decreases (6, 19, 26, 42, 63) in plasma [NO<sub>2</sub><sup>-</sup>] during exercise. In addition to exercise, the  
108 metabolism of NO and its derivatives are known to be influenced by intracellular PO<sub>2</sub> and the  
109 fraction of inspired oxygen (FIO<sub>2</sub>). *In vitro*, endothelial NOS (eNOS) expression and eNOS-  
110 derived NO production in human endothelial cells are reduced in hypoxia (25, 53). However,  
111 *in vivo*, eNOS expression and activity can be up- or down-regulated by hypoxia, with both  
112 decreased (58) and increased (44, 48) NO bioavailability being reported in hypoxia.  
113 Characterizing the kinetic changes in [NO<sub>2</sub><sup>-</sup>] during exercise and recovery at different FIO<sub>2</sub>  
114 may offer insight into NO metabolism during exercise in normoxia and hypoxia. This  
115 understanding may have important implications for athletes exercising in hypoxic  
116 environments.

117 Considering that the  $\text{NO}_3^-$ - $\text{NO}_2^-$ - $\text{NO}$  pathway is facilitated in hypoxic conditions (48), we  
118 reasoned that BR supplementation may modulate the changes in  $[\text{NO}_2^-]$  during exercise and  
119 recovery and may help to ameliorate the negative effects of hypoxia on exercise tolerance.  
120 The primary aim of this study was to investigate the effects of BR supplementation on  
121 physiological responses (plasma  $[\text{NO}_2^-]$  dynamics, pulmonary  $\dot{V}\text{O}_2$  and muscle oxygenation)  
122 and exercise tolerance, in both normoxia and hypoxia. We hypothesized that the reduction of  
123  $[\text{NO}_2^-]$  during exercise would be greater in hypoxia compared to normoxia but that  $[\text{NO}_2^-]$   
124 would be higher at the same iso-time during exercise following BR compared to PL  
125 supplementation. We also hypothesized that BR supplementation would improve moderate-  
126 intensity exercise economy and severe-intensity exercise tolerance in both hypoxia and  
127 normoxia, with greater effects being evident in hypoxia.

## 128 **Methods**

### 129 *Subjects*

130 Twelve physically active male subjects (mean  $\pm$  SD; age =  $22 \pm 4$  yr, height =  $1.80 \pm 0.06$  m,  
131 body mass =  $78 \pm 6$  kg,  $\dot{V}\text{O}_{2\text{peak}} = 58.3 \pm 6.3$  mL $\cdot$ kg $^{-1}\cdot$ min $^{-1}$ ) volunteered to take part in this  
132 study. The protocol and procedures used in this study were approved by the Institutional  
133 Research Ethics Committee. All subjects gave written, fully informed consent prior to  
134 commencement of the study, once the experimental protocol, associated risks, and potential  
135 benefits of participation had been outlined. Subjects were instructed to arrive at the  
136 laboratory, at least 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding  
137 each testing session. Subjects were asked to refrain from caffeine and alcohol intake 6 and 24  
138 h before each test, respectively, and to consume the same light pre-exercise meal of their  
139 choice 4-5 h before testing. In addition to this, subjects were asked to abstain from using  
140 antibacterial mouthwash and chewing gum for the duration of the study since this has been  
141 shown to blunt the conversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  in the oral cavity (27). Subjects were also  
142 instructed to maintain their normal dietary intake for the duration of the study. All exercise  
143 tests were performed at the same time of day ( $\pm 1$  h) for each subject.

### 144 *Procedures*

145 Subjects were required to attend the laboratory on six occasions over a 4-wk period. All  
146 exercise tests were performed using an electronically braked cycle ergometer (Lode  
147 Excalibur Sport, Groningen, the Netherlands). During *visit 1*, subjects completed a ramp

148 incremental test to exhaustion for the determination of the maximal O<sub>2</sub> uptake ( $\dot{V}_{O_{2peak}}$ ) and  
149 the gas exchange threshold (GET). Subjects performed 3 min of baseline cycling at 20 W and  
150 80 rpm, after which the power output was increased at a rate of 30 W·min<sup>-1</sup> in a linear fashion  
151 until volitional exhaustion. The height and configuration of the saddle and handlebars were  
152 recorded and reproduced in subsequent tests. The breath-by-breath pulmonary gas-exchange  
153 data were collected continuously during the incremental test and averaged over 10-s periods.  
154  $\dot{V}_{O_{2peak}}$  was determined as the highest mean  $\dot{V}_{O_2}$  during any 30-s period. The GET was  
155 determined from a number of measurements, including: 1) the first disproportionate increase  
156 in CO<sub>2</sub> production ( $\dot{V}_{CO_2}$ ) from visual inspection of individual plots of  $\dot{V}_{CO_2}$  and  $\dot{V}_{O_2}$ ; and 2)  
157 an increase in expired ventilation ( $\dot{V}_E/\dot{V}_{O_2}$ ) with no increase in  $\dot{V}_E/\dot{V}_{CO_2}$ . Power outputs  
158 representing moderate- and severe-intensity exercise for each individual were calculated,  
159 with account taken of the mean response time for  $\dot{V}_{O_2}$  during ramp exercise (i.e., two-thirds  
160 of the ramp rate was deducted from the power output at GET).

161 All subjects were familiar with laboratory exercise testing procedures, having previously  
162 participated in studies employing cycle ergometry in our laboratory. *Visit 2* served as a  
163 familiarization to exercising in normobaric hypoxia. Following completion of the  
164 familiarization session, subjects were randomly assigned to receive 3 days of dietary  
165 supplementation with 140 ml·d<sup>-1</sup> of NO<sub>3</sub><sup>-</sup>-rich BR or 140 ml·d<sup>-1</sup> of NO<sub>3</sub><sup>-</sup>-depleted BR  
166 concentrate as a placebo (PL), (see ‘Supplementation’ below), prior to the subsequent  
167 exercise trials.

168 During *visits 3-6*, the subjects completed step-transition, cycling exercise for the  
169 determination of pulmonary  $\dot{V}_{O_2}$  and plasma [NO<sub>2</sub><sup>-</sup>] kinetics. In total, there were four  
170 different experimental conditions: 1) Hypoxia-BR (H-BR); 2) Hypoxia-PL (H-PL); 3)  
171 Normoxia-BR (N-BR); and 4) Normoxia-PL (N-PL). Trial order was randomly assigned in a  
172 balanced fashion such that three subjects started on H-BR, three started on H-PL, three  
173 started on N-BR and three started on the N-PL condition.

174 Upon arrival at the laboratory, a cannula (Insyte-W<sup>TM</sup> Becton-Dickinson, Madrid, Spain)  
175 was inserted into the subject’s antecubital vein to enable frequent blood sampling before,  
176 during and after the exercise protocol. Prior to the exercise protocol, subjects lay in a supine  
177 position for 10 min breathing normoxic inspire. A further 10-min period elapsed with  
178 subjects breathing either the hypoxic or normoxic inspire. The exercise protocol involved  
179 two 5-min bouts of moderate-intensity cycling at 80% GET, and one bout of severe-intensity

180 cycling at 75%  $\Delta$  (a power output representing GET plus 75% of the difference between the  
181 power outputs at GET and  $\dot{V}_{O_{2peak}}$ ) (65) which was continued to volitional exhaustion. Each  
182 exercise bout involved an abrupt transition to the target power output initiated from a 20 W  
183 baseline, with the three exercise bouts separated by 6 min of passive recovery. The severe-  
184 intensity exercise bout was continued until task failure as a measure of exercise tolerance.  
185 The time to exhaustion was recorded when the pedal rate fell by > 10 rpm below the 80 rpm  
186 pedal rate. In these bouts, the subjects were verbally encouraged to continue for as long as  
187 possible. Following exhaustion, a further 10-min recovery period elapsed with subjects  
188 continuing to breathe either the hypoxic or normoxic inspirate.

189 The  $\dot{V}_{O_2}$  responses for the two moderate bouts were averaged before analysis to reduce  
190 breath-to-breath noise and enhance confidence in the parameters derived from the modelling  
191 process (36). Blood was sampled pre-exercise (prior to any exercise and breathing of  
192 experimental inspirate), then during the baseline 20 W cycling preceding the first moderate  
193 transition (ModBL) and at 1 (Mod1), 3 (Mod3) and 5 (Mod5) min of the first moderate-  
194 intensity exercise bout. Further samples were drawn during the 20 W baseline preceding the  
195 severe transition (SevBL) and after 1 (Sev1) and 3 (Sev3) min of severe-intensity exercise  
196 and at exhaustion (Exh). Finally, samples were drawn during recovery from the severe bout at  
197 1.5 (Rec1.5), 3 (Rec3) and 10 (Rec10) min.

### 198 *Inspirate*

199 The inspirate was generated using a Hypoxico HYP 100 filtration system (Sporting Edge UK  
200 Ltd, Basingstoke, UK), with the generator supplying the inspirate via an extension conduit to  
201 a 150 L Douglas Bag (Cranlea & Co., Birmingham, UK). This acted as a reservoir and  
202 mixing chamber, and had a separate outlet tube feeding into a two-way breathing valve  
203 system (Hans Rudolph, Cranlea & Co.). The two-way valve was connected to the mouthpiece  
204 which provided a constant, unidirectional flow rate and ensured that no re-breathing of  
205 expired air occurred. The  $O_2$  and  $CO_2$  concentration of the inspirate was monitored during  
206 each test using a Servomex 5200 High Accuracy Paramagnetic  $O_2$  and  $CO_2$  Analyzer  
207 (Servomex, Crowborough, UK). The gas analyzer was calibrated prior to each test with a  
208 16.0%  $O_2$ , 8.0%  $CO_2$  and 76.0%  $N_2$  gas mix (BOC Special Gases, Guildford, UK). For the N-  
209 PL and N-BR trials, the Hypoxico HYP-100 generator was switched to normoxic mode (i.e.  
210 all  $O_2$  filters were turned off so that no  $O_2$  was removed from the ambient air). However,



211 during the H-PL and H-BR trials, the generator was set to maximum O<sub>2</sub> filtration, which  
212 supplied an FIO<sub>2</sub> of 0.131 ± 0.02, and an FICO<sub>2</sub> of 0.004 ± 0.00.

### 213 *Supplementation*

214 After completion of the non-supplemented *visits 1 and 2*, subjects were assigned in a double-  
215 blind, randomized, crossover design to receive a course of dietary NO<sub>3</sub><sup>-</sup> supplementation  
216 before *visits 3-6*. The supplements were either concentrated, NO<sub>3</sub><sup>-</sup>-rich BR (2 x 70 mL·d<sup>-1</sup> of  
217 BR providing ~8.4 mmol NO<sub>3</sub><sup>-</sup> per day; Beet it, James White Drinks, Ipswich, UK) or  
218 concentrated, NO<sub>3</sub><sup>-</sup>-depleted PL (2 x 70 mL·d<sup>-1</sup> of PL providing ~0.006 mmol NO<sub>3</sub><sup>-</sup> per day;  
219 Beet it, James White Drinks, Ipswich, UK). The PL beverage was created by passing the  
220 juice, before pasteurization, through a column containing Purolite A520E ion exchange resin,  
221 which selectively removes nitrate ions. The PL was identical to the BR in appearance, taste  
222 and smell. Subjects were instructed to consume the beverages in the morning and afternoon  
223 of days 1 and 2 of supplementation, and then in the morning and 2.5 h before the exercise test  
224 on day 3. A washout period of at least 72 h separated each supplementation period. Subjects  
225 were instructed to follow their normal dietary habits throughout the testing period and to  
226 replicate their diet and timing of supplementation across conditions. Subjects were informed  
227 that the supplementation may cause beeturia (red urine) and red stools temporarily but that  
228 this side effect was harmless.

### 229 *Measurements*

230 Blood samples were drawn into 5-ml lithium-heparin tubes (Vacutainer, Becton-Dickinson,  
231 New Jersey, USA). 200 µl of blood was immediately hemolyzed in 200 µl of cold Triton X-  
232 100 buffer solution (Triton X-100, Amresco, Salon, OH) and analyzed to determine blood  
233 [lactate] and [glucose] (YSI 2300, Yellow Springs Instruments, Yellow Springs, OH). Blood  
234 samples for the determination of plasma [NO<sub>2</sub><sup>-</sup>] and [NO<sub>3</sub><sup>-</sup>] were collected into lithium-  
235 heparin tubes and immediately centrifuged at 4000 rpm and 4 °C for 8 min. Plasma was  
236 extracted and immediately frozen at -80 °C for later analysis of [NO<sub>2</sub><sup>-</sup>] and [NO<sub>3</sub><sup>-</sup>].

237 Prior to and regularly during analysis, all glassware, utensils, and surfaces were rinsed with  
238 deionized water to remove any residual NO<sub>2</sub><sup>-</sup>. Plasma [NO<sub>2</sub><sup>-</sup>] and [NO<sub>3</sub><sup>-</sup>] were analysed using  
239 gas phase chemiluminescence. This initially required NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> to be reduced to NO gas.  
240 For reduction of NO<sub>2</sub><sup>-</sup>, undiluted plasma was injected into a glass purge vessel containing 5  
241 ml glacial acetic acid and 1ml NaI solution. For NO<sub>3</sub><sup>-</sup> reduction, plasma samples were

242 deproteinized in an aqueous solution of zinc sulphate (10% w/v) and 1 M sodium hydroxide,  
243 prior to reduction to NO in a solution of vanadium (III) chloride in 1 M hydrochloric acid  
244 (0.8% w/v). Quantification of NO was enabled by the detection of light emitted during the  
245 production of nitrogen dioxide formed upon reaction of NO with ozone. Luminescence was  
246 detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a  
247 Sievers gas-phase chemiluminescence NO analyzer (Sievers NOA 280i, Analytix Ltd,  
248 Durham, UK). The concentrations of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  were determined by plotting signal area  
249 (mV) against a calibration plot of 25nM to 1 $\mu$ M sodium nitrite and 100nM to 10 $\mu$ M sodium  
250 nitrate respectively. The rate of change in plasma  $[\text{NO}_2^-]$  during the severe exercise bout was  
251 calculated as the difference between pre-exercise baseline and exercise  $[\text{NO}_2^-]$  values relative  
252 to exercise duration.

253 During all laboratory exercise tests, pulmonary gas exchange and ventilation were measured  
254 continuously with subjects wearing a nose clip and breathing through a mouthpiece and  
255 impeller turbine assembly (Triple V, Jaeger, Hoechburg, Germany). The inspired and expired  
256 gas volume and gas concentration signals were continuously sampled at 100 Hz, the latter  
257 using paramagnetic ( $\text{O}_2$ ) and infrared ( $\text{CO}_2$ ) analyzers (Oxycon Pro, Jaeger, Hoechburg,  
258 Germany) via a capillary line connected to the mouthpiece. Pulmonary gas exchange  
259 variables were calculated and displayed breath-by-breath. Heart rate (HR) and arterial oxygen  
260 saturation ( $\text{SaO}_2$ ) were continuously measured during the test protocol using a pulse oximeter  
261 device (Rad-87, Masimo, Irvine, CA), which was attached to the subject's right index finger.

262 The oxygenation status of the *m. vastus lateralis* of the right leg was monitored via near  
263 infrared spectroscopy (NIRS) (NIRO 200, Hamamatsu Photonics KK, Hamamatsu-City,  
264 Japan) during the exercise protocol, as described previously (4). Deoxyhemoglobin  
265 concentration ( $[\text{HHb}]$ ), oxyhemoglobin concentration ( $[\text{HbO}_2]$ ), total hemoglobin  
266 concentration ( $[\text{Hb}_{\text{tot}}]$ ) and tissue oxygenation index (TOI) were measured.

#### 267 *Data analysis*

268 The breath-by-breath  $\dot{V}_{\text{O}_2}$  data from each exercise test were initially examined to exclude  
269 errant breaths caused by coughing and swallowing with those values lying more than four SD  
270 from the local mean being removed. The breath-by-breath data were subsequently linearly  
271 interpolated to provide second-by-second values, and, for each individual, identical  
272 moderate-intensity repetitions were time-aligned to the start of exercise and ensemble-  
273 averaged. This approach enhances the signal-to-noise ratio and improves confidence in the

274 parameters derived from the modelling process. The first 20 s of data after the onset of  
275 exercise (the phase I response) were deleted, and a non-linear least squares algorithm was  
276 used to fit the data thereafter. A single-exponential model was used to characterize the phase  
277 II  $\dot{V}_{O_2}$  responses to both moderate- and severe- intensity exercise, as described in following  
278 equation:

$$279 \quad \dot{V}_{O_2}(t) = \dot{V}_{O_2 \text{ baseline}} + A_p [1 - e^{-(t-TD_p/\tau_p)}] \quad \text{Eqn.1}$$

280 Where  $\dot{V}_{O_2}(t)$  represents the absolute  $\dot{V}_{O_2}$  at a given time  $t$ ;  $\dot{V}_{O_2 \text{ baseline}}$  represents the mean  $\dot{V}_{O_2}$   
281 over the final 60 s of baseline cycling;  $A_p$ ,  $TD_p$ , and  $\tau_p$  represent the amplitude, time delay  
282 and time constant, respectively, describing the phase II increase in  $\dot{V}_{O_2}$  above baseline. An  
283 iterative process was used to minimize the sum of the squared errors between the fitted  
284 function and the observed values. The end-exercise  $\dot{V}_{O_2}$  was defined as the mean  $\dot{V}_{O_2}$   
285 measured over the final 30 s of exercise.

286 The fitting strategy was subsequently used to identify the onset of any ‘slow component’ in  
287 the  $\dot{V}_{O_2}$  response to severe-intensity exercise as previously described (56). The fitting window  
288 was lengthened iteratively until the exponential model-fit demonstrated a discernible  
289 departure from the measured response profile. Identification, via visual inspection, of the flat  
290 residual plot profile (signifying a good fit to measured data) systematically differing from  
291 zero, gave indication of the delayed slow component onset. The magnitude of the slow  
292 component for  $\dot{V}_{O_2}$  was measured as the difference between the phase II steady state  
293 amplitude and the final  $\dot{V}_{O_2}$  value, averaged over the last 30 s of exercise.

294 To obtain information on muscle oxygenation, the [HHb] response to exercise was also  
295 modelled, as described previously (4). The [HHb] kinetics for moderate- and severe-intensity  
296 exercise were determined using a single-exponential model similar to that described above  
297 (Eqn. 1), with the exception that the fitting window commenced at the time at which the  
298 [HHb] signal increased 1 SD above the baseline mean (18). For moderate-intensity exercise,  
299 the fitting window was constrained to the point at which mono-exponentiality became  
300 distorted, consequent to a gradual fall in [HHb], as determined by visual inspection of the  
301 residual plots. For severe-intensity exercise, the [HHb] fast and slow phase responses were  
302 determined as described above for the  $\dot{V}_{O_2}$ . The [HbO<sub>2</sub>], [Hb<sub>tot</sub>] and TOI responses were not  
303 modelled as they do not approximate an exponential. Rather, the changes in these variables  
304 were assessed by determining the [HbO<sub>2</sub>], [Hb<sub>tot</sub>] and TOI at baseline (60 s preceding step

305 transition), at 120 s and at end-exercise during moderate exercise and at baseline, 60 s, 120 s  
306 and exhaustion for severe exercise.

### 307 *Statistical analyses*

308 Differences in the cardio-respiratory, NIRS-derived, pulse-oximetry and exercise tolerance  
309 variables between conditions were analyzed using two-way (supplement x FIO<sub>2</sub>) repeated  
310 measures ANOVA. Blood metabolites were analyzed via two-way (condition x time)  
311 repeated measures ANOVA, during moderate-, severe-intensity- and in recovery from-  
312 exercise (Condition refers to H-BR, H-PL, N-BR or N-PL). Significant effects were further  
313 explored using simple contrasts with Fisher's LSD. One-tailed paired *t*-tests were used to  
314 compare differences in exercise tolerance between BR and PL treatments in hypoxia and  
315 normoxia. Correlations were assessed via Pearson's product-moment correlation coefficient  
316 between physiological and performance variables. All data are presented as mean ± SD with  
317 statistical significance being accepted when  $P < 0.05$ .

### 318 **Results**

319 Self-reported compliance to the supplementation regimen was 100% and subjects' food  
320 diaries confirmed that the timing of supplement taken on the morning of the laboratory tests  
321 was consistent across the experimental conditions. No deleterious side-effects were reported.

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

323 Pre-exercise, plasma [NO<sub>2</sub><sup>-</sup>] was significantly elevated in H-BR compared to H-PL (H-BR:  
324 301 ± 89 vs. H-PL: 88 ± 56 nM;  $P = 0.02$ ) and N-BR relative to N-PL (N-BR: 401 ± 276 vs.  
325 N-PL: 61 ± 28 nM;  $P = 0.01$ ) but did not differ between H-BR and N-BR ( $P = 0.54$ ) or H-PL  
326 and N-PL ( $P = 0.66$ ).

327 Plasma [NO<sub>3</sub><sup>-</sup>] was significantly elevated at all time-points following BR compared to PL in  
328 both hypoxia and normoxia although no differences were evident in the kinetic response  
329 during exercise and recovery (data not shown).

330 The group mean kinetic profiles of plasma [NO<sub>2</sub><sup>-</sup>] during moderate- and severe- intensity  
331 exercise and subsequent recovery are presented in Figure 1.

332 *Moderate exercise.* ANOVA revealed there were significant main effects by condition and  
333 time on plasma [NO<sub>2</sub><sup>-</sup>] during moderate-intensity exercise. BR supplementation significantly

334 elevated plasma  $[\text{NO}_2^-]$  across all time points compared to PL in both hypoxic and normoxic  
335 conditions (all  $P < 0.05$ ). In N-BR, plasma  $[\text{NO}_2^-]$  was significantly decreased after 5 min of  
336 moderate-intensity exercise (Mod5) compared to ModBL (ModBL:  $332 \pm 184$  vs. Mod5:  $290$   
337  $\pm 207$  nM,  $P = 0.04$ ). However, the decrease in plasma  $[\text{NO}_2^-]$  in H-BR only showed a trend  
338 towards a reduction (ModBL:  $306 \pm 109$  vs. Mod5:  $270 \pm 125$  nM,  $P = 0.10$ ). The rate of  
339 decline in plasma  $[\text{NO}_2^-]$  from ModBL to Mod5 was not significantly different in H-BR ( $-7 \pm$   
340  $11.7$  nM $\cdot$ min $^{-1}$ ) compared to N-BR ( $-10.6 \pm 15.9$  nM $\cdot$ min $^{-1}$ ), H-PL ( $-3.9 \pm 6.1$  nM $\cdot$ min $^{-1}$ )  
341 compared to N-PL ( $-2.1 \pm 4$  nM $\cdot$ min $^{-1}$ ), H-BR ( $-7 \pm 11.7$  nM $\cdot$ min $^{-1}$ ) compared to N-PL ( $-2.1 \pm$   
342  $4$  nM $\cdot$ min $^{-1}$ ) or N-BR ( $-10.6 \pm 15.9$  nM $\cdot$ min $^{-1}$ ) compared to N-PL ( $-2.1 \pm 4$  nM $\cdot$ min $^{-1}$ ).

343 *Severe exercise.* There were significant main effects by condition and time and an interaction  
344 effect for plasma  $[\text{NO}_2^-]$  during severe-intensity exercise to exhaustion. BR supplementation  
345 significantly elevated plasma  $[\text{NO}_2^-]$  across all time points compared to PL in both hypoxic  
346 and normoxic conditions (all  $P < 0.05$ ). In N-BR, plasma  $[\text{NO}_2^-]$  significantly decreased after  
347 3 min of severe-intensity exercise (Sev3) and at exhaustion, compared to SevBL (SevBL:  $271$   
348  $\pm 177$ ; Sev3:  $206 \pm 129$ ;  $P = 0.01$ ; Exhaustion:  $132 \pm 117$  nM,  $P = 0.00$ ). In H-BR, plasma  
349  $[\text{NO}_2^-]$  decreased from SevBL ( $277 \pm 142$  nM) to Sev1 ( $229 \pm 123$  nM,  $P = 0.01$ ), Sev3  
350 ( $n=10$ ,  $164 \pm 64$  nM,  $P = 0.03$ ) and exhaustion ( $171 \pm 115$  nM,  $P = 0.00$ ). The absolute  
351 decline in plasma  $[\text{NO}_2^-]$  from SevBL to exhaustion showed a trend toward being smaller in  
352 H-BR ( $106 \pm 60$  nM) compared to N-BR ( $138 \pm 79$  nM,  $P = 0.10$ ). In N-PL, plasma  $[\text{NO}_2^-]$   
353 decreased from SevBL ( $40 \pm 23$  nM) to exhaustion ( $22 \pm 19$  nM,  $P = 0.02$ ). This decrease  
354 was not significant in H-PL (SevBL:  $53 \pm 65$  vs. Exhaustion:  $37 \pm 45$  nM,  $P = 0.52$ ). The  
355 rate of decline in plasma  $[\text{NO}_2^-]$  was significantly greater from SevBL to exhaustion in H-BR  
356 compared to H-PL (H-BR:  $-30 \pm 22$  vs. H-PL:  $-7 \pm 10$  nM $\cdot$ min $^{-1}$ ,  $P = 0.00$ ) and in N-BR  
357 compared to N-PL (N-BR:  $-26 \pm 19$  vs. N-PL:  $-1 \pm 6$  nM $\cdot$ min $^{-1}$ ,  $P = 0.00$ ), but was not  
358 different between N-BR and H-BR ( $P = 0.66$ ) or N-PL and H-PL ( $P = 0.13$ ), (Figure 1).

359 *Recovery.* During the 10-min recovery from exhaustive exercise, ANOVA revealed  
360 significant main effects by condition and time and an interaction effect for plasma  $[\text{NO}_2^-]$   
361 (Figure 1). BR supplementation significantly elevated plasma  $[\text{NO}_2^-]$  across all time points  
362 compared to PL in both hypoxic and normoxic conditions (all  $P < 0.05$ ). In N-BR, plasma  
363  $[\text{NO}_2^-]$  was significantly lower at exhaustion compared to 3 min into the recovery period ( $P =$   
364  $0.05$ ), with a significant difference also evident between Rec1.5 and Rec3 ( $P = 0.01$ ). Plasma  
365  $[\text{NO}_2^-]$  was significantly higher in H-BR compared to N-BR at Rec1.5 ( $P = 0.04$ ). In N-PL,  
366 recovery of plasma  $[\text{NO}_2^-]$  was evident between exhaustion and Rec10 ( $P = 0.04$ ), with a

367 significant increase in  $[\text{NO}_2^-]$  from Rec3 to Rec10 also evident ( $P = 0.04$ ). In H-PL, plasma  
368  $[\text{NO}_2^-]$  tended to recover between Rec1.5 and Rec3 ( $P = 0.06$ ), with a further increase evident  
369 between Rec3 and Rec10 ( $P < 0.00$ ).

370 Blood [glucose] was significantly reduced in H-BR compared to N-BR at Rec1.5 (H-BR:  $4.3$   
371  $\pm 1.0$  mmol·L vs. N-BR:  $5.5 \pm 1.2$  mmol·L;  $P = 0.01$ ), Rec3 (H-BR:  $4.5 \pm 1.1$  mmol·L vs. N-  
372 BR:  $5.6 \pm 1.3$  mmol·L;  $P = 0.02$ ) and Rec10 (H-BR:  $4.7 \pm 1.0$  mmol·L vs. N-BR:  $5.3 \pm 1.0$   
373 mmol·L;  $P = 0.03$ ). No differences were evident between PL and BR conditions.

374

### 375 *Arterial O<sub>2</sub> saturation and heart rate*

376 The SaO<sub>2</sub> data at rest and during moderate- and severe-intensity exercise are reported in  
377 Table 1. Resting SaO<sub>2</sub> and HR prior to the administration of inspirate were not significantly  
378 different between conditions. However, ANOVA revealed a significant main effect by FIO<sub>2</sub>  
379 following 10 min of breathing the hypoxic or normoxic inspirate, with SaO<sub>2</sub> being  
380 significantly reduced in H-PL compared to N-PL ( $P = 0.00$ ) and H-BR compared to N-BR ( $P$   
381  $= 0.00$ ). HR was significantly elevated in H-PL compared to N-PL ( $P = 0.00$ ) and H-BR  
382 compared to N-BR ( $P = 0.02$ ) in the final 30 s of gas inspiration.

383 *Moderate exercise.* During moderate-intensity exercise, SaO<sub>2</sub> was significantly reduced in  
384 both hypoxic conditions compared to the normoxic conditions (both  $P = 0.00$ ) (Table 1). HR  
385 was significantly elevated in both hypoxic conditions compared to the normoxic conditions in  
386 the final 30 s of exercise (both  $P = 0.00$ ), with H-BR being lower than H-PL ( $P = 0.05$ ) over  
387 the entire 6-min duration.

388 *Severe exercise.* SaO<sub>2</sub> was significantly lower in H-PL compared to N-PL ( $P = 0.00$ ) and in  
389 H-BR compared to N-BR ( $P = 0.00$ ) at exhaustion following severe-intensity exercise. There  
390 were no differences in SaO<sub>2</sub> between BR and PL in either hypoxia or normoxia. Also, there  
391 were no differences in HR between conditions (Table 1).

### 392 *$\dot{V}_{\text{O}_2}$ kinetics*

393 Pulmonary  $\dot{V}_{\text{O}_2}$  responses across the four experimental conditions are presented in Figures 2  
394 and 3, and the parameters derived from the model fits are summarized in Table 2.

395 *Moderate exercise.* ANOVA revealed a significant main effect by supplement and an  
396 interaction effect on the  $\dot{V}_{\text{O}_2}$  response to moderate-intensity exercise. The  $\dot{V}_{\text{O}_2}$  in the final 30 s

397 of exercise in H-BR was significantly lower compared to H-PL ( $P = 0.02$ ) and N-PL ( $P =$   
398  $0.01$ ). BR supplementation also resulted in a reduced  $\dot{V}_{O_2}$  during baseline (20 W) exercise in  
399 hypoxia compared to PL ( $P = 0.02$ ). The  $\dot{V}_{O_2}$  phase II  $\tau$  tended to be increased (i.e., slower  
400 kinetics) in hypoxia ( $P = 0.07$ ). Post-hoc analyses revealed that the  $\dot{V}_{O_2}$  phase II  $\tau$  was smaller  
401 (i.e., faster kinetics) in H-BR compared to H-PL ( $P = 0.04$ ).

402 *Severe exercise.* During severe-intensity exercise, the  $\dot{V}_{O_2}$  slow component amplitude ( $P =$   
403  $0.00$ ) and  $\dot{V}_{O_2}$  at exhaustion ( $P = 0.00$ ) were significantly reduced as a result of the hypoxic  
404 inspire in both PL and BR (Table 2). In hypoxia, BR tended to further reduce the end-  
405 exercise  $\dot{V}_{O_2}$  compared to H-PL ( $P = 0.07$ ), while BR had no effect upon end-exercise  $\dot{V}_{O_2}$  in  
406 normoxia.

#### 407 *NIRS*

408 The [HHb], [HbO<sub>2</sub>], [Hb<sub>tot</sub>] and TOI values measured during moderate- and severe-intensity  
409 exercise are shown in Table 3.

410 *Moderate exercise.* During moderate-intensity exercise, ANOVA revealed a significant main  
411 effect by FIO<sub>2</sub>. The modelled [HHb] amplitude was significantly greater in hypoxia  
412 compared to normoxia in both supplemented conditions across all time points (all  $P < 0.05$ ).  
413 The end-exercise [HbO<sub>2</sub>] was lower in H-BR compared to N-BR ( $P = 0.02$ ) and H-PL  
414 compared to N-PL ( $P = 0.01$ ). TOI at baseline and throughout exercise was also significantly  
415 reduced in hypoxia compared to normoxia ( $P < 0.05$ ). Post-hoc analyses revealed that BR  
416 tended to offset the negative effects of hypoxia on TOI when compared with PL ( $P = 0.08$ ).

417 *Severe exercise.* During severe-intensity exercise, ANOVA revealed a significant main effect  
418 by FIO<sub>2</sub>. [HHb] was significantly increased in H-BR and H-PL compared to N-BR and N-PL  
419 ( $P < 0.05$ ), whereas the [HHb] slow phase amplitude was larger in normoxia compared to  
420 hypoxia ( $P < 0.05$ ). [HbO<sub>2</sub>] was reduced in hypoxia compared to normoxia ( $P < 0.05$ ) and  
421 TOI was lower as a result of hypoxia throughout exercise ( $P < 0.05$ ). No differences in NIRS  
422 data between BR and PL were evident during severe-intensity exercise.

#### 423 *Exercise tolerance*

424 ANOVA revealed that hypoxia resulted in a significant reduction in exercise tolerance when  
425 compared to normoxia in both PL (H-PL:  $197 \pm 28$  vs. N-PL:  $431 \pm 124$  s,  $P = 0.00$ ) and BR  
426 conditions (H-BR:  $214 \pm 43$  vs. N-BR  $412 \pm 139$  s,  $P = 0.00$ ). Although the unspecific  $F$ -test

427 for interaction effect across all four conditions did not attain significance at the 95% level, it  
428 should be noted that the specific test for a difference between exercise tolerance in H-BR and  
429 H-PL was significant (H-BR:  $214 \pm 43$  vs. H-PL:  $197 \pm 28$  s,  $P = 0.04$ ), whereas the  
430 comparison between N-BR and N-PL was not (N-BR:  $412 \pm 139$  vs. N-PL:  $431 \pm 124$  s,  $P =$   
431  $0.50$ ). The change in severe-intensity exercise tolerance was correlated with the change in  
432 moderate steady-state  $\dot{V}_{O_2}$  following BR supplementation in hypoxia ( $r = -0.96$ ;  $P = 0.00$ ).

### 433 **Discussion**

434 Consistent with previous findings, the decline of plasma  $[\text{NO}_2^-]$  during exercise was greater  
435 following BR compared to PL supplementation. However, in contrast to our experimental  
436 hypothesis, the decline of plasma  $[\text{NO}_2^-]$  during exercise was similar or slightly smaller in  
437 hypoxia compared to normoxia. Nonetheless, 3 days of BR supplementation significantly  
438 speeded  $\dot{V}_{O_2}$  kinetics and lowered the steady-state  $\dot{V}_{O_2}$  during moderate-intensity cycle  
439 exercise in hypoxia, but not normoxia. Furthermore, BR supplementation improved severe-  
440 intensity exercise tolerance in hypoxia ( $P < 0.05$ ), but not normoxia ( $P > 0.05$ ). These  
441 findings suggest that BR is more effective at improving exercise economy and exercise  
442 tolerance in hypoxia than normoxia.

#### 443 *Effects of BR supplementation on the kinetic profile of plasma $[\text{NO}_2^-]$*

444 Plasma  $[\text{NO}_2^-]$  increased significantly following BR supplementation compared with PL, at  
445 rest and prior to administration of the inspirate. These findings are consistent with previous  
446 research which has consistently reported elevations in plasma  $[\text{NO}_2^-]$  (3, 4, 33, 34, 51, 62,  
447 67), following BR supplementation.

448 Previous studies have suggested that baseline plasma  $[\text{NO}_2^-]$  and/or the change in the  
449 concentrations of this metabolite during exercise may be associated with exercise  
450 performance (19, 53, 61, 68). This study is the first to characterise  $[\text{NO}_2^-]$  dynamics during  
451 and following exercise of different intensities in hypoxia and normoxia with and without  
452  $\text{NO}_3^-$  supplementation. The results suggest that the metabolism of NO and its derivatives are  
453 altered by exercise and  $\text{NO}_3^-$  supplementation and, to a lesser extent,  $\text{FIO}_2$ . The interpretation  
454 of these data is not straightforward, however.  $\text{NO}_3^-$  can be reduced *in vivo* to bioactive  $\text{NO}_2^-$   
455 and further to NO (47) and this reduction of  $\text{NO}_2^-$  to NO is expected to be facilitated in  
456 hypoxia (13). However,  $\text{NO}_2^-$  is also an oxidation product of NO generation via the NOS  
457 pathway (30) with plasma  $[\text{NO}_2^-]$  providing a sensitive marker of NO production through



458 NOS (43). Therefore, the dynamics of plasma  $[\text{NO}_2^-]$  over the exercise bouts is likely  
459 reflective of the dynamic balance between NOS-derived NO and  $\text{NO}_2^-$  reduction to NO. In  
460 the present study, plasma  $[\text{NO}_2^-]$  declined during both moderate- and severe-intensity  
461 exercise (Figure 1) with the magnitude and rate of plasma  $[\text{NO}_2^-]$  decline being significantly  
462 greater in the BR trials compared to PL trials, in both normoxia and hypoxia. These findings  
463 suggest that the reduction of  $\text{NO}_2^-$  to NO appeared to outweigh the synthesis of NO through  
464 NOS during exercise.

465 The rate of plasma  $[\text{NO}_2^-]$  decline over the 5-min moderate-intensity bout was not  
466 significantly different between N-BR and H-BR, and N-PL and H-PL. However, following  
467 5-min of moderate-intensity exercise, plasma  $[\text{NO}_2^-]$  had fallen significantly below ModBL in  
468 N-BR; whereas, there was only a trend for a lower plasma  $[\text{NO}_2^-]$  in H-BR. Similarly, the rate  
469 of plasma  $[\text{NO}_2^-]$  decline over the severe-intensity exercise bout was not significantly  
470 different between N-BR and H-BR or N-PL and H-PL, but the absolute fall in plasma  $[\text{NO}_2^-]$   
471 tended to be less in H-BR than in N-BR, in spite of a longer exercise duration in N-BR. These  
472 results are contrary to our hypothesis and suggest that, in hypoxia, the contribution of NOS to  
473 NO production (30), and subsequently to the regulation of muscle perfusion and matching of  
474  $\text{O}_2$  supply, may be greater (12).

475 During the 10-min passive recovery from exhaustive exercise, plasma  $[\text{NO}_2^-]$  increased in a  
476 similar fashion in H-PL and N-PL. Specifically, plasma  $[\text{NO}_2^-]$  increased after 3 min of  
477 recovery and plateaued after 10 min. The increases in plasma  $[\text{NO}_2^-]$  may represent an  
478 increase in NO oxidation (as NO is continuing to contribute to muscle perfusion and  
479 matching of  $\text{O}_2$  supply and demand; 12) during recovery. Following BR supplementation, the  
480 recovery profile of plasma  $[\text{NO}_2^-]$  was slightly different between normoxia and hypoxia.  
481 Plasma  $[\text{NO}_2^-]$  was higher in H-BR than N-BR following 1.5 min of recovery, although the  
482 difference between Exh and 1.5Rec was not different between conditions. It is important to  
483 note that differences in plasma  $[\text{NO}_2^-]$  dynamics between hypoxia and normoxia were not  
484 substantial either during exercise or in recovery.

#### 485 *Effects of BR supplementation on the physiological response to moderate-intensity exercise*

486 BR supplementation significantly reduced the  $\text{O}_2$  cost of sub-maximal cycle exercise in  
487 hypoxia.  $\dot{V}\text{O}_2$  during baseline cycling in H-BR was reduced by 10% compared to H-PL and  
488 by 4% compared to N-PL. Furthermore, a 7% reduction in the end-exercise (steady-state)  $\dot{V}\text{O}_2$   
489 was found in H-BR compared to H-PL. These findings are consistent with previous studies

490 which have reported reductions in submaximal cycling  $\dot{V}_{O_2}$  in varying severities of hypoxia.  
491 For example, Masschelein et al. (50) reported a 4% reduction in steady state  $\dot{V}_{O_2}$  with an FIO<sub>2</sub>  
492 of 0.11 during cycle exercise at 45% peak  $\dot{V}_{O_2}$  and Muggeridge et al. (51) reported a ~6-8%  
493 reduction in steady-state  $\dot{V}_{O_2}$  at an FIO<sub>2</sub> of 0.15 during cycle exercise at 60% of maximum  
494 work rate, following BR supplementation. A reduction in muscle metabolic perturbation (i.e.  
495 slower rates of change of muscle pH and phosphocreatine (PCr) and inorganic phosphate  
496 concentrations) during severe-intensity knee-extensor exercise in hypoxia has also been  
497 reported following BR supplementation (64).

498 In the present study, the  $\dot{V}_{O_2}$  phase II  $\tau$  during moderate-intensity exercise was reduced by BR  
499 supplementation in hypoxia. This finding is consistent with a recent study in older  
500 individuals, where the  $\dot{V}_{O_2}$  mean response time was speeded with BR supplementation (32).  
501 This may be related to the slower  $\dot{V}_{O_2}$  kinetics that is typically found in older individuals and  
502 the potential to abate this through enhancing muscle O<sub>2</sub> delivery (57), via increasing NO  
503 bioavailability. Similarly, hypoxia tended to slow  $\dot{V}_{O_2}$  kinetics in the young healthy  
504 participants in the present study. Specifically, the phase II  $\tau$  tended to be slowed in hypoxia  
505 compared to normoxia (from ~22 to ~31 s; Table 2). This observation is consistent with  
506 previous reports of slower  $\dot{V}_{O_2}$  kinetics in hypoxia (29, 59). BR supplementation speeded the  
507 phase II  $\tau$  in hypoxia toward values recorded in normoxia, thereby helping to reverse the  
508 detrimental effect of a reduced FIO<sub>2</sub> on  $\dot{V}_{O_2}$  kinetics. These findings are consistent with a  
509 recent study which showed that muscle PCr recovery kinetics, which reflects the maximal  
510 rate of mitochondrial ATP resynthesis and is influenced by O<sub>2</sub> availability, were speeded by  
511 BR supplementation in hypoxia (64). These data suggest that, in addition to reducing O<sub>2</sub>  
512 demand during exercise (50, 51, present study), BR may enhance skeletal muscle O<sub>2</sub>  
513 availability in hypoxia.

514 In contrast to some (3, 4, 14, 40, 41, 62), but not all (5, 8, 32, 65), previous studies, 3-days of  
515 BR supplementation did not significantly reduce  $\dot{V}_{O_2}$  during sub-maximal exercise in  
516 normoxia. Previous studies have typically reported reductions in steady state  $\dot{V}_{O_2}$  of ~3-5%  
517 following several days of NO<sub>3</sub><sup>-</sup> supplementation (4, 40, 62). The mechanistic bases for this  
518 lower O<sub>2</sub> cost of exercise have been suggested to include improved mitochondrial efficiency  
519 (39) and/or reductions in the ATP cost of muscle force production (3) which may be linked to  
520 enhanced Ca<sup>2+</sup>-related muscle contractility (28). NO is involved in the regulation of  
521 mitochondrial O<sub>2</sub> consumption and it is well established that NO has a strong affinity for  
522 cytochrome-*c* oxidase (COX) (9). It has been suggested that competition for the COX binding

523 site between NO and O<sub>2</sub> may be responsible, in part, for the reduced O<sub>2</sub> cost of exercise  
524 following NO<sub>3</sub><sup>-</sup> supplementation (4, 41), with this initiating a signalling cascade resulting in  
525 mitochondrial protein changes which collectively enhance respiratory chain efficiency (39).  
526 Interestingly, hypoxia, *per se*, may also result in an acute, reversible inhibition of COX (10).  
527 The combination of hypoxia and BR supplementation may therefore make it more likely for  
528 these effects to be manifest. It is also noteworthy that reductions in  $\dot{V}_{O_2}$  during moderate-  
529 intensity exercise were recently reported to be evident following acute supplementation with  
530 16.8 mmol NO<sub>3</sub><sup>-</sup> (4 x 70 ml BR shots), tended to be evident with 8.4 mmol NO<sub>3</sub><sup>-</sup> (2 x 70 ml  
531 BR shots), but were not evident with 4.2 mmol NO<sub>3</sub><sup>-</sup> (1 x 70 ml BR shot) (67). It is therefore  
532 possible that an insufficient NO<sub>3</sub><sup>-</sup> dose was consumed immediately prior to the tests to  
533 significantly influence the  $\dot{V}_{O_2}$  response to exercise in normoxia in the present study.  
534 Furthermore, the inter-individual differences in the  $\dot{V}_{O_2}$  response to exercise in normoxia  
535 evident in the current study, may have also contributed to the lack of statistically significant  
536 effects. It may be concluded that BR supplementation can (3, 4, 14, 40, 41, 62), but does not  
537 always (present study, 5, 8, 32, 66), alter the O<sub>2</sub> cost of exercise in normoxia.

538 Indices of muscle oxygenation measured with NIRS were altered as a result of the  
539 manipulation of FIO<sub>2</sub> during moderate-intensity exercise but BR supplementation did not  
540 significantly influence this response. Consistent with a previous study (49), [HHb] was  
541 greater in hypoxia indicating that muscle fractional O<sub>2</sub> extraction was increased, while  
542 [HbO<sub>2</sub>] and TOI were significantly reduced in hypoxia compared to normoxia. Although not  
543 significant, BR supplementation tended to ameliorate the negative effects of hypoxia upon  
544 TOI during moderate-intensity exercise in the current study (a 3.6% increase in TOI), in a  
545 similar fashion to that reported by Masschelein et al. (50) (a 4% increase in TOI). These  
546 effects are consistent with observations that the arterial-venous nitrite difference is associated  
547 with limb vasodilatation and increased skeletal muscle blood flow during exercise performed  
548 in hypoxia (20). The trend for an improved TOI with BR supplementation indicates better  
549 muscle oxygenation (24) which may have been responsible for the speeding of the  $\dot{V}_{O_2}$   
550 kinetics observed in hypoxia. Consistent with a possible improvement in oxygenation status,  
551 the typical compensatory rise in HR in hypoxia was attenuated by BR compared to PL during  
552 moderate-intensity exercise. Specifically, HR was 5-6 b·min<sup>-1</sup> lower in the H-BR compared to  
553 the H-PL condition. There were no differences between H-BR and H-PL in indices of muscle  
554 oxygenation or HR during severe-intensity exercise.

555 Whether the reduction in cardiac work (lower HR) and metabolic requirement (lower  $\dot{V}_{O_2}$ )  
556 with BR observed in the present study might translate into enhanced performance during  
557 prolonged low-intensity exercise at altitude remains to be determined. Furthermore, older age  
558 and a number of disease conditions including peripheral arterial disease, diabetes, COPD and  
559 anaemia are associated with tissue hypoxia. A reduced  $O_2$  cost of moderate-intensity exercise  
560 (i.e. walking) and reduced muscle metabolic perturbation during physical activity may  
561 improve the quality of life in individuals with these diseases (34, 64). However, further  
562 research is required to explore the effects of BR supplementation on health and functional  
563 capacity in patient populations.

#### 564 *Effects of BR supplementation on the physiological response to severe-intensity exercise*

565 The end-exercise  $\dot{V}_{O_2}$  was significantly reduced in hypoxia compared to normoxia. Moreover,  
566  $[HbO_2]$  and TOI of the *m. vastus lateralis* were significantly reduced, while  $[HHb]$  and HR  
567 were significantly increased in hypoxia compared to normoxia, consistent with previous  
568 findings (50). There was a trend toward a reduction in end-exercise  $\dot{V}_{O_2}$  with BR compared to  
569 PL supplementation in hypoxia of  $\sim 6\%$ . This finding indicates the  $\dot{V}_{O_{2peak}}$  may be reduced by  
570  $NO_3^-$  supplementation and is consistent with some (6, 42) but not all previous studies (4, 33,  
571 62) conducted in normoxia.

572 Tolerance to severe-intensity cycle exercise in hypoxia in the present study was significantly  
573 improved (9%,  $P < 0.05$ ) following BR supplementation. This finding is consistent with  
574 earlier studies which reported that BR supplementation increased exercise tolerance during  
575 constant-work-rate (64) and incremental (50) exercise protocols and also enhanced cycling  
576 time-trial performance (51) in hypoxia. However, in contrast to previous findings (3, 4, 8, 33,  
577 37), we found no effect of BR supplementation on exercise tolerance in normoxia. An  
578 interesting observation in the present study was the significant correlation between the  
579 reduction in steady-state  $\dot{V}_{O_2}$  and the improvement in exercise tolerance following BR  
580 supplementation in hypoxia ( $r = -0.96$ ). Therefore, the lack of effect on  $\dot{V}_{O_2}$  during sub-  
581 maximal exercise in normoxia following BR supplementation may explain the lack of effect  
582 on exercise tolerance. Further research is required to address the physiological bases for  
583 responders and non-responders to dietary nitrate supplementation.

#### 584 *Perspectives*

585 This study provides the first description of the influence of FIO<sub>2</sub> and BR supplementation on  
586 plasma [NO<sub>2</sub><sup>-</sup>] dynamics during moderate- and severe-intensity exercise and subsequent  
587 recovery in humans. The greater rate of decline of plasma [NO<sub>2</sub><sup>-</sup>] during exercise following  
588 BR compared to PL supplementation suggests that elevating plasma [NO<sub>2</sub><sup>-</sup>] prior to exercise  
589 may promote NO production through the nitrate-nitrite-NO pathway. In hypoxia, but not  
590 normoxia, BR supplementation reduced the O<sub>2</sub> cost of moderate-intensity exercise, speeded  
591  $\dot{V}_{O_2}$  kinetics, and improved severe-intensity exercise tolerance. These findings may have  
592 important implications for individuals exercising at altitude.

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595 technical support, and Beet It Ltd. for providing the beverages used in this study, gratis.

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598 **Figure Legends**

599 **Figure 1.** Plasma  $[\text{NO}_2^-]$  response during moderate- and severe-intensity exercise and  
600 recovery following BR and PL, in normoxia and hypoxia. Error bars indicate SE. H-BR was  
601 greater than H-PL at each time point and N-BR was greater than N-PL at each time point.  $a =$   
602  $P < 0.05$  for N-BR compared to H-BR;  $b = P < 0.05$  compared to moderate baseline;  $c = P <$   
603  $0.05$  compared to severe baseline. Where error bars are not visible, the size of the data point  
604 exceeds the error.

605 **Figure 2.** Pulmonary  $\text{O}_2$  uptake ( $\dot{V}_{\text{O}_2}$ ) responses during a step increment to a moderate-  
606 intensity work rate, following PL and BR supplementation. Responses following BR are  
607 represented as solid circles, with the PL responses being shown as open circles. The dotted  
608 vertical line denotes the abrupt ‘step’ transition from baseline to moderate-intensity cycling  
609 exercise. Error bars indicate the SE. *A*: Group mean response to moderate-intensity exercise  
610 in normoxia ( $\sim 21\%$   $\text{FIO}_2$ ); *B*: Group mean response to moderate-intensity exercise in hypoxia  
611 ( $\sim 13.2\%$   $\text{FIO}_2$ ); \* =  $P < 0.05$  compared to H-PL.

612 **Figure 3.** Pulmonary  $\text{O}_2$  uptake ( $\dot{V}_{\text{O}_2}$ ) responses and time-to exhaustion during a step  
613 increment to a severe-intensity work rate, following PL and BR supplementation. Responses  
614 following BR are represented as solid circles, with the PL responses being shown as open  
615 circles. The dotted vertical line denotes the abrupt ‘step’ transition from baseline to moderate-  
616 intensity cycling exercise. Error bars indicate the SE. *A*: Group mean response to severe-  
617 intensity exercise in normoxia ( $\sim 21\%$   $\text{FIO}_2$ ); *B*: Group mean response to severe-intensity  
618 exercise in hypoxia ( $\sim 13.2\%$   $\text{FIO}_2$ ). \* = Time to exhaustion greater in H-BR compared to H-PL  
619 ( $P < 0.05$ ; one-tailed t-test).

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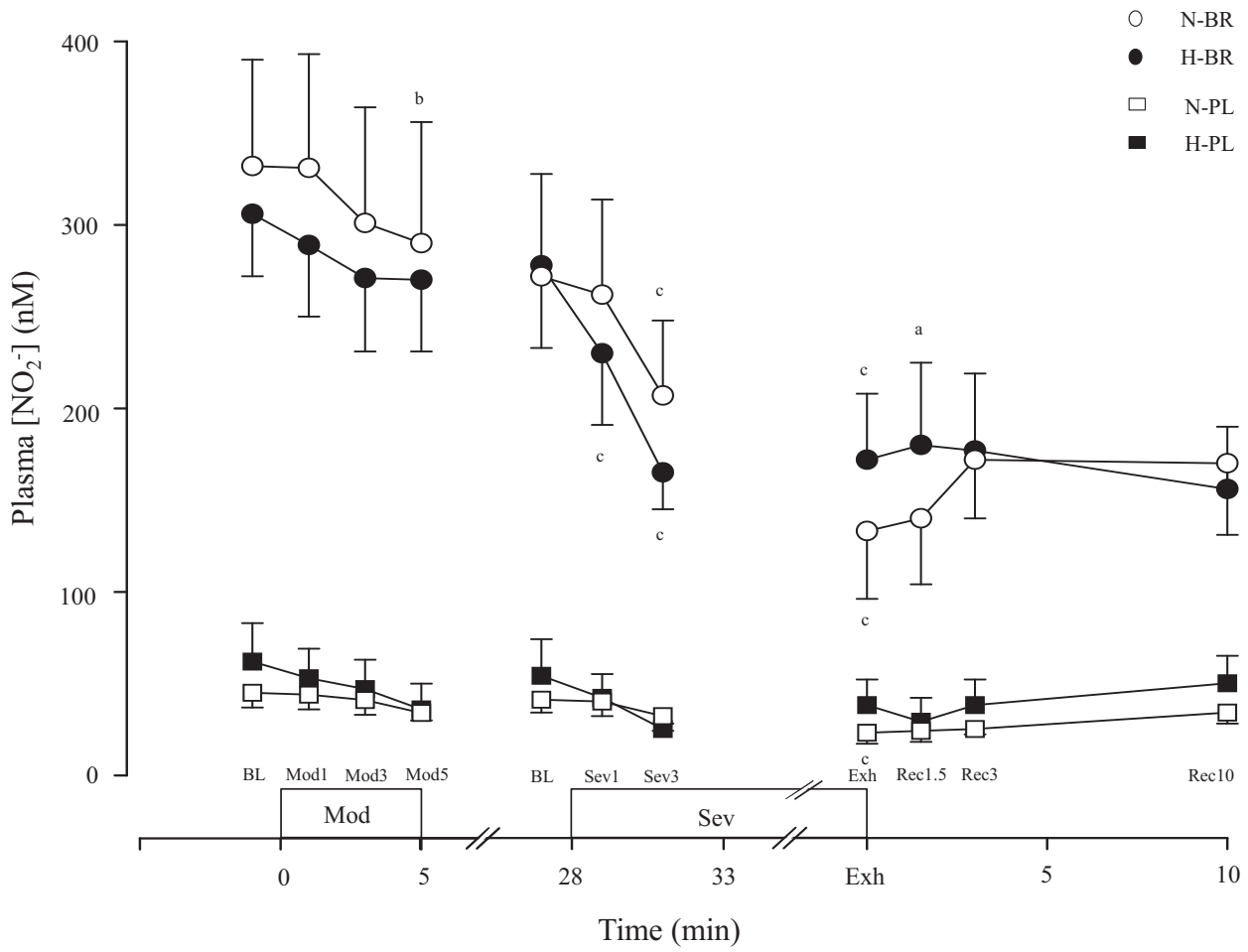
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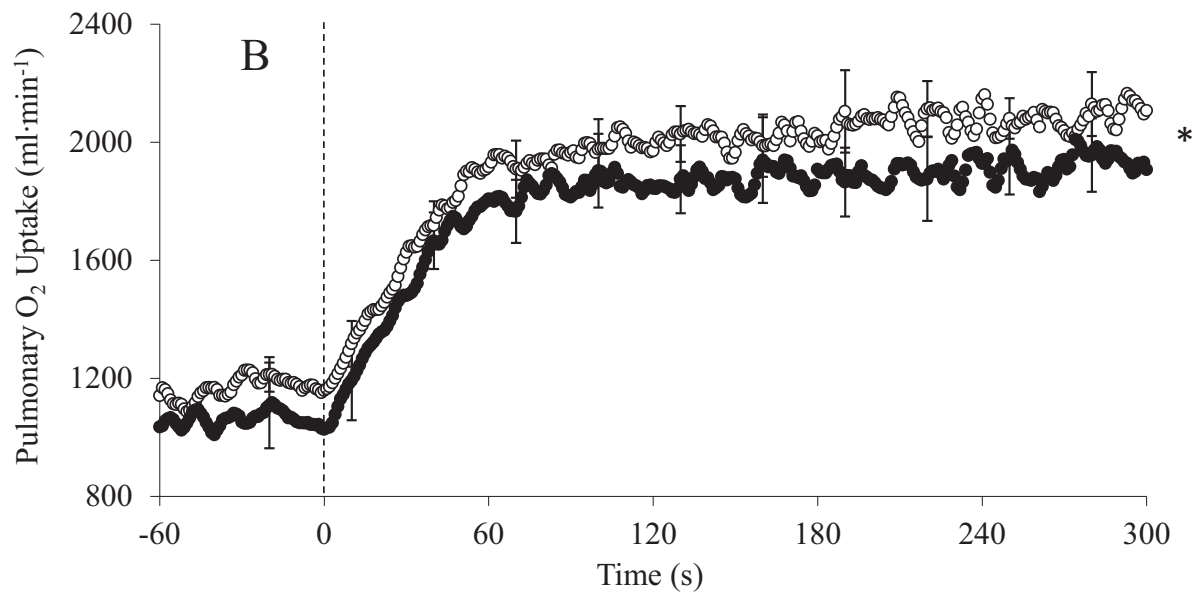
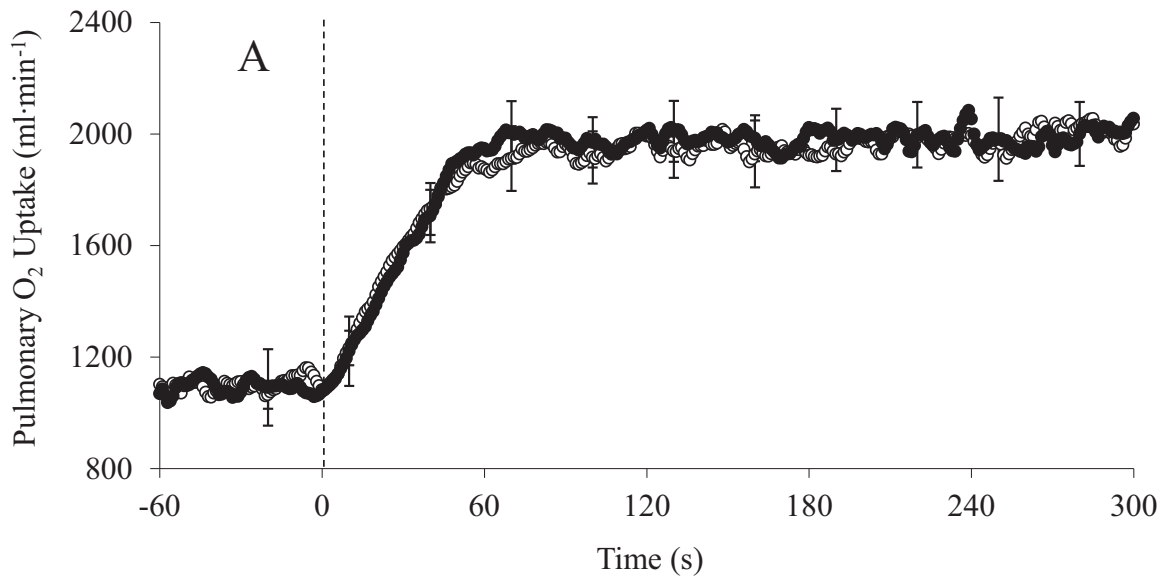
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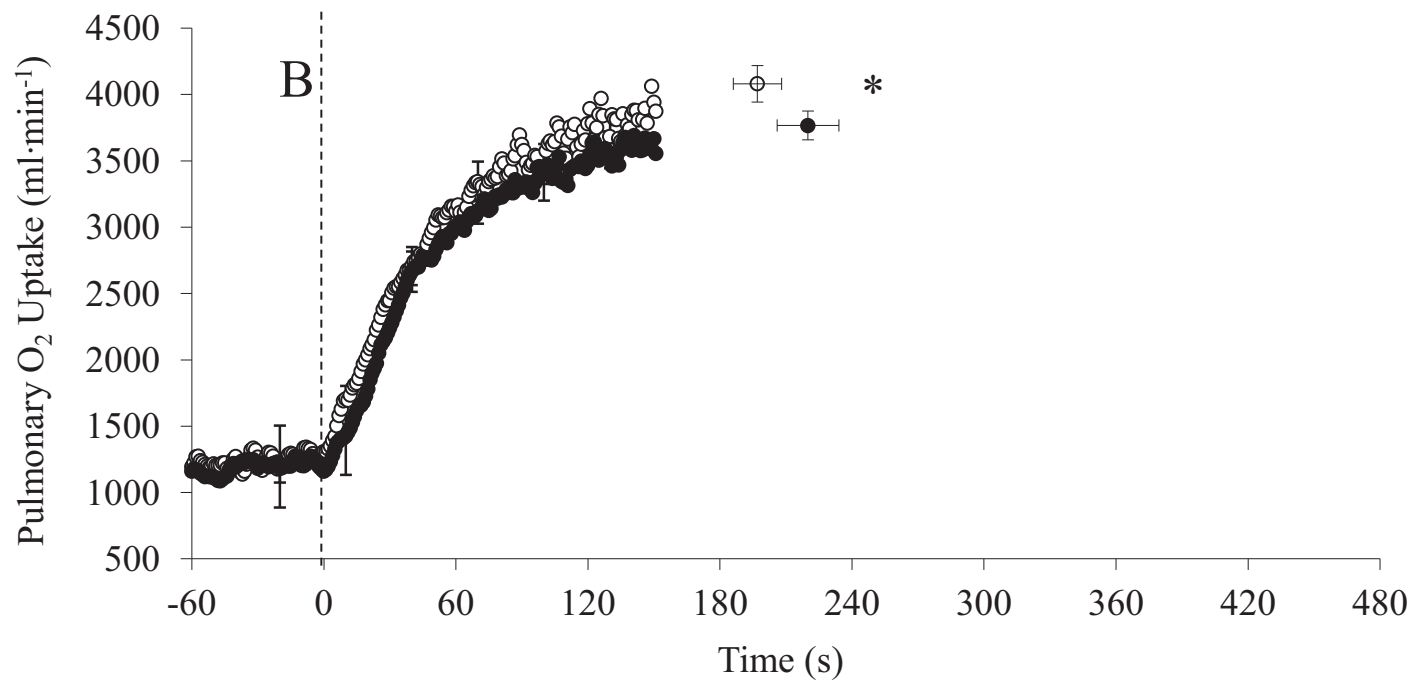
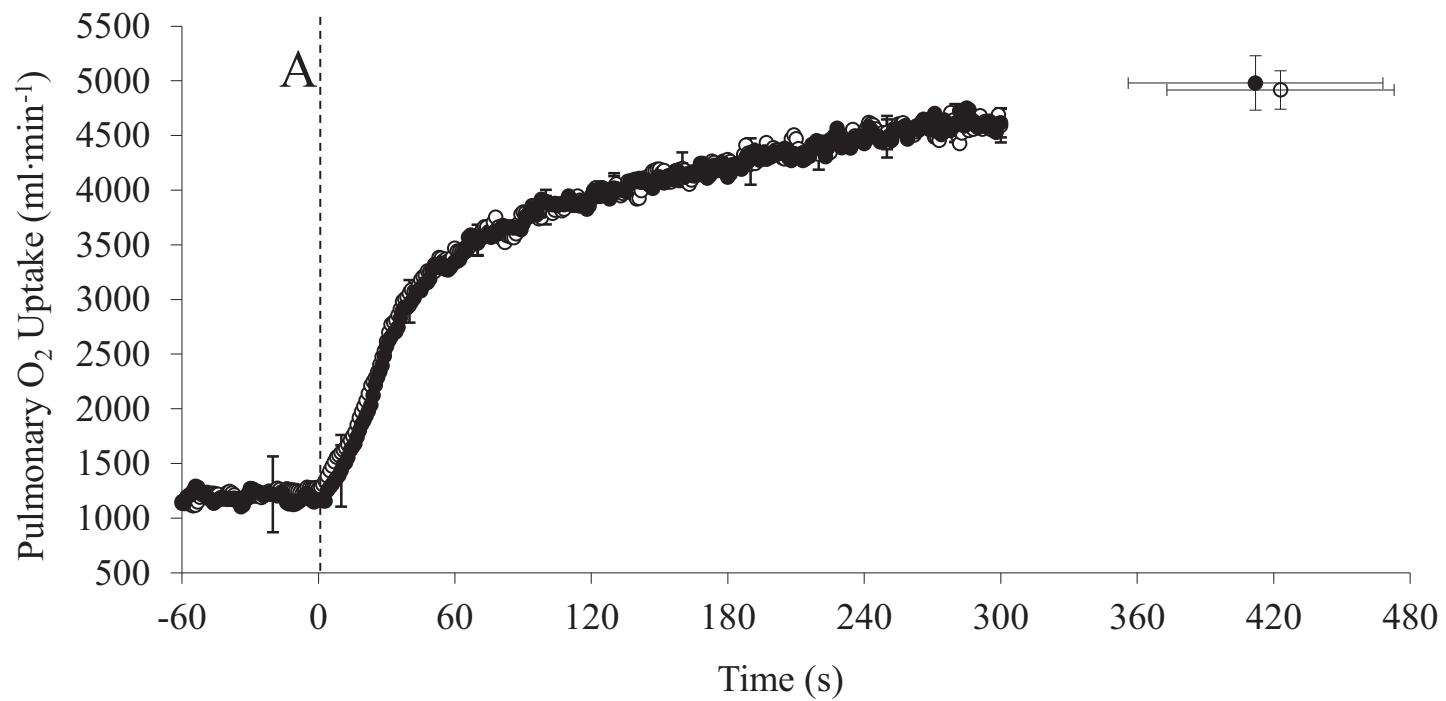
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**Table 1. Arterial oxygen saturation and heart rate during rest and in response to moderate- and severe-intensity exercise.**

	N-PL	N-BR	H-PL	H-BR
<i>Resting without inspire</i>				
<i>SaO<sub>2</sub> (%)</i>				
10 min period	99 ± 1	99 ± 1	99 ± 1	99 ± 1
End	99 ± 1	99 ± 1	99 ± 1	99 ± 1
<i>HR (b/min)</i>				
10 min period	59 ± 9	61 ± 10	61 ± 10	61 ± 9
End	60 ± 9	61 ± 9	61 ± 10	61 ± 9
<i>Resting with inspire</i>				
<i>SaO<sub>2</sub> (%)</i>				
10 min period	99 ± 1	99 ± 1	93 ± 2†	93 ± 2*
End	99 ± 1	99 ± 1	90 ± 3†	91 ± 1*
<i>HR (b/min)</i>				
10 min period	58 ± 9	60 ± 11	68 ± 11	66 ± 10 <sup>#</sup>
End	60 ± 8	60 ± 11	68 ± 11†	66 ± 10*
<i>Moderate-intensity exercise</i>				
<i>SaO<sub>2</sub> (%)</i>				
Baseline	97 ± 3	98 ± 2	87 ± 4	85 ± 4
6 min period	97 ± 3	98 ± 2	83 ± 3†	84 ± 4*
End	97 ± 3	97 ± 3	81 ± 4†	82 ± 5*
<i>HR (b/min)</i>				
Baseline	82 ± 10	86 ± 12	101 ± 16	94 ± 13
6 min period	102 ± 15	107 ± 15	122 ± 15	117 ± 19 <sup>#</sup>
End	105 ± 16	111 ± 17	130 ± 15†	124 ± 19*
<i>Severe-intensity exercise</i>				
<i>SaO<sub>2</sub> (%)</i>				
Baseline	98 ± 2	97 ± 3	86 ± 4	87 ± 4
Exhaustion	94 ± 4	94 ± 4	80 ± 3†	80 ± 4*
<i>HR (b/min)</i>				
Baseline	97 ± 9	103 ± 12	113 ± 9	114 ± 12
Exhaustion	179 ± 4	180 ± 5	172 ± 6	171 ± 6

<sup>#</sup>  $P < 0.05$  compared to H-PL; \*  $P < 0.05$  compared to N-BR; †  $P < 0.05$  compared to N-PL.

**Table 2. Oxygen uptake kinetics in response to moderate- and severe-intensity exercise in hypoxic and normoxic conditions.**

	N-PL	N-BR	H-PL	H-BR
<i>Moderate-intensity exercise</i>				
<i><math>\dot{V}O_2</math> (ml/min)</i>				
Baseline	1102 ± 156	1010 ± 343	1167 ± 123	1056 ± 133 <sup>#</sup>
End Exercise	1970 ± 251	1908 ± 340	2049 ± 247	1905 ± 275 <sup>#</sup>
Phase II $\tau$ , (s)	22 ± 10	17 ± 4 <sup>#</sup>	31 ± 11	24 ± 13 <sup>#</sup>
Primary amplitude	868 ± 210	899 ± 256	882 ± 214	849 ± 208
<i>Severe-intensity exercise</i>				
<i><math>\dot{V}O_2</math> (ml/min)</i>				
Baseline	1212 ± 179	1205 ± 158	1244 ± 175	1193 ± 177
End Exercise	4814 ± 470	4721 ± 434	3986 ± 300 <sup>†</sup>	3751 ± 249 <sup>*</sup>
Phase II $\tau$ , (s)	30 ± 6	28 ± 9	35 ± 14	31 ± 11
Primary amplitude	2716 ± 398	2636 ± 486	2450 ± 497	2264 ± 386
Slow Component Amplitude	886 ± 235	881 ± 259	302 ± 290 <sup>†</sup>	301 ± 274 <sup>*</sup>

<sup>#</sup>  $P < 0.05$  compared to H-PL; <sup>\*</sup>  $P < 0.05$  compared to N-BR; <sup>†</sup>  $P < 0.05$  compared to N-PL.

**Table 3. Near-infrared spectroscopy- derived HHb, HbO<sub>2</sub>, Hb<sub>tot</sub> and TOI dynamics during moderate- and severe-intensity exercise.**

	N-PL	N-BR	H-PL	H-BR
<i>Moderate-intensity exercise</i>				
<i>[HHb] (AU)</i>				
Baseline	7 ± 5	6 ± 5	11 ± 5†	10 ± 5*
120 s	11 ± 8	11 ± 7	18 ± 8†	17 ± 10*
End Exercise	12 ± 8	11 ± 7	20 ± 8†	18 ± 10*
Time Constant, (s)	23 ± 7	19 ± 6	22 ± 9	23 ± 7
Amplitude	5 ± 4	6 ± 4	8 ± 5†	7 ± 6*
<i>[HbO<sub>2</sub>] (AU)</i>				
Baseline	2 ± 6	3 ± 6	2 ± 5	2 ± 7
120 s	1 ± 6	2 ± 6	-2 ± 4	-2 ± 8
End Exercise	4 ± 5	5 ± 5	0 ± 3†	-2 ± 9*
<i>[Hb<sub>tot</sub>] (AU)</i>				
Baseline	1 ± 0	1 ± 0	1 ± 0	1 ± 0
120 s	1 ± 0	1 ± 0	1 ± 0	1 ± 0
End Exercise	1 ± 0	1 ± 0	1 ± 0	1 ± 0
<i>TOI (AU)</i>				
Baseline	65 ± 3	65 ± 4	61 ± 4†	63 ± 4*
120 s	61 ± 5	60 ± 6	52 ± 5†	54 ± 6*
End Exercise	62 ± 7	61 ± 7	52 ± 6†	54 ± 6*
<i>Severe-intensity exercise</i>				
<i>[HHb] (AU)</i>				
Baseline	5 ± 5	5 ± 5	10 ± 6†	10 ± 6*
120 s	19 ± 13	18 ± 11	25 ± 12†	24 ± 14*
End Exercise	22 ± 14	21 ± 12	26 ± 12†	26 ± 14*
Time Constant, (s)	13 ± 5	11 ± 5	11 ± 3	12 ± 6
Primary amplitude	14 ± 10	14 ± 8	14 ± 9	14 ± 10
Slow phase amplitude	3 ± 2	3 ± 2	2 ± 2†	2 ± 2*
<i>[HbO<sub>2</sub>] (AU)</i>				
Baseline	7 ± 7	8 ± 7	6 ± 5†	5 ± 8*
120 s	-4 ± 7	-3 ± 7	-9 ± 4 †	-10 ± 8*
End Exercise	-7 ± 9	-7 ± 7	-11 ± 5†	-12 ± 7 *
<i>[Hb<sub>tot</sub>] (AU)</i>				
Baseline	1 ± 0	1 ± 0	1 ± 0	1 ± 0
120 s	1 ± 0	1 ± 0	1 ± 0	1 ± 0
End Exercise	1 ± 0	1 ± 0	1 ± 0	1 ± 0
<i>TOI (AU)</i>				
Baseline	70 ± 5	69 ± 4	64 ± 4	64 ± 4
120 s	52 ± 12	51 ± 10	44 ± 9	44 ± 10
End Exercise	48 ± 11	47 ± 9	41 ± 9†	41 ± 8*

Deoxygenated hemoglobin concentration ([HHb]), oxygenated hemoglobin concentration ([HbO<sub>2</sub>]), total hemoglobin concentration ([Hb<sub>tot</sub>]) and total oxygenation index (TOI) are shown. \*  $P < 0.05$  compared to N-BR; †  $P < 0.05$  compared to N-PL. AU = arbitrary units.