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Beetroot supplementation improves the physiological responses to incline walking --Manuscript Draft--

Manuscript Number:	EJAP-D-17-00916R2
Full Title:	Beetroot supplementation improves the physiological responses to incline walking
Article Type:	Original Article
Keywords:	Nitrate; efficiency; energy cost; blood pressure.
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Order of Authors Secondary Information:	
Funding Information:	
Abstract:	<p>Purpose: We investigated the effects of an acute 24-h nitrate-rich beetroot juice supplement (BR) on the energy cost, exercise efficiency and blood pressure responses to intermittent walking at different gradients. Methods: In a double-blind, cross-over design, eight participants were provided with a total of 350 ml of nitrate-rich (~20.5 mmol nitrate) BR or placebo (PLA) across 24-h before completing intermittent walking at 3 km/h on treadmill at gradients of 1%, 5%, 10%, 15% and 20%. Results: Resting mean arterial pressure (MAP) was ~4.1% lower after BR (93 vs. 89 mmHg; P = 0.001), as well as during exercise (102 vs. 99 mmHg; P = 0.011) and recovery (97 vs. 94 mmHg; P = 0.001). Exercising (1227 vs. 1129 ml/min P < 0.001) and end-stage (1404 vs. 1249 ml/min; P = 0.002) oxygen uptake (VO₂) was lower in BR compared to PLA, which was accompanied by an average reduction in phase II VO₂ amplitude (1067 vs. 940 ml/min; P = 0.025). Similarly, recovery VO₂ (509 vs. 458 ml/min; P = 0.001) was lower in BR. Whole-blood potassium concentration increased from pre-post exercise in PLA (4.1 ± 0.3 vs. 4.5 ± 0.3 mmol/L; P = 0.013) but not BR (4.1 ± 0.31 vs. 4.3 ± 0.2 mmol/L; P = 0.188). Conclusions: Energy cost of exercise, recovery of VO₂, MAP and blood markers were ameliorated after BR. Previously reported mechanisms explain these findings, which are more noticeable during less efficient walking at steep gradients (15-20%). These findings have practical implications for hill-walkers.</p>
Response to Reviewers:	<p>Reviewers' comments:</p> <p>Reviewer #1: The manuscript is much improved. I just have two minor comments in the conclusion that need to be addressed:</p> <ol style="list-style-type: none"> 1) It is the reduction of NO₂- to NO not NO₃- to NO 2) You have referenced the Whitfield paper to support a mitochondrial effect for the lower VO₂ with nitrate, but these authors observed a lower VO₂ without alterations in mitochondrial function. Just delete the sentence starting mechanistically... as this is

not needed.

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We hope the changes made are suitable. Thank you, once again, to the reviewers and Editor for their time and feedback on the manuscript.

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1 **Title:** Beetroot supplementation improves the physiological responses to incline walking

3 **Authors:**

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27 **Abstract**

1
2
3 28 **Purpose:** We investigated the effects of an acute 24-h nitrate-rich beetroot juice supplement
4
5 29 (BR) on the energy cost, exercise efficiency and blood pressure responses to intermittent
6
7
8 30 walking at different gradients. **Methods:** In a double-blind, cross-over design, eight
9
10 31 participants were provided with a total of 350 ml of nitrate-rich (~20.5 mmol nitrate) BR or
11
12 32 placebo (PLA) across 24-h before completing intermittent walking at 3 km/h on treadmill at
13
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21
22 37 lower in BR compared to PLA, which was accompanied by an average reduction in phase II
23
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26 39 ml/min; P = 0.001) was lower in BR. Whole-blood potassium concentration increased from
27
28 40 pre-post exercise in PLA (4.1 ± 0.3 vs. 4.5 ± 0.3 mmol/L; P = 0.013) but not BR (4.1 ± 0.31
29
30 41 vs. 4.3 ± 0.2 mmol/L; P = 0.188). **Conclusions:** Energy cost of exercise, recovery of $\dot{V}O_2$,
31
32 42 MAP and blood markers were ameliorated after BR. Previously reported mechanisms explain
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34 43 these findings, which are more noticeable during less efficient walking at steep gradients (15-
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36 44 20%). These findings have practical implications for hill-walkers.
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46 **Key words:** Nitrate; efficiency; energy cost; blood pressure.

51	Abbreviations
52	Adenosine triphosphate (ATP)
53	Beetroot juice (BR)
54	Blood lactate concentration (B[La])
55	Blood potassium concentration ([K ⁺])
56	Blood pressure (BP)
57	Coefficient of variation (CV%)
58	Diastolic (DP)
59	Energy cost of walking (C _w)
60	Glucose concentration ([Glu])
61	Gross efficiency (GE%)
62	Heart rate (HR)
63	Mean arterial pressure (MAP)
64	Nitrate (NO ₃ ⁻)
65	Nitrite (NO ₂ ⁻)
66	Nitric oxide (NO)
67	Oxygen uptake ($\dot{V}O_2$)
68	Placebo (PLA)
69	Rating of perceived exertion (RPE)
70	Sarcoplasmic reticulum calcium (SR-Ca ²⁺)
71	Systolic pressure (SP)
72	Ventilatory threshold (V _T)
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78 **Introduction**

79 Supplementation of inorganic nitrate (NO_3^-), in the form of a highly-concentrated beetroot
80 juice (BR) supplement (5.2 - 8.2 mmol of NO_3^- per day), improves endurance performance
81 (Bailey et al. 2009, 2010, 2015; Bryan et al. 2008; Lansley et al. 2011a; Wylie et al. 2013).

82 These effects are thought to be facilitated by the oxygen independent reductions of NO_3^- to
83 nitrite (NO_2^-) and nitric oxide (NO) (Bailey et al. 2012). The NO_3^- - NO_2^- -NO cascade
84 increases circulating NO_2^- , which can be further reduced to NO under certain physiological
85 conditions (Bryan et al. 2008). Thus, the availability of NO can be preserved when enzymatic
86 synthesis of NO via nitric oxide synthase (NOS) has been compromised (Bryan et al. 2008).
87 The role of the NO_3^- - NO_2^- -NO pathway is potentiated during intense exercise, in particular,
88 owing to the mild hypoxic and/or acidic conditions induced in the working skeletal muscle
89 (Jones et al. 2016).

90
91 Nitric oxide is a key regulator of blood pressure (BP) and a signalling molecule for smooth
92 muscle surrounding the endothelium (Gilchrist et al. 2011). These mechanisms permit
93 vasodilation of blood vessels and enhance blood flow to the working musculature (Ferguson
94 et al. 2013). Accordingly, reduced BP is common after NO_3^- supplementation during rest
95 (Bailey et al. 2009; Bond et al. 2013, 2014; Kapil et al. 2010; Vanhatalo et al. 2010; Webb et
96 al. 2008) and exercise (Bond et al. 2013, 2014; Choi et al. 2016; Kenjale et al. 2011; Oggioni
97 et al. 2017). The combination of increased muscle blood flow and reduced oxygen extraction
98 at the site of the working musculature leads to enhanced exercise tolerance (Bailey et al.
99 2010). The latter finding has been attributed to an enhanced efficiency of oxidative adenosine
100 triphosphate (ATP) turnover in the mitochondria (Larsen et al. 2011). Mammalian studies
101 have demonstrated that other factors, such as improved sarcoplasmic reticulum calcium (SR-
102 Ca^{2+}) handling, might also contribute to improved exercise tolerance following NO_3^-

103 supplementation (Hernández et al. 2011). However, recent studies have demonstrated that
104 longer (> 7 days) supplementation periods would be necessary to induce such changes in
105 calcium handling proteins and point to alternative mechanisms, such as increased emission of
106 mitochondrial reactive oxygen species and their possible effects on muscle contractile
107 apparatus (Whifield et al. 2016). Nevertheless, these mechanisms contribute to a decreased
108 ATP turnover for a given power output (Bailey et al. 2010), thus improving exercise
109 economy across a variety of work-rates, with a notable 12 % reduction in the O₂ cost ($\dot{V}O_2$
110 amplitude) of walking at fixed gradients of 1% (Lansley et al. 2011b) and preferable
111 alterations in the pulmonary oxygen uptake ($\dot{V}O_2$) kinetics of older adults (Kelly et al. 2013).

112
113 BR supplementation has not improved endurance performance or lowered exercising $\dot{V}O_2$ in
114 all cases, particularly when administered to elite endurance athletes (Christensen et al. 2012;
115 Peacock et al. 2012; Boorsma et al. 2014). The inconsistent findings have been ascribed to
116 the aerobic fitness level of the athletes (Porcelli et al. 2015) and might relate to the lower
117 reliance on the NO₃⁻-NO₂⁻-NO pathway or the presumed higher proportions of type I muscle
118 fibre among well-trained endurance athletes. This is consistent with the evidence that the
119 effects of NO₃⁻ are potentiated in type II muscle fibre (Hernández et al. 2011; Ferguson et al.
120 2013). As such, certain forms of locomotion that mandate the recruitment of type II muscle
121 fibre could be affected differently by BR supplementation compared to those without,
122 particularly when performed by non-elite athletes.

123
124 Incline walking is a popular and effective mode of exercise for physical fitness (Ainslie et al.
125 2002, 2005; Gottschall and Kram 2006) but will present a challenge to certain populations or
126 vocations. The energy cost of walking (C_w) increases as a function of surface incline (Minetti
127 et al. 2006). The external work done to walk on gradients between 1% and 20% comprises a

1 128 combination of negative and positive contributions (Minetti et al. 2006). On extreme uphill
2 129 slopes (20%), this work becomes entirely positive and reaches a mechanical efficiency (i.e.
3
4 130 ratio of mechanical work for vertical displacement to the energy expended) equivalent to
5
6 131 concentric muscle contraction (Margaria et al. 1963). Negative work is more efficient (~
7
8
9 132 80%), yet diminishes as a function of walking gradient, meaning there is a greater reliance on
10
11 133 less efficient (~ 25%) positive work (Minetti et al. 2006). When walking uphill at
12
13 134 progressively steeper inclines, muscle recruitment strategies are proportionally altered to
14
15 135 coincide with the leading leg performing longer and more forceful concentric muscle
16
17 136 contractions (i.e. more positive work) (Gottschall and Kram 2006). Consequently, greater
18
19 137 lower-limb muscle activation (Vernillo et al. 2017), anaerobic metabolism (Sloniger et al.
20
21 138 1997; Staab et al. 1992) and perception of effort (Kolkhorst et al. 1996) is evident during
22
23 139 uphill locomotion, compared to level exercise at the same speed. These observations reflect a
24
25 140 change in the muscle recruitment patterns of the lower limbs and a logical reliance on type II
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27 141 muscle fibres to support the more forceful and concentrically-biased muscle contraction.
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36 143 Whilst it is known that dietary NO_3^- can enhance muscle efficiency in humans, rodent studies
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38 144 have shown that NO_3^- supplementation can improve muscle O_2 delivery in musculature with a
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40 145 high type II fibre distribution (Ferguson et al. 2013), where it also improves SR- Ca^{2+} storage
41
42 146 and release (Hernández et al. 2011). It has recently been proposed that the ergogenic
43
44 147 properties of NO_3^- are fibre type dependent (Jones et al. 2016). In support of this, Breese et al.
45
46 148 (2013) reported faster $\dot{V}\text{O}_2$ kinetics and improved time to exhaustion during step-exercise,
47
48 149 designed to elicit the recruitment of higher order motor units. It is therefore feasible that
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50 150 consumption of NO_3^- can improve the efficiency of uphill walking, owing to the constrained
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52 151 changes in gait pattern toward a less efficient form of locomotion and an increased reliance
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54 152 on type II muscle fibres as the gradient increases. Indeed, a recent study demonstrated
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153 lowered $\dot{V}O_2$ after BR supplementation during loaded uphill walking at 10% gradients in
154 hypoxic (fraction of inspired $O_2 = 11.7\%$) conditions (Shannon et al. 2017). However, the
155 effects of BR supplementation on the physiological responses to incline walking at different
156 gradients have not been systematically investigated among unloaded subjects in normoxic
157 conditions. Accordingly, this study aimed to assess the effects of an acute 24-h NO_3^- -rich BR
158 supplement on the energy cost, exercise efficiency and BP responses to intermittent walking
159 at different gradients. We hypothesized that the BR supplement would reduce the cost of
160 exercise as a function of the exercise intensity and ameliorate recovery between exercise
161 bouts, including a reduction in BP during rest and exercise.

163 **Methods**

165 *Participants and design*

166 Eight participants (5 male (age 27 ± 2 years; stature 1.74 ± 0.09 m; body mass 81.4 ± 7.3 kg;
167 $\dot{V}O_{2max}$ 56.8 ± 7.8 ml/kg/min) and 3 female (age 29 ± 5 years; stature 166 ± 4 cm; body mass
168 60.0 ± 6.1 kg; $\dot{V}O_{2max}$ 38.0 ± 10.4 ml/kg/min) provided written informed consent to take part
169 in this study. The participants were all recreationally active and regularly hill-walked as part
170 of their occupation or leisure activities. The participants were all naive to the effects of
171 beetroot juice on exercise. After baseline measures and familiarisation, in a double-blind,
172 randomized cross-over design, the participants were either supplemented with a total of 350
173 ml of BR, in the form of beetroot shots, which were consumed at three evenly-spaced periods
174 in the 24-h before the exercise trial, or a placebo (PLA), one week apart. After 24-h of
175 supplementation, the participants completed a randomized, intermittent, inclined walking
176 protocol on a treadmill, while their physiological ($\dot{V}O_2$, blood lactate concentration (B[La]),
177 blood potassium concentration ($[K^+]$), heart rate (HR) and continuous BP), perceptual (rating

178 of perceived exertion; RPE) and gross mechanical work was measured. The participants were
179 instructed to arrive at least 3-h post prandial, abstain from heavy exercise, caffeine intake,
180 anti-bacterial mouthwash and NO₃⁻-rich foods in the 48-h before and during the study. Food
181 diaries were completed to ensure compliance with these instructions. This study was granted
182 institutional ethical approval and was conducted in accordance with the ethical standards laid
183 down in the 1964 Declaration of Helsinki.

184

185 **Procedure**

186 *Baseline measurements*

187 The participants visited the laboratory on three occasions. On their first visit, the participants
188 were screened to ensure low-risk stratification, including assessment of resting BP and the
189 completion of extended health questionnaires (Exercise Sport Science Australia). Resting BP
190 was measured from the left brachial artery, using an automated sphygmomanometer (Omron
191 M7 BP Monitor, OMRON Healthcare Europe, Hoofddrop, Netherlands) after sitting for 15-
192 min in a quiet room. The average systolic (SP), diastolic (DP) and mean arterial pressures
193 (MAP) of three measurements was recorded, calculated as: (MAP) (MAP = DBP + 0.33
194 (SBP-DBP)). This method of BP measurement was adopted at baseline, for screening
195 purposes, whereas continuous measurements were performed for all subsequent visits. A HR
196 monitor was strapped across the participants' chest and synchronized with a wrist watch
197 (V800, Polar, Kempele, Finland) to record resting HR. Whilst seated in the same position,
198 baseline venous blood was drawn from the participants' right arm at the antecubital fossa,
199 using a hypodermic needle and a lithium-heparinized vacutainer (4 ml). The whole blood was
200 immediately tested for blood glucose ([Glu]) and [K⁺] concentrations using a mobile analyser
201 (iSTAT analyser, Abbott Point of Care Inc, Princeton, NJ) with a manufacturer-reported
202 coefficient of variation (CV%) of 1.1%. The remaining and whole blood was then centrifuged

203 at 4,000 r/min and 4 °C for 10-min, after which the plasma was immediately stored at -80 °C.

204 Blood plasma was later thawed and analysed for NO₃⁻ using a colorimetric assay, with an

205 intra-assay CV% of 2.7, and an ELISA with an intra-assay CV% of 6.3, respectively (see

206 http://www.abnova.com/products/products_detail.asp?catalog_id=KA1342). Plasma insulin

207 was measured using a solid phase two-site enzyme immunoassay, with an intra-assay CV%

208 of 6.4 and an inter-assay CV of 8.5 (see

209 http://www.abnova.com/products/products_detail.asp?catalog_id=KA0921).

210

211 The participants also completed an incremental, maximal treadmill test on their first visit, to

212 establish $\dot{V}O_{2max}$ and ventilatory threshold (V_T). An adapted Bruce protocol was performed on

213 a calibrated motorized treadmill (Mercury® Med, hpcosmos sports & medical gmbh;

214 Nussdorf-Traunstein, Germany), beginning at 2.7 km/h and 0% gradient and increasing by

215 speed and gradient every 3-min until volitional exhaustion. This test was selected to

216 familiarize the participants to gradient walking, as well as incorporating a warm-up stage into

217 the test and providing a measurement of $\dot{V}O_{2max}$. Pulmonary gas was measured continuously

218 using a breath-by-breath gas analyser (Jaegar, Oxycon Pro, Viasys Healthcare, Hoechberg,

219 Germany). The gas analyser and flow turbine were calibrated before each test using a known

220 gas mixture (15% O₂ and 5% CO₂) and a 3-L syringe, respectively (Hans Rudolph, Kansas

221 City, KS). $\dot{V}O_{2max}$ was determined as the mean value recorded over the final 30-s of the test.

222 The same gas analyser was used throughout the study and calibrated identically, with a CV%

223 of 4.3. The V_T was determined using the V-slope method, which has previously been used

224 during 3-min incremental stages (Amman et al. 2004). The vertical mechanical power output

225 (W) was calculated at the V_T according to Minetti et al. (2006) as:

226

227
$$\dot{W}_{vert} = gv \sin(\arctan[i]) \quad [\text{Eq. 1}]$$

228

229 where g = gravity (m/s/s), v = treadmill velocity (m/s) and i = the treadmill gradient.

230

231 The power output was subsequently used to delineate moderate and heavy exercise domains.

232 The aim of the final experimental protocol was to exercise participants below the heavy

233 exercise domain, at fixed gradients and speeds. As such, the power output at V_T was

234 measured to ensure that it was higher than the highest power output used in the final test

235 protocol. The mean of the highest power output to be performed during the experimental trial

236 was calculated *a-priori* as 108 ± 18 W, while the mean power output at V_T was 165 ± 29 W,

237 with no participants achieving a V_T power output lower than the highest power output in the

238 experimental protocol. Therefore, all participants were exercising below the heavy intensity

239 domain during the study. The mean $\% \dot{V}O_{2\max}$ during the highest intensity (20 % gradient)

240 trials was 52 ± 14 % and 48 ± 13 % in the PLA and BR conditions, respectively.

241

242 ***Experimental protocol***

243 At baseline testing, the participants were randomly assigned to one of the two conditions and

244 returned to the laboratory 24-h later (am) to receive their first of three supplements, in the

245 form of two NO_3^- -rich BR shots (2 x 70 ml ~ 4.1 mmol NO_3^- per shot; Beet it, James White

246 Drinks Ltd., Ipswich, UK) or PLA (isocaloric fruit juice and maltodextrin mixture). The

247 drinks were opened 10-min before their arrival at the laboratory and provided in identical

248 opaque containers. The drinks were consumed within 30-s by all participants. The

249 participants visited the laboratory to take their supplements on two further occasions across

250 the subsequent 24-h period, with the second beverage (2 x 70 ml ~ 4.1 mmol NO_3^- per shot or

251 PLA) consumed mid-way between the first and final serving. The final drink (1 x 70 ml ~ 4.1

252 mmol NO_3^- per shot or PLA) was consumed 1.5-h prior to testing (~2-2.5-h prior to exercise).

1
2 254 On the morning of each visit to the laboratory, resting BP, HR was measured and venous
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5 255 blood was drawn after 15-min of seated rest. The participants then mounted the treadmill and
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7 256 were fitted with a continuous BP monitor (Finometer MIDI, Finapres Medical Systems,
8
9
10 257 Amsterdam, The Netherlands), designed to measure brachial BP using photoelectric
11
12 258 plethysmography of the finger pressure waveform (Wesseling 1995). The Finapres® device
13
14 259 was fitted to the left arm of each participant. Specifically, the device was fitted by securing an
15
16
17 260 inflatable plastic bladder (50 µm and 55 mm length) between the distal and proximal inter-
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19 261 phalangeal joint of the middle finger, which housed an infrared light-emitting diode (950 nm
20
21
22 262 emission) and detector. This was connected to a front-end unit via an air hose. The front-end
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24 263 unit was attached to the left wrist using a Velcro strap. The front end was connected to the
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26
27 264 main unit and pump inside the Finometer MIDI. A height correction unit was also fitted to
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29 265 the front-end unit to automatically correct for hydrostatic pressure changes due to movement
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31
32 266 in hand position relative to the heart. During the experimental protocol, the participants
33
34 267 walked with a natural arm carriage, with their fingers relaxed and without holding on to the
35
36
37 268 treadmill. A laptop was connected to the Finometer MIDI and the raw 200 Hz data were
38
39 269 analysed using the BeatScope® software (Version 1.1, Finapres Medical Systems BV,
40
41 270 Arnhem, Netherlands). BeatScope® software was used to reconstruct mean brachial pressure
42
43
44 271 from the finger-pulse pressure waveform. The Finapres® was activated 1-min prior to
45
46 272 recording to allow for calibration. The calibration is performed automatically through the
47
48
49 273 PhysioCal software in the device, which was activated during the trials. Any interruption of
50
51 274 the recording owing to calibration was removed from the data set. No further filtering was
52
53
54 275 applied to the data. MAP was reported during exercising and resting conditions. The inter-
55
56 276 day walking MAP CV of this device ranged from 1.6-3.2 %, increasing as a function of
57
58 277 exercise intensity (i.e. gradient).

1
 2 279 Prior to exercise, a finger-prick capillary sample of blood was then drawn from the index
 3
 4
 5 280 finger of the participant whilst standing on the treadmill, which was measured for B[La]
 6
 7 281 using a hand-held analyser (Lactate Pro; Arkray KDK Corp., Kyoto, Japan), which has a
 8
 9
 10 282 CV% of 3.1. The participants were then fitted with the face mask of the breath-by-breath gas
 11
 12 283 analyser, where 3-min measurements of $\dot{V}O_2$, respiratory exchange ratio (RER), HR, and
 13
 14 284 continuous BP were recorded during quiet standing. These measurements were continued for
 15
 16
 17 285 the remainder of the protocol. After quiet standing measurements, the participants began the
 18
 19 286 intermittent walking trial, which comprised 5×5 -min bouts of walking at randomized 1%,
 20
 21
 22 287 5%, 10%, 15% or 20% inclines, at a fixed speed of 3 km/h, with each exercise stage
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 24 288 interspersed by 5-min of quiet standing. No talking was permitted by participants during the
 25
 26
 27 289 trial. B[La] was measured 1-min post exercise bout, while RPE was provided by the
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 29 290 participants in the final 15-s of each bout. Final venous blood draws were performed no more
 30
 31
 32 291 than 5-min after the final exercise stage.

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 35
 36 293 The rest-recovery configuration of the protocol permitted the measurement of on-pulmonary
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 38
 39 294 $\dot{V}O_2$ kinetics during intermittent exercise and prevented interference of $\dot{V}O_2$ responses
 40
 41
 42 295 between repeated exercising bouts. Raw $\dot{V}O_2$ data were filtered to remove errant breaths more
 43
 44 296 than 4 SD from the local mean. After interpolating breath-by-breath data to 1-s values, the
 45
 46
 47 297 $\dot{V}O_2$ on-kinetics were modelled using a non-linear least squares fitting procedure in a custom
 48
 49 298 programme (Microsoft Excel). After excluding the first 20-s of data on-transients, a mono-
 50
 51
 52 299 exponential model was used to characterize all trials, as they were performed below the
 53
 54 300 heavy intensity domain. On-transients were modelled based eq. 2:

301

$$302 \quad \Delta\dot{V}O_2(t) = \text{base}\dot{V}O_2 + A [1 - e^{-(t - TD)/\tau}] \quad [\text{Eq. 2}]$$

303

1
2 304 Where $\Delta\dot{V}O_2(t)$ = absolute $\dot{V}O_2$ at a given time t ; $\text{base}\dot{V}O_2$ = baseline resting $\dot{V}O_2$; τ =
3
4
5 305 fundamental time constant; TD = time delay and A = $\dot{V}O_2$ amplitude (i.e. $\Delta\dot{V}O_2 > \text{base}\dot{V}O_2$).
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10 307 The overall mean response time (MRT) was resolved by fitting a mono-exponential model to
11
12 308 the $\dot{V}O_2$ data from 0-s to end exercise, without adding the time delay. The external
13
14
15 309 mechanical work (W) was calculated based on published equations (Minetti et al. 2006; see
16
17 310 Eq.1) and was used to determine gross efficiency (GE%) as the ratio of mechanical work
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19
20 311 done and metabolic energy, as measured via gas analysis. The energy cost of walking (Cw)
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22 312 was expressed as in joules (J) / kg body mass / min (J/kg/min), using the thermal equivalent
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24 313 of oxygen (20.9 J/L O_2) (Minetti et al. 2006).
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29 315 **Statistical analyses**

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32 316 All data are presented as means \pm SD, unless otherwise stated. A two-way repeated measures
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34 317 analysis of variance was used to evaluate the effects of condition (BR or PLA), gradient (1%,
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36 318 5%, 10%, 15% and 20%) and their interaction. If tests of Sphericity were violated, the
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39 319 Greenhouse-Geisser correction was used. In the event a statistical difference was identified, a
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41 320 *post-hoc* Bonferroni test was used to identify differences. An alpha level of $P < 0.05$ was set
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43
44 321 for all analyses. Effect sizes (d), calculated as the mean difference divided by the pooled
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46 322 standard deviation of scores, were included for pairwise comparisons to provide information
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49 323 on the magnitude of any effect. Effect sizes were defined as: trivial = 0.2; small = 0.21–0.6;
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51 324 moderate = 0.61–1.2; large = 1.21–1.99; very large > 2.0 (Hopkins et al. 2009). Statistical
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53 325 analysis was conducted through IBM SPSS (Software V22.0, IBM, New York, USA).
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328 **Results**

329 *Plasma [NO₃]*

330 BR significantly elevated plasma [NO₃] (340 ± 58 μM) prior to exercise compared to
331 baseline (90 ± 11 μM, P < 0.001; d = 5.3) and PLA (89 ± 12 μM, P = 0.001; d = 5.3). After
332 the exercise protocol, plasma [NO₃] was significantly reduced in BR (309 ± 45 μM) but
333 remained higher than PLA (86 ± 11 μM, P < 0.001; d = 6.0).

334
335 ******Insert Table 1 here******

336 *MAP during rest and exercise*

337 Resting MAP was ~4.1% lower after BR consumption compared to PLA (P = 0.001, d = 1.5).
338 There were also main effects (independent of the exercise stage) for recovery (P = 0.001) and
339 exercise MAP (P = 0.011), which in both cases was lower (average ~3.9% and ~2.4%,
340 respectively) in BR than PLA (Figure 1). The interaction between exercise stage and
341 supplement for exercise MAP approached significance (P = 0.056), with the highest mean
342 difference in MAP present at a gradient of 1% (~4.1%; d = 0.98) and lowest at 20% (~1.7%;
343 d = 0.3).

344
345 ******Insert Figure 1 here******

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348
349 *Cardiorespiratory measurements*

350 There were no differences between BR and PLA for resting (P = 0.699), exercise (P = 0.257)
351 or recovery HR (P = 0.512) (Table 1). Pulmonary $\dot{V}O_2$ kinetics are presented in Table 1 and
352 Figure 2, whilst average $\dot{V}O_2$ during each exercise stage and subsequent recovery is shown in
353 Figure 3. Average (~18%, P < 0.001) and end-stage (~11%, P = 0.002) $\dot{V}O_2$ during each

1 354 exercise stage was lower in BR compared to PLA. This was accompanied by an average
2
3 355 reduction in phase II $\dot{V}O_2$ amplitude (~12%, $P = 0.025$), and a trend for a lower phase II time
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5 356 constant (~12%, $P = 0.07$) with BR in response to exercise. There was an effect of the incline
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7 357 on the MRT ($P = 0.04$) and a trend for BR ($P = 0.08$), but no pairwise differences or
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10 358 interactions ($P > 0.05$) were found. There were also main effects for recovery $\dot{V}O_2$ ($P =$
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12 359 0.001), which on average was lower in BR (~10%).

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18 362 *****Figure 2 here*****

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25 366 *****Figure 3 here*****

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30 369 The energy cost of exercise was significantly lower across each exercise stage with BR (~8%,
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32
33 370 $P < 0.001$). There was also a significant interaction between exercise gradient and condition
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35 371 for gross efficiency ($P < 0.001$), which was lower with BR when walking at 10% ($P < 0.001$;
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38 372 $d = 1.79$), 15% ($P = 0.001$; $d = 1.79$) and 20% ($P = 0.002$; $d = 1.05$) gradients, but not
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40 373 different to PLA at any other stage (Figures 4 and 5).

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46 376 *****Figure 4 here*****

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53 381 *****Figure 5 here*****

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2 386 **Blood measurements**

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5 387 BR had no effect on blood lactate ($P = 0.122$), which increased across each exercise stage (P
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7 388 $= 0.002$). Plasma insulin did not change significantly from the start to the end of the exercise
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9 389 protocol ($P = 0.104$) and was not different between BR and PLA ($P = 0.332$). Similarly,
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11 390 blood glucose did not change across gradients ($P = 0.799$) or between conditions ($P = 0.379$).
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14 391 However, there was a significant interaction ($P = 0.005$) for K^+ , which was higher after
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16 392 exercise (4.5 ± 0.3 mmol/L) than before (4.1 ± 0.3 mmol/L) in PLA ($P = 0.013$; $d = 1.33$), but
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19 393 did not change in BR from pre-to-post exercise (4.1 ± 0.3 *cf.* 4.3 ± 0.2 mmol/L respectively,
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22 394 $P = 0.188$).

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26 396 *****Table 1 here*****

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30 398 **Discussion**

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32 399 The main findings of this study further the current understanding of the physiological
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35 400 responses to exercise mediated by NO_3^- supplementation. In support of our hypothesis, we
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37 401 have demonstrated that the O_2 cost of exercise, the exercise-induced increase in blood $[K^+]$
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40 402 and both resting and exercising MAP was reduced, alongside favourable alterations in the
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42 403 pulmonary $\dot{V}O_2$ kinetics during rest-to-exercise transitions, after consuming NO_3^- -rich BR
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45 404 across a 24-h period. Collectively, these findings support, and extend upon, the reported
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47 405 physiological effects conferred by BR supplementation, which have many potential
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50 406 implications for people walking on inclined surfaces as part of their daily tasks. Furthermore,
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52 407 our findings suggest that the form of locomotion (uphill walking) permits the physiological
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54 408 changes induced by dietary NO_3^- to manifest during exercise, despite the moderate intensity
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57 409 of the task. The $MAP/\dot{V}O_2$ at higher external intensities (i.e. 15-20% inclines) also reveals a
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60 410 phenomenon previously unreported after dietary NO_3^- supplementation.

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2 412 We have demonstrated that acute supplementation of a NO₃⁻-rich BR supplement lowered
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5 413 MAP at baseline (rest) and during inclined walking, with seemingly larger effects at gradients
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7 414 of 1% - 10%, as well as during post-exercise recovery (Figure 1). However, there were no
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10 415 differences in HR between conditions (Table 1). These findings imply that the net systemic
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12 416 vasodilatory effect of NO on blood vessels was more active during rest and lower-intensity
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14 417 exercise ($Cw < \sim 7$ J/kg/min) than when exercise intensity was increased ($Cw = > \sim 8$
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17 418 J/kg/min) via inclined walking. Indeed, since MAP was also lower during rest, the net
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19 419 increase in MAP during 15% and 20% inclines was actually larger in the BR compared to the
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22 420 PLA condition. We did not anticipate these responses and, consistent with most studies
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24 421 (Bond et al. 2013; Choi et al. 2016; Kenjale et al. 2011), it was hypothesized that MAP would
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27 422 be systematically lower throughout the rest and exercise protocol.

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32 424 Oggioni et al. (2017) reported very similar findings to that of the current study, albeit among
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34 425 older adults (64.7 ± 3.0 years), where reductions in diastolic BP were observed at lower
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36 426 external cycling power output of 20 W – 80 W after BR consumption. During their
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39 427 subsequent exhaustive trial, both systolic and diastolic BP was equal between PLA and BR
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41 428 conditions. However, other studies have reported a consistent reduction in BP with increasing
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44 429 exercise intensity (Bond et al. 2013; Choi et al. 2016; Kenjale et al. 2011). The reasons for
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46 430 conflicting findings between studies is unclear, but it is noteworthy that each of the studies
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49 431 with contrasting data to ours used manual sphygmomanometry to measure BP responses,
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51 432 which rely on human operation and only provide a single caption of end-exercise values,
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53 433 rather than continuous recordings. Indeed, ambulatory BP recordings have previously been
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56 434 reduced among hypertensive individuals (Kapil et al. 2015), which avoids the technical
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58 435 expertise needed to measure manual exercising BP. One explanation for our MAP

1 436 observations could be a greater increase in cardiac output (via stroke volume) in the BR
2 437 condition at higher gradients; however, changes in cardiac output have not been observed
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4 438 after BR supplementation (Oggioni et al. 2017). Redistribution of blood flow towards the
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7 439 working skeletal musculature has also been demonstrated following BR supplementation
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10 440 (Horiuchi et al. 2017) and the seemingly necessary exercise-induced net increase in central
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12 441 BP might also be explained by a similar mechanism. Further investigations are needed to
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14 442 explore such mechanisms associated with nitrate-induced changes in MAP during different
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17 443 exercise tasks.

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21 445 Consistent with other studies (Bailey et al. 2009; Larsen et al. 2011; Lansley et al. 2011b), we
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24 446 found a reduction in the O₂ cost of exercise, which translated into improvements in GE at
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27 447 10%, 15% and 20% gradients, among the BR condition compared to PLA (Figure 5). These
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29 448 findings are most comparable with the reported 12%-14% reductions in O₂ cost of exercise
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31 449 (Lansley et al. 2011b), despite the lower treadmill velocity (3 km/h vs. 4 km/h). The only
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34 450 other study to use an inclined treadmill protocol (Kenjale et al. 2011) did not increase the
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37 451 incline beyond 6% and reported minimal effects of BR on $\dot{V}O_2$ responses, despite exercising
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39 452 to exhaustion. We attribute our findings to the type of exercise being performed at higher
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41 453 gradients.

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46 455 When walking uphill at progressively steeper inclines, muscle recruitment strategies are
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49 456 proportionally altered, such that the leading leg performs longer and more forceful concentric
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51 457 muscle contractions (i.e. more positive work) (Gotschall and Kram 2006). Based on
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54 458 Henneman's size principle, the leading legs would require recruitment from a greater number
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56 459 of larger motor units (type II fibre). These muscle fibres provide more force but are less
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59 460 fatigue-resistant and are recruited during a period of increased metabolic demand (Minetti et

1 461 al. 2006). Given the lower-limb forces required to walk on inclined terrain, coupled with the
2 462 above-mentioned changes in gait, it is feasible that intermittent occlusion of blood flow will
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4 463 also result in metabolic perturbation at the working musculature. The increased metabolic
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6 464 cost, intensity and duration of contractions, coupled with an intermittent occlusion of local
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8 465 blood flow that occurs during uphill walking, would lead to a mildly hypoxic and acidic
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10 466 environment, which has been suggested to potentiate the effects of NO at the muscle (Jones et
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12 467 al. 2016).

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19 469 Plasma insulin and glucose concentrations were unaffected by the BR condition compared to
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21 470 PLA, which has been reported before (Gilchrist et al. 2013), despite the previously described
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23 471 improvements in glucose homeostasis induced by dietary NO_3^- after intense exercise (Wylie
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25 472 et al. 2013). Our findings are most likely related to the lower intensity of the exercise or the
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27 473 health status of the participants, who did not present with symptoms of metabolic disease.
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29 474 There were also no differences in B[La] between conditions after each trial. However, owing
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31 475 to the organization of the protocol, B[La] was recorded in the 1-min after exercise cessation,
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33 476 which is unlikely to permit a larger accumulation of lactate in the blood. There was an
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35 477 interaction between time and condition for $[\text{K}^+]$, indicating that BR supplementation
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37 478 attenuated its exercise-induced increase. This confirms the findings of other analyses (Wylie
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39 479 et al. 2013), which were explained by the accelerated NO_3^- - NO_2^- -NO pathway in acidic
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41 480 environments, potentially blunting the rise in extracellular $[\text{K}^+]$ in this state. We also
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43 481 speculate that the increased ATP availability (Larsen et al. 2011) for Na^+/K^+ pumping or
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45 482 increases in muscle blood flow (Ferguson et al. 2013) found with NO_3^- consumption would
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47 483 facilitate clearance of K^+ . We are the first to show this during moderate intensity exercise and
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49 484 also attribute this finding to the type of exercise performed. Plasma $[\text{K}^+]$ is increased after
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51 485 exercise, reflecting a transient disturbance in muscle ion homeostasis and reduced muscle
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1 486 excitability. Intermittently walking at 1%-20% inclines for a total of 55-min at an intensity
2 487 below the heavy domain was, therefore, sufficient to induce K^+ flux into the blood. The
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4 488 recruitment of muscle fibres when fixed into an inefficient gait cycle is likely to be type II
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7 489 preferential, thus relying upon musculature that is more fatigable owing to the lower O_2
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10 490 muscle tension (Jones et al. 2016). The observed increase in $[K^+]$ in this study can be
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12 491 considered as a marker of peripheral fatigue that is increased during inclined walking and
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14 492 attenuated by BR supplementation. Future studies should explore the mechanisms through
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17 493 which BR exerts this effect.
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22 495 $\dot{V}O_2$ on-kinetics were improved with BR supplementation. Reductions in the amplitude and
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24 496 non-significant ($P = 0.07$), yet small effect sizes ($d = 0.2 - 0.5$), for phase II time constant
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26 497 from rest to exercise support the findings of others during different exercise types (Bailey et
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29 498 al. 2009, 2010; Lansley et al. 2011b). To the best of the authors' knowledge, this is the first
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31 499 study to demonstrate enhanced recovery of pulmonary O_2 after BR supplementation.
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34 500 Recovery was improved by reducing the resting $\dot{V}O_2$ during the intermittent 5-min recovery
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36 501 periods. These results were accompanied by a lower resting MAP, reflecting reduced
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39 502 cardiorespiratory stress in response to exercise and, thus, improved exercise tolerance after
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41 503 BR supplementation.
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46 505 There are a variety of occupations in which employees are required to regularly perform
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48 506 physically demanding tasks. For example, the daily duties of outdoor occupations, such as
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51 507 military, farming and emergency services, comprise walking activities performed over-
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53 508 ground of varying topography (Ruby et al. 2002). Similarly, physically challenging outdoor
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56 509 activities, such as mountaineering and hiking, are increasingly popular recreational pursuits,
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59 510 requiring prolonged sub-maximal hill-walking (Ainslie et al. 2002, 2005; Gottschall and
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1 511 Kram 2006). For people engaged in such habitual activities, uphill locomotion is perhaps one
2 512 of their most challenging tasks and can lead to an earlier onset of fatigue (Vernillo et al.
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4 513 2017). Indeed, there is a significant metabolic cost associated with hill-walking, which
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6 514 increases proportionally with the gradient of the slope and limits the capacity for mechanical
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8 515 energy transfer (Gottschall and Kram 2006; Minetti et al. 2006). Interventions that increase
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10 516 hill-walking performance and presumably delay the onset of fatigue via reduced metabolic
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12 517 costs delay the onset of fatigue, without the need for long-term training adaptation, are
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14 518 therefore applicable to habitual hill-walkers. Beyond those employed in hazardous
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16 519 occupations, the capacity to improve the physiological responses to daily exercise, such as
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18 520 commuting, might also confer a functional and health benefit. Given its apparent capacity to
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20 521 reduce the appearance of fatigue-related metabolites and lower the O₂ cost of exercise, acute
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22 522 NO₃⁻ supplementation (BR) could provide a practical intervention for such populations.
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32 524 **Conclusion**

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34 525 The Cw of walking is reduced and muscle efficiency is increased, particularly at steeper
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36 526 gradients (10%-20%), after acute BR supplementation. Intermittent recovery of pulmonary
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38 527 O₂ uptake and MAP is ameliorated and selected blood markers of peripheral fatigue are
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40 528 attenuated by BR supplementation. BP was particularly lowered whilst walking at smaller
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42 529 gradients (1-10%) after BR supplementation but approaches PLA conditions at higher (15-
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44 530 20%) gradients, despite a lower O₂ cost. We collectively attribute these findings to the
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46 531 inefficient form of locomotion that is required to walk at these gradients, necessitating the
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48 532 recruitment of more fatigable muscle fibre types during a period of increased metabolic
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50 533 demand and intermittently hypoxic or mildly acidic environment, known to augment the
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52 534 reduction of NO₂⁻ to NO. However, further research is required to confirm these suggestions.
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1 535 These findings have implications for those exercising on terrain of varying topography in
2 536 their daily tasks.
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14 540 **Compliance with Ethical Standards**

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20 542 **Conflict of Interest:** The authors declare that they have no conflict of interest.
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23 543 **Ethical approval:** All procedures were performed in accordance with the ethical standards of
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25
26 544 the institutional and/or national research committee and with the 1964 Helsinki declaration
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29 545 and its later amendments or comparable ethical standards.
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32 546 **Informed consent:** Informed consent was obtained from all individual participants included
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34 547 in the study
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737 **Figure legends**

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Figure 1. Mean arterial pressure responses to walking at 1%, 5%, 10%, 15% and 20% inclines and intermittent recovery in beetroot and placebo conditions ($n = 8$). *Significant ($P < 0.05$) main effect of BR supplementation

Figure 2. Pulmonary $\dot{V}O_2$ (L/min) responses of a representative subject walking at 1%, 5%, 10%, 20% and 15% inclines and intermittent recovery in beetroot and placebo conditions ($n = 8$). Data are shown in 5-s averages.

Figure 3. Mean pulmonary $\dot{V}O_2$ (ml/min) responses to walking at 1%, 5%, 10%, 15% and 20% inclines and intermittent recovery in beetroot and placebo conditions ($n = 8$). *Significant ($P < 0.05$) main effect of BR supplementation

Figure 4. Energy cost (J/kg/min) of walking at 1%, 5%, 10%, 15% and 20% inclines in beetroot and placebo conditions ($n = 8$). *Significant ($P < 0.05$) main effect of BR supplementation

Figure 5. Gross efficiency (%) of walking at 1%, 5%, 10%, 15% and 20% inclines in beetroot and placebo conditions ($n = 8$). *Significant ($P < 0.01$) interaction between BR and PLA.

Table title

Table 1. Cardiorespiratory responses to walking at 1%, 5%, 10%, 15% and 20% inclines in beetroot (BR) and placebo (PLA) conditions ($n = 8$). *Significant ($P < 0.05$) main effect of BR supplementation

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Figure 1

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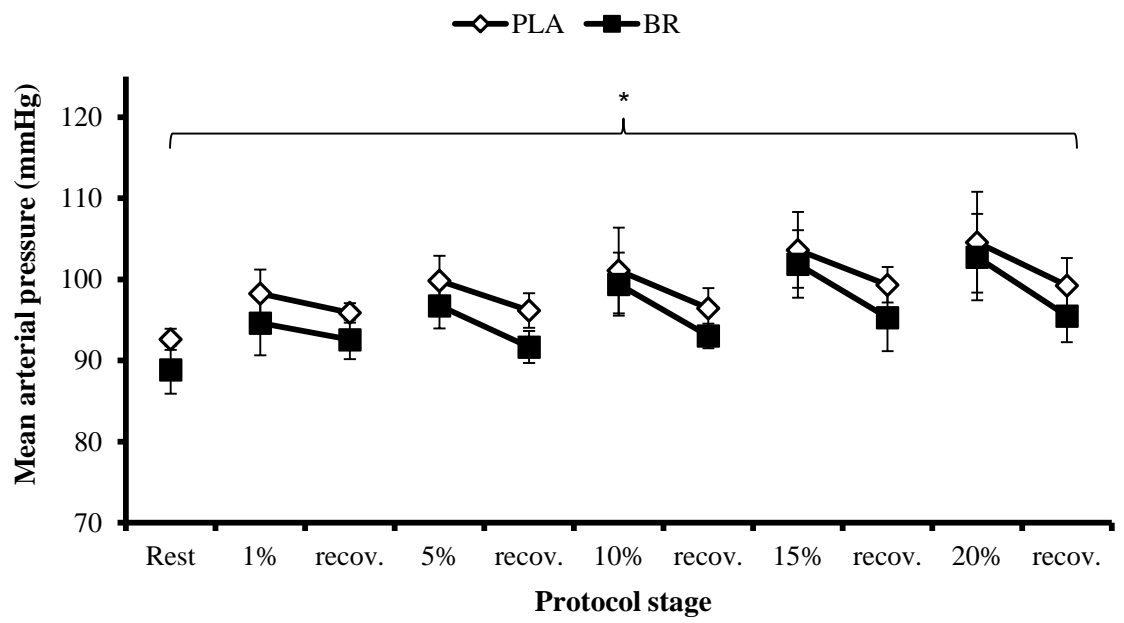


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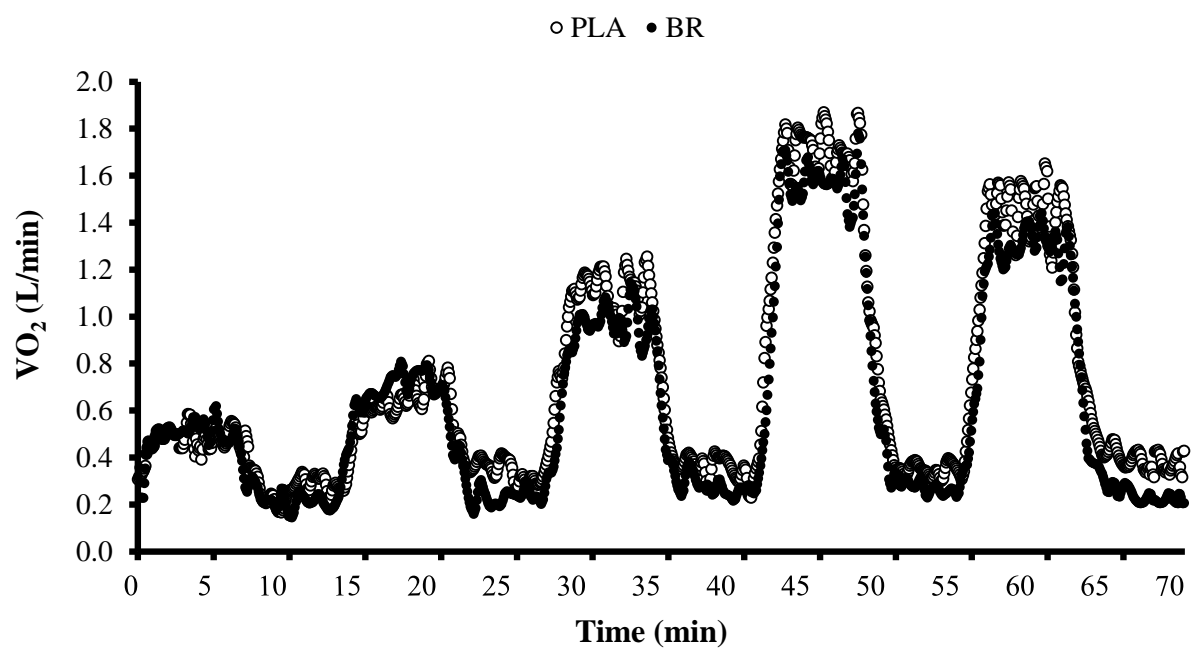


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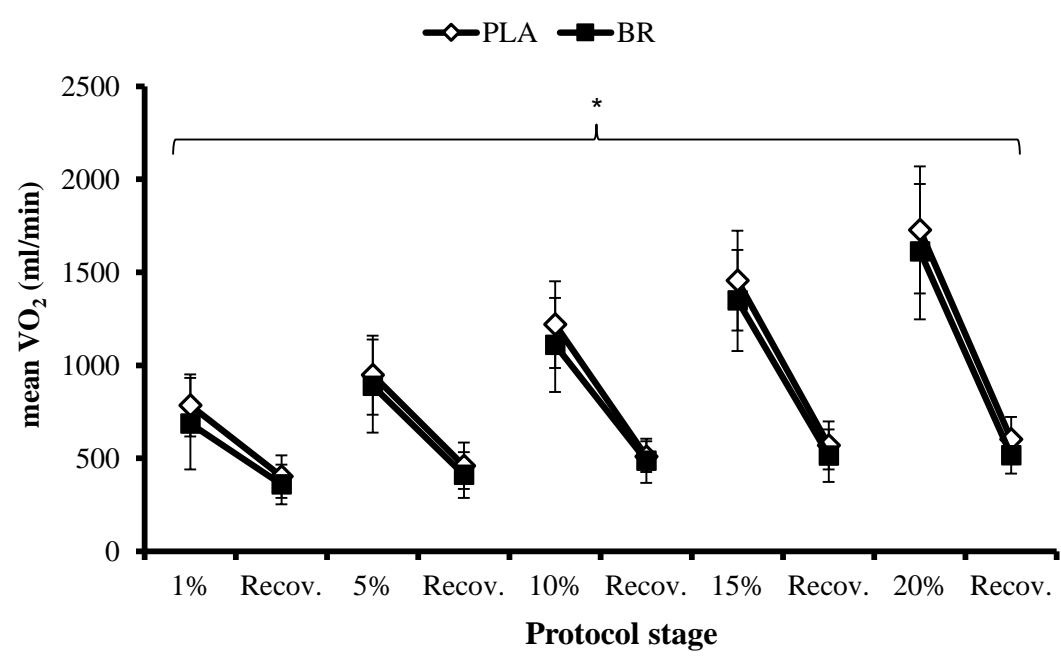


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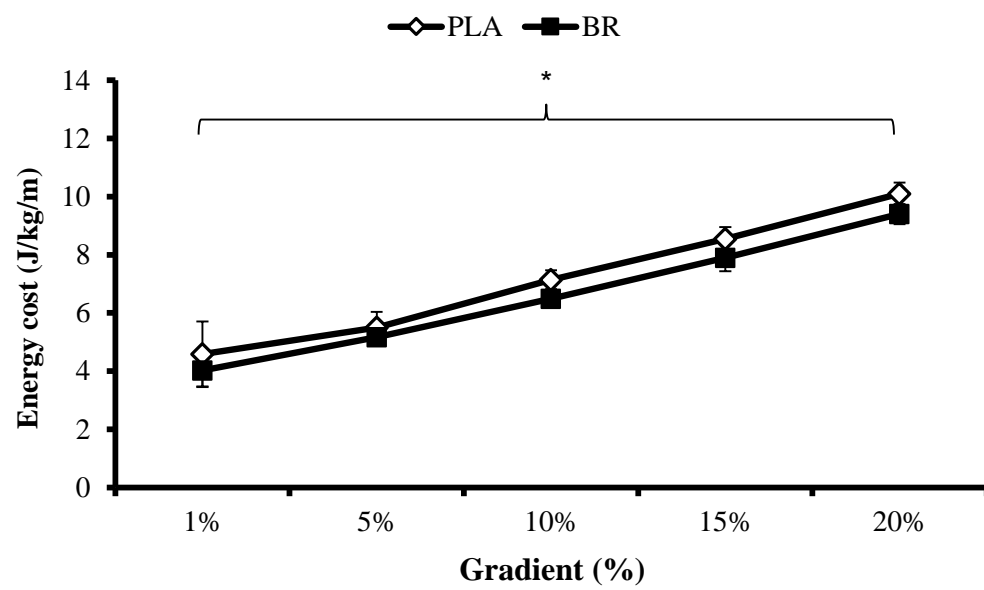


Table 1

	PLA	BR	PLA	BR	PLA	BR	PLA	BR	PLA
	1%		5%		10%		15%		
End exercise VO ₂ (ml/min)*	794 ± 204	643 ± 137	1076 ± 277	931 ± 221	1363 ± 285	1249 ± 264	1707 ± 287	1550 ± 310	2083 ± 38
On phase II amplitude (ml/min)*	472 ± 125	349 ± 101	742 ± 265	591 ± 237	1078 ± 303	1086 ± 383	1319 ± 291	1158 ± 278	1722 ± 30
On phase II time constant (s)	30 ± 16	26 ± 13	35 ± 12	29 ± 7	43 ± 11	38 ± 16	24 ± 10	26 ± 11	37 ± 18
Mean response time (s)	41 ± 18	39 ± 14	47 ± 12	42 ± 9	56 ± 12	52 ± 16	32 ± 11	37 ± 13	48 ± 18
Exercising Heart rate (b/min)	80 ± 11	79 ± 12	88 ± 12	86 ± 13	98 ± 13	97 ± 12	113 ± 19	109 ± 20	120 ± 17
Resting heart rate (b/min)	71 ± 10	68 ± 12	78 ± 12	76 ± 10	81 ± 13	77 ± 11	87 ± 20	79 ± 14	89 ± 18

MW conceived and designed research. MW, JH and CL conducted experiments. MW, JH and LW analyzed data. MW, JH, LW and AG wrote the manuscript. All authors read and approved the manuscript.