Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O_2 uptake dynamics during exercise in hypoxia and normoxia

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Running Head: Nitrate supplementation in hypoxia and normoxia

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28   **Abstract**

29   We investigated the effects of dietary nitrate (NO_3^-) supplementation on the concentration of

30   plasma nitrite ($[NO_2^-]$), oxygen uptake ($\dot{V}_{O_2}$) 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% O_2) and twice in H (13.1% O_2). Subjects ingested either 140

33    $ml·d^{-1}$ of NO_3^--rich beetroot juice (8.4 mmol NO_3; BR) or 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 $[NO_2^-]$ 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 $[NO_2^-]$ was greater during severe-

37   intensity exercise in H-BR (-30±22 $nM·min^{-1}$, 95% *CI*; -44, -16) compared to H-PL (-7±10

38    $nM·min^{-1}$, 95% *CI*; -13, -1; *P* = 0.00) and in N-BR (-26±19 $nM· min^{-1}$, 95% *CI*; -38, -14)

39   compared to N-PL (-1±6 $nM· min^{-1}$, 95% *CI*; -5, 2; *P* = 0.00). During moderate-intensity

40   exercise, steady-state pulmonary $\dot{V}_{O_2}$ was lower in H-BR (1.91±0.28 $L·min^{-1}$, 95% *CI*; 1.77,

41   2.13) compared to H-PL (2.05±0.25 $L·min^{-1}$, 95% *CI*; 1.93, 2.26, *P* = 0.02) and $\dot{V}_{O_2}$ kinetics

42   was faster in H-BR (τ: 24±13 s, 95% *CI*; 15, 32) compared to H-PL (31±11 s, 95% *CI*; 23,

43   38; *P* = 0.04). NO_3^- supplementation had no significant effect on $\dot{V}_{O_2}$ 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 NO_3^- in hypoxia (H-PL:

46   197±28; 95% *CI*; 173, 220 vs. H-BR: 214±43 s, 95% *CI*; 177, 249; *P* = 0.04) but not

47   normoxia. The metabolism of NO_2^- during exercise is altered by NO_3^- supplementation,

48   exercise and to a lesser extent, hypoxia. In hypoxia, NO_3^- supplementation enhances $\dot{V}_{O_2}$

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.

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Introduction

Nitric oxide (NO) is a ubiquitous, water soluble, free radical gas which plays a crucial role in many biological processes. Effective NO production is important in normal physiological functioning, from the regulation of blood flow, muscle contractility and mitochondrial respiration, to host defence, neurotransmission and glucose and calcium homeostasis (11, 17, 60). NO production via the oxidation of L-arginine, in a process catalysed by nitric oxide synthase (NOS), may be blunted in conditions of reduced O_2 availability (52). It is now widely accepted that NO can also be generated via an alternative pathway, whereby inorganic nitrate (NO_3^-) is reduced to nitrite (NO_2^-) and further to NO. This NOS- and O_2- independent NO_3^- -NO_2^- -NO pathway represents a complementary system for NO synthesis spanning a broad range of redox states (49). In addition to being produced endogenously, the body's NO_3^- stores can be increased via the diet, with green leafy vegetables and beetroot being particularly rich in NO_3^-. Upon ingestion, inorganic NO_3^- is absorbed from the gut and passes into the systemic circulation where ~25% of it is concentrated in the saliva (50). Commensal bacteria in the oral cavity then reduce the NO_3^- to NO_2^- (21). Some salivary NO_2^- is converted into NO when swallowed into the acidic environment of the stomach (7), whilst the remainder is absorbed, increasing circulating plasma NO_2^- concentration [NO_2^-]. This NO_2^- may be reduced to NO via a number of enzymatic and non-enzymatic pathways (e.g., xanthine oxidoreductase and deoxyhemoglobin), which are potentiated in hypoxic environments, such as may be evident in contracting skeletal muscle (55).

NO plays a key role in the physiological response and adaptation to hypoxia. A reduced fraction of O_2 in inspired air results in reductions in arterial O_2 concentration and intracellular partial pressure of O_2 (PO_2). The development of muscle hypoxia leads to increased metabolic perturbation (46) and reduced functional capacity at altitude (2) and in several disease conditions (22, 34). In order to restore sufficient O_2 supply, local blood flow is increased via hypoxia-induced vasodilatation with NO being implicated as a major mediator of this process (12). NO_2^- may also promote hypoxic vasodilatation in an NO-independent manner (16).

Dietary NO_3^- supplementation, in the form of nitrate salts and nitrate-rich beetroot juice (BR), represents a practical method of increasing circulating plasma [NO_3^-] (31, 42, 67) and [NO_2^-] (4, 33, 62). NO_3^- supplementation has been shown to reduce resting blood pressure (3, 33, 42) and oxygen uptake ($\dot{V}O_2$) during submaximal exercise (4, 39, 40, 41, 62, 67), and to improve

exercise performance in young, healthy individuals exercising in normoxic conditions (14, 38), but not necessarily in well trained athletes (5-6, 66). These changes may be related to NO-mediated alterations in mitochondrial efficiency (39), muscle contractile function (3, 28) and enhanced muscle blood flow, with preferential distribution to type II fibers (23). These physiological alterations could be particularly beneficial when normal O_2 availability (~21%) is reduced. Indeed, NO_3^- supplementation in the form of BR has recently been shown to reduce muscle metabolic perturbation during exercise in hypoxia and to restore constant-work-rate exercise tolerance and post-exercise indices of oxidative function to values observed in normoxia (64). BR supplementation has also been shown to extend incremental exercise tolerance, improve arterial and skeletal muscle oxygenation (50), and to enhance cycling economy and time-trial performance (51), in hypoxia. However, while these studies suggest that BR can improve physiological responses and exercise performance in hypoxia, it has yet to be determined whether the effects BR are more pronounced in hypoxia relative to normoxia.

The dose-response and pharmacodynamic relationships of BR supplementation have recently been investigated in normoxia (67) and provides a guide to enable optimal timing and dosing of BR intake to elicit peak circulating plasma $[NO_2^-]$ values. However, the kinetics of plasma $[NO_2^-]$ during hypoxic exercise and subsequent recovery, and possible changes elicited by BR supplementation, are presently not known. It was recently reported that during high-intensity, intermittent running exercise, plasma $[NO_2^-]$ declined significantly during exhaustive exercise and showed a tendency to recover back to baseline following 15 min of passive rest (68). Previous research has reported increases (1, 54) but, more commonly, decreases (6, 19, 26, 42, 63) in plasma $[NO_2^-]$ during exercise. In addition to exercise, the metabolism of NO and its derivatives are known to be influenced by intracellular PO_2 and the fraction of inspired oxygen (FIO_2). *In vitro*, endothelial NOS (eNOS) expression and eNOS-derived NO production in human endothelial cells are reduced in hypoxia (25, 53). However, *in vivo*, eNOS expression and activity can be up- or down-regulated by hypoxia, with both decreased (58) and increased (44, 48) NO bioavailability being reported in hypoxia. Characterizing the kinetic changes in $[NO_2^-]$ during exercise and recovery at different FIO_2 may offer insight into NO metabolism during exercise in normoxia and hypoxia. This understanding may have important implications for athletes exercising in hypoxic environments.

117   Considering that the $NO_3^- $-$NO_2^-$-NO pathway is facilitated in hypoxic conditions (48), we

118   reasoned that BR supplementation may modulate the changes in $[NO_2^-]$ 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 $[NO_2^-]$ dynamics, pulmonary $\dot{V}_{O_2}$ and muscle oxygenation)

122   and exercise tolerance, in both normoxia and hypoxia. We hypothesized that the reduction of

123    $[NO_2^-]$ during exercise would be greater in hypoxia compared to normoxia but that $[NO_2^-]$

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 ± SD; age = 22 ± 4 yr, height = 1.80 ± 0.06 m,

131   body mass = 78 ± 6 kg, $\dot{V}_{O_2peak}$ = 58.3 ± 6.3 mL·kg^{-1}·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 NO_3^- to 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 (± 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_2 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^{-1} 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_2 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^{-1} of NO_3^--rich BR or 140 ml·d^{-1} of NO_3^--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 $[NO_2^-]$ 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 ^{TM} 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 inspirate. A further 10-min period elapsed with

178   subjects breathing either the hypoxic or normoxic inspirate. 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% Δ (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_2 filtration, which

212   supplied an FIO_2 of 0.131 ± 0.02, and an $FICO_2$ of 0.004 ± 0.00.

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_3^- supplementation

216   before *visits 3-6*. The supplements were either concentrated, NO_3^--rich BR (2×70 mL·d^{-1} of

217   BR providing ~8.4 mmol NO_3^- per day; Beet it, James White Drinks, Ipswich, UK) or

218   concentrated, NO_3^--depleted PL (2×70 ml·d^{-1} of PL providing ~0.006 mmol NO_3^- 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.

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_2^-] and [NO_3^-] 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_2^-] and [NO_3^-].

237   Prior to and regularly during analysis, all glassware, utensils, and surfaces were rinsed with

238   deionized water to remove any residual NO_2^-. Plasma [NO_2^-] and [NO_3^-] were analysed using

239   gas phase chemiluminescence. This initially required NO_2^- and NO_3^- to be reduced to NO gas.

240   For reduction of NO_2^-, undiluted plasma was injected into a glass purge vessel containing 5

241   ml glacial acetic acid and 1ml NaI solution. For NO_3^- 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 NO_2^- and NO_3^- were determined by plotting signal area

249   (mV) against a calibration plot of 25nM to 1μM sodium nitrite and 100nM to 10μM sodium

250   nitrate respectively.The rate of change in plasma $[NO_2^-]$ during the severe exercise bout was

251   calculated as the difference between pre-exercise baseline and exercise $[NO_2^-]$ 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 (O_2) and infrared (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 (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 ([HHb]), oxyhemoglobin concentration ($[HbO_2]$), total hemoglobin

266   concentration ($[Hb_{tot}]$) and tissue oxygenation index (TOI) were measured.

267   *Data analysis*

268   The breath-by-breath $\dot{V}O_2$ 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

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

280   Where $\dot{V}_{O_2}(t)$ represents the absolute $\dot{V}_{O_2}$ at a given time t; $\dot{V}_{O_2\ baseline}$ represents the mean $\dot{V}_{O_2}$

281   over the final 60 s of baseline cycling; A_p, TD_p, and τ_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$_2$], [Hb$_{tot}$] 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$_2$], [Hb$_{tot}$] 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_2) 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_2^-] and [NO_3^-]*

323   Pre-exercise, plasma [NO_2^-] 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_3^-] 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_2^-] 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_2^-] during moderate-intensity exercise. BR supplementation significantly

elevated plasma $[NO_2^-]$ across all time points compared to PL in both hypoxic and normoxic conditions (all $P < 0.05$). In N-BR, plasma $[NO_2^-]$ was significantly decreased after 5 min of moderate-intensity exercise (Mod5) compared to ModBL (ModBL: 332 ± 184 vs. Mod5: 290 ± 207 nM, $P = 0.04$). However, the decrease in plasma $[NO_2^-]$ in H-BR only showed a trend towards a reduction (ModBL: 306 ± 109 vs. Mod5: 270 ± 125 nM, $P = 0.10$). The rate of decline in plasma $[NO_2^-]$ from ModBL to Mod5 was not significantly different in H-BR (-7 ± 11.7 nM·min^{-1}) compared to N-BR (-10.6 ± 15.9 nM·min^{-1}), H-PL (-3.9 ± 6.1 nM·min^{-1}) compared to N-PL (-2.1 ± 4 nM·min^{-1}), H-BR (-7 ± 11.7 nM·min^{-1}) compared to N-PL (-2.1 ± 4 nM·min^{-1}) or N-BR (-10.6 ± 15.9 nM·min^{-1}) compared to N-PL (-2.1 ± 4 nM·min^{-1}).

Severe exercise. There were significant main effects by condition and time and an interaction effect for plasma $[NO_2^-]$ during severe-intensity exercise to exhaustion. BR supplementation significantly elevated plasma $[NO_2^-]$ across all time points compared to PL in both hypoxic and normoxic conditions (all $P < 0.05$). In N-BR, plasma $[NO_2^-]$ significantly decreased after 3 min of severe-intensity exercise (Sev3) and at exhaustion, compared to SevBL (SevBL: 271 ± 177; Sev3: 206 ± 129; $P = 0.01$; Exhaustion: 132 ± 117 nM, $P = 0.00$). In H-BR, plasma $[NO_2^-]$ decreased from SevBL (277 ± 142 nM) to Sev1 (229 ± 123 nM, $P = 0.01$), Sev3 (n=10, 164 ± 64 nM, $P = 0.03$) and exhaustion (171 ± 115 nM, $P = 0.00$). The absolute decline in plasma $[NO_2^-]$ from SevBL to exhaustion showed a trend toward being smaller in H-BR (106 ± 60 nM) compared to N-BR (138 ± 79 nM, $P = 0.10$). In N-PL, plasma $[NO_2^-]$ decreased from SevBL (40 ± 23 nM) to exhaustion (22 ± 19 nM, $P = 0.02$). This decrease was not significant in H-PL (SevBL: 53 ± 65 vs. Exhaustion: 37 ± 45 nM, $P = 0.52$). The rate of decline in plasma $[NO_2^-]$ was significantly greater from SevBL to exhaustion in H-BR compared to H-PL (H-BR: -30 ± 22 vs. H-PL: -7 ± 10 nM·min^{-1}, $P = 0.00$) and in N-BR compared to N-PL (N-BR: -26 ± 19 vs. N-PL: -1 ± 6 nM· min^{-1}, $P = 0.00$), but was not different between N-BR and H-BR ($P = 0.66$) or N-PL and H-PL ($P = 0.13$), (Figure 1).

Recovery. During the 10-min recovery from exhaustive exercise, ANOVA revealed significant main effects by condition and time and an interaction effect for plasma $[NO_2^-]$ (Figure 1). BR supplementation significantly elevated plasma $[NO_2^-]$ across all time points compared to PL in both hypoxic and normoxic conditions (all $P < 0.05$). In N-BR, plasma $[NO_2^-]$ was significantly lower at exhaustion compared to 3 min into the recovery period ($P = 0.05$), with a significant difference also evident between Rec1.5 and Rec3 ($P = 0.01$). Plasma $[NO_2^-]$ was significantly higher in H-BR compared to N-BR at Rec1.5 ($P = 0.04$). In N-PL, recovery of plasma $[NO_2^-]$ was evident between exhaustion and Rec10 ($P = 0.04$), with a

367 significant increase in [NO_2^-] from Rec3 to Rec10 also evident ($P = 0.04$). In H-PL, plasma

368 [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_2 saturation and heart rate*

376 The SaO_2 data at rest and during moderate- and severe-intensity exercise are reported in

377 Table 1. Resting SaO_2 and HR prior to the administration of inspirate were not significantly

378 different between conditions. However, ANOVA revealed a significant main effect by FIO_2

379 following 10 min of breathing the hypoxic or normoxic inspirate, with SaO_2 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_2 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_2 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_2 between BR and PL in either hypoxia or normoxia. Also, there

391 were no differences in HR between conditions (Table 1).

392 $\dot{V}_{O_2}$ *kinetics*

393 Pulmonary $\dot{V}_{O_2}$ 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 $\dot{V}_{O_2}$ response to moderate-intensity exercise. The $\dot{V}_{O_2}$ 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 τ 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 τ 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   inspirate 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$_2$], [Hb$_{tot}$] 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$_2$. 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$_2$] 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$_2$. [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$_2$] 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 ± 28 vs. N-PL: 431 ± 124 s, $P = 0.00$) and BR

426   conditions (H-BR: 214 ± 43 vs. N-BR 412 ± 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 ± 43 vs. H-PL: 197 ± 28 s, $P = 0.04$), whereas the

430 comparison between N-BR and N-PL was not (N-BR: 412 ± 139 vs. N-PL: 431 ± 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; $P = 0.00$).

Discussion

434 Consistent with previous findings, the decline of plasma $[NO_2^-]$ during exercise was greater

435 following BR compared to PL supplementation. However, in contrast to our experimental

436 hypothesis, the decline of plasma $[NO_2^-]$ 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.

Effects of BR supplementation on the kinetic profile of plasma $[NO_2^-]$

444 Plasma $[NO_2^-]$ 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 $[NO_2^-]$ (3, 4, 33, 34, 51, 62,

447 67), following BR supplementation.

448 Previous studies have suggested that baseline plasma $[NO_2^-]$ 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 $[NO_2^-]$ dynamics during

451 and following exercise of different intensities in hypoxia and normoxia with and without

452 NO_3^- supplementation. The results suggest that the metabolism of NO and its derivatives are

453 altered by exercise and NO_3^- supplementation and, to a lesser extent, FIO_2. The interpretation

454 of these data is not straightforward, however. NO_3^- can be reduced *in vivo* to bioactive NO_2^-

455 and further to NO (47) and this reduction of NO_2^- to NO is expected to be facilitated in

456 hypoxia (13). However, NO_2^- is also an oxidation product of NO generation via the NOS

457 pathway (30) with plasma $[NO_2^-]$ providing a sensitive marker of NO production through

458   NOS (43). Therefore, the dynamics of plasma $[NO_2^-]$ over the exercise bouts is likely

459   reflective of the dynamic balance between NOS-derived NO and NO_2^- reduction to NO. In

460   the present study, plasma $[NO_2^-]$ declined during both moderate- and severe-intensity

461   exercise (Figure 1) with the magnitude and rate of plasma $[NO_2^-]$ 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 NO_2^- to NO appeared to outweigh the synthesis of NO through

464   NOS during exercise.

465   The rate of plasma $[NO_2^-]$ 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 $[NO_2^-]$ had fallen significantly below ModBL in

468   N-BR; whereas, there was only a trend for a lower plasma $[NO_2^-]$ in H-BR. Similarly, the rate

469   of plasma $[NO_2^-]$ 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 $[NO_2^-]$

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   O_2 supply, may be greater (12).

475   During the 10-min passive recovery from exhaustive exercise, plasma $[NO_2^-]$ increased in a

476   similar fashion in H-PL and N-PL. Specifically, plasma $[NO_2^-]$ increased after 3 min of

477   recovery and plateaued after 10 min. The increases in plasma $[NO_2^-]$ may represent an

478   increase in NO oxidation (as NO is continuing to contribute to muscle perfusion and

479   matching of O_2 supply and demand; 12) during recovery. Following BR supplementation, the

480   recovery profile of plasma $[NO_2^-]$ was slightly different between normoxia and hypoxia.

481   Plasma $[NO_2^-]$ 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 $[NO_2^-]$ 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 O_2 cost of sub-maximal cycle exercise in

487   hypoxia. $\dot{V}_{O_2}$ 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) $\dot{V}_{O_2}$

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_2

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_2 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 τ 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_2 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 τ 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 τ in hypoxia toward values recorded in normoxia, thereby helping to reverse the

508   detrimental effect of a reduced FIO_2 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_2 availability, were speeded by

511   BR supplementation in hypoxia (64). These data suggest that, in addition to reducing O_2

512   demand during exercise (50, 51, present study), BR may enhance skeletal muscle O_2

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_3^- supplementation (4, 40, 62). The mechanistic bases for this

518   lower O_2 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^{2+}-related muscle contractility (28). NO is involved in the regulation of

521   mitochondrial O_2 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_2 may be responsible, in part, for the reduced O_2 cost of exercise

524   following NO_3^- 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_3^- (4 x 70 ml BR shots), tended to be evident with 8.4 mmol NO_3^- (2 x 70 ml

531   BR shots), but were not evident with 4.2 mmol NO_3^- (1 x 70 ml BR shot) (67). It is therefore

532   possible that an insufficient NO_3^- 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_2 cost of exercise in normoxia.

538   Indices of muscle oxygenation measured with NIRS were altered as a result of the

539   manipulation of FIO_2 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_2 extraction was increased, while

542   [HbO$_2$] 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^{-1} 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 ~ 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*

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585   This study provides the first description of the influence of FIO_2 and BR supplementation on

586   plasma $[NO_2^-]$ dynamics during moderate- and severe-intensity exercise and subsequent

587   recovery in humans. The greater rate of decline of plasma $[NO_2^-]$ during exercise following

588   BR compared to PL supplementation suggests that elevating plasma $[NO_2^-]$ 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_2 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.

593   *Acknowledgements*

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598 **Figure Legends**

599 **Figure 1.** Plasma [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 O_2 uptake ($\dot{V}_{O_2}$) 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 (~21% FIO_2); *B*: Group mean response to moderate-intensity exercise in hypoxia
611 (~13.2 FIO_2); * = *P* < 0.05 compared to H-PL.

612 **Figure 3.** Pulmonary O_2 uptake ($\dot{V}_{O_2}$) 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 (~21% FIO_2); *B*: Group mean response to severe-intensity
618 exercise in hypoxia (~13.2 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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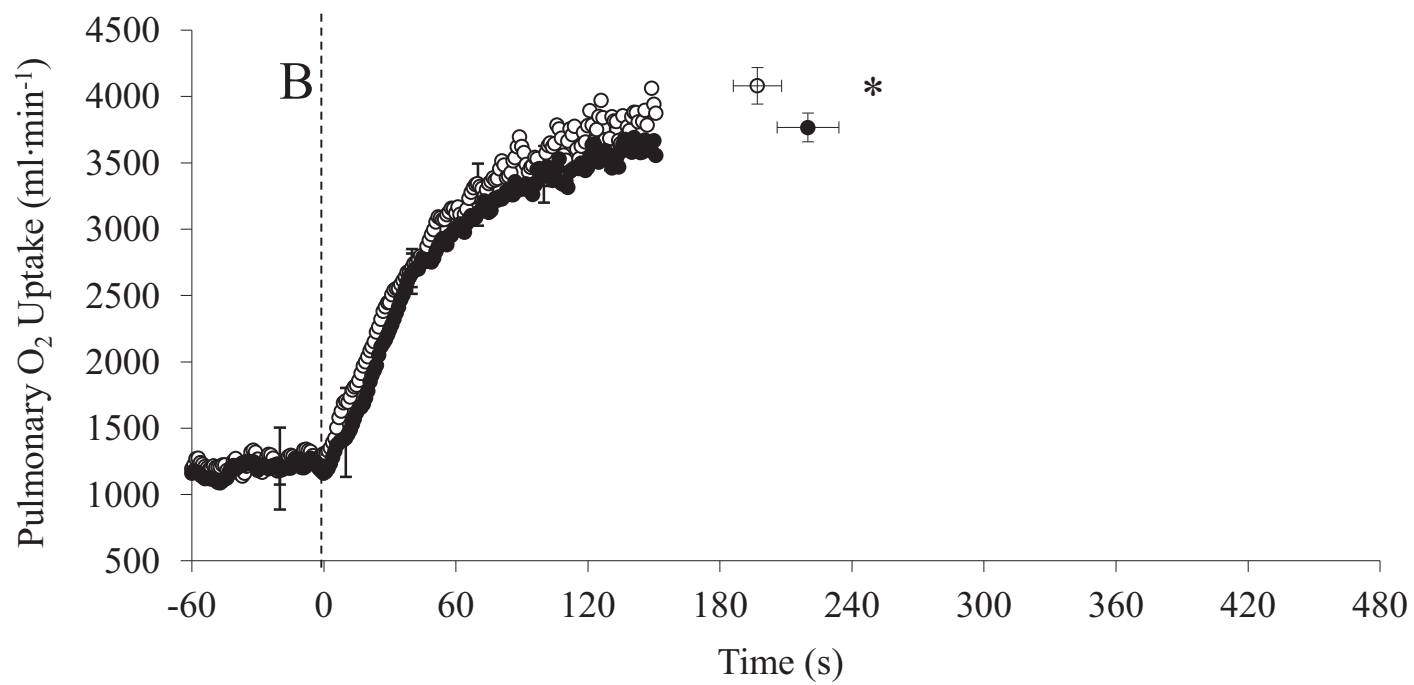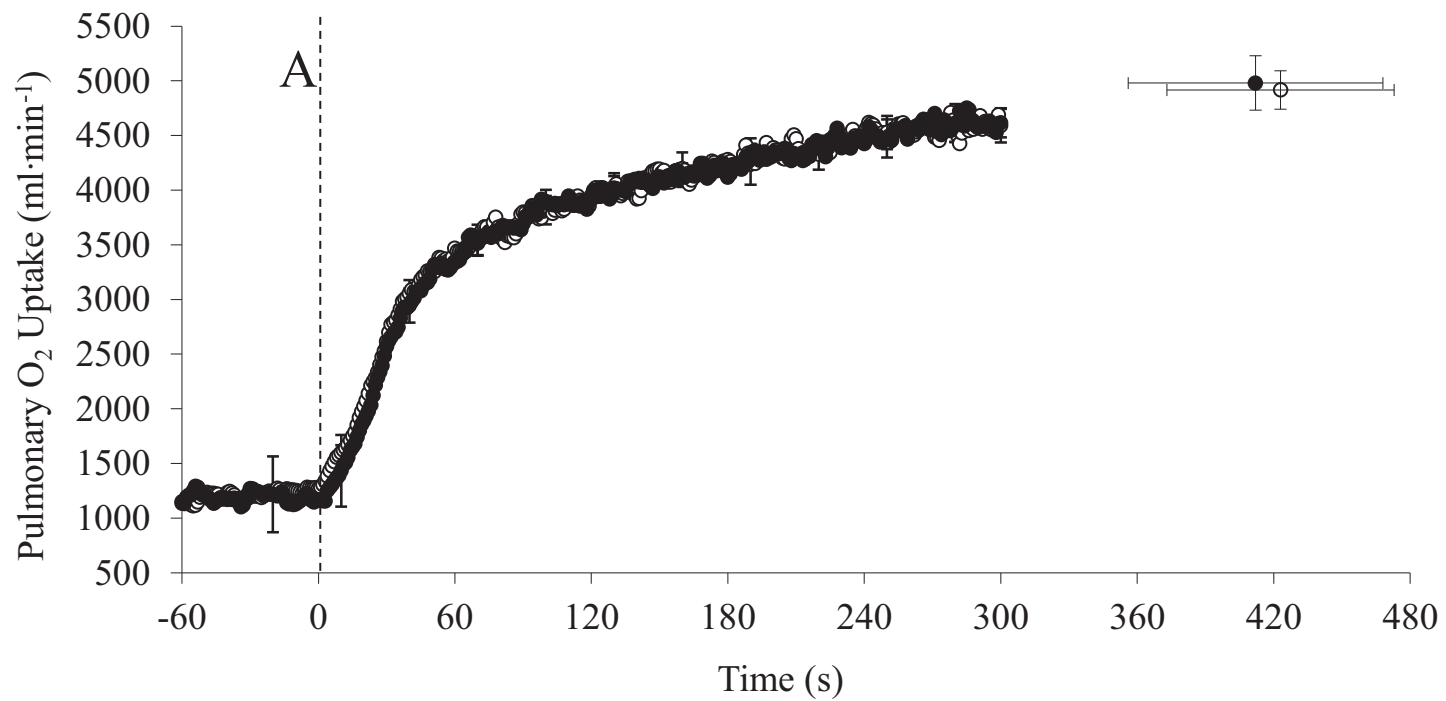
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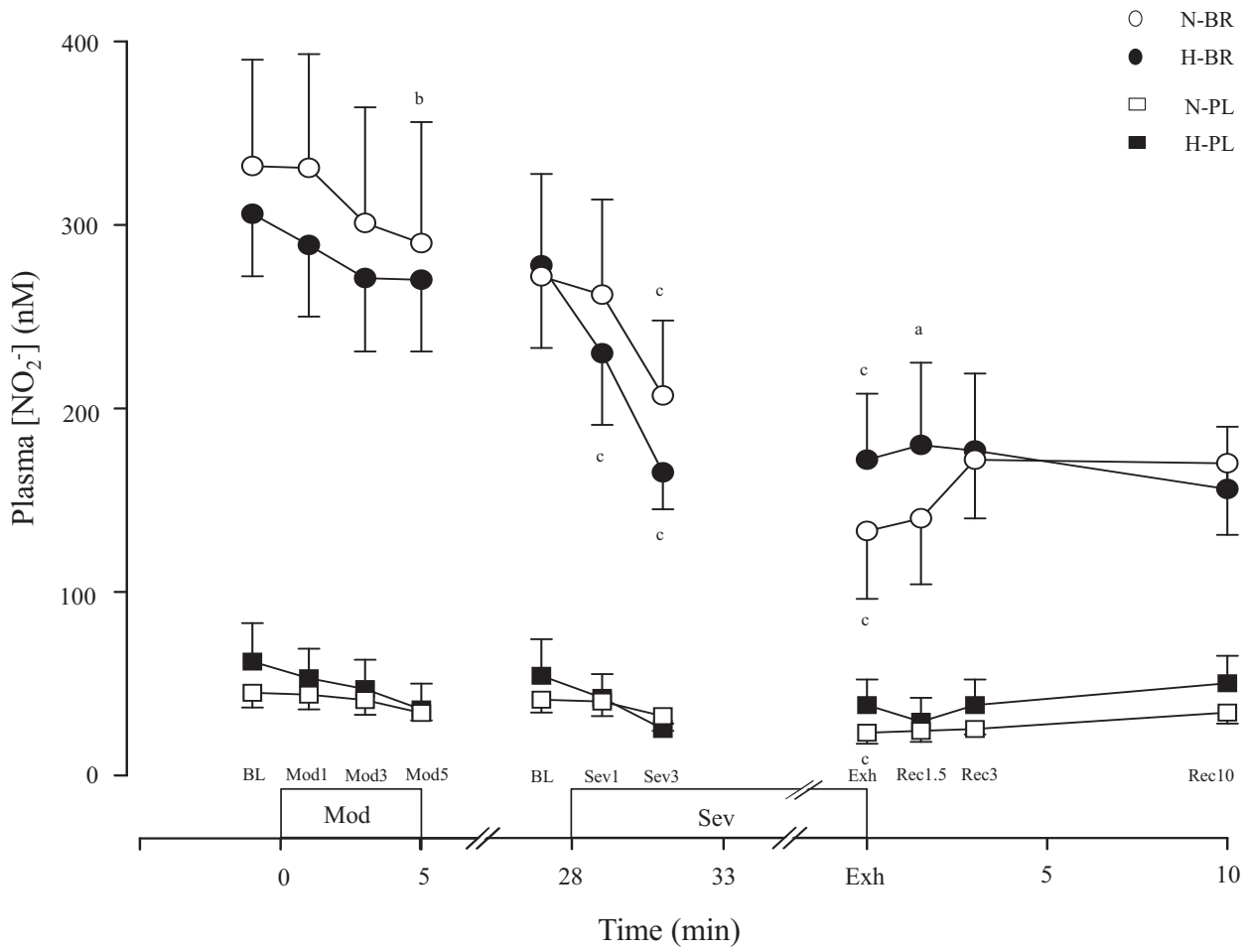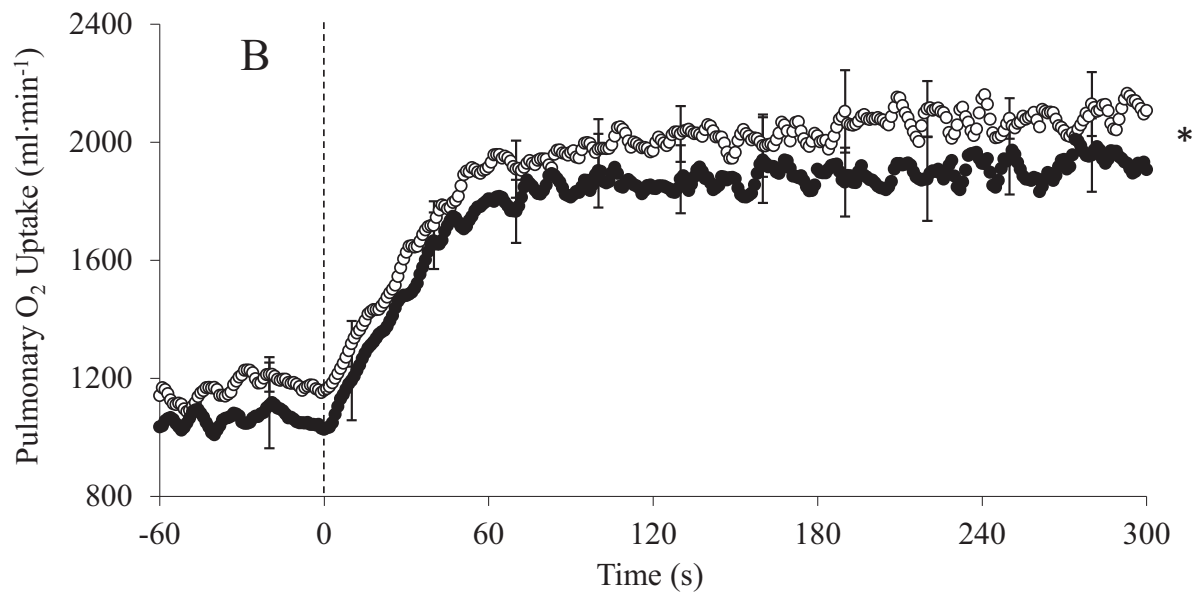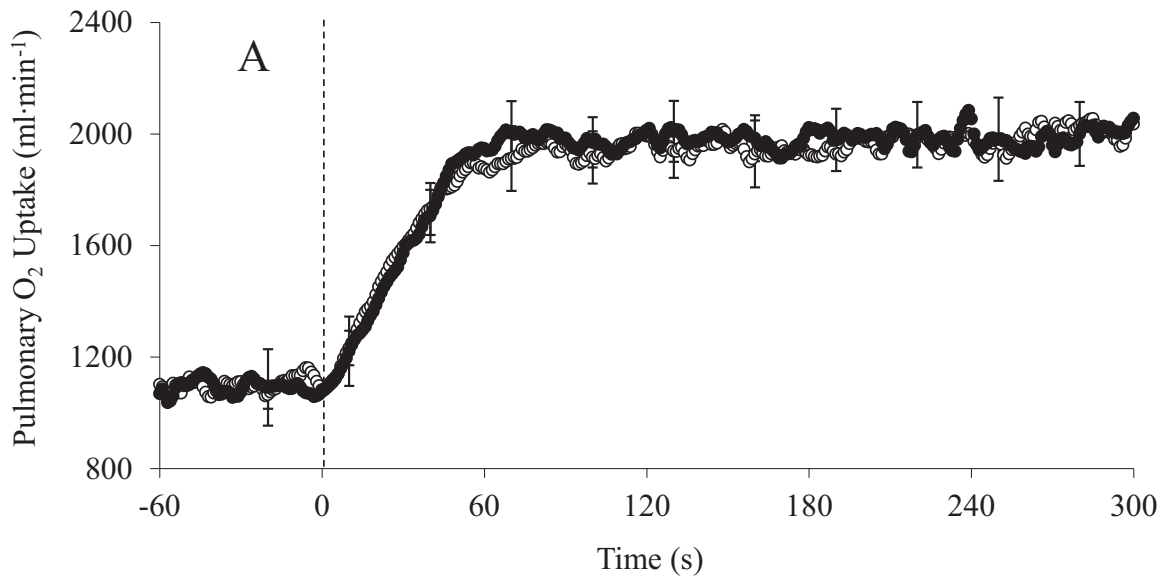
Table 1. Arterial oxygen saturation and heart rate during rest and in response to moderate- and severe-intensity exercise.

	N-PL	N-BR	H-PL	H-BR
Resting without inspirate				
SaO₂ (%)				
10 min period	99 ± 1	99 ± 1	99 ± 1	99 ± 1
End	99 ± 1	99 ± 1	99 ± 1	99 ± 1
HR (b/min)				
10 min period	59 ± 9	61 ± 10	61 ± 10	61 ± 9
End	60 ± 9	61 ± 9	61 ± 10	61 ± 9
Resting with inspirate				
SaO₂ (%)				
10 min period	99 ± 1	99 ± 1	93 ± 2†	93 ± 2*
End	99 ± 1	99 ± 1	90 ± 3†	91 ± 1*
HR (b/min)				
10 min period	58 ± 9	60 ± 11	68 ± 11	66 ± 10#
End	60 ± 8	60 ± 11	68 ± 11†	66 ± 10*
Moderate-intensity exercise				
SaO₂ (%)				
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*
HR (b/min)				
Baseline	82 ± 10	86 ± 12	101 ± 16	94 ± 13
6 min period	102 ± 15	107 ± 15	122 ± 15	117 ± 19#
End	105 ± 16	111 ± 17	130 ± 15†	124 ± 19*
Severe-intensity exercise				
SaO₂ (%)				
Baseline	98 ± 2	97 ± 3	86 ± 4	87 ± 4
Exhaustion	94 ± 4	94 ± 4	80 ± 3†	80 ± 4*
HR (b/min)				
Baseline	97 ± 9	103 ± 12	113 ± 9	114 ± 12
Exhaustion	179 ± 4	180 ± 5	172 ± 6	171 ± 6

# $P < 0.05$ 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
Moderate-intensity exercise				
$\dot{V}O_2$ *(ml/min)*				
Baseline	1102 ± 156	1010 ± 343	1167 ± 123	1056 ± 133[#]
End Exercise	1970 ± 251	1908 ± 340	2049 ± 247	1905 ± 275[#]
Phase II τ, (s)	22 ± 10	17 ± 4[#]	31 ± 11	24 ± 13[#]
Primary amplitude	868 ± 210	899 ± 256	882 ± 214	849 ± 208
Severe-intensity exercise				
$\dot{V}O_2$ *(ml/min)*				
Baseline	1212 ± 179	1205 ± 158	1244 ± 175	1193 ± 177
End Exercise	4814 ± 470	4721 ± 434	3986 ± 300†	3751 ± 249*
Phase II τ, (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†	301 ± 274*

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

Table 3. Near-infrared spectroscopy- derived HHb, HbO$_2$, Hb$_{tot}$ and TOI dynamics during moderate- and severe-intensity exercise.

	N-PL	N-BR	H-PL	H-BR
Moderate-intensity exercise				
[HHb] (AU)				
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*
[HbO$_2$] (AU)				
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*
[Hb$_{tot}$] (AU)				
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
TOI (AU)				
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*
Severe-intensity exercise				
[HHb] (AU)				
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*
[HbO$_2$] (AU)				
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 *
[Hb$_{tot}$] (AU)				
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
TOI (AU)				
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$_2$]), total hemoglobin concentration ([Hb$_{tot}$]) and total oxygenation index (TOI) are shown. * $P < 0.05$ compared to N-BR; † $P < 0.05$ compared to N-PL. AU = arbitrary units.