1 # Dietary nitrate supplementation reduces the O_2 cost

2 # of low-intensity exercise and enhances tolerance to

3 # high-intensity exercise in humans

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Abstract

Pharmacological sodium nitrate supplementation has been reported to reduce the O_2 cost of sub-maximal exercise in humans. In this study, we hypothesised that dietary supplementation with inorganic nitrate in the form of beetroot juice (BR) would reduce the O_2 cost of sub-maximal exercise and enhance the tolerance to high-intensity exercise. In a double-blind, placebo-controlled, crossover study, eight males (aged 19-38 yr) consumed 500 mL per day of either beetroot juice (BR, containing 11.2 ± 0.6 mM of nitrate) or blackcurrant cordial (as a placebo, PL, with negligible nitrate content) for six consecutive days, and completed a series of 'step' moderate-intensity and severe-intensity exercise tests on the last 3 days. On days 4-6, plasma [nitrite] was significantly greater following dietary nitrate supplementation compared to placebo (BR: 273 ± 44 *vs*. PL: 140 ± 50 nM; $P<0.05$) and systolic blood pressure was significantly reduced (BR: 124 ± 2 *vs*. PL: 132 ± 5 mmHg; $P<0.01$). During moderate exercise, nitrate supplementation reduced muscle fractional O_2 extraction (as estimated using near infra-red spectroscopy). The gain of the increase in pulmonary $\dot{V}_{O_2}$ following the onset of moderate exercise was reduced by 19% in the BR condition (BR: 8.6 ± 0.7 *vs*. PL: 10.8 ± 1.6 mL·min^{-1}·W^{-1}; $P<0.05$). During severe exercise, the $\dot{V}_{O_2}$ slow component was reduced (BR: 0.57 ± 0.20 *vs*. PL: 0.74 ± 0.24 L.min-1; $P<0.05$) and the time-to-exhaustion was extended (BR: 675 ± 203 *vs*. PL: 583 ± 145 s; $P<0.05$). The reduced O_2 cost of exercise following increased dietary nitrate intake has important implications for our understanding of the factors which regulate mitochondrial respiration and muscle contractile energetics in humans.

Running Head: Dietary nitrate and $\dot{V}_{O_2}$ kinetics

Key Words: exercise economy, muscle efficiency, O_2 uptake, exercise performance, $\dot{V}_{O_2}$ kinetics, fatigue

Introduction

A fundamental tenet of human exercise physiology is a predictable oxygen (O_2) cost for a given sub-maximal work rate. Upon the initiation of moderate-intensity exercise (i.e. exercise performed at work rates below the gas exchange threshold, GET), pulmonary O_2 uptake ($\dot{V}_{O_2}$), which closely reflects O_2 consumption in the skeletal muscles (2, 28, 38), rises in an exponential fashion to attain a 'steady-state' within approximately 2-3 min in healthy humans (64). The steady-state increase in $\dot{V}_{O_2}$ is linearly related to the increase in external work rate; is essentially independent of factors such as age, health status or aerobic fitness; and approximates 10 mL O_2 per minute per Watt of external power output during cycle ergometry (i.e., 10 mL·min^{-1}·W^{-1}; 36). During supra-GET exercise, $\dot{V}_{O_2}$ dynamics become more complex owing, in part, to the development of a delayed-onset $\dot{V}_{O_2}$ 'slow component' which elevates the O_2 cost of exercise above 10mL·min^{-1}·W^{-1} (36, 64).

Whereas it is known that interventions such as training and the inspiration of hyperoxic gas can reduce the O_2 cost of heavy (above the GET but below critical power, CP, 52) and severe (>CP) exercise by reducing the amplitude of the $\dot{V}_{O_2}$ slow component, the steady-state $\dot{V}_{O_2}$ during moderate exercise is unaffected by these and other interventions in healthy humans (1, 15, 36, 51, 65). Surprisingly, however, it was recently reported that 6 days of dietary supplementation with pharmacological sodium nitrate reduced the O_2 cost of sub-maximal cycling at work rates expected to require 45-80% $\dot{V}_{O_2}$ max (45). That this effect occurred without any increase in estimated non-oxidative energy production (as reflected by an unchanged blood [lactate]) suggested that sodium nitrate ingestion improved the efficiency of muscle oxidative metabolism. It is known that tolerance to high-intensity exercise is, in certain respects, a function of $\dot{V}_{O_2}$ max and sub-maximal exercise economy (20). Therefore, assuming that $\dot{V}_{O_2}$ max is not altered by dietary nitrate, it is feasible that dietary nitrate supplementation might enhance exercise tolerance. However, this possibility has not been investigated.

The nitrate anion (NO_3^-) is relatively inert, and thus any biological effects are likely conferred via its conversion to the bioactive nitrite anion (NO_2^-). Inorganic nitrate is rapidly absorbed from the gut and is concentrated in saliva at least 10-fold. In the mouth, facultative anaerobic

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83   bacteria on the surface of the tongue reduce NO_3^- to NO_2^- (23). Nitrite can be converted to

84   nitric oxide (NO) in the stomach (6, 47) but it is also clear that some is absorbed to increase

85   circulating plasma [nitrite] (21, 46). We and several other groups have shown that NO_2^- can be

86   converted to NO under appropriate physiological conditions (6). The requisite one electron

87   reduction has variously been reported to be catalysed via xanthine oxidoreductase,

88   haemoglobin, myoglobin, endothelial nitric oxide synthase, and the mitochondrial electron

89   transfer complexes (61).

90

91   There are at least two mechanisms by which NO derived from NO_2^- (rather than from the much

92   better known synthesis of NO from L-arginine by the NO synthase family of enzymes) might

93   influence O_2 utilization by contracting skeletal muscle. Firstly, as all of the known

94   mechanisms for NO_2^- reduction are facilitated by hypoxia, it may be that more NO (which is a

95   potent vasodilator) is generated in parts of muscle which are receiving less or using more O_2,

96   and therefore this mechanism would help to match local blood flow to O_2 requirement,

97   providing a more homogenous distribution of O_2 within skeletal muscle. However, while this

98   might be beneficial in terms of muscle function, it would not explain a reduced O_2 cost during

99   exercise. A second possible mechanism involves the roles of NO_2^- and NO as regulators of

100   cellular O_2 utilization. For example NO is known to be an important inhibitor of cytochrome

101   oxidase activity (10). More recently, it has been suggested that NO might enhance the

102   efficiency of oxidative phosphorylation by reducing 'slippage' of the mitochondrial proton

103   pumps (17). There is also evidence that NO_2^- can serve as an alternative electron acceptor,

104   theoretically replacing the role of O_2 in respiration (3).

105

106   The diet constitutes the main source of NO_3^- in humans, with vegetables accounting for 60-

107   80% of daily NO_3^- intake in a Western diet (67). Given the reported ability of pharmacological

108   sodium nitrate to reduce the O_2 cost of exercise (45), we sought to determine whether similar

109   effects are observed when the NO_3^- dose is administered in the form of nitrate-rich beetroot

110   juice (BR). This is important because sodium nitrate is a pharmaceutical product whereas BR

111   is a natural food product that can be readily ingested as part of the normal diet. We therefore

112   investigated the influence of BR ingestion on plasma [nitrite], blood pressure (BP), muscle

113   oxygenation (assessed with near infra-red spectroscopy, NIRS) and the $\dot{V}O_2$ response to step

114 transitions to moderate and severe intensity exercise. We hypothesized that dietary BR

115 supplementation would reduce the O_2 cost of moderate-intensity exercise and increase exercise

116 tolerance (assessed as the time to task failure) during severe-intensity exercise.

117

118 **Methods**

119

120 *Subjects*

121

122 Eight healthy males (mean ± SD, age 26 ± 7 yr, height 180 ± 3 cm, body mass 82 ± 6 kg;

123 maximal O_2 uptake ($\dot{V}O_2$ max) 49 ± 5 mL·kg^{-1}·min^{-1}) who were recreationally active in sporting

124 activities volunteered to participate in this study. None of the subjects were tobacco smokers

125 or users of dietary supplements. All subjects were fully familiar with laboratory exercise

126 testing procedures, having previously participated in studies employing cycle ergometry in our

127 laboratory. The procedures employed in this study were approved by the Institutional Research

128 Ethics Committee. All subjects gave their written informed consent prior to the

129 commencement of the study, after the experimental procedures, associated risks, and potential

130 benefits of participation had been explained. Subjects were instructed to arrive at the

131 laboratory in a rested and fully hydrated state, at least 3 h postprandial, and to avoid strenuous

132 exercise in the 24 h preceding each testing session. Each subject was also asked to refrain from

133 caffeine and alcohol intake 6 and 24 h before each test, respectively. All tests were performed

134 at the same time of day (± 2 hours).

135

136 **Procedures**

137

138 The subjects were required to report to the laboratory on seven occasions, over a 4-5 week

139 period. During the first visit to the laboratory, subjects performed a ramp incremental exercise

140 test for determination of the $\dot{V}O_{2peak}$ and gas exchange threshold (GET). All cycle tests were

141 performed on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, the

142 Netherlands). Initially, subjects completed 3 min of 'unloaded' baseline cycling, after which

143 the work rate was increased at a rate of 30 W·min^{-1} until the subject was unable to continue.

144 The participants cycled at a self-selected pedal rate (70-90 rpm), and this pedal rate along with

145 saddle and handle bar height and configuration were recorded and reproduced in subsequent

146 tests. The breath-by-breath pulmonary gas-exchange data were collected continuously during

147 the incremental tests and averaged over consecutive 10-s periods. The $\dot{V}_{O2peak}$ was taken as the

148 highest 30-s average value attained prior to the subject's volitional exhaustion. The GET was

149 determined as described previously (1, 5). The work rates that would require 80% of the GET

150 (moderate exercise) and 70% Δ (70% of the difference between the power output at the GET

151 and $\dot{V}_{O2\ peak}$, severe exercise) were subsequently calculated with account taken of the mean

152 response time for $\dot{V}_{O2}$ during ramp exercise (i.e., two thirds of the ramp rate was deducted from

153 the power output at GET and peak; 63).

154

155 Following completion of the ramp test, subjects were randomly assigned in a cross-over

156 design, to receive 6 days of dietary supplementation with either nitrate (NO_3^-; 5.5

157 mmol·day^{-1}; administered as 0.5 L organic beetroot juice per day^{-1}; Beet It, James White

158 Drinks Ltd., Ipswich) or "placebo" (low-calorie blackcurrant juice cordial with

159 negligible nitrate content). The subjects were instructed to sip the beverages at regular

160 intervals throughout the day. A 10 day washout separated the supplementation periods.

161 The order between the nitrate and placebo supplementation periods was balanced. The

162 subjects were provided with a list of foods rich in nitrates and asked to abstain from

163 consuming these foods for the duration of the study. The subjects were not aware of the

164 experimental hypotheses to be tested but were informed that the purpose of the study was

165 to compare the physiological responses to exercise following the consumption of two

166 commercially available beverages. The personnel administering the exercise tests were

167 not aware of the type of beverage being consumed by the subjects.

168

169 On days 4, 5 and 6 of the supplementation periods, the subjects completed 'step' exercise tests

170 from a 20 W baseline to moderate and severe intensity work rates for the determination of

171 pulmonary $\dot{V}_{O2}$ dynamics. On the fourth day of supplementation, subjects completed two bouts

172 of moderate cycling, while on days five and six the subjects completed one bout of moderate

173 cycling and one bout of severe cycling. The two bouts of exercise on each day were separated

174 by 25 min of passive recovery. All exercise bouts were of 6 min duration, with the exception

175 of the severe exercise bout on the final day which was continued until task failure as a measure

176   of exercise tolerance. The time to task failure was recorded when the pedal rate fell by >10

177   rpm below the self-selected pedal rate. In these bouts, the subjects were verbally encouraged

178   to continue for as long as possible. The $\dot{V}_{O_2}$ responses to the four moderate and two severe

179   exercise bouts were averaged prior to analysis to reduce breath-to-breath noise and enhance

180   confidence in the parameters derived from the modeling process (44). Before each exercise

181   bout, blood pressure was measured and venous blood samples were collected for subsequent

182   determination of plasma [nitrite] (see 'Measurements').

183

184   **Measurements**

185

186   During all tests, pulmonary gas exchange and ventilation were measured continuously using a

187   portable metabolic cart (MetaMax 3B, Cortex Biophysik, Leipzig, Germany), as described

188   previously (1). A turbine digital transducer measured inspired and expired airflow while an

189   electro-chemical cell O_2 analyzer and an infrared CO_2 analyzer simultaneously measured

190   expired gases. Subjects wore a nose clip and breathed through a low dead-space, low-

191   resistance mouthpiece that was securely attached to the volume transducer. The inspired and

192   expired gas volume and gas concentration signals were continuously sampled via a capillary

193   line connected to the mouthpiece and displayed breath-by-breath. Heart rate (HR) was

194   measured during all tests using short-range radiotelemetry (Polar S610, Polar Electro Oy,

195   Kempele, Finland). During one of the transitions to moderate and severe exercise, for both

196   supplementation periods, a blood sample was collected from a fingertip into a capillary tube

197   over the 20 s preceding the step transition in work rate and within the last 20 s of exercise. A

198   capillary blood sample was also collected at the limit of tolerance for the severe bout

199   performed on day 6 of each supplementation period. These whole blood samples were

200   subsequently analyzed to determine blood lactate concentration ([lactate]) (YSI 1500, Yellow

201   Springs Instruments, Yellow Springs, OH) within 30 s of collection. Blood lactate

202   accumulation (Δ blood [lactate]) was calculated as the difference between blood [lactate] at

203   end-exercise and blood [lactate] at baseline.

204

205   The oxygenation status of the *m. vastus lateralis* of the right leg was monitored using a

206   commercially available NIRS system (model NIRO 300, Hamamatsu Photonics KK, Hiugashi-

207   ku, Japan). The system consisted of an emission probe that irradiates laser beams and a

208   detection probe. Four different wavelength laser diodes provided the light source (776, 826,

209   845, and 905 nm), and the light returning from the tissue was detected by a photomultiplier

210   tube in the spectrometer. The intensity of incident and transmitted light was recorded

211   continuously at 2 Hz and used to estimate concentration changes from the resting baseline for

212   oxygenated, deoxygenated, and total tissue hemoglobin/myoglobin. Therefore, the NIRS data

213   represent a relative change based on the optical density measured in the first datum collected.

214   The [HHb] signal can be regarded as being essentially blood-volume insensitive during

215   exercise and so was assumed to reflect the balance between local O_2 supply and utilization and

216   to provide an estimate of changes in O_2 extraction in the field of interrogation (1, 22, 24, 30).

217   The leg was initially cleaned and shaved around the belly of the muscle, and the probes were

218   placed in the holder, which was secured to the skin with adhesive at 20 cm above the fibular

219   head. To secure the holder and wires in place, an elastic bandage was wrapped around the

220   subject's leg. The wrap helped to minimize the possibility that extraneous light could

221   influence the signal and also ensured that the optodes did not move during exercise. Pen marks

222   were made around the probes to enable precise reproduction of the placement in subsequent

223   tests. The probe gain was set with the subject at rest in a seated position with the leg extended

224   at down stroke on the cycle ergometer before the first exercise bout, and NIRS data were

225   collected continuously throughout the exercise protocols. The data were subsequently

226   downloaded onto a personal computer, and the resulting text files were stored on disk for later

227   analysis.

228

229   Blood pressure of the brachial artery was measured with subjects in a rested, seated position

230   prior to each exercise bout using an automated sphygmomanometer. Three measurements

231   were taken at each sample point with the mean of the second and third blood pressure

232   measurements being recorded. Venous blood samples were also drawn into lithium-heparin

233   tubes prior to each exercise bout and centrifuged at 4000 rpm and 4°C for 10 min, within 3 min

234   of collection. Plasma was subsequently extracted and immediately frozen at -80°C, for later

235   analysis of nitrite (NO_2^-) via chemiluminescence (4).

236

237   All glass wear, utensils and surfaces were rinsed with deionized water to remove residual NO_2^-

238   prior to analysis. After thawing at room temperature, plasma samples were initially

239   deproteinised prior to analysis using the procedures of Higuchi and Motomizu (33). Initially,

240   100 μl of sample was placed in a microcentrifuge tube along with 200 μl of deionised H_2O and

241   300 μl 0.3 N NaOH, and left to stand at room temperature for 5 min. Then, 300 μl of 5% by

242   weight aqueous $ZnSO_4$ was added to the mixture, after which the sample was vortexed and left

243   to stand at room temperature for a further 10 min. Thereafter, samples were centrifuged at

244   4000 rpm for 15 min and the supernatant was removed for subsequent analysis. The $[NO_2^-]$ of

245   the deproteinised plasma samples was determined by its reduction to NO in the presence of

246   5ml glacial acetic acid and 1% NaI under nitrogen at room temperature in a gas-sealed purging

247   vessel. Samples were introduced to the vessel via injection into the septum at the top of the

248   vessel. The NO content was quantified by a chemiluminescence nitric oxide analyzer (Sievers,

249   280i nitric oxide analyzer, CO, USA). The reaction of NO with ozone in the chemiluminescent

250   reaction chamber yielded electronically excited NO_2 (nitrogen dioxide) which emits light at the

251   infra-red region of the electromagnetic spectrum. Ozone was generated from an O_2 supply via

252   an electrostatic ozone generator and high voltage transformer. To minimize the interference of

253   the chemiluminescent reactions of sulfur-containing compounds an optical filter transmitted

254   only red wavelengths (>600 nm), since the light emitted by sulfur-containing compounds is of

255   shorter wavelengths. The intensity of the filtered infra-red light was quantified by a red-

256   sensitive photomultiplier tube and amplified producing an analog mV output signal. The $[NO_2^-]$

257   ] was derived from the integral of the NO-generated mV signal over time compared to those

258   obtained for $NaNO_2^-$ standards.

259

260   **Data Analysis Procedures**

261

262   The breath-by-breath $\dot{V}_{O_2}$ data from each test were initially examined to exclude errant breaths

263   caused by coughing, swallowing, sighing, etc., and those values lying more than four standard

264   deviations from the local mean were removed. The breath-by-breath data were subsequently

265   linearly interpolated to provide second-by-second values and, for each individual, identical

266   repetitions were time-aligned to the start of exercise and ensemble-averaged. The two severe

267   exercise bouts were of different duration (6 min for the first bout and > 6 min in the second

268 bout which was performed to task failure) and so, at this intensity, only the first 6 min of data

269 were averaged together and modeled. The first 20 s of data after the onset of exercise (i.e., the

270 phase I response) were deleted and a nonlinear least-square algorithm was used to fit the data

271 thereafter. A single-exponential model was used to characterize the $\dot{V}_{O_2}$ responses to moderate

272 exercise and a bi-exponential model was used for severe exercise, as described in the following

273 equations:

274

275 $\dot{V}_{O_2}(t) = \dot{V}_{O_2\ baseline} + A_p (1-e^{-(t-TDp/\tau p)})$ (moderate) (Eqn. 1)

276 $\dot{V}_{O_2}(t) = \dot{V}_{O_2\ baseline} + A_p (1-e^{-(t-TDp/\tau p)}) + A_s (1-e^{-(t-TDs/\tau s)})$ (severe) (Eqn. 2)

277

278 where $\dot{V}_{O_2}(t)$ represents the absolute $\dot{V}_{O_2}$ at a given time t; $\dot{V}_{O_2baseline}$ represents the mean $\dot{V}_{O_2}$ in

279 the baseline period; A_p, TD_p, and τ_p represent the amplitude, time delay, and time constant,

280 respectively, describing the phase II increase in $\dot{V}_{O_2}$ above baseline; and A_s, TD_s, and τ_s

281 represent the amplitude of, time delay before the onset of, and time constant describing the

282 development of, the $\dot{V}_{O_2}$ slow component, respectively.

283

284 An iterative process was used to minimize the sum of the squared errors between the fitted

285 function and the observed values. $\dot{V}_{O_2baseline}$ was defined as the mean $\dot{V}_{O_2}$ measured over the

286 final 90 seconds of baseline pedaling. The end-exercise $\dot{V}_{O_2}$ was defined as the mean $\dot{V}_{O_2}$

287 measured over the final 30 seconds of exercise. Because the asymptotic value (A_s) of the

288 exponential term describing the $\dot{V}_{O_2}$ slow component may represent a higher value than is

289 actually reached at the end of the exercise, the actual amplitude of the $\dot{V}_{O_2}$ slow component at

290 the end of exercise was defined as A_s'. The A_s' parameter was compared at the same iso-time

291 (360-s) under both supplementation periods. The amplitude of the slow component was also

292 described relative to the entire $\dot{V}_{O_2}$ response. In addition, the functional 'gain' (G) of the

293 fundamental $\dot{V}_{O_2}$ response was computed by dividing A_p by the Δ work rate. The functional

294 gain of the entire response (i.e., end-exercise 'gain') was calculated in a similar manner. To

295 determine the overall kinetics of the $\dot{V}_{O_2}$ response to both moderate- and severe-intensity

296 exercise, data were fit with a mono-exponential model from 0-s to end-exercise, without time

297 delay.

298

299   To provide information on muscle oxygenation, we also modeled the [HHb] response to

300   exercise. Mono- and biexponential models, similar to those described above, were applied to

301   the ensemble averaged data with the exception that the fitting window commenced at the time

302   at which the [HHb] signal increased 1 SD above the baseline mean (22). The [HHb] kinetics

303   for moderate exercise were determined by constraining the fitting window to the point at which

304   mono-exponentiality became distorted, consequent to a gradual fall in [HHb] (1), as

305   determined by visual inspection of the residual plots. The [HHb] kinetics for severe exercise

306   were determined by fitting a bi-exponential model from the first data point, which was 1 SD

307   above the baseline mean through the entire response. The [HHb] TD and τ values were

308   summed to provide information on the overall [HHb] response dynamics in the fundamental

309   phase of the response. The [HbO_2] and [Hbtot] responses do not approximate an exponential

310   (22) and were, therefore, not modeled. Rather, we assessed changes in these parameters by

311   determining the [HbO2] and [Hbtot] at baseline (90-s preceding step transition), 120 s, and end

312   exercise (average response over the final 30 s of exercise).

313

314   We also modeled the HR response to exercise in each condition. For this analysis, HR data

315   were linearly interpolated to provide second-by-second values, and, for each individual,

316   identical repetitions from like transitions were time aligned to the start of exercise and

317   ensemble averaged. Nonlinear least squares mono-exponential and bi-exponential models

318   without TD were used to fit the data to moderate and severe-intensity exercise, respectively,

319   with the fitting window commencing at $t = 0$ s. The HR τ_p so derived provides information on

320   the overall HR response dynamics.

321

322   **Statistics**

323

324   Differences in the cardiorespiratory and NIRS-derived variables between conditions were

325   analyzed with two-tailed, paired-samples t-tests. Alterations in blood pressure and plasma

326   [NO_2^-] were determined via a two-tailed, two-way (supplement × time) repeated-measures

327   ANOVA.   Significant effects were further explored using simple contrasts with the alpha level

328   adjusted via a Bonferroni correction. Correlations were assessed via Pearson's product

329 moment correlation coefficient. Data are presented as mean ± SD, unless otherwise stated.

330 Statistical significance was accepted when $P<0.05$.

331

332 **Results**

333

334 The BR supplementation regimen implemented in this study was well tolerated with no

335 deleterious side effects. Subjects did, however, report beeturia (red urine) and red stools,

336 consistent with a previous study (62).

337

338 **Plasma [NO_2^-] and blood pressure**

339

340 The group mean plasma [NO_2^-] values obtained at the two sample points on each of days 4, 5

341 and 6 of the BR and PL supplementation periods are illustrated in Fig 1. Participants showed

342 elevations in plasma [NO_2^-] during the BR supplementation compared to PL at all sample

343 points (Fig 1). On average, across the six sample points, BR ingestion increased plasma [NO_2^-]

344 by 96%. The BR-induced elevations in plasma [NO_2^-] were not different across days 4-6.

345

346 The group mean systolic blood pressure values measured at the six BR and PL sample points

347 are shown in Fig 2. The ingestion of BR significantly reduced systolic BP at five of the six

348 sample points, relative to placebo (Table 1). Overall, systolic blood pressure was reduced by 6

349 mmHg across the six samples points (Fig 2); however, similar to plasma [NO_2^-], the BR-

350 induced reductions in systolic BP were not significantly different among days 4-6. The

351 systolic BP was significantly related to the plasma [NO_2^-] on day 5 ($r = -0.71$, $P < 0.05$) but no

352 relationships were detected between systolic BP and plasma [NO_2^-] on days 4 or 6. Diastolic

353 BP ($\sim 72 \pm 8$ mmHg) and mean arterial pressure ($\sim 91 \pm 5$ mmHg) were not significantly

354 affected by BR ingestion.

355

356

NIRS measurements

Moderate exercise

The [O_2Hb], [HHb] and [Hbtot] values measured during moderate exercise are shown in Table 1 and the group mean responses are shown in Fig 3. A 13% reduction in the [HHb] amplitude was observed following BR ingestion, indicating that fractional O_2 extraction was reduced (PL: 88 ± 38 *vs.* BR: 78 ± 34 AU; $P<0.05$; Fig 3). The [O_2Hb] within the microvasculature was increased at baseline and at 2 min into exercise, but was not significantly different at the end of exercise (Table 1). BR ingestion elevated [Hbtot] (an index of vascular red blood cell content) at baseline; however, this effect was not maintained during exercise (Table 1).

Severe exercise

The [O_2Hb], [HHb] and [Hbtot] values measured during severe exercise are shown in Table 1 and the group mean response is shown in Fig 4. In contrast to the BR-induced changes in indices of muscle oxygenation during moderate exercise, the [HHb], [O_2Hb], and [Hbtot] parameters were unaffected by BR ingestion during severe exercise.

Oxygen uptake dynamics and exercise tolerance

Moderate exercise

The pulmonary $\dot{V}_{O_2}$ response during moderate exercise is highlighted in Fig 5 and the parameters derived from the model fit are presented in Table 2. Dietary BR supplementation resulted in a 19% reduction in the amplitude of the pulmonary $\dot{V}_{O_2}$ response, relative to PL, following a step increment to the same absolute moderate-intensity cycling work rate (Pl: 640 ± 146 *vs.* BR: 521 ± 153 ml·min^{-1}; $P<0.01$; Fig 5), with there being no difference in $\dot{V}_{O_2}$ during the baseline period of very low intensity (20 W) cycling. Accordingly, the functional 'gain' (i.e., the ratio of the increase in O_2 consumed per minute to the increase of external power output) was reduced from 10.8 mL·min^{-1}·W^{-1} following PL supplementation to 8.6 mL·min^{-1}·W^{-1} following BR supplementation. The absolute $\dot{V}_{O_2}$ value over the final 30-s of moderate exercise was also significantly lower following BR ingestion (PL: 1517 ± 123 *vs.* BR: 1448 ±

388   129 ml·min^{-1}; $P<0.01$; Fig 5). The phase II time constant was not significantly altered by BR

389   supplementation (PL: 26 ± 7 *vs*. BR: 29 ± 6 s; $P>0.05$). The 95% confidence interval for the

390   estimation of the phase II time constant was 3 ± 1 s for both conditions. The baseline and end-

391   exercise values of $\dot{V}_{CO_2}$, $\dot{V}_E$, respiratory exchange ratio (RER), HR and blood [lactate] were not

392   significantly between the conditions (Tables 2 and 3).

393

394   *Severe exercise*

395

396   The pulmonary $\dot{V}_{O_2}$ response during severe exercise is shown in Fig 6 and the parameters

397   derived from the bi-exponential fit are presented in Table 2. In contrast to the effects observed

398   for moderate exercise, the primary $\dot{V}_{O_2}$ amplitude during severe exercise was significantly

399   elevated following BR supplementation (PL: 2158 ± 168 *vs*. BR: 2345 ± 179 ml·min^{-1};

400    $P<0.05$). Additionally, the phase II time constant was significantly greater following BR

401   supplementation relative to placebo (PL: 33 ± 11 *vs*. BR: 40 ± 13 s; $P<0.05$; Table 2). The

402   95% confidence intervals for the estimation of the phase II time constant were 5 ± 2 s and 6 ± 3

403   s for the PL and BR conditions, respectively. The amplitude of the $\dot{V}_{O_2}$ slow component was

404   significantly smaller following BR supplementation (PL: 739 ± 242 *vs*. BR: 568 ± 195 ml·min^{-}

405   ^{1}; $P<0.05$), and therefore represented a smaller proportion of the overall $\dot{V}_{O_2}$ response (PL: 25 ±

406   6 *vs*. BR: 19 ± 6 %; $P<0.05$). The $\dot{V}_{O_2}$ attained at task failure was not different either between

407   the conditions or from the $\dot{V}_{O_2}$ max recorded during the initial ramp incremental test. Exercise

408   tolerance was enhanced following BR supplementation as demonstrated by the increased time

409   to task failure (PL: 583 ± 145 *vs*. BR: 675 ± 203 s; $P<0.05$; Table 2). However, the increased

410   time to task failure was not correlated with the reduction of the $\dot{V}_{O_2}$ slow component (r = -0.14;

411   P = 0.70). The baseline and end-exercise values of $\dot{V}_{CO_2}$, $\dot{V}_E$, RER and HR were not

412   significantly between the conditions (Tables 2 and 3). Blood [lactate] at 6 min of exercise and

413   at task failure was also not significantly different between the conditions (Table 3).

414

415

Discussion

The principal original finding of this investigation is that three days of dietary supplementation with nitrate-rich beetroot juice (which doubled the plasma [nitrite]) significantly reduced the O_2 cost of cycling at a fixed sub-maximal work rate and increased the time to task failure during severe exercise. These findings were consistent with our experimental hypotheses. The O_2 cost of cycling at a fixed moderate work rate is known to be highly consistent in human populations irrespective of factors such as age and training status (36, 49). That an acute nutritional intervention (i.e., dietary supplementation with a natural food product that is rich in nitrate) can reduce the O_2 cost of a given increment in work rate by ~20% is therefore remarkable.

Short-term (i.e. 4-6 days) dietary supplementation with BR increased plasma $[NO_2^-]$ by ~96% in this investigation. Consistent with these findings, dietary NO_3^- supplementation has previously been shown to increase plasma $[NO_2^-]$ when administered as either sodium nitrate (45) or BR (62). Importantly, interrupting the entero-salivary circulation by spitting out saliva thwarted the rise in plasma $[NO_2^-]$ (62), while administration of antibacterial mouthwash prior to sodium nitrate ingestion also prevented the rise in plasma $[NO_2^-]$ by decreasing the NO_3^- reducing bacteria counts in the oral cavity (29). Collectively, these data highlight the dependence of the NO_3^- to NO_2^- conversion pathway on the commensal bacterial nitrate reductases present in the human oral cavity. The bacterially-derived NO_2^- can increase circulating plasma NO_2^- and undergo reduction to yield NO in hypoxia or acidosis (21, 46).

Effects of dietary nitrate on blood pressure

In the present study, dietary supplementation with BR reduced systolic BP by an average of 6 mm Hg, but without altering diastolic BP or MAP. In contrast, both systolic and diastolic BP was reduced following 3 days of dietary NO_3^- supplementation in the study of Larsen et al. (45). Recent data indicate that, following BR ingestion, peak reductions in systolic and diastolic BP are observed 2.5 and 3 hrs post-ingestion, respectively (62). Furthermore, the BR-induced reduction in systolic BP persisted for 24 hrs post-ingestion, while diastolic BP had

15

447 returned towards baseline (62). Collectively, these data suggest that systolic BP is more

448 amenable to nitrate-induced change than is diastolic BP. The reduced BP observed with a diet

449 rich in nitrates suggests that this 'natural' approach has the potential to maintain or enhance

450 aspects of cardiovascular health.

451

452 The NO_3^--induced reduction in systolic BP is likely mediated via its conversion to NO_2^-, and

453 thence NO. Nitric oxide is known to be an important endothelial relaxing factor, through its

454 role as a secondary messenger in cyclic guanosine mono-phosphate (cGMP) synthesis,

455 culminating in smooth muscle relaxation (31). Conventionally, NO production was considered

456 to be derived, universally, via the NO synthase family of enzymes in humans. These enzymes

457 produce NO through catalyzing the five electron oxidation of L-arginine in a reaction requiring

458 oxygen (O_2) and NADPH (9). More recent research has identified an O_2-independent pathway

459 for the generation of NO, via the reduction of nitrite (NO_2^-) to NO in an acidic milieu (6).

460 Importantly, this pathway preserves blood and tissue NO production during hypoxia when the

461 enzymatic activity of the NO synthases are rate-limited by the lack of O_2 availability. As such,

462 a considerable body of evidence now supports a biological role for NO_2^- in hypoxic

463 vasodilation (26) which serves to protect tissues from ischemia and reperfusion injury (27).

464

465 *Effects of dietary nitrate on the physiological responses to moderate exercise*

466

467 Increased dietary NO_3^- consumption altered indices of muscle oxygenation as investigated

468 using NIRS. During moderate exercise, [Hbtot] was elevated at baseline and [O_2Hb] was

469 elevated both at baseline and over the first 120 s of exercise following BR ingestion. The

470 increased blood volume in the region of interrogation at baseline following BR ingestion is

471 presumably a consequence of enhanced muscle vasodilatation resulting from increased NO

472 production from NO_2^-. The NIRS-derived [HHb] response reflects the balance between local

473 O_2 delivery and utilization and has been used previously as an index of muscle fractional O_2

474 extraction (1, 22, 24, 30). In the present study, the amplitude of the [HHb] response was

475 reduced by 13% following BR ingestion. Conversely, inhibition of NO synthesis via L-NAME

476 administration has been shown to increase muscle O_2 extraction in the exercising horse (40).

477 By the Fick equation, for the same $\dot{V}_{O_2}$, an increased muscle O_2 delivery would be expected to

478 enable a reduced muscle fractional O_2 extraction. However, in the present study in which $\dot{V}_{O_2}$

479 was reduced by dietary BR supplementation, an alternative interpretation is that less O_2

480 extraction was required consequent to a reduced aerobic energy turnover or muscle energy

481 utilization.

482

483 Perhaps the most striking finding of the present investigation was the significant reduction in

484 the O_2 cost of sub-maximal exercise following increased dietary NO_3^- intake. While the $\dot{V}_{O_2}$

485 response during the unloaded baseline cycle was unaffected, a 19% reduction in the amplitude

486 of the pulmonary $\dot{V}_{O_2}$ response, relative to placebo, was evident following NO_3^-

487 supplementation during a step increment to the same absolute moderate-intensity cycling work

488 rate. Accordingly, the functional 'gain' (i.e., the ratio of the increase in O_2 consumed per

489 minute to the increase of external power output and the reciprocal of delta efficiency; 64) was

490 reduced from 10.8 mL·min^{-1}·W^{-1} following placebo supplementation to 8.6 mL·min^{-1}·W^{-1}

491 following NO_3^- supplementation. Moreover, the gross O_2 cost of exercise (comprising resting

492 metabolic rate, the O_2 cost of moving the limbs during baseline pedaling, and the O_2 cost of

493 muscle contraction to meet the imposed work rate) was reduced by ~5%. Importantly, the

494 reduction in $\dot{V}_{O_2}$, and thus of ATP re-synthesis through oxidative phosphorylation, was not

495 compensated by elevations in glycolytic ATP provision, as inferred, albeit crudely, from the

496 similar blood [lactate] values between the BR and placebo conditions. These findings extend

497 those of Larsen et al. (45) by demonstrating that the O_2 cost of sub-maximal exercise is

498 reduced following NO_3^- ingestion in the form of a natural food product. That heart rate (HR)

499 and minute ventilation ($\dot{V}_E$) were not significantly different between treatments suggests that

500 the reduction in $\dot{V}_{O_2}$ originated from the skeletal muscles, and not from alterations in the

501 energetic cost of cardio-respiratory support processes. Moreover, the similar RER between the

502 conditions indicates that substrate utilization (which can influence the O_2 cost of exercise) was

503 not altered by the intervention.

504

505 The mechanistic bases for the reduced O_2 cost of sub-maximal exercise following increased

506 NO_3^- intake either by pharmacological (45) or natural dietary means (present study) are

507 unclear. The inhibition of NO synthesis has previously been shown to increase steady-state $\dot{V}_{O_2}$

508 in dogs (56) but not humans (38) or horses (41). It is widely accepted, however, that NO is

17

509   involved in the regulation of mitochondrial O_2 consumption. In particular, it has been

510   established that NO has a strong affinity for cytochrome c oxidase (CytOX; 10), but there is

511   also evidence that NO has the potential to modulate other aspects of mitochondrial and muscle

512   contractile function (11, 57, 58).

513

514   A reduction in the O_2 cost of mitochondrial ATP re-synthesis would require either more

515   protons pumped per O_2 molecule reduced, or the use of an alternative terminal electron

516   acceptor in place of O_2. It has been proposed that mitochondrial efficiency is intimately linked

517   to the process of uncoupled respiration in which mitochondrial proton leak results in energy

518   dissipation as heat instead of conversion to ATP (8). In this regard, the improved O_2 efficiency

519   noted in the present study following BR ingestion might be related to a reduction of

520   mitochondrial proton leak or proton pump slippage. There is evidence to suggest that NO

521   increases the efficiency of oxidative phosphorylation in isolated mitochondria by reducing

522   slipping of the proton pumps (17). Another possibility is that NO_2^- could be acting in place of

523   O_2 as the final electron acceptor in the respiratory chain, thereby reducing the requirement for

524   O_2 consumption (3). An intra-mitochondrial NO_2^- regeneration pathway would be critical in

525   this scenario, given the limited NO_2^- concentration within the mitochondria. Under conditions

526   of low electron flux, NO can inhibit CytOX by binding to the Cu^{2+} active site yielding

527   nitrosonium (NO^+) which is subsequently hydrated to NO_2^- (18). One possible scenario is that

528   the NO_2^- so produced could be reduced to NO by accepting an electron from CytOX, and this

529   NO could subsequently bind to the Cu^{2+} active site, completing the cyclical process to

530   regenerate NO_2^-. This possibility is intriguing as the hydration of NO^+ to NO_2^- yields an

531   electron which can be redistributed within CytOX (19, 60). Subsequently, this electron may be

532   accepted by NO_2^-, potentially utilizing the electron derived from its synthesis, and could

533   contribute to proton pumping and ATP synthesis, in an efficiently-coupled process.

534

535   The reduction in O_2 consumption with increased dietary NO_3^- intake could also be attributed, in

536   part, to a reduced ATP cost of force production, requiring less flux through oxidative

537   phosphorylation. One of the most energetically costly processes during skeletal muscle

538   contraction is sarcoplasmic reticulum (SR) Ca^{2+} pumping, which may account for up to 50% of

539   the total ATP turnover (7). The presence of reactive oxygen species (ROS) increases the

540   opening probability of the SR Ca^{2+} release channels (48), and the active reuptake of the

541   elevated cytosolic Ca^{2+} would present a considerable energetic challenge (7). NO donors

542   which invoke small elevations in NO might protect the channel against oxidation-induced Ca^{2+}

543   release, without significantly altering channel function (53). Therefore, the BR-induced

544   elevations in NO may have prevented an excess of Ca^{2+} release, and subsequently reduced the

545   considerable energetic cost of its re-sequestration. These suggestions are naturally speculative

546   at the present time and await further investigation.

547

548   *Effects of dietary nitrate on the physiological responses to severe exercise*

549

550   The physiological responses during severe exercise were different to those observed during

551   moderate exercise, suggesting that the influence of NO_2^- and/or NO on muscle function are

552   specific to the exercise intensity domain being investigated. Firstly, there were no significant

553   differences in NIRS-derived indices of muscle oxygenation between the NO_3^- and placebo

554   conditions during severe exercise, though this might be a function of the fact that severe

555   exercise always followed moderate exercise in our experiments. Moreover, in contrast to the

556   reduced steady-state $\dot{V}_{O_2}$ observed during moderate exercise following increased NO_3^-

557   consumption, the amplitude of the primary or fundamental component $\dot{V}_{O_2}$ response during

558   severe exercise was increased (by ~7%) and the amplitude of the subsequent $\dot{V}_{O_2}$ slow

559   component was reduced (by ~23%), with the $\dot{V}_{O_2}$ at the point of task failure being not

560   significantly different between the conditions. These changes in $\dot{V}_{O_2}$ kinetics during high-

561   intensity exercise following dietary NO_3^- supplementation resemble those which are observed

562   following an initial 'priming' bout of high-intensity exercise (15, 35, 45), effects which have

563   been attributed, either separately or in combination, to increased muscle O_2 delivery, increased

564   oxidative metabolic enzyme activity and carbon substrate availability, and altered motor unit

565   recruitment patterns (15, 35, 45). We have previously reported that NOS inhibition with L-

566   NAME significantly increased the amplitude of the $\dot{V}_{O_2}$ slow component and speculated that

567   this might be related to changes in muscle O_2 delivery or its distribution and/or to (related)

568   changes in motor unit recruitment patterns (39). It is presently unclear which, if any, of the

569   above-named mechanisms contributed to the altered $\dot{V}_{O_2}$ kinetics during severe exercise

570   following dietary NO_3^- supplementation. Myocytes in close proximity to a capillary are

571 advantaged with respect to muscle O_2 availability, whereas myocytes situated further away are

572 increasingly less well supplied with O_2, creating an O_2 pressure gradient within the contracting

573 muscles. The hypoxic and acidic milieu within and surrounding the distal myocytes might

574 stimulate NO_2^- reduction to NO facilitating vasodilatation, and thus delivery of O_2. The NO so

575 produced is capable of diffusion and may inhibit mitochondrial O_2 consumption in the

576 myocytes proximal to the capillary bed, promoting deeper diffusion of the available O_2 (32, 59)

577 and therefore enabling a more appropriate matching of local O_2 delivery to O_2 requirement.

578 Another possibility is that the increased NO availability following increased dietary NO_3^-

579 intake promotes mitochondrial biogenesis (16, 50). However, irrespective of the mechanism(s)

580 involved, the return of $\dot{V}_{O_2}$ kinetics *towards* first-order linear system dynamics during severe

581 exercise will likely limit the rate at which metabolites which have been associated with the

582 fatigue process (e.g., H^+, P_i, ADP) accumulate in skeletal muscle (14), effects which would be

583 expected, in turn, to portend enhanced exercise tolerance.

584

585 The τ_p was significantly longer (i.e. phase II pulmonary $\dot{V}_{O_2}$ kinetics was slower) following

586 NO_3^- supplementation relative to placebo for severe exercise (group mean τ_p 40 *vs*. 33 s for BR

587 and placebo, respectively). This is consistent with our previous reports that the τ_p was

588 significantly faster when NOS activity was inhibited by L-NAME (38, 39, 66). The τ_p was also

589 slightly longer during moderate exercise (group mean τ_p 29 *vs*. 26 s for BR and placebo,

590 respectively), but this difference was not statistically significant. While it is acknowledged that

591 changes in muscle blood flow, which might occur with greater or lesser NO availability, have

592 the potential to dissociate the normally close relationship between muscle and pulmonary $\dot{V}_{O_2}$

593 kinetics (2), our data suggest that NO might have an important regulatory influence on the

594 inertia of $\dot{V}_{O_2}$ dynamics following the onset of exercise (36, 51). It is of interest that NO_3^-

595 supplementation resulted in changes in the amplitudes of the $\dot{V}_{O_2}$ response to exercise (i.e.,

596 lower steady-state $\dot{V}_{O_2}$ during moderate exercise and higher $\dot{V}_{O_2}$ fundamental component and

597 reduced $\dot{V}_{O_2}$ slow component during severe exercise) while also slowing the phase II $\dot{V}_{O_2}$

598 kinetics. While the mechanistic bases of this effect is not clear, it is possible that increased NO

599 availability results, simultaneously, in a slowing of the rate at which $\dot{V}_{O_2}$ rises following the

600 onset of exercise (via competitive inhibition of cytochrome c oxidase; 10, 38, 39, 41, 56) and

601 changes in the amplitudes of the fundamental and slow components of $\dot{V}_{O_2}$ (through effects on

602 the efficiency of muscle oxidative metabolism and/or contractile function; 3, 11, 17, 57).

603

604 The kinetics of $\dot{V}_{O_2}$ is considered to be an important determinant of exercise tolerance (14, 36).

605 However, in this study, we observed a 16% improvement in the time to task failure during

606 severe exercise in the NO_3^- condition despite the slower phase II $\dot{V}_{O_2}$ kinetics. Another

607 parameter of $\dot{V}_{O_2}$ dynamics considered to influence exercise tolerance is the slow component

608 rise in $\dot{V}_{O_2}$ observed during supra-GET exercise since this parameter is associated with greater

609 utilization of the finite phosphocreatine (55) and glycogen (42) reserves. Indeed, a reduction in

610 the $\dot{V}_{O_2}$ slow component amplitude has been associated with improved exercise tolerance (1,

611 14). However, in the present study, the improvement in severe exercise tolerance with NO_3^-

612 supplementation was not significantly correlated with the reduction of the $\dot{V}_{O_2}$ slow component

613 amplitude. The $\dot{V}_{O_2}$ at task failure (which was equivalent to the pre-established $\dot{V}_{O_2}$ max) was

614 not different between conditions suggesting that the $\dot{V}_{O_2}$ max was reached more slowly and/or

615 could be sustained for longer following NO_3^-supplementation. It is noteworthy that the $\dot{V}_{O_2}$

616 max during severe exercise was not impaired with NO_3^- supplementation although the steady-

617 state $\dot{V}_{O_2}$ was reduced during sub-maximal exercise. These results differ from those obtained

618 with NOS inhibition. With the latter, $\dot{V}_{O_2}$ max and exercise tolerance are impaired during both

619 ramp incremental exercise (37) and supra-maximal step exercise (66). Although the

620 mechanism for the enhanced performance observed in the present study is uncertain, an

621 interesting possibility is that an elevation of tissue s-nitrosothiols with increased dietary nitrate

622 intake (13) prevents thiols undergoing irreversible oxidative modification as a consequence of

623 the production of ROS (12, 54), an effect which is known to compromise skeletal muscle force

624 production (25). We wish to stress here that while the 16% improvement in the time to task

625 failure during severe constant-work-rate exercise is impressive, the magnitude of effect would

626 be expected to be much smaller during time-trial exercise tasks in which a given distance is

627 completed in the shortest possible time (34). Nevertheless, it is possible that the effect might

628 still be meaningful in terms of performance enhancement.

629

630

631   *Applications and conclusions*

632

633   A short period of dietary NO_3^- supplementation through a natural food product resulted in

634   increased plasma [nitrite] and reduced systolic blood pressure in the normotensive young adult

635   males who participated in our study. During exercise at a fixed moderate work rate, increased

636   NO_3^- intake resulted in improvements in NIRS-derived indices of muscle oxygenation and a

637   significant reduction in pulmonary $\dot{V}_{O_2}$. It should be stressed that the remarkable reduction in

638   the O_2 cost of sub-maximal exercise following dietary supplementation with inorganic nitrate

639   in the form of a natural food product cannot be achieved by any other known means, including

640   long-term endurance exercise training (1, 15, 49, 65). Although not directly tested in the

641   present study, the results suggest that increased dietary NO_3^- intake has the potential to enhance

642   exercise tolerance during longer-term endurance exercise. Moreover, in certain human

643   populations (including the senescent and those with cardiovascular, respiratory or metabolic

644   diseases), the activities of daily living are physically difficult because they have an energy

645   requirement that represents a high fraction of the $\dot{V}_{O_2}$ max. A reduction in the $\dot{V}_{O_2}$ associated

646   with such activities following dietary nitrate supplementation therefore has the potential to

647   improve exercise tolerance and the quality of life in these groups. During exercise at a fixed

648   severe work rate, BR ingestion reduced the amplitude of the $\dot{V}_{O_2}$ slow component and increased

649   the time to task failure by ~16%, suggesting that dietary nitrate supplementation might

650   enhance high-intensity exercise performance. Further research is required to investigate the

651   mechanistic bases for the reduced O_2 cost of sub-maximal exercise observed with increased

652   dietary nitrate intake in this study and previously (45; i.e., reduced ATP cost of force

653   production and/or increased mitochondrial P/O ratio). Finally, the possible ergogenicity of

654   dietary nitrate supplementation during different types of exercise in humans is likely to be a

655   fertile area for further research.

656

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Figure Legends

Figure 1: Plasma nitrite concentration ($[NO_2^-]$) following 4-6 days of dietary nitrate or placebo supplementation. The upper panel shows the group mean (± SEM) values of plasma NO_2^- on days 4, 5 and 6 of supplementation with either nitrate (black circles) or placebo (grey squares). The blood samples for $[NO_2^-]$ determination were taken before each of the six exercise bouts that were completed in each condition (bouts 1, 2, 3 and 5 were moderate, and bouts 4 and 6 were severe; see text for further details). Note the significantly greater plasma $[NO_2^-]$ following dietary nitrate supplementation: # and * denote significant difference from placebo at corresponding time point at the 5% and 1% levels of significance, respectively. The lower panel shows the individual (dashed grey lines) and mean ± SEM (solid black line) values for plasma $[NO_2^-]$ measured over days 4, 5 and 6.

Figure 2: Systolic blood pressure (SBP) following 4-6 days of dietary nitrate or placebo supplementation. The upper panel shows the group mean (± SEM) values of SBP on days 4, 5 and 6 of supplementation with either nitrate (black circles) or placebo (grey squares). Note the significantly lower SBP following dietary nitrate supplementation: # and * denote significant difference from placebo at corresponding time point at the 5% and 1% levels of significance, respectively. The lower panel shows the individual (dashed grey lines) and mean ± SEM (solid black line) values for SBP measured over days 4, 5 and 6.

Figure 3: Group mean changes in the parameters of muscle oxygenation following nitrate and placebo supplementation before and during a step increment to a moderate-intensity cycle work rate. Responses following nitrate supplementation are shown as filled circles, while the placebo responses are shown as open circles. The dashed vertical line represents the abrupt imposition of a moderate work rate from a baseline of 'unloaded' cycling. Panel A: [HHb]; Panel B: $[O_2Hb]$; Panel C: [Hbtot]. For each individual, the responses to four like-transitions were averaged together prior to analysis. Note the reduction in the amplitude of the [HHb] response and the greater $[HbO_2]$ and [Hbtot] before and during moderate exercise following dietary nitrate supplementation. Error bars not shown for clarity but are see Table 1 for further details.

Figure 4: Group mean changes in the parameters of muscle oxygenation following nitrate and placebo supplementation before and during a step increment to a severe-intensity cycle work rate. Responses following nitrate supplementation are shown as filled circles, while the placebo responses are shown as open circles. The dashed vertical line represents the abrupt

939   imposition of a severe work rate from a baseline of 'unloaded' cycling. Panel A: [HHb]; Panel

940   B: [O₂Hb]; Panel C: [Hbtot]. For each individual, the responses to two like-transitions were

941   averaged together prior to analysis. The NIRS-derived parameters were not appreciably

942   different before or during severe exercise following dietary nitrate supplementation. Error bars

943   not shown for clarity but are see Table 1 for further details.

944

945   **Figure 5:** Pulmonary oxygen uptake ($\dot{V}_{O_2}$) response following nitrate and placebo

946   supplementation during a step increment to a moderate-intensity work rate. Responses

947   following nitrate supplementation are shown as filled circles, while the placebo responses are

948   shown as open circles. The dashed vertical line represents the abrupt imposition of the

949   moderate work rate from a baseline of 'unloaded' cycling. The upper panel shows the $\dot{V}_{O_2}$

950   response of a representative individual (data are shown at 5 s intervals). The middle panel

951   shows group mean $\dot{V}_{O_2}$ response with error bars shown every 30 s for clarity. The oxygen cost

952   of moderate exercise was significantly reduced following beetroot supplementation. The lower

953   panel shows individual changes in the amplitude of the $\dot{V}_{O_2}$ response to moderate exercise

954   following nitrate supplementation (dashed grey lines) along with the group mean change (solid

955   black line). For each individual, the responses to four like-transitions were averaged together

956   prior to analysis. Note that the effect was observed in all participants.

957

958   **Figure 6**: Pulmonary oxygen uptake ($\dot{V}_{O_2}$) response following nitrate and placebo

959   supplementation during a step increment to a severe-intensity work rate. Responses following

960   nitrate supplementation are shown as filled circles, while the placebo responses are shown as

961   open circles. The dashed vertical line represents the abrupt imposition of the severe work rate

962   from a baseline of 'unloaded' cycling. The upper panel shows the $\dot{V}_{O_2}$ response of a

963   representative individual (data are shown at 5 s intervals). The data are plotted as a fraction of

964   the $\dot{V}_{O_2}$ fundamental component amplitude to more clearly illustrate the slower phase II $\dot{V}_{O_2}$

965   kinetics and reduced $\dot{V}_{O_2}$ slow component following nitrate supplementation. The middle panel

966   shows group mean $\dot{V}_{O_2}$ response with error bars shown every 30 s for clarity. The group mean

967   ± SEM $\dot{V}_{O_2}$ at task failure is also shown. The lower panel shows individual changes in the

968   tolerance of severe exercise following nitrate supplementation (dashed grey lines) along with

969   the group mean change (solid black line).

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974 **Table 1. Mean ± SD NIRS-derived deoxygenated haemoglobin (HHb), oxygenated**
975 **haemoglobin (O₂Hb) and total haemoglobin (Hbtot) dynamics during moderate- and**
976 **severe-intensity exercise following supplementation with nitrate and placebo.**
977

	Placebo	Nitrate
Moderate-intensity exercise		
[HHb]		
[HHb] Baseline (AU)	-132 ± 84	-131 ± 96
[HHb] 120-s (AU)	-41 ± 56	-54 ± 74
[HHb] End (AU)	-51 ± 55	-55 ± 83
[HHb] Mean Response Time (s)	32 ± 8	29 ± 9
[HHb] Amplitude (AU)	88 ± 38	78 ± 34#
[O₂Hb]		
[O₂Hb] Baseline (AU)	-29 ± 74	21 ± 51#
[O₂Hb] 120-s (AU)	-80 ± 72	-15 ± 30#
[O₂Hb] End (AU)	-5 ± 67	25 ± 39
[Hbtot]		
[Hbtot] Baseline (AU)	-160 ± 129	-110 ± 89#
[Hbtot] 120-s (AU)	-47 ± 89	-29 ± 70
[Hbtot] End (AU)	-57 ± 92	-30 ± 81
Severe-intensity exercise		
[HHb]		
[HHb] Baseline (AU)	-142 ± 95	-104 ± 89
[HHb] 120-s (AU)	176 ± 107	202 ± 125
[HHb] End (AU)	215 ± 110	246 ± 126
[HHb] Primary time constant (s)	9 ± 2	11 ± 2
[HHb] Primary time delay (s)	8 ± 1	8 ± 2
[HHb] Primary amplitude (AU)	300 ± 70	287 ± 103
[HHb] Slow phase amplitude (AU)	63 ± 27	67 ± 16
[O₂Hb]		
[O₂Hb] Baseline (AU)	96 ± 119	57 ± 91
[O₂Hb] 120-s (AU)	-147 ± 66	-176 ± 68
[O₂Hb] End (AU)	-133 ± 59	-166 ± 65
[Hbtot]		
[Hbtot] Baseline (AU)	-46 ± 116	-47 ± 69
[Hbtot] 120-s (AU)	29 ± 121	26 ± 105
[Hbtot] End (AU)	82 ± 110	80 ± 104

978
979   # = significantly different from placebo ($P<0.05$).
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985　**Table 2. Mean ± SD ventilatory and gas exchange dynamics during moderate- and**
986　**severe-intensity exercise following supplementation with nitrate and placebo.**
987

	Placebo	**Nitrate**
Moderate-intensity exercise		
Oxygen Uptake ($\dot{V}_{O_2}$)		
Baseline (L·min^{-1})	0.91 ± 0.09	0.93 ± 0.05
End-exercise (L·min^{-1})	1.52 ± 0.12	1.45 ± 0.13*
Phase II Time Constant (s)	26 ± 7	29 ± 6
Mean Response Time (s)	39 ± 8	45 ± 4
Primary Amplitude (L·min^{-1})	0.64 ± 0.15	0.52 ± 0.15*
Primary Gain (ml·min^{-1}·W^{-1})	10.8 ± 1.6	8.6 ± 0.7#
Expired Carbon Dioxide ($\dot{V}_{CO_2}$)		
Baseline (L·min^{-1})	0.85 ± 0.08	0.84 ± 0.05
End-exercise (L·min^{-1})	1.31 ± 0.15	1.32 ± 0.15
Minute Ventilation ($\dot{V}_E$)		
Baseline (L·min^{-1})	25 ± 2	24 ± 1
End-exercise (L·min^{-1})	36 ± 4	34 ± 3
Respiratory Exchange Ratio		
Baseline	0.93 ± 0.07	0.90 ± 0.07
End-exercise	0.90 ± 0.05	0.91 ± 0.02
Severe-intensity exercise		
Oxygen Uptake ($\dot{V}_{O_2}$)		
Baseline (L·min^{-1})	0.99 ± 0.10	0.96 ± 0.07
End-exercise (L·min^{-1})	3.87 ± 0.29	3.82 ± 0.28
Phase II Time Constant (s)	33 ± 11	40 ± 13#
Primary Amplitude (L·min^{-1})	2.19 ± 0.17	2.35 ± 0.18#
Primary Gain (ml·min^{-1}·W^{-1})	9.0 ± 0.7	9.4 ± 0.6
Slow phase amplitude (L·min^{-1})	0.74 ± 0.24	0.57 ± 0.20#
Slow component amplitude (%)	25 ± 6	19 ± 6#
Overall Gain (ml·min^{-1}·W^{-1})	11.6 ± 0.9	10.8 ± 0.8*
Overall Mean Response Time (s)	75 ± 16	71 ± 16
Expired Carbon Dioxide ($\dot{V}_{CO_2}$)		
Baseline (L·min^{-1})	0.85 ± 0.23	0.86 ± 0.10
End-exercise (L·min^{-1})	3.99 ± 0.32	4.03 ± 0.43
Minute Ventilation ($\dot{V}_E$)		
Baseline (L·min^{-1})	24 ± 7	25 ± 3
End-exercise (L·min^{-1})	140 ± 14	139 ± 21
Respiratory Exchange Ratio		
Baseline	0.86 ± 0.17	0.89 ± 0.09
End-exercise	1.04 ± 0.05	1.05 ± 0.05

988
989　# = significantly different from placebo ($P<0.05$); * = significantly different from placebo
990　($P<0.01$).
991

992

Table 3. Mean ± SD heart rate and blood lactate responses to moderate- and severe-intensity exercise following supplementation with nitrate and placebo.

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996

	Placebo	Nitrate
Moderate-intensity exercise		
Heart Rate		
Baseline (b·min^{-1})	81 ± 9	81 ± 8
End (b·min^{-1})	98 ± 12	98 ± 13
Time Constant (s)	28 ± 12	31 ± 16
Amplitude (b·min^{-1})	17 ± 6	16 ± 7
Blood [Lactate]		
Baseline (mM)	1.0 ± 0.5	0.9 ± 0.3
End (mM)	1.2 ± 0.7	1.1 ± 0.2
Δ (mM)	0.2 ± 0.2	0.2 ± 0.2
Severe-intensity exercise		
Heart Rate		
Baseline (b·min^{-1})	85 ± 8	88 ± 8
End (b·min^{-1})	170 ± 8	170 ± 8
Time Constant (s)	16 ± 10	17 ± 5
Blood [Lactate]		
Baseline (mM)	1.0 ± 0.2	1.1 ± 0.5
End (mM)	6.9 ± 1.6	6.9 ± 1.2
Δ (mM)	5.9 ± 1.6	5.7 ± 1.0
Exhaustion (mM)	10.0 ± 1.9	10.0 ± 1.7

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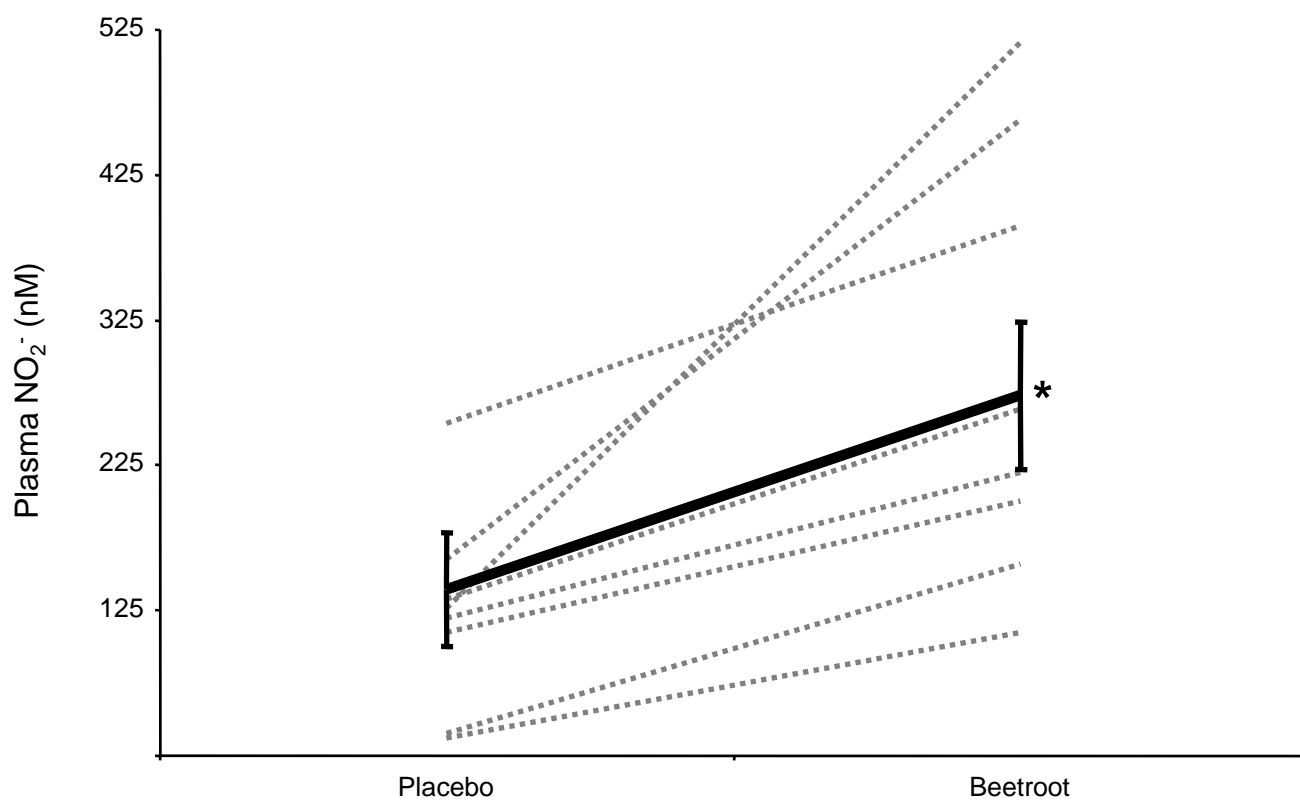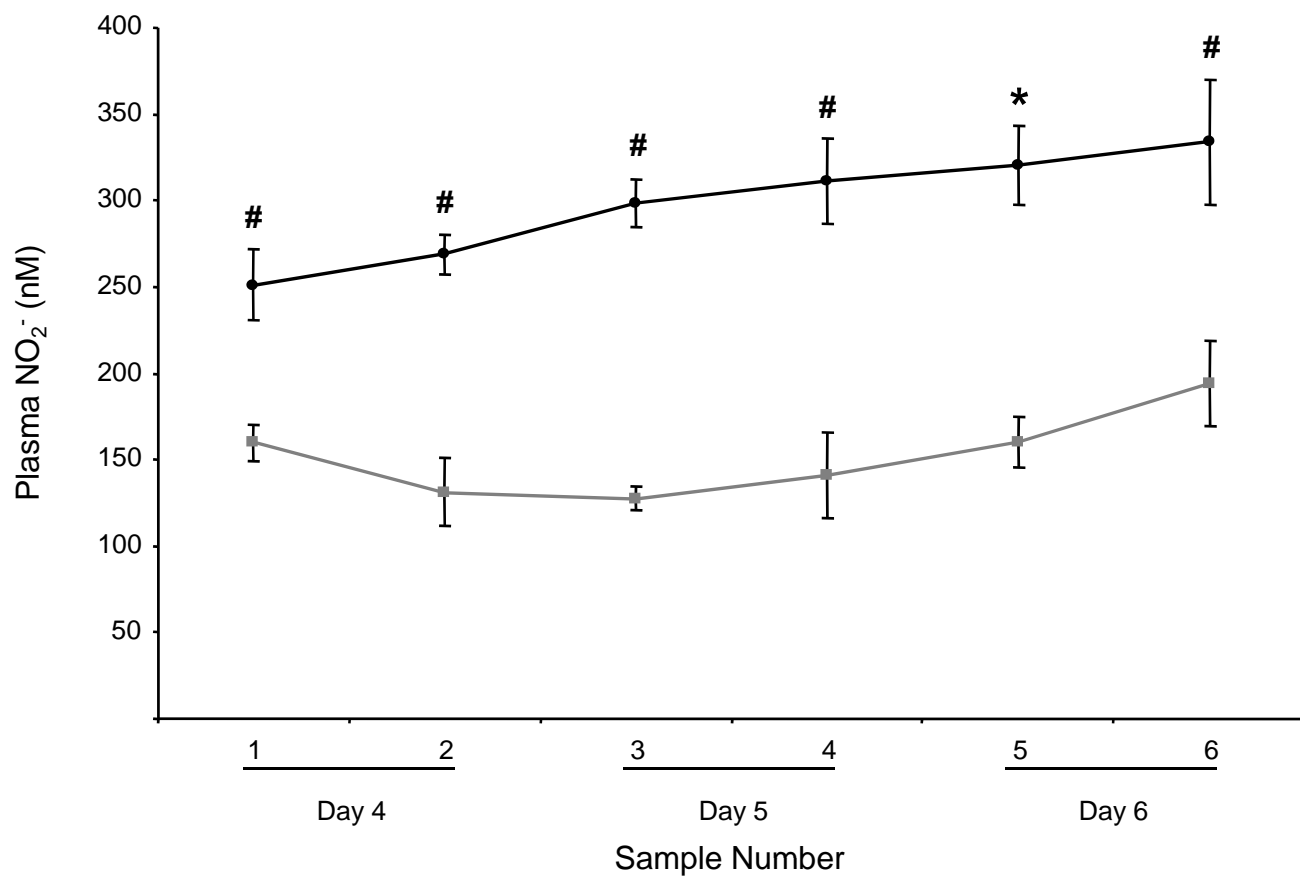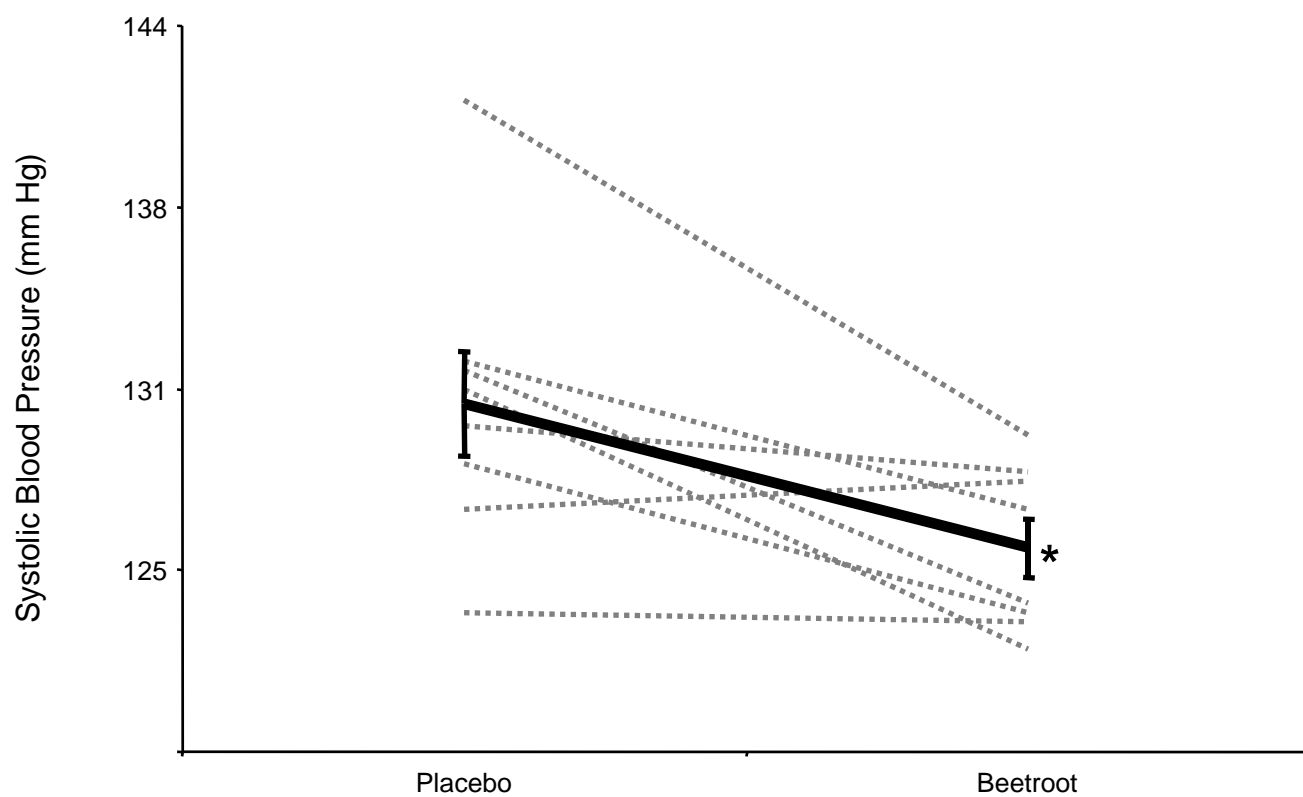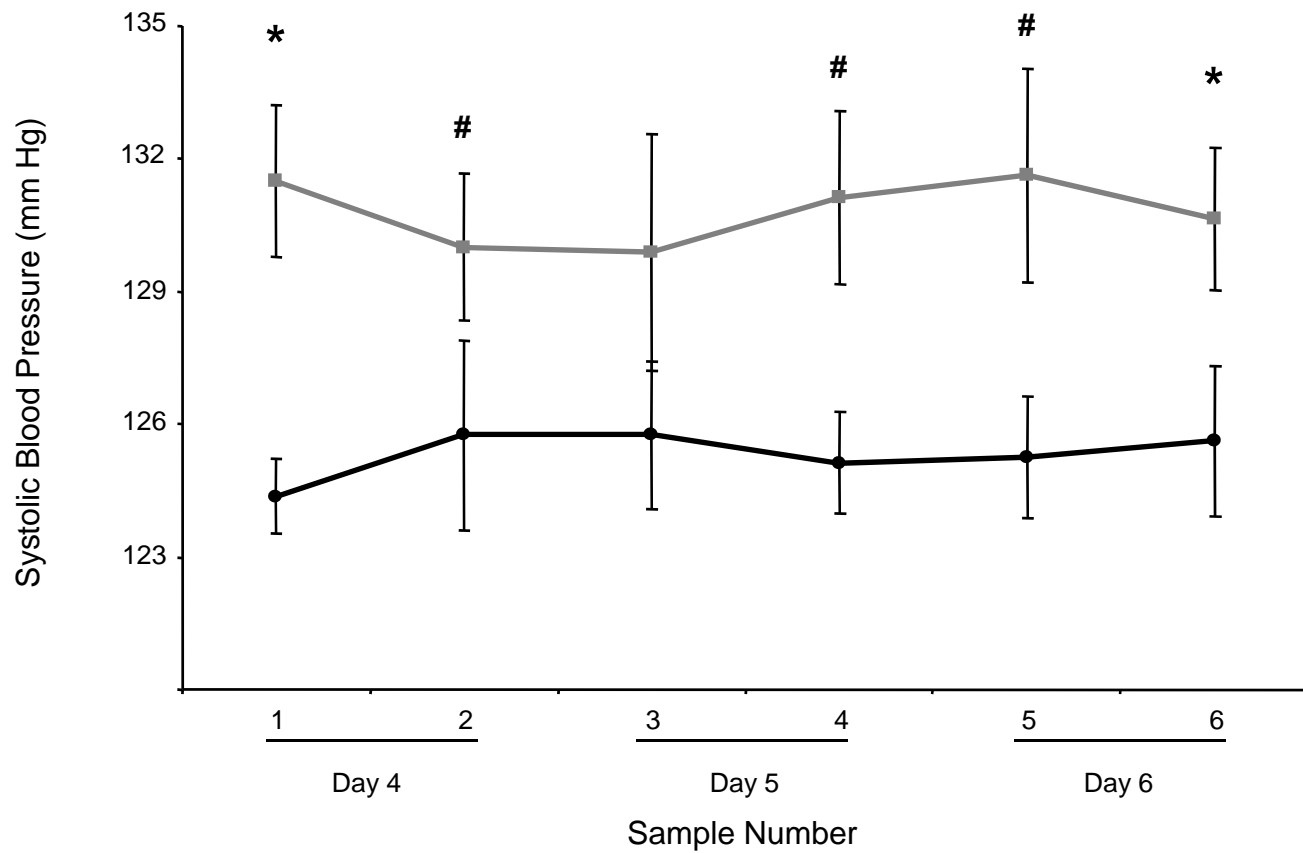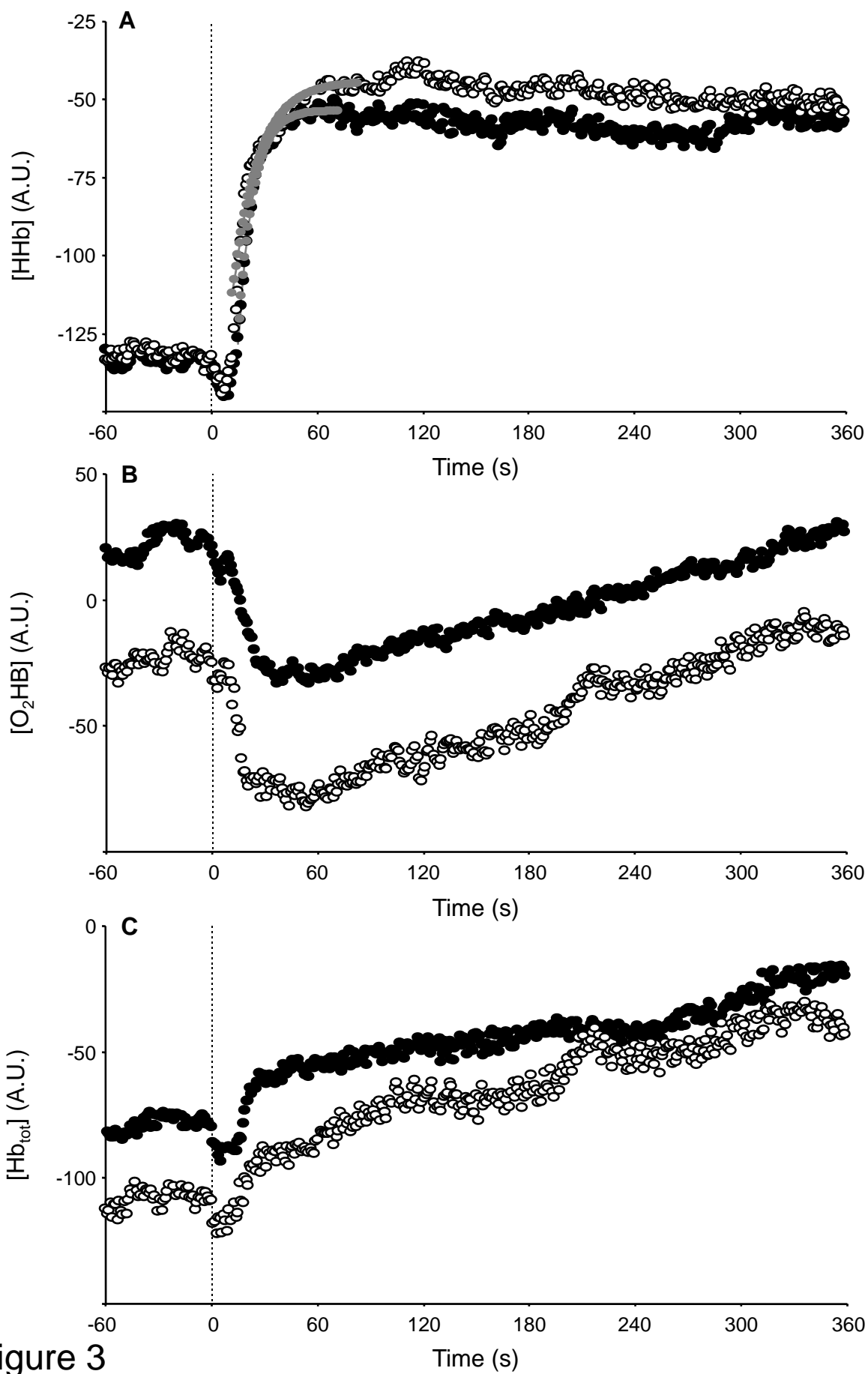
Figure 1

Figure 2

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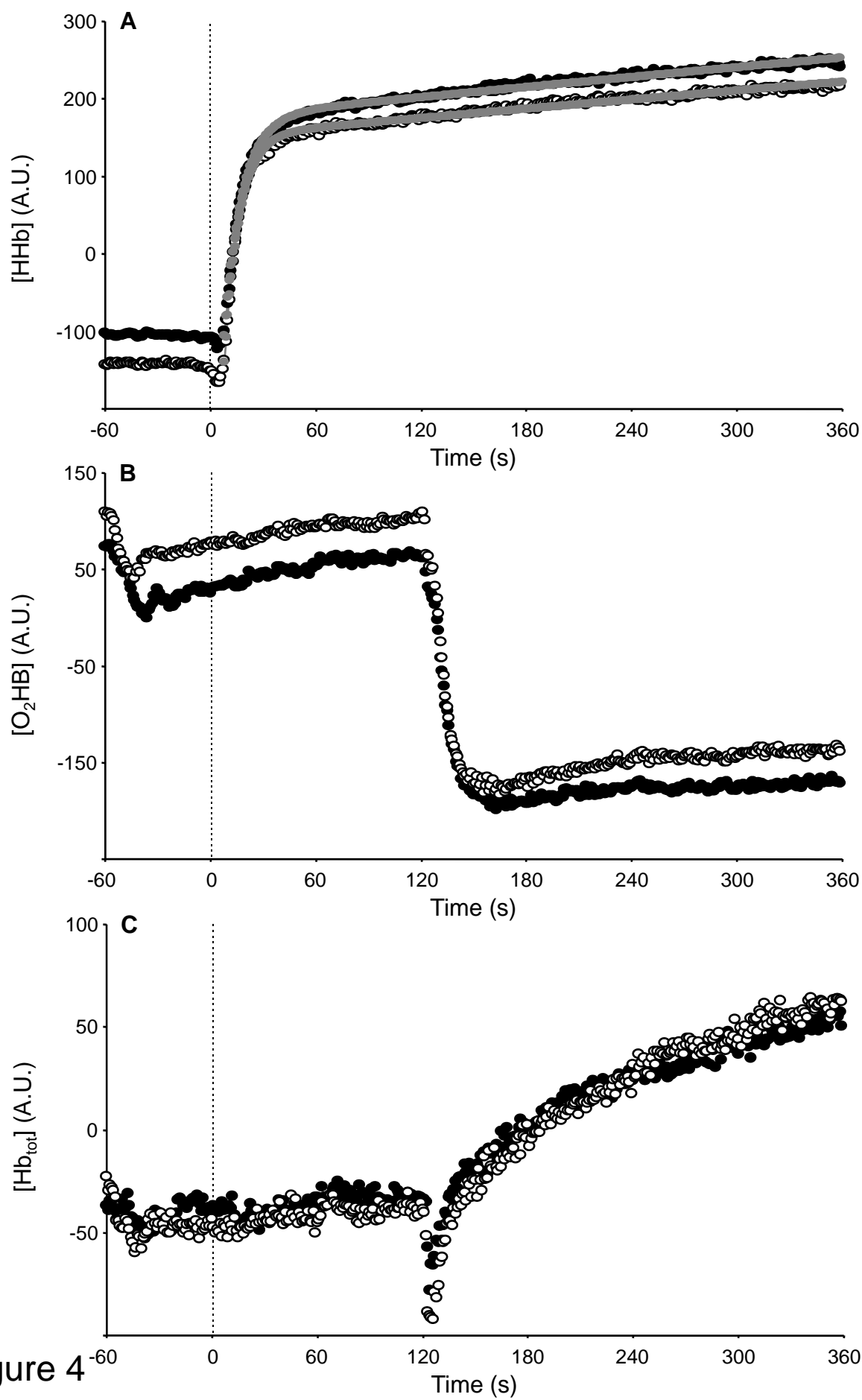
Figure 4

Figure 5

Figure 6