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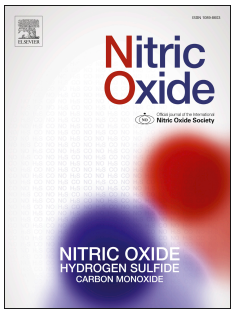

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Title

Multiple-day high-dose beetroot juice supplementation does not improve pulmonary or muscle deoxygenation kinetics of well-trained cyclists in normoxia and hypoxia

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Declarations of interest: EW is a co-applicant on patents related to the therapeutic use of nitrate and nitrite. Other authors, none.

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Abstract

Dietary nitrate (NO_3^-) supplementation via beetroot juice (BR) has been reported to lower oxygen cost (i.e., increased exercise efficiency) and speed up oxygen uptake (VO_2) kinetics in untrained and moderately trained individuals, particularly during conditions of low oxygen availability (i.e., hypoxia). However, the effects of multiple-day, high dose (12.4 mmol NO^-_3 per day) BR supplementation on exercise efficiency and VO_2 kinetics during normoxia and hypoxia in well-trained individuals are not resolved. In a double-blinded, randomized crossover study, 12 well-trained cyclists (66.4 ± 5.3 ml $min^{-1}·kg^{-1}$) completed three transitions from rest to moderate-intensity (~70% of gas exchange threshold) cycling in hypoxia and normoxia with supplementation of BR or nitrate-depleted BR as placebo. Continuous measures of VO_2 and muscle (vastus lateralis) deoxygenation (ΔHHb, using near-infrared spectroscopy) were acquired during all transitions. Kinetics of VO_2 and deoxygenation (ΔHHb) were modelled using mono-exponential functions. Our results showed that BR supplementation did not alter the primary time constant for VO_2 or ΔHHb during the transition from rest to moderate-intensity cycling. While BR supplementation lowered the amplitude of the VO_2 response (2.1%, p=0.038), BR did not alter steady state VO_2 derived from the fit (p=0.258), raw VO_2 data (p=0.231), moderate intensity exercise efficiency (p=0.333) nor steady state ΔHHb (p=0.224). Altogether, these results demonstrate that multiple-day, high-dose BR supplementation does not alter exercise efficiency or oxygen uptake kinetics during normoxia and hypoxia in well-trained athletes.

Keywords: Nitric oxide; beetroot juice; oxygen kinetics; hypoxia; muscle oxygenation;

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1.1 Introduction

For work rates within the moderate-intensity domain, and below the lactate threshold (LT) or gas exchange threshold (GET), pulmonary oxygen uptake (VO_2) rises rapidly to attain a new steady-state level (27). This process is tightly regulated and defined by the mono-exponential kinetics of VO_2 (27).

The amplitude of the VO_2 response is mainly determined by the work rate and exercise efficiency, such that a lower amplitude at a given power output reflects improved exercise efficiency. The time constant (τ) of VO_2 defines the capability for upregulation of oxidative phosphorylation, and faster kinetics (lower τ) is accompanied by reduced reliance on anaerobic energy turnover at exercise onset and during intensity transitions (27, 35). Therefore, strategies to improve exercise efficiency and VO_2 kinetics are of great interest in improving exercise tolerance and performance.

Nitrate (NO_3^-) supplementation, typically in the form of concentrated beetroot juice (BR), has been reported to lower the amplitude of VO_2 during submaximal exercise, in some (3, 15, 31, 38, 39, 44, 59) but not all studies (8, 16, 40, 50, 51). Also, BR has been reported to speed up VO_2 kinetics during submaximal cycling in some (11, 30, 31) but not all studies (3, 16). The discrepancy in the literature is likely influenced by several factors, including environmental conditions (oxygen availability), study population, and supplementation strategy (28). Specifically, the effects of BR have been proposed to be augmented in conditions of lower oxygen availability (i.e., hypoxia) (59, 60). Kelly et al. (31) showed that, in physically active individuals (58.3 ml $min^{-1} \cdot kg^{-1}$), BR lowered the amplitude of

the VO_2 response and reduced $VO_2\tau$ during moderate-intensity cycle exercise in hypoxia, but not in normoxia.

The majority of studies reporting beneficial effects of NO_3^- on VO_2 kinetics have been conducted in untrained or moderately trained individuals (VO_2max < 60 ml $min^{-1} \cdot kg^{-1}$) (3, 37, 44), while the studies conducted in well-trained individuals (VO_2max > 60 ml $min^{-1} \cdot kg^{-1}$) show minor (7, 15, 52, 59) or no effects (1, 5, 8, 16, 51). Relative to less trained individuals, well-trained individuals have elevated resting levels of NO_3^-, which may partly explain the attenuated effects of BR in this population (16, 55, 56). Further, a larger dosage of NO_3^- may be required to elicit the benefits of the supplementation in this population (26). Therefore, several studies propose a supplementation strategy including several days of NO_3^- loading, with a higher NO_3^- dose to raise plasma levels of NO_3^- and NO_2^-, and enhance the benefits of BR supplementation (26, 58, 62, 66). Previous studies examining VO_2 kinetics and exercise efficiency in well-trained athletes, have used either a single dose (50) or multiple-day, lower dosage supplementation (9, 16).

Recently, we showed that 4-7 days of a high dose BR supplementation improved 10 km cycling performance of well-trained individuals (66.4 ml $min^{-1} \cdot kg^{-1}$) in both normoxia and hypoxia (58). The factors responsible for improved time trial performance after BR supplementation are not resolved, but enhanced exercise efficiency, improved oxygen uptake kinetics as well as optimized blood flow distribution may all contribute (20, 28, 58). Near-Infrared spectroscopy (NIRS) can provide insights about the interaction between O_2 delivery and utilization at

the level of the exercising muscle (22). Changes in deoxygenated hemoglobin (ΔHHb) during rest-to-exercise transitions reflect the balance between O_2 delivery and O_2 utilization at the muscle level (22). Further, the rate constant of ΔHHb kinetics represents an index of local muscle oxygen extraction during exercise transients (34). Linking ΔHHb and VO_2, the ratio of ΔHHb-to-VO_2 is proposed to reflect the dynamic relationship between O_2 extraction and O_2 utilization during the adjustment phase at exercise onset (45, 47). As such, a reduction in the ΔHHb-to-VO_2 ratio suggests improved microvascular O_2 delivery and reduced reliance on O_2 extraction for a given VO_2 (45, 47, 61).

To our knowledge, no previous study has examined the effects of multiple-day high-dose NO_3^- supplementation on exercise efficiency, VO_2 and muscle deoxygenation kinetics in normoxia and hypoxia in well-trained individuals. The purpose of the present study was, therefore, to test the hypotheses that multiple days of high-dose, BR supplementation would lower the amplitude of VO_2 and reduce the $VO_2\tau$ in hypoxia and normoxia, during transitions from rest to moderate-intensity cycling, in well-trained individuals. Also, we hypothesized that BR supplementation would lower the ΔHHb-to-VO_2 ratio in hypoxia and normoxia, suggesting that BR improves microvascular O_2 delivery during exercise onset.

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2.1 Materials and Methods

2.1.1 Study design

The study design has previously been reported (58). Briefly, 12 well-trained cyclists (66.4 ± 5.3 ml $min^{-1} \cdot kg^{-1}$) reported to the laboratory on five separate occasions. The first visit consisted of a habituation trial and an incremental maximal exercise test to determine GET and VO_2max. Visits 2-5 all involved experimental trials. Each experimental trial consisted of three step transitions from rest to moderate intensity cycling at a power output corresponding to 70% of the GET (measured in normoxia). Each six-minute transition was separated by six minutes of rest. The step transitions were performed in conditions of normoxia (20.9%) or hypoxia (15%), with supplementation of BR or nitrate-depleted BR as a placebo (PLA). The experimental trials were randomized in a counterbalanced-crossover design and double-blinded for supplementation and single-blinded for inspiratory conditions. The protocol and procedures used in the current study were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Northern Jutland (N-20150049). All participants signed informed consent prior to enrollment. Experimental setup and descriptive data from these participants have previously been reported, with a different aim (58)

2.1.2 BR supplementation

Participants ingested BR or PLA for seven consecutive days. Specifically, participants consumed 140ml of concentrated BR (~12.4 mmol nitrate) or 140ml of nitrate-depleted BR (PLA; ~0 mmol nitrate) (Beet It Sport, James White Drinks Ltd., Ipswich, UK) per day; one dose (70 ml) in the morning and one dose (70 ml) in the evening. On the days of the experimental trials (i.e., days four and seven),

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participants were instructed to consume the total dose (i.e., 140 ml) 2-h before arriving at the laboratory (~2.5h before commencing the step transitions). Further, subjects were asked to refrain from using antibacterial mouthwash.

Experimental trials 2.1.3

Each experimental trial started with a blood sample taken from the antecubital vein. Determination of plasma nitrate and nitrite was performed according to the method described by Hezel et al. (25). Resting blood pressure (BP) was measured three times (Omron M4-I, Omron Matsusaka, Japan) and the average was used for further analysis. Participants then rested 5-minutes on the bike ergometer while breathing the gas mixture corresponding to the condition for that specific trial before commencing exercise.

VO_2 kinetics 2.1.4

Pulmonary VO_2 was measured using a metabolic cart (Jaeger, Vyntus CPX, Carefusion). Breath-by-breath data obtained during the step transitions were examined and data points lying more than four SDs away from the local mean were considered outliers and removed. The data were interpolated on a second-by-second basis and then averaged across the three transitions. This approach enhances the signal-to-noise ratio and improves confidence in the parameters derived from the modeling process (64). Further, the first 20s of data (the initial cardiopulmonary phase) was removed and VO_2 kinetics was modeled using the following mono-exponential function(16):

$$VO_2(t) = \text{Baseline} + A_P\,(1-e^{-(t-TD/\tau)})$$

where $VO_2(t)$ reflects absolute VO_2 for a given time in seconds. The baseline was calculated as the mean VO_2 from 90-30s before the onset of exercise. A_P, TD, and

τ were amplitude, time delay, and time constant, respectively, describing the fundamental response in VO_2 above baseline. The average of three step transitions for an exemplar participant is presented in Figure 1.

NIRS kinetics 2.1.5

Measures of oxygenated (HbO_2), deoxygenated (HHb), and total (THb) hemoglobin were recorded continuously at 2 Hz (Oxymon MK III, Artinis Medical Systems, Netherlands). The probe was placed over the belly of the Vastus Lateralis muscle of the right leg using double-sided adhesive tape and identical placement was ensured between tests by marking the placement with a permanent pen. The data were expressed as relative changes (Δ) from the baseline value.

The kinetics of ΔHHb in response to exercise was modeled using a mono-exponential function, similar to the function used for VO_2 kinetics(18, 46). At the onset of exercise, the ΔHHb profile consists of a time delay, followed by a mono-exponential increase in ΔHHb(18, 46). The time delay for ΔHHb (ΔHHb_{TD}) was determined by the time interval between onset of exercise to the nadir ΔHHb just before a systematic increase in the ΔHHb. The fitting of ΔHHb commenced from the end of the ΔHHb_{TD} and was constrained to 90s for each transition (18, 46). The τΔ[HHb] described the time course for the increase in ΔHHb, while the overall time course of ΔHHb from the onset of the exercise was described by the effective τ'Δ[HHb] (ΔHHb_{TD}+ τΔHHb)(18, 46). The average of three step transitions for an exemplar participant is presented in Figure 2. Kinetics of ΔHbO_2 and ΔTHb do not approximate a mono-exponential model, and were therefore reported as changes from baseline to the averages of the entire work period (0-360s), and the last minute (300-360s) of the work period.

The overall ΔHHb-to-VO_2 ratio for the adjustment during the early stages of the exercise transition was derived by first normalizing (0-100%) the second-by-second ΔHHb and VO_2 data, such that 0% corresponded to the baseline value while 100% reflected the steady-state value. Hereafter, VO_2 data was left-shifted by 20s to appropriately time-align the VO_2 and NIRS-derived signal. Hereby, we account for the phase I component of the VO_2 signal due to the inherent circulatory transit time lag between the exercising muscles and the lung (46). The normalized and left-shifted data were averaged into 5s bins and the overall ratio was then calculated as the mean of the 5s bins from 20-120s of the transition (46, 47).

Mean VO_2 for the last 2-minutes from each step-transition was used to determine gross mechanical efficiency (GE) calculated as:

GE= external bike load (kJ/min) / energy turnover (kJ/min) × 100%

With energy turnover being estimated as VO_2 multiplied by the energetic value of O_2, accounting for the oxidation of fat and carbohydrates determined from the RER-values(54).

Statistical analysis 2.1.6

Differences in physiological parameters were examined using linear mixed models for repeated measures. We used this method to analyse our data, as it has the advantage of preventing listwise deletion due to missing data. For clarification, the number of missing data (MD) for each analysis has been noted in Tables 1 and 2. The variable of interest was entered into the model as the dependent variable. Supplementation (BR vs. PLA), condition (hypoxia vs. normoxia), and supplementation-by-condition were entered as fixed effects in the

model, while subject id was included as a random effect. All data are presented as means ± SE, unless stated otherwise, with statistical significance being accepted when $p<0.05$. All statistical tests were performed using SPSS 25 (IBM Corp., Armonk, USA) or STATA (Texas, USA) version SE 13.

3.1 Results

3.1.1 Plasma nitrate, nitrite and BP

Results for plasma NO_3^- and NO_2^- have been reported previously (58). Briefly, there were significant main effects of supplementation on NO_3^- and NO_2^- (both $p<0.001$) such that BR elevated NO_3^- (PLA 34 ± 4 vs. BR 713 ± 39 µm) and NO_2^- (PLA 0.246 ± 0.03 vs. BR 0.669 ± 0.07 nm) with no effects of condition ($p\geq0.542$), supplementation-by-condition ($p\geq0.687$) or differences between supplementation for 4 or 7 days ($p\geq0.231$).

Resting blood pressure was unchanged with BR (systolic: BR 126 ± 3.1 vs. PLA 124.2 ± 3.1 mm Hg, p=0.283; diastolic: BR 70.2 ± 2.2 vs. PLA 70.5 ± 2.2 mm Hg, p=0.852)

3.1.2 Moderate-intensity exercise

The moderate-intensity exercise elicited oxygen uptake corresponding to ~60-62% VO_2max with no significant effects of condition (p=0.377), supplementation (p=0.210) or supplementation-by-condition (p=0.860). There was a significant effect of condition on HR and SpO_2 (Table 1), such that hypoxia increased HR and decreased SpO_2 during moderate-intensity cycling, with no effects of supplementation and no supplementation-by-condition interactions.

247 *3.1.3 VO_2 kinetics*

248 Data from analysis of VO_2 kinetics are presented in Table 1 and Figures 1 and 3.
249 There were significant effects of condition on τVO_2, VO_2TD, VCO_2, VE and RER
250 such that hypoxia increased τVO_2, VCO_2, VE and RER, while VO_2TD was
251 reduced in hypoxia. There were no effects of supplementation or
252 supplementation-by-condition interactions for these variables.
253 The amplitude of the VO_2 response derived from the mono-exponential fit was
254 significantly reduced with BR, despite no significant effects of supplementation
255 on steady-state VO_2 derived from the fit, steady-state VO_2 derived from the raw
256 VO_2 data, baseline VO_2 or exercise efficiency (GE). There were no effects of
257 condition or supplementation-by-condition interactions for any of these variables
258 (Table 1).

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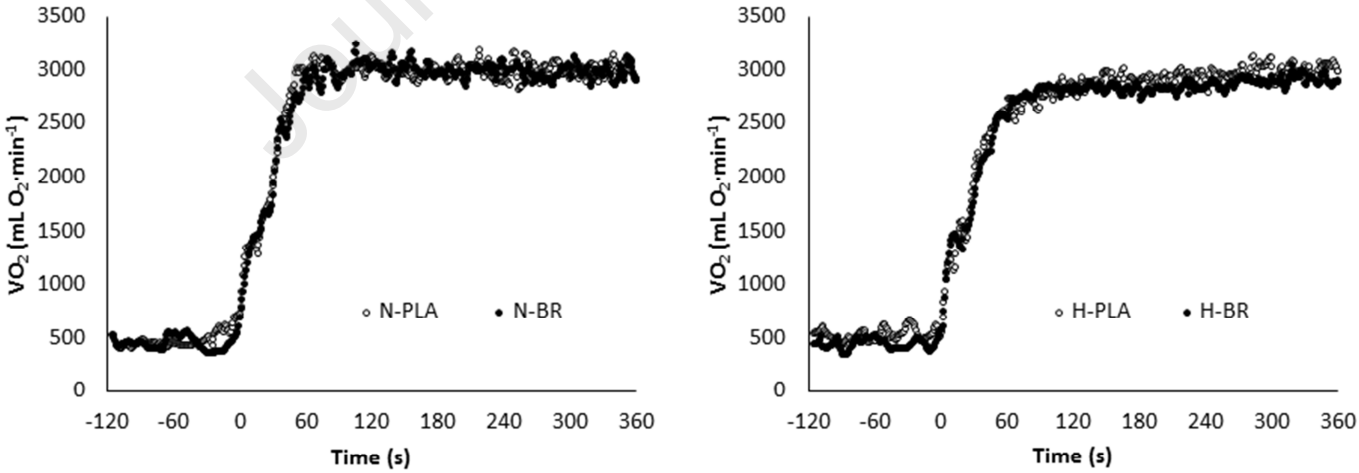

Fig 1. Pulmonary oxygen uptake (VO_2) averaged across the three step transitions for an exemplar participant with placebo (open circles) and beetroot (filled circles) in normoxia (left) and hypoxia (right).

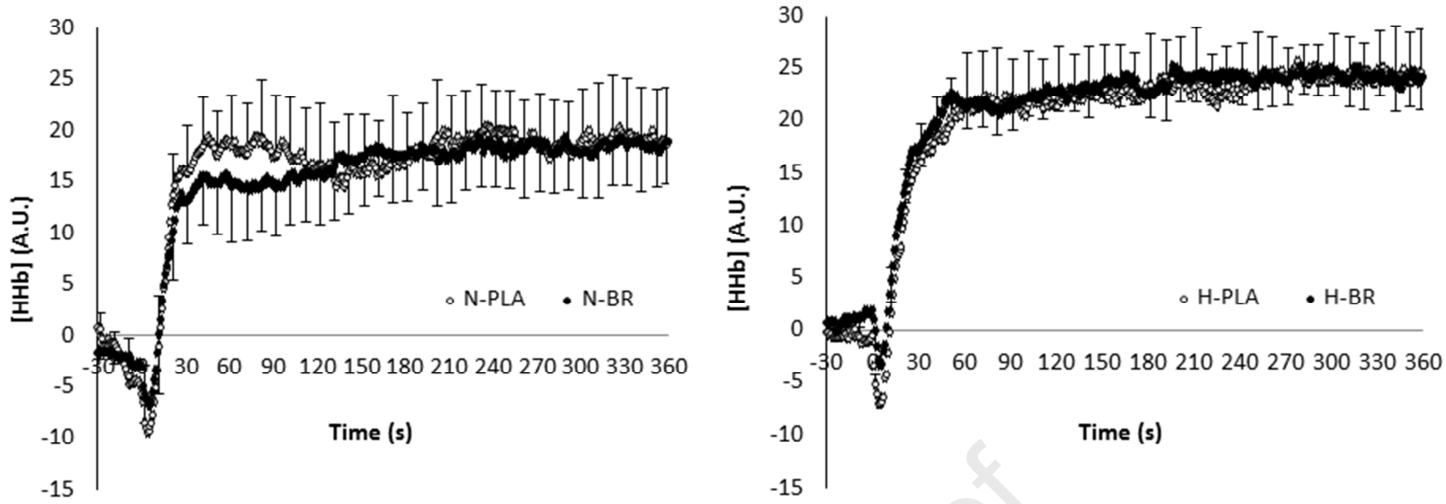

Fig 2. Muscle deoxyhemoglobin (HHb) averaged across the three step transitions for an exemplar participant with placebo (open circles) and beetroot (filled circles) in normoxia (left) and hypoxia (right). 0 represents exercise onset. Standard error bars show intra-subject variability for the exemplar participant with placebo (plus) and beetroot (minus). AU, arbitrary units.

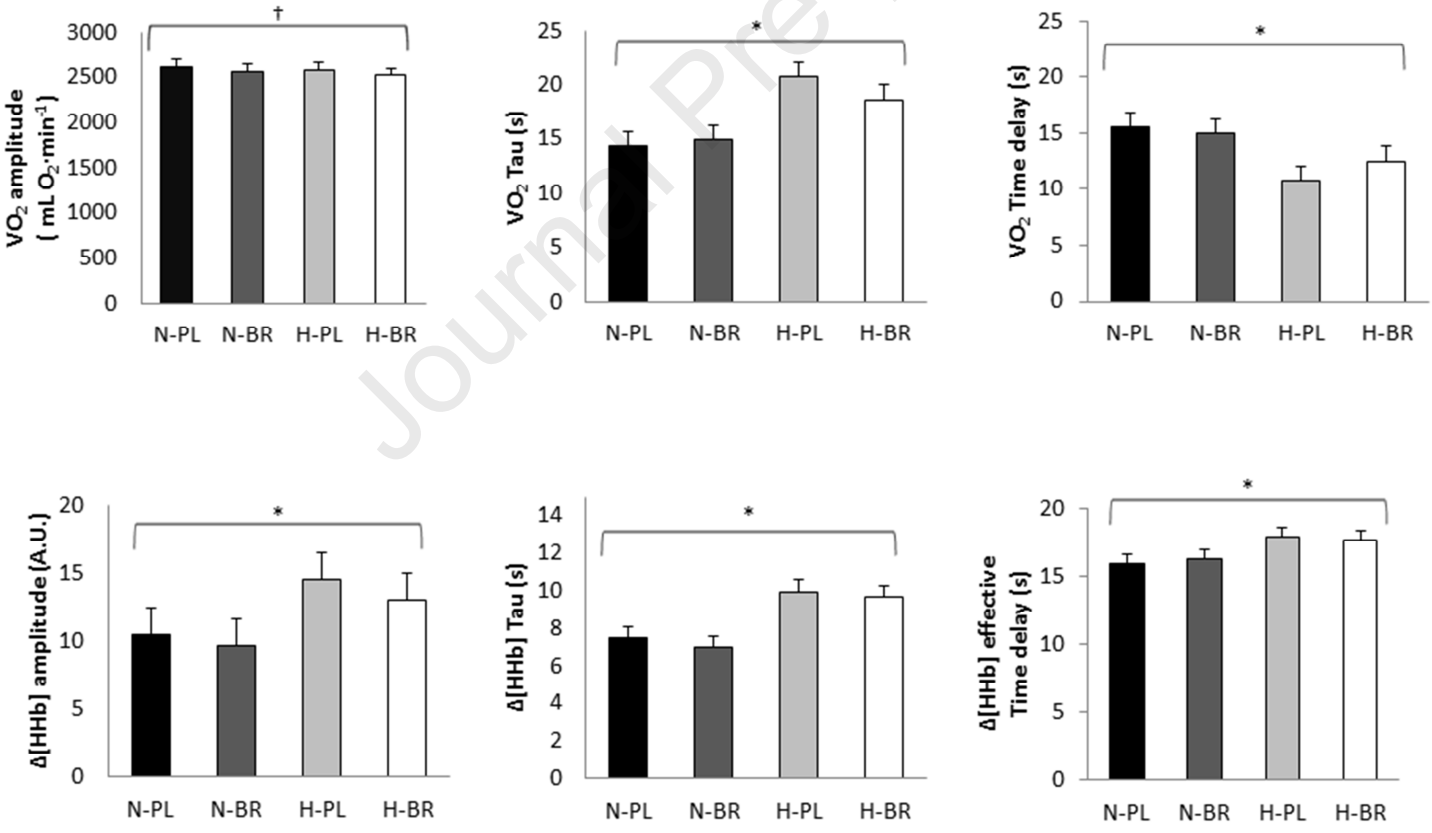

Fig 3. Parameters from the mono-exponential fit of pulmonary oxygen kinetics (top) and muscle oxygen kinetics (bottom) averaged across the three step transitions with placebo (PL) and beetroot (BR) in hypoxia (H) and normoxia (N). *Significant effect of condition. †Significant effect of supplementation.

	MD	N-PL	N-BR	H-PL	H-BR	Linear mixed model effects		
						Supplement	Condition	Interaction
τVO_2, s	7	14.4 ± 1.3	14.9 ± 1.3	20.7 ± 1.4	18.6 ± 1.4	p=0.342	p=0.000	p=0.140
VO_2Ap, ml·min^{-1}	7	2615 ± 80	2568 ± 80	2584 ± 80	2523 ± 81	p=0.038	p=0.131	p=0.777
VO_2Base, ml·min^{-1}	7	521 ± 25	539 ± 23	535 ± 25	575 ± 25	p=0.139	p=0.183	p=0.578
VO_2TD, s	7	15.5 ± 1.3	15.0 ± 1.2	10.8 ± 1.3	12.5 ± 1.3	p=0.300	p=0.001	p=0.101
VO_2 (fit), ml·min^{-1}	7	3137 ± 79	3107 ± 78	3114 ± 79	3096 ± 79	p=0.258	p=0.417	p=0.779
VO_2 (raw), ml·min^{-1}	7	3092 ± 76	3069 ± 76	3117 ± 76	3084 ± 77	p=0.231	p=0.253	p=0.802
VCO_2, ml·min^{-1}	7	2859 ± 83	2888 ± 82	2985 ± 83	3000 ± 85	p=0.399	p=0.007	p=0.796
VE, L·min^{-1}	7	71.1 ± 3.6	71.7 ± 3.5	84.0 ± 3.6	84.3 ± 3.7	p=0.711	p<0.001	p=0.936
RER,	7	0.93 ± 0.0	0.94 ± 0.0	0.96 ± 0.0	0.97 ± 0.0	p=0.153	p=0.019	p=0.876
GE, %	7	18.0 ± 0.4	18.2 ± 0.4	17.6 ± 0.4	17.9 ± 0.4	p=0.333	p=0.112	p=0.656
HR, · min^{-1}	4	131.3 ± 3.8	132.2 ± 3.7	145.6 ± 3.8	145.7 ± 3.8	p=0.689	p<0.001	p=0.711
SpO_2, %	10	98.9 ± 0.9	99.2 ± 0.9	85.0 ± 0.9	84.7 ± 0.9	p=0.988	p<0.001	p=0.778

Table 1. Ventilatory and cardiopulmonary data averaged across the three transitions. MD denotes the number of missing datapoints (total number of datapoints = 48). Values are means ± SE.

3.1.4 NIRS measurements

Data from NIRS measurements are presented in Table 2 and Figures 2 and 3. There were significant effects of condition on $\tau\Delta$[HHb], τ'Δ[HHb], ΔHHb_{TD}, ΔHHb_{end}, ΔHHb_{avg}, ΔHbO_{2end}, ΔHbO_{2avg} such that hypoxia increased $\tau\Delta$[HHb], τ'[HHb], ΔHHb_{end}, ΔHHb_{avg}, while ΔHbO_{2end} and ΔHbO_{2avg} were reduced in hypoxia. There were no significant effects of supplementation or any supplementation-by-condition interactions for any of the NIRS measurements.

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	MD	N-PL	N-BR	H-PL	H-BR	Linear mixed model effects		
						Supplement	Condition	Interaction
τΔ[HHb], s	3	7.5 ± 0.6	7.0 ± 0.6	10.0 ± 0.6	9.7 ± 0.6	p=0.258	p<0.0001	p=0.836
ΔHHb_{TD}, s	3	8.4 ± 0.7	9.1 ± 0.7	7.9 ± 0.7	8.1 ± 0.7	p=0.392	p=0.005	p=0.385
τ'Δ[HHb], s	3	15.9 ± 0.7	16.3 ± 0.7	17.9 ± 0.7	17.8 ± 0.7	p=0.776	p=0.0001	p=0.581
ΔHHb-to-VO_2	13	0.94 ± 0.03	0.95 ± 0.03	0.96 ± 0.03	0.96 ± 0.03	p=0.573	p=0.032	p=0.629
ΔHbO_2 end, AU	4	- 14.3 ± 1.6	-13.1 ± 1.6	-17.8 ± 1.6	-17.4 ± 1.6	p=0.357	p=0.005	p=0.684
ΔHbO_2 avg, AU	4	-16.3 ± 1.7	-15.1 ± 1.6	-18.5 ± 1.7	-18.2 ± 1.6	p=0.433	p=0.027	p=0.638
ΔHHb end, AU	4	10.5 ± 2.0	9.7 ± 1.9	14.5 ± 2.0	13.1 ± 1.9	p=0.231	p=0.004	p=0.725
ΔHHb avg, AU	4	9.1 ± 2.0	8.2 ± 2.0	11.9 ± 2.0	10.8 ± 1.9	p=0.224	p=0.040	p=0.902
ΔTHb end, AU	4	-1.5 ± 0.8	-1.3 ± 0.8	-2.0 ± 0.8	-0.7 ± 0.8	p=0.257	p=0.936	p=0.415
ΔTHb avg, AU	4	-2.1 ± 0.8	-2.3 ± 0.8	-2.9 ± 0.8	-2.4 ± 0.7	p=0.780	p=0.411	p=0.528

Table 2. NIRS data including steady staty measurements and ΔHHb kinetics averaged across the three step transitions. MD denotes the number of missing datapoints (total number of datapoints = 48). Values are means ± SE.

Discussion 4.1

To our knowledge, this is the first study to examine the effects of multiple-day, high-dose BR supplementation on exercise efficiency, pulmonary VO_2 kinetics, and local muscle deoxygenation kinetics during moderate intensity cycling in normoxia and hypoxia in well-trained individuals. The main findings were that 1) BR supplementation did not alter VO_2 or muscle ΔHHb kinetics, 2) BR supplementation lowered the amplitude of the VO_2 response, while steady-state VO_2, exercise efficiency, and steady-state ΔHHb were unaffected. Taken together, these results show that multiple days of high-dose BR supplementation does not alter oxygen uptake kinetics or exercise efficiency during moderate intensity exercise in normoxia and hypoxia, in well-trained individuals.

290 *4.1.1 Supplementation strategy*

291 The majority of studies conducted with BR supplementation in well-trained
292 athletes have not used an optimized supplementation strategy. The use of a
293 multiple-day, high dose BR supplementation strategy, in the present study,
294 elicited markedly elevated levels of NO_3^- and NO_2^-, as described previously (58).
295 Levels of plasma NO_3^- and NO_2^- were markedly higher than plasma levels
296 reported in studies using single dose (1, 44, 50, 59) or multiple-day, lower
297 dosages (1, 9, 16, 23, 44, 65) of NO_3^-. Theoretically, this approach would favor
298 nitrate storage capacity in muscle (48) and increase the availability of NO_3^- and
299 NO_2^- and therefore enhance the possibility of detecting physiological effects of
300 BR.

301

302 *4.1.2 Steady-state VO_2*

303 Multiple days of BR supplementation reduced the amplitude of the VO_2 response
304 (derived from the mono-exponential fit) by 53.4 ml (~2.1%). However, BR did
305 not alter steady-state VO_2 (derived from the mono-exponential fit, ~0.7%
306 reduction), steady-state VO_2 (averaged raw data, ~0.9% reduction), or exercise
307 efficiency (~0.1% improvement). Thus, the small, yet significant, reduction in the
308 VO_2 amplitude with BR results from a non-significant higher baseline VO_2 (~5%)
309 combined with the non-significant lower steady-state VO_2. Nonetheless, exercise
310 efficiency and measures of steady-state VO_2 (absolute values) were unaltered
311 indicating that the oxygen cost of submaximal exercise did not change with BR
312 supplementation.

Our findings are consistent with results from studies in normoxia (4, 6, 8, 16, 43, 49, 51) showing no effects of BR supplementation on oxygen cost during submaximal exercise in well-trained athletes. Few studies in well-trained athletes have been conducted in hypoxia, with the majority of studies reporting no effect of BR on oxygen cost (13, 40, 50). However, in a group of individuals with a broad range of training level (VO_2max range 44-77 ml $min^{-1}·kg^{-1}$), Shannon et al. (59) showed that acute high-dose BR supplementation (~15.2 mmol nitrate) lowered oxygen uptake and increased SpO_2 during moderate-intensity running exercise in hypoxia. Notably, 6 of the 12 individuals, in that study, were classified as recreationally or physically active. Thus, their finding of lowered oxygen uptake could be influenced by including less trained individuals. This interpretation is consistent with studies reporting that NO_3^- lowered the oxygen cost of submaximal exercise in untrained and moderately trained individuals (VO_2max < 60 ml $min^{-1}·kg^{-1}$), but not in well-trained individuals (VO_2max > 60 ml $min^{-1}·kg^{-1}$) (14, 55). As we did not find any condition-by-supplementation interactions for measures of oxygen uptake or SpO_2, oxygen availability does not appear to modulate the effects of BR on exercise efficiency or arterial saturation in well-trained individuals during moderate intensity cycling. These results contradict the proposed hypothesis that hypoxia augments the effects of BR supplementation via enhanced reduction of nitrate to nitric oxide (31, 33, 60). However, the lack of effect of hypoxia in the present study may relate to the training status of the participants as well-trained endurance athletes already have higher NO_3^- plasma levels (29, 56) and greater NO release (63), increased NOS activity (42) and a higher percentage of type I fibres (21).

337 *4.1.3 Effects of BR on VO_2 and muscle deoxygenation kinetics*

338 There were no effects of BR on τVO_2, reflecting the rate of oxygen usage from
339 rest to moderate-intensity exercise in well-trained athletes. This finding is
340 consistent with results from previous studies performed in normoxia in both
341 untrained (55), moderately trained (55) and well-trained athletes (6, 16, 55). On
342 the contrary, in physically active men (~58 ml $min^{-1} \cdot kg^{-1}$), Kelly et al. reported
343 faster τVO_2 in hypoxia but not in normoxia during the transition from rest to
344 moderate-intensity cycling after multiple-day high dose BR supplementation. The
345 supplementation strategy and exercise intensity used by Kelly et al. (31) were
346 similar to our approach, suggesting that differences in results between studies are
347 explained by differences in training status of the participants.
348
349 To assess the kinetics of muscle oxygen extraction, we measured changes in
350 ΔHHb from the vastus lateralis muscle at the onset of exercise throughout the 6
351 min bout of moderate-intensity cycling. Consistent with the VO_2 kinetics results,
352 we found no changes in τΔHHb with BR, suggesting that BR did not enhance the
353 rate of muscle O_2 extraction in the vastus lateralis, which is in agreement with
354 results from previous studies (11, 31). Further, BR did not alter the ΔHHb-to-VO_2
355 ratio implying that BR did not improve the matching of O_2 delivery-to-muscle O_2
356 utilization. In addition, BR supplementation did not alter steady-state levels of
357 ΔHHb or relative changes in THb or HbO_2 during moderate-intensity cycling.
358 Together, these results indicate that BR supplementation does not alter the balance
359 between O_2 delivery and utilization at the muscle level during moderate intensity
360 cycling in well-trained individuals. While these results are in agreement with

previous studies in well-trained athletes showing no effects of BR on muscle oxygenation during submaximal whole-body exercise (32, 50), other studies have provided evidence indicating that BR can improve vascular control, and O_2 delivery to the exercising muscle (20, 21, 57). Ferguson et al. (20) showed that BR augmented muscle O_2 delivery predominantly in fast twitch muscle fibers during locomotory exercise in rats. In humans, Richards et al.(57) demonstrated that BR increased muscle blood flow during handgrip exercise via local vasodilation. However, considering differences in muscle fiber type composition (rat versus human) and exercise modality (handgrip exercise versus whole body exercise), these findings may not translate into improved muscle blood flow during cycling exercise.

4.1.4 Effects of hypoxia on VO_2 and muscle deoxygenation kinetics

Our results revealed that hypoxia slowed VO_2 kinetics, which is in agreement with results from previous studies (10, 31, 41, 61). Slowed VO_2 kinetics in hypoxia have been proposed to occur via a) reduced O_2 delivery to the muscle during the transition, b) limitation in O_2 diffusive transport, and/or c) a change in the control of the intracellular metabolic adjustments (10, 19, 36). Accompanying slowed VO_2 kinetics, hypoxia also slowed muscle deoxygenation kinetics (i.e., greater $\tau\Delta$[HHb] and $\tau'\Delta$[HHb]) during the transition from rest to moderate-intensity exercise, which likely contributed to the slowed VO_2 kinetics. Studies have demonstrated that exercise in hypoxia is accompanied by a compensatory increase in muscle blood flow to maintain oxygen extraction and usage (12, 53). However, this compensatory increase in muscle blood flow may not sufficiently

preserve bulk O_2 supply during the adjustment phase (12, 36). In support of a limitation related to O_2 delivery and/or diffusion during hypoxia, we found an increase in the ΔHHb-to-VO_2 ratio with hypoxia. This result implies an increased reliance on O_2 extraction for a given VO_2 during the on-transient in hypoxia (45, 47, 61).

Hypoxia induced a shorter initial time delay (ΔHHb_{TD}) preceding the increase in ΔHHb, suggesting that lower oxygen availability prompts a mismatch between local O_2 delivery and utilization. This could possibly be a consequence of hypoxia 'priming' the intramuscular oxidative metabolic machinery, eliciting a faster onset of deoxygenation and O_2 extraction at exercise onset (19, 24, 46). A shorter time delay suggests that slowed VO_2 kinetics in hypoxia did not occur as a result of a limitation within the control of the intracellular metabolic adjustment. Together these results indicate that the slowed VO_2 kinetics during hypoxia in well-trained athletes is accompanied by impaired O_2 delivery to the active muscle tissue. This interpretation is supported by the results from Spencer et al. (61).

In agreement with results from previous studies (31, 41), hypoxia increased steady-state ΔHHb, and amplified the reduction of ΔHbO_2, indicating that lower oxygen availability, verified by lower levels of SpO_2 (~85%), increased muscle oxygen extraction during cycling at the same submaximal power output.

4.1.5 General experimental considerations

In the same group of participants, we recently showed that BR supplementation improved 10 km cycling performance (58). The current study demonstrates that BR supplementation does not alter exercise efficiency or O_2 kinetics, however

these factors are assessed during transition from rest to moderate intensity cycling, eliciting ~60% VO_2max, and not at higher exercise intensities.

Others have reported beneficial effects of BR on muscle oxygenation and VO_2 kinetics in the transition from moderate to severe-intensity work rates, but not from unloaded to moderate work rates (11, 17), and during cycling with high cadence but not in cycling with low cadence (2). These results suggest that beneficial effect of BR on VO_2 may be more pronounced in conditions with greater involvement of fast-twitch muscle fibers. Notably, unaltered steady state exercise efficiency, with BR, extends our previous findings of unaltered power-to-VO_2 ratio (proxy of exercise efficiency) during time trial cycling (58), reinforcing that the effects of BR on exercise performance, in well trained individuals, are not mediated via improved exercise efficiency.

Time trial cycling (vs. steady state exercise) likely recruits a greater proportion of fast twitch muscles fibers, which may explain why BR supplemenation (via augmented O_2 delivery predominantly in fast twitch fibers (21)) elicits a larger utilization of VO_2max and hence improved exercise performance (58). However, this hypothesis warrants further examination in well-trained individuals.

5.1 Conclusion

In summary, multiple-day, high-dose BR supplementation did not improve muscle O_2 or VO_2 kinetics nor exercise efficiency during moderate-intensity cycling in normoxia and hypoxia in well-trained athletes. These results provide new information demonstrating that an optimized BR supplementation strategy failed to improve exercise efficiency or oxygen uptake kinetics during rest-to-

moderate intensity transitions in well-trained individuals. It is possible, however, that BR may evoke beneficial effects on exercise efficiency and oxygen uptake kinetics during higher exercise intensities involving greater recruitment of fast-twitch muscle fibers.

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7.1 Conflict of interest statement

The authors declare no support from any organization for the submitted work; EW is a co-applicant on patents related to the therapeutic use of nitrate and nitrite. Other authors, none.

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- NO_3^- supplementation does not alter moderate-intensity VO_2 or HHb kinetics
- Oxygen uptake during moderate-intensity cycling were unchanged in trained athletes
- The effects of NO_3^- supplementation were not different between hypoxia and normoxia
- Beetroot juice did not improve exercise efficiency in well-trained athletes
- NO_3^- supplementation did not change muscle deoxygenation kinetics of well-trained

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