Beetroot juice supplementation speeds O₂ uptake kinetics and improves exercise tolerance during severe-intensity exercise initiated from an elevated metabolic rate

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Submitted 14 June 2013; accepted in final form 27 September 2013

Breese BC, McNarry MA, Marwood S, Blackwell JR, Bailey SJ, Jones AM. Beetroot juice supplementation speeds O₂ uptake kinetics and improves exercise tolerance during severe-intensity exercise initiated from an elevated metabolic rate. Am J Physiol Regul Integr Comp Physiol 305: R1441–R1450, 2013. First published October 2, 2013; doi:10.1152/ajpregu.00295.2013.—Recent research has suggested that dietary nitrate (NO₃⁻) supplementation might alter the physiological responses to exercise via specific effects on type II muscle. Severe-intensity exercise initiated from an elevated metabolic rate would be expected to enhance the proportional activation of higher-order (type II) muscle fibers. The purpose of this study was, therefore, to test the hypothesis that, compared with placebo (PL), NO₃⁻-rich beetroot juice (BR) supplementation would speed the phase II V˙O₂ kinetics (τₚ) and enhance exercise tolerance during severe-intensity exercise initiated from a baseline of moderate-intensity exercise. Nine healthy, physically active subjects were assigned in a randomized, double-blind, crossover design to receive BR (140 mL/day, containing ~8 mmol of NO₃⁻ and PL (140 mL/day, containing ~0.003 mmol of NO₃⁻) for 6 days. On days 4, 5, and 6 of the supplementation periods, subjects completed a double-step exercise protocol that included transitions from unloaded to moderate-intensity exercise (U→M) followed immediately by moderate to severe-intensity exercise (M→S). Compared with PL, BR elevated resting plasma nitrite concentration (PL: 65 ± 32 vs. BR: 348 ± 170 nM, P < 0.01) and reduced the V˙O₂τₚ in M→S (PL: 46 ± 13 vs. BR: 36 ± 10 s, P < 0.05) but not U→M (PL: 25 ± 4 vs. BR: 27 ± 6 s, P > 0.05). During M→S exercise, the faster V˙O₂ kinetics coincided with faster near-infrared spectroscopy-derived muscle [deoxyhemoglobin] kinetics (τₚ: PL: 20 ± 9 vs. BR: 10 ± 3 s, P < 0.05) and a 22% greater time-to-task failure (PL: 521 ± 158 vs. BR: 635 ± 258 s, P < 0.05). Dietary supplementation with NO₃⁻-rich BR juice speeds V˙O₂ kinetics and enhances exercise tolerance during severe-intensity exercise when initiated from an elevated metabolic rate.

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The size principle of Henneman and Mendell (29) posits that skeletal muscle fibers are recruited in a hierarchical manner during exercise, according to the requirements for muscle force production. A protocol that has been employed to interrogate the metabolic response of different muscle fiber populations to exercise is the “work-to-work” step exercise test (14, 22, 33). In this protocol, transitions to a higher metabolic rate are divided into two increments in work rate (i.e., lower step and upper step) to manipulate motor unit recruitment and, hence, reveal the metabolic response profiles of different segments of the motor unit pool. For example, a transition from unloaded cycling to a moderate-intensity work rate (U→M) would be expected to mandate the recruitment of muscle fibers that are positioned low in the recruitment hierarchy (i.e., type I fibers), whereas a subsequent transition from a moderate- to a severe-intensity work rate (M→S) would be expected to require the recruitment of muscle fibers positioned higher in the recruitment hierarchy (i.e., type II fibers) (42). Compared with U→M, the VO₂ max during M→S is greater (i.e., VO₂ kinetics are slower) (22, 68). Moreover, compared with a transition from unloaded cycling to a severe-intensity work rate (U→S), the VO₂ max during M→S is greater, and the amplitude of the VO₂ slow component is truncated, such that the overall response reverts toward being “first-order” (20–22, 67, 68). It is possible that the slower VO₂ kinetics in M→S compared with U→M reflects a relative imbalance in muscle O₂ supply relative to demand. Consistent with this, it has been reported that microvascular PO₂ (which reflects the dynamic balance between muscle O₂ delivery and muscle O₂ utilization) declines more rapidly during contractions in predominantly type II compared with type I muscle (10, 51).

Given that NO₃⁻ supplementation has been reported to increase both the absolute and relative distribution of blood flow toward contracting type II muscle (23), this might be expected to improve the local matching of O₂ delivery relative to muscle VO₂ and, therefore, to speed phase II VO₂ kinetics during M→S. While NO₃⁻ supplementation does not reduce the VO₂ max during either U→M or U→S in young, healthy subjects (2, 5, 44), the effect of NO₃⁻ supplementation on the VO₂ max during M→S has yet to be investigated.

Therefore, the purpose of this study was to investigate the effects of short-term dietary NO₃⁻ supplementation on VO₂ kinetics during work-to-work exercise transitions, i.e., U→M followed immediately by M→S. We used the muscle deoxy-hemoglobin concentration ([HHb]) signal from NIRS measurements to explore the mechanistic bases for any NO₃⁻-induced changes in phase II VO₂ kinetics. The kinetics (τ) of muscle [HHb] following the onset of exercise resembles that of mixed venous [O₂] (28, 38) and approximates the reduction in microvascular PO₂ during transitions from rest to electrically stimulated contractions (36). The [HHb] signal is, therefore, considered to provide an index of local O₂ extraction (19, 27) and, hence, to reflect the balance between muscle O₂ delivery and muscle O₂ utilization. We hypothesized that NO₃⁻ supplementation would reduce the VO₂ max and increase the muscle [HHb] τ in M→S but not U→M. We also hypothesized that these kinetic changes following NO₃⁻ supplementation would enhance severe-intensity exercise tolerance.

### METHODS

**Participants.** Nine healthy subjects (four male: mean ± SD, age 30 ± 6 years; body mass 77 ± 11 kg; stature 1.78 ± 0.06 m; and five female: age 30 ± 6 years; body mass 58 ± 4 kg; stature 1.66 ± 0.02 m) volunteered to participate in the study. The participants were all recreationally active, but not highly trained. Prior to testing, participants were informed of the protocol and risks and gave written consent to participate in the study. All procedures were approved by Swansea University Ethics Committee and were conducted in accordance with the Declaration of Helsinki. Participants were asked to arrive at the exercise physiology laboratory at Swansea University in a rested state, at least 2 h postprandial and to avoid strenuous exercise in the 24 h preceding each testing session. Participants were also asked to refrain from caffeine and alcohol for 6 and 24 h before each test, respectively. The participants also refrained from the use of antibacterial mouthwash throughout the duration of the study because this has been shown to markedly attenuate the oral bacteria which are necessary for the conversion of nitrate to nitrite (26). All tests were performed at the same time of day (± 0.5 h).

**Procedures.** Participants were required to visit the laboratory on seven occasions over a 4-week period. On the first visit, participants completed a ramp incremental exercise test for determination of the VO₂ peak and GET. The test included 3 min of baseline cycling at 15 W, after which the work rate was increased at a rate of 20 W/min for females and 30 W/min for males until the limit of tolerance. The participants were asked to maintain a cadence of 70–80 rpm. Breath-by-breath pulmonary gas-exchange data were collected continuously during the incremental tests and averaged over consecutive 5-s periods (Oxycon Pro, Jaeger, Germany). The VO₂ peak was taken as the highest 10-s mean value attained before the subject’s volitional exhaustion in the test. The GET was determined using the V-slope method (9) as the first disproportionate increase in CO₂ production (VCO₂) relative to the increase in VO₂, and subsequently verified by an increase in the ventilatory equivalent for VO₂ (VE/VO₂) with no increase in V̇E/VO₂. The work rates that would require 90% of the GET (moderate-intensity exercise) and 70% of the difference (Δ) between the GET and VO₂ peak (severe-intensity exercise, Δ70%) were subsequently determined, with account taken of the mean response time for VO₂ during ramp exercise, i.e., two-thirds of the ramp rate was deducted from the work rate at the GET and peak VO₂ (65).

Following the ramp incremental test, participants were randomly assigned in a crossover, double-blind design to receive 6 days of dietary supplementation with NO₃⁻-rich beetroot juice (BR) (140 ml/day; ~8 mmol NO₃⁻; Beet It, James White Drinks, Ipswich, UK) or NO₃⁻-depleted BR as a placebo (PL; ~0.0034 mmol NO₃⁻; Beet It, James White Drinks, Ipswich, UK). The placebo NO₃⁻-depleted BR beverage was identical in color, taste, smell, and texture to the experimental NO₃⁻-rich BR beverage. The BL beverage was created by passage of the juice, before pasteurization, through a column containing Purilite A520E ion exchange resin, which selectively removes NO₃⁻ ions (44). Five participants began with the BR condition, and the other four participants began with the PL condition. The subjects were instructed to consume the beverages (70 ml in the morning and afternoon) on days 1–3 of the supplementation period. On days 4–6, the subjects were instructed to consume the beverages over a 10-min period, 2 h prior to the start of the exercise test (see below), based on recent evidence that plasma [NO₃⁻] peaks at ~2–2.5 h postadministration of BR containing 8.4 mmol NO₃⁻ (71). A 7-day washout period separated each supplementation period. Throughout the study, subjects were instructed to maintain their normal daily activities and food intake.

On days 4, 5, and 6 of the supplementation periods, subjects completed a series of step exercise tests for the determination of VO₂ and muscle [HHb] kinetics. The protocol, which was performed on three consecutive days, consisted of 3-min “unloaded” pedaling at 15 W, followed by 4-min of moderate-intensity cycling (U→M), and...
then 6-min of severe-intensity cycling (M→S). The tests were performed on separate days because it is known that prior exercise can alter the \(V_O^2\) response to exercise (3). A schematic illustration of the experimental protocol is shown in Fig. 1. On day 6 of each supplementation period, the M→S bout was continued until task failure. The participants were blinded to the elapsed exercise time in both the BR and PL conditions. The time to task failure was used as a measure of exercise tolerance and was recorded when the pedal rate fell by >10 rpm below the required pedal rate. In total, the participants completed three bouts of U→M and M→S exercise following BR and PL ingestion, with the \(V_O^2\) data being subsequently ensemble-averaged prior to curve-fitting to enhance the signal-to-noise ratio.

**Measurements.** Venous blood samples (≈4 ml) were drawn into lithium-heparin tubes (7.5 ml Monovette lithium heparin; Sarstedt, Leicester, UK), which have very low levels of NO\(_3^–\) and NO\(_2^–\), on each of days 4–6. Within 3 min of collection, the samples were centrifuged at 2,700 g and 4°C for 10 min. Plasma was extracted and immediately frozen at −80°C for later analysis of [NO\(_2^–\)] using a modification of the chemiluminescence technique (7). All glassware, utensils, and surfaces were rinsed with deionized water to remove residual NO\(_2^–\) prior to analysis. Following defrosting at room temperature, the [NO\(_2^–\)] of the undiluted (nonproteinized) plasma was determined by its reduction to NO in the presence of glacial acetic acid and 4% (wt/vol) aqueous NaI. The spectral emission of electronically excited nitrogen dioxide product, from the NO reaction with ozone, was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence nitric oxide analyzer (Sievers NOA 280i; Analytix, Durham, UK). The [NO\(_2^–\)] was determined by plotting signal (mV) area against a calibration plot of 100 nM to 1 \(\mu \text{M}\) of sodium nitrite.

Throughout all exercise tests, participants wore a face mask and breathed through a low dead space (90 ml), low-resistance (0.75 mmHg·l\(^{-1}·\text{s}^{-1}\) at 15 l/s) impeller turbine assembly (Jaeger Triple V, Hoechberg, Germany). The inspired and expired gas volumes and gas concentration signals were continuously sampled at 100 Hz, the latter using paramagnetic (O\(_2\)) and infrared (CO\(_2\)) analyzers (Jaeger Oxycyon Pro, Hoechberg, Germany) via a capillary line connected to the mouthpiece. These analyzers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated using a 3-liter syringe (Hans Rudolph, Kansas City, MO). The volume and concentration signals were time aligned by accounting for the delay in capillary gas transit and analyzer rise time relative to the volume signal. Breath-by-breath fluctuations in lung gas stores were corrected for by computer algorithms (8). A Reynolds Lifecard CF digital Holter recorder (Spacelabs Medical, Hertford, UK) was used to record a three-lead ECG continuously throughout the tests. The ECG leads were positioned in the modified V5, CC5, and modified V5R electrode configuration. This system provided ECG data with a sample accuracy of 2.5 \(\mu \text{V}\) and 1,024-Hz sampling frequency. During one of the U→M and M→S transitions, for both supplementation periods, a blood sample was collected from a fingertip into a capillary tube over the 20 s preceding the step transition in work rate and within the last 20 s of exercise. A capillary blood sample was also collected at the limit of tolerance for the M→S bout performed on day 6 of each supplementation period. The blood samples were subsequently analyzed to determine [lactate] (YSI 1500; Yellow Springs Instruments, Yellow Springs, OH) within 30 s of collection. Blood lactate accumulation was calculated as the difference between blood [lactate] at end-exercise and blood [lactate] at baseline.

NIRS was used to monitor changes in oxygenation status of the musculus vastus lateralis of the right leg during step exercise (NIRS; OxiplexTS; ISS, Champaign, IL). The NIRS probe was affixed over the midpoint between the greater trochanter and lateral epicondyle of the right leg using adhesive tape and secured by elastic Velcro strapping to ensure the device remained stationary and to minimize the interference of extraneous light during exercise. Source (NIR) light was emitted into the muscle at wavelengths of 690 and 830 nm, and detection was sampled at 2 Hz to measure absolute concentrations (\(\mu \text{M}\)) of oxyhemoglobin (HbO\(_2\)) and deoxyhemoglobin (HHb) within the microcirculation of the interrogated muscle region. Light source-detector separation distances of 1.50–3.04 cm for each wavelength were used with cell water concentration assumed to be constant at 70%. The NIRS probe was calibrated before each testing session using a calibration block of known absorption and scattering coefficients. Calibration was then cross-checked using a second block of known, but distinctly different, absorption and scattering coefficients. Each of these procedures was performed according to the manufacturer’s recommendations. The contribution of myoglobin (Mb) to the NIRS signal is generally accepted to be relatively small (50, 62) but is currently unresolved. The [HHb] signal reported herein should, therefore, be considered to reflect the combined concentrations of both deoxygenated Hb and Mb.

**Data analysis procedures.** The breath-by-breath \(V_O^2\) data from each step exercise bout were initially examined to exclude “errant” breaths by removing values lying more than four standard deviations from the local mean determined using a 5-breath rolling average. Filtered \(V_O^2\) data were subsequently linearly interpolated to provide second-by-second values and, for each individual, identical repetitions of each exercise condition were time aligned to the start of exercise and averaged together to form a single data set for analysis.

For each step transition, the first 20 s of data after the onset of exercise were deleted to remove the phase I (cardiodynamic) response and a monoexponential model with time delay (Eq. 1) was then fitted to the averaged \(V_O^2\) data.

**Fig. 1.** Schematic of the step exercise test protocol performed on days 4–6 of the supplementation period.
where $\Delta V\dot{O}_2$ is the increase in $V\dot{O}_2$ at time $t$ above the baseline value (calculated as the mean $V\dot{O}_2$ from the first 45 s of the last minute of basal pedaling), and $A_1$, $\delta_1$, and $\tau_1$ are the primary component amplitude, time delay (which was allowed to vary freely), and time constant, respectively. Kinetic variables ($\lambda_1$, $\delta_1$, and $\tau_1$) and their 95% confidence intervals were determined by least squares non-linear regression analysis (GraphPad Prism, GraphPad Software, San Diego, CA). A monoexponential model was ultimately used for both moderate and severe-intensity exercise because, for the $M-S$ transition, a biexponential model (Eq. 2) produced an inferior and ambiguous fit based on analysis of the model residuals.

$$\Delta V\dot{O}_2(t) = A_1 \cdot (1 - e^{-(t-\delta_1)/\tau_1}) + A_2 \cdot (1 - e^{-(t-\delta_2)/\tau_2})$$

Given the failure of the biexponential model to adequately describe the $V\dot{O}_2$ response during $M-S$, the onset of the $V\dot{O}_2$ slow component was determined using purpose-designed LabVIEW software, which iteratively fits a monoexponential function to the $V\dot{O}_2$ data until the window encompasses the entire response. The estimated $\tau$ for each fitting window was plotted against time and the onset of the $V\dot{O}_2$ slow component was identified as the point at which the estimated $\tau$ consistently deviated from the previously “flat” profile (61). The amplitude of the $V\dot{O}_2$ slow component was subsequently determined by calculating the difference between the end exercise $V\dot{O}_2$ and the sum of the primary amplitude and baseline $V\dot{O}_2$. This was expressed both in absolute terms and relative to the end-exercise $V\dot{O}_2$. The functional gain of the primary $V\dot{O}_2$ response during $U-M$ and $M-S$ was also calculated by dividing the primary phase amplitude by the change in work rate. Finally, the mean response time (MRT) for both $U-M$ and $M-S$ was calculated by fitting a single exponential curve to the data with no time delay from the onset to the end of exercise. The NIRS-derived [Hb] response to exercise was also modeled to provide information on muscle oxygenation. The responses to each transition were interpolated to 1-s intervals, time aligned, and averaged to produce a single data set. Since the [Hb] signal increased after a short delay in response to step exercise, the time of onset for the exponential-like increase. For $M-S$, the model fitting window was constrained to the onset of the [Hb] slow component determined using the iterative curve-fitting procedure, as described for $V\dot{O}_2$ above. The primary [Hb] amplitude was divided by the phase II $V\dot{O}_2$ asymptote to determine the $\Delta$[Hb]/$\Delta V\dot{O}_2$ ratio as an index of the change in fractional muscle O2 extraction required to elicit a given $\Delta V\dot{O}_2$ during the primary phase. In addition, we assessed changes in total blood volume by summing the [HbO2] and [Hb] signals to provide an estimate of the total [Hb] in the area under investigation. Specifically, we determined the mean value at baseline (30 s preceding each transition), at 60-s intervals throughout exercise (15-s bins centered on each time point), and at end exercise (final 30 s) to facilitate comparisons between conditions. Finally, heart rate (HR) kinetics was modeled for each condition with the $TD$ parameter in Eq. 1 fixed to $t = 0$ s (i.e., monoexponential with no delay) and with the fitting window constrained to the onset of the $V\dot{O}_2$ “slow component.” Statistics: Gaussian distribution was confirmed by the Shapiro-Wilks test. Following this, the pulmonary $V\dot{O}_2$, HR, and NIRS-derived variables were analyzed using two-way repeated-measures ANOVA with “exercise intensity” ($U-M$ and $M-S$) and “supplement” (BR vs. PL) included as within-subject factors. Differences in BP and plasma [NO2] were determined using two-way (supplement $\times$ time) repeated-measures ANOVA. Subsequent paired samples t-tests were employed as appropriate to identify the location of statistically significant effects. Pearson product moment correlation coefficients were used to analyze the degree of association between key variables. All statistical analyses were conducted using PASW Statistics 18 (SPSS, Chicago, IL). Data are presented as means ± SD. Statistical significance was accepted when $P \leq 0.05$. RESULTS The subjects’ peak $V\dot{O}_2$ was 3.73 ± 0.46 l/min for men and 2.69 ± 0.52 l/min for women with the GET occurring at 2.08 ± 0.41 l/min and 1.71 ± 0.41 l/min, respectively. The peak work rate attained from the incremental test was 327 ± 32 W for men and 263 ± 38 W for women. The work rates calculated to require 90% of the GET and $\Delta$70% were 100 ± 26 and 215 ± 37 W, respectively. Plasma [NO2]. There was a main effect for supplement on plasma [NO2] at rest over the last 3 days of the supplementation period ($F_{(1,8)} = 21.59$, $P = 0.01$). Follow-up paired comparisons revealed that plasma [NO2] was elevated ($P < 0.02$) at each sample point following BR compared with PL ingestion on day 4 (PL: 64 ± 36 vs. BR: 300 ± 141 nm), day 5 (PL: 66 ± 35 vs. BR: 374 ± 149 nm), and day 6 (PL: 65 ± 32 vs. BR: 348 ± 170 nm). Muscle oxygenation. The [Hb] and [HHb] values derived from NIRS interrogation are presented in Table 1. There was no significant main effect for supplement on the [Hb] during $U-M$ and $M-S$ exercise. The [HHb] response during step exercise for a representative subject is illustrated in Fig. 2. Two-way ANOVA revealed a significant interaction effect between exercise intensity and supplement on [HHb] kinetics following the onset of exercise ($F_{(1,6)} = 15.30, P = 0.01$). Specifically, compared with PL, the [HHb] $\tau$ was speeded during $M-S$ following BR supplementation (PL: 20 ± 9 vs. BR: 14 ± 2).
BR: 10 ± 3 s, *P* = 0.05), but there were no differences between PL and BR during U→M (PL: 7 ± 3 s vs. BR: 10 ± 5 s, *P* = 0.17). The [HHb] \( \tau \) was significantly slower for M→S compared with U→M in PL (\( *P* = 0.01 \)), but there was no difference between the upper and lower step in BR (\( *P* = 0.94 \)). There was no significant main effect for supplement on the primary [HHb] amplitude when normalized per unit change in \( \dot{V}O_2 \) during the fundamental exponential phase [\( F(1,6) = 4.81 \), \( P = 0.07 \)].

**HR kinetics.** The HR responses to step exercise are presented in Table 2. There were no differences in the primary HR \( \tau \) between PL and BR for U→M or M→S [\( F(1,8) = 0.10 \), \( P = 0.77 \)]. During M→S, the relative change in the \( \dot{V}O_2 \) \( \tau_p \) was not correlated with the relative change in HR kinetics between conditions (\( r = 0.42 \), \( P = 0.27 \)). There were no significant differences in blood [lactate] between conditions.

**Vo2 kinetics and exercise tolerance.** The \( \dot{V}O_2 \) kinetic parameters derived from the monoexponential fit are presented in Table 3, and the \( \dot{V}O_2 \) response of a representative subject to U→M and M→S is shown in Fig. 2. The group mean \( \dot{V}O_2 \) profile during M→S is presented in Fig. 3. Two-way ANOVA revealed a significant interaction effect between exercise intensity and supplement on phase II \( \dot{V}O_2 \) kinetics following the onset of exercise [\( F(1,8) = 18.54 \), \( P = 0.01 \)]. Compared with PL, the \( \tau_p \) was shorter during M→S following BR ingestion (PL: 46 ± 13 vs. BR: 36 ± 10 s, \( *P* = 0.01 \)), but there were no differences during U→M (PL: 25 ± 4 vs. BR: 27 ± 6 s, \( *P* = 0.25 \)). For the PL condition, the \( \tau_p \) was greater in M→S compared with U→M (\( *P* = 0.001 \)), but there were no significant differences between U→M and M→S in the BR condition (\( *P* = 0.12 \)). During M→S, the speeding of \( \dot{V}O_2 \) \( \tau_p \) was not correlated with the speeding of the primary [HHb] \( \tau \) after BR compared with PL (\( r = -0.16 \), \( P = 0.76 \)). There was no significant main effect for supplement on the primary \( \dot{V}O_2 \) amplitude [\( F(1,8) = 0.01 \), \( P = 0.91 \)] or primary \( \dot{V}O_2 \) gain [\( F(1,8) = 0.05 \), \( P = 0.83 \)] during U→M or M→S. The emergence of a slow phase in \( \dot{V}O_2 \) during M→S occurred after a similar time delay, and there were no differences in the absolute or relative amplitude of the \( \dot{V}O_2 \) slow component between PL and BR (both \( P = 0.44 \)). For M→S, there were no differences between PL and BR in the \( \dot{V}O_2 \) amplitude at end-exercise [\( F(1,8) = 0.60 \), \( P = 0.46 \)] or the total \( \dot{V}O_2 \) gain [\( F(1,8) = 0.14 \), \( P = 0.72 \)].

The \( \dot{V}O_2 \) attained at task failure (PL: 3.12 ± 0.51 vs. BR: 3.09 ± 0.51 l/min) was not different between conditions or compared with the peak \( \dot{V}O_2 \) obtained during the initial ramp incremental test (\( P > 0.66 \)). Compared with PL, the exercise time to task failure was significantly increased during M→S following BR supplementation (PL: 521 ± 158 vs. 635 ± 258 s, \( P = 0.02 \)). The time to task failure was greater in every
participant after BR compared with PL (range = 3% to 54%; Fig. 4). The increased time to task failure was not correlated with the reduction in the $V_{\text{O}_2}$ $\tau_p$ after BR compared with PL ($r = 0.03$, $P = 0.95$).

**DISCUSSION**

The principal novel finding of this investigation was that 6 days of dietary supplementation with NO$_3$-rich BR juice speeded pulmonary $V_{\text{O}_2}$ and muscle [HHb] kinetics and increased the time-to-task failure during $M \rightarrow S$ exercise compared with NO$_3$-depleted PL juice. These results suggest that increasing plasma [NO$_3$] and, thus, the potential for O$_2$-independent NO generation after BR supplementation, can speed the $V_{\text{O}_2}$ $\tau_p$ in $M \rightarrow S$ such that it is not significantly different from the $V_{\text{O}_2}$ $\tau_p$ in $U \rightarrow M$. It is possible that this faster rate of ATP resynthesis through oxidative metabolism can account, at least in part, for the improved exercise tolerance observed during $M \rightarrow S$ exercise after BR supplementation. Given that $M \rightarrow S$ would be expected to recruit a population of muscle fibers that are positioned higher in the recruitment hierarchy (i.e., type II) compared with $U \rightarrow M$ (29, 39), these results suggest that BR supplementation may have specific effects on metabolic and/or vascular control in type II muscle fibers in humans, consistent with previous reports in rodent models (23, 32).

In the present study, short-term dietary supplementation with NO$_3$-rich BR juice markedly increased plasma [NO$_3$]. Surprisingly, however, this was not associated with a reduced steady-state $V_{\text{O}_2}$ during $U \rightarrow M$. This finding contrasts with previous studies in young, recreationally active subjects (2, 5, 44–47, 63), but it is consistent with other studies in which the participants were well trained (11, 55). Training status does not provide an explanation for the lack of effect of BR ingestion on steady-state $V_{\text{O}_2}$ during moderate-intensity exercise in the present study because the participants were not well trained (48 and 46 ml·kg$^{-1}·$min$^{-1}$ for males and females, respectively). In a recent study investigating the dose-response relationship between acute NO$_3$ intake and the physiological responses to exercise (71), it was reported that steady-state $V_{\text{O}_2}$ during moderate-intensity exercise was significantly reduced following the consumption of 280 ml of BR (~16 mmol NO$_3$) but not 70 ml BR (~4 mmol NO$_3$) or 140 ml BR (~8 mmol NO$_3$). While this suggests that a higher NO$_3$ dose than the 8 mmol employed in the present study might have been required to elicit an altered O$_2$ cost of exercise, it should be noted that significant reductions in steady-state $V_{\text{O}_2}$ with 5–8 mmol NO$_3$ supplementation (administered as BR) have been reported previously (5, 44, 64). The explanation for the lack of effect of BR on steady-state $V_{\text{O}_2}$ during moderate-intensity exercise in the present study is, therefore, obscure.

While NO$_3$ has traditionally been considered as an inert product of NO oxidation (53), recent studies have shown that NO$_3$ can be recycled back into bioactive NO (48). Moreover, in contrast to the generation of NO through the oxidation of L-arginine in a reaction catalyzed by nitric oxide synthase, the reduction of NO$_2$ to NO is O$_2$-independent (17) and is potentiated by acidosis (52). Since pH and microvascular PO$_2$ decline more rapidly in contracting type II muscle (10, 51), NO$_2$ reduction to NO may be a more effective pathway for NO generation in, and within the microvasculature surrounding, type II muscle fibers during contractions.

In this study, we have shown for the first time that, compared with PL, BR ingestion speeded phase II $V_{\text{O}_2}$ kinetics in $M \rightarrow S$ exercise whereas, consistent with previous research (5, 44), BR did not alter phase II $V_{\text{O}_2}$ kinetics during $U \rightarrow M$. The intensity-dependent effects of dietary NO$_3$ intake with BR on phase II $V_{\text{O}_2}$ kinetics may be due, at least in part, to differences in muscle fiber activation patterns in $U \rightarrow M$ and $M \rightarrow S$. In accord with an orderly “size” principle of motor unit recruitment (31), $M \rightarrow S$ would be predicted to activate a fraction of the total muscle fiber pool positioned higher in the recruitment hierarchy.

## Table 2. Blood [lactate] and heart rate dynamics during moderate- and severe-intensity exercise following BR and PL supplementation

<table>
<thead>
<tr>
<th></th>
<th>PL</th>
<th>BR</th>
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<tbody>
<tr>
<td><strong>Unloaded-to-Moderate-Intensity Exercise</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline HR, beats/min</td>
<td>$83 \pm 11$</td>
<td>$82 \pm 10$</td>
</tr>
<tr>
<td>Primary HR time constant, s</td>
<td>$30 \pm 9$</td>
<td>$29 \pm 10$</td>
</tr>
<tr>
<td>End-exercise HR, beats/min</td>
<td>$119 \pm 14$</td>
<td>$118 \pm 14$</td>
</tr>
<tr>
<td>Baseline blood [lactate], mM</td>
<td>$1.9 \pm 0.6$</td>
<td>$1.7 \pm 0.4$</td>
</tr>
<tr>
<td>End-exercise blood [lactate], mM</td>
<td>$3.0 \pm 0.9$</td>
<td>$2.6 \pm 0.8$</td>
</tr>
<tr>
<td>$\Delta$ blood [lactate], mM</td>
<td>$1.1 \pm 1.4$</td>
<td>$1.0 \pm 0.9$</td>
</tr>
</tbody>
</table>

**Moderate-to-Severe-Intensity Exercise**

<table>
<thead>
<tr>
<th></th>
<th>PL</th>
<th>BR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline HR, beats/min</td>
<td>$117 \pm 14^*$</td>
<td>$116 \pm 13^*$</td>
</tr>
<tr>
<td>Primary HR time constant, s</td>
<td>$48 \pm 19^*$</td>
<td>$47 \pm 12^*$</td>
</tr>
<tr>
<td>HR at 360 s, beats/min</td>
<td>$170 \pm 13^*$</td>
<td>$171 \pm 13^*$</td>
</tr>
<tr>
<td>HR mean response time, s</td>
<td>$73 \pm 20$</td>
<td>$67 \pm 17$</td>
</tr>
<tr>
<td>Baseline blood [lactate], mM</td>
<td>$3.0 \pm 0.9^*$</td>
<td>$2.6 \pm 0.8^*$</td>
</tr>
<tr>
<td>Blood [lactate] at 360 s, mM</td>
<td>$11.0 \pm 3.0$</td>
<td>$10.7 \pm 3.1$</td>
</tr>
<tr>
<td>$\Delta$ blood [lactate], mM</td>
<td>$8.0 \pm 2.2^*$</td>
<td>$8.1 \pm 2.4^*$</td>
</tr>
<tr>
<td>Blood [lactate] at exhaustion, mM</td>
<td>$10.8 \pm 2.8$</td>
<td>$10.9 \pm 2.3$</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. HR, heart rate; $\Delta$, change. Significantly different from moderate exercise within condition: $^* P < 0.01$.

## Table 3. Pulmonary $O_2$ uptake responses to moderate- and severe-intensity exercise following BR and PL supplementation

<table>
<thead>
<tr>
<th></th>
<th>PL</th>
<th>BR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unloaded-to-Moderate-Intensity Exercise</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline $V_{\text{O}_2}$, l/min</td>
<td>$0.76 \pm 0.13$</td>
<td>$0.76 \pm 0.15$</td>
</tr>
<tr>
<td>Phase II time constant, s</td>
<td>$25 \pm 4$</td>
<td>$27 \pm 6$</td>
</tr>
<tr>
<td>Primary amplitude, l/min</td>
<td>$0.91 \pm 0.28$</td>
<td>$0.95 \pm 0.33$</td>
</tr>
<tr>
<td>Primary gain, ml·min$^{-1}·$W$^{-1}$</td>
<td>$10.8 \pm 1.4$</td>
<td>$11.1 \pm 1.3$</td>
</tr>
<tr>
<td>End-exercise $V_{\text{O}_2}$, l/min</td>
<td>$1.67 \pm 0.37$</td>
<td>$1.70 \pm 0.39$</td>
</tr>
<tr>
<td>Mean response time, s</td>
<td>$40 \pm 12$</td>
<td>$40 \pm 6$</td>
</tr>
</tbody>
</table>

**Moderate-to-Severe-Intensity Exercise**

<table>
<thead>
<tr>
<th></th>
<th>PL</th>
<th>BR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline $V_{\text{O}_2}$, l/min</td>
<td>$1.66 \pm 0.38^*$</td>
<td>$1.69 \pm 0.39^*$</td>
</tr>
<tr>
<td>Phase II time constant, s</td>
<td>$46 \pm 13^*$</td>
<td>$36 \pm 10^†$</td>
</tr>
<tr>
<td>Primary amplitude, l/min</td>
<td>$1.18 \pm 0.25$</td>
<td>$1.14 \pm 0.26$</td>
</tr>
<tr>
<td>Primary gain, ml·min$^{-1}·$W$^{-1}$</td>
<td>$10.3 \pm 1.1$</td>
<td>$9.9 \pm 0.8$</td>
</tr>
<tr>
<td>Slow phase time delay, s</td>
<td>$163 \pm 27$</td>
<td>$157 \pm 21$</td>
</tr>
<tr>
<td>Slow phase amplitude, l/min</td>
<td>$0.24 \pm 0.11$</td>
<td>$0.26 \pm 0.12$</td>
</tr>
<tr>
<td>Slow phase relative amplitude, %</td>
<td>$17 \pm 7$</td>
<td>$18 \pm 8$</td>
</tr>
<tr>
<td>Total gain, ml·min$^{-1}·$W$^{-1}$</td>
<td>$12.4 \pm 0.9^#$</td>
<td>$12.3 \pm 1.2^#$</td>
</tr>
<tr>
<td>$V_{\text{O}_2}$ at 360 s, l/min</td>
<td>$3.08 \pm 0.55^*$</td>
<td>$3.10 \pm 0.54^*$</td>
</tr>
<tr>
<td>Mean response time, s</td>
<td>$76 \pm 14^*$</td>
<td>$69 \pm 11^*$</td>
</tr>
<tr>
<td>$V_{\text{O}_2}$ at exhaustion, l/min</td>
<td>$3.12 \pm 0.51$</td>
<td>$3.09 \pm 0.51$</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. Significantly different from moderate exercise within condition: $^* P < 0.01$, $^# P < 0.05$. Significantly different from PL: $^† P < 0.05$. 

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*AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00295.2013 • www.ajpregu.org*
chy compared with U→M. Empirical evidence to support this postulate is provided by the study of Krustrup et al. (42). These authors reported that PCR and glycogen content were lowered more in type II compared with type I muscle fibers when subjects cycled at an intensity corresponding to 80% \( V\dot{O}_2_{\text{max}} \), whereas the reverse was true at 50% \( V\dot{O}_2_{\text{max}} \) (42). The steady-state \( V\dot{O}_2 \) amplitude in the U→M step in the present study was \( \sim 54\% \) of \( V\dot{O}_2_{\text{max}} \), suggesting that type I muscle fibers were principally activated in the lower-step transition. Conversely, the longer \( V\dot{O}_2 \) mean response time and increased total \( V\dot{O}_2 \) gain observed during M→S in the PL condition is consistent with what would be expected if a greater proportional activation of type II muscle fibers occurred in the upper step (6, 40, 41, 58). Our findings, therefore, suggest that the faster \( V\dot{O}_2 \) kinetics observed following BR supplementation during M→S might be related to specific effects of NO\(_3\) treatment on higher-order (i.e., type II) muscle fibers.

To explore the mechanisms responsible for any alterations in \( V\dot{O}_2 \) in M→S, the NIRS-derived muscle [HHb] signal was used to provide information on the dynamic (im)balance between microvasculature \( O_2 \) delivery and metabolic demand (19, 27). For the same \( V\dot{O}_2 \) kinetics, enhanced muscle \( O_2 \) supply relative to muscle \( O_2 \) demand would be expected to result in a longer muscle [HHb] \( \tau \), whereas faster \( V\dot{O}_2 \) kinetics alongside unchanged [HHb] kinetics would be interpreted as a proportionally similar increase in the rate of muscle \( O_2 \) delivery to \( V\dot{O}_2 \). However, in the present study, faster \( V\dot{O}_2 \) kinetics in M→S with BR was accompanied by a shorter [HHb] \( \tau \) during which [HB\(_{\text{tot}}\)] (and by inference blood volume) in the interrogated muscle area was not different compared with PL. This suggests that BR may have speeded \( V\dot{O}_2 \) kinetics, in part, by enhancing muscle \( O_2 \) extraction. It has been reported that muscle \( O_2 \) demand exceeds microvascular \( O_2 \) delivery in muscle comprising predominantly type II fibers (10, 51) and that BR increases muscle bulk blood flow and promotes a greater distribution of blood flow to type II muscle fibers (23). If absolute or relative perfusion of type II fibers was greater after BR ingestion, this might have facilitated enhanced muscle \( O_2 \) extraction, as suggested by the faster muscle [HHb] kinetics and, therefore, permitted faster \( V\dot{O}_2 \) kinetics in M→S. However, the faster \( V\dot{O}_2 \) \( \tau \) with BR compared with PL was not significantly correlated with the reduction in the [HHb] \( \tau \). It is, therefore, also possible that BR speeded \( V\dot{O}_2 \) kinetics, by altering metabolic control in type II fibers during the transition from M→S. Given that short-term NO\(_3\) supplementation does not increase markers of mitochondrial biogenesis in skeletal muscle (45) or speed the recovery of [PCR] following intense exercise (44), which would reflect increased muscle oxidative capacity (12), the faster \( V\dot{O}_2 \) kinetics in M→S is unlikely to have resulted from an increase in mitochondrial volume. Increased intracellular calcium content ([Ca\(^{2+}\)]\(_i\)) has been observed during tetanic contractions of type II, but not type I, muscle fibers excised from mice supplemented with NO\(_3\) (32). As well as activating the muscle contractile apparatus, Ca\(^{2+}\) has also been suggested to signal the activation of oxidative phosphorylation (30). Therefore, it is possible that increased [Ca\(^{2+}\)]\(_i\) and parallel activation of the contractile and oxidative metabolic machinery might have contributed to the faster muscle [HHb] and \( V\dot{O}_2 \) kinetics reported in this study.

It has been reported previously that the tolerable duration of severe-intensity exercise initiated from an unloaded cycling or resting baseline can be enhanced after a period of BR supplementation (2, 5, 35, 44). The findings of this study extend these earlier reports by showing that the tolerable duration of severe-intensity cycle exercise initiated from a moderate-intensity baseline work rate can also be improved (by \( \sim 22\% \) on average). Recent studies show that performance is also enhanced during high-intensity intermittent exercise (13, 72), which...
would also be expected to engender significant recruitment of type II muscle fibers (39). It has been reported (using multichannel NIRS) that there is marked intersite heterogeneity in matching of O2 delivery to V\textsubscript{O2} within the quadriceps muscle during high-intensity cycling (37). One possibility is that NO might inhibit O2 utilization in some well-oxygenated muscle fibers (15), whereas the hypoxic and acidic environment within and surrounding muscle fibers receiving less O2 might stimulate NO\textsubscript{2} reduction to NO and, thus, increase microvascular O2 supply (29). Faster phase II V\textsubscript{O2} kinetics during M\textrightarrow S after BR might, therefore, have resulted from a more homogenous distribution of O2 relative to metabolic demand within contracting muscle. Interventions that speed V\textsubscript{O2} kinetics have been previously shown to improve the tolerable duration of severe-intensity exercise (3, 4). A faster adjustment of V\textsubscript{O2} during M\textrightarrow S would be expected to spare expenditure of the finite anaerobic reserves (i.e., from PCr breakdown and anaerobic glycolysis) and reduce the accumulation of metabolites that have been implicated in the development of skeletal muscle fatigue (1, 16, 54). However, in the present study, while an increased time to task failure with BR was accompanied by a shorter V\textsubscript{O2} \tau\textsubscript{p} compared with PL, the two were not significantly correlated.

Dietary supplementation with NO\textsubscript{3}-rich BR juice has been reported to improve exercise tolerance in concert with attenuated skeletal muscle ATP turnover, PCr hydrolysis, and P\textsubscript{i} and ADP accumulation during high-intensity exercise (2). Perturbations of skeletal muscle Ca\textsuperscript{2+} handling and membrane excitability are also hallmarks of skeletal muscle fatigue (1). In this respect, it is interesting that mice receiving NO\textsubscript{3} treatment had an improved capacity for sarcoplasmic Ca\textsuperscript{2+} release and increased tetanic force production in type II muscle (32). In humans, BR supplementation appears to blunt the accumulation of extracellular K\textsuperscript{+}, possibly preserving muscle excitability, during intense intermittent exercise (72). As discussed earlier, improvements in muscle blood flow and a greater distribution of blood flow to type II muscle fibers with BR (23) might also have contributed to the improved exercise performance in this study. The enhanced exercise tolerance observed during M\textrightarrow S in the present study might, therefore, be consequent to a conflation of alterations in skeletal muscle metabolism, excitation-contraction coupling, and perfusion. Additional studies are required to address these issues.

It is of interest that, in vitro, NO may inhibit oxidative ATP flux by competing with O2 for the O2-binding site at cytochrome-c oxidase (COX) in the electron transport chain (15). If NO\textsubscript{3} supplementation and the associated increased NO production significantly inhibited COX, then an increased ATP contribution from anaerobic metabolism would be expected for the same work rate. However, we have reported previously that muscle PCr utilization is reduced, and pH is not changed after NO\textsubscript{3} supplementation (2), which argues against this possibility. NO has many physiological effects, and it is possible that any inhibition of COX by NO is offset by other, positive, effects. For example, COX inhibition of fibers nearest a capillary might allow O2 to diffuse to fibers further from the capillary, which might be O2-deficient (thereby increasing "global" oxidative ATP production across a muscle) (29). There is also evidence that greater NO production via NO\textsubscript{3} supplementation might improve matching of O2 supply to O2 utilization and increase the O2 driving pressure within contracting muscle (23, 24), increase the mitochondria P/O ratio (45), and improve mitochondrial function in hypoxia (64). Therefore, while the effects of NO on oxidative metabolism are complex, the existing evidence suggests that NO\textsubscript{3} supplementation has a beneficial rather than a detrimental effect on oxidative function.

**Perspectives and Significance**

In this study, we showed that 6 days of dietary supplementation with NO\textsubscript{3}-rich BR juice speeded pulmonary V\textsubscript{O2} and muscle [HHb] kinetics and increased the tolerable duration of severe-intensity cycling in M\textrightarrow S compared with PL. It remains to be determined whether longer periods of supplementation might elicit greater, or lesser, physiological and performance effects. It has previously been reported that V\textsubscript{O2}\textsubscript{max} and peak power output during incremental exercise were increased, and that acute reductions of resting blood pressure and the O2 cost of moderate-intensity exercise were maintained, after 15 days of BR supplementation (63). This indicates that subjects do not develop tolerance to inorganic nitrate intake, at least up to 15 days of supplementation.

In addition to containing NO\textsubscript{3}, BR is also rich in several other compounds, including betaine, antioxidants, and polyphenols (including quercetin and resveratrol) (70), that might influence the physiological responses to exercise. At present, we cannot rule out the possibility that these compounds might exert independent effects or that NO\textsubscript{3} operates synergistically with one or more of them. Moreover, ascorbate and polyphenols facilitate the reduction of nitrite to NO (49), which might augment NO production. However, in a previous study, we reported that the physiological responses to exercise and exercise tolerance were only improved when BR contained NO\textsubscript{3} (44). When placebo BR (which has negligible amounts of NO\textsubscript{3}) was administered, the physiological responses were not different to those measured in a control (nonsupplemented) condition (44). It is also important to note that similar effects on plasma NO\textsubscript{2} and exercising V\textsubscript{O2} have been reported when subjects have consumed nitrate salts (45, 47). This strongly suggests that the physiological effects of BR consumption can be attributed, in large part, to its high NO\textsubscript{3} content. Nevertheless, it would be beneficial for future research to compare the physiological effects of dietary inorganic NO\textsubscript{3} supplementation with those of other NO donors, such as nitroglycerin, and for changes in NO availability to be more directly assessed.

The results of the present study have important implications for competitive sports and also provide insight into the mechanisms by which BR supplementation may improve performance during simulated competition (18, 43), as well as during high-intensity intermittent exercise (13, 72). Continuous athletic events, such as cycling and running races, are rarely completed at an even pace but are often stochastic with frequent "surges" in speed (i.e., step transitions in metabolic rate) throughout the competition. The results of the present study, which indicate faster V\textsubscript{O2} kinetics in the transition from a lower to a higher metabolic rate, suggest that BR supplementation has the potential to enhance performance in such events. This provides further support to the notion that short-term BR supplementation may be conducive to exercise performance, at least in recreationally active participants.
DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
B.C.B., S.J.B., and A.M.J. conception and design of research; B.C.B., M.A.M., and S.M. performed experiments; B.C.B., M.A.M., and J.R.B. analyzed data; B.C.B., M.A.M., S.J.B., and A.M.J. interpreted results of experiments; B.C.B. and M.A.M. prepared figures; B.C.B. drafted manuscript; B.C.B., M.A.M., S.J.B., and A.M.J. edited and revised manuscript; B.C.B., M.A.M., S.J.B., and A.M.J. approved final version of manuscript.

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