Beetroot juice ingestion *during* prolonged moderate-intensity exercise attenuates progressive rise in O_2 uptake

Original Article

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Abstract

Nitrate-rich beetroot juice (BR) supplementation has been shown to increases biomarkers of nitric oxide bioavailability with implications for the physiological responses to exercise. We hypothesized that BR supplementation before and during prolonged moderate-intensity exercise would: maintain an elevated plasma nitrite concentration ($[NO_2^-]$), attenuate the expected progressive increase in $\dot{V}O_2$ over time, and improve performance in a subsequent time trial (TT). In a double-blind, randomized, crossover design, 12 males completed 2-h of moderate-intensity cycle exercise followed by a 100 kJ TT in three conditions: 1) BR before and 1-h into exercise (BR+BR); 2) BR before and placebo (PL) 1-h into exercise (BR+PL); and 3) PL before and 1-h into exercise (PL+PL). During the 2-h moderate-intensity exercise bout, plasma $[NO_2^-]$ declined by ~17% in BR+PL but increased by ~8% in BR+BR such that, at 2-h, plasma $[NO_2^-]$ was greater in BR+BR than both BR+PL and PL+PL ($P<0.05$). $\dot{V}O_2$ was not different between conditions over the first 90 min of exercise, but was lower at 120 min in BR+BR (1.73 ± 0.24 $L \cdot min^{-1}$) compared to BR+PL (1.80 ± 0.21 $L \cdot min^{-1}$; $P=0.08$) and PL+PL (1.83 ± 0.27 $L \cdot min^{-1}$; $P<0.01$). The decline in muscle [glycogen] over the 2-h exercise bout was attenuated in BR+BR (~28% decline) compared to BR+PL (~44% decline) and PL+PL (~44% decline; n = 9, $P<0.05$). TT performance was not different between conditions ($P>0.05$). BR supplementation before and during prolonged moderate-intensity exercise attenuated the progressive rise in $\dot{V}O_2$ over time and appeared to reduce muscle glycogen depletion but did not enhance subsequent TT performance.

Keywords: nitric oxide, efficiency, glycogen depletion, substrate utilization, oxygen consumption, performance

Introduction

Nitric oxide (NO) is recognized as a ubiquitous signaling molecule fundamental to regulating many physiological functions including vasodilation (14), skeletal muscle contraction (49), mitochondrial respiration (8), and glucose uptake (3). In humans, NO bioavailability can be increased through exogenous consumption of inorganic nitrate (NO_3^-) which can be reduced to nitrite (NO_2^-) by bacterial NO_3^- reductases in the oral cavity and further reduced into NO and other reactive nitrogen species under appropriate physiological conditions (39). In addition to reducing resting blood pressure (54), dietary NO_3^- supplementation has been reported to reduce the O_2 cost of exercise (2, 38, 53) and to enhance skeletal muscle contractile function (22, 24, 55), effects which might be expected to result in improved exercise performance.

Several studies indicate that NO_3^- supplementation can enhance short duration (<30 min) exercise performance (1, 2, 11, 35, 48). However, the efficacy of NO_3^- supplementation in improving longer duration exercise performance is less clear (6, 11, 12, 34, 57). This disparity in the efficacy of NO_3^- supplementation in shorter vs. longer endurance exercise may be related to the metabolism of NO_3^- and NO_2^- during exercise. The pre-exercise elevation in plasma [NO_2^-] following NO_3^- supplementation has been shown to be associated with the magnitude of performance enhancement during long duration cycling (57). However, following NO_3^- supplementation, plasma [NO_2^-] declines over the course of short duration moderate- and severe-intensity exercise (32, 50), as well as during repeated sprints (51, 52, 59). Indeed, this decline in plasma [NO_2^-] with time during exercise, which may reflect the use of nitrite as a 'substrate' for NO production, is correlated with enhanced high-intensity exercise performance following NO_3^- supplementation (52, 59). It is possible, therefore, that long duration endurance exercise results in a progressive, and perhaps substantial, depletion of plasma [NO_2^-] such that the potential benefits of NO_3^- supplementation on performance later in exercise are no longer elicited (12, 57). Ingesting NO_3^- *during* longer duration exercise might maintain plasma [NO_2^-] at an elevated level and provide the potential for performance to be improved.

During prolonged, constant-work-rate exercise, an upward drift in pulmonary O_2 uptake ($\dot{V}O_2$) is typically observed (9, 25). The O_2 cost of such exercise may increase with time due to a shift in

substrate utilization towards fat oxidation, a progressive recruitment of type II muscle fibers, or a decline in skeletal muscle mitochondrial and/or contractile efficiency (29). Muscle glycogen depletion during prolonged exercise may also contribute to the loss of efficiency over time (43). Dietary NO_3^- supplementation has the potential to lower O_2 demand during prolonged exercise (2, 27). Specifically, NO_3^- supplementation has been reported to enhance the mitochondrial P/O ratio (37; cf. 55) and to reduce the ATP cost of muscle force production (1). In animal studies, NO_3^- supplementation has been reported to improve intracellular calcium (Ca^{2+}) handling and increase force production at low frequencies of contraction in type II muscle fibers (24) and to lead to preferential blood flow (and O_2) distribution to type II muscle (15, 16). Given that: 1) fatigue development and the progressive increase in $\dot{V}O_2$ during prolonged exercise may be related, at least in part, to the recruitment of type II muscle fibers (33); and that 2) NO_3^- supplementation positively impacts muscles comprised predominantly of type II fibers (28); it is possible that ingesting NO_3^- during as well as before such exercise may be better than pre-exercise NO_3^- ingestion alone in limiting fatigue development, minimizing $\dot{V}O_2$ and enhancing performance.

Another mechanism by which NO_3^- supplementation might potentially alter the O_2 cost of exercise is via effects on carbohydrate metabolism. NO has been shown to play an important role in regulating skeletal muscle glucose uptake (3). Wylie et al. (59) reported lower blood [glucose] during high-intensity intermittent exercise following NO_3^- supplementation, which might suggest enhanced skeletal muscle glucose uptake; however, this was not confirmed during longer duration moderate-intensity exercise (6). It therefore remains unclear whether dietary NO_3^- supplementation before, and especially *during*, prolonged exercise can affect carbohydrate metabolism or muscle glycogen utilization. A lower metabolic cost of exercise as reflected by a lower $\dot{V}O_2$ and/or increased muscle glucose uptake from the blood might reduce muscle glycogen utilization during prolonged exercise and enhance endurance performance.

The purpose of the present study was, therefore, to investigate whether ingestion of NO_3^--rich beetroot juice (BR) before, and also *during*, 2 h of moderate-intensity cycle exercise influences physiological responses and improves performance in a subsequent target-work (100 kJ) cycling performance test relative to a placebo condition. We hypothesized that BR supplementation

before and during 2-h moderate-intensity exercise would: 1) preserve an elevated plasma [NO_2^-]; 2) attenuate the expected progressive increase in $\dot{V}O_2$ with time; 3) reduce muscle glycogen depletion; and, therefore, 4) improve TT performance.

Methods

Subjects

Twelve recreationally-active males (mean ± SD: age 21 ± 1 years, body mass 78 ± 11 kg, height 1.77 ± 0.07 m, $\dot{V}O_{2peak}$, 45 ± 4 $mL \cdot kg^{-1}min^{-1}$) volunteered to participate in this study, nine of whom volunteered for invasive measurements (muscle biopsies and blood sampling). The protocol, risks, and benefits of participating were explained prior to obtaining written informed consent. This study was approved by the Institutional Research Ethics Committee and conformed to the code of ethics of the Declaration of Helsinki.

Experimental overview

Subjects reported to the laboratory on 5 separate occasions over a 5-week period. On the first visit, subjects completed a ramp incremental exercise test for the determination of $\dot{V}O_{2peak}$ and gas exchange threshold (GET). During the second visit, subjects were familiarized to the exercise testing procedures, including completion of a moderate-intensity exercise bout (at a work rate of 80% of the GET) for 30 min before completing a target-work (100 kJ) cycling performance test designed to simulate a 4-km TT.

For the duration of the study, subjects were asked to avoid consuming NO_3^--rich foods such as spinach, rocket (arugula), kale, and beetroot, and to refrain from taking any other dietary supplements or using antibacterial mouthwash as the latter affects the commensal bacteria in the oral cavity, resulting in the inhibition of NO_3^- reduction into NO_2^- (21). In a double-blind, randomized, crossover design, subjects were assigned to receive dietary supplementation for 3 days. On day 3 of each supplementation period (See Supplementation), subjects reported to the laboratory to complete the experimental protocol. Experimental visits were performed at the same time of day (± 2-h). Subjects recorded their activity and diet during the 24-h prior to the first experimental visit and were asked to repeat these for subsequent visits. Subjects were also

instructed to arrive at the laboratory following a 10-h overnight fast, having avoided strenuous exercise and alcohol in the 24-h preceding, and caffeine in the 8-h preceding, each experimental visit. The subjects were provided with a standardized breakfast consisting of 2 porridge oats sachets (Quaker Oats Ltd, Leicester, UK; containing 54 g of oats, 200 kcal, 4.2 g fat, 31.8 g carbohydrate, 5.6 g fibre, 6.0 g protein) mixed with 180 mL of water, 1-h prior to exercising.

Supplementation

Subjects were randomly assigned to three 3-day supplementation periods in which they consumed 2 x 70 mL doses per day of either NO_3^--rich BR: (~6.2 mmol NO_3^- per 70 mL; Beet it, James White Drinks Ltd., Ipswich, UK) or a NO_3^--depleted placebo (PL: ~0.04 mmol NO_3^- per 70 mL; Beet it, James White Drinks Ltd., Ipswich, UK) separated by a 5-day wash-out period. The three supplementation conditions were: 1) BR supplementation both before and at 1-h into exercise (BR+BR); 2) BR supplementation before and PL at 1-h into exercise (BR+PL); and 3) PL before and at 1-h into exercise (PL+PL). Each 70 mL beverage contained 72 kcal energy and 15.4 g of carbohydrate. On the first two days of each supplementation period, subjects consumed one 70 mL beverage in the morning and one in the evening, whereas on the experimental day, subjects consumed 2 x 70 mL of their allocated beverage in the morning 2.5-h prior to the exercise and 1 x 70 mL of their allocated beverage at 1-h into exercise. This 3-day protocol was chosen to simulate the approach to supplementation that an athlete might take prior to competition with the time frame for supplement ingestion on the final morning selected because peak plasma [NO_2^-] occurs ~2-3-h following NO_3^- intake (54, 59).

Exercise procedures

All exercise tests were performed on an electronically-braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). On the first visit, subjects completed a ramp incremental test, involving 3 min of baseline cycling at 20 W, after which the work rate was increased by 30 W/min until task failure. Task failure was recorded once the pedal rate fell by >10 rpm below the target cadence. The self-selected cadence (70-90 rpm) and seat height and handle bar configuration were recorded and reproduced on subsequent visits. Breath-by-breath pulmonary gas exchange data were collected continuously during the incremental test and averaged over 10-s periods. $\dot{V}O_{2peak}$ and GET were determined as previously described (53). Heart rate (HR) was

measured during all tests using short-range radio telemetry (Polar S610, Polar Electro, Kempele, Finland).

During the experimental visits, subjects performed baseline cycling at 20 W for 3 min. Following this, subjects completed 2-h of cycling at 80% GET (91 ± 24 W) at their self-selected cadence. A 1-min rest period followed the end of the 2-h bout during which a muscle biopsy was obtained (see *Muscle Biopsy*). The 100 kJ TT commenced immediately after the 1-min period. Subjects were provided with a 5-s countdown prior to the commencement of all cycling trials. The resistance on the pedals during the TT was set for each individual using the linear mode of the Lode ergometer so that the subject would attain the power output associated with GET plus 65% of the difference between GET and peak power output (65%Δ) on reaching a cadence of 90 rpm (35). Subjects were deprived of visual performance cues and did not receive notification on elapsed time but they received consistent verbal encouragement for each TT and were informed when 75, 50, 25 and 10 kJ of work remained to be completed. Pulmonary gas exchange was measured for discrete 6-min time periods (from 0-6 min, 27-33 min, 60-66 min, 87-93 min, and 114-120 min) during the 2-h exercise bout (the first 2 min of each period was not used in analysis), and continuously during the TT.

Measurements

Muscle biopsy

Skeletal muscle samples were obtained from two incisions made in the m. *vastus lateralis* under local anesthesia (1% lidocaine) using the percutaneous Bergström needle biopsy technique with suction (5). Muscle samples were obtained at rest (10 min prior to the start of the 2-h moderate-intensity exercise bout), within 15 s of the completion of the 2-h exercise bout and within 15 s of the completion of the TT. Muscle samples were immediately snap-frozen in liquid nitrogen before being stored at -80°C for subsequent analysis.

Muscle metabolites

Muscle samples were freeze-dried and dissected to remove visible fat, blood, and connective tissue using forceps. 200 µL of 3 M perchloric acid was added to ~2 mg dry weight (DW) of muscle tissue. Samples were incubated on ice for 30 min, then centrifuged for 3 min at 4000

rpm. 170 µL of supernatant was transferred over to a fresh microcentrifuge tube, and 255 µL of cooled 2 M potassium hydrogen carbonate ($KHCO_3$) was added. This was centrifuged, and the supernatant was analyzed for [PCr], [ATP], and [lactate] by fluorometric assays as described by Black et al. (7).

Muscle glycogen

~ 1 mg DW muscle tissue was hydrolysed in 500 µL of 1 M hydrochloric acid at 100 °C for 3-h to release glycosyl units, and immediately measured using an automated glucose analyzer (YSI 2900 Biochemistry Analyzer, Yellow Springs Instruments, Yellow Springs, OH). The precision of this method of analysis within this physiological range (0.05 to 0.55 mmol/L) was checked by measuring the glucose concentration across a range of solutions made up using glucose diluted in hydrochloric acid; the measured vs. expected values lay on the line of identity with an R^2 of 0.99.

Blood analysis

Venous blood was sampled at baseline, 30, 60, 90 and 120 min during the 2-h moderate-intensity exercise bout, and immediately following the completion of the TT. All blood samples were obtained from a cannula (Insyte-W^{TM} Becton-Dickinson, Madrid, Spain) that was inserted in the subject's antecubital vein, and were drawn into 6 mL lithium-heparin vacutainers (Becton-Dickinson, New Jersey, USA). For blood [lactate] and [glucose] analysis, 200 µL of blood was immediately hemolyzed into 200 µL of cold Triton X-100 buffer solution (Triton X-100, Amresco, Salon, OH) and then measured using an automated glucose and lactate analyzer (YSI 2300, Yellow Springs Instruments, Yellow Springs, OH). The remaining whole blood samples were centrifuged within 2 min of collection at 4000 rpm and 4°C for 10 min and then the plasma was immediately extracted and frozen at -80°C. Before the analysis of plasma [NO_3^-] and [NO_2^-], samples were deproteinized using cold ethanol precipitation. Specifically, thawed samples were centrifuged at 14000 g for 10 min, before 200 µL of sample was added to 400 µL of chilled ethanol and incubated on ice for 30 min. After further centrifugation at 14000 g for 10 min, the supernatant was removed for the subsequent determination of [NO_3^-] and [NO_2^-] via gas phase chemiluminescence as described by Wylie et al. (59).

Statistical Analysis

A two-way (condition x time) repeated measures analysis of variance (ANOVA) was used to analyze differences in physiological and performance responses during the 2-h moderate-intensity exercise bout and the TT. Significant main and interaction effects were further explored using Fisher's Least Significant Difference test. In addition, one-way repeated measures ANOVAs were used to determine physiological and performance differences in the mean and change values from pre- to post- 2h moderate exercise, and post-TT. The relationship between $\dot{V}O_2$ and muscle [glycogen] was explored using the Pearson product moment correlation coefficient. Statistical significance was accepted at $P \leq 0.05$. Results are presented as mean ± SD unless otherwise stated.

Results

All subjects reported consuming all servings of each supplement at the correct times and confirmed that they had maintained their exercise and dietary habits prior to each testing visit. There were no reports of gastrointestinal distress or discomfort following the ingestion of BR or PL either before or during exercise.

Plasma [NO_3^-] and [NO_2^-]

There was an interaction effect (condition x time) ($P<0.01$), main effect of time ($P<0.01$), and main effect of condition ($P<0.01$) for plasma [NO_3^-] (Fig. 1A). At baseline, plasma [NO_3^-] was significantly elevated in BR+BR (315 ± 57 µM; $P<0.01$) and BR+PL (302 ± 88 µM; $P<0.01$) compared to PL+PL (16 ± 7 µM). Plasma [NO_3^-] in BR+BR and BR+PL were elevated at all time points compared to PL+PL. In PL+PL, plasma [NO_3^-] was unchanged throughout exercise. In BR+PL, plasma [NO_3^-] was unchanged from baseline to 90 min ($P>0.05$). However, compared to baseline, plasma [NO_3^-] in BR+PL decreased by ~16% at 120 min (254 ± 56 µM, $P<0.05$). In BR+BR, plasma [NO_3^-] was unchanged from baseline to 60 min (317 ± 52 µM; $P>0.05$) but then increased by ~41% at 90 min (448 ± 51 µM, $P<0.0001$) and remained elevated until 120 min (463 ± 70 µM, $P>0.05$). Plasma [NO_3^-] was significantly elevated at 90 min, 120 min, and post-TT in BR+BR compared to BR+PL ($P<0.01$).

There was an interaction effect (condition x time) ($P<0.05$) and main effect of condition

(P<0.01) for plasma [NO_2^-] (Fig. 1B). At baseline, plasma [NO_2^-] was significantly greater in BR+BR (482 ± 211 nM; P<0.01) and BR+PL (484 ± 188 nM; P<0.01) compared to PL+PL (203 ± 63 nM), with no significant difference between BR+BR and BR+PL. Plasma [NO_2^-] was unchanged throughout exercise in PL+PL. In BR+PL, plasma [NO_2^-] tended to decrease by ~17% from baseline to 120 min (P=0.07). In contrast, in BR+BR, plasma [NO_2^-] increased by ~8% from baseline to 120 min. Plasma [NO_2^-] tended to be elevated at 90 min in BR+BR (491 ± 157 nM) compared to BR+PL (405 ± 188 nM, P=0.09), and was significantly elevated at 120 min in BR+BR (519 ± 152 nM) compared to BR+PL (400 ± 158 nM, P<0.05). Plasma [NO_2^-] fell significantly (by ~35%) from 120 min to post-TT in BR+BR (P<0.001), BR+PL (P<0.01) and PL+PL (P<0.05).

Pulmonary gas exchange during prolonged moderate-intensity exercise

$\dot{V}O_2$ measured at baseline was not different between conditions (P>0.05). There was a main effect of time (P<0.01) and an interaction effect (condition x time) for $\dot{V}O_2$ (P<0.05; Fig. 2A). *Post hoc* analyses revealed that the change in $\dot{V}O_2$ from 30 min to 120 min (P<0.05) was lower in BR+BR compared to PL+PL (P<0.05) and tended to be lower compared to BR+PL (P=0.07, Fig. 2B); there was no difference between BR+PL and PL+PL (P>0.05). At 120 min, $\dot{V}O_2$ was lower in BR+BR compared to PL+PL (P<0.01), and tended to be lower than BR+PL (P=0.08); (P>0.05). There was a main effect of time on RER (P<0.01), with RER declining from ~0.93 at 30 min to ~0.89 at 120 min, but no effect of condition and no interaction (P>0.05). Mean RER was not significantly different between conditions at 30 min (PL+PL: 0.93 ± 0.04 vs. BR+PL: 0.92 ± 0.04 vs. BR+BR: 0.93 ± 0.03), 60 min (PL+PL: 0.90 ± 0.03 vs. BR+PL: 0.89 ± 0.02 vs. BR+BR: 0.89 ± 0.03), 90 min (PL+PL: 0.91 ± 0.04 vs. BR+PL: 0.90 ± 0.06 vs. BR+BR: 0.91 ± 0.04) or 120 min (PL+PL: 0.90 ± 0.04 vs. BR+PL: 0.89 ± 0.03 vs. BR+BR: 0.90 ± 0.04). Similarly, there was a main effect of time (P<0.05) but no effect of condition or interaction for HR or minute ventilation. There was a main effect of time (P<0.05) but no effect of condition or interaction for blood [glucose] (P>0.05; Table 1). There was no effect of time or condition and no interaction effect for blood [lactate] (P>0.05; Table 1).

Muscle metabolic variables

There was a main effect of time (P<0.01) and a trend for an interaction effect (P=0.06) on muscle [glycogen] measured at baseline, 120 min, and post-TT (Fig. 3). At baseline, there was no significant difference in muscle [glycogen] between conditions (BR+BR: 383 ± 105 vs. BR+PL: 383 ± 144 vs. PL+PL: 412 ± 121 mmol·kg^{-1} DW, P>0.05). *Post hoc* tests revealed that in all conditions, muscle [glycogen] was significantly lower at 120 min compared to resting baseline (P<0.01), and at post-TT compared to 120 min (P<0.01). At 120 min, muscle [glycogen] tended to be greater in BR+BR (283 ± 103 mmol·kg^{-1} DW) compared to BR+PL (215 ± 102 mmol·kg^{-1} DW; P=0.08) and PL+PL (226 ± 90 mmol·kg^{-1} DW; P=0.08) There was no difference between conditions at post-TT (BR+BR: 161 ± 79 vs. BR+PL: 127 ± 65 vs. PL+PL: 132 ± 69 mmol·kg^{-1} DW, P>0.05). The absolute muscle [glycogen] at 120 min was inversely correlated with the absolute $\dot{V}O_2$ at 120 min (r = -0.71; P<0.01). There was a trend for a main effect of condition in the change in muscle [glycogen] from baseline to 120 min (P=0.09), where the ~28% decline in BR+BR was significantly less compared to the ~44% decline in PL+PL (P<0.05) and tended to be less than the ~44% decline in BR+PL (P=0.07). The change in muscle [glycogen] from 120 min to post-TT were not significantly different between conditions (P>0.05).

There was a main effect of time on muscle [PCr] (P<0.01; Fig 4A.), [ATP] (P<0.01; Fig. 4B) and [lactate] (P<0.01; Fig. 4C). Baseline muscle [PCr] and [ATP] were not different between conditions (P>0.05). There was no effect of condition and no interaction for muscle [PCr] or [ATP] (P>0.05). *Post hoc* tests revealed that in all conditions, muscle [PCr] declined from baseline to 120 min (P<0.05), and from 120 min to post-TT (P<0.01). The mean [PCr] tended to be greater in BR+BR compared to PL+PL (P=0.08) but there was no difference between BR+BR and BR+PL or between BR+PL and PL+PL (P>0.05). Muscle [ATP] declined significantly from 120 min to post-TT in BR+BR (P<0.01) and BR+PL (P<0.05) but not PL+PL. Muscle [lactate] was not significantly different between conditions at 120 min but, compared to 120 min, muscle [lactate] increased significantly post-TT in all conditions (P<0.01).

TT performance

TT completion time, mean $\dot{V}O_2$ and mean power output during the TT were not significantly different between conditions (all P>0.05, Fig. 5). Similarly, maximal HR, blood [lactate] and

blood [glucose] were not different between conditions (P>0.05; Table 1).

Discussion

This is the first study to investigate the effect of BR ingestion *during* exercise, in addition to pre-exercise, on the physiological responses to prolonged moderate-intensity exercise, and subsequent TT performance. The major novel findings of this study were that, compared to pre-exercise BR supplementation alone, a 'top-up' dose of BR consumed during exercise: 1) maintained the elevation of plasma [NO_2^-]; 2) better maintained the lowered O_2 cost of exercise; 3) tended to attenuate the fall in muscle [glycogen] over 2-h of moderate-intensity cycling; but, 4) did not alter simulated 4-km TT performance. Although TT performance was not significantly improved, our findings indicate that the ingestion of BR during prolonged exercise, in addition to short-term BR supplementation, may attenuate the rise in $\dot{V}O_2$ that typically develops during such exercise.

Plasma [NO_3^-] and [NO_2^-] during prolonged moderate-intensity exercise

It is well established that pre-exercise BR supplementation elevates resting plasma [NO_3^-] and [NO_2^-] (2, 32, 53), and the results of the present study were consistent with these previous reports. After reaching peak values at ~2-3 h following ingestion, plasma [NO_2^-] then declines with time (54, 59) as well as during exercise (32, 52). Assuming that plasma [NO_2^-] reflects the potential for O_2-independent NO synthesis in the vasculature and skeletal muscle (20, 54), a decline in plasma [NO_2^-] over time and during exercise may impact on the efficacy of BR supplementation in long-duration exercise bouts. Changes in plasma [NO_2^-] during exercise may reflect the utilization of NO_2^- to produce NO, conversion of NO_2^- to NO_3^- or other reactive nitrogen species, or transport to other body compartments including skeletal muscle (47). In the present study, when BR was only consumed pre-exercise (i.e., in the BR+PL condition), both plasma [NO_3^-] (by 16%; P<0.05) and [NO_2^-] (by 17%; P=0.07) declined from baseline to 120 min. However, when BR was also consumed at 60 min into exercise (i.e. in the BR+BR condition), plasma [NO_3^-] was increased above baseline by 41% at 90 min and 120 min and plasma [NO_2^-] was increased above baseline by 8% at 120 min (Fig. 1). Plasma [NO_2^-] was therefore significantly greater at 120 min in BR+BR compared to BR+PL. These results indicate

that, following pre-exercise BR supplementation, prolonged moderate-intensity exercise can lead to a substantial reduction in plasma [NO_3^-] and [NO_2^-], but that this decline can be negated by BR ingestion during exercise. The results of the present study demonstrate, for the first time, that BR ingestion during exercise can lead to relatively rapid changes in plasma [NO_3^-] and [NO_2^-]. The pharmacodynamics and pharmacokinetics of plasma [NO_3^-] and [NO_2^-] following dietary NO_3^- ingestion have been described at rest (54, 59) but not during exercise, and further research is warranted to determine whether, and to what extent, the NO_3^- - NO_2^- - NO pathway is impacted by exercise and its sequelae (including, for example, changes in metabolic rate, core and oral temperature, distribution of cardiac output, and salivary flow rate).

Influence of BR on metabolic responses during prolonged moderate-intensity exercise

In the present study, $\dot{V}O_2$ was not significantly different between conditions until 120 min of exercise, at which point it was lower in BR+BR compared to BR+PL and PL+PL. The increase in $\dot{V}O_2$ as exercise progressed in BR+PL and PL+PL was therefore attenuated in BR+BR (Fig. 2). An increasing O_2 cost of maintaining the same work rate during long-duration exercise may be related to an increased O_2 cost of mitochondrial ATP production and/or an increased ATP cost of force production and could reflect changes over time in substrate utilization, mitochondrial function and motor unit recruitment (29).

Dietary NO_3^- supplementation has been reported to reduce the O_2 cost of exercise in many (1, 2, 36, 37, 38, 53, 56), though not all (6, 52) studies, but the mechanistic basis for this effect is not fully resolved. Larsen et al. (37) reported that $NaNO_3$ supplementation enhanced mitochondrial P/O ratio *in vitro* and found that this was significantly correlated with the reduction in the O_2 cost of cycling *in vivo*. In contrast, Whitfield et al. (56) reported that, while BR reduced the O_2 cost of exercise, it did not alter indices of mitochondrial efficiency. Another explanation for a lower O_2 cost of exercise following NO_3^- supplementation is a reduced ATP cost of muscle contraction. Consistent with this, it has been reported, using ^{31}P magnetic resonance spectroscopy, that muscle PCr depletion is reduced during exercise following BR supplementation (2, 18). In the present study, muscle [PCr] determined from biopsy samples tended to be higher at 120 min of moderate-intensity exercise in BR+BR compared to PL+PL (P=0.08). Given that the depletion of PCr during exercise reflects the energy cost of contraction

(31), these results suggest that BR supplementation may have reduced the metabolic cost of force production. For the same mitochondrial P/O, a lower ATP requirement at the same power output would dictate a lower $\dot{V}O_2$ (58).

It has been reported in rodents (24, 26) and in humans (13, 22, 55), that muscle contractile force is increased following NO_3^- supplementation. However, the mechanism responsible for this effect remains to be elucidated given that modifications to key contractile proteins related to intracellular Ca^{2+} handling have been observed in rodents (24) but not humans (55). Whitfield et al. (56) reported an increased emission of hydrogen peroxide following BR supplementation, suggesting a potential role for redox signaling in augmenting contractile efficiency (17). Moreover, at least in rodents, BR supplementation preferentially increases blood flow to (15), and increases microvascular O_2 pressure surrounding (16), type II muscle fibers, which could contribute to enhanced contractile function. It is possible that, collectively, these effects lower the O_2 cost of long-duration exercise by reducing or delaying the recruitment of motor units that are higher in the recruitment hierarchy and that may be less efficient (4, 29).

In the present study, we found that muscle glycogen declined by ~28% over 120 min of exercise in BR+BR, compared to ~44% decline in both BR+PL and PL+PL (Fig. 3). This tendency for muscle glycogen sparing could be reflective of a reduction in overall metabolic demand (from mitochondrial and/or contractile efficiency improvements), and therefore a lower absolute requirement for carbohydrate oxidation. This is supported by the existence of a significant negative correlation between the absolute $\dot{V}O_2$ and muscle [glycogen] measured at 120 min of exercise. It has been reported that muscle glycogen content is positively correlated with sarcoplasmic reticulum Ca^{2+} release rate, which may affect skeletal muscle contractile function (43). The tendency for muscle glycogen sparing in the BR+BR condition of the present study suggests a possible new mechanism by which dietary NO_3^- might enhance efficiency during long-duration exercise, with implications for exercise performance in such events, and is worthy of further investigation.

There was no difference in RER or blood [glucose] between conditions in the present study. In some previous studies, RER has been observed to be slightly (1, 37) or significantly (59) higher

following NO_3^- compared to PL supplementation, although most studies have not found significant differences in RER (2, 6, 12, 53, 56). Wylie et al. (60) reported a lower blood [glucose] during high-intensity intermittent exercise following BR compared to PL supplementation and suggested that this may be due to an increased skeletal muscle glucose uptake. It is possible that this effect is intensity-dependent given that other studies have reported no effect of BR on glucose handling during moderate-intensity exercise (6, 12). Given that we did not observe differences between conditions in blood [glucose] or RER, the sparing of muscle glycogen in BR+BR would appear to be related to a reduced overall muscle metabolic demand as reflected in the lower O_2 cost of exercise. Alternatively, the tendency for muscle [PCr] to be somewhat better maintained during exercise in BR+BR compared to PL+PL, which is consistent with the lower $\dot{V}O_2$ in BR+BR (1), indicates that muscle energy charge may have been higher when BR was ingested such that the stimulation of glycogenolysis was reduced (23). In contrast to our findings, Betteridge et al. (6) reported no effect of pre-exercise BR supplementation on muscle [glycogen] (or $\dot{V}O_2$) during 60 min of moderate-intensity cycling. The reason for this difference is unclear but, in addition to the longer exercise duration and the inclusion of BR ingestion *during* as well as pre-exercise, our subjects consumed 12.4 mmol NO_3^- per day for 3 days whereas the subjects in the study of Betteridge et al. (6) consumed an acute 8 mmol dose of NO_3^- 2.5 hours pre-exercise. The dose and duration of NO_3^- supplementation is one factor that is likely to influence efficacy (27) since it may influence NO_3^- and NO_2^- storage in skeletal muscle as well as blood (44, 47, 61). Recent studies indicate that rat (47) and human (44) skeletal muscle has high [NO_3^-] relative to the blood, that the muscle NO_3^- store decreases substantially during exercise in rats (46) and that muscle [NO_3^-] can be modulated by dietary NO_3^- content (19, 44).

Influence of BR on metabolic responses and performance during TT exercise

Plasma [NO_2^-] declined markedly during the TT (Fig. 1B). This greater rate of decline in plasma [NO_2^-] from 120 min to post-TT is in contrast to the more gradual decline in plasma [NO_2^-] observed from baseline to 120 min in the BR+PL condition, which may suggest an exercise-intensity dependency of plasma [NO_2^-] dynamics. Indeed, previous research has reported significant reductions in plasma [NO_2^-] following high-intensity exercise of shorter duration (32, 50, 52, 60). It is possible that the greater degree of hypoxia and acidosis that would be expected to develop in skeletal muscle during high-intensity exercise, such as TT, compared to moderate-

intensity exercise, facilitates or dictates a greater reduction of NO_2^- to NO (42). Moreover, a greater recruitment of type II muscle fibers, which have a lower microvascular O_2 pressure compared to type I fibers (16), during higher intensity exercise may also result in a greater reduction of NO_2^- to NO.

It is perhaps surprising that, despite evidence that the metabolic cost of the initial long-duration exercise bout was reduced in BR+BR (i.e. lower end-exercise $\dot{V}O_2$ and trends for a sparing of muscle [PCr] and [glycogen]), subsequent simulated 4-km TT performance was not different between the three conditions. Our results are consistent, in part, with those of Christensen et al. (12) who reported that performance in a 400-kcal cycle TT, which began after a 2-h moderate-intensity 'pre-load', was not significantly altered by BR compared to PL in elite cyclists (18.3 vs. 18.6 min, respectively). The influence of NO_3^- supplementation on TT performance is controversial (10, 11, 12, 34, 35, 41, 45, 50, 57) and whether or not NO_3^- ingestion is performance-enhancing appears to depend on factors such as subject training status, the dose and duration of NO_3^- supplementation, and the intensity, duration, and modality of exercise (27). Positive effects of NO_3^- supplementation are more likely to be exhibited in tests of exercise capacity rather than TT efforts (40). When observed, the ergogenic effect of NO_3^- supplementation on TT performance, while relatively small (~2%; 10, 35, 45, 50), may be meaningful in terms of competitive performance. However, as is the case for the majority of putative nutritional ergogenic aids, the magnitude of this effect is within the sensitivity of most laboratory tests (30) and may be obscured by intrinsic variability in performance as well as subject motivation. It is possible that the apparently positive effects of BR on some physiological variables during prolonged exercise that we found were simply too small to impact on TT performance. However, it is also possible that a greater exercise pre-load, resulting in greater glycogen depletion, and/or the inclusion of a longer duration TT, or a higher-sensitivity test of exercise capacity (40), might have enabled the detection of a beneficial effects of BR on exercise performance. Administering the top-up dose of BR earlier than 60 min and/or increasing the duration of the moderate-intensity exercise bout might have enabled plasma [NO_2^-] to reach a higher value prior to the TT and perhaps resulted in a performance benefit.

Experimental Considerations

Although there was no significant difference in muscle [glycogen] between conditions at 120 min of exercise, the decline in muscle [glycogen] between resting baseline and 120 min was significantly attenuated in BR+BR compared to PL+PL. The changes in muscle [PCr] and $\dot{V}O_2$ during exercise were also significantly smaller in BR+BR compared to PL+PL. Although statistical significance was not attained, the changes in muscle [glycogen], muscle [PCr] and $\dot{V}O_2$ over time also tended to be smaller in BR+BR compared to BR+PL. The significant inverse correlation across conditions between the absolute $\dot{V}O_2$ and the absolute muscle [glycogen] at 120 min lends confidence to the interpretation that the sparing of muscle glycogen utilization was related to changes in oxidative metabolic demand following BR ingestion. However, it should be acknowledged that the extent of the sparing of muscle glycogen utilization between baseline and 120 min in BR+BR (~100 $mmol \cdot kg^{-1}$ DW) compared to PL+PL (~186 $mmol \cdot kg^{-1}$ DW) and BR+PL (~168 $mmol \cdot kg^{-1}$ DW) was much greater than would be expected based on the comparatively small differences in $\dot{V}O_2$ and [PCr] we measured. There is the possibility, therefore, that the differences in muscle [glycogen] may have been overestimated in the present study. Additional studies are required to investigate the influence of pre- and in-exercise NO_3^- supplementation on changes in muscle [glycogen] in a larger sample and in trained as well as untrained participants.

If a glycogen sparing effect of BR ingestion during exercise can be confirmed, this may have important implications not just for single long-endurance events but also for multi-day endurance events such as cycle tours and expeditions, wherein muscle [glycogen] may fall progressively over consecutive days of exercise. It is also possible that consuming BR during arduous endurance training programs might attenuate fatigue development related to glycogen availability and enable additional training to be completed.

Conclusion

A single dose of BR ingested *during* exercise in addition to pre-exercise BR supplementation increased plasma [NO_3^-] and preserved an elevated plasma [NO_2^-] during prolonged moderate-intensity exercise. This was associated with an attenuated upward drift in the O_2 cost of exercise, and a tendency for a sparing of muscle glycogen and PCr, effects which might be expected to predispose to enhanced exercise tolerance. In conclusion, BR supplementation *during* exercise

516 can modulate plasma [NO_3^-] and [NO_2^-] dynamics and attenuate the progressive rise in $\dot{V}O_2$
517 during prolonged moderate-intensity exercise. However, under the conditions of the present
518 study, subsequent TT performance was not enhanced by BR supplementation.
519

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Figure Legends

Figure 1. Mean ± SE plasma nitrate (panel A) and nitrite (panel B) concentrations over 120 min of moderate-intensity cycle exercise and a subsequent 4-km TT following: PL+PL: placebo consumed before and during exercise (solid triangle and dotted line); BR+PL: NO_3^- -rich beetroot juice consumed before and placebo consumed during exercise (open circle and solid line); and BR+BR: NO_3^- -rich beetroot juice before and during exercise (solid circle and solid line), (n = 9). * = significantly different from PL+PL, ** = BR+BR significantly different from BR+PL, # = significantly different from 120 min to end of TT in BR+BR, † = significantly different from 60 min to end of TT in BR+PL, ‡ = significantly different from 90 min to TT in PL+PL.

Figure 2. Mean ± SE O_2 uptake over 120 min of moderate-intensity cycle exercise (panel A) and the change in O_2 uptake from 30 min to 120 min (panel B) following PL+PL, PL+BR and BR+BR. * = significantly different in BR+BR compared to PL+PL.

Figure 3. Mean ± SE muscle [glycogen] at rest (PRE), after 120 min moderate-intensity exercise (POST), and after the 4-km time trial (TT), (n = 9). * = significantly different from PRE to POST, ** = significantly different from POST to TT. There were no significant differences between the three conditions at any discrete time point but the change in muscle [glycogen] was significantly less in BR+BR compared to PL+PL ($P<0.05$; see text for details).

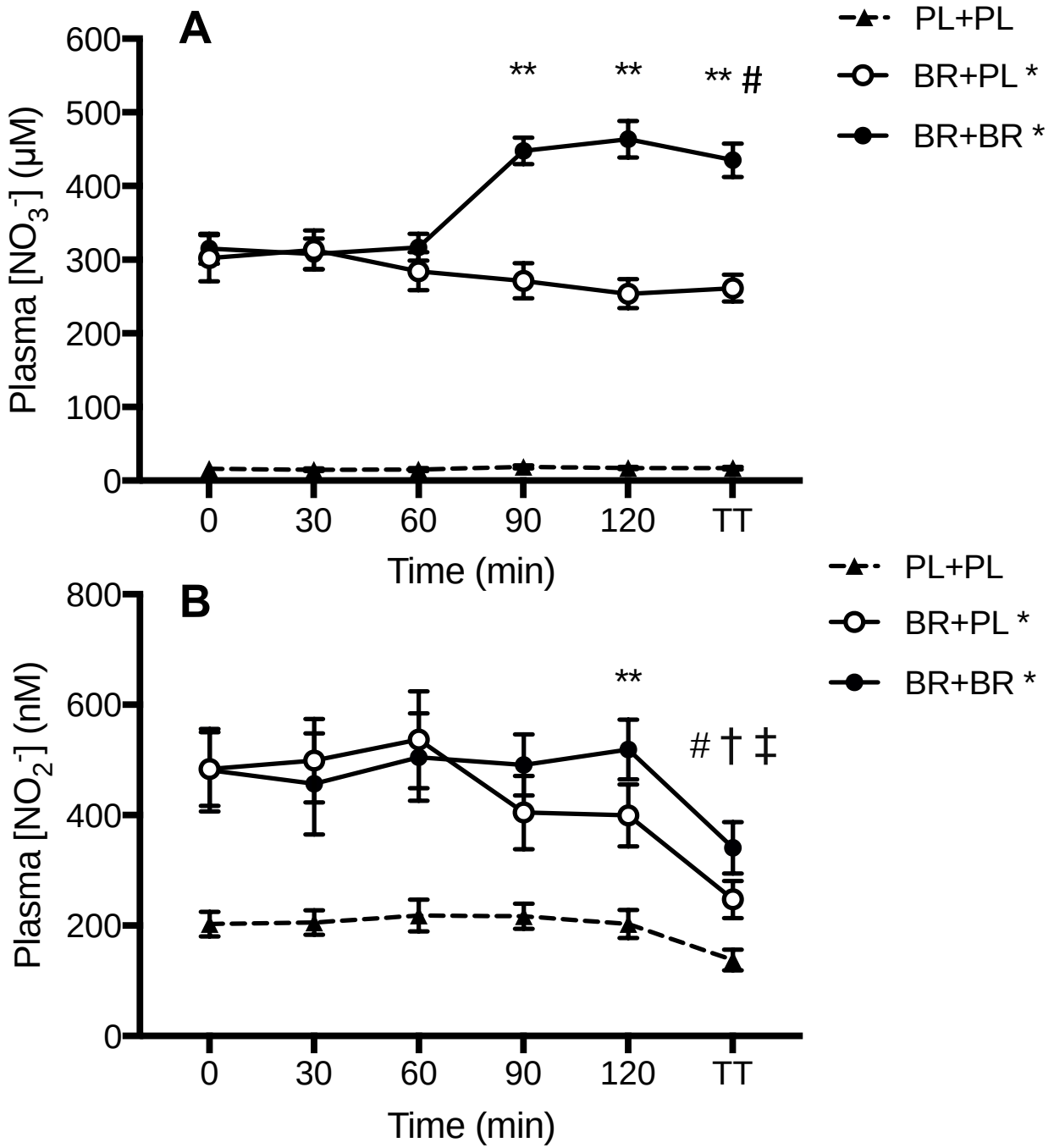
Figure 4. Mean ± SE muscle [PCr] (panel A), [ATP] (panel b), and [lactate] (panel C) at rest (PRE), after 120 min moderate-intensity exercise (POST), and after the 4-km time trial (TT), (n = 9). * = significantly different from PRE to POST, ** = significantly different from POST to TT.

Figure 5. Mean ± SE O_2 uptake (panel A), power output (panel B), and completion time (panel C) over the 4-km time trial in PL+PL (black bars), BR+PL (grey bars) and BR+BR (white bars). Completion times for individual subjects shown in grey lines.

Table 1. Blood [glucose] and [lactate] during 2-h moderate-intensity exercise and at the end of a simulated 4-km time trial.

	0	30	60	90	120	TT
Blood [glucose]						
PL+PL	4.2 ± 0.8	4.0 ± 0.3	4.3 ± 0.5	4.3 ± 0.4	4.0 ± 0.6	4.3 ± 0.8
BR+PL	3.8 ± 0.9	3.8 ± 0.3	4.0 ± 0.5	4.1 ± 0.6	4.3 ± 0.6	4.7 ± 1.0
BR+BR	3.9 ± 0.6	3.7 ± 0.6	3.9 ± 0.5	4.2 ± 0.4	4.3 ± 0.5	4.3 ± 0.4
Blood [lactate]						
PL+PL	1.1 ± 0.3	1.1 ± 0.2	1.0 ± 0.3	1.1 ± 0.3	1.2 ± 0.4	8.0 ± 2.3*
BR+PL	1.1 ± 0.4	1.1 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.3 ± 0.4	8.0 ± 2.2*
BR+BR	1.1 ± 0.3	1.1 ± 0.5	1.1 ± 0.4	1.2 ± 0.4	1.3 ± 0.4	7.2 ± 2.0*

Values are mean ± SD (n = 9). BR+BR, dietary nitrate supplementation before and during exercise; BR+PL, dietary nitrate supplementation before and placebo during exercise; PL+PL, placebo before and during exercise. *Significantly different from 120 min (P<0.001).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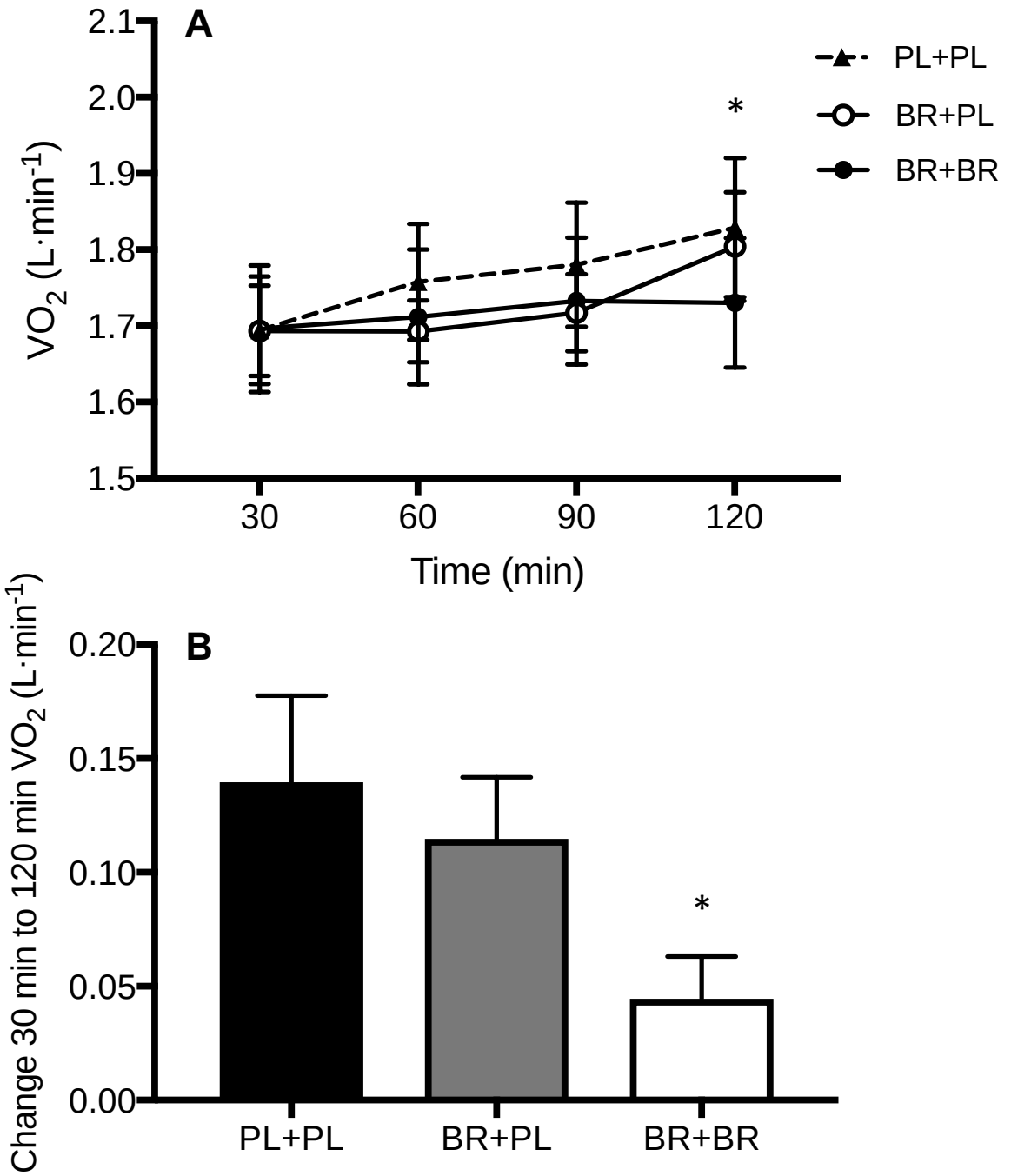
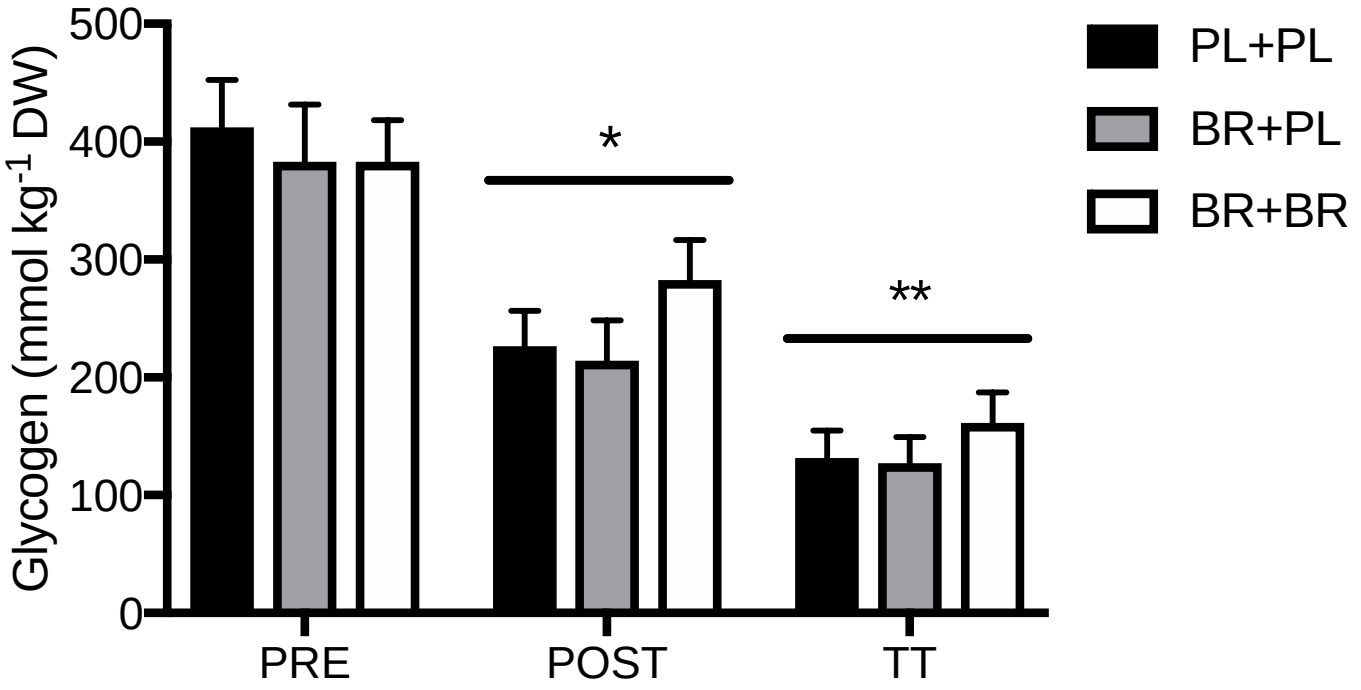
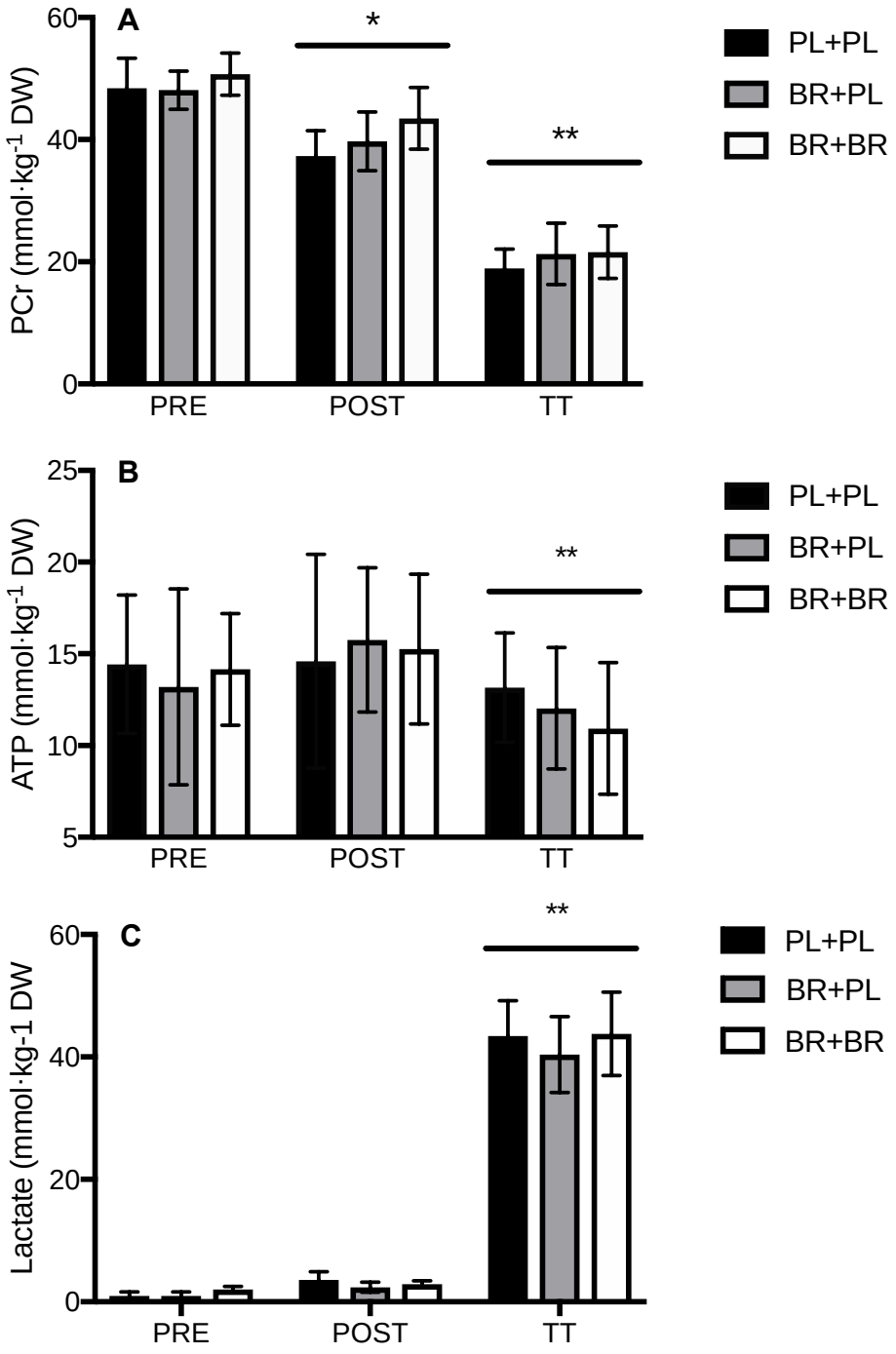
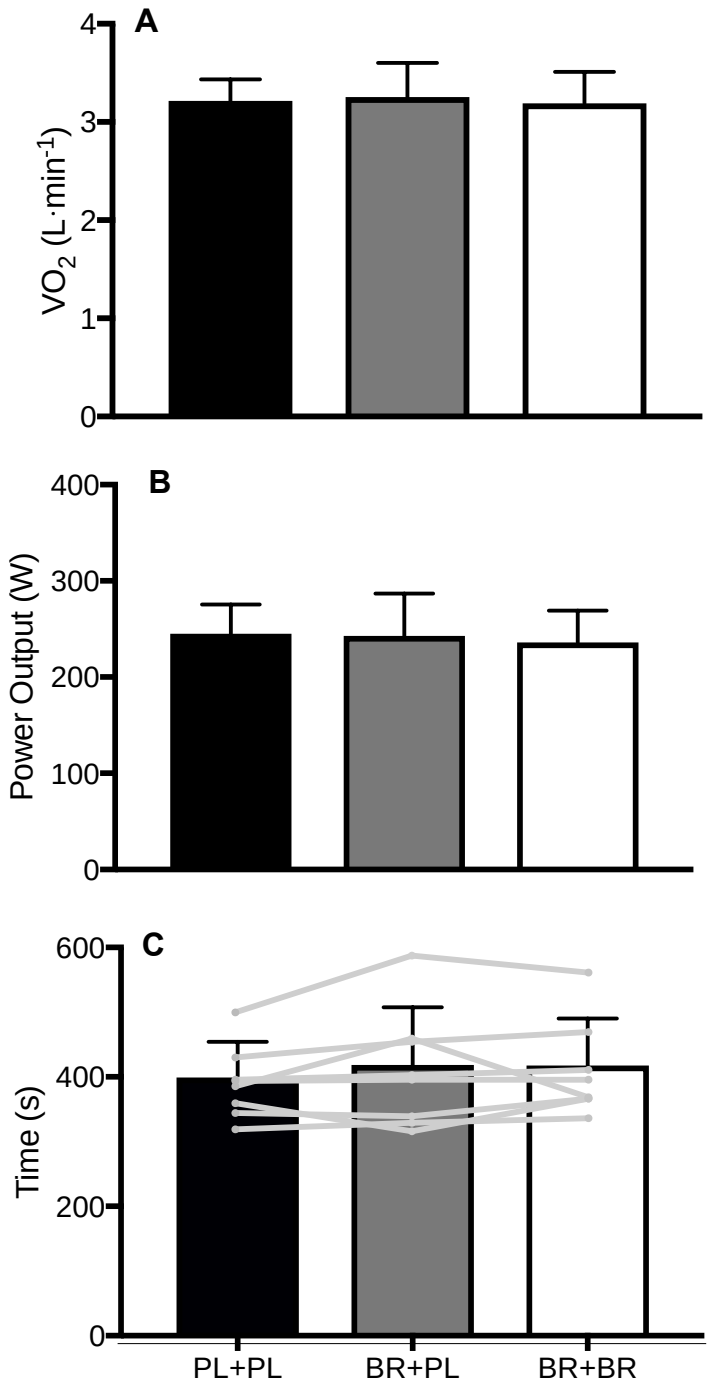
PL+PL
BR+PL *
BR+BR *


A
2.1
2.0
1.9
1.8
1.7
1.6
1.5
VO2 (L·min-1)
30
60
90
120
Time (min)
*
PL+PL
BR+PL
BR+BR
B
0.20
0.15
0.10
0.05
0.00
Change 30 min to 120 min VO2 (L·min-1)
PL+PL
BR+PL
BR+BR
*


Glycogen (mmol kg-1 DW)
500
400
300
200
100
0
PRE
POST
TT
*
**
PL+PL
BR+PL
BR+BR


A
PCr (mmol·kg-1 DW)
60
40
20
0
*
**
PRE
POST
TT
PL+PL
BR+PL
BR+BR
B
ATP (mmol·kg-1 DW)
25
20
15
10
5
**
PRE
POST
TT
PL+PL
BR+PL
BR+BR
C
Lactate (mmol·kg-1 DW
60
40
20
0
**
PRE
POST
TT
PL+PL
BR+PL
BR+BR


A
4
3
2
1
0
VO_2 ($L \cdot min^{-1}$)
B
400
300
200
100
0
Power Output (W)
C
600
400
200
0
Time (s)
PL+PL
BR+PL
BR+BR