Beetroot Juice Does Not Enhance Supramaximal Intermittent Exercise Performance in Elite Endurance Athletes.

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Beetroot juice does not enhance supramaximal intermittent exercise performance in elite endurance athletes

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ABSTRACT

Objective: Nitrate (NO_3^-)-rich beetroot juice (BR) is recognized as an ergogenic supplementation that improves exercise tolerance during submaximal to maximal intensity exercise in recreational and competitive athletes. A recent study has investigated the effectiveness of BR on exercise performance during supramaximal intensity intermittent exercise (SIE) in Olympic-level track cyclists, but studies conducted in elite endurance athletes are scarce. The present study aimed to determine whether BR supplementation enhances the tolerance to SIE in elite endurance athletes.

Methods: Eleven elite endurance athletes (age: 21.7±3.7 years, maximal oxygen uptake ($\dot{V}O_{2max}$): 71.1±5.2 $mL \cdot kg^{-1} \cdot min^{-1}$) performed a SIE test until exhaustion following either a 3-day BR supplementation (340 mg/day) or a placebo (PL) supplementation (<2,5 mg/day) in a randomized, single blind, placebo-controlled and crossover study. The exercise test consisted of 15-s cycling exercise bouts at 170% of the maximal aerobic power interspersed with 30-s passive recovery periods. Gas exchange was measured during SIE tests as local muscle O_2 delivery and extraction were assessed by near infrared spectroscopy.

Results: The number of repetitions completed was not significantly different between BR (13.9±4.0 reps) and PL conditions (14.2±4.5 reps). BR supplementation did not affect oxygen uptake ($\dot{V}O_2$) during SIE tests (BR: 3378.5±681.8 $mL \cdot min^{-1}$, PL: 3466.1±505.3 $mL \cdot min^{-1}$). No significant change in the areas under curves was found for local muscle total hemoglobin

(BR: 6816.9±1463.1 arbitrary units (a.u.), PL: 6771.5±3004.5 a.u.) and deoxygenated hemoglobin (BR: 6619.7±875.8 a.u., PL: 6332.7±1336.8 a.u.) during time-matched work+recovery periods from SIE tests following BR supplementation.

Conclusion: BR supplementation does not enhance the tolerance to supramaximal intensity intermittent exercise in elite endurance athletes and does affect neither $\dot{V}O_2$ nor local muscle O_2 delivery and extraction.

Keywords: dietary nitrate; high aerobic fitness; exercise tolerance; endurance athletes; intermittent exercise

Abbreviations: AUC: Area Under the Curve; ATP: adenosine triphosphate; BR: beetroot juice; DBP: diastolic blood pressure; Hb: hemoglobin; HbO_2: muscle oxygenated hemoglobin; HHb: muscle deoxygenated hemoglobin; NIRS: near infrared spectroscopy; Mb: myoglobin; NO: nitric oxide; NO_3^-: nitrate; NO_2^-: nitrite; NO_x: nitrate+nitrite; PL: placebo; RER: respiratory exchange ratio; SBP: systolic blood pressure; SIE: supramaximal intensity intermittent exercise; THb: muscle total hemoglobin; TM: time-matched data; $\dot{V}E$: minute ventilation; $\dot{V}CO_2$: carbon dioxide output; $\dot{V}O_2$: oxygen uptake; $\dot{V}O_{2max}$: maximal oxygen uptake.

Introduction

Nitric oxide (NO) is a ubiquitous signaling molecule that possesses the ability to improve vascular function, mitochondrial efficiency and respiration, glucose homeostasis and muscle contractility (1). NO is enzymatically produced by the nitric oxide synthase (NOS) that is dependent on L-arginine and oxygen availability, and thus becomes limited in hypoxia (2). However, a relevant alternative source of NO has emerged through the consumption of nitrate (NO_3^-)-rich vegetables such as lettuce, spinach and beetroot (3). Dietary NO_3^- are absorbed from the small intestine, and after an entero-salivary recirculation and concentration into the saliva, are converted into nitrite (NO_2^-) by oral NO_3^- reductase bacteria (3). The swallowed salivary NO_2^- are either reduced to NO and other nitrogen species in the acidic stomach or absorbed from the intestine, and thus increase endogenous circulating NO pool (3).

The consumption of dietary NO_3^- is recognized as a nutritional aid to improve tolerance to submaximal to maximal intensity exercise (4). The increase in exercise tolerance has been mostly attributed to decreased ATP cost of muscle contraction (5), and improved oxidative efficiency (6). Some studies reported that NO_3^--rich beetroot juice (BR) supplementation did not change any indices of mitochondrial coupling and respiratory efficiency (7) and did not alter in vivo muscle oxidative efficiency in recreationally active males (8). In these studies, the authors found decreased whole-body and muscle $\dot{V}O_2$ during submaximal cycling and isometric handgrip exercise, respectively (7,8). Thus, BR supplementation may act through divergent mechanisms than sodium nitrate (9) by improving skeletal muscle contractile efficiency (7,10) and/or local tissue perfusion (8,11). However, a number of studies did not show any change in exercise performance during time-trial tests following NO_3^- supplementation (12–16), particularly in elite endurance athletes. In situations in which muscle O_2 supply may be restricted, Shannon et al showed that well-trained individuals can

also experience benefits from dietary NO_3^- supplementation during high-intensity exercise (17).

Moreover, mixed findings have especially been reported regarding the effect of NO_3^- supplementation on supramaximal intensity exercise (SIE) performance (12,18–21). This type of exercise is known to recruit a high proportion of type II, predominantly glycolytic, muscle fibers (22) that are characterized by lower O_2 availability than type I, highly oxidative, muscle fibers (23). As such, the reduction of NO_3^- to NO may be enhanced in type II muscles fibers given that it preferentially occurs in both acidic and hypoxic conditions (3). Running time over a single 180m sprint was shown to be improved (18), and we also reported improved tolerance to the repetition of supramaximal intensity bouts of exercise (≥170% power output at $\dot{V}O_{2max}$) in moderately fit subjects ($\dot{V}O_{2max}$ between 40 and 55 $mL \cdot kg^{-1} \cdot min^{-1}$) following dietary NO_3^- supplementation (19). However, in contrast to findings during submaximal intensity exercise, enhanced tolerance to SIE was not related to any decrease in oxygen uptake ($\dot{V}O_2$) or in anaerobic energy production (19,20). Apart from improving exercise tolerance, we found an increase in local muscle total hemoglobin during SIE after 3-day beetroot juice (BR) supplementation (19). By better maintaining O_2 delivery to the working muscle, NO_3^- supplementation likely delays the depletion of muscle phosphocreatine (PCr) and the accumulation of adenosine diphosphate (ADP) and phosphate ion (Pi) metabolites (24), and promotes PCr resynthesis, dependent on oxidative phosphorylation (25). In combination, these effects can attenuate the development of muscle fatigue and contribute to sustain power output between supramaximal intensity exercise bouts. To date, no investigation was assessed local muscle O_2 delivery and extraction in elite endurance athletes conducting SIE tests after BR supplementation.

There is mounting evidence suggesting that the level of aerobic fitness of the athletes modulates the effects of NO_3^- supplementation on performance (26,27). Porcelli et al.

demonstrated that the magnitude of increase in exercise performance during a 3000-meter running race time trial was inversely correlated to $\dot{V}O_{2max}$, with no benefit for athletes with $\dot{V}O_{2max}$ above 60 $mL \cdot kg^{-1} \cdot min^{-1}$. Conversely, most studies that reported enhanced performance were conducted in non-endurance athletes or moderately trained subjects ($\dot{V}O_{2max}$ ~50 $mL \cdot kg^{-1} \cdot min^{-1}$) (5,19,28–33). The reasons for a lower effectiveness are still unclear, but may be related to characteristics of athletes with high $\dot{V}O_{2max}$, such as already high synthesis of NO through the enzymatic pathway (34), and a high proportion of type I muscle fibers, which are less sensitive to an increase in NO bioavailability than type II muscle fibers (23).

Nevertheless, dietary NO_3^- supplementation may be of great interest to endurance athletes that will engage in high-intensity training and/or events. Although mean exercise intensity is submaximal in endurance sports, the time spent beyond power output corresponding to $\dot{V}O_{2max}$ can exceed 15% of the total race duration in activities such as cycling or triathlon (35). Moreover, the time spent at these supramaximal intensities is distributed into short bouts of exercise across the duration of the event (36). The exercise performance may be impacted by the capacity to maintain efforts in successive high-intensity exercise intervals over a long time period (37). Furthermore, middle-distance athletes (800 to 5000-10 000m), who are also characterized by high $\dot{V}O_{2max}$, select pacing strategies during running competition events so that they regularly run at speeds well above the $\dot{V}O_{2max}$ intensity (38). Interval training protocols at supramaximal intensity allow to maximally strain aerobic and anaerobic metabolism (39) and to enhance physiological determinants of exercise performance even in athletes that have already elicited cardiorespiratory adaptations to chronic exercise training (38,40,41).

Our purpose was therefore to determine whether dietary NO_3^- supplementation enhances tolerance to supramaximal intensity intermittent exercise (SIE) in elite endurance athletes

having high aerobic fitness ($\dot{V}O_{2max}$ > 65 mL·kg^{-1}·min^{-1}). We hypothesized that dietary NO_3^- supplementation would increase plasma NO_x levels without improving tolerance to exercise.

Material and Methods

Population

Subjects' characteristics are presented in Table 1. Subjects were contacted in track and field and triathlon clubs and were all engaged in intense endurance exercise training and competitive running or triathlon. Eleven subjects were initially recruited for the study and two subjects were excluded after completing the preliminary visit because they did not meet the criteria for inclusion ($\dot{V}O_{2max}$ > 65 mL·kg^{-1}·min^{-1}). Subjects were not taking any nutritional supplement or medication known to affect NO metabolism. All subjects were informed about the study protocol, its potential risks and benefits, and gave written consent to participate in this study. Subjects were concealed to the true aim of study and were told that the purpose of trials was to compare the ergogenic effects of two beverages. The protocol of the study complied with the Declaration of Helsinki and was approved by the Institutional Ethics Committee of the Haute Ecole Provinciale du Hainaut.

- Table 1 here -

Incremental maximal exercise test

At least one week before the first experimental visit, subjects performed an incremental exercise test until volitional exhaustion to ensure their eligibility for the study. $\dot{V}O_{2max}$ and Power Output at $\dot{V}O_{2max}$ were measured during a graded exercise test until volitional exhaustion performed on a mechanically braked cycle ergometer (894E, MONARK EXERCISE TM, Sweden). The test was initiated at 100 W for 3 min and increased by 35 W increments every 2 min. Subjects were invited to maintain a pedaling frequency of 80 rpm

and were strongly encouraged throughout the test to perform a maximal effort. We considered that $\dot{V}O_{2max}$ was achieved when at least three of the following criteria were established: volitional exhaustion, theoretical maximal heart rate (220-age ±10 beats·min^{-1}), Respiratory Exchange ratio (RER, $\dot{V}CO_2/\dot{V}O_2$) above 1.1, and a $\dot{V}O_2$ plateau (≤ 2.1 mL·kg^{-1}·min^{-1}) between the last two stages. Maximal Aerobic Power Output ($W_{aerobic\ max}$) was determined as the sum of the power output during the last stage fully completed (W_{final}) and the work rate increment (WR) multiplied by the fraction of time spent (t) in the final unfinished stage as follows:

$$W_{aerobic\ max} = W_{final} + \left(\frac{t}{120} \times WR\right)$$

Dietary supplementation conditions

Subjects were supplemented either in dietary NO_3^- with beetroot juice with an average nitrate content of 680 mg·L^{-1} or apple-black currant juice used as a placebo had a nitrate content <5 mg·L^{-1} in a randomized, single blind, placebo controlled, crossover study. We used a random number table to assign the order of supplementation conditions to each participant. The experimenter engaged in the randomization process did not take part in the experimental visits. Because the placebo juice differs in taste, smell and appearance compared to beetroot juice supplementation, the purpose of the study was communicated to the participants as the comparison of exercise performance and physiological responses after the consumption of two vegetable beverages. There was a minor difference in the energy provided by BR (185 kcal) and placebo (225 kcal). Even although beetroot is rich in antioxidants, polyphenols and flavonoids, nitrate appears to be the active compounds responsible for the physiologic effects following beetroot juice supplementation. Lansley et al. found no reduction in $\dot{V}O_2$ during moderate- and severe-intensity exercise in healthy, recreationally active subjects following

nitrate-depleted beetroot juice using an ion-exchange resin that selectively removes NO_3^- ions (42). The two beverages were purchased from PAJOTTENLANDER TM (Belgium). Drinks were distributed by an independent technician, not involved in the process of exercise testing. In the morning of the two days preceding each experimental visit, subjects ingested either 500mL of beetroot juice or 500mL of apple-black currant juice. On the day of each experimental visit, subjects were instructed to ingest 500 mL of BR or placebo 2 h before the time of their appointment at the laboratory so that the start of SIE tests corresponded to plasma nitrite peak. Pharmacokinetic data suggest that plasma nitrite will peak 2.5–3 h after ingestion of a single dose of BR (43). Subjects were instructed to refrain from caffeine and alcohol 6 and 24 h before the experimental visits. Participants were not asked to refrain consuming NO_3^--rich food products, but they were instructed to replicate their food intake over the supplementation days between the two conditions and to complete a daily food diary for each supplementation period. In addition, subjects were finally instructed not to brush their teeth or to use mouthwash for 3 h after each BR or placebo intake (44).

Supramaximal intermittent exercise tests

After the preliminary visit, subjects performed cycling SIE tests during the two sessions 180-min after the last dose of either ingested dietary nitrate or placebo supplementation. The exercise tests were completed on the same cycle ergometer than during the preliminary visit and separated by at least 2 weeks. Subjects warmed up for 5 min at 50% of maximal aerobic power output measured during the preliminary visit and were instructed to maintain a cycling cadence of 70 rpm. After a 2 min recovery period, subjects started the test consisting of work periods repetitions composed as follows: 15-sec cycling bout at 170% of maximal aerobic power output interspersed with 30-sec passive recovery periods. Before the test, subjects were

instructed to reach as quickly as possible the targeted pedaling frequency of 90 rpm during each 15-s cycling exercise bouts. Subjects started pedaling after a 3 sec countdown by one of the experimenters, when they reached 50 rpm the resistance corresponding to 170% of maximal aerobic power output was automatically applied with the Monark Software (Monark Anaerobic Test, MONARK EXERCISE TM, Sweden). Subjects recovered passively between each work period. The test was stopped when subjects indicated volitional exhaustion or when they were no longer able to maintain a pedaling frequency above 87 rpm during the 5 last seconds of a 15-sec cycling bout.

Gas exchange measurement

$\dot{V}O_2$, CO_2 output ($\dot{V}CO_2$), Respiratory Exchange Ratio (RER: $\dot{V}CO_2/\dot{V}O_2$), Minute Ventilation ($\dot{V}E$) were measured breath-by-breath through a gas exchange measurement system ($K4b_2$, COSMED TM, Italy) during the graded maximal exercise test and SIE tests. Before each test, the gas analyzer was calibrated with ambient air and a gas mixture of known concentration (O_2: 16%, CO_2: 5%), and the turbine flowmeter was calibrated with a 3 L syringe. Gas exchange data were recorded on a computer for later analysis. Heart rate was continuously recorded with a heart rate monitor (S810, POLAR TM, Finland).

Local muscle parameters

The changes in local muscle microvascular oxygenation, deoxygenation and total-hemoglobin volume of the vastus lateralis muscle were continuously measured during SIE tests by NIRS (Oxymon, ARTINIS MEDICAL SYSTEM TM, Netherlands). Briefly, the NIRS system measures the change in light absorption by hemoglobin (Hb) and myoglobin (Mb), which is related to Hb and Mb O_2 saturation. The two wavelengths emitting (780 and 850nm) and

receiving optodes were spaced out of 4 cm and were placed at midway between the lateral condyle of the knee and the greater trochanter of the femur. Optodes were taped to the skin with adhesive strapping, covered with a dense black cloth to minimize exogenous light contamination, and wrapped with an elastic bandage to avoid any movement during the exercise tests. The location of the optodes was marked with a permanent pen during the first experimental visit to place them at the same location during the second experimental visit. NIRS signal acquisition was performed at 10 Hz with the Oxysoft software (Oxysoft, ARTINIS MEDICAL SYSTEM TM, Netherlands), and data were exported at 1 Hz for later analysis. Three indexes of muscle oxy-, deoxy-, and total-hemoglobin volume were derived from NIRS measurements of the vastus lateralis muscle: changes from the resting baseline in oxygenated hemoglobin (HbO_2) and deoxygenated hemoglobin (HHb) were respectively estimates of muscle oxygenation and fractional O_2 extraction; total hemoglobin (THb) was calculated as the sum of HbO_2 and HHb and was an estimate of the change in muscle microvascular blood volume.

Blood pressure measurement

Upon their arrival at the laboratory, subjects rested for 10 min in supine position on a medical exam table. Resting Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP) and heart rate were measured in duplicate with an electronic sphygmomanometer (IntelliSense, OMRON TM, Netherlands) and then were averaged for analysis.

Blood sampling and assessment

After blood pressure measurements, blood sampling was performed through a catheter inserted in an antecubital vein. A first sample (5 mL) was collected 15 minutes before supramaximal intermittent exercise tests in an EDTA tube with subjects in a semi recumbent

position. Twenty-five minutes after the end of exercise tests, a second sample was drawn with the same procedure. Samples were centrifuged at 3600 g and 4°C for 10 min, and plasma was aliquoted and stored at -80°C for later analysis.

Plasma NO_x concentrations were determined by the Griess method with a commercial kit (CAYMAN NO_3^- colorimetric assay kit, Bertin Pharma TM, France).

Data analysis

Given each subject completed a number of repetitions different between BR and PL conditions, the experimental condition with the lowest number of repetitions completed was used as the trial reference and was compared to time matched data of the other experimental condition with the highest number of repetitions. The time-matched gas exchange and NIRS variables compared between the two conditions are denoted TM-throughout the Results and Discussion sections. Mean gas exchange values were calculated over the whole exercise duration, only TM-work bouts, only TM-recovery bouts and combined TM-work and recovery periods, respectively. The amplitude of change for $\dot{V}O_2$, $\dot{V}CO_2$, and $\dot{V}E$ ($\Delta\dot{V}O_2$, $\Delta\dot{V}CO_2$, and $\Delta\dot{V}E$, respectively) between work and recovery periods during TM-repetitions was also calculated.

All NIRS variables were expressed as relative changes from baseline values. Baseline values for HbO_2, HHb, and THb were measured during the last 30-s of a 2-min resting period with subjects in a seated position before the warm-up for the exercise test. The maximal values for HHb and THb, and the minimal and maximal values for HbO_2 (HbO_{2min} and HbO_{2max}, respectively) were determined during each experimental condition. The areas under the curves (AUC) for HbO_2 (AUC-HbO_2+), HHb (AUC-HHb), and THb (AUC-THb) were calculated by the trapezoidal method over the duration of combined TM-work and recovery periods. The

AUC were calculated to provide an integrated index of muscle oxy-, deoxy-, and total-hemoglobin volume. During exercise, changes in HbO_2 occur below the baseline level; the AUC for HbO_2 was also determined below the baseline level (AUC-HbO_2-).

Statistical analysis

Statistical analyses were run with R software (R version 3.2.5, R Foundation for Statistical Computing, Vienna, Austria). Data are expressed as mean ± standard deviation (SD) unless otherwise stated. Normal distribution of the data was assessed by the Kolmogorov–Smirnov test. A bilateral Student paired t-test was used to compare the dependent variables (gas exchange values, NIRS-derived indexes and exercise performance parameters) from SIE tests between the experimental conditions (independent variables: beetroot juice supplementation vs. placebo). Plasma NO_x levels were analyzed by two-way ANOVA for repeated measures to compare the experimental conditions (beetroot juice supplementation vs. placebo) before and after the exercise test. Post-hoc analysis was then performed for dependent samples, as appropriate. Pearson's product moment correlation coefficients were calculated to assess the relationships between the change in NO_x and the changes in exercise tolerance and physiological parameters. Statistical significance was set at $p < 0.05$.

Results

Plasma NO_x levels

Plasma NO_x levels were significantly higher in BR condition compared to PL condition before (BR: 91.05±30.01 μM vs. PL: 21.41±7.59 μM; $p < 0.01$) and after supramaximal intermittent exercise (BR: 78.23±14.67 μM vs. PL: 20.17±6.17 μM; $p < 0.01$).

Exercise performance

Individual number of repetitions is displayed in figure 1. The number of work periods completed was not significantly different between the two conditions (BR: 13.9±4.0 reps, PL: 14.2±4.5 reps). The targeted power output representing 170% of maximal aerobic power output was 555.3±50.3 W. Mean Power Output were 579.2±57.7 W during the BR condition and 578.9±54.3 W during the placebo condition. There was no difference for total work performed during the two conditions (BR: 121±38 kJ, PL: 121±29 kJ). The number of work bouts completed was not significantly associated with plasma NO_x levels in any of the two conditions both before (BR: r=-0.05, PL: r=-0.44) and after (BR: r=-0.26, PL: r=-0.45) SIE test. The order of the experimental sessions did not significantly affect exercise performance (1^{st} session: 13.2±3.7 reps, 2^{nd} session: 14.8±4.6 reps).

-Figure 1 here-

Gas exchange and heart rate

There were no significant differences for $\dot{V}O_2$, $\dot{V}CO_2$ and $\dot{V}E$ between the BR and placebo conditions for the whole exercise, the first three work and recovery periods, and TM-work and recovery periods (see Figure 2). Heart rate was also similar between the two conditions for the whole exercise (BR: 159.9±9.0 bpm, PL: 156.9±15.4 bpm) and for TM-periods (BR: 170.4±14.7 bpm, PL: 172.0±7.1 bpm). There was no significant association between the changes in plasma NO_x levels and the changes of $\dot{V}O_2$ in any of the two conditions both before (BR: r=0.12, PL: r=0.19 for time-matched work+recovery periods) and after (BR: r=0.244, PL: r=0.325 for time-matched work+recovery periods) SIE test. All correlations between plasma NO_x levels and $\dot{V}O_2$ changes are reported in Supplemental online Table 3. Results for gas exchange parameters and heart rate during exercise tests periods between BR and PL conditions are shown in Supplemental online Table 1.

- Figure 2 here –

NIRS-derived parameters and resting blood pressure

Results from NIRS measurements are displayed in Table 2. At baseline, HbO_2, HHb, and THb were not significantly different between the two conditions. During exercise, the maximal values of HbO_2, HHb, and THb were not significantly different between the two conditions. AUC-HbO_2+ and AUC-HbO_2-, AUC-HHb, and AUC-THb did not differ between the BR condition and placebo. The changes in plasma NO_x levels were not significantly associated with the changes in fractional O_2 extraction (before SIE test: BR r=0.37, PL: r=0.39; after SIE test: BR r=0.34, PL: r=-0.03) and muscle microvascular blood volume (before SIE test: BR r=0.22, PL: r=0.32; after SIE test: BR r=0.15, PL: r=-0.19) during time-matched

work+recovery periods in any of the two conditions. All correlations between plasma NO_x levels and changes in NIRS-derived parameters are reported in Supplemental online Table 3. There was no significant effect of BR supplementation on SBP, DBP, and heart rate at rest (Supplemental online Table 2).

- Table 2 here –

Discussion

The main finding of our study is the lack of improvement in tolerance to SIE after dietary nitrate supplementation in athletes with a high level of aerobic fitness ($\dot{V}O_{2max}$>65 mL·kg^{-1}·min^{-1}). The present study, to our knowledge, is the first to investigate the exercise tolerance to SIE and to measure gas exchange and local muscle O_2 delivery and extraction in elite endurance athletes. Regardless of the aerobic fitness status of participants, a few studies have questioned whether NO_3^- supplementation increases tolerance to SIE. These studies showed either an impaired (20), unchanged (12,18,20,21) or improved performance (19,21).

High aerobic fitness is likely a major factor contributing to the lack of effect of NO_3^- supplementation on exercise performance (26,27). Previous studies showed that 1-day (45–49) and 6-days of dietary NO_3^- supplementation (12,49,50) did not improve exercise performance during running (45,46,48,50) and cycling time-trials (12,47,49) in endurance athletes with $\dot{V}O_{2max}$ above ~60 mL·kg^{-1}·min^{-1}. Few nutritional supplementations have showed good benefits for exercise performance in elite athletes that are already well adapted to sport events due to chronic training adaptations (51). Investigations that address the effectiveness of dietary NO_3^- supplementation during supramaximal intensity exercise in elite endurance athletes are rare to date, with no improved exercise performance during 180m running time-trial in elite cross-country skiers (48) and during repeated sprint capacity (6 x 20 s sprints, recovery 100 s) in elite cyclists (12). It is striking that, with the same exercise protocol as in the present study, team sports athletes with moderate aerobic fitness ($\dot{V}O_{2max}$<55 mL·kg^{-1}·min^{-1}) had a 20% increase in the mean number of repetitions completed when supplemented with dietary NO_3^- compared to placebo (19). In this exercise protocol, we fixed the recovery time at 30 s that is sufficiently long to allow the PCr pool to be resynthesized and the lactic acid accumulation to be limited (38). When subjects performed repeated-sprints with 30 s of

recovery between each sprint, the power output was maintained by ATP that was derived mainly from PCr degradation and an increased contribution of aerobic metabolism (52). PCr recovery kinetics have been shown to be dependent on O_2 availability in human skeletal muscle (53). The reasons for the absence of change in exercise tolerance in the present study may be related to characteristics of elite endurance athletes such a high proportion of type I muscle fibers (54), greater skeletal muscle capillary density (55) and increased oxidative capacity (56). Tesch et al. showed a similar PCr degradation between type I and type II muscle fibers in vastus lateralis muscle in active men following 30 maximal voluntary knee extensions (57). However, after 60 seconds of recovery, type I muscle fibers had a significant greater level of PCr relative to the initial level than type II muscle fibers (57). These results suggest that elite endurance athletes may already have an optimal rate of PCr resynthesis during recovery periods that could not be affected by dietary NO_3^- supplementation.

Hernández et al. well showed that NO_3^- supplementation increased force production in type II, predominantly glycolytic, muscle fibers, but not in type I, highly oxidative, muscle fibers in mouse (58). The same authors reported an increase in the expression of the sarcoplasmic reticulum Ca^{2+}-handling proteins only in type II muscle fibers of NO_3^- supplemented mouse. Further study is required to address whether NO_3^- supplementation elicits comparable specific physiological responses of type II muscle fibers to exercise in humans. One purpose of the present study was therefore to investigate subjects with high aerobic fitness who are known to have a high proportion of type I muscle fibers and a small proportion of type II muscle fibers (54). The unaltered exercise tolerance in the present study provides support to the hypothesis that increased NO bioavailability may have selective effects in subjects with a high proportion of type II muscle fibers (19,59), or during exercise that elicits a high recruitment of type II muscle fibers (23,60).

There may be a need for higher NO_3^- dose than what we used to elicit significant physiological effects and subsequent increase in exercise tolerance in athletes with high aerobic fitness (14,26). Such athletes have indeed been shown to have already high plasma NO_x levels (34,61), and NO_3^- dose that are effective in population with lower aerobic fitness may be ineffective to increase NO_x levels in this population. Indeed, highly trained rowers improved 2000-meter rowing performance when supplemented with 8.4 mmol, as did not with 4.2 mmol of NO_3^- (62). Nitrate supplementation in the present study (5.2 $mmol \cdot day^{-1}$) being only slightly higher than the lowest dose used by Hoon et al. may have been insufficient to elicit significant physiological effects (62). It is however to note that when using NO_3^- dose as high as 19.5 mmol either acutely or during 8 days, Boorsma et al. showed no effect on 1500-m time trial performance of elite middle distance runners (14). Furthermore, the duration of NO_3^- supplementation being ingested could be considered. Vanhatalo et al. showed that maximal aerobic power output of healthy, but not highly trained subjects, was significantly improved relative to placebo after 15 days of NO_3^- supplementation, whereas there were no significant differences with a 5-day or acute supplementation (63). Studies with supplementation duration of at least 15 days have to our knowledge not been conducted in athletes with high aerobic fitness. However, studies comparing 6 to 8 days NO_3^- supplementation in athletes with high $\dot{V}O_{2max}$ showed no additional benefit (14,15), and NO_3^- and NO_2^- plasma levels were not greater after the long NO_3^- supplementation duration than the shorter one (15). In addition, a recent meta-analysis showed that features of the NO_3^- supplementation (the dose of NO_3^- supplementation, the number of days of supplementation and the total amount ingested of NO_3^-) were not associated with changes in physical performance in both non-athlete and athlete individuals (64). In all, further research is required to establish the real sufficient dose of NO_3^- being ingested, if any, in order to enhance the responsiveness to NO_3^- supplementation in elite endurance athletes.

Regarding the present study, plasma NO_x levels after dietary NO_3^- supplementation were lower than in previous studies in subjects with high aerobic fitness (13,14,18,65). However, Porcelli et al. who also ultrafiltrated plasma samples and used the Griess assay, found levels similar to those that we report in the placebo and NO_3^- supplemented conditions (26). It is to note that many studies do not report ultrafiltration of plasma samples, and that proteins may interfere with NO_3^-/NO_2^-, resulting in erroneously high NO_x values (66). Finally, a number of studies have reported that resting plasma NO_2^- levels were similar in elite athletes and non-athletes (26,65), and that acute dietary NO_3^- supplementation in elite athletes with dose similar to that used in non-athletes (614 mg) were able to elicit significant increase in plasma NO_3^-/NO_2^-, but no change in performance (13,18).

A second and important finding of the present study was that $\dot{V}O_2$, local muscle oxy-, deoxy-, and total-hemoglobin volume were unaffected by NO_3^- supplementation. These findings are in contrast to the repeatedly reported decrease in $\dot{V}O_2$ during submaximal intensity exercise in subjects with lower $\dot{V}O_{2max}$ (5,28,29,32,67,68). Unchanged $\dot{V}O_2$ was previously reported in studies investigating the effects of NO_3^- supplementation during supramaximal intensity exercise (19–21). The percentage of $\dot{V}O_{2max}$ elicited in the present study (~75%) was similar to that reported by Martin et al. who showed no improvement in exercise tolerance with NO_3^- supplementation (20). In contrast, the team sports players that we previously investigated reached on average 85% $\dot{V}O_{2max}$ and had a significant increase in exercise tolerance with NO_3^- supplementation (19). In the latter study, we also observed that NO_3^- supplementation was associated with significantly higher $\dot{V}E$, and lower $\dot{V}O_2$ and $\dot{V}CO_2$ fluctuations. These responses to NO_3^- supplementation were not present in the current study. Even though it is unclear how these physiological changes contributed to increased exercise tolerance in previous study, their absence in the present study provide additional evidences that increasing NO bioavailability has different effects in endurance athletes with aerobic fitness levels. We

however cannot fully exclude that NO_3^- supplementation would be effective in elite endurance athletes during intermittent exercise with work:recovery ratio periods that would elicit a higher percentage of $\dot{V}O_{2max}$ (21).

It has been established that high-intensity intermittent exercise heavily recruits type II muscle fibers (22), which are more sensitive to fatigue than type I muscle fibers (69). BR supplementation improves blood flow distribution toward type II muscle fibers (70) and increases microvascular O_2 pressure in type II muscle fibers during exercise in rats (71). It is thus hypothesized that dietary NO_3^- supplementation may improve exercise tolerance during high-intensity intermittent exercise by better matching blood flow to metabolic rate across the working muscle (72). In accordance with this perspective, we have previously reported that 3-day BR supplementation, similar to that used in the present study, enhances tolerance to exercise at supramaximal intensity with increased microvascular total-hemoglobin in the working muscle (19). However, to date, there has been no investigation of local muscle O_2 delivery and extraction in athletes with high $\dot{V}O_{2max}$ and a low proportion of type 2 muscle fibers supplemented in dietary NO_3^- during SIE. Using NIRS-derived indexes, we show that O_2 extraction and local blood volume in muscle vastus lateralis were unaltered during SIE. Furthermore, the lack of differences for HHb parameters, in conjunction with similar changes in HbO_2 and THb between BR and placebo conditions, provides additional supports that dietary NO_3^- did not affect muscle microvascular perfusion in elite endurance athletes during SIE. Our present findings were supported by a recent study that reported no effect of single dose of BR supplementation on muscle fractional O_2 extraction and muscle $\dot{V}O_2$ during sustained isometric exercise in moderately trained subjects (8). In the latter study, the lack of effect of BR supplementation was evident even when blood flow was obstructed to working muscles (8).

Together, the absence of change in exercise tolerance and physiological responses after BR supplementation in elite endurance athletes could be a consequence of many adaptations resulting of chronic training such as enhanced NO bioavailability by an upregulation of NO synthase activity (34,73), greater skeletal muscle capillary density (55), increased content of skeletal muscle Ca^{2+}-handling proteins (74) and a lower proportion of type II muscle fibers (54).

Study considerations

The low number of subjects can be considered as a limitation of our study. We cannot exclude that a study including a higher number of subjects would have yielded different results. However, rather than including a large number of subjects, we recruited a well characterized population of healthy young men based on $\dot{V}O_{2max}$ above 65 $mL \cdot kg^{-1} \cdot min^{-1}$ and participation to endurance competitive sports.

Another limitation of the study was the small dose of nitrate (5.2 $mmol \cdot day^{-1}$) used in the present study. It has been suggested that the minimal effective dose required to enhance exercise performance in most individuals is ≥ 5 mmol of NO_3^- per serving (75,76). However, a recent meta-analysis reported that the dose of NO_3^- supplemented and the total amount of NO_3^- ingested were not associated with changes in exercise performance in non-athletes and athletes (64). Thus, further research is needed to determine whether higher NO_3^- intake than in our study is required in athletes with high $\dot{V}O_{2max}$ to improve the tolerance to supramaximal intensity intermittent exercise, although few beetroot juice products intended for athletes effectively provide ≥ 5 mmol of NO_3^- per serving (76)

Conclusion

Three days of NO_3^--rich beetroot juice supplementation (5.2 mmol·day^{-1}) did not increase the tolerance to supramaximal intensity intermittent exercise in elite endurance athletes with high $\dot{V}O_{2max}$, and did not affect $\dot{V}O_2$ and local muscle O_2 delivery and extraction. This finding provides additional support to the hypothesis that beetroot juice supplementation is less effective to improve performance in endurance-trained athletes, even in the case of exercise modalities that are known to promote the effectiveness of dietary NO_3^- supplementation by recruiting a greater proportion of NO-sensitive type II muscle fibers.

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Conflicts of interest and source of funding

The authors do not have any conflicts of interest to disclose. No funding was received for this study.

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Tables

Table 1. Characteristics of the subjects included in the study (*n*=9)

Parameters	**Mean±SD**
Age (years)	21.7±3.7
Body Mass (kg)	66.8±8.5
Height (m)	1.80±0.07
Body Fat (%)	11.1±2.4
Maximal aerobic power (W)	327±30
$\dot{V}O_{2max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	71.1±5.2

Values are mean±SD.

Table 2. Effects of a 3-day dietary nitrate supplementation (beetroot juice) on NIRS-derived parameters of the vastus lateralis muscle during supramaximal intensity intermittent exercise tests.

		Beetroot Juice	Placebo
HbO_2	Baseline	39.3±14.1	40.3±14.1
	Maximal	14.3±7.9	12.7±3.8
	TM-AUC+	1710.8±1083.7	1911.1±1026.3
	Minimal	-14.5±6.4	-15.0±7.3
	TM-AUC-	3178.5±1597.5	4013.3±2166.7
HHb	Baseline	45.1±18.2	44.9±17.7
	Maximal	25.6±12.7	21.8±6.5
	TM-AUC	6619.7±875.8	6332.7±1336.8
THb	Baseline	83.0±33.4	85.2±31.2
	Maximal	15.4±12.3	15.6±5.2
	TM-AUC	6816.9±1463.1	6771.5±3004.5

Values are mean±SD. AUC: Area Under the Curve, HbO_2: oxygenated hemoglobin, HHb: deoxygenated hemoglobin, THb: total hemoglobin, TM- denotes time-matched comparison for 3 successive work+recovery periods, TM-AUC+ and TM-AUC- indicate the areas under curves calculated for HbO_2 data above and below the resting baseline values, respectively.

Figures

Figure 1. Individual data points for the number of repetitions completed before volitional exhaustion during supramaximal intensity intermittent exercise tests following a placebo and a beetroot juice supplementation. Red squares indicate the mean number of work periods completed for each condition.

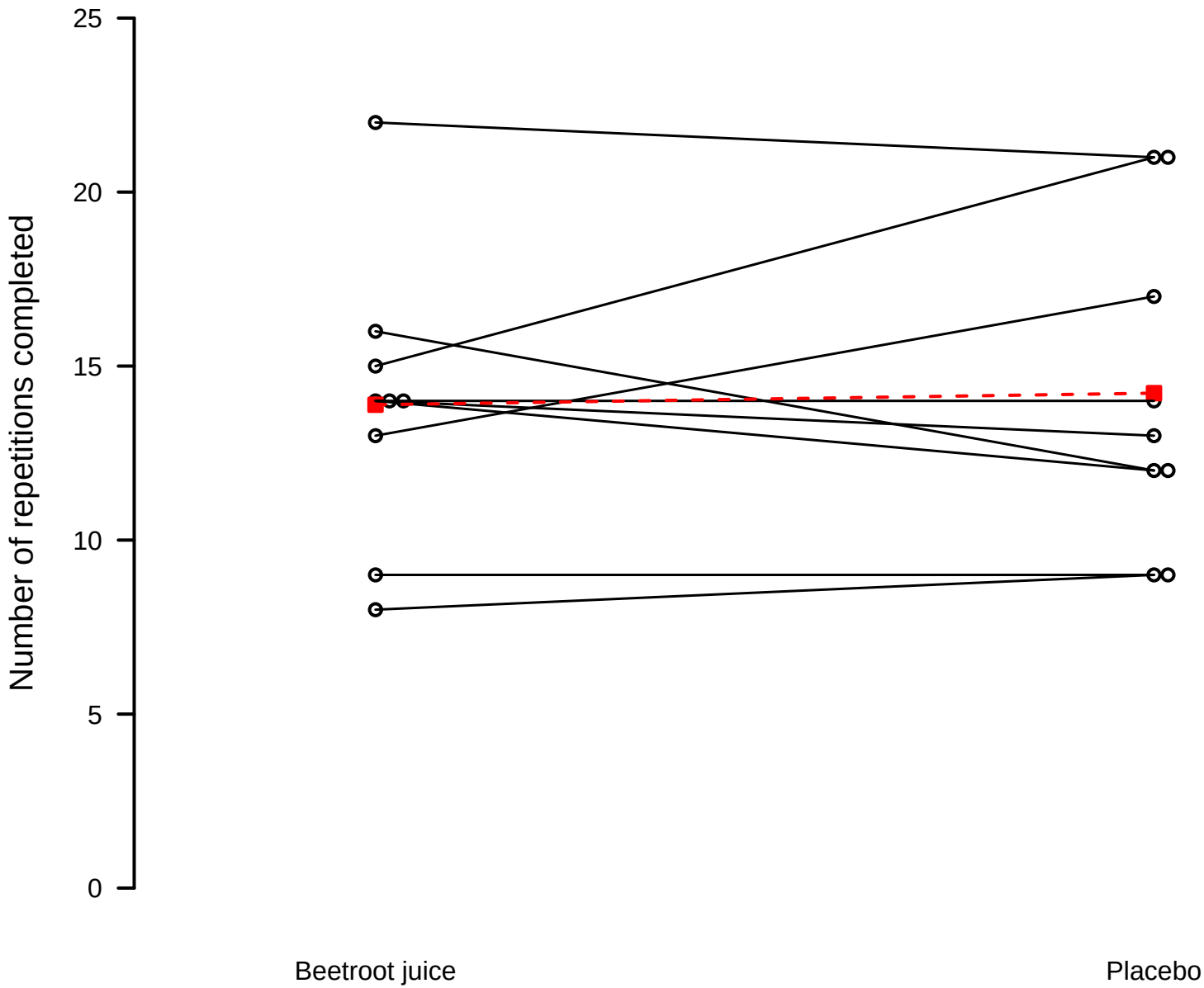
Figure 2. Bar plots of gas exchange and heart rate parameters during supramaximal intensity intermittent exercise tests following a placebo (white bars) and a beetroot juice supplementation (grey bars). Data were calculated over the whole duration (Whole session), the first three work and recovery periods (First 3 bouts), and TM-work and recovery periods (Last 3 bouts) for $\dot{V}O_2$ (A), $\dot{V}CO_2$ (B), $\dot{V}E$ (C) and heart rate (D).

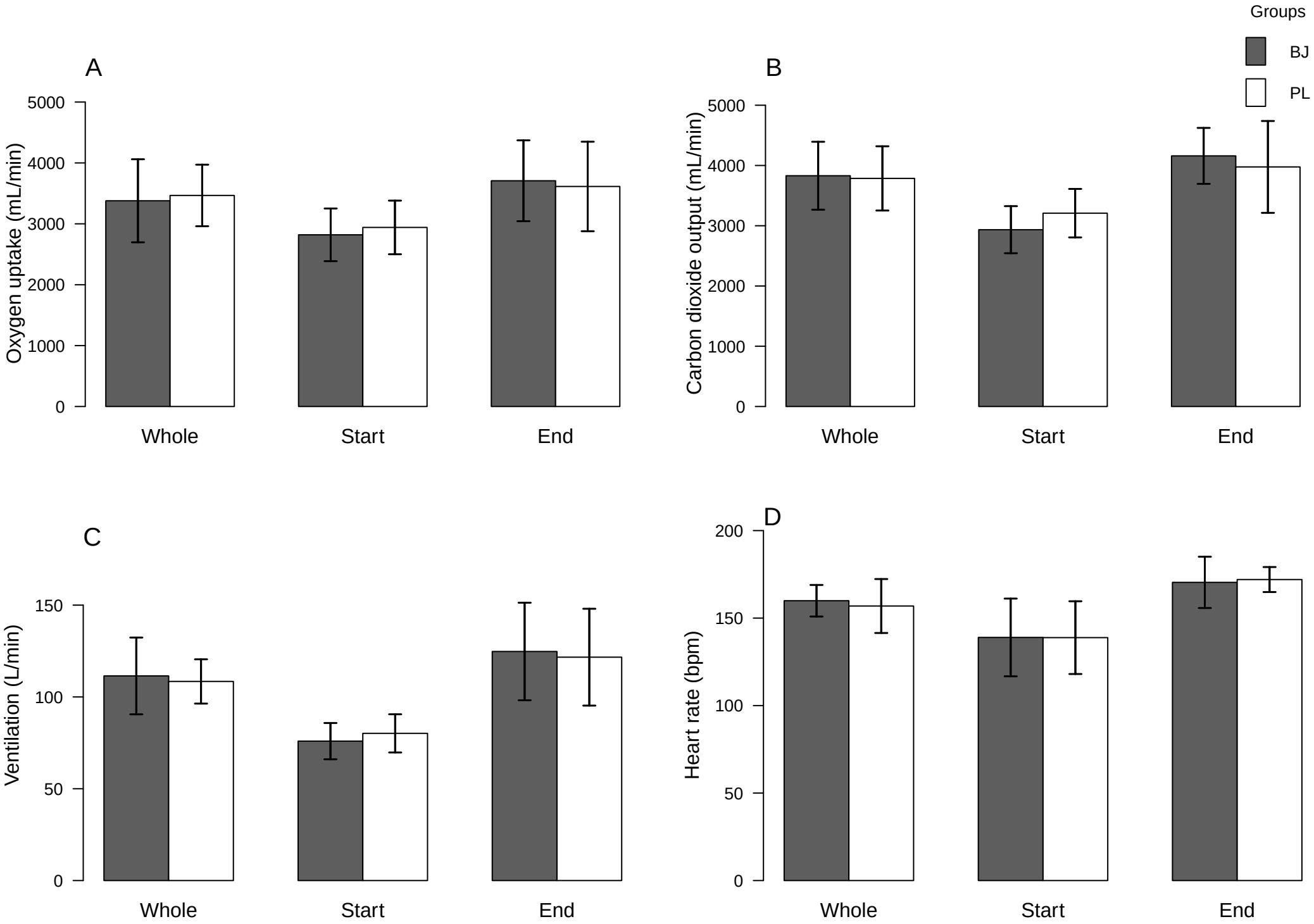
Supplemental Online Data

Supplemental Online Table 1. Effects of a 3-day dietary nitrate supplementation (beetroot juice) on fluctuations of gas exchange parameters and heart rate during supramaximal intensity intermittent exercise tests.

Supplemental Online Table 2. Effects of a 3-day dietary nitrate supplementation (beetroot juice) on resting heart rate and blood pressure.

Supplemental Online Table 3. Pearson correlation coefficients of plasma NO_x levels with changes in $\dot{V}O_2$ and NIRS-derived parameters before and after supramaximal intermittent exercise (SIE) tests.


Number of repetitions completed
25
20
15
10
5
0
Beetroot juice
Placebo


A
B
C
D
Groups
BJ
PL
Oxygen uptake (mL/min)
Carbon dioxide output (mL/min)
Ventilation (L/min)
Heart rate (bpm)
0
1000
2000
3000
4000
5000
50
100
150
200
Whole
Start
End