

## Effect of dietary nitrate supplementation on tolerance to supramaximal intensity intermittent exercise



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### ABSTRACT

Dietary nitrate ( $\text{NO}_3^-$ ) supplementation has been shown to increase exercise tolerance and improve oxidative efficiency during aerobic exercise in healthy subjects. We tested the hypothesis that a 3-day supplementation in beetroot juice (BJ) rich in  $\text{NO}_3^-$  would improve the tolerance to supramaximal intensity intermittent exercise consisting of 15-s exercise periods at 170% of the maximal aerobic power interspersed with 30-s passive recovery periods. The number of repetitions completed before reaching volitional exhaustion was significantly higher in the BJ than in the placebo condition ( $26.1 \pm 10.7$  versus  $21.8 \pm 8.0$  respectively,  $P < 0.05$ ). In contrast to previous findings during exercise performed at intensity below the peak oxygen uptake ( $\text{VO}_{2\text{peak}}$ ), oxygen uptake ( $\text{VO}_2$ ) was unaffected (BJ:  $2735 \pm 345 \text{ mL kg}^{-1} \text{ min}^{-1}$  vs. placebo:  $2787 \pm 346 \text{ mL kg}^{-1} \text{ min}^{-1}$ , NS). However, the Area Under the Curve for microvascular total hemoglobin (AUC-THb) in the vastus lateralis muscle assessed by near infrared spectroscopy during 3 time-matched repetitions was significantly increased with  $\text{NO}_3^-$  supplementation (BJ:  $9662 \pm 1228 \text{ a.u.}$  vs. placebo:  $8178 \pm 1589 \text{ a.u.}$ ;  $P < 0.05$ ). Thus, increased  $\text{NO}_3^-$  (BJ:  $421.5 \pm 107.4 \mu\text{M}$  vs placebo:  $39.4 \pm 18.0 \mu\text{M}$ ) and  $\text{NO}_2^-$  (BJ:  $441 \pm 184 \text{ nM}$  vs placebo:  $212 \pm 119 \text{ nM}$ ) plasma levels ( $P < 0.001$  for both) are associated with improved muscle microvascular Red Blood Cell (RBC) concentration and  $\text{O}_2$  delivery during intense exercise, despite no effect on resting femoral artery blood flow, and vascular conductance. Maximal voluntary force during an isometric leg extensor exercise, and blood lactate levels were also unaffected by  $\text{NO}_3^-$  supplementation.

To conclude, dietary  $\text{NO}_3^-$  supplementation enhances tolerance to exercise at supramaximal intensity, with increased microvascular total RBC concentration in the working muscle, in the absence of effect on contractile function and resting hemodynamic parameters.

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### 1. Introduction

Nitric oxide (NO) is a gaseous signaling molecule with a wide range of physiological effects, that include the regulation of skeletal muscle oxidative efficiency, contractile properties and blood flow [1–3]. Circulating NO is short lived and rapidly converted to nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ), which is considered as the main body pool of NO [4,5]. Besides NO synthesis from L-arginine and oxygen by the endothelial, neural and inducible isoforms of NO synthases,

the ingestion of dietary inorganic nitrate ( $\text{NO}_3^-$ ) is also able to significantly increase  $\text{NO}_3^-$  and  $\text{NO}_2^-$  plasma levels [3,6–8]. Once ingested and absorbed from the gastrointestinal tract into the blood, approximately 25% of dietary  $\text{NO}_3^-$  are taken up by the salivary gland, concentrated into the saliva, and converted to  $\text{NO}_2^-$  by oral nitrate reductase bacteria [3]. Once swallowed, salivary  $\text{NO}_2^-$  is either being reduced to NO and other nitrogen species in the acidic stomach or absorbed from the intestine [3,9]. During exercise in the aerobic domain, supplementation with inorganic nitrate translates to effects such as increased time to exhaustion [6], higher work rate achievement [10] and remarkable decrease in whole body  $\text{VO}_2$  for a given work rate [6,11,12]. Larsen et al. also clearly showed a tight relationship between the reduction of the oxygen cost of cycling exercise and the increase of the ratio of ATP produced to oxygen consumed in skeletal muscle mitochondria from

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subjects supplemented with sodium nitrate [2]. There are evidences supporting the inhibition of enzyme cytochrome c oxidase activity by NO and inhibition of uncoupled respiration as candidate factors responsible for the increased mitochondrial respiratory efficiency that prevent low O<sub>2</sub> availability from becoming a limiting factor to respiration [13]. In contrast, it is less evident to date that enhanced exercise performances can be mediated by the vasodilator effect of NO. Nitrite can be reduced to NO by various mechanisms involving deoxyhemoglobin, deoxymyoglobin, xanthine oxidoreductase, complexes of the mitochondrial transport chain, ascorbate, polyphenols and protons [3]. Among these mechanisms, vasodilation has been shown to particularly increase under hypoxic condition in response to enhance reduction of NO<sub>2</sub><sup>-</sup> to NO and other nitric oxide species by deoxyhemoglobin [5]. Similarly when intracellular pO<sub>2</sub> decreases in muscle cells, the enhanced conversion of NO<sub>2</sub><sup>-</sup> to NO by deoxymyoglobin increases the inhibition of mitochondrial respiration [4].

Athletes are frequently engaged in exercise of intermittent character, where the power output during working periods is above their power output at VO<sub>2max</sub>. During recovery periods, local muscle blood flow and O<sub>2</sub> delivery to skeletal muscle determine the rate of PCr resynthesis by oxidative phosphorylation and therefore the ability to repeat exercise bouts at high work rates [14,15]. Considering the pronounced increase of deoxyhemoglobin [5] in regions of poor oxygenation [16], skeletal muscle could provide a enabling environment to the generation of NO generation from NO<sub>2</sub><sup>-</sup> during intense exercise. A facilitated NO generation from NO<sub>2</sub><sup>-</sup> in acidic condition at pH typically encountered in heavy working skeletal muscle is another factor that could increase NO availability [17]. Considering that the formation of NO occurs in a nitrite concentration-dependent manner at low pH levels, increasing circulating nitrite levels by a dietary nitrate supplementation may delay fatigue development by facilitating local O<sub>2</sub> supply to active skeletal muscle.

Altogether, these data suggest that tolerance to supramaximal intermittent exercise may be particularly sensible to NO bioavailability due to specific physicochemical conditions occurring with its achievement: important type II fibers recruitment (greater force production), ischemia and hypoxia (imbalance between O<sub>2</sub> demand and delivery), and intracellular acidosis (low muscle pH). However, previous studies that examined the effects of NO<sub>3</sub><sup>-</sup> supplementation during supramaximal exercise have yielded conflicting results, ranging from improved [18], to impaired performances without effects on oxygen uptake [19] during repeated sprints.

The aim of the present study was therefore to determine in young men the effects of a 3-day supplementation in beetroot juice rich in NO<sub>3</sub><sup>-</sup> on the tolerance to supramaximal intermittent exercise, oxygen uptake, the change of muscle microvascular THb concentration and oxygen extraction. Our hypotheses after dietary NO<sub>3</sub><sup>-</sup> supplementation were as follow: 1) tolerance to exercise would be significantly increased, 2) VO<sub>2</sub> during exercise would be significantly decreased, 3) and muscular O<sub>2</sub> delivery (characterized by microvascular RBC concentration) would be significantly increased during intermittent exercise.

## 2. Material and methods

### 2.1. Subjects

The characteristics of the 12 male subjects who were recruited for the study are presented in Table 1. All subjects were informed of the study protocol, its potential risks and benefits, and signed an informed consent form before their inclusion to the study. Subjects were included after completing a preliminary visit where they performed a maximal aerobic test until volitional exhaustion to ensure that they met the inclusion criteria of a VO<sub>2max</sub> between 40

**Table 1**  
Subjects characteristics (n = 12).

| Parameters  | Mean ± SD   |
|---|-------------|
| Age (years)   | 22.8 ± 3.1  |
| Body mass (kg)  | 75.1 ± 10.1 |
| Height (m)  | 1.82 ± 0.06 |
| Body fat (%)  | 13.6 ± 3.9  |
| Aerobic peak power (W)                                      | 268 ± 32    |
| VO <sub>2max</sub> (mL kg <sup>-1</sup> min <sup>-1</sup> ) | 46.6 ± 3.4  |

and 55 mL kg<sup>-1</sup> min<sup>-1</sup>. Seventeen subjects attended to the preliminary visit and 5 were excluded because their VO<sub>2max</sub> either exceeded 55 mL kg<sup>-1</sup> min<sup>-1</sup> (n = 4) or was lower than 40 mL kg<sup>-1</sup> min<sup>-1</sup> (n = 1). All subjects were non smokers and participating to team sports but none of them was engaged in intense endurance exercise training. Subjects were not taking any nutritional supplements or medications that could affect NO metabolism. 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.

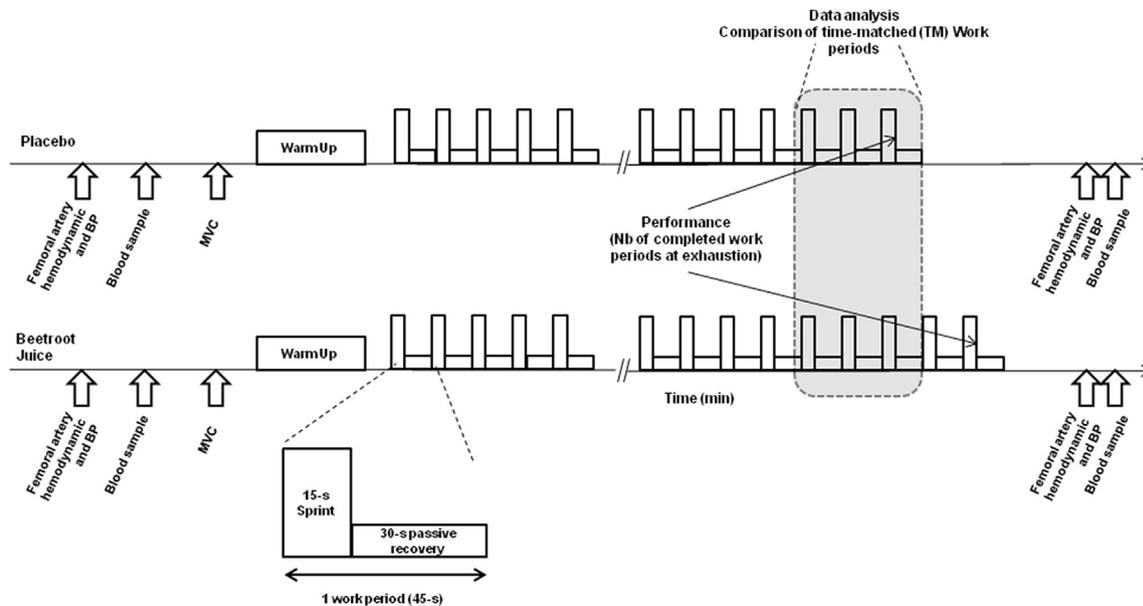
### 2.2. Study design

During the preliminary visit, subjects performed a maximal incremental exercise test. Subjects were included in the study according to the inclusion criteria and attended to the laboratory for two experimental visits where they performed a supramaximal intensity intermittent exercise trial. During each experimental visit, performance was assessed by the number of work periods completed before reaching volitional exhaustion. The experimental visits were carried out at the same time of the day and were separated by at least one week and at most 2 weeks. A randomized crossover design single blinded to the subjects was used in this study and subjects were provided either with beetroot juice (BJ condition) or apple-black currant juice (placebo condition) for a 3-day supplementation before each of the two visits.

The following measurements were performed during the two visits: resting blood pressure and common femoral artery diameter and blood flow, arterial compliance, vascular conductance, maximal isometric voluntary contraction of the quadriceps, gas exchange measurements (indirect calorimetry), lactate measurement, changes in local muscle oxygenation, and microvascular THb concentration measured by NIRS, as well as plasma NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> levels. The experimental exercise protocol is depicted in Fig. 1. Because the number of completed work periods was different between the 2 conditions, the trial with the lowest number of repetitions was used as the reference for the selection of data (gas exchange and NIRS parameters) that were compared during 3 time-matched repetitions. The variables compared during the 3 time-matched repetitions are denoted TM-throughout the paper.

### 2.3. Incremental maximal exercise test

VO<sub>2peak</sub> and Aerobic Peak Power were measured during a graded exhaustive cycling exercise test performed one week before the first experimental visit. The initial power of 70 W was maintained during 3 min and followed by 35 W increments every 2 min. Subjects were asked to maintain a pedaling frequency of 70 rpm and were strongly encouraged by experimenters throughout the test to perform a maximal effort. Criteria for the achievement of VO<sub>2peak</sub> were subjective exhaustion with heart rate above 220-age ± 10 beats min<sup>-1</sup> and/or Respiratory Exchange ratio (RER, VCO<sub>2</sub>/VO<sub>2</sub>) above 1.1 and/or when VO<sub>2</sub> plateaued. All tests were performed on a mechanically braked cycle ergometer (829E, Monark



**Fig. 1.** Experimental protocol of the two experimental conditions, with either a 3-day beetroot juice or a placebo supplementation. BP: Blood Pressure, MVC: Maximal Voluntary Contraction. Sprints were performed at 170% of the Aerobic Peak Power determined during a preliminary visit. Gas exchange data and Near Infrared Spectroscopy derived parameters in Placebo and Beetroot Juice were averaged over three repetitions (gray area) and matched for repetition number (time-matched, denoted TM). Because the number of completed work periods was different between conditions, the trial with the lowest number of repetitions was used as the reference for data selection.

exercise, Sweden).  $V_E$ ,  $VO_2$  and  $VCO_2$  were measured breath-by-breath through a facemask connected to flowmeter,  $O_2$  and  $CO_2$  analyzers (K4b<sub>2</sub>, Cosmed, Italy). Aerobic Peak Power was determined as the sum of the power output from the last stage completed and the fraction of the time spent during the last stage multiplied by 35 W. Calibration of gases analyzers was performed before each test with commercial gases of known concentration. Gas exchange parameters were averaged every 30 sec. Before each test, the  $O_2$  and  $CO_2$  analyzer were calibrated with ambient air and a standard gas mixture ( $O_2$ : 16 %,  $CO_2$ : 5 %) and the turbine flowmeter was calibrated using a 3 L syringe. Heart rate was continuously monitored using a polar heart rate monitor (S810, Polar, Finland).

#### 2.4. Experimental visits

On the experimental visit days subjects arrived to the exercise laboratory at the Haute Ecole Provinciale du Hainaut (Tournai, Belgium) between 1100am and 0300pm. The appointment time for each subject was the same for the two experimental visits.

#### 2.5. Nitrate and placebo supplementation

Beetroot juice (BJ) was used for dietary  $NO_3^-$  supplementation and had an average  $NO_3^-$  content of  $680 \text{ mg L}^{-1}$  and apple-black currant juice used as a placebo had a nitrate content  $<5 \text{ mg L}^{-1}$ . The two beverages were purchased from Pajottenlander (Belgium). The subjects were neither informed of the potential effects of dietary nitrate, nor of the study hypothesis. Drinks were distributed by an independent technician, not involved in the process of exercise testing. Participants were not informed that the purpose nitrate supplements would be tested and compared to the baseline trial. After the completion of the two experimental sessions, subjects were questioned again to confirm that they did not become aware of the research hypotheses during the course of the study. In the morning at breakfast time during the two days preceding experimental visits subjects ingested 500 mL of beetroot juice or

500 mL of apple-black currant juice. On the day of the experimental visit subjects were instructed to ingest 500 mL of BJ or placebo 2 h before the time of their appointment at the laboratory. There was a minor difference in the energy provided by BJ (185 kcal) and placebo (225 kcal). Subjects were instructed not to consume any drink containing caffeine or alcohol during the 24 h before the experimental visits. In addition, subjects were asked to not to brush their teeth for 3 h after each dietary supplementation or to use mouthwash throughout the study period [20].

#### 2.6. Resting blood pressure and femoral artery hemodynamic

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

Measurements of arterial diameters and mean blood velocity of the common femoral artery (2–3 cm proximal to the femoral bifurcation) were performed using Doppler ultrasonography (Vivid i, GE Medical Systems, USA). B-mode imaging was performed at 10 MHz emitting frequency, whereas blood flow measurements were performed in full duplex Doppler mode at 4.4 MHz pulsed Doppler emitting frequency. Diastolic and systolic artery diameters (mm) were measured as the distance between the leading edges of the intima–lumen interface of the near-wall and the lumen-intima interface of the far wall. Time-averaged mean velocity ( $\text{cm s}^{-1}$ ) was recorded at the same level, by pulsed wave Doppler with a 45–60° insonation angle. Measurements were corrected for the insonation angle, and the pulsed Doppler sample volume was adjusted to cover the entire width of the vessel.

Femoral blood flow ( $\text{mL min}^{-1}$ ) was calculated as:  $[Acsa \times MBFv]$ , where  $Acsa$  is mean artery cross-sectional area and  $MBFv$  is mean blood flow velocity. Artery compliance ( $\text{mm}^2 \text{ mmHg}^{-1}$ ) was calculated as:  $[(\pi \times (AD_{sys}^2 - AD_{dia}^2)) / 4 \times \Delta P]$ , where  $AD_{sys}$  and  $AD_{dia}$  are systolic and diastolic arterial diameters, respectively, and  $\Delta P$  is pulse pressure. Vascular

conductance ( $\text{mL min}^{-1} \text{mmHg}^{-1}$ ) was calculated as:  $[\text{BF}/\text{MAP}]$ , where BF is blood flow and MAP is mean arterial pressure, calculated as  $[(\text{SBP} + 2 \times \text{DBP})/3]$ .

### 2.7. Maximal isometric voluntary contraction of the quadriceps

Maximal knee extensor torque was measured during an isometric maximal voluntary contraction performed on a dynamometer (Cybex II, Biodex, USA). After calibration of the dynamometer, subjects were seated on the dynamometer chair in the upright position. The thigh was secured to the chair using a Velcro strap and the ankle was secured to the lever arm by an ankle pad. The axis of rotation of the dynamometer was aligned with the lateral femoral condyle and the knee joint angle was set at  $45^\circ$  flexion. Subjects performed three 5sec repetitions separated by 3 min of recovery. Experimenters provided verbal encouragement during the maximal voluntary contraction. The best performance was retained during each of the two experimental conditions.

### 2.8. Supramaximal intermittent exercise tests

The exercise tests started ~180 min after the time of beetroot juice or placebo ingestion and were completed on a mechanically braked stationary cycle ergometer (Monark 829E, Monark exercise, Sweden). After a 5 min warm up at 50 % of aerobic peak power at 70 rpm measured during the preliminary visit and resting for 2 min, subjects performed an intermittent exercise trial to volitional exhaustion consisting of work periods repetitions composed as follows: 15 sec active sprint at 170 % of aerobic peak power interspersed with 30 sec passive recovery periods. Subjects were instructed to rapidly reach and maintain a pedaling frequency of 90 rpm during each bout of active sprint. In order to do so, subjects started unloaded pedaling after a 3 s countdown by one of the experimenter and the resistance was automatically applied when subjects reached 50 rpm. Subjects recovered passively between active sprints. The exercise was interrupted when subjects were no longer able to maintain a pedaling frequency above 87 rpm during the 5 last seconds of an active sprint or when subjects declared that they were no longer able to perform. Oxygen uptake,  $\text{VCO}_2$ ,  $\text{V}_E$  and heart rate were measured as above-mentioned for the incremental maximal exercise test. Gas exchange values were averaged over the whole duration of the supramaximal intermittent exercise, and during work and passive recovery periods separately. The differences for  $\text{VO}_2$ ,  $\text{VCO}_2$  and  $\text{V}_E$  ( $\Delta\text{VO}_2$ ,  $\Delta\text{VCO}_2$  and  $\Delta\text{V}_E$  respectively) between work and recovery periods during TM-repetitions were also calculated.

### 2.9. Muscle oxygenation and microvascular red blood cell concentration

The change in local muscle oxygenation and microvascular RBC concentration of the vastus lateralis was estimated using Near Infrared Spectroscopy (Oxymon, Artinis Medical system BV, Netherlands). Briefly, NIRS measures the change in near infrared light absorption by hemoglobin (Hb) and myoglobin (Mb), which is related to Hb and Mb  $\text{O}_2$  saturation. The two wavelengths emitting (780 and 850 nm) and receiving optodes were placed at mid-distance between the lateral condyle of the knee and the greater trochanter of the femur. Optodes were taped to the skin using adhesive strapping (Tensoplast, BSN Medical, France), covered with black cloth to avoid 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 visit in order to place them at the same location during the second visit. The interoptode distance was 4 cm. NIRS data were

stored on a computer and later analyzed using the Oxysoft software (Oxysoft, Artinis Medical system BV, Netherlands). NIRS signal acquisition was performed at 10 Hz and data were subsequently averaged over 1-sec periods.

Three indexes of muscle oxygenation and microvascular RBC concentration were derived from NIRS measurements in the region of interest of exercising skeletal muscles. Changes in oxygenated hemoglobin ( $\text{HbO}_2$ ) and deoxygenated hemoglobin (HHb) were used as estimates of muscle  $\text{O}_2$  delivery and extraction. Total hemoglobin (THb) was calculated as the sum of  $\text{HbO}_2$  and HHb and to estimate the change in total microvascular RBC concentration in the vastus lateralis muscle.

### 2.10. Blood samples and nitrate/nitrite assessment

After blood pressure and hemodynamic measurements, and before the assessment of maximal isometric voluntary contraction of the quadriceps, a catheter was placed in an antecubital vein. A first sample (5 mL) was immediately drawn on an EDTA vacutainer tube with subjects placed in a semi recumbent position. Twenty five minutes after the end of exercise after hemodynamic and blood pressure measurements a second sample was drawn with the same procedure. Samples were centrifugated at 3600 g for 10 min at  $4^\circ\text{C}$ , and the supernatant was aliquoted in eppendorf tubes. Aliquots were stored in a freezer at  $-80^\circ\text{C}$  until analysis. Plasma  $\text{NO}_3^-$  concentrations were determined by the Griess method using a commercial kit (Cayman  $\text{NO}_3^-$  colorimetric assay kit, Bertin Pharma, France). Plasma  $\text{NO}_2^-$  levels were determined by fluorometry according to the protocol by Nüssler et al. [21] with a fluorescence spectrophotometer (F-2710, Hitachi, Japan).

### 2.11. Capillary blood lactate measurement

Whole blood lactate levels were determined using a portable lactate analyzer and lactate strips (Lactate Scout, SensLab GmbH, Germany). During the incremental maximal exercise test, a capillary blood sample was taken 3 min after the end of the test. During the experimental visits, capillary blood was sampled from the finger tip immediately after the 10th bout of exercise and subsequently after every 10 bouts until volitional exhaustion. Two last capillary blood sample were taken at the end and 3 min after the end of the exercise test.

### 2.12. Data analysis

Gas exchanges and NIRS-derived parameters were compared between the two conditions to determine the effect of the  $\text{NO}_3^-$  supplementation. Because the number of completed work periods was different between conditions, the data during the last three repetitions (work + recovery) during the condition with the lowest number of repetitions completed was used as the reference, and compared to time-matched (TM) data during the condition with the highest number of repetitions completed. Gas exchange data are reported for the whole exercise, and for TM-work and passive recovery periods separately.

The maximal values for HHb and THb and the minimal and maximal values for  $\text{HbO}_2$  ( $\text{HbO}_{2\text{min}}$  and  $\text{HbO}_{2\text{max}}$  respectively) were determined during each trial. The Area Under the Curve (AUC) for  $\text{HbO}_2$  (AUC- $\text{HbO}_{2+}$ ), HHb (AUC-HHb) and THb (AUC-THb) was calculated over the duration of TM-repetitions by the trapezoidal method after subtracting the baseline. Baseline values were determined from a 2-min measurement performed at rest when subjects were in a seated position before the warm-up for the intermittent exercise test. The AUC were calculated to provide an integrated index of muscle oxygen and microvascular RBC

concentration, as HbO<sub>2</sub>, HHb and THb constantly change with work and recovery periods. The minimal values for HbO<sub>2</sub> and AUC below baseline level (AUC-HbO<sub>2</sub><sup>-</sup>) were also reported because there are variations for HbO<sub>2</sub> both below and above the baseline level. AUC-THb for a representative subject is presented in Fig. 2.

### 2.13. Statistical analysis

Data were expressed as mean ± standard deviation (SD) unless otherwise stated. Normal distribution of the data was assessed by the Kolmogorov–Smirnov test. To determine the effect of NO<sub>3</sub><sup>-</sup> supplementation, experimental variables were compared using a bilateral paired t-test. The effect of dietary NO<sub>3</sub><sup>-</sup> supplementation on pre and post-test NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> plasma levels were analyzed using a 2-way analysis of variance (2-way ANOVA). Statistical analyses were performed using Statview software. The level of significance was  $P < 0.05$ .

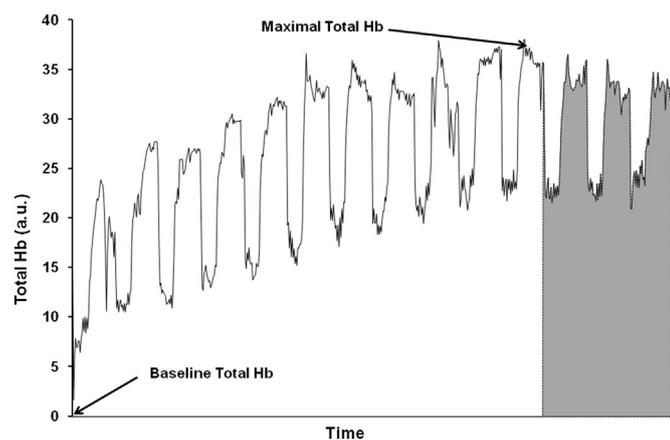
## 3. Results

### 3.1. Plasma nitrate and nitrite concentrations

Plasma NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> concentrations were significantly increased in the BJ condition at baseline (NO<sub>3</sub><sup>-</sup>, BJ: 421.5 ± 107.4 μM vs. placebo: 39.4 ± 18.0 μM; NO<sub>2</sub><sup>-</sup>, BJ: 441 ± 184 nM vs. placebo: 212 ± 119 nM;  $P < 0.001$  for both) and during the recovery period (NO<sub>3</sub><sup>-</sup>, BJ: 405.1 ± 116.1 μM vs. placebo: 39.4 ± 20.9 μM; NO<sub>2</sub><sup>-</sup>, BJ: 327 ± 129 nM vs. placebo: 153 ± 68 nM;  $P < 0.001$  for both). There was no significant effect of time, or interaction between time and supplementation, on NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> plasma levels. There was no significant association between the increase in NO<sub>2</sub><sup>-</sup> levels and the increase in the number of work periods completed during the intermittent exercise test.

### 3.2. Isometric maximal knee extensor torque

Force production during isometric maximal voluntary contraction was unchanged following dietary NO<sub>3</sub><sup>-</sup> supplementation (BJ: 272.25 ± 54.90 N vs. placebo: 277.44 ± 67.04 N, NS).



**Fig. 2.** Data of a representative subject for the change of Total Hb during 14 repetitions of 15-sec work and 30-sec passive recovery periods. The dark gray area indicates the Area Under the Curve during the 3 last repetitions used for comparison between the 2 experimental conditions (beetroot juice supplementation versus placebo). Baseline Total Hb determined at rest (not represented).

### 3.3. Exercise performance

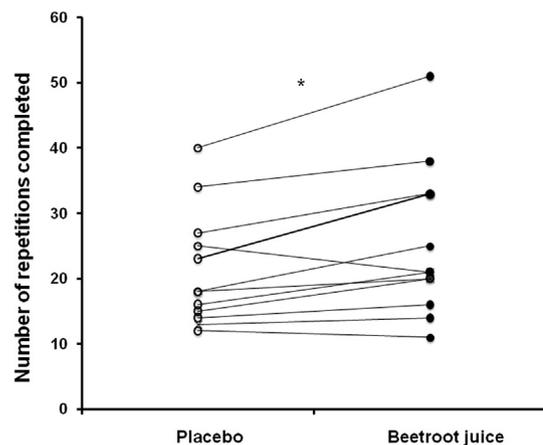
The mean power output during active sprints bouts was 472.5 ± 42.5 W during the BJ condition and 468.8 ± 43.4 W during the placebo condition (NS), and was close to the targeted power output (461.7 ± 64.5 W) calculated as 170% of the Maximal Aerobic Power. The total work performed in the BJ condition (168.1 ± 60.2 kJ) was significantly higher than in the PL condition (142.0 ± 46.8 kJ;  $P < 0.05$ ). The number of work periods completed until volitional exhaustion and duration of exercise were significantly increased after the BJ supplementation (BJ: 26.1 ± 10.7 reps and 19.6 ± 8.1 min vs. placebo: 21.8 ± 8.0 reps and 16.4 ± 6.0 min,  $P < 0.05$ ). Furthermore, there was no order effect on the number of work periods completed (1st session: 22.0 ± 8.8 reps, 2nd session: 24.0 ± 9.9, NS). Individual results are reported in Fig. 3.

### 3.4. Gas exchange

Whole exercise mean VO<sub>2</sub> and VCO<sub>2</sub> were unaffected by the NO<sub>3</sub><sup>-</sup> supplementation (Table 2). There were no significant differences for VO<sub>2</sub> and VCO<sub>2</sub> during both work and recovery periods between the BJ and placebo conditions during TM-repetitions. However, TM-ΔVO<sub>2</sub> was significantly lower during the BJ than during the placebo condition ( $P < 0.05$ ), and there was a trend for lower TM-ΔVCO<sub>2</sub> during the BJ condition ( $P = 0.09$ ). There was a strong trend toward higher V<sub>E</sub> in the BJ condition compared to the placebo condition ( $P = 0.053$ ). Data for VO<sub>2</sub> and V<sub>E</sub> during the first six work periods and TM-work periods at volitional exhaustion are presented in Figs. 4 and 5. Oxygen uptake at the end of the 6th active sprint represented 80.2 ± 6.0 %VO<sub>2peak</sub> in the BJ condition and 81.8 ± 6.9 %VO<sub>2peak</sub> in the placebo condition (NS), and 85.9 ± 11.6 %VO<sub>2max</sub> and 86.7 ± 7.8 %VO<sub>2peak</sub> in the BJ and placebo conditions respectively during TM-work periods (NS). There were no significant associations between the change in plasma NO<sub>3</sub><sup>-</sup> or NO<sub>2</sub><sup>-</sup> levels and the change of VO<sub>2</sub> between the placebo and BJ conditions.

### 3.5. Resting hemodynamics, local muscle oxygenation and muscle microvascular THb concentration during exercise

Nitrate supplementation had no significant effect on resting SBP, DBP, heart rate as well as on diameter and hemodynamic of the femoral artery (Table 3). Results from NIRS measurements are



**Fig. 3.** Individual data point with a placebo (empty circles) and a dietary NO<sub>3</sub><sup>-</sup> (beetroot juice) supplementation (full circles) for the number of repetitions completed before volitional exhaustion during an intermittent exercise test at supramaximal intensity. \* indicates a significant difference between the beetroot juice and placebo conditions ( $P < 0.05$ ).

**Table 2**  
Effects of a 3-day dietary nitrate supplementation (beetroot juice) on gas exchange parameters during exercise.

|  |  | Beetroot juice | Placebo      |
|--|--|----------------|--------------|
| $V_E$ (L min <sup>-1</sup> )             | Whole exercise                           | 98.3 ± 10.9*   | 95.2 ± 7.3   |
|  | TM-Work periods                          | 122.9 ± 16.7   | 127.5 ± 20.0 |
|  | TM-Recovery periods                      | 101.0 ± 12.8   | 96.8 ± 10.8  |
|  | Δrecovery-work                           | -20.7 ± 17.2   | -29.5 ± 19.2 |
|  | VO <sub>2</sub> (mL min <sup>-1</sup> )  | 2735 ± 345     | 2787 ± 346   |
| VO <sub>2</sub> (mL min <sup>-1</sup> )  | Whole exercise                           | 2735 ± 345     | 2787 ± 346   |
|  | TM-Work periods                          | 2936 ± 436     | 3031 ± 435   |
|  | TM-Recovery periods                      | 2777 ± 330     | 2759 ± 331   |
|  | Δrecovery-work                           | -132 ± 161*    | -284 ± 200   |
|  | VCO <sub>2</sub> (mL min <sup>-1</sup> ) | 2994 ± 278     | 3045 ± 313   |
| VCO <sub>2</sub> (mL min <sup>-1</sup> ) | Whole exercise                           | 2994 ± 278     | 3045 ± 313   |
|  | TM-Work periods                          | 2912 ± 326     | 2903 ± 296   |
|  | TM-Recovery periods                      | 3344 ± 409     | 3498 ± 493   |
|  | Δrecovery-work                           | 413 ± 336      | 589 ± 348    |
|  | RER                                      | 1.10 ± 0.07    | 1.10 ± 0.07  |
| RER                                      | Whole exercise                           | 1.10 ± 0.07    | 1.10 ± 0.07  |
|  | TM- Work periods                         | 1.14 ± 0.09    | 1.15 ± 0.10  |
|  | TM-Recovery periods                      | 1.06 ± 0.07    | 1.06 ± 0.07  |

Data are mean ± SD.

\* Different from placebo condition ( $P < 0.05$ ), TM-denotes time-matched comparison for 3 successive work + recovery periods, Δ indicates difference between work and passive recovery periods.

**Table 3**  
Effects of a 3-day dietary nitrate supplementation (beetroot juice) on resting hemodynamic parameters.

|   | Beetroot juice | Placebo       |
|---|----------------|---------------|
| Diastolic pressure (mmHg)                                       | 63.9 ± 5.1     | 64.0 ± 6.1    |
| Systolic pressure (mmHg)  | 127.2 ± 9.8    | 128.5 ± 7.3   |
| Heart rate (bpm)  | 69.1 ± 8.7     | 70.1 ± 9.5    |
| Femoral artery diameter (mm)                                    | 8.85 ± 0.98    | 8.91 ± 0.88   |
| Femoral artery blood flow (mL min <sup>-1</sup> )               | 911 ± 223      | 846 ± 166     |
| Arterial compliance (mm <sup>2</sup> mmHg <sup>-1</sup> )       | 0.108 ± 0.037  | 0.091 ± 0.023 |
| Vascular conductance (mL min <sup>-1</sup> mmHg <sup>-1</sup> ) | 10.70 ± 2.54   | 9.98 ± 2.27   |

Data are mean ± SD.

presented in Table 4. Baseline and maximal HbO<sub>2</sub>, HHb and THb, and minimal HbO<sub>2</sub> were not significantly different between the two conditions. Nevertheless, TM-AUC-THb was significantly higher in the BJ than in the placebo condition ( $P < 0.05$ ), indicating higher muscle microvascular RBC concentration. There were no differences between the two conditions regarding TM-AUC-HHb and TM-AUC-HbO<sub>2</sub>. There was no significant association between the increase in NO<sub>3</sub><sup>-</sup> or NO<sub>2</sub><sup>-</sup> plasma levels and the change in NIRS-derived parameters between the two conditions, although a trend was observed for the increase in plasma NO<sub>2</sub><sup>-</sup> levels and the increase of AUC-THb ( $r = 0.54$ ,  $P = 0.08$ ) between the placebo and BJ conditions.

**Capillary blood lactate** There was no significant difference in capillary blood lactate concentration at the end of the 10th repetition (BJ: 8.4 ± 1.3 mmol L<sup>-1</sup> vs. placebo: 9.1 ± 2.7 mmol L<sup>-1</sup>, NS), 20th repetition (BJ: 11.0 ± 2.0 mmol L<sup>-1</sup> vs. placebo: 10.6 ± 1.2 mmol L<sup>-1</sup>, NS) and 3 min post exercise trial (BJ: 11.5 ± 2.6 mmol L<sup>-1</sup> vs. placebo: 11.7 ± 2.3 mmol L<sup>-1</sup>, NS). Owing to the small number of subjects who completed more than 20 work periods in the two conditions, data for capillary blood lactate are not reported after the 20th active sprint.

**4. Discussion**

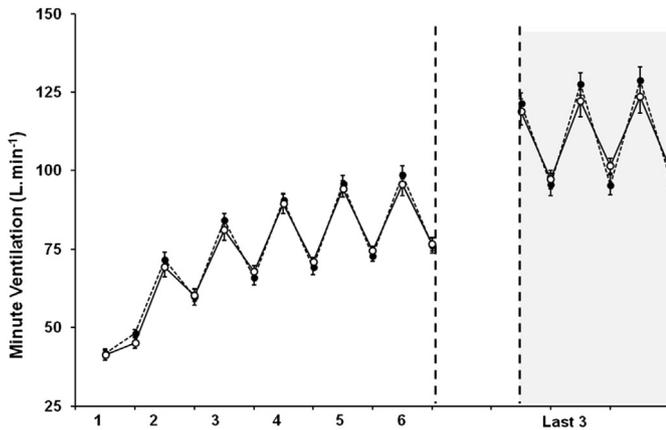
There are three main findings from this study which aimed at exploring the effect of NO<sub>3</sub><sup>-</sup> supplementation on the tolerance of exercise at supramaximal intensity. Firstly, the number of sprints completed before reaching volitional exhaustion increased on average by 30 %, which is in the high range of improvement reported during moderate to maximal intensity exercise at a fixed work rate [22]. Secondly, this study confirms that dietary NO<sub>3</sub><sup>-</sup> do not decrease VO<sub>2</sub> during supramaximal intensity intermittent exercise [19], in contrast to the improvement of oxidative efficiency

**Table 4**  
Effects of a 3-day dietary nitrate supplementation (beetroot juice) on NIRS derived indexes of muscle oxygenation and microvascular RBC concentration.

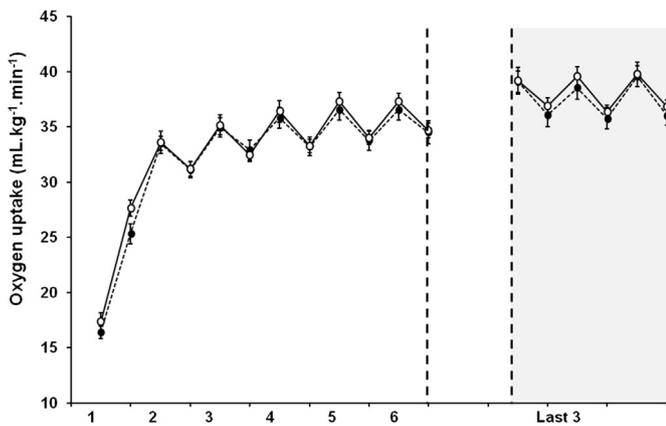
|                  |          | Beetroot juice | Placebo      |
|------------------|----------|----------------|--------------|
| HbO <sub>2</sub> | Baseline | 1.5 ± 4.5      | -0.5 ± 2.6   |
|                  | Maximal  | 14.0 ± 10.0    | 11.8 ± 10.1  |
|                  | TM-AUC+  | 1659 ± 1730    | 1511 ± 1354  |
|                  | Minimal  | -17.8 ± 8.2    | -19.2 ± 10.3 |
|                  | TM-AUC-  | -5177 ± 2535   | -5008 ± 2579 |
| HHb              | Baseline | -0.3 ± 8.3     | 0.8 ± 4.6    |
|                  | Maximal  | 33.2 ± 17.9    | 37.1 ± 24.0  |
|                  | TM-AUC   | 9199 ± 1752    | 8772 ± 2104  |
| THb              | Baseline | 0.8 ± 6.9      | 0.4 ± 5.1    |
|                  | Maximal  | 26.8 ± 12.9    | 27.1 ± 15.0  |
|                  | TM-AUC   | 9662 ± 1228*   | 8178 ± 1589  |

Data are mean ± SD, TM-denotes time-matched comparison for 3 successive work + recovery periods.

\* Different from placebo condition ( $P < 0.05$ ).



**Fig. 4.** Minute ventilation (L min<sup>-1</sup>) during 15-sec active and 30-sec passive recovery periods. Empty circles: placebo, full circles: beetroot juice. Data are from the first six repetitions and the 3 time-matched repetitions at volitional exhaustion (shaded area). Values are mean ± S.E.M.



**Fig. 5.** Oxygen uptake (VO<sub>2</sub>, mL kg<sup>-1</sup> min<sup>-1</sup>) during 15-sec work and 30-sec passive recovery periods. Empty circles: placebo, full circles: beetroot juice. Data are from the first six repetitions and the 3 time-matched repetitions at volitional exhaustion (shaded area). Values are mean ± S.E.M.

reported at lower exercise intensities. Thirdly, microvascular RBC concentration of the working muscle for TM-work periods was maintained at a higher-level following  $\text{NO}_3^-$  supplementation, and may have contributed to improved exercise tolerance.

#### 4.1. Effect of $\text{NO}_3^-$ supplementation on plasma $\text{NO}_3^-/\text{NO}_2^-$ levels and gas exchange

The increase in plasma  $\text{NO}_2^-$  after the 3-day beetroot juice supplementation were within the range of values reported in previous studies in young and healthy subjects [6,18,23,24]. Plasma  $\text{NO}_3^-$  levels were slightly higher than those reported in previous studies with dietary  $\text{NO}_3^-$  supplementation, and this may be explained by the use of the Griess method, whereas chemiluminescence analysis has been used in many other studies [2,12,25,26]. There are limited data available regarding the effect of  $\text{NO}_3^-$  supplementation on tolerance to supramaximal intermittent exercise and its associated  $\text{VO}_2$ . Wylie et al. showed a lesser decrease in running speed in moderately trained men who performed an intermittent running test [18], but Christensen et al. [27] reported unchanged power output during repeated sprints in elite cyclists. However, none of these two studies reported  $\text{VO}_2$  measurements during supramaximal intensity exercise. In contrast, Martin et al. who examined the effect of  $\text{NO}_3^-$  supplementation on the ability to repeat 8-sec sprints at 200 % of the Aerobic Peak Power with 30-sec active recovery periods, reported impaired performances with unaffected  $\text{VO}_2$  [19]. Despite, a similar design for the intermittent exercise in the latter study, that also elicited  $\text{VO}_2 > 75\% \text{VO}_{2\text{max}}$  and a population with a similar level of  $\text{VO}_{2\text{max}}$  ( $49.6 \pm 11.8 \text{ mL kg}^{-1} \text{ min}^{-1}$ ) the effect of  $\text{NO}_3^-$  supplementation on performance outcomes was opposite to those of the present paper. The lower intensity that we used (170 % of the Aerobic Peak Power), with passive rather than active recovery periods, may have been sufficient to increase PCr resynthesis, and the ability to complete a higher number of exercise bouts, via an NO-dependent effect. However, similarly to Martin et al., we also observed that  $\text{NO}_3^-$  supplementation had no effect on average  $\text{VO}_2$  and  $\text{VCO}_2$  during exercise [19], and this is a major difference with studies showing better exercise tolerance during submaximal to maximal intensity exercise. At these intensities,  $\text{NO}_3^-$  supplementation is associated with higher oxidative efficiency, as indicated either by a lower  $\text{VO}_2$  for a given power output [8,11,23,24] or an unchanged  $\text{VO}_2$  with a higher power output [28,29]. One possible explanation is that the significant increase in oxidative efficiency usually observed during exercise of intensities up to  $\text{VO}_{2\text{max}}$  may be mitigated in the present study and in the study by Martin et al. [19] by a higher fast twitch muscle fibers recruitment, which have a higher  $\text{O}_2$  cost of contraction [30,31]. It is however unlikely that the improved exercise tolerance was caused by an increased contribution of the anaerobic glycolysis to energy expenditure as capillary blood lactate after the 10th, 20th work periods and at volitional exhaustion were similar between the BJ and placebo condition.

We observed a strong trend ( $P = 0.053$ ) toward higher ventilation after  $\text{NO}_3^-$  supplementation despite unchanged  $\text{VO}_2$ . Larsen et al. who reported decreased  $\text{VO}_{2\text{max}}$  with maintained maximal aerobic power showed that  $\text{VE}/\text{VO}_2$  was significantly increased in subjects who received a sodium nitrate treatment [11]. It is excluded that  $V_E$  was increased in response to  $\text{CO}_2$  production as  $\text{VCO}_2$  was similar between the two conditions.

#### 4.2. Effect of $\text{NO}_3^-$ supplementation on muscle $\text{O}_2$ delivery and utilization

Compared to exercise in the submaximal to maximal intensity domains, an exercise performed at supramaximal intensity requires

a more important recruitment of fast twitch muscle fibers, where microvascular perfusion is highly sensitive to changes in NO availability [32–34]. An important finding from the present study is that  $\text{NO}_3^-$  supplementation resulted in significantly higher AUC-THb over TM-work periods. This may indicate that increased NO availability could increase muscle microvascular RBC concentration, facilitate the transition of muscle microvascular hematocrit level toward systemic level, and ultimately improve the blood myocyte  $\text{O}_2$  flux [35]. With the exception of a study by Kenjale et al. [36] in a pathological population with peripheral artery disease, which is characterized by muscle hypoxia, there was to date no evidence in humans of higher muscle microvascular RBC concentration and  $\text{O}_2$  delivery during exercise following  $\text{NO}_3^-$  supplementation. However, in the only study where THb was reported in healthy subjects, THb was unaffected by dietary  $\text{NO}_3^-$  supplementation during submaximal and maximal exercise performed in hypoxia [29].

The change in Hhb is considered to appropriately reflect the balance between  $\text{O}_2$  delivery and extraction in the microvascular bed of skeletal muscle [37]. The lack of difference for TM-AUC-Hhb between the two conditions indicates that local muscle deoxygenation was not reduced with  $\text{NO}_3^-$  supplementation during the work periods despite the higher microvascular RBC concentration, and is consistent with the lack of change for  $\text{VO}_2$ . However, despite a significantly higher TM-AUC-THb, maximal THb was not significantly higher during the  $\text{NO}_3^-$  condition, indicating that  $\text{NO}_3^-$  supplementation may have allowed a more stable RBC concentration throughout the succession of active and recovery periods, which is consistent with significantly smaller variations of  $\text{VO}_2$  between work and recovery. Regarding muscle microvascular blood flow, a limitation of the study is that NIRS only provides an estimate of the change in RBC concentrations, but not velocity in the muscle region of interest. A slowing down of RBC velocity, and consequently an increased capillary transit time would facilitate muscle oxygen extraction [38]. However, the lack of difference for maximal Hhb and AUC-Hhb do not suggest any difference in oxygen extraction and do not support the hypothesis of increased capillary transit time.

#### 4.3. Resting hemodynamic and blood pressure

In order to determine whether  $\text{NO}_3^-$  supplementation had vasodilatory effects independently of exercise, we performed resting hemodynamics measurements of the common femoral artery before exercise. Measurements of the femoral artery blood flow and conductance confirmed that there was no enhanced vasodilation during the pre-exercise period. Hence,  $\text{NO}_3^-$  supplementation affects muscle microvascular RBC concentration during exercise more than large vessels hemodynamic parameters under resting condition. This supports a role for the local hypoxic and low-pH in muscle environment during supramaximal exercise, that creates favorable conditions for a higher rate of conversion of  $\text{NO}_2^-$  to NO, thereby providing a stimulus for vasodilation and hyperemia during recovery periods. Using NIRS, Bailey et al. [6] reported a local increase in resting blood volume of the vastus lateralis, suggesting a peripheral microvascular vasodilation and providing an explanation to the significantly decreased systolic blood pressure, but this study showed did not show any effect of  $\text{NO}_3^-$  supplementation on muscle microvascular THb concentration once subjects were exercising.

#### 4.4. Effect of $\text{NO}_3^-$ supplementation on muscle contractile function

By using a supramaximal intensity of exercise in the present study, we sought to elicit an important activation of fast twitch

muscle fibers which may be more sensible than slow twitch muscle fibers to the effect of increased  $\text{NO}_2^-$  levels [39]. To determine whether the expected tolerance to exercise could be partly related to an effect of  $\text{NO}_3^-$  supplementation on muscle contractile function, we measured force during a single knee extensor isometric contraction. As previously shown,  $\text{NO}_3^-$  supplementation did not affect maximal isometric muscle force [1,40]. However, Coggan et al. indicated that dietary  $\text{NO}_3^-$  significantly increased peak power at high angular velocity, which may better reflect the muscle contraction regimen of a cycling exercise [1]. In addition, the force levels developed by the quadriceps muscle during an exercise at 170 % of the aerobic peak power as in the present study are submaximal since the force produced during a pedal thrust at 100%  $\text{VO}_{2\text{max}}$  reaches at most 25% of the quadriceps force during maximal voluntary contraction [41]. Thus, even though we did not observe any effect of  $\text{NO}_3^-$  supplementation on maximal isometric force during a leg extensor exercise, it is not excluded that  $\text{NO}_3^-$  supplementation has a favorable effect on submaximal muscle contractile function during a dynamic exercise, such as cycling, that may have delayed the development of fatigue.

#### 4.5. Experimental and methodological considerations

A limitation of NIRS lies in its inability to separate between the signals from the oxygenated or deoxygenated forms of myoglobin and hemoglobin. Some consider that the hemoglobin to myoglobin ratio above >5 in the region of interest almost exclusively reflects change in Hb oxygenation, whereas others have indicated that Mb could represent between 35 and 50 % of the NIRS signal [42]. In the present study, the combined signal for Mb and Hb is only a minor limitation and the results that we report rather represent an integrated view of the effect of dietary  $\text{NO}_3^-$  supplementation on both muscle oxygen delivery and utilization. Moreover, as total myoglobin concentration does not change in the region of interest, our conclusion regarding the effect of  $\text{NO}_3^-$  supplementation on THb are unaffected by the relative contribution of Mb to NIRS signals.

In the present study, the change in exercise tolerance was the primary criteria to describe the effect of dietary nitrate supplementation, and secondary criteria included the measurement of oxygen uptake, local muscle oxygenation and RBC volume (physiological parameters) to provide objective assessment of physiological effects of dietary nitrate. With the exception of muscle RBC volume, the other physiological parameters were unaffected and are unlikely to explain the higher number of exercise bouts completed when supplemented with dietary nitrate. Because exercise tolerance is determined both by peripheral and central fatigue, we cannot exclude that increased  $\text{NO}_2^-$  bioavailability affected cerebral  $\text{O}_2$  extraction as shown by Thompson et al. (2015), even though the effects on perceived exertion and mental fatigue are yet to be demonstrated. In addition, the assessment of muscle force decline and muscle glycogen depletion after the intermittent exercise test were not performed in the present study, but may have provided additional insight into the mechanisms that resulted in increased exercise tolerance. Otherwise, we considered that performance can also decline with the repetition of exhaustive exercises and subjects did not perform familiarization trials. This is a potential limitation, because the familiarization of subjects with the repeated-sprint test can also improve the reliability of work and power measures [43]. However, with regard to the lack of difference in the number of work periods completed between the 1st and the 2nd session, and given that the subjects took the  $\text{NO}_3^-$  supplementation and placebo in a randomized order, we are confident that the learning effect was minimal on the performance

outcomes. Two subjects out of twelve performed less repetitions following dietary  $\text{NO}_3^-$  supplementation, despite increased plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  levels consistent with those of the whole population. This is similar to previous studies, which also showed an overall increase in time to exhaustion following dietary nitrate supplementation, but also that some individuals have unchanged or even decreased exercise tolerance [29]. Improved exercise tolerance results from the interaction of NO effects at multiple levels rather than uniquely improved mitochondrial oxidative efficiency or muscle perfusion during exercise. It cannot be excluded that in some individuals detrimental effects of NO, such as increased nitrosative stress, may override the beneficial effect of increased  $\text{NO}_2^-$  levels and result in unchanged or impaired performance. Although improved exercise tolerance has been reported with time interval between beetroot juice ingestion and the beginning of the exercise test similar to the present study [10,26,44,45], it is also possible that a time interval shorter than 3 h would have yielded different results regarding exercise tolerance or gas exchanges, as plasma nitrite levels tend to peak around 2 h after the dietary nitrate ingestion before slowly declining [18,46].

Finally, a limitation of the study is that with the exception of the significant difference for AUC-THb between the two conditions, and a trend for an association between the change for  $\text{NO}_2^-$  plasma levels and the change for AUC-THb, we observed no significant change in any other parameter that could explain the improved exercise tolerance. This is in contrast to studies showing improved exercise tolerance or performance with dietary nitrate supplementation for exercise in the aerobic intensity domain, that also report a reduced  $\text{O}_2$  cost of exercise [6,10]. Nevertheless, despite the cross-over design of our study which eliminated the among participant variability, the sample size may have been too small to detect statistically significant effects of dietary nitrate on oxygen uptake and other physiological parameters.

## 5. Conclusions

To summary, our findings indicate that  $\text{NO}_3^-$  supplementation via beetroot juice ingestion is associated with higher exercise tolerance during supramaximal intermittent exercise in subjects with  $\text{VO}_{2\text{max}}$  between 40 and 55  $\text{mL kg}^{-1} \text{min}^{-1}$ , that is aerobic fitness level typical of subjects participating to team sports, and sensitive to the effects of dietary nitrate on exercise tolerance [47]. In contrast to exercise ranging from low intensity up to 100%  $\text{VO}_{2\text{max}}$  this occurred in the absence of significant effects on exercise  $\text{VO}_2$ , but with higher muscle microvascular RBC concentration. These results are thought to be related to a strong contribution to the muscle work of fast twitch muscle fibers, which are more sensitive to changes in NO availability. Resting blood pressure and femoral artery hemodynamic were unaffected. This suggests that the biochemical (hypoxia and acidosis) conditions present in skeletal muscle during strenuous work may be needed for a significant conversion of  $\text{NO}_2^-$  to NO with effects on muscle microvascular function. Future studies in subjects with either a high or a low proportion of fast twitch muscle fibers are required to confirm the respective effects on  $\text{NO}_3^-$  supplementation on muscle force production, motor unit activation and muscle microvascular RBC concentration. However, it is unlikely that increased  $\text{NO}_3^-$  and  $\text{NO}_2^-$  plasma levels increased the contribution of anaerobic glycolysis to energy expenditure.

## Funding and conflicts of interest

The authors declare that they have no conflict of interest. No funding was received for this study.

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J.A., F.X.G., J.B. and G.C. designed the research (project conception, development of overall research plan, and study oversight); J.A., F.X.G., J.B., G.C. and M.P.C. conducted the research (hands-on conduct of the experiments and data collection); J.A., F.X.G., J.B. and M.P.C. analyzed the data or performed statistical analysis; J.A., F.X.G., J.B. and M.P.C. wrote the paper; and J.A., J.B., and F.X.G. have primary responsibility for the final content.

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