

Beetroot juice supplementation reduces whole body oxygen consumption but does not improve indices of mitochondrial efficiency in human skeletal muscle

J. Whitfield¹, A. Ludzki¹, G. J. F. Heigenhauser², J. M. G. Senden³, L. B. Verdijk³, L. J. C. van Loon³, L. L. Spriet¹ and G. P. Holloway¹

¹Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

²Department of Medicine, McMaster University, Hamilton, Ontario, Canada, L8N 3Z5

³Department of Human Movement Sciences, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, 6200 MD Maastricht, The Netherlands

Key points

- Oral consumption of nitrate (NO_3^-) in beetroot juice has been shown to decrease the oxygen cost of submaximal exercise; however, the mechanism of action remains unresolved.
- We supplemented recreationally active males with beetroot juice to determine if this altered mitochondrial bioenergetics.
- Despite reduced submaximal exercise oxygen consumption, measures of mitochondrial coupling and respiratory efficiency were not altered in muscle.
- In contrast, rates of mitochondrial hydrogen peroxide (H_2O_2) emission were increased in the absence of markers of lipid or protein oxidative damage.
- These results suggest that improvements in mitochondrial oxidative metabolism are not the cause of beetroot juice-mediated improvements in whole body oxygen consumption.

Abstract Ingestion of sodium nitrate (NO_3^-) simultaneously reduces whole body oxygen consumption (\dot{V}_{O_2}) during submaximal exercise while improving mitochondrial efficiency, suggesting a causal link. Consumption of beetroot juice (BRJ) elicits similar decreases in \dot{V}_{O_2} but potential effects on the mitochondria remain unknown. Therefore we examined the effects of 7-day supplementation with BRJ (280 ml day⁻¹, ~26 mmol NO_3^-) in young active males ($n = 10$) who had muscle biopsies taken before and after supplementation for assessments of mitochondrial bioenergetics. Subjects performed 20 min of cycling (10 min at 50% and 70% $\dot{V}_{\text{O}_{2\text{peak}}}$) 48 h before 'Pre' (baseline) and 'Post' (day 5 of supplementation) biopsies. Whole body \dot{V}_{O_2} decreased ($P < 0.05$) by ~3% at 70% $\dot{V}_{\text{O}_{2\text{peak}}}$ following supplementation. Mitochondrial respiration in permeabilized muscle fibres showed no change in leak respiration, the content of proteins associated with uncoupling (UCP3, ANT1, ANT2), maximal substrate-supported respiration, or ADP sensitivity (apparent K_m). In addition, isolated subsarcolemmal and intermyofibrillar mitochondria showed unaltered assessments of mitochondrial efficiency, including ADP consumed/oxygen consumed (P/O ratio), respiratory control ratios and membrane potential determined fluorometrically using Safranin-O. In contrast, rates of mitochondrial hydrogen peroxide (H_2O_2) emission were increased following BRJ. Therefore, in contrast to sodium nitrate, BRJ supplementation does not alter key parameters of mitochondrial efficiency. This occurred despite a decrease in exercise \dot{V}_{O_2} , suggesting that the ergogenic effects of BRJ ingestion are not due to a change in mitochondrial coupling or efficiency. It remains to be determined if increased mitochondrial H_2O_2 contributes to this response.

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Corresponding author G. P. Holloway: Human Health and Nutritional Sciences, University of Guelph, 491 Gordon St., Guelph, ON, Canada, N1G 2W1. Email: ghollowa@uoguelph.ca

Abbreviations ANT, adenine nucleotide translocase; BRJ, beetroot juice; IMF, intermyofibrillar mitochondria; PDH, pyruvate dehydrogenase; PmFB, permeabilized muscle fibre bundles; P/O ratio, ADP consumed/oxygen consumed; RCR, respiratory control ratio; ROS, reactive oxygen species; SOD, superoxide dismutase; SS, subsarcolemmal mitochondria; UCP3, uncoupling protein 3.

Introduction

Nitric oxide (NO) is an important signalling molecule; it has been implicated in the regulation of many biological processes, including blood flow (as reviewed by Joyner & Tschakovsky, 2003), skeletal muscle excitation–contraction coupling (Andrade *et al.* 1998) and mitochondrial bioenergetics (Clerc *et al.* 2007; Boushel *et al.* 2012). While NO was originally thought to be exclusively generated from nitric oxide synthase-mediated oxidation of L-arginine (Moncada & Higgs, 1993), it is now acknowledged that NO can also be generated from the sequential reduction of nitrate (NO_3^-) and nitrite (NO_2^-) in a process dependent on bacterial nitrate reductases produced by facultative anaerobes in the oral cavity (Spiegelhalder *et al.* 1976; Govoni *et al.* 2008).

Given the diverse biological effects of NO, the oral consumption of NO_3^- has been investigated as a modality to alter metabolic responses to exercise. Indeed, amongst dietary supplements, nitrate is unique in that it decreases the oxygen cost of submaximal exercise in humans (Larsen *et al.* 2007, 2011; Bailey *et al.* 2009, 2010; Vanhatalo *et al.* 2010; Lansley *et al.* 2011; Cermak *et al.* 2012). While the mechanism(s) of action remain unresolved, one prominent working model is an improvement in mitochondrial coupling efficiency (Larsen *et al.* 2011). This possibility is supported by the *in vitro* observations that NO directly increases oxidative phosphorylation efficiency (Clerc *et al.* 2007), particularly when cytochrome oxidase is reduced (Bolaños *et al.* 1994; Brown & Cooper, 1994; Cleeter *et al.* 1994; Giuffrè *et al.* 2000). In addition, dietary supplementation with sodium nitrate for 3 days in humans improves various parameters within isolated mitochondria, including assessments of efficiency (P/O ratios and thermodynamic coupling) (Larsen *et al.* 2011). Altogether, sodium nitrate consumption and NO appear to have the capacity to improve mitochondrial bioenergetics through alterations in coupling efficiency.

Several vegetables have high NO_3^- concentrations, creating the potential for a natural food product to exert similar beneficial effects to sodium nitrate. To date, beetroot juice (BRJ) has been shown to reduce blood pressure (Webb *et al.* 2008; Bailey *et al.* 2009, 2010; Vanhatalo *et al.* 2010; Lansley *et al.* 2011), oxygen consumption during exercise (Bailey *et al.* 2009, 2010; Vanhatalo *et al.* 2010; Wylie *et al.* 2013a), and improve

exercise performance (Lansley *et al.* 2011a,b; Bond *et al.* 2012; Cermak *et al.* 2012; Wylie *et al.* 2013b; Peeling *et al.* 2015), vascular control (Ferguson *et al.* 2013), and muscle contractile function in humans (Fulford *et al.* 2013; Haider & Folland, 2014). Indirect assessments of muscle metabolism via ^{31}P -magnetic resonance spectroscopy suggest that BRJ consumption reduces ATP turnover during muscle contraction, which probably contributes to the observed attenuation in oxygen consumption during exercise (Bailey *et al.* 2010). It remains to be determined if BRJ, similar to sodium NO_3^- , improves mitochondrial oxidative phosphorylation efficiency to further explain the observed reduction in whole body oxygen consumption.

In addition to exercise performance, NO_3^- supplements are being investigated as potential therapeutic interventions for several clinical populations, including chronic obstructive pulmonary disease (Kerley *et al.* 2015), hypertension (Kapil *et al.* 2015), heart failure (Zamani *et al.* 2015; Ormerod *et al.* 2015) and insulin resistance (Nyström *et al.* 2012). Understanding the mechanism(s) by which BRJ alters excitation–contraction coupling/metabolism is therefore essential to ensure that undesirable ‘off-target’ effects do not occur. For instance, the reported reduction in adenine nucleotide translocase (ANT) proteins and uncoupling protein 3 (UCP3), and the increase in coupling efficiency following sodium nitrate consumption (Larsen *et al.* 2011), may increase proton motive force and mitochondrial reactive oxygen species (ROS) emission. ANT has previously been shown to contribute between one-half and two-thirds of basal mitochondrial leak respiration (Brand *et al.* 2005) and a reduction in ANT protein has been associated with impairments in submaximal ADP sensitivity and oxidative stress (Smith *et al.* 2013).

Therefore, the primary aim of the current study was to determine if BRJ altered various indices of mitochondrial bioenergetics. The current data suggest that an improvement in mitochondrial oxidative efficiency is not required for BRJ-mediated reductions in whole body \dot{V}_{O_2} . However, an increase in mitochondrial H_2O_2 emission was observed in both permeabilized muscle fibres (PmFB) and in isolated mitochondria following supplementation, suggesting BRJ can modify mitochondrial bioenergetics.

Methods

Ethics approval

Thirteen healthy, recreationally active males (23.6 ± 0.6 years, 79.4 ± 1.5 kg, $\dot{V}_{O_{2peak}} 49.6 \pm 1.4$ ml⁻¹ min⁻¹ kg⁻¹) were initially recruited for this study, of which 10 underwent biopsy trials both prior to, and following, supplementation. Prior to participation, written informed consent was obtained from all subjects. All procedures were approved by the Research Ethics Boards of the University of Guelph (Guelph, Ontario) and McMaster University (Hamilton, Ontario), and conform to the *Declaration of Helsinki*.

Pre-experimental tests

Prior to supplementation, subjects underwent a continuous incremental cycling test to exhaustion on an electronically braked cycle ergometer (Lode, Groningen, the Netherlands) for determination of pulmonary $\dot{V}_{O_{2peak}}$, as well as a 20 min cycle test with 10 min at a workload corresponding to 50% $\dot{V}_{O_{2peak}}$ followed by an immediate step increase to a workload corresponding to 70% $\dot{V}_{O_{2peak}}$ for an additional 10 min. For familiarization, subjects performed a practice trial of the 20 min test, which also served to confirm subjects were at the correct workloads. Trials were separated by 48 h. Ventilation and expired oxygen and carbon dioxide concentrations were measured throughout all tests using a metabolic measurement system (MOXUS Modular VO₂ System, AEI Technologies, Pittsburgh, PA, USA), and the last 5 min at each power output was used for analysis (Fig. 1).

Study design

Following the completion of pre-experimental tests (≤ 48 h) and an overnight fast, 10 subjects had four biopsies taken from the vastus lateralis as previously described (Bergstrom, 1975). A small portion of the first biopsy was taken and immediately used for the preparation of permeabilized muscle fibres (PmFB; described below). The remaining portion was combined with the second and third biopsies for the isolation of mitochondrial sub-fractions. The fourth biopsy was flash-frozen for Western blotting. Due to limited tissue yield, mitochondrial isolations and Western blotting could only be performed on a subset of five participants. Following the biopsies, subjects began supplementing with concentrated BRJ (2×6.5 mmol NO₃⁻/70 ml Beet It Sport; James White Drinks, Ipswich, UK; taken twice daily for a total daily intake of ~ 26 mmol NO₃⁻) for 7 days in an effort to maintain elevated blood plasma NO₃⁻ and NO₂⁻ levels. On day 5 of the supplementation period, subjects repeated the submaximal cycling exercise at 50 and 70% of $\dot{V}_{O_{2peak}}$.

On the morning of day 7 after an overnight fast, subjects consumed $2 \times$ Beet It Sport shots 90 min prior to having four biopsies taken from the vastus lateralis of the contralateral leg for post-supplementation measurements. Subjects were asked to refrain from consuming foods rich in nitrates for the duration of the study, and to abstain from the use of antibacterial mouthwash, as this has been shown to attenuate the conversion of nitrate to nitrite by commensal bacteria (Govoni *et al.* 2008).

Preparation of permeabilized fibres

A small portion of each biopsy was placed in ice-cold BIOPS (50 mM MES, 7.23 mM K₂EGTA, 2.77 mM CaK₂EGTA, 20 mM imidazole, 0.5 mM DTT, 20 mM taurine, 5.77 mM ATP, 15 mM PCr, and 6.56 mM MgCl₂.H₂O; pH 7.1) and separated under a microscope into bundles using fine-tipped forceps as described previously (Perry *et al.* 2012). Fibre bundles were then treated with 30 μ g ml⁻¹ saponin for 30 min at 4°C, then either washed for 15 min in MiR05 respiration buffer (0.5 mM EGTA, 10 mM KH₂PO₄, 110 mM sucrose, and 1 mg ml⁻¹ fatty acid-free bovine serum albumin (BSA); pH 7.1) for respiration analysis, or buffer Z (105 mM K-Mes, 30 mM KCl, 1 mM EGTA, 10 mM K₂HPO₄, 5 mM MgCl₂.H₂O, 0.005 mM pyruvate, 0.002 mM malate with 5 mg ml⁻¹ fatty acid-free BSA; pH 7.4) for measurements of H₂O₂ emission as described below.

Mitochondrial isolation

Mitochondrial subsarcolemmal (SS) and intermyofibrillar (IMF) populations were isolated by differential centrifugation as described previously (Campbell *et al.* 2004) with minor modifications (Matravadia *et al.* 2014). Muscle was quickly minced and diluted in 1 ml mg⁻¹ isolation buffer (100 mM sucrose, 100 mM KCl, 50 mM Tris HCl, 1 mM KH₂PO₄, 0.1 mM EGTA, 2 mg ml⁻¹ fatty acid-free BSA, 1 mM ATP; pH 7.4) and homogenized using a Polytron with a Teflon head. SS mitochondria underwent centrifugation at 800 g for 10 min and the supernatant was pelleted at 10,000 g for 10 min before being washed in Mg²⁺-absent MiR05 buffer (0.5 mM EGTA, 60 mM potassium lactobionate, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, 1 mg ml⁻¹ BSA; pH 7.1), and spun again at 10,000 g for 10 min. After the initial 800 g spin, the IMF mitochondrial pellet was resuspended 10-fold in isolation buffer and treated with 0.025 ml g⁻¹ protease for 5 min before being diluted 10-fold in isolation buffer and spun again at 5000 g for 5 min. The IMF pellet was resuspended in isolation buffer and pelleted at 800 g for 10 min after which the supernatant was pelleted at 10,000 g for 10 min before being washed in Mg²⁺-absent MiR05 buffer, and spun again at 10,000 g for 10 min. The final pellets for both

SS and IMF isolations were resuspended in Mg^{2+} -absent MiR05 buffer prior to respiration, H_2O_2 emission and membrane potential measurements. Protein content was quantified spectrophotometrically using bicinchoninic acid.

Mitochondrial respiration

Measurements of O_2 consumption were performed in MiR05 respiration medium on prepared permeabilized fibres using an Oxygraph-2 K (Oroboros Instruments, Innsbruck, Austria) at $37^\circ C$ in the presence of $25 \mu M$ blebbistatin as previously described (Perry *et al.* 2011). Mitochondrial kinetics in PmFB were analysed using three separate titration protocols. ADP kinetics were analysed in the presence and absence of 20 mM creatine. Both ADP titrations were initiated with 2 mM malate and 10 mM pyruvate, and ADP was titrated at various concentrations. Pyruvate kinetics were determined in the presence of 5 mM ADP, 5 mM malate, and 20 mM creatine while pyruvate was titrated in at various concentrations. Glutamate (10 mM) and 10 mM succinate were added following the pyruvate and ADP titrations to determine maximum mitochondrial respiration. Finally, $10 \mu M$ cytochrome *c* was added, with <10% increase in respiration in all measurements indicating outer mitochondrial membrane integrity. All fibres were recovered from the respirometer, freeze-dried, and weighed for normalization to muscle bundle weight. The apparent K_m for pyruvate and ADP were determined as previously described (Perry *et al.* 2012).

In isolated mitochondria, respiration experiments were performed at $25^\circ C$ as described previously (Lally *et al.* 2013). State IV respiration was determined in the presence of 5 mM pyruvate and 2 mM malate. To determine P/O ratios, 50 μM ADP was added followed by two boluses of 100 μM ADP. The change in oxygen concentration in the chamber following each of the two 100 μM ADP boluses was averaged and used for the calculation of P/O ratios. Maximal state III respiration was determined by the addition of 5 mM ADP, with 10 mM glutamate and

10 mM succinate added to determine maximal complex I and maximal complex I + II respiration, respectively.

H_2O_2 emission

Mitochondrial H_2O_2 emission in PmFB was measured in buffer Z (105 mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K_2HPO_4 , 5 mM $MgCl_2 \cdot H_2O$, 0.005 mM pyruvate, 0.002 mM malate with 5 mg ml^{-1} fatty acid-free BSA; pH 7.4) fluorometrically (Lumina; Thermo Scientific, Waltham, MA, USA) at $37^\circ C$ in the presence of 25 μM blebbistatin. Cuvettes contained 10 $\mu g ml^{-1}$ oligomycin, 10 μM Amplex Red (Invitrogen, Carlsbad, CA, USA), 0.5 U ml^{-1} horseradish peroxidase, and in both the presence and absence of 40 U ml^{-1} superoxide dismutase (SOD). In isolated mitochondria, H_2O_2 emission was measured in MiR05 at $25^\circ C$ in cuvettes containing 10 $\mu g ml^{-1}$ oligomycin, 10 μM Amplex Red (Invitrogen), 0.5 U ml^{-1} horseradish peroxidase, and 40 U ml^{-1} SOD. The reaction for both PmFB and isolated mitochondria was initiated by the addition of 10 mM succinate. The rate of H_2O_2 emission was calculated from the slope (absorbance/minutes) after subtracting the background, from a standard curve established with the same reaction conditions. In PmFB, H_2O_2 rates were normalized to fibre dry weight as performed previously (Anderson *et al.* 2009), and in isolated mitochondria were normalized to protein content.

Membrane potential

Mitochondrial membrane potential was measured fluorometrically at $25^\circ C$ in cuvettes filled with MiR05 and 2 μM Safranin-O as described previously (Goo *et al.* 2013), with some modifications. Briefly, mitochondria were added to cuvettes followed by the addition of 10 mM pyruvate and 5 mM malate (state IV), 2.5 mM ADP (state III), 10 mM glutamate (maximal complex I), and 10 mM succinate (maximal complex I + II). Mitochondria were uncoupled

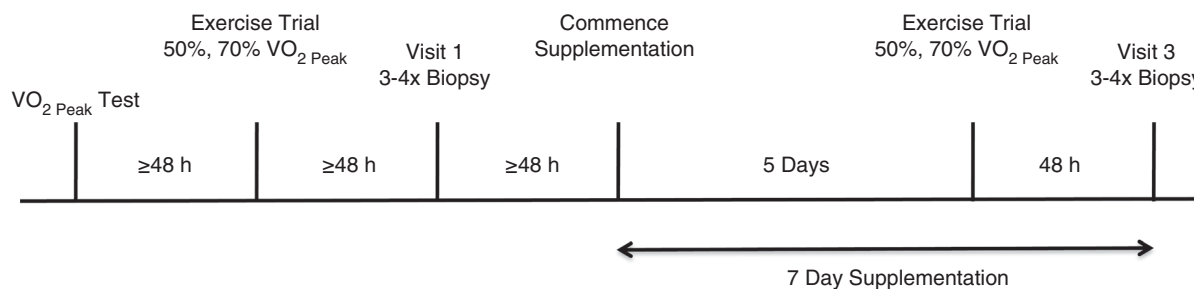


Figure 1. Schematic overview of the experimental protocol

Subjects had biopsies taken at rest and on the morning of day 7 following the completion of 7 days of beetroot juice supplementation. On day 5 of the supplementation period, subjects performed submaximal cycling exercise at 50 and 70% of VO_2 .

by the addition of 2 μM 2,4-dinitrophenol (DNP), followed by the addition of Safranin-O ($2 \times 1 \mu\text{M}$) for calibration of the fluorescence signal. Membrane potential was then estimated using the Nernst equation:

$$\Delta\Psi = \frac{RT}{zF} \ln \left(\frac{[\text{Safranin}]_{\text{out}}}{[\text{Safranin}]_{\text{in}}} \right)$$

where R is the universal gas constant, F is Faraday's constant, T is absolute temperature, and z is the valency (+1) of Safranin.

Western blotting

Western blotting was performed on whole muscle homogenate using methods described previously (Herbst *et al.* 2014). Samples were loaded equally for α -tubulin (Abcam, Cambridge, UK), UCP3 (Millipore, Billerica, MA, USA), ANT1 (Mitosciences, Eugene, OR, USA), ANT2 (Abcam), SOD2 (Abcam), catalase (Abcam), 4-hydroxynonenal (4HNE; Alpha Diagnostics, Antonio, TX, USA), pyruvate dehydrogenase (PDH) subunit E1 α (Molecular Probes, Eugene, OR, USA), oxidative phosphorylation (OXPHOS; Mitosciences), and for the Oxyblot protein oxidation detection kit (Millipore) and N3-nitrosylation (Cayman Chemical, Ann Arbor, MI, USA). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and were incubated in blocking solution, primary antibody, and the corresponding secondary antibody as specified by the supplier. All samples were detected from the same Western blot by cutting gels and transferring onto a single membrane to limit variability, and all gels were loaded with 10 μg protein. Linearity of signal was confirmed from 5–15 μg of protein. Membrane proteins were detected by enhanced chemiluminescence (Perkin Elmer, Woodbridge, ON, Canada) and quantified by densitometry (Alpha Innotech Fluorchem HD2; Fisher Scientific, Ottawa, ON, Canada).

Plasma analyses

Five separate subjects repeated the supplementation protocol for the collection of blood plasma. Subjects reported to the lab following an overnight fast, and had blood samples taken immediately prior to consuming their first bolus of BRJ. Samples were then taken again 1.5 and 3 h after ingestion. This process was repeated again in the evening, and on day 6 of the supplementation period. Blood samples (8 ml) were collected in EDTA-containing tubes and centrifuged at 1000 g for 10 min at 4°C. Plasma aliquots were frozen and stored at -80°C for subsequent analysis of plasma nitrate (NO_3^-) and nitrite (NO_2^-) by chemiluminescence using a Sievers gas-phase chemiluminescence NO analyser (Sievers NOA

280i; Analytix, Durham, UK) (Lundberg & Govoni, 2004).

Statistics

A two-way analysis of variance was used to detect differences between day 1, day 6, and sampling time-points for blood plasma NO_3^- and NO_2^- measurements. If significance was detected, a Fischer LSD *post hoc* test was applied. A Student's paired t test (two-way) was used to detect differences between pre- and post-supplementation data in submaximal exercise trials, and in isolated mitochondria and permeabilized muscle fibre experiments. Significance was determined at $P < 0.05$ with confidence intervals $\geq 95\%$.

Results

Whole body oxygen consumption and energy expenditure

We first characterized the pulmonary \dot{V}_{O_2} responses during exercise to ensure that the supplementation with BRJ coincided with the expected metabolic effect. Oxygen consumption was unaltered while exercising at 50% $\dot{V}_{\text{O}_{2\text{peak}}}$ (Fig. 2A). In contrast, oxygen consumption while exercising at 70% $\dot{V}_{\text{O}_{2\text{peak}}}$ decreased ~ 100 ml following BRJ consumption, representing $\sim 3\%$ of oxygen consumption (Fig. 2B). It is worth noting that of the 13 participants tested in the exercise trials, 8 showed a decrease in pulmonary \dot{V}_{O_2} at 50% (Fig. 2A), and 12 of 13 subjects decreased at 70% (Fig. 2B). Despite the change in oxygen consumption at 70% $\dot{V}_{\text{O}_{2\text{peak}}}$, there was no change in respiratory exchange ratio (Pre, 0.99 ± 0.01 ; Post, 0.99 ± 0.01), indicating that there was no change in fuel preference following BRJ consumption.

Respiration in permeabilized fibres

Given the change in oxygen consumption, we next aimed to determine if BRJ supplementation altered various indices of mitochondrial bioenergetics. Supplementation did not alter leak respiration (non-ADP-stimulated respiration; Fig. 3A) or maximal substrate-supported respiration (Fig. 3A). In support of a lack of change in maximal respiration, the content of various electron transport chain proteins and PDH was not altered (Fig. 3B and C). A previous paper found that sodium nitrate supplementation decreased ANT protein content (Larsen *et al.* 2011), and therefore we next examined if BRJ altered mitochondrial ADP sensitivity. Titrating ADP in the presence of substrates revealed an expected Michaelis–Menten curve (insets in Fig. 4A and D) that was utilized to estimate the ADP sensitivity (apparent K_m). BRJ supplementation did not alter maximal respiration (Fig. 4B and E) or the apparent K_m (Fig. 4C and E) in

the presence or absence of creatine. In support of this, ANT1, ANT2 and UCP3 protein contents were unaltered following supplementation (Fig. 4G and H). To further examine the potential effect of BRJ supplementation on electron transport function we determined the kinetic properties of pyruvate to stimulate respiration in the presence of saturating ADP concentrations. Unfortunately some of the permeabilized fibre bundles could not be recovered for dry weight assessments following

respiration experiments, and therefore only kinetic properties were examined and not maximal respiration. Pyruvate displayed a typical Michaelis–Menten curve (Fig. 5A); however, BRJ did not alter the apparent sensitivity of mitochondria to pyruvate (Fig. 5B). Altogether these data suggest that BRJ, while decreasing oxygen consumption during exercise, does not improve mitochondrial respiratory measures in permeabilized muscle fibres.

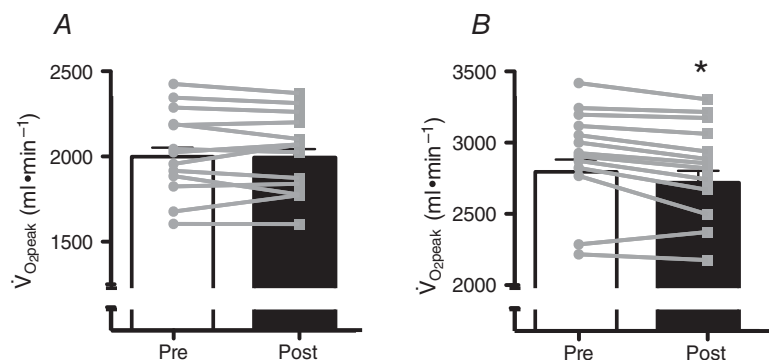


Figure 2. Oxygen uptake for subjects cycling at 50% (A) and 70% (B) of $\dot{V}O_{2\text{peak}}$ prior to supplementation (Pre) and on day 5 of supplementing (Post) with beetroot juice. Inset represents the individual responses. Values are means \pm SEM. * $P < 0.05$. $n = 13$.

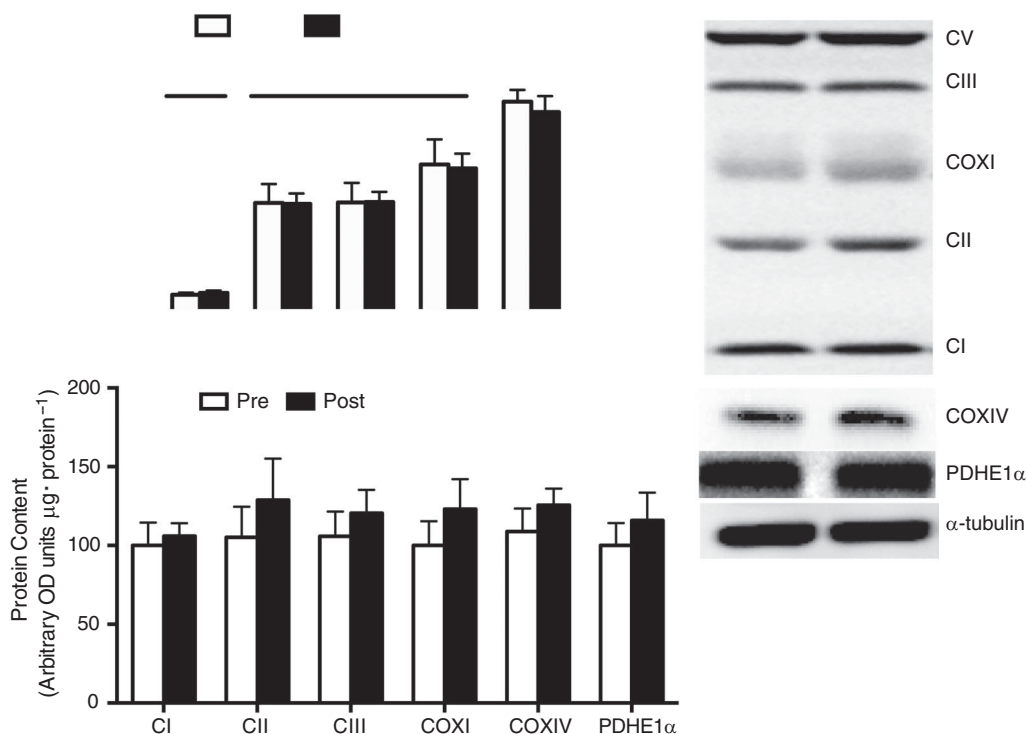


Figure 3. Mitochondrial capacity in permeabilized fibres (A) and the expression of oxidative phosphorylation (OXPHOS) proteins (B and C)

In A, $J\dot{O}_2$ represents mitochondrial O_2 flux. In C, OXPHOS proteins are expressed in arbitrary optical density (OD) units (μg total protein) $^{-1}$. PM: 10 mM pyruvate + 2 mM malate either without (leak, $-ADP$) or with (state III, $+ADP$) 5 mM ADP. PMG: state III maximal complex 1 linked, 10 mM pyruvate + 2 mM malate + 5 mM ADP + 10 mM glutamate. PMGS: max complex 1 and 2 linked, 10 mM pyruvate + 2 mM malate + 5 mM ADP + 10 mM glutamate + 10 mM succinate. Values are means \pm SEM. $n = 10$ for PmFB, $n = 6$ for Western blot data.

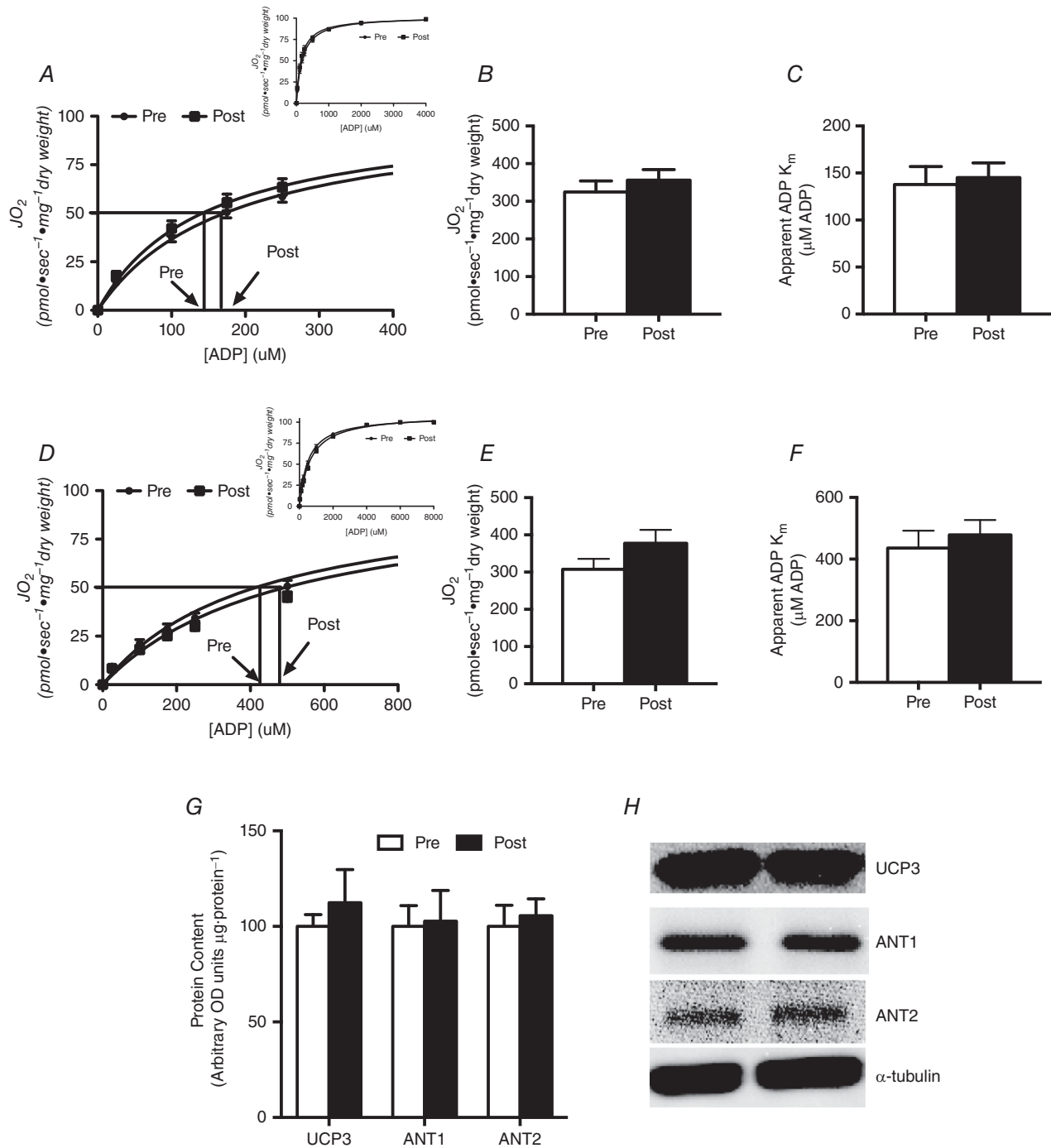


Figure 4. ADP titrations either in the presence (A–C) or absence (D–F) of creatine with pyruvate and malate present in the respiration media, and the expression of protein targets contributing to mitochondrial membrane potential (G and H)

The corresponding V_{max} (B and E) indicated there was no change in mitochondrial capacity, and no change in the apparent K_m indicating no change in sensitivity to ADP (C and F). Values are means ± SEM. $n = 10$ for PmFB, $n = 6$ for Western blot data.

Respiration and membrane potential in isolated mitochondria

Given the lack of change in mitochondrial respiration, in a subset of participants ($n = 5$) both subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial subpopulations were isolated to directly examine mitochondrial coupling efficiencies. BRJ supplementation did not alter the P/O ratio (Fig. 6A and B), or the respiratory control ratio (RCR) (Fig. 6C and D) in SS or IMF mitochondria. Furthermore, there was no correlation between the measured coupling ratios in either subpopulation and the change in whole body \dot{V}_{O_2} at either power output (50%: $R^2 = 0.04$ SS, 0.003 IMF; 70%: $R^2 = 0.008$ SS, 0.119 IMF). In addition, changes in membrane potential ($\Delta\Psi$) were estimated fluorometrically using Safranin-O. In both SS and IMF mitochondria, $\Delta\Psi$ decreased following the addition of substrates and ADP (Fig. 7A and B); however, there were no differences in either fraction following supplementation. These data further support the interpretation that a reduction in whole body oxygen consumption during exercise is independent of improvements in mitochondrial respiration/coupling.

H₂O₂ emission and oxidative stress

Given that NO can inhibit cytochrome oxidase (Bolaños *et al.* 1994; Brown & Cooper, 1994; Cleeter *et al.* 1994; Giuffrè *et al.* 2000), we also examined the possibility that BRJ could increase the propensity of mitochondria to produce ROS. In permeabilized fibres, maximal succinate-induced mitochondrial H₂O₂

emission increased ~50% following supplementation in the absence (Fig. 8A) and presence (Fig. 8B) of exogenous superoxide dismutase (SOD). The presence of exogenous SOD did not increase H₂O₂ emission (Fig. 8A and B), suggesting the Qo site of complex III does not release superoxide into the intermembrane space or contribute to succinate-induced ROS in humans. This supports the notion that reverse electron flow to complex I represents the majority of superoxide production in the presence of succinate. However, there was no correlation between the propensity to produce H₂O₂ and the change in whole body \dot{V}_{O_2} either with ($R^2 = 0.03$ at 50%, 0.115 at 70%) or without ($R^2 = 0.08$ at 50%, 0.209 at 70%) exogenous SOD. Within IMF mitochondria, which represent ~80% of the mitochondria within muscle (Hoppeler *et al.* 1985; Ferreira *et al.* 2010), there was also a strong trend for an increase in H₂O₂ emission ($P = 0.07$). However, there was no increase within SS mitochondria (Fig. 8C and D). The increased propensity to produce ROS within permeabilized fibres did not result from a reduction in either catalase or SOD2 (Fig. 8E and F) and did not manifest with overt oxidative stress, as indicated by a lack of change in lipid peroxidation (4HNE), protein carbonylation or nitrotyrosine residues in muscle homogenates (Fig. 8G and H).

Temporal effects of beetroot juice consumption on blood nitrate and nitrite concentrations

Despite the observed decrease in whole body \dot{V}_{O_2} and increase in muscle mitochondrial ROS emission, given the lack of response with respect to mitochondrial coupling

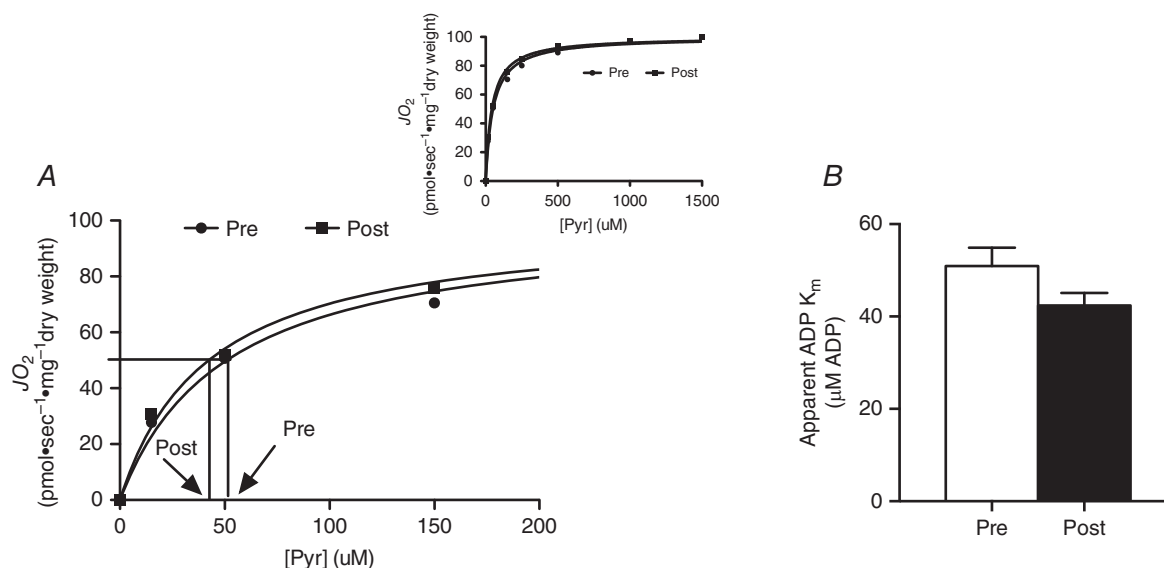


Figure 5. Pyruvate titrations with malate and ADP present in the respiration media

There was no change in the apparent K_m indicating no change in sensitivity to pyruvate. Values are means \pm SEM. $n = 10$.

we wanted to confirm that the dosing protocol resulted in increases in plasma NO_3^- and NO_2^- . Therefore in separate participants we re-ran the study to only measure plasma responses. In these individuals we characterized the blood NO_3^- and NO_2^- responses during the first and

last full day of the supplementation period. In general, both NO_3^- (Fig. 9A) and NO_2^- (Fig. 9B) concentrations increased ($P < 0.05$) within 90 min, and remained elevated 2 h following ingestion in the morning and in the evening. The basic response to BRJ consumption was

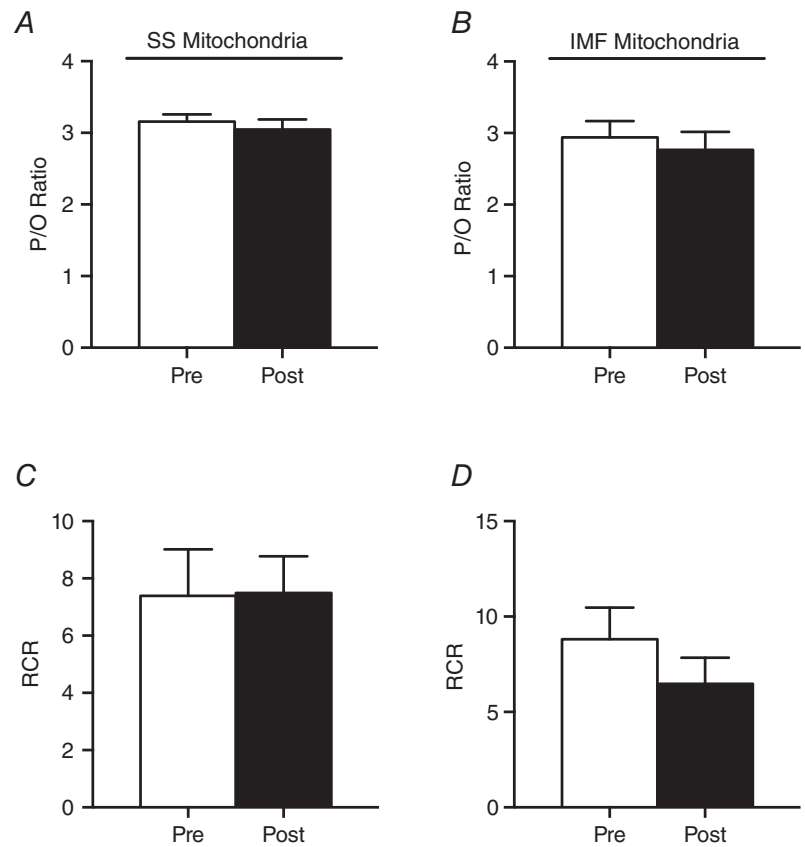


Figure 6. Measures of mitochondrial efficiency (P/O ratio, A and B) and coupling (RCR, C and D) in SS and IMF mitochondria
Values are means \pm SEM. $n = 5$.

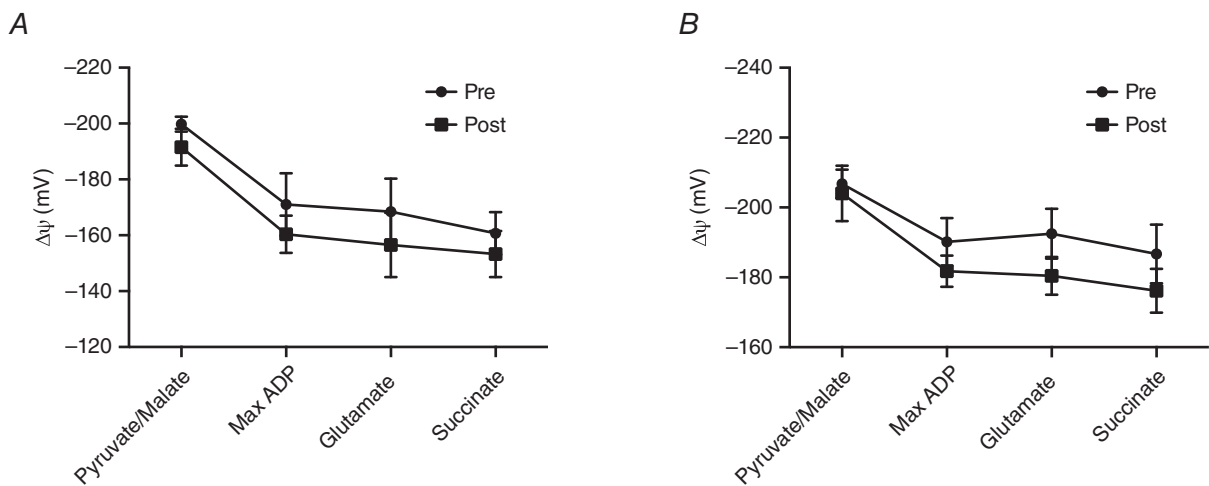


Figure 7. Membrane potential measured using Safranin-O in SS (A) and IMF (B) mitochondrial sub-populations
Pyruvate/malate: 10 mM pyruvate + 5 mM malate. ADP: 10 mM pyruvate + 5 mM malate + 2.5 mM ADP. Glutamate: 10 mM pyruvate + 5 mM malate + 2.5 mM ADP + 10 mM glutamate. Succinate: 10 mM pyruvate + 5 mM malate + 2.5 mM ADP + 10 mM glutamate + 10 mM succinate. Values are means \pm SEM. $n = 5$.

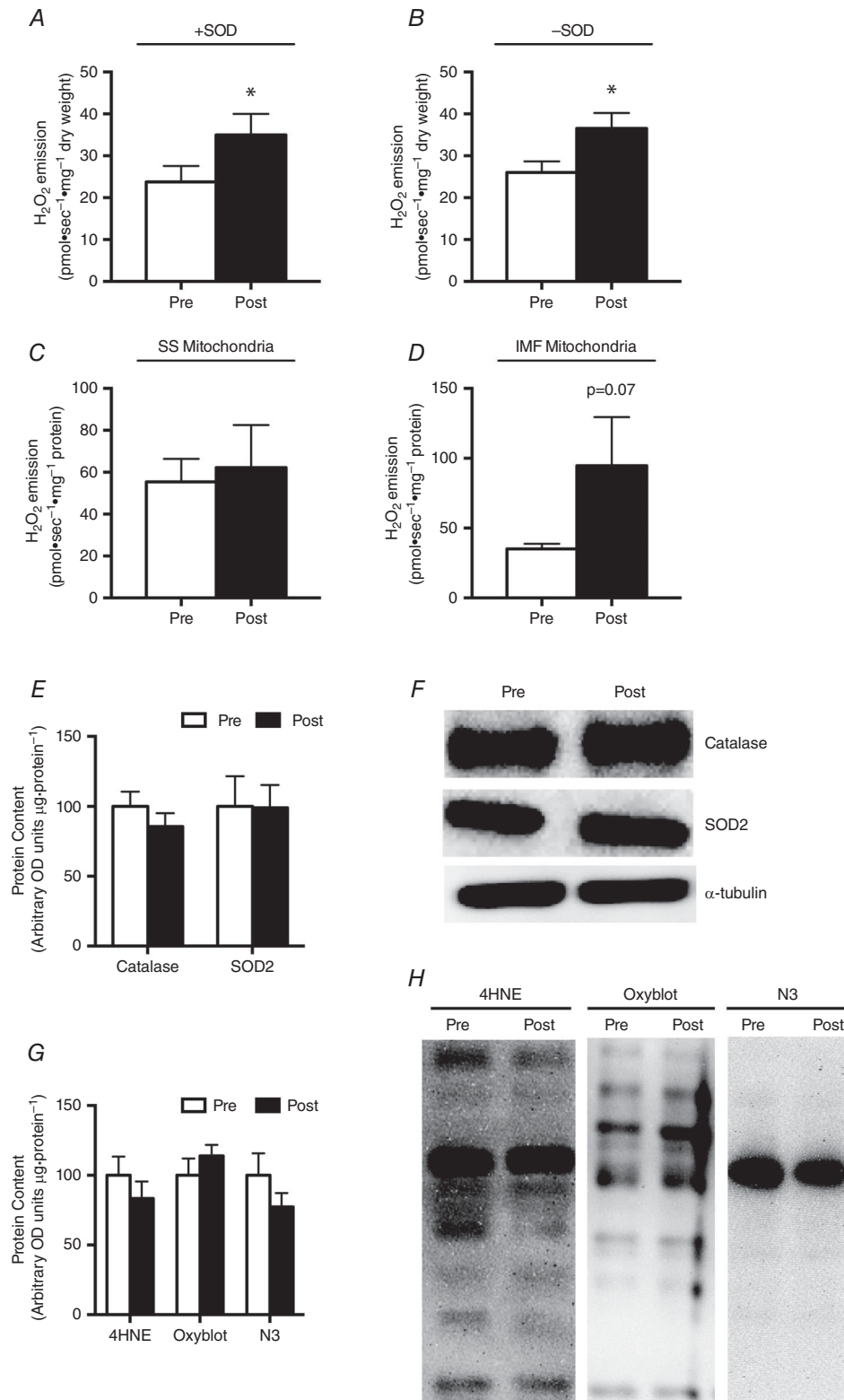


Figure 8. Propensity for succinate-supported mitochondrial ROS emission before (Pre) and after (Post) beetroot juice supplementation

Permeabilized fibre traces were performed in the presence (A) and absence (B) of 40 U/ml exogenous SOD with 20 mM succinate and 25 μM blebbistatin present in the media. Isolated mitochondria traces for SS (C) and IMF (D) were performed in the presence of 40 U/ml of exogenous SOD and 8 mM succinate. Values are means ± SEM. **P* < 0.05. *n* = 10 for PmFB, *n* = 5 for isolated mitochondria.

not dramatically altered when measured following 6 days of supplementation. However, NO_3^- levels were higher in the morning on day 6 (Fig. 9A), such that NO_3^- was higher in the morning at all time points examined, in association with a strong trend ($P = 0.07$) for an increase in NO_2^- (Fig. 9B) 90 min following ingestion. In the evening, NO_3^- was not dramatically different on day 6, while NO_2^- was lower 120 min after the consumption of BRJ (Fig. 9A and B). Therefore, the plasma responses indicate that the dosing protocol used in the current study was sufficient to maintain elevated plasma NO_3^- and NO_2^- concentrations throughout the experiment.

Discussion

In the current study we show that BRJ consumption, while decreasing the oxygen utilization of submaximal exercise, did not alter various indices of mitochondrial coupling/respiratory responses. Specifically, state IV and III respirations were not altered in either isolated SS and IMF mitochondria or permeabilized muscle fibres, and coupling (P/O) ratios and proton motive force were also unaffected by BRJ consumption. There was also no correlation between the measured coupling ratios in either subpopulation and the change in whole body $\dot{V}\text{O}_2$ at either power output. These data are in stark contrast to a previous publication utilizing sodium nitrate consumption, which reported improved coupling efficiency within pooled isolated mitochondria (Larsen *et al.* 2011). This previous paper argued that a reduction in UCP3 and ANT protein content accounted for the improvement in the observed P/O ratios. However, in the current study UCP3 and ANT proteins were not reduced and the apparent mitochondrial ADP respiratory sensitivity remained unaltered. Therefore, it appears that although

sodium nitrate and BRJ both reduce whole body oxygen consumption during exercise, they may work through divergent mechanisms of action.

Previous work using ^{31}P -magnetic resonance spectroscopy (Bailey *et al.* 2010) suggested that BRJ reduces ATP turnover rate during exercise. Combined with the current observation that BRJ does not alter mitochondrial coupling, these results strongly suggest an improvement in excitation–contraction efficiency (i.e. increased work/ATP consumption) is responsible for the reduction in $\dot{V}\text{O}_2$ following BRJ. Considering NO has been shown to increase calcium ATPase (SERCA) efficiency (Ca^{2+} uptake/ATPase activity; Ishii *et al.* 1998), and oral NO_3^- consumption increases intracellular calcium handling and force production in rodents (Hernández *et al.* 2012) as well as low-frequency force generation and rates of relaxation in humans (Haider & Folland, 2014), future work should focus on the potential for BRJ to alter SERCA properties. While we have not provided a mechanism of action in the current study to account for the reduction in $\dot{V}\text{O}_2$ during exercise, the present data strongly suggest that alterations in mitochondrial efficiency are not required for the observed whole body responses.

While indices of mitochondrial oxygen consumption were not altered following BRJ consumption, we have provided evidence that BRJ supplementation increases the capacity for mitochondrial ROS emission. The functional consequence of an increased propensity for mitochondrial H_2O_2 emission following BRJ consumption is not currently known. Furthermore, in resting muscle neither BRJ (current study) nor sodium nitrate (Larsen *et al.* 2014) increased measures of overt oxidative stress within skeletal muscle, and therefore it remains unclear if the observed increase in the *in vitro* capacity for ROS emission

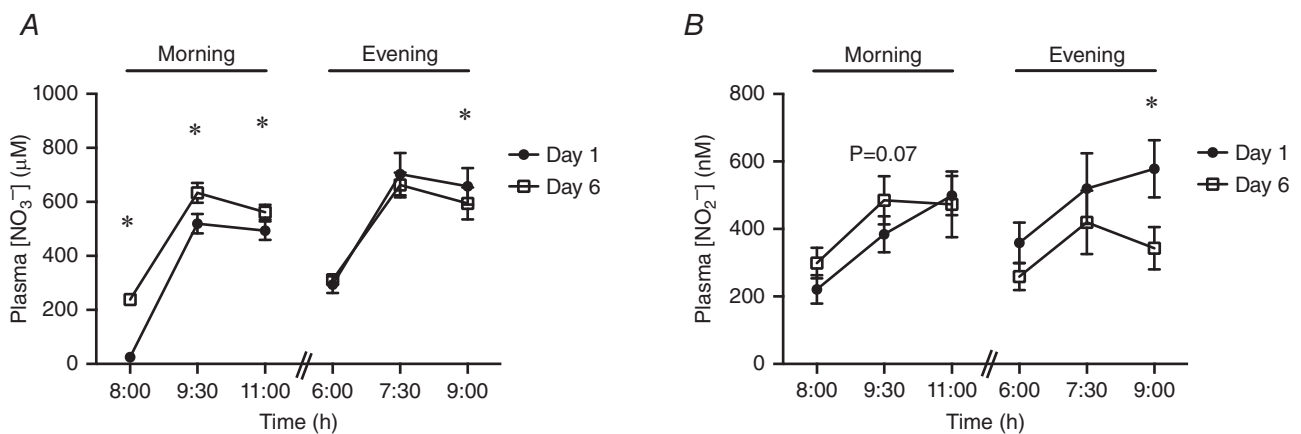


Figure 9. Plasma nitrate concentration ($[\text{NO}_3^-]$); A) and nitrite concentration ($[\text{NO}_2^-]$); B) following consumption of beetroot juice (13 mmol NO_3^-) in the morning and in the evening

Baseline samples were taken prior to the morning and evening supplementation (8.00 am and 6.00 pm, respectively), and at 90 min and 3 h post-consumption. Values are means \pm SEM. * $P < 0.05$ vs. same time-point on day 1. $n = 6$.

translates to a functional effect *in vivo*. This is further emphasized by the fact that the change in whole body \dot{V}_{O_2} and the increase in H_2O_2 emission did not significantly correlate. However, the study of redox biology is complex and ROS-induced signalling can occur in the absence of overt oxidative damage. As a result, it remains possible that an increase in mitochondrial ROS could contribute to the observed reduction in whole body \dot{V}_{O_2} following BRJ consumption. In support of this, H_2O_2 has been shown to directly interact with the contractile apparatus to increase force production in the absence of changes in cytosolic calcium concentrations (Andrade *et al.* 1998), and H_2O_2 can directly increase the mechanical efficiency of skeletal muscle (Andrade *et al.* 2001). However, H_2O_2 has also been shown to decrease myofibrillar calcium sensitivity and muscle force production (Moopanar & Allen, 2005), a process that appears to require thiol alterations (Moopanar & Allen, 2006). Therefore, while the potential mechanistic role of increased mitochondrial H_2O_2 in the observed reduction in whole body \dot{V}_{O_2} during exercise remains speculative, it is possible that the redox signalling initiated in resting muscle following BRJ supplementation may augment the response to exercise. Clearly, future work is required to elucidate how BRJ alters whole body \dot{V}_{O_2} during exercise; however, if ROS contributes as a signalling molecule in these responses this may explain the relative insensitivity to BRJ in elite athletes (Bescós *et al.* 2012; Christensen *et al.* 2013; Boorsma *et al.* 2014), as these individuals have increased skeletal muscle antioxidant enzyme content (Powers & Jackson 2008).

Previous literature has shown that acute BRJ consumption containing ~ 16 mmol NO_3^- is ideal with respect to peak blood NO_3^- and NO_2^- , responses that are normalized over a 12 h period (Wylie *et al.* 2013a). Therefore, in the current study we used this dosing intervention every 12 h to maintain chronically elevated NO_3^- and NO_2^- plasma profiles over the entire 7 day study. The plasma profiles on the final day of the intervention show that while NO_3^- was higher in the morning, the concentration of NO_2^- was $\sim 50\%$ lower in the evening. This is in contrast to previous work showing similar responses over 15 days with a lower dose of NO_3^- (Vanhatalo *et al.* 2010). However, intriguingly, on day 8 plasma NO_2^- was not significantly different from the pre-supplementation baseline, suggesting the response observed in the current study on day 6 may be transient. Carlström and colleagues (2010, 2014) have shown that 8–10 weeks of supplementation with sodium nitrate is followed by a return to baseline in plasma $[NO_3^-]/[NO_2^-]$ in rodents suggesting that it may theoretically be possible to become resistant to prolonged high plasma $[NO_3^-]/[NO_2^-]$. This may contribute to the relative insensitivity in elite athletes (Peacock *et al.* 2012; Christensen *et al.* 2013; Boorsma *et al.* 2014) to the otherwise consistent finding with recreationally active

subjects that BRJ lowers \dot{V}_{O_2} during exercise, as elite athletes exhibit higher baseline plasma $[NO_3^-]$ when compared with untrained individuals (Poveda *et al.* 1997; Vassale *et al.* 2003). In the current study whole body \dot{V}_{O_2} measurements were made 48 h prior to biopsies, and therefore it remains possible that mitochondrial efficiency was altered 2 days earlier, especially since Larsen and colleagues (2011) examined mitochondrial efficiency after 3 days of sodium nitrate. However, this remains an unlikely explanation, since reductions in steady-state pulmonary \dot{V}_{O_2} are seen 2.5 h after an acute dose of BRJ (Vanhatalo *et al.* 2010).

Limitations

A limitation of this study was the time delay between measurements, as whole body oxygen consumption, muscle biopsies and the collection of blood were taken on different days. While we could have performed the exercise trials on the same experimental day as the biopsies, out of necessity this would dictate that the exercise and biopsies occurred at different times following the consumption of the BRJ (i.e. biopsies at 90 min and exercise 120–150 min post-consumption). Instead, we opted to standardize the time that all measurements were taken following the final morning's dose of BRJ, which dictated that measurements were taken on different days. This decision was made as we are unaware of data to indicate that the response to BRJ would be different between days 5 and 7; however, the acute response to beetroot juice is known to vary considerably across time. Nevertheless, the reader should be aware of the time delay between measures.

Unfortunately the subjects recruited for blood measurements represent a different cohort, and therefore should only be interpreted as proof-of-principle that the current dosing protocol resulted in the expected increase in plasma NO_2^- and NO_3^- . Given the results of our blood measures, we are confident our dosing protocol was sufficient to result in a prolonged elevation in plasma NO_2^- and NO_3^- in the first cohort of subjects, and therefore the lack of an effect is not the result of our supplementation regime. This is further corroborated by our results at 70% $\dot{V}_{O_{2peak}}$ exhibiting the well-documented decrease in whole body \dot{V}_{O_2} following NO_3^- supplementation (Larsen *et al.* 2007, 2011; Bailey *et al.* 2009, 2010; Vanhatalo *et al.* 2010; Lansley *et al.* 2011; Cermak *et al.* 2012). This shows that our dosing protocol is still eliciting an ergogenic effect, but the underlying mechanism is not an alteration in mitochondrial coupling, and thus lies elsewhere.

Another possible perceived limitation was the small sample size, particularly for the isolated mitochondria experiments. However, in order to detect a 5% change in mitochondrial coupling ratios with 80% power, for SS mitochondria alone we would need to recruit an

additional 50 subjects. Furthermore, the results of our mitochondrial measurements support what was seen within the larger group in PmFB experiments. We therefore felt we could not ethically justify recruiting more subjects and performing biopsies just to increase the sample size when we did not anticipate seeing an effect, as the tissue requirement for isolated mitochondria is significant.

Conclusions

Altogether, in the current study we provide evidence that improvements in mitochondrial coupling efficiency are not required for the reduction in whole body oxygen utilization during exercise following BRJ supplementation in recreationally active subjects. Instead, we provide evidence that mitochondrial H_2O_2 is increased; however, it remains to be determined if this contributes to the observed reduction in $\dot{V}O_2$. This is in contrast to the results found following 3 days of sodium nitrate supplementation (Larsen *et al.* 2011). This is surprising given that nitrate appears to be the active ingredient in both supplements, as a nitrate-depleted beetroot juice placebo has been shown to have no effect on pulmonary responses to exercise in both moderate- and severe-intensity exercise, as well as blood pressure (Lansley *et al.* 2011). It is therefore possible that these two supplements act through different mechanisms, or elicit effects beyond simply increasing dietary nitrate intake. Future work should compare both BRJ and sodium nitrate directly with respect to mitochondrial adaptations and H_2O_2 emission to further our understanding of how these supplements may alter metabolism.

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Additional information

Competing interests

None declared.

Author contributions

J.W. and G.P.H. designed experiments, interpreted the data, and wrote the manuscript. L.L.S. designed experiments and interpreted the data. J.W., A.L., G.J.F.H., J.M.G.S., L.B.V. and L.J.C.v.L. performed experiments. All authors edited the manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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