

# Marching to the Beet: The effect of dietary nitrate supplementation on high altitude exercise performance and adaptation during a military trekking expedition

Anna R Marshall<sup>a\*</sup>, Joanna E Rimmer<sup>b</sup>, Nishma Shah<sup>a</sup>, Kyo Bye<sup>b</sup>, Courtney Kipps<sup>a</sup>, David R Woods<sup>b,c,d</sup>, John O'Hara<sup>c</sup>, Christopher J Boos<sup>c,e,f</sup>, Matthew Barlow<sup>c</sup>

<sup>a</sup>Department of Surgical and Interventional Sciences, Institute of Sport, Exercise & Health, UCL, London, WC1E 6B

<sup>b</sup>Defence Medical Services, Lichfield, WS14 9PY

<sup>c</sup>Carneige School of Sport, Leeds Beckett University, Leeds, LS16 5LF

<sup>d</sup>Northumbria and Newcastle NHS Trusts, Wansbeck General and Royal Victoria Infirmary, Newcastle, NE66 2NS

<sup>e</sup>Department of Cardiology, Poole Hospital NHS Foundation trust, Poole, BH15 2JB

<sup>f</sup>Department of Postgraduate Medical Education, Bournemouth University, Bournemouth, BH1 3LT

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## ARTICLE INFO

## ABSTRACT

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### Abstract

**Purpose:** The aim was to investigate the effect of dietary nitrate supplementation (in the form of beetroot juice, BRJ) for 20 days on salivary nitrite (a potential precursor of bioactive nitric oxide), exercise performance and high altitude (HA) acclimatisation in field conditions (hypobaric hypoxia).

**Methods:** This was a single-blinded randomised control study of 22 healthy adult participants (12 men, 10 women, mean age  $28 \pm 12$  years) across a HA military expedition. Participants were randomised pre-ascent to receive two 70ml dose per day of either BRJ (~12.5mmol nitrate per day; n=11) or non-nitrate calorie matched control (n=11). Participants ingested supplement doses daily, beginning 3 days prior to departure and continued until the highest sleeping altitude (4800m) reached on day 17 of the expedition. Data were collected at baseline (44m altitude), at 2350m (day 9), 3400m (day 12) and 4800m (day 17).

**Results:** BRJ enhanced the salivary levels of nitrite ( $p=0.007$ ). There was a significant decrease in peripheral oxygen saturation and there were increases in heart rate, diastolic blood pressure, and rating of perceived exertion with increasing altitude ( $p<0.001$ ). Harvard Step Test fitness scores significantly declined at 4800m in the control group ( $p=0.003$ ) compared with baseline. In contrast, there was no decline in fitness scores at 4800m compared with baseline ( $p=0.26$ ) in the BRJ group. Heart rate recovery speed following exercise at 4800m was significantly prolonged in the control group ( $p<0.01$ ) but was unchanged in the BRJ group ( $p=0.61$ ). BRJ did not affect the burden of HA illness ( $p=1.00$ ).

**Conclusions:** BRJ increases salivary nitrite levels and ameliorates the decline in fitness at altitude but does not affect the occurrence of HA illness.

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\*Corresponding Author  
Dr Anna Ruth Marshall

## 1. Introduction

Exposure to a high altitude (HA) environment for sporting, recreational and occupational purposes is becoming more prevalent [1]. With increasing altitude, the partial pressure of oxygen ( $PO_2$ ) decreases because of reducing atmospheric pressure (hypobaric hypoxia). Above 2500m, this impacts on the overall content of oxygen in the arterial circulation ( $CaO_2$ ) [2], reducing both the tissue diffusion gradient and the delivery of oxygenated blood, leading to impaired physical performance [3-5]. For this reason, a reduction in exercise performance relative to sea level is ubiquitous at HA. Acclimatisation describes physiological adaptations to offset the fall in  $CaO_2$ . The rate of acclimatisation varies amongst individuals and failure to acclimatise can lead to HA illnesses which can be fatal. Successful acclimatisation involves regulation of multiple physiological processes which are linked to nitric oxide (NO) bioavailability [1,6,7-9].

NO is a signalling molecule responsible for regulating oxygen delivery and utilisation, implicated in blood flow distribution [7,10,11], mitochondrial respiration [12], endothelial functioning [13], microcirculatory function [11], neurotransmission [14], muscle metabolism [15,16], contractile efficiency [17-19] and muscle recovery [17,19]. Increased NO metabolites are associated with successful acclimatisation in both lowlanders and native highlanders, ascending or dwelling at HA [1,2,9,10,20-23]. Native highlanders possess higher concentrations of these compounds and exhaled NO ( $E_{NO}$ ) [10,11,20,21,23], one explanation for their remarkable ability to cope in extreme hypoxia [10,11,23,24]. Suppressed NO bioavailability has been linked to the development of HA illness [21,22].

Endogenous NO is generated via two pathways that can be targeted. Firstly, an oxygen-dependent pathway involving oxidation of amino acid L-arginine catalysed by the nitric oxide synthase (NOS) enzymes can produce NO,  $NO_2^-$  and  $NO_3^-$ . In vitro evidence has shown hypoxia alters antisense transcript which could influence expression of NOS enzymes, possibly altering NO synthesis in such conditions [25]. A second pathway involves serial reduction of dietary  $NO_3^-$  to  $NO_2^-$  to NO [6]. Following ingestion, a proportion of  $NO_3^-$  enters the entero-salivary circulation where it is concentrated in the salivary glands. This can be recirculated to into the mouth where it is reduced by oral commensal bacteria to  $NO_2^-$  later transformed to NO in the stomach. The majority of ingested  $NO_3^-$  however, reaches the intestine where it enters systemic circulation where it is reduced by enzymes and non-enzymatic catalysts. This pathway could be promoted in hypoxic conditions, as oxygen inhibits reduction processes [1,6,26].

Dietary nitrate ( $NO_3^-$ ) supplementation in the form of beetroot juice (BRJ) has been shown to increase endogenous production of bioactive nitric oxide (NO) [1,4,6,27]. BRJ also reduces  $O_2$  consumption during exercise [7,28] and augments hypoxic exercise capacity and performance [26,29-32]. Although these findings have not been replicated in all studies in simulated hypoxia [12,26,27,33-36] meta-analyses conclude that dietary nitrate supplementation lowers the  $O_2$  cost of exercise [36] and improves endurance exercise performance in hypoxia [38]. Only relatively few studies have explored the effects of BRJ on acclimatisation to terrestrial HA [9,13,20,24,39]. The majority conclude that BRJ supplementation is safe at terrestrial altitude [9,13,22,24]. One investigation found no effect of 3 days of BRJ preloading (0.10-0.18mmol/kg/day equivalent to approximately 10mmol/day divided over 3 doses) and supplementing throughout a 5 day residence at 4559m on microcirculatory response [9] and concluded that, despite this result, there may be a benefit of BRJ on exercise performance at terrestrial HA [9]. In addition, 3 days of preloading BRJ (0.10-0.18mmol/kg/day equivalent to approximately 10mmol/day) and continued supplementation over ascent and 5 days residence at 4559m showed no effect on peripheral oxygen saturations ( $SpO_2$ ), heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP) or acute mountain sickness (AMS) [20]. However, these two studies do not realistically capture HA trekking, where people typically combine low, moderate and high intensity exercise in variable levels of hypoxia for differing timeframes depending on the ascent profile.

One study to date has evaluated the effects of BRJ on acute mountain sickness across a trekking ascent to HA [24]. This study included 40 young males aged 14-18yrs old who were given BRJ supplementation

(10mmol/day) for 6 days across an 11-day trek to 5300m. In this study, BRJ had no apparent effect on acute mountain sickness symptoms (AMS) [24]. The study did not measure circulating NO metabolite concentrations and was unable to confirm that BRJ supplementation regime enhanced NO metabolite levels [24]. Meta-analysis results show that chronic nitrate exposure (defined as  $\geq 8$  days) may be more beneficial on exercise performance in comparison to the acute exposure used in this study [38]. Thus, further research is required to establish the effects of BRJ on whole body performance in hypoxia using a higher dosing strategy for a longer time-period.

## **2. Purpose**

This is the first study to investigate the effects of daily dietary nitrate supplementation in the form of BRJ for 20 days during a trekking expedition at terrestrial HA on physiological functioning, exercise performance, acclimatisation, and the burden of HA illness. It was hypothesised that BRJ would improve exercise performance and reduce the burden of HA related illnesses.

## **3. Methods**

### *3.1. Population and Study Design*

This was a single-blinded randomised controlled two-group trial of British Military adults (12 men and 10 women) taking part in the RAF 100 Himalayan Venture 18 expedition. Inclusion criteria required participants to be aged  $>18$  yrs and low altitude dwellers. All participants engaged in regular physical activity and were fully fit for military duty. A modified version of the Centre of Aviation Medicine (RAF Henlow) health questionnaire was used for the health screening of the participants prior to their inclusion. Exclusion criteria included those self-administering Acetazolamide for prophylaxis and the use of mouthwash or Chlorhexidine containing products as they can inhibit oral bacteria. The participants were recruited from two trekking teams performing planned identical ascent profiles (figure 1), with staggered starts. This was a 23-day expedition starting from Kathmandu (1400m) trekking to a maximum altitude of 5755m (Tepsi-Lapsa Pass).

Following baseline measures and assessment at sea level (44m), the cohort was randomised to two groups (BRJ (n=11) or control (n=11)) using [www.randomizer.org](http://www.randomizer.org). The groups were blinded and randomised on a matched pairs basis from each trekking team to ensure the intervention and control groups had matched ascent profiles.

### *3.2. Supplementation Protocol*

This study compared the effects of a NO<sub>3</sub><sup>-</sup> containing supplement in the form of BRJ (Beet-It Sport, James White Drinks Ltd) to a control supplement containing negligible nitrate content. For 3 days prior to departure from the UK and for each trek day to the highest sleeping altitude at 4800m (day 17) participants in the intervention group were given 140ml concentrated BRJ (~12.5mmol nitrate, equivalent to 800mg as specified by the supplier) in the form of one 70ml dose with breakfast and one with evening meal. The control group received a calorie-, colour- and volume-matched dose which contained 15.4g Maltodextrin, 2.8g protein powder, 14mls blackcurrant cordial with negligible phytochemical content and 70mls Buxton water. The placebo had been used in a previous study, which established negligible phytochemical and

nitrate content [40]. Although the supplements were distinguishable by taste, participants were told the effects of two different antioxidant supplements were being investigated to prevent any bias.

### 3.3. Data Collection

Baseline data was collected at approximate sea level (44m) 15 days prior to departure. Physiological parameters and HA illness-related symptoms were recorded twice daily before breakfast and before dinner for 17 days across the expedition up to the highest sleeping altitude (4800m). SpO<sub>2</sub>, HR, SBP and DBP were measured after participants seated at rest for 3-5 minutes. SpO<sub>2</sub> was measured at the fingertip using a Nonin Onyx Series II pulse oximeter (Plymouth, MN 55441, USA). HR, DBP and SBP were measured using a brachial cuff USCOM BP+ Device (Uscom, Sydney, NSW, Australia). Participants were asked not to speak during recordings. A brachial cuff was applied to the dominant arm and measures of blood pressure were made in triplicate, with the lowest of the three readings recorded.

Before breakfast daily salivary NO<sub>2</sub><sup>-</sup> levels were measured using a testing strip based on the well-established modified Greiss reagent reaction (manufacturer; Nitric Oxide Test Strips, Berkeley Life, Chicago, USA). The salivary pad was placed on the tongue making contact with saliva then was gently pressed to the test pad for 5 seconds. The test pad changes colour in relation to NO<sub>2</sub><sup>-</sup> concentration, using the Berkeley test mobile phone App (Nitric Oxide Test, Berkeley Fit LLC) colour change was calibrated to provide a numeric value for NO<sub>2</sub><sup>-</sup> concentration (micromolar).

HA illness related symptoms were recorded twice daily before breakfast and evening meal using the updated 2018 Lake Louise Score (LLS) [41] and Acute Mountain Sickness – cerebral score (AMSc) [42]. LLS defined a participant with AMS if they scored ≥3 points including one point for headache at an altitude of > 2000m [43]. An AMSc score ≥0.7 is indicative of AMS [42,44,45].

Daily rating of perceived exertion (RPE) was recorded after the evening meal on each expedition day using the Borg scale. RPE was collected retrospectively at the end of the day and reflected the overall effort of that trek day. This is a quantified 15-point scale from 6-20 with 6 representing the resting state and 20 representing exhaustive exercise [46]. RPE measurements at high altitude have previously been found to correlate with LLS, AMS, SpO<sub>2</sub>, anxiety scores measured by GAD-7 and sleep scores [47,48]

Exercise capacity was assessed using the Harvard step test at baseline and 4800m. The Harvard step is a validated method to measure physical fitness and has been shown to be predictive of VO<sub>2</sub> max [49,50]. This is a 3-minute step test on a 35cm step at a rate of 96 steps per minute using alternate feet dictated by a metronome. Subsequently, heart rate recovery was measured using a Garmin chest heart rate monitor (HRM model) as HR at 1, 2 and 3 minutes post-test [51,52]. Testing took place in the morning before breakfast. A fitness index score was calculated using HR recovery data by the equation; [51,53]. Time is the duration of exercise period in seconds, (ΣHR) is the sum of 3 half minute pulse counts during recovery.

$$\text{Fitness Index} = \frac{\text{Time} \times 100}{(\Sigma\text{HR}) \times 2}$$

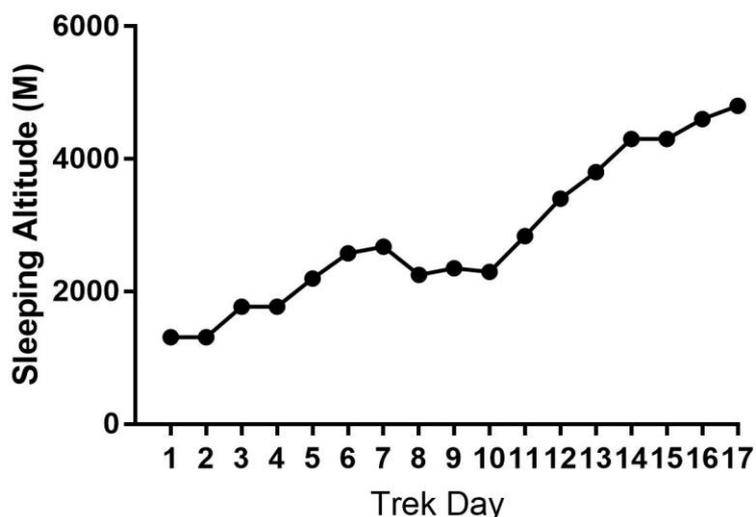
Fitness index is a continuum, <55 indicative of poor fitness and > 90 indicative of excellent fitness [53]. A worked example of the fitness calculation can be seen below;

$$\text{Fitness index} = \frac{180 \times 100}{(30 + 34 + 38) \times 2}$$

Answer = 88.2

The ascent profile was the same for each team, figure 1. Data from baseline (44m), HA 2350m (day 9) and 3400m (day 12) and 4800m (day 17) were analysed. Medication use during the expedition was

recorded. Subjects were still included if they were prescribed Acetazolamide as a treatment for HA illness during the expedition, this variable being analysed as a cofounder.



**Fig. 1.** Ascent profile of trekking expedition

### 3.4. Ethics

Ministry of Defence Research Ethics Committee granted ethical approval. All participants received an information sheet and provided written consent. Participants were told that the research was assessing the effects of two independent supplements on adaptation to altitude. Participants were blinded to fact that one supplement could be ergogenic and enhance physiological responses to altitude and one was a control. On day 18 of the expedition, participants were informed that one supplement was a control for BRJ. With this information, participants were asked if they wished to remain in the study and were re-consented for the inclusion of their data.

### 3.5. Statistical analysis and sample size calculation

The sample size was constrained by the number of military personnel on the expedition undertaking the ascent profile, and a convenience sample was selected. Significant findings of BRJ supplementation on exercise performance in hypoxia have been found with participant groups as small as  $n = 9$  [7] and  $n=10$  [26]. Two groups of  $n=11$  were recruited for this study.

Categorical data was compared using Fisher's Exact Test. Independent 2 group comparisons of continuous and ordinal data were analysed using an unpaired  $t$  test or Mann-Whitney test for parametric or non-parametric data respectively. Comparisons of continuous data from  $\geq 3$  time points of parametric and non-parametric data were performed using a one-way ANOVA or Friedman test respectively. The comparisons of continuous data from  $\geq 3$  times points between the two groups was performed by a two-way repeated measures ANOVA (rmANOVA) for parametric or Kruskal-Wallis test for non-parametric data. A p-value of  $< 0.05$  was considered significant for all comparisons.

## 4. Results

### 4.1. Demographics

The two groups were similar in terms of their sex, age and ethnicity. 56% participants were male in BRJ and control groups. The average age (years), BRJ  $27 \pm 12$  and control  $30 \pm 13$  ( $p=0.59$ ). 100% participants were white European and non-smokers. There was no difference their baseline SpO<sub>2</sub> ( $p=0.62$ ), heart rate ( $p=0.69$ ), SBP ( $p=0.20$ ), DBP ( $p=0.16$ ) or salivary nitrite ( $p=1.00$ ). The baseline fitness index BRJ 67.00 and control 66.30 ( $p=0.88$ ). The baseline fitness index BRJ 67.00 and control 66.30 ( $p=0.88$ ).

### 4.2. Salivary Nitrite ( $\mu\text{M}$ )

There was no difference in baseline pre-supplement salivary NO<sub>2</sub><sup>-</sup> at sea level between BRJ ( $47.90 \pm 30.29 \mu\text{M}$ ) and control groups ( $39.40 \pm 22.49 \mu\text{M}$ ,  $p=1.00$ ), figure 2. Salivary NO<sub>2</sub><sup>-</sup> was significantly higher at high altitude for BRJ (2350m:  $447.6 \pm 321.7 \mu\text{M}$ , 3400m:  $213.3 \pm 221.1 \mu\text{M}$ , 4800m:  $357.4 \pm 363.5 \mu\text{M}$ ) compared with control (2350m:  $152.2 \pm 110.6 \mu\text{M}$ ,  $p=0.012$ , 3400m:  $51.1 \pm 44.0 \mu\text{M}$ ,  $p=0.054$ , 4800m:  $60.3 \pm 94.8 \mu\text{M}$ ,  $p=0.014$ ). There was a main effect of time ( $p<0.001$ ), salivary NO<sub>2</sub><sup>-</sup> was significantly higher at all altitudes ( $p=0.003$ ,  $p=0.052$ ,  $p=0.025$ ) compared with sea level in the BRJ group, whilst in the control group salivary nitrite was significantly higher at 2350m ( $p=0.007$ ).

### 4.3. Physiological Parameters

Resting SpO<sub>2</sub>, HR, SBP, DBP and RPE at sea level were not different between BRJ and control (Table 2). There was a significant decrease in SpO<sub>2</sub> with increasing altitude for both BRJ ( $p<0.001$ ) and control groups ( $p<0.001$ ). Resting HR and RPE increased with altitude, being significantly higher at 4800m compared with sea level for both BRJ ( $p=0.004$ ,  $p=0.057$ ) and control groups ( $p=0.009$ ,  $p=0.006$ ). There was a significant increase in diastolic blood pressure at 3400 and 4800m compared with sea level in the BRJ ( $p=0.031$ ,  $p=0.001$ ) and control groups ( $p=0.097$ ,  $p=0.005$ ). However, high altitude exposure did not affect systolic blood pressure for either group ( $p>0.05$ ,  $p>0.05$ ). There were no significant differences in resting SpO<sub>2</sub>, HR, SBP, DBP and RPE between BRJ and control groups at high altitude (Table 2).

### 4.4. Harvard Step Test

There was no difference in heart rate recovery following the Harvard Step Test at sea level and at 4800m between the BRJ and control groups across all three-time points ( $p=0.16$  and  $p=0.90$ , respectively, Table 3). Heart rate across all 3 time points was significantly higher at 4800m compared with sea level for the control group. In contrast, there was no difference in heart rates in the BRJ group at 4800m compared with sea level.

The fitness index at sea level and 4800m for BRJ ( $66.30 \pm 13.8$  and  $61.90 \pm 12.9$ ) was similar compared with control ( $67.00 \pm 8.8$ ,  $p=0.88$  and  $56.92 \pm 7.5$ ,  $p=0.23$ ), figure 2. The fitness index was significantly decreased at 4800m compared with sea level for control ( $p=0.003$ ). In contrast there was no significant difference in the fitness index for BRJ at 4800m compared with sea level ( $p=0.26$ ).

### 4.5. High Altitude illness

Four participants developed symptoms of AMS at high altitude (1 at 2350m, 1 at 3400m, 2 at 4800m). There was no significant difference in LLS or AMSc scores between BRJ and control groups at a given high altitude, table 4. In addition, there was no significant difference in the use of Acetazolamide between BRJ (36%) and control (45%) at 4800m ( $p=0.67$ ) or the average number of treatment days ( $1.18 \pm 1.25$  vs.  $0.91 \pm 1.14$ ,  $p=0.56$ ).

**Table 1:** Comparison of changes in physiological parameters within increasing high altitude for both beetroot juice and control.

	<b>Beetroot</b>	<b>Control</b>	<b>P value</b> (between conditions)	<b>P value</b> (within conditions Vs altitude)
SpO <sub>2</sub> (%) - Sea Level - 2350m - 3400m - 4800m	98.6 ± 1.03 94.6 ± 1.69 <i>acd</i> 91.5 ± 1.64 <i>abd</i> 85.0 ± 3.80 <i>abc</i>	97.9 ± 1.38 94.8 ± 1.54 <i>acd</i> 91.0 ± 2.32 <i>ab</i> 87.7 ± 4.36 <i>ab</i>	0.28	<0.01 <sup>a,b,c,d</sup>
HR (beats/min) Sea Level - 2350m - 3400m - 4800m	77 ± 13.57 77 ± 10.70 76 ± 10.70 88 ± 13.68 <i>abc</i>	69 ± 10.20 75 ± 12.15 75 ± 10.84 89 ± 14.57 <i>abc</i>	0.51	<0.01 <sup>a,b,c</sup>
Systolic blood pressure (mmHg) Sea Level - 2350m - 3400m - 4800m	125 ± 16.84 123 ± 15.53 <i>d</i> 125 ± 14.65 127 ± 14.96 <i>b</i>	123 ± 9.42 117 ± 6.00 <i>d</i> 125 ± 13.45 124 ± 11.47 <i>b</i>	0.67	<0.01 <sup>b,d</sup>
Diastolic blood pressure (mmHg) Sea Level - 2350m - 3400m - 4800m	75 ± 8.46 <i>d</i> 74 ± 7.77 <i>cd</i> 83 ± 5.13 <i>abd</i> 88 ± 7.59 <i>abc</i>	78 ± 6.05 <i>d</i> 77 ± 5.97 <i>cd</i> 83 ± 8.34 <i>abd</i> 89 ± 5.56 <i>abc</i>	0.51	<0.01 <sup>b,c,d</sup> <0.05 <sup>a</sup>
Borg (RPE) Sea Level - 2350m - 3400m - 4800m	8.4 ± 0.92 12.4 ± 0.73 12.1 ± 1.39 13.8 ± 2.11	8.1 ± 1.04 11.7 ± 1.50 11.3 ± 1.38 <i>d</i> 14.3 ± 0.95 <i>b,c</i>	0.57	<0.01 <sup>b,c</sup>

*a* denote significantly different to sea level, *b* denotes significantly different to 2350m, *c* denotes significantly different to 3400m, *d* denotes significantly different to 4800m

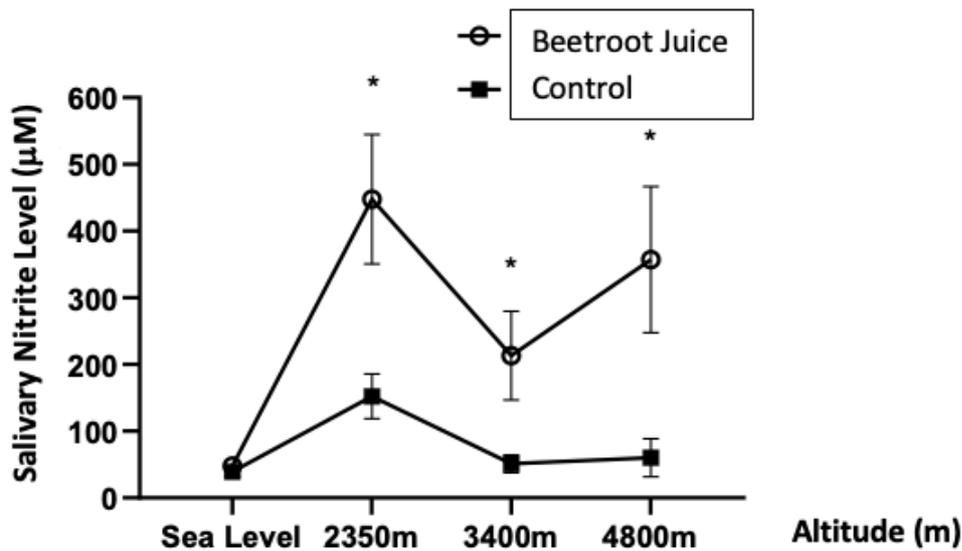
**Table 2:** Heart rate recovery at sea level and 4800m following the Harvard Step Test for both beetroot juice and control.

	Sea Level	4800m	P value
<b>BRJ</b>			
Minute 1	100 ± 21.41	113 ± 21.94	0.10
Minute 2	90 ± 20.21	94 ± 19.41	0.53
Minute 3	91 ± 16.71	94 ± 20.36	0.61
<b>Control</b>			
Minute 1	101 ± 14.17	118 ± 17.41	< 0.01
Minute 2	89 ± 14.71	102 ± 15.69	<0.05
Minute 3	83 ± 12.11	101 ± 12.88	<0.01

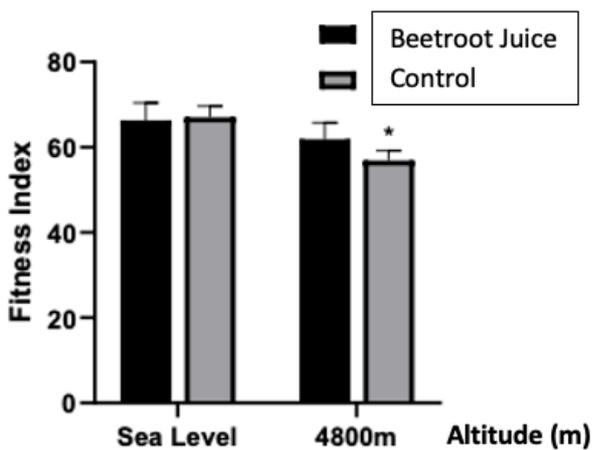
**Table 3:** Lake Louise and acute mountain sickness-cerebral scores at high altitude for both beetroot juice and control.

	Beetroot	Control	P value (between conditions)	P Value (within conditions Vs altitude)
LLS - 2350m - 3400m - 4800m	0.64 ± 1.03 1.00 ± 1.18 1.73 ± 1.42	0.82 ± 2.40 0.36 ± 0.67 <i>c</i> 1.36 ± 1.12 <i>b</i>	0.60	<0.01 <sup>b,c</sup>
AMSc - 2350m - 3400m - 4800m	0.28 ± 0.52 0.03 ± 0.67 0.11 ± 0.09	0.13 ± 0.34 0.26 ± 0.65 0.52 ± 0.42	0.31	<0.05

*a* denote significantly different to 2350m, *b* denotes significantly different to 3400m, *c* denotes significantly different to 4800m



**Fig. 2.** Salivary nitrite at sea level and high altitude for both beetroot juice and control. \*denotes beetroot juice being significantly higher than control ( $p < 0.0001$ ).



**Fig. 3.** Fitness Index measured following the Harvard Step Test at sea level and 4800m for both beetroot juice and control. \*denotes 4800m being significantly lower than sea level ( $p = 0.003$ ).

## 5. Discussion

To our knowledge, this is the first prospective randomised single study to examine the impact of BRJ supplementation for  $> 8$  days on HA exercise performance and acclimatisation at terrestrial HA. In this study, we have shown that BRJ supplementation has a positive effect on exercise during a HA trekking expedition by improving heart rate recovery and possibly protecting against decline in fitness. Despite increased salivary  $\text{NO}_2^-$ , BRJ did not have a significant effect on resting RPE, HR, DBP, SBP or  $\text{SpO}_2$ . In addition, BRJ was not associated with a reduction in burden of HA illness.

Salivary  $\text{NO}_2^-$  levels increased in response to BRJ supplementation. This is consistent with previous research and supports the theory that BRJ increases NO-generating capacity via the nitrate-nitrite-NO pathway [5,8,31,35]. At 2350m the levels of salivary  $\text{NO}_2^-$  were significantly higher in both groups compared with baseline. This effect was not maintained in control group at 3400m or 4800m. Research has

previously shown that in unacclimatised individuals, NO levels (measured by  $E_{NO}$  and NO-derived liquid phase molecules) decreased on arrival to altitude but then increased above baseline after 5 days at HA, possibly reflecting acclimatisation [21]. Our study showed that dietary nitrate supplementation resulted in higher salivary levels  $NO_2^-$  across all expedition points, the extent to which this reflects increased bioactive NO levels has not been determined and critical to our understanding of hypoxic response [54].

BRJ failed to affect SBP, DBP, HR or  $SpO_2$  in the present study. These results contrast with research at sea level and simulated altitude, where  $NO_3^-$  reduced SBP [28,55,56], DBP [56] and enhanced vascular compliance [26]. In fact,  $NO_3^-$  supplementation has been shown to abolish the reduction in endothelial functioning caused by acute terrestrial altitude 3700m (measured by flow mediated dilation) [13]. In addition,  $NO_3^-$  supplementation has been shown to increase arterial oxygenation by 1-4% during acute exposure to simulated normobaric hypoxia between 2500m - 5000m [5,26,32] and to increase muscle oxygenation [5,18,32]. However, our negative data are comparable to those from studies involving prolonged high altitude terrestrial exposure > 7 days [1,9,11,13,29]. Similar to our study, no effect of  $NO_3^-$  supplementation was found on  $SpO_2$ , HR, DBP, SBP across an 11-day trek to 5300m [24]. These negative results are potentially important as they could reflect that, physiologically, the stimulus of terrestrial altitude is different to simulated hypoxia and/or that the acute versus chronic responses differ.

The effect of BRJ in this study on SBP, DBP, HR or  $SpO_2$  may have been offset by chronic exposure to altitude and endogenous NO production via other mechanisms during acclimatisation. The physiological parameters did change in response to altitude, as expected and more advantageous effects of BRJ on physiology might be seen in acute HA exposure or when ascent is rapid. Possibly, increasing dietary nitrate may not directly translate to improved bioavailability of NO or to the alteration of physiological responses measured (SBP, DBP, HR,  $SpO_2$ ). The Xtreme Alps study found that significant increases in salivary nitrite measurements did not correlate with measurable microcirculatory responses [9]. Measuring NO levels in vivo is challenging and therefore studies to date have not been able to measure the interaction between nitrite and NO levels. To date, the concentration of NO that is optimal under physiological conditions to promote adaptation to hypoxia remains unknown. The dose-response relationship may also differ between individuals, ages, training and health status. Fasting measurements of salivary  $NO_2^-$  have been shown to remain lower than baseline across one expedition to 4559m suggesting timing of samples also needs consideration [9]. To expand our understanding of acclimatisation and role of NO metabolism, the NO inactivation mechanism, renal system and excretion of NO-metabolites warrants further investigation.

It is also important to recognise there are additional NO-derived and related factors which influence acclimatisation and could explain differences between increased salivary  $NO_2^-$  and physiological responses. We know that responses to altitude include hyperventilation, global circulatory changes (increased cardiac output, increased HR), increased red blood cell count and haemoglobin changes [2]. The role of haemoglobin in oxygen transport, binding and scavenging NO as oxygen tensions decrease is an area of consideration [57] and these mechanisms are shown to influence catalytic NOS enzyme kinetics [58]. The Bohr effect demonstrates respiratory alkalosis increases the affinity of Hb for oxygen and acidotic conditions promote oxygen dissociation and, hence, supply of oxygen to tissues. Tibetan highlanders have been shown to have lower levels of oxygen-saturated haemoglobin which may reflect a physiological adaptation. There is a suggestion in research that NO production via oxidation reactions is reduced in hypoxia. However in vitro evidence suggests that in levels of 21% oxygen much lower than physiological levels of hypoxia experienced at altitude, did not affect nNOS activity [59]. This study also found that NO operates physiologically at subnanomolar concentrations. [59]. Furthermore, there was no change in evoked NO generation by nNOS in brain slices at differing oxygen partial pressures suggesting it is not as oxygen dependent as isolated enzyme studies suggest [59]. Our research supports that NOS pathway remains functional and contributes to adaptation. In vitro studies also support this, endothelial NOS deficient mice develop more severe pulmonary hypertension and exaggerated pulmonary artery remodelling in the setting of hypoxia [60] and upregulation of NOS was shown when mice were exposed to hypoxia [61]. It maybe that NOS-dependant pathway is dependent on other physiological responses to hypoxia and as such, is altitude and time exposure dependent. Whether pre-loading with  $NO_3^-$  for use on

rapid ascent profiles may be more beneficial to provide substrates thus promoting earlier physiological acclimatisation could be investigated in future research [11,62].

A novel finding of this study was that chronic dietary nitrate supplementation ameliorated the decline in fitness associated with increasing altitude, measured by fitness index scores. Heart rate was significantly increased at each recovery time point in the control group between baseline and 4800m whereas this effect was absent in the BRJ group. The rate of post-exercise HR recovery is an indicator of cardiac-vagal reactivation [63]. Faster HR recovery/enhanced parasympathetic neural tone is an adaptation found in native highlanders [63]. Our findings support previous evidence that BRJ has a positive effect on exercise performance in hypoxia [5,12,17,26,27,31,38] and further suggests a neural or cardiac mechanism. Our study differs from one other study where acute supplementation (6 days) had no effect on exercise performance [24]. Our exercise performance findings could reflect that chronic dietary nitrate promotes physiological conditions for improving both function and recovery of large muscle groups, cardiac functioning or whole-body performance.

The trained status of individual participants was not stratified in this study and could have affected the homogeneity of the two groups. BRJ may be more beneficial to less trained individuals travelling to high altitude. Although the effect of exercise training on nitric oxide metabolism is not fully understood, a greater effect of dietary nitrate in untrained and moderately trained individuals compared to well-trained athletes has been reported [4,26,30]. The oral nitrate-reducing capacity has also been positively correlated to aerobic fitness levels [64]. Exercise training upregulates NOS expression which may promote physiological changes including angiogenesis and vascular endothelial functioning [64,65]. Highly trained individuals are known to possess more microcirculatory changes in their muscles, similar to those found in native highlanders [9,64]. Significantly increased muscle capillary density is a physiological adaptation seen in elite mountaineers and native highlanders and it probably contributes to their exceptional performance at altitude [9].

The study showed that BRJ supplementation was not effective at reducing RPE or the occurrence of HA illness compared with a control group. This is comparable to other reports of no effect of  $\text{NO}_3^-$  on HA-illness at simulated or terrestrial HA [1,5,13,24,67]. The lack of BRJ effect detected in this study could be due to the low prevalence of HA-illness (6%) compared with rates of 51% in a study of 150 trekkers at the same altitude (4500-5000m) [68]. The expedition was planned to maximise acclimatisation through a gradual ascent profile (<400m per day) and, as such, was effective at keeping the prevalence of HA illness low. In addition, the RPE data support the low prevalence of HA illness. Even though RPE significantly increased with altitude the average exertion level at the highest sleeping altitude (4800m) averaged at a 'somewhat hard' pace (RPE 13) [46]. This reflects exertion rates being moderated across the expedition and is lower than RPE scores (>15) during trekking at high altitude, that have been associated with high AMS scores [47]. These considerations explain the low rates of HA illness in the present study. Whether BRJ would be effective at reducing HA-illness during a more provocative ascent profile is unknown.

## 6. Strengths and Limitations

To our knowledge, this is one of the largest studies to investigate the effects of dietary nitrate supplementation under field conditions at terrestrial altitude. This study ensured participants were blinded to supplements (intervention and control), investigators supervised consumption of supplements and ensured 100% compliance across the expedition and the recording of complete data. Our study power was strengthened by participant testing occurring at multiple altitude points and data collection included a wide range of objective physiological markers and self-rated outcomes. These factors added reliability to our study despite the many challenges field research presents. Our study measured exercise capacity through the Harvard Step Test, heart rate recovery data and calculation of fitness index scores. This method was possible in field conditions. A gold standard measurement of fitness would have been through  $\text{VO}_2$  max, time to exhaustion or time trial performance.

The manufacturer provided data of the dosage of nitrate within each supplement volume. The nitrate content of the concentrate used to produce supplements is measured and the final product batch tested at random. To add to the reliability of this, the exact dosage of nitrate participants received in the supplements could have been measured using gold-standard techniques (chemiluminescence or HPLC). Participants were randomised and not informed whether they were in control or intervention group. A de-nitrised beetroot juice supplement would have minimised any possible placebo effect on non-objective data in the scenario that a participant was aware of the ergogenic effects of BRJ. The use of antibiotics was not prohibited, and one participant required antibiotics treatment.

Salivary nitrite was measured using test strips, in future studies a more accurate measurement of nitrite would be to collect salivary samples for analysis using gold standard techniques (chemiluminescence or HPLC). Participants were provided with uniform water bottles and educated on importance of hydration prior to expedition. To control for the effect of individuals hydration status on results, urine specific gravity could have been measured with a urine specific portable refractometer.

The expedition was planned to maximise success and minimise illness which included a graduated ascent profile. The graduated ascent and chronic exposure resulted in low rates of HA illness potentially introducing risk of type 2 error.

## 7. Conclusion

In a group of 22 adults, dietary nitrate supplementation may ameliorate the decline in fitness associated with altitude and promoted faster heart rate recovery following submaximal exercise at altitude, without affecting acclimatisation measured by physiological parameters or the burden of HA illness. BRJ may influence whole body performance by the cumulative effect of multiple physiological changes resulting in detectable and significant change in exercise performance. Despite the practical difficulties of exercise testing in field conditions, our results should encourage further investigation of the potential benefits of nitrate supplementation on HA performance. Future research could compare the effects of NO<sub>3</sub>-supplementation in acute terrestrial altitude exposure and in those undertaking more rapid ascent profiles where endogenous NO bioavailability may be reduced.

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4. *Institute of Risk and Disaster Management Reduction, Faculty of Mathematical and Physical Sciences, University College London, South Wing, Main Quadrangle, London, WC1E 6BT.*

5. *871 Penn Squadron ATC, Herts and Bucks Wing ATC HQ, Station Learning Centre, RAF Halton, Aylesbury, Buckinghamshire, HP22 5PG*

6. *7 Squadron England, RAF Odiham, Hook, Hampshire, RG29 1QT.*

7. *The University of Edinburgh, Old College, South Bridge, Edinburgh EH8 9YL*

8. *University of Glasgow, University Avenue, Glasgow, G12 8QQ*

9. *Imperial College London, South Kensington Campus, London SW7 2AZ*

10. *Wolfson Institute for Biomedical Research, University College London, The Cruciform Building, Gower Street, London, WC1E 6BT.*