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Nitrate-containing beetroot juice reduces oxygen consumption during submaximal exercise in low but not high aerobically fit male runners

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[Purpose] To examine the effect of a 4-day NO₃⁻ loading protocol on the submaximal oxygen cost of both low fit and high fit participants at five different exercise intensities.

[Methods] Eleven (6 high fit, VO_{2max} 60.1 ± 4.6ml/kg/min; 5 low fit, VO_{2max} 42.4 ± 3.2ml/kg/min) participants were initially assigned to a placebo (PL; negligible NO₃⁻) or inorganic nitrate-rich (NR; 6.2 mmol nitrate/day) group using a double-blind, placebo-controlled, crossover design. Participants completed three trials (T1, T2 and T3). T1 included a maximal aerobic capacity (VO_{2max}) treadmill test. A 6-day washout, minimizing nitrate consumption, preceded T2. Each of the four days prior to T2 and T3, participants consumed either PL or NR with the final dose 2.5 hours prior to exercise. A 14-day washout followed T2. T2 and T3 consisted of 5-minute submaximal treadmill bouts (45, 60, 70, 80 and 85% VO_{2max}) determined during T1.

[Results] Low fit nitrate-supplemented participants consumed less oxygen (p<0.05) at lower workloads (45% and 60% VO_{2max}) compared to placebo trials; changes were not observed in high fit participants. The two lowest intensity workloads of 45 and 60% VO_{2max} revealed the greatest correlation (r=0.54, p=0.09 and r=0.79, p<0.05; respectively) between VO_{2max} and change in oxygen consumption. No differences were found between conditions for heart rate, respiratory exchange ratio or rating of perceived exertion for either fitness group.

[Conclusion] Nitrate consumption promotes reduced oxygen consumption at lower exercise intensities in low fit, but not high fit males. Lesser fit individuals may receive greater benefit than higher fit participants exercising at intensities <60% VO_{2max}.

[Key words] Nitric Oxide, Dietary Nitrate, Plasma Nitrite, Aerobic Fitness, Exercise Nutrition Physiology, Submaximal Oxygen Cost.

INTRODUCTION

Foods containing dietary nitrate (NO₃⁻) have recently emerged as a potential ergogenic aid for endurance athletes¹⁻³. Dietary inorganic nitrate can be found in a variety of foods such as root and green leafy vegetables, as well as some cured/processed meats⁴. Additionally, acute supplementation can elevate concentrations of plasma nitrate and nitrite ([NO₃⁻] and [NO₂⁻]; respectively) as early as two and a half hours post-ingestion. Like vegetable food sources, supplementation with sodium nitrate has also been shown to elevate both [NO₃⁻] and [NO₂⁻] in as little as two days of supplementation⁵. Dietary NO₃⁻ may enhance athletic performance⁶⁻⁸ via the rise in [NO₂⁻], a biomarker of increased nitric oxide (NO) availability^{9,10}. Interestingly, NO₃⁻ supplementation has been reported to reduce oxygen consumption at pre-established submaximal exercise intensities, which suggests dietary nitrate improves oxygen utilization^{5,7,11}. Despite this evidence linking NO₃⁻ supplementation with reduced oxygen cost, a recreationally trained cohort appear to be those receiving the greatest benefit (VO_{2max} 40-60 ml/kg/min)^{1,3,7}. In contrast, many of the studies examining well-trained or highly fit participants (VO_{2max} > 60 ml/kg/min) supplementing with nitrate found no change in submaximal oxygen cost and no improvement in performance compared to placebo conditions¹²⁻¹⁴. Together, as alluded to by others, these data suggest dietary and supplemental NO₃⁻ affect athletic performance to a magnitude largely influenced by training status^{15,16}. The purpose of this study was to examine the effect of a 4-day NO₃⁻ loading protocol on the submaximal oxygen cost of both low fit and high fit participants at five different exercise intensities. We hypothesized that increasing [NO₂⁻] via dietary NO₃⁻ supplementation would reduce oxygen consumption at 45, 60, 70, 80, and 85% VO_{2max} in low but not high fit participants. We also hypothesized that NO₃⁻ supplementation would alter oxygen consumption in a manner dependent on aerobic fitness, with low fit participants experiencing the greatest increase in metabolic efficiency.

This study was conducted with treadmill exercise while other stud-

Table 1. Participant descriptive data.

Participants	Group	Age (yr)	HT (cm)	WT (kg)	BMI	VO _{2max} (ml/kg/min)
n = 5	Low fit	23 ± 3	178.02 ± 6.02	80.53 ± 19.18	25.30 ± 5.25	42.4 ± 3.2
n = 6	High fit	24 ± 4	182.03 ± 4.30	80.21 ± 9.64	24.18 ± 2.54	60.1 ± 4.6

Values are means ± SD. Height, HT (cm); weight, WT (kg); body mass index, BMI (kg/m²); maximal oxygen consumption, VO_{2max} (ml/kg/min).

ies have examined the effects of nitrate via cycling protocols. Cycling and running exercise are inherently different in terms of muscle recruitment which creates difficulty with direct comparison of results. This study also differs from previous literature by examining the changes in oxygen consumption in both high and low fit participants following identical supplementation protocols. It is difficult to compare results of other studies with either high or low fit cohorts as supplementation protocols may not be similar. While other studies have, in large part, examined a single workload, this study examined 5 workloads to better understand the efficacy of dietary nitrate supplementation over a range of exercise intensity relative to each participant.

METHODS

Eleven healthy men (6 high fit with VO_{2max}: 60.1±4.6 ml/kg/min and age 23±3; 5 low fit with VO_{2max}: 42.4±3.2 ml/kg/min and age 24±4; Table 1) provided written consent for this study as approved by the University of New Mexico's institutional review board (#13-288). Participants were excluded if they indicated any known cardiovascular, pulmonary or metabolic disease on a health history questionnaire. Participants were classified with either high or low aerobic fitness based on their VO_{2max} (>85% or <65% of age and male gender norms; respectively) according to previously published fitness categories for maximal aerobic power¹⁷. Participants who achieved a VO_{2max} between 65 and 85% of age and male gender norms were excluded from the study to better create heterogeneity between high and low fit groups.

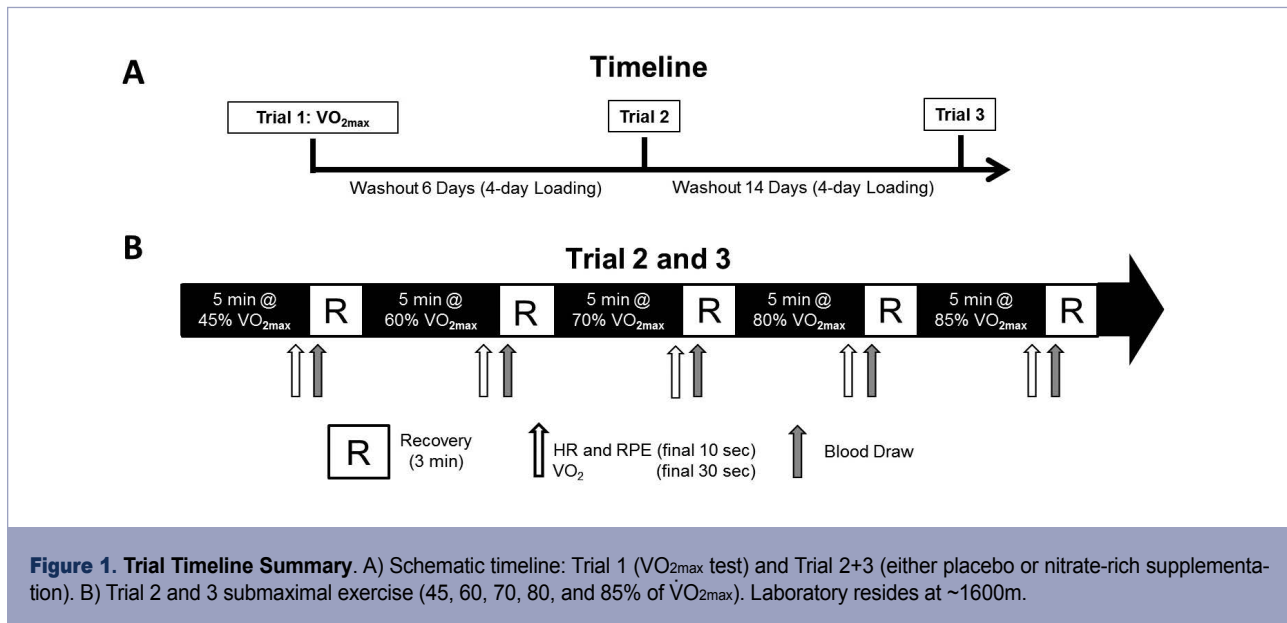
All tests were conducted on a treadmill with the velocity and grade controlled manually (Precor C966i, Woodinville, WA). During trial 1 (T1), all participants completed a standardized VO_{2max} treadmill test to exhaustion; the protocol varied for participants prescreened as fit or unfit based on self-reported weekly physical activity. Following an initial 2-minute slow walk at 1% (1.34 and 1.79 m/s for unfit and fit; respectively) the velocity increased by 0.67 m/s every minute and a half for the first 6 stages. Treadmill grade then increased by 3% every 1.5 min. Breath-by-breath metabolic gases were continuously collected (True One, ParvoMedics, Sandy, Utah, USA) and averaged over 11-breath sequences. The VO_{2max} was recorded as the highest value collected over the 30 seconds prior to the participant's volitional exhaustion. Oxygen consumption data were then plotted against treadmill velocity during the first 6 stages (grade at 1%) to establish

the velocity corresponding to 5 different intensities (45, 60, 70, 80, and 85% VO_{2max}) to be used during trial 2 (T2) and trial 3 (T3). During T2 and T3, metabolic gasses were analyzed as described above to assess VO₂ over the final 30 seconds of each five-minute submaximal exercise bout. Heart rate (HR) was measured via short range radiotelemetry (Polar Electro T31, New York, USA). HR and rating of perceived exertion (RPE; 6-20 scale)¹⁸ were recorded during the final 10 seconds of each exercise bout. Figure 1 depicts the testing sequence including washout and supplement loading periods between trials as well as T2 and T3 details.

Following T1, in a randomized, double-blind, placebo-controlled, crossover fashion, participants were initially assigned to a placebo (PL) or nitrate-rich (NR) beverage group. Each participant received four 70ml beverages (either four placebo or four NR) and instructions to consume one beverage per day following the designated washout period and prior to T2. The opposite treatment beverage was consumed prior to T3. Beverages were consumed at approximately the same time of day with the final dose consumed 2.5 hours prior to commencing exercise. The PL beverage was black-currant juice with negligible NO₃⁻ content^{4, 6, 11} and the NR trial consisted of a commercially available, inorganic nitrate-rich product, (Beet It with 6.2 mmol NO₃⁻, James White Drinks Ltd, Ipswich, UK). Beverages were similar in color and were provided to participants in identical packaging (bottles) to minimize indicators of treatment (PL or NR). During the dietary nitrate washout periods (Figure 1), participants were asked to minimize their consumption of nitrate-rich foods and supplements containing nitrate/l-arginine⁴ based on a list of items provided to each participant.

Participants were asked to avoid strenuous exercise, alcohol, chewing gum, and mouthwash 24 hours prior to each trial and caffeine 12 hours before each trial as such items may attenuate the rise in [NO₂⁻] following dietary NO₃⁻ supplementation¹⁹. Participants self-recorded their dietary intake prior to T2 and then consumed the same diet prior to T3.

Upon arrival to the lab for T2 and T3, an intravenous catheter (Venflon IV cannula; Becton-Dickinson, Franklin Lakes, New Jersey, USA) was placed in a prominent forearm vein. Blood samples (~10 ml) were drawn and collected into a tube containing lithium heparin before exercise and following each intensity (45%, 60%, 70%, 80%, and 85% of VO_{2max}) for analysis of [NO₂⁻] during both T2 and T3 (Figure 1B). Prior to each blood draw, ~3 ml sample was drawn and discarded to clear the line of saline. A 5 ml saline flush was administered following the



10 ml blood draw. The catheter was removed following the post-85% $\dot{V}O_{2max}$ blood draw.

All tubes were inverted approximately ten times and immediately centrifuged at 4,000 rpm at 4°C for 6 minutes. Plasma samples were then stored at -80°C for subsequent analysis. Plasma nitrite was measured in duplicate using a commercially available microplate-based colorimetric assay kit (Cayman Chemical, USA). The inter-assay coefficient of variation was within the range as indicated by the manufacturer.

Results are presented as mean \pm standard deviation. Data were analyzed using a two-way analysis of variances (ANOVA) to examine variables between condition (NR or PL) at each intensity (45%, 60%, 70%, 80%, and 85% of $\dot{V}O_{2max}$). Significant findings were determined by $p < 0.05$ using a Bonferoni post-hoc when an interaction effect was observed. The magnitude of linear association between oxygen consumption at a given intensity and $\dot{V}O_{2max}$ was measured with the Pearson's coefficient of correlation.

RESULTS

We began our analyses by verifying that dietary NO_3^- increased $[\text{NO}_2^-]$. As expected, $[\text{NO}_2^-]$ was consistently greater in the NR group in both low fit and high fit participants (Figure 2A and 2B, respectively). Next, we measured the effect of dietary NO_3^- supplementation on oxygen cost and observed that low fit participants exhibited a significantly reduced oxygen cost at 45% and 60% $\dot{V}O_{2max}$ (Figure 2C). Conversely, there were no differences between the NR and PL trials for the high fit participants (Figure 2D). High and low fit groups exhibited no differences in RER values between treatment (NR and placebo) at all intensities (Figure 4C and D).

In addition, the lowest intensity workloads of 45 and 60% revealed the greatest correlation ($r=0.54$, $p=0.09$ and $r=0.79$, $p<0.05$; respectively) between aerobic fitness and the change in oxygen consumption following nitrate consumption, suggesting NR beverages may possess greatest benefits at moderately intense exercise regardless of aerobic fitness (Figure 3).

High and low fit participants' HR responses were similar between their respective NR and placebo trials at all intensities (Figure 4A and B). There were also no differences in HR responses for any submaximal bout during the placebo and NR trials based on aerobic fitness comparisons. Likewise, RPE increased in an exercise intensity-dependent manner, but was more pronounced for the low fit participants. Regardless, no differences in perceived exertion between NR and PL trials at any of the five submaximal intensities were found for the low and high fit groups (data not shown).

DISCUSSION

This study is one of the first to investigate the influence of aerobic fitness ($\dot{V}O_{2max}$ level (<65% or >85% normative values for men) in which participants completed the same supplementation protocol and exercised at 5 different submaximal intensities ranging from 45% to 85% of each participant's respective $\dot{V}O_{2max}$. While our loading protocol (Figure 1A) was successful in elevating $[\text{NO}_2^-]$ levels, the varying degree to which it did highlights the heterogeneity of our subject pool and may have precluded our ability to report statistically significant differences for oxygen cost reductions across all submaximal intensities.

The four-day loading protocol concluded with the final beverage ingested 2.5 hour prior to the first bout of submaximal exercise. Supplementation within two to three

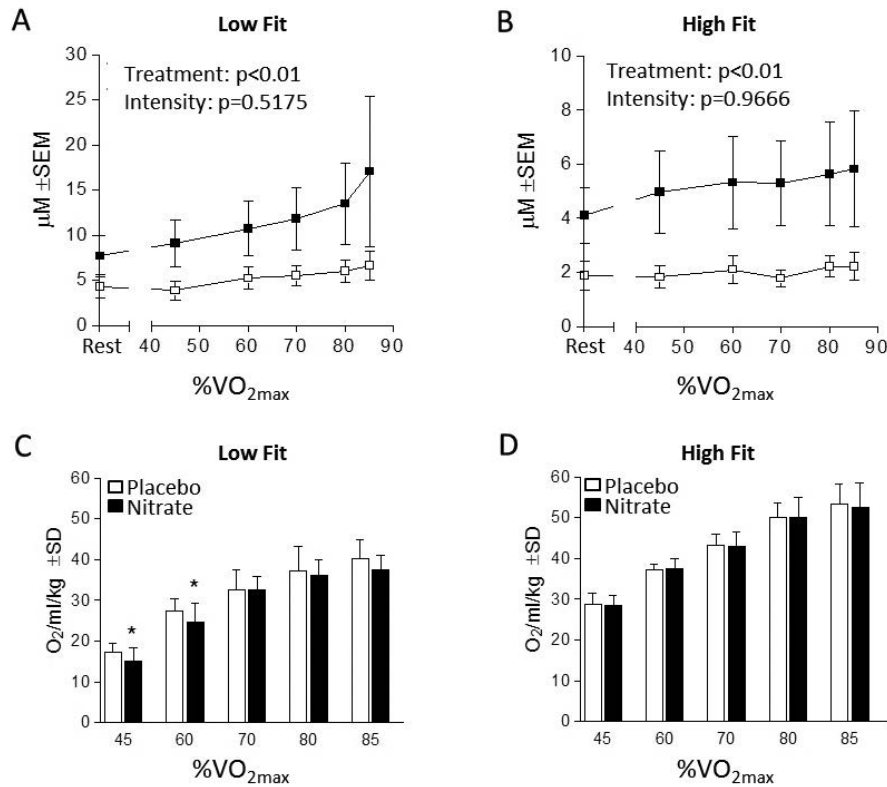


Figure 2. Plasma Nitrite and Submaximal Oxygen Costs. A) Plasma nitrite (μM) of low fit participants during submaximal exercise at baseline (Rest), 45%, 60%, 70%, 80% and 85% $\text{VO}_{2\text{max}}$ after placebo (white) and nitrate-rich (black) supplementation. B) Plasma nitrite from high fit participants during the same submaximal exercise intensities described for panel A. C) Oxygen consumption of low fit participants ($\text{ml}/\text{kg}/\text{min}$) during submaximal exercise at 45%, 60%, 70%, 80% and 85% $\text{VO}_{2\text{max}}$ after placebo (white bars) and nitrate-rich (black bars) supplementation 2.5 hours prior to exercise. D) Oxygen consumption of high fit participants ($\text{ml}/\text{kg}/\text{min}$) during the previously described submaximal exercise during intensities. *Significance at $p < 0.05$.

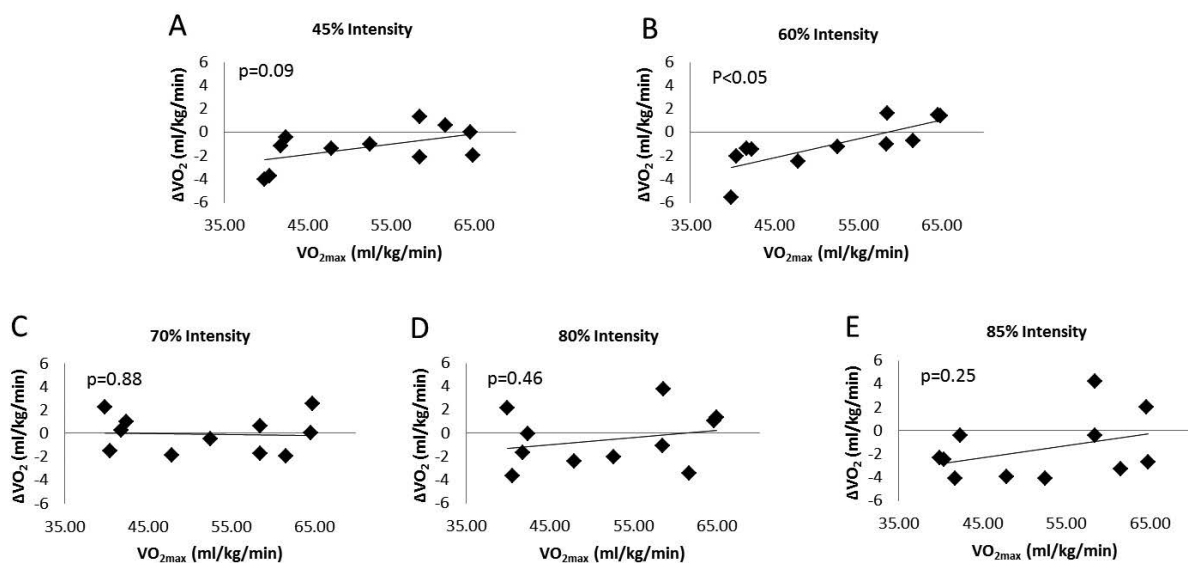


Figure 3. Aerobic fitness and change in VO_2 following nitrate supplementation. Panels represent the correlation between participants' aerobic fitness and change in VO_2 following nitrate supplementation at the intensities indicated. A) 45% Intensity, $r = 0.54$, $p = 0.09$ B) 60% Intensity, $r = 0.79$, $p < 0.05$ C) 70% Intensity, $r = -0.05$, $p = 0.88$ D) 80% Intensity, $r = 0.25$, $p = 0.46$ E) 85% Intensity, $r = 0.38$, $p = 0.25$

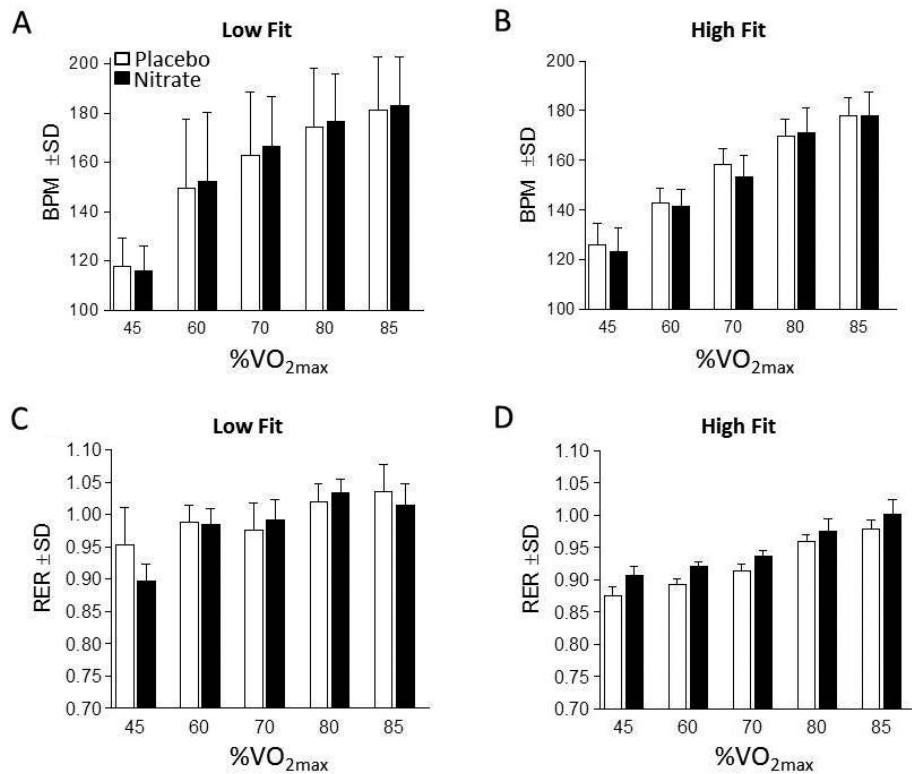


Figure 4. Submaximal Exertion Response A) Heart rate (HR) of low fit participants in beats per minute (BPM) during submaximal exercise at 45%, 60%, 70%, 80% and 85% VO_{2max} after placebo (white bars) and nitrate-rich (black bars) supplementation 2.5 hours prior to exercise. B) Participant HR of high fit participants during submaximal exercise during intensities described in panel A. C) Respiratory exchange ratio (RER). D) Participant RER of high fit participants during submaximal exercise during intensities described in panel A.

hours of exercise⁶⁻⁸ after a loading period as short as two⁵ or three³ days has been shown effective in increasing [NO₂⁻]. As expected, we observed increases in the average [NO₂⁻] values at baseline and throughout the NR trials as compared to PL for both our high fit and low fit participants (Figure 2A and 2B). As others have reported, the decrease in the O₂ cost of exercise is negatively correlated with VO_{2max}, such that aerobically fit individuals may experience less reduction in oxygen cost compared to a lesser fit cohort. In addition, the increase in NO₂⁻ plasma levels following dietary NO₃⁻ supplementation has been found to be lower in subjects with high VO_{2max}. Both of these findings are consistent with our data^{20,21}. Dietary nitrate supplementation increases NO bioavailability via the nitrate-nitrite-nitric oxide pathway^{9,10} and may, therefore, improve performance²²⁻²⁴, perhaps most notably in populations with lower aerobic fitness as explained below.

The current study results support previous investigations reporting that individuals who are more aerobically fit (as evidenced by a higher VO_{2max}) incur a lesser reduction in submaximal oxygen cost when compared to less fit individuals^{15,20}. Porcelli et al.²⁰ examined changes in oxygen cost at 80% of individual ventilatory thresholds (reported as moderate-intensity exercise), while the

current study examined 5 different training intensities relative to individual VO_{2max} (ranging from 45 to 85% VO_{2max}). Dietary NO₃⁻ supplementation for our high fit participants (VO_{2max}: 60.1±4.6 ml/kg/min) failed to produce a statistically significant reduction in oxygen cost at any of the submaximal intensities (Figure 2B). In addition, the findings of our study support studies that examined changes in participants classified as low fit or recreationally active (VO_{2max} between 40 and 60 ml/kg/min). In lesser fit participants, others have also found a reduction in oxygen cost followed dietary nitrate supplementation^{2,3,7}. Therefore, findings for our low fit group (VO_{2max}: 42.4±3.2 ml/kg/min), were expected, although interestingly the significant reductions in oxygen cost only occurred during the lower intensity exercise bouts (45 and 60% VO_{2max}). While oxygen consumption was reduced for our low fit cohort, no differences in RER were found between PL and NR treatments for either the high or low fit group. Given the nature of physical activity, RER may not reflect macronutrient oxidation as other factors such as hyperventilation and lactate buffering may contribute to non-metabolic-created CO₂. Future studies may wish to examine blood lactate in conjunction with metabolic gases to better elucidate the changes in oxygen consumption

and macronutrient catabolism.

In a meta-analysis examining the effect of nitrate supplementation on exercise performance in healthy individuals, Hoon et al.¹⁵ reported, "Qualitative analysis suggested that performance benefits are more often observed in inactive to recreationally active individuals and when a chronic loading of NO_3^- over several days is undertaken." Given the number of studies published recently in addition to our findings, it is becoming evident that the benefits of dietary NO_3^- may be limited to those individuals who have not already received the benefit of chronic physiological adaptation to aerobic exercise training^{15,16}. Dietary nitrate has been implicated in improved mitochondrial efficiency, which may provide some indication of the mechanism through which some individuals receive benefit²⁵⁻²⁷. Although speculative, dietary nitrate may alter blood flow distribution including vasodilation of non-working muscle vascular beds as dietary nitrate does not prioritize a site-specific vasodilation to muscles engaged in exercise. In fact, blood flow distribution may, therefore, be reduced to working muscles within the limits of maximal cardiac output. In this manner, reduced blood flow may reduce oxygen delivery and transport to those muscles which need it most. Future research should also examine the changes in mitochondrial content and efficiency following dietary NO_3^- controlling for aerobic fitness to better ascertain the benefits of this supplement alongside natural adaptations in response to chronic aerobic exercise.

The efficacy of dietary NO_3^- may also be dependent on the amount consumed daily. Previous research found 3 of 10 participants who consumed 4 mmol NO_3^- 2.5 hours prior to exercise were classified as "non-responders" as they did not demonstrate a marked increase in $[\text{NO}_2^-]$ ²⁸. When those 10 participants consumed 8 mmol and 10 mmol, however, the number of "non-responders" were reduced to 2 and 1, respectively. As is evident in Figure 2A and 2B, our results yielded large standard deviations during the NR supplementation trials; this lends support to the possibility that the supplement dosage administered in this study was too low for some in our sample, although ≤ 6.2 mmol has been used by others in as little as 2.5 hours prior to exercise^{7,29,30}. Therefore, a number of factors should be considered when determining the potential benefit of dietary NO_3^- supplementation. The amount of dietary NO_3^- consumed in this study may impact benefits and doses larger than 6.2 mmol may elicit different outcomes. As determined in the current study, aerobic fitness may be another factor which may alter supplement outcomes. Our low fit participants experienced a reduction in oxygen cost during low to moderate intensity exercise ($\leq 60\%$ $\text{VO}_{2\text{max}}$) that was not found in high fit participants, consistent with other publications as previously described.

While the focus of this investigation was on the changes in oxygen cost during submaximal exercise, we also evaluated indicators of exertion including HR responses during each of the exercise bouts. A unique feature of our

study was the inclusion of RPE during each of the five submaximal treadmill exercise bouts. While some elite and other aerobically trained participants may experience benefits from dietary NO_3^- ³¹, it would appear that the current trend is that less benefit is received by elite and highly trained participants as compared to those with lower aerobic capacities^{15,16,20}.

Dietary nitrate may increase bioavailability of NO and therefore may also be responsible for a number of changes including the following: increased blood flow via vascular smooth muscle relaxation and cyclic GMP accumulation, improved or increased exercise induced glucose uptake in skeletal muscle, neurotransmission, immune response, regulation of mitochondrial respiration, and glycolysis as mediated by AMPK^{9,10}. Dietary NO_3^- has been shown to improve endothelial function in healthy individuals with a lesser effect in older individuals (>60 years of age) as well as those with cardiovascular disease risk factors³². Future research examining the effect of dietary nitrate on endothelial function and arterial stiffness controlling for aerobic fitness may better explain the mechanisms for dietary nitrate and the cost of oxygen during submaximal exercise. Other areas which may also benefit from further research include the augmentation of performance of events using different muscle fiber types (type I vs. type II), as well as diseased and aging populations.

A greater benefit from NO_3^- may, therefore, be observed in those participants who are less fit and have not received natural physiologic adaptation from aerobic training. The implications of our findings suggest that low fit men ($\text{VO}_{2\text{max}}$ lower than 65% of their age-specific normative values) may experience greater reduction in submaximal oxygen consumption following NO_3^- supplementation compared to individuals who have a higher fitness level ($\text{VO}_{2\text{max}}$ greater than 85% of their age-specific normative values).

In summary, our findings support conclusions of others which have also reported that high fit individuals may receive less benefit than low fit individuals who consume dietary NO_3^- supplements prior to physical activity³³. In a commentary on this topic, Porcelli also reported on the effects of nitrate supplementation on oxygen consumption with consideration of aerobic fitness level. Our findings support Porcelli's conclusions that the effects of nitrate supplementation are in fact significantly related to individual aerobic fitness levels, "with no benefits observed on highly trained subjects ($\text{VO}_{2\text{max}} >60$ ml/kg/min)." While more evidence is necessary, the same findings may also be true at higher elevations where high fit cyclists and runners alike did not receive exercise benefits following dietary NO_3^- supplementation^{34,35}.

Men with $\text{VO}_{2\text{max}}$ lower than 65% of their age-specific normative values appear to be more likely to incur reduced oxygen consumption following dietary NO_3^- supplementation during low to moderate intensity exercise (below 60% $\text{VO}_{2\text{max}}$). Further, the greater benefit from dietary NO_3^- supplementation may occur in individuals who have not received natural physiologic adaptation from

aerobic training. Men with a VO_{2max} greater than 85% of their age-specific normative values may incur lesser reduction in submaximal oxygen cost following dietary nitrate supplementation when compared to low fit individuals.

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