The Effects of Acute and Chronic Beetroot Juice Supplementation on
Exercise Economy and Time Trial Performance in Recreationally
Active Females

by

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ABSTRACT

THE EFFECTS OF ACUTE AND CHRONIC BEETROOT JUICE SUPPLEMENTATION ON EXERCISE ECONOMY AND TIME TRIAL PERFORMANCE IN RECREATIONALLY ACTIVE FEMALES

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Beetroot juice (BRJ) is a supplement that reduces the oxygen cost of submaximal exercise and improves performance in recreationally active males, both acutely and chronically. However, evidence supporting the effects of BRJ in females is lacking. This thesis examined the effects of acute and chronic BRJ supplementation in 12 recreationally active females ($V_{O_2\text{peak}}$: $40.6 \pm 1.2$ mL O$_2$/kg/min). Using a randomized, double-blind, placebo-controlled, crossover design, subjects supplemented with 280 mL BRJ or a nitrate-free placebo for 8 days separated by a 9 $\pm$ 1 days washout period. On days 1 (acute) and 8 (chronic), subjects completed a submaximal cycling protocol and a 4 kJ/kg time trial. Acute and chronic BRJ supplementation had no effect on $V_{O_2}$ at 50 or 70% $V_{O_2\text{peak}}$, despite large increases in plasma nitrate and nitrite. Additionally, there was no difference in time trial completion. To conclude, recreationally active females may not benefit from acute or chronic BRJ supplementation.
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NOS - Nitric Oxide Synthase
NO\textsubscript{2} - Nitrite
O\textsubscript{2} - Oxygen
PCr - Phosphocreatine
PGC1a - Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-
Alpha
PLA - Placebo
PO - Power Output
P/O Ratio - Phosphate/Oxygen Ratio (ATP synthesis/Oxygen utilization)
ROS - Reactive Oxygen Species
RPE - Rating of Perceived Exertion
SBP - Systolic Blood Pressure
SERCA - Sarcoplasmic Reticulum Calcium ATPase
SOD - Superoxide Dismutase
TFAM - Mitochondrial Transcription Factor A
TT - Time Trial
UCP-3 - Uncoupling Protein 3
UVA - Ultraviolet A
VO\textsubscript{2} - Oxygen Uptake
VO\textsubscript{2max} - Maximal Oxygen Uptake
VO\textsubscript{2peak} - Peak Oxygen Uptake
W - Watts
Nitric oxide (NO) is a gaseous signalling molecule that elicits potent effects on numerous biological tissues and is involved in an array of physiological processes including, but not limited to: vasodilation, calcium handling, mitochondrial respiration, and neurotransmission (Bailey et al. 2011).

NO can be produced endogenously and obtained from exogenous dietary sources. Endogenously, NO is produced through the oxidation of L-arginine to L-citrulline and NO (Figure 1). This reaction requires oxygen (O$_2$) and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as a co-substrate as well as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH$_4$), heme, and calmodulin as cofactors and substrates and is catalyzed by the enzyme nitric oxide synthase (NOS) (Bailey et al. 2011). NOS exists in several isoforms depending on its location and role within the biological system of interest. These isoforms include neuronal NOS, endothelial NOS, and inducible NOS. These isoforms will not be discussed in detail as they are beyond the scope of this literature review.

In 1994, it was acknowledged by Benjamin et al. (1994) as well as Lundberg et al. (1994) that NO can be produced through an alternative exogenous pathway that does not rely on O$_2$. This anaerobic pathway involves the sequential one electron reduction of nitrate (NO$_3^-$) to nitrite (NO$_2^-$) and further to NO (Figure 1). This allows the formation of NO in situations where the NOS enzyme may be impaired such as hypoxia or disease. This pathway has important implications for cardiovascular disease as well as exercise performance. The NO$_3^-$ necessary for this reaction is obtained primarily from dietary sources such as green leafy vegetables (spinach,
lettuce, kale, arugula) and root vegetables (beetroot, yams, turnips, carrots). Spinach, lettuce and beetroot have the highest dietary NO\textsubscript{3}\textsuperscript{−} concentrations (Hord et al. 2009).

Approximately 75% of ingested dietary NO\textsubscript{3}\textsuperscript{−} is excreted in the urine, with only ~25% entering the enteral-salivary circulation (Webb et al. 2008). Dietary NO\textsubscript{3}\textsuperscript{−} is swallowed and subsequently absorbed from the stomach and small intestines. Once it enters the circulation, it is concentrated in the salivary glands. NO\textsubscript{3}\textsuperscript{−} is secreted from the salivary glands into the mouth via saliva, and anaerobic bacteria facilitate the reduction of NO\textsubscript{3}\textsuperscript{−} to NO\textsubscript{2}\textsuperscript{−}. The NO\textsubscript{2}\textsuperscript{−} is swallowed again and is further reduced to NO in the acidic environment of the stomach (Bailey et al. 2011).

**Figure 1:** The oxygen (O\textsubscript{2})-independent (left) and O\textsubscript{2}-dependent (right) pathways for nitric oxide (NO) production. The O\textsubscript{2}-independent pathway relies on dietary nitrate (NO\textsubscript{3}\textsuperscript{−}) to undergo sequential reduction to nitrite (NO\textsubscript{2}\textsuperscript{−}) and to NO. The O\textsubscript{2}-dependent pathway relies on a number of substrates and cofactors including nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine nucleotide (FAD), tetrahydrobiopterin (BH\textsubscript{4}), haem, and calmodulin as well as the enzyme nitric oxide synthase (NOS). This figure also depicts an array of the putative biological targets of NO (Bailey et al. 2011).

A final and newly regarded pathway by which the body generates NO is through ultra violet A (UVA) exposure (Oplander et al. 2009; Liu et al. 2014; Muggeridge et al. 2015). The NOS enzyme is active in a number of skin cells such as keratinocytes, melanocytes, fibroblasts
and endothelial cells (Bruch-Gerharz et al. 1998). The NO produced by skin cell NOS are converted to nitroso compounds, NO$_2^-$ or NO$_3^-$ (Oplander et al. 2009). Remarkably, when UVA rays penetrate the skin they can convert these compounds back to NO. This may help explain why blood pressure is shown to be lower in countries that are closer to the equator and may partially explain why blood pressure is typically lower in the summer months compared to the winter (Oplander et al. 2009).

A plethora of nutritional supplements have been used in an attempt to augment the human body’s production of NO. L-arginine is able to accumulate in the blood, but there is equivocal evidence as to whether it increases NO production in healthy individuals (Bode-Böger et al. 1998; Liu et al. 2009; Bailey et al. 2010; Alvares et al. 2012). Furthermore, it has been shown that citrulline, the co-product of NO synthesis, can be enzymatically converted back to arginine to produce NO (Schwedhelm et al. 2007; Bryk et al. 2008). These supplements provide substrate for the endogenous production of NO via the oxidation of L-arginine, however a greater body of literature is required to support the effectiveness of these supplements. Concentrated beetroot juice (BRJ) is a potent source of dietary NO$_3^-$ containing 6.5 mmol (mM) or 400 mg per 70 mL dose. BRJ facilitates the production of NO through the exogenous O$_2$-independent NO$_3^-$-NO$_2^-$-NO pathway and has been shown to elevate plasma NO$_2^-$ in a number of studies (Vanhatalo et al. 2010; Wylie et al. 2013; Bailey et al. 2015; Betteridge et al. 2016). Similarly, sodium nitrate (NaNO$_3$) and potassium nitrate (KNO$_3$) administered as supplemental forms of NO$_3^-$ can augment the NO$_3^-$-NO$_2^-$-NO pathway. NaNO$_3$ has been shown to elevate plasma NO$_2^-$ in a number of studies (Larsen et al. 2010; Bescós et al. 2012; Gasier et al. 2017), and KNO$_3$ has also been shown to increase plasma NO$_2^-$ following supplementation (Kapil et al. 2010). Therefore, the literature supports the use of dietary NO$_3^-$ to increase plasma [NO$_3^-$] and [NO$_2^-$].
NO is an unstable molecule and has a very short half-life in the blood of merely a few milliseconds (Kelm 1999). NO is rapidly oxidized to NO$\textsubscript{2}^-$, and this is the largest circulating pool of NO$\textsubscript{3}^-$ products in the blood (Cosby et al. 2003). NO$\textsubscript{2}^-$ can be converted back to NO for cellular signalling or can be further oxidized to NO$\textsubscript{3}^-$. NO can act on several cellular targets including proteins that contain transition metals or thiol groups (Tengan et al. 2013). These mechanisms will be addressed in detail in a subsequent section of this literature review.

**BRJ Pharmacokinetics, Dose and Duration**

**Dietary NO$\textsubscript{3}^-$ Pharmacokinetics**

Pharmacokinetic profiles are essential to nutritional intervention studies as they provide a framework to validate the accumulation of the treatment in the blood, demonstrate the time course with which the treatment rises and peaks in the blood, and depict the rate of clearance from the body. Previous research has demonstrated that plasma NO$\textsubscript{3}^-$ levels begin to rise ~30 min post-ingestion and peak ~1.5 h following the ingestion of BRJ (Webb et al. 2008; Wylie et al. 2013) or KNO$\textsubscript{3}$ (Kapil et al. 2010). Plasma NO$\textsubscript{3}^-$ remains elevated for up to 24 h following a single dose of dietary NO$\textsubscript{3}^-$ (Wylie et al. 2013; Kapil et al. 2010). Furthermore, plasma NO$\textsubscript{2}^-$ levels begin to rise ~1.5 h post-ingestion, peaking ~2.5 h post-ingestion, and have been shown to remain elevated for an additional 3 h (Webb et al. 2008; Kapil et al. 2010; Wylie et al. 2013). Both plasma NO$\textsubscript{3}^-$ and NO$\textsubscript{2}^-$ have been shown to return to baseline concentrations ~24 h following a large, acute dose of dietary NO$\textsubscript{3}^-$ (Webb et al. 2008; Kapil et al. 2010; Wylie et al. 2013). However, plasma NO$\textsubscript{2}^-$ levels have been shown to decrease by 12 h post-ingestion with a moderate dose (8.4 mM) of dietary NO$\textsubscript{3}^-$ (Wylie et al. 2013). Interestingly, the slight lag time for peak plasma NO$\textsubscript{2}^-$ compared to plasma NO$\textsubscript{3}^-$ likely reflects the time required for NO$\textsubscript{3}^-$ to enter the enterosalivary circulation and undergo the sequential reduction from NO$\textsubscript{3}^-$ to NO$\textsubscript{2}^-$. The results
from this study indicate that dietary NO\textsuperscript{3−} should be administered every 12 h to ensure plasma [NO\textsuperscript{3−}] and [NO\textsuperscript{2−}] remain chronically elevated above baseline concentrations.

The conversion in the enterosalivary circulation can be disrupted by spitting (Webb et al. 2008) or destruction of the anaerobic bacteria via antibacterial mouthwash (Govoni et al. 2008; McDonagh et al. 2015; Woessner et al. 2016). As expected, when the enterosalivary circulation is disrupted following a dose of dietary NO\textsuperscript{3−} there is a concomitant termination in the reduction of NO\textsuperscript{3−} to NO\textsuperscript{2−}, as represented by no change in plasma [NO\textsuperscript{2−}] despite a significant increase in plasma [NO\textsuperscript{3−}]. Furthermore, since plasma [NO\textsuperscript{2−}] is correlated with NO production, the disruption of the enterosalivary circulation eliminates the beneficial biological effects associated with dietary NO\textsuperscript{3−} supplementation (Webb et al. 2008). This demonstrates that oral bacteria are fundamental to the rise in plasma [NO\textsuperscript{2−}] and subsequent biological effects following a dose of dietary NO\textsuperscript{3−}.

**Dose Response: Plasma Levels, Blood Pressure, Oxygen Uptake (VO\textsubscript{2}) and Performance**

To date, only two studies have investigated the dose-response relationship associated with dietary NO\textsuperscript{3−} supplementation. Kapil et al. (2010) investigated the dose-response associated with supplementing acutely with 4, 12 and 24 mM of KNO\textsubscript{3} in 21 healthy subjects. These authors found a 7-fold increase in peak plasma [NO\textsuperscript{3−}] following 4 mM KNO\textsubscript{3}, a 27-fold increase following 12 mM KNO\textsubscript{3}, and a 35-fold increase following 24 mM KNO\textsubscript{3} (Kapil et al. 2010). The peak plasma [NO\textsuperscript{3−}] occurred 3 h post-ingestion. In a similar manner, these authors found a 1.3-fold increase in peak plasma [NO\textsuperscript{2−}] following 3 mM KNO\textsubscript{3}, a 2-fold increase following 12 mM KNO\textsubscript{3}, and a 4-fold increase following 24 mM KNO\textsubscript{3}. The peak plasma [NO\textsuperscript{2−}] occurred 2.5 h post-ingestion. Interestingly, this dose-response relationship translated to dose-dependent reductions in systolic blood pressure (SBP) and diastolic blood pressure (DBP). When 4 mM
KNO₃ was administered, the peak reduction in SBP was 2 mmHg and the peak reduction in DBP was 4 mmHg. When 12 mM KNO₃ was administered, the peak reduction in SBP was 6 mmHg and the peak reduction in DBP was 4 mmHg. Lastly, when 24 mM KNO₃ was administered, the peak reduction in SBP was 6 mmHg and the peak reduction in DBP was 5 mmHg (Kapil et al. 2010). The results from this study demonstrate that plasma NO₃⁻ and NO₂⁻ increase in a dose-dependent manner which correlates with reductions in SBP and DBP.

Wylie et al. (2013) investigated the dose-response relationship of 4.2, 8.4, and 16.8 mM of dietary NO₃⁻ administered as an acute dose of BRJ in 10 recreationally active males. These authors found an 8-fold increase in peak plasma [NO₃⁻] following 4.2 mM NO₃⁻, a 12-fold increase following 8.4 mM NO₃⁻, and a 24-fold increase following 16.8 mM NO₃⁻ (Figure 2). The peak plasma [NO₃⁻] occurred 1 h post-ingestion following 4.2 and 8.4 mM dietary NO₃⁻ and 2 h post-ingestion for 16.8 mM dietary NO₃⁻. Similarly, these authors found a 3.3-fold increase in peak plasma [NO₂⁻] following 4.2 mM NO₃⁻, a 5-fold increase following 8.4 mM NO₃⁻, and a 7.8-fold increase following 16.8 mM NO₃⁻. The peak plasma [NO₂⁻] occurred 2 h post-ingestion following 4.2 and 8.4 mM dietary NO₃⁻ and 4 h post-ingestion for 16.8 mM dietary NO₃⁻.

Similar to the results presented by Kapil et al. (2010), these authors found a dose-dependent relationship in the reductions of SBP, DBP, and mean arterial pressure (MAP). An acute dose of 4.2 mM NO₃⁻ resulted in peak reductions of 4 mmHg in SBP, 1 mmHg in DBP, and 2 mmHg in MAP. When an acute 8.4 mM NO₃⁻ dose was administered, peak reductions were 8 mmHg in SBP, 3 mmHg in DBP, and 4 mmHg in MAP. Lastly, when an acute 16.8 mM NO₃⁻ dose was administered, peak reductions were 10 mmHg in SBP, 4 mmHg in DBP, and peak 5 mmHg in MAP (Wylie et al. 2013).
The same authors also investigated the dose-response relationship with regard to the reductions in baseline and end-exercise VO2 during moderate (~105 watts (W)) and severe intensity (~300 W) exercise to exhaustion. There was no change in baseline VO2 with moderate or severe intensity exercise. There was no change in end-exercise VO2 with an acute dose of 4.2 mM NO3⁻, however when an acute dose of 8.4 mM NO3⁻ was administered there was a 2% reduction in end-exercise VO2 during moderate intensity exercise, and the acute dose of 16.8 mM resulted in a 3% reduction in end-exercise VO2 with moderate intensity exercise (Wylie et al. 2013). There was no effect of any dose of BRJ supplementation on end-exercise VO2 during the severe exercise bout. Interestingly, an acute dose of 8.4 mM NO3⁻ resulted in a significant improvement in time to exhaustion during severe intensity exercise compared to placebo (PLA) (8.4 mM: 570 ± 153 vs. PLA: 498 ± 113 s). Time to exhaustion was also significantly improved with an acute dose of 16.8 mM NO3⁻ (552 ± 117 s) compared to PLA (493 ± 114 s). The results from this study indicate that an acute dose of at least 8.4 mM NO3⁻ is required to elicit positive effects on BP, the O2 cost of exercise, and to improve time to exhaustion.
Figure 2: The pharmacokinetic profile and dose response relationship of 4.2, 8.4 and 16.8 mM dietary nitrate (NO$_3^-$) on plasma NO$_3^-$ and nitrite (NO$_2^-$). Filled circles, control treatment; filled triangles, 4.2 mM; filled squares, 8.4 mM; and filled diamonds, 16.8 mM (Wylie et al. 2013).

Acute vs. Chronic Supplementation: Plasma Levels, VO$_2$ and Performance

Few studies have directly compared the effects of different durations of dietary NO$_3^-$ supplementation on blood parameters, health, and performance (Vanhatalo et al. 2010; Fulford et al. 2013; Wylie et al. 2016). Vanhatalo et al. (2010) investigated the effects of acute and chronic BRJ supplementation on BP and exercise performance in 8 healthy participants (5 males). These authors found that mean plasma [NO$_2^-$] measured 2.5 h post-ingestion increased by 36% following an acute dose of 5.2 mM BRJ, by 59% following 12 days of supplementation, and by 46% following 15 days of supplementation. SBP was lowered by ~5 mmHg with BRJ supplementation at all time points, DBP was lowered by ~4 mmHg, and MAP was lowered by ~5 mmHg. Furthermore, moderate intensity (~90 W) end-exercise VO$_2$ was consistently reduced by ~5% following acute supplementation, 5 days of supplementation and 15 days of
supplementation. Fulford et al. (2013) explored the effects of dietary NO\textsubscript{3} on skeletal muscle force production both acutely and chronically in 8 healthy males. These authors demonstrated that plasma [NO\textsubscript{2}•] measured at 2.5 h post-ingestion increased by a similar amount following an acute dose of 10.2 mM NO\textsubscript{3}, and after 5 and 15 days of supplementation. Interestingly, there was no impact of acute or chronic BRJ supplementation on skeletal muscle contractile force production (Fulford et al. 2013). Unfortunately, these authors did not measure VO\textsubscript{2}.

Wylie et al. (2016) reported the pharmacokinetic profiles and exercise effects associated with different durations and doses of dietary NO\textsubscript{3} supplementation in 34 recreationally active subjects (19 males). These authors found that with daily doses of both 3 and 6 mM of dietary NO\textsubscript{3}, there was no significant difference within conditions for plasma [NO\textsubscript{3}] or [NO\textsubscript{2}•] following an acute dose, 7 days of supplementation or 30 days of supplementation. However, plasma [NO\textsubscript{3}] and [NO\textsubscript{2}•] were significantly greater in the 6 mM condition compared to the 3 mM condition, where plasma [NO\textsubscript{3}] increased by 313% in the 3 mM dietary NO\textsubscript{3} condition and by 867% in the 6 mM dietary NO\textsubscript{3} condition (Wylie et al. 2016). Plasma [NO\textsubscript{2}•] increased by 165% in the 3 mM condition and 579% in the 6 mM condition (Wylie et al. 2016). There was no effect of the 3 mM condition on end-exercise VO\textsubscript{2} during moderate intensity exercise (~90 W). Conversely, the rise in plasma [NO\textsubscript{2}•] in the 6 mM condition was associated with a 3% reduction in end-exercise VO\textsubscript{2} during moderate intensity exercise. These authors also investigated the effects of chronic supplementation without an acute dose administered 2 h prior to blood sampling and exercise testing. Without the acute dose, plasma [NO\textsubscript{3}] returned to baseline levels by 24 h post-ingestion in the 3 mM condition and declined significantly in the 6 mM condition. However, plasma [NO\textsubscript{3}] was still significantly elevated above baseline in the 6 mM condition 24 h following the last dose of BRJ. Without the acute dose, plasma [NO\textsubscript{2}•] returned to baseline by
24 h post-ingestion in the 3 and 6 mM conditions. Similar to the chronic supplementation with the acute dose administered prior to testing, there was no change in end-exercise VO\textsubscript{2} in the 3 mM condition. Interestingly, when the dose was 6 mM per day the 3% reduction in VO\textsubscript{2} was conserved despite no acute dose administered 2 h prior to testing.

From these studies, it appears that subjects do not develop a tolerance to dietary NO\textsubscript{3}\textsuperscript{-} with up to 30 days of supplementation, and the effects on blood parameters, health, and exercise performance are similar both acutely and chronically. Further corroborating these findings, Kelly et al. (2013) had subjects visit the laboratory over 5 consecutive days during days 3 through 7 of dietary NO\textsubscript{3}\textsuperscript{-} supplementation and reported no difference in plasma [NO\textsubscript{2}\textsuperscript{-}] measured 2.5 h post-ingestion across the 5 days, with BRJ consistently improving time to exhaustion to the same extent (~13%) during severe intensity exercise tests (Kelly et al. 2013).

**The Effects of BRJ on Exercise Economy and Athletic Performance**

**Moderate Intensity Submaximal Exercise Economy**

For the purpose of this thesis, exercise economy is defined as a reduction in the O\textsubscript{2} cost of exercise at a fixed workload. A basic tenet of human exercise physiology states that the O\textsubscript{2} cost of submaximal exercise at a given work rate is fixed regardless of age, health and fitness status (Poole and Richardson 1997). Furthermore, this tenet suggests that the O\textsubscript{2} cost of submaximal exercise is unaltered by physical, nutritional or pharmacological interventions (Poole and Richardson 1997). It has been demonstrated that O\textsubscript{2} uptake increases linearly as a function of work rate, where O\textsubscript{2} consumption is ~9 to 11 mL O\textsubscript{2}/W/min during moderate intensity exercise (Poole and Richardson 1997). BRJ supplementation challenges this dogma by consistently demonstrating reductions, typically between 3 to 5%, in the O\textsubscript{2} cost of moderate intensity submaximal exercise in recreationally active males as reviewed by Affourtit et al. 2015.
Moderate intensity exercise is characterized by low production and adequate clearance of blood lactate, and therefore is generally classified as exercise that falls below the lactate threshold (Xu and Rhodes 1999). The lactate threshold is defined as the blood lactate concentration at which lactate production exceeds its clearance and accumulation occurs. The onset of blood lactate accumulation occurs at an arterial lactate concentration of 4 mM/L and typically occurs around 75% maximal oxygen uptake (VO_{2max}) (Ghosh 2004). The VO_{2} kinetics associated with moderate intensity exercise can be divided into three major components. Phase I is depicted by the early and rapid rise in VO_{2} that occurs in the first 15 to 25 s of exercise, and this has been linked to the rise in cardiac output and blood flow at the onset of exercise (Xu and Rhodes 1999) (Figure 3). Phase II is represented by an exponential rise in VO_{2} towards steady state and reflects shifts in skeletal muscle metabolism and substrate utilization. Phase III is achieved after ~3 min of exercise and is defined as steady state VO_{2}, which has been described previously as ~9 to 11 mL O_{2}/W/min and is indicated by a heavy reliance on aerobic metabolism. BRJ supplementation has been shown to influence VO_{2} kinetics by decreasing the primary VO_{2} amplitude and ultimately decreasing end-exercise VO_{2} in recreationally active groups composed of predominantly male subjects (Bailey et al. 2009; Vanhatalo et al. 2010; Lansley et al. 2011) (Figure 3).

Interestingly, these reductions in the O_{2} cost of exercise are present merely 2.5 h following an acute dose of dietary or inorganic NO_{3}⁻ and persist for up to 30 days of chronic supplementation. Furthermore, these effects are seen with doses ranging from 5.2 mM to 16.8 mM dietary or inorganic NO_{3}⁻. BRJ supplementation has shown to be effective at reducing the O_{2} cost of exercise at intensities ranging from 45 to 75% VO_{2peak} and these reductions have been elicited across a range of exercise modalities including walking (Lansley et al. 2011), running

**Figure 3**: An illustration of the effects of beetroot juice (nitrate) supplementation on oxygen uptake (VO$_2$) kinetics from rest to moderate intensity exercise in recreationally active males. Nitrate significantly reduced the oxygen cost of exercise by reducing the primary amplitude of VO$_2$ kinetics (Lansley et al. 2011).

**Severe and Maximal Intensity Exercise Economy**

Severe intensity exercise exceeds the lactate threshold. Although phase II kinetics still exist in this exercise domain, VO$_2$ kinetics are complicated by the VO$_2$ slow component that develops after a few minutes at this intensity (Xu and Rhodes 1999). During severe intensity exercise, VO$_2$ steady state (phase III) is not achieved and the slow component continues to increase until task failure (Xu and Rhodes 1999). Two studies have investigated VO$_2$ kinetics during severe intensity exercise (Bailey et al. 2009; Breese et al. 2013). These groups define severe intensity exercise as 70% of the difference between the gas exchange threshold (GET) and peak oxygen consumption (VO$_{2\text{peak}}$) (Bailey et al. 2009, ~275 W; Breese et al. 2013, ~215 W).
Bailey et al. (2009) found a significant reduction in the VO$_2$ slow component amplitude with 6 days of BRJ supplementation, which translated to a 16% improvement in time to exhaustion in 8 healthy men. These authors also found a significantly decreased primary VO$_2$ amplitude following BRJ supplementation compared to PLA (Bailey et al. 2009). Conversely, Breese et al. (2013) found no significant difference in the VO$_2$ slow component amplitude or the primary VO$_2$ amplitude following 6 days of BRJ supplementation compared to PLA in 9 healthy men. These discrepancies may exist because of differing exercise protocols in which Bailey et al. (2009) transitioned from very low intensity exercise to severe intensity exercise, whereas Breese et al. (2013) transitioned from moderate intensity exercise to severe intensity exercise. A greater body of literature is required to elucidate the VO$_2$ kinetic profiles associated with BRJ supplementation and severe intensity exercise.

To date, three studies have investigated the effects of BRJ supplementation on VO$_{2\text{max}}$ (Larsen et al. 2007; 2010; Vanhatalo et al. 2010). The initial study by Larsen et al. (2007) demonstrated no significant effect of 3 days of NaNO$_3$ supplementation on VO$_{2\text{max}}$ in 9 well-trained males. However, in 2010 these authors found a significant reduction in VO$_{2\text{max}}$ following 3 days of NaNO$_3$ supplementation in 9 healthy volunteers (7 males) (Larsen et al. 2010). A reduced VO$_{2\text{max}}$ would likely indicate impaired exercise performance. However, Vanhatalo et al. (2010) demonstrated an increase in VO$_{2\text{max}}$ following 15 days of BRJ supplementation in 8 recreationally active individuals (5 males). It is difficult to reconcile the array of outcomes seen across these studies and a greater body of research is required to determine if dietary NO$_3^-$ substantially impacts maximal exercise performance.
Time Trial Performance

Time trial performance is typically measured as a set distance, set amount of work, or set time allocated for exercise performance. Time trials hold the greatest real-world validity when measuring exercise performance in athletes and are the most reliable and reproducible protocols (Currell and Jeukendrup 2008). There are no distinguishable differences in variability between time trial exercise modalities where the coefficient of variation (CV) is below 5% for cycling, running, and rowing (Currell and Jeukendrup 2008).

Aerobic Cycling Time Trial Performance

A few studies have demonstrated an improvement in aerobic cycling time trial performance following dietary NO3− supplementation. Although some of these studies did not achieve statistical significance, they fall below the smallest worthwhile change for cycling time trial performance in elite cyclists as described by Hopkins (2004). This statistical inference suggests that the average CV for cycling time trials is ~0.6% for trained cyclists, and any change that exceeds this may be deemed worthwhile (Paton and Hopkins 2006). Lansley et al. (2011) found a 2.8% improvement in 4 km time trial performance, and a 2.7% improvement in 16.1 km time trial performance following an acute dose of BRJ in 9 competitive male cyclists. Wilkerson et al. (2012) found a 0.8% improvement on a 50 mile time trial following an acute dose of BRJ in 8 trained male cyclists, and Cermak et al. (2012) found a 1.3% improvement in 10 km time trial performance following 6 days of BRJ supplementation in 13 trained male cyclists and triathletes. Lastly, Lee et al. (2015) found a 2.1% improvement in 20.15 km time trial performance following acute supplementation with a NO containing lozenge in 16 trained cyclists (8 males). All of these studies utilized a single-blinded testing protocol for the subjects or a double-blinded testing protocol. However, none of these studies administered a post-testing
questionnaire to confirm if the subjects were successfully blinded to the supplementation regimes.

Interestingly, there are also a number of studies that demonstrated no significant effect on aerobic cycling time trial performance following dietary NO\textsubscript{3}\textsuperscript{-} supplementation (Cermak et al. 2012; Lane et al. 2013; Christensen et al. 2013; Glaister et al. 2015; McQuillan et al. 2016; Nyakayiru et al. 2016; Callahan et al. 2017). It is important to note that although dietary NO\textsubscript{3}\textsuperscript{-} supplementation may not elicit a beneficial effect on time trial performance, it has not shown to be detrimental to athletic performance. Furthermore, it is important to consider that these time trial studies were conducted in trained athletes, and their training status may contribute to the controversial findings on athletic performance following dietary NO\textsubscript{3}\textsuperscript{-} supplementation. This will be addressed in a subsequent section.

Aerobic Running Time Trial Performance

There is a limited body of evidence investigating the effects of dietary NO\textsubscript{3}\textsuperscript{-} supplementation on aerobic running time trial performance and the outcomes are equivocal (Murphy et al. 2012; Peacock et al. 2012; Boorsma et al. 2014). Murphy et al. (2012) found a significant improvement in 5 km time trial performance, where running velocity was increased by 5% following an acute dose of BRJ in 11 healthy individuals (5 males). Conversely, Peacock et al. (2012) found no effect of an acute dose of KNO\textsubscript{3} on 5 km running time trial performance in 10 elite male cross-country skiers. Similarly, Boorsma et al. (2014) found no significant effect of acute or chronic BRJ supplementation on 1500 m running performance in 10 elite male distance runners. The smallest worthwhile change for elite athletes in these events is a 1.1% improvement in performance as described by Hopkins (2004). Neither Peacock et al. (2012) or Boorsma et al. (2014) found performance benefits greater than these parameters. Again, training status may
have confounded the performance benefits associated with dietary NO$_3^-$ supplementation, as Peacock et al. (2012) and Boorsma et al. (2014) recruited elite runners whereas, Murphy et al. (2012) recruited recreationally active individuals.

**Water Sport Time Trial Performance**

There is some evidence to suggest that BRJ may improve performance in water sports such as rowing, kayaking, and swimming. The CV for elite rowing time trial performance is noted to be ~ 0.4%, therefore any improvement greater than the CV is predicted to be a worthwhile improvement (Hopkins 2004). Hoon et al. (2012) showed that acute BRJ supplementation was possibly beneficial (~2 s improvement) to 2000 m rowing performance in 10 highly trained male rowers. Bond et al. (2012) demonstrated that 6 days of BRJ supplementation was likely beneficial during 6 x 500 m rowing sprints, where the benefit was almost certain in sprints 4 through 6. The CV for elite kayaking time trial performance is ~ 0.6% (Hopkins 2004) and Peeling et al. (2015) found a 0.7% improvement in the distance covered during a 4 min kayaking test in 6 national level male kayakers and found a 1.7% improvement in 500 m kayaking time trial performance in 5 international level female kayakers. However, Muggeridge et al. (2013) saw no difference in 1 km kayaking time trial performance despite a significantly reduced submaximal VO$_2$ (3.1%) when kayaking for 15 min at 60% maximal workload ($W_{max}$) following an acute dose of BRJ in 8 trained male kayakers. Pospieszna et al. (2016) investigated the effects of 8 days of BRJ supplementation on 6 x 50 m sprint swimming performance, and 800 m endurance swimming performance in 11 female collegiate swimmers. These authors noted a statistically significant improvement in sprints 4 through 6 following BRJ supplementation as well as improvements in 800 m time trial performance.
Time to Exhaustion

A number of studies have reported improved time to exhaustion following BRJ supplementation in healthy, recreationally active males. These improvements have been reported with both acute supplementation and chronic supplementation lasting up to 15 days. Furthermore, time to exhaustion has been enhanced across a range of exercise modalities including: cycling (Bailey et al. 2009; Larsen et al. 2010; Wylie et al. 2013; Kelly et al. 2013; Thompson et al. 2014; Bailey et al. 2015), running (Lansley et al. 2011), and knee extension exercise (Bailey et al. 2010; Hoon et al. 2015). The reported improvements have been between 7 and 20%.

Sprint and Team Sport Performance

To date, only two studies have investigated the effects of BRJ supplementation on sprint cycling performance. Rimer et al. (2016) found that acute BRJ supplementation increased peak power during 4 sets of 3 – 4 s sprints, however there was no benefit of BRJ supplementation on peak or mean power during a 30 second Wingate in 13 competitively trained athletes from an array of sports (11 males). Conversely, after an acute dose of BRJ, Dominguez et al. (2017) noted a significant increase in peak power during a 30 s Wingate, but no difference in mean power in 15 recreationally active males. Since little literature exists, the results are equivocal and require further investigation.

Due to the complexity and multifaceted nature of team sports, it is difficult to quantify team sport performance in a controlled setting. One commonly employed method to bypass the complexity, uncertainty, and spontaneity of stop-and-go sporting events is to create a simulated environment that closely mimics gameplay. Stop-and-go team sports are typically characterized by intermittent and repeated sprints, and protocols such as the running Loughborough
Intermittent Shuttle Test or the Yo-Yo Intermittent Recovery Test have been highly correlated with movement patterns utilized in team sports (Currell and Jeukendrup 2008). There is mixed support for the benefit of BRJ supplementation on team sport performance as assessed by repeated sprint efforts. It has been shown that acute (Wylie et al. 2013) and chronic (Thompson et al. 2016) BRJ supplementation can increase the distance covered during the Yo-Yo Intermittent Recovery Test by ~ 4% in male recreational team-sport athletes. Another study demonstrated a significant increase in mean power during 24 sets of 6 s sprints, however there was no improvement during 7 sets of 30 s sprints, or 6 sets of 60 s sprints with 5 days of BRJ supplementation in 34 male recreationally active team-sport athletes (Wylie et al. 2016). On the contrary, Buck et al. (2015) failed to demonstrate an improvement in repeated sprint performance with acute BRJ supplementation in 13 female amateur team-sport athletes. Although it appears that BRJ supplementation may be beneficial for team sport performance, a greater body of literature is required to validate and confirm these findings.

**Strength Performance**

There is minimal research published regarding the effects of BRJ supplementation on strength performance. Mosher et al. (2016) demonstrated that acute supplementation with BRJ improved the number of repetitions to failure and consequently the total weight lifted at failure during bench press performed at 60% of 1 repetition maximum in 12 recreationally active males. Conversely, Flanagan et al. (2016) found no significant effect of 3 days of BRJ supplementation on the maximum number of repetitions performed at 60, 70, 80 or 90% 1 repetition maximum in 14 resistance-trained males.
Performance During Hypoxia

Exercise in simulated hypoxic environments is characterized by a reduced inspired O$_2$ concentration, ultimately resulting in reduced arterial O$_2$ content, reduced oxidative capacity of contracting muscles, and impaired exercise tolerance (Vanhatalo et al. 2011; Kelly et al. 2014). The reduction in inspired O$_2$ can compromise the L-arginine pathway for NO production since it relies on O$_2$ as a substrate (Lundberg et al. 2010). Therefore, BRJ supplementation may be important in hypoxic conditions because it augments the sequential reduction of NO$_3^-$ to NO which does not rely on O$_2$. Most of the literature studying BRJ supplementation in hypoxia (15% O$_2$) demonstrates a profound 6 – 8% decrease in the O$_2$ cost of exercise during moderate intensity cycling (~135 – 200W) (Kelly et al. 2014; Muggeridge et al. 2014) (Figure 4), and significant improvements in time to task failure (Vanhatalo et al. 2011; Kelly et al. 2014) and TT performance (Muggeridge et al. 2014). Interestingly, Vanhatalo et al. (2011) found that BRJ supplementation significantly attenuated the rate of metabolic perturbation associated with high intensity knee extension exercise in a hypoxic environment (~15% O$_2$), and restored exercise tolerance to normoxic conditions in 9 recreationally active subjects (7 males). Specifically, after 60 s and 120 s of exercise, phosphocreatine (PCr) had fallen to a greater extent in the PLA condition compared to the BRJ condition. Consequently, inorganic phosphate (Pi) accumulation and pH reduction were greater in the PLA condition compared to the BRJ condition, ultimately indicating a greater reliance on aerobic metabolism following BRJ supplementation in hypoxic conditions. There was no difference in these parameters between the hypoxic BRJ condition and the control normoxic condition indicating a scenario by which BRJ restored exercise tolerance.
Figure 4: An illustration of the effects of beetroot juice (BRJ) supplementation (nitrate) on oxygen uptake kinetics during moderate intensity exercise (~135 W) in simulated altitude (15% oxygen (O$_2$)) in recreationally active males. There was an ~8% reduction in the O$_2$ cost of exercise with BRJ during hypoxia compared to a 3% reduction in the O$_2$ cost of exercise with BRJ during normoxia. (Kelly et al. 2014).

Conclusion

BRJ supplementation has been shown to elicit profound effects on exercise economy and athletic performance. Specifically, BRJ supplementation has been shown to reduce the O$_2$ cost of moderate intensity exercise and improve time to exhaustion. These findings are exacerbated in hypoxic situations. The effects of BRJ supplementation on time trial, sprint, team sport, and strength performance are equivocal and often confounded by a lack of literature in the field. Alternatively, it is often suggested that an athlete’s training status may impact their response to dietary NO$_3^-$ supplementation. This will be addressed in the following section.

Individual Responses to BRJ Supplementation

There is inherently individual variability in the physiological responses to nutritional interventions, and the likelihood that an individual is going to respond positively to a nutritional intervention is influenced by a multitude of factors. Furthermore, the magnitude of responses is influenced by these very same factors and can be visualized as a continuum. When considering
the factors that influence the response to BRJ supplementation, it is important to consider differences in the fitness status, dietary habits, and digestive pathway of the subjects. A “non-responder” is identified as an individual who consistently does not display a reduction in the \( O_2 \) cost of exercise following BRJ supplementation. Conversely, a “responder” is an individual who consistently demonstrates a reduction in the \( O_2 \) cost of exercise that is greater than the coefficient of variance for the measurement.

Interestingly, genetics may also influence an individual’s physiological responses to NO. A recent study by Emdin et al. (2017) demonstrated that certain individuals may have a genetic predisposition for enhanced NO signalling. This was associated with a reduced risk of coronary heart disease and stroke. Interestingly, individuals who had rare genetic variants that inactivated certain NO signalling genes demonstrated significantly elevated blood pressure and a 3-fold increased risk of coronary heart disease (Emdin et al. 2017). Future studies should investigate the possibility that individuals who have enhanced endogenous NO signalling may be less likely to respond to dietary NO\(_3^-\) supplementation.

**Fitness Status**

There is substantial evidence to suggest that highly trained individuals may not respond to dietary NO\(_3^-\) supplementation and therefore do not demonstrate a reduced \( O_2 \) cost of submaximal exercise (Peacock et al. 2012; Wilkerson et al. 2012; Christensen et al. 2013; Boorsma et al. 2014; Lee et al. 2015; McQuillan et al. 2016; Nyakayiru et al. 2016). A study by Porcelli et al. (2015) investigated the effects of chronic NaNO\(_3\) supplementation on plasma [NO\(_3^-\)] and [NO\(_2^-\)], the \( O_2 \) cost of exercise and time trial performance in 21 males with either low, moderate, or high aerobic fitness. It was discovered that individuals with high aerobic fitness had higher baseline plasma [NO\(_3^-\)] compared to individuals with low or moderate aerobic fitness.
fitness. Furthermore, the individuals with low or moderate aerobic fitness demonstrated the greatest rises in both relative and absolute plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ following chronic supplementation with NaNO$_3$. These authors identified that individuals with low and moderate aerobic fitness elicited a 9.8% and 7.3% reduction in the O$_2$ cost of exercise at 80% GET, respectively. Interestingly, chronic NaNO$_3$ supplementation also resulted in a significant improvement in 3 km running time trial performance for individuals with low and moderate aerobic fitness. However, there was no difference in the O$_2$ cost of exercise or 3 km running time trial performance for individuals with high aerobic fitness. It is possible that the individuals with low and moderate aerobic fitness may experience performance benefits with dietary NO$_3^-$ due to the dramatic elevation in plasma $[\text{NO}_2^-]$ following supplementation compared to individuals with high aerobic fitness. Similar results were obtained by Carriker et al. (2016) who found a reduced O$_2$ cost of exercise at 45% (~90 W) and 60% (~150 W) VO$_{2\text{max}}$ in 5 males with low aerobic fitness (VO$_{2\text{max}}$, 42.4 ± 3.2 mL/kg/min). However, these results were not found in 6 males with high aerobic fitness levels (VO$_{2\text{max}}$, 60.1 ± 4.6 mL/kg/min) (Carriker et al. 2016).

Jonvik et al. (2016) collected diet records from hundreds of elite athletes to assess their habitual dietary NO$_3^-$ intake. These authors hypothesized that elite athletes may have a blunted response to dietary NO$_3^-$ supplementation because they may already have diets rich in these foods. However, it was discovered that the average dietary NO$_3^-$ intake was only 106 mg/d, where a typical dose of BRJ is at least 400 mg of dietary NO$_3^-$. Furthermore, only 10% of the athletes consumed greater than 400 mg/d of dietary NO$_3^-$. This suggests that there are other physiological mechanisms at play with elite athletes and their blunted response to dietary NO$_3^-$ supplementation. Interestingly, Jonvik et al. (2016) reported that female athletes typically ingested a larger absolute amount of dietary NO$_3^-$ per day than male athletes, despite having
lower total energy intake. This was attributed to a greater consumption of vegetables by female athletes.

**Diet**

It is possible that individuals who habitually consume high levels of dietary NO$_3^-$ may not respond to BRJ supplementation since they already express elevated plasma [NO$_3^-$] and [NO$_2^-$]. However, it is expected that these differences would manifest as variations in individual plasma [NO$_3^-$] and [NO$_2^-$]. It has been shown that traditional Japanese diets are high in dietary NO$_3^-$, and adherence to this diet is associated with lower SBP and DBP (Gee and Ahluwalia 2016). Furthermore, when the same Japanese population was placed on a western diet they experienced significant increases in SBP and DBP (Gee and Ahluwalia 2016). It has been suggested that the high dietary NO$_3^-$ in the Japanese diet may be a major contributor to the population’s longevity and low rates of cardiovascular disease (Gee and Ahluwalia 2016). Similarly, other authors argue that individuals who follow the Dietary Approaches to Stop Hypertension (DASH) diet experience reductions in blood pressure, which is partially attributed to the high leafy green, and consequently high dietary NO$_3^-$ content, of this diet (Ashworth et al. 2015).

**Digestive Pathway**

It is expected that there is interindividual variability in the way we absorb, process, and store nutrients. When analyzing the potential variability in the responses to BRJ supplementation, it is important to consider interindividual differences that may exist in the enterosalivary circulation. First, it is possible that there are differences in the amount of NO$_3^-$ that is absorbed from the stomach and small intestines. This may mean that there is interindividual variability in the fraction of dietary NO$_3^-$ that is excreted from the body. Second, the rate of and magnitude of NO$_3^-$ concentration into the salivary glands may differ between
individuals. Next, there are likely differences in the amount of and activity of the bacteria that reduce NO$_3^-$ to NO$_2^-$ inside the mouth. Lastly, there may be interindividual differences in the storage, conversion and degradation of dietary NO$_3^-$ that is absorbed as NO$_2^-$. There is currently no literature focusing on these aspects as they are difficult to quantify and measure. However, it is expected that these differences would manifest as variations in individual plasma [NO$_3^-$] and [NO$_2^-$]. Interestingly, Kapil et al. (2010) found that plasma [NO$_2^-$] rose 2-fold higher in female subjects (n = 12) compared to males (n = 8) following an acute dose of KNO$_3$. This trend still existed when plasma [NO$_2^-$] was normalized to body weight. These authors suggest that sex differences may exist in the way nitrate loads are processed.

**BRJ Improves Exercise Economy and Performance – Potential Mechanisms of Action**

**Are We Sure It’s Nitrate?**

The early BRJ studies were performed before the development of a NO$_3^-$-free PLA which is manufactured using ion resin exchange (Lansley et al. 2011). This PLA was first introduced in the BRJ literature by Lansley et al. in 2011. Up until that point, it was difficult to ascertain whether the beneficial effects of BRJ supplementation were strictly from its dietary NO$_3^-$ content or if there were other bioactive properties in the beverage. It is now abundantly clear that the main bioactive in BRJ is dietary NO$_3^-$ since the beneficial effects are only reported with the NO$_3^-$-rich beverage compared to the NO$_3^-$-free placebo. However, some authors continue to argue that other bioactives within BRJ are a major contributor to its positive biological effects. A number of recent publications have attributed the effects of BRJ supplementation to its high antioxidant status. BRJ is high in betalain pigment, an antioxidant that gives the vegetable its deep red colour (Clifford et al. 2016b). Specifically, BRJ is high in betanin, a subclass of the betalain pigment. Clifford et al. (2016a; 2016b; 2017) have continually explored the antioxidant effects of
BRJ supplementation on muscle damage, recovery and performance in recreationally active males. They have consistently shown significant reductions in muscle soreness as well as attenuated performance decrements assessed using a countermovement jump following fatiguing exercise with 3 days of BRJ supplementation (Clifford et al. 2016a; 2016b). However, these authors have failed to show a benefit with respect to running performance or changes in biomarkers of inflammation (Clifford et al. 2016b; 2017a). Furthermore, despite the high betanin content of BRJ, these authors failed to demonstrate an increase in plasma betanin over an 8 h period following acute BRJ supplementation suggesting that this antioxidant may not play an ergogenic role (Clifford et al. 2017b). A major limitation of these studies is that they cannot differentiate the potential anti-inflammatory effects of NO$_3^-$ compared to betalain or betanin since a NO$_3^-$-free PLA was not utilized. However, in a separate set of experiments, Hoorebeke et al. (2016) demonstrated that concentrated betalain supplementation improved perceived exertion during submaximal treadmill running and improved 5 km time trial performance in 15 recreationally active males. Similarly, Montenegro et al. (2017) found that concentrated betalain supplementation improved performance on a 10 km time trial, and a 5 km time trial performed the next day in 22 triathletes (9 males). It is important to note, that neither of these studies demonstrated a reduction in the O$_2$ cost of exercise following supplementation suggesting betalain is not responsible for this physiological response to BRJ supplementation (Hoorebeke et al. 2016; Montenegro et al. 2017). Cumulatively, although it is important to recognize that there are other bioactives in BRJ that may contribute to its beneficial effects, the antioxidant properties associated with betalain fail to explain the consistent reduction in the O$_2$ cost of exercise seen with dietary NO$_3^-$ interventions.
BRJ supplementation may improve exercise performance through a variety of mechanisms. The potential mechanisms for the reduced $O_2$ cost of exercise range from improved $O_2$ delivery and waste removal to improved contractile or mitochondrial efficiency. It has also been proposed that BRJ may improve exercise performance by attenuating skeletal muscle damage or facilitating mitochondrial biogenesis.

**Improved Blood Flow**

NO is renowned for its role as a potent vasodilator. This occurs through the interaction of NO with guanylyl cyclase (Gc) which ultimately increases cyclic guanosine monophosphate (cGMP) and facilitates the removal of $Ca^{2+}$ from the smooth muscle cell resulting in relaxation. Furthermore, NO has also been shown to improve skeletal muscle blood flow. Ferguson et al. (2013a) performed a series of studies utilizing rats and BRJ supplementation. The first study demonstrated a significant increase in hindlimb skeletal muscle blood flow and vascular conductance during treadmill running in rats undergoing 5 days of BRJ supplementation. The second study demonstrated that 5 days of BRJ supplementation attenuated the decline in $O_2$ delivery/$O_2$ utilization that occurs between contractions, meaning that the drive for $O_2$ was elevated in the periods between contractions providing enhanced delivery (Ferguson et al. 2013b). In humans, Richards et al. (2017) found increased forearm blood flow following acute BRJ supplementation during a dynamic handgrip task in 18 healthy subjects (8 males). Although this does not explain the reduced $O_2$ cost of exercise, it may be an important factor related to the performance benefits of BRJ since this may augment $O_2$ delivery as well as $CO_2$ and waste removal. Future research needs to investigate the effects of BRJ supplementation with direct measures of blood flow in human subjects.
Improved Mitochondrial Function

Reduction in the \( \text{O}_2 \) Cost of Mitochondrial Adenosine Triphosphate (ATP) Resynthesis

The electron transport chain (ETC) is comprised of a series of complexes that use electrons from NADH and FADH\(_2\) to facilitate the movement of protons (hydrogen (H\(^+\)) ions) from the mitochondrial matrix to the intermembrane space creating an electrochemical gradient that drives the synthesis of ATP via mitochondrial membrane potential (Farrell et al. 2012). Importantly, the final electron acceptor in the ETC is \( \text{O}_2 \), and ATP is resynthesized from adenosine diphosphate (ADP) and inorganic phosphate via the enzyme ATP synthase (Farrell et al. 2012). The efficiency of ATP resynthesis depends on the rate of proton leak and slippage (Farrell et al. 2012). Proton leak occurs when H\(^+\) ions are utilized via alternative pathways that do not contribute to the generation of ATP such as uncoupling protein 3 (UCP-3). Slippage occurs when electrons are moved without the pumping of protons, ultimately resulting in a decreased membrane potential to drive ATP resynthesis (Farrell et al. 2012). The efficiency of mitochondrial respiration is often expressed as the phosphate/\( \text{O}_2 \) (P/O) ratio and is defined as the rate of ATP synthesis divided by the rate of \( \text{O}_2 \) consumption (Larsen et al. 2011).

It has been proposed that dietary \( \text{NO}_3^- \) supplementation can improve the efficiency of ATP resynthesis by reducing proton leak and slippage (Clerc et al. 2007; Larsen et al. 2011). Larsen et al. (2011) associated the reduced \( \text{O}_2 \) cost of exercise following 3 days of Na\( \text{NO}_3^- \) supplementation with an improved P/O ratio. Specifically, these authors found a significant reduction in state 4 respiration, which is characterized by availability of respiratory substrate but not ADP and estimates the back leakage of protons through the inner mitochondrial membrane (Larsen et al. 2011). This indicates more efficient mitochondrial ATP synthesis since a larger portion of the membrane potential is contributing to ATP synthesis as opposed to uncoupling.
processes. The reduction in membrane leak was attributed to a significant reduction in ANT protein expression and a strong trend for a reduction in UCP-3 protein expression (Larsen et al. 2011). However, Whitfield et al. (2016) found dramatically different results where 7 days of BRJ supplementation showed no effect on mitochondrial efficiency as expressed by no change in leak respiration, UCP-3 or ANT protein content, and the P/O ratio. Although it is possible there are different mechanisms of action for NaNO₃ compared to BRJ supplementation, this is highly unlikely and more research is required to understand these stark differences, and to elucidate the effects of dietary NO₃⁻ on mitochondrial coupling and efficiency.

Alternatively, it has been suggested that NO is capable of reversibly inhibiting complex IV of the ETC by competing with O₂ at its binding site (Brown and Cooper 1994; Cleeter et al. 1994; Bolaños et al. 1994; Guiffrè et al. 2000). By changing the stoichiometry between O₂ used and ATP produced, this would result in a decrease in O₂ utilization and would ultimately decrease the O₂ cost of exercise.

Mitochondrial Biogenesis

A few cell culture studies have identified NO as a stimulus for mitochondrial biogenesis. These studies have demonstrated an increase in mitochondria DNA, mitochondrial markers such as cytochrome oxidase IV (COXIV) and cytochrome C, and increased O₂ consumption following incubation with NO (Nisoli et al. 2003; 2004). It is thought that NO acts through Gc and cGMP to activate peroxisome proliferator-activated receptor gamma coactivator a-alpha (PGC-1α), the master regulator of mitochondrial biogenesis (Nisoli et al. 2004; Lira et al. 2010). However, these findings have not been replicated with BRJ supplementation in humans. Larsen et al. (2011) found no change in mitochondrial density or mRNA content for proteins related to mitochondrial biogenesis (PGC-1α, mitochondrial transcription factor A (TFAM), COXIV)
following 3 days of NaNO₃ supplementation. These results are not surprising as the time course for significant mitochondrial biogenesis to occur is likely longer than this supplementation period. Furthermore, since significant reductions in the O₂ cost of exercise occur merely 2.5 h following BRJ supplementation it is likely that the mechanism of action is at least partially independent of increased protein content as this does not occur on this timescale. Moreover, Whitfield et al. (2016) found no change in mitochondrial protein content following 7 days of BRJ supplementation in recreationally active males, suggesting that BRJ supplementation may not influence mitochondrial biogenesis in humans.

**Improved Contractile Efficiency**

**Reduction in the ATP Cost of Skeletal Muscle Force Production**

The ATP cost of skeletal muscle force production is dominated by sarcoplasmic reticulum calcium ATPase (SERCA) and myosin ATPase which account for ~25 – 30% and 70% of the total cost of contraction respectively (Barclay et al. 2007). Na⁺-K⁺ ATPase is shown to account for ~5% of the ATP cost of skeletal muscle force production (Barclay et al. 2007).

Therefore, due to their robust contributions to the ATP cost of exercise, it was a logical step to investigate the effect of dietary NO₃⁻ supplementation on the ATP cost associated with SERCA and myosin ATPase.

Recently, a number of studies have contributed to the hypothesis that BRJ supplementation may reduce the ATP cost of exercise. However, the exact mechanism of action remains to be elucidated. Bailey et al. (2010) designed an elegant study to indirectly tease apart the potential mechanisms of action associated with the reduced O₂ cost of exercise following BRJ supplementation. These authors used magnetic resonance spectroscopy (MRS) to determine the total ATP cost of low and high intensity knee extension exercise, and estimated the relative
ATP contributions from PCr, glycolysis, and oxidative phosphorylation. These authors found a 
significant reduction in total ATP hydrolysis during low intensity and high intensity exercise 
with BRJ supplementation and demonstrated a 25% improvement in exercise tolerance during 
high intensity exercise to exhaustion following BRJ supplementation. These findings suggest that 
the O$_2$ cost of submaximal exercise may be reduced through an improvement in skeletal muscle 
contractile efficiency. Using a similar methodology, Fulford et al. (2013) also found a significant 
decrease in the relative contribution of PCr to ATP hydrolysis during knee extension exercise. It 
is important to note that these findings are based on calculations and have inherent error built 
into them. Furthermore, Fulford et al. (2013) did not calculate the ATP contribution from aerobic 
or glycolytic metabolism making it difficult to interpret whether BRJ supplementation decreased 
total ATP turnover or shifted fuel utilization.

Other studies have used force frequency curves to assess the effects of BRJ 
supplementation on skeletal muscle force production and contractile efficiency. Hernandez et al. 
(2012) found a significant increase in low frequency force production at 10 Hz following 7 days 
of NaNO$_3$ supplementation in mice. This was accompanied by an increase in intracellular 
calcium and an increase in the expression of calsequestrin and dihydropyridine receptor proteins, 
both associated with calcium-handling. Haider and Folland (2014) found an increase in force 
production at a low stimulation frequency of 10 Hz following 7 days of BRJ supplementation in 
healthy males. Whitfield et al. (2017) reported similar findings where low stimulation frequency 
force production was increased at 10 Hz in healthy males suggesting improved contractile 
efficiency. However, unlike Hernandez et al. (2012), this was not associated with changes in 
calcium-handling protein expression suggesting BRJ may influence the force produced via 
myosin ATPase during skeletal muscle contraction. Conversely, Hoon et al. (2015) found no
effect of 4 days of BRJ supplementation on skeletal muscle force production in 19 healthy adults (13 males). The discrepancies in the outcomes from these studies may be explained by the variation in study model as well as the supplementation and electrical stimulation protocols, and by the small magnitude of the expected changes.

Since dietary NO$_3^-$ supplementation can reduce the oxygen cost of exercise merely 2.5 h following an acute dose, it is unlikely that protein content changes in this time frame. Therefore, it still remains to be elucidated if dietary NO$_3^-$ supplementation results in post-translational modifications that may alter the activity of calcium-handling or contractile proteins. There is evidence to suggest that dietary NO$_3^-$ supplementation may slow cross bridge cycling to allow for greater force production per ATP hydrolyzed. Using skinned skeletal muscle fibers from rabbits and differentiated myotubes from mice, Nogueira et al. (2009) demonstrated that NO was able to s-nitrosylate myosin on multiple cysteine thiols. This was accomplished by adding reactive nitrogen species to the reaction mixtures. These authors found that exposure to NO resulted in decreased myosin ATPase activity but no change in the affinity of myosin for actin suggesting a slowing of cross bridge cycling (Nogueira et al. 2009). This was the first evidence that NO may play a role in increasing skeletal muscle force production via altering myosin ATPase function. This work was promptly followed up by Evangelista et al. (2010) who confirmed that exposure of rat skeletal muscle myosin to NO donors can s-nitrosylate cysteine residues on myosin which caused a slowing of cross bridge cycling and ultimately doubled force production.

Reactive Oxygen Species (ROS)

A study by Whitfield et al. (2016) demonstrated increased intermyofibrillar (IMF) mitochondrial ROS production following 7 days of BRJ supplementation. These authors
suggested that increased ROS production may influence the skeletal muscle contractile machinery by improving mechanical efficiency and ultimately increasing force production during cross bridge cycling. Alterations in mitochondrial ROS production is an attractive theory for the mechanism underlying BRJ supplementation because it helps explain the acute effects and the diminished effect or lack of effect seen in elite athletic populations. These populations have increased superoxide dismutase (SOD) content and activity which may buffer the mitochondrial ROS production and mitigate any performance benefits of BRJ supplementation. Unfortunately, these authors found no correlation between increased mitochondrial ROS production and the decrease in whole body VO$_2$ during submaximal exercise, suggesting that the in vitro experimental results may not explain the whole-body effects of BRJ supplementation. Clearly future work needs to investigate the in vivo effect of BRJ supplementation and mitochondrial ROS production, and the effects on calcium-handling as well as skeletal muscle contractile machinery. It is possible that ROS produced at the level of the mitochondria may influence the ATP cost of the contractile apparatus.

**Women – Addressing the Gender Gap**

Throughout history women have been consistently underrepresented in sport medicine and exercise research, and only recently has this gender gap begun to be attenuated. Costello et al. (2014) indicated that between 2011 and 2013, women still only represented 39% of research participants across 1382 studies. Furthermore, it is widely accepted that sexual dimorphism exists in human physiology and metabolic responses to exercise making it extremely valuable to study sex specific responses to exercise interventions. Unfortunately, a major barrier to including women in exercise studies is the added complexity of the menstrual cycle and the hormonal profiles associated with this process. Hormonal contraceptive use is a method used to minimize
hormonal fluctuations throughout the menstrual cycle, however this only became common practice in the 1980s (Liao 2012) further contributing to the delay in active recruitment of female research participants.

**Characterization of the Menstrual Cycle**

A typical female menstrual cycle is ~28 days in duration and is typically characterized by the early follicular phase (days 1 – 7), late follicular phase (days 7 – 14), early luteal phase (days 14 – 21), and late luteal phase (days 21 – 28). Menses is classified as day 1 of the menstrual cycle. Estrogen and progesterone levels follow an inverse relationship throughout the menstrual cycle (Figure 5). During the early follicular and late follicular phase estrogen levels continue to rise until a peak at ovulation (day 14). Following ovulation estrogen levels decline and progesterone levels begin to rise until menses occurs again (Jonge et al. 2003). Research utilizing female participants is further complicated by abnormal menstrual cycle durations, assumption of normal hormone fluctuations, and the individual variability associated with menstrual cycle phases (Jonge et al. 2003). The most reliable method to confirm menstrual cycle phase is by measuring plasma hormone concentrations using commercially available kits (Jonge et al. 2003).

**Figure 5**: Hormonal profile throughout a normal menstrual cycle with significant surges in estrogen (estradiol) and progesterone (Sherman and Korenman 1975).
Some work has investigated the variation in NO production that occurs naturally throughout the menstrual cycle and there are discrepancies in the literature as to which female sex hormone, estrogen or progesterone, is the main regulator of NO production. Some authors suggested that NO metabolites are higher in the follicular phase and peak at ovulation when estrogen concentrations are at their highest (Cicinelli et al. 1996). Alternatively, it has also been proposed that NO production is inhibited by progesterone which peaks during the luteal phase (Giusti et al. 2002; Teran et al. 2002). In line with these hypotheses, Williams et al. (2001) demonstrated that brachial artery flow mediated dilatation was higher during the follicular phase, peaking near ovulation and dropping off sharply in the early luteal phase. This suggests a possible positive correlation between estrogen concentrations and NO production, as well as a potential inhibitory effect of progesterone on NO production. Lastly, when exhaled NO is measured there appears to be either no correlation of sex hormones with NO metabolites (Morris et al. 2002), or a relationship where estrogen decreases NO production and progesterone increases NO production (Mandhane et al. 2009). It is possible that these discrepancies exist because exhaled NO is a less reliable measurement of NO metabolites than blood parameters.

Cell culture studies have proposed a role of estrogen in stimulating the production of NO as there are estrogen receptors located on NOS (Xia and Krukoff 2004; Townsend et al. 2011). Xia and Krukoff (2004) showed that the addition of estrogen to neuroblastoma cells resulted in the activation of eNOS and nNOS, and ultimately facilitated the production of NO. Similarly, Townsend et al. (2011) demonstrated that when human bronchial epithelial cells were exposed to estrogen there was a rapid increase in NO production, whereas administration of an estrogen receptor antagonist resulted in inhibition of NO production. These authors proposed that estrogen
activates caveolin-1 which is able to increase intracellular Ca$^{2+}$ concentrations and can ultimately activate NOS to produce NO (Townsend et al. 2011). It still remains to be elucidated whether this relationship exists in skeletal muscle.

**Pharmacokinetics of Hormonal Contraceptives**

Studies have sought to characterize the estrogen and progesterone profiles in women actively using hormonal contraceptives and it is clear that hormonal contraceptives providing synthetic estrogen and progesterone prevent ovulation by attenuating the surge in estrogen that typically occurs around day 14 of a natural menstrual cycle (Gaspard et al. 1983; Kuhl et al. 1985; Jung-Hoffmann et al. 1988; Christin-Maitre et al. 2010). Hormonal contraceptive use ultimately results in stable and low estrogen levels throughout the menstrual cycle (Christin-Maitre et al. 2010) (Figure 6).

![Figure 6](image-url)  

**Figure 6:** Low estrogen (estradiol) and progesterone levels throughout the menstrual cycle in women actively using two different forms of hormonal birth control (Christin-Maitre et al. 2010).
Previous Female BRJ Literature

There is a major gender gap in BRJ exercise research with only 5 studies that specifically investigated female populations. This is in stark comparison to the more than 45 studies with strictly male populations. Of the 5 female studies that exist, there is a diverse range of exercise modalities, fitness levels, and supplementation protocols.

The first study providing insight that women may respond positively to acute and chronic dietary NO$_3^-$ supplementation was performed by Vanhatalo et al. (2010) who demonstrated a ~5% reduction in the O$_2$ cost of moderate intensity exercise in a group of 5 males and 3 females following 2.5 h, 5 days and 15 days of supplementation. Although these authors do not provide the individual data points or comment on potential sex differences in the responses to BRJ supplementation, it is highly likely that most of the female subjects responded positively to this intervention.

Three studies in strictly female populations have investigated the effects of BRJ supplementation on exercise performance in trained athletes. However, none of these studies measured the O$_2$ cost of submaximal exercise. One study explored the effects of acute BRJ supplementation, sodium phosphate and the combination on repeated sprint performance and simulated game play in 13 female amateur team-sport athletes (Buck et al. 2015). The subjects ingested 6 mM dietary NO$_3^-$ 3 h prior to the exercise protocol. Despite a significant rise in plasma NO$_3^-$, there was no effect of BRJ supplementation on repeated sprint performance. These authors did not measure plasma NO$_2^-$ to give an indication whether NO$_3^-$ was being converted to NO$_2^-$ via the enterosalivary circulation. It is possible that these women did not respond to BRJ supplementation because the dose was too low to elicit a positive effect on exercise performance or the women may have been too trained.
Similarly, Glaister et al. (2015) explored the effects of acute BRJ, caffeine, and the combination on 20 km cycling TT performance in 14 trained female cyclists. Approximately 2.5 h prior to exercise, the subjects consumed ~7.3 mM dietary NO$_3^-$ . Despite significant rises in plasma NO$_3^-$ and NO$_2^-$ , there was no effect of BRJ supplementation on TT performance. Interestingly, these authors found plasma NO$_2^-$ to increase from 92 to 297 nM, whereas Lansley et al. (2011) administered an acute dose of 6.2 mM dietary NO$_3^-$ and found plasma NO$_2^-$ to increase from 293 to 575 nM. In contrast to the work of Glaister et al. (2015), Lansley et al. (2011) demonstrated a significant improvement in 16.1 km cycling TT performance in trained males. This may suggest that there is a plasma [NO$_2^-$] threshold that dietary NO$_3^-$ supplementation must surpass in order to elicit a biological effect.

Lastly, Pospieszna et al. (2016) explored the effects of chronic BRJ supplementation on 6 sets of 50 m swimming sprints, and 800 m swimming performance in 11 female collegiate swimmers. The subjects participated in two, 8 day supplementation periods where they supplemented with either 0.5 L of BRJ and chokeberry juice or 0.5 L of carrot juice with added KNO$_3$. Each supplement delivered 10.5 mM of dietary NO$_3^-$ per day. A major flaw of this study was that no PLA treatment was utilized for comparison and proper blinding. Nonetheless, these authors found that both chronic BRJ and chokeberry supplementation as well as carrot juice supplementation with added KNO$_3$ improved repeated sprint performance by 3.13% and 2.09% respectively. Specifically, both juices improved performance on sprints 4 through 6. The improvements were significantly greater with BRJ and chokeberry supplementation compared to carrot juice with added KNO$_3$ supplementation. Furthermore, both juices significantly reduced the time to complete the 800 m swim, with greater improvements seen following carrot juice supplementation with added KNO$_3$. It is possible that dietary NO$_3^-$ elicited a positive effect on
performance in this study since the daily dose was significantly greater than that of Buck et al. (2015) and Glaister et al. (2015) and the supplementation protocol lasted for 8 days instead of an acute dose. However, we cannot rule out the possibility of a placebo effect influencing the outcome of this study.

To date, only two studies have investigated the effects of BRJ supplementation on exercise economy and performance in recreational or sedentary female populations. First, Bond Jr et al. (2014) explored the effects of acute BRJ supplementation on submaximal VO$_2$ and blood pressure in 12 sedentary African American women (VO$_{2\text{peak}}$, 26.1 ± 3.3 mL/kg/min). The women were provided with either ~12 mM dietary NO$_3^-$ or orange juice (negligible NO$_3^-$ content) 2 h prior to blood sampling and exercise testing for 5 min each at 40, 60 and 80% VO$_{2\text{peak}}$. Blood pressure was measured during exercise. These authors found significant increases in plasma NO following acute BRJ supplementation. Furthermore, there was a significant reduction in SBP at rest as well as during exercise at 40, 60 and 80% VO$_{2\text{peak}}$. BRJ supplementation had no effect on VO$_2$ at rest, but reduced VO$_2$ by ~15% at 40, 60, and 80% VO$_{2\text{peak}}$ (as inferred from a figure in the manuscript). A major limitation of this study is the lack of a NO$_3^-$-free PLA, and these authors failed to report the mean VO$_2$ values associated with rest and exercise at 40, 60 and 80% VO$_{2\text{peak}}$.

Rienks et al. (2015) was the only other group to investigate the effects of acute BRJ supplementation on VO$_2$ during exercise. The major outcome of this study was to determine the effects of acute BRJ supplementation on work performed during a rating of perceived exertion (RPE) clamp protocol in 10 recreationally active females (VO$_{2\text{peak}}$, 36.1 ± 4.7 mL/kg/min). To do this, the subjects ingested 12.9 mM dietary NO$_3^-$ 2.5 h prior to cycling exercise. These authors measured VO$_2$ during a 20 min cycling protocol where subjects exercised at a self-selected
intensity that elicited an RPE of 13 (somewhat hard). A secondary outcome of this study was to determine the effect of acute BRJ supplementation on submaximal $O_2$ uptake. This was assessed by 5 min at 75 W to measure constant workload $VO_2$ following a 5 min cooldown after the 20 min RPE clamp protocol. There was no effect of BRJ supplementation on the work completed or total $VO_2$ during the 20 min RPE clamp protocol. However, acute BRJ supplementation resulted in a weak but statistically significant 4% reduction in $VO_2$ while cycling at 75 W ($p = 0.048$).

Interestingly, only Bond Jr et al. (2014) and Buck et al. (2015) attempted to control for menstrual cycle phase, and the selected phases differed between these two studies where Bond Jr et al. (2014) utilized the luteal phase and Buck et al. (2015) utilized the follicular phase.

Furthermore, Pospieszna et al. (2016) was the only group to investigate chronic BRJ supplementation, and no study to date has combined acute and chronic supplementation into the same study protocol with strictly female participants. Lastly, only Rienks et al. (2015) recruited recreationally active subjects when the bulk of the literature suggests the reductions in $VO_2$ are present in recreationally active males compared to their trained counterparts. Therefore, of the 5 studies, three show improvement following BRJ supplementation. However, the results and validity of these studies are clouded by poor study design and poorly presented data.

**Conclusion**

Ultimately, there is very little research exploring the effects of BRJ supplementation in female populations making it difficult to draw comprehensive conclusions on exercise economy and performance. It is important to have a number of studies from a variety of laboratories to validate and support the existing research findings in sedentary, recreationally active as well as trained female subjects. Furthermore, it is important to explore the effectiveness and mechanisms behind BRJ supplementation in female populations as these have yet to be examined.
Chapter 2: Statement of the Problem

Rationale

Over the last decade, a wealth of research has surfaced exploring the effects of NO$_3^-$-rich BRJ on athletic performance across a multitude of exercise modalities. The vast majority of this research has been conducted on male participants, and it appears that the greatest benefits are seen in recreationally active males. Moreover, there are only 5 BRJ studies that have considered exclusively female subjects, and only 2 of those studies measured submaximal O$_2$ uptake. Only one of these studies recruited recreationally active women, however their primary outcome was performance during a RPE clamp protocol. Furthermore, none of these studies assessed both acute and chronic BRJ supplementation in the same protocol. Therefore, the purpose of this study was to determine the effects of acute and chronic BRJ supplementation on submaximal cycling economy, and performance on a 4 kJ/kg time trial in recreationally active females.

BRJ was used instead of sodium NO$_3^-$ or potassium NO$_3^-$ because BRJ is a commercially available product that athletes from around the globe, and across a range of calibres, use to potentially enhance their athletic performance.

The subjects supplemented with BRJ and PLA both acutely and chronically to determine if short-term and long-term supplementation periods had differing effects on submaximal exercise economy and time trial performance in recreationally active women. Beneficial effects have been seen in recreationally active males both acutely and chronically.

The subjects were required to ingest 13 mmol NO$_3^-$ approximately 2.5 h before the exercise protocol on the mornings of the experimental trials. This dose has been shown to reduce the O$_2$ cost of exercise and improve time trial performance in recreationally active males, whereas doses smaller than 4.2 mmol NO$_3^-$ have not shown these same benefits (Wylie et al.)
Secondly, pharmacokinetic profiles have demonstrated that NO\textsuperscript{3−} levels peak in the blood approximately 2.5 h after consumption of BRJ (Wylie et al. 2013). On the chronic supplementation days, the subjects were instructed to consume 26 mmol NO\textsuperscript{3−} as 13 mmol in the morning and 13 mmol in the evening. Following a dose of 13 mmol NO\textsuperscript{3−} plasma NO\textsuperscript{3−} concentrations have been shown to return to baseline 12 h post-ingestion, therefore the subject’s plasma NO\textsuperscript{3−} concentrations were constantly elevated during this supplementation regime. This dose was selected as it is higher than any other female BRJ study to ensure adequate accumulation in the blood.

The subjects completed a submaximal exercise protocol at wattages that elicited 50% \textit{VO}_\text{2peak} and 70% \textit{VO}_\text{2peak} to assess exercise economy at multiple submaximal workloads. Secondly, the subjects completed a 4 kJ/kg time trial as a measure of athletic performance. A time trial was used instead of a time to exhaustion protocol because of greater repeatability across trials and greater ecological validity (Currell and Jeukendrup 2008). The 4 kJ/kg time trial was selected instead of a fixed time or fixed distance time trial to normalize the subjects’ work to their body weight.

Overall, the testing protocol was carefully selected to allow for a comprehensive analysis of the effects of BRJ supplementation on exercise economy and time trial performance in recreationally active females.

**Hypotheses**

1) Plasma [NO\textsuperscript{3−}] and [NO\textsuperscript{2−}] will be significantly elevated following acute and chronic supplementation with NO\textsuperscript{3−}-rich BRJ compared to baseline and PLA in recreationally active females.
2) Acute and chronic supplementation with NO$_3^-$-rich BRJ will significantly decrease the O$_2$ cost of submaximal cycling at 50% VO$_{2peak}$ and 70% VO$_{2peak}$ in recreationally active females.

3) Acute and chronic supplementation with NO$_3^-$-rich BRJ will improve performance on a 4 kJ/kg time trial in recreationally active females.

Chapter 3: Methods

Subjects

Twelve recreationally active females were recruited to participate in this study (mean ± SEM, age: 23 ± 0.4 years; weight: 65.0 ± 3.0 kg; height: 169 ± 2 cm; VO$_{2peak}$: 40.7 ± 1.2 mL/kg/min). Subjects were included in the study if they were healthy, recreationally active females, between the ages of 18 and 30, actively using hormonal contraceptives for 6 months prior to participation, and consumed less than 300 mg NO$_3^-$ per day. Subjects were considered recreationally active if they were physically active 3 – 4 times per week and had a VO$_{2peak}$ of ~ 35 – 45 mL/kg/min. Subjects reported being physical active 4 ± 0.2 days per week and performed 4.5 ± 1.0 h of exercise/week in the 6 months leading up to participation in this study. The subjects were informed of the study requirements and potential risks prior to providing oral and written consent. This study was approved by the University of Guelph Research Ethics Board (#REB17-02-008).

Study Design

This study was comprised of 7 laboratory visits (Figure 7). The first visit consisted of a VO$_{2peak}$ test to determine the subject’s aerobic capacity, and familiarization trials were performed during visits two and three. Then, using a double-blind, randomized, crossover design, the subjects supplemented with BRJ (6.5 mmol NO$_3^-$/70 mL; Beet It Sport, James White Drinks, Ipswitch, UK) or a NO$_3^-$-free placebo (PLA, 0.006 mmol NO$_3^-$/70 mL; Beet It Sport) for 1 day
(acute) and 8 days (chronic). The treatment conditions were separated by a washout period (9 ± 1 days). This time course had been previously validated to allow sufficient time for plasma NO$_3^-$ and NO$_2^-$ levels to return to baseline following chronic supplementation with exogenous NO$_3^-$ (Nyakayiru et al. 2017). The PLA was manufactured using an ion resin exchange that selectively removed NO$_3^-$ from the original product (Lansley et al. 2011). The BRJ and PLA were indistinguishable by taste, smell, appearance, and packaging. The supplementation order was counterbalanced, where 6 subjects began supplementing with BRJ and 6 began supplementing with PLA. To investigate the effects of acute and chronic BRJ supplementation on exercise performance, the subjects completed a submaximal cycling protocol and an aerobic-based time trial (TT) on days 1 and 8 of supplementation. The testing began with subjects consuming 140 mL of BRJ (13 mmol NO$_3^-$) or PLA 2.5 h prior to submaximal cycling and the TT. In the evening on day 1, the subjects consumed an additional 140 mL of BRJ (13 mmol NO$_3^-$) or PLA. On days 2 – 7, the subjects consumed 280 mL of BRJ or PLA. The first dose of 140 mL was consumed in the morning with breakfast and the second 140 mL dose was consumed in the evening with dinner.

**Figure 7**: A schematic of the experimental design consisting of 7 laboratory visits. Subjects completed 2 x 8 days supplementation phases separated by a washout of 9 ± 1 days. The subjects came to the laboratory on days 1 (acute trial) and 8 (chronic trial) of each supplementation phase to perform a cycling protocol consisting of submaximal cycling and an aerobic-based time trial.

**Pre-Experimental Tests**

To determine VO$_{2\text{peak}}$, and the cycling intensity to elicit 50% and 70% VO$_{2\text{peak}}$, each subject performed an incremental exercise test to volitional exhaustion on a cycle ergometer.
(Lode Excalibur Sport, Groningen, The Netherlands). The subjects were equipped with a heart rate (HR) monitor (POLAR H10, Oulu, Finland) before warming up for 2 min at 60 W, then the power output (PO) was increased to 100 W. Once the subjects achieved steady state VO₂ (~ 2 min) at each stage, the PO was increased by 25 W. Steady state was defined as two x 20 s VO₂ measurements that were within 50 mL O₂/min of each other. This process was repeated until the subjects reached volitional exhaustion (VO₂peak was determined as the highest value obtained during the test). Time, HR, VO₂, and PO were recorded at the end of each step increment. VO₂ and carbon dioxide production (VCO₂) were measured (Moxus Modular VO₂ System, Pittsburgh, USA) in 20 s intervals during the test.

The subjects completed two familiarization trials prior to the experimental trials. The subjects arrived to the laboratory and were asked to provide a urine sample for determination of urine specific gravity (USG). Then, the subjects were weighed and equipped with a HR monitor. In the first familiarization trial the subjects were set up comfortably on the ergometer and the position was documented for subsequent trials. During the familiarization trials, the subjects completed the entire experimental exercise protocol consisting of a 5 min warm up at 60 W, 10 min at 50% VO₂peak, 10 min at 70% VO₂peak, and a 4 kJ/kg body mass (BM) aerobic time-based TT. In the first familiarization trial the subjects consumed water ad libitum. The subjects were weighed after the trial for determination of body mass loss through sweat. If the subject lost or gained mass compared to the initial weigh in, then the volume of fluid provided during the exercise protocol was adjusted prior to the second familiarization trial. The volume of water consumed in the second familiarization trial was replicated for the subsequent experimental trials. The subjects moved to the experimental trials once there was less than 5% variability
between the TT performances (Currell and Jeukendrup 2008). All subjects’ TT performances achieved this, therefore no subject was required to complete a third familiarization trial.

Diet, Sleep and Exercise Standardization

The subjects recorded their dietary intake, sleep and exercise in the 48 h leading up to the familiarization and experimental trials. Subjects were asked to replicate their diet, sleep and exercise habits for the duration of the study. In the 24 h prior to the familiarization and experimental trials, the subjects were asked to refrain from intense exercise and alcohol, to abstain from caffeine in the 12 h before the trials. Subjects were not instructed to abstain from NO³-rich foods, as the pre-study screening indicated that they did not consume diets high in dietary NO³. The use of antibacterial mouthwash and chewing gum was prohibited during the experimental trials, as there is evidence suggesting that these substances destroy the anaerobic bacteria in the mouth necessary for the conversion of NO₃⁻ to NO₂⁻ and NO (McDonagh et al. 2015). Furthermore, the subjects were instructed to avoid brushing their teeth for 2 h before and after the ingestion of the BRJ or PLA.

For the experimental trials, the subjects arrived to the laboratory in an overnight fasted state. A baseline blood sample (9.5 mL) was drawn for analysis of serum estrogen and progesterone as well as plasma [NO₃⁻] and [NO₂⁻]. The subjects were then provided with a standardized breakfast (consumed in < 15 min) that consisted of a banana, a SoLo GI® bar, and 140 mL of BRJ or PLA. This breakfast was intentionally carbohydrate-rich to ensure that carbohydrate was not limiting during exercise (79 g CHO, 16.5 g protein, 7.7 g fat). Following the standardized breakfast, the subjects were required to wait 2 h before a second blood sample was drawn (6 mL) for determination of plasma [NO₃⁻] and [NO₂⁻]. During this period, the
subjects were provided with water to ensure adequate hydration prior to exercise. The volume of water consumed at rest in the first experimental trial was replicated for subsequent trials.

Experimental Exercise Protocol

Immediately after the second blood sample, the subjects were escorted to the exercise laboratory (21.4 ± 0.3°C, 56.0 ± 3.0%). The subjects were asked to provide a urine sample for determination of USG and to void their bladder. They were then weighed and equipped with a HR monitor. Exercise testing occurred ~ 2.5 h post-beverage ingestion (Figure 8). The exercise protocol was designed to make VO\(_2\) measurements at two different submaximal exercise intensities, followed by an aerobic TT. The subjects completed a 5 min warmup at 60 W, followed by 10 min at 50% VO\(_{2\text{peak}}\) (81 ± 4 W) and 10 min at 70% VO\(_{2\text{peak}}\) (131 ± 5 W). The subjects were instructed to maintain a cadence of 80 revolutions per minute (rpm) throughout the submaximal cycling protocol. The subjects then rested for 3 min before completing a 4 kJ/kg BM TT (260 ± 12 kJ). The ergometer was set to linear mode, and the subjects started cycling at a resistance equal to 70% VO\(_{2\text{peak}}\) at 80 rpm. The subjects were able to increase their PO by increasing their rpms and were only shown the number of kJ completed during the TT.

The rating of perceived exertion (RPE) was recorded every 5 min throughout the submaximal exercise protocol using the Borg Scale (6 – 20) (Borg 1982). Respiratory measurements were recorded over 20 s intervals for the final 4 min of each submaximal exercise intensity. Time, PO, rpm and RPE were recorded with the completion of each 20% of the TT. Verbal encouragement was provided with each 20% completed. HR was recorded continuously throughout the exercise protocol. Following the time trial, subjects completed a blinding and adverse side effects questionnaire.
Figure 8: Experimental trial exercise protocol. Red arrows indicate recording of rating of perceived exertion (RPE), power output (PO) and revolutions per minute (rpm) at 5 min intervals during the submaximal exercise protocol. Blue arrows indicate collection of oxygen uptake for the final 4 min of each submaximal exercise intensity. Black arrows indicate recording of RPE, PO, rpm and time elapsed with every 20% of the 4 kJ/kg body mass (BM) time trial completed.

Blood Sampling and Analysis

On the mornings of the experimental trials, the subjects arrived to the laboratory and had a baseline blood sample drawn from the antecubital vein. This blood sample was ~9.5 mL, of which 3.5 mL was collected into a serum separator tube and 6 mL was collected into a K$_2$EDTA-coated tube. The second 6 mL blood sample was collected 2 h later into a K$_2$EDTA-coated tube. Immediately after collection, all blood tubes were thoroughly mixed.

Immediately following collection, the K$_2$EDTA-coated tubes were centrifuged for 10 min at 2600 rpm and 4°C. The plasma was then aliquoted equally into two 1.8 mL cryovials. The serum separator tube was left for 30 min at room temperature to coagulate and centrifuged for 10 min at 3000 rpm and 4°C. The serum was aliquoted equally into two 1.8 mL cryovials. All plasma and serum samples were immediately stored at -80°C for future analysis.

Serum samples were analyzed for estradiol (pg/mL) and progesterone (ng/mL) concentrations using commercially available ELISA kits (Abcam, Item Nos: ab108667, ab108654, USA). For determination of serum estradiol concentrations, the serum samples were thawed on ice and vortexed for 5 s before 25 µL of sample or standard was added to the 96 well plate and 200 µL of Estradiol-horseradish peroxidase (HRP) conjugate was added to each well.
The plate was covered and incubated for 2 hr at 37°C to allow the Estradiol-HRP to compete
with the estradiol within the sample for antibody binding sites. The wells were then aspirated and
washed three times with 300 µL of 1x wash solution to remove unbound material and 100 µL
substrate was added to each well. The substrate was catalyzed by 3,3’,5,5’-Tetramethylbenzidine
(TMB) to produce blue colouration. The plate was incubated for 30 min at room temperature
before 100 µL stop solution was added to each well. The absorbance was read at 450 nm.

For determination of serum progesterone concentrations, the serum samples were thawed
on ice and vortexed for 5 s before 20 µL of sample or standard was added to the 96 well plate.
Next, 200 µL of Progesterone-HRP conjugate was added to each well. The plate was covered
and incubated for 1 hr at 37°C to allow the Progesterone-HRP to compete with the progesterone
within the sample for antibody binding sites. The wells were then aspirated and washed three
times with 300 µL of 1x washing solution to remove unbound material and 100 µL substrate was
added to each well. The substrate was catalyzed by TMB substrate to produce blue colouration.
The plate was incubated at room temperature for 15 min before 100 µL stop solution was added
to each well. The absorbance was read at 450 nm.

Plasma samples were analyzed for NO$_3^-$ and NO$_2^-$ concentrations using
chemiluminescence. To quantify NO$_2^-$, the plasma sample was passed with a gastight syringe
into a reduction solution containing 45 mmol/L potassium iodide and 10 mmol/L iodine in
glacial acetic acid. The reduction solution was kept at 56°C and continuously bubbled with
nitrogen. Sample aliquots were incubated with sulfanilamide for 15 min. Sulfanilamide forms a
stable diazonium ion that traps NO$_2^-$ and prevents it from further reduction to NO. The quantity
of NO$_2^-$ was determined by calculating the area under the curve for NO$_2^-$ from the aliquots
treated with sulfanilamide and subtracting this from the NO$_2^-$ area under the curve for the
untreated aliquots. Plasma NO$_3^-$ was determined by reducing NO$_3^-$ to NO via vanadium (III) chloride. The amount of NO$_3^-$ was quantified by subtracting the previously determined NO$_2^-$ concentration from the amount of NO$_3^-$ reduced to NO (Lundberg and Govoni 2004).

**Compliance**

Each subject’s adherence to the chronic supplementation protocol was assessed through the return of the empty supplemental beverage containers, and through the analysis of plasma NO$_3^-$ and NO$_2^-$ concentrations. Subjects were instructed to return all empty supplement containers at the time of their chronic supplementation trial.

**Statistical Analysis**

Diet records were analyzed using Food Processor Nutrition Analysis Software (ESHA Research, Salem, USA). Statistical analyses were performed using Prism 7 (GraphPad Software, San Diego, USA). Statistical significance was achieved when P < 0.05. Data are presented as mean ± SEM. The statistical tests for serum estradiol and progesterone, pre-trial characteristics (48 h diet and exercise record, pre-trial sleep), environmental conditions (temperature, humidity), hydration measures (pre-fluid intake, USG, during-fluid intake), submaximal exercise respiratory variables (VO$_2$, VCO$_2$, RER, V$_E$, V$_T$, and f), energy expenditure, and submaximal exercise performance (HR, RPE, and rpm) were conducted using a two-way analysis of variance (ANOVA) and Tukey’s multiple comparisons post-hoc test.

Time trial learning effect was assessed with a one-way ANOVA and Tukey’s multiple comparisons post-hoc test. Time trial performance (HR, RPE, PO, rpm, and elapsed time) were analyzed using a three-way ANOVA (BRJ vs. PLA, acute vs. chronic, time) and Tukey’s multiple comparisons post hoc test.
Responders to BRJ supplementation were arbitrarily defined as individuals who demonstrated a $\geq 3\%$ reduction in $\text{VO}_2$ as this adheres with previous research and is greater than the coefficient of variation (CV) between the second familiarization trial, Acute PLA condition, and Chronic PLA condition. Responders to BRJ supplementation were also arbitrarily defined as individuals who demonstrated a $\geq 4.2\%$ improvement in time trial performance as this is greater than the CV between the second familiarization trial, Acute PLA condition, and Chronic PLA condition.

Chapter 4: Results

Blood Parameters

*Estradiol and Progesterone*

Serum estradiol and progesterone concentrations were reported for 10 subjects. The data for 2 subjects was lost during analysis due to values below the limit of detection. Serum estradiol concentrations were not significantly different across the trials (Acute PLA, $0.01 \pm 0.003$: Chronic PLA, $0.02 \pm 0.01$: Acute BRJ, $0.01 \pm 0.01$: Chronic BRJ, $0.01 \pm 0.01$ pg/mL). Serum progesterone concentrations were not significantly different across the trials (Acute PLA, $0.98 \pm 0.36$: Chronic PLA, $0.47 \pm 0.13$: Acute BRJ, $1.39 \pm 0.97$: Chronic BRJ, $1.14 \pm 0.76$ ng/mL).

*Nitrate (NO$_3^-$)*

Baseline plasma NO$_3^-$ concentrations were not different in the Acute PLA ($36 \pm 4 \mu M$), Chronic PLA ($36 \pm 3 \mu M$), and Acute BRJ trials ($44 \pm 3 \mu M$) (Figure 9). However, plasma NO$_3^-$ was significantly elevated at baseline in the Chronic BRJ trial ($548 \pm 57 \mu M$) compared to all other conditions ($p < 0.0001$). There was no significant difference in plasma NO$_3^-$ 2 h post-beverage ingestion in the Acute PLA ($49 \pm 5 \mu M$) and Chronic PLA ($40 \pm 3 \mu M$) trials. Conversely, there was a significant rise in plasma NO$_3^-$ concentrations 2 h post-beverage
ingestion in the Acute BRJ (776 ± 33 μM) and Chronic BRJ (1125 ± 65 μM) trials (p < 0.0001).

Plasma NO$_3^-$ concentrations were significantly greater in the Acute BRJ trial compared to Acute PLA and Chronic PLA 2 h post-beverage consumption (p < 0.0001). Similarly, 2 h post-beverage consumption, plasma NO$_3^-$ concentrations were significantly greater in the Chronic BRJ trial compared to Acute PLA and Chronic PLA (p < 0.0001). Interestingly, plasma NO$_3^-$ concentrations were significantly greater in the Chronic BRJ compared to the Acute BRJ trial 2 h post-beverage consumption (p < 0.0001).

**Figure 9:** Plasma nitrate (NO$_3^-$) at baseline (B) and two hours post-ingestion (2 h) of 140 mL beetroot juice (BRJ) or NO$_3^-$-free placebo (PLA) both acutely and chronically. Values are mean ± SEM. *, significantly different than all other conditions.

Plasma NO$_2^-$ concentrations were not significantly different at baseline across all trials (Acute PLA, 231 ± 28: Chronic PLA, 216 ± 27: Acute BRJ, 240 ± 26: Chronic BRJ, 328 ± 20).
There was no change in plasma NO$_2^-$ concentrations 2 h post-beverage ingestion in the Acute PLA (266 ± 23 nm) and Chronic PLA (280 ± 24 nm) trials. The increase in plasma NO$_2^-$ 2 h post-beverage ingestion was significant in both the Acute BRJ (729 ± 80 nm) and Chronic BRJ (706 ± 95 nm) trials (p < 0.0001). However, there was no significant difference in plasma NO$_2^-$ between the Acute BRJ and Chronic BRJ trials 2 h post-beverage ingestion.

Figure 10: Plasma nitrite (NO$_2^-$) at baseline (B) and two hours post-ingestion (2 h) of 140 mL beetroot juice (BRJ) or nitrate-free placebo (PLA) both acutely and chronically. Values are mean ± SEM. †, significantly greater than conditions with no symbol.

Pre-Trial Characteristics

The subjects successfully maintained their dietary habits throughout the study as there was no significant difference in energy intake in the 48 h prior to the experimental trials (Acute PLA, 2447 ± 210; Chronic PLA, 2150 ± 104: Acute BRJ, 2155 ± 190: Chronic BRJ, 2316 ± 179 kcal). Similarly, there was no change in physical activity in the 48 h prior to each trial. Lastly,
there was no difference in sleep patterns the night prior to each trial (Acute PLA, 7.6 ± 0.2: Chronic PLA, 7.3 ± 0.2: Acute BRJ, 7.4 ± 0.2: Chronic BRJ, 7.2 ± 0.3 h).

Environmental Characteristics

The average temperature in the exercise laboratory during the exercise tests was 21.4 ± 0.3°C and the average relative humidity was 56.0 ± 3.0%. Temperature did not differ between any of the trials (Acute PLA, 21.4 ± 0.3: Chronic PLA, 21.5 ± 0.4: Acute BRJ, 21.3 ± 0.2: Chronic BRJ, 21.6 ± 0.3°C), and there was no significant difference in relative humidity across the trials (Acute PLA, 55.3 ± 2.9: Chronic PLA, 53.8 ± 3.4: Acute BRJ, 58.6 ± 2.8: Chronic BRJ, 56.2 ± 3.1%).

Hydration Status

On average, the subjects consumed 665 ± 49 mL of water in the 2 h prior to the exercise trial. The volume of water consumed prior to exercise did not differ between the trials (Acute PLA, 677 ± 43: Chronic PLA, 674 ± 47: Acute BRJ, 658 ± 51: Chronic BRJ, 651 ± 58 mL). The subjects arrived to the exercise laboratory very well hydrated with an average USG of 1.005 ± 0.0012. USG was not different across trials (Acute PLA, 1.003 ± 0.001: Chronic PLA, 1.005 ± 0.002: Acute BRJ, 1.005 ± 0.001: Chronic BRJ, 1.005 ± 0.001). On average, the subjects consumed 569 ± 56 mL of water throughout the exercise protocol. The volume of water consumed during the exercise protocol was unchanged across trials (Acute PLA, 576 ± 56: Chronic PLA, 558 ± 57: Acute BRJ, 584 ± 62: Chronic BRJ, 560 ± 54 mL). The subjects were successful at replenishing their sweat loss with water, as the average change in body mass was + 0.1 ± 0.1%. The percent change in BM was the same across trials (Acute PLA, + 0.1 ± 0.1: Chronic PLA, + 0.2 ± 0.1: Acute BRJ, + 0.2 ± 0.1: Chronic BRJ, + 0.1 ± 0.1%).

Exercise Economy 50% VO_2peak
There was no change in mean VO\textsubscript{2} between the trials when the subjects cycled at an intensity that elicited 50% VO\textsubscript{2peak} (Acute PLA, 1254 ± 45: Chronic PLA, 1267 ± 45: Acute BRJ, 1259 ± 46: Chronic BRJ, 1252 ± 42 mL/min O\textsubscript{2}) (Figure 11). Interestingly, 25% of the subjects were defined as responders to Acute BRJ supplementation and 42% of the subjects were defined as responders to Chronic BRJ supplementation at 50% VO\textsubscript{2peak} (Figure 12). A responder was defined as a subject who demonstrated a ≥ 3% reduction in VO\textsubscript{2} following BRJ supplementation compared to PLA. This is in accordance with previous research (Wylie et al. 2013) and outside of the calculated CV for VO\textsubscript{2} at 50% VO\textsubscript{2peak} (2.4%). In addition, V\textsubscript{E}, V\textsubscript{T}, f, and RER were unchanged across trials (Table 1). Furthermore, there was a main effect of condition where there was a small but significant increase in VCO\textsubscript{2} in the chronic trials (Chronic PLA, 1160 ± 35: Chronic BRJ, 1172 ± 36 mL/min) compared to the acute trials (Acute PLA, 1147 ± 33: Acute BRJ, 1155 ± 43 mL/min) (p = 0.01) (Table 1). HR was not significantly different between conditions or from 5 - 10 min at 50% VO\textsubscript{2peak} (Table 2). RPE was not significantly different between conditions, however there was a main effect of time where RPE was significantly increased from 5 - 10 min at 50% VO\textsubscript{2peak} (p < 0.0001) (Table 2). The subjects successfully maintained a cadence of 80 rpm at 50% VO\textsubscript{2peak} during each of the trials (Table 2). There was no change in calculated energy expenditure between trials (Acute PLA, 62 ± 2: Chronic PLA, 63 ± 2: Acute BRJ, 62 ± 2: Chronic BRJ, 62 ± 2 kcal).
Figure 11: Mean oxygen uptake (VO₂) at 50% peak oxygen uptake (VO₂peak) following acute and chronic supplementation with beetroot juice (BRJ) or a nitrate-free placebo (PLA). Data reported as mean ± SEM.

Exercise Economy 70% VO₂peak

Mean VO₂ remained unchanged at 70% VO₂peak between trials (Acute PLA, 1804 ± 60: Chronic PLA, 1814 ± 54; Acute BRJ, 1791 ± 53; Chronic BRJ, 1789 ± 54 mL/min O₂) (Figure 13). Interestingly, 25% of the subjects were defined as responders to Acute BRJ supplementation.
and 33% of the subjects were defined as responders to Chronic BRJ supplementation (Figure 14). A responder was defined as a subject who demonstrated a ≥ 3% reduction in VO₂ following BRJ supplementation compared to PLA. This is in accordance with previous research (Wylie et al. 2013) and outside of the calculated CV for VO₂ at 70% VO₂peak (2.0%). Regardless of trial, VCO₂, V̇ₜ, f, and RER were unchanged (Table 1). However, there was a very small but significant decrease in V̇ₑ in the BRJ trials (Acute BRJ, 42.4 ± 1.5: Chronic BRJ, 42.6 L/min) compared to PLA (Acute PLA, 43.4 ± 1.6: Chronic PLA, 44.4 ± 1.4 L/min) (p = 0.002). HR was not significantly different between conditions or from 5 - 10 min at 70% VO₂peak (Table 2). RPE was not significantly different between conditions, however there was a main effect of time where RPE increased from 5 - 10 min at 70% VO₂peak (p < 0.0001) (Table 2). The subjects successfully maintained a cadence of 80 rpm at 70% VO₂peak (Table 2). Energy expenditure was unaltered between trials (Acute PLA, 90 ± 3: Chronic PLA, 90 ± 3: Acute BRJ, 89 ± 3: Chronic BRJ, 89 ± 3 kcal).

![Graph](image)

**Figure 13**: Mean oxygen uptake (VO₂) at 70% peak oxygen uptake (VO₂peak) following acute and chronic supplementation with beetroot juice (BRJ) or a nitrate-free placebo (PLA). Data reported as mean ± SEM.
Figure 14: Individual oxygen uptake (VO$_2$) at 70% peak oxygen uptake (VO$_{2\text{peak}}$) following acute and chronic supplementation with beetroot juice (BRJ) or a nitrate-free placebo (PLA).

**Figure 14**

**Figure 15**: Time Trial Performance

There was no learning effect associated with time trial completion (Trial 1, $1857 \pm 107$; Trial 2, $1873 \pm 112$; Trial 3, $1913 \pm 117$; Trial 4, $1912 \pm 120$ s) (Figure 15). There was no significant difference in time trial completion across the trials (Acute PLA, $1845 \pm 71$; Chronic PLA, $1869 \pm 75$; Acute BRJ, $1912 \pm 93$; Chronic BRJ, $1892 \pm 98$ s) (Figure 16). Similarly, there was no difference in the time to complete each 20% split between trials (Figure 17). There was a main effect of time in which the final split was significantly faster than the other splits ($p = 0.0001$). No difference was observed for HR throughout the time trial (Figure 18). However, a main effect of time was demonstrated, where HR was significantly higher in the final split compared to the rest of the time trial ($p < 0.0001$). There was no effect of condition on RPE, but RPE was significantly higher in the final split compared to the rest of the time trial ($p < 0.0001$) (Figure 19). There was no significant difference in rpm or PO between conditions during the time trial (Figure 20). However, there was a main effect of time in which rpm ($p < 0.0001$) and PO ($p < 0.0001$) were significantly increased in the final split compared to the rest of the time trial.
Figure 15: Time trial learning effect. Data reported as mean ± SEM.

Figure 16: Mean time trial completion. Values reported as mean ± SEM.
Figure 17: Mean time to complete each 20% split of the time trial. Values reported as mean ± SEM. *, significantly faster than the previous splits.

Figure 18: Mean heart rate (HR) at the end of each 20% split during the time trial. Values reported as mean ± SEM. *, significantly higher than previous splits.
Figure 19: Mean rating of perceived exertion (RPE) at the end of each 20% split during the time trial. Values reported as mean ± SEM. a, significantly greater than 20%; b, significantly greater than 20% and 40%; c, significantly greater than every other split.

Figure 20: Mean power output at the end of each 20% split during the time trial. Values reported as mean ± SEM. *, significantly higher than previous splits.

Compliance, Blinding and Adverse Events

The subjects were 100% compliant to the supplementation protocol as assessed through the return of empty supplemental beverage containers, and plasma NO$_3^-$ and NO$_2^-$ analysis. The results from the blinding and adverse events questionnaire indicated that 100% of the subjects were successfully blinded to the treatments they received, and 58% of the subjects experienced mild adverse effects acutely and chronically with both treatments. Nausea was the most
commonly reported side effect (41%), 17% reported gastrointestinal upset, 17% reported beeturia, and 8% reported acid reflux.

Table 1: Respiratory variables at 50% and 70% VO$_{2_{peak}}$ following acute and chronic supplementation with beetroot juice (BRJ) or a nitrate-free placebo (PLA). Values reported as mean ± SEM. *, Chronic trials demonstrated significantly higher carbon dioxide production (VCO$_2$) compared to acute trials. **, BRJ significantly decreased ventilation (V$_E$) compared to PLA. V$_T$, tidal volume.

<table>
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<tr>
<th>VO$_2$ (mL/min)</th>
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<th>70% VO$<em>2</em>{peak}$</th>
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<td>Chronic PLA</td>
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<td>Acute BRJ</td>
<td>27.8 ± 1.1</td>
<td>42.6 ± 1.4 **</td>
</tr>
<tr>
<td>Chronic BRJ</td>
<td>28.1 ± 1.0</td>
<td>42.4 ± 1.5 **</td>
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<table>
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<td>Acute BRJ</td>
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<td>Chronic BRJ</td>
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Table 2: Heart rate (HR), rating of perceived exertion (RPE) and revolutions per minute (rpm) recorded at 5 and 10 min at 50% and 70% peak oxygen consumption ($\text{VO}_{2\text{peak}}$) following acute and chronic supplementation with beetroot juice (BRJ) or a nitrate-free placebo (PLA). Values reported as mean ± SEM.

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<td><strong>RPE</strong></td>
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<td><strong>rpm</strong></td>
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<td>81 ± 1.0</td>
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Chapter 5: Discussion

The principle findings of this study were that acute and chronic dietary NO$_3^-$ supplementation, in the form BRJ, did not improve submaximal exercise economy or aerobic TT performance in recreationally active females. This study is novel as it is the first to investigate the effects of acute and chronic BRJ supplementation in females and is the first to address the O$_2$ cost of submaximal exercise in recreationally active females at multiple intensities.

Comparison to Previous Work in Women

There has been little previous BRJ literature in female populations and the results have produced equivocal findings. The first study to provide insight that women may respond positively to BRJ supplementation was performed by Vanhatalo et al. (2010). The study cohort consisted of 5 healthy males and 3 healthy females (VO$_{2peak}$ ~ 47 mL O$_2$/kg/min), and these authors found a 5% reduction in the O$_2$ cost of moderate intensity submaximal exercise on days 1, 5 and 15 of supplementation with 5.2 mM dietary NO$_3^-$. Interestingly, this group demonstrated significantly elevated plasma NO$_2^-$ levels prior to supplementation (~454 nM) compared to the current study (~250 nM). These baseline differences may be attributed to dietary habits or fitness status. Additionally, the subjects had a smaller rise in plasma NO$_2^-$ following supplementation (~650 nM) compared to the current study (~725 nM). The differences in the rise in plasma NO$_2^-$ likely reflect the differences in the BRJ doses between the studies. The dramatically different VO$_2$ outcomes between this study and the current study may reflect the inclusion of predominantly male subjects in the study by Vanhatalo et al. (2010).

To date, three studies have investigated the effects of BRJ supplementation in trained female populations. Glaister et al. (2015) found no effect of acute BRJ supplementation on 20 km cycling TT performance (~35 min) in trained female cyclists. Similar to the results from the
current study, we found no effect of BRJ supplementation on TT performance lasting ~30 min. However, it is also important to consider that Glaister et al. (2015) utilized a significantly smaller dose of BRJ (5.1 mM) which may explain the lack of effect in their study. Similar to Glaister et al. (2015), Buck et al. (2015) found no effect of acute BRJ supplementation on a 1 h exercise circuit involving repeated sprints in trained female team sport athletes. Again, it is possible that the BRJ dose was too small (6 mM) to elicit an ergogenic effect. Both Glaister et al. (2015) and Buck et al. (2015) obtained plasma samples for determination of NO$_3^-$ and NO$_2^-$ levels. Unsurprisingly, plasma NO$_3^-$ and NO$_2^-$ levels were significantly lower following supplementation compared to the current study. Lastly, Pospieszna et al. (2016) found a significant improvement in repeated sprint swimming performance as well as 800 m swimming performance in college level female swimmers with 8 days of BRJ supplementation. These authors utilized a larger dose of BRJ (10.2 mM) and this is the only study to employ a chronic supplementation protocol in female subjects. However, it is difficult to determine the validity of this study as the authors did not use a placebo supplement and no plasma samples were collected. Bond Jr et al. (2014) measured cycling exercise economy in sedentary, overweight, African American women. These authors found a significant reduction in the O$_2$ cost of exercise at 40, 60, and 80% VO$_{2\text{peak}}$ following acute BRJ supplementation (~12 mM). This dose is comparable to the acute 13 mM dose administered in the current study. This may further emphasize the importance of administering a large enough dose of dietary NO$_3^-$ in order to ensure a biological effect is elicited. However, these authors failed to report the VO$_2$ data adding question to the validity of their results. Rienks et al. (2015) found no effect of acute BRJ supplementation (12.9 mM) on exercise performance during a 20 min RPE clamp protocol in recreationally active women. However, as a secondary outcome, these authors measured exercise
economy at 75 W for 5 min immediately following the RPE clamp protocol and found a weak, but statistically significant ~4% reduction in the O$_2$ cost of exercise (p = 0.048). The findings from these two studies are in contrast to the results from the current study, but inherent study design flaws and weak statistical significance make it difficult to be confident in the findings from these studies. Future work from across a number of laboratories is required to determine the true biological effect of dietary NO$_3^-$ supplementation in female populations.

Interestingly, the findings from the current study are in stark contrast to the consistent reports of a reduced O$_2$ cost of exercise in recreationally active men following both acute and chronic BRJ supplementation (Bailey et al. 2009; Vanhatalo et al. 2010; Lansley et al. 2011; Wylie et al. 2013). Although the exact mechanism still remains to be elucidated, it is proposed that dietary NO$_3^-$ supplementation improves exercise economy and TT performance by improving contractile, calcium-handling, or mitochondrial efficiency through the provision of NO (Bailey et al. 2010; Larsen et al. 2011; Hernandez et al. 2012; Whitfield et al. 2016). The results from this study suggest that there may be differences in the way women utilize dietary NO$_3^-$ compared to men. It is possible that NO$_3^-$ supplementation fails to elicit a response in recreationally active women because there may be differences in the way dietary NO$_3^-$ is digested and handled or differences in the efficiency of NO production. Alternatively, sex differences in skeletal muscle composition, and the role of estrogen on mitochondria and contractile machinery may play an important role in determining the effectiveness of NO$_3^-$ supplementation.

**Nitrate Digestion**

It is unlikely that recreationally active women have impaired function of the NO$_3^-$-NO$_2^-$-NO pathway. A couple of insights can be drawn by comparing the data from this study to another
study from our laboratory that utilized the same absolute dose of BRJ in recreationally active males as well as the same NO$_3^-$ and NO$_2^-$ analytic techniques (Whitfield et al. 2016). In this study, the relative dose of dietary NO$_3^-$ was 0.40 mM/kg, whereas Whitfield et al. 2016 implemented a relative dose of 0.33 mM/kg dietary NO$_3^-$. In the current study, baseline plasma NO$_3^-$ and NO$_2^-$ levels were 44 uM and 240 nM respectively and Whitfield et al. (2016) found baseline plasma NO$_3^-$ and NO$_2^-$ to be ~25 uM and ~200 nM respectively. Perhaps unsurprisingly, following acute BRJ supplementation we found plasma NO$_3^-$ and NO$_2^-$ levels to increase to 776 uM and 729 nM respectively, whereas Whitfield et al. (2016) reported plasma NO$_3^-$ and NO$_2^-$ levels to increase to ~500 uM and ~500 nM respectively. This supports the notion that the greater relative dose of dietary NO$_3^-$ in the current study resulted in a greater increase in plasma NO$_3^-$ and NO$_2^-$ and suggests that women do not have an impaired ability to convert dietary NO$_3^-$ to NO$_2^-$. This is an interesting finding as 58% of the participants in this study reported mild adverse side effects associated with BRJ supplementation, with 42% of participants specifically reporting gastrointestinal (GI) upset. Beets are considered a high Fermentable Oligosaccharides Disaccharides Monosaccharides and Polyols (FODMAP) food due to their oligosaccharide content (Marcason 2012). High FODMAP foods are known to cause GI upset due to their poor digestibility (Magge and Lembo 2012). Women are more likely to be diagnosed with GI conditions such as irritable bowel syndrome (Anbardan et al. 2012; Oshima and Miwa 2015) therefore it was hypothesized that women may have more adverse responses to high FODMAP foods and may not be reaping the benefits of BRJ supplementation due to poor digestibility. However, it is important to consider that this may be confounded by the fact that young women are more likely to report GI discomfort and are more likely to consult a physician than young men (Wang et al. 2013) and based on the dramatic increases in plasma NO$_3^-$ and NO$_2^-$ following
acute and chronic BRJ supplementation in this study it is unlikely that women have an impaired
ability to process dietary NO$_3^-$.

Nitrate and Nitrite Levels

When comparing the baseline plasma NO$_3^-$ and NO$_2^-$ levels in this study to the findings of Whitfield et al. (2016) there are no discernable differences between the sexes. This is in contrast to findings by Kapil et al. (2010) who noted that although men and women did not differ in baseline plasma NO$_3^-$, women had significantly higher plasma NO$_2^-$ levels compared to men. Independent of plasma NO$_3^-$ and NO$_2^-$ increases, we cannot rule out the possibility that women may rely more heavily on endogenous NO production via NOS or that women may have different dietary habits than men. First, it is possible that women rely more heavily on endogenous NO production since previous literature suggests a positive link between estrogen and endogenous NO production (Cicinelli et al. 1996). This would mean a greater reliance on endogenous NO production and decreased reliance on exogenous provision of dietary NO$_3^-$. Second, it’s possible that women have different dietary habits than men. A study by Jonvik et al. (2017) demonstrated that absolute dietary NO$_3^-$ intake was higher in female athletes compared to male athletes, and when dietary NO$_3^-$ intake was expressed relative to total energy intake these differences were more pronounced. However, it is unlikely that the women in this study had different dietary habits than the men in the study by Whitfield et al. (2016) as the baseline plasma NO$_3^-$ and NO$_2^-$ levels were similar. Furthermore, our exclusion criteria stated that participants could not consume a high dietary NO$_3^-$ diet (> 300 mg dietary NO$_3^-$/day). It is unclear if Whitfield et al. (2016) controlled for dietary NO$_3^-$ habits.
Antioxidant Capacity

It has been suggested that estrogen plays an important role in enhancing antioxidant capacity in women by regulating the activity of antioxidant enzymes. A number of studies have demonstrated that women have lower plasma levels of markers of oxidative stress than men (Ide et al. 2002; Bloomer and Fisher-Wellman 2008). Furthermore, a study by Barp et al. (2002) demonstrated that female rats have lower levels of oxidative stress in heart tissue than male rats, and this was associated with female rats having greater enzyme activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx). Interestingly, it is likely that estrogen drives this relationship as it has been shown that women who undergo hysterectomies (Barp et al. 2002; Borras et al. 2003; Bellanti et al. 2013) as well as post-menopausal women (Singh et al. 2016) have reduced SOD enzyme content (Borras et al. 2003; Bellanti et al. 2013; Singh et al. 2016) and activity (Barp et al. 2002; Borras et al. 2003).

It is possible that the increased antioxidant capacity seen in young, healthy women buffers the reactive nitrogen species and subsequent s-nitrosylation associated with BRJ supplementation. However, it is important to consider that the antioxidant capacity relationships described above were observed in plasma blood samples as well as heart, liver and brain tissue and may not reflect skeletal muscle. If these sex differences in antioxidant capacity exist in skeletal muscle, women may have minimized effects of BRJ supplementation on altering skeletal muscle contractile function, calcium-handling, or mitochondrial efficiency. Future work is needed to elucidate whether these differences exist at the level of the skeletal muscle and if they manifest in significant metabolic and physiological outcomes.
Differences in Skeletal Muscle Fiber Types and Capillarization

A number of studies have performed histochemical analysis on human skeletal muscle fibers to determine sex differences in fiber type composition. These studies consistently demonstrate that women have an ~5% greater proportion of type I oxidative muscle fibers, whereas men have a greater proportion of type II glycolytic muscle fibers (Staron et al. 2000; Haizlip et al. 2014). BRJ supplementation is purported to elicit its beneficial effects predominantly in type II muscle fibers. This is attributed to the fact that the NO$_3^-$-NO$_2^-$-NO pathway is an O$_2$ independent pathway that elicits its effects in scenarios of reduced O$_2$ delivery (Vanhatalo et al. 2011). BRJ has been suggested to facilitate the delivery of O$_2$ to type II muscle fibers that are less oxygenated than type I muscle fibers (Wilkerson et al. 2012). Therefore, it is possible that women are less likely to respond to BRJ supplementation due to a greater proportion of type I muscle fibers, a more oxygenated skeletal muscle environment, and therefore a decreased reliance on the O$_2$ independent NO$_3^-$-NO$_2^-$-NO pathway. Although this is an interesting hypothesis, it is unclear if a 5% difference in fiber type composition is a large enough difference to drive these differences in response to BRJ supplementation.

However, this hypothesis is supported in two different ways. First, as previously described, there is a positive link between estrogen and NOS activity (Cicinelli et al. 1996; Townsend et al. 2011) suggesting that women may place a heavier reliance on the endogenous NO production than men, and that the exogenous NO$_3^-$-NO$_2^-$-NO pathway may provide a less robust contribution to NO production in women than men. Secondly, women have greater skeletal muscle capillarization (Roepstorff et al. 2006; Hoeg et al. 2009) suggesting they have enhanced O$_2$ delivery to skeletal muscle compared to men. This would further support the notion of a decreased reliance on the NO$_3^-$-NO$_2^-$-NO pathway in women compared to men due to a more
oxygenated muscle environment. Conversely, it is important to consider the fact that men have
greater hemoglobin levels (Murphy 2014) and skeletal muscle mass (Janssen et al. 2000), and
these are larger driving factors for $O_2$ delivery and exercise performance than skeletal muscle
capillarization and fiber type differences. Furthermore, it is unlikely that $O_2$ delivery is hindered
at 50% or 70% VO$_{2\text{peak}}$ making this hypothesis feasible but unlikely to explain the potential sex
differences seen in this study with BRJ supplementation.

**Mitochondrial Efficiency**

There is scientific literature suggesting that women have a greater reliance on fat for fuel
during submaximal exercise (Blatchford et al. 1985; Horton 1998; Tarnopolsky et al. 1990). It is
possible that since women rely more heavily on oxidative metabolism, have a greater capillary
density and have a greater proportion of type I muscle fibers that they may exhibit more efficient
mitochondrial coupling compared to men. This notion is in part supported by evidence
suggesting that the livers and brains from female rats produce less mitochondrial H$_2$O$_2$ compared
to males indicating the potential for improved mitochondrial coupling (Borras et al. 2003).

Mitochondrial H$_2$O$_2$ production is increased in situations of elevated electron slippage (Wong et
al. 2017). Therefore, it is possible that a lower level of mitochondrial H$_2$O$_2$ in female rats is
indicative of decreased electron slippage and ultimately improved mitochondrial coupling.

Interestingly, this relationship is likely driven by estrogen as Borras et al. (2003) found increased
mitochondrial H$_2$O$_2$ production in ovariectomized rats and that estrogen replacement therapy
reduced mitochondrial H$_2$O$_2$ production to basal levels. Furthermore, a recent study by Acaz-
Fonseca et al. (2017) showed that cerebral cortex mitochondria of male mice have greater UCP-2
expression compared to female mice. It is possible that this is a metabolic adaptation to deal with
greater mitochondrial ROS in male mice compared to females. Although these studies did not
investigate these relationships in skeletal muscle, it would be interesting to see if these same trends exist in this tissue. Based off of these speculations, it is possible that women have a decreased reliance on the NO$_3^-$-NO$\_2^-$-NO pathway due to more efficient mitochondrial coupling and ultimately oxidative metabolism. Therefore, it’s possible that any effects of dietary NO$_3^-$ supplementation that may affect mitochondrial efficiency may be lost in healthy women who may exhibit more efficient mitochondrial coupling compared to men. Although mitochondrial H$_2$O$_2$ production and UCP protein content are not the only indicators of mitochondrial coupling, they may be interesting avenues for future research to consider in skeletal muscle.

A recent study by Miotto et al. (2018) investigated sex differences in mitochondrial respiratory kinetics at rest in human skeletal muscle. These authors noted that women have decreased ADP sensitivity, as well as greater sensitivity for malonyl CoA inhibition of palmitoyl CoA supported respiration compared to men. The functional role of these findings remains to be determined, but this is the first piece of evidence supporting sex differences in mitochondrial function in human skeletal muscle and therefore may support the notion of studying sex differences in mitochondrial efficiency with respect to BRJ supplementation.

**Calcium-Handling Proteins**

A final difference that may explain why women do not respond to BRJ supplementation is the possible differences that may exist in skeletal muscle calcium-handling. Intriguingly, the time to peak tension and rate of relaxation are faster in the muscle of human males compared to females (Wüst et al. 2008). Time to peak tension is used as an indirect measure of calcium release at the onset of skeletal muscle contraction and rate of relaxation is used as a proxy for the reuptake of calcium following skeletal muscle contraction. This suggests that men may have a
greater ability to release and resequester calcium than women and may implicate sex differences in skeletal muscle calcium-handling protein content or activity.

A study by Welle et al. (2008) demonstrated that healthy women have a 3.6-fold greater gene expression of RyR3 in skeletal muscle than healthy men. Although mRNA does not always translate to protein content, it is possible that women have a greater ability to release calcium via RyR in skeletal muscle. This seems counterintuitive as it would be expected that if women had greater RyR content they would have greater Ca^{2+} release compared to men and likely faster time to peak tension but, this theory does not account for differences in RyR protein activity or the responsiveness of female contractile elements to Ca^{2+} influx. However, if more Ca^{2+} is released via RyR with no differences in SERCA or CASQ content or activity, it may take longer to resequester this Ca^{2+} helping corroborate slower relaxation times seen in women. This would also result in a greater ATP cost from SERCA to pump the additional Ca^{2+}. Perhaps most importantly, it is important to readdress the role of fiber type differences between men and women. Women have a greater proportion of type I fibers, which are known to have slower time to peak tension and half relaxation times than type II fibers (Buchthal and Schmalbruch 1970). Although these fiber type differences are around 5% they likely account for the differences in contractile properties to a greater extent than potential sex differences in skeletal muscle contractile protein content or activity.

Individual Responders

Although mean VO_{2} and time trial performance were unaltered by acute and chronic BRJ supplementation, individual responders were identified at 50% VO_{2peak}, 70% VO_{2peak} and for TT performance. Interestingly, 25% of the subjects were defined as responders to Acute BRJ supplementation and 42% of the subjects were defined as responders to Chronic BRJ
supplementation at 50% VO_{2peak}. A responder was defined as a subject who demonstrated a ≥ 3% reduction in VO_{2} following BRJ supplementation compared to PLA. This is in accordance with previous research (Wylie et al. 2013) and is outside of the calculated CV for VO_{2} at 50% VO_{2peak} (2.4%). Furthermore, 25% of the subjects were defined as responders to Acute BRJ supplementation and 33% of the subjects were defined as responders to Chronic BRJ supplementation at 70% VO_{2peak}. Cumulatively, 2 participants responded to BRJ supplementation in 3 of the 4 conditions, and 1 participant responded in all BRJ conditions. A TT responder was defined as a subject who demonstrated a ≥ 4.2% reduction in time to complete the 4 kJ/kg TT as this was the CV for this measurement. None of the participants demonstrated this level of improvement, however one subject who demonstrated a VO_{2} response both acutely and chronically to BRJ supplementation also demonstrated a 3.3% improvement in TT performance acutely and a 3.8% improvement chronically. These results are in agreement with studies by Christensen et al. (2013) and Boorsma et al. (2014) who identified individual responders to BRJ supplementation in males. In the current study, responders could not be identified based on baseline or rises in plasma NO_{3}⁻ and NO_{2}⁻ levels. This is in accordance with the findings by Boorsma et al. (2014). It is unclear why the individuals in this study responded positively to BRJ supplementation since they had similar dietary habits, exercise regimes, and fitness statuses compared to the other participants. Future research is required to elucidate the mechanisms behind individualized responses to BRJ supplementation. Nonetheless, these findings are important because despite no change in mean VO_{2}, it is still possible for some women to respond positively to acute and chronic BRJ supplementation, and it is possible for the improvement in submaximal exercise economy to translate to slight aerobic TT performance benefits in some individuals.
Limitations

The metabolic cart used to collect the data in this study has up to 3% error in VO\textsubscript{2} measurement, therefore it is possible that the current methodology was not sensitive enough to detect small reductions in VO\textsubscript{2} following BRJ supplementation. Typically, the reduction in VO\textsubscript{2} following BRJ supplementation is in the range of 3 – 5%. In the current study, the CV for VO\textsubscript{2} was 2.4% at 50% VO\textsubscript{2peak} and 2.0% at 70% VO\textsubscript{2peak}. Therefore, it is possible that some of the change in VO\textsubscript{2} associated with BRJ supplementation may have been masked by inherent error associated with the metabolic cart.

The present study used a 4 kJ/kg TT (~30 min) to assess exercise performance. This style of TT has been validated to have greater ecological validity compared to time to exhaustion protocols (Currell and Jeukendrup 2008). However, this may not be a valid measure of performance in recreationally active individuals who do not train for TT performance. We ensured each participant was adequately familiarized with the TT protocol twice and that there was less than 5% difference in TT completion before they graduated to the experimental trials. Interestingly, the CV for TT performance between the second familiarization trial, Acute PLA and Chronic PLA trials, where there presumably there was no effect of condition, was 4.24%, which is in agreement with Currell and Jeukendrup (2008) suggesting < 5% variability in TT performance for trained individuals. This indicates that this protocol had high repeatability despite the recreational status of the participants.

Future Directions

Following the completion of this study, a number of questions remain unanswered regarding sex differences that may exist with BRJ supplementation. Future work should include a study similar to that of Porcelli et al. (2015) to verify the findings from the current study and to
determine if the effect of training status on the response to BRJ supplementation exists in women. Second, future work should directly compare the responses to acute and chronic BRJ supplementation in recreationally active male and female populations. If consistent effects are seen with VO₂ and exercise performance, then muscle biopsies should be obtained to delve into the mechanisms underpinning the potential sex differences that may exist. Lastly, future work should investigate the effects of BRJ supplementation in sedentary male and female populations and should determine if improved fitness status via aerobic exercise training can remove the potential beneficial effect of BRJ supplementation. Specifically, VO₂ should be measured at rest, 50% and 70% VO₂peak at baseline, following 4 weeks of training, and again following 10 weeks of aerobic training. Clearly, incorporating women into BRJ research is in its infancy and there are a number of avenues yet to be explored, it will be fascinating to see how the field grows over the next few years and what studies stem from the work presented in this thesis.

**Conclusion**

The main findings from the current study were that acute and chronic supplementation with NO₃⁻-rich BRJ did not improve submaximal cycling exercise economy or aerobic TT performance in recreationally active women. These findings are novel because it is the first study to investigate the effects of acute and chronic BRJ supplementation in women and is the first study to measure exercise economy at multiple exercise intensities in recreationally active women. Although we did not perform any mechanistic measures in this study, we can speculate that there may be sex differences associated with BRJ supplementation. Future research should continue to investigate the potential sex differences that may exist with dietary NO₃⁻ supplementation and should seek to determine the mechanisms underpinning these differences.
References


