Title of Article: Dose effects of New Zealand blackcurrant on substrate oxidation and physiological responses during prolonged cycling

Authors: Matthew David Cook[1,2], Stephen David Myers[1], Mandy Lucinda Gault[1], Victoria Charlotte Edwards[1], Mark Elisabeth Theodorus Willems[1]

Affliliation: [1]University of Chichester
Department of Sport & Exercise Sciences
College Lane
Chichester, PO19 6PE
United Kingdom

[2]University of Worcester
Institute of Sport & Exercise Science
Henwick Grove
Worcester, WR2 6AJ
United Kingdom

Corresponding author: Professor Mark Willems
University of Chichester
Department of Sport & Exercise Sciences
College Lane
Chichester, PO19 6PE
United Kingdom

Phone: +44 (0)1243 816468

Fax: +44 (0)1243 816080

Email: m.willems@chi.ac.uk

Abstract

Purpose It has been previously shown that New Zealand blackcurrant (NZBC) extract increased fat oxidation during short duration cycling. The present study examined the effect of different doses of NZBC extract on substrate oxidation and physiological responses during prolonged cycling.

Methods Using a randomized counterbalanced Latin square design, 15 endurance trained male cyclists (age: 38±12 yrs, height: 187±5 cm, body mass: 76±10 kg, $\dot{V}O_{2max}$: 56±8 mL·kg^{-1}·min^{-1}, mean±SD) completed four separate 120 minutes cycling bouts at 65% $\dot{V}O_{2max}$ after ingesting no dose, or one of three doses (300, 600 or 900 mg·day^{-1}) of NZBC extract (CurraNZ^{TM}) for 7-days.

Results A dose effect (P<0.05) was observed for average fat oxidation (0, 300, 600 and 900 mg·day^{-1} values of 0.63±0.21; 0.70±0.17; 0.73±0.19 and 0.73±0.14 g·min^{-1}) and carbohydrate oxidation (0, 300, 600, 900 mg·day^{-1} values of 1.78±0.51, 1.65±0.48, 1.57±0.44, and 1.56±0.50 g·min^{-1}). The individual percentage change of mean fat oxidation was 21.5% and 24.1% for 600 and 900 mg·day^{-1} NZBC extract, respectively, compared to no dose. Heart rate, $\dot{V}O_2$, $\dot{V}CO_2$, plasma lactate and glucose were not affected.

Conclusion Seven-days intake of New Zealand blackcurrant extract demonstrated a dose-dependent effect on increasing fat oxidation during 120 minutes cycling at 65% $\dot{V}O_{2max}$ in endurance-trained male cyclists.

Keywords Substrate oxidation · New Zealand blackcurrant · Anthocyanins · Polyphenols · Sports nutrition · Cycling

Abbreviations

ACC	acetyl-CoA carboxylase

AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
FAT/CD36	fatty acid translocase/cluster of differentiation 36
FMD	flow-mediated dilation
GTE	green tea extract
NZBC	New Zealand blackcurrant
RER	respiratory exchange ratio
$\dot{V}O_2$	oxygen consumption
$\dot{V}CO_2$	carbon dioxide production
$\dot{V}O_{2max}$	maximum oxygen uptake
WR_{max}	maximum work rate

INTRODUCTION

Among berries, blackcurrant (*Ribes nigrum*) has one of the highest concentrations of the polyphenol, anthocyanin, and typically contains delphinidin-3-rutinoside, delphinidin-3-glucoside, cyanidin-3-rutinoside and cyanidin-3-glucoside. Anthocyanins are flavonoids that have been associated with health benefits acting through inflammatory or antioxidant activity (Pojer et al. 2013). Increased peripheral blood flow during typing activity was shown with blackcurrant intake (Matusmoto et al. 2005), potentially through anthocyanin-induced vasodilation and vasorelaxation (Ziberna et al. 2013).

Studies on New Zealand blackcurrant (NZBC) during exercise have observed that 7-days intake (~105 $mg \cdot day^{-1}$ of anthocyanins) had no effect on rating of perceived exertion during repeated high intensity treadmill running sprints (Perkins et al. 2015) or maximum oxygen uptake ($\dot{V}O_{2max}$) during cycling (Willems et al. 2015). However, an increased 16.1 km cycling time trial (Cook et al. 2015) and intermittent running performance (Perkins et al. 2015), a

greater absolute lactate decrease following exercise (Cook et al. 2015, Perkins et al. 2015) and an increase in lactate threshold (Willems et al. 2015) were observed. In addition, a 27% higher fat oxidation rate was observed at 65% of $\dot{V}O_{2max}$ during 10 minutes cycling, with no changes in heart rate, oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$) and plasma lactate at 45, 55 and 65% of $\dot{V}O_{2max}$ (Cook et al. 2015). However, the metabolic and physiological responses during prolonged exercise (i.e. greater than 60 minutes) with intake of NZBC are not known. The physiological responses influencing fat oxidation during prolonged exercise are different to those during short duration exercise. For example, fatty acid translocase/cluster of differentiation 36 (FAT/CD36) on the mitochondrial membrane is increased at 120 minutes after exercise, but not after 30 minutes (Holloway et al. 2006). In addition, prolonged cycling exercise causes a gradual decrease in insulin concentration, a gradual increase in plasma free fatty acid and glycerol concentration (Jeukendrup et al. 1999) and a lowering of intramuscular glycogen stores (Vøllestad and Blom 1985). Therefore, the substrate oxidation and the physiological responses may be different with NZBC during prolonged exercise of 120 minutes, to those reported during shorter duration steady state exercise, however this has not been examined.

Evidence that polyphenols may increase fat oxidation is also provided by studies using green tea extract (GTE), which is rich in the polyphenol catechins. Venables et al. (2008) observed that a 24-hour GTE ingestion (366 mg·day^{-1}) increased fat oxidation rate by 17% (placebo: 0.35±0.03 vs. GTE: 0.41±0.03 g·min^{-1}) during 30-minutes of cycling at 60% $\dot{V}O_{2max}$ in young healthy men (26±2 yrs). A similar effect was observed when dosing chronically for 3 months with catechins (218 mg·day^{-1}) in healthy men (range 26-42 yrs), a 24% higher fat oxidation rate was observed (control: 3956±1205 vs. catechin: 5217±904 kcal·day^{-1}) during 30-minutes of treadmill walking at 5 km·hr^{-1} compared to a control of no catechins (Ota et al., 2005). However, a lower dose of GTE containing 160 mg·day^{-1} catechins [of which 70 mg·day^{-1} was

epigallocatechin gallate] for three weeks did not effect fat oxidation during 120-minutes cycling at 50% of maximum work rate in endurance trained men (Eichenberger et al 2009). Taken together, these studies suggest that increases in fat oxidation during exercise from catechin polyphenols within GTE are dose-dependent.

Following anthocyanin intake, vascular function has also demonstrated dose-dependent responses. For example, in healthy individuals, Rodriguez-Mateos et al. (2013) reported a dose-dependent increase in flow-mediated dilation (FMD) up to 310 mg anthocyanin and then a plateau above this dose, with additional intake causing no further increases. Previous studies on the effectiveness of New Zealand blackcurrant during exercise (Cook et al., 2015, Perkins et al. 2015, Willems et al., 2015) have not examined if the physiological responses are dose-dependent. However, based upon previous responses to polyphenol intake, dose-dependent changes on physiological responses during exercise may occur. Therefore, this study aimed to examine if dose-dependent changes in physiological responses occur following New Zealand blackcurrant taken for 7-days during prolonged cycling in trained cyclists.

METHODS

Participants

Fifteen endurance-trained men volunteered for the study without payment and provided written informed consent to participate. They were recruited from local cycling clubs with a history of participation of greater than 3 years and were not engaged in a structured training program for the duration of the study but typically performed cycling exercise 6–10 hours a week. Participants were screened for intake of other dietary supplements before commencing participation with all not taking any nutritional supplements. Participant characteristics are presented in Table 1. The study was approved by the University of Chichester Research

Ethics Committee with protocols and procedures conforming to the 2013 Declaration of Helsinki.

Experimental Design

Participants visited the laboratory on 5 occasions at the same time of day. See Fig. 1 for the timeline of experimental sessions and testing. Prior to all visits, participants were instructed to abstain from vigorous exercise for 48 hours, alcohol for 24 hours and caffeine-containing products on the day of testing. On the first visit, participants were measured for position on the electronically controlled cycle ergometer (SRM ergometer, SRM International, Jülich, Germany) with saddle height and setback, handlebar height and drop replicated for all visits. The ergometer was fitted with the participant's saddle and pedals, with participants also using their own cycling shoes.

During the first visit, participants stature (Seca 213, Seca, Birmingham, UK) and body mass (Kern ITB, Kern, Germany) were measured. Subsequently, participants completed an incremental-intensity cycling test until a blood plasma lactate ≥ 4 mmol·L^{-1} (YSI 2300 Stat Plus, Yellow Springs Instruments Co. Inc., Yellow Springs, USA) was reached. After a 15-minute break, participants then completed a maximal cycling test to volitional exhaustion for calculation of $\dot{V}O_{2max}$ and maximum work rate (WR_{max}; the last completed work rate, plus the fraction of time spent in the final non-completed work rate multiplied by the work rate increment).

Participants were assigned, in a randomized, counterbalanced Latin-square design, to three NZBC dose conditions (1, 2 or 3 capsules a day) for 7-days and a no dose condition. Optimal dosing strategy of NZBC is not known, however multiple days of intake have been used previously before exercise testing [e.g. 5 days before (Bell et al. 2015)]. Each 300 mg NZBC capsule contained 105 mg of anthocyanins, consisting of 35-50% delphinidin-3-rutinoside, 5-20% delphinidin-3-glucoside, 30-45% cyanidin-3-rutinoside, 3-10% cyanidin-3-glucoside

(CurraNZ™, Health Currancy Ltd, Surrey, UK). The NZBC capsules were independently analysed and confirmed the ingredients present with no presence of caffeine. Participants were instructed to take the capsules, with breakfast (one-a-day), 12 hours apart (two-a-day) and evenly spaced through the day (three-a-day). On the final day of supplementation, participants reported to the laboratory, two hours post-prandial of standard breakfast (i.e. one slice of buttered bread or toast) and all the capsules required for that condition. Participants then completed a 120-minute cycling protocol at a power calculated to elicit ~65%$\dot{V}O_{2max}$, with expired gas samples collected and lactate measured every 15 minutes. Thirteen participants performed the 120-minute cycle at an intensity below lactate threshold. Participants were allowed to drink plain water *ad libitum*, with all exercise tests conducted in a temperature-controlled laboratory at 18°C with a fan in front of participants to limit unwanted heat storage. Between dosing conditions, there was a 14-day washout period. An anthocyanin intake similar to that of our highest dose for one month showed a return to baseline of biochemical and biomarkers of antioxidant status after 15-days washout (Alvarez-Suarez et al. 2014).

Anthocyanin Consumption, Physical Activity and Dietary Standardization

Participants completed a food frequency questionnaire, which detailed the amount and frequency of anthocyanin containing foods eaten complied from the Phenol Explorer database (Neveu et al 2010). Intake of anthocyanin was calculated as the sum of the consumption frequency of each anthocyanin containing food multiplied by the content of the anthocyanin content for the portion size (Table 1).

Before all experimental visits, participants were instructed to keep their weekly schedule as consistent as possible. Participants recorded their dietary intake on a written food diary 48 hours prior to the first experimental dosing condition (i.e. visit 2) and were then told to replicate this for all subsequent experimental visits (i.e. visits 3, 4, 5) using the first diary as a

guide, while recording on a new diary their dietary intake for that visit. Food diaries were analysed (Nutritics LTD, Dublin, Ireland) for carbohydrate, fat and protein intake and total energy intake (kJ). There were no differences for carbohydrate, fats or protein intake in absolute or relative units between the experimental visits (Table 2). Analysis of the diaries indicated participants reported 100% adherence to the dietary instructions.

Incremental cycling test

The intermittent incremental cycling test performed within visit 1 was completed to establish the relationship between cycling power output and oxygen consumption. The protocol consisted of 4-minute stages of work interspersed with 2 minutes rest where participants rested on the ergometer without pedalling. The protocol began at 50 W and increased by 30 W each stage. At the beginning of the rest stage, a capillary blood sample was taken from the finger and blood plasma lactate concentration analysed. Blood samples were not lysed therefore represent plasma rather than whole blood. The test was terminated when participant's plasma lactate reached a value ≥ 4 mmol·L^{-1}. In the last minute of each exercise stage an expired gas sample was collected in 200 L plastic Douglas bags (Cranlea & Co. Bourneville, Birmingham, UK).

Maximal Rate of Oxygen Uptake

Calculation of $\dot{V}O_{2max}$ was completed following an incremental intensity cycling test to volitional exhaustion. The test began at 50 W for 4 minutes and subsequently increased by 30 W each minute with participants instructed to keep pedal cadence between 70 and 90 rev·min^{-1}, which was displayed on a television screen. In at least the last 3 minutes of the protocol, expired gases were collected in 45 second samples with 30 second samples in the last minute. Expired and inspired fractions of oxygen and carbon dioxide were determined with a gas analyser (Series 1400, Servomex, Crowborough, UK), calibrated using known gases [15.06% O_2, 5.01% CO_2, 79.93% N_2 (Linde Gas UL Ltd., West Bromwich, UK)], and

expired volumes measured using a dry gas meter (6162, Harvard Apparatus Ltd., Edenbridge, UK) and expressed as standard temperature and pressure dry. A finger prick capillary blood sample was taken four minutes after the end of the test and analysed for peak plasma lactate concentration. $\dot{V}O_{2max}$ was considered to be achieved if participants attained at least two of the following $\dot{V}O_{2max}$ criteria; 1) plateau in $\dot{V}O_2$ of <2.1 $mL \cdot kg^{-1} \cdot min^{-1}$ between the last two gas collections, 2) blood plasma lactate >8 $mmol \cdot L^{-1}$, 3) respiratory exchange ratio (RER) ≥1.15 (Howley et al. 1995).

120-minute cycling

The power to oxygen uptake relationship (as a percentage of $\dot{V}O_{2max}$) during the incremental cycling test to a plasma lactate of 4 $mmol \cdot L^{-1}$ was used to establish the power at 65% of participant's $\dot{V}O_{2max}$. This power was a fixed-load for the 120-minute protocol. Participants cycled continually for 120 minutes, keeping a constant pedal cadence between 70 and 90 $rev \cdot min^{-1}$ and they were allowed to consume water *ad libitum*. Finger prick blood sampling for lactate and glucose and one ~60 second expired air sample were collected every 15 minutes (i.e. at 15, 30, 45, 60, 75, 90, 105, 120 minutes of the protocol). Pilot testing indicated variability of the Douglas bag technique in calculating fat oxidation of 6.3% during two 30-minute bouts of cycling at 65% $\dot{V}O_{2max}$ separated by 7-days. Rates of whole body carbohydrate and fat oxidation were calculated using the following stoichiometric equations for moderate intensity exercise with the assumption that protein oxidation during exercise was negligible (Jeukendrup and Wallis 2005):

Fat Oxidation $(g \cdot min^{-1}) = 1.695 \cdot \dot{V}O_2 - 1.701 \cdot \dot{V}CO_2$

Carbohydrate Oxidation $(g \cdot min^{-1}) = 4.210 \cdot \dot{V}CO_2 - 2.962 \cdot \dot{V}O_2$

Statistical Analysis

All statistical analyses were complete using SPSS 20.0 (SPSS, Chicago, USA). Data normality assumptions were assessed using Kolmogorov-Smirnov test. Differences between doses during the 120-minute cycling were analysed using a dose (0 vs. 300 vs. 600 vs. 900 $mg \cdot day^{-1}$) by time-point (15, 30, 45, 60, 75, 90, 105, 120 minutes) repeated measures analysis of variance (ANOVA). A Bonferroni *post hoc* test was used to identify time comparisons. When dose effects were found, average responses over the 120-minute protocol were analysed with a repeated measures one-way ANOVA with *post hoc* pairwise comparisons with Bonferroni correction. Mauchley's Test of Sphericity was conducted to test for homogeneity of data and where violations were present Greenhouse-Geisser adjustments were made. An a-priori power analysis indicated a sample size of 15 would allow detection of a 27% increase in fat oxidation rates with a high statistical power ($1 - \beta = 0.80$: $0.05 = \alpha$ level). To determine the effect size of responses, Cohen's *d* were calculated (Cohen 1988). All data are reported as mean±SD and significance was accepted at $P<0.05$.

RESULTS

Physiological data, energy expenditure and rates of substrate oxidation

There was a time effect for $\dot{V}O_2$ ($F_{(1.4267,19.966)}=7.889$, $P=0.006$), energy expenditure ($F_{(1.521, 21.299)}=6.490$, $P=0.010$) and relative intensity ($F_{(7,98)}=18.062$, $P<0.001$) with no difference between the doses across the eight collections of the 120 minute ride ($P>0.05$) (Table 3). There was no time or dose effect for $\dot{V}CO_2$ ($P>0.05$). Mean relative intensity was not different for the doses ($F_{(2.230,31.223)}=1.101$, $P=0.360$) (0 $mg \cdot day^{-1}$: 63.9±3.9; 300 $mg \cdot day^{-1}$: 64.6±4.3; 600 $mg \cdot day^{-1}$: 64.8±3.7; 900 $mg \cdot day^{-1}$: 64.4±3.5 % $\dot{V}O_{2max}$).

The RER during the 120-minute protocol showed a time ($F_{(3.209,44.924)}=17.445$, $P<0.001$) and dose effect ($F_{(3,42)}=3.984$, $P=0.014$) with no interaction effect ($F_{(21,294)}=0.917$, $P=0.570$) (Fig. 2 a). The mean RER (0 $mg \cdot day^{-1}$: 0.86±0.04, 300 $mg \cdot day^{-1}$: 0.85±0.03, 600 $mg \cdot day^{-1}$:

0.83±0.03, 900 mg·day^{-1}: 0.84±0.02) showed a dose effect ($F_{(3,42)}$=3.984, P=0.014) with 600 (d=1.01) and 900 (d=0.71) mg·day^{-1} decreasing from 0 mg·day^{-1} (P<0.05).

Fat oxidation showed time ($F_{(2.799,39.182)}$=21.271, P<0.001) and dose effects ($F_{(3,42)}$=3.913, P<0.001), with no interaction effect ($F_{(21,294)}$=0.954, P=0.522) (Fig. 2 b). Mean fat oxidation (0 mg·day^{-1}: 0.63±0.20 g·min^{-1}, 300 mg·day^{-1}: 0.70±0.16 g·min^{-1}, 600 mg·day^{-1}: 0.74±0.18 g·min^{-1}, 900 mg·day^{-1}: 0.74±0.13 g·min^{-1}) showed a dose effect ($F_{(3,42)}$=3.913, P=0.015) with *post hoc* testing indicating a group mean (i.e. mean of all individual percentage changes of mean fat oxidation) of 21.5% (13 of 15 participants increased) and 24.1% (13 of 15 participants increased) increase in fat oxidation from 0 mg·day^{-1} for 600 and 900 mg·day^{-1} NZBC, respectively (P<0.05). Between 0 and 300 mg·day^{-1}, fat oxidation was 17.5% higher (11 of 15 participants increased), however, this was not different (P=0.124). The effect sizes for increases in average fat oxidation from 0 mg·day^{-1} were 0.42, 1.03 and 0.75, for 300, 600 and 900 mg·day^{-1} NZBC intake, respectively. Similarly, absolute carbohydrate oxidation during the 120-minute protocol showed a time ($F_{(2.635,36.892)}$=9.831, P<0.001) and dose effect ($F_{(3,42)}$=2.907, P=0.046), with no interaction effect ($F_{(21,294)}$=0.825, P=0.688) (Fig. 2 c). Mean carbohydrate oxidation (0 mg·day^{-1}: 1.78±0.48 g·min^{-1}, 300 mg·day^{-1}: 1.65±0.45 g·min^{-1}, 600 mg·day^{-1}: 1.56±0.41 g·min^{-1}, 900 mg·day^{-1}: 1.56±0.46 g·min^{-1}) showed a dose condition effect ($F_{(3,42)}$=2.907, P=0.046), with *post hoc* testing indicating no differences between the doses (P>0.05).

Blood parameters

There was a main time effect for plasma glucose ($F_{(3.511,2.405)}$=4.049, P=0.009), with no differences between the dosing conditions (P>0.05). There was no dose or time effect for plasma lactate (P>0.05) (Table 3).

Cycling power, economy and heart rate

298 A time effect for cycling economy ($F_{(7,98)}$=3.114, P=0.005) and heart rate
299 ($F_{(1.675,23.446)}$=17.070, P<0.001) was observed with no effect of dose (P>0.05) (Table 3). The
300 power was fixed for the 120-minute protocol, with the average power for the four dosing
301 conditions (0 mg·day^{-1}: 193±31 W, 300 mg·day^{-1}: 193±30 W, 600 mg·day^{-1}: 194±32 W, 900
302 mg·day^{-1}: 193±31 W) not different (P>0.05).
303
304 **DISCUSSION**
305 The principal finding from this study was that there was a dose-dependent effect of NZBC on
306 fat oxidation during 120 minutes cycling in trained male cyclists at ~65%$\dot{V}O_{2max}$. This is the
307 first study to examine the effect of different doses of NZBC on fat oxidation during long
308 duration exercise in trained cyclists. Our results indicate a 21.5% and 24.1% (P<0.05)
309 increase in mean fat oxidation rates, absolute increases of 0.11 and 0.10 grams·min^{-1}, for 600
310 and 900 mg·day^{-1} NZBC intake, respectively with the calculated effect-sizes indicating
311 moderate to large effects.
312 The changes in mean fat oxidation rates observed in this study are lower than the 27%
313 increase (0.37±0.15 placebo vs. 0.44±0.12 NZBC) reported by Cook et al. (2015) during 10
314 minutes cycling at 65% $\dot{V}O_{2max}$ following 300 mg·day^{-1} NZBC (105 mg·day^{-1} anthocyanin).
315 The group mean increases of 21.5% and 24.1% in the present study occurred following 600
316 and 900 mg·day^{-1} of NZBC, doses which are twice and three times that of Cook et al., (2015)
317 with 300 mg·day^{-1} demonstrating no change in average fat oxidation in this study.
318 This may represent a lack of statistical power to detect a difference (i.e. 0 vs. 300 mg·day^{-1}),
319 despite a group mean increase of 17.5%, an identical absolute increase of 0.07 grams·min^{-1}
320 and effect size of 0.42. What is more, the 27% increase observed by Cook et al (2015)
321 occurred during an incremental 30-minute protocol (3 blocks of 10 minutes at 45, 55 and
322 65% $\dot{V}O_{2max}$) and it has been noted that during an incremental protocol, the work completed

in the previous stage may influence fat oxidation in the next stage (Achten et al. 2002). In Cook et al (2005), fat oxidation with NZBC extract at 65% $\dot{V}O_{2max}$ was 0.44±0.12 grams·min^{-1}. The present study used cycling exercise of 120 minutes and this would explain the higher absolute fat oxidation values (e.g. 0.70±0.16 g·min^{-1} with 300 mg·day^{-1}) as fat oxidation increases over time during prolonged exercise (Romijn et al. 1993). As the power was fixed for the 120-minute protocol, the observations of a time effect for heart rate can be explained by the cardiovascular drift effect observed during prolonged exercise (Fritzsche et al. 1999). Similarly, the time effect for $\dot{V}O_2$ would explain the time effect for relative intensity and cycling economy and is likely to result from $\dot{V}O_2$ drift caused by an increase in body temperature, recruitment of additional muscle fibres and fat oxidation (Ishijima et al. 2011). Despite this $\dot{V}O_2$ drift, the mean oxygen cost elicited a relative intensity of ~65%$\dot{V}O_{2max}$ with no differences between the doses. This also indicates that intake of NZBC has no adverse physiological responses on $\dot{V}O_2$ that could diminish performance. However, future studies should examine the implications for the performance of endurance exercise modalities from an increase in fat oxidation by NZBC.

The coefficient of variation (CV) of fat oxidation during exercise lasting greater than one hour is reported between 3-6% (Hodgson et al. 2013) with the day-to-day variation reported to be as high as 9.6% (Achten and Jeukendrup 2003). The much larger group mean 21.5% and 24.1% increases from 600 mg·day^{-1} and 900 mg·day^{-1} NZBC was, therefore, attributed to the NZBC intake. This may result from effects of the anthocyanins in NZBC on fat metabolism. For example, in C57BL/6J mice fed a high fat diet, blackcurrant anthocyanins increased the mRNA expression of 633 genes involved in energy expenditure and mitochondrial biogenesis including peroxisome proliferator-activated receptor alpha, proliferator-activated receptor delta, uncoupling protein 2 and 3 and mitochondrial transcription factor A (Benn et al. 2014). Anthocyanin has also been observed to increase

AMP-activated protein kinase (AMPK) in skeletal muscle of mice (Takikawa et al. 2010) and fatty acid oxidation of human HepG2 cells following *in vitro* incubation (Guo et al. 2012). The activity of acetyl-CoA carboxylase (ACC) 1 and ACC-2 is inhibited by AMPK, which leads to increased fatty acid oxidation and decreased fatty acid synthesis (Towler et al. 2007). Using biopsies, Roepstorff et al. (2005) demonstrated that following 60 minutes cycling at 65%$\dot{V}O_{2max}$ in moderately trained men, there was a decrease in muscle malonyl-CoA concentration, which was associated with an increased activity of AMPK and inhibition of acetyl-CoA carboxylase resultant from its phosphorylation by AMPK. It has also been reported that AMPK activation can induce translocation of FAT/CD36 allowing increased fatty acid uptake (Luiken et al. 2003). It is therefore possible that the interaction of the physiological responses during exercise and alterations in fat oxidation mechanisms following anthocyanin intake lead to increased fat oxidation during exercise. However, various factors should be considered when comparing these studies to *in vivo* human conditions. For example, Takikawa et al. (2010) fed mice a very high intake of anthocyanins (10g/kg diet), while Guo et al. (2012) incubated for one hour with only cyanidin-3-glucoside. Blackcurrant can increase peripheral blood flow during an MVC of the trapezius muscle following typing activity (Matsumoto et al. 2005) Therefore, anthocyanin induced increases in peripheral blood flow may also explain the higher fat oxidation rates through greater delivery of free fatty acids, as an increase in plasma fatty acids has shown to increase fat oxidation (Romijn et al. 1995). The increase in peripheral blood flow may occur by increasing nitric oxide availability, as shown by anthocyanins ability to inhibit NADPH oxidase (Rodriguez-Mateos et al. 2013). It should be noted however, that mode of exercise, intensity and tissue mass within the study by Matsumoto et al (2005) were very different compared to the present study. To develop a greater understanding of the potential

mechanisms involved, future studies should examine plasma glycerol as an indirect marker of lipolysis and free fatty acids during exercise following intake of NZBC.

Rodriguez-Mateos et al. (2016) observed non-linear dose-dependent changes in FMD to cranberry polyphenols, with 409 mg of polyphenols having no effect, whereas responses to 787 mg and 1238 mg increased linearly and plateaued after 1238 mg. The results in this study with NZBC indicate similar dose-dependent changes, as it appears there may be a minimum NZBC dose required to elicit physiological effects. For example, fat oxidation was only increased after 600 mg·day^{-1} (210 mg·day^{-1} anthocyanin) and 900 mg·day^{-1} (315 mg·day^{-1} anthocyanin), with no difference between 600 and 900 mg·day^{-1}. These responses may also represent that an upper limit in substrate utilisation changes by NZBC was reached, or that changes in substrate utilisation were limited because mechanisms for anthocyanin absorption were limited (Kurilich et al. 2005).

Upon ingestion, anthocyanins are reported to have poor bioavailability, with studies reporting uptake of 12.4±1.4% of the ingested dose (Czank et al. 2013). However, beneficial vascular responses following anthocyanin intake have been associated with a peak in phenolic metabolites such as ferulic acid, isoferulic acid, vanillic acid, 2-hydroxybenzoic acid, benzoic acid and caffeic acid in the plasma (Rodriguez-Mateos et al. 2013). Furthermore, anthocyanin metabolites have been observed in plasma up to 48 hours following intake (Kay et al. 2005), therefore a 7-day intake, as in this study, may represent a build-up of anthocyanin metabolites over time which resulted in the increased fat oxidation. The use of multiple days of supplementation before an exercise with a supplement taken on the day of the test is consistent with previous studies supplementing with cherry anthocyanins (Bell et al. 2015). However, this approach does not allow separation of the acute or chronic effects of the supplementation. Furthermore, as anthocyanins may act synergistically with other dietary polyphenols (Niki et al. 1988), future studies may implement anthocyanin wash out periods

before testing similarly to Bell et al., (2015) (i.e. no fruits, vegetables, tea coffee, alcohol, chocolate, cereals, whole meal bread and grains 4 days before and 3 days post exercise). However, this approach should be used with caution as such experimental design would be problematic for ecological validity. The duration responses (i.e. if changes occur following one day of supplementation) to NZBC intake on fat oxidation are unknown. This is an area where future research should focus on, considering that complete anthocyanins are detectable in the plasma at one hour after consumption (Zhu et al. 2011).

Anthocyanins are a sub-class of flavonols. The daily intake of anthocyanins calculated from the food frequency questionnaire was 67±47 $mg \cdot day^{-1}$. This is comparable to the intake of flavonols (including anthocyanins) in men within the United Kingdom of 51 $mg \cdot day^{-1}$ (Zamora-Ros et al., 2011). It also highlights the lowest dose of NZBC (105 $mg \cdot day^{-1}$) used is likely to be considerably higher than their habitual intake and represents a substantial increase in daily consumption of anthocyanins. However, this dose results in no changes in substrate utilisation but an even higher dose of 600 $mg \cdot day^{-1}$ NZBC extract is required, indicating that these effects would be difficult to achieve from consuming unprocessed blackcurrants, whereby each capsule was equivalent to ~80 blackcurrants.

Conclusions

Seven days intake of New-Zealand blackcurrant increases fat oxidation during 120 minutes cycling at ~65% $\dot{V}O_{2max}$ in endurance trained individuals and this occurs in a dose dependent manner. High dose intake of New Zealand blackcurrant does not have adverse physiological effects in trained cyclists. To elucidate mechanisms of the observed findings from this study, future research should examine fat oxidation with measures of circulating fatty acids and peripheral blood flow.

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FIGURE LEGENDS

Fig. 1 Experimental design and timeline of the 5 visits

Fig. 2 respiratory exchange ratio (RER) (a), fat oxidation (b) and carbohydrate oxidation (c) during 120 minutes cycling at ~65% $\dot{V}O_{2max}$ following 0, 300, 600 and 900 mg·day^{-1} New Zealand blackcurrant extract. Vales are presented as mean±SD. a, 0 and 300 mg·day^{-1} different; b, 0 and 600 mg·day^{-1} different; c, 0 and 900 mg·day^{-1} different (P<0.05).

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