Title of Article: Cardiovascular Function during Supine Rest in Endurance Trained Males with New Zealand Blackcurrant: A Dose-Response Study

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ABSTRACT

Purpose Blackcurrant contains anthocyanins that could alter cardiovascular function and reduce cardiovascular disease risk. We examined dose responses of New Zealand blackcurrant (NZBC) extract on cardiovascular function during supine rest.

Methods Fifteen endurance trained male cyclists (age: 38±12 years, height: 178±5 cm, body mass: 76±10 kg, $\dot{V}O_{2max}$: 56±8 mL·kg^{-1}·min^{-1}, mean±SD) were randomly assigned using a counterbalanced Latin square design to complete four conditions, a control of no NZBC, or one of three doses (300, 600 or 900 mg·day^{-1}) of NZBC extract (CurraNZ^{TM}) for seven-days with a fourteen-day washout. Cardiovascular function (i.e. blood pressure, heart rate, ejection time, cardiac output, stroke volume and total peripheral resistance) during supine rest was examined (Portapres® Model 2).

Results Systolic and diastolic blood pressure, heart rate and ejection time were unchanged by NZBC. A dose effect (P<0.05) was observed for cardiac output, stroke volume and total peripheral resistance. A trend for a dose effect was observed for mean arterial blood pressure. Cardiac output increased by 0.6±0.6 L·min^{-1} (15%) and 1.0±1.0 L·min^{-1} (28%) and stroke volume by 5±8 mL (7%) and 6±17 mL (18%) between control and 600, and 900 mg·day^{-1}, respectively. Total peripheral resistance decreased by 4±3 mmHg·L^{-1}·min^{-1} (20%) and 5±9 mmHg·L^{-1}·min^{-1} (20%) for 600, and 900 mg·day^{-1}.

Conclusion Seven-days intake of New Zealand blackcurrant extract demonstrated dose-dependent changes on some cardiovascular parameters during supine rest in endurance-trained male cyclists.

Keywords: Cardiovascular function; New Zealand blackcurrant; anthocyanins; sports nutrition; polyphenols.

Abbreviations:

FMD	flow-mediated dilation
NADPH	Nicotinamide-adenine dinucleotide phosphate
NZBC	New Zealand blackcurrant
$\dot{V}O_{2max}$	Maximal rate of oxygen uptake
WR_{max}	Maximum work rate

INTRODUCTION

Blackcurrant (*Ribes nigrum*) is a rich source of flavonoids, especially the anthocyanins delphinidin-3-rutinoside, delphinidin-3-glucoside, cyanidin-3-rutinoside and cyanidin-3-glucoside (Kähkönen et al. 2003). In animal studies, anthocyanins induced vasodilation and relaxation in thoracic aortic rings in male Wistar rats, and prevented loss of endothelium-dependent relaxation by exposure to exogenous reactive oxygen species in porcine arteries (Bell and Gochenaur 2006). Such observations in humans may, in the long term, reduce cardiovascular risk factors. Indeed, numerous epidemiological studies indicate that consumption of foods high in flavonoids can reduce the risk of cardiovascular disease (Huxley and Neil 2003; Mink et al. 2007).

In *in vitro* animal studies, physiological responses have shown dose-response effects to anthocyanins. For example, blackcurrant concentrate induced dose-dependent relaxation on norepinephrine contracted rat aorta (Nakamura et al. 2002) and incubation of bovine arterial cells with cyanidin-3-glucoside increased endothelial nitric oxide synthase (eNOS) expression in a dose-dependent manner (Xu et al. 2004a). However, caution is required to generalise findings from *in vitro* observations with anthocyanins on arteries and myocardium to *in vivo* human conditions due to the low bioavailability of anthocyanins and possible additional cardiovascular effects by the anthocyanin metabolites. Increases in circulating anthocyanin metabolites were linked with a dose-dependent increase in flow-mediated dilation (FMD) up to 310 mg of blueberry anthocyanins with higher doses having no further increases (Rodriguez-Mateos et al. 2013).

However, studies that highlighted a dose-response effect of intake of berry anthocyanins on cardiovascular parameters were executed in healthy untrained subjects (Rodriguez-Mateos et al. 2013, 2016). We observed in endurance trained athletes that a daily intake of New Zealand blackcurrant powder for seven days increased stroke volume and cardiac output by 25% and 26%, respectively, and total peripheral resistance was decreased by 16% with no changes in systolic, diastolic or mean arterial blood pressure during supine rest (Willems et al. 2015). This observation was with a daily intake of 138.6 $mg \cdot day^{-1}$ of blackcurrant anthocyanins and it is not known whether there is dose-dependent effect on cardiovascular function during supine rest. The dose-dependent cardiovascular responses to berry anthocyanin intake are unknown for those regularly undertaking endurance training, which possess already cardiovascular adaptations by the endurance training (for a review see Hellsten and Nyberg 2015). It is possible that an endurance trained cardiovascular system may not clearly respond to dose effects of anthocyanin intake. We therefore hypothesized that there would be no dose-response effects

of a rich berry anthocyanin-containing extract on cardiovascular function during supine rest in trained male cyclists. The aim of the present study was to examine the dose-response effects of New Zealand blackcurrant extract on cardiovascular function at supine rest in trained male cyclists.

METHODS

Participants

Fifteen endurance trained men (age: 38±12 years, height: 178±5 cm, body mass: 76±10 kg, $\dot{V}O_{2max}$: 57±8 $mL \cdot kg^{-1} \cdot min^{-1}$, WR_{max}: 378±55 W) provided written informed consent to participate in the study. Participants were recruited from local cycling clubs with a history of cycling participation of greater than 3 years and were not involved in a structured training programme for the study duration, but typically performed cycling exercise for 6 to 10 hours a week. All participants were non-smokers and they were taking no nutritional supplements. 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 for 5 visits at the same time of day (8:00am). Before arrival, participants were instructed to abstain from vigorous exercise for 48 hours, alcohol for 24 hours and caffeine-containing products on the day of testing. Before commencing data collection on that visit, participants verbally acknowledged compliance to the experimental requirements. During the first visit, stature (Seca 213, Seca, Birmingham, UK), body mass (Kern ITB, Kern, Balingen, Germany) and body fat (Tanita BC418 Segmental Body Composition analyzer, Tanita, Illinois, USA) were measured. Subsequently, participants completed an incremental intensity maximal cycling test to volitional exhaustion for calculation of maximal oxygen uptake ($\dot{V}O_{2max}$) and maximum work rate (WR_{max}; the last complete work rate, plus the fraction of time spent in the final non-completed work rate multiplied by the work rate) on an electronically controlled cycle ergometer (SRM ergometer, SRM International, Jülich Germany).

Participants were assigned, in a randomised, counterbalanced Latin-square design, to three NZBC doses (i.e. 1, 2 or 3 capsules a day) for seven-days and one control condition of no dose. The 300 mg active cassis capsules 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). Participants were instructed to take the capsules, with breakfast (one capsule per day, 300 mg·day^{-1} condition), 12 hours apart (two capsules per day, 600 mg·day^{-1} condition) and evenly spaced through the day (three capsules per day, 900 mg·day^{-1} condition). Optimal dosing duration of NZBC extract is not known. However, previous studies on the effectiveness of berry juice intake in humans also used multiple days of intake (Connolly et al. 2006; Howatson et al. 2010).

On the final day of supplementation, participants reported to the laboratory, two hours post-prandial of a standard breakfast (i.e. one slice of buttered bread or toast ~840 kJ, ~30 g carbohydrate, ~6 g protein and ~7 g fat) and all the capsules required for that condition. Between laboratory visits, there was a fourteen-day washout period. An anthocyanin intake for one month similar to highest dose in the present study returned biochemical and biomarkers of antioxidant status to baseline of after a fifteen-day washout (Alvarez-Suarez et al. 2014). The NZBC capsules were independently analysed and confirmed the ingredients present with an absence of caffeine. Participants then rested for 5 minutes in a supine position before beat-to-beat blood pressure (Portapres® Model 2, Finapres Medical Systems BV, Amsterdam, The Netherlands) was recorded for 20-minutes during supine rest (see below). Cardiovascular responses in rest are affected by posture position (Nishiyasu et al. 1998).

Anthocyanin Consumption, Physical Activity and Dietary Standardization

Participants completed a food frequency questionnaire that listed the amount and frequency of anthocyanin containing foods and drinks compiled from the Phenol Explorer database (Neveu et al. 2010). Daily anthocyanin intake was calculated as the sum of consumption frequency of each food multiplied by the anthocyanin content for the portion size. Daily intake of anthocyanins was calculated to be 67±47 mg·day^{-1}.

Participants were instructed to keep their weekly exercise schedule as consistent as possible. All participants recorded their dietary intake and exercise on a written diary 48 hours prior to the first experimental 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 and exercise for that visit. Food diaries were analysed using Nutritics (Nutritics LTD, Dublin, Ireland) for carbohydrate, fat and protein intake and total energy intake (kJ).

There were no differences (P>0.05) in absolute or relative per kilogram of body mass values for carbohydrate, fat, protein, or total energy for 48 hours prior to each experimental visit (Table 1).

Analysis of the food diaries identified that all participants reported 100% adherence to the dietary instructions 48 hours prior to each visit.

Maximal Rate of Oxygen Uptake

$\dot{V}O_{2max}$ and WR_{max} were determined with 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}. Expired air samples were collected using the Douglas bag technique with separate air samples collected for a minimum of 3-minutes before participants reached volitional exhaustion. Expired air was analysed with a three-pointed calibrated gas analyser (Series 1400, Servomex, Crowborough, UK), and volume measured (Harvward Apparatus Ltd., Edenbridge, UK). Gas volumes were calculated using Haldane transformation and standardisation to STPD conditions, with consideration of inspired fraction of oxygen and carbon dioxide measured within the laboratory during the protocol. $\dot{V}O_{2max}$ and WR_{max} were measured in visit 1.

Cardiovascular Function Measurements

Cardiovascular responses were recorded using a beat-to-beat blood pressure monitoring system during 20 minutes of rest in a supine position using the arterial volume clamp method (Penaz 1973). The Portapres® is a beat-to-beat finger blood pressure analyser that allows the non-invasive continuous measurement of haemodynamic parameters. The cardiac output calculated by the Portapres has shown to be significantly correlated (r=0.87, P<0.01) with cardiac output measurements by ultrasound Doppler from rest up to 130% of the ventilatory threshold during semi-supine cycling (Sugawara et al. 2003). The finger cuff was positioned around the same finger of the left hand. Cardiovascular measures were averaged over 10 consecutive beats, with the lowest systolic blood pressure and associated measures recorded. Systolic blood pressure, diastolic blood pressure, mean arterial blood pressure, heart rate, ejection time, cardiac output, stroke volume and total peripheral resistance were recorded (Beatscope 1.1a., Finapres Medical Systems BV, Amsterdam, The Netherlands).

Statistical Analysis

An a-priori power analysis indicated a sample size of 15 would allow a detection of a 26% increase in cardiac output with a high statistical power ($1 - \beta = 0.95$: $0.05 = \alpha$ level). Statistical analyses were completed using SPSS 20.0 (SPSS, Chicago, USA). Differences between the dosing conditions (0 vs. 300 vs. 600 vs. 900 mg·day^{-1}) were analysed with a one-way within subjects analysis of variance

(ANOVA) with between dose condition difference examined with a paired samples *t*-test. Mauchley's Test of Sphericity was conducted to test for homogeneity of data and where violations were present Greenhouse-Geisser adjustments were made. To determine the effect size of responses, Cohen's *d* were calculated with Cohen (1988) describing an effect size of <0.2 as trivial, 0.2-0.39 as a small, 0.4-0.69 as a moderate and ≥0.7 as a large magnitude of change. Statistical significance was accepted at $P<0.05$. Interpretation of $0.05 \geq P \leq 0.1$ as a trend was according to guidelines by Curran-Everett and Benos (2004).

RESULTS

There were no differences between the dosing conditions for systolic blood pressure ($P=0.35$), diastolic blood pressure ($P=0.60$), heart rate ($P=0.56$) and ejection time ($P=0.52$) (Figures 1 a, b, c and d, respectively). There was a dose effect of NZBC on mean arterial pressure ($P=0.023$), cardiac output ($P<0.001$), stroke volume ($P=0.014$) and total peripheral resistance ($P=0.012$) (Figures 1 e, f, g and h, respectively).

Mean arterial pressure (Fig. 1e) exhibited a decrease of 7±9 mmHg (8%, 11 of 15 participants decreased, $d=0.76$) between 0 and 600 mg·day^{-1} and 5±7 mmHg (6%, 14 of 15 participants decreased, $d=0.69$) between 300 and 900 mg·day^{-1} ($P<0.05$). There was a trend for a lower mean arterial pressure of 5±11 mmHg (6%) ($P=0.1$) between 0 and 900 mg·day^{-1} and 7±12 mmHg (7%) ($P=0.05$) between 300 and 600 mg·day^{-1}. NZBC increased cardiac output by 0.6±0.6 L·min^{-1} (15%, 14 of 15 participants increased, $d=0.93$), 1.0±1.0 L·min^{-1} (28%, 11 of 15 participants increased, $d=0.94$) and 0.6±0.9 L·min^{-1} (15%, 13 of 15 participants increased, $d=0.67$) between 0 and 600 mg·day^{-1}, 0 and 900 mg·day^{-1} and 300 and 900 mg·day^{-1} (all $P<0.05$), respectively (Fig. 1f). Between 0 and 600 mg·day^{-1} and 0 and 900 mg·day^{-1}, stroke volume (Fig. 1g) increased by 5±8 mL (7%, 13 of 15 participants increased, $d=0.70$) and 6±17 mL (18%, 13 of 15 participants increased, $d=0.95$), respectively. For total peripheral resistance (Fig. 1h), a decrease of 4±3 mmHg·L^{-1}·min^{-1} (20%, 13 of 15 participants decreased, $d=1.29$), 5±9 mmHg·L^{-1}·min^{-1} (20%, 13 of 15 participants decreased, $d=0.60$) and 3±4 mmHg·L^{-1}·min^{-1} (15%, 11 of 15 participants, $d= 0.78$) was observed between 0 and 600 mg·day^{-1}, 0 and 900 mg·day^{-1} and 300 and 900 mg·day^{-1} ($P<0.05$), respectively.

DISCUSSION

This is the first study to examine the dose-response effects of NZBC extract on cardiovascular function during supine rest in trained male cyclists. The principle finding from the present study was that NZBC extract increased cardiac output and stroke volume, and decreased total peripheral resistance in a dose-dependent manner in endurance trained male cyclists, with changes having moderate and large effect sizes. There was a trend for a dose effect for mean arterial blood pressure.

Willems et al. (2015) also observed no changes in systolic or diastolic blood pressure and heart rate following seven-days intake of NZBC powder in trained male and female triathletes. However, increases in cardiac output by 25%, stroke volume by 26%, and a decrease in total peripheral resistance by 16% were observed (Willems et al. 2015). The present study observed similar group mean increases, but following a dose almost three times that of Willems et al (2015) (~139 vs ~315 mg·day^{-1} anthocyanin). This may have resulted from the different way in which NZBC was delivered. Willems et al (2015) used NZBC powder dissolved in water while the present study used capsulated NZBC extract which may affect absorption rate of anthocyanin and also bypasses the potentially degrading properties of saliva (Kamonpatana et al. 2012). Additionally, Willems et al (2015) observed no change in mean arterial pressure, whereas in this study differences between 0 and 600 and 900 mg·day^{-1} were observed with large and moderate effect sizes, respectively. This indicates that higher intakes of anthocyanins are associated with reduced mean arterial pressure (Jennings et al. 2012).

The dose-dependent cardiovascular function responses during supine rest in endurance trained individuals in the present study support those studies examining the dose-response relationships of anthocyanin on FMD in healthy untrained individuals. For example, Rodriguez-Mateos et al (2013) reported a dose-dependent increase in FMD up to 310 mg anthocyanin, and then a plateau above this dose. The present study observed no significant increases between 600 and 900 mg·day^{-1} NZBC (210 and 315 mg·day^{-1} anthocyanin, respectively) on any cardiovascular parameter, indicating a levelling off in cardiovascular responses during supine rest with a dose of 600 mg·day^{-1} NZBC extract. However, the responses above 900 mg·day^{-1} NZBC extract are unknown. It is possible, however, that a plateau on cardiovascular function exists in a similar fashion to the results of the study by Rodriguez-Mateos et al (2013), as uptake of higher intakes of NZBC extract may be limited by mechanisms for anthocyanin absorption (Kurilich et al. 2005).

Upon ingestion, anthocyanins have poor bioavailability (Czank et al. 2013). Their uptake is affected by gut microflora [for review see Kemperman et al. (2010)], with inter-individual variations in gene

content of gut bacterial species of 13% observed (Zhu et al. 2015). Furthermore, George et al (2012) observed that expression of the Glu298Asp single nucleotide polymorphism in the endothelial nitric oxide synthase gene differentially affects the endothelium-dependent vasodilation response to a fruit and vegetable puree drink. Taken together, such factors may explain the inter-individual variation for NZBC extract on cardiovascular function responses during supine rest.

Blackcurrant anthocyanins are quickly absorbed and excreted with values reaching maximum plasma concentrations within 2 hours (Matsumoto et al. 2001). Therefore, metabolites of anthocyanins, or synergistic action of metabolites, could lead to the cardiovascular responses during supine rest. In addition, metabolites have been shown to remain within the plasma for 48 hours following intake (Czank et al. 2013). Therefore, a build-up of metabolites over the 7-day supplementation period within the present study and effects of the metabolites may have caused the altered cardiovascular function during supine rest. However, we cannot exclude that the cardiovascular responses during supine rest in the present study may have been caused by acute responses to the anthocyanin intake as measurements were taken 2 hours after intake. In both Willems et al. (2015) and the present study, the last intake across the seven days was taken 2 hours before the recording of cardiovascular function during supine rest. This is supported by observations that increases in FMD have occurred 1-2 hours following an intake of blueberry polyphenols and coincides 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), but anthocyanin composition of blueberries differ from blackcurrant with potential consequences for the occurrence of plasma metabolites. Similarly, Kent et al. (2016) observed that a single serving of cherry juice (~207 mg anthocyanins) reduced systolic and diastolic blood pressure and heart rate 2 hours following intake and this coincided with a peak in caffeic acid. Therefore, future studies should examine the acute responses for cardiovascular function during supine rest to NZBC extract intake with measurement of phenolic metabolites. It is possible that these phenolic metabolites maybe responsible for the possible mechanisms for the observed effect in the present study. For example, they have been observed to influence human vascular smooth muscle cell behaviour *in vitro* (Keane et al. 2016a) and may also increase nitric oxide availability, as shown by inhibiting NAPH oxidase (Rodriguez-Mateos et al. 2013) and increasing endothelial nitric oxide synthase expression (Xu et al. 2004b). While these effects upon expression and activity of nitric oxide would potentially result in vascular responses, Keane et al. (2016b) observed plasma nitrite and nitrate

(surrogate markers for nitric oxide production) to be unaffected by cherry anthocyanins. Therefore, the effects of anthocyanin metabolites on vascular smooth cell behaviour seems the most likely mechanism for the cardiovascular responses, which lead to a decrease in total peripheral resistance and mean arterial pressure in the present study. Whilst indirect, the decrease in total peripheral resistance also suggests an increased peripheral blood flow during supine rest as changes in arterial diameter influence blood flow (Mayet and Hughes 2003), an observation which has been previously been made following intake of blackcurrant anthocyanins (Matsumoto et al. 2005). However, the combination of decreased total peripheral resistance and mean arterial pressure with increased cardiac output and stroke volume with no change in heart rate and systolic or diastolic blood pressure suggests more complex mechanisms. For example, an elevation of mean arterial pressure can only result from an increase in cardiac output, an increase in total peripheral resistance, or both (Mayet and Hughes 2003). However, a decreased mean arterial pressure and total peripheral resistance as in this study indicates greater venous return resulting in the increased cardiac output from a larger end diastolic filling during the cardiac cycle.

Limitations

For the present study, various limitations should be considered. Firstly, the short time frame of the present study does not indicate benefits for longer-term consumption and cardiovascular health. Secondly, the study population consisted of healthy men who regularly participate in cycling exercise and observations cannot be extended to the general population, and further work is required to identify whether similar cardiovascular responses would occur in women, untrained populations and those with cardiovascular disease conditions. However, future work should examine the potential consequences of increased cardiac output in rest on cardiomyocyte oxygen consumption. Thirdly, the present study supplemented with capsules of NZBC extract. Therefore, these results are limited to this delivery mechanism and it is unknown if similar responses are observed from whole unprocessed blackcurrant intake. Finally, in present study, dietary intake was controlled for 48 hours before each visit, with no differences observed, but the total polyphenol intake was not measured. Therefore, we cannot exclude that the intake of dietary polyphenols including anthocyanins acted synergistically with the NZBC anthocyanin intake in the present study.

Conclusion

In conclusion, New Zealand blackcurrant extract taken in capsules for seven-days increased cardiac output and stroke volume, and decreased mean arterial pressure and total peripheral resistance during supine rest in a dose-dependent manner up to a daily intake of 900 $mg \cdot day^{-1}$ (315 $mg \cdot day^{-1}$ anthocyanin) in endurance trained male cyclists. While anthocyanins have been shown to influence cardiovascular responses in diseased and untrained populations, these findings indicate that anthocyanins also alter cardiovascular function during supine rest in endurance trained cyclists in a dose-dependent manner. In a previous study with the lowest dose of New Zealand blackcurrant as used in the present study, we did not observe differences in cardiovascular responses between 40% and 80% of maximum power (Willem et al. 2015). Future work should examine whether higher doses of New Zealand blackcurrant affects the cardiovascular responses during exercise.

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Conflict of Interest

The authors declare no other conflicts of interest.

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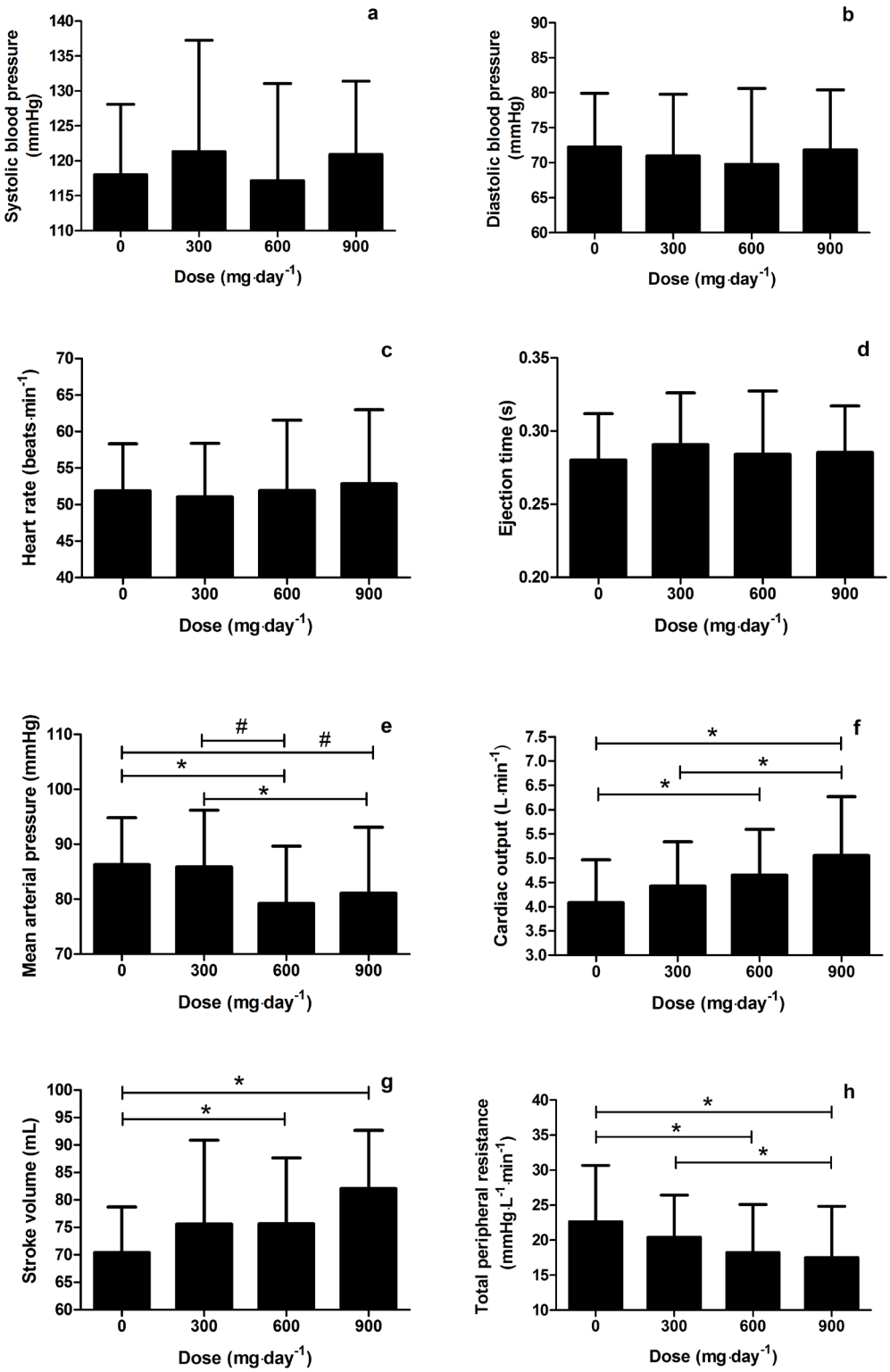

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420 **Fig. 1**. a: Systolic blood pressure, b: Diastolic blood pressure, c: Heart rate, d: Ejection time, e: Mean

421 arterial pressure, f: Cardiac output, g: Stroke volume, h: Total peripheral resistance during supine rest

422 following 0 or 300, 600 and 900 mg·day^{-1} of New Zealand blackcurrant extract in 15 endurance trained
423 male cyclists. Data are mean±SD. * indicates difference between doses (P<0.05), # indicates a trend
424 between doses

425

426 Table 1. Dietary intake 48 hours before each visit for each treatment condition.

	0 mg·day^{-1}	300 mg·day^{-1}	600 mg·day^{-1}	900 mg·day^{-1}
Carbohydrate (g)	494±91	495±90	479±85	490±101
(g·kg body mass^{-1})	6.7±1.8	6.7±1.7	6.5±1.6	6.6±1.9
Fats (g)	228±68	228±68	230±65	235±73
(g·kg body mass^{-1})	3.1±1.0	3.1±0.9	3.1±0.9	3.1±1.0
Protein (g)	216±59	221±58	217±56	220±60
(g·kg body mass^{-1})	2.9±0.9	3±0.9	2.9±0.8	3.0±0.9
Total Energy Intake (kJ)	20654±2950	20804±3080	20724±2805	20709±2835
(kJ·body mass^{-1})	279±63	280±59	279±56	278±54

427 Intake of dietary variables for the different NZBC dosing conditions of 0, 300, 600 and 900 mg·day^{-1}.
428 All values were collected from 48-hour food diaries before each experimental visit. Data reported as
429 mean ± SD from 15 endurance trained male cyclists.
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