The effect of acute dark chocolate consumption on carbohydrate metabolism and performance during rest and exercise

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Abstract

Consumption of cocoa-enriched dark chocolate (DC) has been shown to alter glucose and insulin concentration during rest and exercise compared to an iso-caloric/carbohydrate (CHO) cocoa-depleted control (CON). However, the impact of DC consumption on exercise metabolism and performance is uncertain. Therefore the current aim was to investigate CHO metabolism via stable isotope tracer techniques during exercise after subjects ingested either DC or CON. Sixteen overnight-fasted male cyclists (30 ± 6 yrs; 72.8 ± 6.0 kg, 56.3 ± 5.7 ml/kg/min VO_2max) performed two trials in a single blinded, randomized, cross-over design, after consuming either DC (561 kcal; 33.3 g CHO; 11.4 g cocoa) or CON (544 kcal; 27.5 g CHO; <0.5 g cocoa) 2 h prior to 2.5 h of steady-state (SS) exercise (~45% VO_{2peak}). This was followed by a ~15 min time-trial (TT) and 60 min of recovery. [6,6-$^2H_2$]Glucose and [U-^{13}C]glucose were continuously infused during SS to assess glucose rate of appearance (R_a) and disappearance (R_d). After DC consumption, plasma (-)-epicatechin, theobromine, glucose and insulin concentrations were significantly ($p<0.001$) elevated throughout vs. CON. During SS, there was no difference in [6,6-$^2H_2$]glucose R_a between treatments, but towards the end of SS (last 60 min) there was a ~16% decrease in R_d in DC vs. CON ($p<0.05$). Accordingly, after DC there was a ~18% significant decrease in plasma glucose oxidation (trial effect; $p=0.032$), and a ~15% increase in tracer derived muscle glycogen utilization ($p=0.045$) late during SS exercise. This was without effect on TT performance. The higher blood glucose concentrations during exercise and recovery after DC consumption coincided with high concentrations of polyphenol and/or methylxanthine compounds but not caffeine. In summary, DC consumption altered muscle CHO partitioning, between muscle glucose uptake and glycogen oxidation, but this was without any effect on cycling TT performance.

Key words: chocolate, cocoa extract, exercise, time-trial, blood glucose

Introduction

As early as 1552, chocolate and/or cocoa have been consumed as a medicinal and cultural treatment for fatigue in Mexican Mayan and Aztec cultures (Dillinger et al. 2000). However, very few studies have examined metabolic and anti-fatigue effects of dark chocolate. Recent studies have shown that 100 g of dark chocolate increased the concentration of plasma glucose (Davison et al. 2011), and chronic dark chocolate consumption caused a ~21% increase in plasma free-fatty acids (Allgrove et al. 2011), during prolonged exercise compared to a iso-fat/calorie/carbohydrate (CHO) control. But, these studies did not examine mechanisms associated with these shifts in metabolism during exercise, nor performance outcomes.

Beyond CHO and lipids, dark chocolate also contains cocoa derived phytochemicals, among which are many potentially bioactive compounds (Bruinsma et al. 1999). These compounds intrinsic to cocoa include the methylxanthines such as theobromine and caffeine, as well as polyphenols, of which (-)-epicatechin is the most abundant (Schroeter et al. 2006). Methylxanthines (primarily caffeine) have consistently been shown to increase exercise capacity and performance (Burke 2008; Graham et al. 2008)). However, the effects of methylxanthines (mainly caffeine) on glucose disposal (glucose uptake, rate of disappearance (R_d) or glucose oxidation) have been inconsistent. Most studies showed a decrease (Graham, Battram et al. 2008), while others showed no change (Roy et al. 2001; Hulston et al. 2008) or increase in glucose oxidation during exercise (Yeo et al. 2005). Another methylxanthine (theophylline) has also shown a significant decrease in glucose R_d during exercise (Raguso et al. 1996). These results suggest that component(s) within dark chocolate could have a potential impact on fatigue and performance.

The causes of fatigue and limits to performance during prolonged endurance exercise are multi-factorial in nature. However, one mechanism for prolonged exercise performance appears to be the maintenance of blood glucose levels (for review see: (Jeukendrup 2004)). The importance of euglycemic blood glucose concentrations for enhanced performance during prolonged exercise, together with the recent findings of a better maintenance of

plasma glucose with dark chocolate consumption during prolonged exercise (Davison, Callister et al. 2011), which could provide substrate to maintain carbohydrate oxidation, provided the rationale for the current study. Our primary aim was to characterize glucose turnover using continuous [6,6-$^{2}H_{2}$]glucose and [U-^{13}C]glucose dual-tracer infusion with breath and blood measurements to demonstrate the mechanisms by which cocoa-rich dark chocolate consumption results in associated increases in blood glucose during steady-state exercise and recovery. Accordingly, we assessed glucose rate of appearance, disappearance, oxidation and determined indirect measurements of gluconeogenesis and glycogen utilization throughout the entire steady-state exercise period. Secondly, we incorporated a cycling time-trial to ascertain whether a potentially enhanced maintenance of blood glucose after DC consumption would be associated with greater exercise performance.

Materials and Methods

Volunteers

Sixteen healthy male cyclists (4-20 h/ week of training) participated in this study and are characterized in Table 1. Volunteers were excluded if having a nut allergy, smokers or on regular medication and were informed verbally and in writing about the nature and potential risks of the experimental procedures before written informed consent was obtained. The study was approved by the ethics committee of the University of Lausanne, Switzerland. The clinical trial was conducted following the principles of International Conference on Harmonization guideline for Good Clinical Practice (GCP). This study was conducted from March 2008 until January 2010.

General study design and treatment composition

After pre-experimental testing, each volunteer performed two trials separated by at least one week in a single blinded, randomized, cross-over design. In each trial, volunteers either consumed 561 kcal of dark chocolate (DC; Nestlé Noir Intense- 70% Cocoa) or closely matched CHO, fat and calorie control chocolate (CON; Table 2) 2 h prior to exercise. Volunteers then cycled for 2.5 h at ~45% VO_{2peak} in steady-state conditions (SS), followed by a ~15 min time-trial (TT; Fig. 1). Recovery measurements were then made as subjects rested over another 60 min (RECOV). To characterize whole-body and glucose metabolism, frequent breath and blood (n=17) measurements were made and [6,6-$^{2}H_{2}$]glucose and [U-^{13}C]glucose tracers were infused prior to SS and TT cycling, as well as during RECOV.

Pre-experimental trial testing

At least 10 days prior to the first experimental trial, volunteers performed a continuous incremental cycling test on a cycle ergometer (Excalibur-Lode, Groningen, The Netherlands) to exhaustion to determine peak oxygen uptake (VO_{2peak}; VMax 29C, SensorMedics, Bilthoven, The Netherlands) and maximal workload capacity (W_{max}; Table 1). On the first visit, the cycle ergometer was individually adjusted for each subject and was set accordingly for all subsequent tests. Heart rate (HR) was monitored every 5 sec by near infrared

telemetry (Polar NV, Polar Electro, Kempele, Finland). The VO_{2peak} test started at 100W for 5min, followed by 50W increases every 2.5 min until HR>160 beats/min. Subjects then increased 25W every 2.5 min until exhaustion (RQ>1.1; HR max; plateau and/or exhaustion). W_{max} was then calculated as $W_{max} = W_{out} + (t/150) * 25$, where W_{out} equaled the last workload (wattage attained), and t was the time (sec) at the last workload (Jeukendrup et al. 1996).

At least 7 days prior to the first experimental session, the subjects underwent a full familiarization trial for the 2.5h exercise protocol, which confirmed the power output of ~45% VO_{2peak} required for each subject and TT familiarization. Previous investigations have shown that serial TTs, without distance or time feedback, result in progressively improved performances, being nearly optimal by the second or third blinded TT (Mauger et al. 2009). Thus, a practice familiarization TT was implemented to ensure that subjects had learned the best possible pacing strategy prior to the experimental trials so that any differences would be due to the experimental conditions.

Activity and diet prior to experimental trials

All subjects were instructed to maintain their usual physical activity patterns throughout the experimental period. However, for the 48 h prior to all pre-testing and experimental trials subjects abstained from any heavy exercise. Starting one week prior to the two experimental trials, subjects were placed on dietary restrictions including no alcohol, corn starch, colas or high caffeine/ polyphenol containing foods or beverages. Dietary instructions and food lists restricted products with a high natural abundance of ^{13}C-carbohydrates (e.g. C_4 plants such as maize or sugar cane) to reduce ^{13}C background shift from endogenous substrate stores to allow for better accuracy of the [U-^{13}C]glucose tracer. Volunteers were instructed to document all food intake and exercise for the 48 h prior to the first full experimental trial, and this diet was then duplicated prior to the second experimental session, of which 100% compliance was reported. Only water was allowed from 9 pm the night before each trial.

Experimental trials

Figure 1 provides a schematic drawing of the entire experimental protocol. Volunteers arrived overnight fasted at 8:00am, weighed, and completed a 24 h food/ beverage questionnaire to confirm compliance to the dietary controls. Teflon catheters (B/BRAUN 18 gauge, Melsungen A.G.) were then inserted into each antecubital vein for blood sample drawing and for subsequent infusion of labeled glucose at the beginning of exercise (see *Tracer infusion* section below for full details). Thereafter, the first resting blood sample was taken and expired breath sample was collected into a vacutainer tube. The subject then received the randomized treatment of either DC or CON to consume within a 10min period. Although the researchers and subjects were blinded to the identity of the treatment, the treatments were not visually identical. Upon completion of the treatment, another blood and breath sample was taken (time point = 0min), and additional resting state blood and breath samples were taken at 15, 30, 45, 60, 90 and 120min after the time 0. The tracer infusion was started at 90min after the treatment, which was 30min prior to the commencement of exercise with bolus prime, followed by a 4.5 h continuous infusion of [6,6-$^{2}H_{2}$]glucose and [U-^{13}C]glucose throughout exercise (SS and TT) and the recovery period. During the 2.5 h SS cycling segment the power output on the cycle ergometer was manually set to 142.0±15.9 W to elicit 43.5±4.5% VO_{2peak} so that the relative fuel utilization and metabolism of each subject could be assessed. Within 5 min after the completion of the 2.5 h SS portion of the protocol the cycling ergometer was switched from manual to linear mode so the subject controlled power output by varying the pedal cadence for the TT according to the formula $W = L \times (RPM)^2$. The L (linear alpha factor) was based on the individual subjects W_{max} and preferred cycling cadence during the VO_{2peak} test. As described previously (Jeukendrup, Saris et al. 1996), the individual amount of work (kJ) to complete was based on the individual subjects 90% W_{max}, with a required TT duration of ~10-12min, according to the formula:

$$\text{Required amount of work (kJ)} = (0.90 \times W_{max}\ (W) \times 600\ (sec)) / 1000$$

The aim of the TT test was for subjects to complete a set amount of work (203±15 kJ) as quickly as possible. During the TT the only information the subject was given was the amount of work done and left to complete by a moving bar graph on a screen in front of him.

No other objective external cues were available and no encouragement was given. One volunteer could not finish one of the TT's, and his data have been omitted. Immediately after the TT, recovery measurements were then made as subjects rested over another 60 min (RECOV). Continuous blood and breath samples (n=17) were taken every 15-20 min throughout the entire SS, TT and RECOV periods (see Fig 1; Blood and breath analysis below).

Tracer infusion

Initially, volunteers received a 5 mL intravenous bolus prime of $NaH^{13}CO_3$ (165.04 µmol/mL; Eurisotope, France), [6,6-$^2H_2$]glucose (421 µmol/mL) and [U-^{13}C]glucose (57.7 µmol/mL) within 2 min. Thereafter, a continuous infusion of sterile pyrogen free dual-labeled tracer solution containing [6,6-$^2H_2$]glucose and [U-^{13}C]glucose was delivered via a calibrated ARGUS 707V pump (Codan Medical AG, Baar, Switzerland) at the rates of 2.240±0.168 and 0.032±0.002 µmol/kg/min, respectively. Measured glucose concentrations in the infusates averaged 35.15 and 4.78 µmol/ml for the [6,6-$^2H_2$]glucose and [U-^{13}C]glucose, respectively.

Blood and breath analysis

Fourteen blood samples of 12 mL each (taken at -10, 15, 30, 45, 60, and then 90, 120, 140, 160, 180, 210, 240, 300 and 360 min) and 3 drawings of 7 mL (taken at 0, 270 and 330 min) went to immediate separation for plasma. Then, aliquots of 0.5 mL were taken and stored. One aliquot was acidified for polyphenol analysis. Additionally, ten breath samples were taken at 120, 140, 160, 180, 210, 240, 270, 300, 330 and 360 min.

Breath samples were analyzed for $^{13}C/^{12}C$ ratio by gas chromatography continuous flow isotope ratio mass spectrometer (GC-IRMS, Thermo Fischer, Bremen, Germany). The reference gas used was normalized with calibration gases of known isotopic ratio (Eurisotope, France).

Isotopic enrichment of deuterated plasma glucose was measured by gas chromatography-mass spectrometry (GC-MS) (Agilent, Santa Clara, USA). The analysis of the glucose penta-acetate derivative was performed using chemical ionization mode with methane gas with selective monitoring at mass-to-charge ratios (*m/z*) 331 and 333. Briefly, 100 µL of plasma was deproteinized using 0.3 N barium hydroxide and 0.3 N zinc sulphate. After centrifugation, the supernatant was purified on ion exchange resins (AG50W-X8 and AG 1-X8). To determine deuterated glucose isotopic enrichment (tracer over tracee ratio (TTR)) by GC-MS, half of the supernatant was dried under nitrogen gas and derivatized with a freshly prepared solution of acetic anhydride/pyridine (Wolfe et al. 2005). The remainder of the supernatant was diluted with water and filtered with a 0.2 µm filter (Waters, New Milford, MA, USA) and directly injected into an LC-IRMS to measure $^{13}C/^{12}C$ isotopic enrichment of plasma glucose (Godin et al. 2011).

Aliquots of plasma samples were used for glucose, insulin, glucagon, triglyceride (TG), free-fatty acid (FFA), noradrenaline, adrenaline, flavanols and methylxanthines quantification. Glucose and TG were assessed enzymatically by a Siemens Dimension clinical chemistry system (Siemens Healthcare Diagnostics Inc, Newark, USA), with D40 Dimension and DF69A Dimension clinical chemistry kits for glucose and TG, respectively. Glucagon and insulin were quantified by an immunoassay ELISA test kit (Roche Diagnostics, Germany) and RIA test kit (Euro-Diagnostica, Sweden), respectively. FFA was determined via an enzymatic colorimetric method assay (Wako Chemicals, GmbH). Noradrenaline, adrenaline, flavanols and methylxanthines (theobromine and caffeine) were assessed by reverse phase HPLC using previously published methods (Georgia et al. 2001; Westermann et al. 2002; Ritter et al. 2010).

Calculations

VO_2 and VCO_2 were recorded on a breath by breath basis over a 5 to 8 min period prior to sampling time, and from these respiratory measures, total fat and carbohydrate oxidation rates were calculated using the non-protein respiratory quotient (Peronnet et al. 1991).

Fat oxidation rate = 1.695 VO_2 – 3.22 VCO_2 *(Eq.1)*

Carbohydrate oxidation rate = 4.585 VCO_2 – 3.22 VO_2 *(Eq.2)*

VO_2 and VCO_2 are reported in liters per minute (L/min) and oxidation rates in grams/minute (g/min).

The $^{13}C/^{12}C$ isotopic ratio is expressed as $\delta^{13}C$ values calibrated against an international standard (Vienna Pee Dee Belemnite, VPDB; (Slater et al. 2001)). The delta notation is defined as $\delta^{13}C/^{12}C_{sample}$, ‰ = ($R_s$ / R_{st} - 1) × 1000, where R_s is the ratio of $^{13}C/^{12}C$ in the sample and R_{st} is the ratio of ratio of the international standard used.

The $\delta\ ^{13}C/^{12}C$ value was transformed into TTR according to *equation 3*.

$$TTR = R_{istd}\left(\delta_{sample} - \delta_{blank}\right) \times [(1 - A^n)/1000] \quad \textit{(Eq. 3)}$$

Where R_{istd} is 0.011237, δ_{sample} and δ_{blank} are $\delta\ ^{13}C/^{12}C$ of sample after infusion of tracer and $\delta^{13}C$ of sample (at natural abundance) before the infusion of the tracer, respectively. ‘A’ is the skew factor (equivalent to “1-R_{istd}”) and “n” is the total number of labeled atoms (n=6, for [U-^{13}C]glucose).

The total glucose rate of appearance of glucose (R_a, *Eq. 4*), rate of disappearance of glucose (R_d, *Eq. 5*), glucose clearance rate *(Eq. 6)*, gluconeogenesis (GNG; or glucose carbon recycling, *Eq. 7*), plasma glucose oxidation (*Eq.8*), fat and total glucose oxidation and muscle glycogen oxidation (*Eq. 9*) were calculated using the single pool non steady state model of Steele (Wolfe and Chinkes 2005) and as described by Jeukendrup *et al.* (Jeukendrup et al. 1999).

$$\text{Total Ra} = \frac{\text{F} - \text{V}\left[\frac{\text{C2}+\text{C1}}{2}\right]\left[\frac{\text{E2}-\text{E1}}{\text{t2}-\text{t1}}\right]}{\frac{\text{E2}+\text{E1}}{2}} \quad \textit{(Eq. 4)}$$

$$\text{Total Rd} = \text{Ra x V}\left[\frac{\text{C2}-\text{C1}}{\text{t2}-\text{t1}}\right] \quad \textit{(Eq. 5)}$$

Glucose clearance rate = Rd / ((C2 + C1)/2) *(Eq. 6)*

Where F is the infusion rate (µmol/kg/min), V is the volume of distribution (160 mL/kg), C2 and C1 are the glucose concentrations at consecutive time points (e.g. t_2 and t_1) and E2 and E1 are the plasma glucose enrichments at consecutive time points (e.g. t_2 and t_1).

GNG = R_a ([6,6-$^2H_2$]glucose) – R_a ([U-^{13}C]glucose) *(Eq. 7)*

Plasma glucose and muscle glycogen oxidation (µmol/kg/min) were calculated according to the following equations (Jeukendrup, Wagenmakers et al. 1999):

Plasma glucose oxidation = (R_d glucose) x (%glucose tracer oxidized) (*Eq. 8*)

Where % infused tracer oxidized was calculated as ($V^{13}CO_2$ x k^{-1}) / (infusion rate of [U-^{13}C]glucose) x 100, with k at 0.747. $V^{13}CO_2$ (µmol/kg/min) was calculated as $\Delta^{13}CO_2$ (isotopic enrichment of breath CO_2 at a given time corrected by CO_2 at natural abundance as background without any tracer) multiplied by VCO_2.

Muscle glycogen oxidation = Total CHO oxidation – plasma glucose oxidation *(Eq. 9)*

Statistics

A mixed-model repeated measurement analysis was performed with F-tests on the time-treatment interactions. A mixed model allowed for correcting for time period residuals as well as any missing data points. When data was not normally distributed, log-transformation was performed, and if still not normally distributed non-parametric tests were applied. Treatment differences at each time point were calculated according to Hodges-Lehmann and a Wilcoxon sign-rank test was performed. The area-under-the-curve (AUC) for the different outcomes was calculated using Kinetica software (Thermo Fisher Scientific Inc., MA, USA) and analyzed via paired t-tests. The statistical analysis was performed with SAS 9.1. (SAS Institute, Cary, NC, USA). All data are reported as mean ± standard error measurement (SEM).

Results

Plasma metabolite, hormone, (-)-epicatechin and theobromine concentrations

Plasma glucose, insulin, glucagon and triglycerides are shown in figure 2. Time and treatment interaction was observed ($p<0.0001$), indicating that blood glucose post DC treatment was greater than CON (Fig. 2A). The glucose area under the curve (gAUC) was significantly greater during the SS, TT and RECOV periods after consuming DC vs. CON (Fig. 2B). Accordingly, over the entire measurement period, DC had a 5% greater gAUC compared to CON ($p=0.0045$). DC led to higher insulin levels during the pre-exercise rest than CON (Fig 2C). The greatest differences between DC and CON insulin concentrations were observed during the later stages of the SS period. Insulin calculated AUC was higher ($p<0.005$) after DC compared to CON during the RECOV period (Fig. 2D). Conversely, plasma glucagon was lower after DC compared to CON ($p<0.0001$; Fig. 2E), with the greatest difference between treatments at the end of the SS phase (Fig. 2F). Despite the similar fat content in the chocolate samples (42.7 and 47.6g for DC and CON, respectively) a significantly greater amount of TG over time was demonstrated with DC compared to CON (Fig. 2G, $p<0.0001$), with significantly higher TG values for DC vs. CON from 90 to 180 min after consumption (end of the resting and commencement of SS cycling phase). However, there were no differences found between treatments for plasma FFA throughout rest, SS, TT or recovery time periods (*data not shown*). At rest, plasma FFA was 140±40 μmol/L for both treatments. Exercise caused a 5-fold increase in FFA, with peak values found at the end of the SS exercise period (790±101 μmol/L). Plasma adrenaline and noradrenaline values were also assessed during the entire study. Adrenaline values did not show any difference between treatments ($p=0.98$). Conversely, noradrenaline plasma levels showed a small, but significant, elevation in the CON trial vs. DC at the end of the time trial ($P<0.01$; *data not shown*). Plasma theobromine, caffeine, and (-)-epicatechin metabolites were analyzed (Fig. 3). Only data from the DC treatment are plotted, as the CON chocolate contained only trace levels of theobromine and flavanols (Table 2). After DC consumption, plasma theobromine concentrations peaked at 180 min, and by the start of the TT phase theobromine concentration was still within ~90% of the peak value, whereas plasma caffeine

concentrations remained nearly at the baseline level (Fig. 3A). Plasma concentrations of (-)-epicatechin metabolites also peaked at 190 min (Fig. 3B). By the start of the TT phase, all flavanol metabolites had appreciably decreased to ~65% of peak concentrations, declining further during RECOV.

Plasma tracer kinetics in the SS phase

The trial effect of DC on glucose rate of appearance (R_a) was not different between treatments whether measured via [6,6-$^2H_2$]glucose (p=0.263) or [U-^{13}C]glucose (Table 3), but the calculated glucose rate of disappearance (R_d) values for DC showed a ~16% reduction late during SS exercise (last 60 min) compared to CON with both [6,6-$^2H_2$]glucose and [U-^{13}C]glucose tracers (p<0.05; Table 3). Accordingly, calculated plasma glucose oxidation was significantly reduced by ~18% during last 60min of SS after DC compared to CON (trial effect; p=0.032; Table 3 & Fig. 4A), with a time and treatment interaction at 240 min (p=0.013). This was also reflected by significantly reduced calculated glucose clearance rate in the last hour of SS exercise (p<0.05; Figure 4C). However, there was no significant difference in [6,6-$^2H_2$]glucose R_a-R_d AUC during SS exercise between treatments (p=0.71). There was a compensatory ~15% increase in calculated muscle glycogen oxidation after DC consumption versus CON (trial effect p=0.045; Fig. 4B). The difference between the glucose R_a between the two tracers ([6,6-$^2H_2$]glucose and [U-^{13}C]glucose) can assess the glucose recycling rate and can be used as an indirect estimation of gluconeogensis (Jeukendrup, Wagenmakers et al. 1999). The values obtained for R_a via [6,6-$^2H_2$]glucose and [U^{13}-C]glucose were not significantly different (Table 3), thus glucose recycling, or estimated gluconeogensis, during the SS portion of exercise was minimal (p=0.72).

Cardio-respiratory measures and substrate source utilization

Regardless of the treatment, there were significant and similar increases in VO_2, VCO_2, ventilation (VE), heart rate (HR) and ratings of perceived exertion (RPE) from rest to SS exercise, and again from SS to TT. The average values of VO_2 for the SS phase were 24.7±0.6 ml/Kg/min (44±4% of VO_{2peak}) and 24.3±0.6 ml/Kg/min (43±5% of VO_{2peak}) for DC

and CON, respectively. Throughout the SS exercise period, there was a progressive decrease in the respiratory exchange ratio (RER), as highlighted by increases in fat oxidation and corresponding decreases in carbohydrate oxidation (Supp. Table 1).

Cycling Time-Trial Performance and recovery period

There was no difference ($p=0.39$) in TT performance between treatments (11.5 ± 1.6 min vs. 11.6 ± 1.1 for DC and CON, respectively). Seven volunteers showed a better TT performance after DC consumption, while seven performed better after CON, and one subject showed no difference between treatments. In the recovery period, blood glucose and plasma insulin were significantly higher in the DC than in CON treatment ($p<0.05$; Fig 2A,B,C&D). DC treatment also resulted in lower plasma concentrations of glucagon vs. CON in the early phase of recovery post TT, with the difference becoming insignificant by the end of recovery.

Discussion

To our knowledge this is the first study to characterize the altered whole-body glucose metabolism associated with cocoa-rich dark chocolate (DC) consumption using stable isotope methodologies during steady-state (SS) cycling exercise. These results demonstrate that the consumption of DC augments blood glucose concentrations during low-intensity exercise compared to a nearly iso-caloric and iso-carbohydrate cocoa-deplete control treatment (CON). The increased plasma glucose coincided with high concentrations of polyphenol and methylxanthine (primarily theobromine, but not caffeine) compounds found in DC that could potentially attenuate plasma glucose uptake. However, this augmented plasma glucose concentrations with DC treatment did not impact upon cycling time-trial performance.

The primary aim of this study was to clarify potential mechanism(s) associated with augmented glucose after DC consumption as found previously (Davison, Callister et al. 2011). We also demonstrated a significant ~8% increase in blood glucose during the SS and TT phases of the protocol (Fig 2A&B) after DC consumption compared to CON despite the lower SS exercise intensity in the present study (~45% VO_{2peak} vs. ~60% VO_{2peak} (Davison, Callister et al. 2011)). To gain insight into the physiological mechanism(s) behind these altered blood glucose responses the present study incorporated continuous [6,6-$^{2}H_2$]glucose and [U-^{13}C]glucose tracers to ascertain glucose kinetics (rate of appearance, R_a; rate of disappearance, R_d) and to estimate rates of glucose clearance and gluconeogensis (Jeukendrup, Wagenmakers et al. 1999; Wolfe and Chinkes 2005). There are two possible mechanisms that are potentially responsible for the increased plasma glucose during the SS phase: 1) an increase in the glucose R_a via exogenous (oral consumption) and/or endogenous (increased gluconeogenesis) carbohydrate provision and 2) a decrease in glucose R_d into primarily exercising skeletal muscle and, to a lesser extent, liver and other organs. The current data demonstrated no change in R_a between treatments ($p=0.261$; Table 3). Notwithstanding the technical constraints in perfect iso-caloric and carbohydrate matching between treatments (27 vs. 33g CHO in DC and CON, respectively), it is highly

unlikely that the extra 6g of CHO provision from the DC treatment on glucose R_a would cause any impact during the 2h pre-exercise resting period, which is supported by near identical glucose concentrations postprandially (Fig 2A). To examine the role of endogenous glucose production in glucose R_a, we included two glucose tracers ([U-^{13}C] vs. [6,6-$^{2}H_2$]glucose) in the study. Similar to Jeukendrup *et al.* (Jeukendrup, Wagenmakers et al. 1999), the current study showed that the calculated R_a were similar regardless of the glucose tracer used ([U-^{13}C] vs. [6,6-$^{2}H_2$]glucose), and that during steady state conditions ~90-100% of the glucose disposal was through oxidation (Table 3). Under these conditions the glucose recycling rate was minimal; suggesting that glucose R_a via endogenous glucose output (liver derived gluconeogenesis) was negligible. However, tracers have considerable measurement variability that may have masked any small physiological differences in gluconeogenesis. Nevertheless, Cordero-Herrera *et al.* recently demonstrated that a cocoa phenolic extract or (-)-epicatechin directly suppressed glucose production in HepG2 liver cells (Cordero-Herrera et al. 2013). Thus, further studies examining liver derived glucose production in humans with dark chocolate consumption are required.

However, the current study did demonstrate a significant treatment difference in glucose R_d for both [6, 6-^{2}-H_2]glucose and [U-^{13}C]glucose tracers, with a ~16% reduction in R_d late during SS exercise (last 60 min) after DC consumption compared to CON, which was further reflected in significantly reduced glucose clearance in the DC treatment (Fig. 4C). The corresponding calculated plasma glucose oxidation was ~18% lower during SS exercise after DC consumption (Fig. 4A), with a compensatory ~15% increase in muscle glycogen oxidation (Fig. 4B). Taken together, DC consumption altered muscle CHO partitioning, between muscle glucose uptake and glycogen oxidation and our data suggests that augmented plasma glucose during exercise found with DC was due to an unaltered total glucose R_a and a decreased glucose R_d, the combination of which resulted in a subsequent increase in plasma glucose concentration and increase in calculated muscle glycogen utilization.

The cocoa liquor found in the DC treatment is rich in (-)-epicatechin and the methylxanthine theobromine (240 and 690 mg, respectively) After DC consumption, (-)-epicatechin metabolite concentrations peaked at ~700 nM at approximately 2.5 h after consumption (Fig. 3A&B), which is in agreement with previous studies (Richelle et al. 1999). In contrast, circulating theobromine concentrations only reached 70 μM range, which is 100 fold higher than that of (-)-epicatechin metabolites. However, the effect of caffeine after DC consumption was negligible because caffeine concentrations remained nearly at the baseline levels (Fig 3). The timing of the peak in (-)-epicatechin concentration coincides with both the first significant increase in plasma glucose in the DC compared to CON condition as well as to exercise onset. The primary means of skeletal muscle glucose uptake (glucose R_d) is via intracellular translocation of the glucose transporter-4 (GLUT4) to the plasma membrane, which is mediated via both insulin and exercise/contraction mechanisms (Thorell et al. 1999). Intriguingly, several recent *in vitro* experiments in rodent cell lines have shown a competitive inhibition of GLUT4 by some classes of polyphenols (i.e. quercetin, myricetin and catechin-gallate; (Strobel et al. 2005)), including blocking the insulin-dependent GLUT4 translocation by flavanols (Nomura et al. 2008). However, given that a contraction mediated GLUT4 translocation mechanisms would have been dominate given our exercise protocol, and that we did not quantify GLUT4 from muscle biopsies, the mechanism(s) supporting our finding of augmented glucose due to decreased glucose R_d remains elusive.

Theobromine has been shown to be a phosphodiesterase inhibitor and adenosine receptor antagonist (Daly et al. 1987). We quantified a very high and similar theobromine concentration as found previously after the ingestion of chocolate (Richelle, Tavazzi et al. 1999), with peak values of nearly 70 μM, sufficient to antagonize the adenosine receptor (Fig. 3A). The only previous study to directly examine the stimulatory and subjective effects of pure caffeine compared to theobromine in humans found that a 560 mg dose of theobromine did cause significant stimulatory effects compared to a placebo, but less than caffeine (Mumford et al. 1994). Although the mechanistic effects of acute administration of theobromine in humans has not been previously studied, several studies have demonstrated

that the acute increase of other plasma methylxanthine concentrations (e.g. caffeine) have consistently been shown to interfere with skeletal muscle plasma glucose uptake both at rest and exercise in healthy humans. Acute caffeine ingestion has been shown to decrease leg glucose uptake by ~50% (Thong et al. 2002), while theophylline significantly decreased glucose R_d during exercise (Raguso, Coggan et al. 1996). The latter study also found significant increases in plasma glucose concurrently with decreased glucose R_d. The effect of the methylxanthine theobromine on elevated glucose levels in the DC group might be secondary to its adenosine receptor antagonizing activity, as Vergauwen *et al.* (Vergauwen et al. 1994) have previously demonstrated that adenosine receptor antagonists inhibited electrical stimulated glucose transport in the presence of insulin in perfused skeletal muscle. This supports the present results and previous findings (Davison, Callister et al. 2011) that theobromine from DC increased plasma glucose only after the onset of an exercise stress. Similarly, theophylline infusion only decreased glucose R_d during exercise and not at rest (Raguso, Coggan et al. 1996). Recently, Neufingerl et al. reported that theobromine may be the main cocoa constituent responsible for the HDL cholesterol raising effect of dark chocolate (Neufingerl et al. 2013). Although both theobromine and epicatechins have been shown to regulate glucose homeostasis, the 100-fold greater Cmax of theobromine than (-)-epicatechin metabolites after DC consumption (Fig. 3) makes theobromine a dominate candidate for the altered glucose handling found in the present study.

The current study also showed a significant increase in plasma triglycerides in the DC treatment vs. CON, which nearly mimicked the plasma kinetic response of measured (-)-epicatechin (Fig. 2G & Fig. 3B). The high concentrations of plasma triglyceride cannot be explained by the amount of triglycerides in the chocolate treatments as both had similar amounts of total triglycerides. Yoshikawa *et al.* (Yoshikawa et al. 2002) reported that (-)-epicatechin inhibits rat lipoprotein lipase (LPL) activity with the IC50 at 81 mg/L (279 µM). Inhibition of LPL activity can prolong the triglyceride clearance in the plasma, which could be the mechanism explaining the elevated triglycerides in the DC group. However, whether acute DC consumption as found within this study directly has an LPL inhibitory effect remains

to be determined. The recent studies by Allgrove *et al.* (Allgrove, Farrell et al. 2011) and Davison *et al.* (Davison, Callister et al. 2011) both showed significant increases in plasma FFA throughout moderate intensity exercise that were greater after dark chocolate consumption than control. In contrast, the current study found no differences in FFA concentrations during rest or exercise between groups.

Despite differences in plasma glucose between treatments all measured glucose responses were euglycemic (>4 mmol), and thus it may not be surprising that the current study found no differences in ~11.5 min TT performance, which is consistent with a recent publication showing no change in a time to exhaustion performance test over ~6.5 min after chronic and acute dark chocolate consumption (Allgrove, Farrell et al. 2011). Consequently, perhaps a more taxing and prolonged exercise stimulus may have altered the performance outcomes. *A priori* there were several reasons to believe that physical performance might be enhanced with cocoa-rich DC supplementation. First, during prolonged exercise the maintenance of blood glucose levels can delay fatigue and improve performance (Coyle et al. 1983). Since a previous study showed an increased maintenance of plasma glucose late during prolonged exercise with pre-exercise cocoa-rich dark chocolate consumption, compared to an iso-carbohydrate control (Davison, Callister et al. 2011), there could be the potential to improve performance after prolonged cycling exercise. Secondly, the methylxanthines, such as caffeine, have a long history of enhancing physical and mental performance via primarily central nervous system mechanisms (Burke 2008). Interestingly, theobromine has been shown to increase time to exhaustion by 38% in rats (Durham et al. 1993). Despite the fact that theobromine is a compound with ~3-fold lower locomotion stimulatory effect than caffeine (Snyder et al. 1981), the long half-life (~7 h) and large concentration of theobromine in the plasma found in the current study (~70 µM), it could be hypothesized that it may give as similar stimulatory effect as ~1 to 2 mg caffeine/kg B.W. Nevertheless, the acute effect of theobromine on exercise or resting metabolism or performance in humans remains to be assessed and the 690 mg dose in the DC did not have an impact on the short-duration performance test used in this study.

In summary, through the utilization of glucose-tracer methodologies this is the first study to characterize the increases in glycemia and insulinemia during rest, exercise and recovery after dark chocolate consumption. This augmented plasma glucose response was not due to difference in glucose R_a between treatments. However, during the later period of SS exercise there was a ~16% decrease in R_d, which was reflected in a ~18% decrease in plasma glucose oxidation, with a compensatory ~15% increase in muscle glycogen utilization ($p=0.045$) after DC consumption vs. CON during SS exercise. The molecular mechanisms behind our findings remain elusive but either flavanol inhibition of skeletal muscle GLUT4 translocation and/or methylxanthine adenosine antagonism of glucose uptake may play a significant role. However, these metabolic changes did not impact upon performance. Given the large dietary intakes of both polyphenols and methylxanthines from varying dietary sources (Cooper et al. 2008), further scientific examination of their apparent unique bioactive metabolic properties, or potential synergies for improving performance is warranted.

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The authors' responsibilities were as follows: TS, JPG, CJC, DG, ABR, KC, EP, AR and GW: designed the study; TS, JPG, ABR, KC and MB: organized the clinical trial which was executed by SOA, ABL, AL, MC, AFM, AZC and MB; JPG, LAG, AFM, AZC ,IM and DG: performed sample processing and analyses; TS, JPG, CJC, DG, ABR GW and LAG: interpreted the data, wrote and edited the manuscript. All authors reviewed and approved the manuscript.

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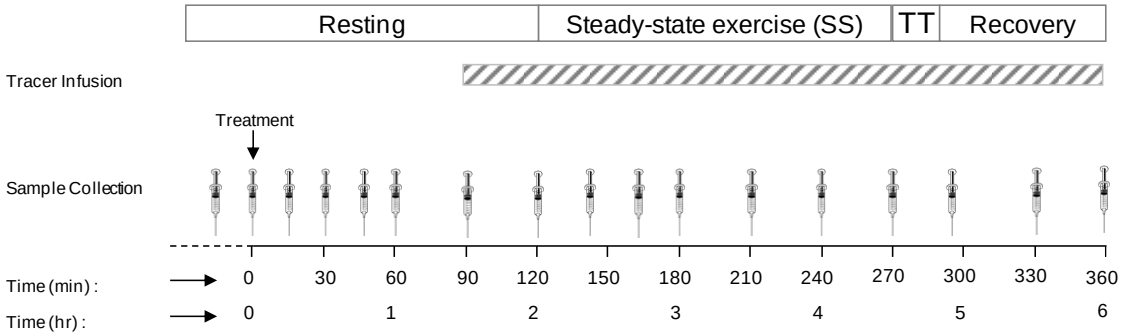
Figure 1. Overview of the study protocol.



Time course of the study

Figure 2. Glucose, insulin, glucagon & triglycerides concentrations.

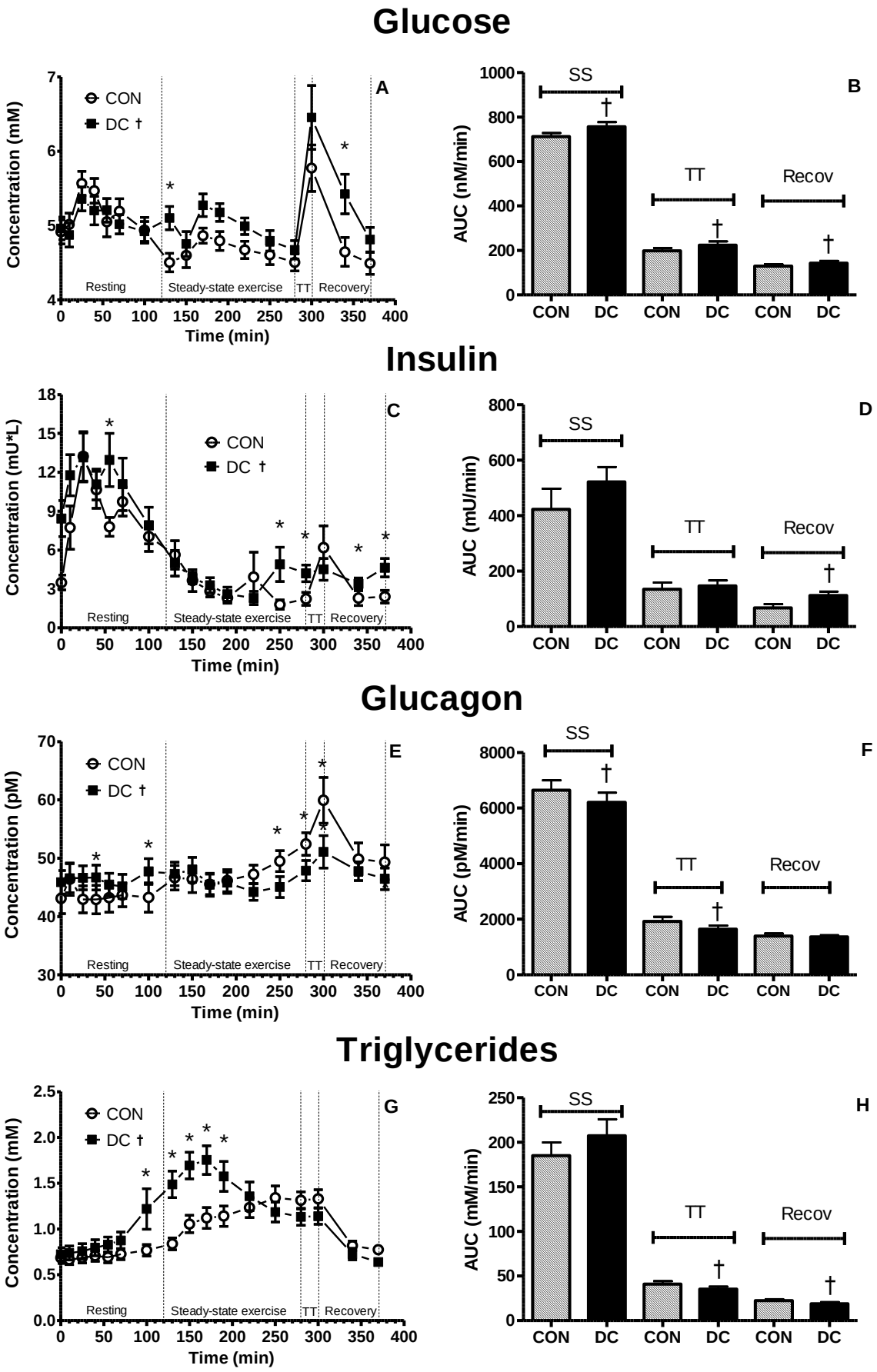


Figure 3. Plasma theobromine and caffeine **(A)** and total (-)-epicatechin concentrations **(B)** after DC consumption.

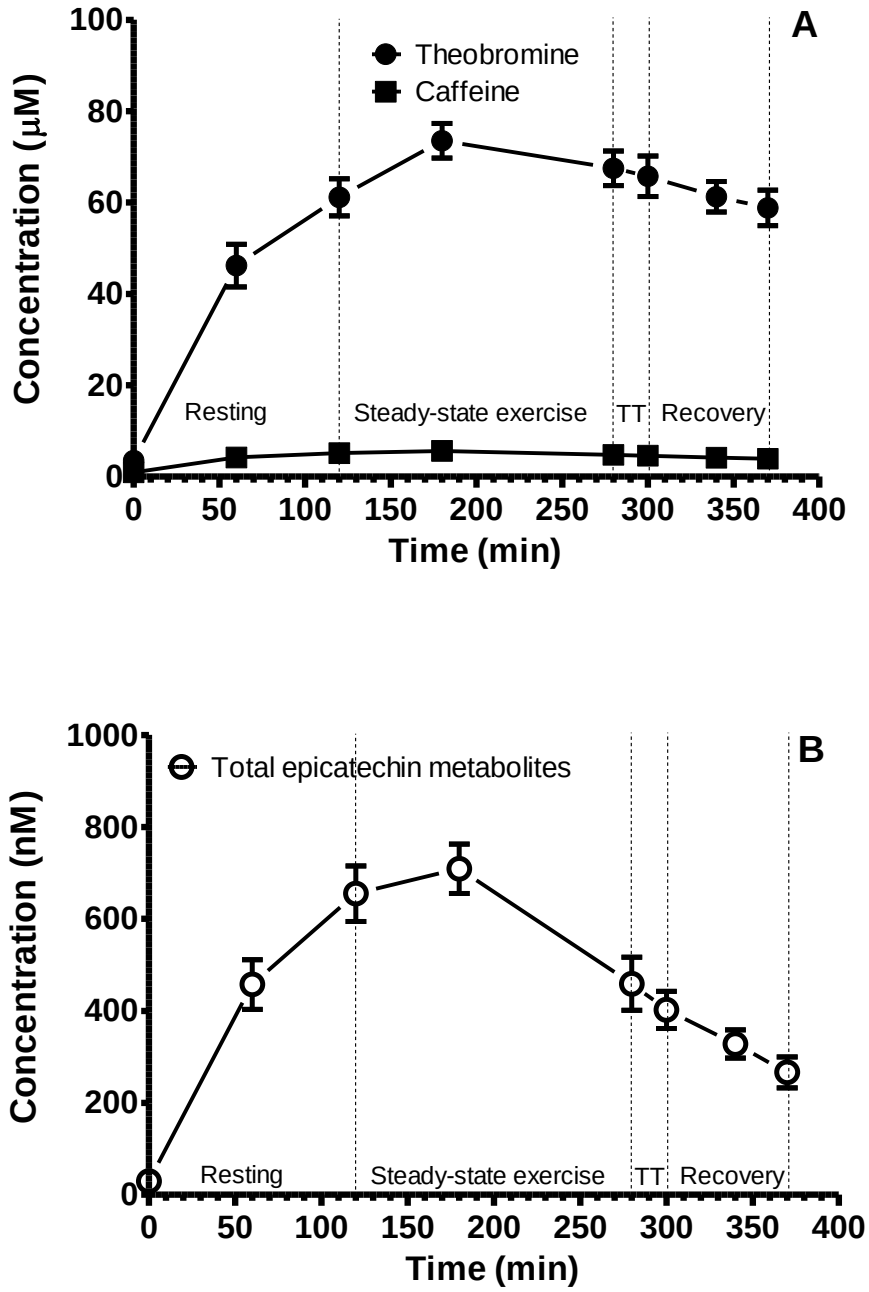


Figure 4. Plasma glucose **(A)** glycogen **(B)** and glucose clearance **(C)**.

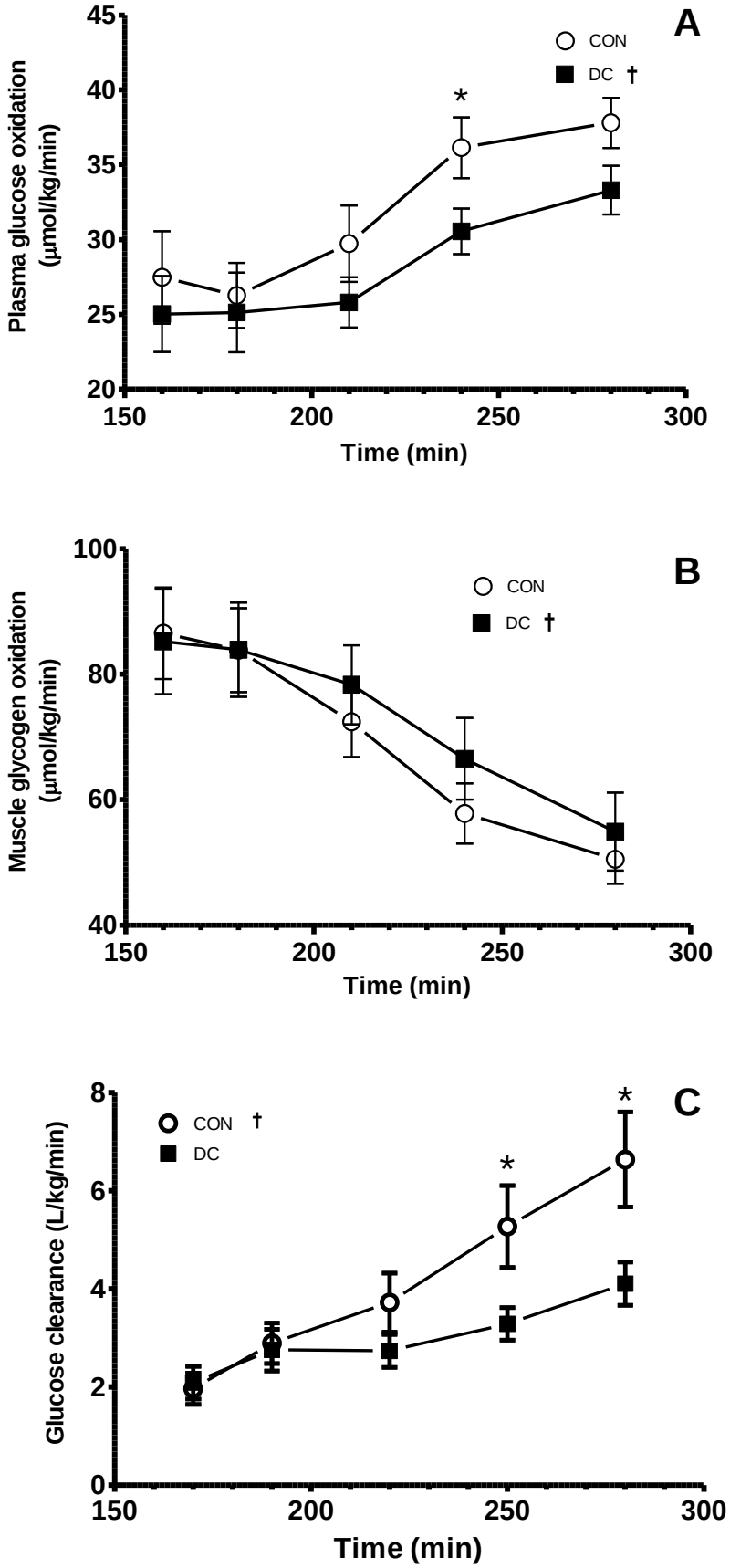


Figure 1. Overview of the study protocol. Resting blood and breath samples were taken prior to and after either 561 kcal of dark chocolate (DC) or nearly iso-caloric and carbohydrate low cocoa control chocolate (CON) consumption. Continuous [6,6-$^{2}H_{2}$]-glucose and [U-^{13}C]-glucose dual-tracer infusion, breath and blood measurements were made throughout the end of the rest period and during 2.5h of cycling at 45% VO_{2peak} (steady-state, SS), time-trial (TT), and recovery periods.

Figure 2. Plasma glucose **(A&B),** insulin **(C&D),** glucagon **(E&F)** and triglyceride **(G&H)** concentrations and AUC, respectively, at rest, during 2.5h steady-state (SS) cycling at ~45%VO_{2peak}, a ~15min time-trial (TT) and recovery (RECOV) after the consumption of either 561 kcal of dark chocolate (DC) or nearly iso-caloric and carbohydrate low cocoa control chocolate (CON) 2h prior to exercise. Values are means ± SEM *n*=16. † Significant trial effect of DC different from CON (P<0.05). * DC significantly different from CON at the corresponding time point (P<0.05).

Figure 3. Plasma theobromine and caffeine **(A)** and total (-)-epicatechin metabolites **(B**; sum of aglycone, 3'-*O*-methyl-epicatechin and 4'-*O*-methyl-epicatechin) at rest, during 2.5h steady-state (SS) cycling at ~45%VO_{2peak} and a ~15min time-trial (TT) after the consumption of 561 kcal of dark chocolate (DC) 2h prior to exercise. Values are means ± SEM *n*=16.

Figure 4. Plasma glucose **(A)** glycogen **(B)** and glucose clearance **(C)** during 2.5h steady-state (SS) cycling at ~45%VO_{2peak} after the consumption of either 561 kcal of dark chocolate (DC) or nearly iso-caloric and carbohydrate low cocoa control chocolate (CON) 2h prior to exercise. Values are means ± SEM *n*=16. † Significant trial effect: DC different from CON (P<0.05). * DC significantly different from CON at the corresponding time point (P<0.05).

Table 1: Volunteer characteristics (*n*=16)

Characteristics	n	Mean	SD	Min	Max
Age (Years)	16	30.0	6.1	19	38
Height (m)	16	179.9	7.8	1.69	1.97
Weight (Kg)	16	72.8	6.0	65	90
BMI (Kg/m^2)	16	22.5	1.4	19.3	24.1
Cycling per week (hrs)	16	9.4	4.6	4	20
VO_{2peak} (ml/kg/min)	16	56.3	5.7	45	66
Cadence (rev/min)	16	89.1	6.1	75	100
Maximum Wattage (Watts)	16	376.8	27.3	310	418
SS Power-Output (Watts)	16	142.0	15.9	110	165
Required Work (kJ) for TT	16	203.6	14.7	168	226

Body mass index (BMI) is calculated by dividing body mass by height^2; SS, 2.5 hr steady-state cycling period at ~45% VO_{2peak}; TT, ~15min time-trial.

Table 2: Overview of the dietary composition for the dark chocolate (DC) and control chocolate (CON) treatments.

Composition	Dark chocolate	Control chocolate
Energy (Kcal)	561	544
Energy (Kjoules)	2329	2251
Fat (g)	43.7	47.9
Saturates	24.3	27.1
Mono unsaturates	12.7	13.6
Polyunsaturates	1.1	1.2
Carbohydrates (g)	33.3	27.5
Glucose	<0.1	<0.1
Fructose	0.1	<0.1
Lactose	0.2	<0.1
Sucrose	27.3	23.4
Maltose	<0.1	<0.1
Cocoa extract (g)		
Fiber	11.4	<0.5
Protein	8.8	0
Theobromine	0.69	<0.01
Caffeine	0.09	0.01
Cocoa polyphenols (mg)		
Epicatechin	89	<0.05
Catechin	24	<0.05
Procyanidin B2	55	<0.05
Procyanidin B5	15	<0.05
Trimer C	37	<0.05
Tetramer D	20	<0.05

Table 3: Tracer kinetics and calculated tracer substrate source utilization during steady-state (SS) cycling at ~45%VO_{2peak} after the consumption of either 561 kcal of dark chocolate (DC) or nearly iso-caloric and carbohydrate low cocoa control chocolate (CON) 2h prior to exercise.

Parameter	***Treatment***	***Time (min)***				
		160	***180***	***210***	***240***	***270***
Enrichment [6,6-$^{2}H_{2}$-glucose] *(%)(x100)*	CON	1.21±0.07	1.04±0.08	0.91±0.06	0.75±0.06	0.67±0.05
	DC	1.10±0.08	0.99±0.06	0.98±0.07	0.88±0.06	0.78±0.07
Enrichment [U-^{13}C-glucose] *(%)(x100)*	CON	0.15±0.01	0.14±0.01	0.13±0.01	0.10±0.01	0.09±0.01
	DC	0.14±0.01	0.13±0.01	0.13±0.01	0.11±0.01	0.10±0.00
Ra [6,6-$^{2}H_{2}$-glucose] *(µmol/kg/min)*	CON	25.9±2.4	28.3±2.7	29.8±2.6	36.2±3.0	39.0±3.2
	DC	29.1±2.9	28.0±2.8	26.6±2.7	30.5±2.9	33.4±2.9
Rd [6,6-$^{2}H_{2}$-glucose] *(µmol/kg/min)*	CON	23.6±2.3	28.7±2.4	30.7±2.7	36.5±3.0	39.6±3.1
	DC†	25.0±2.6	28.8±2.8	27.6±2.6	31.6±2.8	34.1±3.0
Ra [U-^{13}C-glucose] *(µmol/kg/min)*	CON	27.0±2.3	26.7±2.0	29.2±2.5	35.8±2.2	38.9±2.3
	DC	28.5±2.0	27.4±2.2	27.5±1.6	30.8±1.4	34.6±1.3
Rd [U-^{13}C-glucose] *(µmol/kg/min)*	CON	24.8±2.1	27.3±1.9	29.8±2.7	36.2±2.2	39.5±2.3
	DC†	24.3±1.5	28.2±2.6	28.5±1.5	31.9±1.4	35.2±1.3
Gluconeogenesis *(µmol/kg/min)*	CON	0.34±2.20	−2.25±4.90	0.91±6.43	−0.41±5.10	0.80±6.2
	DC	−0.35±4.44	0.62±6.44	0.59±11.04	−0.89±5.97	−0.33±7.4
Tracer infused oxidized *(%)*	CON	106.9±5.0	95.1±4.0	100.7±4.3	101.7±4.7	97.9±3.9
	DC	99.4±4.4	87.6±3.6	90.1±3.1	95.8±3.0	95.5±4.5
Plasma glucose oxidation *(µmol/kg/min)*	CON	27.5±3.2	26.3±2.2	29.7±2.6	36.1±2.1	37.8±1.7
	DC†	25±2.6	25.1±2.8	25.8±1.7	30.6±1.6*****	33.3±1.7
Muscle glycogen oxidation *(µmol/kg/min)*	CON	86.5±7.3	83.8±6.7	72.4±5.6	57.8±4.8	50.5±3.9
	DC†	85.2±8.4	83.9±7.5	78.3±6.3	66.5±6.5	54.9±6.2

Values are expressed as means ± SEM, *n*=16. R_a, rate of appearance; R_d, rate of disappearance; R_{ox}, rate of oxidation. **†** significant trial effect: DC different from CON (P<0.05); ***** DC significantly different from CON trial at the corresponding time point (P<0.05).

Supplementary Table 1: Cardio-respiratory measures at rest, during 2.5h steady-state (SS) cycling at ~45%VO_{2peak}, a ~15min time-trial (TT) and recovery (Recov) after the consumption of either 561 kcal of dark chocolate (DC) or nearly iso-caloric and carbohydrate low cocoa control chocolate (CON) 2h prior to exercise.

Parameter	***Treatment***	***Phase***							
		Rest	***SS***	***SS***	***SS***	***SS***	***SS***	***Recov***	***Recov***
		Time							
		120	***140***	***160***	***180***	***210***	***240***	***330***	***360***
VO_2	CON	3.1±0.2	24.2±0.6	23.8±0.7	23.6±0.8	24.2±0.9	24.7±0.8	2.8±0.1	2.9±0.2
(ml/kg/min)	DC	3.6±0.2	23.9±0.6	24.1±0.6	24.6±0.6	24.9±0.6	25.1±0.6	2.7±0.3	2.8±0.3
VCO_2	CON	2.7±0.2	21.8±0.6	21.0±0.7	20.6±0.8	20.8±0.8	20.9±0.7	2.0±0.1	2.1±0.1
(ml/kg/min)	DC	3.0±0.2	21.4±0.5	21.0±0.6	21.3±0.6	21.4±0.6	21.2±0.6	1.8±0.2	2.1±0.2
RER	CON	0.86±0.02	0.90±0.01	0.88±0.01	0.87±0.01	0.86±0.01	0.84±0.00	0.70±0.01	0.74±0.02
	DC	0.85±0.02	0.90±0.01	0.87±0.01	0.87±0.01	0.86±0.01	0.84±0.01	0.69±0.02	0.74±0.01
CHO oxid.	CON	0.16±0.03	1.70±0.08	1.48±0.07	1.44±0.08	1.34±0.06	1.23±0.06	−0.010±0.01	0.03±0.02
(g/min)	DC	0.31±0.15	1.54±0.11	1.44±0.10	1.43±0.08	1.36±0.08	1.26±0.08	−0.031±0.02	0.03±0.02
Fat oxid.	CON	0.05±0.28	0.01±0.07	0.34±0.09	0.36±0.09	0.41±0.11	0.48±0.12	0.10±0.03	0.09±0.02
(g/min)	DC	0.08±0.29	0.02±0.08	0.39±0.10	0.41±0.11	0.45±0.12	0.49±0.13	0.12±0.03	0.09±0.02
VE	CON	8.46±0.39	37.8±1.06	37.9±1.15	37.4±1.32	38.0±1.25	38.5±1.19	7.93±0.26	8.17±0.38
(L/min)	DC	8.87±0.39	38.5±0.81	38.4±0.95	39.1±0.83	39.1±0.97	39.4±0.96	8.11±0.49	8.22±0.24
HR	CON	119±4	122±4	123±4	124±4	129±4	129±5		
(bpm)	DC	119±4	125±4	125±4	127±4	128±4	127±7		
RPE	CON	9.5±0.5	10.1±0.5	10.3±0.5	10.8±0.5	11.6±0.5	11.1±0.5		
	DC	9.6±0.4	9.9±0.5	10.0±0.5	10.4±0.5	11.1±0.5	10.9±0.4		

Values are expressed as means± SE, *n*=16, (P<0.05). VO_2, rate of oxygen consumption; VCO_2, rate of carbon dioxide expiration; RER, respiratory exchange ratio; Oxid., oxidation; HR, heart rate; and RPE, rate perceive exhaustion.