Title page

Title: One week CF intake increases prefrontal cortex oxygenation at rest and during moderate-intensity exercise in normoxia and hypoxia

Running head: Cocoa flavanols intake and exercise in hypoxia

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Abstract

Introduction: During exercise in hypoxia, O_2 delivery to brain and muscle is compromised and oxidative stress is elicited. Cocoa flavanols (CF) have antioxidant capacities and can increase blood flow by stimulating endothelial function. We aimed to examine the effects of 7-day CF intake on oxidative stress, nitric oxide production and tissue oxygenation in response to exercise in normobaric hypoxia (14.3 % O_2).

Methods: In a randomized, double blind, cross-over study, 14 well-trained male cyclists completed 4 trials: exercise in normoxia or hypoxia, after 7-day CF or placebo intake. Flow-mediated dilation (FMD) was measured before intake of the last dose CF or placebo. One hundred minutes later, 20-minute steady-state (SS, 45% VO_2max) and 20-minute time trial (TT) (cycling) were performed. Blood samples were taken. Prefrontal and muscular oxygenation were assessed by near-infrared spectroscopy.

Results: At baseline, FMD was increased by CF. Hypoxia increased exercise-induced elevations in lipid peroxidation and antioxidant capacity. CF suppressed exercise-induced lipid peroxidation, but did not influence antioxidant capacity. At rest and during SS, prefrontal and muscular oxygenation were decreased by hypoxia. CF elevated prefrontal oxygenation, but did not impact muscular oxygenation. During TT, hypoxia accelerated the exercise-induced decrease in prefrontal oxygenation, but not in muscular oxygenation. During TT, CF didn't alter prefrontal and muscular oxygenation. CF did not change plasma nitrite, nitrate and arginine:citrulline.

Conclusion: During high-intensity exercise, CF did neither improve tissue oxygenation, nor performance in well-trained athletes. At rest and during moderate-intensity exercise, CF reduced exercise-induced lipid peroxidation and partially restored the hypoxia-induced decline in prefrontal oxygenation.

Keywords: cocoa, altitude, exercise, oxidative stress, endothelial function

New and noteworthy:

For the first time, we showed that CF had beneficial effects on endothelial function at rest, as well as on prefrontal oxygenation at rest and during moderate-intensity exercise, both in

normoxia and hypoxia. Moreover, we showed that CF intake inhibited oxidative stress during exhaustive exercise in hypoxia.

Glossary:

- O_2 Oxygen
- F_IO_2 Fraction of inspired oxygen
- NO Nitric oxide
- eNOS Endothelial nitric oxide synthase
- ROS Reactive Oxygen Species
- CF Cocoa flavanols
- FMD Flow mediated dilation
- EC Epicatechin
- PL Placebo
- H Hypoxia
- N Normoxia
- SS Steady State
- TT Time trial
- HR Heart rate
- SaO2 Oxygen saturation
- AMS Acute mountain sickness
- NIRS Near-Infrared Spectroscopy
- DPF Differential pathlength factor
- TSI Tissue saturation index
- TEAC Trolox equivalent antioxidant capacity
- MDA Malondialdehyde
- MAP Mean arterial pressure
- UA Uric acid
- Arg:citr Arginine over citrulline ratio

Introduction

Several sports such as skiing, mountaineering, and sometimes cycling and running involve exercise at altitude. The lower barometric pressure at altitude reduces the partial pressure of inspired oxygen, which results in reductions of O_2 delivery to the active muscles and the brain (38) and elicits the formation of reactive oxygen species (ROS) (26). This leads to a faster development of peripheral and central fatigue, resulting in decreased exercise performance (43). Thus, enhancing O_2 delivery by improving blood flow at altitude could improve tolerance to physical exercise and recovery thereafter.

One of the key molecules regulating blood flow is nitric oxide (NO). NO is endogenously produced by the conversion of arginine into citrulline by endothelial NO synthase (eNOS), in the presence of O_2. NO exerts its vasodilatory function via stimulating guanylate cyclase and relaxing smooth muscle cells. eNOS-dependent NO production can be limited in conditions of low O_2 availability and high levels of oxidative stress (29, 33). Besides, oxidative stress decreases NO availability by increased NO degradation through the reaction of NO with superoxide, the precursor of most other ROS, to form peroxynitrite (5). Both exercise (32) and hypoxia (26) independently elicit the formation of ROS. While ROS have important roles in cell signalling, apoptosis, gene expression and ion transport, excessive ROS leads to oxidative modification and damage of DNA, RNA, proteins and lipids. In the context of exercise, especially at altitude, the excessive ROS formation can lead to impaired muscle contractile and mitochondrial function, resulting in faster development of exercise-induced muscle fatigue and a decreased NO availability (32).

It has been reasoned that modulating NO metabolism by nutritional interventions may influence physiological responses to exercise and thus exercise performance in both normoxia and hypoxia

(9). Dietary nitrate supplementation, for example via beetroot juice, has beneficial effects on NO availability, muscle oxygenation and exercise performance (9), but other supplements hold promise to increase NO availability, too. The intake of cocoa flavanols (CF), a subgroup of polyphenols with antioxidant capacities causes NO-mediated vasodilatation, clinically measured by flow-mediated dilation (FMD) (16, 19). *In vitro* and *in vivo* data showed that (-)-epicatechin (EC), the main bioactive constituent of cocoa, increased nitrite concentration, an indirect marker of eNOS dependent NO production (6, 23). Furthermore, CF and/or their metabolites are strong antioxidants, by directly scavenging superoxide, inhibiting NADPH oxidase (37) and/or by modulating the endogenous antioxidant defence (34).

Despite the existing evidence of the beneficial effects of CF on endothelial function and oxidative stress, few studies investigated the possibilities of CF to modulate exercise-induced oxidative stress and/or exercise performance (2, 8, 13, 15, 31, 41, 42, 44). Because of the large variety of study designs, subject samples, type of exercise and timing and dosages of CF intake and used in these studies, results concerning the CF-induced decrease in oxidative stress after exercise are inconsistent. These studies were all performed at sea level. However, it may be conceivable that this nutritional strategy is more efficient in hypoxia, where O_2 delivery is reduced and where ROS formation is exaggerated.

Consequently, the objectives of this study were to investigate the effects of a 7-day CF intake on (i) selected plasma markers of NO availability and oxidative stress, (ii) muscle and cerebral oxygenation in response to an acute exercise bout in normoxia (sea level) and normobaric hypoxia (simulated altitude of 3000 m, 14.3 % O_2) and (iii) on the implications for exercise performance. We hypothesized that, compared to placebo (PL), CF intake would increase NO

availability, decrease oxidative stress and increase cerebral and muscular oxygenation during exercise in normoxia and hypoxia and enhance exercise performance.

Materials and methods

Participants

A sample size calculation, based on the results of Allgrove et al.(1), Patel et al. (31) and Wiswedel et al.(44), indicated that 14 subjects were required to detect differences at P value p<0,05 with 90 % power. The recruitment started in January 2016. Subjects were excluded when *(i)* younger than 18 years or older than 36 years, *(ii)* smoking or smoking in the past, *(iii)* took antioxidant supplementation, *(iv)* trained less than 10 h per week, *(v)* had stayed at high altitude (> 2000m) for more than 3 weeks during the last 6 months, or *(vi)* if the medical examination prior to the experiment revealed they were hypertensive or had cardiovascular disease. Fifteen healthy well-trained male cyclists were selected for participation in this study. One subjects dropped out because of an injury (knee injury). The study was approved by the UZ Brussel Ethics Committee and was in accordance with the declaration of Helsinki. The experimental procedures and potential risks were explained to the participants and a written informed consent was provided and signed before the start of the study. This trial was registered at clinicaltrials.gov as NCT03135314 and this manuscript is compliant with CONSORT (Consolidated Standards for Reporting Trials).

Study design

A randomized, placebo controlled, counter-balanced, cross-over study design was used. The study was conducted at the Department human physiology of the Vrije Universiteit Brussel (VUB, Brussels, Belgium) from March 2016 until July 2016. On the first lab visit, subjects

underwent a complete medical screening (including skinfolds measures) and performed a maximal incremental cycle test on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). During this test, initial work rate was set at 80W and work rate was then increased every 3 minutes by 40 W until volitional exhaustion. Maximal oxygen uptake (VO_2max) was determined using the Metalyzer cortex (Biophysik GmbH, Germany) and peak power output (PPO) was determined.

Subsequently, subjects visited the lab once every 2 weeks for 8 weeks (4 visits): each visit was preceded by a 1-week wash-out (except for the first visit) and a 1-week nutritional intervention (PL or CF). The sequence of the 4 nutritional interventions was randomly assigned for each participant by using a computer-based randomly permutated block method. The allocation list was generated by CT (co-author), recruitment of participants was conducted by LD (first author) and allocation of participants was conducted by a third author (EL). Participants and researchers involved in data collection, outcome assessment and statistical analysis were blinded to the nutritional intervention. Participants and all researchers, except for LD, were blinded for FIO_2.

Subjects performed 4 interventional trials in randomised order: (1) exercise in (normobaric) hypoxia (H) (3000 m; 14.3 % O_2) preceded by 7 days of CF intake [H-CF], (2) exercise in H (3000 m; 14.3 % O_2) preceded by 7 days of PL intake [H-PL] (3) exercise in normoxia (N) (0 m; 21.0 % O_2) preceded by 7 days of CF intake and [N-CF] (4) exercise in N (0 m; 21 % O_2) preceded by 7 days of PL intake [N-PL]. All experimental trials were conducted in 20° C and relative humidity was kept between 30 and 40 %.

Supplementation

Subjects were asked to consume the provided supplements (PL or CF, Naturex, Avignon, France) every morning at breakfast during the 6 days prior to the testing day. On the testing day, subjects consumed the last dose of supplements upon arrival in the lab. The daily dose of CF consisted of 4 capsules, containing a total of 1765 mg cocoa extract of which 100 mg EP, 23 mg catechin, 119 mg theobromine and 17 mg caffeine (**Table 1**). The dose and duration of the supplementation were based on the finding that 1-week of CF intake enhances vascular function in a dose-dependent manner, with an optimal effect of 100 mg EP (16) and on the pooled results from a recent meta-analysis where 1 week of polyphenol intake increases performance (36). The PL capsule contained 1765 mg maltodextrine and was matched with the CF capsule in colour and shape, theobromine and caffeine content. From the blinding check, it was clear that subjects were unable to distinguish the 2 interventions. The nutritional intervention was double-blinded and counter-balanced. Subjects were provided with a list of foods rich in polyphenols which they should avoid throughout the 8-week study. They were asked to abstain from caffeine during the last 24 h prior to each intervention trial and to repeat the same nutritional regimen during the last 24 h prior to each intervention trial. Subjects completed a 24h-food recall on 3 random days during the study, to check for a potential influence of polyphenol intake on the measurements.

The four interventional trials

Subjects were asked to keep a training diary for the entire duration of the study and to repeat the same weekly training regimen (volume and intensity) for the duration of the study. They were instructed to abstain from intensive training the last 24 h prior to each intervention trial. On each visit, subjects arrived at the lab at the same time of the day in a 3-h fasted state. The entire protocol is depicted in **Figure 1**. First, a baseline FMD measurement took place. Subsequently, a catheter was placed in a forearm vein and a first venous blood sample was collected. Subjects

then consumed the last dose of their supplementation, together with a carbohydrate rich meal, which was carefully selected by a nutritionist to contain 600 kcal, 85% carbohydrates, 10% proteins and 5% fat. After the meal, subjects entered the isobaric hypoxic chamber, which was pre-set at the desired % O_2. Subjects were asked to sit down and relax. It was shown that the maximal concentration of plasma flavanols is reached 100 minutes after acute CF intake and the plasma concentration of flavanols remains at a maximum for 50 minutes (35). Therefore, ninety-five minutes after the last supplement intake, a second blood sample was taken. Subsequently, the participants started a 20-minute steady state (SS) cycling exercise, one hundred minutes after the last dose. During the SS, power output was fixed at 45% of their PPO. SS was followed by five minutes passive rest in seated position and a blood sample was taken. The 20-minute TT then started at 75% of PPO, but subjects were free to increase or decrease their power output as desired from the outset. The goal was 'to perform as much as possible during 20 minutes'. Subjects received information on the time lapsed, but did not receive any feedback regarding power output or heart rate (HR). HR and saturation (SaO_2) were recorded continuously during the experimental trial using a chest belt and Polar HR monitor and a pulse oximeter which was positioned on the participants' left index finger (Medlab, Germany). Rating of perceived exertion (RPE) was measured at the start and after 5, 10, 15 and 20 minutes of the SS and the TT. Blood lactate was enzymatically determined in a capillary blood sample from the ear lobe (Ekf, Biosen 5030, Magdeburg, Germany), at the start, after 10 and 20 minutes of the SS, and the TT. During the 20-minute TT, the completed work (kJ) was used as the main outcome parameter of exercise performance. The occurrence of acute mountain sickness (AMS) was assessed using the Lake Louise Questionnaire at the end of each trial, but none of the subjects experienced any symptom of AMS.

Measurements

Primary outcome measures were muscle and cerebral oxygenation, markers of oxidative stress and exercise tolerance and performance. Secondary outcome measures included flavanol metabolites, markers of NO availability, FMD and mean arterial pressure.

Flow-mediated dilation (FMD) and mean arterial pressure (MAP)

Upon arrival at the lab, subjects were instructed to relax in supine position for 10 minutes, during which MAP was measured automatically (Medisana BU510, Kerkrade, Netherlands). Then, arterial endothelial function was assessed by FMD. The operator, blinded to PL or CF condition, took images of the vessel upon arrival of the participants (baseline measure) with a 5-10 Hz linear array probe. The scan of the right brachial artery, approximately 2-7 cm above the antecubital fosse (marked on the first visit, ensuring that measurement occurred at the same place for each scan), was evaluated in the longitudinal image. The sphygmomanometer placed around the forearm (distal) was inflated 50 mmHg above the systolic blood pressure for 5 min. A continuous scan of the brachial artery was then performed during 90 s after the rapid deflation of sphygmomanometer, which induced shear-stress and endothelium-dependant dilation. Each brachial artery diameter was manually measured three times at end of diastole, 60 s after the cuff deflation. The FMD (%) was calculated as (hyperaemic diameter – pre-inflation diameter)/pre-inflation diameter)*100.

Muscle and cerebral oxygenation during exercise

Near-infrared spectroscopy (NIRS) (Portalite continuous-wave NIRS system (Artinis, Elst, Netherlands), a non-invasive optical imaging technique, was used to assess changes in oxygenation status of the *prefrontal cerebral cortex* and the *M. vastus lateralis* during exercise.

The use of NIRS to assess tissue oxygenation, including its limitations, has been extensively described (11).

Upon entrance in the isobaric hypoxic chamber, one emitters/receptor optode pair was positioned over the left prefrontal cortical area between Fp1 and F3, according to the modified international EEG 10-20 system. One emitters/receptor optode pair was attached to the (shaved) skin on the lower third of the belly of the right *M. vastus lateralis* (middle between the lateral epicondyle and trochanter). Skinfold measures during the medical screening assured that the adipose tissue thickness was well below 1.5 cm to allow the NIRS photons to penetrate into the muscle (11). The inter-optode distance for both probes was 4 cm and the probes were covered with a black cloth to minimize intrusion of extraneous light. A dark elastic band was wrapped around the head and the leg to keep the NIRS-optode pairs in place.

NIRS data collection was started 20 minutes prior to the start of exercise. To collect a baseline NIRS value, the mean of a 2-minute period during which subjects sat still without speaking or moving, was calculated. The tissue saturation index (TSI) was determined by spatially resolved spectroscopy and offers a surrogate measure of the fraction of O_2 saturated haemoglobin and myoglobin, reflecting a tissue oxygenation status in percentage (%) (11). Data were collected at a sampling frequency of 5 Hz and were down-sampled with factor 5 for analysis. During exercise, mean TSI was calculated per 30 second window. NIRS values of the following 30-sec epochs were used for data analysis: 0, 5, 10, 15 and 20 minutes of the SS and TT.

Blood analyses

Venous blood samples were collected at baseline, and at the start and end of the SS and the TT. Blood was collected into 5 ml EDTA tubes, 5 ml heparinized tubes and 8 ml anticoagulant-free

tubes and were centrifuged immediately to obtain plasma or after 30 minutes at room temperature to allow clotting to obtain serum (10 minutes at 704 *g*, 4° C). Plasma and serum were aliquoted and stored at -80° C until further analyses. Values were corrected for changes in plasma volume using the haematocrit and haemoglobin concentration according to Dill and Costill (7). Hemoglobin concentration was measured in duplibcate using an azidemethemoglobin double wavelength photometer method (Hemocue Hb201+, Angelholm, Sweden), while haematocrit was determined by microcentrifugation in triplicate (Heraeus Pico 17, Germany).

Serum flavanols

Serum samples were analysed for EP and catechin concentrations as described by Neukam et al (27). In detail, 0.5 ml of serum was mixed with 1.0 ml phosphate buffer (100 mM, pH 5, containing ascorbic acid (20 mg/ml), EDTA (1.5 mg/ml)) and 20 µl glucuronidase/sulfatase (100,000 and 7500 units/ml, respectively) and incubated at 37 °C for 30 min to hydrolyse glucuronate and sulfate conjugates of epicatechin and catechin. Then, 5 ml tertbutylmethylether was added and vortexed for 1 min. For phase separation, the mixture was centrifuged at 10 °C for 5 min at 1957 *g*. The organic phase was transferred to a new tube and after a second extraction with 5 ml tertbutylmethylether, the combined extracts were dried under a stream of nitrogen and stored at −80 °C. For HPLC analyses, the dry residues were reconstituted in 200 µl methanol, vortex-mixed and centrifuged at 5 °C at 15 339 *g* for 5 min. 20 µl of the supernatant were injected onto the HPLC-column. For HPLC analysis, a reversed-phase RP18 end-capped column (Lichrospher 100, 5 µm, 250 × 4 mm; Merck) coupled with a guard column (Lichrospher 100, 5 µm, 4 × 4 mm; Merck) was used. Detection was accomplished at an excitation wavelength of 280 nm and an emission wavelength of 310 nm. Data were recorded by HPLC- System Manager software (Merck/Hitachi; Darmstadt, Germany). Samples were eluted from the column at 20 °C

using a step-gradient as follows: from 0 to 18 min 53% acetonitrile/H_2O/acetic acid (150:846:4)/47% H_2O/acetic acid (1000:5), from 18 to 27 min 80% acetonitrile/H_2O/ acetic acid (150:846:4/20% H_2O/acetic acid (1000:5). To elute retained compounds the column was flushed from 27 to 60 min with 100% acetonitrile/acetic acid (1000:4) and subsequent equilibration from 60 to 80 min with starting conditions. Flow rate was 1.5 ml/min. Under these conditions the analytes elute with retention times of 15.4 min for catechin and 24.9 min for epicatechin. Peak areas of catechin and epicatechin were used to calculate the concentrations applying the external standard method. Standard curve linearity was observed in the range from 0.125 to 20 μM for both compounds.

Plasma nitrite and nitrate

Plasma nitrite (NO_2^-) and nitrate (NO_3^-) were analysed by gas phase chemiluminescence analysis. Plasma was deproteinised with ice-cold ethanol. For NO_2^- analysis, samples were injected into a glass purge vessel containing 5ml glacial acetic acid and 1ml NaI solution, which reduces NO_2^- to nitric oxide (NO) gas, that is carried into the NO detector in inert nitrogen. For NO_{3-} analysis, samples were reduced in a solution of vanadium (III) chloride in 1 M hydrochloric acid (0.8% w/v). Quantification of NO was enabled by the detection of light emitted during the production of nitrogen dioxide formed upon reaction of NO with ozone. Luminescence was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence nitric oxide analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). The concentrations of NO_2^- and NO_3^- were determined by plotting signal area (mV) against a calibration plot of 25nM to 1μM sodium nitrite and 100nM to 10μM sodium nitrate respectively.

315 The NO_3^- concentration was corrected by deduction of the NO_2^- value, since the vanadium
316 chloride solution also reduces nitrite.

317 *Plasma arginine and citrulline*

318 150 µL of internal standard (50 µM arginine, methanol mixture) was added to 10 µL of
319 heparinized plasma and centrifuged (13000 rpm, 10 minutes, 4°C) to remove the precipitated
320 proteins. Supernatant was collected and dried under a stream of nitrogen at 70°C. The dried
321 extract was dissolved in 100 µL of a butanol solution containing 3N HCl and kept at 70°C for 40
322 minutes. The solvent was removed by evaporation under nitrogen flow at 70°C. The sample was
323 then dissolved in 2.5 mL of water-methanol (90:10, v/v) containing 0.1% formic acid and 5 µL
324 was injected into an analytical column (Kinetex C18 (5µm, 2.1x100mm)). Mass spectrometric
325 analysis was performed using an UFLC-XR Shimadzu coupled with an QTRAP® 5500 hybrid
326 system, equipped with a Turbo VTM ion197 source (AB Sciex, Foster City, CA, USA). Multiple
327 reaction monitoring (MRM) measurement was performed using optimal cone and collision
328 energy values. Each run was performed at a flow rate of 500 µL/min at 30°C, lasting 9 minutes in
329 total. A gradient profile consisted of solution A (water with 0.1% (v/v) formic acid) and solution
330 B (methanol with 0.1% (v/v) formic acid). The percentage of organic solution B was changed
331 gradually as follows: 0 min, 2%; 4 minutes, 7%; 6 minutes, 50%; 7 minutes, 2 %; 9 minutes, 2%.
332 Data were acquired with Analyst Software version 1.5.2. Calibration curves, performed in water,
333 were obtained adding increasing concentrations of arginine and citrulline from 12.5 to 125 µM.

334 *Quantification of total antioxidant capacity (TEAC), uric acid (UA), malondialdehyde (MDA) in*
335 *plasma*

Plasma antioxidant capacity was quantified as trolox equivalent antioxidant capacity (TEAC) according to Fischer et al.(12). In this procedure, the decolorization of the preformed green-blue 2,2`-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical by the heparinized plasma within a fixed time reflects the antioxidant capacity of the sample. To correct plasma TEAC values for individual differences in uric acid (UA) concentrations, the most abundant antioxidant in blood, UA plasma concentrations were quantified by HPLC (34). MDA, a marker of lipid peroxidation as a result of oxidative stress, was quantified in EDTA-plasma samples after derivatization with thiobarbituric acid by using HPLC with fluorometric detection as described by Lepage et al. (22).

Statistical analyses

A power calculation to determine the minimal sample size required to determine whether CF intake would affect exercise-induced markers of oxidative stress and exercise performance (TT) (n=15, p= 0.05, power= 0.8) was based on the results of Allgrove, Patel and Wiswedel (2, 31, 44).

Statistical analyses were performed with IBM SPSS Statistics (version 22; IBM Corp, Armonk, USA) and considered significant at $p < 0.05$. Data are presented as mean ± standard deviation (SD) for n=13, except when otherwise indicated. Normality and sphericity of the data were assessed by the Kolmogorov-Smirnov test and Mauchly's test. To follow the absorption and metabolism of EP and catechin after 6-day CF intake and after intake of the final dose of CF, a three-way repeated measures ANOVA (F_IO_2 x supplement x time (baseline, start SS, start TT, end TT)) was used. Two-way repeated measures ANOVA (fraction of inspired O_2 (F_IO_2) x supplement) at *baseline* was employed to assess the baseline differences in nitrite, nitrate, arg:citr, TEAC, UA, MDA, MAP and FMD between 6-day CF and PL intake, with interpretation

of the main effect of supplement. Two-way repeated measures ANOVAs (*F_IO) x supplement*) were used to assess differences in nitrite, nitrate, arg:cit, TEAC, UA, MDA and muscular and prefrontal TSI between CF and PL in H and N at start of SS (acute effect *supplement* and *F_IO2*) and start of TT. For significant interactions between F_IO_2 and supplement, pairwise comparisons were performed using the post hoc Bonferroni correction. Two three-way repeated measures ANOVAs (*F_IO_2 x supplement x time)* were used to assess differences between CF and PL in H and N during exercise (1 for SS, 1 for TT) for the following outcome parameters: nitrite, nitrate, arginine:citrulline ratio (arg:citr ratio), TEAC, UA, MDA, work performance during TT, muscular and prefrontal TSI. The effects of H and CF intake on TT performance (work performed after 20 min) was assessed by a two-way repeated measures ANOVA (F_IO_2 x supplement).Significant interactions in the three-way repeated measures ANOVA were further analysed by two-way repeated measures ANOVA with subsequent paired t-tests to interpret the effect of the supplement over time at each F_IO_2 (N and H) and the effect of F_IO_2 over time after CF or PL intake. If no significant interaction effects were observed, main effects were immediately interpreted through pairwise comparisons with the Bonferroni correction. Significant interactions in the two-way repeated measures ANOVA (supplement x F_IO_2) were further analysed by paired t-tests to interpret the effect of the supplement at each F_IO_2 (N and H) and the effect of F_IO_2 after each supplementation. RPE was not normally distributed and was therefore analysed by Friedman tests and Wilcoxon signed rank tests. A Pearson correlation was used to assess correlations between baseline concentrations of nitrite and nitrate and relative increases in nitrite and nitrate concentration after 6 days of CF intake.

Results

Subject characteristics

The 14 well-trained athletes included in this study were 30.7 ± 3.1 years old, had a height of 1.80 ± 0.05 m, weight of 73.4 ± 7.4 kg and BMI of 22.5 ± 1.5. They had a VO_2max of 62.9 ± 5.8 mL/min*kg and PPO of 366 ± 45 W.

Effects of CF intake on (-)-epicatechin and (+)-catechin

At baseline, there was no significant difference between 6-day CF and PL intake on serum EP concentration. Three-way repeated measures ANOVA showed a significant *supplement x time* interaction ($F=14.70$, $p<0.001$). Post-hoc analysis showed that 100 minutes after acute CF intake, serum EP was elevated compared to baseline (+ 238 ± 43 %, H and N pooled, $p<0.05$). After CF intake, serum EP further increased during SS exercise (+ 359 ± 32 %, H and N pooled, $p<0.001$ compared to baseline), while a plateau phase in serum EP was reached during the TT (no further increase post-TT compared to pre-TT) (**Figure 2A**). After PL intake, EP did not change over time. Serum catechin was not affected by CF intake, compared to PL intake, at any time points and in both N and H (**Table 2** for baseline values, other data not shown).

Effect of CF intake on NO availability during exercise in H

eNOS dependent NO production

eNOS dependent NO synthesis was reflected by plasma nitrite and nitrate concentrations and by the plasma ratio of arg:citr (39). Plasma nitrite and nitrate did not change during exercise and were not affected by H (**Figure 2B**). Arg:citr ratio significantly decreased at the end of exercise compared to pre-exercise (main effect of time: $F=14.1$, $p=0.003$) and was significantly higher in H compared to N (main effect of F_IO_2: $F=10.0$, $p=0.008$)(**Figure 3A**). CF intake did not

404 significantly change plasma nitrite, nitrate and arg:citr ratio, neither before (thus at "baseline"),
405 nor after the final dose. CF intake did not change plasma nitrite, nitrate and arg:citr ratio after
406 exercise. However, a significant negative correlation was found between baseline nitrite (after 6
407 days of PL intake) and the relative increase in nitrite concentration after 6 days of CF intake (R^2
408 = 0.67, $p<0.001$, Pearson correlation).

409 *Oxidative stress and antioxidant capacity*

410 Two-way repeated measures ANOVAs at baseline and pre-exercise showed that MDA was
411 neither affected by 6-day CF intake (Table 2) nor by the final dose of CF, in rest. Three-way
412 repeated measures ANOVA during exercise revealed a significant interaction effect of *time x*
413 *supplementation* (F=7.95 p=0.018): the significant exercise-induced increases in plasma MDA
414 concentrations after PL intake (+ 12.2 ± 5.5 %, p=0.047 in N and +19.0 ± 6.8 %, p=0.016 in H),
415 were suppressed by CF intake in both N (+ 2.9 ± 4.4 %, NS) and H (+ 2.0 ± 4.4 %, NS) (**Figure**
416 **3B**).

417 Two-way repeated measures ANOVAs at baseline and pre-exercise showed that total plasma
418 antioxidant capacity, measured as TEAC, was neither affected by the intake of 6 day CF intake,
419 nor by the last dose of CF in N and H in rest. Three-way repeated measures ANOVA revealed
420 that the exercise-induced increase in TEAC was larger in H than in N (time x F_IO_2: F= 4.83
421 p=0.05), but was not affected by CF intake (**Figure 3C**). To correct for individual differences in
422 UA, the most abundant plasma antioxidant that contributes to plasma TEAC, UA concentrations
423 were quantified in every sample. Two-way repeated measures ANOVAs showed that UA was
424 neither affected by 6-day CF intake, nor by intake of the final dose of CF at rest. Three-way
425 repeated measures ANOVA showed that UA concentrations were elevated after exercise, and in

H compared to N (main effect of time: F=25.26, p<0.001; main effect of F_IO_2: F=6.48 p=0.026), while CF did not influence this response (**Figure 3D**).

Vasoreactivity

At baseline, MAP was not different between 6-day CF and PL intake (Table 2). FMD was significantly increased after 6 days of CF intake compared to PL (main effect of supplement: F=5.59, p=0.042) (Table 2). The change in FMD after 6 days of CF intake compared to 6 days of PL was not correlated with the relative increases in nitrite and (-)-epicatechin concentration after 6 days of CF intake compared to PL.

Muscle oxygenation during exercise

At the start of SS exercise, TSI in the V. lateralis was not affected by the supplement and H. During SS, a significant interaction *time x* F_IO_2 effect was found for TSI (F=11.95 p<0.001(**Figure 4A**). Post-hoc Bonferroni corrections showed that TSI decreased during the first 5 minutes and then stabilized. This exercise-induced decrease was aggravated in H, compared to N, while CF intake had no effect.

At the start of the TT, TSI was significantly lower in H than in N (main effect of F_IO_2: F=6.96 p=0.02). Three-way repeated measures ANOVAs showed a main effect of time during the TT for TSI (F=71.65 p<0.001): TSI significantly decreased during the first 5 minutes and stabilized during the last 15 minutes. H and CF intake did not influence TSI during the TT.

Prefrontal cortex oxygenation during exercise

At the start of SS exercise, both H and CF influenced TSI (main effect of F_IO_2: F=7.05, p=0.02, main effect of supplement: F=7.66, p=0.017) (**Figure 4B**). TSI was significantly lower in H compared to N. TSI was significantly higher after CF intake compared to PL (Figure 4B).

448 During SS, 3-way repeated measures ANOVA showed a significant main effect of supplement
449 (F= 12.28, p=0.004) and a significant *FIO_2 x time* interaction for TSI (F=24.10 p<0.0001). CF
450 intake significantly increased prefrontal TSI during SS exercise. TSI significantly decreased in H,
451 but not in N.

452 At the start of the TT, TSI was significantly lower in H than in N (*effect of F_IO_2*: F=6.43
453 p=0.026), while CF intake had no significant effect. Three-way repeated measures ANOVA
454 showed a significant *F_IO_2 x supplement x time* interaction effect for TSI during the TT (F=4.11
455 p=0.016). In N, TSI decreased significantly for the entire duration of the TT after both PL and CF
456 intake. However, a larger decrease was observed after CF intake, compared to PL (significant
457 *supplement x time* interaction effect (F=6.38 p<0.001)). In H, TSI enormously decreased during
458 the first 5 minutes and did not change significantly during the remaining 15 minutes after both PL
459 and CF intake and no interaction effect of *supplement x time* was found.

460 *Exercise tolerance and performance*

461 *Steady state*

462 Two-way repeated measures ANOVA showed that at the start of SS, SaO_2 was significantly
463 lower in H than in N (**Table 3**). At rest, CF intake did not alter SaO_2. HR and lactate were similar
464 in H and N and were not different after CF intake, compared to PL. Three-way repeated measures
465 ANOVAs showed a significant *F_IO_2 x time* interaction effect for SaO_2, HR and lactate during SS.
466 An exercise-induced decrease in SaO_2 occurred in H, but not in N. The exercise-induced increase
467 in HR was larger in H than N. In N, lactate decreased during SS, but in H, lactate increased
468 during SS. During SS, RPE was significantly higher in H than in N. The (significant) difference
469 in SaO_2 between CF and PL intake during SS exercise in H (-1.21 ± .48% in CF vs. PL) was

smaller than the accuracy range (2 - 3%) claimed by the distributor of the pulse oximeter used and might thus not be reliable. CF did not influence HR, lactate and RPE during SS exercise.

Time trial

TT performance (work performed during the 20 minute-TT) decreased in H compared to N, but CF intake did not influence TT performance (Table 5). Two-way repeated measures ANOVAs at the start of the TT showed that SaO_2 and lactate were significantly lower (-8 ± 1%, $p<0.001$) and higher (+0.9 ± 0.2 $mmol.L^{-1}$, $p<0.001$) in H compared to N. No significant difference between N and H was observed for HR. CF did not influence SaO_2, lactate or HR. Three-way repeated measures ANOVAs showed a significant F_IO_2 *x time* interaction effect for SaO_2, HR and lactate during the TT. Post hoc analysis showed a larger drop in SaO_2 and a larger increase in lactate during the TT in H compared to N. Post hoc analysis showed a faster elevation of HR, but lower HR_{max} at the end of the TT in H than in N. RPE was significantly higher in H than in N during the first half of the TT, but there was no difference during the second half. CF intake did not influence any of these physiological changes.

Discussion

The important novel findings of this study were that in well-trained cyclists, 1-week CF intake can *i)* increase prefrontal oxygenation at rest and during moderate-intensity exercise and thus can partially restore the hypoxia-induced decline in oxygenation during exercise at altitude and *ii)* reduce exercise-induced oxidative stress, which is substantially higher in hypoxia than in normoxia. CF does not improve exercise performance in normoxia and hypoxia.

It is well documented that CF intake leads to improved endothelial function, as reflected by FMD, in individuals with and without cardiovascular risks (16, 17). We found that this beneficial effect also occurs in well-trained athletes who already have an enhanced endothelial function by regular exercise training. Studies using either NO-synthase inhibitor (L-NMMA) (18) or parallel measure of circulating nitrites (17) suggested that the CF-induced improvement in FMD can originate from an effect of CF on NO metabolism. However, in the current study, nitrite concentration and arg:citr ratio, two indirect markers of eNOS dependent NO production and NO availability (6, 10), were not altered by CF intake and no correlation between the change in FMD and change in nitrite concentration was found. One hypothesis to explain this result could be that CF could also act on other molecules than NO, which would play a role in smooth muscles relaxation. As for example, Grassi et al. (16) showed that 7-day CF supplementation improved FMD and decreased concentrations of endothelin 1, a substance known to act directly on smooth muscles by inducing vasoconstriction in healthy volunteers. Despite a greater CF-induced increase of plasma nitrites in subjects with lower initial levels of nitrite, we did not find greater FMD improvements in those subjects. This result might be an additional argument for a putative role of other dilator substances, besides NO, in CF effects.

CF intake did not affect nitrite, nitrate and arg:citr ratio in response to exercise and hypoxia. Moreover, nitrite and nitrate were neither altered by acute hypoxia, nor by exercise. Similarly, nitrite was similar to pre-exercise levels after a 3h cycling race in the study of Sureda et al. (40) and Kelly et al. (21) found no effect of exercise in normoxia and hypoxia on nitrite after PL intake. However, the interpretation of these data is not straightforward, since plasma nitrite is likely to reflect the dynamic balance between NOS-derived NO production and the reduction from nitrate to nitrite and further NO, which is expected to be facilitated in hypoxia (21). Furthermore, in the current study, the arg:citr ratio was lowered after exercise and the magnitude of this decrease was smaller in hypoxia compared to normoxia. This seems consistent with the notion that enzymatic production of NO depends on the availability of O_2 (29) and that hypoxia triggers superoxide anion generation, causing depletion of tetrahydrobiopterin, the essential eNOS cofactor, which results in eNOS uncoupling and decreased eNOS-dependent NO production (30).

Previously, it has been shown that CF intake leads to inhibition of NADPH oxidase (20). The generation of the superoxide anion radicals by NADPH oxidase results in scavenging of NO, eNOS uncoupling and reduced NO availability, but also triggers oxidative stress. CF intake may decrease oxidative stress after different types and durations of exercise in humans at sea level (2, 8, 13, 44). At altitude, the magnitude of exercise-induced oxidative stress is elevated compared to at sea level (26). For the first time, we demonstrated that 7-day CF intake can inhibit the exercise-induced increase in lipid peroxidation in N, but also in H. Lipid peroxidation, which is the result of a multistep chain reaction where ROS attack lipids in cell membranes (4), was affected by CF intake. However, CF did neither affect plasma UA concentrations, nor the total plasma antioxidant capacity measured as TEAC, in response to exercise and hypoxia. The plasma

antioxidant capacity does not necessarily correlate with changes in lipid peroxidation since hydrophilic antioxidants are not efficient against lipid peroxidation (28). Previous *in vitro* studies showed that CF can directly scavenge free radicals, act as a chain-breaking antioxidant in lipid peroxidation and/or regulate ROS-related enzymes (3, 20, 24). Our results propose that during exercise in hypoxia, CF mainly reduces oxidative stress in the environment of membranes and lipoproteins. The diminished oxidative stress raises the possibility for CF to prevent muscle damage and thus have a beneficial effect on exercise recovery.

Consistent with previous research, the exercise-induced drops in tissue oxygenation were larger in hypoxia than in normoxia during moderate-intensity exercise (14, 25). The decreased muscular oxygenation in hypoxia was paralleled by elevated blood lactate concentration, indicating a higher reliance on anaerobic glycolysis, but was not affected by CF intake. Thus, the effects of hypoxia to inhibit oxidative energy production during moderate-intensity exercise were not suppressed by CF. In contrast to the muscle, CF intake beneficially impacted cerebral oxygenation at rest and during moderate-intensity exercise in hypoxia. Although no other studies have examined muscular nor prefrontal oxygenation changes in response to CF intake, we might speculate that there is a tissue-specific reaction to CF supplementation. Using another supplement (beetroot) during moderate-intensity exercise in hypoxia, Masschelein et al. (25) found a tissue-specific reaction, but in the opposite way with improved muscular oxygenation, but no difference in prefrontal oxygenation. However, beetroot is known to influence the NO metabolism, while we found no differences in nitrate and nitrite concentrations after CF intake. Thus, the specific tissue responsiveness to CF supplementation merits further investigation.

The beneficial effects of CF on prefrontal oxygenation vanished during high-intensity exercise, indicating that the physiological alterations in response to exhaustive exercise largely overruled

any beneficial effects of CF. CF intake could not increase muscular oxygenation and could not prevent greater reliance on anaerobic glycolysis during the TT in hypoxia, as evidenced by the higher blood lactate concentration. Moreover, CF intake did not have ergogenic effects in hypoxia and normoxia.

Future research may address some of the potential limitations of the current study. The measured markers of NO availability and oxidative stress in plasma might not exactly reflect changes in the endothelium, brain and muscle. While NIRS is currently the only method allowing the measurement of muscular and cerebral blood flow and oxygenation continuously during whole-body exercise, it only provides indirect information.

For the first time, we showed that CF intake inhibited oxidative stress during exhaustive exercise in hypoxia. CF had beneficial effects on endothelial function at rest, as well as on prefrontal oxygenation at rest and during moderate-intensity exercise. This is not only relevant for athletes exposed to altitude, but also for hypoxemic patients suffering from a reduced blood oxygenation, as well as for patients suffering from chronic diseases involving increased levels of oxidative stress.

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704

705 *Tables*

706 *Table 1. Composition of cocoa flavanol (CF) and placebo (PL) supplementation (Naturex) (daily dose).*

Content in 4 pills	PL	CF 707
Cocoa extract (mg)	0	1764
Maltodextrine (mg)	1764	136
Total flavanols (mg)	0	530
Total monomers (mg)	0	121
(-)-Epicatechine (mg)	0	100
(+)-Catechine (mg)	0	21
Theobromine (mg)	119	119
Caffeine (mg)	17	17

708

709

710 *Table 2. Baseline measures following 6-day (before intake of last dose) cocoa flavanol (CF) or placebo (PL) intake (n=14).*

	PL	*CF*
(-)-Epicatechin (nM)	35.4 ± 10.8	35.3 ± 9.2
(+)-Catechin (nM)	19.7 ± 10.8	21.7 ± 14.7
Nitrite (nM)	69.9 ± 17.5	81.4 ± 19.9
Nitrate (nM)	43.8 ± 22.9	47.5 ± 21.5
MDA (µmol/L)	1.17 ± 0.25	1.19 ± 0.21
Arg:Citr ratio	1.93 ± 0.56	1.92 ± 0.27
TEAC	442.8 ± 28.7	447.4 ± 49.3
UA (µmol/L)	302.0 ± 39.3	303.5 ± 40.4
MAP (mm Hg)	95.5 ± 7.1	94.0 ± 7.1
FMD (%)	0.56 ± 2.26	2.15 ± 2.19*

711 *MAP: mean arterial pressure, FMD: flow mediated dilation.* $p<0.05$ between CF and PL. Epicatechin and catechin,were*
712 *measured in serum, nitrite, nitrate, malonaldehyde (MDA), Arginine (Arg), Citrullin (Citr), Uric acid (UA) and Trolox Equivalent*
713 *Antioxidant Capacity (TEAC) were measured in plasma.*

Table 3. Effect of 7-day cocoa flavanol intake on physiological changes during moderate and high intensity exercise in hypoxia and normoxia (n=14).

	PL- N	CF- N	PL- H	CF-H	*2-way RM anova (start)*	*3-way RM anova*
SaO_2 - SS						
- Start	97 ± 4	98 ± 1	89 ± 3*	88 ± 6*£	*S: NS*	*O_2 x S: F=5.11 p=0.05*
- 5'	97 ± 2	95 ± 4	79 ± 5*	78 ± 5*£	*O_2: F= 122.00*	*O_2 x T: F=26.90 p<0.0001*
- 10'	97 ± 2	97 ± 1	79 ± 4*	76 ± 4*£	*p<0.001*	
- 15'	95 ± 2	96 ± 1	79 ± 4*	76 ± 3*£		*S: F= 6.48 p=0.027*
- End	96 ± 2	96 ± 2	80 ± 3*	78 ± 3*£		*O_2: F=624.55 p<0.0001*
						T: F=25.20 p<0.0001
SaO_2 - TT						
- Start	97 ± 1	97 ± 2	88 ± 1*	89 ± 4*	*S: NS*	*T x O_2 : F=34.92 p<0.001*
- 5'	93 ± 2	94 ± 2	79 ± 3*$	78 ± 2*	*O_2: F=100.3*	
- 10'	93 ± 2	93 ± 2	79 ± 3*	79 ± 3*	*p<0.001*	*S: NS*
- 15'	93 ± 2	93 ± 2	80 ± 3*	79 ± 3*		*O_2: F=102.61 p<0.0001*
- End	93 ± 2	94 ± 2	80 ± 2*	80 ± 3*		*T: F=113.13 p<0.0001*
HR - SS						
- Start	72 ± 5	69 ± 3	72 ± 3	68 ± 3	*S: NS*	*T x O_2: F=14.54 p<0.001*
- 5'	122 ± 3	123 ± 3	136 ± 3	136 ± 3	*O_2: NS*	
- 10'	127 ± 3	129 ± 3	141 ± 3	143 ± 3		*S: NS*
- 15'	130 ± 3	132 ± 3	142 ± 2	146 ± 4		*O_2: F=44.19 p<0.0001*
- End	131 ± 3	134 ± 2	146 ± 3	148 ± 3		*T: F=530.72 p<0.0001*
HR - TT						
- Start	91 ± 3	95 ± 4	93 ± 2	94 ± 3	*S: NS*	*T x O_2: F=8.06 p<0.001*
- 5'	162 ± 2	165 ± 2	168 ± 3	168 ± 2	*O_2: NS*	
- 10'	170 ± 2	173 ± 2	172 ± 2	171 ± 2		*S: NS*
- 15'	175 ± 2	176 ± 2	173 ± 2	171 ± 2		*O_2: NS*
- End	180 ± 2	181 ± 2	177 ± 1	176 ± 1		*T: F=140.60 p<0.0001*
Lactate - SS						
- Start	1.4 ± .2	1.4 ± .2	1.5 ± .1	1.5 ± .1	*S: NS*	*T x O_2: F=34.34 p<0.001*
- 10'	1.1 ± .1	1.0 ± .1	1.9 ± .2	1.8 ± .2	*O_2: NS*	
- End	.9 ± .1	.8 ± .1	1.9 ± .2	2.0 ± .3		*S: NS*
						O_2: F=30.57 p<0.0001
						T: NS
Lactate - TT						
- Start	.9 ± .1	.8 ± .1	1.7 ± .2	1.7 ± .2	*S: NS*	*T x O_2: F=4.24 p=0.026*
- 10'	4.2 ± .6	4.5 ± .4	6.9 ± .8	7.2 ± .7	*O_2: F= 53.02*	
- End	6.5 ± .8	7.2 ± .8	8.9 ± .6	7.8 ± .7	*p<0.001*	*S: NS*
						O_2: F=22.90 p<0.0001
						T: F=109.44 p<0.0001
RPE- SS					*Wilcoxon*	*Wilcoxon*
- 5'	10 ± 1	11 ± 2	11 ± 2	11 ± 2		
- 10'	10 ± 1	11 ± 2	12 ± 2*	12 ± 2*		
- 15'	11 ± 1	11 ± 2	12 ± 2*	12 ± 2*		
- End	11 ± 1	11 ± 2	12 ± 2*	12 ± 2*		
RPE- TT					*Wilcoxon*	*Wilcoxon*
- 5'	14 ± 1	14 ± 1	16 ± 1*	16 ± 1*		
- 10'	15 ± 1	16 ± 1	17 ± 1*	17 ± 1*		
- 15'	17 ± 1	17 ± 1	18 ± 1	18 ± 1		
- End	18 ± 1	19 ± 1	19 ± 1	19 ± 1		
Work (kJ) performed						
- 5'						
- 10'	79.1 ± 9.2	79.5 ± 10.2	75.2 ± 10.0	74.7 ± 9.1	*S: NS*	*T x O_2: F=46.74 p<0.001*
- 15'	160.9 ± 20.9	162.8 ± 22.8	147.7 ± 18.7*	147.7 ± 19.1*	*O_2:F=46.67*	
- End	244.0 ± 34.0	246.2 ± 36.0	216.9 ± 27.5*	217.2 ± 28.4*	*p<0.0001*	*S: NS*
	327.5 ± 46.7	330.9 ± 49.9	287.2 ± 37.7*	288.3 ± 37.4*		*O_2: F=35.98 p<0.0001*
						T: F=651.3 p<0.0001

*PL: placebo, CF: cocoa flavanol, H: hypoxia, N: normoxia, SS: steady-state, TT: time trial, SaO_2: peripheral oxygen saturation, HR: heart rate, RPE: rate of perceived exertion. *: p<0.05: main effect of F_IO_2 (O_2) (H-PL compared to N-PL and H-CF compared to N-CF); £: p<0.05: main effect of supplement (S) CF compared to PL); $: p<0.05: main effect of time (T) (compared to previous timepoint)*

720 *Legends for figures*

721 *Figure 1. Interventional exercise protocol, twice executed in hypoxia and twice in normoxia, following 7 days of cocoa flavanol*
722 *(CF) or placebo (PL) intake. FMD: Flow mediated dilation. NIRS: Near infrared spectroscopy at M. vastus lateralis and*
723 *prefrontal cerebral cortex. SS: steady state, TT: time trial.*

724 *Figure 2. Effect of 7-day cocoa flavanol (CF, black lines) or placebo (PL, grey lines) supplementation in hypoxia (H, dashed lines*
725 *and normoxia (N, full lines) on plasma epicatechin (A) and plasma nitrite (B) concentrations. BL: baseline, before intake of the*
726 *last dose, Pre-SS: at the start of the 20-minute steady state exercise (45% of peak power output), pre-TT: at the start of the 20-min*
727 *time trial, post-TT: at the end of the 20-min time trial. Mean ± SE presented. £: p<0.05: main effect of supplement.*

728 *Figure 3. Effect of 7-day cocoa flavanol (CF, black lines) or placebo (PL, grey lines) supplementation in hypoxia (H, dashed*
729 *lines) and normoxia (N, full lines) on exercise-induced changes in plasma arginine:citrulline ratio (A), plasma malondialdehyde*
730 *concentration (MDA) (µmol/L) (B), plasma trolox equivalent antioxidant capacity (TEAC) (C) and plasma uric acid*
731 *concentration (UA) (µmol/L) (D). Pre-SS: at the start of the 20-minute steady state exercise (45% of peak power output), Post-*
732 *TT: at the end of the 20-min time trial. Mean ± SE presented. *: p<0.05: main effect of F_IO_2; £: p<0.05: main effect of*
733 *supplement; $: p<0.05: main effect of exercise.*

734 *Figure 4. Effect of 7-day cocoa flavanol (CF, black lines) or placebo (PL, grey lines) supplementation in hypoxia (H, dashed*
735 *lines) and normoxia (N, full lines) on tissue oxygenation (TSI, %) in the M. vastus lateralis (A) and prefrontal cerebral cortex (B).*
736 *0-20 min: steady state exercise (45% of peak power output), 20-25 min: passive rest in seated position on the bike, 25-45 min:*
737 *time trial. Mean ± SE presented. *: p<0.05: main effect of F_IO_2; £: p<0.05: main effect of supplement; $: p<0.05: main effect of*
738 *exercise.*

739