The impact of intensified training with a high or moderate carbohydrate feeding strategy on resting and exercise-induced oxidative stress

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Abstract

Purpose: This study investigated the impact of intensified training (IT) and carbohydrate (CHO) supplementation on resting and exercise-induced oxidative stress.

Methods: Male cyclists (n=13, mean ± SD: age 25 ± 6 years; $\dot{V}O_{2max}$ 72 ± 5 ml/kg/min) undertook two 9-day periods of endurance-based IT. In a counterbalanced, crossover and double-blinded study design, participants completed IT whilst ingesting high (H-CHO) or moderate (M-CHO) CHO beverages before (HCHO: 24g *vs*. M-CHO: 2g), during (HCHO: 60g/hour *vs*. M-CHO: 20g/hour) and after training sessions (HCHO: 44g *vs*. M-CHO: 10g). Participants completed fasted performance trials without CHO on days 2, 6 and 10. Blood samples were taken before and immediately after exercise to assess plasma oxidative stress.

Results: Resting thiol (-SH) and catalase (CAT) activities decreased following 6 days of IT, independent of CHO condition (-SH (μM oxidised NADPH): H-CHO – 14.0 ± 18.8, M-CHO – 20.4 ± 20.3 and CAT (nmol/min/ml): H-CHO – 12.5 ± 12.5, M-CHO – 6.0 ± 4.5; all $p<.05$). Resting total antioxidant capacity (TAC) was reduced after IT in M-CHO. All exercise bouts elicited significant increases in CAT, TAC, protein carbonylation (PC) and lipid hydroperoxides (LOOH), independent of CHO condition ($p<.05$). The magnitude of increase in PC and LOOH was greater on days 6 and 10 compared to day 2 in both conditions.

Conclusions: Short-term IT caused reductions in resting antioxidant capacity in trained cyclists. Exercise-induced increases in PC and LOOH were exaggerated as a result of IT; however, these responses were independent of carbohydrate intake before, during and after the preceding IT sessions.

Introduction

Periods of intensified training (IT) are commonly utilised by athletes throughout a season; however, excessive physical overload and or inadequate recovery can induce a state of overtraining (OT). Exercise of sufficient intensity (21) and duration (3) is accompanied by a transient increase in oxidative stress, whereby the production of reactive oxygen species (ROS) overwhelms their clearance by antioxidants (3, 41). ROS are known to have various roles in regulating tissue function (20, 32) and cellular signalling pathways in response to exercise that are involved in training adaptations (11, 17). Despite this, exercise-induced changes in ROS are thought to parallel the model of hormesis (33), whereby an optimal threshold between subtle control of cellular signals and aberrant oxidation of proteins, lipids and DNA occurs (30, 34). This threshold concept may exist in

OT athletes, where resting and acute exercise-induced oxidative stress are modulated following periods of aerobic (39) and resistance-based IT (22).

Previous studies have reported that resting markers of oxidative stress, such as plasma protein carbonylation (PC) (22, 39, 46) and lipid peroxidation (10, 22, 46) are elevated to a greater extent following IT periods of between 4-12 weeks, when compared with habitual training regimens. The effects of IT on antioxidant defences are more complex, with antioxidant enzyme activities increasing (22, 31) and total plasma antioxidant capacity (TAC) decreasing during these periods of IT (22, 31). Phagocytic cells (e.g. neutrophils, monocytes and macrophages) that infiltrate damaged muscle fibres and other active tissues during exercise may generate large quantities of ROS that deplete extracellular antioxidants, promoting the activity and release of endogenous antioxidants into the circulation. The resulting changes in resting oxidative stress may also modulate the acute oxidative stress response to exercise, with reports of exaggerated (31, 40) and suppressed (39) oxidative stress observed following acute exercise challenges in athletes undergoing IT. These inconsistencies can be attributed to the range of study designs utilised, with respect to the duration and intensity of the IT period, and also the timing of blood measures following the change in overload stimulus. To our knowledge, resting and exercise-induced oxidative stress has not been assessed in the immediate days following a period of training overload.

Nutritional strategies to modulate resting and acute changes in oxidative stress following exercise are common. Carbohydrate (CHO) ingestion is routinely used by athletes before, during and after exercise to improve physical performance, maintain a high average training workload and optimise recovery (15). In addition, CHO ingestion maintains intracellular reduced nicotinamide adenine dinucleotide phosphate (NADPH) concentrations, a coenzyme essential in buffering ROS production and potentially delaying muscle fatigue (9), via the conversion of six to five carbon sugars in the pentose phosphate pathway. Previous studies have reported both no differences (27, 42) and marked attenuations in oxidative stress following single bouts of sub-maximal exercise, whilst ingesting a high versus placebo (non-CHO) beverage (24, 25). These mixed findings may be attributed to the varying doses of CHO administered, along with other aspects of study design. To our knowledge, no studies have assessed the influence of CHO supplementation on markers of resting and exercise-induced oxidative stress during or following a period of IT. Using the recommended guidelines for the upper limit of CHO intake with exercise, Halson et al, 2004 reported that a high CHO intake before (6.4% CHO, providing 32g CHO), during (6.4% CHO, providing 32g CHO per hour) and after (20% CHO, providing 200g CHO) the training sessions of short term IT (i.e. 11 days), attenuated decrements in both physical and

psychological performance compared to a moderate carbohydrate beverage (2% CHO, providing 10g before, 10g per hour during and 20g CHO after exercise, respectively) (1, 13). This study demonstrates the significant physiological and psychological effects that the composition of pre-exercise CHO beverages can confer, beyond comparisons of CHO versus non-CHO placebo. Indeed, athletes now utilise highly specific guidelines (ACSM) on the required macronutrient intake before, during and after highly demanding exercise, to maintain physical workload and optimise performance (35). Accordingly, the aims of the present study were to investigate the impact of a short-term period of IT on resting and exercise-induced oxidative stress, and to assess these responses under conditions of high and moderate CHO intake during the IT period. We tested the hypothesis that IT would increase resting and exercise-induced changes in oxidative stress, with high carbohydrate consumption during the preceding training sessions dampening this response.

Keywords: Overtraining, antioxidant, reactive oxygen species, glucose, performance

Abbreviations:

ANOVA: Analysis of Variance, CAT: Catalase activity, M-CHO: Moderate carbohydrate group, CV: Coefficient of variance, DNA: deoxyribonucleic acid, DNPH: Dinitrophenylhydrazine, ELISA: Enzyme Linked Immunosorbent Assay, FRAP: Ferric Reducing Ability of Plasma, H-CHO: High carbohydrate group, HCl: Hydrochloric acid, HR_{MAX}: Maximum heart rate, HRP: Horseradish Peroxidase, IT: Intensified training, LOOH: Lipid Hydroperoxides, NaCl: Sodium Chloride, NADH: reduced nicotinamide adenine dinucleotide, OT: Overtraining, PC: Protein Carbonyl, ROS: Reactive oxygen species, SD: Standard deviation, TAC: Total antioxidant capacity, TBS: Tris-Buffered Saline, -SH: Total thiol activity, VCO_2: Carbon dioxide production, VO_2: Oxygen consumption, VO_{2MAX}: Maximum oxygen consumption.

Methods

This investigation formed part of a larger investigation assessing parameters of sleep quality, hormonal balance and immune function (18, 19, 38).

Participants

Thirteen healthy, highly trained males (mean ± SD: age 25 ± 6 y; body mass 69.7 ± 6.3 kg; body fat percentage 13.4 ± 4.1%; maximal oxygen consumption ($\dot{V}O_{2\ MAX}$) 72.2 ± 4.9 ml/kg/min) were recruited for this double-blind, placebo-controlled crossover design study. All participants gave their written informed consent and the investigation was approved by the Loughborough University ethical review committee. Inclusion criteria were based on participants being highly trained ($\dot{V}O_{2\ MAX}$ > 64ml/kg/min) experienced cyclists, engaging in at least 6 hours of cycling training per week. Participants were excluded if they were smokers or had a current illness or infection.

Study design

Each participant completed two 9-day periods of intensified training (IT), separated by a 15-day washout period (*Figure 1*). During one period of IT, participants were provided with high CHO beverage before, during and after every training session (H-CHO) with additional protein during recovery (*Table 1*). Participants also undertook an identical period of IT with a taste-matched moderate CHO beverage (M-CHO) with no protein during the recovery period. The two IT periods were completed in a randomised, counter-balanced order. During and after both periods of IT, each participant undertook a standardised 1 hour performance trial (PT) in an overnight-fasted state on days 2, 6 and 10 (i.e. the morning after the final IT session), with resting and acute changes in markers of oxidative stress assessed on those days. Participants were instructed to not take any supplements or anti-inflammatory drugs during the study. During the 15-day washout period, participants were encouraged to recover fully before returning to normal training.

Pre-trial assessments

All participants visited Loughborough University for determination of baseline demographics and $\dot{V}O_{2\ MAX}$. Height and weight were recorded (*Seca Alpha, Hamburg, Germany*) and body composition determined by DEXA at the Gatorade Sports Science Institute. An incremental cycle test to exhaustion on an electronically braked ergometer (Lode Excalibur Sport, Groningen, Netherlands) was used for determination of $\dot{V}O_{2\ MAX}$. Expired gas composition was monitored continually throughout the test and breath-by-breath analysis was performed automatically with a Moxus metabolic systems analyser (AEI Technologies Inc., Naperville, IL, USA). Heart rate (HR) was recorded continually using short range telemetry (Polar RS800CX, Polar, Kempele, Finland) and ratings of perceived exertion were recorded from participants during the final minute of each stage.

A respiratory exchange ratio >1.10 and a plateau in VO_2 despite increasing workload were used to confirm that $\dot{V}O_{2\,MAX}$ had been reached (14).

Participants fulfilling the inclusion criteria were provided with HR monitors (Suunto, Vantaa, Finland) and SRM power meters (SRM Shimano DA7900 PowerMeter). All participants were then requested to train for two weeks according to their normal programmes and to record this information in the training diaries provided (baseline training load). This information was used to generate the IT protocols. Baseline assessments of psychological well-being were also determined, using Daily Analysis of Life Demands of Athletes (DALDA B) (36) and Profile of Mood States (POMS-65) (12) questionnaires. These standardised questionnaires measured the manifestation of stress symptoms and mood state, respectively. No participants exhibited symptoms of overreaching during their pre-trial phase according to the guidelines set by Meeusen and colleagues (4).

Intensified training programme

Participants were prescribed an individualised training plan based on their baseline training load and the results obtained from their $\dot{V}O_{2\,MAX}$ test. During each period of IT, total training volume was doubled compared to baseline. Further, time spent training at high-intensity (>82% HR_{MAX}) was more than trebled. Participants were prescribed two training sessions per day, with every session including a component of high intensity exercise (82-95% HR_{MAX}). On days 1, 2, 5, 6 and 8 of both IT periods, participants attended supervised training sessions at Loughborough University. All other training was performed in their own time on a bicycle equipped with a power meter (SRM PowerMeter Shimano DA7950 compact, SRM International, Jülich, Germany) and HR continuously measured (Suunto, Valaa, Finland). Standardised training sessions were administered the day before each performance test. Mood-state profiles were re-assessed on days 6 and 10.

Nutritional Intervention

During both periods of IT, participants were provided with a specific volume and composition of CHO beverage before (< 15 minutes before exercise), during and after (< 10 minutes after exercise) every training session (*Table 1)*. The participants were instructed not to consume other sports nutrition supplements during the study period. The prescribed carbohydrate intake for the H-CHO condition reflected current ACSM guidelines on the upper range of recommended intakes for endurance exercise training sessions (60 g/h), including a high CHO and protein recovery beverage after training (35). In the M-CHO condition, participants were provided with a taste-matched moderate CHO beverage, providing 30 g/h during all training sessions. In addition, a

placebo pill (1 g cellulose) was provided alongside the post-exercise M-CHO beverage. Participants were informed that the purpose of the study was to monitor the impact of an amino acid recovery pill versus a beverage following exercise only. In non-training periods, participants completed weighed diet diaries for three days prior to (pre-trial and washout) and every day during each experimental period (H-CHO and M-CHO) in order to monitor daily energy and macronutrient intake. Participants were instructed to consume their normal diet throughout each training period and keep their diets as similar as possible between the two conditions.

Performance testing and blood sampling

Participants arrived at the laboratory at a standardised time between 06:30-08:30, following an overnight fast for the PT on days 2, 6, and 10. A rested venous blood sample (pre-exercise) was obtained by venepuncture from the antecubital vein of the arm and blood collected into vacutainers containing K_3EDTA anticoagulant (*Becton, Dickson & Company, Oxford, UK*). Following a 10-minute warm-up at a self-selected intensity, participants performed an all-out one hour PT on their own bicycles on a turbo trainer (CycleOps Flow). Heart rate was recorded continually during each PT using short range telemetry (Suunto, Valaa, Finland) and power was recorded with SRM power meters (SRM Shimano DA7900 PowerMeter). Participants were provided with a clock during the PT, but blinded their power output, cadence and heart rate data. A second blood sample was taken immediately following cessation of the PT (post-exercise). Blood was centrifuged at 15,600rpm (400*g*) for 10 minutes at 4°C. Plasma was then extracted and stored at -80 °C until further analysis.

Analytical Measures

Protein Carbonylation

PC was assessed by ELISA (4, 6). Samples were diluted in sodium carbonate buffer (50 mM, pH=9.2) to a concentration of 0.05 mg/ml. Samples and standards (50 µL) were added in triplicate to a 96 well NUNC maxisorb microtitre plate for 1 hour at room temperature. Bound protein was incubated in the dark for 1 hour at room temperature with 2, 4-dinitrophenylhydrazine (DNPH) (1 mM, in 2 M HCl). All wells were then blocked with TBS Tween (200 µL, 0.1%) overnight at 4°C. Wells were incubated with monoclonal mouse anti-DNP antibody (50 µL, 1:1000) for 2 hours at 37°C, followed by peroxidase conjugated rat anti-mouse IgE conjugated HRP (50µL, 1:5000) for 1 hour at room temperature. Substrate (0.5 M Citrate phosphate buffer (10 ml, pH=5), hydrogen peroxide (8 µl) and Ortho-Phenylenediamine tablet (2 mg); 50 µL) was added to each well and the reaction stopped using sulphuric acid (50 µL, 2 M). The absorbance of each sample was measured at 490 nm

(Varioskan Flash, Thermo Fisher Scientific, *Leicestershire, UK*) and quantified using absorbance values of PC standards made in our lab (1.94-4.91 nmol/mg of protein). Protein concentration was determined using the bicinchoninic assay method (37).

Lipid Hydroperoxides

Lipid hydroperoxide (LOOH) concentrations were assessed using a method originally developed by El-Saadini et al., 1989 (7). Briefly, plasma samples (10 µl) were incubated with a reagent mix (100 µL: 0.2 M Potassium phosphate (pH=6.2), 0.12 M potassium iodide, 0.15 mM sodium azide, Triton X (2g/l), alkylbenzyldimethylammonium (0.1 g/l), 10 µM ammonium molybdate and HPLC grade water) for 30 minutes at room temperature, in the dark. The assay measures the oxidative capacity of LOOH to convert iodide to iodine. Wells were read at 340 nm (Varioskan Flash, Thermo Fisher Scientific, *Leicestershire, UK*) and the concentration of LOOH (nM) determined using the Beer-Lambert Law (extinction co-efficient ε_{340} = 24600 /M/cm) (7).

Total Antioxidant Capacity

TAC was assessed using the Ferric Reducing Ability of Plasma (FRAP) assay, developed by Benzie et al., 1996 (2). Plasma samples (10 µL) and standards (10 µL) were incubated with FRAP reagent mix (300 mM sodium acetate (pH=3.6), 160 mM 2, 4, 6- tripyridyltriazin and 20 mM ferric chloride; 300 µL) for 8 minutes at room temperature, and then absorbance was measured at 650 nm (Varioskan Flash, Thermo Fisher Scientific, *Leicestershire, UK*). TAC values were obtained using absorbance values of known ascorbic acid concentrations (ascorbic acid, 0-1000 µM) and expressed as µM of antioxidant power relative to ascorbic acid (26).

Catalase Activity

The peroxidatic activity of Catalase was measured in plasma (diluted 1:1 with sample buffer [25 mM potassium phosphate, pH = 7.5, 1 mM EDTA and 0.1% Bovine Serum Albumin]), using a commercially available assay kit, according to the manufacturer's instructions (*Cayman Chemical*). Enzyme activity was assessed by incubating samples with methanol (100%), in presence of hydrogen peroxide (0.04 M) and formaldehyde formation measured. Absorbance values were measured at 540 nm (Varioskan Flash, Thermo Fisher Scientific, *Leicestershire, UK*) and compared with values of known formaldehyde concentration (0-75 µM).

Total Thiol Activity

Total thiol (-SH) activity was measured using a commercially available kit according to manufacturer instructions (ab83463, *Abcam, Cambridge, UK*). Briefly, plasma samples were adjusted to the lowest sample protein concentration (0.55 mg/ml protein in assay buffer) and added (50 µL) with a reaction mix (30 µL assay buffer + 8 µL 5,5'-dithiobis (2-nitrobenzoic) acid (DNTB) + 2 µl nicotinamide adenine dinucleotide phosphate (NADPH) per well) to a 96-well microtitre assay plate. Absorbance values were measured at 412 nm every 5 minutes for 25 minutes to determine the kinetics of the enzymatic reaction (Varioskan Flash, Thermo Fisher Scientific, *Leicestershire, UK*). -SH activity was obtained from a standard curve of known 5-thio-2-nitrobenzoic acid (TNB) concentrations (0-50 nmol/well).

Sample size calculation and Statistical Analysis

Power analyses using Gpower3 (8), with significance at .05 and power at .90, were conducted based upon results from previous studies (43–45) and preliminary pilot work assessing markers of oxidative stress following steady state exercise in young adults. Primary outcome measures of protein carbonylation, lipid peroxidation and total antioxidant capacity were used. A sample size of 13 participants was required to detect differences with an effect size of .24 (medium effect size).

Data Reduction and Statistical Analysis

Statistical analyses were performed using SPSS (PASW Statistics, 22.0). Shapiro-Wilk tests were used to investigate normal distribution and differences between variables at baseline were assessed using one-way analyses of variance (ANOVA). Total training volume and time spent exercising at high-intensity were assessed by paired sample t-tests. The influence of CHO ingestion (carbohydrate) on changes in acute (exercise) and resting (day) oxidative stress were assessed by 2*2*3 repeated-measures ANOVA (carbohydrate, exercise, day). Changes in performance (mean power (watts) and maximum heart rate (bpm)) and mood-state profiles (DALDA B and POMS-65 TMD) were assessed by 3*2 repeated-measures ANOVA (day, carbohydrate). Changes in macronutrient consumption (total energy intake (kcal), carbohydrate (grams/day(g/d)), fat (g/d), protein (g/d)) were assessed by 2*2 repeated-measures ANOVA (period (i.e. days 1-6 and days 7-9), carbohydrate). All post hoc analysis of the interaction effects was performed by a test of simple effects by pairwise comparisons, with

Bonferroni correction. Data which was not normally distributed was log transformed prior to statistical analyses (catalase activity and LOOH). Statistical significance was accepted at the $p<0.05$ level.

Results

Changes in exercise volume, macronutrient intake and health & fitness parameters

Total training volume for the 9 day period of IT increased from 9.3 ± 2.4 h/week (baseline training load) to 23.5 ± 3.4 h/week ($p<0.0001$), and time spent exercising at high-intensity (>82% HR_{MAX}) increased from 2.6 ± 2.5 h/week (baseline training load) to 6.5 ± 4.0 h/week ($p<.0001$). No statistical differences in these parameters were observed between M-CHO and H-CHO conditions.

Changes in daily macronutrient intake are reported in Table 3. Daily average CHO and total energy intake were significantly lower in the M-CHO condition compared with the H-CHO condition (carbohydrate effects: all $p<0.039$). Average CHO, sugar, protein, and total energy intake increased during days 7-9, relative to days 1-6 (period effects: $p<0.016$). No differences were observed in fat intake between H-CHO and M-CHO conditions or across the periods assessed. Period*Carbohydrate interaction effects were observed for changes in daily average CHO consumption ($F_{1,9} = 11.2$, $p=0.009$) and total energy intake ($F_{1,9} = 5.7$, $p=0.041$). Pairwise comparisons revealed that average CHO ($p=0.02$) and total energy intake ($p=0.029$) during days 7-9 was significantly greater in H-CHO, relative to M-CHO.

No significant changes in the participants' body mass were noted across either period of IT (mean body mass change: +0.3 ± 1.0 kg (H-CHO) and -0.5 ± 0.9 kg (M-CHO)).

Resting changes in markers of plasma oxidative stress

Changes in resting concentrations of PC, LOOH and TAC, and enzyme activities of CAT and –SH following 9 days of IT are shown for both H-CHO and M-CHO conditions in Figure 2 (panels 1 and 2: A-E). LogCAT ($p<0.0001$) and –SH ($p=0.003$) activities significantly decreased on day 6, relative to day 2 in the H-CHO and M-CHO conditions (day effects, LogCAT: $F_{2,24} = 16.9$, $p<0.0001$ and –SH: $F_{2,24} = 6.1$, $p=0.007$). Only LogCAT remained supressed, relative to day 2 on day 10 in the H-CHO and M-CHO conditions ($p=0.003$). PC and LogLOOH were unaltered in response to IT in both H-CHO and M-CHO conditions. TAC values were significantly lower in the M-CHO condition on day 10 (Day*CHO interaction effect: $F_{2,24} = 3.6$, $p=0.044$), relative to day 2 ($p=0.048$) and day 6 ($p<0.0001$). TAC was lower on day 6 (-53.8 µM, $p=0.160$), relative to day 2 in the H-CHO condition, although this did not reach statistical significance.

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293 ***Changes in exercise-induced plasma oxidative stress***

294 Exercise-induced changes in concentrations of PC, LOOH and TAC, and enzyme activities of CAT and
295 –SH following 9 days of IT are shown for both H-CHO and M-CHO conditions in Figure 2 (panels 1 and 2: A-
296 E). Exercise stimulated increases in PC, LogLOOH, TAC and LogCAT on all days and in both H-CHO and M-
297 CHO conditions (time effects, PC: $F_{1,12} = 16.9$, $p=0.014$; LogLOOH: $F_{2,24} = 6.1$, $p=0.007$; TAC: $F_{2,24} = 16.9$,
298 $p<0.0001$ and LogCAT: $F_{2,24} = 6.1$, $p=0.007$). Acute changes in TAC, CAT and –SH activities were not
299 influenced by IT or CHO condition. Day*Time interaction effects were observed for changes in PC ($F_{2,24} = 7.1$,
300 $p=0.004$) and LogLOOH ($F_{2,24} = 6.0$, $p=0.008$). Pairwise comparisons revealed that the increases in LogLOOH
301 on day 6 ($p=0.001$) and day 10 ($p=0.05$) were greater than on day 2. Increases in PC were greater on day 6
302 ($p=0.001$) than days 2 and 10, however post-exercise PC concentrations on day 6 ($p=0.006$) and day 10
303 ($p=0.050$) were greater than on day 2. A Day*Time*CHO interaction effect was observed for LogLOOH ($F_{2,24} =$
304 4.0, $p=0.032$), although pairwise comparisons revealed no statistical differences in the responses in H-CHO and
305 M-CHO conditions on different days.

306

307 ***Physical performance and psychological mood profiles***

308 Parameters of physical performance are indicated in Table 2. Average power output ($F_{2,24} = .846$,
309 $p=0.442$) during the 1 hour PT was not different on days 2, 6 and 10. Maximum heart rate during the PT was
310 lower on days 6 ($p<0.001$) and 10 ($p=0.011$), relative to day 2 (Day effect: $F_{2,24} = 10.6$, $p<0.001$). All
311 performance and physiological parameters were independent of carbohydrate ingestion during training.

312 IT resulted in significant changes in mood state throughout both IT periods, as assessed by DALDA
313 (Day effect: $F_{2,22} = 21.1$, $p<0.0001$) and POMS-65 TMD scores (Day effect: $F_{2,24} = 12.4$, $p<0.0001$). Both scores
314 increased from day 2 to day 6 (DALDA: +5.1, POMS-65: +19.4, both $p<0.016$) and remained elevated above
315 day 2, at day 10 (DALDA: +5.2, POMS-65: +21.7, both $p<0.0001$). Both these changes were independent of M-
316 CHO and H-CHO conditions.

317

318 **Discussion**

319 Studies investigating changes in oxidative stress in response to IT have assessed the effects of training
320 overload over the course of months, taking either observational approaches (10, 39) or implementing generic IT
321 regimens (22, 40). Based on baseline training loads, the present study implemented personalised IT regimens of

increased volume (h/week: +153%) and intensity (>82% HR_{MAX}: +146%), to assess the short-term effects of training overload on parameters of resting and exercise-induced oxidative stress in trained male cyclists. The influence of high and moderate carbohydrate ingestion before, during and after IT sessions was also assessed. It was shown that a short-term period of IT caused significant reductions in resting antioxidant capacity after 6 days. In addition, the magnitude of post-exercise increases in protein and lipid markers of oxidative stress was greater on days 6 and 10, relative to baseline. These changes were independent of the carbohydrate composition of beverages ingested before, during or after the IT sessions.

Previous studies have reported that periods of IT, or participants described as OT have higher resting levels of PC (22, 39), lipid peroxidation products (10, 22) and antioxidant enzyme activity (22, 31). In addition, many studies have found that plasma TAC is lower than in non-OT participants (22, 31). The current study presents evidence that 9 days of intensified cycling training lowered resting plasma catalase and thiol activities by day 6 and TAC by day 10 ($p<0.05$; M-CHO only); however, no alterations were observed in resting PC or LOOH concentrations (*Figure 2)*. The differential pattern of antioxidant response may be explained, in part, by the wide variety of study designs that have been used, including both endurance (31) and/or resistance based programmes (22). Furthermore, the current study assessed resting markers of oxidative stress much closer to the change in overload stimulus (days, as opposed to weeks or months). Between days 2 and 6 of IT, overload appears to have caused a marked initial rise in ROS that subsequently exhausted catalase enzyme function and depleted plasma thiols.

The maintenance of PC and LOOH concentrations during and following IT may be explained by the training status of the participants. Palazetti et al., showed no changes in resting thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation, in response to 4 weeks of endurance-based IT in highly trained triathletes ($\dot{V}O_{2max}$: 66 ± 4 ml/kg/min) (31). Higher aerobic fitness and endogenous antioxidant capacity in trained athletes (11, 16) may enable a buffering of protein and lipid biomarker formation during IT, with formation greater in less well trained participants (10, 22, 39). The vast array of antioxidant enzymes controlling redox balance makes the precise antioxidant mechanisms modulating PC and LOOH in the current study hard to elucidate. We can speculate that elevated enzyme efficiency as a result of higher training status (11, 16) or activation of antioxidant enzymes not measured (i.e. superoxide dismutase and glutathione peroxidase) might explain the apparent paradoxical depletion in catalase and thiol activity and maintenance of PC and LOOH at baseline levels. It must also be reemphasised that the period of IT assessed was much shorter than previous

studies (22, 31), suggesting that heightened PC and LOOH formation may occur further along the overtraining continuum (10, 22, 39).

The current study assessed exercise-induced changes in oxidative stress in response to three fasted PTs, both during (days 2 and 6) and immediately after the period of IT (day 10). All PTs elicited significant increases in oxidative stress (CAT, TAC, PC and LOOH), with the magnitude of increase in PC and LOOH greater on days 6 and 10, relative to day 2. Some previous investigations have shown that a period of IT can exaggerate acute increases in oxidative stress (31, 40). Indeed, lower resting thiol and catalase activities in the current study may have provided less protection against the formation of PC and LOOH following PTs on days 6 and 10 respectively. Interestingly, the magnitude of increase in PC was greater on day 6, than on day 10, suggesting a degree of habituation to training overload by the end of the IT period. It must be noted that other studies in OT humans (39) and animals (28, 29) have shown supressed oxidative stress following a single bout of exercise, indicative of a maladaptive response. Overtraining is a complex phenomenon, which is hard to classify with a single variable. Indeed, data from the current study reports that IT elicited decrements in psychological mood state, but did not significantly affect exercise performance (Table 2), making it hard to specify whether participants were OT *per se*. Therefore, the discrepancies in findings between various investigations may be explained by fundamental differences in study design and classifications of training overload and overtraining.

An additional aim of the current study was to assess the impact of high (H-CHO) or moderate (M-CHO) CHO ingestion before, during and after IT sessions on resting and exercise-induced oxidative stress. Carbohydrate composition of the IT beverages had no impact on subsequent oxidative stress responses to any of the fasted PT's, supporting some previous work reporting no effect of CHO intake on exercise-induced oxidative stress (27, 42). With regards to changes in resting oxidative stress, we did observe that high CHO consumption before, during and after IT sessions (H-CHO) prevented the decline in resting TAC by day 10. The decrease in TAC between days 6 and 10 in M-CHO suggests that non-enzymatic antioxidants were strongly consumed during this IT period, supporting results from previous IT studies (22, 31). Higher CHO (44 g *vs.* 10 g) and also protein intake (17 g *vs.* 0 g) in the immediate recovery period following each IT session explains, in part, the maintenance of plasma antioxidant status in H-CHO and not M-CHO. Furthermore, daily energy intake and CHO consumption (g/day) between days 7 and 9 was also significantly greater in the H-CHO condition (Table 3). Additional adenosine triphosphate (ATP) supply during IT-induced metabolic stress, may have maintained plasma uric acid concentrations, a degradation product of ATP which largely contributes (approximately 60%) to plasma TAC status (5).

This study is not without limitations. The moderate carbohydrate condition ingested a significant amount of carbohydrate throughout the 9-day period of IT, despite lower daily intakes compared to the high carbohydrate group (M-CHO: 8.21 g/kg of body weight vs. H-CHO: 9.67 g/kg body weight). Given that the period of administered training overload was not deemed overtraining *per se*, this dosage of carbohydrate may have been sufficient to modulate changes in oxidative stress as result of IT. In addition, we must acknowledge that the additional protein added to the recovery beverage in the H-CHO condition only (Table 1) complicated aspects of the study design. No condition-dependent effects were observed with regards to changes in resting markers of oxidative stress between days 2 and 6. However, the additional protein in the H-CHO condition may have aided the regeneration of intracellular antioxidants, such as glutathione following exercise (23). This makes it hard to elucidate the precise mechanism behind the maintenance of plasma TAC in this condition.

Conclusions

The present data demonstrates that a 9-day period of IT, in which training volume and intensity were markedly increased, caused reductions in resting plasma catalase and thiol activities and exacerbated acute increases in protein carbonylation and lipid peroxidation following exhaustive exercise. Importantly, resting and exercise-induced changes in these markers was not influenced by the different carbohydrate beverages ingested before, during and after training sessions.

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Conflict of Interest

None of the authors declare a conflict of interest.

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Table Legends

Table 1: **Composition of the high and moderate carbohydrate training solutions**

	High Carbohydrate			Moderate Carbohydrate		
	Pre	**During**	**Post**	**Pre**	**During**	**Post**
Energy (Kcal)	98	250	250	12	100	50
Carbohydrate (g)	24	60	44	2	20	10
Protein (g)	0	0	17	0	0	0

Table 1 Footnote: Values are in Kcal (energy of solution) and grams (carbohydrate and protein content) for the pre, during and post-exercise solutions.

Table 2: **Changes in performance trial parameters**

	High Carbohydrate			Moderate Carbohydrate		
	Day 2	**Day 6**	**Day 10**	**Day 2**	**Day 6**	**Day 10**
Average Workload (Watts)	254 ± 35	249 ± 33	253 ± 32	247 ± 34	238 ± 36	240 ± 34
Maximum Heart Rate (bpm)	158 ± 13	148 ± 15***	150 ± 13*	159 ± 13	148 ± 15***	158 ± 14*

Table 2 Footnote: Values are means ± standard deviation * indicates significant difference, relative to day 2: * $p<.05$; *** $p<.0001$).

Table 3: **Changes in macronutrient (total energy intake, carbohydrate, fat, sugar and protein) ingestion throughout the 9 day period of IT**

	High Carbohydrate		Moderate Carbohydrate	
	Days 1-6	**Days 7-9**	**Days 1-6**	**Days 7-9**
Total Energy Intake (Kcal/day)	3787 ± 277	4597 ± 372*$^{+}$	3524 ± 237	3864 ± 289*
Carbohydrate (g/day)	611 ± 42	738 ± 50*$^{+}$	552 ± 36	593 ± 38*
Fat (g/day)	91 ± 10	110 ± 12	91 ± 12	104 ± 13
Sugar (g/day)	310 ± 37	379 ± 38*$^{+}$	237 ± 28	261 ± 34*
Protein (g/day)	127 ± 12	155 ± 21*$^{+}$	115 ± 10	134 ± 12*

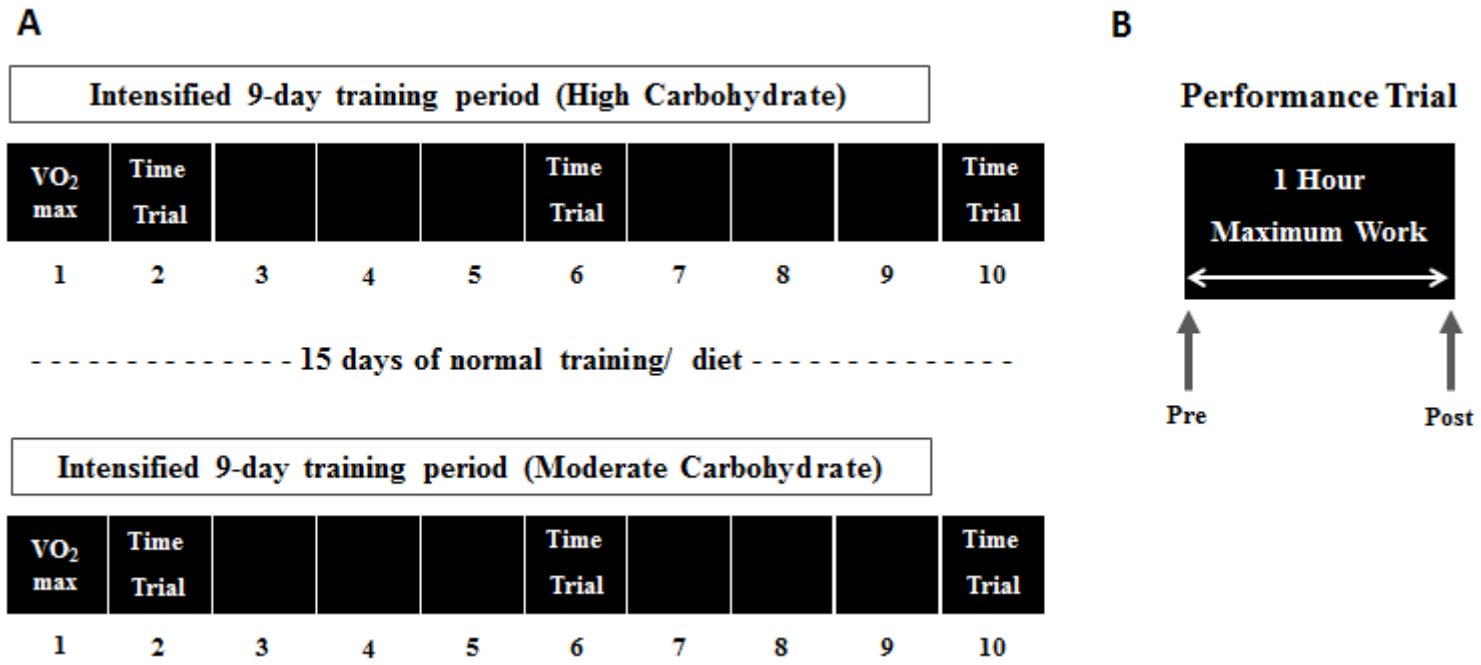
Table 3 Footnote: Values are average daily intake ± standard deviation * indicates significant differences in macronutrient ingestion on days 7-9, relative to days 1-6: * $p<.05$). $^{+}$ indicates significant differences between the H-CHO and M-CHO conditions: $^{+}$ $p<.05$).

Figure Legends

Fig 1: **Schematic representation of the study design. A:** In this randomized crossover and double-blinded study design, participants engaged in two 9-day periods of intensified exercise training whilst ingesting high (H-CHO) or moderate (M-CHO) carbohydrate solutions before, during and after each training session. Conditions were separated by 15 days of normal training/ diet. A VO_{2MAX} test was carried out on day 1. Numbers 1-10 represent days. **B:** Blood samples (↑) were taken before (pre) and immediately after (post) a 1 hour performance trial (days 2, 6 and 10) in a fasted state.

Fig 2: **Changes in resting and exercise-induced changes in plasma markers of oxidative stress**. Bars represent nmol/mg protein (PC, 1A), nM (LOOH, 1B), uM (TAC, 1C), nmol/min/ml (LogCAT, 1D) and uM oxidised NADPH (-SH, 1E) in plasma before (pre-exercise) and after exercise (post-exercise) on day 2 (black bars), day 6 (white bars) and day 10 (grey bars). Values are means ± standard error. $^{+}$ indicates a significant difference in resting levels, relative to day 2: $^{+}$ p<.05; $^{++}$ p<.01; $^{+++}$ p<.0001. $^{\#}$ indicates a significant difference in resting levels, relative to day 6: $^{\#\#\#}$ p<.0001. * indicates significant differences post-exercise, relative to baseline: * p<.05; ** p<.01; *** p<.0001. $^{\$}$ indicates a significant difference between post-exercise levels, relative to day 2: $^{\$}$ p<.05; $^{\$\$}$ p<.01.

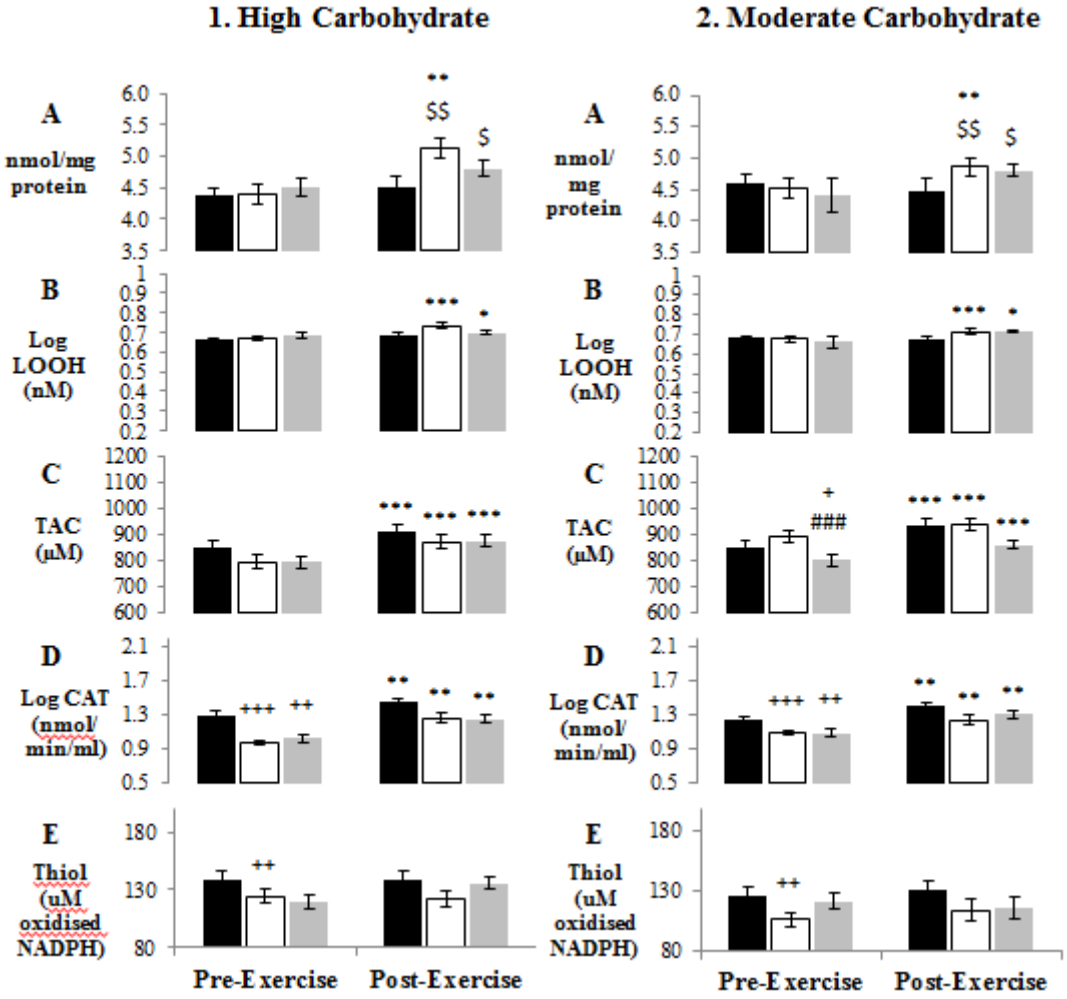
Figure One


A
Intensified 9-day training period (High Carbohydrate)
VO2 max
Time Trial
Time Trial
Time Trial
1 2 3 4 5 6 7 8 9 10
15 days of normal training/ diet
Intensified 9-day training period (Moderate Carbohydrate)
VO2 max
Time Trial
Time Trial
Time Trial
1 2 3 4 5 6 7 8 9 10
B
Performance Trial
1 Hour
Maximum Work
Pre
Post

625

Figure Two

626



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