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Carbohydrate improves exercise capacity but does not affect subcellular lipid droplet morphology, AMPK and p53 signalling in human skeletal muscle

J. Marc Fell, Mark A. Hearris, Daniel G. Ellis, James E. P. Moran [iD], Emily F. P. Jevons [iD], Daniel J. Owens [iD], Juliette A. Strauss [iD], Matthew Cocks [iD], Julien B. Louis, Sam O. Shepherd [iD] and James P. Morton [iD]

Research Institute for Sport and Exercise Sciences (RISES), Liverpool John Moores University, Byrom Street, Liverpool, L3 3AF, UK

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Key points

- Muscle glycogen and intramuscular triglycerides (IMTG, stored in lipid droplets) are important energy substrates during prolonged exercise.
- Exercise-induced changes in lipid droplet (LD) morphology (i.e. LD size and number) have not yet been studied under nutritional conditions typically adopted by elite endurance athletes, that is, after carbohydrate (CHO) loading and CHO feeding during exercise.
- We report for the first time that exercise reduces IMTG content in both central and peripheral regions of type I and IIa fibres, reflective of decreased LD number in both fibre types whereas reductions in LD size were exclusive to type I fibres.
- Additionally, CHO feeding does not alter subcellular IMTG utilisation, LD morphology or muscle glycogen utilisation in type I or IIa/II fibres.
- In the absence of alterations to muscle fuel selection, CHO feeding does not attenuate cell signalling pathways with regulatory roles in mitochondrial biogenesis.

Abstract We examined the effects of carbohydrate (CHO) feeding on lipid droplet (LD) morphology, muscle glycogen utilisation and exercise-induced skeletal muscle cell signalling. After a 36 h CHO loading protocol and pre-exercise meal (12 and 2 g kg^{-1}, respectively), eight trained males ingested 0, 45 or 90 g CHO h^{-1} during 180 min cycling at lactate threshold followed by an exercise capacity test (150% lactate threshold). Muscle biopsies were obtained pre- and post-completion of submaximal exercise. Exercise decreased ($P < 0.01$) glycogen concentration to comparable levels (∼700 to 250 mmol kg^{-1} DW), though utilisation was greater in type I (∼40%) *versus* type II fibres (∼10%) ($P < 0.01$). LD content decreased in type I (∼50%) and type IIa fibres (∼30%) ($P < 0.01$), with greater utilisation in type I fibres ($P < 0.01$). CHO feeding did not affect glycogen or IMTG utilisation in type I or II fibres (all $P > 0.05$). Exercise decreased LD number within central and peripheral regions of both type I and IIa fibres, though reduced LD size was exclusive to type I

J. Marc Fell recently completed his PhD in exercise metabolism at Liverpool John Moores University. The data presented in this paper was collected as part of his PhD programme and he hopes to further study the exercise-induced regulation of carbohydrate and fat metabolism throughout his career. Alongside his research, he is also an accredited sports nutritionist with the UK Sport and Exercise Nutrition Register and is experienced in delivering nutrition support to athletes from team sports, track and field and endurance road cycling.

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fibres. Exercise induced (all $P < 0.05$) comparable AMPK^{Thr172} ($\sim$4-fold), p53^{Ser15} ($\sim$2-fold) and CaMKII^{Thr268} phosphorylation ($\sim$2-fold) with no effects of CHO feeding (all $P > 0.05$). CHO increased exercise capacity where 90 g h^{-1} (233 ± 133 s) > 45 g h^{-1} (156 ± 66 s; $P = 0.06$) > 0 g h^{-1} (108 ± 54 s; $P = 0.03$). In conditions of high pre-exercise CHO availability, we conclude CHO feeding does not influence exercise-induced changes in LD morphology, glycogen utilisation or cell signalling pathways with regulatory roles in mitochondrial biogenesis.

Introduction

Muscle glycogen and intramuscular triglycerides (IMTG) provide important energy substrates during prolonged endurance exercise (Van Loon, 2004). In contrast to glycogen, our understanding of IMTG storage and metabolism during exercise is not as advanced and is often complicated by the contrasting methodologies that have been used to quantify IMTG content, that is, biochemical triacylglycerol extraction, high-resolution electron microscopy or immunofluorescence microscopy. Using the latter method, our laboratory (Shepherd *et al.* 2013; Jevons *et al.* 2020) and others (Van Loon *et al.* 2003*a*,*b*; De Bock *et al.* 2005; Stellingwerff *et al.* 2007*a*) have observed that resting IMTG content is approximately twofold greater in type I fibres compared with type IIa fibres, a reflection of both a greater number and size of IMTG-containing lipid droplets (LD) (Shepherd *et al.* 2013; Jevons *et al.* 2020).

In relation to exercise, reductions in IMTG content predominantly occur in type I fibres with smaller changes in II/IIa fibres (Van Loon *et al.* 2003*a*,*b*; De Bock *et al.* 2005; Stellingwerff *et al.* 2007*a*,*b*; Van Proeyen *et al.* 2011; Shepherd *et al.* 2013; Jevons *et al.* 2020). We recently reported that the reduction of IMTG within type I fibres is underpinned by reduced LD number with no difference in LD size (Jevons *et al.* 2020), though previous researchers have observed reductions in both LD size and number (Stellingwerff *et al.* 2007*a*). From a subcellular perspective, we also observed that prolonged endurance exercise reduces IMTG content in the central region (indicative of the intermyofibrillar region) of type I fibres but not the peripheral (i.e. subsarcolemmal) region (Jevons *et al.* 2020). It is noteworthy, however, that different methodological protocols may affect our interpretation of the subcellular utilisation patterns of IMTG, given that the choice of lipid dye (Oil Red O *versus* BODIPY) and chosen size of peripheral region (i.e. 2 *versus* 5 μm) may contribute additional artefacts upon the quantification of changes in substrate pools during exercise (Jevons *et al.* 2020; Strauss *et al.* 2020). Collectively, such data demonstrate that further research is

required to further characterise the exercise-induced subcellular changes in LD morphology within specific fibre types.

The regulation of muscle fuel selection during exercise has often been studied using exercise protocols commenced in the fasted state (Romijn *et al.* 1993; Van Loon *et al.* 2001). However, given the ergogenic effects of CHO on exercise performance (Hawley *et al.* 1997; Stellingwerff & Cox, 2014), such an approach often contrasts with the nutritional practices adopted by athletic populations. Indeed, athletes will increase CHO availability in the day(s) and hours before competition so as to commence exercise with sufficient muscle and liver glycogen stores (Burke *et al.* 2018). In addition, it is now an established practice for elite endurance athletes to also consume CHO during exercise, with contemporary guidelines recommending CHO ingestion rates up to 90 gh^{-1} when exercise is greater than 2.5–3 h in duration (Thomas *et al.* 2016). Although it is generally accepted that CHO feeding induces ergogenic effects via liver glycogen sparing (as opposed to muscle glycogen sparing; Coyle *et al.* 1986; Gonzalez *et al.* 2015), the effects of CHO feeding on fibre-specific IMTG utilisation during exercise are conflicting. For example, De Bock *et al.* (2005) observed an attenuated use of IMTG in type I muscle fibres when CHO was consumed before ($\sim$150 g) and during 2 h moderate intensity cycling ($\sim$75 g h^{-1}) when compared with exercise completed in the fasted state. In the absence of a pre-exercise meal, however, Stellingwerff *et al.* (2007*a*) observed no effect of CHO feeding ($\sim$50 g h^{-1}) during 3 h of exercise upon net utilisation of IMTG stores in either type I or II muscle fibres. As such, it is possible that the effects of CHO feeding on the down regulation of IMTG utilisation are only apparent in the presence of a pre-exercise meal and/or when a larger absolute CHO intake has been ingested. When taken together, such studies clearly demonstrate the importance of isolating the potential regulatory effects of CHO feeding to that consumed 'during' exercise as opposed to that induced by the pre-exercise meal. Additionally,

the effects of CHO intake during exercise on the subcellular changes in LD morphology have not yet been studied.

Although CHO feeding is well established as ergogenic for exercise performance (Stellingwerff & Cox, 2014), there is a growing body of literature demonstrating that CHO restriction before, during and/or after exercise can up-regulate cell signalling pathways with putative roles in training adaptation (Impey *et al.* 2018). The practical application of such findings has been communicated according to the train-low, compete-high paradigm surmising that beneficial metabolic adaptations may be induced by carefully selected periods of CHO restriction around certain training sessions but that competition (and indeed, high-intensity training sessions) should always be commenced with high CHO availability (Impey *et al.* 2018). We (Morton *et al.* 2009) and others (De Bock *et al.* 2005; Akerstrom *et al.* 2006; Lee-Young *et al.* 2006) have observed that CHO ingestion before and/or during exercise may attenuate acute skeletal muscle cell signalling and gene expression as well as training-induced oxidative adaptations of human skeletal muscle. However, such regulatory effects of CHO ingestion during exercise may only manifest if the CHO feeding strategy has induced muscle glycogen sparing (Akerstrom *et al.* 2006; Lee-Young *et al.* 2006). Indeed, recent data from our laboratory support the hypothesis that the enhanced adaptations associated with train-low models of CHO restriction are particularly dependent on the absolute post-exercise glycogen concentration (Hearris *et al.* 2019, 2020). Accordingly, it is possible that consuming CHO during exercise induces negligible effects on cell signalling and training adaptation providing that no alterations to muscle fuel selection and utilisation have actually occurred.

With this in mind, the aim of the present study was to test the following hypotheses: (1) CHO feeding during prolonged endurance exercise does not affect IMTG (and LD morphology) and muscle glycogen utilisation in both type I and type II muscle fibres, (2) CHO feeding does not impair the activation of skeletal muscle cell signalling pathways with regulatory roles in training adaptation and, (3) CHO feeding improves exercise capacity in a dose dependent manner. To this end, trained male cyclists completed 180 min of steady-state submaximal exercise (followed by an exhaustive high-intensity capacity test) during which they consumed 0, 45 or 90 g CHO h^{-1}, comprising a mixture of fluids, solids and gels. Importantly, each experimental trial was commenced after a 36-h CHO loading protocol (12 g kg^{-1}) and 3 h after consumption of a pre-exercise meal that was rich in CHO (2 g kg^{-1}). In this way, our experimental approach allowed us to isolate the potential regulatory effects of CHO to that consumed during exercise as opposed to that induced by pre-exercise feeding.

Methods

Ethical approval

All participants gave written informed consent prior to participation after all experimental procedures and potential risks had been fully explained. The study was approved by the Ethics Committee of Liverpool John Moores University (Ethics No: 16 SPS 030) and conformed to the standards set by the latest revision of the *Declaration of Helsinki* (except for registration in a database).

Participants

Eight endurance-trained amateur male cyclists (mean ± SD: age, 31 ± 7 years; body mass 73.6 ± 6.1 kg; height, 177.0 ± 8.0 cm) volunteered to participate in the study. Mean $\dot{V}_{O_2 max}$, peak power output (PPO) and power output at lactate threshold (LT) were 60.5 ± 5.4 ml $kg^{-1}min^{-1}$, 383 ± 21 W and 208 ± 11 W, respectively. Subjects were defined as trained in accordance with the criteria specified by Jeukendrup *et al.* (2000). None of the subjects had any history of musculoskeletal or neurological disease nor were they under any pharmacological treatment or using any nutritional supplements in the lead up to and during the course of the testing period.

Experimental overview

In a repeated-measures, randomised, cross-over design separated by 7–8 days, participants completed four prolonged endurance-based cycling exercise protocols which consisted of 180 min steady-state submaximal exercise (undertaken at LT) followed by a exercise capacity test to exhaustion (undertaken at 150% of LT). The initial trial was a full familiarisation with the exercise protocol (where water only was consumed during exercise) and in the following three trials, subjects ingested CHO at a rate of 0, 45 or 90 g h^{-1} in the form of solids, gels and fluids during the exercise bouts. These CHO ingestion regimens replicated the typical feeding patterns of professional cyclists during competition (Heikura *et al.* 2019; Muros *et al.* 2019). Each experimental trial was commenced after a 36-h CHO loading protocol (12 g kg^{-1}) and 3 h after consumption of a pre-exercise meal that was rich in CHO (2 g kg^{-1}). Muscle biopsies were obtained from the m. vastus lateralis immediately before and at the end of the 180 min submaximal cycle (the 180 min time point biopsy was obtained prior to commencing the

exercise capacity test). An overview of the experimental design and nutritional protocols are displayed in Fig. 1.

Preliminary testing

At least 7 days prior to experimental trials, all subjects performed a two-part incremental cycle test (Lode Excalibur Sport, Groningen, Netherlands) to determine LT, maximal oxygen consumption ($\dot{V}_{O_2 max}$) and PPO (similar to that described in Newell *et al.* 2015). Briefly, the first part of the incremental cycling test was commenced at 125 W and each stage increased by 25 W every 4 min with a capillary blood sample (fingertip) being obtained for blood lactate concentration analysis by micro-assay (Lactate Pro 2, ArkRay Inc., Kyoto, Japan) during the last 30 s of each stage. The LT and lactate turn-point (LTP) were defined as the first and second sustained increases in blood lactate above baseline values (Carter *et al.* 1999). The mean ($\pm$ SD) lactate concentration at LT was 1.7 ± 0.3 mmol l^{-1} corresponding to an intensity of $55 \pm 3\%$ of PPO and $64 \pm 3\%$ of $\dot{V}_{O_2 max}$. The test end time and power output of the final stage from the second part of the incremental cycling test was used to calculate PPO using the following equation (Kuipers *et al.* 1985):

$$\text{PPO} = W_{final} + ([t/60] \cdot \text{PI}),$$

where W_{final} is the power output of the final completed stage in watts, t is time spent in the final uncompleted stage (seconds), 60 is the duration of each stage (seconds) and PI is the increase in power output between each stage (W). $\dot{V}_{O_2 max}$ was assessed via an automated online gas analyser (Moxus Modular Metabolic System, AEI Technologies, Pittsburgh, PA, USA) and was determined as the highest average $\dot{V}_{O_2}$ captured over a 30 s period. The same automated online gas analyser was used in all subsequent trials.

Familiarisation and experimental protocols

At least 7 days prior to the main experimental trials, subjects undertook a full familiarisation with the experimental trial (detailed below) where they refrained from CHO intake during the submaximal cycling protocol, thus replicating the same nutritional conditions as per the 0 g h^{-1} trial (without blood samples and muscle biopsies being taken). The familiarisation trial served to
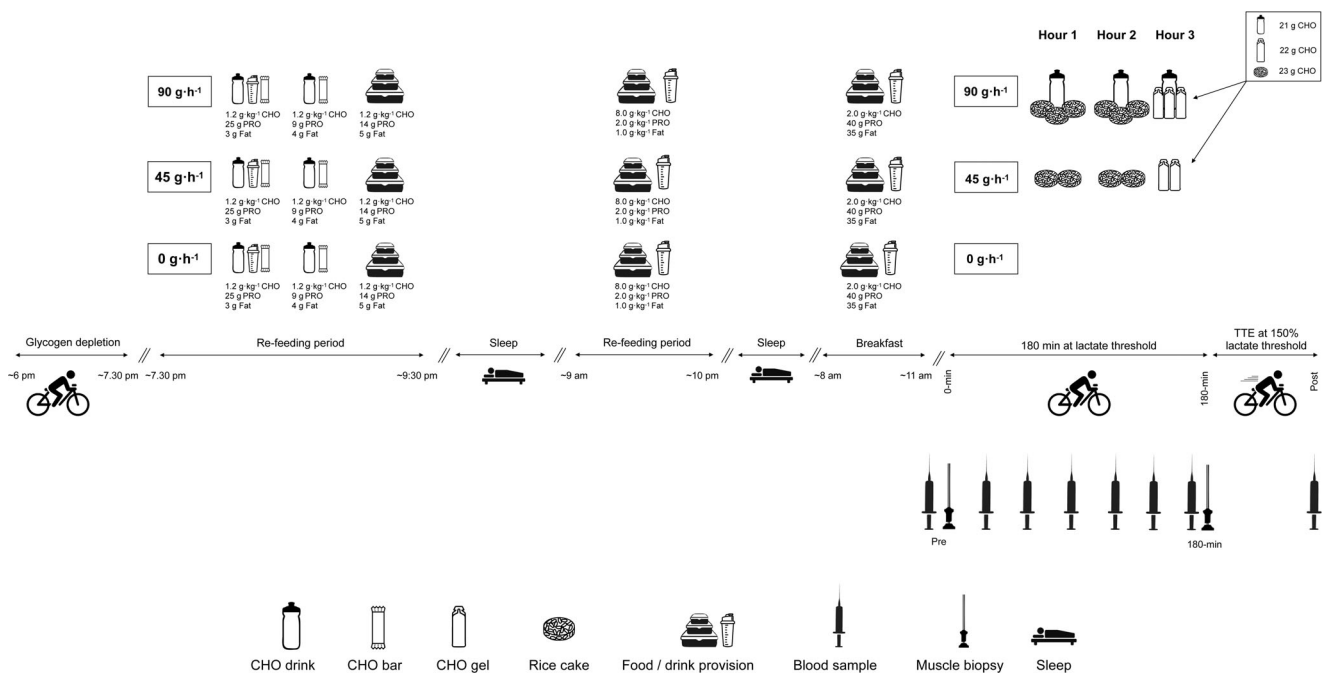
Figure 1. Schematic overview of the experimental protocol employed in each trial
On the evening of day 1, participants completed a glycogen depleting cycling protocol followed by a high carbohydrate (CHO) diet in the hours before sleep. Throughout the entirety of day 2, participants consumed a high CHO diet. During the main experimental trial on day 3, subjects consumed a high CHO pre-exercise meal before undertaking 180 min steady-state submaximal exercise whereby they consumed 0, 45 or 90 g h^{-1} CHO followed by a time to exhaustion (TTE) exercise capacity test. Muscle biopsies were obtained immediately pre-exercise and at the end of the 180 min submaximal exercise. PRO, protein.

introduce participants to the research design, verify the subject's ability to complete the prescribed intensity of the 180 min submaximal ride and also provide baseline data for the exercise capacity test.

Day 1: glycogen depletion protocol

At 36 h before the main experimental trial (i.e. evening of Day 1), subjects reported to the laboratory at approximately 18.00 to perform a 90 min bout of intermittent glycogen depleting cycling, as previously described with in our laboratory (Taylor *et al.* 2013). The activity pattern and total time to exhaustion (TTE) for the glycogen depleting ride was recorded for each participant during their first trial and replicated for the remainder of the trials. This intermittent pattern of exercise was employed in an attempt to induce glycogen depletion in both type I and II fibres. To facilitate the goal of CHO loading, subjects consumed high CHO snacks/fluids (SiS GO Energy, SiS REGO Protein, SiS GO Bars, Science in Sport, Blackburn, UK) and food (pasta and tomato-based sauce) immediately and at 1 h and 2 h post glycogen depletion exercise (see Fig. 1).

Day 2: CHO loading regimen

Over the course of the following day (i.e. Day 2), participants were provided with a pre-packaged standardised high CHO diet (i.e. breakfast cereals, breads, pasta, rice, confectionary products) to consume throughout the entirety of that day (see Fig. 1) and also refrained from any strenuous physical activity and alcohol consumption. Fluid intake was restricted to water only and was allowed *ad libitum*.

Day 3: familiarisation and experimental trials

On the morning of the main experimental trials at ~08.00 h, subjects reported to the laboratory in a fasted state and were immediately provided with a standardised high-CHO breakfast (see Fig. 1). At 3 h post-prandial and immediately before the beginning of the cycling protocol, an indwelling cannula (Safety Lock 22G, BD Biosciences, West Sussex UK) was inserted into the antecubital vein in the anterior crease of the forearm and a resting blood sample drawn. After a resting blood sample was taken, the cannula was flushed with ~5 ml of sterile saline (Kays Medical supplies, Liverpool, UK) to keep the cannula patent and sterile, this procedure was repeated after each subsequent blood draw. Participants then completed a 10 min warm-up at 100 W and began the 180 min cycle preload at LT (208 ± 11 W). LT was chosen as the exercise intensity given it has been suggested as an appropriate method of matching metabolic stress

between participants when compared to exercising at a percentage of $\dot{V}_{O_2\,max}$ (Baldwin *et al.* 2000). Heart rate (Polar H7, Kempele, Finland), RPE and cycling cadence were obtained at 30 min intervals throughout the submaximal cycle. Expired gas was collected for 5 min at 30 min intervals in order to calculate substrate oxidation. Venous blood samples were also collected at 30 min intervals throughout the 180 min submaximal exercise.

During the 180 min submaximal ride, subjects consumed 0, 45 or 90 g h^{-1} CHO (see Fig. 1). During the 0 g h^{-1} trial, subjects received 125 ml of a lemon flavoured drink containing no CHO (SiS GO Hydro, Science in Sport, Blackburn, UK) at 20 min intervals, beginning at 0 min (2×125 ml doses was provided at 60 and 120 min). During the 45 g h^{-1} trial subjects received the same fluid intake pattern as the 0 g h^{-1} trial but also consumed a solid homemade CHO rice cake every 30 min beginning at 0 min for the first 120 min of exercise followed by a commercially available CHO gel (SiS GO Isotonic Gel, Science in Sport) every 30 min in the final hour of exercise, of which the final gel was consumed at 150 min. During the 90 g h^{-1} trial, a solid homemade CHO rice cake was provided at 20 min intervals beginning at 0 min for the first 120 min of exercise followed by a commercially available CHO gel at 20 min intervals during the final hour of exercise, of which the final gel was consumed at 160 min. Additionally, 125 ml of a bespoke lemon flavoured 4% CHO drink (Science in Sport) was consumed at 20 min intervals throughout the whole duration of the 90 g h^{-1} trial (2×125 ml doses was provided at 60 and 120 min). Immediately following the 180 min submaximal cycle and after obtaining the post-exercise muscle biopsy, participants had a brief warmup (5 min at 100 W) and then immediately began the exercise capacity test whereby they cycled at 150% LT (312 ± 15 W) to volitional exhaustion. No music was played and the only available information to the participants was the fixed power output and cadence. No performance results were shown to the subjects until they had completed all of the experimental trials. All exercise tests were performed at the same time of day ($11:00 \pm 1$ h) under normal laboratory conditions ($20-21°C$ and $50-60\%$ humidity) using the same electrically braked cycle ergometer (Lode Excaliber Sport, Groningen, Netherlands) and automated gas analyser machine Moxus Modular Metabolic System (AEI Technologies, IL, USA). During all exercise trials, subjects were cooled with a floor standing fan to minimise thermal stress. Each experimental trial was separated by 7–8 days and subjects were asked to continue with their habitual training schedule during this period which equated to $03:21 \pm 02:14$ (hh:min) and 95 ± 67 km (excluding the 90 min glycogen depletion and ~180 min cycling protocol). Full breakdown of nutritional compositions of

the CHO feeding strategies are provided in the Appendix available online in Supporting information.

Estimates of whole-body substrate oxidation and total energy expenditure

Rates of whole-body CHO and fat oxidation (g min^{-1}) were calculated at 30 min intervals from each steady-state gas sample collected throughout the 180 min sub-maximal cycle (Jeukendrup & Wallis, 2005). Total energy expenditure was estimated for each trial assuming an energy yield of 17.57 kJ and 39.33 kJ for 1 g of CHO and fat, respectively.

Blood sampling and analysis

Venous blood samples were collected into vacutainers containing K_2 EDTA, lithium heparin or serum separation tubes (BD Biosciences, UK). K_2 EDTA and lithium heparin tubes were stored on ice while serum separation tube vacutainers were stored at room temperature, both for 1 h before centrifugation at 1500 *g* for 15 min at 4°C. Following centrifugation, plasma and serum was separated into 4 aliquots and stored at −80°C for subsequent analysis. Samples were later analysed for plasma glucose, lactate, non-esterified fatty acids (NEFA), glycerol and β-hydroxybutyrate (β-OHB) using commercially available enzymatic spectrophotometric assays (RX Daytona Analyser, Randox, Co. Antrim, UK) as per the manufacturer's instructions.

Muscle biopsies

Skeletal muscle biopsies (100–150 mg) were obtained from the lateral portion of the m. vastus lateralis immediately before (0 min) and immediately upon completion of the 180 min steady-state submaximal cycle (180 min) but before the exercise capacity test. Muscle biopsies were obtained using the Weil-Blakesley conchotome technique under local anaesthesia (0.5% Marcaine) and were taken from the same region and depth on alternating legs, with incisions separated by ∼5 cm with care to avoid damage from multiple biopsies. Once collected, samples were quickly dissected from fat and connective tissue and divided into multiple pieces on an irradiated sterile Petri dish using surgically sterile tweezers and a scalpel. A portion of the muscle tissue was then prepared for immunohistochemical analysis (∼50 mg) by embedding in Tissue-Tek OCT compound (Sakura Finetek Europe, The Netherlands) on a cork board which was immediately frozen in liquid nitrogen-cooled iso-pentane (Sigma-Aldrich, Dorset, UK) before being stored in aluminium cryotubes (LuBio Science, Switzerland) which were subsequently submerged in liquid nitrogen

and stored at −80°C for later analysis. The remaining portions (for glycogen and signalling proteins) of the muscle biopsy were also immediately frozen in liquid nitrogen and stored at −80°C for later analysis. The time elapsed between the finish of the 180 min submaximal exercise, having the muscle biopsy taken and returning to the bike ergometer for the exercise capacity test was ∼5 min.

Muscle glycogen concentration

Whole muscle glycogen concentrations were determined according to the acid hydrolysis method described by Van Loon *et al.* (2000). Approximately 2–5 mg of freeze-dried muscle tissue was powdered, dissected of all visible blood and connective tissue and subsequently hydrolysed by incubation in 500 μl of 1 M HCl for 3 h at 100°C. After cooling to room temperature, samples were neutralised by the addition of 250 μl 0.12 mol l^{-1} Tris-2.1 mol l^{-1} KOH saturated with KCl. Following centrifugation, 200 μl of the supernatant was analysed in duplicate for glucose concentration according to hexokinase method using a commercially available kit (GLUC-HK; Randox Laboratories, Antrim, UK).

Intramuscular triglyceride immunofluorescence staining and analysis

Cryosections (5 μm) were cut using a microtome (Bright Instrument Company Ltd, Huntingdon, UK) housed within a cryostat at −25°C. The sections were collected onto uncoated, ethanol-cleaned glass slides (VWR International Ltd, Leicestershire, UK). Sections from both the 0 min and 180 min exercise time point from one participant were placed on a single slide but samples between trials were placed on separate slides to account for any variation in staining intensity between sections. Fibre type-specific IMTG content was quantified using immunofluorescence staining as described previously from within our laboratory (Strauss *et al.* 2020). Full details are provided in the Appendix (Supporting information). For fluorescence immunohistochemistry, antibodies targeting myosin heavy chain type I (MHCI; a4.840c developed by Dr Blau, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA) and myosin heavy chain type IIa (MHCIIa; n2.261c, DSHB) were combined with secondary antibodies Alexa Fluor goat anti-mouse (GAM) IgM 546 to visualise anti-MHCI and Alexa Fluor GAM IgG 405 to visualise anti-MHC IIa. Additionally, wheat germ agglutinin (WGA) Alexa Fluor 633 conjugate (Invitrogen, Paisley, UK) was used to identify the cell membrane (and used as a cell border for the purposes of analysis) and BODIPY 493/503

(Invitrogen, Paisley, UK, D3922) was used to visualise IMTG.

Images of cross-sectionally orientated sections, used to investigate fibre type-specific IMTG content and lipid droplet (LD) morphology, were captured using an inverted confocal microscope (Zeiss LSM710; Carl Zeiss AG, Oberkochen, Germany) with a 63× (1.4 NA) oil immersion objective. Type I and type IIa fibres were identified through positive staining, and any fibres without positive staining for either MHCI or MHCIIa were assumed to be type IIx fibres. Although, some images were captured for type IIx fibres, there was an insufficient number to include within the data. Approximately 20 images were captured per time point, aiming for an even split across type I and IIa fibres. Overall, 10 ± 1 fibres were analysed per time point and condition for type I fibres and 9 ± 1 fibres for type IIa fibres, equating to approximately 111 ± 30 fibres per participant. Recent work from our laboratory has revealed that imaging of at least 10 fibres per fibre type per time point will generate a reliable estimate of IMTG content which will be within 10% of a larger number of fibres (i.e. 20 type I and 15 type IIa fibres) (Strauss *et al.* 2020). Given that in most acute and chronic training interventions the changes expected in IMTG content will exceed 10% (Van Loon *et al.* 2003*a*,*b*; Shepherd *et al.* 2013; Jevons *et al.* 2020), we deemed that 10 fibres per fibre type per time point would be time efficient without compromising the validity of the data generated. Image processing was completed using Image-Pro Plus 5.1 software (Media Cybernetics, MD, USA). To assess IMTG content and LD morphology on a fibre type-specific basis, the fibre was first separated into a peripheral region to measure subsarcolemmal LD (first 2 μm from the cell border) and the central region to measure intermyofibrillar LD (remainder of the cell) (see Fig. 2.). This approach of using a fixed 2 μm distance from the membrane to represent the subsarcolemmal region has been utilised previously to examine IMTG content in differing populations (Van Loon, 2004; Jevons *et al.* 2020). An intensity threshold was uniformly selected to represent a positive signal for IMTG. The content of IMTG was expressed as the positively stained area relative to the total area of the peripheral or central region of each muscle fibre. LD number was calculated as the number of IMTG objects relative to area. The mean area of individual IMTG objects (LDs) was used as a measure of LD size.

Muscle glycogen fibre-type histochemistry and analysis

Fibre type-specific glycogen depletion was quantified by combining a brightfield periodic acid-Schiff stain (PAS) with an immunofluorescence myosin heavy chain (MHC) stain on single cross-sections similar to the methodology

described elsewhere (Schaart *et al.* 2004). Full details are provided in the Appendix (Supporting information). PAS was used to visualise muscle glycogen; however, the Schiff reagent (Sigma-Aldrich) was diluted to 25% in PBS as a result of prior optimisation, given that when we applied the reagent without any dilution the dye intensity saturated the signal in both the 0 min and 180 min exercise time point samples. For fluorescence immunohistochemistry, a primary antibody targeting myosin heavy chain type I (MHCI; a4.840c developed by Dr Blau, DSHB) was combined with a secondary antibody Alexa Fluor GAM IgM 488. Images of sections used to investigate fibre type-specific muscle glycogen were captured using a Leica DM6 B upright microscope coupled to a Leica DFC7000 T camera and LAS X software (Leica, Wetzlar, Germany), with images captured at ×40,000 magnification. PAS stained sections were captured in bright field with the same exposure time, gain, brightness, contrast and offset settings brightness across all samples, whilst corresponding fluorescence images for determining fibre type were captured by a simple shift

Figure 2. Region-specific image analysis
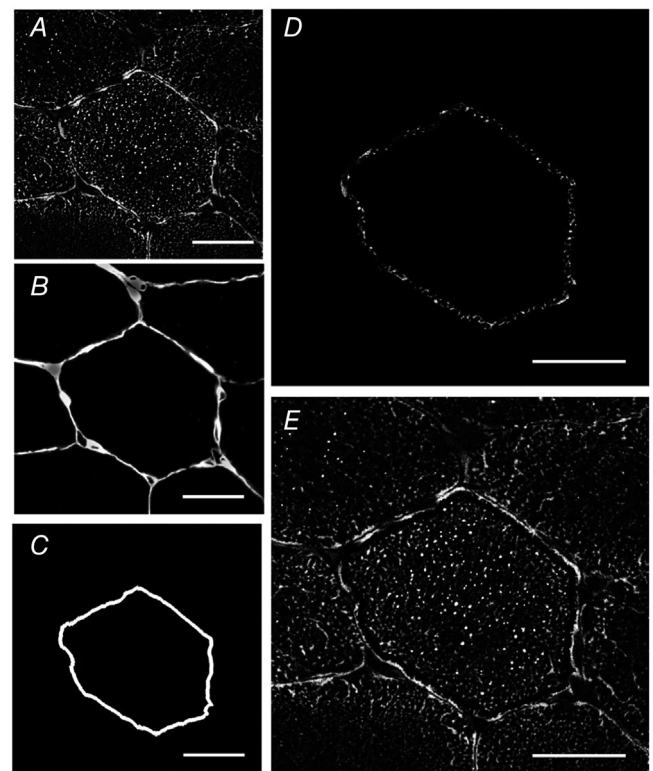A grey scale image was created of both BODIPY 493/503 staining (*A*) and the cell membrane (labelled using WGA Alexa Fluor 633 conjugate) (*B*). A 2 μm region was identified within the inside of the membrane (*C*). LD were then identified in this 2 μm (peripheral) region (*D*) in addition to the remainder of the cell (central region) (*E*). Scale bars represent 30 μm.

in filters with images captured using the excitation filter (465–495 nm) in the green channel.

To assess fibre-specific muscle glycogen content, fibres that were positively stained for myosin heavy chain type I were classified as type I fibres and all remaining fibres negatively stained were classified as type II fibres. Unfortunately, we could not acquire a positive stain for type IIa/x fibres due to interaction effects between the current dye and antibodies used. The entire available muscle cross-section per biopsy sample was imaged in order to capture as many muscle fibres as possible. Image processing was completed using Image-Pro Plus 5.1 software (Media Cybernetics, MD, USA). To assess muscle glycogen content, the bright-field image of the PAS stain was converted *post hoc* to 8-bit greyscale values (0–255). The mean optical density of the PAS-stained muscle fibres was determined by averaging the optical density measured in every pixel in the cell, corrected for the mean optical density of the background stain, containing no muscle fibres. The relative staining intensity of each muscle fibre was classified into 4 separate sections based of optical density increments of 25% in order to visualise the relative scale of semi-quantitative glycogen content (empty, partially empty, partially full and full). The 25% increment sections were calculated from the lowest to the highest recorded optical density value within the current data set. For PAS analysis, on average per subject, a total of 154 ± 101 muscle fibres were analysed (85 ± 54 type I, 76 ± 51 type II). This number of analysed muscle fibres was in line with previous studies that detected significant differences in fibre-specific muscle glycogen utilisation during prolonged endurance-based exercise with and without CHO supplementation (De Bock *et al.* 2007; Stellingwerff *et al.* 2007*a*).

SDS page and western blot analysis

Approximately 20 mg of frozen tissue was homogenised in 200 μl of ice-cold 1× RIPA lysis buffer (Sigma Aldrich, UK) supplemented with protease and phosphatase inhibitors (Pierce, Thermo Scientific, Denmark), rotated end over end at 4°C for 1 h and centrifuged at 12,000 *g* for 10 min at 4°C. Following the determination of protein quantification via BCA assay (Pierce, Thermo Scientific, Denmark), each sample was resuspended in 4× Laemmli buffer, denatured by boiling for 5 min at 95°C and separated by SDS-PAGE using 12% TGX Precast Mini Protein stain-free gels After electrophoresis, stain-free gels were activated according to the manufacturer's instructions and semi-dry transferred to nitrocellulose membranes (TransBlot Turbo, Bio-Rad, UK). Following transfer, a stain-free image was obtained for total protein loading normalisation. Membranes were subsequently blocked in Tris-buffered saline-Tween (TBST; 0.19 M

Tris pH 7.6, 1.3 M NaCl, 0.1% Tween-20) containing 5% non-fat milk for 1 h and incubated in primary antibodies for AMPKα (CST: 2532), p-AMPK[Thr172] (CST: 2531), p38MAPK (CST: 9212), p-p38MAPK[Thr180/Tyr182] (CST: 9211), CaMKII (CST: 3362), p-CaMKII[Thr268] (CST: 3361), p53 (CST: 9282), p-p53[Ser15] (CST: 9281), CREB (CST: 9197), p-CREB[Ser133] (CST: 9198) before incubation in a relevant secondary antibody (anti-rabbit: CST: 7074) at a concentration of 1:2000 at room temperature the following morning. After washing (3 × 5 min in TBST), membranes were incubated in a chemiluminescence substrate (Thermo Fisher Scientific, UK) and visualised using a Chemi-doc MP system (Bio-Rad, UK) with band densities quantified using Image Lab software (Bio-Rad, UK). The volume density of each target band was normalised to the total amount of protein loaded into each lane using Stain-Free technology.

Statistical analysis

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS Statistics Version 26, IBM, USA). Changes in exercise capacity, total substrate oxidation, and post-exercise capacity test blood metabolites were analysed using a one-way repeated-measures general linear model (GLM), where the within-subject factor was condition. Metabolic (i.e. blood metabolites and muscle glycogen), molecular (i.e. activity of signalling molecules), physiological and perceptual responses during exercise (i.e. heart rate, RPE, oxidation rates) were analysed with a two-way repeated-measures GLM, where the within factors were time and condition. To assess differences in IMTG, we hypothesised a *priori* that IMTG content would be significantly different between type I and IIa fibres, as based on previous work (Van Loon, 2004; Stellingwerff *et al.* 2007*a*; Jevons *et al.* 2020). Thus, we used a three-factor repeated measures ANOVA for comparisons of pre-exercise IMTG content (% area stained) and LD morphology where the within factors were condition, region and fibre. Similarly, changes in IMTG content (% area stained), LD morphology and the relative distribution of IMTG content between sub-cellular regions within each fibre during exercise were also analysed with a three-factor repeated measures ANOVA, where the within factors were condition, region and time. Moreover, comparisons of IMTG breakdown were also analysed with a three-factor repeated measures ANOVA, where the within factors were condition, region and fibre. Linear mixed modelling was used to examine the dependent variable, fibre-specific skeletal muscle glycogen content, at immediately before and after the 180 min submaximal cycle with data separated into the three different experimental conditions (0, 45 and

Table 1. Heart rate, RPE, exercise intensity (%$\dot{V}_{O_2 max}$) and energy expenditure during the 180 min submaximal steady-state cycling protocol

	Time (min)					
	30	60	90	120	150	180
Heart rate (beats min^{-1})						
0 gh^{-1}	149 ± 13	151 ± 14	153 ± 16	155 ± 14[a,c]	160 ± 10[a,c]	160 ± 10[a,c]
45 gh^{-1}	148 ± 10	150 ± 12	150 ± 9	153 ± 9[a,c]	157 ± 7[a,c]	159 ± 8[a,c]
90 gh^{-1}	153 ± 11	156 ± 14	157 ± 11	159 ± 14[a,c]	159 ± 13[a,c]	159 ± 14[a,c]
RPE (AU)						
0 gh^{-1}	13 ± 2	14 ± 1[a]	14 ± 2[a,b]	15 ± 1[a,b,c]	17 ± 2[a,b,c,d]	18 ± 2[a,b,c,d,e]
45 gh^{-1}	13 ± 2	13 ± 2[a]	14 ± 2[a,b]	15 ± 2[a,b,c]	16 ± 2[a,b,c,d]	17 ± 2[a,b,c,d,e]
90 gh^{-1}	13 ± 2	14 ± 2[a]	14 ± 2[a,b]	15 ± 2[a,b,c]	16 ± 2[a,b,c,d]	16 ± 3[a,b,c,d,e]
% $\dot{V}_{O_2 max}$						
0 gh^{-1}	66 ± 4	66 ± 5	68 ± 5[a]	68 ± 4[a,b]	69 ± 4[a,b,c]	69 ± 5[a]
45 gh^{-1}	67 ± 5	67 ± 6	68 ± 5[a]	69 ± 4[a,b]	70 ± 5[a,b,c]	70 ± 5[a]
90 gh^{-1}	67 ± 3	68 ± 4	69 ± 4[a]	69 ± 4[a,b]	69 ± 4[a,b,c]	67 ± 4[a]
Energy expenditure (kJ·min^{-1})						
0 gh^{-1}	61.5 ± 3.1	61.6 ± 3.8	62.0 ± 4.1	62.2 ± 3.8	62.6 ± 3.5	61.6 ± 5.4
45 gh^{-1}	62.8 ± 4.1	62.8 ± 4.3	62.8 ± 4.6	63.5 ± 4.1	63.4 ± 4.2	63.2 ± 4.6
90 gh^{-1}	62.9 ± 4.3	62.8 ± 4.9	63.4 ± 5.3	63.5 ± 5.4	63.3 ± 6.0	62.1 ± 5.0

[a] Significant difference from 30 min time point,
[b] significant difference from 60 min time point,
[c] significant difference from 90 min time point,
[d] significant difference from 120 min time point and
[e] significant difference from 150 min time point, $P < 0.05$.

90 g h^{-1} CHO). All main effects and interactions were tested using a linear mixed-effects model, with random intercepts to account for repeated measurements within subjects to examine differences between experimental conditions, fibre type and time. *Post hoc* LSD tests were used in all statistical models where significant main effects and interactions were observed in order to locate specific differences. All data in text, figures and tables are presented as means ± SD, with P values ≤ 0.05 indicating statistical significance.

Results

CHO feeding alters circulating metabolites and whole-body substrate utilisation

Heart rate, oxygen uptake and RPE increased during exercise ($P = 0.027$, $P < 0.001$ and $P < 0.001$, respectively) though no differences were apparent between trials ($P = 0.349$, 0.725 and 0.328, respectively) (see Table 1). Plasma lactate ($P < 0.001$), NEFA ($P < 0.001$), glycerol ($P < 0.001$) and β-OHB ($P < 0.001$) all displayed significant changes during exercise (see Fig. 3). In accordance with the increased dose of CHO, there was a significant difference between conditions for plasma glucose ($P < 0.001$), NEFA ($P = 0.005$), glycerol ($P < 0.001$) and β-OHB ($P = 0.002$) (see Fig. 3).

Rates of whole-body CHO oxidation progressively decreased during exercise ($P < 0.001$) with a significant difference also evident between conditions ($P = 0.005$) (see Fig. 4). Total CHO oxidation was significantly different between trials ($P = 0.005$) and was 315 ± 74, 386 ± 67 and 412 ± 69 g for 0, 45 and 90 g h^{-1} trial, respectively, of which the 0 g h^{-1} trial was significantly different from the 45 and 90 g h^{-1} ($P = 0.030$ and 0.004) (see Fig. 4). Fat oxidation increased during exercise ($P < 0.001$) with a significant difference between conditions ($P = 0.034$) (see Fig. 4). Total fat oxidation was significantly different between trials ($P = 0.035$) and was 139 ± 23, 113 ± 17 and 104 ± 22 g for 0, 45 and 90 g h^{-1}, respectively, of which the 0 g h^{-1} trial was significantly different from 90 g·h^{-1} ($P < 0.001$) (see Fig. 4). RER significantly decreased during exercise ($P < 0.001$) with a significant difference between conditions ($P = 0.019$). Rates of energy expenditure did not significantly change during exercise ($P = 0.420$) or differ between conditions ($P = 0.386$) (see Table 1).

During the first hour of exercise, the contribution of CHO towards total energy expenditure was significantly greater than fat ($P < 0.001$) and there was no significant difference between trials ($P = 0.07$) (see Fig. 4). During the second hour of exercise, CHO also contributed a greater proportion of energy expenditure than fat ($P = 0.029$) and furthermore, CHO contributed a greater energy yield

in the 45 and 90 g h^{-1} trials compared with the 0 g h^{-1} trial ($P = 0.014$ and $P = 0.005$, respectively) (see Fig. 4). During the final hour of exercise, however, there was no significant difference between the contribution of CHO and fat towards total energy expenditure ($P = 0.06$) though CHO again contributed a greater energy yield in the 45 and 90 g h^{-1} trials compared with the 0 g h^{-1} trial ($P = 0.015$ and $P = 0.001$, respectively) (see Fig. 4). Visual representation of the 'crossover point' (i.e. where lipid provided the largest contribution
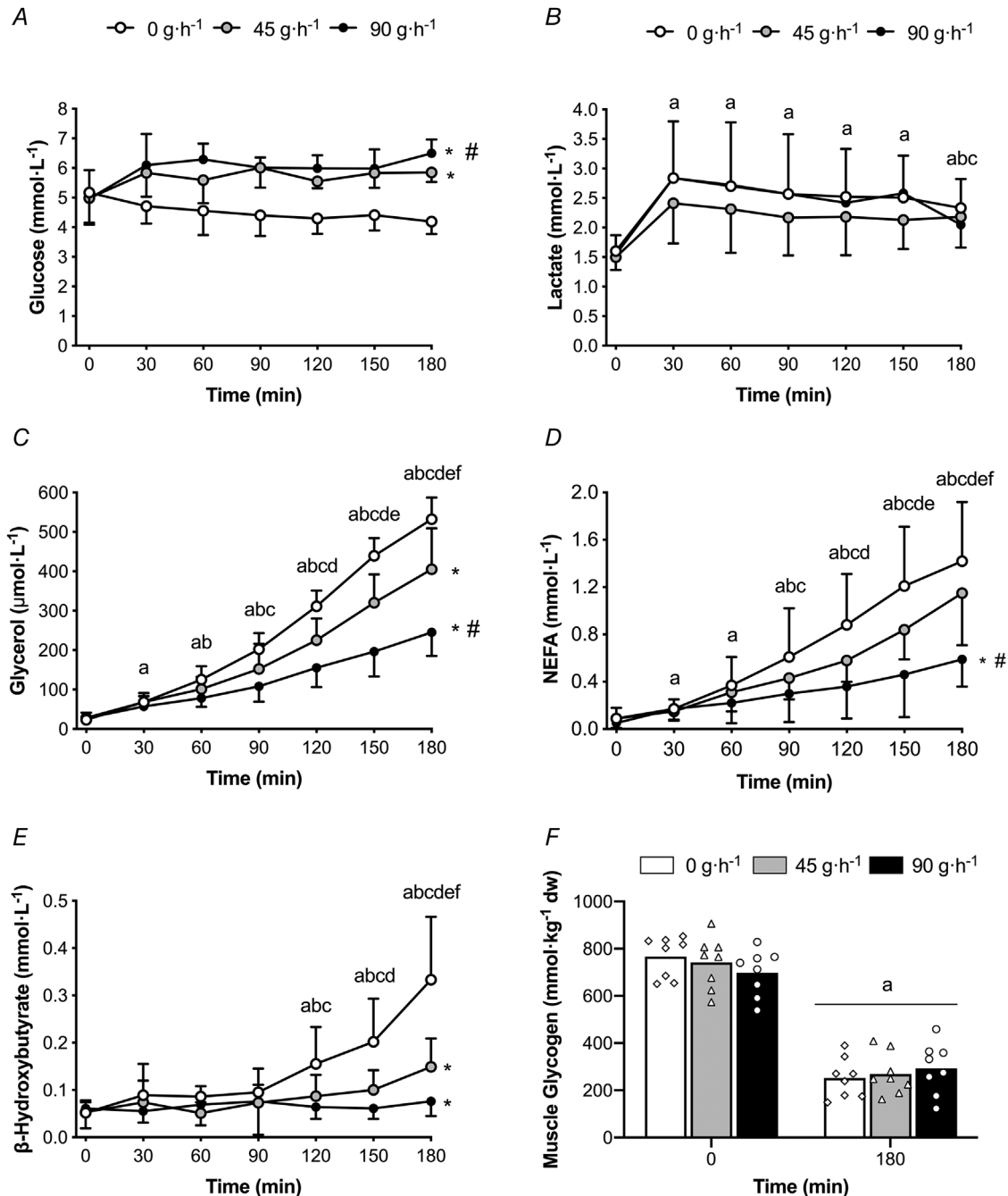

Figure 3. Effects of exercise and carbohydrate feeding on circulating metabolites and whole muscle glycogen
Plasma glucose (*A*), lactate (*B*), glycerol (*C*), NEFA (*D*) and β-OHB (*E*) responses before and during the 180 min submaximal exercise. *F*, whole-muscle glycogen concentration before and after the 180 min submaximal exercise protocol. *Significant difference from 0 g h^{-1}, #significant difference from 45 g h^{-1}, $P < 0.05$. [a]Significant difference from 0 min, [b]significant difference from 30 min, [c]significant difference from 60 min, [d]significant difference from 90 min, [e]significant difference from 120 min and [f]significant difference from 150 min, $P < 0.05$.
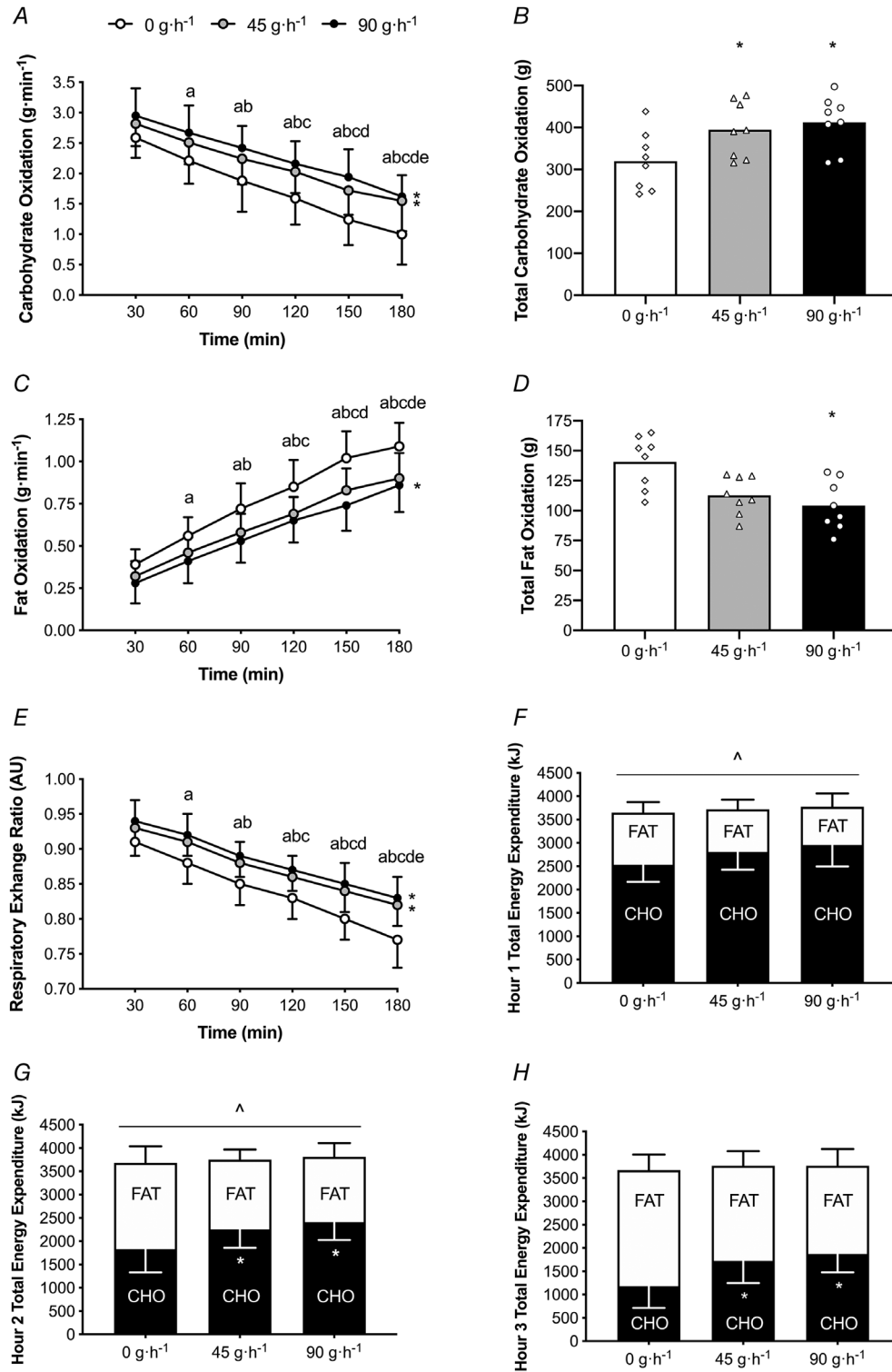
Figure 4. Effects of exercise and carbohydrate feeding on whole-body substrate utilisation

A, carbohydrate oxidation rates during exercise; *B*, total carbohydrate oxidation from exercise; *C*, fat oxidation rates during exercise; *D*, total fat oxidation from exercise; *E*, respiratory exchange ratio (RER) during exercise; *F*, total energy expenditure during the first hour of exercise; *G*, total energy expenditure during the second hour of exercise; *H*, total energy expenditure during the third hour of exercise. ˜Significant main effect between carbohydrate and fat contribution to total energy expenditure, *significant difference from 0 g h^{−1}, $P < 0.05$. [a]Significant difference from 30 min, [b]significant difference from 60 min, [c]significant difference from 90 min, [d]significant difference from 120 min, [e]significant difference from 150 min, $P < 0.05$.

towards total energy contribution) is also presented in Fig. 5.

CHO feeding does not alter subcellular and muscle fibre-specific IMTG use during exercise

Pre-exercise IMTG content and LD morphology. Type I muscle fibres had greater IMTG content compared to type IIa fibres ($P < 0.001$) (see Table 2). IMTG content was more concentrated in the periphery of the cell (within the 2 μm border) compared with the central region ($P = 0.003$; see Table 2). Overall, though, the majority of IMTG was observed in the central compared with the peripheral region of the cell ($P < 0.001$; see Table 3). There was also a greater number ($P = 0.001$; see Table 2) and size of LD ($P = 0.012$; see Table 2) in type I fibres compared with type IIa fibres. Thus, pre-exercise fibre type

differences in IMTG content were explained by differences in both LD number and size.

Effects of CHO feeding and exercise on IMTG and LD morphology. Exercise decreased IMTG content in type I ($P < 0.001$) and type IIa fibres ($P = 0.002$), the magnitude of which was not affected by CHO feeding during exercise (type I: $P = 0.489$; type IIa: $P = 0.841$) (see Fig. 6). As such, there was no difference in the net breakdown of absolute IMTG between trials (main condition effect; $P = 0.152$; see Fig. 6). A greater net IMTG breakdown was also observed in type I compared to type IIa fibres ($P < 0.001$; see Fig. 6).

When examining exercise-induced changes on a subcellular-specific basis, IMTG content was significantly reduced in both the peripheral ($P < 0.001$; type IIa: $P = 0.016$) and central regions (type I: $P < 0.001$; type IIa: $P = 0.001$) of both fibres (see Fig. 6). Additionally, the relative contribution of IMTG across the subcellular
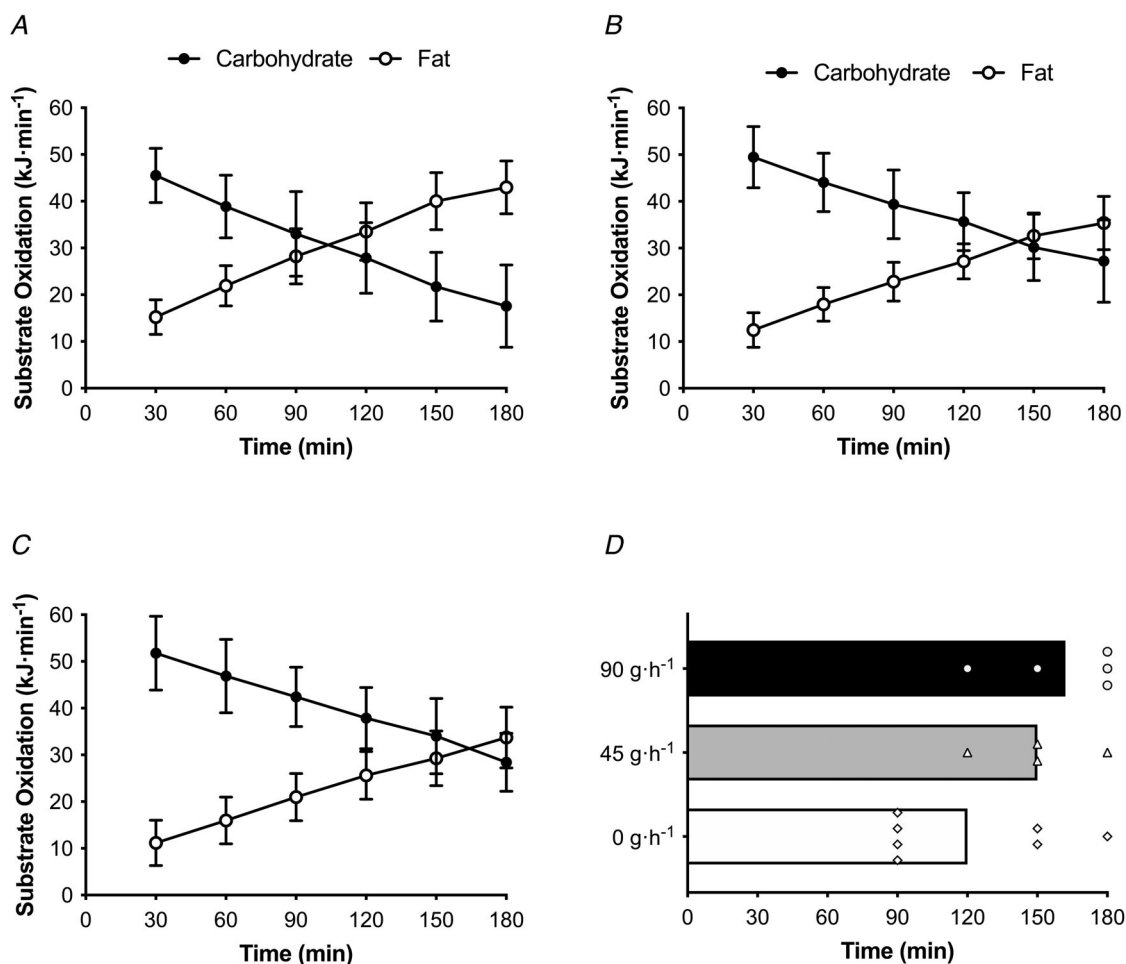
Figure 5. Rates of energy provision from carbohydrate and fat oxidation during exercise
Rates of energy provision from carbohydrate (CHO) and fat oxidation during the 0 (*A*), 45 (*B*) and 90 g h^{-1} (*C*) trials. *D*, individual time points at which the crossover point occurred whereby fat provided the predominant contribution to energy expenditure (note, no individual data points are provided for those individuals who displayed no crossover contribution time point within a specific trial).

Table 2. Pre-exercise IMTG content and LD morphology

| | 0 gh⁻¹ | | | | 45 gh⁻¹ | | | | 90 gh⁻¹ | | | | P value | | |
| | Type I Fibres | | Type IIa fibres | | Type I fibres | | Type IIa fibres | | Type I fibres | | Type IIa fibres | | | | |
	Peripheral	Central	Peripheral	Central	Peripheral	Central	Peripheral	Central	Peripheral	Central	Peripheral	Central	Condition	Fibre	Region
IMTG Content (% area stained)	7.60 ± 2.16*	5.40 ± 2.20*	5.03 ± 1.63	3.68 ± 2.16	7.65 ± 2.16*	5.82 ± 2.93*	5.24 ± 2.43	3.42 ± 2.18	7.39 ± 2.54*	5.60 ± 1.77*	4.60 ± 1.18	3.65 ± 1.02	$P = 0.944$	$P < 0.001$	$P = 0.003$
LD Size (μm²)	0.365 ± 0.076*	0.364 ± 0.118*	0.326 ± 0.117	0.324 ± 0.098	0.346 ± 0.056*	0.348 ± 0.072*	0.289 ± 0.062	0.301 ± 0.086	0.351 ± 0.067*	0.390 ± 0.138*	0.304 ± 0.048	0.384 ± 0.066	$P = 0.600$	$P = 0.012$	$P = 0.112$
LD number (LD·μm²)	0.209 ± 0.054*	0.143 ± 0.028*	0.156 ± 0.041	0.104 ± 0.037	0.215 ± 0.048*	0.159 ± 0.063*	0.165 ± 0.062	0.104 ± 0.059	0.209 ± 0.077*	0.150 ± 0.050*	0.148 ± 0.025	0.096 ± 0.027	$P = 0.781$	$P = 0.001$	$P < 0.001$

*Significantly greater in type I fibres, $P < 0.05$. Intramuscular triglycerides (IMTG) content and lipid droplet (LD) number are expressed relative to the area of the peripheral or central region

regions within type I fibres decreased in the central region ($P = 0.001$), with a reciprocal increase in the relative distribution of IMTG within the peripheral region ($P = 0.001$) (see Table 3). In contrast, the relative distribution within type IIa fibres remained similar after exercise ($P = 0.141$) (see Table 3).
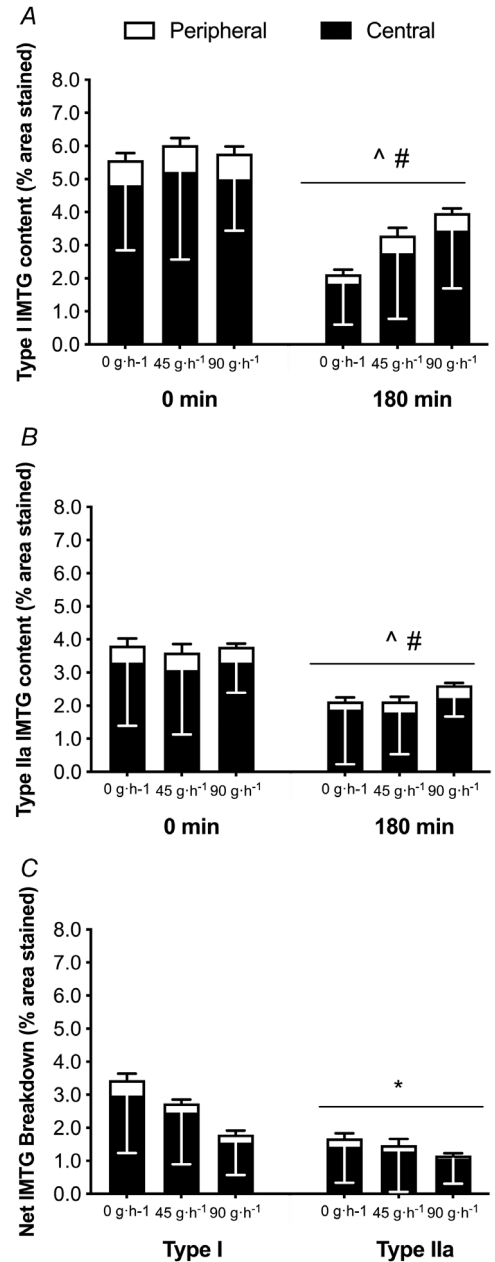
Figure 6. Fibre type and subcellular-specific changes in IMTG content before and after the 180 min submaximal exercise protocol
IMTG content in each region was normalised to total cell area. Shown are IMTG content in type I (A) and type IIa (B) muscle fibres and net IMTG breakdown (C). #Significant reduction in IMTG content from 0 min, $P < 0.05$. ^Significant region × time interaction from 0 min, $P < 0.05$. *Significantly different from type I fibres, $P < 0.05$.

Table 3. Relative distribution of IMTG between subcellular regions before and after the 180 min submaximal cycling exercise protocol

		Percentage of IMTG			
		Type I fibres		Type IIa fibres	
		Peripheral	Central*	Peripheral	Central*
0 min					
	0 gh^{-1}	16 ± 6	84 ± 6	19 ± 7	81 ± 7
	45 gh^{-1}	16 ± 5	84 ± 5	18 ± 6	82 ± 6
	90 gh^{-1}	14 ± 3	86 ± 3	14 ± 4	86 ± 4
180 min					
	0 gh^{-1}	22 ± 15[#]	78 ± 15[#]	18 ± 7	82 ± 7
	45 gh^{-1}	25 ± 13[#]	75 ± 13[#]	22 ± 13	78 ± 13
	90 gh^{-1}	19 ± 7[#]	81 ± 7[#]	15 ± 5	85 ± 5

*Significant effect of region across all time points, $P < 0.05$.
[#]Significantly different from 0 min time point within same condition, $P < 0.05$.

When examining changes in LD morphology, LD number reduced significantly in both type I ($P < 0.001$) and type IIa muscle fibres ($P = 0.001$) with no effect of CHO feeding in either fibre type (type I: $P = 0.417$; type IIa: $P = 0.975$) (see Fig. 7). LD number decreased in both the central and peripheral regions within both type I ($P < 0.001$) and type IIa fibres ($P = 0.001$) (see Fig. 7). However, LD size was significantly smaller after exercise in type I muscle fibres only ($P = 0.004$), with reductions in both the peripheral ($P = 0.011$) and central ($P = 0.005$) regions (see Fig. 7).

Thus, IMTG utilisation during exercise could be explained by a decrease in LD number within the peripheral and central regions of both type I and IIa fibres but a reduction in LD size occurred exclusively in type I fibres. Figure 8 depicts a visual representation of IMTG content in type I and IIa fibres at 0 min and after 180 min of submaximal cycling exercise in the 0, 45 and 90 g h^{-1} condition.

CHO feeding does not alter whole-muscle or fibre-specific muscle glycogen during exercise

In accordance with the 36 h dietary standardisation protocol, subjects presented as CHO loaded where there was no difference in pre-exercise muscle glycogen concentration between trials ($P = 0.137$; 0 g h^{-1}: 767 ± 87, 45 g h^{-1}: 742 ± 109, 90 g h^{-1}: 698 ± 98 mmol kg^{-1} DW). Exercise induced significant decreases in muscle glycogen to comparable levels immediately after the submaximal cycle ($P < 0.001$; 0 g h^{-1}: 252 ± 85, 45 g h^{-1}: 269 ± 88, 90 g h^{-1}: 293 ± 109 mmol·kg^{-1} DW) and no differences were apparent between conditions ($P = 0.783$) (see Fig. 3). Muscle glycogen was reduced to a greater extent in type I compared to type II fibres ($P < 0.001$) and CHO feeding did not alter glycogen utilisation in either

fibre type ($P = 0.309$) (see Fig. 9). Figure 10 depicts a visual representation of glycogen content in type I and II fibres at 0 min and after 180 min of submaximal cycling exercise in the 0, 45 and 90 g h^{-1} conditions. Muscle glycogen content was reduced by 41 ± 16, 35 ± 12 and 30 ± 18% in type I fibres and by 7 ± 8, 11 ± 11 and 9 ± 14% in type II fibres for the 0, 45 and 90 g h^{-1} trial, respectively. Further representation of the relative scale of glycogen depletion within type I and type II fibres is presented in Fig. 11.

CHO feeding does not attenuate skeletal muscle cell signalling pathways with regulatory roles in promoting training adaptation

Exercise significantly increased the phosphorylation of AMPK^{Thr172} ($P < 0.001$) although increases (~4-fold) were similar between conditions ($P = 0.851$) (see Fig. 12). Similarly, exercise increased the phosphorylation of both CaMKII^{Thr286} (2-fold; $P = 0.003$) and p53^{Ser15} (2-fold; $P = 0.025$) independently of CHO availability ($P = 0.360$ and $P = 0.784$, respectively) (see Fig. 12). In contrast, neither p38 MAPK$^{Thr180/Tyr182}$ (main effect of condition; $P = 0.645$, time; $P = 0.064$) nor CREB^{Ser133} (main effect of condition; $P = 0.601$, time; $P = 0.733$) (see Fig. 12) phosphorylation was significantly altered by either exercise or CHO availability, which both displayed large inter-individual variance in their responses. Representative western blots are displayed in Fig. 12.

CHO feeding improves exercise capacity in a dose dependent manner

Exercise capacity was increased in a dose dependent manner where the 90 g h^{-1} trial (233 ± 133 s) was

greater than the FAM (87 ± 63 s; $P = 0.005$: 95% CI for differences $= 62$ to 232 s), 0 g h^{-1} (108 ± 54; $P = 0.013$: 95% CI for differences $= 35$ to 215 s) and 45 g h^{-1} (156 ± 66 s; $P = 0.06$: 95% CI for differences $= -4$ to 158 s) trials (see Fig. 13). The 45 g h^{-1} dose was also longer than the FAM ($P = 0.035$: 95% CI for differences $= 6$ to 133 s) and 0 g h^{-1} ($P = 0.029$: 95% CI for differences $= 7$ to 89 s) trial, whilst there was no significant difference between the 0 g h^{-1} and FAM ($P = 0.299$: 95% CI for differences $= -24$ to 68 s) trial (see Fig. 13). There was a significant difference in capacity times when data were analysed for a trial order effect ($P = 0.05$) but this was due to a significant increase in capacity time between FAM and the first experimental trial ($P = 0.005$: 95% CI for differences $= 45$ to 179 s) with no other pairwise differences present (see Fig. 13). Plasma glucose ($P = 0.004$), NEFA ($P = 0.003$), glycerol ($P = 0.001$) and β-OHB ($P = 0.002$) were all significantly different between conditions at exhaustion (see Fig. 14), whilst lactate ($P = 0.277$) was not different between trials.

Discussion

Confirming our hypothesis, our data demonstrate that CHO feeding during prolonged endurance exercise does not alter IMTG and glycogen utilisation in either type I or type II/IIa muscle fibres. Moreover, we also present novel data on the subcellular-specific changes in IMTG content and LD morphology during exercise, in that we observed for the first time that IMTG content was reduced in both the peripheral and central regions of both fibres, an effect independent of CHO feeding. The reduction of IMTG content was a function of a reduction in LD number in both type I and IIa fibres, whereas a reduction in LD size was only evident in type I fibres. In accordance with similar absolute glycogen utilisation and depletion, CHO feeding did not attenuate activation of acute cell signalling pathways with regulatory roles in modulation of skeletal muscle mitochondrial biogenesis. Finally, CHO feeding improved exercise capacity in a dose dependent manner, an effect likely to be related to maintenance of
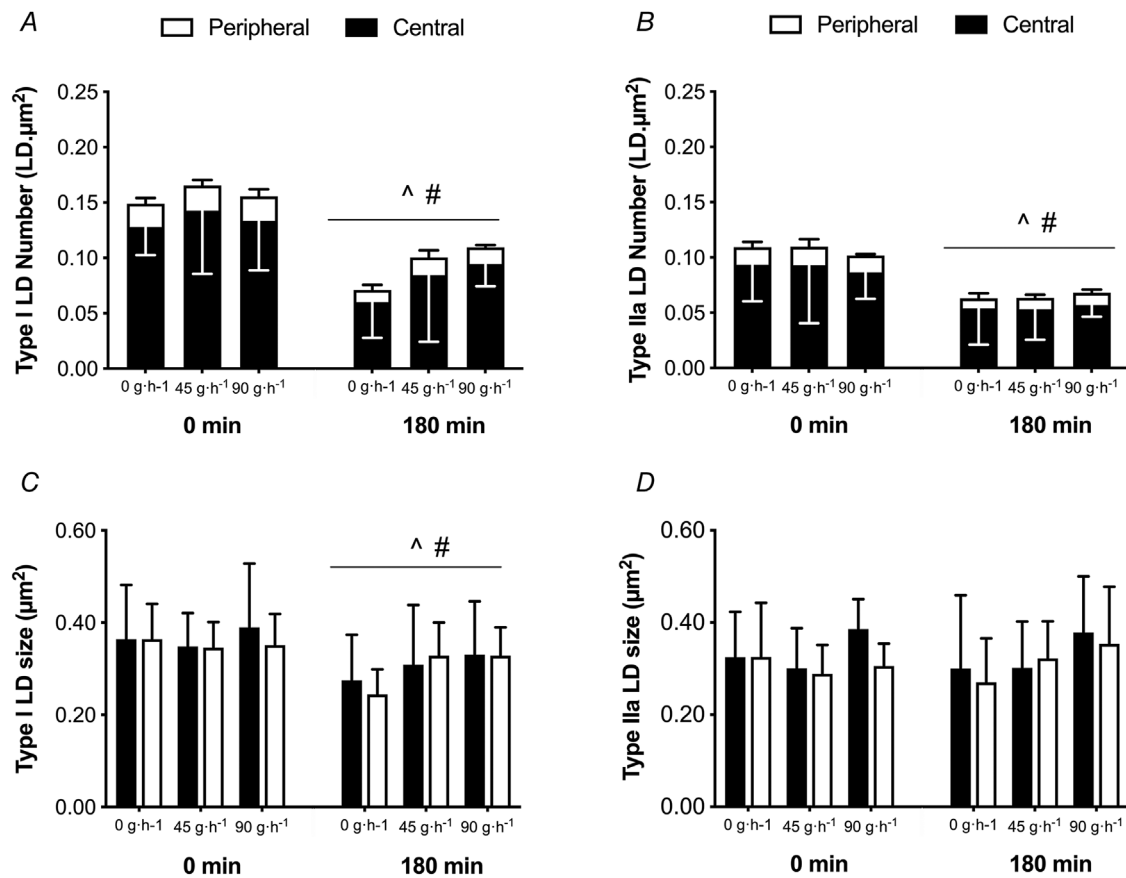

Figure 7. Fibre type and subcellular-specific changes in lipid droplet (LD) morphology before and after the 180 min submaximal exercise protocol
LD number in each region was normalised to total cell area. Shown are LD number in type I (*A*) and type IIa (*B*) muscle fibres, and LD size in type I (*C*) and type IIa (*D*) muscle fibres. [#]Significant reduction from 0 min, $P < 0.05$. [^]Significant region $\times$ time interaction from 0 min, $P < 0.05$.

circulating glucose availability and whole-body rates of CHO oxidation.

We employed an experimental design that evaluated muscle fuel selection in a manner that is aligned with the nutritional practices often adopted by endurance athletes, i.e. CHO availability was increased in the day(s) and hours before competition so as to commence exercise with sufficient muscle and liver glycogen stores (Burke *et al.* 2018). In accordance with the trained status of our participants and the 36 h CHO loading protocol, our model was successful in achieving highly elevated pre-exercise glycogen stores, as evidenced by whole muscle glycogen concentrations (see Fig. 3) and the dark

PAS staining in both type I and type II muscle fibres (see Fig. 10). In agreement with previously documented storage patterns of IMTG (Van Loon *et al.* 2003*a*,*b*; De Bock *et al.* 2005; Stellingwerff *et al.* 2007*a*; Shepherd *et al.* 2013; Jevons *et al.* 2020), we also observed that resting IMTG stores were twofold greater in type I fibres compared to type IIa fibres (see Table 2). Additionally, IMTG content was more concentrated in the periphery of the cell compared with the central region, as also documented in previous studies utilising immuno-fluorescence analysis with a similar peripheral region classification (a fixed 2 μm region from the cell border) (Jevons *et al.* 2020). The fibre type specific difference in
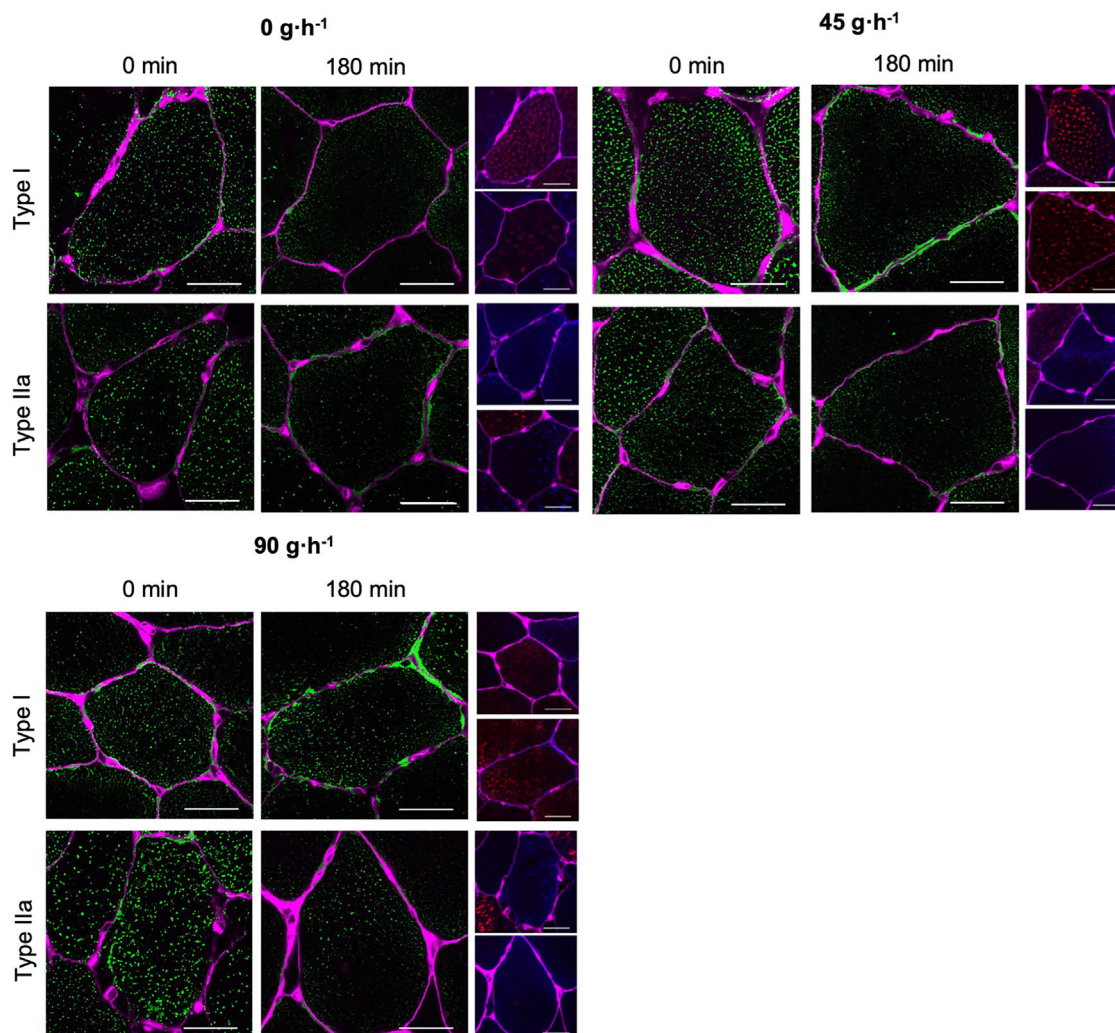

Figure 8. Representative immunofluorescence images of intramuscular triglycerides (IMTG) in response to prolonged endurance exercise with and without carbohydrate (CHO) feeding during exercise in type I and IIa muscle fibres
Sections were stained for IMTG (stained using BODIPY 493/503; green), fibre type (type I: primary antibody MHCI – A4.840c; secondary antibody goat anti-mouse IgM 546, red; type IIa: MHCIIa – N2.261c; secondary antibody goat anti-mouse goat anti-mouse IgG 405, blue), and wheat germ agglutinin Alex Fluor 633 to identify cell border (stained pink). Images depict IMTG content in type I (stained red) and IIa fibres (stained blue) at 0 min and after 180 min of submaximal cycling exercise. Scale bars represent 30 μm.

resting IMTG was a function of a greater number and size of LDs in type I fibres compared with type IIa fibres (see Table 2), also consistent with recent observations from our laboratory in male endurance trained participants (Jevons *et al.* 2020). Importantly, the resting storage pattern of both IMTG and muscle glycogen was not different between trials, thus demonstrating that the 36 h exercise and dietary protocol was successful in

A

□ 0 g·h⁻¹   ▨ 45 g·h⁻¹   ■ 90 g·h⁻¹

B

C

Figure 9. Fibre type changes in muscle glycogen content before and after the 180 min submaximal exercise protocol
Muscle glycogen content in type I (*A*) and type II (*B*) fibres before and after the 180 min submaximal cycling exercise protocol, as determined on periodic acid-Schiff-stained muscle cross-sections. *C*, net muscle glycogen utilisation. #Significantly different from 0 min, $P < 0.05$. *Significantly different from type I fibres, $P < 0.05$.

ensuring similar substrate availability prior to the main experimental trial. Nonetheless, we acknowledge that our dietary standardisation protocol could be considered sub-optimal in relation to dietary fat intake (i.e. 1 g kg⁻¹) since it has previously been shown that ∼2 g kg⁻¹ day⁻¹ is required to fully replenish IMTG stores within 48 h of the last exercise bout (Van Loon *et al.* 2003*b*). However, we chose to replicate previous dietary approaches from our laboratory (Impey *et al.* 2016), given that our focus was to primarily maximise CHO availability for the main experimental trial.

Our submaximal exercise protocol induced an approximate 50% decrease in IMTG content within type I fibres, a magnitude of utilisation that agrees well with previous studies also utilising cycling protocols lasting ≥ 3 h (Van Loon *et al.* 2003*b*; Stellingwerff *et al.* 2007*a,b*; Jevons *et al.* 2020). Although previous studies have reported IMTG degradation to be negligible within type II (Van Loon *et al.* 2003*a,b*; Stellingwerff *et al.* 2007*a,b*) and IIa (De Bock *et al.* 2005; Van Proeyen *et al.* 2011; Jevons *et al.* 2020) fibres, we observed an approximate 30% utilisation of IMTG stores within type IIa fibres. Our data therefore agree with observations from well-trained cyclists whereby a 32% reduction in IMTG content in type II fibres was reported following 3 h of steady-state cycling at 62% $\dot{V}_{O_2 max}$ (Stellingwerff *et al.* 2007*b*). Additionally, an approximate 45% depletion of IMTG has also been observed in type IIa fibres during 2 h of prolonged cycling when examined after 6 weeks of consistent endurance training in the fasted state (Van Proeyen *et al.* 2011).

From a subcellular-specific perspective, we provide novel data by demonstrating that the exercise-induced decrease in IMTG content in both fibre types occurred within both the central and peripheral regions. This pattern of utilisation is in contrast to previous reports within our laboratory using a similar lipid dye (BODIPY 493/503) where Jevons *et al.* (2020) observed that only the central region type I fibres displayed reduced IMTG content. The reduction in IMTG content observed in the present study was also due to a reduction in LD number in both type I and IIa fibres whereas a reduction in LD size was only evident in type I fibres. Our data are in agreement with previous observations that have also reported a reduction in both LD size and number in type I fibres following 1 h (Shepherd *et al.* 2013), 2 h (Van Loon *et al.* 2003*a*) and 3 h (Stellingwerff *et al.* 2007*a*) of moderate intensity cycling exercise. In contrast, Jevons *et al.* (2020) observed that the decrease in IMTG content in type I fibres after a 4 h cycling protocol at ∼56 $\dot{V}_{O_2 max}$ was the consequence of a reduction in LD number with no difference in LD size. Interestingly, the reduction in LD number primarily underpinning the decrease in IMTG content in type IIa fibres has not been previously reported in the literature
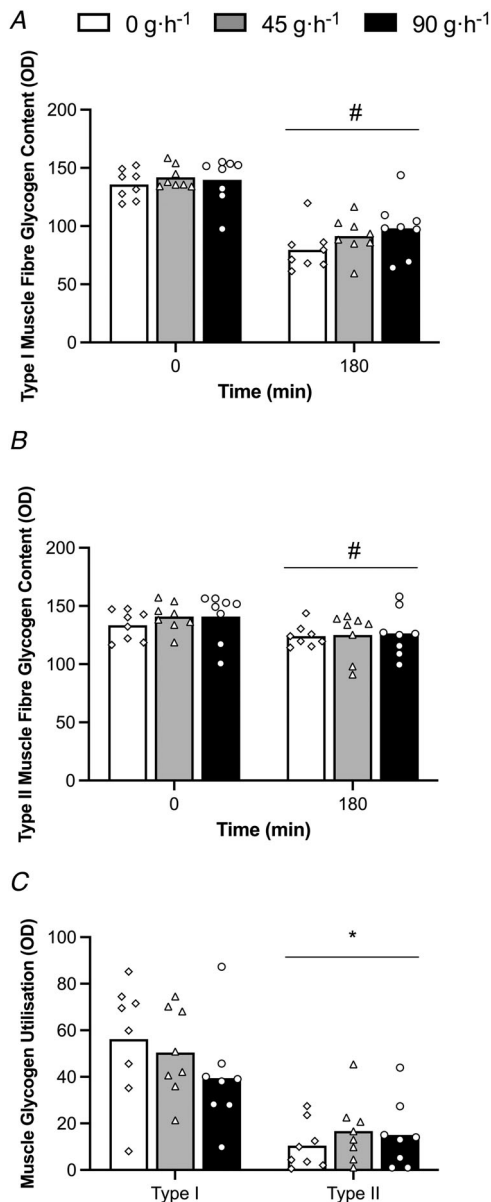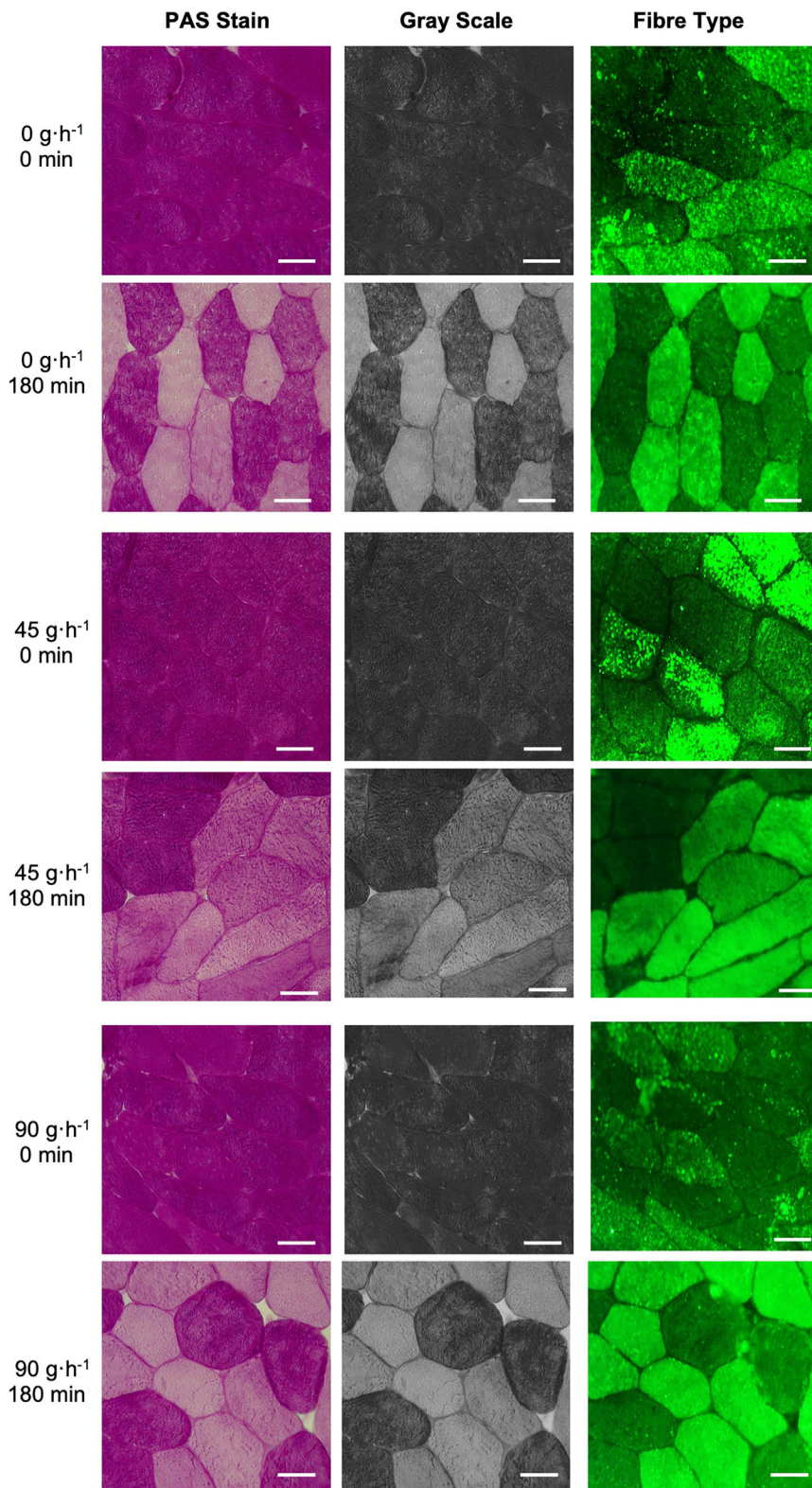
Figure 10. Representative images of muscle glycogen in response to prolonged endurance exercise with and without carbohydrate (CHO) feeding during exercise
Sections were stained for glycogen (periodic acid-Schiff (PAS)), fibre type (primary antibody MHCI – A4.840c; secondary antibody goat anti-mouse IgM 488, green). Images depict glycogen content in type I and II fibres at 0 and 180 min in the 0, 45 and 90 g h^{-1} conditions. Scale bars represent 50 μm.

following prolonged moderate intensity exercise (>2 h). As such, it is difficult to compare this finding with previous research, but such observations may be partially explained by the oxidative nature of type IIa fibres within endurance-trained individuals compared to untrained or less trained individuals. Moreover, there is also a large level of recruitment of the m. vastus lateralis during prolonged cycling, which would therefore require high energy demand from both fibre types. When taken together, our data present novel findings in relation to IMTG utilisation in type IIa fibres as well as subcellular-specific

IMTG utilisation. Such differences between the present and aforementioned studies may be methodological in nature and a reflection of different immunohistochemical staining techniques. Indeed, most of these aforementioned studies assessed IMTG content through the use of ORO lipid dye (Van Loon *et al.* 2003*a*,*b*; De Bock *et al.* 2005; Stellingwerff *et al.* 2007*a*,*b*) whilst the present study utilised an alternative dye, BODIPY 493/503. The different lipid dyes have important methodological considerations given that ORO does not exclusively stain IMTG but also labels all neutral lipids (e.g. phospholipids within
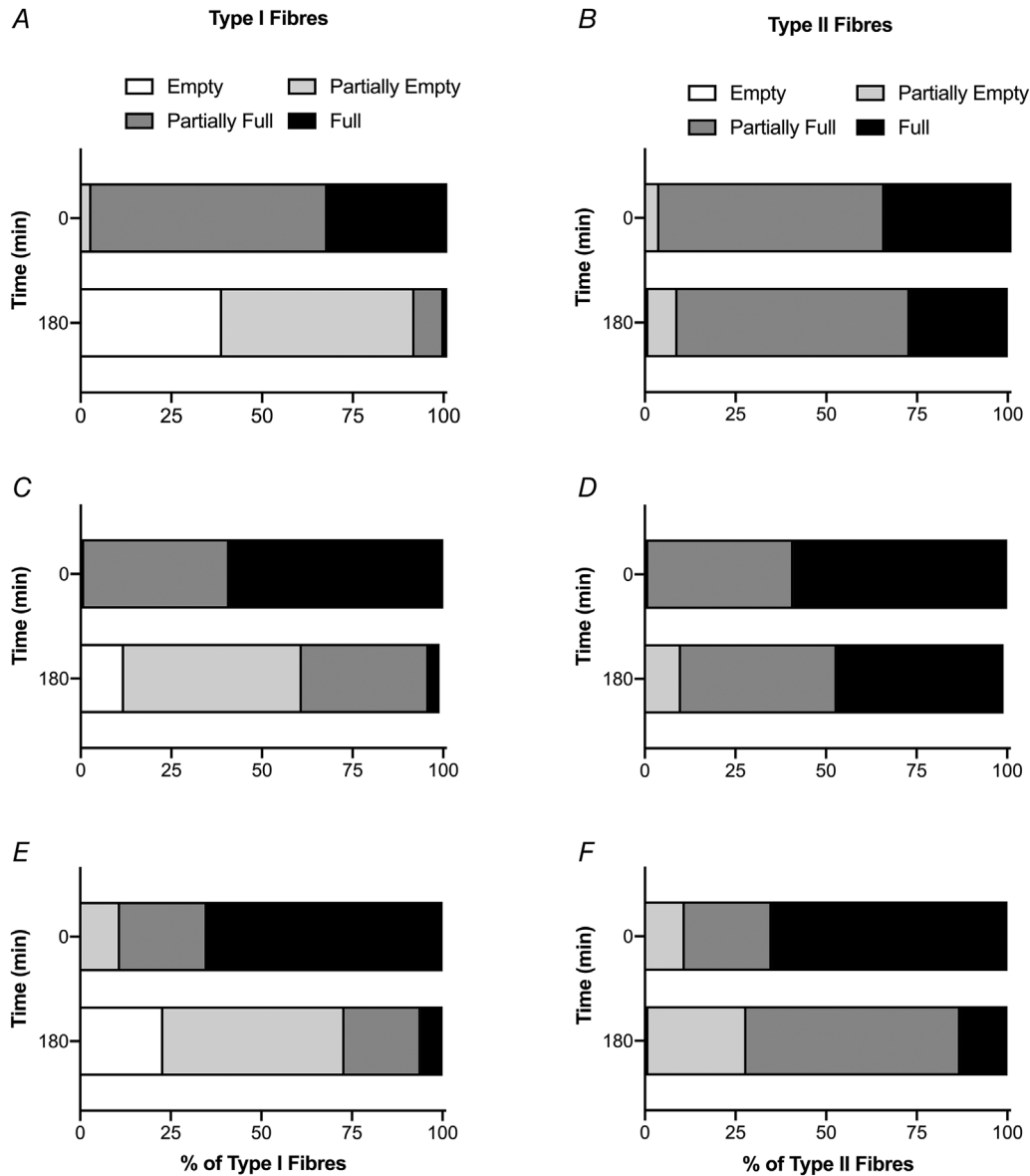

Figure 11. Relative fibre-specific muscle glycogen content before and after the 180 min submaximal exercise protocol
Relative glycogen content in 0 and 180 min sections in 0 g h^{-1} type I (*A*), 0 g h^{-1} type II (*B*), 45 g h^{-1} type I (*C*), 45 g h^{-1} type II (*D*), 90 g h^{-1} type I (*E*), and 90 g h^{-1} type II (*F*) muscle fibres. Data are presented as percentage of fibres categorised within optical density increments of 25% from the lowest recorded optical density to the highest value across all samples.

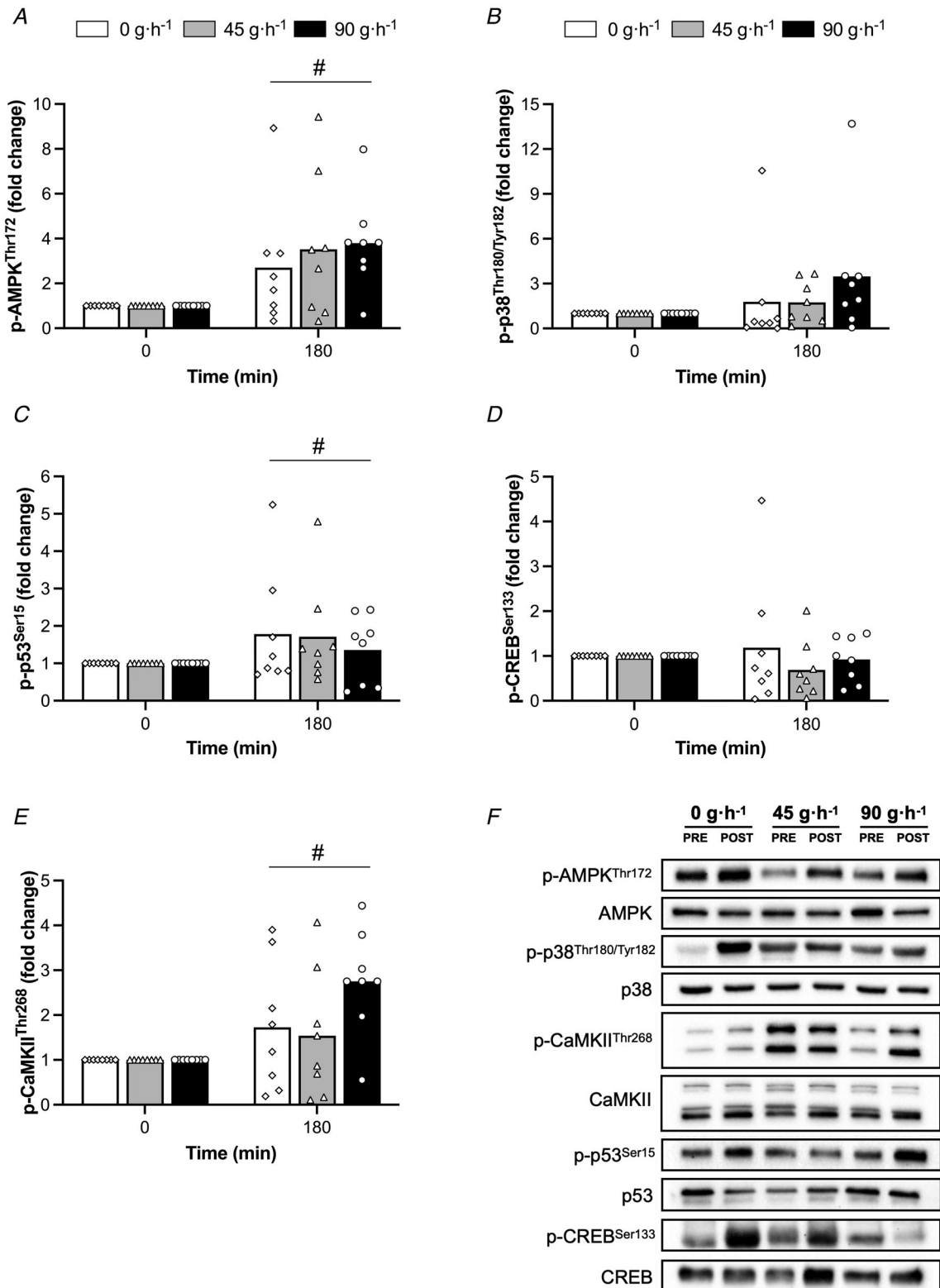**Figure 12. Effects of exercise and carbohydrate feeding on skeletal muscle cell signalling pathways**
AMPK^{Thr172} phosphorylation (*A*), p38$^{Thr180/Tyr182}$ phosphorylation (*B*), p53^{Ser15} phosphorylation (*C*), CREB^{Ser133} phosphorylation (*D*) and CaMKII^{Thr286} phosphorylation (*E*) at 0 and 180 min. *F*, representative western blot images.
[#]Significantly different from 0 min, $P < 0.05$.

membranes) and recent comparisons between the two suggest that BODIPY detects greater amounts of IMTG content compared to ORO in all fibre types (Strauss *et al.* 2020). Additionally, differences in the quantification of the subcellular utilisation of IMTG are also evident with the use of the different dyes, as comparisons showed IMTG utilisation to be specific to the peripheral region when examined using ORO whilst utilisation was of a similar magnitude in the peripheral and central region with the use of BODIPY 493/503 (Strauss *et al.* 2020).

Despite examining both central and peripheral IMTG utilisation, it is currently difficult to ascertain the precise functional role of these subcellular pools. Nonetheless, it has been suggested that the central IMTG pool is the preferred fuel source for the central mitochondria, thus providing energy for muscle contraction (Hood, 2001). The central mitochondria also contain increased levels of oxidative phosphorylation proteins and increased activity of the respiratory chain complex (Ferreira *et al.* 2010). As such, the central mitochondria appear specialised towards energy production for contractile function and may therefore rely on the central IMTG pool. In contrast, the peripheral IMTG pool presumably supply the peripheral mitochondria and potentially provide energy for membrane related processes (Hood, 2001). Future work utilising transmission electron microscopy (TEM) (given its greater magnification capabilities and resolving power) is warranted to further investigate LD utilisation and mitochondrial content within these subcellular regions.

We also observed that the magnitude of IMTG degradation in both type I and IIa fibres was not affected by CHO feeding during exercise, thus demonstrating that an IMTG sparing effect did not occur (see Fig. 6). These findings are consistent with previous suggestions that the ingestion of 50 g h^{-1} of glucose during exercise does not alter skeletal muscle IMTG utilisation during 3 h of moderate intensity cycling exercise (Stellingwerff *et al.*

2007*a*). In contrast, De Bock *et al.* (2005) observed an attenuated use of IMTG in type I fibres with 2.5 g kg^{-1} ($\sim$150 g) and 1 g kg h^{-1} ($\sim$75 g h^{-1}) of CHO intake before and during 2 h of a more intense cycling protocol ($\sim$75% of $\dot{V}_{O_2 max}$) compared to completing the exercise trial after an overnight fast. It is possible that the consumption of a pre-exercise meal across all trials in the present study may have offset any potential regulatory effects of CHO feeding during the exercise bout itself. In this way, the metabolic differences between trials (e.g. hormonal milieu and substrate availability) during the initial 60–90 min of exercise may not have been sufficient to alter the pattern of IMTG use. This is especially relevant given that IMTG utilisation appears to predominate during the first 2 h of exercise, with an attenuated use thereafter (Romijn *et al.* 1993; Watt *et al.* 2002). Indeed, although a progressive difference in plasma NEFA availability and delivery during the third hour of exercise (see Fig. 3) may have suppressed IMTG mobilisation and/or oxidation (Watt *et al.* 2002; Van Loon *et al.* 2003*a*, 2005; Watt *et al.* 2004*a*), it is possible that the predominant IMTG utilisation had already occurred during the first 1–2 h of exercise. Such a hypothesis is supported by the observation that whole body rates of fat oxidation did not differ between trials until the second and third hour of exercise (see Fig. 4). It is also noteworthy that although glucose consumption during exercise has previously been shown to suppress skeletal muscle hormone sensitive lipase activity, this was not associated with alteration to estimated IMTG oxidation (Watt *et al.* 2004*b*).

Similar to IMTG, we observed greater relative glycogen depletion in type I fibres compared with type II muscle fibres, the magnitude of which was not affected by CHO feeding during exercise (see Fig. 9). The absence of a glycogen sparing effect is in contrast to previous reports demonstrating that CHO feeding exerts glycogen sparing in type I (Stellingwerff *et al.* 2007*a*) and
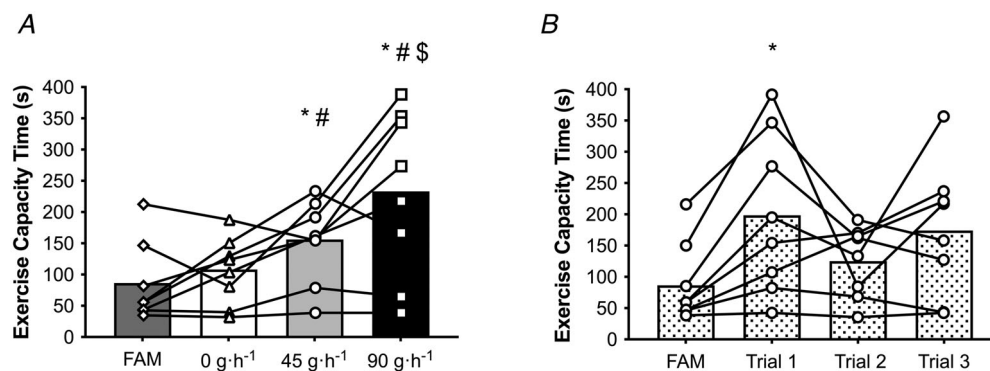

Figure 13. Effects of carbohydrate feeding on exercise capacity
Exercise capacity time (time to exhaustion) during the familiarisation (FAM), 0, 45 and 90 g h^{-1} trials (*A*), and trial order exercise capacity time (*B*). *Significantly different from FAM, $P < 0.05$. #Significantly different from 0 g h^{-1}, $P < 0.05$. $Significantly different from 45 g h^{-1}, $P = 0.06$. Bars represent group means and symbols represent individual data points.

type II fibres (De Bock *et al.* 2007; Stellingwerff *et al.* 2007*a*). Such discrepancies between studies may be underpinned by methodological differences that are related to muscle glycogen availability, pre-exercise CHO feeding, exercise duration and timing of assessment of substrate metabolism. In relation to the latter, Stellingwerff *et al.* (2007*a*) observed that the glycogen sparing effect in type I and type II fibres was due to a reduction in muscle glycogen utilisation in the first hour of exercise, as evidenced by stable isotope methodology. These researchers also used a dietary protocol in which subjects commenced exercise in the fasted state and after

consuming ∼6 g kg^{-1} CHO per day for 2 days. Given the greater CHO availability associated with the present dietary protocol (i.e. a true CHO loading protocol as well as consumption of a pre-exercise high CHO meal), it is possible that any potential regulatory effects of consuming CHO 'during' exercise are offset by the effects of higher pre-exercise muscle glycogen availability on absolute muscle glycogen utilisation (Hargreaves *et al.* 1995; Arkinstall *et al.* 2004). Despite using similar dietary conditions to those adopted here (i.e. consumption of a pre-exercise CHO meal of 2.5 g kg^{-1} in the CHO fed trial), De Bock *et al.* (2007) observed a glycogen

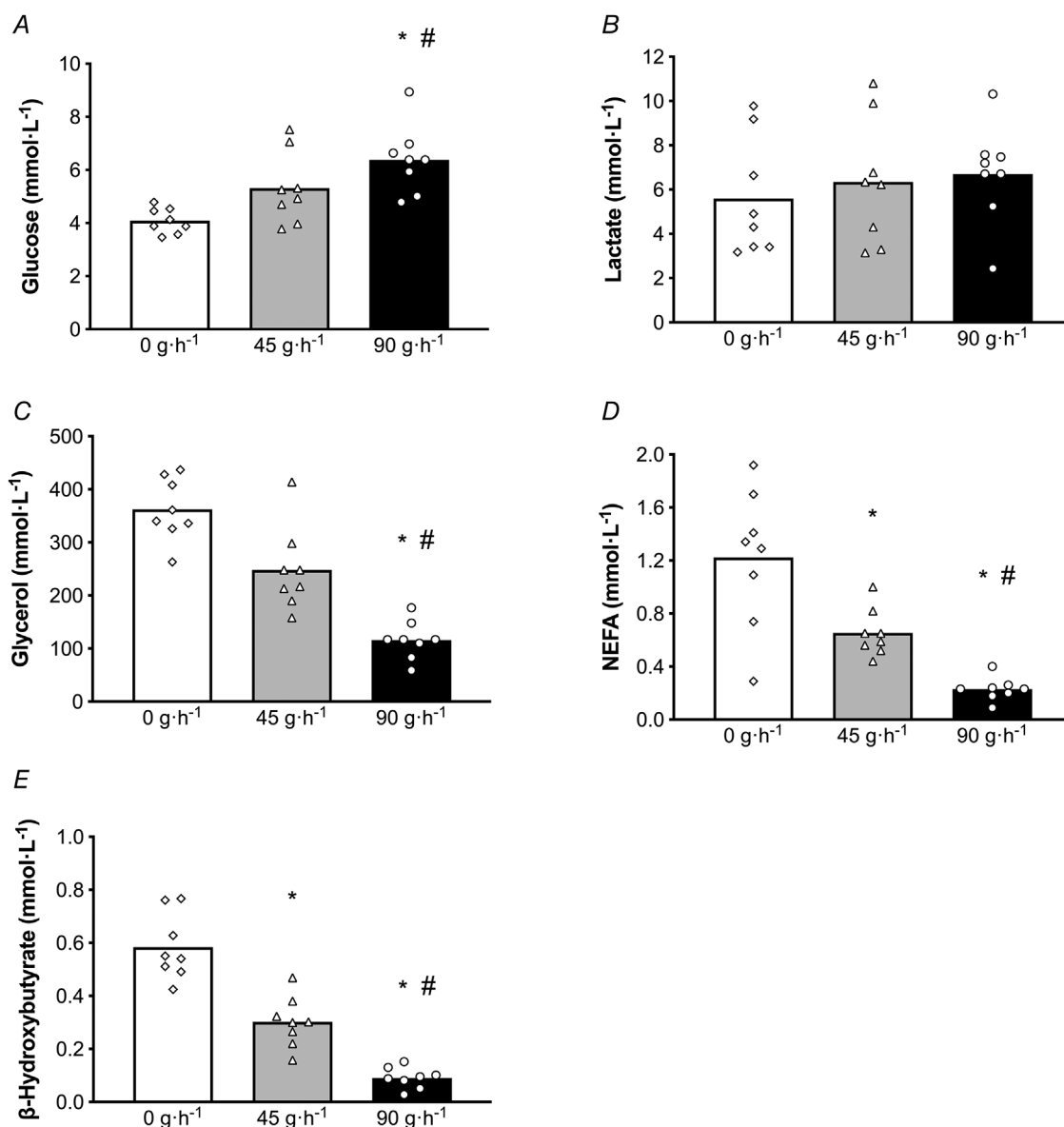**Figure 14. Circulating metabolite responses immediately after the exercise capacity test**
Plasma glucose (*A*), lactate (*B*), glycerol (*C*), NEFA (*D*), β-OHB (*E*) responses immediately after the exercise capacity test. *Significant difference from 0 g h^{-1}, #significant difference from 45 g h^{-1}, $P < 0.05$. Bars represent group means and symbols represent individual data points.

sparing effect in type IIa fibres whereas we observed no such effect. Given the well documented effects of exercise duration on glycogen utilisation (Areta & Hopkins, 2018) coupled with the selective recruitment of type II fibres as type I fibres become depleted (Gollnick et al. 1973), it is possible that discrepancies between studies in this instance are simply a reflection of a greater exercise duration (i.e. 3 vs. 2 h cycling protocol). Such findings highlight the need for future studies to assess the time course of fibre-specific muscle glycogen use during prolonged endurance exercise. In addition, the assessment of glycogen utilisation within distinct sub-cellular pools of both type I and II muscle fibres (through the use of quantitative TEM) is also warranted to provide a more complete understanding of glycogen utilisation with CHO feeding. This is especially relevant given the recent findings that endurance capacity is associated with intra-myofibrillar glycogen content within type I fibres and that supercompensation of the subsarcolemmal glycogen pool (via CHO loading) reduces the utilisation of the intra-myofibrillar glycogen pool during exercise (Jensen et al. 2020).

In relation to cell signalling pathways, CHO feeding did not affect exercise-induced activation of upstream (e.g. AMPK^{Thr172} and CaMKII^{Thr268} phosphorylation) or downstream signalling (e.g. p53^{Ser15} phosphorylation) proteins with regulatory roles in mitochondrial biogenesis. In contrast to AMPK, we did not observe any change in the phosphorylation status of p38 MAPK$^{Thr180/Tyr182}$ in response to exercise or CHO feeding, though we note the large inter-individual variability between subjects. When considered with previous data demonstrating that p38 MAPK phosphorylation is unaltered by muscle glycogen concentration (Yeo et al. 2010; Bartlett et al. 2013; Hammond et al. 2019) or pre-exercise CHO feeding (Stocks et al. 2019), our data provide further evidence to support the notion that CHO availability does not readily modulate the phosphorylation of p38 MAPK in whole muscle homo-genate. We have previously observed that both AMPK and p53 related signalling is enhanced when exercise is commenced and finished with high (~500 and ~300 mmol kg^{-1} DW, respectively) versus low (~150 and ~50 mmol kg^{-1} DW, respectively) muscle glycogen (Bartlett et al. 2013). More recently, we also observed that comparable cell signalling responses occur if exhaustive (Hearris et al. 2019) and non-exhaustive (Hearris et al. 2020) exercise is finished with similar muscle glycogen concentrations (i.e. <300 mmol kg^{-1} DW), despite commencing exercise with graded levels of muscle glycogen concentration. On this basis, it was suggested that in the context of manipulating CHO availability to potentiate cell signalling, the absolute post-exercise muscle glycogen concentration is a more influential factor than pre-exercise muscle glycogen concentration.

Such a line of thinking is accordance with the present data as well as independent studies (Akerstrom et al. 2006; Lee-Young et al. 2006) demonstrating that CHO feeding does not affect cell signalling when alterations to muscle glycogen utilisation (i.e. glycogen sparing) has not occurred. Such findings are likely to be underpinned by the physical tethering of AMPK to the glycogen granule via its ß-subunit (Steinberg et al. 2006). In this way, comparable exercise-induced glycogen depletion may result in dissociation of AMPK, thus allowing for similar kinase phosphorylation. Additionally, our observation of comparable CaMKII phosphorylation between trials may also be underpinned by similar absolute levels of glycogen utilisation and depletion, that is, any potential glycogen mediated impairments in Ca^{2+} release from the sarcoplasmic reticulum (Gejl et al. 2014) would be similar between trials, thus providing a similar stimulus for Ca^{2+} mediated signalling.

In considering exercise capacity, we observed that CHO feeding improved exercise capacity in a dose dependent manner such that 90 g h^{-1} > 45 g h^{-1} > 0 g h^{-1}. Importantly, we utilised a CHO feeding protocol consisting of solids, gels and fluid (in accordance with the feeding practices of elite endurance athletes) as well as inclusion of a full familiarisation trial. Whilst it is difficult to directly compare between studies (given the differences in training status, exercise protocol and CHO source and blend), our observation that 90 g h^{-1} is an ergogenic dose agrees with several recent dose-response studies examining multiple source blends. For example, Smith et al. (2013) estimated that 78 g h^{-1} CHO was the optimal dose to maximise performance when a wide range of doses (0 to 120 g h^{-1}) was investigated. Indeed, using a multiple site research protocol, these researchers used a variety of doses of CHO (comprising a 1:1:1 ratio of glucose, fructose and maltodextrin solution consumed during 2 h steady-state ride at 95% onset of blood lactate accumulation) and observed that the magnitude of performance improvement during a subsequent 20 km time trial (TT) was curvilinear in nature. More recently, King et al. (2018) observed that ingestion of 90 g h^{-1} of a glucose-fructose (2:1 ratio) solution during a 2 h sub-maximal ride at 77% $\dot{V}_{O_2 \, max}$ produced the highest mean power output during a subsequent 30 min self-paced TT when compared with 60 and 75 g h^{-1} of a glucose only solution and 112.5 g h^{-1} of a glucose-fructose (2:1 ratio) solution. When the same group extended exercise duration beyond 3 h but of a lower exercise intensity (60% $\dot{V}_{O_2 \, max}$), the researchers reported that ingestion of 90 g h^{-1} of glucose and fructose (2:1 ratio) solution increased power output during a 30 min self-paced TT by 6.8% and 4% when compared with 100 g h^{-1} and 80 g h^{-1}, respectively (King et al. 2019). When such studies are considered with the present data, it becomes apparent that where exercise duration exceeds 2.5–3 h,

the ergogenic effects of CHO feeding are likely to be most predominant with multiple source blends comprising 75–90 g h^{-1}.

In contrast to a muscle glycogen sparing effect, the observed exercise capacity improvements are most likely related to maintenance of high CHO availability, plasma glucose concentrations and ultimately, high rates of CHO oxidation (Coyle *et al.* 1986; Smith *et al.* 2010; Newell *et al.* 2018). Indeed, although we acknowledge that our study design did not represent a true double-blind design (given that subjects were consciously aware of consuming more solids in the 90 g h^{-1} trial), examination of substrate availability and oxidation support the hypothesis that maintenance of high rates of CHO oxidation (at the expense of lipid oxidation) underpinned the exercise capacity improvements observed here. Such a hypothesis is especially evident when examining the 'crossover point' at which lipid oxidation comprised the largest proportion of energy production during exercise. Indeed, these data demonstrate that consuming 90 g h^{-1} of CHO delayed the crossover point by ∼10 and ∼40 min when compared with the 45 and 0 g h^{-1} trials, respectively (see Fig. 5). The magnitude of improvement in exercise capacity between trials is also of physiological significance given that professional cycling races (e.g. the cycling Grand Tours) can be won or lost with seconds.

Aside from an exercise performance perspective, our data provide greater insights into how athletes can implement nutritional strategies that promote training quality without sacrificing cellular adaptation. Indeed, there is often a belief that nutritional strategies to promote training intensity (i.e. promotion of adequate CHO availability) are mutually exclusive to those promoting training adaptation (i.e. CHO restriction). However, the present data demonstrate that trained male athletes can still achieve these fundamental adaptive goals of training even in the presence of high CHO availability before and during training. Our data clearly demonstrate that high CHO availability (i.e. high glycogen availability, pre-exercise feeding and CHO intake during exercise) does not impair the activation of cell signalling pathways with regulatory roles in stimulating hallmark adaptations to endurance training such as mitochondrial biogenesis. Importantly, these findings were observed during an exercise challenge that adopted an exercise duration (i.e. > 3 h) and intensity (i.e. above lactate threshold) that is of 'real world' physiological relevance to 'elite' endurance athletes.

In summary, we demonstrate that CHO feeding does not alter fibre and subcellular-specific IMTG content, LD morphology, muscle glycogen utilisation or exercise-induced cell signalling in human skeletal muscle. In relation to IMTG metabolism, we report for the first time that the exercise-induced decrease in IMTG content occurred in both the peripheral and central regions of both type I and IIa muscle fibres. Additionally, we observed a reduction in LD number in both type I and IIa fibres whereas a reduction in LD size was only evident in type I fibres. In the absence of alterations to muscle fuel selection, we also observed that CHO feeding does not attenuate cell signalling pathways with regulatory roles in mitochondrial biogenesis. Finally, the data demonstrate that CHO feeding improves exercise capacity in a dose dependent manner, an effect that was associated with maintenance of circulating glucose availability and whole-body rates of CHO oxidation. Importantly, our assessment of substrate metabolism was conducted under experimental conditions that are considered best nutritional practice (i.e. after CHO loading and consumption of CHO before and during exercise) for elite endurance athletes.

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Additional information

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

J.P.M. is a consultant for Science in Sport (SiS). SiS, GlaxoSmithKline (GSK) and Lucozade Ribena Suntory (LRS) have funded his previous research on glycogen metabolism and exercise.

Author contributions

J.M.F., J.B.L., S.O.S., J.P.M.: substantial contributions to the conception or design of the work; All authors: the acquisition, analysis, or interpretation of data for the work; J.M.F., M.A.H., J.A.S., S.O.S., J.P.M.: drafting the work or revising it critically for important intellectual content; All authors: final approval of the version to be published; All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Keywords

glycogen, intramuscular triglyceride, vastus lateralis

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Document

Supplementary appendix