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Local depletion of glycogen with supramaximal exercise in human skeletal muscle fibres

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Key points

- Glycogen is stored in local spatially distinct compartments within skeletal muscle fibres and is the main energy source during supramaximal exercise.
- Using quantitative electron microscopy, we show that supramaximal exercise induces a differential depletion of glycogen from these compartments and also demonstrate how this varies with fibre types.
- Repeated exercise alters this compartmentalized glycogen depletion.
- The results obtained in the present study help us understand the muscle metabolic dynamics of whole body repeated supramaximal exercise, and suggest that the muscle has a compartmentalized local adaptation to repeated exercise, which affects glycogen depletion.

Abstract Skeletal muscle glycogen is heterogeneously distributed in three separated compartments (intramyofibrillar, intermyofibrillar and subsarcolemmal). Although only constituting 3–13% of the total glycogen volume, the availability of intramyofibrillar glycogen is of particular importance to muscle function. The present study aimed to investigate the depletion of these three subcellular glycogen compartments during repeated supramaximal exercise in elite athletes. Ten elite cross-country skiers (aged 25 ± 4 years, $\dot{V}_{O_2max}$: 65 ± 4 ml kg^{-1} min^{-1}; mean ± SD) performed four ∼4 min supramaximal sprint time trials (STT 1–4) with 45 min of recovery. The subcellular glycogen volumes in musculus triceps brachii were quantified from electron microscopy images before and after both STT 1 and 4. During STT 1, the depletion of intramyofibrillar glycogen was higher in type 1 fibres [−52%; (−89:−15%)] than type 2 fibres [−15% (−52:22%)] ($P = 0.02$), whereas the depletion of intermyofibrillar glycogen [main effect: −19% (−33:0%), $P = 0.006$] and subsarcolemmal glycogen [main effect: −35% (−66:0%), $P = 0.03$] was similar between fibre types. By contrast, only intermyofibrillar glycogen volume was significantly reduced during STT 4, in both fibre types [main effect: −31% (−50:−11%), $P = 0.002$]. Furthermore, for each of the subcellular compartments, the depletion of glycogen during STT 1 was associated with the volumes of glycogen before STT 1. In conclusion, the depletion of spatially distinct glycogen compartments differs during supramaximal exercise. Furthermore, the depletion changes with repeated exercise and is fibre type-dependent.

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Abbreviations $_{est}$CE, estimate of coefficient of error; CHO, carbohydrate; CI, confidence interval; CrP, creatine phosphate; DIA, diagonal stride; DP, double-pooling subtechnique; E–C, excitation contraction; GE, gross efficiency; MHC, myosin heavy chain; SR, sarcoplasmic reticulum; STT, sprint time trial; TEM, transmission electron microscopy; $\dot{V}_{O_2}$, oxygen uptake; $\dot{V}_{O_2max}$, maximal oxygen uptake.

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Introduction

Early studies have demonstrated strong associations between the depletion of muscle glycogen and impaired muscle function during exercise at moderate to high intensities in humans (Bergström *et al.* 1967; Hermansen *et al.* 1967; Bangsbo *et al.* 1992). Although the detailed mechanisms underlying this link remain unknown, studies in both animals and humans have demonstrated that key steps in the excitation–contraction (E–C) coupling [i.e. sarcoplasmic reticulum (SR) Ca^{2+} handling and Na^{+}-K^{+} transport] are disturbed by reductions in muscle glycogen (James *et al.* 1999; Duhamel *et al.* 2006; Ørtenblad *et al.* 2011; Gejl *et al.* 2014; Ørtenblad & Nielsen 2015).

To gain insight into the role of glycogen, its subcellular localization in human muscle fibres has been examined both qualitatively (Oberholzer *et al.* 1976; Sjöström *et al.* 1982; Fridén *et al.* 1985; Fridén 1989) and semiquantitatively by transmission electron microscopy (TEM) (Marchand *et al.* 2007; Nielsen *et al.* 2011). Muscle glycogen is heterogeneously distributed between three separated compartments: below the sarcolemma (subsarcolemmal glycogen), between the myofibrills (intermyofibrillar glycogen) and within the myofibrils (intramyofibrillar glycogen) (Marchand *et al.* 2002). Stereological glycogen quantification of TEM images has revealed that highly trained endurance athletes generally deposit 8–11% as subsarcolemmal glycogen, 77–84% as intermyofibrillar glycogen and 3–13% as intramyofibrillar glycogen (Nielsen *et al.* 2011; Nielsen *et al.* 2012). Importantly, this distribution varies with training status, in response to acute exercise (Nielsen *et al.* 2011) and even between different fibre types at rest. For example, type 1 fibres from musculus triceps brachii of elite cross-country skiers have been shown to contain twice as much intramyofibrillar glycogen as type 2 fibres, whereas , in the other compartments, levels are similar (Nielsen *et al.* 2011).

The relative degradation of glycogen during exercise differs between the three subcellular compartments (Oberholzer at al. 1976; Sjöstrøm *et al.* 1982; Fridén *et al.* 1985; Nielsen *et al.* 2011). This is of particular importance because the volume of intramyofibrillar glycogen has been shown to correlate with the SR Ca^{2+} release rate subsequent to prolonged exercise and, similarly, half-relaxation time has been shown to correlate with the volume of intermyofibrillar glycogen in rats (Nielsen *et al.* 2009) (Ørtenblad *et al.* 2011). This emphasizes that certain subcellular compartments of glycogen regulate important parts of E–C coupling.

Although all glycogen compartments are reduced after moderate to high-intensity exercise, the results of previous studies investigating their relative degradation are inconsistent (Oberholzer at al. 1976; Sjöstrøm *et al.* 1982; Fridén *et al.* 1985; Nielsen *et al.* 2011). Employing semiquantitative methods, Marchand *et al.* (2007) demonstrated very low levels of intramyofibrillar glycogen after exhaustive cycling exercise, and we have shown previously that, after 1 h of exhaustive exercise, the intramyofibrillar compartment is depleted to a relatively greater extent compared to glycogen in the other compartments in both type 1 and 2 fibres (Nielsen *et al.* 2011). Therefore, the link between exhaustion of endogenous glycogen stores and muscle fatigue after prolonged exercise may originate from the intramyofibrillar compartment. To date, compartmentalized glycogen depletion has been described in relation to prolonged exercise (Marchand *et al.* 2007; Nielsen et al., 2011, 2012), whereas the effects of short-term high-intensity exercise have not yet been investigated by quantitative methods. Furthermore, it is unclear whether the utilization of glycogen at its various subcellular compartments during short-term high-intensity exercise is fibre type-specific. From biochemical measurements of whole muscle glycogen concentration, it has been reported that a single bout of short-term exhaustive exercise (3–5 min) reduces the level of glycogen by 25–35% (Essen 1978; Bangsbo *et al.* 1991; Bangsbo *et al.* 1992; Bangsbo *et al.* 1994; Graham *et al.* 2001) and, if exercise is repeated, the depletion of glycogen is attenuated (Karlsson & Saltin, 1971; Bangsbo *et al.* 1992). Interestingly, low levels of muscle glycogen have been associated with impairments of performance even during short-term exhaustive exercise (Bangsbo *et al.* 1992; Balsom *et al.* 1999; Skein *et al.* 2012). To understand how short-term high-intensity exercise influences glycogen metabolism and muscle function, it is important to determine the utilization of glycogen in its distinct subcellular compartments during this type of exercise.

The present study therefore aimed to characterize compartmentalized glycogen utilization by type 1 and 2 fibres during repeated supramaximal exercise in elite athletes. On the basis of previous findings, our hypothesis was that intramyofibrillar glycogen would be depleted preferentially in both fibre types during supramaximal exercise.

Methods

Ethical approval

The project was approved by the Regional Ethics Review Board in Umeå, Sweden (#2013-59-31) and the experiments conformed with the standards of the *Declaration of Helsinki*. All subjects were fully informed of any risk associated with the experiments and provided their written consent before participation. The present study is part of a larger project, and biopsy material from musculus vastus lateralis was obtained contemporary with the biopsies used here from musculus triceps brachii. Biopsy material from the thigh and remaining parts

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of the biopsy material from the arms were stored for supplementary studies (Andersson *et al.* 2016; Gejl *et al.* 2016).

Study design

The effect of repeated sprint cross-country skiing on subcellular glycogen compartmentalization was examined in 10 elite cross-country skiers. The skiers were tested on a treadmill in the laboratory on separate days over 3 weeks, with at least 48 h between test days (Andersson *et al.* (2016)). Preliminary measurements, including submaximal gross efficiency, height, body mass and maximal oxygen uptake ($\dot{V}_{O_2max}$), were obtained during the initial visits, whereas the final visit before the sprint time trial (STT) performance test involved the testing of maximal velocity and familiarization with the STT protocol. The performance test involved four supramaximal 1300 m STTs (~ 4 min each) interspersed with 45 min of recovery (i.e. a simulated sprint race in cross-country skiing) (Fig. 1). Subjects were instructed to refrain from activities of moderate to high intensity during the 48 h prior to testing. Dietary intake was standardized from 24 h prior to the first STT, as well as throughout the STT protocol.

Subjects

Ten male elite cross-country skiers participated in the study; age 25 ± 4 years, height 183 ± 7 cm, body mass 79.2 ± 5.9 kg, $\dot{V}_{O_2max}$: 5.1 ± 0.5 l min^{-1} or 64.9 ± 4.1 ml kg^{-1} min^{-1} with diagonal stride (DIA) and 5.0 ± 0.5 l min^{-1} or 62.5 ± 4.2 ml kg^{-1} min^{-1} with the double-pooling technique (DP) (mean ± SD). Muscle tissue from musculus triceps brachii contained 39 ± 6% myosin heavy chain (MHC) type 1 fibres and 62 ± 6% MHC type 2 fibres (mean ± SD). Skiers participated in national and/or international sprint and distance races and trained 8–11 h per week during the testing period. All participants had experienced treadmill roller skiing.

Race simulation

The race simulation consisted of four successive 1300 m STTs (~4 min each), interspersed with 45 min of recovery with carbohydrate intake (CHO) (see below). A 5 min warm-up and 5 min cool-down were performed before and after each STT. Each 1300 m STT consisted of three DP sections (1°) and two DIA sections (7°), and skiers were encouraged to perform maximally during each trial. Treadmill velocity and $\dot{V}_{O_2}$ were monitored continuously during the four STTs, and the data were averaged. Heart rate was measured continuously throughout each STT and blood lactate was collected from the fingertip at 1 min following each STT. Room temperature (~22°C) and humidity (~56%) were maintained throughout the course of the experiment.

Estimation of aerobic and anaerobic energy contributions

The modified gross efficiency (GE) method was used to estimate the supramaximal O_2 deficit during STT 1–4. The supramaximal GE was estimated based on extrapolation of the submaximal relationships for skiing velocity and incline *vs.* GE during the preliminary test (Andersson *et al.* 2016). These relationships were then used to estimate the supramaximal GE during STT 1–4. The total metabolic rate required during the STT was obtained by dividing the supramaximal power output by the GE and converting it to a $\dot{V}_{O_2}$ requirement. The O_2 deficit was then computed by subtracting the accumulated $\dot{V}_{O_2}$ from the accumulated $\dot{V}_{O_2}$ requirement (Andersson *et al.* 2016).

Dietary restrictions

Dietary intake was controlled and calculated throughout the experiments, based on body mass. Subjects received standardized CHO-enriched meals [three meals and three snacks; 8 g of CHO (kg body weight^{-1}) day^{-1}] during

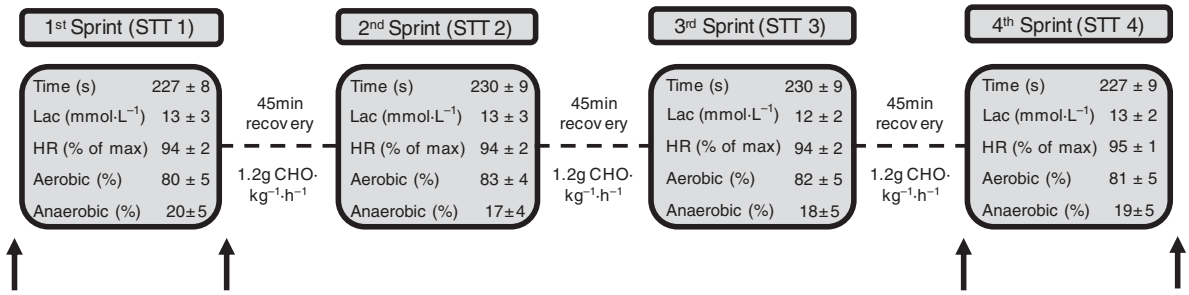

Figure 1. Study design
Schematic illustration of the study design together with relevant data from each sprint. These data have been published previously and are presented as the mean ± SD (Andersson *et al.* 2016; Gejl *et al.* 2016). Only data from subjects included in the glycogen analysis are presented for STT 1 and 4 (n = 10 and n = 9, respectively). Data from all subjects are included in STT 2 and 3 (n = 10). The arrows indicate the time points at which muscle biopsies were obtained.

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the 24 h prior to the race simulation. On average, the meals contained 18 000 kJ (55% carbohydrates, 30% fat and 15% protein). The last meal was consumed upon reporting to the laboratory 120 min before the warm-up. In each of the three recovery periods from STT 1 to 4, subjects received CHO beverages and energy gels, ensuring that they consumed 1.2 g CHO (kg body weight^{-1}) h^{-1}. On average, subjects consumed 70 g of carbohydrates during the 45 min recovery period (40 g of sport drink mixed with water and 30 g of energy gel), in addition to water *ad libitum*. This CHO intake corresponded to the recommendations from the American College of Sports Medicine (Rodriguez *et al.* 2009).

Maximal oxygen uptake

Respiratory variables were measured using an ergospirometry system (AMIS 2001 model C; Innovision A/S, Odense, Denmark). The gas analysers were calibrated with a mixture of 16.0% O_2 and 4.0% CO_2 (Air Liquide, Kungsängen, Sweden) and calibration of the flow meter was performed at low, medium and high flow rates, with a 3 L air syringe (Hans Rudolph, Kansas City, MO, USA). Ambient conditions were monitored with an external apparatus (Vaisala PTU 200; Vaisala Oy, Helsinki, Finland). Blood samples (20 μl) were obtained from the fingertip and used for determining blood lactate concentration by Biosen 5140 (EKF Diagnostic GmbH, Magdeburg, Germany). This apparatus was calibrated with a standard solution of lactate (12 mmol l^{-1}) prior to each analysis. Heart rate was measured continuously throughout the tests, using a heart rate monitor (S810; Polar Electro OY, Kempele, Finland).

Two incremental roller skiing $\dot{V}_{O_2max}$ tests were performed in a random order and at different slopes, with either DP or DIA (DP at 1° and DIA at 7°). The two tests were separated by a 5 min cool-down and a 15 min passive recovery. The $\dot{V}_{O_2max}$ tests started at 21 km h^{-1} during DP and at 9 km h^{-1} during DIA. The velocity was then increased by 1 km h^{-1} every 60 s until exhaustion in DP, and by 0.5 km h^{-1} every 45 s until exhaustion in DIA. The average of the three highest 10 s consecutive values was analysed to determine $\dot{V}_{O_2max}$ with each technique. Blood lactate was collected from the fingertip at 1, 3 and 10 min, respectively, on completion of the test.

Muscle biopsies

In the present study, tissue was obtained from arm muscle (musculus triceps brachii) before and after the first and fourth STT (Fig. 1) because this muscle is highly active during cross-country skiing (Holmberg *et al.* 2005). The Bergström needle biopsy technique was used to obtain muscle samples from the mid-section of the musculus triceps brachii (in a random order of the left and right arm) at near identical depth and vicinity of location. The pre-STT 1 biopsy was obtained 20 min before STT 1, and the post-biopsies were obtained immediately after STT 1 and STT 4 (within 5 min post-race). The pre-STT 4 biopsy was obtained 10–12 min before STT 4 (i.e. prior to warm-up). The muscle sample was placed on a filter paper, on an ice-cooled ∼0°C Petri dish, and divided into five specimens. For the purpose of the present study, one part was used for TEM imaging and another part for determining fibre type distribution.

MHC distribution

MHC composition was determined from homogenate using gel electrophoresis as described previously (Danieli Betto *et al.* 1986) and modified for humans (Ørtenblad 2000). In brief, muscle homogenate (80 μL) was mixed with 200 μL of sample-buffer (10% glycerol, 5% 2-mercaptoethanol and 2.3% SDS, 62.5 mM Tris and 0.2% bromophenolblue at pH 6.8.), boiled in water for 3 min, and loaded with three different quantities of protein (10–40μL) on a SDS-PAGE gel [6% poly-acrylmide (100:1 acrylmid:bis-acrylmid), 30% glycerol, 67.5 mM Tris-base, 0.4% SDS and 0.1 M glycine]. Gels were run at 80 V for at least 42 h at 4 °C and MHC bands made visible by staining them with Coomassie. The gels were scanned (Linoscan 1400 scanner; Linoscan Heidelberg, Germany) and MHC bands were quantified densiometrically (Phoretix 1D, non-linear; Phoretix International Ltd, Newcastle, UK) as an average of the two to three loaded protein amounts, giving clear MHC bands. MHC II was identified by western blotting, using monoclonal antibody (M 4276; Sigma, St Louis, MO, USA), with the protocol Xcell IITM (Invitrogen, Carlsbad, CA, USA).

TEM

Muscle biopsy specimens were prepared for glycogen visualization using TEM, as described previously (Nielsen *et al.* 2010). In brief, specimens were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 24 h, and subsequently rinsed four times in 0.1 M sodium cacodylate buffer. After rinsing, muscle specimens were post-fixed with 1% osmium tetroxide (OsO_4) and 1.5% potassium ferrocyanide [$K_4Fe(CN)_6$] in 0.1 M sodium cacodylate buffer for 90 min at 4 °C. After post-fixation, the muscle specimens were rinsed twice in 0.1 M sodium cacodylate buffer at 4 °C, dehydrated through a graded series of alcohol at 4–20 °C, infiltrated with graded mixtures of propylene oxide and Epon at 20 °C and embedded in 100% Epon at 30 °C. Ultrathin sections were cut in three depths separated by 150 nm to obtain as many fibres as possible (Ultracut UCT ultramicrotome; Leica Microsystems, Wetzlar, Germany).

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The sections were contrasted with uranyl acetate and lead citrate, and photographed with a pre-calibrated EM 208 transmission electron microscope (Philips, Eindoven, The Netherlands) and a Megaview III FW camera (Olympus Soft Imaging Solutions, Münster, Germany). All longitudinally-orientated fibres from each depth were included, resulting in an average of of seven fibres per biopsy (range: 6–11). Twenty-four fibres images were obtained from each fibre at 10 000× magnification, in a random systematic order, including 12 from the subsarcolemmal region and six from both the superficial and the central region of the myofibrillar space. The variation in the parameters between images was used to estimate a coefficient of error ($_{est}$CE), as proposed for stereological ratio estimates by Howard and Reed (2005). Based on intermyofibrillar mitochondrial volume and Z-line width, myofibres were classified as being type 1 or 2 (Sjöström *et al.* 1982). Intermyofibrillar mitochondrial volume was plotted against Z-line width from all fibres (n = 4–11) from each biopsy. The fibres thereafter demonstrating the highest mitochondria volume fraction and thickest Z-line width were classified as type 1 fibres, and vice versa for type 2 fibres. Only distinct type 1 and 2 fibres were included, whereas intermediate fibres were discarded. In the final analysis, two or three fibres of each type were analysed per biopsy, except in the case of one biopsy, where only one type 2 fibre was clear enough for analysis. All analyses were carried out by the same investigator. Unfortunately, two samples were mixed during preparation and one sample failed quality control. Glycogen volume and particle size in the three compartments were therefore only estimated in nine and eight subjects before and after STT 4, respectively.

Glycogen quantification

To validate the stereological quantification of glycogen, inter-investigator variability between the present investigator and an investigator possessing great experience with the quantification of glycogen from TEM images was evaluated. There was no large bias between the two investigators (<5%) and the coefficient of variation was low (<5%), having being evaluated as proposed by Bland & Altman (1986).

Within the myofibre, glycogen was quantified into three distinct localizations: (1) the intermyofibrillar space; (2) the intramyofibrillar space; and (3) the subsarcolemmal space. Based on stereological counting of glycogen in each space, and by taking section thickness into account, glycogen volume fraction (V_v) was calculated as proposed by Weibel: $V_v = A_A - t\,\{(1/\pi)\cdot B_A - N_A\cdot[(t\cdot H)/(t + H)]\}$, where A_A is glycogen area fraction, t is the section thickness (60 nm), B_A is the glycogen boundary length density, N_A is the number of particles per area and H is the average glycogen particle diameter (Weibel 1980). Glycogen particles were assumed to be spherical (Melendez-Hevia *et al.* 1993). The glycogen area fraction was estimated by point counting, and the average glycogen molecule diameter was measured using iTEM, version 5.0 (Olympus Soft Imaging Solutions). Intermyofibrillar glycogen was expressed relative to the myofibrillar space, and counted with a grid size of 180 and 300 nm, respectively. The myofibrillar space consists of myofibrils (intramyofibrillar space), mitochondria, SR, t-system and lipids). The intramyofibrillar glycogen was expressed relative to the intramyofibrillar space and counted with a grid size of 60 and 180 nm, respectively. The subsarcolemmal glycogen was expressed relative to the muscle fibre surface (estimated from direct length measurement) providing assurance that the results were unbiased, from differences in other subsarcolemmal organelles (e.g. mitochondria, nuclei and lipids). Grid size during the counting of subsarcolemmal glycogen was 180 nm. In muscle fibres with high glycogen volumes, we often observed a clustering of intermyofibrillar glycogen, with glycogen particles covering each other. We therefore investigated the potential underestimation of glycogen volume by plotting the different subfractions of glycogen in type 1 and 2 fibres against whole muscle glycogen concentration. This revealed an underestimation of intermyofibrillar glycogen volume in type 1 fibres in muscles with high glycogen concentrations. Therefore, all intermyofibrillar glycogen volumes were corrected for this underestimation by extrapolating the linear relationship observed at moderate glycogen concentrations to high glycogen concentrations.

The variation in the parameters between images was used to estimate a coefficient of error ($_{est}$CE) as proposed for stereological ratio-estimates by Howard & Reed (2005). The $_{est}$CE were 0.13, 0.17 and 0.20, respectively, in intermyofibrillar, intramyofibrillar and subsarcolemmal glycogen. Quantification of total glycogen volume from electron microscopy pictures was weighed and based on individual fibre type distribution. Glycogen quantifications from homogenates and electron microscopy images were well associated (r^2 = 0.48, $P < 0.05$) (Fig. 2).

Biochemical determination of muscle glycogen

Total muscle glycogen content was determined spectrophotometrically (DU 650; Beckman Coulter, Fullerton, CA, USA). Freeze dried muscle tissue (1.5 mg) was boiled in 0.5 ml of 1 M HCL for 150 min, before it was rapidly cooled, whirl-mixed and centrifuged at 3500 g for 10 min at 4 °C. Next, 40 μl of boiled muscle sample and 1 ml of reagent solution containing Tris-buffer (1 M), distilled water, ATP (100 mM), $MgCl_2$ (1 M), $NADP^+$ (100 mM) and glucose 6-phosphate dehydrogenase were mixed before the process was initiated

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by adding 10 μl of diluted hexokinase. Absorbance was recorded for 60 min before the glycogen content was calculated. Muscle glycogen was expressed as mmol kg dry weight^{-1}.

Statistical analysis

Statistical analyses were performed using Stata, version 13.1 (StataCorp LP, College Station, TX, USA). All interactions or main effects were tested using a linear mixed-effects model, with subjects and time as random effects and with time and fibre type as fixed effects. Variables with skewed distributions were log-transformed before analysis. Associations between variables were evaluated using Pearson's correlation coefficient. Values are presented as geometric means and 95% confidence intervals (CI), unless stated otherwise. The significance level was set at $\alpha = 0.05$.

Results

Performance and energy contribution

Completion time, average heart rate, acute lactate concentrations and contribution from aerobic and anaerobic metabolism to energy production during each STT are shown in Fig. 1. These data have previously been published elsewhere, and are included to characterize the work load employed (Andersson *et al.* 2016). Biochemical measurement showed that total muscle glycogen was reduced by 22% (−129 mmol kg dry weight^{-1}) and 24% (−93 mmol kg dry weight^{-1}), respectively, during STT 1 and STT 4 (575 ± 14 to 446 ± 16 mmol kg dry weight^{-1} and 383 ± 14 to 290 ± 16 mmol kg dry weight^{-1}; $P < 0.01$ for both reductions and $P = 0.03$ for STT 1 *vs.* STT 4).

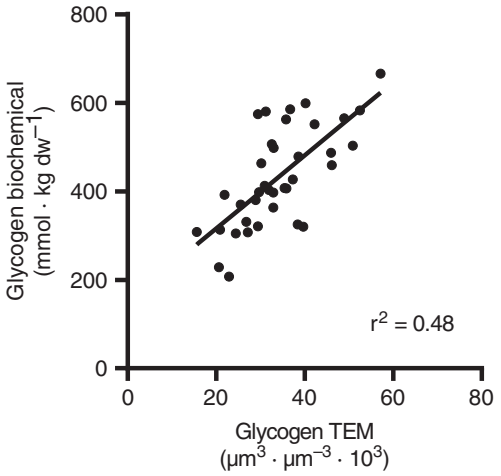

Figure 2. Linear correlation between biochemically measured glycogen and glycogen estimated with TEM method
Data from all time points (before and after STT 1 and 4) are included ($n = 10$ and $n = 10$; $n = 9$ and $n = 8$ in STT 1 and 4, respectively).

Subcellular distribution of glycogen prior to exercise

Before exercise, the total muscle glycogen volume did not differ between fibre types. However, type 1 fibres contained more intramyofibrillar glycogen [+24%, (3:46%); $P = 0.02$], with there being no difference in the case of intermyofibrillar and subsarcolemmal glycogen. Representative TEM images illustrating glycogen distributions are shown in Fig. 3.

Utilization of glycogen subfractions during supramaximal exercise (STT 1)

Intramyofibrillar glycogen volume decreased in type 1 fibres (−52% [−89:–15%]), but not in type 2 fibres [−15% (−52:22%); time × fibre type interaction: $P = 0.02$]. By contrast, intermyofibrillar glycogen volume was reduced in both fibre types [type 1: −15% (−32:1%); type 2: –23% (−39:−7%); main effect: −19% (−33:0), $P = 0.006$; time × fibre type interaction: $P = 0.41$] (Fig. 4*A* and *B*), as was the level of subsarcolemmal glycogen volume [type 1: −29% (−69:11%); type 2: −42% (−81:−2%); main effect: −35% (−66:0%), $P = 0.03$; time × fibre type interaction: $P = 0.62$] (Fig. 4*C*).

The fibre type-specific utilization of intramyofibrillar glycogen during STT 1 was further emphasized by the observation of intramyofibrillar glycogen being used to a relatively large extent in comparison to the other compartments in type 1 fibres but not in type 2 fibres ($P = 0.01$) (Table 1).

Utilization of glycogen compartments during subsequent supramaximal exercise (STT 4)

During STT 4, the overall volume of intermyofibrillar glycogen was reduced by 31% [type 1: −55% (−60: −11%); type 2: –26% (−50:−1%); main effect: −31% (−50:−11%), $P = 0.002$; time × fibre type interaction: $P = 0.24$] (Fig. 5*A*), whereas intramyofibrillar and subsarcolemmal glycogen volumes were not significantly altered (Fig. 5*B* and *C*). Accordingly, the relative distribution between the three glycogen compartments changed in a similar manner in the two fibre types during STT 4, with both demonstrating that intermyofibrillar glycogen was depleted preferentially (Table 1).

An evaluation of the overall effect of the 4 repeated sprints reveals that, independent of fibre type each of the subcellular compartments were reduced from pre STT 1 to post STT 4 ($P < 0.0001$) (Figs 4 and 5) and that the relative distribution of glycogen between the three compartments was not significantly changed (Table 1).

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Relationship between pre-exercise glycogen availability and net glycogen depletion

No associations between the biochemically-measured baseline concentration of glycogen and glycogen depletion (pre – post) were observed in neither STT 1 ($r^2 = 0.12$, $P = 0.33$), nor STT 4 ($r^2 = 0.04$, $P = 0.63$). However, when data from both STT 1 and STT 4 were combined, there was a weak association ($r^2 = 0.26$, $P = 0.03$). By contrast, in each of the compartments and in both fibre types, the glycogen depletion (pre – post) was closely associated with the glycogen volume before the exercise in STT 1 but not in STT 4 (Figs 6 and 7).

Particle volume

During STT 1, the volume of intermyofibrillar and subsarcolemmal glycogen particles declined in a fibre type independent manner [−18% (−31:−5%), $P = 0.006$; −15% (−35:4), $P = 0.02$, respectively], with there being a similar tendency in the case of intramyofibrillar glycogen particles [−13% (−27:1%), $P = 0.075$] (Table 2). In STT 4, the volume of intermyofibrillar and intramyofibrillar glycogen particles in both fibre types were significantly reduced [−20% (−38:−1%), $P = 0.04$; −18% (−34:−1%), $P = 0.04$, respectively], with there being a similar tendency in the case of subsarcolemmal glycogen particles [−16% (−34:1%), $P = 0.07$] (Table 2). In all three glycogen compartments, the volume of glycogen particles was 16–20% larger in type 2 compared to type 1 fibres both before and after STT 4 (Table 2).

Glycogen particle volume was significantly reduced in all subcellular compartments of both fibre types from pre STT 1 to post STT 4 (−26 to −42%; $P < 0.0001$) (Table 2). However, the volumes of intermyofibrillar and intramyofibrillar glycogen particles were reduced to a greater extent in type 1 fibres (38% and 42%, respectively) than in type 2 fibres (26% and 32%, respectively), whereas

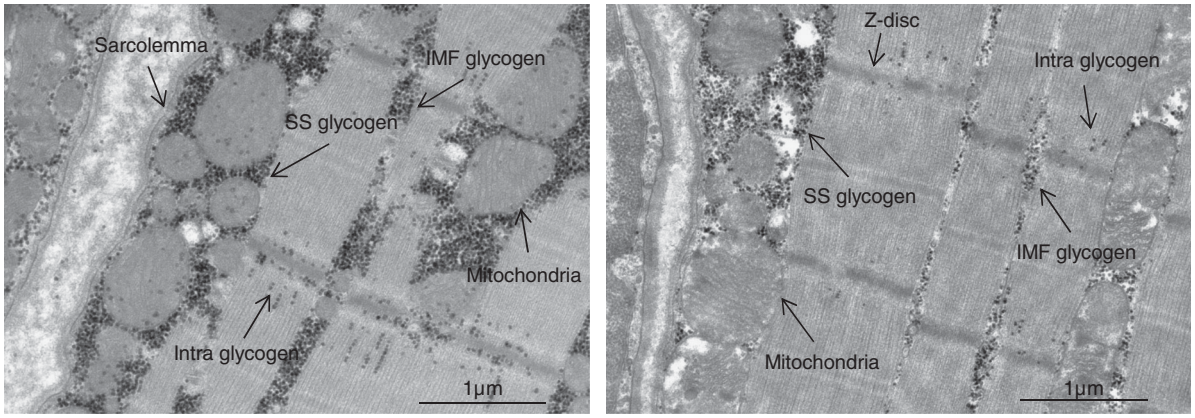

Figure 3. Representative EM images
Images illustrate subcellular glycogen compartments and other cellular constituents of the muscle fibre before STT 1 (left) and after STT 4 (right). Both images show type 1 fibres from musculus triceps brachii. The black dots are glycogen particles. SS, subsarcolemmal glycogen; IMF, intermyofibrillar glycogen; Intra, intramyofibrillar glycogen.

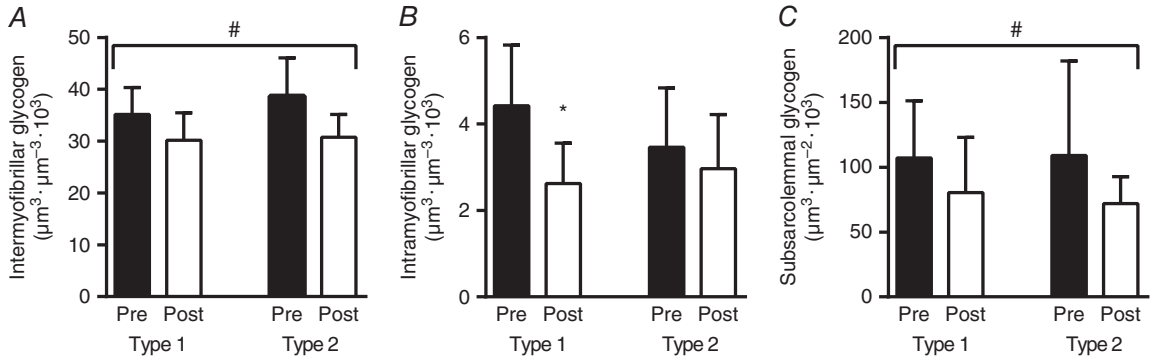

Figure 4. Changes in glycogen volume with supramaximal exercise
Glycogen volume in the intermyofibrillar (*A*), intramyofibrillar (*B*) and subsarcolemmal (*C*) compartments of type 1 and type 2 fibres in skeletal muscle of the arm (musculus triceps brachii) before (pre, $n = 10$) and after (post, $n = 10$) STT 1. The bars and vertical lines represent the geometric mean ± 95% CI, respectively. ∗Time × fibre type interaction, $P < 0.05$ compared to pre. #Main time effect, $P < 0.05$.

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Table 1. The relative distribution (%) of glycogen in the intermyofibrillar, intramyofibrillar and subsarcolemmal compartments of type 1 and 2 fibres before (Pre) and after (Post) STT 1 and 4

	Type 1		Type 2	
STT 1	Pre	Post	Pre	Post
Subjects	10	10	9	8
Intermyofibrillar	80.2 (76.1:84.5)	82.7 (80.0:85.6)	81.5 (75.4:88.0)	83.3 (80.4:86.4)
Intramyofibrillar	6.2 (4.6:8.3)	4.5 (3.1:6.6)(*)	4.7 (3.6:6.3)	5.4 (3.9:7.7)§
Subsarcolemmal	12.2 (9.4:16.0)	11.0 (8.0:15.2)	11.5 (8.0:16.5)	9.8 (7.5:12.7)
	Type 1		Type 2	
STT 4	Pre	Post	Pre	Post
Subjects	10	10	9	8
Intermyofibrillar	85.5 (83.9:87.2)	82.7 (80.0:85.6)	84.6 (81.9:87.5)	83.6 (81.6:85.8)#
Intramyofibrillar	4.0 (3.1:5.1)	4.9 (3.0:8.1)	3.9 (2.9:5.2)	3.9 (2.5:6.1)
Subsarcolemmal	10.2 (9.3:11.2)	11.0 (8.7:13.9)	10.8 (8.5:13.6)	11.7 (9.9:13.8)

§Time × fibre type interaction, $P < 0.05$. #Main effect of time for both fibre types, $P < 0.05$. (*) *vs.* Pre, $P = 0.10$. Values are the geometric mean and 95% CI.

subsarcolemmal glycogen particle volume was lowered to approximately the same extent in both fibre types (37% *vs.* 31%) (time × fibre type interactions: intermyofibrillar glycogen: $P = 0.05$; intramyofibrillar glycogen: $P = 0.02$; subsarcolemmal glycogen: $P = 0.32$).

Discussion

In the present study, we have demonstrated that 4 min of exercise at supramaximal intensity resulted in a marked reduction in the volume of intramyofibrillar glycogen in type 1 fibres only, whereas the volumes of intermyofibrillar and subsarcolemmal glycogen were reduced to the same extent in both fibre types. Thus, with this type of supramaximal exercise, the two main fibre types of human skeletal muscles show differential utilization of spatially distinct glycogen stores. Interestingly, following STT 2 and 3 and the subsequent 45 min recovery periods, this fibre type difference disappeared during the subsequent fourth STT, where both type 1 and type 2 fibres utilized intermyofibrillar glycogen preferentially. Consequently, the alterations in the relative distribution of glycogen between subcellular compartments observed following STT 1 were no longer evident after STT 4 where the distribution had returned to resting condition (pre-STT 1).

Therefore, the hypothesis that intramyofibrillar glycogen would be used preferentially during supramaximal exercise was confirmed in type 1 fibres, although not in type 2 fibres and not with repeated sprint exercise in either type 1 or 2 fibres. Because the hypothesis was based on a preferential utilization of intramyofibrillar glycogen during prolonged (~1 h) high-intensity exercise (Nielsen *et al.* 2011), and because intramyofibrillar glycogen was used to a lesser extent during STT 4, these results indicate

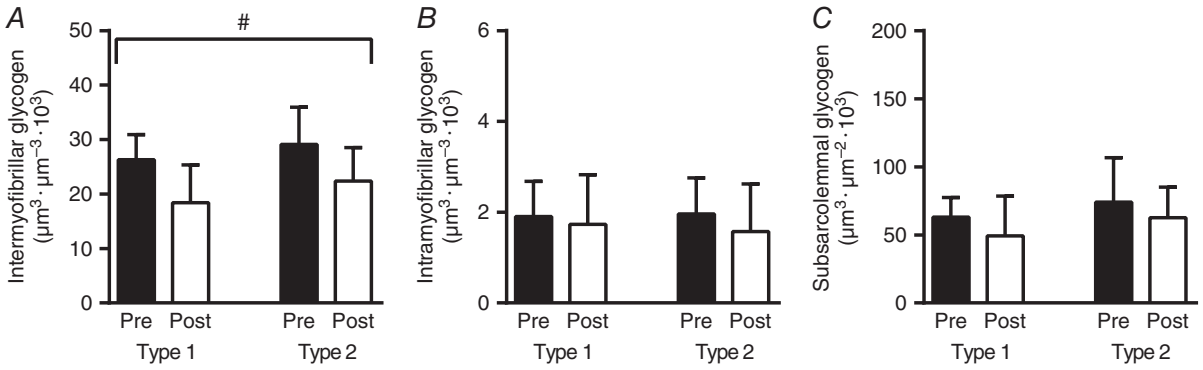

Figure 5. Changes in glycogen volume with subsequent exercise
Glycogen volume in the intermyofibrillar (*A*), intramyofibrillar (*B*) and subsarcolemmal (*C*) compartments of type 1 and type 2 fibres in skeletal muscle of the arm (musculus triceps brachii) before (pre, $n = 9$) and after (post, $n = 8$) STT 4. The bars and vertical lines represent the geometric mean ± 95% CI, respectively. #Main time effect, $P < 0.05$.

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that the use of intramyofibrillar glycogen in type 1 fibres during high-intensity exercise generally occurs during the first period of exercise, with a subsequent levelling-off. In the present study, the average rate of reduction in intramyofibrillar glycogen volume in type 1 fibres was 0.48 μm^3 μm^{-3} 10^3 min^{-1} during STT 1, whereas 60 min of exhaustive cross-country skiing revealed a markedly lower reduction rate of only 0.10 μm^3 μm^{-3} 10^3 min^{-1} (Nielsen *et al.* 2011). This marked difference is not only explained by dissimilarities in exercise intensity and energy demands, but also perhaps by a gradual reduction in the utilization rate of glycogen as exercise progresses and the availability of intramyofibrillar glycogen is considerably reduced. In line with this, the results from STT 4 show that the contribution from intramyofibrilar glycogen in type 1 fibres was markedly reduced as exercise progressed (0.04 μm^3 μm^{-3} 10^3 min^{-1}).

The 22% lower resting volume of intramyofibrillar glycogen in type 2 fibres compared to type 1 fibres could at least partly explain why there was only a small non-significant reduction in this subfraction of type 2 fibres during STT 1. This basal fibre type difference in intramyfibrillar glycogen volume was also reported in our previous study in cross-country skiers (Nielsen *et al.* 2011), and it probably affects the rate of reduction in intramyofibrillar glycogen volume. It has been suggested that the subcellular glycogen phosphorylation rate is dependent upon the glycogen availability (Hespel & Richter, 1992) and this could explain why type 2 fibres demonstrate a less pronounced rate of reduction in intramyofibrillar glycogen volume compared to type 1 fibres (Figs 4, 5, 6 and 7).

Overall, we observed an attenuation of glycogen utilization from STT 1 to STT 4, in agreement with previous studies involving repeated exercise (Karlsson & Saltin, 1971; Bangsbo *et al.* 1992), and which might be related to a lower initial glycogen availability. Certain earlier studies have reported positive associations between biochemically-measured glycogen concentration and glycogen utilization (Hespel & Richter 1992; Hargreaves

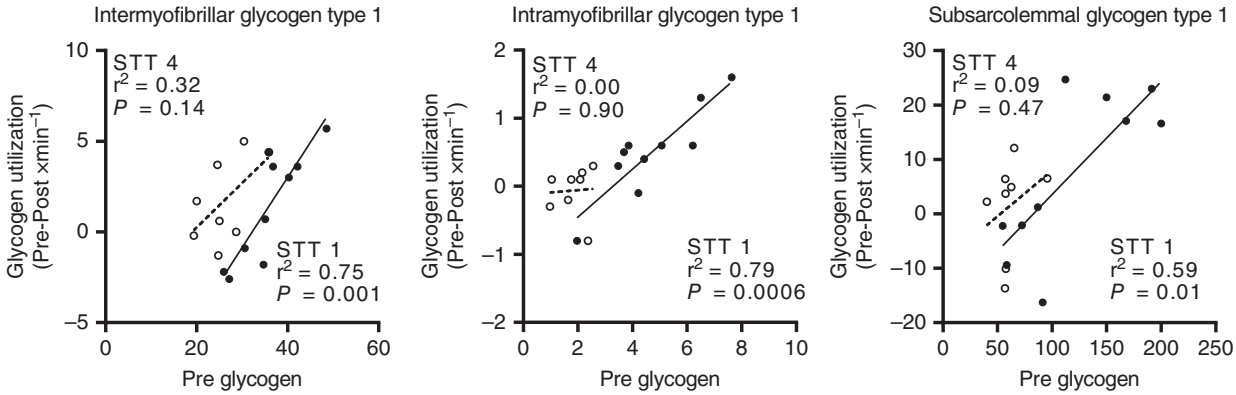

Figure 6. Glycogen availability and utilization in type 1 fibres
Correlations between glycogen depletion (pre - post) and pre-exercise glycogen volume in intermyofibrillar, intramyofibrillar and subsarcolemmal compartments of type 1 fibres. The data points and fitted lines for STT 1 (filled circles and full line) and STT 4 (open circles and dashed line) are shown.

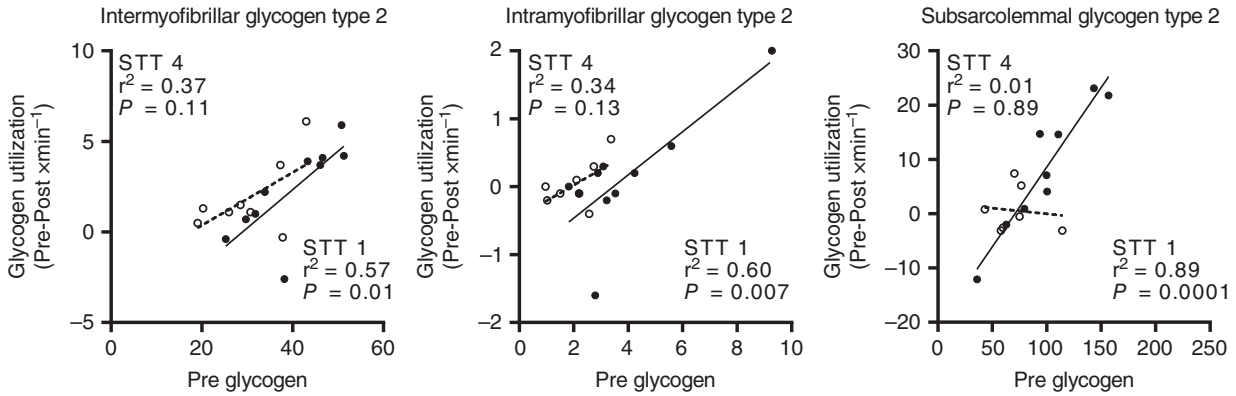

Figure 7. Glycogen availability and utilization in type 2 fibres
Correlations between glycogen depletion (pre - post) and pre-exercise glycogen volume in intermyofibrillar, intramyofibrillar and subsarcolemmal compartments of type 2 fibres. The data points and fitted lines for STT 1 (filled circles and full line) and STT 4 (open circles and dashed line) are shown.

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Table 2. Changes in the volumes (nm^3) of intermyofibrillar, intramyofibrillar and subsarcolemmal glycogen particles in type 1 and 2 fibres during STT 1 and 4

	Type 1		Type 2	
STT 1	Pre	Post	Pre	Post
Subjects	10	10	9	8
Intermyofibrillar	12094 (10773:13578)	10219 (8691:12018)	12893 (11539:14404)	10645 (8818:12851)*
Intramyofibrillar	12412 (11134:13837)	10319 (8499:12528)	12368 (10461:14623)	11530 (9573:13887)(*)
Subsarcolemmal	11494 (10068:13121)	9862 (8339:11663)	13135 (11593:14883)	10709 (8974:12780)*#
	Type 1		Type 2	
STT 4	Pre	Post	Pre	Post
Subjects	10	10	9	8
Intermyofibrillar	9409 (7578:11683)	7446 (5977:9276)	11266 (9439:13447)	9477 (7709:11650)*#
Intramyofibrillar	8737 (6934:11008)	7186 (5591:9234)	10285 (8550:12372)	8448 (6717:10626)*#
Subsarcolemmal	8832 (7339:10628)	7251 (5654:9298)	10427 (8870:12258)	9063 (7744:10608)(*)#

*Overall time effect, $P < 0.05$. (*)Trend to overall time effect, $P = 0.07$. #Overall group effect, different from Type 1, $P < 0.05$. Values are the geometric mean (nm^3) and 95% CI.

et al. 1995; Arkinstall *et al.* 2004), although Bangsbo *et al.* (1992) found no such association. In the present study, there was no association between biochemically-measured glycogen concentration and glycogen breakdown when investigating STT 1 and 4 separately, although there was a weak association when these data were combined. Interestingly, we observed strong associations between the availability of glycogen in all subcellular pools prior to STT 1 and subsequent glycogen utilization in both type 1 and 2 fibres (Figs 6 and 7). However, these associations were no longer present during STT 4. The lack of significant associations between glycogen availability and the breakdown of it during STT 4 could be explained by a narrow spectrum of both glycogen depletion and pre-exercise glycogen volume of STT 4 compared to STT 1 (Figs 6 and 7).

Interestingly, the relative distribution of glycogen between the three compartments returned to the resting condition following STT 4, so that, despite fluctuations during STT 1, the overall relative glycogen utilization in the subcellular compartments was identical during STT 1–4. This re-setting to resting conditions was caused by preservation of intramyofibrillar and subsarcolemmal glycogen during STT 4 and, consequently, intermyofibrillar glycogen was consumed preferentially. Therefore, the attenuation of whole muscle glycogen utilization (as measured biochemically) observed during the fourth STT can be ascribed to a lower degree of utilization of intramyofibrillar and subsarcolemmal glycogen. Because the overall metabolic rate during STT 1 and STT 4 did not vary (Andersson *et al.* 2016), this attenuation of glycogen utilization must be the result of either a change in the anaerobic/aerobic energy production ratio (i.e. ATP production per mM glycogen) or the replacement of glycogen by another substrate. There was no difference in the blood lactate levels after STT 1 and 4, or in the relative contribution of anaerobic energy production to the overall energy requirements (Fig. 1), indicating that the attenuation of glycogen utilization cannot be explained by a change in the anaerobic/aerobic energy production ratio. The possibility of a higher turnover of other substrates during the fourth STT was not investigated in the present study. However, Sahlin *et al.* (1997) have demonstrated a 13% overshoot in creatine phosphate (CrP) of type 1 fibres following 5 min recovery from fatiguing exercise. Interestingly, creatine kinase is located in the M-band within the intramyofibrillar space, where it has been shown to be functionally coupled to the myosin ATPase (Ventura-Calpier *et al.* 1998). That means that an increased availability of CrP could potentially reduce the amount of glycogen phosphorylation needed to meet the energy requirements from the myosin ATPase during exercise, thus sparing intramyofibrillar glycogen. Type 2 fibres have been shown to contain ∼20% more CrP compared to type 1 fibres (Sahlin *et al.* 1997), which may explain the difference in intramyofibrillar glycogen availability and utilization during STT 1. In addition to CrP, a higher glucose uptake and oxidation with repeated exercise could also explain the sparing of both intramyofibrillar and subsarcolemmal glycogen during STT 4 (Marliss *et al.* 1991).

Implications for muscle function

Previous studies have suggested that depletion of total glycogen concentration to below ∼250–300 mmol kg dry weight^{-1} (Bangsbo *et al.* 1992; Duhamel *et al.* 2006*a*; Duhamel *et al.* 2006*b*; Ørtenblad *et al.* 2011; Gejl *et al.*

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2014) or of intramyofibrillar glycogen volume to below 2–3 μm^3 μm^{-3} 10^3 (Nielsen *et al.* 2009; Ørtenblad *et al.* 2011; Nielsen *et al.* 2014) impairs muscle performance. At no point during the present study did either of these parameters fall below these critical levels, which explains why performance was maintained during the repeated time trials (Fig. 1). Importantly, during the 45 min recovery periods between time trials, the athletes ingested a high amount of carbohydrates, which probably led to significant re-synthesis of glycogen, thereby avoiding extensive net depletion during the time course of the repeated time trials (Van Hall *et al.* 2000). By contrast, Bangsbo *et al.* (1992) observed a reduction in time to exhaustion during a second bout of one-legged knee extension exercise, when subjects consumed only water during recovery, under which circumstances glycogen concentration was reduced below the critical limit (~200 mmol kg dry weight^{-1}).

All subcellular glycogen compartments can be depleted to lower levels than observed in the present study, and eventually impair the E–C coupling and muscle function (Nielsen *et al.* 2011; Ørtenblad *et al.* 2011; Nielsen *et al.* 2012). The preservation of intramyofibrillar and subsarcolemmal glycogen during STT 4 could be interpreted as a safety mechanism to avoid critical reductions in these subfractions. The exact role(s) of glycogen in maintaining muscle function remain to be fully clarified. However, low intramyofibrillar glycogen volume has been shown to be associated with a reduced SR Ca^{2+} release rate (Nielsen *et al.* 2009; Ørtenblad *et al.* 2011; Nielsen *et al.* 2014) and intermyofibrillar glycogen is associated with SR Ca^{2+} uptake (Nielsen *et al.* 2009). The subsarcommal glycogen compartment is suggested to carry out other roles in the E–C coupling including support both the active ion transport across the sarcolemma and the superficial part of the contractile apparatus close to the surface membrane (Fridén *et al.* 1985; Fridén *et al.* 1989).

Glycogen particle volume

The relationship between glycogen particle volume and its content of glycosyl units is slightly exponential when investigating particles in a wide spectra of volumes (500–40 000 nm^3) (Shearer & Graham 2004). When comparing alterations in particle volume between very small and very big particles, one must therefore take into account that large particles contain more glycosyl units per volume. However, in the present study, 95% of all values were within the range 4.930–17.275 nm^3. Within this range, the relationship between particle volume and the content of glycosyl unit is linear, as shown previously by Shearer and Graham (2004). This linear assumption is further supported by the observed linear relationship between biochemically measured glycogen concentration and total glycogen volume estimated by the electron microscopy method ($r^2 = 0.48$, $P < 0.05$) (Fig. 2). However, a major limitation of the TEM method employed in the present study is the inability to detect glycogen particles smaller than ~10 nm, demonstrating that the estimate of the average glycogen particle volume is semi-quantitative and represents only the visible particles. However, because the small particles only contribute with a very little amount of glycogen, the inability to detect the small particles does not compromise the estimate of glycogen volume (Shearer and Graham 2004).

In the present study, we show that exercise reduces the size of the glycogen particles at all subcellular locations and in both fibre types (Table 2), in agreement with previous observations (Marchand *et al.* 2007; Nielsen *et al.* 2012). A full understanding of whether glycogen depletion is ascribed by a homogenous and partly degradation of all particles or a heterogeneous pattern where only some particles are fully degraded (loss of particles) is beyond the scope of the present investigation as a result of the above mentioned methodological limitations. The much larger decrements in glycogen volume than particle volume within the intramyofibrillar space (in type 1 fibres only) and subsarcolemmal space during STT 1 and within the intermyofibrillar space during STT 4 can therefore be explained by a degradation of some particles below our detection limit.

Despite no significant depletion of intramyofibrillar and subsarcolemmal glycogen volume during STT 4 (Fig. 5), there was a decrease in the size of the glycogen particles (Table 2). This mismatch can be explained by a higher statistical precision of the estimates of particle volume than glycogen volume, although it also suggests that both intramyofibrillar and subsarcolemmal glycogen were indeed utilized during STT 4. However, the decrease in the relative contribution of intermyofibrillar glycogen volume to total glycogen volume (Table 1) still demonstrates a preferential depletion of this subfraction during STT 4.

Interestingly, before STT 4, the glycogen particle volume of all three compartments was ~16% smaller in type 1 fibres compared to type 2 fibres (Table 2). Because the glycogen volume and the reduction in particle volume during the exercise bouts were not different between fibre types, this indicates fibre type-specific strategies for glycogen re-synthesis during the recovery periods. Although type 2 fibres appear to enhance the size of particles, type 1 fibres prioritize to enhance the number of glycogen particles. Interestingly, rodent type 1 fibres were reported to contain four-fold more glycogen branching enzyme than rodent type 2 fibres (Murphy *et al.* 2012), which would give a more glucose-dense packed glycogen particle with fewer tiers and a poorer capacity for storing glucose (Meléndez-Hevia *et al.* 1993) and, in turn, a need for a higher number of particles. At baseline, however, the glycogen particles of type 1 fibres were only marginally smaller than those of type 2 fibres, so

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future studies should investigate the roles of exercise and post-exercise recovery, as well as the interaction with different fibre types on glycogen particle size and glycogen associated proteins (i.e. glycogen branching enzyme).

Conclusions

In conclusion, a semi-quantitative estimation of local glycogen depletion in muscle subcellular compartments during supramaximal exercise revealed that intramyofibrillar glycogen was depleted in type 1 fibres but not in type 2 fibres, whereas intermyofibrillar and subsarcolemmal glycogen were depleted to the same extent in both fibre types. If exercise was repeated, intermyofibrillar glycogen was preferentially depleted in both fibre types during the fourth sprint, resulting in similar overall relative reductions in all subcellular compartments during STT 1–4. This shift in local glycogen depletion from different compartments indicates the existence of a mechanism, which, during repeated supramaximal exercise or cumulated exercise, regulates the spatial depletion of glycogen. Because the spatial distribution of glycogen in skeletal muscle fibres is important for muscle function, such a mechanism may influence the role of glycogen during exercise.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

KDG, NØ, EA, HCH and JN were responsible for the conception or design of the study. KDG, NØ, EA, PP, HCH and JN were responsible for acquisition, analysis or interpretation of data. KDG, NØ, EA, PP, HCH and JN were responsible for drafting the paper or revising it critically for important intellectual content. All authors have approved the final version of the manuscript. All persons designated as authors qualify for authorship. Experiments were carried out at Swedish Winter Sports Research Centre, Department of Health Sciences, Mid Sweden University, Östersund, Sweden. Electron microscopy analysis was carried out at the Department of Pathology and Department of Sports Science and Clinical Biomechanics at the University of Southern Denmark, Odense, Denmark.

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