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Relationship between muscle water and glycogen recovery after prolonged exercise in the heat in humans

Valentín E. Fernández-Eliás¹ · Juan F. Ortega¹ · Rachael K. Nelson² · Ricardo Mora-Rodriguez¹

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Abstract

Purpose It is usually stated that glycogen is stored in human muscle bound to water in a proportion of 1:3 g. We investigated this proportion in biopsy samples during recovery from prolonged exercise.

Methods On two occasions, nine aerobically trained subjects ($\dot{V}O_{2\max} = 54.4 \pm 1.05 \text{ mL kg}^{-1} \text{ min}^{-1}$; mean \pm SD) dehydrated $4.6 \pm 0.2 \%$ by cycling 150 min at 65 % $\dot{V}O_{2\max}$ in a hot-dry environment ($33 \pm 4 \text{ }^\circ\text{C}$). One hour after exercise subjects ingested 250 g of carbohydrates in 400 mL of water (REH_{LOW}) or the same syrup plus water to match fluid losses (i.e., $3170 \pm 190 \text{ mL}$; REH_{FULL}). Muscle biopsies were obtained before, 1 and 4 h after exercise.

Results In both trials muscle water decreased from pre-exercise similarly by $13 \pm 6 \%$ and muscle glycogen by $44 \pm 10 \%$ ($P < 0.05$). After recovery, glycogen levels were similar in both trials (79 ± 15 and $87 \pm 18 \text{ g kg}^{-1}$ dry muscle; $P = 0.20$) while muscle water content was higher in REH_{FULL} than in REH_{LOW} (3814 ± 222 vs. $3459 \pm 324 \text{ g kg}^{-1} \text{ dm}$, respectively; $P < 0.05$; ES = 1.06). Despite the insufficient water provided during REH_{LOW}, per each gram of glycogen, 3 g of water was stored in muscle (recovery ratio 1:3) while during REH_{FULL} this ratio was higher (1:17).

Conclusions Our findings agree with the long held notion that each gram of glycogen is stored in human muscle with at least 3 g of water. Higher ratios are possible (e.g., during REH_{FULL}) likely due to water storage not bound to glycogen.

Keywords Sweating · Muscle water · Muscle glycogen storage · Oral rehydration · Carbohydrates

Abbreviations

$\dot{V}O_{2\max}$	Maximal oxygen consumption
h	Hours
yrs	Years
Usg	Urine specific gravity

Introduction

During prolonged exercise in the heat, muscle glycogen is oxidized at a higher rate than when exercising in a thermoneutral environment (Febbraio et al. 1994). During recovery from muscle-glycogen-depleting exercise, 40 % of muscle glycogen is restored in the first four hours if enough carbohydrates are provided soon after exercise ceases (Ivy et al. 1988a, b). Furthermore, during prolonged exercise in the heat, muscle water content is maintained but declines when fluid compartments equilibrate soon after exercise [i.e., 1 h (Mora-Rodriguez et al. 2014)]. We have recently reported that muscle water deficit could be restored within 4 h after exercise when enough fluid is provided (Fernandez-Eliás et al. 2014a). Thus both, muscle glycogen and water can be restored after prolonged depleting exercise. It has been proposed that the post-exercise recovery of water and glycogen in human muscle obeys a coordinated response (Olsson and Saltin 1970). Specifically, it is commonly stated

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Table 1 Subjects characteristics

Variable	Value
Age	24 ± 3
Body mass (kg)	68.9 ± 6.9
Height (cm)	173 ± 7
Body fat (%)	14.4 ± 1.5
$\dot{V}O_{2\max}$ (mL Kg ⁻¹ min ⁻¹)	54.4 ± 1.1
HR _{max} (beats min ⁻¹)	191 ± 10
W _{max} (watts)	366 ± 35

Maximal oxygen uptake ($\dot{V}O_{2\max}$), maximal heart rate (HR_{max}) and maximal work load (W_{max}). Data are mean ± SD

that each gram of glycogen is stored in human muscle with at least 3 g of water.

It was first observed in rabbits undergoing starvation-refeeding diets that liver glycogen increased along with liver weight in a 1:4 ratio. Others found similar results when measuring both water and glycogen directly in rodent livers (MacKay and Bergman 1932; Puckett and Wiley 1932), while Bridge and Bridges found no relationship (Bridge and Bridges 1931). Thus, the ratio of liver water to glycogen contents during periods of changing nutrition remained controversial. Geddes and co-workers (Geddes et al. 1977) analysed liver glycogen molecular size in vitro and found that each gram of glycogen had 1.1 g of water associated. However, they confessed that their hydration conditions increased the effective radius of the particles by 40 %, and thus the amount of water associated with glycogen under different hydrodynamic conditions may be different.

Hepatocytes and myocytes comprise the main glycogen depots in rodents and mammals. However, being structurally and metabolically different tissues, it is unclear if they store water and glycogen in similar fashions. In 1982, Sherman and co-workers (1982) carried out an experiment in rats' muscles to ascertain if the glycogen-to-water relationship was consistent with the proposed 1:3 ratio. They compared a control group with an exhausted group (i.e., exercise, glycogen-depleted) and an exhausted-recovered group (3 days recovery with food and water ad libitum). They reported a lack of relationship between glycogen and water in rat skeletal muscle of different fiber composition (i.e., gastrocnemius, red vastus and white vastus).

In humans, Olsson and Saltin (1970) found a parallel increase in muscle glycogen and whole body water after muscle-glycogen-depleting exercise when exercise was followed by a high-carbohydrate diet to refill muscle glycogen stores. Their calculations corroborated the data in rat liver since there was a relationship between glycogen resynthesis and whole body water gains of 1–4 g. However, water was not measured directly in the muscle but at the whole body level using tritium-labelled water. In humans Neuffer and

co-workers (1991) found that dehydrated muscle resynthesized glycogen at the same rate than when muscle is rehydrated. They concluded that muscle glycogen resynthesis during recovery from exercise is not limited by a reduction in muscle water content and thus their data argues against a fixed ratio between glycogen and water in human muscle.

Despite contradictory study results, the ratio of 1:3 g of glycogen to water recovery is well accepted (Brooks and Fahey 1984). Furthermore, given that muscle glycogen recovery determines performance during aerobically intense exercise (Bergström et al. 1967) it is commonly advised the ingestion of sufficient amount of fluid along with solid carbohydrates to enhance muscle glycogen recovery (Coggan and Swanson 1992). The 1:3 relationship between glycogen and water has been used to explain mismatch between estimates and actual results in weight-loss programs (Kreitzman et al. 1992). The loss of water associated with glycogen could explain the fast reduction in body weight without a parallel body fat loss when people undergo a hypocaloric low-carbohydrate diet. Likewise, the rapid weight regain when discontinuing the low-carbohydrate diet could be explained by the restoration of glycogen and its associated water (Kreitzman et al. 1992).

The purpose of this study was to investigate the relationship between water and glycogen restoration following prolonged exercise in the heat designed to reduce both muscle glycogen and muscle water content. This information is important to re-address current nutritional advices regarding glycogen recovery to improve athletic performance as well as to explain body weight fluctuations in people who adopt a low-carbohydrate diet. To achieve this goal we measured the recovery of muscle glycogen and water 4 h after prolonged exercise in the heat that reduced both muscle glycogen and muscle water content. Importantly, in order to avoid estimations errors when extrapolating to the whole body level, both water and glycogen changes were measured in the same muscle biopsy in each assessment. A randomized, cross-over study design was used to investigate the role of rehydration in muscle glycogen recovery in subjects who underwent two trials with the same provision of carbohydrate but very different volumes of water ingested.

Methods

Participants

Nine endurance-trained male cyclists, who routinely cycled at least 2 h day⁻¹, 4–7 days week⁻¹ during the last 3 years, were recruited. Participant's physical characteristics are shown in Table 1. Subjects were fully informed about the experimental procedures and the possible risks and discomforts associated with the experiment before they gave their

written informed consent to participate. This study was approved by the Virgen de la Salud Hospital Research Ethics Committee in accordance with the latest version of the Declaration of Helsinki.

Preliminary testing

One week prior to their participation in the study, subjects underwent a physical examination including rest and exercise ECG (Cosmed T12, Italy). Subjects underwent graded exercise testing (GXT) in an electromagnetically braked cycle ergometer (Cardiostest 100, Seca, Germany) to determine their maximal aerobic capacity (i.e., $\dot{V}O_{2\max}$). After a 5-min warm-up at 100 W, participants began cycling at 125 W with increments of 25 W each minute until exhaustion. $\dot{V}O_2$ and $\dot{V}CO_2$ were measured using an automated breath by breath indirect calorimetry system (Quark b2, Cosmed, Italy) and averaged every 10 s. $\dot{V}O_{2\max}$ was defined as the highest oxygen consumption value that occurred despite increases in workload. Maximal heart rate (from ECG recordings) and maximal power output (W_{\max} ; Arts et al. 1993) were determined as the highest values recorded during the GXT.

Experimental design

All participants underwent two experimental trials in a random counterbalanced order separated 1 week between them. Each trial started by dehydrating subjects with 150 min of continuous cycling at 65 % $\dot{V}O_{2\max}$ in a hot-dry environment (33 ± 4 °C, 25 % relative humidity, air flow 2.5 m s^{-1}) without fluid replacement. After dehydration, subjects rested supine in a thermoneutral environment (22 ± 2 °C and 40 % relative humidity) while ingesting 250 g of carbohydrate (i.e., 250 g glucose plus fructose with 125 mg of Na^+ , 30 mg of K^+ and 1.5 mg of Cl^- ; Powerade®) dissolved in 400 mL of water (i.e., REH_{LOW} trial), or ingested the same amount of carbohydrate syrup (i.e., 250 g) plus a volume of water that matched their individual fluid losses (i.e., ~3170 mL; REH_{FULL} trial).

Experimental protocol

The day before the onset of each experimental trial, participants refrained from exercising, followed a diet rich in carbohydrates ($6\text{--}9 \text{ g kg}^{-1}$ per day) and drank liberally to ensure full glycogen storage. Experimental trials started at the same time-of-day to avoid circadian variation in physiological variables (Krauchi and Wirz-Justice 1994). Participants arrived at the laboratory after an overnight fast and were provided with a standardized breakfast (i.e., 3 g kg^{-1} of body mass consisting of milkshake, bread and cacao) and 500 mL of water to ensure euhydration. One hour after breakfast

subjects voided and their nude body weight was measured using a sensitive scale (i.e., ± 0.05 kg; Seca 764, Germany). Urine was analysed for specific gravity (U_{SG}) to confirm euhydration [i.e., < 1.020 ; (Fernandez-Elias et al. 2014b; Sawka et al. 2007)]. Then, subjects rested on a stretcher and a muscle biopsy was obtained from the vastus lateralis. Following the muscle biopsy, participants dressed in shorts and cleated cycling shoes and entered in the climatic chamber where they started cycling. Upon completion of the dehydrating exercise, participants towed dry and their post-exercise nude body mass was measured again. Then, participants lay down on a stretcher for an hour to ensure that body fluids were evenly redistributed among fluid compartments (Mora-Rodriguez et al. 2014) and subsequently a second muscle biopsy was obtained (i.e., post-exercise biopsy). Then participants drank either 250 g of carbohydrates in 400 mL of water (REH_{LOW}) or the same syrup plus as much water as needed to replace body fluids losses (REH_{FULL}). Subjects were encouraged to drink it within 20 min. Subjects rested supine for 3 h after which a third biopsy was obtained. During recovery, urine was collected and its volume measured using a graduated cylinder (Symax, Proton, Spain). At the end of the recovery period, participant's body weight was measured again. A timeline of the trials is shown in Fig. 1.

Muscle biopsy

Muscle biopsy samples were taken from the vastus lateralis using the suction-modified Bergstrom technique (Tarnopolsky et al. 2011). Skin was prepared with Povidone-iodine (Betadine, MEDA, France), followed by injection of 2 % lidocaine without epinephrine (Braun 2 %, Braun Medical, Spain). Then, the skin and underlying tissues were surgically opened (scalpel blade number 10, Braun, Germany) and muscle tissue obtained using a 5 mm internal diameter Bergstrom biopsy needle. Samples were immediately cleaned of connective tissue and rapidly frozen in liquid nitrogen for subsequent analysis of water and glycogen. The incision was closed using adhesive strips (Steri-Strip™, 3M, USA) covered with an adhesive dressing pad (Tegaderm™ + Pad, 3M, USA) and compressive dressing (IcoVenda, Novico medica, Spain) during the next 24 h.

To limit participants' burden in terms of number of biopsies (three per trial) only one pre-exercise biopsy was obtained from each participant out of the two trials. Since we standardized diet and physical activity before trials, and because trials were separated one week to allow full recovery, we assumed that participants started both trials with the same muscle glycogen and water level. In each trial all biopsies were taken from the same leg using a different incision for each biopsy. The incisions were spaced ~5 cm in between and were taken advancing distally (Costill et al. 1988).

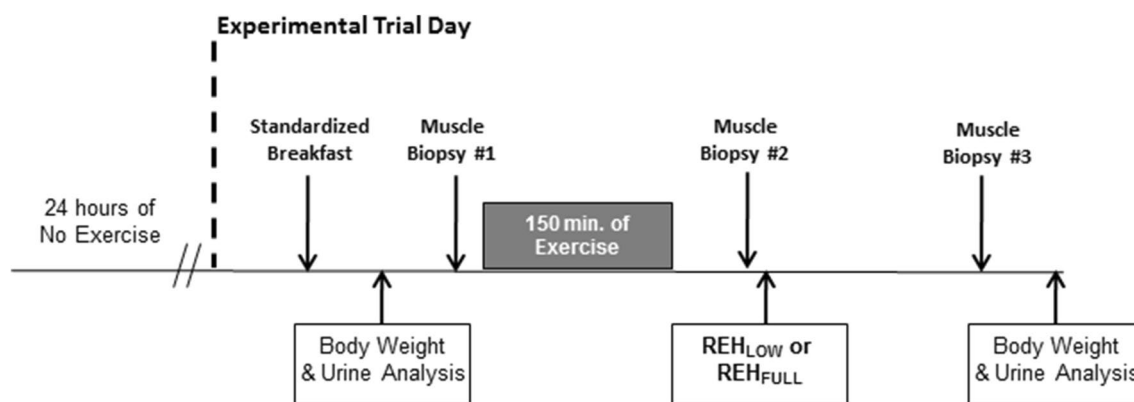


Fig. 1 Timeline description of the experimental design

Measurement of water and glycogen in biopsy samples

All samples from a given subject were analyzed in the same assay batch. Frozen samples were weighed on an electronic balance with a sensitivity of 0.1 μg (XB220A, Precisa, Switzerland). Elapsed time from sample removal from the freezer until weighing was recorded to permit correction for tissue water evaporation. Samples were freeze dried in a thermoelectric freeze-dryer (Cryodos-50, Telstar, Spain) for 6 h at $-50\text{ }^{\circ}\text{C}$ and at a vacuum of 10^{-2} Torr. In brief, this apparatus freezes the liquid in the sample to then sublimate it with a potent vacuum pump at a high flux rate (83 L min^{-1}). Samples were then re-weighed in the same precision scale and the difference in weight (i.e., water) was expressed per 100 g of dry tissue. Data from one subject that underwent 10 resting biopsies within 8 weeks in a euhydrated state ($U_{\text{sg}} < 1.020$ and body weight ± 0.25 kg) revealed a high reproducibility (i.e., 5 % CV) in the measurement of muscle water content using this technique in our laboratory.

Glycogen content was determined from the measurement of glucose after acid hydrolysis analysis (Passonneau and Lauderdale 1974). Briefly, muscle samples (~ 20 mg) were homogenized using a glass-on-glass system on ice with deionized water. Then, samples were hydrolyzed in 2 N hydrochloric acid and heated for 2 h at $100\text{ }^{\circ}\text{C}$ (Tembloc, JP Selecta, Spain). Finally, samples were neutralized to pH 6.5–7.5 with 1 N sodium hydroxide, and glucose concentration was analyzed by standard enzymatic assay. Glycogen from bovine liver (Sigma, Spain) was used as internal standard.

Statistical analysis

Data is presented as mean \pm SD. Shapiro–Wilk test was used to confirm normal distribution of data. Differences between treatments were identified by using student's

Table 2 Body weight loss during exercise and fluid replacement and urine production during recovery

	REH _{LOW}	REH _{FULL}
Pre-exercise body mass (kg)	68.98 \pm 7.0	68.93 \pm 6.9
Mass loss post-exercise (kg)	3.33 \pm 0.26	3.17 \pm 0.17
Ingested H ₂ O (mL)	400 [†]	3170 \pm 190
Urine production (mL)	186 \pm 65 [†]	1027 \pm 356
Mass regained (kg)	0.24 \pm 0.38 [†]	2.01 \pm 0.87
Post-recovery body mass (kg)	65.92 \pm 6.7 [†]	67.77 \pm 6.7

Data are mean \pm SD

[†] Different from REH_{FULL} ($P < 0.05$)

paired t test. Data collected repeatedly over time were analyzed using two-way (time \times treatment) repeated measures ANOVA. After a significant F ratio (Greenhouse–Geisser adjustment for sphericity), pair-wise differences were identified using Tukey's (HSD) *post hoc* procedure. The level of significant was set at $P < 0.05$. Cohen's formula for effect size (ES) was used and the results were based on the following criteria: >0.70 large effect, 0.30 – 0.69 moderate effect, and <0.30 small effect (Cohen 1988). Data analysis was performed using SPSS software v.19 (IBM, USA). Data are presented as mean \pm SD.

Results

Body weight loss and fluid replacement

Dehydrating exercise resulted in similar body weight loss in both trials (3.33 ± 0.26 vs. 3.17 ± 0.17 kg; $P = 0.48$; Table 2). During the 4 h after exercise, subjects recovered 2.01 ± 0.87 kg in the REH_{FULL} trial. However, as expected, body weight remained at dehydrated levels (65.9 ± 6.7 kg) in the REH_{LOW} trial since the 400 mL ingested were

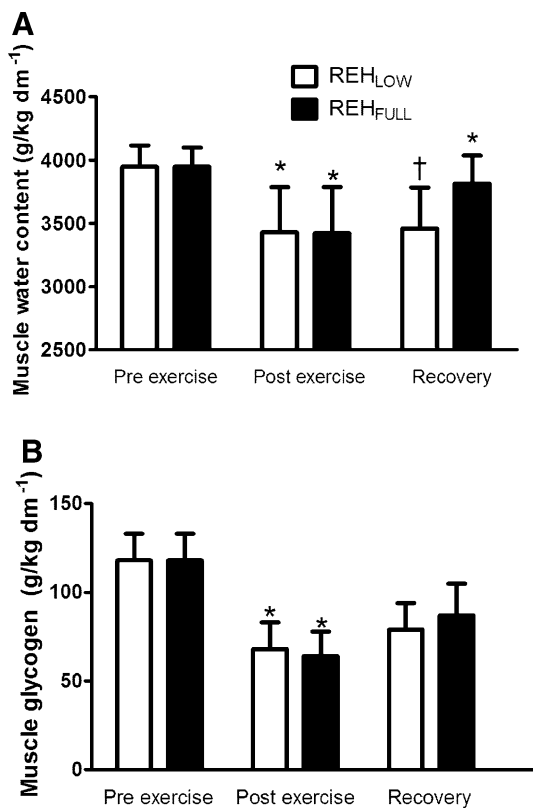


Fig. 2 a Muscle water content before and after dehydrating exercise and after 3 h recovery period. b Muscle glycogen content before and after dehydrating exercise and after 4 h recovery period. Data are presented as mean ± SD. *Different from previous time point. †Different from REH_{FULL} ($P < 0.05$)

calculated to offset the loss of insensible sweat and respiratory water during the 4 h recovery. Urine production during recovery was 186 ± 65 and 1027 ± 356 mL for REH_{LOW} and REH_{FULL}, respectively ($P < 0.05$; Table 2).

Muscle glycogen and muscle water content changes

One hour after the 150 min of dehydrating exercise, muscle glycogen and muscle water were reduced to the same extent in both trials (Fig. 2). Muscle glycogen decreased by 42 ± 11 and 46 ± 12 % during REH_{LOW} and REH_{FULL}, respectively (lower than pre-exercise values; $P < 0.05$; Fig. 2). Muscle water decreased by 13 ± 6 and 13 ± 7 % during REH_{LOW} and REH_{FULL} (lower than pre-exercise values; $P < 0.05$; Fig. 2). Hence, in both trials, the participants had similar depletion of glycogen and water content in their vastus lateralis before the experimental intervention began (i.e., LOW or FULL rehydration).

After the 3 h of rehydration-recovery period, muscle glycogen increased to a similar value in both trials (79 ± 15 and 87 ± 18 g kg⁻¹ dm, for REH_{LOW} and REH_{FULL} respectively; Fig. 2). Net glycogen resynthesis was not different

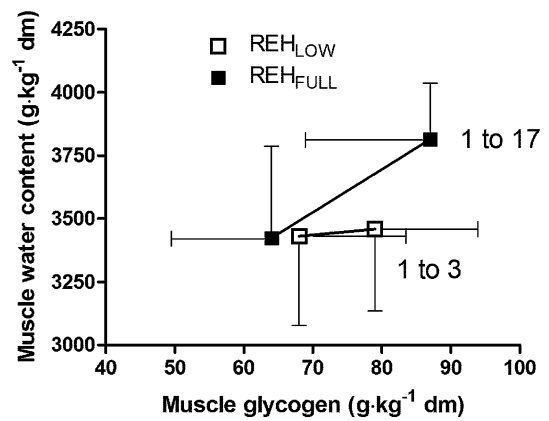


Fig. 3 Muscle glycogen and water restoration during the recovery period in the REH_{LOW} and REH_{FULL} trials. Data are presented as mean ± SD

between trials, although it tended to be higher in REH_{FULL} than in REH_{LOW} (i.e., 11 ± 8 vs. 23 ± 22 g kg⁻¹ dm, respectively; $P = 0.15$; ES = 0.79). As intended, muscle water content after the recovery period was significantly lower in REH_{LOW} compared to REH_{FULL} (3459 ± 324 vs. 3814 ± 222 g kg⁻¹ dm respectively; $P < 0.05$; Fig. 2). Net water recovery was significantly lower in REH_{LOW} (i.e., 34 ± 46 g kg⁻¹ dm) than in REH_{FULL} (i.e., 391 ± 249 g kg⁻¹ dm; $P < 0.05$).

Ratio of glycogen to water recovery

The ratio of glycogen resynthesis to water restoration in muscle biopsies was 1:3 in the REH_{LOW} and 1:17 in REH_{FULL}, a difference that almost reached statistical significance ($P = 0.059$; ES = 0.057; Fig. 3).

Discussion

The main purpose of this study was to determine the relationship between glycogen and water recovery after dehydrating and glycogen-depleting exercise in skeletal muscle. It is commonly stated that 3–4 g of water are bound to each gram of glycogen in skeletal muscle (Olsson and Saltin 1970). Our direct measurement of both water and glycogen recovery in muscle biopsies revealed that this ratio is variable and seems to depend on the amount of fluid provided. In fact, we found that the ratio of glycogen and water storage in muscle can be reduced almost sixfold (from 1:17 to 1:3; Fig. 3) when rehydration is insufficient (i.e., REH_{LOW} trial) resulting in a ratio similar to what Olson and Saltin proposed back in 1970 (i.e., 1:3 glycogen to water).

Initially, our data could be interpreted to support that these two phenomena (i.e., muscle glycogen and water

replenishment) occur at different rates during recovery from prolonged exercise and, therefore, are variable and independent of each other. However, when fluid was given to only offset respiratory and insensible water losses during the 3 h of recovery and subjects remained dehydrated (i.e., REH_{LOW}; Table 2) the glycogen-to-water recovery ratio was 1:3 (Fig. 3). The ingested solution provided during REH_{LOW} contained a 1:1.6 glucose-to-water ratio (i.e., 250 g of carbohydrate in 400 mL of water) and thus passive transport of fluid and carbohydrate into the muscle does not explain the 1:3 ratio found. We think that this 1:3 ratio during REH_{LOW} is not an accidental finding, but rather suggests that each gram of glycogen is bound to at least 3 g of water in muscle. Then, the 1:17 ratio measured during REH_{FULL} reflects that possibility of higher water storage not associated with glycogen.

Olsson and Saltin (1970) proposed in 1970 that 3–4 g of water are bound to each gram of glycogen when stored in human skeletal muscle. In that study, subjects underwent glycogen-depleting exercise followed by 2 days of a low-carbohydrate diet switching over to a high-carbohydrate diet from day 3 to 5. During the high-carbohydrate diet (day 3–5) they found a parallel increase in muscle glycogen (i.e., 500 g) and whole body water (i.e., 2.2 L measured with tritium-labelled water) corresponding to a 1:3 ratio. Nonetheless, water recovery was measured at the whole body level while glycogen resynthesis was measured in a small portion of the musculature (i.e., biopsy sample). The authors extrapolated glycogen resynthesis to all the musculature in the body estimating muscle mass from potassium excretion. Furthermore, it was assumed that all muscles lost the same amount of glycogen than the sampled muscles. Although these assumptions could have distorted the calculation of this ratio, our data measuring both water and glycogen in the same muscle sample coincides with their data in the 1:3 ratio (i.e., only in REH_{LOW}).

The ratio of 1:3 in glycogen to water content was first found in livers of rodent undertaking nutritional manipulations (fast and refeeding). From its proposal, the concept of a coordinated storage of water and glycogen has raised debate among researchers (Peters and Lavietes 1933). Beyond liver data, this ratio has been investigated in rat muscles (Sherman et al. 1982). Sherman and co-workers measured water and glycogen in muscles of a group of rats 45 min after exhausting exercise (i.e., glycogen depleted) and compared it to another group that was allowed to consume water and food ad libitum during 3 days (i.e., recovered). Although muscle glycogen was different between the exhausted and the recovered rats, muscle water was not different. Unlike humans, rats do not rely on sweat to dissipate the heat produced by exercise but rather they regulate their temperature by constricting or expanding blood

vessels in their tails (Vanhoutte et al. 2002). In this light, their lack of difference in muscle water between the glycogen-depleted and the glycogen-recovered rats is not that surprising, since none of the groups may have incurred significant fluid deficit.

The second objective of our study was to address if muscle water restoration would limit the rates of muscle glycogen recovery. This question has been previously investigated in humans. Neuffer and co-workers (Neuffer et al. 1991) used prolonged exercise to lower muscle glycogen and sauna to reduce muscle water inducing similar dehydration level to the ones reported presently (5 vs. 4.6 %). They also measured glycogen and water content in muscles biopsies and were first to find that water deprivation does not prevent normal muscle glycogen resynthesis. We corroborated their findings, since there was no significant reduction in the amount of glycogen stored when subjects remained dehydrated (11 ± 8 vs. 23 ± 22 g kg⁻¹ dm; $P = 0.15$).

In contrast to the data in humans (Neuffer et al. 1991), data in animals suggest that fluid modulates the rate of glycogen restoration in muscle. In rat myotubes, glycogen synthesis is reduced when cell water is reduced by exposure to a hyperosmotic media. Furthermore, glycogen synthesis is increased after swelling induced by exposure to hypotonic media (Low et al. 1996). In horses, fluid ingestion after exercise affects glycogen restoration. Waller and co-workers found that glycogen recovery is accelerated in Standardbred horses when after depleting-dehydrating exercise (70 min intervallic exercise) horses are provided with an amount of water and electrolytes similar to the volume (8000 mL) and composition of sweat lost during exercise (Waller et al. 2009). Thus, more studies in humans are needed to confirm the lack of effect of muscle hydration on the rates of glycogen resynthesis.

Our study has several limitations. First our sample size ($n = 9$) was perhaps concealing a real difference in glycogen recovery between REH_{LOW} and REH_{FULL} (i.e., type II error) since we found a large effect size (i.e., 0.79). Due to the variable response of one subject (higher glycogen recovery in REH_{LOW} than REH_{FULL}) a sample of 58 individuals would have been needed to reach statistical significance. Second, to avoid subject attrition due to the excessive number of muscle biopsies we collected only one pre-exercise sample biopsy and assumed that due to our careful standardization of diet and exercise, the measured levels of muscle water and glycogen could be extrapolated to both trials. Data in one subject who underwent 10 resting biopsies within 8 weeks in a euhydrated, well-fed rest state, revealed a 5 % CV in muscle water and a 4 % CV in muscle glycogen. Nevertheless, it is possible that this low variability was not present in all subjects.

Finally, our water-measuring technique does not allow differentiation between the water stored associated (linked) with glycogen and the gains in water not associated with glycogen. It has been long argued that water could be linked to other solutes in muscle overestimating the relationship between water and glycogen recovery (Peters and Lavietes 1933). After endurance exercise consisting of 2 h of pedalling, whole body amino acid net balance is slightly negative when carbohydrates are provided (Howarth et al. 2009). Thus, it is unlikely that protein synthesis and its associated water in muscle were causing water overestimation in our short-term recovery study (i.e., 4 h). Nevertheless, we recognize that the ratios between glycogen and water presented are only estimations of the coordinated response of the restoration of these substrates in human skeletal muscle.

In conclusion, the results of our short-term recovery (i.e., 4 h) experiment sampling human skeletal muscle do not oppose the frequently quoted statement that with every gram of glycogen 3 g of water are stored. Although we have not directly measured the water bound to the skeletal muscle glycogen molecule, we have measured the coordinated increases in both water and glycogen in the same muscle biopsy piece. When we restrict oral rehydration after prolonged dehydrating exercise the ratio of vastus lateralis glycogen to water recovery is 1:3. Our data suggest that at least 3 g of water are stored for each gram of glycogen in muscles previously depleted of both by prolonged dehydrating exercise.

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Conflict of interest The authors report no conflicts of interest.

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