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The effect of acute hypohydration on glycemic regulation in healthy adults: a randomized crossover trial

Running title: Effect of hydration status on glycemia

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Abbreviations: ACTH, adrenocorticotropic hormone; ANOVA, analysis of variance; AUC, area under the curve; AVP, arginine vasopressin; CI, confidence interval; CSMA, cross-sectional muscle area; EI, energy intake; HYPO, hypohydrated trial arm; iAUC, incremental area under the curve; OGTT, oral glucose tolerance test; pQCT, peripheral quantitative computer tomography; RE, rehydrated trial arm; *SD*, standard deviation; USG, urine specific gravity; VR, vasopressin receptor

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Abstract

The aim of this study was to investigate the acute effect of hydration status on glycemic regulation in healthy adults and explore underlying mechanisms. In this randomized crossover trial, 16 healthy adults (8 male) underwent an oral glucose tolerance test (OGTT) when hypohydrated and rehydrated, after four days of pre-trial standardization. One day pre-OGTT, participants were dehydrated for 1-h in a heat-tent with subsequent fluid restriction (HYPO) or replacement (RE). The following day, an OGTT was performed with metabolic rate measures and pre- and post-OGTT muscle biopsies. Peripheral quantitative computer tomography thigh scans were taken pre- and post-intervention to infer changes in cell volume. HYPO (but not RE) induced 1.9±1.2% body mass loss, 2.9±2.7% cell volume reduction, and increased urinary hydration markers, serum osmolality, and plasma copeptin concentration (all $p \leq 0.007$). Fasted serum glucose (HYPO 5.10±0.42 mmol·l^{-1}; RE 5.02±0.40 mmol·l^{-1}; $p=0.327$) and insulin (HYPO 27.1±9.7 pmol·L^{-1}; RE 27.6±9.2 pmol·L^{-1}; $p=0.809$) concentrations were similar between HYPO and RE. Hydration status did not alter the serum glucose ($p=0.627$) or insulin ($p=0.200$) responses during the OGTT. Muscle water content was lower pre-OGTT after HYPO compared to RE (761±13 g·kg^{-1} wet weight *versus* 772±18 g·kg^{-1} RE), but similar post-OGTT (HYPO 779±15 g·kg^{-1} *versus* RE 780±20 g·kg^{-1}; time $p=0.011$; trial*time $p = 0.055$). Resting energy expenditure was similar between hydration states (stable between -1.21 and 5.94 kJ·kg^{-1}·d^{-1}; trial $p=0.904$). Overall, despite acute mild hypohydration increasing plasma copeptin concentrations and decreasing fasted cell volume and muscle water, we found no effect on glycemic regulation.

23 **New and Noteworthy**

24 We demonstrated for the first time that an acute bout of hypohydration does not impact blood
25 sugar control in healthy adults. Physiological responses to mild hypohydration (< 2 % body
26 mass loss) caused an elevation in copeptin concentrations similar to that seen in those with
27 diabetes, as well as reducing cell volume by ~3 %; both these changes had been hypothesized
28 to cause a higher blood sugar response.

29

Introduction

Whilst it is well-established that several dietary factors are implicated in glycemic regulation, research into the effects of hydration status is lacking. Observationally, plain water intake is typically associated with better glycemic regulation (6, 7, 26) which suggests that this could represent an inexpensive and time-efficient health intervention. However, rather than demonstrating a causal relationship, such associations may reflect water intake being a marker of a generally healthy lifestyle encompassing higher fibre intake and physical activity (19).

Understanding how hydration status impacts glycemia has consequences for both clinical practice and research, whereby fasted glucose concentrations and oral glucose tolerance tests (OGTTs) are commonly used for diabetes diagnostics or to ascertain the efficacy of an intervention. Standardization of food and fluid intake prior to these measures is required to prevent confounding influences, yet hydration status is not uniformly controlled for, despite studies in adults with type 1 and 2 diabetes finding higher glycemic responses when participants were hypohydrated *versus* euhydrated (5, 18). One study has also shown a similar deterioration in fasting glucose in healthy adults when manipulating extracellular osmolality, which mimics some of the physiological effects of hypohydration (20). Yet, the effect of directly manipulating hydration status on glycemic control in healthy adults has, to our knowledge, never been investigated.

There are several causal mechanisms through which hydration status could influence glycemia (6). Briefly, hypohydration decreases cell volume which has been hypothesized to influence glucoregulation (16, 20). Serum osmolality and arginine vasopressin (AVP) concentrations concurrently increase, potentially stimulating hepatic glucose output via

V1aR-binding in the liver (21, 29), and/or via adrenocorticotropic hormone (ACTH) and cortisol secretion. Accordingly, high plasma copeptin concentrations (a surrogate marker of AVP) concentrations (≥10.70 pmol·L^{-1} in males and ≥6.47 pmol·L^{-1} in females) have been associated with worse cardiometabolic health outcomes compared to those with low copeptin concentrations (≤4.59 pmol·L^{-1} and ≤2.71 pmol·L^{-1}, respectively) (10, 11).

Considering the implications for clinical practice, research, and public health, we conducted a pilot study (n=5) whereby ~12-h hypohydration (sauna plus fluid restriction) induced a higher glycemic response to an OGTT compared to sauna plus fluid replacement (8). Such findings warranted further exploration in a tightly controlled study. Therefore we aimed to investigate the role of hydration status in glycemic regulation as well as examine key mechanisms (change in cell volume and AVP secretion), hypothesizing that compared to rehydration, hypohydration would cause a higher glycemic response to an OGTT.

Methods

Participants

Sixteen healthy adults volunteered to participate (n = 8 males), with a mean (± standard deviation; *SD*) age of 30 ± 9 y, body mass of 71.7 ± 9.6 kg, and body mass index of 24.0 ± 3.4 $kg \cdot m^{-2}$. Participants were randomized using simple randomization (no strata) by HAC upon consent using a random number generator (Excel 2013, Microsoft Corp, US). Exclusion criteria were: aged < 18 y or ≥ 60 y, metabolic disease (no body mass restrictions, except self-reported weight loss > 5 kg in previous 6 mo), drug dependency, or pregnant/breastfeeding; thus all participants were considered healthy and not taking medication or necessary supplements (except contraceptives). Females not taking continuous hormonal contraceptives were tested during their estimated follicular phase (3-10 days after onset of menses). Data were collected in South West England between June 2016 and January 2017, inclusive.

The sample size estimate was based on our pilot project in five participants (8) showing the largest magnitude of effect at 45 minutes post-glucose ingestion (D = 1.1 $mmol \cdot L^{-1}$). The standard deviation at this time point in the control (rehydration) group was also 1.1 $mmol \cdot L^{-1}$, resulting in an effect size (*dz*) of 1. To provide a 95 % power (beta) to detect this effect at an alpha (*p*) of ≤ 0.05 using a two-tailed paired t-test required 16 participants.

Experimental design

This was a randomized crossover trial, with 5-35 d washout to account for the menstrual cycle where applicable. Each trial arm consisted of three days of between-trial physical activity and diet replication ('pre-trial monitoring phase'), a within-participant standardized 'intervention

day', and a full 'laboratory testing day' (as detailed below and in **Figure 1**; trial registration can be found Clinicaltrials.gov: NCT02841449 and Open Science Framework: osf.io/ptq7msee; deviations to registered protocol are explained in full in the published dataset). The research received ethical approval from the NHS Health Research Authority Frenchay (ref: 16/SW/0057) and was conducted in accordance with the Declaration of Helsinki.

Pre-trial monitoring phase

Three days pre-trial, participants were asked to replicate their food/fluid intake (weighed food and fluid intake diaries; analysed using Nutritics Nutrition Analysis Software, Nutritics LTD, Dublin) and physical activity (combined heart rate and accelerometry; ActiHeart™; CamNtech, Cambridge, England), morning body mass (Inner scan; body composition monitor, model BC-543, TANITA corp. Japan) and urine specific gravity (**Table 1**). On the third monitoring day, participants were instructed to limit activity and to consume ≥ 40 $ml \cdot kg^{-1}$ lean body mass (assessed via bioelectrical impedance) of non-alcoholic fluid to ensure euhydration before starting the intervention. This is in line with previous research aiming to achieve euhydration (9, 22). No restrictions on caffeinated beverages were placed during this phase, though it was emphasised to participants that they would need to replicate their fluid intake in the subsequent trial arm.

Diet diaries were analysed by the same coder within-participant, and the accuracy was verified by the lead author. Coding discrepancies were shared between all diet analysts to help ensure consistency between-participants. Nutrient intakes for each day of diet recording were taken from the software, and an average created for each participant. Physical activity energy

115 expenditure was analysed using ActiHeart 4 software which utilises a two-branch equation to
116 estimate physical activity energy expenditure based on heart rate and accelerometry. Each
117 ActiHeart™ was calibrated against measured resting metabolic rate for each participant and
118 energy expenditure calculated using the 'Group Cal JAP 2007' model. Physical activity
119 energy expenditure for each day was then averaged for each participant.

120

121 Experimental protocol

122 *Intervention day*

123 After the pre-trial monitoring phase, participants came to the laboratory between 0600-1000 h
124 in a euhydrated state, following overnight fluid and food abstention from 2200 h the previous
125 day. A peripheral quantitative computer tomography (pQCT; Stratec, Pforzheim, Germany)
126 scan of a cross-section of the midpoint of their right thigh was taken as a proxy for muscle
127 cell volume, after which a 10 ml euhydrated baseline blood sample was taken from an
128 antecubital vein. Blood analytes from this venepuncture further confirmed compliance to the
129 pre-trial monitoring phase, showing that participants were in a similar metabolic state before
130 starting each trial arm (**Table 2**).

131

132 Participants then sat in a heat-tent (HYPO 45.2 ± 1.6°C, RE 44.6 ± 1.3°C; $p = 0.292$) wearing
133 a sweat suit (RDX EVA Nylon Sauna Sweat Suit) for 60 min. Participants were nude weighed
134 three times (Seca 803, Seca, Birmingham, UK) immediately before and after the heat-tent to
135 determine body water losses. Post-heat-tent, participants were provided with a sandwich
136 containing ≥ 1 g salt (Co-Operative Group Limited, UK) of their choosing (standardized
137 within-participant; sandwiches contained 1.5 ± 0.5 g, range 1.1-2.4 g) in order to maximise
138 fluid retention and serum osmolality changes(17), and either 3 $mL \cdot kg^{-1}$ body mass (HYPO),

139 or 40 mL·kg^{-1} lean body mass plus 150% sweat losses (RE) of plain water to replace losses
140 and account for the increased drink-induced diuresis (12). All other fluids were prohibited,
141 including those containing caffeine and alcohol. Participants were only allowed to eat from a
142 list of low-water-content foods (e.g. pizza, biscuits, nuts; avoiding fruit, vegetables, soups and
143 other fluids). Physical activity energy expenditure and nutrient intake profiles were similar
144 during the intervention day (all measured nutrients $p \geq 0.102$), except water intake (HYPO
145 0.52 ± 0.11 L·d^{-1} *versus* RE 3.7 ± 0.8 L·d^{-1}; $p < 0.001$), confirming compliance to the
146 protocol.

147

148 *Laboratory testing day*

149 Participants arrived at the laboratory between 0700-0730 h after overnight food and fluid
150 abstention from at least 2200 h the previous day, provided a urine sample and had their body
151 mass recorded (Inner scan; body composition monitor, model BC-543, TANITA corp. Japan).
152 A second pQCT scan of the midpoint of the right thigh was taken, after which participants
153 were asked to rest semi-supine for 10-15 min. Their resting metabolic rate was recorded via
154 indirect calorimetry from gaseous exchange (13), whereby 2 x 5 min measures were taken.
155 Expired gas samples were collected in a Douglas bag (Hans Rudolph, Kansas City, USA)
156 through falconia tubing (Baxter, Woodhouse and Taylor Ltd, Macclesfield, UK). Inspired air
157 was simultaneously measured to adjust for ambient O_2 and CO_2 concentrations (4). Inspired
158 and expired O_2 and CO_2 concentrations were measured using paramagnetic and infrared
159 analysers (Mini HF 5200, Servomex Group Ltd., Sussex, UK). For metabolic rate data, n = 14
160 as two participants were excluded from all metabolic rate and respiratory exchange ratio
161 analyses due to fasted values > 1, which appeared to be caused by hyperventilation at these
162 time points. In a subgroup of participants (n = 9) an additional 5 min Douglas bag was taken

163 at 25-30 min post-glucose ingestion to establish whether the initial diet induced
164 thermogenesis trend was linear between 0 and 60 min.

165

166 Participants then placed their hand in a hotbox (Medical Engineering Unit, University of
167 Nottingham, UK) set to 55°C for five minutes before an indwelling cannula was fitted in an
168 antecubital vein and a fasted blood sample was drawn. An opt-in muscle biopsy was taken
169 (n = 9), followed by a second fasted blood sample in order to quantify any changes from the
170 expected stress response of the biopsy. The muscle samples were acquired via a ~3 mm
171 incision at the anterior aspect of the thigh under local anaesthetic (Lidocaine 1 %, without
172 adrenaline; Hameln Pharmacueticals Ltd, Brockworth, UK) from the *vastus lateralis* by
173 percutaneous needle biopsy technique (3). Samples were immediately removed from the
174 needle and snap frozen in liquid nitrogen before storage at -80°C.

175

176 A 75 g OGTT (Polycal, Nutricia, England) was subsequently conducted. Arterialized-venous
177 blood samples (10 ml) were drawn at 15 min intervals for 120 min. Expired gas samples (1 x
178 5 min Douglas bag as described previously) were collected hourly. A second muscle biopsy
179 was then taken (for those who had opted-in; n = 7) after 120 min.

180

181 Muscle water content analysis

182 Total muscle water content was determined by weighing the biopsy samples before and after
183 freeze drying (23). Samples were weighed on a high precision (resolution 0.01 mg) electronic
184 balance (Mettler Toledo AE240) whilst frozen (wet weight). The time elapsed between
185 sample removal from storage and weighing was fixed and recorded to allow for
186 standardization of tissue water evaporation. Samples were then freeze dried using a LyoDry

Compact, MechaTech Systems freeze dryer for 24 h at -55°C before being weighed again (dry weight) using the same precision balance. Total muscle water content was calculated as grams of water per kilogram of wet muscle using the following equation: (wet weight - dry weight / wet weight) * 1000.

Biochemical analysis

Six millilitres of blood was decanted into two EDTA tubes and spun for 10 min at 2500-3446 x g at 4°C. The remaining four millilitres of blood were decanted into a serum tube, left for at least 30 min at room temperature and then spun as per the plasma. The plasma and serum were aliquoted into separate Eppendorf tubes and frozen at ‑20°C before being moved to a -80°C freezer for longer term storage.

Metabolites and hormones were measured using commercially available ELISAs (plasma arginine[8] vasopressin, Enzo Life Sciences; serum insulin, Mercodia), ECLIAs (plasma ACTH, Roche), ECLIs (plasma cortisol, Roche), automated immune analyzers (plasma copeptin, ThermoFisher Kryptor Compact Plus) and spectrophotometric assays (serum glucose, RX Daytona, Randox Laboratories). Osmolality was measured using freezing-point depression (serum osmolality, Gonotec Osmomat auto; urine osmolality, Micro-Osmometer 3300) and urine specific gravity was measured using a handheld refractometer (SUR-NE Clinical Refractometer, Atago, Japan).

Statistical analysis

The primary aim of the study was the glycemic response to an OGTT. Secondary outcomes were to investigate the insulin, vasopressin (copeptin), rested energy expenditure, and

212 substrate utilization responses to the OGTT, and the change in urine osmolality, urine specific
213 gravity, cell volume, serum osmolality and muscle water content from the intervention. We
214 were additionally able to investigate ACTH and cortisol.

215

216 Data were analyzed using paired samples *t*-test or 2-way repeated measures (trial, time,
217 trial*time) analysis of variance or appropriate non-parametric tests (SPSS, version 22, IBM).
218 Normality was checked visually via P-P plots, histograms of standardized residuals, and
219 scatterplots of the standardized predicted *versus* residual values. Asphericity was determined
220 using Greenhouse-Geisser epsilon; values < 0.75 were corrected for using Greenhouse
221 Geisser correction and values > 0.75 used Huynh-Feldt correction. Total area under the curve
222 (AUC) and incremental AUC (iAUC) were calculated as per Wolever(30). Analyses were
223 repeated excluding those who had muscle biopsies to explore whether their inclusion skewed
224 the overall findings. All analyses were two-tailed with an alpha level of ≤ 0.05.

225 **Results**

226 Markers of hydration status (body mass, urine specific gravity, urine osmolality, and cross-
227 sectional muscle area) suggested compliance to both the HYPO and RE protocol (**Table 3**).

228

229 *Serum glucose concentration*

230 Fasting serum glucose concentrations were similar between HYPO (5.10 ± 0.42 mmol·L^{-1})
231 and RE (5.02 ± 0.40 mmol·L^{-1}; $p = 0.327$). There were no differences in the glycemic
232 response between HYPO and RE during the OGTT (trial $F = 0.246$, $p = 0.627$; time
233 $F = 41.128$, $p < 0.001$; trial*time $F = 0.944$, $p = 0.430$; **Figure 2a**). No differences were found
234 in the serum glucose iAUC (HYPO 303 ± 121 mmol*120 min·L^{-1}, RE 306 ± 113 mmol*120
235 min·L^{-1}, $p = 0.866$), AUC (HYPO 926 ± 169 mmol*120 min·L^{-1}, RE 934 ± 120
236 mmol*120 min·L^{-1}, $p = 0.819$), or time to peak (HYPO 46 ± 14 min, RE 48 ± 14 min,
237 $p = 0.609$).

238

239 *Serum insulin concentration*

240 Serum insulin concentrations were similar in the fasted state (HYPO 27.09 ± 9.66 pmol·L^{-1},
241 RE 27.62 ± 9.21 pmol·L^{-1}; $p = 0.809$). During the OGTT, there were no differences between
242 HYPO and RE in the insulinemic response (trial $F = 1.800$, $p = 0.200$; time $F= 29.597$,
243 $p < 0.001$; trial*time $F = 0.232$, $p = 0.859$; **Figure 2b**). There were no differences in the
244 iAUC (HYPO 20860 ± 8311, RE 21937 ± 8340 pmol*120 min·L^{-1}, $p = 0.369$), AUC (HYPO
245 23958 ± 9275 pmol*120 min·L^{-1}, RE 25326 ± 8679 pmol*120 min·L^{-1}, $p = 0.359$), or time to
246 peak (HYPO 54 ± 25 min, RE 58 ± 26 min, $p = 0.633$) serum insulin concentrations.

247

Serum osmolality

There was an increase from baseline in serum osmolality during HYPO of 9 ± 6 mOsm·kg^{-1} with relative stability from baseline during RE (Δ 1 ± 4 mOsm·kg^{-1}; HYPO vs RE $p < 0.001$; Table 3). Similar differences remained throughout the OGTT (trial $F = 74.457$, $p < 0.001$), reflected in a greater AUC during HYPO compared to RE (HYPO 35355 ± 692 mOsm*120 min·L^{-1}, RE 34232 ± 701 mOsm*120 min·L^{-1}, $p < 0.001$).

Plasma copeptin concentration

Due to the difficulties in measuring AVP(24), our data were unreliable, but are available in the published dataset. Plasma copeptin was measured as a reliable marker of AVP secretion(24). Fasted (pre-biopsy) plasma copeptin concentrations increased significantly from baseline after HYPO (Δ 14.32 ± 9.32; $p < 0.001$) but not after RE (Δ 0.46 ± 2.34; $p = 0.457$). Plasma copeptin concentrations were consistently higher throughout the OGTT during HYPO compared to RE (trial $F = 14.193$, $p = 0.002$; time $F = 1.285$, $p = 0.282$; trial*time $F = 1.396$, $p = 0.261$; **Figure 3a**), confirmed by a higher AUC (HYPO 2704 ± 2398 pmol*120 min·L^{-1}, RE 961 ± 1488 pmol*120 min·L^{-1}, $p = 0.001$).

Plasma adrenocorticotropic hormone concentration (ACTH)

There were no differences in the ACTH response between HYPO and RE (trial $F = 2.541$, $p = 0.132$; time $F = 6.120$, $p = 0.025$; trial*time $F = 1.343$, $p = 0.266$) during the OGTT, nor were there differences in the AUC (HYPO 405 ± 195 pmol*120 min·L^{-1}, RE 468 ± 255 nmol*120 min·L^{-1}, $p = 0.121$).

Plasma cortisol concentration

Plasma cortisol concentrations were similar between HYPO and RE (trial F= 0.216, p = 0.649; time F = 19.416, p < 0.001; trial*time F = 0.275, p = 0.674), with no differences in the plasma cortisol AUC (HYPO 35445 ± 17432 nmol*120 min·L^{-1}, RE 36716 ± 24915 nmol*120 min·L^{-1}, p = 0.642).

Cross-sectional muscle area

Cross-sectional muscle area as a proxy for muscle cell volume reduced significantly from baseline after HYPO (Δ -2.9 ± 2.7 %; p = 0.003) but not after RE (Δ 0.0 ± 2.1 %; p = 0.936; Table 3).

Muscle water content

Pre-OGTT muscle biopsies showed that muscle water content was lower during HYPO (760.5 ± 13.2 g·kg^{-1}) compared to RE (771.6 ± 17.8 g·kg^{-1}) but this difference dissipated post-OGTT (HYPO 778.6 ± 15.1 g·kg^{-1}, RE 780.2 ± 20.0 g·kg^{-1}; trial F = 3.183, p = 0.135; time F = 15.36, p = 0.011; trial*time F = 6.265, p = 0.055; **Figure 4**).

Resting energy expenditure

Resting metabolic rate was similar between trial arms in the fasted (HYPO 96.32 ± 11.94 kJ·d^{-1}·min^{-1}, RE 95.11 ± 13.09 kJ·kg^{-1}·d^{-1}, p = 0.400) and postprandial (trial F = 0.015, p = 0.904; time F = 10.130, p = 0.001; trial*time F = 0.140, p = 0.798) state. Participants had similar fasting respiratory exchange ratios (HYPO 0.84 ± 0.05, RE

0.84 ± 0.06; $p = 0.900$), although carbohydrate oxidation post-glucose ingestion had a tendency to increase to a greater extent in the RE trial at 60 (HYPO 0.88±0.04, RE 0.93±0.09) and 120 min (HYPO 0.87 ± 0.07, RE 0.91 ± 0.07; trial $F = 3.650$, $p = 0.078$; time $F = 14.693$, $p < 0.001$; trial*time $F = 3.754$, $p = 0.039$).

Plasma volume

Due to an error in postural control during the baseline (euhydrated) venepuncture, the plasma volume data were invalid. We have included details of how plasma volume was measured and the data obtained in the published dataset.

Sensitivity analysis

Those who had muscle biopsies demonstrated a distinct stress response in their plasma copeptin (**Figure 3b**), ACTH and cortisol concentrations (shown below). Removing those who had any biopsies did not alter the findings.

<u>Copeptin</u>

Removing those who had any biopsies did not meaningfully effect the trend in plasma copeptin concentration throughout the OGTT (n = 7: trial $F = 13.517$, $p = 0.010$; time $F = 4.081$, $p = 0.040$; trial*time $F = 1.489$, $p = 0.262$), though the overall AUC was lower (n = 7: HYPO 11916 ± 1661 pmol*120 min·L^{-1}, RE 390 ± 177 pmol*120 min·L^{-1}, $p = 0.028$).

<u>ACTH</u>

Removing those who had opted-in for muscle biopsies eliminated the time effect and highlighted a small though non-significant trend towards higher ACTH concentrations during RE compared to HYPO (n = 7: trial $F = 4.203$, $p = 0.086$; time $F = 0.989$, $p = 0.361$;

trial*time $F = 1.729$, $p = 0.219$). When biopsy participants were removed, the AUCs were lower (n = 7: HYPO 292 ± 139 pmol*120·L^{-1}, RE 355 ± 86 pmol*120·L^{-1}, $p = 0.176$) but there was still no difference between HYPO and RE.

Cortisol

In accordance with the plasma copeptin and ACTH response, plasma cortisol concentration also increased post-biopsy. Removing these participants did not meaningfully alter the results, though the time effect was no longer evident (n = 7: trial $F = 0.278$, $p = 0.617$; time $F = 5.172$, $p = 0.055$; trial*time $F = 0.260$, $p = 0.686$). Despite a post-biopsy peak in plasma cortisol concentration, the AUC was higher when the biopsy participants were removed (n = 7: HYPO 37920 ± 25008 nmol*120·L^{-1}, RE 40989 ± 37507 nmol*120·L^{-1}, $p = 0.735$), with no difference between HYPO and RE.

Resting metabolic rate

In the subgroup who had the additional measure at 30 min post-glucose ingestion (n = 9), no difference in RMR (trial $F = 0.346$, $p = 0.573$; time $F = 6.087$, $p = 0.009$; trial*time $F = 0.586$, $p = 0.508$) or RER (trial $F = 0.433$, $p = 0.529$; time $F = 17.330$, $p < 0.001$; trial*time $F = 0.467$, $p = 0.607$) was apparent according to hydration status.

Discussion

This randomized crossover trial is the first to show that neither fasted nor postprandial glycemia or insulinemia are influenced by hydration status in healthy adults, contrary to our hypothesis. The key implication of this work is that clinicians and researchers may not have to control for hydration status when investigating glycemic regulation in healthy adults. Participants replicated their diet and activity four days pre-trial, reducing known confounding influences. Average body mass loss during HYPO was 1.9 % which is within the typical range to induce thirst and is not uncommon in the general population (2), increasing the external validity of these findings. In the hypohydrated state, copeptin (a surrogate marker of AVP) concentrations increased from levels seen in healthy adults to levels reported in those at highest risk of metabolic syndrome (10), with an accompanying reduction in muscle cell volume of ~3 %, hypothesized to be detrimental to glycemic regulation (16). Therefore the level of hypohydration achieved was sufficient to induce physiological changes that theoretically have meaningful health implications; such changes did not occur when participants were rehydrated.

Despite these physiological changes hypothesized to cause higher glycemia, fasted and postprandial glycemia was similar between HYPO and RE. These results are in contrast to similar work in those with diabetes (5, 18). In both previous studies, participants were required to withdraw from diabetes medication; accordingly Burge *et al.* (5) found higher glucosuria when euhydrated compared to hypohydrated potentially explaining the lower glycemic response. As this effect of glucosuria has previously been alluded to (31), it is a possible (at least partial) explanation for the findings of Johnson *et al.* (18). This hypothesis could be tested by comparing glycemic regulation in those with diabetes during medication

withdrawal *versus* prescription. Glucosuria should not occur in healthy adults, potentially explaining why we did not find a lower glycemia during RE compared to HYPO.

Similarly, our findings conflict with research in healthy adults. Keller *et al.* (20) administered intravenous saline and/or desmopressin to induce changes specifically in extra-cellular osmolality, finding higher fasted glucose concentrations during hyperosmolality. This method of dehydration however is not representative of whole body water losses, potentially explaining the discordance between our findings. The present study also contradicts our own pilot work (8), most likely because of the lack of rigorous pre-trial standardization of diet (verbal 24-h recall), physical activity (self-reported replication), and hydration status (no pre-intervention measures) in the pilot.

A key mechanism by which elevations in AVP induce poor glycemic control is through the hypothalamic-pituitary-adrenal (HPA) axis, stimulating ACTH and cortisol secretion. Despite the increase we observed in plasma copeptin concentrations, plasma ACTH and cortisol concentrations were not different between HYPO and RE suggesting that in healthy adults, short-term hypohydration is not a sufficient stimulus to induce a stress response along the HPA axis. This theory is in accordance with earlier work highlighting that the role of AVP in ACTH secretion is predominantly a response to physical stress (15). Nevertheless, we did not find a difference in ACTH or cortisol by hydration status in those who had the muscle biopsies, despite higher copeptin responses during HYPO. In previous work in those with type 2 diabetes, cortisol concentrations did increase 45 min post-glucose ingestion when hypohydrated (18), perhaps suggesting an interaction between hydration status and feeding in

383 those with metabolic impairments. However, as copeptin did not increase postprandially in
384 our study, the mechanism for this cortisol response is unlikely to be AVP-mediated.

385

386 Another mechanism proposed to link hypohydration to higher glycemia is changes to cell
387 volume influencing insulin and glucagon secretion, though a limitation of our study is that
388 glucagon was not measured. Muscle cell volume reduced by ~3 % from baseline, compared to
389 no change when participants were rehydrated—a reduction that may deteriorate glucose
390 tolerance (16, 20). The pQCT data were confirmed by the muscle biopsy samples which
391 showed lower pre-OGTT total muscle water content in HYPO *versus* RE. Interestingly post-
392 OGTT the muscle water content difference diminished suggesting the introduction of glucose
393 to the cell created a strong enough stimulus to move water from other compartments into
394 skeletal muscle. Conversely, muscle water content did not change pre- to post-OGTT during
395 RE, perhaps showing greater overall water balance as no extra water was needed
396 intracellularly for glycogen storage (25).

397

398 Hydration status could influence metabolic health over longer time periods by altering
399 thermogenesis. Whilst it has been shown, albeit inconsistently, that water ingestion induces
400 greater thermogenesis (27), our study did not support these findings, supporting evidence that
401 the thermogenic effect is from consuming (cold) water rather than altering hydration status
402 (27). There was a tendency for higher carbohydrate utilization during RE compared to HYPO,
403 perhaps indicating greater hepatic glucose output during RE to account for the higher
404 carbohydrate utilization; however these findings should be interpreted cautiously as the study
405 was not powered for these outcomes.

406

There are several other speculative theories that could explain our null findings which we did not test. Firstly, it could be that healthy adults have a greater capacity to handle metabolic challenges such as acute bouts of hypohydration, and whilst physiological changes occur to handle this flux, such deviations ensure maintenance of homeostasis and therefore minimize metabolic disruption. Accordingly, both serum osmolality and plasma copeptin concentrations increased during HYPO *versus* RE, suggesting higher AVP secretion to help maintain homeostasis. Considering the level of HYPO we induced is not uncommon in the population (2) and caused an increase in copeptin concentrations associated with poor cardiometabolic health (10, 11), it may be that such commonly achieved levels of hypohydration have detrimental longer terms health effects not captured by our acute study design.

A further hypothesis is that the conflicting roles of vasopressin receptors (VR) in adipose tissue insulin sensitivity led to a null effect on glucose tolerance during HYPO. Specifically, $V1aR^{-/-}$ mice have reduced adipose tissue glucose tolerance, whereas $V1bR^{-/-}$ mice have increased adipose tissue insulin sensitivity (1, 14, 28). If these findings translate into humans (and assuming a similar propensity for VR binding), this could mean that HYPO-induced AVP secretion, such as that achieved in our study, leads to AVP binding on both V1aR and V1bR resulting in net zero effect on glycemic regulation. However, the present study was not equipped to examine this speculative theory.

Overall, despite physiologically and clinically meaningful increases in plasma copeptin and serum osmolality, and a notable reduction in cell volume supporting a systemic difference in hydration status between trial arms, we demonstrated for the first time that acute hypohydration did not alter the glycemic response. Our findings suggest that when conducting

OGTTs in healthy adults, hydration status may not necessarily need to be strictly controlled for. Although longer-term research is needed to understand the glucoregulatory effects of chronic hypohydration, these data suggest that acute manipulations of hydration status in healthy adults do not impact fasted or postprandial glycemic regulation.

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548 **Figure legends**

549 Figure 1. Protocol schematic. *muscle biopsies were taken only in those who opted in.
550 **RMR at 30 min taken in subsample (n = 9). Abbreviations: OGTT, oral glucose tolerance
551 test; pQCT, peripheral quantitative computer tomography scan; RMR, resting metabolic rate

552

553 Figure 2. (a) Serum glucose (trial $F = 0.246$, $p = 0.627$; time $F = 41.128$, $p < 0.001$; trial*time
554 $F = 0.944$, $p = 0.430$) and (b) insulin (trial $F = 1.800$, $p = 0.200$; time $F = 29.597$, $p < 0.001$;
555 trial*time $F = 0.232$, $p = 0.859$) responses to a 75 g oral glucose tolerance test (n = 16). Data
556 are means and normalized 95% confidence intervals. Abbreviations: HYPO, hypohydrated
557 trial arm; RE, rehydrated trial arm

558

559 Figure 3. (a) Plasma copeptin response during an oral glucose tolerance test (n = 16; trial $F =$
560 14.193, $p = 0.002$; time $F = 1.285$, $p = 0.282$; trial*time $F = 1.396$, $p = 0.261$). (b) Plasma
561 copeptin response during an oral glucose tolerance test separating those who had muscle
562 biopsies on both trials (n = 7) to those who had no biopsies on neither trial (n = 7). For those
563 who had biopsies, a fasted blood sample was taken before the biopsy (time point 'Pre-
564 biopsy') and after the biopsy immediately before the glucose was ingested (time point '0'),
565 and for those who did not have the biopsies, only one fasted sample was taken (time point
566 '0'). *$p < 0.050$, **$p < 0.010$ comparing HYPO and RE (trial effect) after Bonferroni
567 adjustment for multiple comparisons. Error bars: normalized 95% confidence intervals.
568 Abbreviations: HYPO, hypohydrated trial arm; RE, rehydrated trial arm

569

570 Figure 4. Average (thick lines) and individual (thin lines) muscle water content pre-OGTT
571 and post-OGTT during HYPO and RE (n = 6; trial $F = 3.183$, $p = 0.135$; time $F = 15.36$, $p =$

572 0.011; trial*time $F = 6.265$, $p = 0.055$). *p-value Bonferroni adjusted for multiple
573 comparisons. Black lines are HYPO; grey lines are RE. Abbreviations: HYPO, hypohydrated
574 trial arm; OGTT, oral glucose tolerance test; RE, rehydrated trial arm

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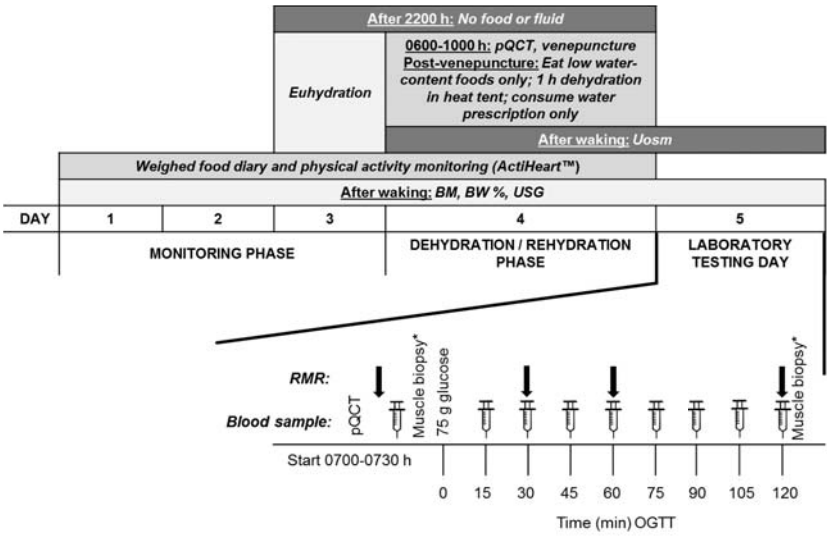
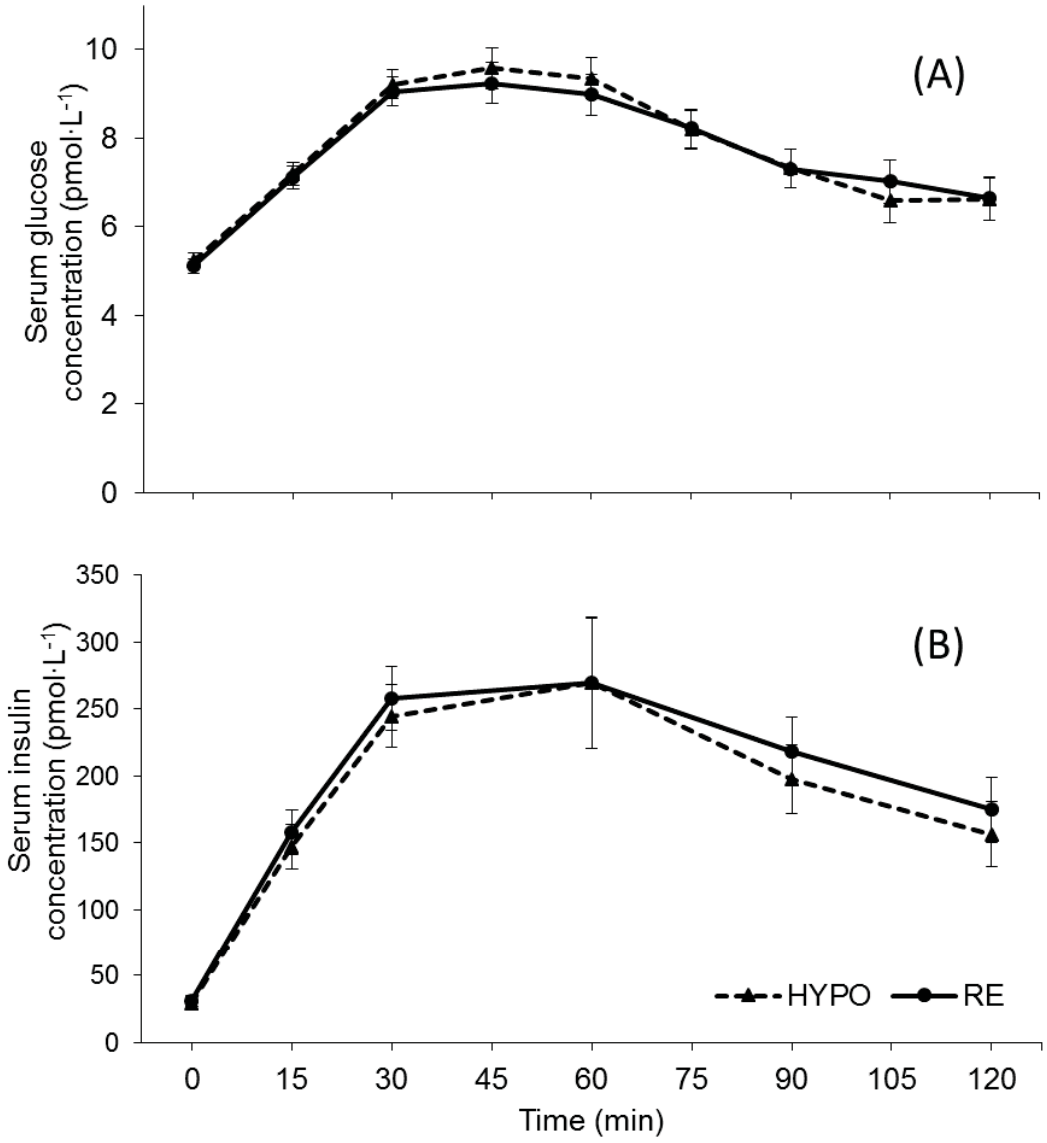
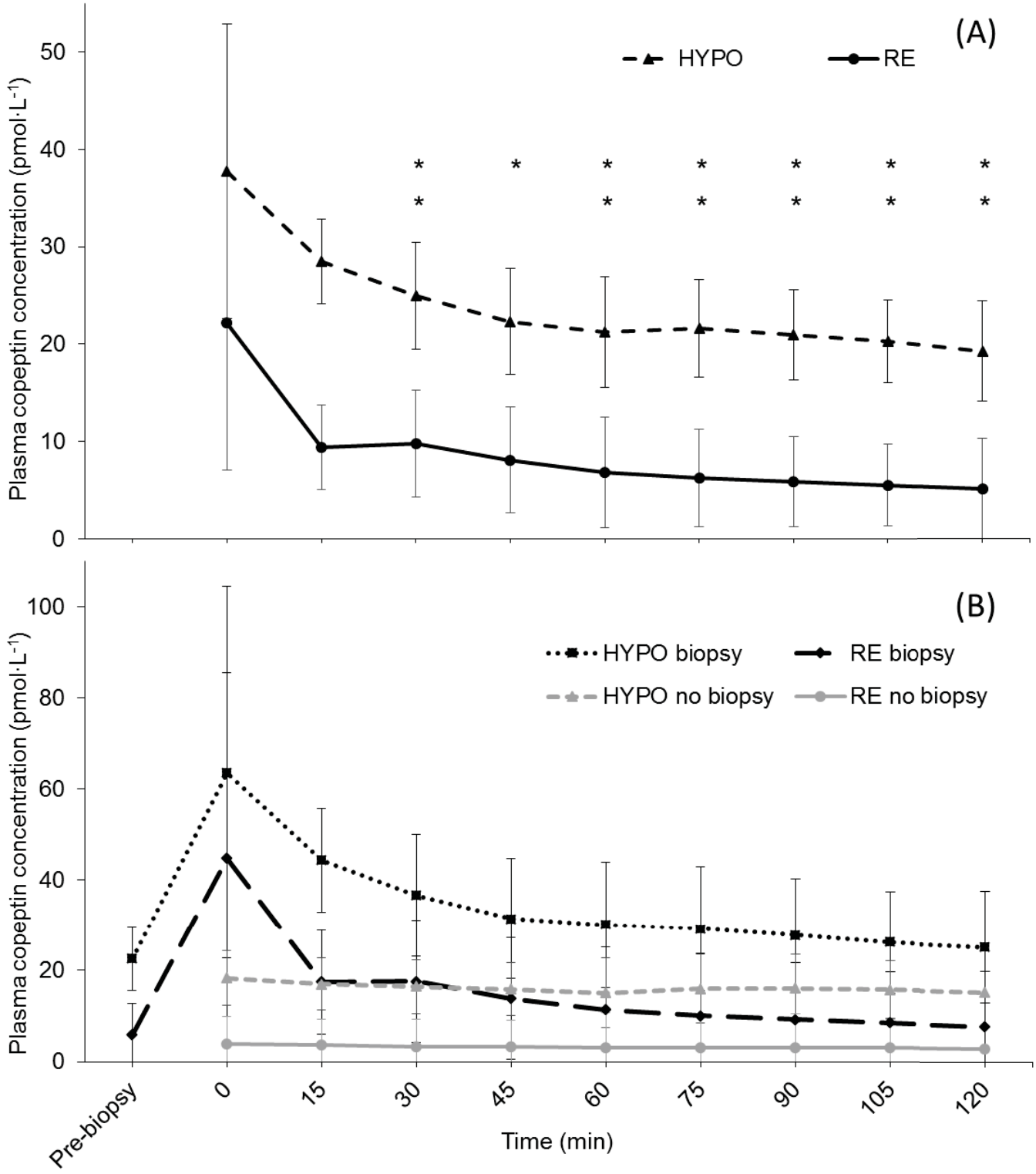
576







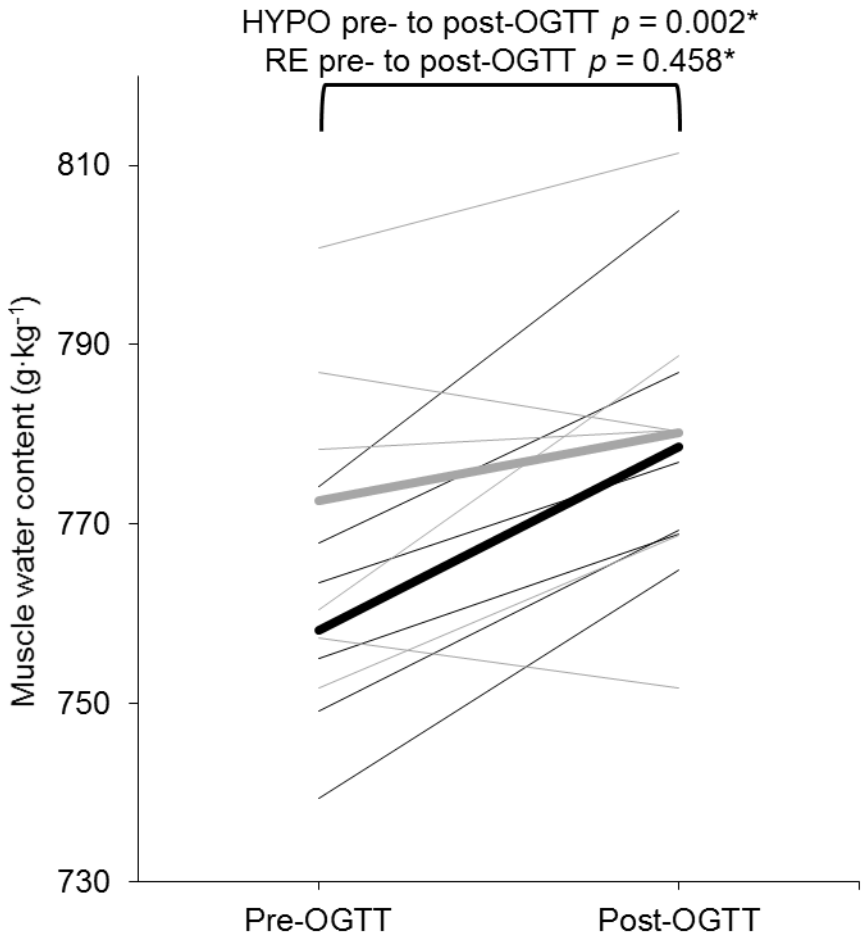


Table 1: Mean ± *SD* of lifestyle factors and markers of hydration status during the 3-day pre-trial monitoring phase (n = 16)

	Hypohydration	Rehydration	
	Mean ± *SD*	Mean ± *SD*	*p*-value [1]
Energy intake (kJ·day^{-1})	9777 ± 3765	10091 ± 3513	0.541
Carbohydrate (g·day^{-1})	274 ± 125	271 ± 125	0.829
Fat (g·day^{-1})	84 ± 51	93 ± 53	0.258
Protein (g·day^{-1})	105 ± 52	103 ± 41	0.729
Sodium (mg·day^{-1})	2487 ± 1774	2497 ± 1156	0.970
Potassium (mg·day^{-1})	3486 ± 3417	2778 ± 1347	0.128
Water (L·day^{-1} food + fluid)	3.3 ± 1.5	3.2 ± 1.4	0.315
Physical activity energy expenditure (kJ·day^{-1})	4462 ± 2276	4314 ± 1764	0.381
Body mass (kg)	71.7 ± 9.6	71.8 ± 9.8	0.340
Body mass index (kg·m^{-2})	23.6 ± 4.2	23.2 ± 4.8	0.409
USG	1.018 ± 0.005	1.018 ± 0.005	0.932

[1] Statistical significance calculated using two-tailed paired *t*-test.

Abbreviations: *SD*, standard deviation; USG, urine specific gravity

Table 2: Overnight fasted concentrations of blood hormones and metabolites in a euhydrated state at baseline before each trial arm (n = 16)

	Pre-hypohydration	Pre-rehydration	
	Mean ± *SD*	Mean ± *SD*	*p*-value[1]
Plasma ACTH (pmol·L-1)	3.54 ± 1.98	3.61 ± 1.48	0.841
Plasma copeptin (pmol·L^{-1})	4.45 ± 2.01	4.25 ± 1.83	0.462
Plasma cortisol (nmol·L^{-1})	316 ± 162	330 ± 269	0.687
Serum glucose (mmol·L^{-1})	5.04 ± 0.30	4.98 ± 0.46	0.493
Serum insulin (pmol·L^{-1})	30.4 ± 11.3	29.8 ± 11.0	0.852
Serum osmolality (mOsm·kg^{-1})	287 ± 6	285 ± 4	0.152

[1] Statistical significance calculated using two-tailed paired *t*-test.

Abbreviations: ACTH, adrenocorticotropic hormone; *SD*, standard deviation

Table 3: Mean ± *SD* markers of hydration status between the morning of the intervention day (euhydrated state) and full laboratory testing day

	Hypohydration			Rehydration				
	BL (Mean ± *SD*)	FT (Mean ± *SD*)	Δ BL to FT HYPO (95 % CI)	BL (Mean ± *SD*)	FT (Mean ± *SD*)	Δ BL to FT RE (95 % CI)	$p_{difference}$ BL HYPO vs RE[1]	$p_{difference}$ FT HYPO vs RE[1]
Body mass (kg)	71.6 ± 9.8	70.3 ± 9.7	-1.3 (-1.8, -0.8)	71.5 ± 9.8	71.4 ± 9.9	-0.1 (-0.3, -0.1)	0.675	< 0.001
Body water (kg)	37.3 ± 7.0	36.8 ± 6.5	-0.4 (-0.8, 0.0)	37.6 ± 5.8	37.8 ± 5.3	0.1 (-0.3, 0.6)	0.669	0.226
CSMA (mm^2)	12773 ± 2829	12408 ± 2662	-365 (-587, -138)	12682 ± 2684	12688 ± 2739	6 (-137, 148)	0.304	0.002
USG	1.017 ± 0.005	1.027 ± 0.003	0.010 (0.007, 0.014)	1.013 ± 0.005	1.016 ± 0.003	0.003 (0.000, 0.005)	0.017	< 0.001
Urine osmolality ($mOsm \cdot kg^{-1}$)	554 ± 185	965 ± 84	442 (355, 529)	419 ± 147	532 ± 126	93 (9, 178)	0.023	< 0.001

[1] Statistical significance calculated using paired *t*-test.

Abbreviations: BL, euhydrated baseline measure from the morning of the intervention day; CI, confidence interval; CSMA, cross-sectional muscle area; HYPO, hypohydrated trial arm; FT, full trial day; RE, rehydrated trial arm; *SD*, standard deviation; USG, urine specific gravity