



Carroll, H. A., Templeman, I., Chen, Y.-C., Edinburgh, R. M., Burch, E. K., Jewitt, J. T., Povey, G., Robinson, T. D., Dooley, W. L., Jones, R., Tsintzas, K., Gallo, W., Melander, O., Thompson, D., James, L. J., Johnson, L., & Betts, J. A. (2019). Effect of acute hypohydration on glycemic regulation in healthy adults: a randomized crossover trial. *Journal of Applied Physiology*, 126(2), 422-430.
<https://doi.org/10.1152/jappphysiol.00771.2018>

Peer reviewed version

Link to published version (if available):
[10.1152/jappphysiol.00771.2018](https://doi.org/10.1152/jappphysiol.00771.2018)

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

Running title: Effect of hydration status on glycemia

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Author contributions: Conception, HAC; design, HAC, DT, LJJ, LJ, JAB; conducted the experiment, HAC, IT, YCC, RME, EKB, JTJ, GP, TDR, WLD, JAB; data analysis and interpretation, HAC, IT, YCC, RJ, KT, WG, OM, LJJ, JAB

Abbreviations: ACTH, adrenocorticotrophic 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

Trial registration: Clinicaltrials.gov: NCT02841449
Open Science Framework: osf.io/ptq7m

Dataset available from: <https://doi.org/10.15125/BATH-00547>

1 **Abstract**

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

22

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

30 **Introduction**

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

37

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

49

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

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

59

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

67 **Methods**

68 Participants

69 Sixteen healthy adults volunteered to participate ($n = 8$ males), with a mean (\pm standard
70 deviation; *SD*) age of 30 ± 9 y, body mass of 71.7 ± 9.6 kg, and body mass index of
71 24.0 ± 3.4 kg·m⁻². Participants were randomized using simple randomization (no strata) by
72 HAC upon consent using a random number generator (Excel 2013, Microsoft Corp, US).
73 Exclusion criteria were: aged < 18 y or ≥ 60 y, metabolic disease (no body mass restrictions,
74 except self-reported weight loss > 5 kg in previous 6 mo), drug dependency, or
75 pregnant/breastfeeding; thus all participants were considered healthy and not taking
76 medication or necessary supplements (except contraceptives). Females not taking continuous
77 hormonal contraceptives were tested during their estimated follicular phase (3-10 days after
78 onset of menses). Data were collected in South West England between June 2016 and January
79 2017, inclusive.

80

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

86

87 Experimental design

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

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

97

98 *Pre-trial monitoring phase*

99 Three days pre-trial, participants were asked to replicate their food/fluid intake (weighed food
100 and fluid intake diaries; analysed using Nutritics Nutrition Analysis Software, Nutritics LTD,
101 Dublin) and physical activity (combined heart rate and accelerometry; ActiHeart™;
102 CamNtech, Cambridge, England), morning body mass (Inner scan; body composition
103 monitor, model BC-543, TANITA corp. Japan) and urine specific gravity (**Table 1**). On the
104 third monitoring day, participants were instructed to limit activity and to consume ≥ 40
105 $\text{ml}\cdot\text{kg}^{-1}$ lean body mass (assessed via bioelectrical impedance) of non-alcoholic fluid to ensure
106 euhydration before starting the intervention. This is in line with previous research aiming to
107 achieve euhydration (9, 22). No restrictions on caffeinated beverages were placed during this
108 phase, though it was emphasised to participants that they would need to replicate their fluid
109 intake in the subsequent trial arm.

110

111 Diet diaries were analysed by the same coder within-participant, and the accuracy was
112 verified by the lead author. Coding discrepancies were shared between all diet analysts to help
113 ensure consistency between-participants. Nutrient intakes for each day of diet recording were
114 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 abstinence 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 $45.2 \pm 1.6^\circ\text{C}$, RE $44.6 \pm 1.3^\circ\text{C}$; $p = 0.292$) 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 $3 \text{ mL}\cdot\text{kg}^{-1}$ body mass (HYPO),

139 or $40 \text{ mL}\cdot\text{kg}^{-1}$ 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 $0.52 \pm 0.11 \text{ L}\cdot\text{d}^{-1}$ versus RE $3.7 \pm 0.8 \text{ L}\cdot\text{d}^{-1}$; $p < 0.001$), 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 abstinence 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 Pharmaceuticals 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

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

191

192 Biochemical analysis

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

198

199 Metabolites and hormones were measured using commercially available ELISAs (plasma
200 arginine⁸ vasopressin, Enzo Life Sciences; serum insulin, Mercodia), ECLIAs (plasma
201 ACTH, Roche), ECLIs (plasma cortisol, Roche), automated immune analyzers (plasma
202 copeptin, ThermoFisher Kryptor Compact Plus) and spectrophotometric assays (serum
203 glucose, RX Daytona, Randox Laboratories). Osmolality was measured using freezing-point
204 depression (serum osmolality, Gonotec Osmomat auto; urine osmolality, Micro-Osmometer
205 3300) and urine specific gravity was measured using a handheld refractometer (SUR-NE
206 Clinical Refractometer, Atago, Japan).

207

208

209 Statistical analysis

210 The primary aim of the study was the glycemic response to an OGTT. Secondary outcomes
211 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 ($5.10 \pm 0.42 \text{ mmol}\cdot\text{L}^{-1}$)
231 and RE ($5.02 \pm 0.40 \text{ mmol}\cdot\text{L}^{-1}$; $p = 0.327$). 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 $303 \pm 121 \text{ mmol}\cdot\text{120 min}\cdot\text{L}^{-1}$, RE $306 \pm 113 \text{ mmol}\cdot\text{120}$
235 $\text{min}\cdot\text{L}^{-1}$, $p = 0.866$), AUC (HYPO $926 \pm 169 \text{ mmol}\cdot\text{120 min}\cdot\text{L}^{-1}$, RE 934 ± 120
236 $\text{mmol}\cdot\text{120 min}\cdot\text{L}^{-1}$, $p = 0.819$), or time to peak (HYPO $46 \pm 14 \text{ min}$, RE $48 \pm 14 \text{ min}$,
237 $p = 0.609$).

238

239 *Serum insulin concentration*

240 Serum insulin concentrations were similar in the fasted state (HYPO $27.09 \pm 9.66 \text{ pmol}\cdot\text{L}^{-1}$,
241 RE $27.62 \pm 9.21 \text{ pmol}\cdot\text{L}^{-1}$; $p = 0.809$). 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 $21937 \pm 8340 \text{ pmol}\cdot\text{120 min}\cdot\text{L}^{-1}$, $p = 0.369$), AUC (HYPO
245 $23958 \pm 9275 \text{ pmol}\cdot\text{120 min}\cdot\text{L}^{-1}$, RE $25326 \pm 8679 \text{ pmol}\cdot\text{120 min}\cdot\text{L}^{-1}$, $p = 0.359$), or time to
246 peak (HYPO $54 \pm 25 \text{ min}$, RE $58 \pm 26 \text{ min}$, $p = 0.633$) serum insulin concentrations.

247

248 *Serum osmolality*

249 There was an increase from baseline in serum osmolality during HYPO of $9 \pm 6 \text{ mOsm}\cdot\text{kg}^{-1}$
250 with relative stability from baseline during RE ($\Delta 1 \pm 4 \text{ mOsm}\cdot\text{kg}^{-1}$; HYPO vs RE $p < 0.001$;
251 Table 3). Similar differences remained throughout the OGTT (trial $F = 74.457$, $p < 0.001$),
252 reflected in a greater AUC during HYPO compared to RE (HYPO $35355 \pm$
253 $692 \text{ mOsm}\cdot 120 \text{ min}\cdot\text{L}^{-1}$, RE $34232 \pm 701 \text{ mOsm}\cdot 120 \text{ min}\cdot\text{L}^{-1}$, $p < 0.001$).

254

255 *Plasma copeptin concentration*

256 Due to the difficulties in measuring AVP(24), our data were unreliable, but are available in
257 the published dataset. Plasma copeptin was measured as a reliable marker of AVP
258 secretion(24). Fasted (pre-biopsy) plasma copeptin concentrations increased significantly
259 from baseline after HYPO ($\Delta 14.32 \pm 9.32$; $p < 0.001$) but not after RE ($\Delta 0.46 \pm 2.34$; $p =$
260 0.457). Plasma copeptin concentrations were consistently higher throughout the OGTT during
261 HYPO compared to RE (trial $F = 14.193$, $p = 0.002$; time $F = 1.285$, $p = 0.282$; trial*time $F =$
262 1.396 , $p = 0.261$; **Figure 3a**), confirmed by a higher AUC (HYPO $2704 \pm 2398 \text{ pmol}\cdot 120$
263 $\text{min}\cdot\text{L}^{-1}$, RE $961 \pm 1488 \text{ pmol}\cdot 120 \text{ min}\cdot\text{L}^{-1}$, $p = 0.001$).

264

265 *Plasma adrenocorticotrophic hormone concentration (ACTH)*

266 There were no differences in the ACTH response between HYPO and RE (trial $F = 2.541$,
267 $p = 0.132$; time $F = 6.120$, $p = 0.025$; trial*time $F = 1.343$, $p = 0.266$) during the OGTT, nor
268 were there differences in the AUC (HYPO $405 \pm 195 \text{ pmol}\cdot 120 \text{ min}\cdot\text{L}^{-1}$, RE
269 $468 \pm 255 \text{ nmol}\cdot 120 \text{ min}\cdot\text{L}^{-1}$, $p = 0.121$).

270

271 *Plasma cortisol concentration*

272 Plasma cortisol concentrations were similar between HYPO and RE (trial $F = 0.216$,
273 $p = 0.649$; time $F = 19.416$, $p < 0.001$; trial*time $F = 0.275$, $p = 0.674$), with no differences in
274 the plasma cortisol AUC (HYPO 35445 ± 17432 nmol*120 min·L⁻¹, RE 36716 ± 24915
275 nmol*120 min·L⁻¹, $p = 0.642$).

276

277 *Cross-sectional muscle area*

278 Cross-sectional muscle area as a proxy for muscle cell volume reduced significantly from
279 baseline after HYPO ($\Delta -2.9 \pm 2.7$ %; $p = 0.003$) but not after RE ($\Delta 0.0 \pm 2.1$ %; $p = 0.936$;
280 Table 3).

281

282 *Muscle water content*

283 Pre-OGTT muscle biopsies showed that muscle water content was lower during HYPO
284 (760.5 ± 13.2 g·kg⁻¹) compared to RE (771.6 ± 17.8 g·kg⁻¹) but this difference dissipated post-
285 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
286 $F = 15.36$, $p = 0.011$; trial*time $F = 6.265$, $p = 0.055$; **Figure 4**).

287

288 *Resting energy expenditure*

289 Resting metabolic rate was similar between trial arms in the fasted (HYPO
290 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 $F =$
291 0.015 , $p = 0.904$; time $F = 10.130$, $p = 0.001$; trial*time $F = 0.140$, $p = 0.798$) state.
292 Participants had similar fasting respiratory exchange ratios (HYPO 0.84 ± 0.05 , RE

293 0.84 ± 0.06 ; $p = 0.900$), although carbohydrate oxidation post-glucose ingestion had a
294 tendency to increase to a greater extent in the RE trial at 60 (HYPO 0.88 ± 0.04 , RE 0.93 ± 0.09)
295 and 120 min (HYPO 0.87 ± 0.07 , RE 0.91 ± 0.07 ; trial $F = 3.650$, $p = 0.078$; time $F = 14.693$,
296 $p < 0.001$; trial*time $F = 3.754$, $p = 0.039$).

297

298 *Plasma volume*

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

302

303 *Sensitivity analysis*

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

307

308 Copeptin

309 Removing those who had any biopsies did not meaningfully effect the trend in plasma
310 copeptin concentration throughout the OGTT ($n = 7$: trial $F = 13.517$, $p = 0.010$; time
311 $F = 4.081$, $p = 0.040$; trial*time $F = 1.489$, $p = 0.262$), though the overall AUC was lower
312 ($n = 7$: HYPO 11916 ± 1661 pmol*120 min·L⁻¹, RE 390 ± 177 pmol*120 min·L⁻¹, $p = 0.028$).

313

314 ACTH

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

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

321

322 Cortisol

323 In accordance with the plasma copeptin and ACTH response, plasma cortisol concentration
324 also increased post-biopsy. Removing these participants did not meaningfully alter the results,
325 though the time effect was no longer evident ($n = 7$: trial $F = 0.278$, $p = 0.617$; time
326 $F = 5.172$, $p = 0.055$; trial*time $F = 0.260$, $p = 0.686$). Despite a post-biopsy peak in plasma
327 cortisol concentration, the AUC was higher when the biopsy participants were removed
328 ($n = 7$: HYPO 37920 ± 25008 nmol*120·L⁻¹, RE 40989 ± 37507 nmol*120·L⁻¹, $p = 0.735$),
329 with no difference between HYPO and RE.

330

331 Resting metabolic rate

332 In the subgroup who had the additional measure at 30 min post-glucose ingestion ($n = 9$), no
333 difference in RMR (trial $F = 0.346$, $p = 0.573$; time $F = 6.087$, $p = 0.009$; trial*time $F =$
334 0.586 , $p = 0.508$) or RER (trial $F = 0.433$, $p = 0.529$; time $F = 17.330$, $p < 0.001$; trial*time
335 $F = 0.467$, $p = 0.607$) was apparent according to hydration status.

336 Discussion

337 This randomized crossover trial is the first to show that neither fasted nor postprandial
338 glycemia or insulinemia are influenced by hydration status in healthy adults, contrary to our
339 hypothesis. The key implication of this work is that clinicians and researchers may not have to
340 control for hydration status when investigating glycemetic regulation in healthy adults.

341 Participants replicated their diet and activity four days pre-trial, reducing known confounding
342 influences. Average body mass loss during HYPO was 1.9 % which is within the typical
343 range to induce thirst and is not uncommon in the general population (2), increasing the
344 external validity of these findings. In the hypohydrated state, copeptin (a surrogate marker of
345 AVP) concentrations increased from levels seen in healthy adults to levels reported in those at
346 highest risk of metabolic syndrome (10), with an accompanying reduction in muscle cell
347 volume of ~3 %, hypothesized to be detrimental to glycemetic regulation (16). Therefore the
348 level of hypohydration achieved was sufficient to induce physiological changes that
349 theoretically have meaningful health implications; such changes did not occur when
350 participants were rehydrated.

351

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

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

362

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

371

372 A key mechanism by which elevations in AVP induce poor glycemic control is through the
373 hypothalamic-pituitary-adrenal (HPA) axis, stimulating ACTH and cortisol secretion. Despite
374 the increase we observed in plasma copeptin concentrations, plasma ACTH and cortisol
375 concentrations were not different between HYPO and RE suggesting that in healthy adults,
376 short-term hypohydration is not a sufficient stimulus to induce a stress response along the
377 HPA axis. This theory is in accordance with earlier work highlighting that the role of AVP in
378 ACTH secretion is predominantly a response to physical stress (15). Nevertheless, we did not
379 find a difference in ACTH or cortisol by hydration status in those who had the muscle
380 biopsies, despite higher copeptin responses during HYPO. In previous work in those with
381 type 2 diabetes, cortisol concentrations did increase 45 min post-glucose ingestion when
382 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

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

417

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

426

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

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

435

436 **Acknowledgements**

437 We would like to thank Dr Oliver Perkin for his invaluable technical help with the pQCT
438 scanner and interpreting these results. Neither funding source had any role in the study design,
439 data analysis, or writing or publishing of this article.

440

441 **Grants**

442 This work was supported by the Economic and Social Research Council (grant no.:
443 ES/J50015X/1) and the European Hydration Institute Graduate Research Grant.

444

445 **Disclosures**

446 This work was supported by the European Hydration Institute Graduate Research Grant. HAC
447 has accepted conference fees from Danone. OM has received consultancy honoraria from
448 Danone Research. LJJ has previously received funding for hydration-related research from
449 PepsiCo Inc., the European Hydration Institute and Volac International Ltd and has performed
450 consultancy work for Lucozade Ribena Suntory. LJ has received funding from Kellogg
451 Europe and Danone Baby Nutrition. JAB has received funding from LucozadeRibenaSuntory,
452 PepsiCo, and Kenniscentrum Suiker. No other authors declared a conflict of interest.

453

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547

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

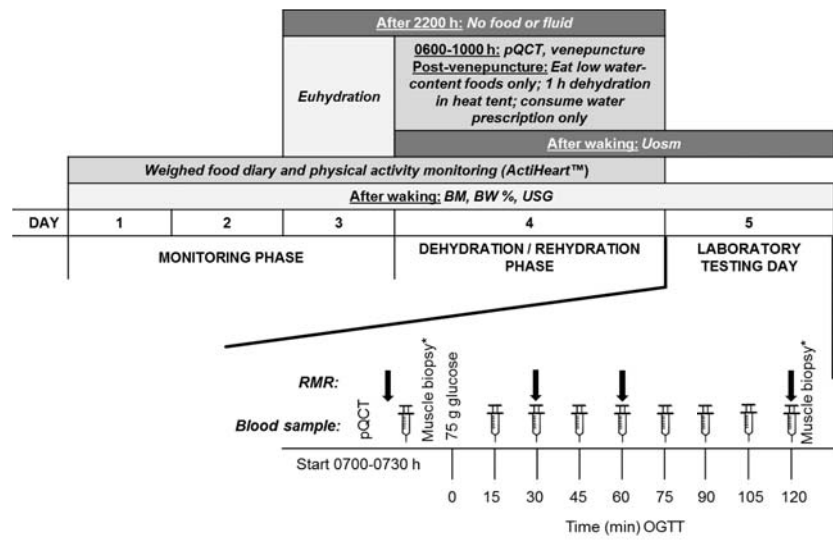
570 Figure 4. Average (thick lines) and individual (thin lines) muscle water content pre-OGTT

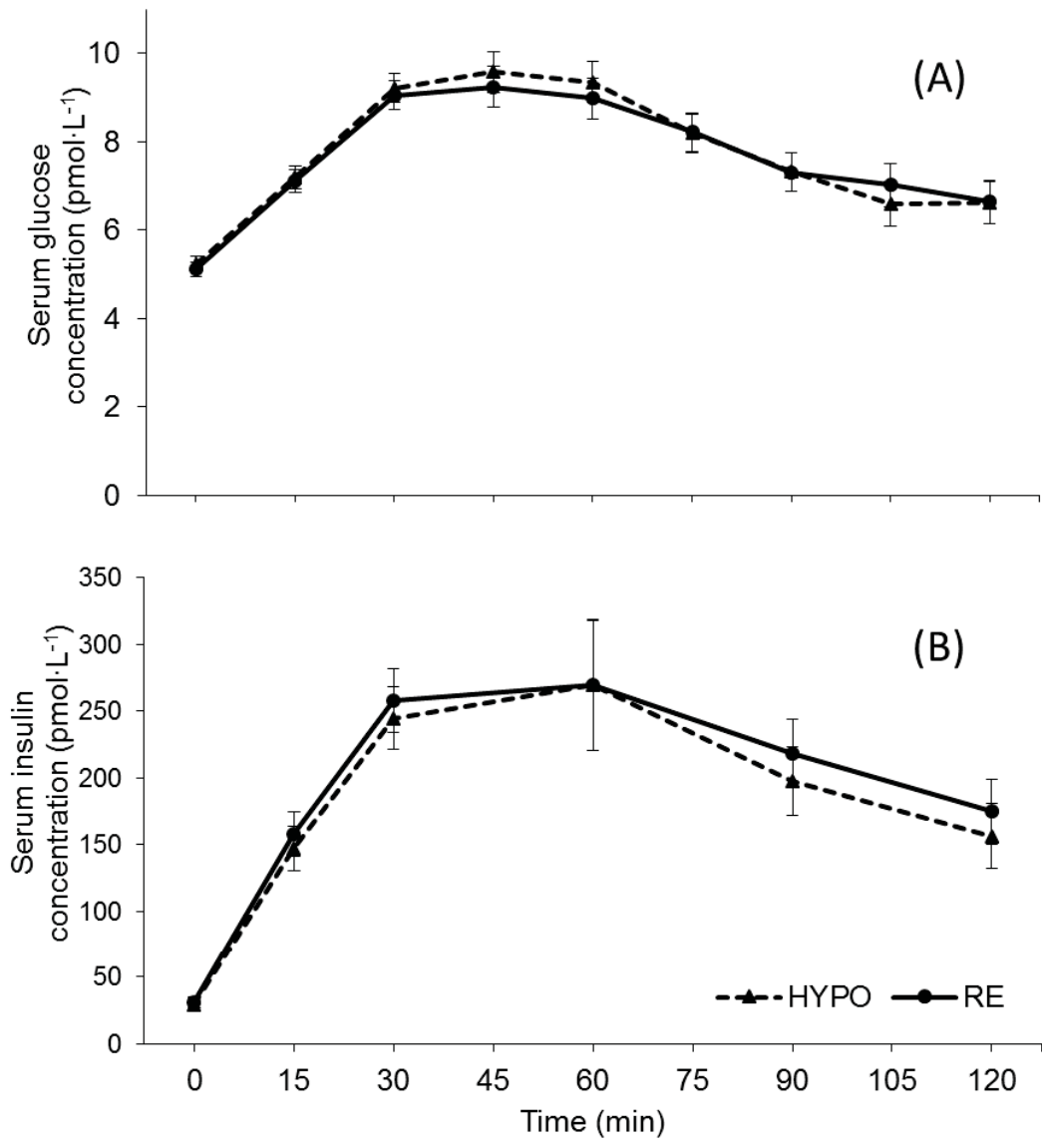
571 and post-OGTT during HYPO and RE (n = 6; trial $F = 3.183$, $p = 0.135$; time $F = 15.36$, $p =$

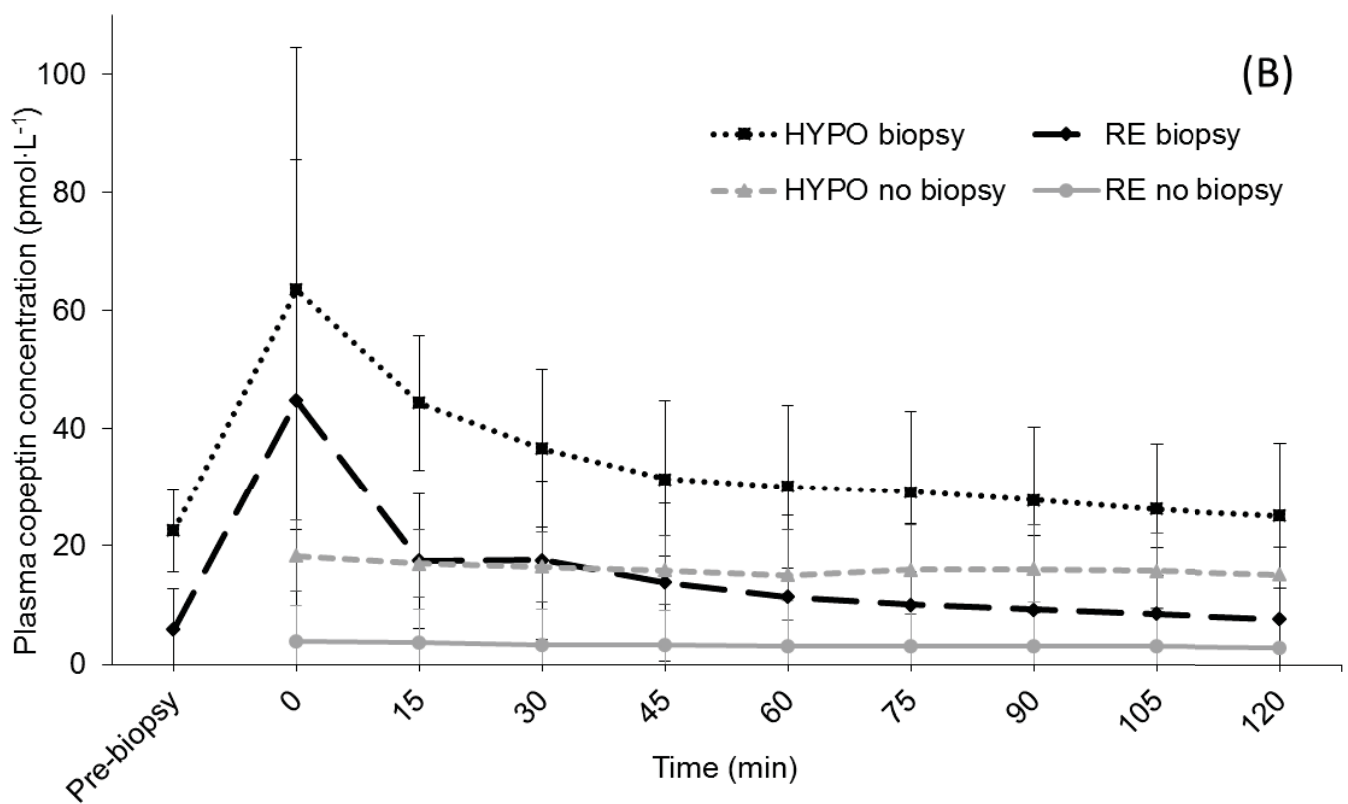
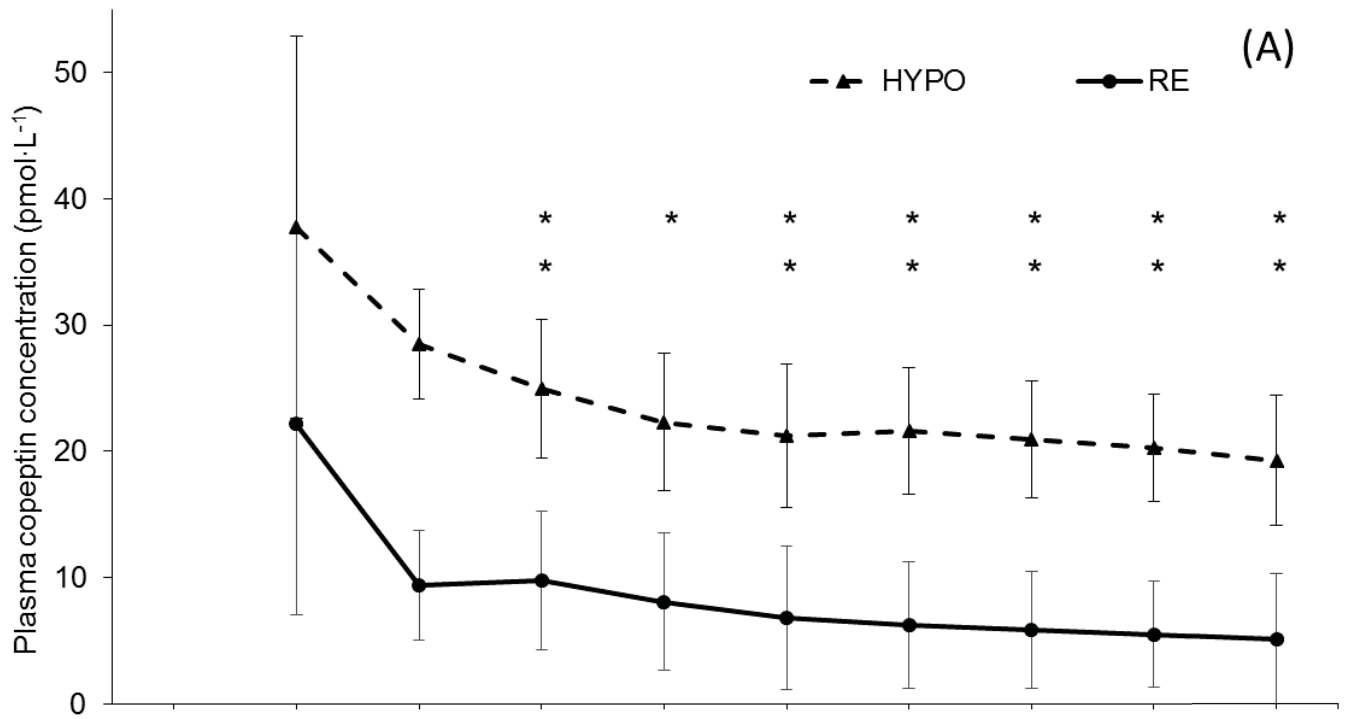
572 0.011; trial*time $F = 6.265$, $p = 0.055$). * p -value Bonferroni adjusted for multiple
573 comparisons. Black lines are HYPO; grey lines are RE. Abbreviations: HYPO, hypohydrated
574 trial arm; OGTT, oral glucose tolerance test; RE, rehydrated trial arm

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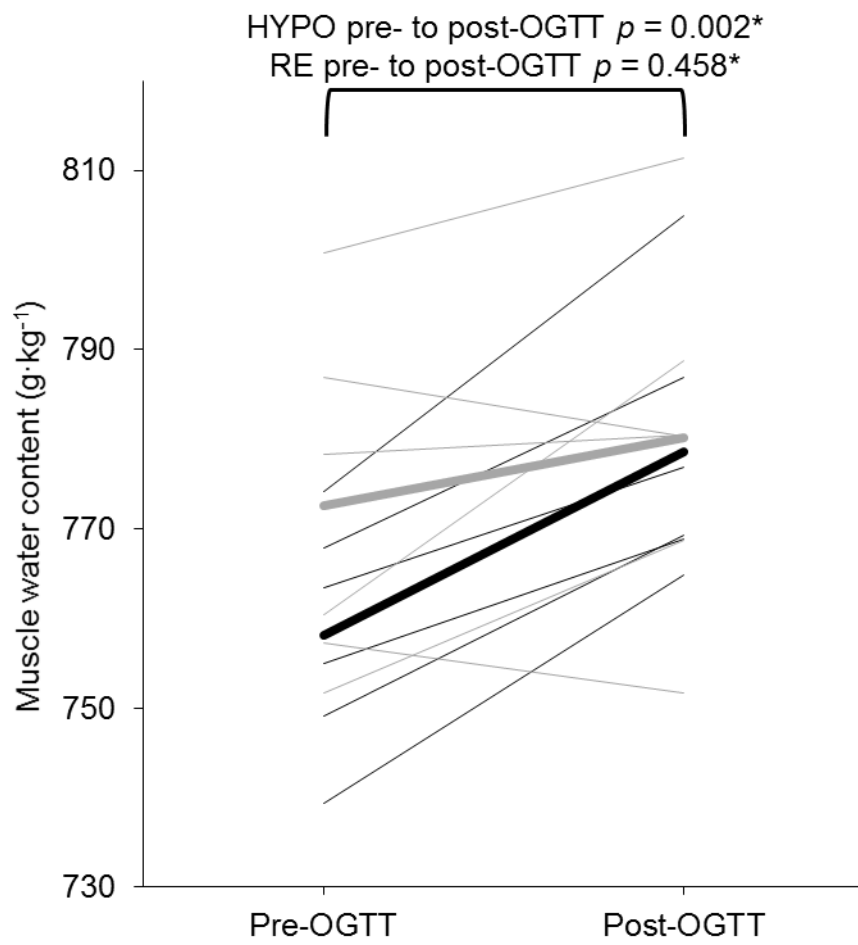


Table 1: Mean \pm *SD* of lifestyle factors and markers of hydration status during the 3-day pre-trial monitoring phase (n = 16)

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

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

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