1 **The acute and chronic effects of hot water immersion on inflammation and**

2 **metabolism in sedentary, overweight adults**

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11 Running head: Inflammatory and metabolic responses to hot water immersion

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Abstract

Regular exercise-induced acute inflammatory responses are suggested to improve the inflammatory profile and insulin sensitivity. As body temperature elevations partly mediate this response, passive heating might be a viable tool to improve the inflammatory profile. This study investigated the acute, and chronic effects of hot water immersion on inflammatory and metabolic markers. Ten sedentary, overweight males (BMI: 31.0 ± 4.2 kg/m^2) were immersed in water set at 39°C for 1 h (HWI) or rested for 1 h at ambient temperature (AMB). Venous blood was obtained prior to, immediately post and 2 h post-session for assessment of monocyte intracellular heat shock protein 72 (iHsp72) and plasma concentrations of extracelullar heat shock protein 72 (eHsp72), interleukin-6 (IL-6), fasting glucose, insulin and nitrite. Thereafter, participants underwent a 2-week intervention period, consisting of 10 hot water immersion sessions (INT). Eight BMI-matched participants (BMI: 30.0 ± 2.5 kg/m^2) were included as control (CON). Plasma IL-6 and nitrite concentrations were higher immediately following HWI compared to AMB (IL-6 $p<0.001$, HWI: 1.37 ± 0.94 to 2.51 ± 1.49 pg/ml; nitrite $p=0.04$, HWI: 271 ± 52 to 391 ± 72 nM), while iHsp72 expression was unchanged ($p=0.57$). In contrast to resting iHsp72 expression ($p=0.59$), fasting glucose ($p=0.04$, INT: 4.44 ± 0.93 to 3.98 ± 0.98 mmol/l), insulin ($p=0.04$, INT: 68.1 ± 44.6 to 55.0 ± 29.9 pmol/l) and eHsp72 ($p=0.03$, INT: $17\pm41\%$ reduction) concentrations were lowered after INT compared to CON. HWI induced an acute inflammatory response and increased nitric oxide bioavailability. The reductions in fasting glucose and insulin concentrations following the chronic intervention suggest that hot water immersion may serve as a tool to improve glucose metabolism.

Passive heating; chronic low-grade inflammation; heat shock protein; interleukin-6; glucose metabolism

New and noteworthy

A single hot water immersion (HWI) session induces an acute increase in plasma interleukin-6 and nitrite concentrations, but does not acutely elevate heat shock protein 72 expression in monocytes (iHsp72). A chronic HWI intervention reduces fasting glucose and insulin concentrations in the absence of changes in resting iHsp72. Therefore, HWI shows potential as a strategy to combat chronic low-grade inflammation and improve glucose metabolism in individuals without the physical capacity to do so using exercise.

Introduction

Passive heating interventions have been linked to several positive health outcomes, such as improved vascular function (4), mental health (11), weight loss (33) and enhanced insulin sensitivity (42). Although observations of a lowering in fasting glycosylated haemoglobin and blood glucose concentrations following hot water immersion (HWI) in individuals with type 2 diabetes supports the notion of improved insulin sensitivity following HWI (33), the mechanisms that underlie this beneficial effect are currently unclear. Chronic low-grade inflammation has been implicated in the aetiology of insulin resistance (9), as evidenced by the positive association between pro-inflammatory proteins and insulin resistance (9, 39), while the body of evidence for a causal relationship of these proteins with insulin resistance is growing (35). Moreover, it is well documented that exercise training can counteract chronic low-grade inflammation (57) and improve insulin sensitivity (29). However, since it is not feasible for all populations to adhere to the recommended exercise guidelines due to a low physical capacity or health conditions that hinder exercise participation, the development of alternative strategies that can reduce chronic low-grade inflammation in populations without the capacity to engage in sufficient volumes of exercise is warranted to mitigate risk factors for insulin resistance and non-communicable diseases.

The acute inflammatory response provoked by a physical stressor, such as exercise, can induce a subsequent protracted anti-inflammatory response. For instance, elevations in circulating interleukin (IL)-6 concentrations immediately following exercise activate the release of anti-inflammatory cytokines such as IL-1ra and IL-10, typically 1 to 4 h following the exercise bout (57). In addition, recent studies have identified an enhanced acute inflammatory response following exercise when body temperature is augmented (43). Increasing body temperature therefore likely serves as an independent stressor able to induce the acute inflammatory responses needed to reduce chronic low-grade inflammation in the

110 long term. This is supported by Welc et al. (66), showing that passive heating for 1 h at

111 42.4°C can activate heat shock factor 1, which in turn upregulates the production of IL-6 and

112 intracellular heat shock protein 72 (iHsp72) in mice skeletal muscle.

113 In humans, 1-2 h of hot water immersion (HWI), at a temperature 2-3°C higher than

114 resting core temperature, has been reported to acutely elevate IL-6, IL-1ra (45), extracellular

115 Hsp72 (eHsp72) (16) and monocyte intracellular Hsp72 (iHsp72) (54). Elevations in iHsp72

116 can block the inflammatory actions of c-jun amino terminal kinase (JNK) and nuclear factor

117 κB (NF-κB), resulting in enhanced insulin sensitivity (31). In contrast to the beneficial

118 functions of iHsp72, Hsp72 found in plasma (i.e. eHsp72) can activate circulating monocytes,

119 resulting in an increase in pro-inflammatory cytokine release (1). Although the transient

120 increase in eHsp72 following an acute bout of exercise is suggested to be part of the

121 beneficial inflammatory response to exercise (67), a reduction in resting eHsp72 is suggestive

122 of an improved inflammatory profile and may improve insulin sensitivity (41).

123 In addition to modulating inflammation, an increase in body temperature has been

124 linked to increased nitric oxide (NO) production through enhanced NO synthase (NOS) (4,

125 36), possibly mediated by an increased expression of Hsp90 (70). It is well documented that

126 NO impacts a myriad of biological processes, including tissue glucose uptake (19, 20, 58, 60).

127 Therefore, an increase in NO synthesis following HWI might contribute to changes in insulin

128 sensitivity resulting from this intervention. Moreover, an acute increase in NO bioavailability

129 exerts an anti-inflammatory effect on human leukocytes (58) and increases the iHsp72

130 expression in peripheral mononuclear blood cells (63), indicating cross-talk between NO and

131 the immune system. However, the extent to which acute and chronic HWI influences NO

132 synthesis and its role in chronic low-grade inflammation and insulin sensitivity is presently

133 unclear.

134   Although there is now evidence for the potential of HWI to induce an *acute*

135   inflammatory response (16, 45, 54), *chronic* intervention studies in humans are scarce.

136   Notwithstanding, the reduction in fasting blood glucose concentrations in patients with

137   diabetes (33) and resting plasma IL-6 concentrations in patients with chronic heart failure (55)

138   are promising initial results. These studies, however, focussed on clinical populations, did not

139   address the mechanistic link between inflammatory and metabolic markers and provided little

140   detail on the acute (thermo-)physiological responses to HWI. For instance, while animal

141   studies have provided compelling evidence for the potential of HWI to chronically elevate

142   basal iHsp72 levels (26, 6, 61), it is not known whether this holds true in humans. The smaller

143   acute core temperature increases reported in human compared to animal studies might make

144   HWI less effective as a strategy to elevate resting iHsp72 levels in humans (27).

145   Therefore, the present study investigated the acute inflammatory response to a single

146   HWI session as well as the potential of a chronic HWI intervention to improve the

147   inflammatory and metabolic profile at rest. It is hypothesised that an HWI session induces

148   acute increases in plasma IL-6 concentrations, NO bioavailability as well as iHsp72

149   expression in monocytes. Chronically, the 2-week HWI intervention is hypothesised to

150   increase resting levels of iHsp72, while reducing IL-6 and eHsp72 concentrations. Finally, in

151   line with Hooper et al. (33), the intervention period is expected to result in reductions in

152   fasting glucose and insulin concentrations.

153   **Methods**

154   *Participants*

155   Participants were sedentary (<2 hours exercise/week), overweight (body mass index >27

156   kg/m^2), otherwise healthy males (Table 1). Exclusion criteria were the usage of anti-

157   inflammatory medication and contra-indications to engage in HWI. The latter was assessed

158 with a medical health questionnaire according to the American College for Sport and Exercise

159 Medicine guidelines for exercise testing and prescription (32). Engagement in structured

160 exercise was reported prior to and following the chronic intervention period, using the

161 International Physical Activity Questionnaire (8). Participants gave informed consent after

162 being instructed about the procedures of the study, which were approved by the Local Ethical

163 Committee of Loughborough University, in accordance with the declaration of Helsinki.

164 *Procedures*

165 An outline of the procedures for the intervention group is given in Fig. 1. Participants

166 visited the laboratory for a HWI (HWI$_{pre}$) and control trial (AMB) in a counterbalanced order,

167 with a minimum of 72 h between the visits. Participants refrained from exercise, alcohol and

168 caffeine and standardised their diet using a food diary in the 24 hours prior to the visits. All

169 visits started between 8-10 am, with the starting consistently applied for each individual to

170 account for a possible circadian rhythm in any of the outcome measures. After an overnight

171 fast, nude body mass, height, hip and waist circumference were measured and skinfold

172 thickness was assessed at four sites (biceps, triceps, subscapular and supra iliac) (14) for the

173 estimation of body fat percentage.

174 ***** Insert Figure 1 around here *****

175 Thereafter, participants underwent 15 min of seated rest in an environmental chamber (27°C,

176 40% humidity) for baseline measurements (21). Following the "pre" blood sample,

177 participants entered the water tank for the HWI$_{pre}$ or remained seated for another hour in the

178 same conditions as AMB. This control condition (instead of immersion in thermoneutral

179 water) was chosen because this study was designed to evaluate the effects of HWI as a stand-

180 alone health intervention rather than to investigate the effects of an increase in body

181 temperature per se. Evidence suggests that the effects of hydrostatic pressure on inflammatory

182 markers are negligible (43).

183   During HWI_{pre}, participants were immersed up to the neck for 1 hour in water set at

184 39°C. Participants sat in an upright position and were allowed to drink water *ad libitum*.

185 During both HWI_{pre} and AMB, measurements were taken every 15 min. Blood pressure

186 (Microlife BP3AC1-1, Cambridge, UK) was measured in duplicate at the level of the heart,

187 while thermal sensation, thermal comfort (21) and basic affect using the Feeling Scale (68)

188 were reported. Expired air was collected for 3 min into Douglas bags for the determination of

189 oxygen uptake ($\dot{V}O_2$) using a Servomex 1440 gas analyser (Servomex Ltd, Crowborough,

190 UK). Tympanic temperature was measured with a tympanic temperature probe (Squirrel,

191 Grant Instruments, Shepreth, UK), using cotton wool to cover the external canal of the ear.

192 Rectal temperature (T_{rec}) was recorded every 5 min throughout the trials, using a rectal probe

193 (YSI 400 series, Ohio, USA) that was inserted 10 cm beyond the anal sphincter. Heart rate

194 (HR) (Polar RS400, Kempele, Finland) was continuously measured throughout.

195   Immediately on completion of the session, a "post" blood sample was taken and

196 participants rested seated in the environmental chamber for 30 min. Thereafter, nude body

197 weight was measured and a breakfast snack was provided (Sainsbury breakfast biscuits; 212

198 kcal, 5.8 g fat, 34.3 g carbohydrates, 4.0 g protein). The change in nude body weight and

199 water consumed was used to estimate sweat loss. Participants were then allowed to rest and

200 perform light work such as reading. Two hours after completion of the session, the "post 2 h"

201 blood sample was taken following 15 min of seated rest.

202 Following the first two visits, participants enrolled in an intervention period consisting of

203 ten HWI sessions, all executed within fourteen days. The first five sessions of this period

204 lasted 45 minutes, while the last five lasted 60 minutes. As pilot work suggested that the HWI

205 sessions can be experienced as uncomfortable, this progression was chosen to avoid drop-out

206   during the intervention period. In all sessions the temperature of the water was set at 39°C and

207   participants were immersed up to their neck. During the ten sessions, HR, tympanic

208   temperature, thermal sensation, thermal comfort and basic affect were assessed every 15 min.

209   Three days after completion of the last session of the intervention period, an acute trial

210   (HWI$_{post}$) was conducted to study the effects of the intervention period on the acute

211   inflammatory response to HWI. The procedures during this session were identical to HWI$_{pre}$.

212   The "pre" blood sample of the first session (either HWI$_{pre}$ or AMB) and HWI$_{post}$ were used to

213   study the chronic effects of the intervention period. Eight individuals matched for body

214   composition, age and physical activity levels were included as control for the chronic arm of

215   the study (CON). These participants visited the laboratory for two resting blood samples only,

216   with the time between both samples held equal to the intervention group. In the intervention

217   group, an additional resting blood sample was taken one week following HWI$_{post}$ to

218   investigate whether any adaptations detected following the intervention period would remain

219   after one week.

220   *Biochemical analyses*

221   Blood was collected in K$_3$EDTA (plasma markers) and sodium heparin (flow cytometry)

222   monovettes. The K$_3$EDTA tubes were spun down immediately for 5 min at 1500 g and 4°C,

223   and plasma was stored at -80°C until batch analysis. Flow cytometry was used to assess

224   changes in iHsp72 in monocytes and the distribution in monocyte subsets. In addition,

225   changes in the expression of iHsp72 in the respective monocyte subsets were assessed. Sixty

226   µL of whole blood was incubated together with 5 µL of PerCP-conjugated cluster of

227   differentiation (CD)14 and 2.5 µL of PE-conjugated CD16 antibodies in the dark at room

228   temperature for 15 min. Thereafter, samples were lysed (750 µL; Facs lysing solution (BD

229   biosciences, San Diego, US), washed (1.5 mL phosphate buffered saline) and fixed using

230   Leucoperm (60 µL; BD biosciences). Following permeabilisation (60 µL; Leucoperm, BD

231   biosciences) samples were incubated with 4 µL of FITC-conjugated Hsp70 antibody or

232   isotype control for 30 min. Finally, samples were washed and resuspended in phosphate

233   buffered saline prior to running through the Flow Calibur (BD biosciences). All antibodies

234   except CD16 (BD biosciences) were purchased from Miltenyi Biotech (Teterow, Germany).

235   Cell Quest software (BD biosciences) was used for the analysis, collecting 100,000 events per

236   sample. Compensation of the flow cytometer prior to the study was performed manually using

237   a whole blood sample of a male volunteer not participating in the study. Monocytes were

238   selected based on positive CD14 expression, whereafter the percentage of monocyte subsets

239   (CD14++CD16- classical monocytes, CD14+CD16+ intermediate monocytes and CD14-

240   CD16++ non-classical monocytes) was determined using the trapezoid method (68). The

241   iHsp72 expression in monocytes was determined using the geometric mean fluorescence

242   intensity (GMFI) following subtraction of the isotype control GMFI.

243   All glassware, utensils, and surfaces were rinsed with deionized water to remove residual

244   NO intermediates prior to plasma [nitrite] analysis. Plasma samples were introduced to a gas-

245   tight purge vessel via 200 uL injections into the septum at the top of the vessel. The [nitrite]

246   of the undiluted (non-deproteinized) plasma was determined by its reduction to NO in the

247   presence of glacial acetic acid and aqueous sodium iodide (4% w/v). The spectral emission of

248   electronically excited nitrogen dioxide, derived from the reaction of NO with ozone, was

249   detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers

250   gas-phase chemiluminescence nitric oxide analyzer (Sievers NOA 280i, Analytix Ltd,

251   Durham, UK). The [nitrite] was determined by plotting signal (mV) area against a calibration

252   plot of sodium nitrite standards. Interleukin-6 (High-sensitivity, RnD systems, Abington,

253   UK), eHsp72 (Amp`d HSP70 high-sensitivity, Enzo life sciences, Farmingdale, US) and

254   insulin (Mercodia AB, Uppsala, Sweden) were measured in plasma, in duplicate, using

255 enzyme linked immunosorbent assays (ELISA). For the determination of eHsp72

256 concentrations, plasma samples were diluted 1:4 prior to running the ELISA. The intra-assay

257 coefficients of variation were 7.0%, 6.2% and 2.5% for IL-6, eHsp72 and insulin,

258 respectively. A Biosen C-line (Biosen, Barleben, Germany) was used to determine blood

259 glucose concentrations in whole blood (52). A whole blood count was obtained using a

260 Yumizen H500 cell counter (Horiba Medical, Montpellier, France) for the determination of

261 leukocyte subsets, haematocrit and haemoglobin. The latter two were used to correct the post

262 and post+2h plasma IL-6 and eHsp72 concentrations for changes in plasma volume (10).

263 *Statistical analyses*

264 All values are given as mean ± standard deviation. Normality of the data was checked using

265 the Shapiro-Wilk test and a log transformation was performed when non-normality was

266 detected. Log transformation was performed on the eHsp72 data. Analysis of variance

267 (ANOVA) with repeated measures where appropriate was used to detect differences in the

268 acute responses between AMB and HWI_{pre} , HWI_{pre} and HWI_{post} as well as the effects of the

269 intervention period on baseline measures compared to CON. Due to a difference in baseline

270 plasma nitrite concentrations between HWI_{pre} and AMB, a one-way ANCOVA was employed

271 to detect differences between HWI and AMB at "post" and "post+2h" using nitrite

272 concentrations at "pre" as a covariate. *R*, the fold change in the eHsp72/iHsp72 ratio, was

273 determined for the acute as well as chronic arm of the study (41). The homeostasis model

274 assessment for insulin resistance (HOMA-IR) was determined using fasting glucose and

275 insulin concentrations (47). For all analyses, a Bonferroni corrected post-hoc test was used for

276 exploration of the differences at every time point when significance was detected. Effect sizes

277 (ES) (Cohen`s *d*) and their 95% confidence intervals were calculated where appropriate,

278 whereby an ES of 0.20, 0.50 and 0.80 refers to a small, moderate or large effect, respectively

279   (7). The effect sizes for a Time x Group (T x G) or Time x Condition (T x C) interaction were

280   calculated by comparing the pre-post change scores in each group or condition. Correlations

281   were computed using Pearson`s *r*. As the latter was an explorative analysis, the risk for a type

282   II error was not deemed problematic, and no Bonferroni correction was applied (56). The 23rd

283   version of the statistical package SPSS (SPSS inc, Chicago, US) was used for all analyses and

284   statistical significance was set at *p*<0.05.

285   **Results**

286

287   *Participants*

288   Baseline characteristics of the participants in the intervention group (INT) and CON can be

289   seen in Table 1. Apart from a trend towards a larger hip circumference in the control group,

290   there were no differences in anthropometrics and physical activity levels between the groups.

291   ***** Insert Table 1 around here *****

292   *Acute responses to hot water immersion*

293   The physiological and perceptual responses during HWI_{pre} and AMB are given in Table 2.

294   During HWI_{pre}, rectal temperature increased from 37.1±0.6°C to 38.7±0.4°C (Fig 2).

295   Folllowing the intervention period, diastolic blood pressure was lower at the end of HWI_{post}

296   when compared to HWI_{pre} (F: 25.4, $p = 0.001$). Thermal sensation at the end of HWI_{post} was

297   lower than at the end of HWI_{pre} (F: 14.3, $p = 0.01$) and sweat loss during HWI was increased

298   from 1.1±0.6 (HWI_{pre}) to 1.7±0.6 L (HWI_{post}) (F: 26.5, $p = 0.001$).

299   ***** Insert Table 2 and Figure 2 around here *****

300   Plasma concentrations of IL-6 were higher compared to AMB immediately following

301    $HWI_{pre,}$ (T x C; F: 14.5, $p<0.001$, ES: 1.71 (1.31 – 2.07)). However, this was not accompanied

302   by a rise in either eHsp72 (T x C; F: 1.9, $p = 0.16$) or iHsp72 in total monocytes (T x C; F:

303   0.5, $p = 0.57$) directly post or 2 h post-HWI$_{pre}$ (Fig. 3). The same was true for the expression

304   of iHsp72 in classical monocytes (T x C: F: 1.7, $p = 0.22$), intermediate monocytes (T x C; F:

305   2.3, $p = 0.19$) and non-classical monocytes (T x C; F: 1.5, $p = 0.25$). R did not differ between

306   HWI$_{pre}$ and AMB (T x C; pre-post F: 0.6, $p = 0.48$; pre-post+2h F: 0.1, $p = 0.76$).

307   ***** Insert Figure 3 around here *****

308   The distribution of monocyte subsets changed immediately after HWI$_{pre}$, with an increase

309   of the intermediate (T x C; F: 9.0, $p = 0.004$, ES: 1.39 (0.36 – 2.03)) and non-classical

310   monocytes (T x C; F: 11.8, $p = 0.001$, ES: 1.34 (0.32 – 1.24)). The proportion of classical

311   monocytes, however, was not reduced (T x C; F: 2.5, $p = 0.10$) (Table 3). Lymphocyte

312   numbers increased to a larger extent directly following HWI$_{pre}$ compared to AMB (T x C; F:

313   11.0, $p = 0.003$, ES: 1.97 (0.84 – 2.94)). There was no difference between HWI$_{pre}$ and AMB

314   in the acute elevation of total monocyte (T x C; F: 0.8, $p = 0.56$), leukocyte (T x C; F: 2.0, $p = $

315   0.16) or neutrophil numbers (T x C; F: 2.7, $p = 0.08$). The increase in plasma nitrite

316   concentration directly following HWI$_{pre}$ was larger compared to AMB (F: 11.2, $p = 0.04$,

317   ES:1.82 (0.71 – 2.77); Fig. 2).

318   ***** Insert Table 3 around here *****

319   The IL-6, eHsp72 and iHsp72 response did not differ following HWI$_{post}$ when compared

320   with HWI$_{pre}$ (T x C; IL-6 F: 0.3, $p = 0.80$, eHsp72 F: 0.9, $p = 0.45$, iHsp72 F: 0.1, $p = 0.71$).

321   The same was true for Hsp72 expression in classical (T x C; F: 1.7, $p = 0.22$), intermediate (T

322   x C; F: 2.2, $p = 0.17$) and non-classical monocytes (T x C; F: 1.5, $p = 0.25$). In contrast to

323   HWI$_{pre}$, the percentage of intermediate monocytes was not elevated following HWI$_{post}$ (Time;

324   F: 3.4, $p = 0.06$; Table 3). There were no differences in the acute change between HWI$_{pre}$ and

325   HWI$_{post}$ for total leukocyte (T x C; F: 1.3, $p = 0.36$), monocyte (T x C; F: 0.2, $p = 0.92$),

326   lymphocyte (T x C; F: 1.9, $p = 0.17$) and neutrophil (T x C; F: 0.8, $p = 0.56$) numbers.

327   Finally, the acute change in plasma nitrite concentration was similar between HWI_{pre} and

328   HWI_{post} (T x C; F: 1.3, $p = 0.30$) (Fig. 3).

329   *Chronic effects of the hot water immersion intervention period*

330   Table 4 shows the physiological responses during the HWI sessions of the intervention

331   period. Body mass did not change in INT following the intervention period (92.1±9.2 kg to

332   92.3±9.5 kg, F: 0.01, $p = 0.92$). Both systolic (T; F: 5.1, $p = 0.05$, ES: 0.60 (0.34 – 1.44))️ and

333   diastolic blood pressure (T; F: 14.3, $p = 0.003$, ES: 0.64 (0.32 – 1.47))️ were lowered

334   following the intervention period. Resting HR (T; F: 0.3, $p = 0.54$) and Trec (T; F: 0.4, $p = $

335   0.22) were not affected by the intervention period (Table 2). Physical activity levels were not

336   different from habitual physical activity (as reported at the start of the intervention period)

337   during the intervention period (T; F: 0.2, $p = 0.64$).

338   **** Insert Table 4 around here ****

339   The effect of the intervention period on resting IL-6, iHsp72 and eHsp72 levels is

340   presented in Fig. 4. Resting levels of IL-6 and iHsp72 in total monocytes were not altered

341   following the intervention period (T x G; IL-6 F: 0.1, $p = 0.87$, iHsp72 F: 0.2, $p = 0.59$). The

342   same was true for the expression of iHsp72 in the monocyte subsets (T x G; classical

343   monocytes F: 1.8, $p = 0.14$; intermediate monocytes F: 1.2, $p = 0.39$; non-classical monocytes

344   F: 0.3, $p = 0.78$). Extracellular Hsp72 was lowered in INT compared to CON (difference in

345   fold change between groups; F: 6.8; $p = 0.03$, ES: 1.00 (0.73 – 1.26))️. This resulted in a lower

346   *R* in INT as compared to CON (G; F: 6.0, $p = 0.04$, ES: 0.34 (0.21 – 0.51))️. The change in the

347   distribution of monocytes subsets in the circulation at rest was not different in INT compared

348   to CON (T x G; classical monocytes F: 0.8, $p = 0.52$, intermediate monocytes F: 1.1, $p = 0.23$,

349   non-classical monocytes F: 1.8, $p = 0.14$) (Fig. 4).

350   ***** Insert Figure 4 around here *****

351   Fasting blood glucose concentrations were lower in INT compared to CON following the

352   intervention period (T x G; F: 5.0, p = 0.04, ES: 0.68 (0.42 – 0.97); Fig. 5). Fasting insulin

353   concentrations did not change in INT compared to CON (T x G; F: 1.3, p = 0.30, ES: 0.50

354   (-0.46 – 1.42)). However, following inspection of the individual data an outlier was detected

355   (Fig. 5, grey line), which was confirmed using the methods for outlier detection postulated by

356   Leys et al. (46). After removing the insulin data of this participant, there was a larger decrease

357   in fasting insulin in INT compared to CON (T x G; F: 4.8, p = 0.04, ES: 1.06 (0.02 – 2.00)).

358   HOMA-IR was also reduced to a larger extend in INT compared to CON (T x G; F: 5.5, p =

359   0.03, ES: 1.07 (0.08 – 2.06)). Finally, there was no difference in the change of resting plasma

360   nitrite concentrations between INT and CON (INT 321±69 nM to 234±64 nM; CON 230±57

361   nM to 262±77 nM; T x G; F: 1.7, p = 0.17).

362   ***** Insert Figure 5 around here *****

363   One week following the post blood sample, resting iHsp72 (pre: 307±53 GMFI, post:

364   309±69, post+1week: 358±116; T; F: 1.8, p = 0.22), IL-6 (pre: 1.22±0.52 pg/ml, post:

365   1.31±0.53, post+1week: 1.12±0.65; T; F: 0.2, p = 0.67), the percentage of classical monocytes

366   (pre: 94.4±1.8%, post: 91.9±4.5%, post+1week: 94.1±1.3%; T; F: 1.7, p = 0.18), intermediate

367   monocytes (pre: 1.25±0.38%, post: 1.69±0.73%, post+1week: 1.47±0.51%; T; F: 1.0. p =

368   0.27) and non-classical monocytes (pre: 2.70±0.92%, post: 3.10±1.09%, post+1week:

369   3.39±1.35%; T; F: 1.0, p = 0.28) were not changed compared to either pre or post. Resting

370   concentrations of eHsp72 were elevated compared to post (fold change pre-post: 0.83±0.41,

371   fold change pre-post+1 week: 1.28±0.34, T; F: 5.8, p = 0.03, ES: 0.83 (0.20 – 1.84)). The

372   lowering of fasting blood glucose following the intervention period was still present at post+1

373   week (pre: 4.44±0.93 mmol/L, post: 3.98±0.98 mmol/L, post+1week: 3.89±0.77 mmol/L, T;

374 F: 25.1, $p = 0.001$, ES: 0.61 (0.08 – 1.32). However, fasting insulin was elevated at post+1

375 week compared to post (pre: 68.10±44.65 pmol/l, post: 51.7±27.3 pmol/l, post+1week:

376 72.6±56.3 pmol/l, T; F: 4.5, $p = 0.05$, ES: 0.53 (0.05 – 1.08), returning to the insulin

377 concentrations found prior to the intervention (pre- post+1 week, T; F: 1.1, $p = 0.21$). There

378 was no difference in HOMA-IR between post+1 week compared with post (pre: 13.91±11.09,

379 post: 8.99±7.89, post+1 week: 12.40±10.01, T; F: 4.1, $p = 0.06$) or pre (T; F: 0.8, $p = 0.47$).

380 Plasma nitrite concentrations were not changed at post+1 week compared to pre or post (pre:

381 314±61 nM, post: 247±66 nM, post+1week: 304±91 nM; T; F: 3.9, $p = 0.09$).

382 *Correlations*

383 During HWI$_{pre,}$ there was no correlation between the peak core temperature attained and

384 the acute change in iHsp72 expression ($r = -0.11$, $p = 0.77$), plasma IL-6 ($r = 0.23$, $p = 0.55$)

385 or nitrite concentrations ($r = 0.04$, $p = 0.91$). Following the chronic intervention, there was a

386 negative correlation between plasma insulin concentration at baseline and its change

387 following the intervention ($r = -0.45$, $p = 0.01$). There was no relationship with insulin at

388 baseline and the change in blood glucose concentrations ($r = 0.23$, $p = 0.33$). No correlation

389 was observed between baseline blood glucose concentration and the chronic change in insulin

390 ($r = -0.28$, $p = 0.27$) or glucose concentrations ($r = 0.29$, $p = 0.25$). In addition, there was no

391 correlation between the fold change in eHsp72 following the intervention and the change in

392 insulin ($r = 0.61$, $p = 0.06$) or glucose concentrations ($r = 0.03$, $p = 0.94$). Finally, there was

393 no correlation between the chronic change in iHsp72 expression and the chronic change in

394 insulin ($r = -0.16$, $p = 0.66$) or glucose concentrations ($r = 0.21$, $p = 0.56$).

395

Discussion

This study investigated the acute inflammatory response to HWI as well as the potential of chronic HWI to improve inflammatory and metabolic profiles at rest. Acute HWI evoked elevated plasma IL-6 and nitrite concentrations, and an increase in the percentage of intermediate and non-classical monocytes. This was however not accompanied by an increase in iHsp72 expression. Two weeks of chronic HWI reduced fasting glucose, insulin and eHsp72 concentrations. Together, this indicates that HWI may be a useful strategy to improve aspects of the inflammatory profile and glucose metabolism in individuals without the physical capacity to do so using exercise training.

Acute responses to hot water immersion

Our observation that one hour of HWI in water set at 39°C induced a significant increase in plasma IL-6 concentrations corroborates with the notion that increases in body temperature can serve as an independent stressor to induce an acute inflammatory response. Previous studies employing 1 h of HWI have shown comparable increases in plasma IL-6 concentrations to the current study (16, 45), while 2 h of HWI results in a more marked IL-6 response (43). Consistent with exercise studies (17), this suggests that the IL-6 response to HWI is dose dependent. In line with this, a more intense HWI protocol than used in the present study (i.e. longer duration or warmer water) may be required to induce changes in iHsp72 or eHsp72. Oehler et al. (54) reported an acute increase in iHsp72 following HWI of 2 h in water set at 39.5°C, while a session of 1 h did not result in elevated iHsp72 expression (50). On the other hand, Faulkner et al. (16) reported acute increases in eHsp72 following immersion up to the waistline for 1 h in water set at 40°C, resulting in a ~1°C increase in core temperature. As the acute inflammatory response to HWI seems dose dependent, it is conceivable that there may exist a threshold in core or muscle temperature or time accrued

420   above this threshold that needs to be reached in order to induce an iHsp72 response. Using

421   exercise as a stressor, Gibson et al. (24) have suggested that at least ~27 min above a core

422   temperature of 38.5°C is needed to induce the upregulation of Hsp72 mRNA. In the current

423   study, participants` rectal temperature exceeded 38.5°C for ~15 min only. This may also

424   explain why an acute increase in iHsp72 following passive heating is a consistent finding in

425   animal studies (28, 64), but not in human studies (50), as the endogenuous heat stress imposed

426   in the former is much higher compared with the present and other studies in humans. Of note,

427   the required heat stress might need to be even higher to induce acute increases in circulating

428   eHsp72 concentrations (23).

429   Although the HWI protocol used in this study did not elevate iHsp72 expression, the

430   acute increase in IL-6 concentrations indicates that in analogy to exercise, passive heating can

431   also induce an acute inflammatory response, possibly leading to the circulating anti-

432   inflammatory milleau postulated by Petersen and Pedersen as one of the benefits of exercise

433   (57). While it is now widely acknowledged that contracting skeletal muscle is the main source

434   of IL-6 during acute exercise (17), it is not clear whether this is also the case for HWI.

435   However, skeletal muscle is suggested to secrete IL-6 in response to increases in local

436   temperature (66). HWI for 1 h in water set at 40°C leads indeed to a muscle temperature

437   increase of ~2.5°C (16). Suggested mechanisms for the acute inflammatory response

438   following passive heating are the influx of calcium via the opening of the thermosensitive

439   transient receptor potential 1 (53) and the activation of heat shock factor 1, which can both

440   result in the production of IL-6 and Hsp72 (66). In addition, circulating monocytes are potent

441   producers of cytokines and might be a source of IL-6 found in the circulation following HWI

442   (1). The acute recruitment of intermediate and non-classical monocytes seen following HWI

443   in this study could indeed have led to increased IL-6 secretion into the circulation as these

444   subsets are known to release more IL-6 in response to an in-vitro stimulant such as

445 lipopolysaccharide (30). However, since monocytes only represent a small percentage of

446 leukocytes, it is not known what the impact of acute changes in circulating monocyte subsets

447 on circulating cytokines is (65). Nevertheless, since the proportion of relatively inflammatory

448 monocytes (i.e. intermediate and non-classical monocytes) at rest are positively associated

449 with the risk for a range of chronic diseases (69), the acute shift following HWI found in this

450 study provides rationale for further research in the potential of HWI interventions to

451 chronically alter the distribution of monocyte subsets in the circulation.

452　　　　While the interest in HWI to reduce chronic low-grade inflammation is a relatively

453 recent phenomenon, its potential to increase blood flow and enhance vascular function is

454 more established (13). Nevertheless, we show for the first time an acute increase in the

455 bioavailability of the vasodilator NO in response to HWI in humans, possibly mediated by the

456 enhanced activation of eNOS in response to the increase in shear stress and/or local

457 temperature (19). Additionally, as Hsp90 acts as an agonist for NO production by eNOS, the

458 acute increase in NO bioavailability may have been mediated by an increased expression of

459 Hsp90 (22). Future studies are therefore needed to identify the potential of HWI to increase

460 Hsp90 expression. Since the acute increase in NO following HWI has the potential to aid

461 tissue blood flow and is implicated in the translocation of GLUT4 to the plasma membrane of

462 skeletal muscle cells during exercise (59), HWI has the potential to facilitate glucose disposal

463 in skeletal muscle and other tissues (2, 20). In support, animal studies suggest GLUT4

464 translocation (25) and enhanced insulin sensitivity in skeletal muscle (27) following an acute

465 HWI session. Of note, in the current study the acute effects of HWI on glucose disposal were

466 not assessed and the implications of an acute increase in NO bioavailability on glucose

467 disposal are therefore only speculative. Indeed, the chronic reduction in fasting glucose and

468 insulin found in the current study occured independently of changes in resting plasma nitrite

469 concentrations.

470   If passive heating is to be successfully introduced as a health promoting intervention

471   in practice, it is important to assess perceptual responses to provide insight into its potential to

472   influence adherence rates to the intervention (68). In the current study, the perceptual

473   responses during 1 h of HWI of indicated profound feelings of discomfort similar to those

474   reported during high-intensity interval training (32, 38). This implies that further increases in

475   water temperature or session duration would result in an activity that is difficult to adhere to

476   (15). Therefore, although more intense HWI sessions than the one used in the current study

477   seems to be needed to induce an acute Hsp72 response, the practical application of HWI

478   sessions such as the one applied in the study of Oehler et al. (54; 2 h at 39.5°C) in the general

479   population is questionable. Moreover, the absence of more positive affective responses during

480   HWI_{post} as compared to HWI_{pre} suggests that no short-term improvements in the perceptual

481   responses can be expected as a result of regular engagement in HWI. Therefore, future studies

482   could test different HWI protocols in an attempt to optimise the balance between delivering a

483   HWI stimulus that evokes the neccessary inflammatory and metabolic benefits without

484   eliciting negative affective responses that have the potential to limit adherence to the

485   intervention. Finally, although HWI did not induce acute changes in Hsp72, we did observe

486   acute elevations of nitrite and IL-6 in addition to chronic improvements in fasting glucose,

487   insulin and eHsp72. This suggests that there may be no need to further increase the thermal

488   load of the HWI sessions to improve metabolic health and that the focus could be directed

489   towards the improvement of the perceptual responses during HWI. A titration study in which

490   the thermal load is gradually reduced may be useful to gain insight in the minimal passive

491   heat stress needed to induce acute changes in factors such as plasma IL-6 concentrations and

492   NO bioavailability and its impact on the perceptual responses during HWI.

493

494

496   As suggested by several authors (34, 42, 48), HWI interventions could serve as a

497   strategy to improve insulin sensitivity, possibly via the elevation of iHsp72 expression and/or

498   reduced chronic low-grade inflammation. In line with this suggestion that emanated from the

499   pilot study by Hooper et al. (33), fasting glucose and insulin concentrations were reduced

500   following the 2-week HWI intervention period applied in the current study. This was

501   accompanied by a reduction in eHsp72 concentrations. However, no changes in resting

502   iHsp72 expression, or plasma IL-6 and nitrite concentrations were found.

503   Animal studies suggest increased basal iHsp72 levels as a mechanism behind the

504   beneficial changes in insulin sensitivity reported following hot water immersion (6, 26).

505   Moreover, Hsp72 knock-out mice are highly insulin resistant and do not experience similar

506   benefits from passive heating strategies compared to mice expressing Hsp72 (12). However,

507   in the current study reductions in fasting glucose and insulin were found in the absence of

508   changes in iHsp72. The reason for this discrepancy might lie in the tissue in which iHsp72

509   was assessed. While most animal studies have investigated iHsp72 in skeletal muscle, in the

510   current study iHsp72 expression was assessed in monocytes. Although the acute iHsp72

511   responses in leukcoytes follow the same pattern as those found in skeletal muscle (64) and in-

512   vitro heat shock upregulates iHsp72 expression in monocytes (62), the chronic adaptations to

513   heat therapy and health interventions in monocytes are less clear. While heat acclimation

514   using exercise can induce increases in monocyte iHsp72 expression (44), trained runners

515   actually express lower levels of iHsp72 in leukocytes compared to their sedentary

516   counterparts (18). More studies that simulatiously measure iHsp72 expression in both tissues

517   following health interventions are therefore needed to resolve the mechanisms for enhanced

518   glucose metabolism after HWI. It should be acknowledged that the chronic intervention may

519   have impacted on other factors implicated in glucose metabolism, as for instance passive

520   lower-limb heating can chronically elevate peroxisome proliferator-activated receptor-gamma

521   coactivator 1-α (PGC-1α) expression (28).

522   Despite no changes in resting iHsp72, eHsp72 concentrations were significantly

523   lowered following the intervention period. When present in the circulation, eHsp72 can

524   activate monocytes via the Toll-like receptor 4/CD14 complex, resulting in the secretion of

525   pro-inflammatory cytokines such as IL-6, tumour necrosis factor-α (TNF-α) and IL-β (1). As

526   the latter cytokines can directly interfere with insulin sensitivity (35), it is suggested that the

527   deletirious effects of eHsp72 on health are exhibited via this mechanism (37). Additionally,

528   the positive change in R in INT might be indicative of an improved inflammatory profile

529   following the intervention period, as suggested by Krause et al. (41). However, the influence

530   of eHsp72 and changes in R on glucose metabolism needs to be studied in more detail.

531   While previous studies have found changes in the inflammatory profile following

532   short-term health interventions, the relatively short duration of the HWI intervention period

533   might have been the reason for the absence of changes in resting levels of iHsp72, IL-6,

534   monocyte subset distrubution and NO bioavailbility. On the other hand, it is striking that only

535   10 HWI sessions resulted in reductions in fasting glucose, insulin and blood pressure in males

536   that were sedentary and overweight, but did not show signs of pre-diabetes or strongly

537   elevated inflammatory markers at baseline. The positive correlation between baseline fasting

538   insulin concentrations and the reduction in fasting insulin following the intervention suggests

539   that those with more impaired metabolic health might benefit most from HWI. The lowered

540   blood pressure following the intervention period supports recent findings by Brunt et al. (4),

541   suggesting that HWI may also be a potent strategy to improve vascular health. While iHsp72-

542   and NO-mediated mechanisms are suggested to play a role in this effect (5), the

543   improvements in blood pressure in the present study were independent of changes in resting

544   levels of both measures.

545   Together, the current study provides a strong rationale to pursue further research on the

546   potential of passive heating strategies to enhance (cardio)metabolic health. For instance,

547   future studies should consider using more robust measures of insulin sensitivity (e.g. oral

548   glucose tolerance testing), implementing longer-term interventions and explore its

549   effectiveness and feasibility in populations that could benefit most from this alternative health

550   intervention (e.g. individuals with a spinal cord injury, frail elderly or those with other

551   conditions that interfere with exercise participation). Additionally, future studies in humans

552   are needed to clarify the role of inflammatory markers in glucose metabolism. In this regard,

553   the relatively modest heat stress imposed in the present study may be considered a limitation.

554   Although here an applicable model of passive heating is presented, future mechanistic studies

555   may consider increasing body temperature to a larger extent and for longer durations. For

556   instance, a passive heating model that is more likely to elevate iHsp72 expression may aid our

557   understanding on the importance of this marker for glucose metabolism in humans. Finally,

558   although there was no acute iHsp72 response following HWI and resting iHsp72 expression

559   in monocytes was not changed following the intervention, an elevated iHsp72 expression in

560   skeletal muscle for up to 7 days has been reported following exercise (51). Therefore, the

561   resting and post-immersion inflammatory and metabolic markers may have been influenced

562   by the potentially elevated iHsp72 expression in skeletal muscle.

563   In summary, a single HWI session induces an acute inflammatory response, indicated

564   by acute elevations in IL-6, changes in the monocyte subset distribution, and increase in NO

565   synthesis, indicated by increased plamsa nitrite concentrations. However, these responses

566   were not accompanied by acute increases in iHsp72 or eHsp72. The 2-week HWI intervention

567   period reduced fasting glucose and insulin, concomitant with lower resting eHsp72

568 concentrations, but independent of iHsp72 expression, plasma IL-6 and nitrite concentrations

569 at rest, as the latter markers did not change following the chronic intervention. Therefore, this

570 study provides support for the use of HWI to improve aspects of the inflammatory profile and

571 enhance glucose metabolism in sedentary, overweight males, and might have implications for

572 improving metabolic health in populations unable to meet the current physical activity

573 recommendations.

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789   Fig. 1 Outline of the study procedures for the intervention group (INT). An acute HWI (HWI_{pre}) and

790   control trial (AMB) were followed by ten HWI sessions within two weeks. A second acute HWI trial

791   (HWI_{post}) was conducted three days after completion of the intervention period and a resting blood

792   sample was taken seven days following HWI_{post} (Post). For the control group (CON), a resting blood

793   sample was taken at the time-points corresponding to visit 1 and 13 of the intervention group.

794

795   Fig. 2 Rectal temperature during and following AMB, HWI_{pre} and HWI_{post} (n = 10). * Significantly

796   different from AMB.

797

798   Fig. 3 The acute changes in plasma IL-6, eHsp72, iHsp72 and nitrite concentrations

799   following AMB, HWI_{pre} and HWI_{post}. Black lines represent individual data points, while the

800   bars represent the group mean (n = 10). *Significant time x trial interaction when compared

801   with AMB.

802

803   Fig. 4 Resting levels of the inflammatory outcome measures before and after the HWI intervention

804   period. INT: intervention group (n = 10), CON: control group (n = 8). The black lines represent

805   individual data points, while the bars represent the group mean. ^ Significant difference between

806   groups.

807

808   Fig. 5 Fasting blood glucose and plasma insulin concentrations for the intervention and control group.

809   INT: intervention group (n = 10), CON: control group (n = 8). The black lines represent individual

810   data points, while the bars represent the group mean. ^ Significant time x group interaction. Participant

811   with grey line does not contribute to the bar representing the group mean.

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813

814   Table 1. Participant characteristics at baseline. Data are presented as mean ± SD.

815

816   Table 2. Physiological responses to the hot water immersion and control trial. Data are presented as
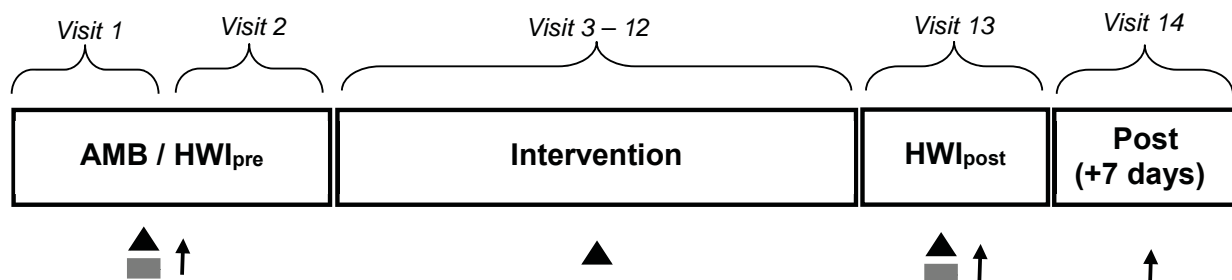
817   mean ± SD.

818

819   Table 3. The distribution of monocyte subsets in whole blood following hot water immersion and the

820   control trial. Data are presented as mean ± SD.

821

822   Table 4. Physiological responses during the sessions of the 2-week intervention period. Data are
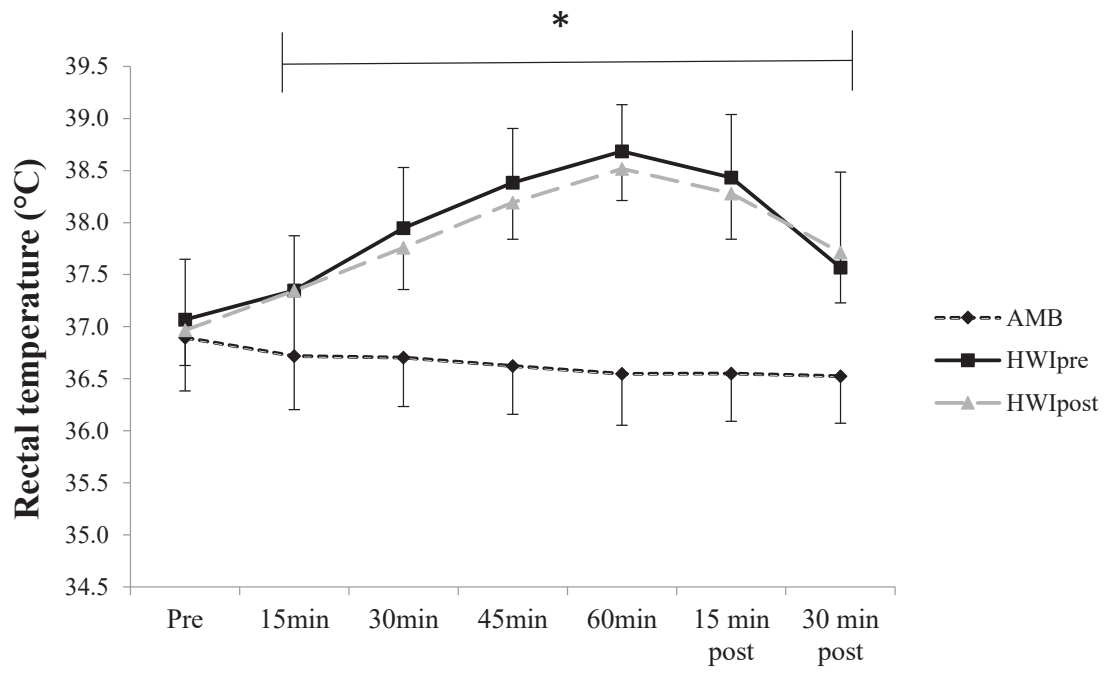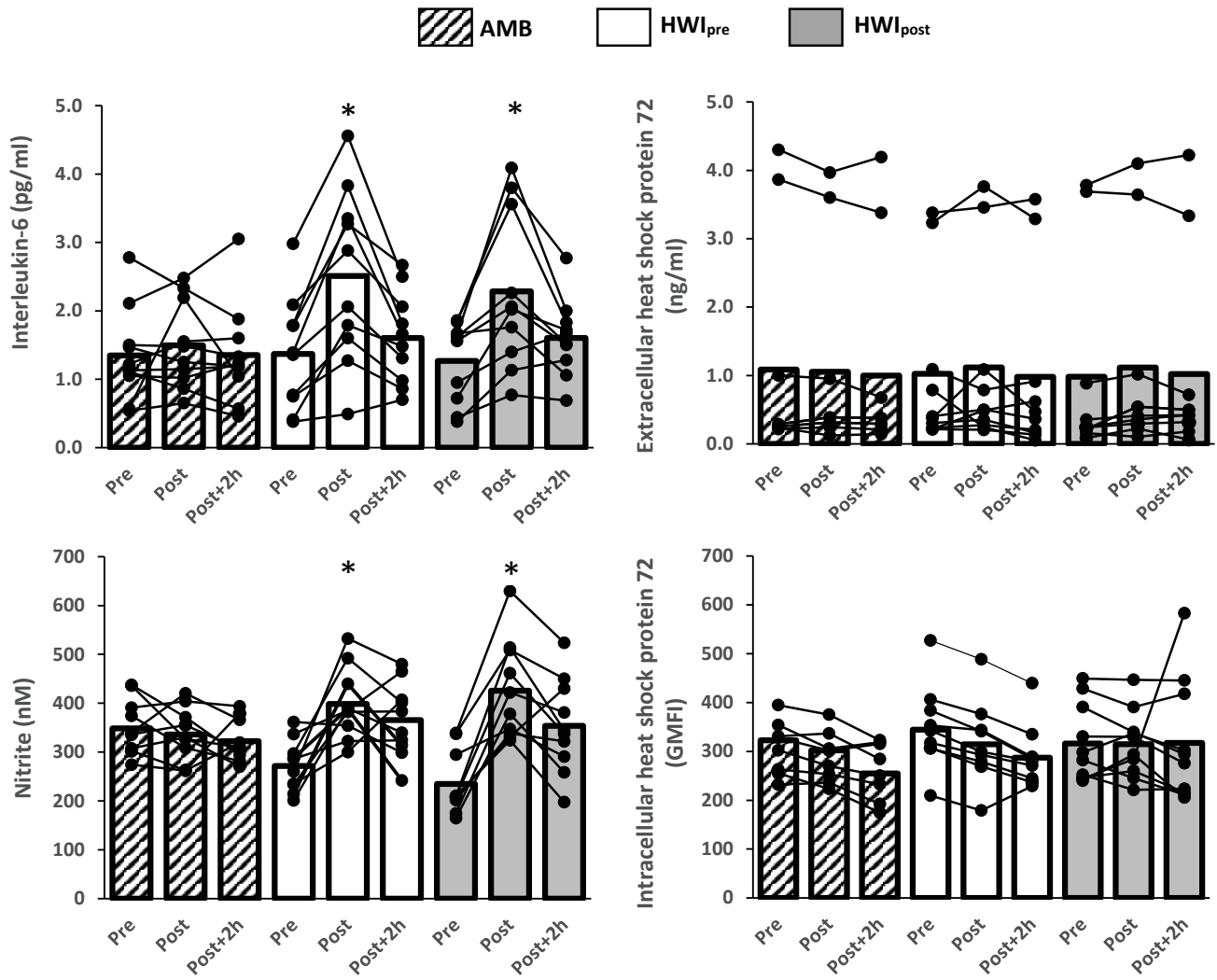
823   presented as mean ± SD.

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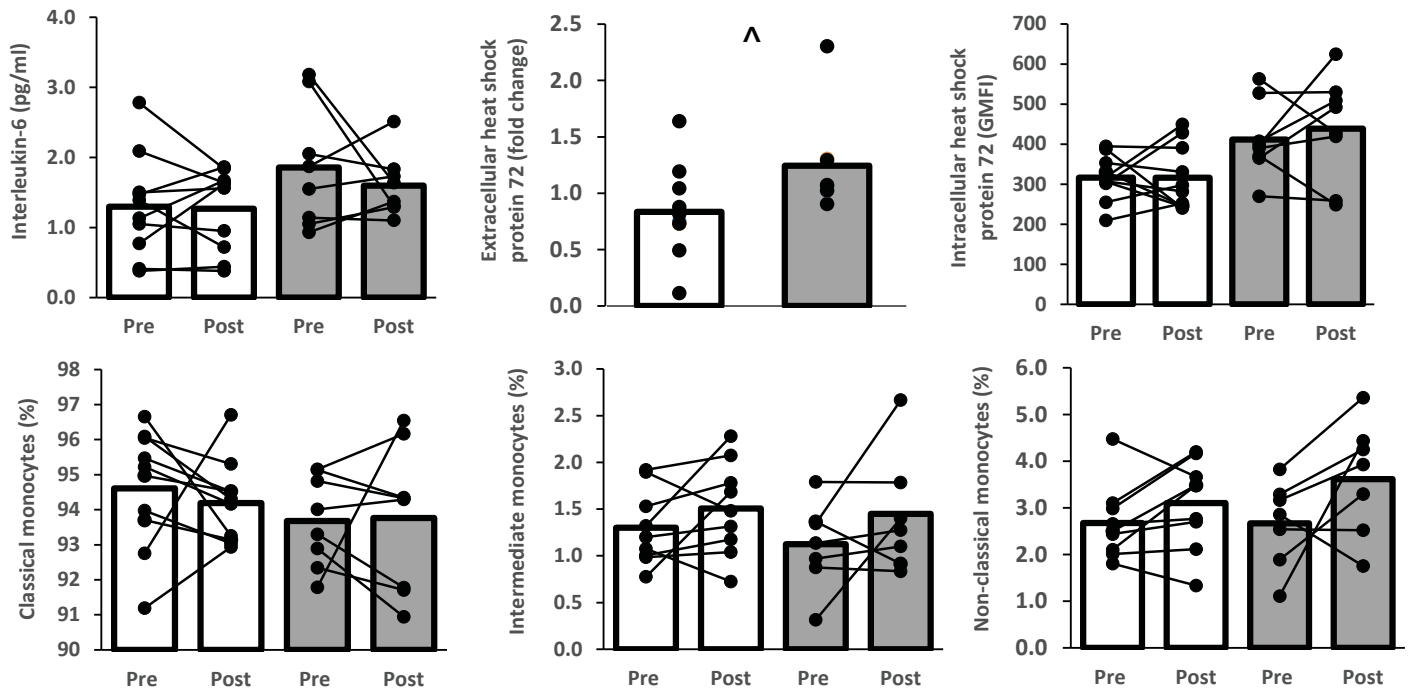| Visit 1	Visit 2	Visit 3 – 12	Visit 13	Visit 14
AMB / HWI$_{pre}$		Intervention	HWI$_{post}$	Post (+7 days)

↑ *Blood sample(s)*

▲ *Heart rate, perceptual responses, core temperature*

▮ *Oxygen uptake, blood pressure*
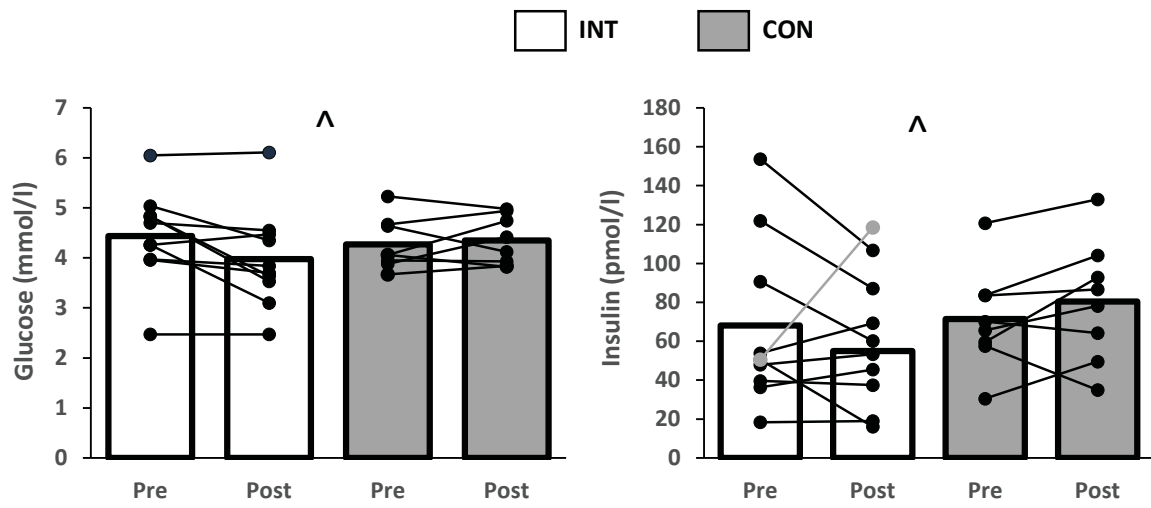
Table 1. Participant characteristics at baseline. Values are given in mean ± SD

Parameter	INT (N=10)	CON (N=8)	*p* (ES)
Age (yrs)	33.2±10.1	32.0±7.5	0.39(0.14)
Height (cm)	173.2±6.9	176.9±4.2	0.17(0.67)
Body mass (kg)	92.1±9.2	93.9±9.4	0.36(0.21)
BMI (kg/m^2)	31.0±4.2	30.0±2.5	0.37(0.30)
Fat percentage (%)	25.1±3.5	25.7±2.3	0.23(0.18)
Waist circumference (cm)	96.9±4.7	100.1±8.4	0.14(0.52)
Hip circumference (cm)	103.9±4.1	109.4±5.1	0.08(1.28)
Structured exercise (min/week)	38±54	29±44	0.49(0.19)

Abbreviations: BMI = body mass index; ES = Cohen`s d effect size, INT = intervention group, CON = control group

Table 2. Physiological responses to the hot water immersion and control trial. Data are presented as mean ± SD.

Parameter	AMB Pre	AMB 60 min	HWI$_{pre}$ Pre	HWI$_{pre}$ 60 min	HWI$_{post}$ Pre	HWI$_{post}$ 60 min
T$_{rec}$ (°C)	36.9±0.5	36.6±0.5	37.1±0.6	38.7±0.4*	37.0±0.3	38.5±0.3*
T$_{tymp}$ (°C)	35.5±0.4	35.2±0.4	35.4±0.7	37.5±0.7*	35.2±0.3	37.6±0.3*
HR (bpm)	69±17	64±15	67±14	105±13*	68±12	104±10*
VO$_2$ (L/min)	0.20±0.04	0.23±0.04	0.21±0.04	0.42±0.10*	0.19±0.05	0.37±0.03**
SBP (mmHg)	127±10	123±12	126±13	138±15*	118±15^	126±13^
DBP (mmHg)	86±9	85±10	83±9	78±9	79±11^	71±12*^
Basic affect (-5 to +5)	0.9±1.4	0.7±1.3	1.3±2.0	-1.1±2.2*	1.1±1.9	-1.2±1.9*
TS (1 to 9)	4.9±0.6	4.8±0.8	5.1±0.9	7.4±1.0*	4.7±0.5	6.7±0.8*^
TC (-5 to +5)	0.0±0.0	0.0±0.5	0.2±0.4	2.2±1.0*	-0.1±0.3	2.3±1.3*
Sweat loss (L)	N/A	0.17±0.19	N/A	1.12±0.56*	N/A	1.65±0.57*^
PV change (%)	N/A	98±3	N/A	95±8	N/A	95±4

Abbreviations: AMB = control trial; HWIpre = hot water immersion session prior to HWI intervention period; HWIpost = hot water immersion session following HWI intervention period; T$_{rec}$ = rectal temperature; T$_{tymp}$: tympanic temperature; HR = heart rate; VO$_2$ = oxygen uptake; SBP = systolic blood pressure; DBP = diastolic blood pressure; TS = thermal sensation; TC = thermal comfort (higher TC scores reflect reduced feelings of thermal comfort), PV = plasma volume
* Significantly different from AMB; ^ Significant difference between HWI$_{pre}$ and HWI$_{post}$

Table 3. The distribution of monocyte subsets in whole blood following hot water immersion and the control trial.

	Classical monocytes (%)	Intermediate monocytes (%)	Non-classical monocytes (%)
AMB pre	94.6±2.3	1.3±0.4	2.6±0.7
AMB post	94.0±4.8	1.3±0.3	2.4±0.5
AMB p2h	94.2±1.9	1.4±0.6	2.6±1.1
HWI$_{pre}$ pre	94.50±4.0	1.3±0.6	2.2±0.9
HWI$_{pre}$ post	91.2±5.8	1.7±0.7*	3.4±1.9*
HWI$_{pre}$ p2h	94.4±2.7	1.3±0.4	2.0±0.4
HWI$_{post}$ pre	94.2±1.2	1.5±0.6	3.1±1.0
HWI$_{post}$ post	93.9±1.7	1.4±0.4^	3.5±0.4*
HWI$_{post}$ p2h	94.7±1.5	1.2±0.5	2.7±1.3

Date are mean±SD. Abbreviations: AMB = control trial; HWIpre = hot water immersion session prior to HWI intervention period; HWIpost = hot water immersion session following HWI intervention period, p2h = 2 hours post hot water immersion

*Significantly different from AMB; ^ Significant difference between HWI$_{pre}$ and HWI$_{post}$

Table 4. Physiological responses during the sessions of the 2-week intervention period.

Parameter	Pre session 1-5	End session 1-5	Pre session 6-10	End session 6-10
Tympanic temperature (°C)	35.3±0.4	37.5±0.2*	35.1±0.3	37.5±0.3*
TS (1 to 9)	4.8±0.5	6.6±0.2*	4.9±0.4	6.7±0.2*
Basic affect (-5 to +5)	1.0±1.0	0.0±2.0	1.2±1.6	-0.7±1.8
HR (bpm)	67±13	105±2*	68±14	105±3*

Abbreviations: TS = thermal sensation; HR = heart rate. Data are means of five sessions during INT. End = measurement taken in the final 30 s of the session. Session 1-5 lasted 45 min, while session 6-10 lasted 60 min.
* Significantly different from Pre session.