

1     **The acute and chronic effects of hot water immersion on inflammation and**  
2                                   **metabolism in sedentary, overweight adults**

3     Hoekstra S.P.<sup>1,2</sup>, Bishop N.C.<sup>2</sup>, Faulkner S.H.<sup>3</sup>, Bailey S.J.<sup>2</sup>, Leicht C.A.<sup>1,2</sup>

4     1. The Peter Harrison Centre for Disability Sport, Loughborough University, Loughborough, United  
5     Kingdom

6     2. The School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough,  
7     United Kingdom

8     3. Department of Engineering, Nottingham Trent University, Nottingham, United Kingdom

9

10

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

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26    **Corresponding author:**

27    Christof A. Leicht

28    The Peter Harrison Centre for Disability Sport; School of Sport, Exercise and Health Sciences

29    Loughborough University

30    Loughborough LE11 3TU, UK

31    [c.a.leicht@lboro.ac.uk](mailto:c.a.leicht@lboro.ac.uk)

32

## 33 **Abstract**

34

35 Regular exercise-induced acute inflammatory responses are suggested to improve the  
36 inflammatory profile and insulin sensitivity. As body temperature elevations partly mediate  
37 this response, passive heating might be a viable tool to improve the inflammatory profile. This  
38 study investigated the acute, and chronic effects of hot water immersion on inflammatory and  
39 metabolic markers. Ten sedentary, overweight males (BMI:  $31.0 \pm 4.2$  kg/m<sup>2</sup>) were immersed  
40 in water set at 39°C for 1 h (HWI) or rested for 1 h at ambient temperature (AMB). Venous  
41 blood was obtained prior to, immediately post and 2 h post-session for assessment of  
42 monocyte intracellular heat shock protein 72 (iHsp72) and plasma concentrations of  
43 extracellular heat shock protein 72 (eHsp72), interleukin-6 (IL-6), fasting glucose, insulin and  
44 nitrite. Thereafter, participants underwent a 2-week intervention period, consisting of 10 hot  
45 water immersion sessions (INT). Eight BMI-matched participants (BMI:  $30.0 \pm 2.5$  kg/m<sup>2</sup>)  
46 were included as control (CON). Plasma IL-6 and nitrite concentrations were higher  
47 immediately following HWI compared to AMB (IL-6  $p < 0.001$ , HWI:  $1.37 \pm 0.94$  to  $2.51 \pm 1.49$   
48 pg/ml; nitrite  $p = 0.04$ , HWI:  $271 \pm 52$  to  $391 \pm 72$  nM), while iHsp72 expression was unchanged  
49 ( $p = 0.57$ ). In contrast to resting iHsp72 expression ( $p = 0.59$ ), fasting glucose ( $p = 0.04$ , INT:  
50  $4.44 \pm 0.93$  to  $3.98 \pm 0.98$  mmol/l), insulin ( $p = 0.04$ , INT:  $68.1 \pm 44.6$  to  $55.0 \pm 29.9$  pmol/l) and  
51 eHsp72 ( $p = 0.03$ , INT: 17±41% reduction) concentrations were lowered after INT compared  
52 to CON. HWI induced an acute inflammatory response and increased nitric oxide  
53 bioavailability. The reductions in fasting glucose and insulin concentrations following the  
54 chronic intervention suggest that hot water immersion may serve as a tool to improve glucose  
55 metabolism.

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

58

59 **New and noteworthy**

60

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

67

68

69

70

71

72

73

74

75

76

77

78

79

80

81

82

83

84

## 85 **Introduction**

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

102           The acute inflammatory response provoked by a physical stressor, such as exercise,  
103 can induce a subsequent protracted anti-inflammatory response. For instance, elevations in  
104 circulating interleukin (IL)-6 concentrations immediately following exercise activate the  
105 release of anti-inflammatory cytokines such as IL-1ra and IL-10, typically 1 to 4 h following  
106 the exercise bout (57). In addition, recent studies have identified an enhanced acute  
107 inflammatory response following exercise when body temperature is augmented (43).  
108 Increasing body temperature therefore likely serves as an independent stressor able to induce  
109 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  $\kappa$ B (NF- $\kappa$ B), 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<sup>2</sup>), 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 ( $\text{HWI}_{\text{pre}}$ ) 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  $\text{HWI}_{\text{pre}}$  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<sub>pre</sub>, 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<sub>pre</sub> 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<sub>post</sub>) 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<sub>pre</sub>.  
212 The “pre” blood sample of the first session (either HWI<sub>pre</sub> or AMB) and HWI<sub>post</sub> 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<sub>post</sub> 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<sub>3</sub>EDTA (plasma markers) and sodium heparin (flow cytometry)  
222 monovettes. The K<sub>3</sub>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  $\mu$ L; BD biosciences). Following permeabilisation (60  $\mu$ L; Leucoperm, BD  
231 biosciences) samples were incubated with 4  $\mu$ L 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<sup>++</sup>CD16<sup>-</sup> classical monocytes, CD14<sup>+</sup>CD16<sup>+</sup> intermediate monocytes and CD14<sup>-</sup>  
240 CD16<sup>++</sup> 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  $\mu$ L 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 (Merckodia 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  $\pm$  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<sub>pre</sub>, HWI<sub>pre</sub> and HWI<sub>post</sub> 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<sub>pre</sub> 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 23<sup>rd</sup>  
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<sub>pre</sub> and AMB are given in Table 2.  
294 During HWI<sub>pre</sub>, rectal temperature increased from  $37.1 \pm 0.6^\circ\text{C}$  to  $38.7 \pm 0.4^\circ\text{C}$  (Fig 2).  
295 Following the intervention period, diastolic blood pressure was lower at the end of HWI<sub>post</sub>  
296 when compared to HWI<sub>pre</sub> (F: 25.4,  $p = 0.001$ ). Thermal sensation at the end of HWI<sub>post</sub> was  
297 lower than at the end of HWI<sub>pre</sub> (F: 14.3,  $p = 0.01$ ) and sweat loss during HWI was increased  
298 from  $1.1 \pm 0.6$  (HWI<sub>pre</sub>) to  $1.7 \pm 0.6$  L (HWI<sub>post</sub>) (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<sub>pre</sub>, (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<sub>pre</sub> (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<sub>pre</sub> 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<sub>pre</sub>, 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<sub>pre</sub> 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<sub>pre</sub> 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<sub>pre</sub> 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<sub>post</sub> when compared  
320 with HWI<sub>pre</sub> (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<sub>pre</sub>, the percentage of intermediate monocytes was not elevated following HWI<sub>post</sub> (Time;  
324 F: 3.4,  $p = 0.06$ ; Table 3). There were no differences in the acute change between HWI<sub>pre</sub> and  
325 HWI<sub>post</sub> 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<sub>pre</sub> and  
328 HWI<sub>post</sub> (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 ( $92.1 \pm 9.2$  kg to  
332  $92.3 \pm 9.5$  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 extent 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+1 week: 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+1 week: 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+1 week: 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+1 week: 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+1 week:  
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+1 week: 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+1 week:  
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+1 week: 304±91 nM; T; F: 3.9,  $p = 0.09$ ).

### 382 *Correlations*

383 During HWI<sub>pre</sub>, 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



## 396 **Discussion**

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

### 405 *Acute responses to hot water immersion*

406 Our observation that one hour of HWI in water set at 39°C induced a significant increase  
407 in plasma IL-6 concentrations corroborates with the notion that increases in body temperature  
408 can serve as an independent stressor to induce an acute inflammatory response. Previous  
409 studies employing 1 h of HWI have shown comparable increases in plasma IL-6  
410 concentrations to the current study (16, 45), while 2 h of HWI results in a more marked IL-6  
411 response (43). Consistent with exercise studies (17), this suggests that the IL-6 response to  
412 HWI is dose dependent. In line with this, a more intense HWI protocol than used in the  
413 present study (i.e. longer duration or warmer water) may be required to induce changes in  
414 iHsp72 or eHsp72. Oehler et al. (54) reported an acute increase in iHsp72 following HWI of 2  
415 h in water set at 39.5°C, while a session of 1 h did not result in elevated iHsp72 expression  
416 (50). On the other hand, Faulkner et al. (16) reported acute increases in eHsp72 following  
417 immersion up to the waistline for 1 h in water set at 40°C, resulting in a ~1°C increase in core  
418 temperature. As the acute inflammatory response to HWI seems dose dependent, it is  
419 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 endogenous 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 milieu 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 occurred 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 necessary 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 leukocytes 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 simultaneously 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- $\alpha$  (PGC-1 $\alpha$ ) 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- $\alpha$  (TNF- $\alpha$ ) and IL- $\beta$  (1). As  
526 the latter cytokines can directly interfere with insulin sensitivity (35), it is suggested that the  
527 deleterious 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 distribution and NO bioavailability. 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 plasma 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.

#### 574 *Acknowledgements*

575 The authors thank Bryany Cornish, Ben Lowry and Tim Lam for their excellent assistance in  
576 the process of data collection. The authors acknowledge the support of the National Institute  
577 for Health Research (NIHR) Leicester Biomedical Research Centre. The views expressed are  
578 those of the authors and not necessarily those of the NHS, the NIHR or the Department of  
579 Health. Most of all, the authors are grateful for the effort and time the participants have  
580 dedicated to this research project.

581

582

583

584

585

586

587

588

589



- 591 1. **Asea A, Rehli M, Kabingu E, Boch JA, Baré O, Auron PE, Stevenson MA, Calderwood**  
592 **SK.** Novel signal transduction pathway utilized by extracellular HSP70. Role of toll-like  
593 receptor (TLR) 2 and TLR4. *J Biol Chem* 277: 15028–15034, 2002.
- 594 2. **Baron AD, Alain D, Johnson A.** Skeletal muscle blood flow independently modulates insulin-  
595 mediated glucose uptake *Am J Physiol Endocrinol Metab* 266:248-253, 1994.
- 596 3. **Bruning RS, Santhanam L, Stanhewicz AE, Smith CJ, Berkowitz DE, Kenney WL,**  
597 **Holowatz LA.** Endothelial nitric oxide synthase mediates cutaneous vasodilation during local  
598 heating and is attenuated in middle-aged human skin. *J Appl Physiol* 112: 2019–2026, 2012.
- 599 4. **Brunt VE, Howard MJ, Francisco MA, Ely BR, Minson CT.** Passive heat therapy improves  
600 endothelial function, arterial stiffness and blood pressure in sedentary humans. *J Physiol* 594:  
601 5329–5342, 2016.
- 602 5. **Brunt VE, Howard MJ, Francisco MA, Ely BR, Minson CT.** Reply from Vienna E. Brunt,  
603 Matthew J. Howard, Michael A. Francisco, Brett R. Ely and Christopher T. Minson. *J Physiol*  
604 595: 3669–3670, 2017.
- 605 6. **Chung J, Nguyen A-K, Henstridge DC, Holmes AG, Chan MHS, Mesa JL, Lancaster GI,**  
606 **Southgate RJ, Bruce CR, Duffy SJ, Horvath I, Mestril R, Watt MJ, Hooper PL, Kingwell**  
607 **BA, Vigh L, Hevener A, Febbraio MA.** HSP72 protects against obesity-induced insulin  
608 resistance. *Proc Natl Acad Sci* 105: 1739–1744, 2008.
- 609 7. **Cohen J.** A Power Primer. *Psychol Bull* 112, 1992.
- 610 8. **Craig CL, Marshall AL, Sjöström M, Bauman AE, Booth ML, Ainsworth BE, Pratt M,**  
611 **Ekelund U, Yngve A, Sallis JF, Oja P.** International physical activity questionnaire: 12-  
612 Country reliability and validity. *Med Sci Sports Exerc* 35: 1381–1395, 2003.
- 613 9. **Dandona P, Aljada A, Bandyopadhyay A.** Inflammation : the link between insulin resistance

- 614 , obesity and diabetes. *Trends Immunol* 25: 4–7, 2004.
- 615 10. **Dill, DB; Costill D.** Calculation of percentage changes in volumes of blood, plasma, and red  
616 cells in dehydration. *J Appl Physiol* 37: 247–248, 1974.
- 617 11. **Dorsey CM, Lukas SE, Teicher MH, Harper D, Winkelman JW, Cunningham SL, Satlin**  
618 **A.** Effects of passive body heating on the sleep of older female insomniacs. *J Geriatr*  
619 *Psychiatry Neurol* 9: 83–90, 1996.
- 620 12. **Drew BG, Ribas V, Le JA, Henstridge DC, Phun J, Zhou Z, Soleymani T, Daraei P, Sitz**  
621 **D, Vergnes L, Wanagat J, Reue K, Febbraio MA, Hevener AL.** HSP72 is a mitochondrial  
622 stress sensor critical for Parkin action, oxidative metabolism, and insulin sensitivity in skeletal  
623 muscle. *Diabetes* 63: 1488–1505, 2014.
- 624 13. **Drummond PD.** Thermoregulatory response to passive body heating in borderline  
625 hypertension. *Clin Auton Res* 3: 233–238, 1993.
- 626 14. **Durnin JVGA, Womersley J.** Body fat assessed from total body density and its estimation  
627 from skinfold thickness : measurements on 481 men and women aged from 16 to 72 years. *Br J*  
628 *Nutr* 32: 77–97, 1973.
- 629 15. **Ekkekakis P, Parfitt G, Petruzzello SJ.** The Pleasure and Displeasure People Feel When they  
630 Exercise at Different Intensities. *Sport Med* 41: 641–671, 2011.
- 631 16. **Faulkner SH, Jackson S, Fatania G, Leicht CA.** The effect of passive heating on heat shock  
632 protein 70 and interleukin-6: A possible treatment tool for metabolic diseases? *Temperature* 4:  
633 1–13, 2017.
- 634 17. **Febbraio MA, Pedersen BK.** Contraction-induced myokine production and release: Is skeletal  
635 muscle an endocrine organ? *Exerc Sport Sci Rev* 33: 114–119, 2005.
- 636 18. **Fehrenbach E, Passek F, Niess AM, Pohla H, Weinstock C, Dickhuth HH, Northoff H.**  
637 HSP expression in human leukocytes is modulated by endurance exercise. *Med Sci Sports*  
638 *Exerc* 32: 592–600, 2000.

- 639 19. **Förstermann U, Sessa WC.** Nitric oxide synthases: Regulation and function. *Eur Heart J* 33:  
640 829–837, 2012.
- 641 20. **Franks PW, Luan J, Barroso I, Brage S, Sanchez JLG, Ekelund U, Ríos MS, Schafer AJ,**  
642 **O’Rahilly S, Wareham NJ.** Variation in the eNOS gene modifies the association between  
643 total energy expenditure and glucose intolerance. *Diabetes* 54: 2795–2801, 2005.
- 644 21. **Gagge AP, Stolwijk JAJ, Hardy JD.** Comfort and thermal sensations and associated  
645 physiological responses at various ambient temperatures. *Environ Res* 1: 1–20, 1967.
- 646 22. **García-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, Sessa**  
647 **WC.** Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* 392: 821–4,  
648 1998.
- 649 23. **Gibson OR, Dennis A, Parfitt T, Taylor L, Watt PW, Maxwell NS.** Extracellular Hsp72  
650 concentration relates to a minimum endogenous criteria during acute exercise-heat exposure.  
651 *Cell Stress Chaperones* 19: 389–400, 2014.
- 652 24. **Gibson OR, Tuttle JA, Watt PW, Maxwell NS, Taylor L.** Hsp72 and Hsp90 $\alpha$  mRNA  
653 transcription is characterised by large, sustained changes in core temperature during heat  
654 acclimation. *Cell Stress Chaperones* 21: 1021–1035, 2016.
- 655 25. **Goto A, Egawa T, Sakon I, Oshima R, Ito K, Serizawa Y, Sekine K, Tsuda S, Goto K,**  
656 **Hayashi T.** Heat stress acutely activates insulin-independent glucose transport and 5'-AMP-  
657 activated protein kinase prior to an increase in HSP72 protein in rat skeletal muscle. *Physiol*  
658 *Rep* 3: 1–10, 2015.
- 659 26. **Gupte AA, Bomhoff GL, Swerdlow RH, Geiger PC.** Heat Treatment Improves Glucose  
660 Tolerance and a High-Fat Diet. *Diabetes* 58: 567–578, 2009.
- 661 27. **Gupte AA, Bomhoff GL, Touchberry CD, Geiger PC.** Acute heat treatment improves  
662 insulin-stimulated glucose uptake in aged skeletal muscle. *J Appl Physiol* 110: 451–457, 2011.
- 663 28. **Hafen PS, Preece CN, Sorensen JR, Hancock CR, Hyldahl RD.** Repeated exposure to heat

- 664 stress induces mitochondrial adaptation in human skeletal muscle. *J Appl Physiol* [In press]
- 665 29. **Hawley JA, Lessard SJ.** Exercise training-induced improvements in insulin action. *Acta*  
666 *Physiol* 192: 127–135, 2008.
- 667 30. **Heine GH, Ortiz A, Massy ZA, Lindholm B, Wiecek A, Martínez-Castelao A, Covic A,**  
668 **Goldsmith D, Süleymanlar G, London GM, Parati G, Sicari R, Zoccali C, Fliser D.**  
669 Monocyte subpopulations and cardiovascular risk in chronic kidney disease. *Nat Rev Nephrol*  
670 8: 362–369, 2012.
- 671 31. **Henstridge DC, Whitham M, Febbraio MA.** Chaperoning to the metabolic party: The  
672 emerging therapeutic role of heat-shock proteins in obesity and type 2 diabetes. *Mol Metab* 3:  
673 781–793, 2014.
- 674 32. **Hoekstra SP, Bishop NC, Leicht CA.** Can intervals enhance the inflammatory response and  
675 enjoyment in upper-body exercise? *Eur J Appl Physiol* 117: 1155–1163, 2017.
- 676 33. **Hooper PL.** Hot-tub therapy for type 2 diabetes mellitus. *N. Engl. J. Med.* 341: 924–925, 1999
- 677 34. **Hooper PL, Balogh G, Rivas E, Kavanagh K, Vigh L.** The importance of the cellular stress  
678 response in the pathogenesis and treatment of type 2 diabetes. *Cell Stress Chaperones* 19: 447–  
679 464, 2014.
- 680 35. **Hotamisligil GS.** Inflammatory pathways and insulin action. *Int J Obes* 27: S53–S55, 2003.
- 681 36. **Ives SJ, Andtbacka RHI, Kwon SH, Shiu Y-T, Ruan T, Noyes RD, Zhang Q-J, Symons**  
682 **JD, Richardson RS.** Heat and  $\beta$ -adrenergic responsiveness in human skeletal muscle feed  
683 arteries: the role of nitric oxide. *J Appl Physiol* 113: 1690–1698, 2012.
- 684 37. **Johnson JD, Fleshner M.** Releasing signals, secretory pathways, and immune function of  
685 endogenous extracellular heat shock protein 72. *J Leukoc Biol* 79: 425–434, 2006.
- 686 38. **Jung ME, Bourne JE, Little JP.** Where does HIT fit? an examination of the affective  
687 response to high-intensity intervals in comparison to continuous moderate- And continuous  
688 vigorous-intensity exercise in the exercise intensity-affect continuum. *PLoS One* 9: 1–18, 2014.

- 689 39. **Karakas M, Koenig W.** CRP in cardiovascular disease. *Herz* 34: 607–613, 2009.
- 690 40. **Kolb H, Mandrup-Poulsen T.** The global diabetes epidemic as a consequence of lifestyle-  
691 induced low-grade inflammation. *Diabetologia* 53: 10–20, 2010.
- 692 41. **Krause M, Heck TG, Bittencourt A, Scmazzon SP, Newsholme P, Curi R, Homem De  
693 Bittencourt PI.** The chaperone balance hypothesis: The importance of the extracellular to  
694 intracellular HSP70 ratio to inflammation-driven type 2 diabetes, the effect of exercise, and the  
695 implications for clinical management. *Mediators Inflamm* 2015, 2015.
- 696 42. **Krause M, Ludwig MS, Heck TG, Takahashi HK.** Heat shock proteins and heat therapy for  
697 type 2 diabetes: Pros and cons. *Curr Opin Clin Nutr Metab Care* 18: 374–380, 2015.
- 698 43. **Laing SJ, Jackson AR, Walters R, Lloyd-Jones E, Whitham M, Maassen N, Walsh NP.**  
699 Human blood neutrophil responses to prolonged exercise with and without a thermal clamp. *J  
700 Appl Physiol* 104: 20–26, 2007.
- 701 44. **Lee BJ, Miller A, James RS, Thake CD.** Cross acclimation between heat and hypoxia: Heat  
702 acclimation improves cellular tolerance and exercise performance in acute normobaric hypoxia.  
703 *Front Physiol* 7: 1–15, 2016.
- 704 45. **Leicht CA, Kouda K, Umemoto Y, Banno M, Kinoshita T, Moriki T, Nakamura T,  
705 Bishop NC, Goosey-Tolfrey VL, Tajima F.** Hot water immersion induces an acute cytokine  
706 response in cervical spinal cord injury. *Eur J Appl Physiol* 115: 2243–2252, 2015.
- 707 46. **Leys C, Ley C, Klein O, Bernard P, Licata L.** Detecting outliers: Do not use standard  
708 deviation around the mean, use absolute deviation around the median. *J Exp Soc Psychol* 49:  
709 764–766, 2013.
- 710 47. **Matthews DR, Hosker JP, Rudenski a S, Naylor B a, Treacher DF, Turner RC.**  
711 Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma  
712 glucose and insulin concentrations in man. *Diabetologia* 28: 412–419, 1985.
- 713 48. **McCarty MF, Barroso-Aranda J, Contreras F.** Regular thermal therapy may promote

- 714 insulin sensitivity while boosting expression of endothelial nitric oxide synthase - Effects  
715 comparable to those of exercise training. *Med Hypotheses* 73: 103–105, 2009.
- 716 49. **Minciullo PL, Catalano A, Mandraffino G, Casciaro M, Crucitti A, Maltese G, Morabito**  
717 **N, Lasco A, Gangemi S, Basile G.** Inflammaging and Anti-Inflammaging: The Role of  
718 Cytokines in Extreme Longevity. *Arch Immunol Ther Exp (Warsz)* 64: 111–126, 2016.
- 719 50. **Morton JP, MacLaren DPM, Cable NT, Campbell IT, Evans L, Bongers T, Griffiths RD,**  
720 **Kayani AC, McArdle A, Drust B.** Elevated core and muscle temperature to levels comparable  
721 to exercise do not increase heat shock protein content of skeletal muscle of physically active  
722 men. *Acta Physiol* 190: 319–327, 2007.
- 723 51. **Morton JP, MacLaren DP, Cable NT, Bongers T, Griffiths RD, Campbell IT, Evans L,**  
724 **Kayani A, McArdle A, Drust B.** Time course and differential responses of the major heat  
725 shock protein families in human skeletal muscle following acute nondamaging treadmill  
726 exercise. *J Appl Physiol*, 101: 176-182, 2006.
- 727 52. **Nowotny B, Nowotny PJ, Strassburger K, Roden M.** Precision and accuracy of blood  
728 glucose measurements using three different instruments. *Diabet Med* 29: 260–265, 2012.
- 729 53. **Obi S, Nakajima T, Hasegawa T, Kikuchi H, Oguri G, Takahashi M, Nakamura F,**  
730 **Yamasoba T, Sakuma M, Toyoda S, Tei C, Inoue T.** Heat induces interleukin-6 in skeletal  
731 muscle cells via TRPV1/PKC/CREB pathways. *J Appl Physiol* : jap.00139.2016, 2016.
- 732 54. **Oehler R, Pusch E, Zellner M, Dungal P, Hergovics N, Homoncik M, Eliassen MM, Brabec**  
733 **M, Roth E.** Cell type-specific variations in the induction of Hsp70 in human leukocytes by  
734 feverlike whole body hyperthermia. *Cell Stress Chaperones* 6: 306–315, 2001.
- 735 55. **Oyama JI, Kudo Y, Maeda T, Node K, Makino N.** Hyperthermia by bathing in a hot spring  
736 improves cardiovascular functions and reduces the production of inflammatory cytokines in  
737 patients with chronic heart failure. *Heart Vessels* 28: 173–178, 2013.
- 738 56. **Perneger TV.** What's wrong with Bonferroni adjustments. *Bmj*, 316: 1236-1238, 1998.

- 739 57. **Petersen AMW, Pedersen BK.** The anti-inflammatory effect of exercise. *J Appl Physiol* 98:  
740 1154–1162, 2005.
- 741 58. **Raubenheimer K, Hickey D, Leveritt M, Fassett R, Munoz JODZ, Allen JD, Briskey D,**  
742 **Parker TJ, Kerr G, Peake JM, Pecheniuk NM, Neubauer O.** Acute effects of nitrate-rich  
743 beetroot juice on blood pressure, hemostasis and vascular inflammation markers in healthy  
744 older adults: A randomized, placebo-controlled crossover study. *Nutrients* 9:1270-1289, 2017.
- 745 59. **Roberts CK, Barnard RJ, Scheck SH, Balon TW.** Exercise-stimulated glucose transport in  
746 skeletal muscle is nitric oxide dependent. [Online]. *Am J Physiol* 273: 220-225, 1997.
- 747 60. **Sansbury BE, Hill BG.** Regulation of obesity and Insulin resistance by Nitric Oxide. *Free*  
748 *Radic Biol Med* : 383–399, 2014.
- 749 61. **Silverstein MG, Ordanes D, Wylie AT, Files DC, Milligan C, Presley TD, Kavanagh K.**  
750 Inducing muscle heat shock protein 70 improves insulin sensitivity and muscular performance  
751 in aged mice. *Journals Gerontol - Ser A Biol Sci Med Sci* 70: 800–808, 2014.
- 752 62. **Simar D, Jacques A, Caillaud C.** Heat shock proteins induction reduces stress kinases  
753 activation, potentially improving insulin signalling in monocytes from obese subjects. *Cell*  
754 *Stress Chaperones* 17: 615–621, 2012.
- 755 63. **Strokov IA, Manukhina EB, Bakhtina LY, Malyshev IY, Zoloev GK, Kazikhanova SI,**  
756 **Ametov AS.** The function of endogenous protective systems in patients with insulin-dependent  
757 diabetes mellitus and polyneuropathy: effect of antioxidant therapy. *Bull Exp Biol Med* 130:  
758 986–990, 2000.
- 759 64. **Tuttle JA, Christmas BCR, Gibson OR, Barrington JH, Hughes DC, Castle PC, Metcalfe**  
760 **AJ, Midgley AW, Pearce O, Kabir C, Rayanmarakar F, Al-Ali S, Lewis MP, Taylor L.**  
761 The Hsp72 and Hsp90a mRNA responses to hot downhill running are reduced following a  
762 prior bout of hot downhill running, and occur concurrently within leukocytes and the vastus  
763 lateralis. *Front Physiol* 8: 1–15, 2017.

- 764 65. **Walsh NP, Gleeson MM, Shephard RJ, Gleeson MM, Woods JA, Bishop NC, Fleshner M,**  
765 **Green C, Pedersen BK, Hoffman-Goetz L, Rogers CJ, Northoff H, Abbasi A, Simon P,**  
766 **Jeffrey MG, Woods A, Bishop NC, Fleshner M, Green C, Pedersen K, Hoffman-Goetz L,**  
767 **Rogers CJ.** Part one : Immune function and exercise. *Exerc Immunol Rev* 17: 6–63, 2011.
- 768 66. **Welc SS, Phillips NA, Oca-Cossio J, Wallet SM, Chen DL, Clanton TL.** Hyperthermia  
769 increases interleukin-6 in mouse skeletal muscle. *AJP Cell Physiol* 303: 455–466, 2012.
- 770 67. **Whitham M, Parker BL, Friedrichsen M, Hingst JR, Hjorth M, Hughes WE, Egan CL,**  
771 **Cron L, Watt KI, Kuchel RP, Jayasooriah N, Estevez E, Petzold T, Suter CM, Gregorevic**  
772 **P, Kiens B, Richter EA, James DE, Wojtaszewski JFP, Febbraio MA.** Extracellular  
773 Vesicles Provide a Means for Tissue Crosstalk during Exercise. *Cell Metab* 27: 237–251.e4,  
774 2018.
- 775 68. **Williams DM, Dunsiger S, Ciccolo JT, Lewis BA, Albrecht AE, Marcus BH.** Acute  
776 affective response to a moderate-intensity exercise stimulus predicts physical activity  
777 participation 6 and 12 months later. *Psychol Sport Exerc* 9: 231–245, 2008.
- 778 69. **Wong KL, Yeap WH, Tai JJY, Ong SM, Dang TM, Wong SC.** The three human monocyte  
779 subsets: Implications for health and disease. *Immunol Res* 53: 41–57, 2012.
- 780 70. **Yoshida M, Xia Y.** Heat shock protein 90 as an endogenous protein enhancer of inducible  
781 nitric-oxide synthase. *J Biol Chem* 278: 36953–36958, 2003.
- 782 71. **Zawada AM, Fell LH, Untersteller K, Seiler S, Rogacev KS, Fliser D, Ziegler-Heitbrock**  
783 **L, Heine GH.** Comparison of two different strategies for human monocyte subsets gating  
784 within the large-scale prospective CARE FOR HOME Study. *Cytom Part A* 87: 750–758, 2015.
- 785  
786  
787  
788



789 Fig. 1 Outline of the study procedures for the intervention group (INT). An acute HWI ( $\text{HWI}_{\text{pre}}$ ) and  
790 control trial (AMB) were followed by ten HWI sessions within two weeks. A second acute HWI trial  
791 ( $\text{HWI}_{\text{post}}$ ) was conducted three days after completion of the intervention period and a resting blood  
792 sample was taken seven days following  $\text{HWI}_{\text{post}}$  (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,  $\text{HWI}_{\text{pre}}$  and  $\text{HWI}_{\text{post}}$  ( $n = 10$ ). \* Significantly  
796 different from AMB.

797

798 Fig. 3 The acute changes in plasma IL-6, eHsp72, iHsp72 and nitrite concentrations  
799 following AMB,  $\text{HWI}_{\text{pre}}$  and  $\text{HWI}_{\text{post}}$ . 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.

812

813

814 Table 1. Participant characteristics at baseline. Data are presented as mean  $\pm$  SD.

815

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

818

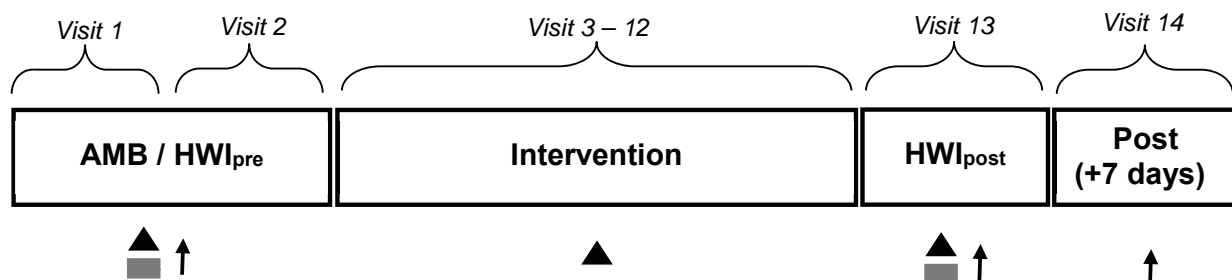
819 Table 3. The distribution of monocyte subsets in whole blood following hot water immersion and the  
820 control trial. Data are presented as mean  $\pm$  SD.

821

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

824

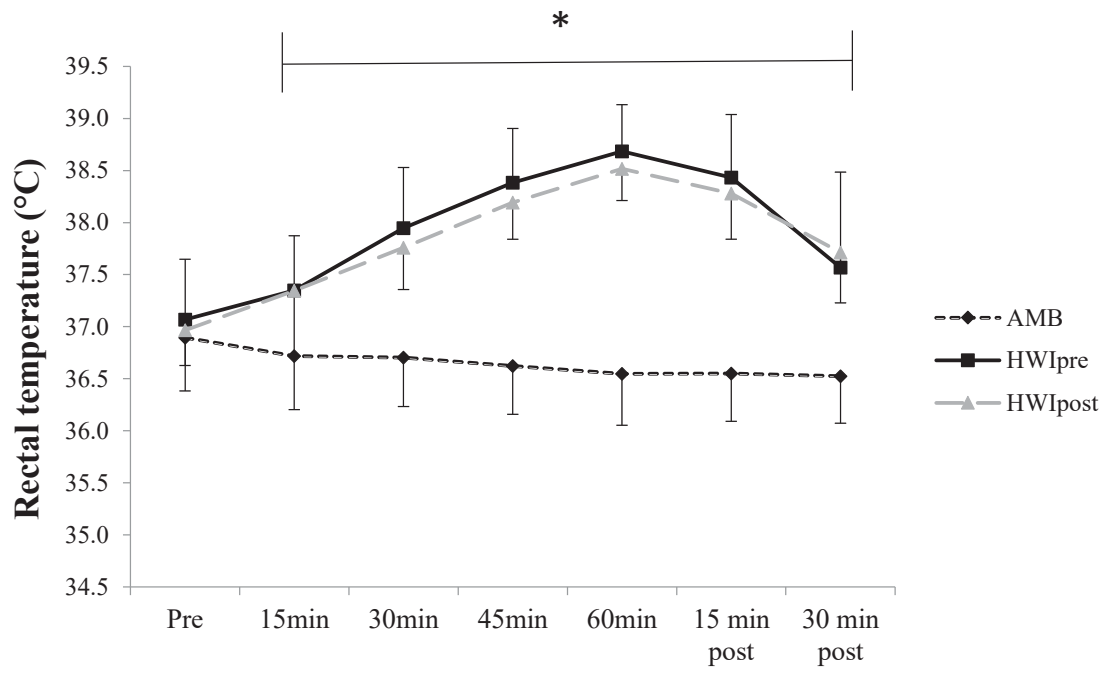
825

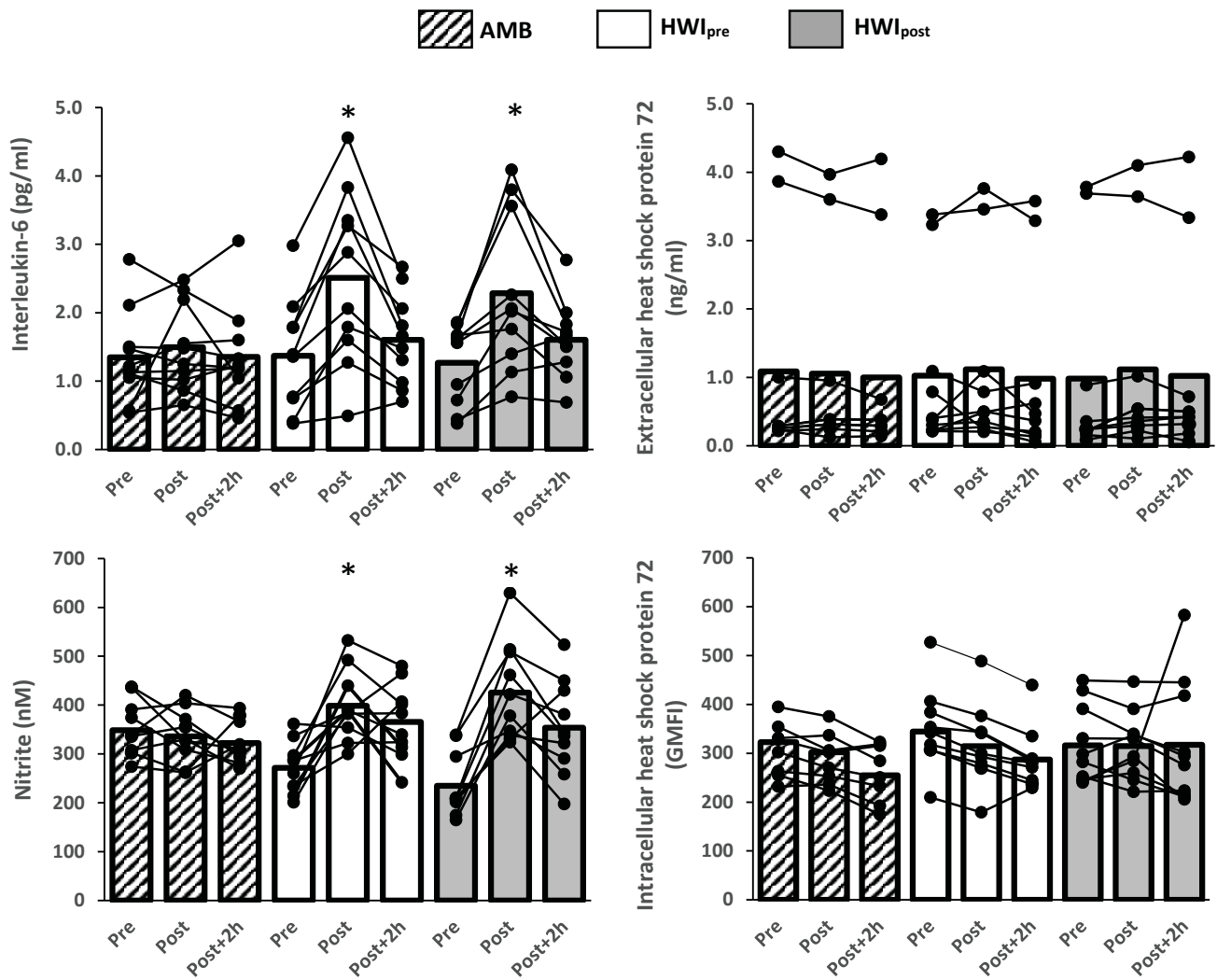


↑ Blood sample(s)

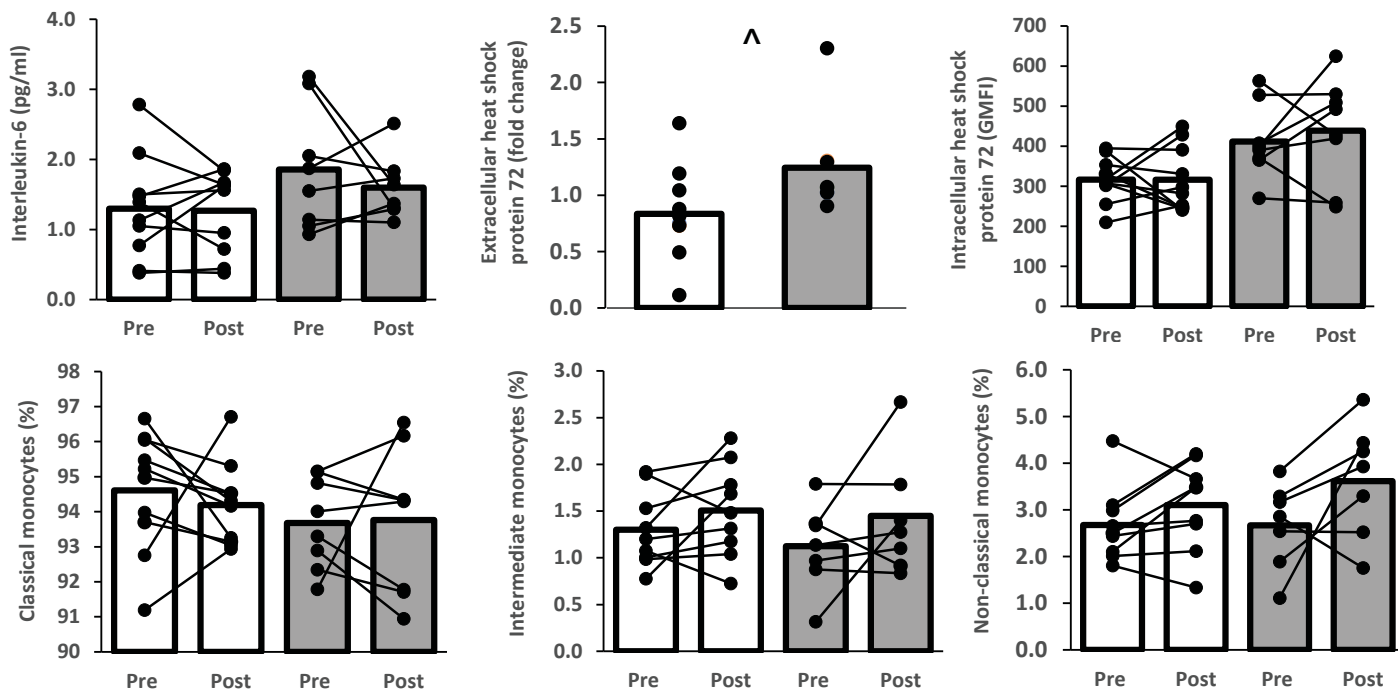
▲ Heart rate, perceptual responses, core temperature

■ Oxygen uptake, blood pressure





INT CON



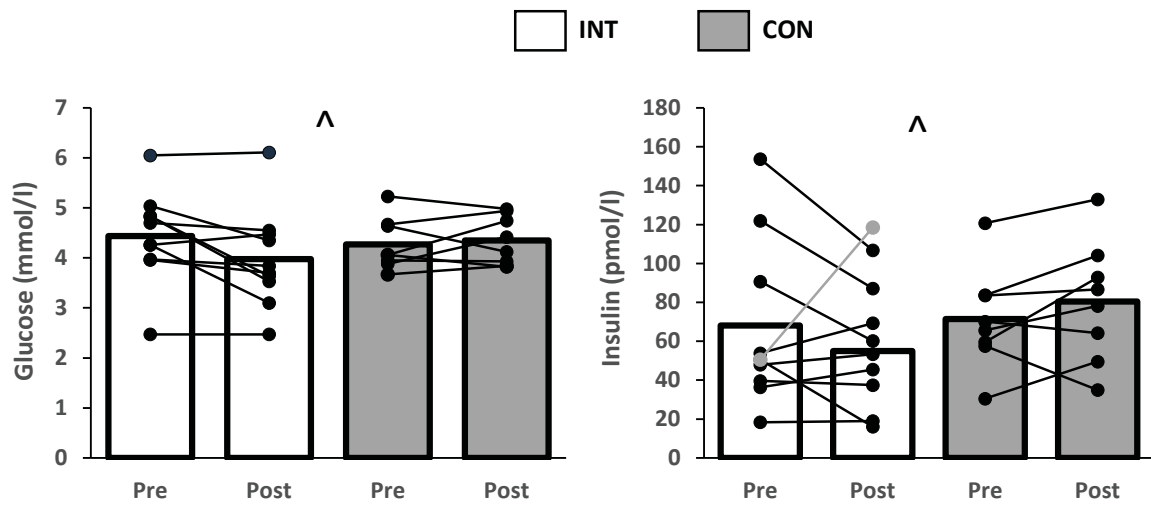


Table 1. Participant characteristics at baseline. Values are given in mean  $\pm$  SD

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

Parameter	AMB Pre	AMB 60 min	HWI <sub>pre</sub> Pre	HWI <sub>pre</sub> 60 min	HWI <sub>post</sub> Pre	HWI <sub>post</sub> 60 min
T <sub>rec</sub> (°C)	36.9 $\pm$ 0.5	36.6 $\pm$ 0.5	37.1 $\pm$ 0.6	38.7 $\pm$ 0.4*	37.0 $\pm$ 0.3	38.5 $\pm$ 0.3*
T <sub>tymp</sub> (°C)	35.5 $\pm$ 0.4	35.2 $\pm$ 0.4	35.4 $\pm$ 0.7	37.5 $\pm$ 0.7*	35.2 $\pm$ 0.3	37.6 $\pm$ 0.3*
HR (bpm)	69 $\pm$ 17	64 $\pm$ 15	67 $\pm$ 14	105 $\pm$ 13*	68 $\pm$ 12	104 $\pm$ 10*
VO <sub>2</sub> (L/min)	0.20 $\pm$ 0.04	0.23 $\pm$ 0.04	0.21 $\pm$ 0.04	0.42 $\pm$ 0.10*	0.19 $\pm$ 0.05	0.37 $\pm$ 0.03**
SBP (mmHg)	127 $\pm$ 10	123 $\pm$ 12	126 $\pm$ 13	138 $\pm$ 15*	118 $\pm$ 15^	126 $\pm$ 13^
DBP (mmHg)	86 $\pm$ 9	85 $\pm$ 10	83 $\pm$ 9	78 $\pm$ 9	79 $\pm$ 11^	71 $\pm$ 12*^
Basic affect (-5 to +5)	0.9 $\pm$ 1.4	0.7 $\pm$ 1.3	1.3 $\pm$ 2.0	-1.1 $\pm$ 2.2*	1.1 $\pm$ 1.9	-1.2 $\pm$ 1.9*
TS (1 to 9)	4.9 $\pm$ 0.6	4.8 $\pm$ 0.8	5.1 $\pm$ 0.9	7.4 $\pm$ 1.0*	4.7 $\pm$ 0.5	6.7 $\pm$ 0.8*^
TC (-5 to +5)	0.0 $\pm$ 0.0	0.0 $\pm$ 0.5	0.2 $\pm$ 0.4	2.2 $\pm$ 1.0*	-0.1 $\pm$ 0.3	2.3 $\pm$ 1.3*
Sweat loss (L)	N/A	0.17 $\pm$ 0.19	N/A	1.12 $\pm$ 0.56*	N/A	1.65 $\pm$ 0.57*^
PV change (%)	N/A	98 $\pm$ 3	N/A	95 $\pm$ 8	N/A	95 $\pm$ 4

Abbreviations: AMB = control trial; HWI<sub>pre</sub> = hot water immersion session prior to HWI intervention period; HWI<sub>post</sub> = hot water immersion session following HWI intervention period; T<sub>rec</sub> = rectal temperature; T<sub>tymp</sub>: tympanic temperature; HR = heart rate; VO<sub>2</sub> = 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<sub>pre</sub> and HWI<sub>post</sub>

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 <sub>pre</sub> pre	94.50±4.0	1.3±0.6	2.2±0.9
HWI <sub>pre</sub> post	91.2±5.8	1.7±0.7*	3.4±1.9*
HWI <sub>pre</sub> p2h	94.4±2.7	1.3±0.4	2.0±0.4
HWI <sub>post</sub> pre	94.2±1.2	1.5±0.6	3.1±1.0
HWI <sub>post</sub> post	93.9±1.7	1.4±0.4^	3.5±0.4*
HWI <sub>post</sub> p2h	94.7±1.5	1.2±0.5	2.7±1.3

Data are mean±SD. Abbreviations: AMB = control trial; HWI<sub>pre</sub> = hot water immersion session prior to HWI intervention period; HWI<sub>post</sub> = hot water immersion session following HWI intervention period, p2h = 2 hours post hot water immersion

\*Significantly different from AMB; ^ Significant difference between HWI<sub>pre</sub> and HWI<sub>post</sub>

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.