

1 **Title**

2 Heat Acclimation attenuates physiological strain and the Hsp72, but not Hsp90α mRNA response to acute
3 normobaric hypoxia

4

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25

26 **Running Head**

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28

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38 **Abstract**

39 Heat acclimation attenuates physiological strain in hot conditions via phenotypic and cellular adaptation.
40 The aim of this study was to determine whether HA reduced physiological strain, and Hsp72 and Hsp90 α
41 mRNA responses in acute normobaric hypoxia.

42

43 Sixteen male participants completed ten 90 min sessions of isothermic heat acclimation (HA;
44 40°C/40%RH) or exercise training (CON; 20°C/40% RH). HA or CON were preceded (HYP1) and proceeded
45 (HYP2) by a 30min normobaric hypoxic exposure (FiO₂=0.12; 10min rest, 10min cycling at 40% $\dot{V}O_{2peak}$,
46 10min cycling at 65% $\dot{V}O_{2peak}$).

47

48 HA induced greater rectal temperatures (T_{rec}), sweat rate (SR) and heart rates (HR) than CON during the
49 training sessions. HA, but not CON, reduced resting T_{rec}, resting HR and increased SR and plasma volume.
50 Haemoglobin mass did not change following HA nor CON. Hsp72 and Hsp90 α mRNA increased in response
51 to each HA session, but did not change with CON.

52

53 HR during HYP2 was lower and O₂ saturation higher at 65% $\dot{V}O_{2peak}$ following HA, but not CON. $\dot{V}O_2$ /HR was
54 greater at rest and 65% $\dot{V}O_{2peak}$ in HYP2 following HA, but was unchanged after CON. At rest, the respiratory
55 exchange ratio reduced during HYP2 following HA, but not CON. The increase in Hsp72 mRNA during HYP1,
56 did not occur in HYP2 following HA. In CON, Hsp72 mRNA expression was unchanged during HYP1 and
57 HYP2. In HA and CON, increases in Hsp90 α mRNA during HYP1 were maintained in HYP2.

58

59 HA reduces physiological strain, and the transcription of Hsp72, but not Hsp90 α mRNA in acute normobaric
60 hypoxia.

61

62 **Keywords**

63 Altitude, Cardiovascular, Cross acclimation, Cross tolerance, Heat Stress, Plasma Volume.

64

65 Introduction

66 Hypoxia increases physiological strain both at rest and during exercise (6), with impairment of exercise
67 performance (72), notably during exercise where aerobic metabolism predominates (3). The physiological
68 advantages and disadvantages of repeated hypoxic/altitude exposures for attenuating the negative effects
69 of hypoxia (2), have been summarised in numerous review articles (20, 46). Altitude/hypoxic training
70 methods are varied, with synergistic interactions between simulated and terrestrial, resting or exercise,
71 and continuous and intermittent exposures, each eliciting different magnitudes of adaptation (46).
72 Irrespective of precise application, hypoxic training requires lengthy durations of exposure over prolonged,
73 repeated periods (typically 14–28 days) for meaningful adaptation (27).

74
75 Heat acclimation, and acclimatization interventions, carried out by repeated exercise in hot conditions (58),
76 reproducibly reduce physiological strain in hot and cooler conditions (32, 33, 39). Recent reviews support
77 a novel adaptive pathway whereby heat acclimation may reduce physiological strain in hypoxia (14, 56,
78 73). Mechanistic pathways can be subdivided into cross acclimation, whereby heat acclimation attenuates
79 physiological strain (73) and cross tolerance, whereby cellular responses to heat acclimation provide
80 cytoprotection during hypoxia (14). Acute physiological responses to hypoxia (2) can be used as criteria
81 for validating heat induced cross acclimation. Heat acclimation reduces glycolysis and metabolic rates
82 during exercise (34), with plasma volume expansion (39, 50) and improved myocardial efficiency (38)
83 preserving cardiac output and skeletal muscle blood flow. Muscle oxygenation is also sustained by heat
84 acclimation induced maintenance of central blood volume via reductions in the core/skin temperature
85 gradient (58) and enhanced evaporative heat loss (51). Improved temperature and haematological
86 regulation facilitate a leftward shift in the oxyhaemoglobin saturation curve (37). Heat acclimation induces
87 expedient and beneficial adaptations within five to fourteen daily sessions demonstrating a greater
88 efficiency of adaptation when compared to altitude/hypoxic interventions (23).

89
90 Crosstolerance has been defined as single or repeated sub-lethal exposures to a stressor eliciting a positive
91 adaptive effect to a subsequent exposure to a different stressor (35). The cellular pathway for this shares
92 commonality with those seen within *in vivo* thermotolerance (47). In this model, cellular thermotolerance
93 accompanies the induction of phenotypic adaptations associated with heat acclimation (43, 45).
94 Thermotolerance confers cytoprotection against subsequent thermal exposure (45, 74) principally by
95 changes in heat shock proteins (31). Heat shock proteins facilitate important cellular processes as protein
96 chaperones (19) and anti-apoptotic mediators (1). In particular, increases in the inducible proteins
97 HSPA1A (Hsp72) and HSPC1 (Hsp90 α) mitigate pathophysiological responses to endogenously stressful
98 stimuli. Both Hsp72 and Hsp90 α augment proportionally to increased cellular stress (increased cellular
99 temperature) in response to *ex vivo* heat shock (45), and have been implicated as important for modulating
100 the adaptive cellular/molecular response to hypoxia (56, 65, 66); this suggests a shared signalling pathway.
101 Both Hsp72 and Hsp90 α mRNA, and protein responses have been used as a marker for identifying the
102 magnitude of stimuli required to initiate the *in vivo* stress response (45). However, not all the Hsp72 mRNA
103 transcripts are translated to Hsp72 protein increase within peripheral blood mononuclear cells following
104 exercise heat stress in humans (44). Basal heat shock protein measurement provides the optimal indication

105 of the acquired capacity to mitigate disruption to cellular homeostasis due to know increases with
106 acclimation (45). The delayed responsiveness of the protein response (16, 17), in comparison to the within
107 session heat shock protein mRNA response (25, 44) emphasises the benefits of the gene transcript as a
108 primary indicator of the magnitude of the stress stimuli, and necessity to signal protein transcription
109 should the stimuli be maintained or repeated. Consequently, the mRNA transcription is appropriate to
110 determine whether the Hsp72 and Hsp90 α responses have been attenuated or mitigated, either in response
111 to reductions in physiological strain, or increased basal protein ultimately highlighting whether cross
112 tolerance may have been conferred.

113

114 Heat acclimation has been evidenced in improving oxygen saturation and heart rates during hypoxic
115 exercise performance (28) with heat acclimation also mitigating increases in Hsp72 protein in hypoxia, due
116 largely to increased basal concentrations of Hsp72 (37). These data support the existence of cross
117 acclimation/tolerance (37) however mechanisms for this interaction are presently unknown (39, 45). The
118 aim of this experiment was to determine whether heat acclimation would reduce physiological strain and
119 the Hsp72 and Hsp90 α mRNA responses to an acute hypoxic exposure (at rest and at various exercise
120 intensities) in comparison to exercise training matched for intensity and duration in temperate conditions.
121 It was hypothesised that heat acclimation would reduce physiological strain in hypoxia via cardiovascular
122 and thermoregulatory adaptations, and that the heat shock protein response to hypoxia would be reduced
123 following heat acclimation.

124

125 **Materials and Methods**

126 *Participants*

127 Sixteen healthy males, who completed various forms of exercise training between three and six times per
128 week, were assigned to matched groups to perform ten days of isothermic heat acclimation (HA; age
129 22.5 \pm 3.5 yrs., nude body mass (NBM) 74.6 \pm 7.9 kg, body surface area 1.95 \pm 0.13 m², peak oxygen uptake (\dot{V}
130 O_{2peak}) 4.32 \pm 0.68 L.min⁻¹, 58.5 mL.kg⁻¹.min⁻¹), or act as a normothermic exercise control (CON; age 26.0 \pm 5.0
131 yrs., NBM 74.6 \pm 4.8kg, body surface area 1.93 \pm 0.13 m², \dot{V} O_{2peak} 4.22 \pm 0.62 L.min⁻¹ 56.6 mL.kg⁻¹.min⁻¹).
132 Confounding environmental and pharmacological variables were all controlled in line with previous work
133 in the field (24, 25). Urine osmolality was used to confirm hydration in accordance with established
134 guidelines prior to each experimental/training session (<700 mOsm.Kg⁻¹ H₂O (57)). This experimental
135 control was not violated for any participant for any experimental/training session. All protocols,
136 procedures and methods were approved by the institutional ethics committee with participants completing
137 medical questionnaires and written informed consent following the principles outlined by the Declaration
138 of Helsinki as revised in 2013.

139

140 *Preliminary Testing*

141 Prior to assessment of \dot{V} O_{2peak}, anthropometric data was collected with NBM measured using digital scales,
142 precise to 0.01 kg (GFK 150, Adam Equipment Inc, Danbury, CT, USA). \dot{V} O_{2peak} (L.min⁻¹) was determined

143 from an incremental test on a cycle ergometer which was used for all subsequent trials (Monark e724,
144 Monark AB, Varberg, Sweden), at a starting intensity of 80W, increasing by 24 W.min⁻¹, in temperate
145 laboratory conditions (20°C, 40% relative humidity (RH)) at sea level (FiO₂ = 0.2093). $\dot{V}O_{2peak}$ was defined
146 as the highest average $\dot{V}O_2$ obtained in any 30 s period with $\dot{V}O_{2peak}$ more appropriately describing the end
147 point of the test due to an absence of $\dot{V}O_2$ plateau in all participants. The confirmation of $\dot{V}O_{2peak}$ was made
148 via the attainment of a heart rate (HR) within 10 b.min⁻¹ of age predicted maximum, and RER >1.1 in all
149 participants. Expired metabolic gas was measured using breath by breath online gas analysis (Metalyser
150 3B, Cortex, Leipzig, Germany). HR was recorded continually during all experimental/training sessions by
151 telemetry (Polar Electro Oyo, Kempele, Finland).

152

153 *Haematological Measures*

154 Twenty four hours prior to hypoxic exposures haemoglobin mass (Hb_{mass}; g) was measured. Hb_{mass}, blood
155 volume (BV; mL) and plasma volume (PV; mL) were determined in accordance with the oCOR-method (59).
156 Participants were seated for 20 min, before being connected to a closed glass spirometer allowing
157 inspiration of a CO (carbon monoxide) bolus of 1.0 mL.kg⁻¹ (68), followed by 2 min rebreathing of a 3.5 L
158 O₂ bolus. Before and 4 min after CO-rebreathing, participants completely exhaled to residual volume into a
159 CO gas meter (Pac 7000, Dräger; Pittsburgh, PA, USA). CO volume not remaining within the body was
160 calculated from the remainder CO in the spirometer and exhaled CO measured immediately after
161 disconnecting the spirometer from the participant (68). Fingertip capillary samples, for determination of
162 carboxyhaemoglobin concentration (%HbCO) were taken immediately before the rebreathing procedure
163 and at 6 and 8 min after the CO bolus was administered. Blood samples were measured immediately in
164 triplicate (69), using an ABL80 CO-OX FlexOXFlex hemoximeter (Radiometer™; Copenhagen, Denmark).
165 Hb_{mass} was calculated from the mean change in %HbCO before and after rebreathing CO (68). At the
166 relevant intervals within the oCOR method, haemoglobin concentration (Hb; g.dL⁻¹) was collected from
167 fingertips in duplicate using a microcuvette and analysed using a B-Haemoglobin Photometer (Hemocue
168 Limited, Ängelholm, Sweden) and haematocrit (Hct; %) was collected in triplicate (~50 µL) with glass
169 capillary tubes and analysed following centrifugation at 14,000 rpm for 3 min (Haemospin 1300
170 Centrifuge, Hawksley & Sons Ltd, West Sussex, UK) (69). The experimenter typical error of measurement
171 for total Hb_{mass} prior to commencing this experiment was ±1.98% (±17.0 g).

172

173 *Hypoxic Exposures*

174 Hypoxic exposures were performed 24 hours prior to commencing the first session of HA or CON (HYP1)
175 and 24 hours following the final HA or CON training session (HYP2). Participants performed a 30 min
176 normobaric hypoxic exposure adapted from Lunt et al., (40). After entering normobaric hypoxic conditions
177 (FiO₂ = 0.12; 18°C, 40%RH) achieved using a purposed built nitrogen-enriched chamber (Altitude Centre,
178 London), participants immediately rested in a supine position for a period of 10 min. Supine rest was
179 followed by two bouts of exercise where participants first cycled at a workload corresponding to 40% of
180 normoxic preliminary $\dot{V}O_{2peak}$ for a period of 10 min (HA=102±27 W, CON=104±26 W) and then

181 immediately proceeded to exercise at a workload corresponding to 65% of normoxic preliminary $\dot{V}O_{2peak}$
182 (HA= 201±41W, CON=192±37W) for a further 10 min. During rest and exercise, HR, oxygen uptake ($\dot{V}O_2$;
183 L.min⁻¹), carbon dioxide production, ($\dot{V}CO_2$; L.min⁻¹), ventilation (V_E ; L.min⁻¹), respiratory exchange ratio
184 (RER) and peripheral arterial oxygen saturation (SpO₂; %) estimated using a fingertip pulse oximeter
185 (Nonin 2500, Nonin Medical Inc, Minnesota, USA) were recorded continuously, with the final 5 min of
186 measures used for analysis. Prior to entry, and following every ten min, participants reported Rating of
187 Perceived Exertion (RPE) and Lake Louise Questionnaire (LLQ) symptoms. Metabolic parameters ($\dot{V}O_2$, \dot{V}
188 CO₂ and \dot{V}_E) was measured using online breath by breath analysis.

189

190 *Heat Acclimation/Exercise Protocols*

191 Each HA or CON training session was conducted at the same time of day (07:00-10:00 h) to control for
192 effects of daily variation in exercise performance (12) and heat shock protein expression (67) inside a
193 purpose built environmental chamber (WatFlow control system; TISS, Hampshire, UK). Temperature and
194 humidity were controlled using automated computer feedback (WatFlow control system; TISS, Hampshire,
195 UK). On arrival to the laboratory, participants provided a mid-flow urine sample for assessment of
196 hydration. Towel-dried NBM was measured before and after the trials, with no fluid consumption
197 permitted between measurements. Sweat rate (SR; L.hr⁻¹), was estimated using the change in NBM from
198 the pre- to post- exercise periods. Participants inserted a rectal thermistor (Henleys Medical Supplies Ltd,
199 Welwyn Garden City, UK, Meter logger Model 401, Yellow Springs Instruments, Yellow Springs, Missouri,
200 USA) 10 cm past the anal sphincter to measure rectal temperature (T_{rec}) and affixed a HR monitor to the
201 chest. Following a 10 min seated stabilisation period in temperate laboratory conditions, at sea level,
202 resting measures (T_{rec} , HR, RPE and thermal sensation; TSS) were recorded and participants immediately
203 entered the environmental chamber (40.2°C±0.4°C, 41.0±6.4% RH) and mounted a cycle ergometer.
204 Participants allocated to the HA group performed ten 90 min sessions involving a combination of cycling
205 exercise and rest in accordance with established isothermic HA protocols (25, 26). HA participants initially
206 exercised, at a workload corresponding to 65% $\dot{V}O_{2peak}$ until the isothermic target T_{rec} of ≥38.5°C had been
207 achieved, and, upon the attainment of a T_{rec} ≥38.5°C, rested in a seated position on the cycle ergometer
208 within the environmental chamber. Participants resumed exercise when their T_{rec} fell below 38.5°C and
209 continued cycling until the target T_{rec} was attained. Participants in the CON group performed ten 90 min
210 sessions copying the exercise-rest prescription of HA in controlled conditions (19.8°C±0.2°C, 28.5±2.7%
211 RH). CON participants initially cycled at an intensity corresponding to 65% $\dot{V}O_{2peak}$ with the workload
212 adjusted to match the total work, and exercise intensity and duration of the whole session (exercise and
213 rest) and exercise components of the HA group, see figure 1 for mean sessional T_{rec} throughout HA and
214 CON. To accurately match the exercise intensity and duration of exercise of the HA group, the exercise
215 requirement of CON was prescribed with progressive increases in the mean exercise duration and intensity
216 implemented throughout the 10 day regime. This progression was derived from the rolling mean of HA

217 participants who had already completed that given day of the intervention. Any resumption of exercise,
218 necessary to increase T_{rec} in HA, was added to the end of the initial exercise bout for CON participants, this
219 strategy was necessary to account for the intermittent nature of subsequent exercise both between, and
220 within HA participants. During each HA or CON session HR, T_{rec} , power output (W), RPE and TSS were
221 recorded every 5 min with adjustments in power (including the cessation of exercise) only made following
222 each completed 5 min period. In compliance with ethical approval, HA was terminated if a subject attained
223 a T_{rec} of 39.7°C (zero incidences), data describing the prescription, physiological and perceptual responses
224 to HA and CON is contained in Table 1.

225

226 *Blood Sampling and RNA extraction*

227 Venous blood samples were taken immediately pre- and post- HYP1 and HYP2, and pre- and post- the first
228 (Day1) and tenth (Day10) of HA or CON with RNA extraction performed using a validated method (9).
229 Briefly, blood samples were drawn from the antecubital vein into 6 mL EDTA tubes (Greiner BIO-one,
230 Stonehouse, UK). Venous blood (1 mL) was pipetted into 10 mL of 1 in 10 red blood cell lysis solution (10X
231 Red Blood Cell Lysis Solution, Miltenyi Biotech, Bisley, UK). Samples were incubated for 15 min at room
232 temperature before isolation via 5 min centrifugation at 400G then washed twice in 2 mL PBS and
233 centrifugation at 400G for 5 min. Due to belonephobia, one participant from HA was excluded from blood
234 sampling and mRNA analyses. The TRIzol method was then used to extract RNA from the leukocytes in
235 accordance with manufacturer instructions (Invitrogen, Life Technologies, Carlsbad, USA). Quantity was
236 determined at an optical density of 260 nm, while quality was determined via the 260/ 280 and 260/ 230
237 ratios using a nanodrop spectrophotometer (Nanodrop 2000c Thermo Scientific, Waltham, MA, USA).

238

239 *One step reverse transcription quantitative polymerase chain reaction (RT-QPCR)*

240 Hsp72 and Hsp90 α relative mRNA expression was quantified using RT-QPCR. Primers β 2-Microglobulin
241 (β 2-M; NCBI Accession number:NM_004048; Forward:CCGTGTGAACCATGTGACT,
242 Reverse:TGCGGCATCTTCAAACCT), Hsp72 (NCBI Accession number:NM_005345;
243 Forward:CGCAACGTGCTCATCTTTGA, Reverse:TCGCTTGTCTGGCTGATGT), and Hsp90 α (NCBI Accession
244 numbers:NM_001017963 (variant 1) & NM_005348 (variant 2); Forward:AAACTGCGCTCCTGTCTTCT,
245 Reverse:TGCGTGATGTGTCGTCATCT) were designed using primer design software (Primer Quest and
246 Oligoanalyzer - Integrated DNA technologies, Coralville, IA, USA) (70). Relative quantification of mRNA
247 expression for each sample was assessed by determining the ratio between the cycling threshold (CT) value
248 of the target mRNA and β 2-M CT values. Fold change in relative mRNA expression was calculated using the
249 $2^{-\Delta\Delta CT}$ method.

250

251 *Statistical Analysis*

252 *A priori* power analysis for key heat acclimation dependent variables selecting conventional α (0.05) and β
253 (0.20) levels, observed eight participants were required in each experimental group. Prior to statistical
254 analysis, all outcome variables were checked for normality using Kolmogorov-Smirnov and sphericity using
255 the Greenhouse Geisser method prior to further analysis. Protocol specific and physiological data for
256 HA/CON were compared using independent samples T-Tests. Two-way mixed-design ANOVA was

257 performed to determine differences between HA and CON and Day1/Pre and Day10/post. Two-way mixed-
258 design ANOVA was performed to determine differences between HA and CON, as well as HYP1 with HYP2;
259 thus rest, 40% $\dot{V}O_{2peak}$ and 65% $\dot{V}O_{2peak}$ conditions within each HYP were analysed independently from one
260 another. Three-way mixed-design ANOVA was performed on the Hsp72 and Hsp90 α mRNA data to
261 determine differences between pre- and post- value (repeated measures – within subjects) on different
262 days (repeated measures – within subjects) from the two interventions (between subjects). Adjusted
263 Bonferroni comparisons were used as post hoc analyses for all ANOVA. Effect sizes (Cohen's d (*d*; small =
264 0.2, medium = 0.5, large = 0.8) or partial eta squared (np^2 ; small = 0.01, medium = 0.06, large = 0.13) were
265 calculated to analyse the magnitude and trends with data. All data are reported as mean \pm SD. For all analysis
266 two-tailed significance was accepted at $p<0.05$.

267

268 **Results**

269 *Heat Acclimation/ Exercise Interventions*

270 HA and CON were successfully matched for exercising duration ($t=0.635$; $p<0.001$; $d=0.34$), work done
271 ($t=-0.168$; $p=0.869$; $d=0.09$), and session intensity ($t=-0.355$; $p=0.728$; $d=0.19$) (Table 1).

272

273 Differences were observed for mean T_{rec} ($t=9.138$; $p<0.001$; $d=4.88$), rate T_{rec} increase ($t=6.876$; $p<0.001$;
274 $d=3.68$), duration $T_{rec}\geq 38.5^{\circ}C$ ($t=14.106$; $p<0.001$; $d=7.54$), between HA and CON interventions, with mean
275 T_{rec} different between HA and CON ($t=55.619$; $p<0.001$; $np^2=0.799$) 30 and 90 min (figure 1). Additionally,
276 SR ($t=7.254$; $p<0.001$; $d=3.88$), mean HR ($t=3.444$; $p=0.004$; $d=1.84$), mean RPE ($t=2.918$; $p=0.011$; $d=1.56$),
277 and mean TSS ($t=8.394$; $p<0.001$; $d=4.49$) were greater in HA compared to CON interventions (Table 1).

278

279 ***INSERT FIGURE 1 APPROXIMATELY HERE***

280

281 ***INSERT TABLE 1 APPROXIMATELY HERE***

282

283 *Adaptation to Heat Acclimation*

284 An interaction effect was observed between HA and CON and day 1 and day 10 for resting T_{rec} ($f=11.507$;
285 $p=0.004$; $np^2=0.451$), resting HR ($f=20.579$; $p<0.001$; $np^2=0.595$), SR ($f=7.146$; $p=0.018$; $np^2=0.338$), plasma
286 volume ($f=23.501$; $p<0.001$; $np^2=0.627$) and blood volume ($f=25.582$; $p<0.001$; $np^2=0.646$) in HA, but not
287 CON (Table 2). SR was greater on day 1 and 10 in HA than CON ($p<0.001$), but resting T_{rec} ($p=0.007$) and
288 resting HR ($p=0.033$) were lower on day 10 in HA compared to CON (Table 2). No difference was observed
289 between days ($f=0.275$; $p=0.608$; $np^2=0.019$) or days*groups ($t=0.237$; $p=0.634$; $np^2=0.017$) for Hbmass
290 (Table 2).

291

292 ***INSERT TABLE 2 APPROXIMATELY HERE***

293

294 *Hsp72 mRNA and Hsp90 α mRNA during HA/CON*

295 An interaction effect was observed for Hsp72 mRNA ($f=20.428$; $p=0.001$; $np^2=0.611$) and Hsp90 α mRNA
296 ($f=10.282$; $p=0.007$; $np^2=0.422$). No difference was observed between HA or CON at pre Day1 or Day10
297 ($p=0.396$ and $p=0.180$), but a difference was observed post HA in comparison to CON ($p=0.004$ and
298 $p=0.012$). Hsp72 mRNA and Hsp90 α mRNA increased pre to post HA ($p<0.001$ and $p<0.001$) in HA, but not
299 CON ($p<0.051$ and $p=0.394$).

300

301 *Hypoxic Tolerance Tests*

302 At rest in hypoxia, there was an interaction effect between groups and HYP1 and HYP2 for $\dot{V}O_2/HR$
303 ($f=6.852$; $p=0.020$; $np^2=0.329$) and RER ($f=5.078$; $p=0.041$; $np^2=0.266$). In HYP2, at rest differences
304 occurred following HA for $\dot{V}O_2/HR$ ($p=0.039$; Figure 2) and RER ($p=0.045$; Figure 3), but not CON ($p>0.05$).
305 No difference was observed in HR ($f=0.820$; $p=0.381$; $np^2=0.055$) nor SpO $_2$ ($f=2.123$; $p=0.167$; $np^2=0.132$)
306 at rest in hypoxia (Figure 2).

307

308 When exercising at 40% $\dot{V}O_{2peak}$ in hypoxia, no differences were observed in the within the group*HYP
309 comparison for HR ($f=1.575$; $p=0.230$; $np^2=0.101$), SpO $_2$ ($f=0.000$; $p=1.000$; $np^2=0.000$), $\dot{V}O_2/HR$ ($f=2.651$;
310 $p=0.126$; $np^2=0.126$) or RER ($f=0.047$; $p=0.831$; $np^2=0.003$) (Figure 2). When exercising at 65% $\dot{V}O_{2peak}$ in
311 hypoxia, differences were observed for HR ($f=4.751$; $p=0.047$; $np^2=0.253$), SpO $_2$ ($f=5.616$; $p=0.033$;
312 $np^2=0.286$) and $\dot{V}O_2/HR$ ($f=10.584$; $p=0.006$; $np^2=0.431$) within the group*HYP comparison. In HYP2, at
313 65% $\dot{V}O_{2peak}$ differences occurred following HA for HR ($p=0.001$), SpO $_2$ ($p=0.006$) and $\dot{V}O_2/HR$ ($p=0.006$),
314 but not CON ($p>0.05$) see Figure 2. No difference was observed in RER ($f=0.248$; $p=0.626$; $np^2=0.017$) when
315 exercising at 65% $\dot{V}O_{2peak}$ (Figure 2).

316

317 No differences ($p>0.05$) were observed between HYP1 and HYP2 trials, at rest, 40% $\dot{V}O_{2peak}$ or 65% $\dot{V}O_{2peak}$
318 during HA or CON for $\dot{V}O_2$, $\dot{V}E$, B_f , RPE or LLQ (Table 3).

319

320 ***INSERT TABLE 3 APPROXIMATELY HERE***

321

322 *Hsp72 mRNA and Hsp90 α mRNA during Hypoxic Tolerance Tests*

323 Hsp72 mRNA increased during HYP1 ($f=17.005$; $p=0.001$ $np^2=0.567$). In the HA group, an increase in Hsp72
324 mRNA was observed following HYP1 ($p=0.006$), but not HYP2 ($p=0.440$). This was supported by the
325 observation that Hsp72 mRNA was greater post HYP1 in comparison to HYP2 ($p=0.021$). No changes in the
326 pattern of Hsp72 mRNA expression were observed in CON. Hsp90 α mRNA increased pre to post HYP1 and
327 HYP2 ($f=17.110$; $p=0.001$ $np^2=0.568$). However, no differences were observed between HYP1 and HYP2,
328 nor between HA and CON at any time.

329

330 **Discussion**

331 This experiment observed that heat acclimation reduced physiological strain and the Hsp72 mRNA
332 response to an acute hypoxic exposure combining rest and exercise. The adaptation pathway was likely
333 mediated in part by PV expansion which improved $\dot{V}O_2/HR$ at rest and exercise in hypoxia, as well as
334 attenuating HR responses and preservation of SpO₂ during exercise in hypoxia. Resting RER reduced after
335 HA, an observation not true of CON, suggesting greater fat oxidation at rest in hypoxia post intervention. At
336 a cellular level, HA, mitigated the group specific Hsp72 mRNA increase, but not the Hsp90 α mRNA response
337 to hypoxia. The Hsp90 α mRNA response also increased comparably to HA before and after CON, however
338 no increase in Hsp72 mRNA was observed in either trials in this group.

339
340 HA and CON were successfully matched for the prescribed training parameters (duration, absolute
341 intensity and work done; Table 1) with the equality of these training parameters giving confidence that
342 adaptations were induced by the increased physiological/thermal strain of the hot environment of HA, in
343 comparison to the temperate conditions of CON (Table 1). The eloquent experimental design of Lorenzo et
344 al., (39) is most closely representative of ours. In agreement with previous data (26), the magnitude of
345 adaptation induced by our isothermic HA regimen is at least equal to that observed by their fixed intensity
346 heat acclimation regimen (39), which improved physiological responses and exercise performance in hot
347 and cool conditions. The authors (39) reported similar absolute changes in T_{rec} (-0.5°C; our data = -0.49°C),
348 HR (-15 b.min⁻¹; our data = -18 b.min⁻¹) and SR (+0.4L.hr⁻¹; our data = +0.4L.hr⁻¹). Additionally, over the
349 same number of heat acclimation sessions we observed a larger expansion of PV (+6.5%; our data = +15%).
350 Despite a cascade of mechanisms well attributed to PV expansion including increased vascular filling to
351 support cardiovascular stability, increased specific heat capacity of blood, and attenuated skin blood flow
352 responses, the observable magnitude of these adaptive responses may be finite, or demonstrate an
353 exponential decay beyond moderate levels of hypervolemia (58). These responses are agreeable with the
354 consensus that isothermic protocols controlling hyperthermia to a core temperature of at least 38.5°C
355 should be implemented to optimize adaptations (53), due to maintenance of the endogenous thermal
356 stimuli for adaptation (50). Increased core temperature (Figure 1), leading to elevated and sustained
357 sweating, are the fundamental potentiating stimuli initiating phenotypic responses known as heat
358 acclimation (54), consequently, in HA, increased mean T_{rec} (+0.8°C), and the duration where T_{rec} exceeded
359 the isothermic threshold of 38.5°C (47 min) (18), induced greater adaptation than the normothermic
360 training of CON (Table 1). Greater heat dissipation through evaporation in hot conditions was evidenced
361 by three fold elevation in sweat rates in HA compared to CON (51). Increased heat storage in HA is the
362 stimuli for observed increases in HR, RPE and TSS for the same exercise prescription as CON (21). HA
363 increased BV (+500 mL) compared to CON (Table 2). No change in Hb_{mass} indicates HA induced
364 hypervolemia was a response to increases in extracellular fluid, with increases in PV (+446 mL)
365 approximate to the absolute change in BV, reaffirming this as a primary adaptation to heat (61), and an
366 established mechanism for the reduction in HR during exercise. Implementation of isothermic methods
367 (50) for HA are the most probable causes for greater PV expansion (+15%) compared with others utilising
368 similar participants, protocol length and environmental conditions (6.5% (39), 9.0% (50), and 11.1% (7)).

369 It remains to be experimentally elucidated whether maintaining lower intensity exercise which matches
370 heat production to evaporative heat loss, thus closely controlling T_{rec} at 38.5°C, rather than implementing
371 passive rest following T_{rec} exceeding the target of 38.5°C would augment even more favourable adaptations
372 resulting from higher sweat rates and elevated cardiovascular response. As such, despite a large magnitude
373 of adaptation observed within this experiment, this is a potential limitation of the implemented
374 experimental design. With no change in gross efficiency as indicated by similar $\dot{V}O_2$, the $\dot{V}O_2/HR$ ratio
375 becomes more efficient after HA (Figure 2). Hypohydration from increased sweating
376 (HA=2.9%NBM.session⁻¹, CON=1.0%NBM.session⁻¹), and the sustained endogenous stimuli (increased T_{rec})
377 of isothermic heat acclimation (26) increases PV expansion via increased plasma albumin and the Renin-
378 Angiotensin-Aldosterone system (50). Large PV has been proposed as maladaptive due to haemodilution
379 (14), where maintenance of cardiac output may be potentially confounded by a reduced relative O₂ carrying
380 capacity of blood. Improved SpO₂ (+3%) following HA suggests that a 15% increase in PV is beneficial in
381 hypoxia, even if optimal PV expansion is currently unknown. Maintenance of SpO₂ following HA (Figure 2)
382 occurs as a reduction in HR and blood viscosity affords a greater erythrocyte alveolar transit time,
383 facilitating more complete re-saturation within the pulmonary system (11). This is important in hypoxia,
384 and for more well-trained individuals, due to a greater reduction in SpO₂ resulting from a typically larger
385 cardiac output, and reduced pulmonary gas exchange at higher exercise intensities (52).

386

387 ***INSERT FIGURE 2 APPROXIMATELY HERE***

388

389 The reduction in T_{rec} and increased SR (Table 2) following HA has a dual role in facilitating enhanced heat
390 balance. Reduced T_{rec} mediates a greater spectrum for temperature increase, whilst increased SR is
391 facilitated by an earlier sweat onset even when accounting for decreased T_{rec} (55). Within HYP1/HYP2, the
392 heat stress was moderate (10, 39) and would appear compensable (8), thus reduced T_{rec} following HA as a
393 mechanism for prolonging permissible physiological strain and exercise performance in temperate hypoxia
394 is not fundamental. Instead, reduced T_{rec} during exercise in hypoxia causes a leftward shift in the
395 oxyhaemoglobin dissociation curve, signifying the potential for enhanced O₂ saturation (73). This
396 thermoregulatory adaptation is relevant in hypoxia vs. normoxia as O₂ utilisation is more greatly
397 compromised. Preservation of SpO₂ observed at 65% $\dot{V}O_{2peak}$ alone is likely a result of the increased demand
398 for O₂ at the muscle at this higher intensity (63). Interestingly, improved physiological response to matched
399 exercise did not augment a reduction in the RPE or LLQ in hypoxia (Table 3), as previously observed
400 regarding TSS in the heat (26). It should be noted that there is potential for the reduction in physiological
401 strain in HYP2 to be a reflection of a reduced relative exercise intensity as heat acclimation has been shown
402 to increase $\dot{V}O_{2max}$ in both cool and hot conditions (39). No data exists stating heat acclimation improves \dot{V}
403 O_{2peak} or $\dot{V}O_{2max}$ in hypoxia, however a post HA $\dot{V}O_{2peak}$ test in the present study would've been able to
404 determine that this is likely to have occurred. Based on this notion, it should be observed that cross
405 acclimation was effective using a model testing an absolute workload equal before and after intervention,

406 which may accurately reflect occupational or military populations completing a fixed task, however it is
407 unknown whether the reduction in physiological strain would have also been observed if workload was
408 derived from the relative exercise intensity of on a post exercise $\dot{V}O_{2\text{peak}}$ test. This perspective being
409 analogous to exercise performance within a given intensity domain.

410

411 Despite no change in our data, hypothetically a sufficient dose of heat acclimation could increase Hb_{mass}
412 (60), via the induction of HIF-1 α (42), as is well established of altitude exposure (27). Trends for increases
413 in erythrocyte volume have been observed following 5 d interventions similar to HA ($4.1\pm 0.9\%$) (22).
414 Conversely, and in agreement with our data, training for 10 d in 30°C at 610 m elicited no change in
415 erythrocyte volume ($+0.4\pm 0.6 \text{ mL}\cdot\text{kg}^{-1}$), whereas training at the same temperature at 2,000 m elicited
416 significant gain ($+1.9\pm 0.4 \text{ mL}\cdot\text{kg}^{-1}$). This suggests long established non-thermal, O_2 sensing pathways are
417 most important for increasing Hb_{mass} (27, 60). Our interventions did not increase Hb_{mass} , thus they did not,
418 or cannot induce sufficient heat strain and/or training load to induct erythropoietin (27). This disparity
419 from comparable research suggests more data are required to elucidate whether heat acclimation can
420 effectively induce changes in Hb_{mass} .

421

422 The metabolic response to altitude is a preferential shift towards glycolysis (48), as such the reduction in
423 RER following HA, an indicator of substrate utilisation was an unexpected observation in hypoxia, though
424 a reduction in metabolism has been observed in response to heat (30). Absent of changes in \dot{V}_E and Bf (Table
425 3), the RER reduction at rest during HYP2 (following HA; Figure 3) appears to be a metabolic response ,
426 rather than an artefact of hyperventilation between trials (5). The present data cannot determine whether
427 hypoxia induced hyperventilation from normoxia occurred, or was reduced following HA. HIF-1 alters
428 metabolism at altitude (48), the typical response being an initial increase in glycolysis upon acute exposure,
429 followed by a reduction with acclimatization/acclimation. HIF-1 is known to increase following heat
430 acclimation (42, 62), thus increases may accelerate the desensitisation, or inhibit the immediate shift in
431 substrate metabolism (49). Another theory implicates Hsp72 as having a therapeutic role in glycogen
432 regulation amongst other metabolic disorders e.g. type II diabetes, obesity (29). Transgenic mice
433 overexpressing Hsp72 evidence increased fatty acid oxidation and reduced mitochondrial dysfunction,
434 alongside increased $\dot{V}O_2$ and exercise capacity (29). At present these mechanisms are speculative, however
435 responses within our data warrant further investigation to authenticate or refute this observation.

436

437 ***INSERT FIGURE 3 APPROXIMATELY HERE***

438

439 Observations, that Hsp72 mRNA increases (+2.5 fold) are sustained during isothermic heat acclimation are
440 supported by our data ($+2.0\pm 1.0$ fold), which also supports similar sustained increases in Hsp90 α mRNA
441 ($+2.4\pm 1.5$ fold) (25). It is notable that this data is supportive of others observing no daily change in resting
442 Hsp72 or Hsp90 α mRNA (25, 44, 70), thus following an initial stress response, the removal of the stress
443 stimuli is sufficient to remove the necessity for transcription back to basal quantities within 24 hours. It is

444 this observation that reaffirms that the Hsp72 mRNA response to exercise is a potential marker of
445 acclimation, however resting levels do not provide sufficient discrimination (44), as basal intracellular
446 Hsp72 protein do (44, 45). These data highlight that isothermic heat acclimation could provide a stimuli
447 for increases in Hsp72 and Hsp90 α protein at both the onset and culmination of the regimen (Figure 4).
448 Endogenous physiological and cellular strain induced by CON was insufficient to induce the respective gene
449 transcripts for Hsp72 (+0.5 \pm 0.2 fold) and Hsp90 α (+0.4 \pm 0.1 fold), likely due to the failure to induce
450 sufficient changes in T_{rec} or oxidative stress. Thus, this exercise prescription is unlikely to increase basal
451 protein within the cell, a requirement of cross-tolerance.

452

453 ***INSERT FIGURE 4 APPROXIMATELY HERE***

454

455 Increased Hsp72 and Hsp90 α mRNA in hypoxia highlights the sensitivity of each gene to both exercise and
456 endogenous environmental stimuli (temperature and oxidative stress induced by hypoxia). Increased gene
457 expression in HYP1 suggests our protocol was effective at providing potentiating stimuli for a heat shock
458 protein response, albeit with smaller expression than the HA sessions suggesting inferior endogenous
459 stimuli for transcription. Reductions in Hsp72 mRNA in HYP2 following HA, evidences either reduced
460 physiological strain during HYP2 (cross acclimation) or an HA induced increase in intracellular Hsp72 (41)
461 (cross tolerance) mitigating requirements for further gene transcription. Neither of these mechanistic
462 pathways are true of Hsp90 α mRNA which shares a similar response between HYP1 (+0.3 \pm 0.4 fold) and
463 HYP2 (+0.4 \pm 0.4 fold) (Figure 5). A longer hypoxic exposure may have elicited a greater magnitude of heat
464 shock protein mRNA responses, especially for participants in CON who demonstrated no increase in Hsp72
465 mRNA. This prolonged protocol may have also further enhanced observable differences in Hsp72 mRNA
466 before and after HA. Maintenance of increased Hsp90 α in HYP2 may relate to the a specific role of the
467 protein within recovery and adaptation to cellular stress i.e. control of cellular signalling cascades (64),
468 recovery of global protein synthesis (13), and coordination of cellular repair (15). Hence continued gene
469 transcription may be required. Relative exercise intensity, which is known to change under different
470 environmental conditions, effects the metabolic strain and molecular responses (4, 36). Accumulation of
471 Hsp72 and Hsp90 protein occurs with HA (45) however basal Hsp90 α has been demonstrated as lower
472 than Hsp72 (45). Within HYP1/HYP2, the metabolic strain likely induced protein denaturation activating
473 the heat shock protein response via heat shock factor-1, however it is plausible that basal Hsp72 was
474 sufficient to cope with the hypoxic stress post HA (4), thus transcription was mitigated, however basal
475 Hsp90 α protein remained lower than necessary thus HYP2 induced further mRNA transcription to a similar
476 extent as observed in HYP1. As previously observed (70), the current study cannot suggest that our
477 intervention can translate the mRNA signal into Hsp72 and Hsp90 α mediated thermotolerance or hypoxic
478 cross tolerance within leukocytes (31), because increased mRNA expression is not necessarily reflective of
479 functional steady state basal protein content which may or may not be the most important component for
480 observing cross tolerance (71). Though it is unknown whether HA induced Hsp72 and Hsp90 α protein
481 accumulation, it has previously been stated that observed mRNA increases provides an indication that the
482 heat shock response has been activated, potentiating protein translation (70).

483

484 ***INSERT FIGURE 5 APPROXIMATELY HERE***

485

486 The present data suggest the existence of pathways for transferring adaptations to heat acclimation to
487 other environments (varying temperature/oxygen availability), although future experiments should
488 determine whether the attenuated responses are specific to thermal stimuli alone, or the combined
489 exercise-heat stress training stimuli that our intervention applied. With likely increases in aerobic capacity
490 (39), the absolute workload model, implemented may have also elicited different responses than a relative
491 intensity model based on post acclimation aerobic capacity, thus this is a limitation of the experiment which
492 should be considered relevant to future research in the area. Data pertaining to cross acclimation within
493 this experiment are clear, particularly when cardiovascular stress compromises the individual in a
494 particular environment (14, 73). The true thermal adaptation might be of greater relevance with regards
495 to the heat shock protein response which is known as responsive to different relative exercise intensities
496 (4, 36). Benefits of increased intracellular Hsp72 and Hsp90 α have been recently reviewed (14, 56, 73),
497 however cross tolerance pathways cannot be fully confirmed given the lack of protein data presently
498 available. Implications for heat acclimation induced changes in Hsp72 and Hsp90 α could be confirmed by
499 measuring Hsp72 and Hsp90 α protein pre and post intervention, and then applying hypoxic shock/stress
500 within *ex vivo* and *in vivo* models. Further research is required to determine the benefits of cross
501 acclimation and cross tolerance across a spectrum of simulated and actual altitudes and workloads within
502 those environments.

503

504 **Conclusion**

505 Heat acclimation is an effective intervention for reducing physiological strain associated with acute
506 normobaric hypoxia, primarily through heat acclimation derived PV expansion improving cardiovascular
507 efficiency which reaffirms a cross acclimation mechanism. Normothermic training failed to reduce
508 physiological strain or alter the Hsp72 and Hsp90 α mRNA response to hypoxia The Hsp72 mRNA increase
509 pre HA was attenuated following acute normobaric hypoxia following heat acclimation, giving efficacy to
510 cross tolerance pathways at a cellular/molecular level, however no changes in Hsp90 α mRNA were
511 observed. These data suggest hyperthermia as viable potentiating stimuli for the cross adaptive
512 mechanisms.

513

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520

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522 No conflicts of interest, financial or otherwise, are declared by the author(s).

523 **References**

- 524 1. **Arya R, Mallik M, Lakhotia SC.** Heat shock genes - integrating cell survival and death. *J Biosci* 32:
525 595–610, 2007.
- 526 2. **Bailey DM, Davies B.** Physiological implications of altitude training for endurance performance at
527 sea level: a review. *Br J Sports Med* 31: 183–190, 1997.
- 528 3. **Balsom PD, Ekblom B, Sjödin B.** Enhanced oxygen availability during high intensity intermittent
529 exercise decreases anaerobic metabolite concentrations in blood. *Acta Physiol Scand* 150: 455–6,
530 1994.
- 531 4. **Beckmann RP, Lovett M, Welch WJ.** Examining the function and regulation of hsp 70 in cells
532 subjected to metabolic stress. *J Cell Biol* 117: 1137–50, 1992.
- 533 5. **Braun B, Mawson JT, Muza SR, Dominick SB, Brooks GA, Horning MA, Rock PB, Moore LG,
534 Mazzeo RS, Ezeji-Okoye SC, Butterfield GE.** Women at altitude: carbohydrate utilization during
535 exercise at 4,300 m. *J Appl Physiol* 88: 246–56, 2000.
- 536 6. **Buchheit M, Richard R, Doutreleau S, Lonsdorfer-Wolf E, Brandenberger G, Simon C.** Effect of
537 acute hypoxia on heart rate variability at rest and during exercise. *Int J Sports Med* 25: 264–9,
538 2004.
- 539 7. **Burk A, Timpmann S, Kreegipuu K, Tamm M, Unt E, Oöpik V.** Effects of heat acclimation on
540 endurance capacity and prolactin response to exercise in the heat. *Eur. J. Appl. Physiol.* (March 13,
541 2012). doi: 10.1007/s00421-012-2371-3.
- 542 8. **Cheung SS, McLellan TM, Tenaglia S.** The thermophysiology of uncompensable heat stress.
543 Physiological manipulations and individual characteristics. *Sports Med* 29: 329–59, 2000.
- 544 9. **Chomczynski P, Sacchi N.** Single-step method of RNA isolation by acid guanidinium thiocyanate-
545 phenol-chloroform extraction. *Anal Biochem* 162: 156–9, 1987.
- 546 10. **Corbett J, Neal RA, Lunt HC, Tipton MJ.** Adaptation to Heat and Exercise Performance Under
547 Cooler Conditions: A New Hot Topic. *Sports Med.* (June 19, 2014). doi: 10.1007/s40279-014-
548 0212-8.
- 549 11. **Dempsey JA, Hanson PG, Henderson KS.** Exercise-induced arterial hypoxaemia in healthy
550 human subjects at sea level. *J Physiol* 355: 161–75, 1984.
- 551 12. **Drust B, Waterhouse J, Atkinson G, Edwards B, Reilly T.** Circadian rhythms in sports
552 performance--an update. *Chronobiol Int* 22: 21–44, 2005.
- 553 13. **Duncan RF.** Inhibition of Hsp90 function delays and impairs recovery from heat shock. *FEBS J*
554 272: 5244–56, 2005.
- 555 14. **Ely BR, Lovering AT, Horowitz M, Minson CT.** Heat acclimation and cross tolerance to hypoxia:
556 Bridging the gap between cellular and systemic responses. *Temperature* 1: 107–114, 2014.
- 557 15. **Erlejman AG, Lagadari M, Toneatto J, Piwien-Pilipuk G, Galigniana MD.** Regulatory role of the
558 90-kDa-heat-shock protein (Hsp90) and associated factors on gene expression. *Biochim Biophys*
559 *Acta* 1839: 71–87, 2014.
- 560 16. **Fehrenbach E, Niess AM, Veith R, Dickhuth H.** Changes of HSP72-expression in leukocytes are
561 associated with adaptation to exercise under conditions of high environmental temperature. 69:
562 747–754, 2001.

- 563 17. **Fehrenbach E, Veith R, Schmid M, Dickhuth H-H, Northoff H, Niess AM.** Inverse response of
564 leukocyte heat shock proteins and DNA damage to exercise and heat. *Free Radic Res* 37: 975–82,
565 2003.
- 566 18. **Fox RH, Goldsmith R, Kidd DJ, Lewis HE.** Acclimatization to heat in man by controlled elevation
567 of body temperature. *J Physiol* 166: 530–47, 1963.
- 568 19. **Freeman BC, Morimoto RI.** The human cytosolic molecular chaperones hsp90, hsp70 (hsc70)
569 and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. *EMBO J*
570 15: 2969–79, 1996.
- 571 20. **Fudge BW, Pringle JSM, Maxwell NS, Turner G, Ingham S a, Jones AM.** Altitude training for elite
572 endurance performance: a 2012 update. *Curr Sports Med Rep* 11: 148–54, 2012.
- 573 21. **Galloway SDR, Maughan RJ.** Effects of ambient temperature on the capacity to perform
574 prolonged cycle exercise in man. *Med Sci Sport Exerc* 29: 1240–1249, 1997.
- 575 22. **Garrett AT, Goosens NG, Rehrer NJ, Patterson MJ, Harrison J, Sammut I, Cotter JD.** Short-term
576 heat acclimation is effective and may be enhanced rather than impaired by dehydration. *Am J Hum*
577 *Biol* 26: 311–320, 2014.
- 578 23. **Garrett AT, Rehrer NJ, Patterson MJ.** Induction and decay of short-term heat acclimation in
579 moderately and highly trained athletes. *Sport Med* 41: 757–71, 2011.
- 580 24. **Gibson OR, Dennis A, Parfitt T, Taylor L, Watt PW, Maxwell NS.** Extracellular Hsp72
581 concentration relates to a minimum endogenous criteria during acute exercise-heat exposure. *Cell*
582 *Stress Chaperones* 19: 389–400, 2014.
- 583 25. **Gibson OR, Mee JA, Taylor L, Tuttle JA, Watt PW, Maxwell NS, Taylor L, Watt PW, Maxwell NS.**
584 Isothermic and fixed-intensity heat acclimation methods elicit equal increases in Hsp72 mRNA.
585 *Scand J Med Sci Sports* 25: 259–268, 2015.
- 586 26. **Gibson OR, Mee JA, Tuttle JA, Taylor L, Watt PW, Maxwell NS.** Isothermic and fixed intensity
587 heat acclimation methods induce similar heat adaptation following short and long-term
588 timescales. *J Therm Biol* 49-50: 55–65, 2015.
- 589 27. **Gore CJ, Sharpe K, Garvican-Lewis L a, Saunders PU, Humberstone CE, Robertson EY,**
590 **Wachsmuth NB, Clark S a, McLean BD, Friedmann-Bette B, Neya M, Pottgiesser T,**
591 **Schumacher YO, Schmidt WF.** Altitude training and haemoglobin mass from the optimised
592 carbon monoxide rebreathing method determined by a meta-analysis. *Br J Sports Med* 47 Suppl 1:
593 i31–i39, 2013.
- 594 28. **Heled Y, Peled A, Yanovich R, Shargal E, Pilz-Burstein R, Epstein Y, Moran DS.** Heat
595 Acclimation and Performance in Hypoxic Conditions. *Aviat Space Environ Med* 83: 649–653, 2012.
- 596 29. **Henstridge DC, Whitham M, Febbraio MA.** Chaperoning to the metabolic party: The emerging
597 therapeutic role of heat-shock proteins in obesity and type 2 diabetes. *Mol Metab* 3: 781–93, 2014.
- 598 30. **Hori S.** Adaptation to heat. *Jpn J Physiol* 45: 921–46, 1995.
- 599 31. **Horowitz M.** Heat Acclimation, Epigenetics, and Cytoprotection Memory. *Compr Physiol* 4: 199–
600 230, 2014.
- 601 32. **Karlsen A, Racinais S, Jensen M V., Nørgaard SJ, Bonne T, Nybo L.** Heat acclimatization does
602 not improve VO₂max or cycling performance in a cool climate in trained cyclists. *Scand J Med Sci*
603 *Sports* 25: 269–276, 2015.

- 604 33. **Keiser S, Flück D, Hüppin F, Stravs A, Hilty MP, Lundby C.** Heat training increases exercise
605 capacity in hot but not in temperate conditions: a mechanistic counter-balanced cross-over study.
606 *Am. J. Physiol. Heart Circ. Physiol.* (July 6, 2015). doi: 10.1152/ajpheart.00138.2015.
- 607 34. **Kirwan JP, Costill DL, Kuipers H, Burrell MJ, Fink WJ, Kovaleski JE, Fielding RA.** Substrate
608 utilization in leg muscle of men after heat acclimation. *J Appl Physiol* 63: 31–5, 1987.
- 609 35. **Kregel KC.** Heat shock proteins: modifying factors in physiological stress responses and acquired
610 thermotolerance. *J Appl Physiol* 92: 2177–86, 2002.
- 611 36. **Lamprecht M, Oettl K, Schwabberger G, Hofmann P, Greilberger JF.** Protein modification
612 responds to exercise intensity and antioxidant supplementation. *Med Sci Sports Exerc* 41: 155–63,
613 2009.
- 614 37. **Lee BJ, Mackenzie RWA, Cox V, James RS, Thake CD.** Human Monocyte Heat Shock Protein 72
615 Responses to Acute Hypoxic Exercise after 3 Days of Exercise Heat Acclimation. *Biomed Res Int* 72:
616 1–16, 2014.
- 617 38. **Levi E, Vivi A, Hasin Y, Tassini M, Navon G, Horowitz M.** Heat Acclimation Improves Cardiac
618 Mechanics and Metabolica Performance Duing Ischemia and Reperfusion. *J Appl Physiol* 75: 833–
619 839, 1993.
- 620 39. **Lorenzo S, Halliwill JR, Sawka MN, Minson CT.** Heat acclimation improves exercise
621 performance. *J Appl Physiol* 109: 1140–7, 2010.
- 622 40. **Lunt HC, Barwood MJ, Corbett J, Tipton MJ.** Cross-adaptation: the effect cold habituation has on
623 the physiological responses to acute hypoxia in humans. *J Physiol* 588: 3605–13, 2010.
- 624 41. **Magalhães FDC, Amorim FT, Passos RLF, Fonseca MA, Oliveira KPM, Lima MRM, Guimarães
625 JB, Ferreira-Júnior JB, Martini ARP, Lima NR V, Soares DD, Oliveira EM, Rodrigues LOC.** Heat
626 and exercise acclimation increases intracellular levels of Hsp72 and inhibits exercise-induced
627 increase in intracellular and plasma Hsp72 in humans. *Cell Stress Chaperones* 15: 885–95, 2010.
- 628 42. **Maloyan A, Eli-berchoer L, Semenza GL, Gerstenblith G, Stern MD, Horowitz M, Ger- G.** HIF-
629 1alpha-targeted pathways are activated by heat acclimation and contribute to acclimation-
630 ischemic cross-tolerance in the heart. *Physiol Genomics* 23: 79–88, 2005.
- 631 43. **Maloyan A, Palmon A, Horowitz M.** Heat acclimation increases the basal HSP72 level and alters
632 its production dynamics during heat stress. *Am J Physiol* 276: R1506–15, 1999.
- 633 44. **Marshall HC, Campbell SA, Roberts CW, Nimmo MA.** Human physiological and heat shock
634 protein 72 adaptations during the initial phase of humid-heat acclimation. *J Therm Biol* 32: 341–
635 348, 2007.
- 636 45. **McClung JP, Hasday JD, He J-RR, Montain SJ, Chevront SN, Sawka MN, Singh IS.** Exercise-heat
637 acclimation in humans alters baseline levels and ex vivo heat inducibility of HSP72 and HSP90 in
638 peripheral blood mononuclear cells. *Am J Physiol Regul Integr Comp Physiol* 294: R185–91, 2008.
- 639 46. **Millet GP, Roels B, Schmitt L, Woorons X, Richalet J-P.** Combining Hypoxic Methods for Peak
640 Performance. *Sport Med* 40: 1–25, 2010.
- 641 47. **Moseley PL.** Heat shock proteins and heat adaptation of the whole organism. *J Appl Physiol* 83:
642 1413–1417, 1997.
- 643 48. **Palmer BF, Clegg DJ.** Ascent to altitude as a weight loss method: The good and bad of hypoxia
644 inducible factor activation. *Obesity (Silver Spring)* 22: 311–7, 2014.

- 645 49. **Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC.** HIF-1 mediates adaptation to hypoxia
646 by actively downregulating mitochondrial oxygen consumption. *Cell Metab* 3: 187–97, 2006.
- 647 50. **Patterson MJ, Stocks JM, Taylor NAS.** Sustained and generalized extracellular fluid expansion
648 following heat acclimation. *J Physiol* 559: 327–34, 2004.
- 649 51. **Poirier MP, Gagnon D, Friesen BJ, Hardcastle SG, Kenny GP.** Whole-Body Heat Exchange during
650 Heat Acclimation and Its Decay. *Med. Sci. Sports Exerc.* (May 27, 2014). doi:
651 10.1249/MSS.0000000000000401.
- 652 52. **Powers SK, Lawler J, Dempsey JA, Dodd S, Landry G.** Effects of incomplete pulmonary gas
653 exchange on VO₂ max. *J Appl Physiol* 66: 2491–5, 1989.
- 654 53. **Racinais S, Alonso JM, Coutts AJ, Flouris AD, Girard O, González-Alonso J, Hausswirth C, Jay
655 O, Lee JKW, Mitchell N, Nassis GP, Nybo L, Pluim BM, Roelands B, Sawka MN, Wingo JE,
656 Périard JD.** Consensus recommendations on training and competing in the heat. *Scand J Med Sci
657 Sports* 25: 6–19, 2015.
- 658 54. **Regan JM, Macfarlane DJ, Taylor NA.** An evaluation of the role of skin temperature during heat
659 adaptation. *Acta Physiol Scand* 158: 365–75, 1996.
- 660 55. **Roberts MF, Wenger CB, Stolwijk JA, Nadel ER.** Skin blood flow and sweating changes following
661 exercise training and heat acclimation. *J Appl Physiol* 43: 133–7, 1977.
- 662 56. **Salgado RM, White AC, Schneider SM, Mermier CM.** A Novel Mechanism for Cross-Adaptation
663 between Heat and Altitude Acclimation: The Role of Heat Shock Protein 90. *Physiol J* 2014: 1–12,
664 2014.
- 665 57. **Sawka MN, Burke LM, Eichner ER, Maughan RJ, Montain SJ, Stachenfeld NS.** American College
666 of Sports Medicine position stand. Exercise and fluid replacement. *Med Sci Sports Exerc* 39: 377–
667 90, 2007.
- 668 58. **Sawka MN, Leon LR, Montain SJ, Sonna LA.** Integrated physiological mechanisms of exercise
669 performance, adaptation, and maladaptation to heat stress. *Compr Physiol* 1: 1883–928, 2011.
- 670 59. **Schmidt W, Prommer N.** The optimised CO-rebreathing method: a new tool to determine total
671 haemoglobin mass routinely. *Eur J Appl Physiol* 95: 486–495, 2005.
- 672 60. **Semenza GL, Wang GL.** A nuclear factor induced by hypoxia via de novo protein synthesis binds
673 to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol
674 Cell Biol* 12: 5447–54, 1992.
- 675 61. **Senay LC, Mitchell D, Wyndham CH.** Acclimatization in a hot, humid environment: body fluid
676 adjustments. *J Appl Physiol* 40: 786–796, 1976.
- 677 62. **Shein N a, Horowitz M, Alexandrovich AG, Tsenter J, Shohami E.** Heat acclimation increases
678 hypoxia-inducible factor 1alpha and erythropoietin receptor expression: implication for
679 neuroprotection after closed head injury in mice. *J Cereb Blood Flow Metab* 25: 1456–65, 2005.
- 680 63. **Stringer W, Wasserman K, Casaburi R, Pórszász J, Maehara K, French W.** Lactic acidosis as a
681 facilitator of oxyhemoglobin dissociation during exercise. *J Appl Physiol* 76: 1462–7, 1994.
- 682 64. **Taipale M, Jarosz DF, Lindquist S.** HSP90 at the hub of protein homeostasis: emerging
683 mechanistic insights. *Nat Rev Mol Cell Biol* 11: 515–28, 2010.

- 684 65. **Taylor L, Midgley A, Christmas B.** The effect of acute hypoxia on heat shock protein 72 expression
685 and oxidative stress in vivo. *Eur J Appl Physiol* 109: 849–55, 2010.
- 686 66. **Taylor L, Midgley AW, Christmas B, Hilman AR, Madden LA, Vince R V, McNaughton LR.** Daily
687 hypoxia increases basal monocyte HSP72 expression in healthy human subjects. *Amino Acids* 40:
688 393–401, 2011.
- 689 67. **Taylor L, Midgley AW, Christmas B, Madden LA, Vince R V, McNaughton LR.** Daily quadratic
690 trend in basal monocyte expressed HSP72 in healthy human subjects. *Amino Acids* 38: 1483–8,
691 2010.
- 692 68. **Turner G, Pringle JSM, Ingham SA, Fudge BW, Richardson AJ, Maxwell NS.** The influence of
693 carbon monoxide bolus on the measurement of total haemoglobin mass using the optimized CO-
694 rebreathing method. *Physiol Meas* 35: N11–9, 2014.
- 695 69. **Turner G, Richardson AJ, Maxwell NS, Pringle JSM.** Comparison of total haemoglobin mass
696 measured with the optimized carbon monoxide rebreathing method across different
697 Radiometer™ ABL-80 and OSM-3 hemoximeters. *Physiol Meas* 35: N41–9, 2014.
- 698 70. **Tuttle JA, Castle PC, Metcalfe AJ, Midgley AW, Taylor L, Lewis MP.** Downhill running and
699 exercise in hot environments increase leukocyte Hsp 72 (HSPA1A) and Hsp90α (HSPC1) gene
700 transcripts. *J Appl Physiol* 118: 996–1005, 2015.
- 701 71. **Vissing K, Bayer ML, Overgaard K, Schjerling P, Raastad T.** Heat shock protein translocation
702 and expression response is attenuated in response to repeated eccentric exercise. *Acta Physiol*
703 196: 283–293, 2009.
- 704 72. **Wehrlin JP, Hallén J.** Linear decrease in VO2max and performance with increasing altitude in
705 endurance athletes. *Eur J Appl Physiol* 96: 404–412, 2006.
- 706 73. **White AC, Salgado RM, Schneider S, Loeppky J a, Astorino T a, Mermier CM.** Does Heat
707 Acclimation Improve Exercise Capacity at Altitude? A Cross-tolerance Model. *Int. J. Sports Med.* (
708 May 9, 2014). doi: 10.1055/s-0034-1368724.
- 709 74. **Yamada PM, Amorim FT, Moseley P, Robergs R, Schneider SM.** Effect of heat acclimation on
710 heat shock protein 72 and interleukin-10 in humans. *J Appl Physiol* 103: 1196–204, 2007.

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712

713 **Figure and Table legends**

714

715 Figure 1. Mean \pm SD Rectal Temperature ($^{\circ}\text{C}$) during 10 days of heat acclimation (HA, n =8) and
716 normothermic exercise (EX, n = 8).

717

718 Figure 2. Mean \pm SD Heart rate (HR; top) Oxygen saturation (SpO_2 ; middle) and oxygen pulse ($\dot{\text{V}}\text{O}_2/\text{HR}$;
719 bottom) during rest and whilst exercising at 40% normoxic $\dot{\text{V}}\text{O}_{2\text{peak}}$ and 65% $\dot{\text{V}}\text{O}_{2\text{peak}}$ in hypoxia ($\text{FiO}_2 = 0.12$)
720 before (HYP1; clear bars) and after (HYP2; filled bars) heat acclimation (HA; left, n = 8) or normothermic
721 exercise (CON; right, n = 8). * denotes significant difference from Hyp1 within condition ($p < 0.05$).

722

723 Figure 3. Mean \pm SD Respiratory exchange ratio (RER) during rest and whilst exercising at 40% normoxic
724 $\dot{\text{V}}\text{O}_{2\text{peak}}$ and 65% $\dot{\text{V}}\text{O}_{2\text{peak}}$ in hypoxia ($\text{FiO}_2 = 0.12$) before (HYP1; clear bars) and after (HYP2; filled bars) heat
725 acclimation (HA; left, n = 8) or normothermic exercise (CON; right, n = 8). * denotes significant difference
726 from Hyp1 within condition ($p < 0.05$).

727

728 Figure 4. Mean \pm SD Hsp72 (top) and Hsp90 α (bottom) mRNA pre and post Day 1 (left) and Day 10 (right)
729 of heat acclimation (HA; clear bars, n = 7) and normothermic exercise controls (CON; filled bars, n = 8). *
730 denotes significant difference from pre within Day and Intervention ($p < 0.05$). # denotes significant
731 difference from CON within Time and Day ($p < 0.05$).

732

733 Figure 5. Mean \pm SD Hsp72 (top) and Hsp90 α (bottom) mRNA in hypoxia ($\text{FiO}_2 = 0.12$) before (HYP1; clear
734 bars) and after (HYP2; filled bars) heat acclimation (HA; left, n = 7) or normothermic exercise (CON; right,
735 n = 8). * denotes significant difference from Pre within condition and HYP ($p < 0.05$). † denotes significant
736 difference from Pre overall ($p < 0.05$).

737

738 Table 1. Mean \pm SD. Summary of protocol and physiological data recorded throughout rest and exercise of
739 ten sessions of heat acclimation (HA) or control (CON). * denotes significant difference from CON
740 ($p < 0.05$)

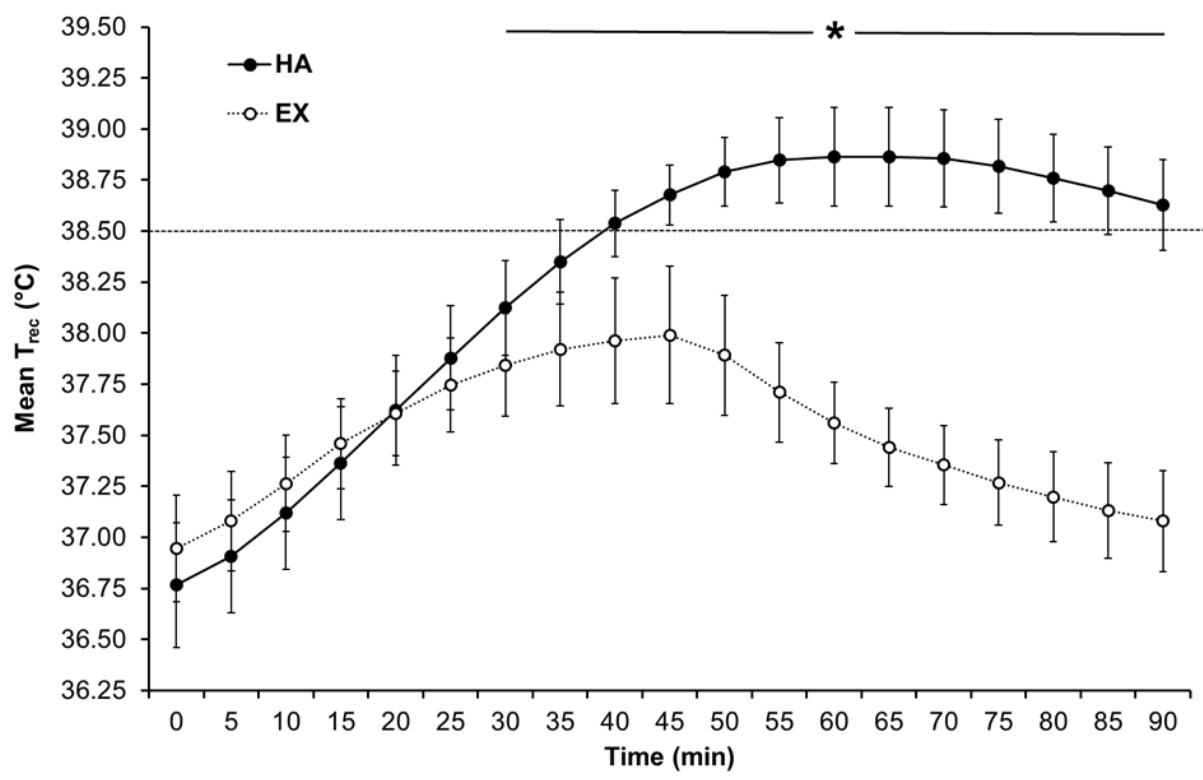
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742 Table 2. Mean \pm SD Comparison of Day 1 and Day 10 (T_{rec} , HR, and SR) and Pre – Post intervention data
743 (Plasma Volume, Hb_{mass} , Blood Volume, Plasma Osmolarity) for Heat Acclimation (HA) and Control (CON)
744 groups. * denotes significant difference from CON within day ($p < 0.05$). # denotes significant difference from
745 day 1 within group ($p < 0.05$)

746

747 Table 3. Mean \pm SD Comparison of physiological and perceptual data at rest and whilst exercising at 40%
748 normoxic $\dot{\text{V}}\text{O}_{2\text{peak}}$ and 65% $\dot{\text{V}}\text{O}_{2\text{peak}}$ in hypoxia ($\text{FiO}_2 = 0.12$) before (Hyp1) and after (Hyp2) heat
749 acclimation (HA, n = 8) or normothermic exercise (CON, n = 8). * denotes significant difference from HYP1
750 within condition ($p < 0.05$).

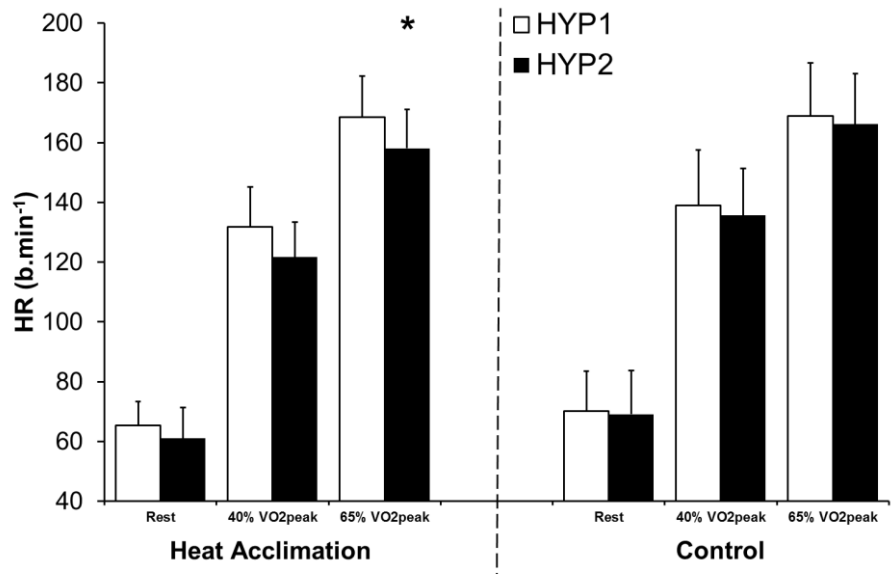
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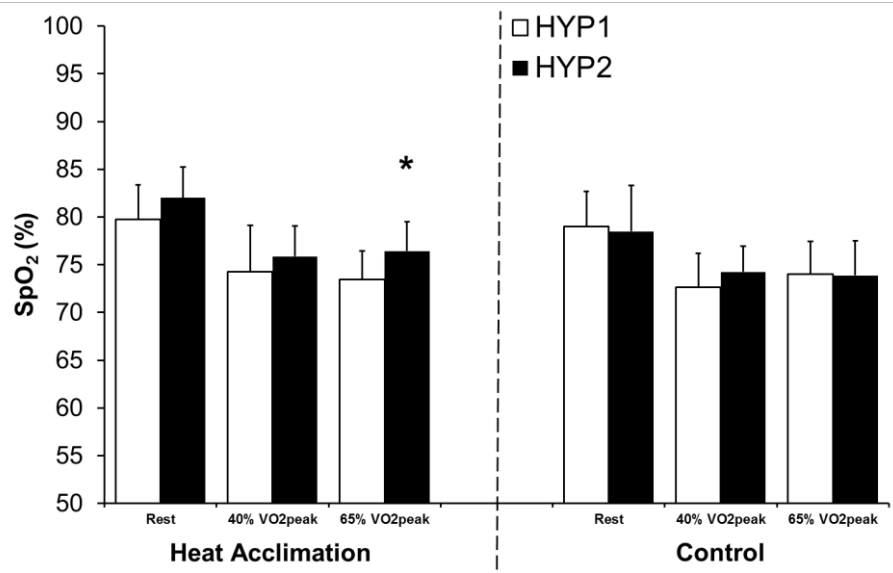
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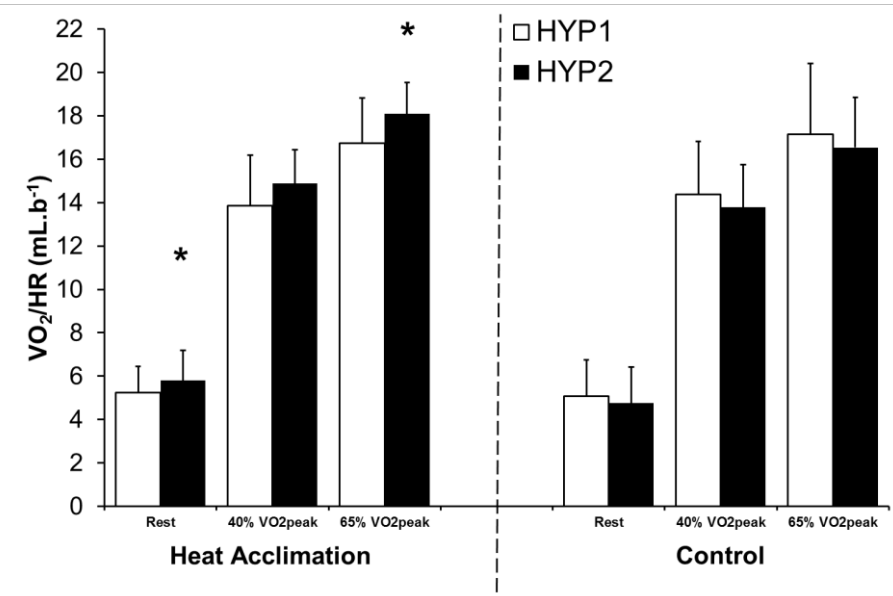
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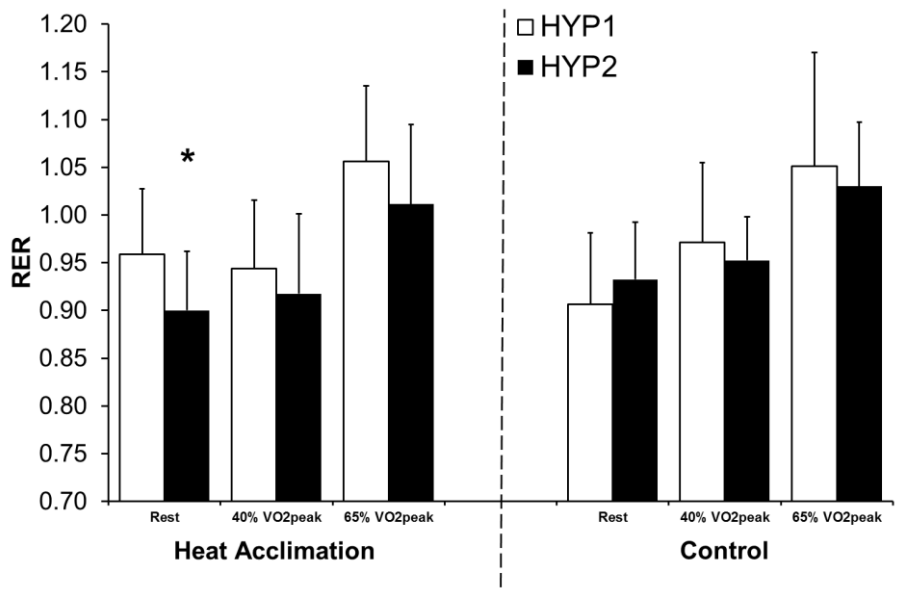
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Figure 2.

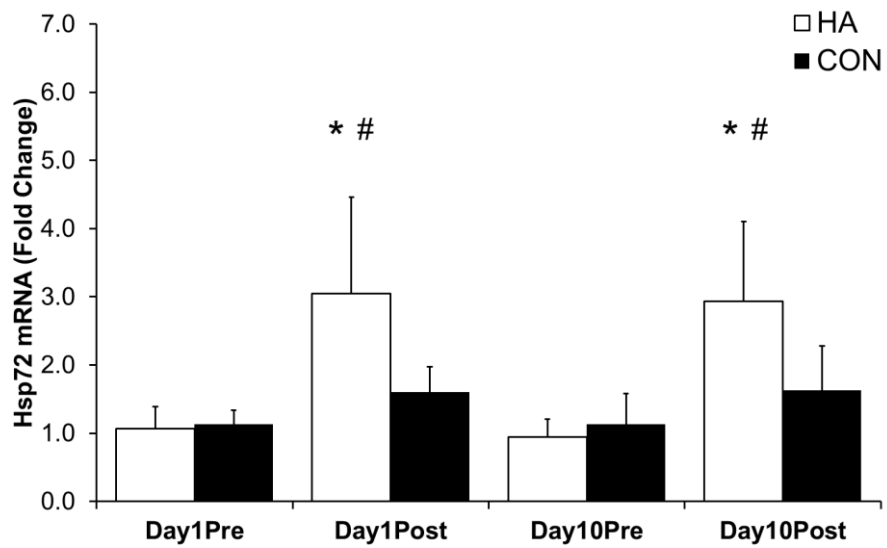


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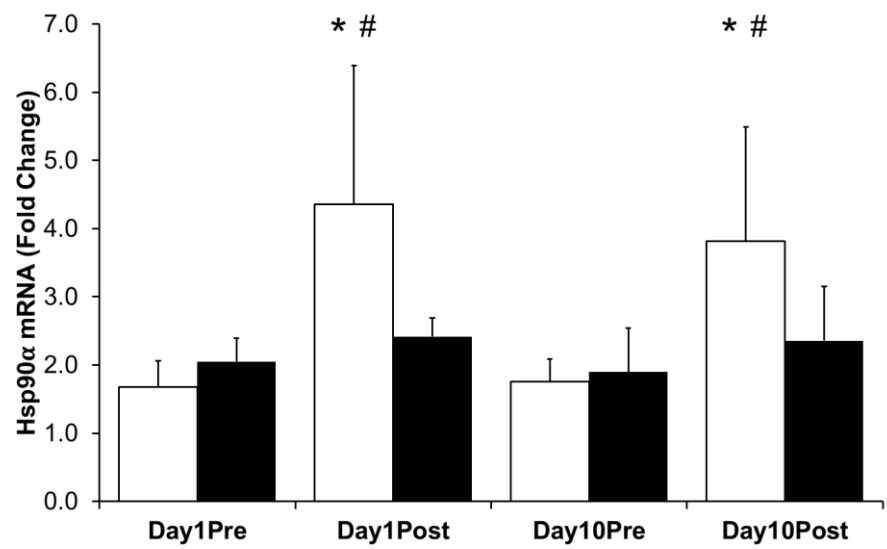
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761 Figure 3.

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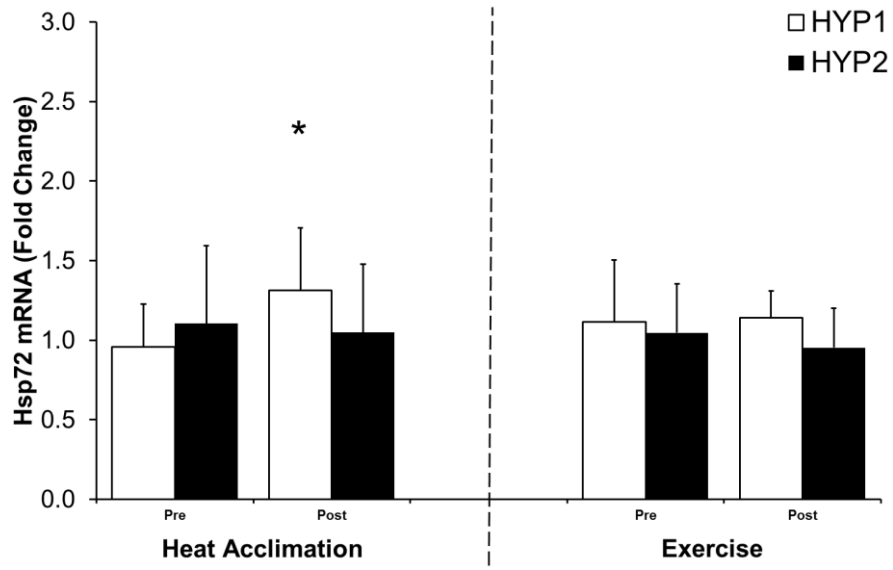
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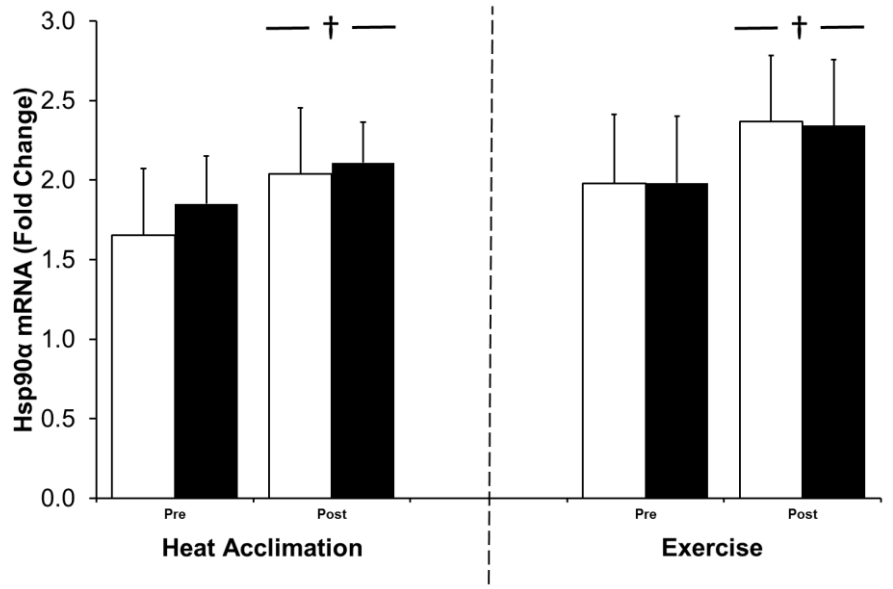
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765 Figure 4.

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768

769 Figure 5.

770 Table 1.

	HA (n = 8)	CON (n = 8)
Exercising Duration (min)	48.4 ± 3.7	47.5 ± 1.1
Total Work Done (kJ)	500.9 ± 101.8	510.0 ± 113.1
Mean T _{rec} (°C)	38.32 ± 0.11 *	37.55 ± 0.21
Duration T _{rec} ≥ 38.5 °C (min)	48.6 ± 9.1*	1.8 ± 2.3
Rate of T _{rec} increase (°C.hr ⁻¹)	2.72 ± 0.39 *	1.38 ± 0.39
Sweat Rate (L.hr ⁻¹)	1.44 ± 0.32 *	0.51 ± 0.18
Mean HR (b.min ⁻¹)	136 ± 14 *	112 ± 14
Mean RPE	11.4 ± 1.2 *	10.0 ± 0.8
Mean TSS	6.4 ± 0.6 *	4.5 ± 0.3

771

772 Table 2.

	Day 1 / Pre		Day 10 / Post	
	HA (n = 8)	CON (n = 8)	HA (n = 8)	CON (n = 8)
Resting T _{rec} (°C)	36.97 ± 0.25	36.99 ± 0.32	36.48 ± 0.29 # *	36.93 ± 0.28
Resting HR (b.min ⁻¹)	74 ± 13	68 ± 14	56 ± 8 # *	66 ± 9
Sweat Rate (L.hr ⁻¹)	1.13 ± 0.28 *	0.45 ± 0.20	1.67 ± 0.42 # *	0.59 ± 0.24
Hb _{mass} (g.kg ⁻¹)	869 ± 92	865 ± 110	869 ± 96	857 ± 126
Plasma Volume (mL)	2981 ± 335	3142 ± 530	3427 ± 335 #	3107 ± 622
Blood Volume (mL)	5627 ± 501	5686 ± 847	6129 ± 550 #	5611 ± 1032

773

774 Table 3.

	HYP1 - Rest		HYP2 - Rest		HYP1 - 40%		HYP2 - 40%		HYP1 - 65%		HYP2 - 65%	
	HA	CON	HA	CON	HA	CON	HA	CON	HA	CON	HA	CON
$\dot{V}O_2$ (L.min ⁻¹)	0.34 ± 0.06	0.34 ± 0.05	0.35 ± 0.05	0.31 ± 0.02	1.82 ± 0.32	1.98 ± 0.44	1.78 ± 0.25	1.85 ± 0.32	2.85 ± 0.45	2.88 ± 0.61	2.85 ± 0.28	2.73 ± 0.38
\dot{V}_E (L.min ⁻¹)	10.5 ± 2.3	10.4 ± 1.8	10.2 ± 1.4	9.9 ± 0.9	54.0 ± 12.5	62.0 ± 16.3	50.7 ± 10.5	57.0 ± 10.4	116.1 ± 27.4	124.6 ± 33.2	108.7 ± 17.6	115.7 ± 19.9
B_f (br.min ⁻¹)	13 ± 3	14 ± 2	14 ± 3	15 ± 1	25 ± 4	29 ± 6	25 ± 2	30 ± 5	40 ± 5	48 ± 12	39 ± 4	48 ± 10
RPE	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	9.4 ± 1.9	12.3 ± 1.8	10.1 ± 1.6	12.6 ± 2.2	16.4 ± 2.2	17.4 ± 1.1	15.8 ± 1.3	17.4 ± 0.9
LLQ	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.4	0.3 ± 0.5	0.1 ± 0.4	0.1 ± 0.4	0.8 ± 1.2	1.0 ± 2.4	0.1 ± 0.4	0.6 ± 1.2

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