Cross acclimation between heat and hypoxia: Heat acclimation improves cellular tolerance and exercise performance in acute normobaric hypoxia

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30   **Abstract**

31   **Background.** The potential for cross acclimation between environmental stressors is not well

32   understood. Thus the aim of this investigation was to determine the effect of fixed-

33   workload heat or hypoxic acclimation on cellular, physiological and performance

34   responses during post acclimation hypoxic exercise in humans. **Method.** Twenty-one

35   males (age 22 ± 5 years; stature 1.76 ± 0.07m; mass 71.8 ± 7.9kg; $\dot{V}O_2$ peak $51 \pm$

36   7mL$\cdot$kg$^{-1}\cdot$min^{-1}) completed a cycling hypoxic stress test (HST) and self-paced 16.1km

37   time trial (TT) before (HST1, TT1), and after (HST2, TT2) a series of 10 daily 60 min

38   training sessions (50% N $\dot{V}O_2$peak) in control (CON, n = 7; 18°C, 35%RH), hypoxic

39   (HYP, n = 7;) or hot (HOT, n = 7; 40°C, 25% RH) conditions. **Results.** TT

40   performance in hypoxia was improved following both acclimation treatments, HYP (-

41        $3{:}16 \pm 3{:}10$ mins:sec; p = 0.0006) and HOT (-$2{:}02 \pm 1{:}02$ mins:sec; p = 0.005), but

42   unchanged after CON (+$0{:}31 \pm 1{:}42$ mins:sec). Resting monocyte heat shock protein

43   72 (mHSP72) increased prior to HST2 in HOT (62 ± 46%) and HYP (58 ± 52%), but

44   was unchanged after CON (9 ± 46%), leading to an attenuated mHSP72 response to

45   hypoxic exercise in HOT and HYP HST2 compared to HST1 (p < 0.01). Changes in

46   extracellular hypoxia-inducible factor 1-α followed a similar pattern to those of

47   mHSP72. Physiological strain index (PSI) was attenuated in HOT (HST1 = $4.12 \pm$

48   0.58, HST2 = 3.60 ± 0.42; p = 0.007) as a result of a reduced HR (HST1 = 140 ± 14

49   b.min^{-1}; HST2 131 ± 9 b.min^{-1} p = 0.0006) and T$_{rectal}$ (HST1 = 37.55 ± 0.18°C; HST2

50   37.45 ± 0.14°C; p = 0.018) during exercise. Whereas PSI did not change in HYP

51   (HST1 = 4.82 ± 0.64, HST2 4.83 ± 0.63). **Conclusion.** Heat acclimation improved

52   cellular and systemic physiological tolerance to steady state exercise in moderate

53   hypoxia. Additionally we show, for the first time, that heat acclimation improved

54   cycling time trial performance to a magnitude similar to that achieved by hypoxic

55   acclimation.

56   **Key words. Heat, hypoxia, cross-acclimation, cycling, heat shock proteins**

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Introduction

Adaptation to one environmental stressor can induce protective responses upon exposure to other stressors as long as they share common adaptive responses (Fregly, 2011). This phenomenon is termed cross-acclimation, when physiological strain is attenuated (Ely et al., 2014), or cross-tolerance, when improved cellular protection is observed (Kregel, 2002). At a cellular level acute heat (Fehrenbach et al., 2001;Lee et al., 2014a;Périard et al., 2015) and hypoxic stress (Taylor et al., 2011;Lee et al., 2014a) induce the heat shock response (HSR; Morimoto 1998), characterized by a transient post exposure increase in the cytoprotective heat shock protein 72 (HSP72). Additionally, acclimation to either heat or hypoxia induces phenotypic alterations that increased expression of genes encoding for cytoprotective HSPs (Maloyan et al., 1999;McClung et al., 2008;Gibson et al., 2015c), leading to greater cellular resilience in the face of subsequent stressful insults (Levi et al., 1993;Hutter et al., 1994).

Enhanced HSP72 following heat acclimation is associated with delayed tissue injury during acute heat stress (Horowitz et al., 2004) and contributes to cross tolerance between heat and ischemic stressors (Maloyan and Horowitz, 2005). In humans supplemented with quercetin - a potent inhibitor of the heat shock response, post heat acclimation thermotolerance was reduced (Kuennen et al., 2011). This was characterized by a diminished cellular stress marker response alongside an attenuated physiological adaptation characterized by physiological parameters, illustrating the functional role of the HSR at a whole body level. However, Hom et al., (2012) demonstrated a heat acclimated phenotype in the absence of HSP72 elevation, suggesting that increased HSP72 alone may not always be present in heat acclimation. In rodent models, increased hypoxia-inducible factor 1-α (HIF1-α), the master regulator of oxygen-regulated genes, and downstream indicators of HIF-1α expression such as erythropoietin (EPO) receptor, EPO and vascular endothelial growth factor (VEGF), have been observed after acute heat stress (Na'ama et al., 2005) and heat acclimation (Maloyan et al., 2005;Tetievsky et al., 2008;Assayag et al., 2012;Umschweif et al., 2013) suggesting an interaction between HIF-1α and the HSR during heat acclimation.

Few studies have investigated the potential for heat acclimation to confer cross acclimation and tolerance to acute hypoxic exposures in a human model (Heled et al., 2012;Lee et al.,

92 2014a;Lee et al., 2014b;Gibson et al., 2015c), with no study examining the response ofHIF1-

93 α to a heat acclimation regimen. Both acute exercising exposures to heat and heat combined

94 with hypoxia have been shown to attenuate physiological strain during hypoxic exercise

95 conducted 24 hours following the initial heat exposure (Lee et al 2014a). The same authors

96 also observed reduced exercise heart rates and rectal temperatures, and increased exercise

97 SpO_2 during hypoxic exercise that was preceded by three days of heat acclimation (Lee et al.,

98 2014b). Longer term heat acclimation programs have also led to a reduction in hypoxic

99 exercise HR alongside increased SpO_2 (Heled et al., 2012; Gibson et al., 2015c), and an

100 improved cardiac efficiency ($\dot{V}O_2$/HR referred to as O_2 pulse; Gibson et al., 2015c). In

101 addition to the improvements seen in systemic function, an increase in resting peripheral

102 blood mononuclear cell (PBMC) HSP72 protein (Lee et al., 2014a, 2014b) and HSP72

103 mRNA (Gibson et al., 2015c) have been observed following heat acclimation. Subsequently,

104 the post hypoxic exercise induced increases in the HSP72 response have been shown to be

105 attenuated in heat-acclimated individuals (Lee et al., 2014b; Gibson et al., 2015c). These data

106 support the existence of both cross-acclimation and cross-tolerance in humans.

107

108 While each of these studies included matched load control groups (Lee et al., 2014b;Gibson

109 et al., 2015c), no study has examined cross-acclimation and cross-tolerance in relation to a

110 matched period of hypoxic acclimation. Neither has the effect of heat acclimation on hypoxic

111 exercise performance been determined. Therefore the aim of the present study was to

112 compare the impact of a period of heat acclimation versus hypoxic acclimation on

113 physiological cross-acclimation and cellular cross-tolerance, and exercise performance during

114 a subsequent exposure to acute normobaric hypoxia. It was hypothesized that a prior period

115 of either heat or hypoxic acclimation would reduce physiological strain and improve physical

116 performance when exercising in moderate normobaric hypoxia, with the effects being

117 greatest following hypoxic acclimation. It was also hypothesized that both heat and hypoxic

118 acclimation would increase resting levels of both mHSP72 and eHIF-1α post acclimation.

119

120 **Methods**

121 **Participant characteristics**

122 Participants (n = 21 males; Figure 1) provided written informed consent to take part in the

123 study, which was approved by the Coventry University Ethics review panel. Established

124 confounding variables of smoking, caffeine, glutamine, quercetin, alcohol, and prior thermal,

125 hypoxic and hyperbaric exposures were all controlled in line with previous work (Taylor et

126 al. 2011; Gibson et al., 2014; Lee et al., 2014). Participants were asked not to undertake any

127 other exercise training in the 72 hours leading up to a testing bout and throughout the

128 intervention period. All data collection was conducted in accordance with the standards set

129 out in the Declaration of Helsinki of 1996.

130

131 **Experimental design**

132 All participants attended the laboratory on 17 separate occasions, as outlined in Figure 1.

133 Two preliminary visits enabled height, body mass, estimated body fat via skinfold

134 measurements, and normoxic (N) and hypoxic (H) $\dot{V}O_2$peak tests, separated by at least 5

135 days, to be conducted. Thereafter participants were split into 3 experimental groups (control,

136 CON; heat acclimation, HOT; hypoxic acclimation, HYP) that were matched for N $\dot{V}O_2$peak

137 and training experience (Figure 1). After both N and H $\dot{V}O_2$peak tests had been conducted all

138 participants undertook two hypoxic stress test (HST) familiarisation (FAM) sessions

139 (described below) at least 4 days apart (Lee et al., 2014a;Lee et al., 2014b). To avoid any

140 confounding acclimation to acute hypoxia FAM was conducted under normoxic conditions.

141 At least 7 days after the final FAM session participants completed the first HST.

142

143 **Preliminary visit measurements**

144 Height was measured in the Frankfurt plane using a Harpenden stadiometer (Harpenden

145 Instruments, Burgess Hill, UK), nude body mass determined on an electronic scale (Seca

146 Body, Cranlea and Company, Birmingham, UK) and sum of skinfolds determined from 4

147 sites using a skinfold caliper (Harpenden Instruments, Burgess Hill, UK) as described by

148 Durnin and Womersley (1974).

149 Peak $\dot{V}O_2$ was determined in both N and H conditions on separate days (preliminary visits 1

150 and 2) using an incremental exercise test to volitional exhaustion on a calibrated SRM cycle

5

151 ergometer (Schoberer Rad Meßtechnik, Welldorf, Germany). Hypoxia was generated via a

152 Hypoxicator unit (Hypoxico HYP123 Hypoxicator, New York, USA), that was used to fill a

153 reservoir of three 1000L Douglas bags in series. Participants inspired via a mouthpiece

154 attached to a two-way non-rebreathable valve (Harvard Ltd, Eldenbridge, UK) connected to

155 the gas reservoir with clear ethylene vinyl tubing.

156 Resting blood lactate (BLa; Biosen C-Line analyser, EKF Diagnostics, Sailauf, Germany)

157 was determined from a finger capillary whole blood sample following a 10-min seated rest

158 period. The test began at a workload of 70 W for 4 min and was then increased by 35 W

159 every 4 min until a blood lactate value of >4 mmol·L^{-1} was reached. Thereafter, workload

160 increased 35 W every 2 min until volitional exhaustion. A cadence of 70 rev·min^{-1} was

161 maintained throughout. Expired gases were collected using 200 L Douglas bags (Cranlea &

162 Co, Birmingham, UK) during the final minute of each stage. Heart rate (Polar FT1, Polar

163 Electro OY, Kempele, Finland) and perceived exertion (Borg, 1976) were recorded at the end

164 of each gas collection. Respiratory gas analysis was completed as previously described (Lee

165 et al., 2014a;Lee et al., 2014b).

166

Familiarisation, hypoxic stress testing (HST) and acclimation procedures

168 On each FAM and HST session, as well as throughout the acclimation period, participants

169 reported to the laboratory after an overnight fast to consume a set breakfast 2 hours prior to

170 the exercise bout. The energy content of the breakfast was 386kcal, made up of 15.6g protein,

171 44.4g carbohydrate and 16.4g fat. Participants drank 400ml of water with the breakfast.

172

173 Each FAM, HST and acclimation session was preceded by 15 min of seated normoxic rest

174 (after instrumentation) to collect baseline data and an additional 15 min of seated rest within

175 the defined environment (N or H). The FAM and HST sessions consisted of 40 min of cycle

176 exercise at 50% N$\dot{V}O_2$peak, a 5 min recovery in which instruments were removed from

177 participants, followed by a 16.1km cycling time trial. The time trial has a CV and TEM of

178 0.63% and 36 seconds respectively following 2 FAM sessions. The smallest worthwhile

179 change in TT time using this protocol is therefore a 46 second difference (Lee et al., 2015).

180 The 10-day acclimation protocol consisted of once daily exposures of cycle ergometer

181 exercise within the defined environment, either CON (18°C, 35% RH), HOT (40°C, 25%

182 RH) or HYP (18°C, 35% RH, $F_IO_2 = 0.14\%$) at 50% N $\dot{V}O_2$peak for 60 minutes (Castle et al.,

183 2011).

184

185 **Physiological measurements**

186 Prior to each testing session participants provided a urine sample for the assessment of urine

187 specific gravity (USG; Atago Refractomer, Jencons Pls, Leighton Buzzard, UK) and urine

188 osmolality (U_{OSMO}; Advanced 3300 Micro-Osmometer, Advanced Inc, Massachusetts, USA),

189 determined their nude body mass (Seca, Bodycare, UK) and inserted a rectal thermistor

190 (Grant Instruments, UK) to a depth of 10cm. Heart rate (HR) was monitored throughout each

191 trial via telemetry (Suunto, T6c, Finland). Blood lactate (Biosen C-Line analyser, EKF

192 Diagnostics, Sailauf , Germany) was determined from a finger capillary whole blood sample

193 at the end of the resting period and at the end of exercise for both HST and acclimation

194 sessions. Heat strain was calculated using the physiological strain index (PSI; (Moran et al.,

195 1998) as follows:

196

197 $PSI = 5(T_{rectalT} - T_{rectal0}) \times (39.5 - T_{rectal0})^{-1} + 5(HR_T - HR_0) \times (180 - HR_0)^{-1}$

198

199 Where $T_{rectal0}$ and HR_0 are the initial T_{rectal} and heart rate respectively, and $T_{rectalT}$ and HR_T

200 were obtained at 10 minute intervals during acclimation sessions or HST with the mean

201 exercise value reported, and at each 1km measurement point throughout the TT. The PSI

202 classifies physiological strain between 0 and 10 units, where 0 represents no or very little

203 strain and 10 represents very high strain (Moran et al., 1998).

204

205 During all hypoxic sessions, arterial oxygen hemoglobin saturation (S_PO_2) was measured

206 throughout via a pulse oximeter (WristOx, Nonin Medical Inc, Minnesota, USA).

207 Hemodynamic indices of stroke volume (SV) and cardiac output ($\dot{Q}$) were estimated via

208 arterial waveform measurements obtained from a pneumatic finger cuff attached to the index

209   finger of the right hand (Portapres Model-2, Finapres Medical Systems, Hogehilweg,

210   Amsterdam). The right arm was supported throughout using a sling, and the index finger

211   positioned at a height equivalent to the aorta via palpation of the third intercostal space.

212   Measurements were taken at the end of each resting phase, and for 120 sec every 10 min

213   during the exercise phase, and calibrations performed at the beginning of the rest period and

214   at 8 minute intervals throughout the HST. Ratings of perceived exertion (RPE; (Borg, 1976)

215   and thermal sensation (TS) were collected at 10 min intervals during the 40 min exercise

216   tolerance phase of the test session with the mean exercise value reported.

217

218   The self-paced 16.1km time trial was completed using the SRMwin software's open-ended

219   mode (Version 6.4.2). Participants were instructed to complete the course as quickly as

220   possible and were given no verbal encouragement during the TT. Participants were only able

221   to see the distance they had covered. Measures of HR, SpO_2, T_{rectal}, and power output were

222   collected every kilometer and a fingertip BLa sample collected immediately upon completion

223   of the TT.

224

225   **Blood sampling**

226   Venous blood samples (~7mL) were collected from an antecubital vein into an EDTA treated

227   vacutainer (Vacuetter, Greiner Bio-One, Stonehouse, UK) following the 15 min seated

228   stabilization period before each HST, and on day 1 and 10 of the acclimation period. Post

229   exercise samples were collected immediately after the exercise phase of the 60 min HST

230   exposure was completed, and immediately upon completion of the aforementioned

231   acclimation sessions. Whole venous blood was used to determine hemoglobin via a calibrated

232   B-Haemoglobin Photometer (Hemocue Ltd, Angleholm, Sweden) and heparinized capillary

233   sample tubes were centrifuged (Hawksley Micro Haematocrit Centrifuge, Hawksley and Son,

234   Lancing, UK) to establish haematocrit using a micro haematocrit reader (Hawksley Micro).

235   Both haemoglobin and haematocrit were assessed in triplicate with the mean value reported.

236   These samples were collected to calculate corrected plasma volumes according to the

237   equations of Dill and Costill (1974). A 100μL aliquot was used for the immediate assessment

238   of monocyte HSP72 (mHSP72; described below). The remaining whole blood was

239   centrifuged at 5000rpm for 10 min and plasma aliquots stored at -80°C until assessment of

240 plasma glucose and lactate (Randox Daytona Rx, County Antrim, Ireland), and extracellular

241 HIF-1α (eHIF-1α; Cusabio, BIOTEK, Newark, New Jersey).

242

mHSP72 determination

244 An IgG1 isotype and concentration-matched FITC-conjugated negative control were used in

245 order to assess non-specific binding. Briefly, cells obtained after red cell lysis were fixed and

246 permeabilised (AbD Serotec, Kidlington, UK) and a negative control (FITC, AbD Serotec,

247 Kidlington, UK) or anti-HSP72 antibody (SPA-810, Enzo lifesciences, Exeter, UK) was

248 added to a final concentration of $100\mu g\cdot ml^{-1}$, this was used to label 1×10^{6} cells according to

249 the manufacturer's instructions and then incubated for 30 min in the dark. Samples were then

250 analysed on a BD FACSCalibur (BD Biosciences, Oxford, UK) by flow cytometry with

251 monocytes gated for forward/side scatter properties and further discriminated by CD14

252 expression (Selkirk et al., 2009). Mean florescence intensity (MFI) was then calculated using

253 CellQuest Pro software (BD Biosciences, Oxford, UK) with a total of 15000 cells counted.

254

Extracellular HIF-1α

256 Extracellular HIF-1α, in EDTA plasma, was measured using a pre-prepared sandwich

257 enzyme-linked immunosorbent assay (ELISA) technique (Cusabio BIOTEK, Newark, New

258 Jersey). 100μL of standards and samples were added to each pre-coated well and incubated

259 for 2 hours at 37°C. Standards and samples were then aspirated and 100μL of biotin-antibody

260 and incubated at 37°C for 1 hour. After three 200μL washes with sodium azide-free wash

261 buffer, 100μL of horse radish peroxidase-avidin was added to each well and incubated for 1

262 hour at 37°C. Following a further three wash steps 90μL of TMB substrate was added and

263 incubated at 37°C in the dark before 50μL of stop solution was added. The plate was then

264 read at 570nm and 450nm to enable wavelength correction. The assay's detection range is

265 $62.5 - 4000pg.mL^{-1}$ and limit of detection is $15.6pg.mL^{-1}$. The intra-assay precision was

266 determined from duplicates of standards within the same plate (6.4%) and inter-assay

267 precision determined from standards assessed across plates (8.2%).

268

269 **Statistical analysis**

270 The primary outcome measures of this study were an assessment of whole body

271 cardiovascular, thermoregulatory, metabolic and mHSP72 and eHIF-1α responses to the

272 HST. Time trial completion time was the main variable of interest during the performance

273 component of the HST. In order to control for the false discovery rate and correct for multiple

274 comparisons four families of hypothesis were tested according to the method of Benjamini

275 and Hochberg (1995); 1) Physiological responses to acclimation; 2) Physiological responses

276 to the hypoxic stress tests; 3) Cellular stress responses; 4) Time trial performance responses.

277 All data were checked for normal distribution prior to analysis and tests employing repeated

278 measures were checked for sphericity before analysis with Mauchly's sphericity test. Where

279 sphericity was broken, *p*-values were corrected using the Huynh-Feldt method. Resting and

280 mean exercise data from day 1 and day 10 of the acclimation period, and HST1 and HST2

281 were analyzed using a 2 (time) x 3 (group) mixed effects linear model, with fixed effects for

282 acclimation day.

283 To enable an exploration of pacing strategies during the TT following the acclimation period

284 power output was averaged over each km of the TT and analysed using linear effects mixed

285 models with fixed effects for time and group. HR, T_{rectal} and PSI during the TT were analysed

286 using the same method. Data are reported as mean ± standard deviation for *n* = 7 in each

287 experimental group, unless otherwise stated. Precise *p*-values are reported, and both Cohen's

288 D (with 95% confidence intervals) and partial eta squared ($P\eta^2$) effect sizes are presented to

289 indicate the magnitude of observed effects (Colquhoun, 2014). Cohens D effect sizes of 0.2,

290 0.5 and 0.8 and partial eta squared ($P\eta^2$) effect sizes of 0.01, 0.06 and 0.13 are considered

291 small, medium and large respectively.

292

293 **Results**

294 **Heat acclimation, hypoxic acclimation and exercise control interventions**

295 Physiological and thermoregulatory variables for each experimental group before and after

296 day 1 and day 10 of acclimation are displayed in Table 1. Participants were hydrated prior to

297 each acclimation session, with no between-day or between group differences observed for

298   pre-trial body mass, U_{osmo} or USG. On day 1 of acclimation, mean exercise HR was greater in

299   HOT ($p = 0.01$) and HYP ($p = 0.02$) compared to CON. Additionally, HOT induced a greater

300   mean exercise T_{rectal} and corresponding PSI compared to HYP (both $p < 0.05$) and CON (both

301    $p < 0.001$), with no difference observed between HYP and CON (Table 1).

302   On day 10 of acclimation an interaction effect was observed for resting plasma volume ($f =$

303   12.336, $p < 0.0001$, $\eta p^2 = 0.58$) which increased in HOT ($d = 3.8$, 95% CI = 1.9 to 5.2),

304   decreased in HYP ($d = -1.7$. 95% CI = -3.2 to -0.6) and remained unchanged in CON ($d =$

305   0.7, 95% CI = -0.5 to 1.7). No main effect for time ($f = 3.346$, $p = 0.084$, $\eta p^2 = 0.16$) or time

306   x group interaction ($f = 2.293$, $p = 0.13$, $\eta p^2 = 0.20$) was observed for resting HR, although a

307   large effect size was noted for HOT ($d = -0.73$, 95% CI = -1.86 to 0.31). Resting T_{rectal} was

308   lower following acclimation in both HYP ($d = -0.72$, 95% CI = -1.69 to 0.45) and HOT ($d = -$

309   2.17, 95% CI = -2.3 to -0.1; $p < 0.05$ for both).

310   Following acclimation PSI was reduced in both HYP ($p = 0.001$, $d = -1.0$, 95% CI = -2.2 to

311   0.1) and HOT ($p = 0.005$, $d = -1.95$, 95% CI = -3.4 to -0.8) and was unchanged in CON ($d = -$

312   0.1, 95% CI = -1.2 to 0.9). The reduction in PSI was mediated by a lower mean exercise HR

313   in HYP ($p = 0.01$, $d = -1.1$, 95% CI = -2.3 to 0.0), and a lower mean exercise HR ($p = 0.04$. d

314    $= -1.3$, 95% CI = -2.3 to 0.0) and T_{rectal} ($p = 0.001$, $d = 4.9$, 95% CI = -5.0 to -1.7) in HOT.

315   No change in these variables was observed in CON. A large effect for rate of T_{rectal} change

316   was observed for HOT ($d = -1.8$, 95% CI = -2.6 to -0.3), and not for CON ($d = -0.3$, 95% CI

317    $= -1.4$ to 0.8) or HYP ($d = -0.1$, 95% CI = -1.1 to 1.0), with no main effect for time (F =

318   4.240, $p = 0.05$, $\eta p^2 = 0.19$) or group x time interaction ($f = 1.526$, $p = 0.244$, $\eta p^2 = 0.15$)

319   observed.

320

321   **Monocyte HSP72 before and after acclimation**

322   No between group difference was observed for mHSP72 prior to acclimation (Figure 2A).

323   Following exercise on day 1, mHSP72 was increased in HOT ($p = 0.0015$, $d = 3.8$, 95% CI =

324   1.9 to 5.2) and HYP ($p = 0.0009$, $d = 1.5$, 95% CI = 0.1 to 2.4), but not CON ($p = 0.14$, $d = -$

325   0.3, 95% CI = -0.5 to 0.7; Figure 2A). An inverse relationship between resting mHSP72 and

326   the magnitude of after exercise expression was observed for HOT ($r = -0.88$, $p = 0.009$), with

327   a weaker relationship observed for HYP ($r = -0.52$, $p = 0.23$).

328   Prior to day 10 of acclimation, resting mHSP72 was increased in HOT ($p = 0.0006$, $d = 4.1$,

329   95% CI = 1.4 to 4.4) and HYP ($p = 0.013$, $d = 1.5$, 95% CI = 0.04 to 2.3), and unchanged in

330   CON ($p = 0.21$, $d = -0.4$, 95% = -1.4 to 0.7; Figure 2 B). As a result of the increased resting

331   concentrations of mHSP72, no further after exercise changes in mHSP72 were observed in

332   HOT ($p = 0.53$) or HYP ($p = 0.24$) on day 10 (Figure 2 B). Consequently, the before

333   acclimation relationship between resting mHSP72 and magnitude in after exercise change

334   was reduced in HOT ($r = -0.05$, $p = 0.924$), although similar in HYP ($r = -0.55$, $p = 0.20$).

335

Extracellular HIF-1α before and after acclimation

337   Figure 3 illustrates eHIF1-α concentrations before and after day 1 and day 10 of acclimation.

338   Following exercise on day 1 of acclimation eHIF-1α was increased in HYP (256 ± 290 %, p

339    $= 0.011$, $d = 1.1$, 95% CI = -0.2 to 2.0) and HOT ($103 \pm 162\%$, $p = 0.076$, $d = 0.8$, 95% CI = -

340   0.4 to 2.0), and unchanged in CON ($8 \pm 29\%$, $p = 0.95$, $d = 0.0$, 95% = -1.1 to 1.0, Figure

341   3A). Prior to day 10 of acclimation eHIF-1α was increased in both HYP ($292 \pm 360\%$, $p =$

342   0.02, $d = 1.2$, 95% CI = -0.2 to 2.0) and HOT ($165 \pm 66\%$, $p = 0.031$, $d = 0.80$, 95% = -0.4 to

343   1.8), and unchanged in CON (5 ± 29 %, $p = 0.55$, $d = -0.1$, 95% -1.1 to 1.0, Figure 3B). On

344   day 10 of acclimation eHIF-1α was no different from rest after exercise in HYP ($21 \pm 79\%$, p

345    $= 0.628$) HOT ($19 \pm 33\%$, $p = 0.112$) or CON ($-5 \pm 17\%$, Figure 3B).

346

Hypoxic tolerance tests

348   Following acclimation and immediately prior to the beginning of the HST trial, plasma

349   volume remained elevated in HOT ($p = 0.022$, $d = 1.0$, 95% CI = -0.3 to 1.9), depressed in

350   HYP ($p = 0.056$, $d = -1.1$, 95% CI = -1.9 to 0.3) and unchanged in CON ($p = 0.41$, $d = -0.2$,

351   95% CI = -1.2 to 0.9). Resting physiological and thermoregulatory parameters are displayed

352   in Table 2. No resting physiological variable was affected as a result of the intervention

353   period ($p > 0.05$, Table 2). Table 3 presents the cardiovascular, metabolic, thermoregulatory

354   and perceptual responses to the HST before and after acclimation. HR was lower in HST2

355   compared to HST1 in HOT ($p = 0.006$, $d = -0.6$, 95% CI = -1.8 to 0.4), and unchanged in

356   CON ($p = 0.44$, $d = 0.2$, 95% CI -0.9 to 1.2) and HYP ($p = 0.38$, $d = -0.1$, 95% CI = -1.1 to

357   1.0). Moderate and large effects were observed for an increased SpO_2 in HOT ($p = 0.0015$, d

358   = 0.70 95% CI = -0.5 to 1.8) and HYP (p = 0.023, d = 0.50, 95% CI = -0.7 to 1.4), with no

359   effect observed in CON (p = 0.36   d = 0.0), neither was an interaction effect observed (f =

360   1.69, p = 0.212). A moderate effect was observed for SV in HOT (p = 0.06, d = 0.40, 95% CI

361   = -0.7 to 1.40) but no effect was observed for CON (p = 0.29, d = -0.1, 95% CI = -1.1 to 1.0)

362   or HYP (p = 0.11, -0.4, 95% CI -1.5 to 0.7), and no interaction effect was evident (f = 2.79, p

363   = 0.08). As a result of the increased SV and decreased HR in HOT, and no changes in either

364   component variable for CON and HYP, no interaction effect was observed for cardiac output

365   (f = 0.50, p = 0.65), with small effects observed for CON (d = -0.1, 95% = -1.2 to 1.0), HYP

366   (d = -0.4, 95% CI = -1.5 to 0.7) and HOT (d = -0.2, 95% CI = -1.3 to 0.8). Cardiac efficiency,

367   as determined by O_2 pulse, was improved in HOT (p = 0.01, d = 0.5, 95% CI = -0.6 to 1.5),

368   and unchanged for CON (p = 0.50, d = 0.0, 95% CI = -1.1 to 1.1) and HYP (p = 0.34, d = -

369   0.1, 95% CI = -1.12 to 0.98), although no interaction effect was observed (f = 3.32, p =

370   0.059). No differences were observed between HST1 and HST2 for $\dot{V}_{Emin}$, $\dot{V}O_2$, $\dot{V}CO_2$ or RER

371   (Table 3).

372   An interaction effect was observed for T_{rectal} (f = 5.58, p = 0.013), with T_{rectal} lower during

373   HST2 for HOT (p = 0.002, d = -0.6, 95% CI = -1.7 to 0.5), and unchanged for CON (p =

374   0.28, d = 0.1, 95% CI = -0.9 to 1.20) and HYP (p = 0.17, d = -0.2, 95% CI = -1.2 to 0.9). The

375   attenuated HR and T_{rectal} observed in HOT resulted in a reduced PSI during HST2 (p = 0.007,

376   d = -0.9, 95% CI = -2.1 to 0.2). PSI was unchanged from HST1 to HST2 in CON (p = 0.30, d

377   = -0.1, 95% CI = -1.2 to 0.9) and HYP (p = 0.47, d = 0.02, 95% CI = -1.0 to 1.1).

378   Additionally, the rate of T_{rectal} change was attenuated in HST2 following HOT (p = 0.026, d =

379   -0.44, 95% CI = -1.4 to 0.7) but similar to HST1 in CON (p = 0.26, d = -0.1, 95% = -1.1 to

380   1.0) and HYP (p = 0.49, d = -0.2, 95% CI = -1.2 to 0.9).

381

382   **Monocyte HSP72 responses to acute hypoxia**

383   Figure 4 illustrates the mHSP72 response to hypoxia before (HST1) and after (HST2) the

384   acclimation intervention. An acute bout of hypoxic exercise lead to increased mHSP72 MFI

385   in all groups prior to acclimation (main effect for time, F = 16.65, p < 0.0001; Figure 4, A),

386   and the inverse relationship between resting mHSP72 and post exercise mHSP72 was

387   observed (r = -0.51, p = 0.019 for the combined cohort, n=21). Following acclimation resting

388   mHSP72 was increased in HYP (58 ± 52%, p = 0.014, d = 1.2, 95% CI = -0.02 to 2.2) and

13

389   HOT (63 ± 46%, p = 0.008, d = 3.8, 95% CI = 0.7 to 3.2), remaining unchanged in CON (10

390   ± 46%, p = 0.83, d = -0.1, 95% CI = -1.1 to 0.1; Figure 4B). Consequently, the mHSP72

391   response following HST2 was blunted for HOT (p = 0.26) and HYP (p = 0.18), and was

392   comparable to HST1 in CON (Figure 4B).

393

394   **Extracellular HIF-1α responses to acute hypoxia**

395   Figure 5 illustrates the eHIF-1α response to hypoxia before (HST1) and after (HST2) the

396   acclimation intervention. Prior to acclimation the HST induced a 171 ± 247%, 197 ± 125%

397   and 266 ± 192% increase in eHIF-1α in CON, HYP and HOT respectively (main effect for

398   time, F = 34.59, p < 0.0001; Figure 5A). Following the 10 day acclimation period resting

399   eHIF-1α was elevated in HYP (220 ± 128%, p = 0.002, d = 1.2, 95% CI = -0.2 to 2.0) and

400   HOT (98 ± 92%, p = 0.017, d = 0.8, 95% CI = -0.4 to 1.8), and unchanged in CON (15 ±

401   76%, p = 0.62, d = 0.0, 95% CI = -1.1 to 1.0) (Figure 5 B). Therefore, after acclimation eHIF-

402   1α increased during exercise from rest in CON (241 ± 193%, p = 0.003, d = 2.3, 95% CI =

403   0.3 to 2.7) and HOT (76 ± 101%, p = 0.07, d = 1.8, 95% CI = -0.1 to 2.2), however this

404   response was attenuated in HYP in comparison to HST1 (33 ± 83%, p = 0.30, d = 0.4, 95%

405   CI = -0.7 to 1.4) (Figure 5B).

406

407   **Time trial performance**

408   Table 4 illustrates performance changes for TT1 and TT2. There was no difference in TT

409   times following the intervention in the CON group (TT1, 43:05 min:sec, 95% CI = 40:18 to

410   45:51 min:sec; TT2, 43:27 min:sec, 95% CI = 40:54 to 45:58 min:sec; d = 0.09). The HYP

411   group were quicker in TT2 (41:32 min:sec, 95% CI = 39:01 to 44:03 min:sec) compared to

412   TT1 (44:48 min:sec, 95% CI = 42:02 to 47:33 min:sec; p = 0.006, d = -1.14). The HOT group

413   were also quicker in TT2 (40:41 min:sec, 95% CI = 38:10 to 43:12 min:sec) compared to

414   TT1 (42:43 min:sec, 95% CI = 39:58 to 45:29 min:sec, p = 0.05, d = -0.70).

415   Power output during TT2 was increased from TT1 in the HYP and HOT groups (p < 0.05,

416   Figure 6). Specifically PO was greater during each kilometer in HYP (Figure 6B), and greater

417   between 1-8km and 14-16km in HOT (Figure 6C). HR and T_{rectal} were no different during the

418   TT pre to post intervention in any experimental group ($p > 0.05$, Table 4). PSI was higher in

419   the post intervention TT in the CON ($p = 0.02$) and HYP groups ($p = 0.03$).

420   Upon completion of acclimation, HST2 and TT2, normoxic $\dot{V}O_2$peak was unchanged from

421   pre-intervention values in all groups (CON: pre 51.4 ± 10.0mL.kg^{-1}.min^{-1}, post 51.9 ± 8.6

422   mL.kg^{-1}.min^{-1}; HYP, pre 50.7 ± 4.7 mL.kg^{-1}.min^{-1}, post 51.4 ± 5.4; HOT, pre 52.3 ± 7.1

423   mL.kg^{-1}.min^{-1}, post 53.4 ± 6.5 mL.kg^{-1}.min^{-1}). Additionally, peak power output and power at

424   lactate threshold also remained unchanged between groups upon completion of the

425   experimental period.

426

427   **Discussion**

428   **Summary of main findings**

429   The main finding of this study is that fixed-work heat acclimation reduces physiological

430   strain, as assessed by exercising HR and T_{rectal}, and improves cycling TT performance in

431   acute normobaric hypoxia. Moreover this effect was comparable to the decreased

432   physiological strain and enhanced performance achieved with hypoxic acclimation and

433   occurred despite no post acclimation improvement in $\dot{V}O_2$peak or lactate threshold in any

434   experimental group. Therefore, the hypothesis pertaining to heat acclimation resulting in

435   improved systemic hypoxic tolerance is accepted. At a cellular level both HOT and HYP

436   increased resting mHSP72 prior to the second HST, supporting the hypothesis that both heat

437   and hypoxic acclimation would enhance cellular tolerance. As a result the cellular stress

438   response to hypoxia was blunted in the HYP and HOT groups. Interestingly, eHIF-1α was

439   elevated in both HYP and HOT immediately post exercise after the initial acclimation

440   session. Thereafter an increased resting concentration was only noted following HYP (48

441   hours post HST). Increased baseline eHIF-1α led to a blunted post hypoxic exercise eHIF-1α

442   response in the HYP group, whereas data from the HOT group was equivocal, indicating that

443   further study on eHIF-1α and related downstream markers regulated by this oxygen sensing

444   gene following heat acclimation are warranted.

445   **Heat and hypoxic cross-acclimation**

446   Heat acclimation induced a greater adaptive stimulus at lower levels of metabolic strain, and

447   in a shorter time frame compared to hypoxic acclimation. This occurred despite the HYP

448   group completing sessions at a higher relative intensity ($61 \pm 0.5\%$ of H $\dot{V}O_2$peak). Exercise

449   durations of up to 90 min, as frequently utilized in acclimation protocols (de Castro

450   Magalhães et al., 2010;Gibson et al., 2015b;Gibson et al., 2015c) increase the variability in

451   trial duration between hypoxia and heat stress (Lee et al., 2014a). Therefore matching both

452   cardiovascular strain and exercise duration during the initial phase of acclimation was

453   achieved by using a workload of 50% N $\dot{V}O_2$ peak for 60 min (Lee et al., 2014a). While

454   cardiovascular strain in the HOT and HYP groups were each higher than CON during the

455   initial phases of acclimation, the total physiological strain was greatest in the HOT group as a

456   result of the elevated T_{rectal} (Table 1).

457   It is accepted that the sudomotor and cardiovascular adaptations to heat stress are completed

458   within 7 – 10 days of daily exposure (Garrett et al., 2009;Castle et al., 2011). The typical

459   indicators of heat acclimation, such as reduced exercising HR and T_{rectal} and increased PV

460   and sweat rate were observed in the present study and were similar in magnitude to previous

461   work using an identical heat acclimation protocol (Castle et al., 2011). We are confident

462   therefore that participants attained a heat acclimated state. Mechanistically, an increased

463   vascular filling time mediated by PV expansion is thought to improve cardiovascular stability

464   during exercise-heat stress (Patterson et al., 2004). The observed PV expansion in the present

465   study was maintained until the second HST (+4%, 48 hours after the final acclimation

466   session). Although causality cannot be determined, it is feasible that the greater PV in HST2

467   mediated the increase in exercise SV thereby reducing HR as observed in the HOT group.

468   Additionally, exercise T_{rectal} and PSI were reduced during HST2, likely as a result of the

469   increased sweat rate. While a reduction in exercise heat gain may improve perceptions of

470   exercise difficulty it is unlikely to impact on exercise performance when conditions are

471   compensable (Cheung et al., 2000). Instead, a reduced exercise T_{rectal} induces a leftward shift

472   in the oxyhemoglobin dissociation curve, theoretically enhancing oxygen saturation during

473   exercise (White et al., 2014;Gibson et al., 2015c), which is a more crucial aspect of hypoxic

474   exercise tolerance than maintaining thermal balance in the compensable hypoxic conditions

475   used in the present study. Our results show an improved exercise SpO_2 in the HOT group

476   post acclimation, occurring alongside greater cardiac stability. Both the reduction in HR, and

477   the reduced blood viscosity (PV expansion) allows for a longer pulmonary system red

478   corpuscle transit time, thereby allowing for a more complete heamoglobin re-saturation

479   (Dempsey et al., 1984). An enhancement in PV prior to an altitude sojourn may also mitigate

480   against the carotid-chemoreceptor dependent diuresis, and subsequent reduced PV and

481   associated reductions in left ventricular filling pressure, stroke volume and cardiac output

482   observed during the initial weeks spent at moderate altitude (Dempsey and Morgan 2015).

483   Our results are comparable to those previously observed following a period of isothermic

484   heat acclimation (Gibson et al., 2015c). Isothermic methods of acclimation are suggested to

485   offer a more complete adaptation because session by session workloads are imposed to

486   achieve a target T_{rectal} >38.5°C, thereby maintaining the adaptive stimulus (Fox et al.,

487   1964;Patterson et al., 2004;Taylor and Cotter, 2006;de Castro Magalhães et al., 2010;Gibson

488   et al., 2015a;Gibson et al., 2015b;Gibson et al., 2015c). However, both fixed work and

489   isothermic models of acclimation have been shown to offer comparable levels of acclimation

490   at a systemic (Gibson et al., 2015b) and gene expression level (Gibson et al., 2015a),

491   indicating each method possesses cross-acclimation and cross-tolerance potential.

492   We observed no change in absolute $\dot{V}O_2$, in addition to a reduction in hypoxic exercise HR 48

493   hours after acclimation, indicating an improvement in gross efficiency as determined from

494   oxygen pulse (O_2 pulse). Gibson et al., (2015c) noted similar improvements in O_2 pulse

495   during hypoxic exercise completed 24 hours after the final isothermic acclimation session.

496   Together these data illustrate that both fixed load and isothermic acclimation methods induce

497   a heat acclimated phenotype which also induces similar reductions in cardiovascular and

498   thermoregulatory strain upon exposure to subsequent normobaric hypoxic exercise. We

499   observed no post acclimation change in N $\dot{V}O_2$peak in all experimental groups, thus the

500   possibility that improved physiological strain occurred as a result of an improved post heat

501   acclimation $\dot{V}O_2$peak and subsequent reduction in relative exercise intensity can be

502   discounted (Lorenzo et al., 2010). Unfortunately we did not conduct a post-acclimation

503   hypoxic $\dot{V}O_2$peak test, so the role heat acclimation has on hypoxic aerobic capacity could not

504   be determined.

505   The duration, frequency and total number of intermittent normobaric hypoxic exposures

506   required to acclimate to later normobaric hypoxia is unclear. Our present data indicates that

507   while SpO_2 was increased during exercise in parallel with a decrease in exercising HR on day

508   10 of acclimation, full hypoxic acclimation was unlikely to have been achieved. The time

509   course required to achieve a more complete adaptation to normobaric hypoxia may therefore

510   require either additional exposures, or an extended daily hypoxic exercise duration. For

511   example, intermittent hypobaric hypoxic exposures have reported a 2-3% increase in exercise

512   SpO$_2$, a 9-20 bpm drop in heart rate, and a 150-160mL drop in $\dot{V}O_2$, and a 6.1 minute (16%

513   improvement) in TT performance following 7 daily 4-hour resting exposures (Beidleman et

514   al., 2008). In contrast, the same authors reported no change in performance following a

515   matched experimental approach utilizing normobaric methods (Beidleman et al., 2009). The

516   discrepancy in results was attributed to a loss of ventilatory acclimation during the 60 hour

517   period between the last acclimation session and follow up testing (Beidleman et al., 2009). In

518   the present study it is possible a loss of ventilatory adaptation occurred during the 48 hour

519   period between the last acclimation session and second HST, accounting for the lack of

520   improved physiological tolerance during HST2. The results suggest that heat acclimation

521   offers a more persistent and time efficient means of improving cardiac stability during

522   subsequent normobaric hypoxic exercise. Furthermore, this was attained at a lower level of

523   metabolic strain compared to when the same absolute exercise intensity was conducted in

524   normobaric hypoxia. The optimal duration and frequency required to elicit adaptation to

525   normobaric hypoxia requires further study to enable additional comparisons between

526   environments.

527   Heat and hypoxic cross-tolerance

528   Our data show that a 10 day period of fixed-work exercise acclimation in both heat and

529   hypoxic conditions enhances basal mHSP72 (Figure 2B). Interestingly, the magnitude of

530   mHSP72 accumulation was similar between HYP and HOT despite the greater total

531   physiological strain accrued during heat acclimation (Figure 2C). The time course of HSP72

532   accumulation throughout a period of heat or hypoxic acclimation has not been studied.

533   Therefore, it cannot be determined whether the different levels of physiological strain

534   observed between conditions in the early phase of acclimation leads to a more rapid or more

535   gradual induction of protective cellular processes. An enhanced reserve of HSP72 is one of

536   the hallmarks of cross tolerance observed in rodent models (Maloyan et al., 1999;Horowitz,

537   2007;Horowitz and Robinson, 2007). In humans, acclimation to both heat and hypoxia has

538   been shown to elicit increases in basal HSP72 (McClung et al., 2008;de Castro Magalhães et

539   al., 2010;Taylor et al., 2011;Taylor et al., 2012) suggesting cross-tolerance potential exists.

540   Increased post exercise mHSP72 is likely mediated by an increase in thermal and

541   physiological strain in conditions of heat stress (Lee et al., 2014a;Périard et al., 2015), and a

542 transient increase in oxidative stress under hypoxic stress (Taylor et al., 2011;Taylor et al.,

543 2012). The results from the HOT group are similar to those reported by others using either

544 fixed workload (Yamada et al., 2007;McClung et al., 2008;Hom et al., 2012) or isothermic

545 heat acclimation methods (de Castro Magalhães et al., 2010). Additionally, we observed

546 increases in resting mHSP72 following HYP acclimation, a response also previously

547 observed following 10 daily resting exposures to a similar magnitude of hypoxia (Taylor et

548 al., 2012). However, we are unable to determine whether the increase in mHSP72 observed

549 following hypoxic acclimation was a result of hypoxia *per se*, and the known increases in

550 oxidative stress that occur in such conditions, or whether the increased relative work-load

551 experienced in HYP was the main driver of the enhanced basal mHSP72. The physiological

552 and cellular strain induced in our control group was not sufficient to induce any changes in

553 mHSP72, which may be due to no substantial exercise induced changes in T_{rectal}, nor any

554 exercise induced increase in oxidative stress.

555 It is well established that the regulation of HSP synthesis is dependent on the levels existing

556 within the cell (Kregel, 2002). Consequently, prior to acclimation we observed an inverse

557 relationship between basal mHSP72 and the magnitude of post exercise increase in this

558 protein. After acclimation this relationship was no longer present as a result of the increased

559 presence of mHSP72 within the cell. Under non-stressed conditions HSP72 is bound to

560 HSF1. When the cell is exposed to one of the myriad of stressors that require HSP72

561 chaperone function, HSP72 binds to denatured proteins, freeing HSF1 to migrate to the

562 nucleus and bind with the heat shock element (HSE). More HSP72 protein is then

563 transcribed, and continues to bind with denatured proteins until equilibrium is restored.

564 Excess HSP72 then binds with the HSF1 and transcription is halted (Morimoto, 1998).

565 Therefore, as acclimation progresses and basal HSP72 is accumulated, the cell becomes more

566 robust to the daily challenge to homeostasis imparted via a fixed model of acclimation. As a

567 result, the stress required to sequester HSP72 from HSF1, to begin further transcription, has

568 to cross a new threshold. It is this mechanism of HSP72 action that makes the constant daily

569 strain imparted by isothermic methods of acclimation an attractive model for imparting

570 cellular tolerance (Taylor and Cotter, 2006;Gibson et al., 2015c). However, as our data show,

571 mHSP72 protein is enhanced after 10 days acclimation using a simple fixed workload model,

572 in agreement with the elegant work of Gibson et al., (2015, 2015a). Furthermore, the elevated

573 basal mHSP72 persisted for at least 48 hours after removal from the heat and hypoxic

574 acclimation stimuli, suggesting achievement of a persistent phenotypic shift towards an

19

575 acclimated state. Subsequently, basal mHSP72 was higher before HST2 compared to HST1

576 in both the experimental groups and the post exercise increase in mHSP72 was attenuated in

577 both HOT and HYP. The role of increased cellular tolerance on physiological function

578 requires greater scrutiny, as we show that while both modes of acclimation enhance cellular

579 reserves of mHSP72, only the heat acclimated group demonstrated improved physiological

580 function in later hypoxic exercise. Our data support previous observations pertaining to

581 improved cellular tolerance following heat and hypoxic acclimation (Levi et al.,

582 1993;Maloyan and Horowitz, 2005;Taylor et al., 2012;Lee et al., 2014b;Gibson et al., 2015c).

583 HIF-1α, the global regulator of cellular and systemic oxygen homeostasis, has also been

584 suggested to play an important role in heat and hypoxic cross tolerance in rodent models (

585 Maloyan et al., 2005), with increased concentrations and related gene transcripts observed

586 following heat acclimation (Maloyan et al., 2005). However the role of HIF-1α during

587 acclimation in humans has not been studied. We show that hypoxic acclimation induced a

588 doubling of HIF-1α in the circulation, an increase that was maintained until the second HST.

589 Subsequently, the post HST2 eHIF-1α response was blunted in HYP. We also show an

590 increase in eHIF-1α after the initial heat acclimation session, suggesting that this pathway

591 may be an important mechanism for both heat acclimation and cross-tolerance in humans.

592 However we acknowledge that the source, function and relationship eHIF-1α has with

593 intracellular HIF-1α (iHIF-1α) is presently unknown. Therefore while these results are novel,

594 caution is required in their interpretation. Further research is required to determine the

595 relationship between eHIF-1α and iHIF-1α and other HIF-1α associated genes and circulating

596 markers of hypoxic adaptation (e.g. erythropoietin). Doing so will establish the utility of

597 eHIF-1α as a biomarker of acclimation and cross-acclimation.

598 **Hypoxic time trial performance before and after acclimation**

599 We show, for the first time, that heat acclimation can improve exercise performance under

600 conditions of acute normobaric hypoxia to levels that were comparable to those observed

601 following hypoxic stress. TT performance may have been enhanced following heat

602 acclimation as a result of greater metabolic efficiency and glycogen sparing during the initial

603 40-minute steady-state pre-load trial (Febbraio et al., 2002;Lorenzo et al., 2010).

604 Unfortunately no measurements of muscle glycogen content were possible in the present

605 investigation. The reduced post exercise BLa in the absence of a change in $\dot{V}O_2$peak or lactate

606 thresholds, in combination with improved exercise efficiency (O_2 pulse) indicates a more

607 efficient aerobic profile. Heat acclimation is known to reduce BLa concentrations for a given

608 intensity (Young et al., 1985;Febbraio et al., 1994). In the present study, mean exercise blood

609 lactate was lower in HST2 following both heat and hypoxic acclimation. It has been

610 suggested that an increase in plasma volume following heat acclimation may have an effect

611 on BLa via an increase in blood flow through the splanchnic circulation, thereby enhancing

612 lactate removal and delaying accumulation (Lorenzo et al., 2010). It is possible that such an

613 effect during HST2 may have led to glycogen sparing via a reduced rate of glycogenesis prior

614 to the TT, thereby preserving glycogen reserves and facilitating a greater maintenance of

615 power output during the TT. Alternatively, the increased TT performance may have occurred

616 as a result of a learning effect following the multiple exercise sessions. However, we took

617 care to ensure that participants were familiarized to the TT protocol in advance. In addition,

618 we validated the pre-loaded TT in our laboratory prior to testing using participants with

619 similar characteristics (Lee et al, 2015). Finally, if performance changes were a result of a

620 learning effect we would expect to see a similar effect in the control group. Instead it appears

621 that the experimental groups had an altered pacing strategy as a result of the acclimation

622 period, with systematic kilometer by kilometer increases in power output, HR, and

623 physiological strain observed in the HYP group, and an altered starting and finishing strategy

624 adopted by the HOT group (Figure 6).

625 **Study limitations**

626 The results of the present investigation are relevant only to those individuals with a moderate

627 aerobic capacity and should not be applied to those with elite physiology. However, the

628 maintenance of SpO_2 following either HOT or HYP acclimation is likely of more importance

629 for more well trained individuals, as they typically experience reduced haemoglobin

630 saturation due to typically larger cardiac outputs and reduced time for gas exchange at higher

631 work-rates (Powers et al., 1989). However, examining physiological responses to hypoxia

632 following acclimation to heat is of interest to athletes that undergo hypoxic training camps.

633 The potential use of heat acclimation to increase ability to tolerate greater work-rates upon

634 arrival to altitude could allow for the maintenance of training volumes and intensities during

635 the initial sessions. However, the role prior acclimation to heat has on longer term hypoxic

636 adaptation has yet to be explored. Additionally, our data only examined normobaric hypoxia.

637 It is possible that responses under ecologically valid hypobaric hypoxic conditions could be

638 different thus future study is required.

Wider application of our results

Our data indicate that heat based exercise offers a more efficient systemic acclimation response to hypoxia in the time frame examined than normobaric hypoxic training offers, which may have relevance to athletes and military personnel requiring a time-effective means of increasing work capabilities in conditions of moderate hypoxia. An enhancement in cardiac efficiency following repeated heat exposures may be desirable in military populations or individuals sojourning to moderate altitude for short durations without the means or time to fully acclimatize before completing work tasks. Additionally, implementing a hyperthermic stimulus to elicit cross-acclimation responses can be achieved with little specialist equipment compared to the chambers or sojourns required to enable altitude adaptation.

Conclusion

We show, for the first time, that heat acclimation can improve exercise performance under conditions of acute normobaric hypoxia to levels that were comparable to those observed following hypoxic acclimation. Fixed work heat acclimation is shown to be an effective intervention when improvements in hypoxic exercise tolerance or endurance performance are required. It was demonstrated that heat acclimation was a more effective and longer term (48 hours post acclimation) means of improving systemic hypoxic tolerance, as quantified by exercise HR and SpO_2, than a matched duration and work period of hypoxic acclimation. Both heat and hypoxic acclimation elicit similar changes at the protein level, with each increasing basal HSP72 and eHIF-1α, along with attenuation of post HST HSP72 and eHIF-1α responses to an acute bout of hypoxic exercise. These data confirm previous findings using isothermic models of acclimation and illustrate that increasing physiological strain via exercise-heat stress is an effective, and simple to administer, intervention for invoking both cross-acclimation and cellular cross-tolerance in humans.

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Table 1. Cardiovascular, thermoregulatory and perceptual responses at rest and during exercise for day 1 and day 10 of the acclimation period. Data are means ± SD for all 21 participants.

Variable	Day 1			Day 10		
	CON	HYP	HOT	CON	HYP	HOT
USG	1.004 ± 0.01	1.020 ± 0.01	1.020 ± 0.01	1.010 ± 0.01	1.010 ± 0.01	1.020 ± 0.01
U_{OSMO} (mOsms·kg^{-1})	553 ± 254	465 ± 239	444 ± 234	320 ± 105	331 ± 176	463 ± 217
Haemoglobin (g/dL^{-1})	15.1 ± 0.9	14.9 ± 0.6	15.5 ± 1.0	14.9 ± 1.0	15.4 ± 0.4	14.8 ± 0.9[*]
Haemotocrit	0.44 ± 0.01	0.45 ± 0.02	0.44 ± 0.02	0.44 ± 0.02	0.43 ± 0.02	0.45 ± 0.01
Plasma volume (%)	55.8 ± 1.2	55.6 ± 2.3	55.9 ± 2.3	56.9 ± 2.0	53.3 ± 1.1	59.3 ± 2.2[#]
HR rest (bts·min^{-1})	71 ± 11	71 ± 13	78 ± 15	70 ± 10	71 ± 14	67 ± 11
T_{rectal} rest (°C)	37.04 ± 0.19	37.09 ± 0.18	37.19 ± 0.12	37.09 ± 0.12	36.96 ± 0.21	36.93 ± 0.26[#]
Mean HR (bts·min^{-1})	135 ± 11	151 ± 13[+]	151 ± 11[^]	135 ± 12	137 ± 9[#]	137 ± 12[*]
Mean T_{rectal} (°C)	37.76 ± 0.25	37.86 ± 0.45	38.26 ± 0.11[+ ψ]	37.73 ± 0.22	37.70 ± 0.21	37.72 ± 0.18[#]
Mean PSI (AU)	4.39 ± 0.84	5.14 ± 0.78	5.91 ± 0.66[^ ψ]	4.26 ± 0.85	4.35 ± 0.61[#]	4.62 ± 0.48[#]
Δ T_{rectal} (°C)	0.72 ± 0.30	0.77 ± 0.44	1.08 ± 0.17[^ ψ]	0.63 ± 0.25	0.77 ± 0.25	0.78 ± 0.22[*]
Δ Body mass (kg)	0.76 ± 0.36	0.84 ± 0.38	1.01 ± 0.60[^ ψ]	0.70 ± 0.31	0.87 ± 0.34	1.90 ± 0.31[#^ ψ]
Mean RPE	12 ± 1	12 ± 2	12 ± 2	11 ± 1	10 ± 2	11 ± 2
Mean TS	4.8 ± 0.2	4.7 ± 0.6	6.3 ± 0.4[+ ψ]	4.4 ± 0.5	4.1 ± 0.6	5.4 ± 0.4[*]

* different from acclimation day 1 (p < 0.05) within group.

different from acclimation day 1 (p < 0.01) within group.

+ different from CON (p < 0.05)

^ different from CON p (< 0.01)

ψ different from HYP (p < 0.05)

860　**Table 2.** Cardiovascular and thermoregulatory measurements at the end of the resting period

861　during HST1 and HST2. Data are means ± SD for all 21 participants.

Variable	HST 1			HST 2		
	CON	HYP	HOT	CON	HYP	HOT
Haemoglobin (g.dL^{-1})	15.2 ± 0.70	14.5 ± 0.3	15.5 ± 1.4	15.2 ± 0.9	15.2 ± 0.8	15.1 ± 1.3
Haematocrit (%)	0.44 ± 0.02	0.45 ± 0.01	0.46 ± 0.03	0.45 ± 0.02	0.44 ± 0.02	0.46 ± 0.02
Plasma volume (%)	55.2 ± 2.3	55.2 ± 2.31	53.9 ± 2.1	55.6 ± 2.1	52.7 ± 3.4*	55.8 ± 2.5*
HR (bts·min^{-1})	79 ± 8	81 ± 11	82 ± 16	82 ± 12	79 ± 18	79 ± 11
Plasma lactate (mmol^{-1})	1.51 ± 0.36	1.73 ± 0.52	1.55 ± 0.47	1.57 ± 0.41	1.44 ± 0.29	1.49 ± 0.57
Plasma glucose (mmol^{-1})	5.01 ± 0.97	4.81 ± 1.36	5.40 ± 0.99	4.87 ± 0.80	4.62 ± 0.52	4.54 ± 0.63
Stroke Volume (mL·bt^{-1})	79.9 ± 9.0	78.2 ± 14.8	78.1 ± 15.0	78.1 ± 11.5	77.2 ± 12.2	78.0 ± 18.2
Cardiac Output (L·min^{-1})	6.3 ± 1.0	6.3 ± 1.1	6.3 ± 1.1	6.4 ± 1.6	6.1 ± 1.5	6.2 ± 1.8
SpO$_2$ (%)	89 ± 2	89 ± 2	89 ± 3	89 ± 4	89 ± 3	91 ± 2
$\dot{V}_E$ (L·min^{-1})	15.4 ± 4.0	13.7 ± 2.0	16.0 ± 2.5	15.4 ± 3.2	14.3 ± 1.6	16.5 ± 2.7
$\dot{V}O_2$ (L·min-1)	0.36 ± 0.11	0.30 ± 0.07	0.36 ± 0.06	0.36 ± 0.11	0.32 ± 0.06	0.38 ± 0.12
T$_{rectal}$ (°C)	37.14 ± 0.15	37.02 ± 0.15	37.11 ± 0.20	37.19 ± 0.17	37.14 ± 0.16	37.08 ± 0.15

862　* different from HST1 ($p < 0.05$) within group.

863 **Table 3.** Mean exercise cardiovascular, metabolic, thermoregulatory and perceptual measurements

864 during HST1 and HST2. Data are mean ± SD for all 21 participants.

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Variable	HST 1			HST 2		
	CON	HYP	HOT	CON	HYP	HOT
HR (bts·min^{-1})	139 ± 5	145 ± 15	140 ± 14	140 ± 8	144 ± 16	131 ± 9[#]
Stroke volume (mL·bt^{-1})	104 ± 12	112 ± 13	99 ± 10	103 ± 11	107 ± 8	103 ± 11
Cardiac output (L·min^{-1})	14.5 ± 1.8	16.1 ± 1.9	13.8 ± 1.3	14.3 ± 1.9	15.8 ± 1.4	13.5 ± 1.1
SpO$_2$ (%)	82 ± 4	82 ± 2	83 ± 3	82 ± 3	83 ± 3[*]	85 ± 2[#]
Oxygen pulse (mL·bt^{-1})	12.6 ± 2.3	11.8 ± 1.3	11.5 ± 1.4	12.6 ± 2.1	11.7 ± 1.4	12.2 ± 1.4[*]
VE BTPS (L·min^{-1})	60.8 ± 10.4	63.2 ± 10.1	60.8 ± 5.0	59.2 ± 12.6	65.0 ± 9.0	58.8 ± 3.2
$\dot{V}O_2$ (L·min^{-1})	1.76 ± 0.34	1.71 ± 0.26	1.60 ± 0.10	1.77 ± 0.35	1.68 ± 0.22	1.60 ± 0.14
$\dot{V}CO_2$ (L·min^{-1})	1.69 ± 0.31	1.71 ± 0.23	1.57 ± 0.13	1.65 ± 0.38	1.66 ± 0.28	1.52 ± 0.12
RER	0.96 ± 0.06	1.00 ± 0.06	0.98 ± 0.06	0.93 ± 0.08	0.98 ± 0.09	0.95 ± 0.06
Plasma lactate (mmol^{-1})	3.44 ± 1.42	3.88 ± 2.08	3.25 ± 1.56	3.31 ± 1.39	3.03 ± 1.09	2.50 ± 0.87
Plasma glucose (mmol^{-1})	4.87 ± 1.13	5.07 ± 0.71	4.50 ± 0.98	4.65 ± 1.08	4.90 ± 1.16	4.77 ± 0.68
T_{rectal} (°C)	37.61 ± 0.14	37.72 ± 0.18	37.55 ± 0.18	37.63 ± 0.11	37.69 ± 0.20	37.40 ± 0.14[#]
ΔT_{rectal} (°C)	0.46 ± 0.19	0.70 ± 0.12	0.44 ± 0.36	0.44 ± 0.20	0.68 ± 0.20	0.32 ± 0.20[*]
PSI (AU)	4.2 ± 0.5	4.8 ± 0.6	4.1 ± 0.6	4.1 ± 0.6	4.8 ± 0.6	3.6 ± 0.4[#]
RPE	13 ± 1	12 ± 2	12 ± 2	12 ± 1	10 ± 2	11 ± 1
TS	3.9 ± 0.5	4.2 ± 0.8	4.3 ± 0.8	3.7 ± 0.4	4.0 ± 0.4	4.0 ± 0.4

866 * different from HST1 to HST2 (p < 0.05) within group

867 ♯ different from HST1 to HST2 (p < 0.01) within group

868   **Table 4.** Individual data and mean ± SD data for performance variables during TT1 and TT2.

	Performance Time (mins)		Percent change	Heart rate (bts.min^{-1})		T_{rectal} (°C)		PSI (AU)	
CON	TT1	TT2		TT1	TT2	TT1	TT2	TT1	TT2
1	42.06	42.08	**0.05%**	161	160	37.94	38.14	4.94	5.73
2	41.03	40.46	**-1.39%**	159	165	37.73	38.10	5.52	6.20
3	51.21	50.59	**-1.21%**	140	156	37.94	37.74	4.14	4.91
4	39.41	38	**-3.58%**	178	183	38.22	38.23	6.62	7.57
5	41.44	44	**6.18%**	148	153	38.03	38.61	4.79	6.14
6	41.54	41	**-1.30%**	173	173	38.00	38.00	6.41	6.25
7	45	48.11	**6.91%**	177	175	38.01	38.04	6.48	6.48
Mean + SD	**43.1 ± 4.2**	**43.5 ± 4.3**	**0.8 ± 3.3**	**162 ± 15**	**166 ± 11**	**37.98 ± 0.14**	**38.12 ± 0.26**	**5.56 ± 0.97**	**6.18 ± 0.80**
HYP									
8	43	42.47	**-1.23%**	131	136	37.83	37.70	5.28	4.76
9	47.48	41.32	**-12.97%**	127	126	37.80	37.95	4.84	6.58
10	41.34	39.3	**-4.93%**	166	170	38.32	38.74	7.06	8.27
11	45.21	41.36	**-8.52%**	154	154	37.94	37.97	5.46	5.94
12	41.55	39.09	**-5.92%**	137	160	37.66	38.31	4.71	5.53
13	51.59	43.19	**-16.28%**	158	157	38.25	38.25	5.57	6.49
14	43.46	44.1	**1.47%**	171	173	38.03	38.12	5.83	6.19
Mean + SD	**45.0 ± 4.0**	**41.5 ± 1.7**	**-6.9 ± 5.5**	**157 ± 13**	**162 ± 15**	**37.98 ± 0.24**	**38.15 ± 0.33**	**5.54 ± 0.78**	**6.25 ± 1.09**
HOT									
15	39	37.35	**-4.23%**	168	170	38.08	38.04	6.94	6.86
16	41.12	40.12	**-2.43%**	174	175	38.05	37.92	6.37	6.27
17	43.4	42	**-3.23%**	162	157	38.23	37.90	6.72	5.82
18	46.45	43.26	**-6.87%**	149	147	38.03	37.93	4.88	5.37
19	45.45	44.25	**-2.64%**	176	184	38.12	38.11	6.26	7.01
20	40.46	38.32	**-5.29%**	151	156	37.80	37.80	4.31	5.35
21	43.25	39.55	**-8.55%**	172	173	38.18	38.33	5.97	6.72
Mean + SD	**42.7 ± 2.9**	**40.7 ± 2.8**	**-4.8 ± 1.7**	**164 ± 11**	**166 ± 13**	**38.07 ± 0.14**	**38.00 ± 0.17**	**5.92 ± 0.97**	**6.20 ± 0.70**

869 **Figure Legends**

870 **Figure 1.** Schematic of the experimental design, anthropometric and physiological characteristics of
871 participants, indicating the typical days on which specific tests were undertaken.

872 **Figure 2.** Monocyte HSP72 responses before and after the acclimation period. **A:** mHSP72 is
873 increased post exercise on day 1 of HYP and HOT, but not CON. **B:** Resting mHSP72 was
874 unchanged in CON and increased in HYP and HOT on day 10 of acclimation compared to day 1 of
875 acclimation. Subsequently, the post exercise mHSP72 response in HYP and HOT was attenuated
876 compared to post exercise on day 1. **C:** The magnitude of change in resting mHSP72 on day 10 of
877 acclimation was no different between HYP and HOT (C). Open bars and shaded bars represent pre
878 and post exercise, respectively. Lines (A and B) and dots (C) represent individual participant
879 responses (n = 21) and bars show the mean group response. The dashed line (C) represents baseline
880 mHSP72. * different from day 1 pre-exercise (p < 0.01). # different from control (p < 0.01).

881 **Figure 3**. Extracellular HIF-1α responses before and after the acclimation period. **A:** eHIF-1α is
882 increased following an acute period of hypoxic exercise and is more variable following HOT. No
883 post exercise changes in eHIF-1α were seen in CON **B:** Resting eHIF-1α was elevated after 10 days
884 of HYP and HOT acclimation, blunting the post exercise response on day 10 of acclimation. No
885 changes in eHIF-1α were observed in CON. **C:** The magnitude of change in resting eHIF-1α on day
886 10 of acclimation was no different between HYP and HOT. Open bars and shaded bars represent pre
887 and post exercise, respectively. Lines (A and B) and dots (C) represent individual participant
888 responses (n = 21) and bars the mean group response. The dashed line (C) represents baseline HIF-
889 1α. + different from day 1 rest (p < 0.05). # different from control (p < 0.01). ^ different from day 1
890 pre exercise (p < 0.10).

891 **Figure 4.** Monocyte HSP72 before and after HST1 and HST2. **A:** mHSP72 is increased after a HST
892 in 20 of 21 participants. **B:** Resting mHSP72 was increased prior to onset of HST2 in HYP and HOT.
893 The post exercise increase in mHSP72 was subsequently only observed in CON. **C:** The magnitude
894 of change in resting mHSP72 prior to HST2 was not different between HYP and HOT and were each
895 elevated in comparison to CON. Lines (A and B) and dots (C) represent individual participant
896 responses and bars the mean group response (n = 21). The dashed line (C) represents baseline
897 mHSP72. † different from pre-exercise (p < 0.01). ¥ different from HST1 pre-exercise (p < 0.05). *
898 different from HST1 pre-exercise (p < 0.01).

899 **Figure 5.** Extracellular HIF-1α responses before and after HST1 and HST2. **A:** eHIF-1α increased in
900 response to exercise in HST1 in all experimental groups. **B**: Prior to HST2 resting levels of eHIF-1α
901 were elevated in HYP when compared to pre HST1, and showed a varied individual response in HOT
902 .eHIF-1α increased in response to exercise in HST2 in CON, but was unchanged in both HYP and
903 HOT, although individual variation in the data is present. **C:** The magnitude of change in resting
904 eHIF-1α was not different between HYP and HOT prior to HST2, and each were elevated in
905 comparison to CON. Lines (A and B) and dots (C) represent individual participant responses and bars
906 the mean group response. The dashed line (C) represents baseline HIF-1α. Different from rest (p <
907 0.01) within trial * different from HST1 pre-exercise (p < 0.01). ¥ different from HST1 pre-exercise
908 (p < 0.05). Ŧ different from HST2 pre-exercise (p < 0.10).

909 **Figure 6.** Mean power output during each kilometer of the 16.1km time trial for CON (A), HYP (B)
910 and HOT (C). * difference from TT1 (p < 0.05).

Figure 1.TIF
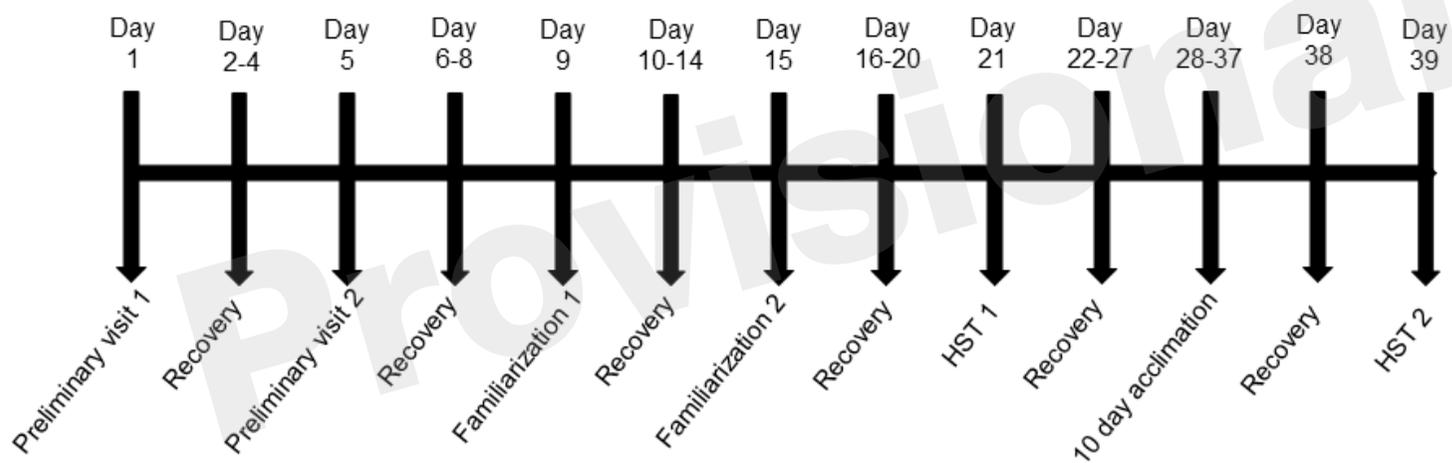
Group	Age (years)	Height (m)	Body mass (kg)	N VO_2peak (mL.kg.min^{-1})	H VO_2peak (mL.kg.min^{-1})	Weekly training (hours)
CON	22 ± 3	1.74 ± 0.08	72.5 ± 11.4	51.4 ± 10.0	41.7 ± 9.8	8.5 ± 3.3
HYP	22 ± 5	1.75 ± 0.06	71.2 ± 2.8	52.3 ± 7.1	40.3 ± 7.1	8.8 ± 3.6
HOT	25 ± 6	1.78 ± 0.08	71.7 ± 9.2	50.7 ± 4.7	41.9 ± 5.7	8.5 ± 3.1

Figure 2.TIF

Group F = 7.999, p = 0.004, Pη^2 = 0.485

Time F = 8.889, p < 0.0001, Pη^2 = 0.343

Group x time F = 4.684, p = 0.001, Pη^2 = 0.355

Figure 3.TIF

Group F = 2.145, p = 0.146, Pη² 0.192

Time F = 8.513, p < 0.0001, Pη² 0.321

Group x time F = 3.569, p = 0.005, Pη² 0.214

Figure 4.TIF

Group F = 0.178, p = 0.838, Pη^2 = 0.0.019

Time F = 16.648, p < 0.0001, Pη^2 = 0.568

Group x time F = 3.544 p = 0.005, Pη^2 = 0.233

Figure 5.TIF

Group F = 0.266, p = 0.769, Pη² = 0.029

Time F = 34.588, p < 0.0001, Pη² = 0.658

Group x time F = 2.544, p = 0.03, Pη² = 0.22

Figure 6.TIF