Title: Effects of 10 days of separate heat and hypoxic exposure on heat acclimation and temperate exercise performance

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Running head: Hypoxia and heat acclimation

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Abstract

Adaptations to heat and hypoxia are typically studied in isolation, but are often encountered in combination. Whether the adaptive response to multiple stressors affords the same response as when examined in isolation is unclear. We examined: i) the influence of overnight moderate normobaric hypoxia on the time course and magnitude of adaption to daily heat exposure; ii) whether heat acclimation (HA) was ergogenic and if this was influenced by an additional hypoxic-stimulus. Eight males ($\dot{V}O_{2max}$=58.5[8.3] mL·kg^{-1}·min^{-1}) undertook two 11-day HA programmes (balanced-crossover design), once with overnight normobaric hypoxia (8[1] h per night; 10 nights; F_IO_2=0.156; S_pO_2=91[2]% [HA_{Hyp}]) and once without (HA_{Con}). Days 1, 6, 11 were exercise-heat stress tests (HST [40°C, 50% RH]); days 2-5 and 7-10 were isothermal-strain (target rectal temperature [T_{re}] ~38.5°C), exercise-heat sessions. A graded exercise test and 30-minute cycle trial were undertaken pre, post and 14-days after HA in temperate-normoxia (22°C, 55% RH; F_IO_2=0.209). HA was evident on day 6 (*e.g.* reduced T_{re}, mean skin temperature [$\bar{T}_{sk}$], heart rate, sweat [Na^+], P<0.05) with additional adaptations on day 11 (further reduced $\bar{T}_{sk}$, heart rate). HA increased plasma volume (+5.9[7.3]%) and erythropoietin concentration (+1.8[2.4] mIU/mL); tHb_{mass} was unchanged. Peak power output (+12[20] W), lactate threshold (+15[18] W) and work done (+12[20] kJ) increased following HA. The additional hypoxic-stressor did not affect these adaptations. In conclusion, a separate moderate overnight normobaric hypoxic-stimulus does not affect the time-course or magnitude of HA. Performance may be improved in temperate-normoxia following HA, but this is unaffected by an additional hypoxic stressor.

Key words (×3-5)

Thermoregulation; Acclimatization; Altitude; Training; Combined-stress

Introduction

Historically, adaptation to environmental stressors has been examined in isolation, yet multiple environmental stressors can be encountered in the natural world, either simultaneously or in close proximity, for instance, heat or cold *and* hypoxia [78]. It cannot be assumed that the adaptive response to multiple stressors affords the same response as when examined in isolation and it has recently been highlighted that three broad types of interaction (additive, synergistic, antagonistic) can occur when combining independent stressors [46]. Consequently, there is a need to better understand adaptations to multiple stressors [78].

Heat acclimation (HA) occurs when core (T_C) and skin temperature (T_{sk}) are frequently and repeatedly elevated to a level challenging thermoeffector responses, commonly as a consequence of exercise-heat stress [*e.g.* 45, 61]. At a systemic level, plasma volume (PV) expansion occurs within ~3 days [74], the resulting hypervolemia increases stroke volume, maximal cardiac output [48], and arterial blood pressure [56], and lowers heart rate for a given work-rate [45, 63]. PV expansion also increases the total specific heat capacity of blood [7], aiding core-skin heat transfer and reducing cutaneous blood-flow requirements [62]. Sudomotor changes (lower threshold and greater sweating sensitivity) are complete after ~10 days [62]. Together these adaptations improve cardiovascular stability [74] and reduce thermal strain (lower T_{sk} and T_C, [21]. There is also evidence of metabolic adaptation, characterized by reduced reliance on carbohydrate metabolism [83] and lower exercise muscle and blood lactate accumulation [48]. At a cellular level, heat exposure activates the heat shock response [42], increasing heat shock protein (HSP70 and HSP90) concentration; these proteins are multi-functional, but are primarily cytoprotective [41, 64]. However, heat exposure may also stimulate the hypoxia inducible factor-1 pathway [4, 44], which primarily controls oxygen-related genes.

Systemic adaptations to hypoxia develop within ~7 to 21 days of living at high-altitude (1,500 m-3,500 m) or intermittent hypoxic exposure [19]. Stimulation of aortic-arch chemoreceptors and carotid bodies increases sympatho-adrenal activity, elevating heart rate, cardiac output, and ventilation [81]. In the early stages of acclimation PV decreases due to diuresis [40] and possibly extra- to intra-cellular fluid shifts [27]. The resultant hypovolemia causes hemoconcentration, increasing oxygen carrying capacity per unit volume [82] and reducing heart rate and cardiac output for a given oxygen demand. Together these effects improve tissue oxygen delivery. With chronic hypoxia, erythropoiesis increases erythrocyte volume (EV) [22], although

reticulocytosis occurs more rapidly [20] and changes in EV may present after removal of the hypoxic stimulus. Metabolically, adaptations to hypoxia may increase reliance on carbohydrate for ATP resynthesis [59] whereas at the cellular-level, hypoxic stress primarily activates the HIF-1 pathway [73], which stimulates a cascade of effects including erythropoiesis, but also induces the heat shock response [42].

Although recent studies have examined the cross-acclimation (attenuated physiological-strain) or cross-tolerance (improved cellular protection) afforded by adaption to heat during subsequent hypoxic exposure [24, 44], the effect of the addition of a hypoxic-stressor on the adaptive response to heat *i.e.* a combined-stressor approach, has received little attention. Although mechanistically important, this question is also practically relevant; athletes often sleep in hypoxic environments (*i.e.* hypoxic tents/nitrogen houses) to try and gain an ergogenic benefit [8], whilst at the same time they may undergo HA prior to competition in a hot environment. Likewise, high ambient temperatures may be encountered at popular high-altitude training venues *e.g.* Colorado (up to 40°C and ~2,000 m). It has been hypothesised that the impact of individual stressors on exercise capacity dictates the interaction; mild stressors producing an additive effect, with a move towards antagonistic interactions as the individual stressors impact increases [46]. Thus, addition of a modest hypoxic stimulus might be hypothesised to potentiate HA. Alternatively it has been suggested that additive effects result from combining stressors with independent mechanisms, whilst interactive effects arise from mechanistically similar stressors [47]. Although there are clearly independent mechanisms by which heat and hypoxic stress elicit adaptation, there are also potential synergies in aspects of the cellular (*e.g.* heat shock response and HIF-1), and systemic (*e.g.* reduced sub-maximal exercise heart rate, improved tissue oxygen delivery) adaptive responses. However, antagonistic effects are also possible; PV is expanded with HA [74], but reduced with hypoxia [68], whereas HA may reduce reliance on glycolysis [37], but this may be increased with hypoxia [29].

An ancillary question which we sought to investigate was whether HA was ergogenic in temperate conditions, and if this was influenced by the addition of hypoxia, *i.e.* a cross-stressor effect between adaptation to heat-hypoxia and performance in temperate-normoxia. Although the ergogenic benefit of hypoxia for endurance exercise is well established [8], the ergogenic potential of HA for prolonged exercise has recently received increased attention (*e.g.* [15]). Although HA could be ergogenic via multiple mechanisms [15] it is suggested that PV expansion is primary among these, due to its positive effect on

cardiac output and $\dot{V}O_{2max}$ [48]. However, other studies have shown no ergogenic effect of HA induced PV expansion [33, 36], possibly due to a hemodilution effect sufficient to offset any increase in cardiac output [16]. Currently it is unclear if the addition of the erythropoietic stimulus of hypoxia is sufficient to offset the hemodilution effect of HA, or whether hypoxia negates normal PV expansion with HA. Although Takeno *et al*. [76] demonstrated increased PV, EV and $\dot{V}O_{2peak}$ with 10-daily exercise bouts (60 min·day^{-1}) in hot, (30°C, 50% RH) hypobaric hypoxic (2,000 m), conditions, these data are limited by the small sample (n=5) and similar adaptations were evident in a cool-normoxic control group, indicating a possible training-effect. Likewise, Buchheit *et al*. [11] reported PV expansion in both normobaric hypoxic (F_IO_2=~0.150; 14±1 h·day^{-1}) and normoxic two-week HA programme (~27 h total heat exposure, ~32°C, 39% RH) although total hemoglobin mass (tHb_{mass}) was increased in the hypoxic condition only. However, these hematological changes were not related to the temperate-normoxic performance improvement following both regimens. More recently, McCleave *et al*. [50] showed a 3.3% improvement in temperate-normoxic 3 km running trial performance three weeks (but not immediately) after completing a 21-day intermittent HA programme. However, the ergogenic effect was absent when normobaric hypoxia was added to the HA programme (F_IO_2=0.144; 13 h·day^{-1}) and although tHb_{mass} did increase with the additional hypoxic stressor, PV expansion was 'possibly less' and the hematological changes were not related to the performance effects.

Accordingly, the aims of the present study were two-fold. First, to examine the addition of a daily hypoxic stimulus on the time course and magnitude of adaption to heat and second, to investigate whether HA was ergogenic under temperate-normoxic conditions, and if this was influenced by the addition of a daily hypoxic stimulus. Our null hypotheses were that the addition of a moderate daily hypoxic stimulus would not affect the time course or magnitude of HA, and would not influence any effect of HA on temperate-normoxic exercise performance.

Materials & Methods

Participants

Sample size was calculated *a priori* using G*Power software; effect size data were derived from the change in exercise T_{re} (η^2=0.16) observed following an identical HA programme (without hypoxia) in our laboratory

[55]. For two-way (Condition × Time) repeated measures analysis of variance with sufficient power ($\beta \geq 0.80$) at an α level of 0.05 a minimum of eight participants was required. Similar sample-size estimates were obtained with effect-size data derived from other key outcome variables, including mean body temperature ($\bar{T}_b$) and heart rate. To account for attrition 12 male participants were recruited; four did not complete the study due to injury (unrelated to study, n=1), illness (n=1) and logistics (n=2). Eight performance level three [17] males (Age: 25[6] years; $\dot{V}O_{2max}$: 58.6[8.9] $mL \cdot min^{-1} \cdot kg^{-1}$; peak power output: 348[53] W) completed this study. Participants were all trained endurance athletes (cyclists/triathletes/runners). The study was approved by the University's Ethics Committee and conformed to the Declaration of Helsinki, and all participants provided written informed consent.

Experimental design

A within-participant, balanced cross-over design was employed, with participants undertaking both control (heat acclimation [HA_{Con}]) and experimental (heat acclimation with hypoxic exposure [HA_{Hyp}]) HA programmes. Each HA programme lasted 11-days and consisted of three bouts of exercise at a fixed external work rate (heat stress test [HST]), undertaken on day 1 (HST_{pre}), day 6 (HST_{mid}) and day 11 (HST_{post}), interspersed with eight isothermal heat strain exercise-heat exposures (ISO). A temperate graded exercise test (GXT) and 30 minute work done trial (T30) were performed before (GXT_{pre}; $T30_{pre}$) and after (GXT_{post}; $T30_{post}$) each HA programme; an additional retention T30 was undertaken 14-days after completing HA ($T30_{ret}$) (

Figure **1**). HA programmes were identical apart from the addition of daily (overnight) normobaric hypoxic exposure in HA_{Hyp}. A minimum three-month wash-out period was prescribed between HA programmes [14] and all testing was completed outside of the UK summertime (average weather conditions: 8.7°C, 77% RH).

INSERT FIGURE 1 HERE

Experimental procedures

Graded Exercise Test

GXTs were performed in a temperate environment (22°C, 50% RH) (pre- and post-HA_{Con} and HA_{Hyp}) on a Lode Excalibur cycle ergometer (Lode B.V. Groningen, the Netherlands). Participants exercised for 20 minutes at 85 or 110 W, dependent upon the estimated fitness of the participant (fixed within-participant for pre-post tests and between-conditions). Thereafter, work-rate was incremented by 25 W every three minutes until blood lactate concentration [Lac] was ≥4 $mmol \cdot L^{-1}$, following which, the participant was given a five minute break before beginning cycling again at 100 W for five minutes. Work-rate was then increased 25 $W \cdot min^{-1}$ until volitional exhaustion. [Lac] was determined from fingertip capillary blood obtained at the end of each exercise stage (Biosen C-line, EKF Diagnostic, Cardiff, UK). Convective cooling was provided at a rate of 3.5 $m \cdot s^{-1}$.

30 Minute maximal cycling trial

T30s were conducted to obtain an index of endurance performance. All trials were performed on a Lode Excalibur cycle ergometer (Lode B.V. Groningen, the Netherlands) in a temperate environment (22°C, 50% RH). After a standardized warm up participants commenced a 30 minute 'all-out' performance trial; 'performance' was defined as the total work done (kJ). A fan provided some convective cooling (3.5 $m \cdot s^{-1}$) to reduce the likelihood of having to end the test early due to reaching withdrawal criteria for T_{re} of 40°C.

Heat Stress Test (HST)

HSTs were completed pre-, mid- and post-HA in both conditions as described previously [54, 55]. Briefly, participants cycled in a hot environment (target ambient conditions: 40°C; 50% RH) on a calibrated COMPUTRAINER™ cycle ergometer (RacerMate Inc., Seattle, WA, USA) for 60 minutes at 35% of peak power output (PPO) reached in the pre-HA GXT. 1.25 L of 3.6% carbohydrate solution (Science in Sport Go Electrolyte drink, Nelson, UK) (drink temperature 20°C) was ingested to replace fluid losses, divided into five equal boluses (0.25 L) and consumed immediately prior to commencing exercise and every 15 minutes thereafter. Convective cooling was provided at a rate of 3.5 $m \cdot s^{-1}$; this prevented participants from reaching the T_{re} withdrawal criteria, whilst maintaining an acceptably high mean skin temperature ($\overline{T}_{sk}$) and allowing thermoeffector responses to be assessed.

Isothermal heat strain sessions (ISO)

178 Participants exercised in a hot environment (target ambient conditions: 40°C; 50% RH) on a calibrated
179 COMPUTRAINER™ cycle ergometer (RacerMate Inc., Seattle, WA, USA), initially selecting a work rate
180 eliciting a rating of perceived exertion (RPE [9]) of 15. This was maintained until T_{re} reached 38.5°C, at
181 which point external power output was adjusted as appropriate to maintain this target temperature (±0.2°C)
182 and a small amount of convective cooling (3 $m \cdot s^{-1}$) was used to facilitate the exercise component and provide
183 some perceptual benefit, whilst maintaining a high T_{sk}. Participants completed eight 90 minute ISO sessions
184 in both the HA_{Con} and the HA_{Hyp} condition and were provided with fluid replacement (7 × 0.25 L, 3.6%
185 carbohydrate, boluses every 15 minutes during ISO sessions).

186 ***Hypoxic exposure***

187 During the HA programme participants in the HA_{Hyp} condition were exposed to nightly moderate normobaric
188 hypoxia (10 nights, 8-10 h exposure per night, $F_IO_2 = 0.156$) comparable to a simulated altitude of ~2,400 m,
189 using 'portable altitude tents' (Hypoxico, New York City, New York, USA). This hypoxic stimulus exceeds
190 the threshold required for erythropoiesis in humans [53], is consistent with the hypoxic stimulus used in
191 previous studies [11, 76] and is similar to the altitude of many popular training camp locations *e.g.* Flagstaff
192 AZ., USA (2,106 m); Sierra Nevada, Spain (2,320 m); Iten, Kenya (2,400 m). Although the hypoxic and
193 heat stimuli were not delivered simultaneously, as might occur with residing at a high altitude training camp,
194 some individuals (athletes) may live or sleep in a hypoxic environment and undertake their training in a
195 normoxic (hot) environment Participants were familiarized with sleeping in the tents (without a reduced PO_2)
196 for several nights prior to commencing HA_{Hyp} to become accustomed to any changes in ambient noise and
197 minimize sleep disturbances. Participants wore a physiological monitoring system (EQUIVITAL™ ,
198 Cambridge, UK) which recorded heart rate (EQO_2 LifeMonitor, EQUIVITAL™, Cambridge, UK) and
199 oxygen saturation (Nonin iPod S_PO_2, EQUIVITAL™, Cambridge, UK) (sampling every 15 seconds, and for
200 two minutes every 10 minutes, respectively) throughout each of the 10-nights.

201 ***General procedures***

202 Participants wore the same clothes on each day, abstained from alcohol throughout the experimental periods
203 or caffeine for 12 hours prior to exercise, consumed a similar diet before each test and drank 0.5 L of water
204 two hours before every attendance. Participants were instructed to maintain their normal high-intensity

205 training (except 24 h before HSTs, GXTs, T30s) and replace an equivalent duration of low/moderate training
206 with that completed in the laboratory to maintain usual training volume. Additionally, participants recorded
207 the number of hours spent in the tent and the evening and morning F_IO_2 (independent reading taken with a
208 calibrated VN202 mkII oxygen analyser, Vandagraph Ltd, Keightly, UK) within the tent each night.

209 To monitor daily hydration status, urine osmolality was assessed prior to exercise (Osmometer 3320,
210 Advanced Instruments Inc., Norwood, MA, USA). Nude body mass (dry) was measured pre- and post- each
211 test session (Industrial Electronic Weight Indicator, Model I10, Ohaus Corporation, Parsippany, NJ, USA);
212 body mass changes were used to determine whole-body sweat rate, adjusted for fluid ingested. Ambient
213 conditions were measured by a WBGT logger (Squirrel 1000, Grant Instruments, Cambridge, UK), T_{re} by a
214 thermistor (Grant Instruments, Cambridge, UK) self-inserted 15 cm beyond the anal sphincter and cardiac
215 frequency (f_c) by short-range telemetry (Polar RS800, Polar Electro, Kempele, Finland). During HSTs and
216 GXTs skin temperature (T_{sk}) was measured using thermistors on the chest, biceps, thigh and calf (Grant
217 Instruments, Cambridge, UK) and local sweat rate at the upper right back (Q-Sweat, WR Medical
218 Electronics, Maplewood, MN, USA) and forearm skin blood flow (MoorLAB, Moor Instruments, Devon,
219 UK) were recorded. During HSTs expired gases (Douglas bag method), RPE [9], thermal sensation [84] and
220 thermal comfort [85] were measured at 15 min intervals. A sample of sweat was collected using a custom
221 patch constructed from TEGADERM™ (TEGADERM™ Dressings, 3M, St. Paul, Minnesota, USA) and
222 PARAFILM® (Bemis NA, Neenah, WI, USA) for determining sodium concentration [Na^+] by flame
223 photometry (Flame Photometer 410, Sherwood Scientific Ltd, Cambridge, UK). During GXTs oxygen
224 uptake was measured breath-by-breath throughout (Quark B2, Cosmed, Rome, Italy).

225 ***Hematological procedures***

226 Immediately before and after ISO1and prior to HSTs a 10 mL venous blood sample was obtained (K2 EDTA
227 blood collection tubes, Beckton Dickson & Company, Plymouth, UK) from the antecubital vein following 15
228 min of seated rest. Whole blood samples were centrifuged (1500 g for 15 min at 4°C, HERAEUS™
229 MULTIFUGE™ 3 S-R, Thermo Electron Corporation, Karlsruhe, Germany) and 20 µL of the resultant
230 plasma was assessed for osmolality (Osmometer 3320, Advanced Instruments Inc., Norwood, MA, USA)
231 and the remainder aliquoted and stored at -80°C for subsequent biochemical analyses using enzyme linked

immunosorbent assays (ELISA). Resting tHb_{mass}, (CV=4.2%), blood volume (BV) (CV=3.4%) and PV (CV=4.4%) were determined using the optimised carbon monoxide rebreathing technique [68] with a 1.0 $mL \cdot kg^{-1}$ body mass CO bolus [79], the day before and after the HA programmes, and 14-days after completion of HA. Fingertip capillary samples were taken in triplicate during the CO rebreathing technique to assess the percentage of carboxyhemoglobin (ABL80 CO-OX Flex Hemoximeter, RADIOMETER™, Copenhagen, Denmark) in the blood. Venous blood samples were also collected to determine hemoglobin concentration [Hb] (201^{+} HEMOCUE®, Ängelholm, Sweden) and hematocrit (Hct) (Hawksley, Lancing, UK) in triplicate. Together, these were used to determine tHb_{mass}, PV and BV, before and after the HA programmes, due to potential for a change in red cells which is not accounted for in the Dill & Costill [18] method.

Data analyses

$\bar{T}_{sk}$ was calculated according to Ramanathan [59] and $\bar{T}_b$ as the weighted mean of T_{re} (0.9) and $\bar{T}_{sk}$ (0.1) according to Jay *et al.* [30]. For GXT data the lactate threshold was defined as the power output at [Lac] of 4 $mmol \cdot L^{-1}$, gross mechanical efficiency was calculated at 185 W (highest work rate below lactate threshold achieved by all participants), and $\dot{V}O_{2max}$ was defined as the highest 15 s $\dot{V}O_2$. Physiological strain index (PSI) was determined according to Moran *et al*. [52] and metabolic heat production (MHP) was calculated according to ISO 8996 Malchaire [49].

Extracellular HIF-1α and erythropoietin (EPO) concentration, in EDTA plasma, were measured using colorimetric sandwich ELISAs (Thermo Fisher Scientific, Waltham, MA, USA, and; Abcam, Cambridge, UK, respectively) and read at 450 nm (450 and 550 nm for EPO) on a plate reader (SPECTRAMAX® i3x, Molecular Devices, Wokingham, UK) with SOFTMAX® Pro (version 6.5.1, Molecular Devices, Wokingham, UK). Results were calculated using the standard curve and the average absorbencies from samples in duplicate. The HIF-1α assay's detection range was 81.92-20,000 pg/mL and limit of detection was <30 pg/mL. The intra-assay precision was determined from duplicates of standards/controls within the same plate (3.2%) and inter-assay precision determined from standards/controls assessed across plates (8.7%). The EPO assays' detection range was 1.6-100 mIU/mL and had a sensitivity of 0.17 mIU/mL, with

an intra-assay precision of 8.0% and an inter-assay precision of 8.6%. Pre-post programme changes in both conditions were assessed on the same plate for each individual.

Statistical analyses

Statistical analyses were undertaken using SPSS (IBM Version 22, IBM, New York, NY, USA). Significance was set *a-priori* at $P \leq 0.05$; data are presented mean(SD) unless otherwise stated. Following Shapiro-Wilk tests for normality, two-way repeated measures ANOVA were used to analyze the main effects, *i.e.* responses over Time (HST: pre/mid/post; GXT and T30: pre/post/ret; ISO: 1-8) and Condition (HA_{Con} vs. HA_{Hyp}), as well as the interaction effect (*i.e.* Time × Condition). Effect sizes are presented using eta squared (η^2, calculated as the sum of squares for an effect/total sum of squares) for ANOVAs (η^2 ≤0.02=small; 0.02-0.13=medium; 0.13-0.26=large effect size). The Huynh-Feldt statistic was employed to account for violations of sphericity; Bonferroni adjusted Students *t*-tests were used *post-hoc* for analysis of main and interaction effects. *Post-hoc* analysis of significant time effects for ISO sessions were made relative to ISO1 only, with alpha adjusted accordingly. A one-way ANOVA was used to assess changes in the daily degree of hypoxic strain, as indicated by overnight oxy-hemoglobin saturation during the HA_{Hyp} condition. Non-parametric tests (Friedman's test for change over time and Wilcoxon signed ranks tests for condition effects at each time point) were used to assess ordinal (RPE) data. Correlations were assessed using Pearson's *r* for parametric data and Spearman's rank comparisons for non-parametric data.

Results

Daily heat and hypoxic exposure

Ambient conditions during ISOs did not differ between conditions (39.6[0.3]°C, 53.3[4.1]% RH, $P>0.05$). Participants sustained a mean power of 105(16) W (not different between conditions, $F_{(1,7)}=0.071$, $P=0.797$, $\eta^2<0.01$) with a 5 minute peak power of 189(40) W (not different between conditions, $F_{(1,7)}=0.379$, $P=0.558$, $\eta^2<0.01$). A T_{re} of 38.5°C was achieved in 31(11) mins (not different between conditions $F_{(1,7)}=0.698$, $P=0.431$, $\eta^2=0.02$) and the average T_{re} for the final 60 minute of each ISO was 38.52(0.17)°C. Power output increased over the eight ISO sessions ($F_{(4.4,30.6)}=2.823$, $P=0.038$, $\eta^2=0.08$) but this did not differ between

conditions ($F_{(1,7)}$=0.071, P=0.797, η^2=0.02). Whole-body sweat rate was increased over time ($F_{(4.0,28.2)}$=18.038, P<0.001, η^2=0.12) and also differed between conditions ($F_{(1,7)}$=15.278, P=0.006, η^2=0.01) although the location of differences could not be located *post-hoc*. Pre-exercise urine osmolality was higher in the HA_{Hyp} condition compared to the HA_{Con} condition ($F_{(1,7)}$=11.142, P=0.012, η^2=0.05) with significant differences between conditions evident on ISO6 only (P=0.024); urine osmolality did not change over the course of HA ($F_{(7,49)}$=0.223, P=0.978, η^2=0.01). An interaction effect was evident for pre-exercise mass ($F_{(7,49)}$=3.316, P=0.006, η^2<0.01) which increased over time in the HA_{Con} condition and decreased in the HA_{Hyp} condition, although *post-hoc* comparisons could not locate these differences (Table 1). The overnight hypoxia (F_IO_2 = 0.156(0.008)) during HA_{Hyp} was sustained for 8(1) hrs on 10 consecutive nights and elicited an average S_pO_2 of 91(2)% (Table 2).

INSERT TABLE 1 HERE

INSERT TABLE 2 HERE

Heat acclimation

Ambient conditions did not differ between the HSTs (39.4(0.5)°C, 50.5(1.6)% RH, P>0.05) and metabolic heat production (8.1(0.8) $W \cdot kg^{-1}$) did not differ throughout HSTs (main effect of time: $F_{(2,14)}$=0.465, P=0.637, η^2=0.01) or between conditions ($F_{(1,7)}$=3.426, P=0.107, η^2=0.06).

Both HA protocols successfully induced HA, with a number of thermophysiological adaptations evident at HST_{mid} and some further adaptations developing by HST_{post} (Figure 2 and Supplemental Table 1). However, the addition of nightly hypoxic exposure to the regimen did not affect HA; no significant interaction effects were observed for parameters measured in the HST (Figure 2 and Supplemental Table 1). Although end exercise f_c recorded in each HST was significantly greater in the HA_{Hyp} condition than then HA_{Con} condition (main effect for condition: $F_{(1,7)}$=13.656, P=0.008, η^2=0.06), Bonferroni corrected *post-hoc t*-tests comparing conditions at each time point could not locate specific differences. No other condition effects were evident.

INSERT FIGURE 2 HERE

Two participants were unable to complete the retention period hematological tests, therefore data in the 3 × 2 (Time × Condition) ANOVA are for n=6. tHb_{mass} was unchanged over time ($F_{(2,10)}$=2.275, P=0.153, η^2=0.03)

and condition ($F_{(1,5)}$=0.852, P=0.398, η^2=0.01) and there were no interaction effects ($F_{(2,10)}$=0.263, P=0.774, η^2=0.01) (**Error! Reference source not found.**3). On the other hand, PV ($F_{(2,10)}$=8.974, P=0.006, η^2=0.10) and BV ($F_{(2,10)}$=8.678, P=0.007, η^2=0.10) changed over time; *post-hoc* comparisons identified a significant decrease from post to retention time points (PV: -8.9[5.2]% (P=0.015); BV: -6.2[4.4%] (P=0.027)), but the pre-HA and retention PV and BV values were not different. PV and BV were also unchanged between conditions and there were no interaction effects (Table 3). To account for the reduced participant number and increased potential for type II error, we undertook a further analysis (*i.e.* a 2 × 2 repeated measures ANOVA), for the time points where n=8 (*i.e.* HA_{pre} *vs*. HA_{post}); with this further analysis both PV (+5.9(7.3)%, $F_{(1,7)}$=10.981, P=0.013, η^2=0.07) and BV (+3.5(5.9)%, $F_{(1,7)}$=10.083, P=0.016, η^2=0.05) were expanded pre to post-HA, but there were no condition or interaction effects.

The concentration of plasma EPO (pre-exercise in HST) was increased over time with HA ($F_{(1,7)}$=6.646, P=0.037, η^2=0.06), *post-hoc* analysis indicated that the increase was significant from HST_{pre} (8.3(3.6) mIU/mL) to HST_{post} (10.1(3.9) mIU/mL). There was no difference between conditions ($F_{(1,7)}$=0.273, P=0.618, η^2<0.01) or interaction effect ($F_{(1,7)}$=0.005, P=0.948, η^2<0.01) (Supplemental Table 1). EPO concentration did not differ following a single bout of overnight hypoxia compared to normoxic exposure ($t_{(7)}$=0.041, P=0.968, d=0.02). HIF-1α was largely undetectable in the plasma at these time points.

INSERT TABLE 3 HERE

Temperate exercise performance following HA

Graded exercise test

Data from the GXTs are shown in Figure 3. No interaction (Time × Condition) effects were reported for the parameters measured ($\dot{V}O_{2max}$, PPO, LT, GME, maximal heart rate) in the temperate GXT completed immediately before and after each HA programme, although a condition effect was detected for PPO ($F_{(1,7)}$=9.632, P=0.017, η^2=0.05), *post-hoc* analysis indicated that this was partly due to a higher baseline PPO in the HA_{Hyp} condition (359(48) W) than the HA_{Con} condition (342(48) W) (P=0.048) as well as following HA (HA_{Hyp}: 373(38) W; HA_{Con}: 353(30) W; P=0.021). PPO and lactate threshold ($F_{(1,7)}$=11.700, P=0.011, η^2=0.02) were improved over time (+12(20) W and +15(18) W, respectively) and f_{Cmax} was reduced (-5(5) $b \cdot min^{-1}$, $F_{(1,7)}$=37.840, P=0.001, η^2=0.17) following the medium-term HA, but GME remained

unchanged with time ($F_{(1,7)}$=1.189, P=0.312, η^2=0.03) or condition ($F_{(1,7)}$=0.394, P=0.550, η^2=0.02). Results for $\dot{V}O_{2max}$ showed different effects depending on whether oxygen uptake was in relative or absolute terms; relative $\dot{V}O_{2max}$ was unchanged with time ($F_{(1,7)}$=0.913, P=0.371, η^2=0.01) or condition ($F_{(1,7)}$=4.641, P=0.068, η^2=0.02). On the other hand, a main effect for condition was reported for absolute $\dot{V}O_{2max}$ ($F_{(1,7)}$=6.735, P=0.036, η^2=0.04); *post-hoc* tests indicated a trend (P=0.094) for a higher $\dot{V}O_{2max}$ at baseline in the HA_{Hyp} (4.36(0.62) L·min^{-1}) condition than the HA_{Con} condition (4.13(0.48) L·min^{-1}), but there was not a main effect over time ($F_{(1,7)}$=0.808, P=0.399, η^2=0.01).

INSERT FIGURE 3 HERE

30 minute work done trial (T30)

Environmental conditions for the T30 were matched between conditions and over time: 22.1(0.2)°C, 52.5(3.0)% RH). Data from the T30 are shown in Figure 4. Two participants in the HA_{Hyp} condition did not complete the retention trial therefore n=6 in the 3 (Time) × 2 (Condition) repeated measures ANOVA. Work done was not different between conditions ($F_{(1,5)}$=3.341, P=0.127, η^2=0.02) and there was no interaction effect ($F_{(2,10)}$=0.505, P=0.618, η^2<0.01) but it was changed over time ($F_{(2,10)}$=5.283, P=0.028, η^2<0.01). Although *post-hoc* comparisons could not locate these differences. We undertook a further analysis (*i.e.* a 2 × 2 repeated measures ANOVA), for the time points where n=8 in both conditions (*i.e.* $T30_{pre}$ and $T30_{post}$), which indicated that work done was improved by +12(20) kJ ($F_{(1,7)}$=5.939, P=0.045, η^2=0.01) immediately following HA. There were no significant differences between conditions ($F_{(1,7)}$=4.102, P=0.082, η^2=0.03) and there was no interaction effect $F_{(1,7)}$=0.036, P=0.854, η^2<0.01). The improvement in work done was not correlated with the increased LT ($r_{(16)}$=0.088, P=0.746) or PPO ($r_{(16)}$=0.476, P=0.062).

INSERT FIGURE 4 HERE

Discussion

This study was the first to examine the effect of adding a moderate overnight hypoxic stimulus on the time course and magnitude of adaption to heat, with an ancillary aim of investigating the ergogenic potential of combined adaptation to heat and hypoxia on exercise performance in a temperate, normoxic environment.

363 The main finding of the present study was that the addition of 80(8) hours normobaric hypoxia did not alter
364 the rate or magnitude of the development of HA, as indicated by key thermophysiological and hematological
365 indices; regardless of the intervention condition some HA was acquired with short-term heat exposure
366 (totaling seven hours over five-days), with a more pronounced heat-acclimated phenotype evident following
367 medium-term heat exposure (totaling 14 hours over 10-days). Furthermore, although there was evidence
368 supporting an ergogenic effect of HA under temperate-normoxic conditions (improved lactate threshold,
369 PPO and work done), this was not affected by the addition of normobaric hypoxia, which did not notably
370 affect the hematological adaptations to HA.

371 Importantly, for our experimental model, thermal-strain, cardiovascular-strain and external work-rate were
372 matched between the HA_{Con} and HA_{Hyp} conditions, whereas oxy-hemoglobin saturation was significantly
373 reduced overnight in HA_{Hyp}. Moreover, the degree of thermal strain experienced by the participants was
374 sufficient to exceed the adaptation threshold [77]; reduced T_{re}, $\overline{T}_{sk}$, $\overline{T}_{b}$, f_c and sweat $[Na^+]$ and augmented
375 sweat rate were evident within five days of HA, with a more developed heat acclimated phenotype
376 (expansion of PV and BV, further reduced $\overline{T}_{sk}$ and f_c) evident after 10-days of HA. Whilst a pronounced
377 adaptive response was evident within five days, the observation that a longer term HA regimen is superior to
378 a shorter regimen is in keeping with a recent meta-analysis [80], whereas the finding that the time-course and
379 magnitude of the adaptive response to heat was unaffected by the addition of 80(8) hours of moderate
380 normobaric hypoxia is novel, although there are some relevant comparison data. For instance, Buchheit *et al*.
381 [11] demonstrated similar reductions in f_c and sweat $[Na^+]$ following a 14-day warm-weather training camp,
382 which was unaffected by the addition of a hypoxic stressor (170 h, $F_IO_{2=}$~0.15), but no measures of body
383 temperature were reported. However, Takeno *et al*. [76] reported reduced esophageal temperature and
384 exercising f_c following 10 (1 $h \cdot day^{-1}$) exercise-heat (30°C, 50% RH) and hypobaric hypoxic (2,000 m
385 altitude) sessions, but surprisingly $\overline{T}_{sk}$ and sweat loss were unchanged and similar adaptation were evident in
386 a cool-normoxic group, indicating that some of this adaptation may have been a training effect [1].

387 A key focus of the present study was the hematological responses to the combined thermal and hypoxic-
388 stressors. Typically, HA is associated with an increase in PV and BV [74], whereas PV and BV are reduced
389 following hypoxic exposure [27, 40]. Our data demonstrated that both PV (+5.9(7.3)%) and BV
390 (+3.5(5.9)%) were increased with HA, irrespective of the additional hypoxic-stressor. This finding is

consistent with Takeno *et al*. [76] who demonstrated ~6% PV and ~5% BV increase following 10-days (1 $h \cdot day^{-1}$) exercise-heat (30°C, 50% RH) and hypobaric hypoxic (2,000 m altitude) and Buchheit *et al*. [11] who reported 6% PV and 4% BV changes following a 14-day warm-weather training camp including ~14(1) $h \cdot day^{-1}$ normobaric hypoxia (F_IO_2=~0.15). Together, these data suggest that the exercise-heat stimulus predominates over the effect of hypoxia on PV and BV, at least for these magnitudes of hypoxic exposure. However, a recent study demonstrated that PV expansion was 'possibly less' when a hypoxic stressor (F_IO_2=0.144; 14 $h \cdot day^{-1}$) was added to a 21 day HA programme, suggesting that a lager hypoxic stimulus could blunt PV expansion [50]. Two-weeks after HA the PV and BV had returned to baseline, in line with the typical decay following HA [58]. tHb_{mass} was unchanged following HA, with or without hypoxic exposure; although some hematological changes can present in a delayed manner following exposure to a hypoxic-stressor [6], there were also no changes in tHb_{mass} evident 14-days after cessation of either intervention. Whilst data supporting the positive effect of adaptation to heat alone on tHb_{mass} are limited [72], tHb_{mass} is typically increased with hypoxic exposure [10], whilst Buchheit *et al*. [11] reported a 3% increase in tHb_{mass} following 14-days and McCleave *et al*. [50] reported a 4% increase following 21-days of combined exercise-heat and hypoxia intervention. However, the erythropoietic effect is proportional to the magnitude of hypoxic stimulus [23, 13] and participants in Buchheit *et al.* [11] and McCleave *et al*. [50] received a greater hypoxic dose than participants in the present study. Moreover, Brugniaux *et al*. [10] have shown that tHb_{mass} increases ~4% with ~100 h hypoxic exposure (~2,500-3,000 m); given the hypoxic dose in the present study, the anticipated increase in tHb_{mass} would have approximated the CV for the CO rebreathing method, possibly limiting detection.

Cross-stressor research has identified commonalities between heat and hypoxic stress in the HSP and HIF-1α pathways, with some evidence for cross-tolerance between environments [24, 44], but the effect on these pathways of concurrent exposure to these stressors is unexplored. Unfortunately, we were unable to detect HIF-1α, with either HA programme, possibly due to the extracellular samples collected and the short half-life of HIF-1α in normoxia [31]. However, the plasma concentration of EPO, a downstream effect following the translocation of HIF-1α and subsequent gene expression in hypoxia [73], was increased following medium-term HA, but this was unaffected by the addition of hypoxia to the programme. Indeed the extent of the increase as a consequence of heat exposure (+28%) was similar to that reported following exposure to

hypoxic stress alone (+42%, five nights, 8-11 h per night, simulated altitude of 2650 m [2]). Our own (unpublished) data indicate that EPO concentration is unchanged by exercise of the same duration and similar intensity to our HA programme when undertaken in cool conditions (11°C), suggesting that the increase was due heat-stress, or the interaction of exercise and heat-stress, rather than a training-effect, or hypoxia. The lack of an additive effect of hypoxia on plasma EPO concentration during HA is not easily explained. It has been suggested that combining mild stressors produces an additive effect, with a move towards antagonistic interactions as the individual stressors impact increases [46], alternatively if EPO production was maximally stimulated as a consequence of the heat stimulus, then the addition of a hypoxic stressor would be of little consequence. Nevertheless, given the increase in EPO it is perhaps surprising that there was no increase in tHb_{mass}. It may be that a greater, or more sustained, change in EPO concentration is required to increase tHb_{mass} and erythrocyte volume [71]. Although reticulocytosis has been demonstrated with exposure to altitude increasing serum EPO by 31-73% [38, 26, 75], other studies reporting similar increases in EPO did not detect increased red blood cell production or tHb_{mass} [2, 3].

There was evidence for an ergogenic effect of HA on performance in a temperate-normoxic environment as shown by an increase in work done in a 30 minute cycling trial (+4%) and GXT PPO (+4%), although it should be noted that the performance benefit in a time trial would be somewhat less given that power is related to cycling velocity with an exponent of between 2.6 and 3 [5]. However, this effect was not influenced by the addition of a hypoxic-stressor and the ergogenic benefits were no longer evident two-weeks after completing the HA programmes. An ergogenic effect of adaptation to heat on temperate-normoxic performance has been demonstrated previously by some (*e.g.* [12, 48, 54]), but not all studies [33, 36], and the ergogenic efficacy of HA is controversial [15, 51, 57]. Similarly, a meta-analysis by Bonetti & Hopkins [8] observed a clear ergogenic effect of adaption to hypoxia on normoxic performance. A relatively small number of studies have previously examined the ergogenic potential of adaptation to heat and hypoxia in combination, but the data are equivocal. For instance, Buchheit *et al*. [11] reported an improvement in temperate-normoxic performance (44% Yo-YoIR2) following HA, which was unaffected by an additional hypoxic exposure. In contrast, McCleave *et al*. [50] showed a 3.3% improvement in temperate-normoxic 3 km running trial performance three weeks (but not immediately) after completing a 21-day intermittent HA

446 programme, but the ergogenic effect was absent when hypoxia was added to the HA programme (3,000 m,
447 13 h·day^{-1}).

448 The reasons for these discrepant findings between studies are uncertain, and where an ergogenic effect has
449 been demonstrated the physiological mechanisms are often unclear. Accordingly, in an attempt to provide
450 insight into any ergogenic effect we also assessed some of the key physiological determinants of
451 performance under temperate-normoxic conditions. Neither $\dot{V}O_{2max}$ nor GME were increased following
452 either programme. Indeed, the evidence supporting an effect of HA on GME is limited, and where an effect
453 has been demonstrated performance was not measured [67]. However, a positive effect of hypoxia on cycling
454 efficiency and running economy has been demonstrated in some studies [25, 66] and is relatively well
455 established [65]. However, the hypoxic dose is typically larger than that included in the present study [34,
456 35] and previous studies demonstrating an effect have not included an additional heat-stressor. A small
457 number of previous studies have shown an effect of HA, with [76], or without [48, 69], an additional
458 hypoxic-stressor on $\dot{V}O_{2max}$. Takeno *et al*. [76] reported an increased $\dot{V}O_{2peak}$, following their combined heat
459 and hypoxic-stressor intervention, but this was not improved to a greater extent than either stressor alone or a
460 cooler control programme, indicating a potential training effect. Similarly, Lorenzo *et al*. [48] reported an
461 increase in $\dot{V}O_{2max}$ following a 10 day HA programme, which they attributed to an increase in PV and a
462 consequent increase in stroke volume and cardiac output [28]. Although PV was expanded to a similar extent
463 in the present study, if the hemodilution effect approximates any increase in cardiac output, then O_2 delivery
464 will be unchanged; this is commonly observed with acute PV expansion in trained individuals [16] and
465 would account for the lack of change in $\dot{V}O_{2max}$ in the present study. However, a significant increase in
466 power at LT (8.6[11.0]%) was evident; whilst the LT does not directly influence performance *per se*, it is
467 well correlated and is typically used as a surrogate of sustainable percentage of $\dot{V}O_{2max}$ [32]. Indeed, Lorenzo
468 *et al*. [48] and Neal *et al*. [54] have demonstrated an increased power at lactate threshold following HA, with
469 possible mechanisms including reduced carbohydrate metabolism [83], increased strength [39] or simply
470 dilution from PV expansion. However, the increased LT was not related to the individual performance
471 improvements in either total work done or GXT PPO, which was also the case in Neal *et al*. [54], whereas
472 Lorenzo *et al*. [48] did not report correlations. Taken together the results of our study and previous studies
473 (*e.g.* [11, 48]) are not able to clearly identify the mechanisms underpinning the ergogenic effect of adaption

to heat (with, or without hypoxia). While it is not possible for us to discount the possibility of either a placebo or training effect, we are able to conclude that the addition of a moderate hypoxic-stressor to a HA programme is of no greater benefit, or harm, than HA alone on temperate-normoxic exercise performance.

The present study was not without limitation. Although we employed a cross-over study design, which is more powerful than a parallel-groups study design, a small sample-size will increase the potential for type II error. Nevertheless, our *a-priori* power calculations indicated that our sample-size would have been sufficient to detect change in our key outcome variables; we detected a number of statistically significant time-effects, whereas the mean between-groups differences in many of our key outcome measures (*e.g.* T_{re}, $\overline{T}_b$, whole body sweat rate) were typically small at each time point and within the normal daily physiological variation (see Supplemental Table 1). Finally, it was not possible to exclude a role of training on the adaptive responses observed in HA_{Con} and HA_{Hyp}. However, our participants were well-trained and maintained their usual training volume by replacing an equivalent duration of low/moderate training with that completed in the laboratory, whereas any training effects will have been similar between groups due to the balanced cross-over study design.

In conclusion, a moderate hypoxic stressor does not affect the time-course or magnitude of thermophysiological or hematological adaptations to heat. Temperate-normoxic endurance performance is improved following longer-term HA, but this is unaffected by the addition of a hypoxic stimulus.

Perspectives and Significance

Adaptations to heat and hypoxia are typically studied in isolation, yet they can be encountered in combination, both in the natural environment, as well as artificially when athletes expose themselves to a hypoxic-stressor in order to gain favorable hematological adaptations, whilst at the same time preparing to compete in a hot environment. Whether the adaptive response to these combined stressors affords the same response as when examined in isolation is unclear and there are potential additive and antagonistic mechanisms by which heat and hypoxic-stress may interact. The present study, using a trained cohort and employing a balanced cross-over design with washout, has shown, for the first time, that the addition of a moderate overnight hypoxic stimulus (equivalent to an altitude of ~2,400 m) to a 10 day HA regimen does

not affect the time-course or magnitude of thermophysiological adaptation to heat. Temperate-normoxic endurance performance is improved following HA, but this is unaffected by a concurrent hypoxic stimulus. Although these findings are mechanistically important, this observation is also practically relevant; athletes preparing for competition in a hot environment should not be concerned about concurrent exposure to a moderate-hypoxic stressor such as that which would occur if sleeping in a hypoxic tent. Future research should seek to characterize the adaptive responses to simultaneous (rather than separate) hypoxia and heat, and over longer time periods, as might as might occur during a prolonged high-altitude sojourn.

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No conflicts of interest, financial or otherwise, are declared by the authors.

Supplementary material: Supplemental Table.

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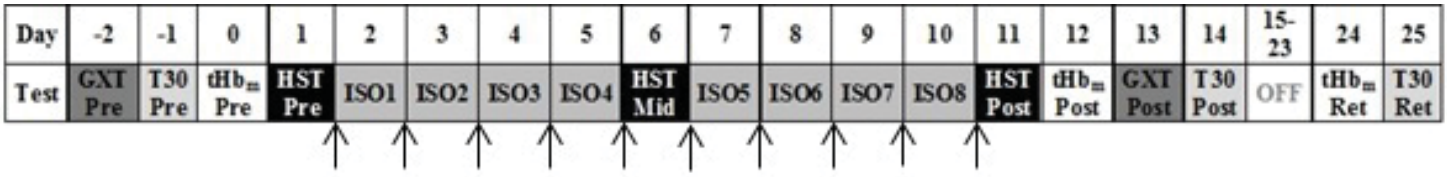
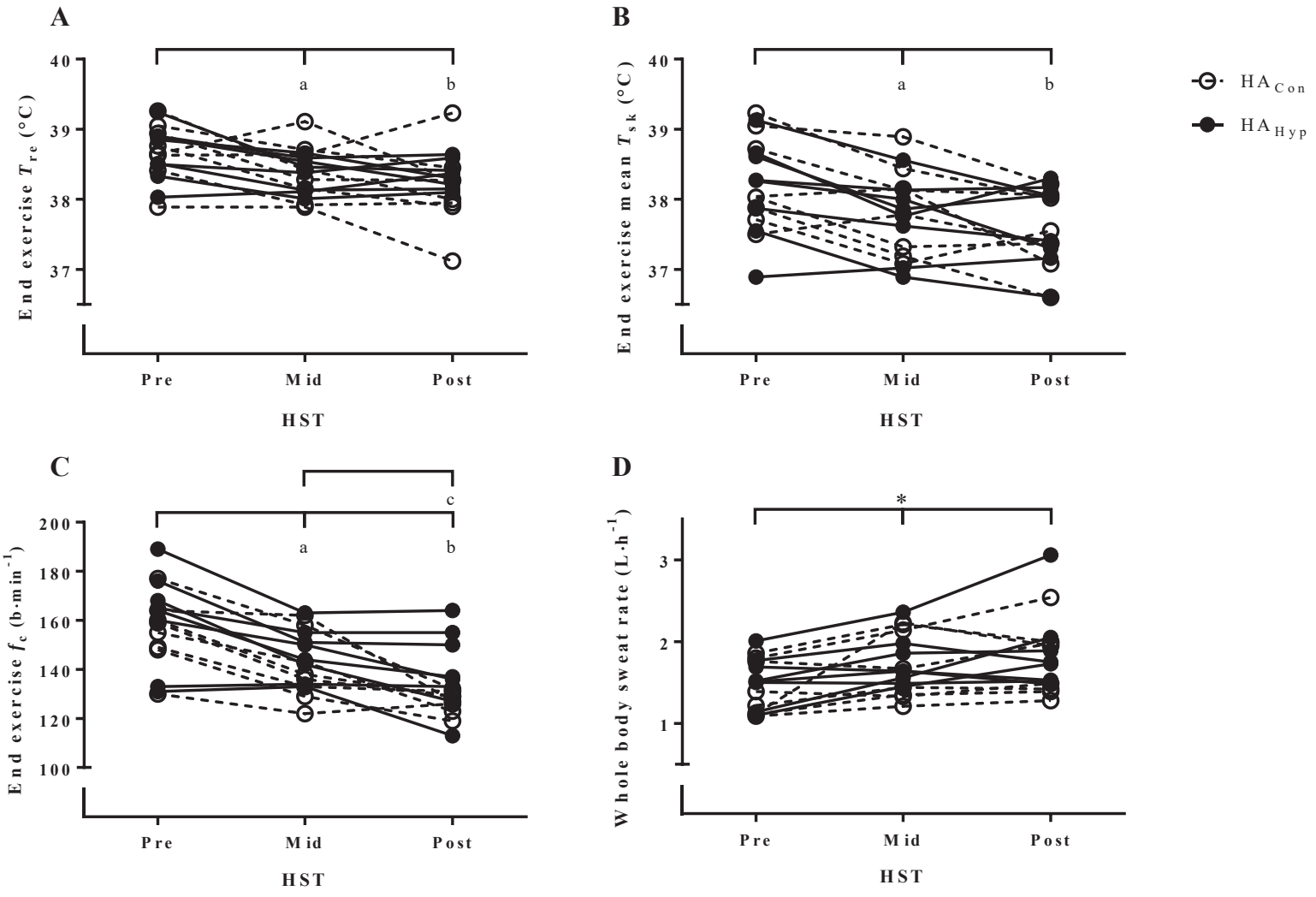
Figure 1 Protocol diagram. Participants completed the heat acclimation protocol with pre/post-tests, twice, in a within-subject balanced crossover design including a three to seven month washout period and two conditions: HA_{Con}: Heat Acclimation Control; HA_{Hyp}: Heat Acclimation with Hypoxia. *GXT*=Graded Exercise Test (22°C, 50% RH); *T30*=30 minute work done trial (22°C, 50% RH); tHb_m=resting measurement of total hemoglobin mass; *HST*=Heat Stress Test (40°C, 50% RH); *ISO*=Isothermal model of heat acclimation (ambient conditions: 40°C, 50% RH; target T_{re}: 38.5°C); ↑ indicates nightly hypoxic exposure in the HA_{Hyp} condition (F_IO_2: 0.156).

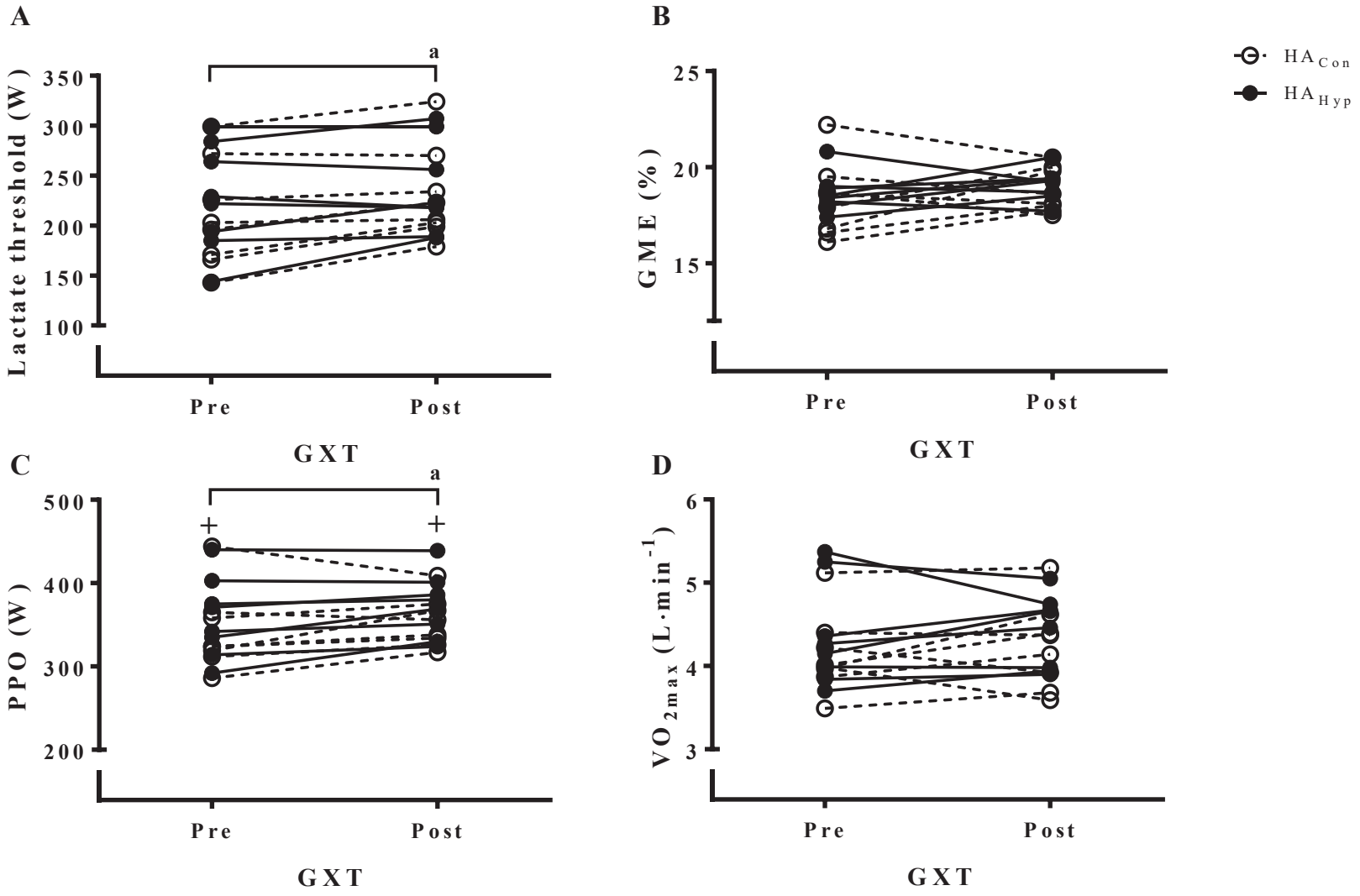
Figure 2 Individual responses (*n*=8) to exercise in the heat stress test (HST) (40°C, 50% RH) before (Pre) and following short- (Mid) and longer-term (Post) heat acclimation, with (HA_{Hyp}, filled circles) and without (HA_{Con}, open circles) overnight normobaric hypoxia, for: *A*: end exercise rectal temperature; *B*: end exercise mean skin temperature; *C*: end exercise cardiac frequency; *D*: whole-body sweat rate. * refers to a significant overall time effect; [a] refers to a change from Pre-Mid, [b] from Pre-Post and [c] from Mid-Post ($P \leq 0.05$).

Figure 3 Individual data (*n*=8) from the graded exercise test (GXT) in a temperate environment (22°C, 50% RH) before (Pre) and after (Post) heat acclimation with (HA_{Hyp}, filled circles) and without (HA_{Con}, open circles) overnight normobaric hypoxia. *A*: lactate threshold; *B*: gross mechanical efficiency (GME); *C*: peak power output (PPO); *D*: maximal oxygen uptake ($\dot{V}O_{2max}$). [a] denotes a pre-post HA change over time; [+] denotes a condition effect, $P \leq 0.05$).

Figure 4 Individual data from the 30 minute work done trial (T30) in a temperate environment (22°C, 50% RH), before (Pre), immediately after (Post) and +14-days after (Retention) heat acclimation with (HA_{Hyp}, filled circles) and without (HA_{Con}, open circles) overnight normobaric hypoxia. *denotes a change over time (over all three time points, *n*=6); [a] denotes a significant change over time (pre-post, *n*=8) ($P \leq 0.05$).


Day
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15-23
24
25
Test
GXT Pre
T30 Pre
tHbm Pre
HST Pre
ISO1
ISO2
ISO3
ISO4
HST Mid
ISO5
ISO6
ISO7
ISO8
HST Post
tHbm Post
GXT Post
T30 Post
OFF
tHbm Ret
T30 Ret


A
B
C
D
End exercise T_{re} (°C)
End exercise mean T_{sk} (°C)
End exercise f_c (b·min^{-1})
Whole body sweat rate (L·h^{-1})
40
39
38
37
200
180
160
140
120
100
3
2
1
a
b
c
*
Pre
Mid
Post
HST
HA$_{Con}$
HA$_{Hyp}$


A
B
C
D
HA Con
HA Hyp
Lactate threshold (W)
GME (%)
PPO (W)
VO2max (L·min-1)
a
Pre
Post
GXT

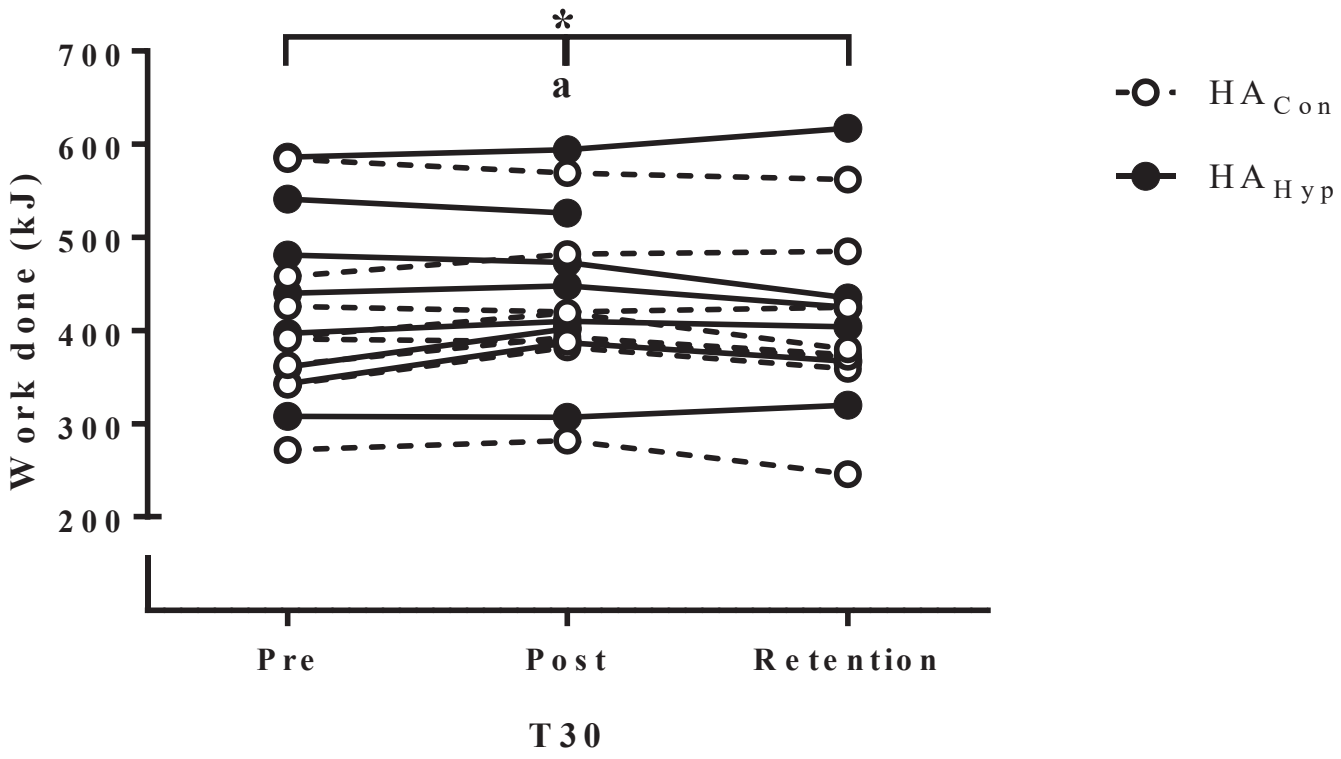

*
a
700
600
500
400
300
200
Work done (kJ)
Pre
Post
Retention
T30
HA Con
HA Hyp

Table 1 Mean(SD) daily exercise responses (n=8) during medium-term heat acclimation with and without overnight hypoxia (HA_{Hyp} and HA_{Con}, respectively). In the case of a main effect for time, [a] refers to a (*post-hoc*) change between ISO1 and ISO8 ($P \leq 0.05$). In the case of a condition effect [b] denotes a significant difference between conditions at ISO6.

	ISO1		ISO2		ISO3		ISO4		ISO5		ISO6		ISO7		ISO8		*P* value		
	HA_{Con}	HA_{Hyp}	HA_{Con}	HA_{Hyp}	HA_{Con}	HA_{Hyp}	HA_{Con}	HA_{Hyp}	HA_{Con}	HA_{Hyp}	HA_{Con}	HA_{Hyp}	HA_{Con}	HA_{Hyp}	HA_{Con}	HA_{Hyp}	Time	Condition	Interaction
Time to target T_{re} (min)	27 (6)	30 (15)	35 (24)	33 (14)	30 (12)	34 (12)	28 (6)	32 (10)	28 (7)	33 (11)	31 (9)	27 (7)	31 (10)	32 (12)	31 (8)	30 (8)	0.556	0.431	0.806
Average T_{re} (°C)	38.65 (0.14)	38.60 (0.23)	38.49 (0.36)	38.49 (0.21)	38.52 (0.15)	38.48 (0.16)	38.55 (0.10)	38.46 (0.10)	38.55 (0.09)	38.47 (0.16)	38.53 (0.14)	38.51 (0.12)	38.55 (0.13)	38.47 (0.12)	38.48 (0.09)	38.45 (0.24)	0.204	0.057	0.802
Average f_c ($b \cdot min^{-1}$)	148 (9)	142 (16)	143 (12)	142 (12)	144 (10)	140 (9)	142 (10)	142 (8)	142 (10)	142 (10)	140 (10)	140 (13)	139 (10)	140 (11)	140 (13)	139 (12)	0.166	0.194	0.419
Average power (W)	97 (18)	97 (29)	99 (20)	101 (13)	108 (18)	108 (14)	111 (22)	114 (15)	100 (17)	110 (11)	106 (12)	106 (15)	107 (11)	103 (13)	111 (16)	107 (11)	0.073	0.797	0.541
5 min peak power (W)	193 (45)	204 (65)	185 (42)	185 (47)	181 (36)	192 (48)	186 (39)	188 (38)	183 (35)	185 (41)	194 (34)	175 (33)	188 (22)	193 (54)	195 (36)	203 (35)	0.375	0.558	0.748
Pre-exercise mass (kg)	73.78 (6.51)	74.99 (7.73)	73.89 (6.69)	74.87 (7.79)	73.89 (6.65)	74.85 (7.88)	73.82 (6.51)	74.83 (7.94)	74.16 (6.82)	74.58 (8.07)	74.32 (6.82)	74.68 (7.76)	74.30 (7.00)	74.42 (7.54)	74.35 (6.80)	74.46 (7.88)	0.996	0.446	0.006
Whole body sweat rate ($L \cdot hr^{-1}$)	1.24 (0.27)	1.28 (0.43)	1.24 (0.34)	1.38 (0.26)	1.33 (0.32)	1.34 (0.29)	1.34 (0.33)	1.40 (0.26)	1.44 (0.36)	1.51 (0.34)	1.49 (0.37)	1.58 (0.41)	1.50 (0.40)	1.62 (0.39)	1.56 (0.38)	1.66 (0.34)	<0.001[a]	0.006	0.681
Urine osmolality ($mOsmo \cdot kg^{-1}$)	509 (353)	652 (335)	577 (387)	652 (335)	544 (204)	560 (274)	534 (324)	501 (288)	502 (329)	607 (249)	383 (245)	597 (289)	385 (265)	677 (323)	379 (203)	632 (290)	0.978	0.019[b]	0.312

ISO: Isothermal strain session; HA_{Con}: Heat Acclimation Control condition; HA_{Hyp}: Heat Acclimation with Hypoxia condition; T_{re}: rectal temperature; f_C: cardiac frequency.

Table 2 Mean(SD) daily overnight responses (n=8) to moderate normobaric hypoxic exposure (15.6[0.9]%). Independent one-way ANOVA were performed and $P \leq 0.05$.

	$HA_{Hyp}1$	**$HA_{Hyp}2$**	**$HA_{Hyp}3$**	**$HA_{Hyp}4$**	**$HA_{Hyp}5$**	**$HA_{Hyp}6$**	**$HA_{Hyp}7$**	**$HA_{Hyp}8$**	**$HA_{Hyp}9$**	**$HA_{Hyp}10$**	***P* value**
Overnight oxyhemoglobin saturation (%)	91 (1)	90 (2)	90 (2)	91 (2)	91 (2)	91 (1)	92 (2)	90 (4)	91 (1)	91 (2)	0.395
Overnight fc ($b \cdot min^{-1}$)	65 (17)	57 (10)	61 (9)	57 (9)	54 (6)	55 (7)	54 (5)	54 (5)	57 (10)	52 (8)	0.263
Hours hypoxic exposure (h)	8.0 (1.0)	7.8 (1.2)	7.4 (1.2)	7.9 (1.5)	7.8 (1.0)	8.3 (1.5)	8.4 (1.4)	8.0 (0.5)	8.2 (0.6)	8.2 (0.8)	0.871

HA_{Hyp}: Heat Acclimation with Hypoxia condition; f_C: cardiac frequency

Table 3 Mean(SD) blood volumes (n=6) calculated using the optimised CO rebreathing technique pre-, post- and retention-HA for both HA_{Con} and HA_{Hyp} conditions. *Post-hoc* pairwise comparisons were performed following a significant main effect for time, [a] represents a significant change from post – retention-HA ($P \leq 0.05$).

	Pre		**Post**		**Retention**		***P* value**		
	HA_{Con}	**HA_{Hyp}**	**HA_{Con}**	**HA_{Hyp}**	**HA_{Con}**	**HA_{Hyp}**	**Time**	**Condition**	**Interaction**
tHb_{mass} ($g \cdot kg^{-1}$)	11.7 (0.6)	11.9 (0.8)	11.6 (0.7)	12.1 (1.0)	11.4 (0.8)	11.7 (0.8)	0.153	0.398	0.774
Plasma volume ($mL \cdot kg^{-1}$)	44.9 (4.3)	44.9 (4.5)	48.0 (6.5)	47.8 (6.5)	43.8 (6.6)	43.4 (6.0)	0.006 [a]	0.889	0.955
Blood volume ($mL \cdot kg^{-1}$)	80.6 (5.6)	81.3 (5.5)	83.4 (8.2)	85.0 (7.9)	78.8 (8.0)	79.1 (7.3)	0.007 [a]	0.731	0.887

HA_{Con}: heat acclimation control condition; HA_{Hyp}; heat acclimation with hypoxia condition; tHb_{mass}: total hemoglobin mass.