

Heat acclimation attenuates physiological strain and the HSP72, but not HSP90 α , mRNA response to acute normobaric hypoxia

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Gibson OR, Turner G, Tuttle JA, Taylor L, Watt PW, Maxwell NS. Heat acclimation attenuates physiological strain and the HSP72, but not HSP90 α , mRNA response to acute normobaric hypoxia. *J Appl Physiol* 119: 889–899, 2015. First published July 23, 2015; doi:10.1152/jappphysiol.00332.2015.—Heat acclimation (HA) attenuates physiological strain in hot conditions via phenotypic and cellular adaptation. The aim of this study was to determine whether HA reduced physiological strain, and heat shock protein (HSP) 72 and HSP90 α mRNA responses in acute normobaric hypoxia. Sixteen male participants completed ten 90-min sessions of isothermic HA (40°C/40% relative humidity) or exercise training [control (CON); 20°C/40% relative humidity]. HA or CON were preceded (HYP1) and proceeded (HYP2) by a 30-min normobaric hypoxic exposure [inspired O₂ fraction = 0.12; 10-min rest, 10-min cycling at 40% peak O₂ uptake ($\dot{V}O_{2\text{ peak}}$), 10-min cycling at 65% $\dot{V}O_{2\text{ peak}}$]. HA induced greater rectal temperatures, sweat rate, and heart rates (HR) than CON during the training sessions. HA, but not CON, reduced resting rectal temperatures and resting HR and increased sweat rate and plasma volume. Hemoglobin mass did not change following HA nor CON. HSP72 and HSP90 α mRNA increased in response to each HA session, but did not change with CON. HR during HYP2 was lower and O₂ saturation higher at 65% $\dot{V}O_{2\text{ peak}}$ following HA, but not CON. O₂ uptake/HR was greater at rest and 65% $\dot{V}O_{2\text{ peak}}$ in HYP2 following HA, but was unchanged after CON. At rest, the respiratory exchange ratio was reduced during HYP2 following HA, but not CON. The increase in HSP72 mRNA during HYP1 did not occur in HYP2 following HA. In CON, HSP72 mRNA expression was unchanged during HYP1 and HYP2. In HA and CON, increases in HSP90 α mRNA during HYP1 were maintained in HYP2. HA reduces physiological strain, and the transcription of HSP72, but not HSP90 α mRNA in acute normobaric hypoxia.

altitude; cardiovascular; cross-acclimation; cross-tolerance; heat stress; plasma volume

HYPOXIA INCREASES PHYSIOLOGICAL strain both at rest and during exercise (6), with impairment of exercise performance (72), notably during exercise where aerobic metabolism predominates (3). The physiological advantages and disadvantages of repeated hypoxic/altitude exposures for attenuating the negative effects of hypoxia (2) have been summarized in numerous review articles (20, 46). Altitude/hypoxic training methods are varied, with synergistic interactions between simulated and

terrestrial, resting or exercise, and continuous and intermittent exposures, each eliciting different magnitudes of adaptation (46). Irrespective of precise application, hypoxic training requires lengthy durations of exposure over prolonged, repeated periods (typically 14–28 days) for meaningful adaptation (27).

Heat acclimation (HA), and acclimatization interventions, carried out by repeated exercise in hot conditions (58), reproducibly reduce physiological strain in hot and cooler conditions (32, 33, 39). Recent reviews support a novel adaptive pathway whereby HA may reduce physiological strain in hypoxia (14, 56, 73). Mechanistic pathways can be subdivided into cross-acclimation, whereby HA attenuates physiological strain (73) and cross-tolerance, whereby cellular responses to HA provide cytoprotection during hypoxia (14). Acute physiological responses to hypoxia (2) can be used as criteria for validating heat-induced cross-acclimation. HA reduces glycolysis and metabolic rates during exercise (34), with plasma volume (PV) expansion (39, 50) and improved myocardial efficiency (38) preserving cardiac output and skeletal muscle blood flow. Muscle oxygenation is also sustained by HA-induced maintenance of central blood volume (BV) via reductions in the core/skin temperature gradient (58) and enhanced evaporative heat loss (51). Improved temperature and hematological regulation facilitate a leftward shift in the oxyhemoglobin saturation curve (37). HA induces expedient and beneficial adaptations within 5–14 daily sessions, demonstrating a greater efficiency of adaptation compared with altitude/hypoxic interventions (23).

Cross-tolerance has been defined as single or repeated sublethal exposures to a stressor, eliciting a positive adaptive effect to a subsequent exposure to a different stressor (35). The cellular pathway for this shares commonality with those seen within *in vivo* thermotolerance (47). In this model, cellular thermotolerance accompanies the induction of phenotypic adaptations associated with HA (43, 45). Thermotolerance confers cytoprotection against subsequent thermal exposure (45, 74), principally by changes in heat shock proteins (31). Heat shock proteins facilitate important cellular processes as protein chaperones (19) and anti-apoptotic mediators (1). In particular, increases in the inducible proteins heat shock protein (HSP) A1A (HSP72) and HSPC1 (HSP90 α) mitigate pathophysiological responses to endogenously stressful stimuli. Both HSP72 and HSP90 α augment proportionally to increased cellular stress (increased cellular temperature) in response to *ex vivo* heat shock (45) and have been implicated as important modulators of the adaptive cellular/molecular response to hypoxia

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(56, 65, 66); this suggests a shared signaling pathway. Both HSP72 and HSP90 α mRNA and protein responses have been used as a marker for identifying the magnitude of stimuli required to initiate the in vivo stress response (45). However, not all of the HSP72 mRNA transcripts are translated to HSP72 protein increase within peripheral blood mononuclear cells following exercise heat stress in humans (44). Basal heat shock protein measurement provides the optimal indication of the acquired capacity to mitigate disruption to cellular homeostasis due to known increases with acclimation (45). The delayed responsiveness of the protein response (16, 17), compared with the within-session heat shock protein mRNA response (25, 44), emphasizes the benefits of the gene transcript as a primary indicator of the magnitude of the stress stimuli and necessity to signal protein transcription should the stimuli be maintained or repeated. Consequently, the mRNA transcription is appropriate to determine whether the HSP72 and HSP90 α responses have been attenuated or mitigated, either in response to reductions in physiological strain, or increased basal protein, ultimately highlighting whether cross-tolerance may have been conferred.

HA has been evidenced in improving oxygen saturation and heart rates (HRs) during hypoxic exercise performance (28), with HA also mitigating increases in HSP72 protein in hypoxia, due largely to increased basal concentrations of HSP72 (37). These data support the existence of cross-acclimation/tolerance (37); however, mechanisms for this interaction are presently unknown (39, 45). The aim of this experiment was to determine whether HA would reduce physiological strain and the HSP72 and HSP90 α mRNA responses to an acute hypoxic exposure (at rest and at various exercise intensities) compared with exercise training matched for intensity and duration in temperate conditions. It was hypothesized that HA would reduce physiological strain in hypoxia via cardiovascular and thermoregulatory adaptations and that the heat shock protein response to hypoxia would be reduced following HA.

MATERIALS AND METHODS

Participants. Sixteen healthy men, who completed various forms of exercise training between three and six times per week, were assigned to matched groups to perform 10 days of isothermic HA [age 22.5 ± 3.5 yr, nude body mass (NBM) 74.6 ± 7.9 kg, body surface area 1.95 ± 0.13 m², peak oxygen uptake ($\dot{V}O_{2\text{ peak}}$) 4.32 ± 0.68 l/min, 58.5 ml·kg⁻¹·min⁻¹], or act as a normothermic exercise control (CON; age 26.0 ± 5.0 yr, NBM 74.6 ± 4.8 kg, body surface area 1.93 ± 0.13 m², $\dot{V}O_{2\text{ peak}}$ 4.22 ± 0.62 l/min, 56.6 ml·kg⁻¹·min⁻¹). Confounding environmental and pharmacological variables were all controlled in line with previous work in the field (24, 25). Urine osmolality was used to confirm hydration in accordance with established guidelines before each experimental/training session [<700 mosmol/kgH₂O (57)]. This experimental control was not violated for any participant for any experimental/training session. All protocols, procedures, and methods were approved by the institutional ethics committee, with participants completing medical questionnaires and written, informed consent following the principles outlined by the Declaration of Helsinki, as revised in 2013.

Preliminary testing. Before assessment of $\dot{V}O_{2\text{ peak}}$, anthropometric data were collected with NBM measured using digital scales, precise to 0.01 kg (GFK 150, Adam Equipment, Danbury, CT). $\dot{V}O_{2\text{ peak}}$ (l/min) was determined from an incremental test on a cycle ergometer, which was used for all subsequent trials (Monark e724, Monark AB, Varberg, Sweden), at a starting intensity of 80 W, increasing by 24 W/min, in temperate laboratory conditions [20°C, 40% relative hu-

midity (RH)] at sea level (inspired O₂ fraction = 0.2093). $\dot{V}O_{2\text{ peak}}$ was defined as the highest average $\dot{V}O_2$ obtained in any 30-s period, with $\dot{V}O_{2\text{ peak}}$ more appropriately describing the end point of the test due to an absence of $\dot{V}O_2$ plateau in all participants. The confirmation of $\dot{V}O_{2\text{ peak}}$ was made via the attainment of a HR within 10 beats/min of age-predicted maximum, and respiratory exchange ratio (RER) >1.1 in all participants. Expired metabolic gas was measured using breath-by-breath online gas analysis (Metalyser 3B, Cortex, Leipzig, Germany). HR was recorded continuously during all experimental/training sessions by telemetry (Polar Electro Oyo, Kempele, Finland).

Hematological measures. Twenty-four hours before hypoxic exposures, hemoglobin mass (Hb_{mass}; g) was measured. Hb_{mass}, BV (ml), and PV (ml) were determined in accordance with the optimized carbon monoxide (CO)-rebreathing method (59). Participants were seated for 20 min, before being connected to a closed glass spirometer, allowing inspiration of a CO bolus of 1.0 ml/kg (68), followed by 2-min rebreathing of a 3.5-liter O₂ bolus. Before and 4 min after CO rebreathing, participants completely exhaled to residual volume into a CO gas meter (Pac 7000, Dräger, Pittsburgh, PA). CO volume not remaining within the body was calculated from the remainder of CO in the spirometer, and exhaled CO was measured immediately after the spirometer was disconnected from the participant (68). Fingertip capillary samples, for determination of carboxyhemoglobin concentration (%HbCO) were taken immediately before the rebreathing procedure and at 6 and 8 min after the CO bolus was administered. Blood samples were measured immediately in triplicate (69), using an ABL80 CO-OX FlexOXFlex hemoximeter (Radiometer, Copenhagen, Denmark). Hb_{mass} was calculated from the mean change in %HbCO before and after rebreathing CO (68). At the relevant intervals within the optimized CO-rebreathing method, hemoglobin concentration (g/dl) was collected from fingertips in duplicate using a microcuvette and analyzed using a B-Hemoglobin Photometer (Hemocue, Ängelholm, Sweden), and hematocrit (%) was collected in triplicate (~ 50 μ l) with glass capillary tubes and analyzed following centrifugation at 14,000 rpm for 3 min (Haemotospin 1300 Centrifuge, Hawksley & Sons, West Sussex, UK) (69). The experimenter typical error of measurement for total Hb_{mass} before commencing this experiment was $\pm 1.98\%$ (± 17.0 g).

Hypoxic exposures. Hypoxic exposures were performed 24 h before commencing the first session of HA or CON (HYP1) and 24 h following the final HA or CON training session (HYP2). Participants performed a 30-min normobaric hypoxic exposure adapted from Lunt et al. (40). After entering normobaric hypoxic conditions (inspired O₂ fraction = 0.12; 18°C, 40% RH) achieved using a purposed built nitrogen-enriched chamber (Altitude Centre, London), participants immediately rested in a supine position for a period of 10 min. Supine rest was followed by two bouts of exercise, where participants first cycled at a workload corresponding to 40% of normoxic preliminary $\dot{V}O_{2\text{ peak}}$ for a period of 10 min (HA = 102 ± 27 W, CON = 104 ± 26 W) and then immediately proceeded to exercise at a workload corresponding to 65% of normoxic preliminary $\dot{V}O_{2\text{ peak}}$ (HA = 201 ± 41 W, CON = 192 ± 37 W) for a further 10 min. During rest and exercise, HR, oxygen uptake ($\dot{V}O_2$; l/min), carbon dioxide production ($\dot{V}CO_2$; l/min), ventilation ($\dot{V}E$; l/min), RER, and peripheral arterial oxygen saturation (SpO₂; %) estimated using a fingertip pulse oximeter (Nonin 2500, Nonin Medical) were recorded continuously, with the final 5 min of measures used for analysis. Before entry, and following every 10 min, participants reported rating of perceived exertion (RPE) and Lake Louise Questionnaire (LLQ) symptoms. Metabolic parameters ($\dot{V}O_2$, $\dot{V}CO_2$, and $\dot{V}E$) was measured using online breath-by-breath analysis.

HA/exercise protocols. Each HA or CON training session was conducted at the same time of day (0700–1000) to control for effects of daily variation in exercise performance (12) and heat shock protein expression (67) inside a purpose-built environmental chamber (Wat-Flow control system; TISS, Hampshire, UK). Temperature and hu-

midity were controlled using automated computer feedback (WatFlow control system; TISS, Hampshire, UK). On arrival to the laboratory, participants provided a midflow urine sample for assessment of hydration. Towel-dried NBM was measured before and after the trials, with no fluid consumption permitted between measurements. Sweat rate (SR; l/h) was estimated using the change in NBM from the pre- to postexercise periods. Participants inserted a rectal thermistor (Hensleys Medical Supplies, Welwyn Garden City, UK; Meter logger model 401, Yellow Springs Instruments, Yellow Springs, MO) 10 cm past the anal sphincter to measure rectal temperature (T_{rec}) and affixed a HR monitor to the chest. Following a 10-min seated stabilization period in temperate laboratory conditions, at sea level, resting measures [T_{rec} , HR, RPE, and thermal sensation (TSS)] were recorded, and participants immediately entered the environmental chamber ($40.2 \pm 0.4^\circ\text{C}$, $41.0 \pm 6.4\%$ RH) and mounted a cycle ergometer. Participants allocated to the HA group performed ten 90-min sessions involving a combination of cycling exercise and rest in accordance with established isothermic HA protocols (25, 26). HA participants initially exercised, at a workload corresponding to $65\% \dot{V}O_{2\text{ peak}}$, until the isothermic target $T_{\text{rec}} \geq 38.5^\circ\text{C}$ had been achieved, and, upon the attainment of a $T_{\text{rec}} \geq 38.5^\circ\text{C}$, rested in a seated position on the cycle ergometer within the environmental chamber. Participants resumed exercise when their T_{rec} fell below 38.5°C and continued cycling until the target T_{rec} was attained. Participants in the CON group performed ten 90-min sessions, copying the exercise-rest prescription of HA in controlled conditions ($19.8 \pm 0.2^\circ\text{C}$, $28.5 \pm 2.7\%$ RH). CON participants initially cycled at an intensity corresponding to $65\% \dot{V}O_{2\text{ peak}}$, with the workload adjusted to match the total work and exercise intensity and duration of the whole session (exercise and rest) and exercise components of the HA group; see Fig. 1 for mean sessional T_{rec} throughout HA and CON. To accurately match the exercise intensity and duration of exercise of the HA group, the exercise requirement of CON was prescribed with progressive increases in the mean exercise duration and intensity implemented throughout the 10-day regime. This progression was derived from the rolling mean of HA participants who had already completed that given day of the intervention. Any resumption of exercise, necessary to increase T_{rec} in HA, was added to the end of the initial exercise bout for CON participants; this strategy was necessary to account for the intermittent nature of subsequent exercise, both between and within HA participants. During each HA or CON session, HR, T_{rec} , power output (W), RPE, and TSS were recorded every 5 min with adjustments in power (including the cessation of exercise) only made following each completed 5-min period. In compliance with ethical

Table 1. Summary of protocol and physiological data recorded throughout rest and exercise of 10 sessions of heat acclimation or control

	HA	CON
<i>n</i>	8	8
Exercising duration, min	48.4 ± 3.7	47.5 ± 1.1
Total work done, kJ	500.9 ± 101.8	510.0 ± 113.1
Mean T_{rec} , $^\circ\text{C}$	$38.32 \pm 0.11^*$	37.55 ± 0.21
Duration $T_{\text{rec}} \geq 38.5^\circ\text{C}$, min	$48.6 \pm 9.1^*$	1.8 ± 2.3
Rate of T_{rec} increase, $^\circ\text{C}/\text{h}$	$2.72 \pm 0.39^*$	1.38 ± 0.39
Sweat rate, l/h	$1.44 \pm 0.32^*$	0.51 ± 0.18
Mean HR, beats/min	$136 \pm 14^*$	112 ± 14
Mean RPE	$11.4 \pm 1.2^*$	10.0 ± 0.8
Mean TSS	$6.4 \pm 0.6^*$	4.5 ± 0.3

Values are means \pm SD; *n*, no. of subjects. HA, heat acclimation; CON, control; T_{rec} , rectal temperature; HR, heart rate; RPE, rating of perceived exertion; TSS, thermal sensation. *Significant difference from CON ($P < 0.05$).

approval, HA was terminated if a subject attained a T_{rec} of 39.7°C (zero incidences); data describing the prescription, physiological, and perceptual responses to HA and CON are contained in Table 1.

Blood sampling and RNA extraction. Venous blood samples were taken immediately before and after HYP1 and HYP2, and before and after the 1st (*day 1*) and 10th day (*day 10*) of HA or CON with RNA extraction performed using a validated method (9). Briefly, blood samples were drawn from the antecubital vein into 6-ml EDTA tubes (Greiner BIO-one, Stonehouse, UK). Venous blood (1 ml) was pipetted into 10 ml of 1 in 10 red blood cell lysis solution (10 \times Red Blood Cell Lysis Solution, Miltenyi Biotech, Biscy, UK). Samples were incubated for 15 min at room temperature before isolation via 5-min centrifugation at 400 *g*, then washed twice in 2 ml PBS and centrifuged at 400 *g* for 5 min. Because of belonephobia, one participant from HA was excluded from blood sampling and mRNA analyses. The TRIzol method was then used to extract RNA from the leukocytes in accordance with manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA). Quantity was determined at an optical density of 260 nm, while quality was determined via the 260/280 and 260/230 ratios using a NanoDrop spectrophotometer (NanoDrop 2000c Thermo Scientific, Waltham, MA).

One step reverse transcription-quantitative polymerase chain reaction. HSP72 and HSP90 α relative mRNA expression was quantified using reverse transcription-quantitative polymerase chain reac-

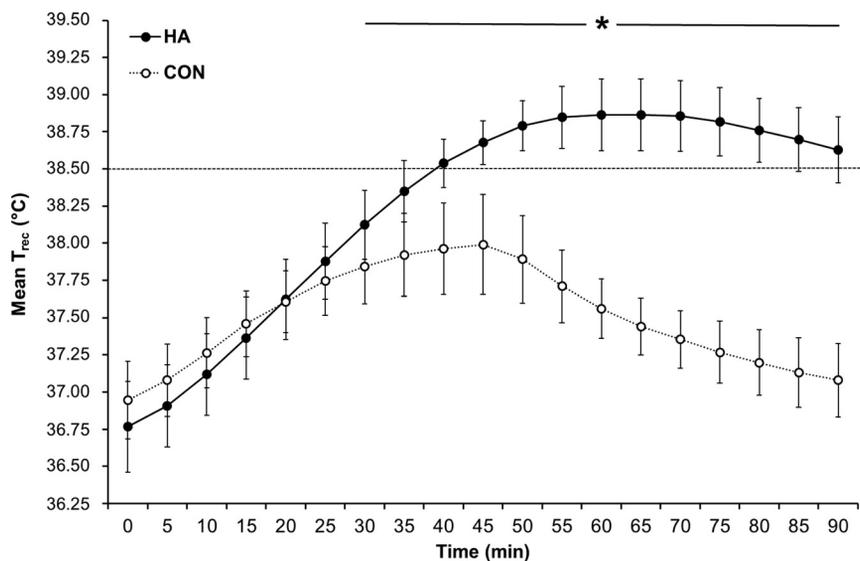


Fig. 1. Rectal temperature (T_{rec} ; $^\circ\text{C}$) during 10 days of heat acclimation (HA; $n = 8$) and normothermic exercise (CON; $n = 8$). Values are means \pm SD. *Significant difference vs. CON ($P < 0.05$).

tion. Primers β 2-microglobulin [National Center for Biotechnology Information (NCBI) accession number: NM_004048; forward: CCGTGTGAACCATGTGACT, reverse: TGCGGCATCTTCAAACCT], HSP72 (NCBI accession number: NM_005345; forward: CGCAACGTGCTCATCTTTGA, reverse: TCGCTTGTCTGGCTGATGT), and HSP90 α [NCBI accession numbers: NM_001017963 (*variant 1*) and NM_005348 (*variant 2*); forward: AAACGTGCGCTCCTGTCTTCT, reverse: TGCGTGATGTGTCGTCATCT] were designed using primer design software (Primer Quest and Oligoanalyzer-Integrated DNA Technologies, Coralville, IA) (70). Relative quantification of mRNA expression for each sample was assessed by determining the ratio between the cycling threshold (CT) value of the target mRNA and β 2-microglobulin CT values. Fold change in relative mRNA expression was calculated using the 2- $\Delta\Delta$ CT method.

Statistical analysis. A priori power analysis for key HA-dependent variables selecting conventional α (0.05) and β (0.20) levels observed that eight participants were required in each experimental group. Before statistical analysis, all outcome variables were checked for normality using Kolmogorov-Smirnov and sphericity tests, using the Greenhouse Geisser method before further analysis. Protocol-specific and physiological data for HA/CON were compared using independent samples *T*-tests. Two-way mixed-design ANOVA was performed to determine differences between HA and CON and *day 1/pre* and *day 10/post*. Two-way mixed-design ANOVA was performed to determine differences between HA and CON, as well as HYP1 with HYP2; thus rest, 40% $\dot{V}O_{2\text{ peak}}$, and 65% $\dot{V}O_{2\text{ peak}}$ conditions within each HYP were analyzed independently from one another. Three-way mixed-design ANOVA was performed on the HSP72 and HSP90 α mRNA data to determine differences between pre- and postvalue (repeated measures – within subjects) on different days (repeated measures – within subjects) from the two interventions (between subjects). Adjusted Bonferroni comparisons were used as post hoc analyses for all ANOVA. Effect sizes [Cohen's *d* (small = 0.2, medium = 0.5, large = 0.8) or partial η^2 (η^2); small = 0.01, medium = 0.06, large = 0.13] were calculated to analyze the magnitude and trends with data. All data are reported as means \pm SD. For all analysis, two-tailed significance was accepted at $P < 0.05$.

RESULTS

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

Differences were observed for mean T_{rec} ($t = 9.138$; $P < 0.001$; $d = 4.88$), rate T_{rec} increase ($t = 6.876$; $P < 0.001$; $d = 3.68$), duration $T_{\text{rec}} \geq 38.5^\circ\text{C}$ ($t = 14.106$; $P < 0.001$; $d = 7.54$), between HA and CON interventions, with mean T_{rec} different between HA and CON ($t = 55.619$; $P < 0.001$; $\eta^2 = 0.799$) 30 and 90 min (Fig. 1). Additionally, SR ($t = 7.254$; $P < 0.001$; $d = 3.88$), mean HR ($t = 3.444$; $P = 0.004$;

$d = 1.84$), mean RPE ($t = 2.918$; $P = 0.011$; $d = 1.56$), and mean TSS ($t = 8.394$; $P < 0.001$; $d = 4.49$) were greater in HA compared with CON interventions (Table 1).

Adaptation to HA. An interaction effect was observed between HA and CON and *day 1* and *day 10* for resting T_{rec} ($f = 11.507$; $P = 0.004$; $\eta^2 = 0.451$), resting HR ($f = 20.579$; $P < 0.001$; $\eta^2 = 0.595$), SR ($f = 7.146$; $P = 0.018$; $\eta^2 = 0.338$), PV ($f = 23.501$; $P < 0.001$; $\eta^2 = 0.627$), and BV ($f = 25.582$; $P < 0.001$; $\eta^2 = 0.646$) in HA, but not CON (Table 2). SR was greater on *day 1* and *day 10* in HA than CON ($P < 0.001$), but resting T_{rec} ($P = 0.007$) and resting HR ($P = 0.033$) were lower on *day 10* in HA compared with CON (Table 2). No difference was observed between days ($f = 0.275$; $P = 0.608$; $\eta^2 = 0.019$) or days \times groups ($t = 0.237$; $P = 0.634$; $\eta^2 = 0.017$) for Hb_{mass} (Table 2).

HSP72 mRNA and HSP90 α mRNA during HA/CON. An interaction effect was observed for HSP72 mRNA ($f = 20.428$; $P = 0.001$; $\eta^2 = 0.611$) and HSP90 α mRNA ($f = 10.282$; $P = 0.007$; $\eta^2 = 0.422$). No difference was observed between HA or CON before *day 1* or *day 10* ($P = 0.396$ and $P = 0.180$), but a difference was observed post-HA compared with CON ($P = 0.004$ and $P = 0.012$). HSP72 mRNA and HSP90 α mRNA increased pre- to post-HA ($P < 0.001$ and $P < 0.001$) in HA, but not CON ($P < 0.051$ and $P = 0.394$).

Hypoxic tolerance tests. At rest in hypoxia, there was an interaction effect between groups and HYP1 and HYP2 for $\dot{V}O_{2\text{ HR}}$ ($f = 6.852$; $P = 0.020$; $\eta^2 = 0.329$) and RER ($f = 5.078$; $P = 0.041$; $\eta^2 = 0.266$). In HYP2, at rest differences occurred following HA for $\dot{V}O_{2\text{ HR}}$ ($P = 0.039$; Fig. 2) and RER ($P = 0.045$; Fig. 3), but not CON ($P > 0.05$). No difference was observed in HR ($f = 0.820$; $P = 0.381$; $\eta^2 = 0.055$) or SpO_2 ($f = 2.123$; $P = 0.167$; $\eta^2 = 0.132$) at rest in hypoxia (Fig. 2).

When exercising at 40% $\dot{V}O_{2\text{ peak}}$ in hypoxia, no differences were observed within the group \times HYP comparison for HR ($f = 1.575$; $P = 0.230$; $\eta^2 = 0.101$), SpO_2 ($f = 0.000$; $P = 1.000$; $\eta^2 = 0.000$), $\dot{V}O_{2\text{ HR}}$ ($f = 2.651$; $P = 0.126$; $\eta^2 = 0.126$), or RER ($f = 0.047$; $P = 0.831$; $\eta^2 = 0.003$) (Fig. 2). When exercising at 65% $\dot{V}O_{2\text{ peak}}$ in hypoxia, differences were observed for HR ($f = 4.751$; $P = 0.047$; $\eta^2 = 0.253$), SpO_2 ($f = 5.616$; $P = 0.033$; $\eta^2 = 0.286$), and $\dot{V}O_{2\text{ HR}}$ ($f = 10.584$; $P = 0.006$; $\eta^2 = 0.431$) within the group \times HYP comparison. In HYP2, at 65% $\dot{V}O_{2\text{ peak}}$, differences occurred following HA for HR ($P = 0.001$), SpO_2 ($P = 0.006$), and $\dot{V}O_{2\text{ HR}}$ ($P = 0.006$), but not CON ($P > 0.05$); see Fig. 2. No difference was observed in RER ($f = 0.248$; $P = 0.626$; $\eta^2 = 0.017$) when exercising at 65% $\dot{V}O_{2\text{ peak}}$ (Fig. 2).

Table 2. Comparison of day 1 and day 10 (T_{rec} , HR, and sweat rate) and pre- and postintervention data (plasma volume, Hb_{mass} , blood volume) for heat acclimation and control groups

	Day 1/Pre		Day 10/Post	
	HA	CON	HA	CON
<i>n</i>	8	8	8	8
Resting T_{rec} , $^\circ\text{C}$	36.97 \pm 0.25	36.99 \pm 0.32	36.48 \pm 0.29*†	36.93 \pm 0.28
Resting HR, beats/min	74 \pm 13	68 \pm 14	56 \pm 8*†	66 \pm 9
Sweat rate, l/h	1.13 \pm 0.28*	0.45 \pm 0.20	1.67 \pm 0.42*†	0.59 \pm 0.24
Hb_{mass} , g/kg	869 \pm 92	865 \pm 110	869 \pm 96	857 \pm 126
Plasma volume, ml	2,981 \pm 335	3,142 \pm 530	3,427 \pm 335†	3,107 \pm 622
Blood volume, ml	5,627 \pm 501	5,686 \pm 847	6,129 \pm 550†	5,611 \pm 1,032

Values are means \pm SD; *n*, no. of subjects. *Significant difference from CON within day ($P < 0.05$). †Significant difference from *day 1* within group ($P < 0.05$).

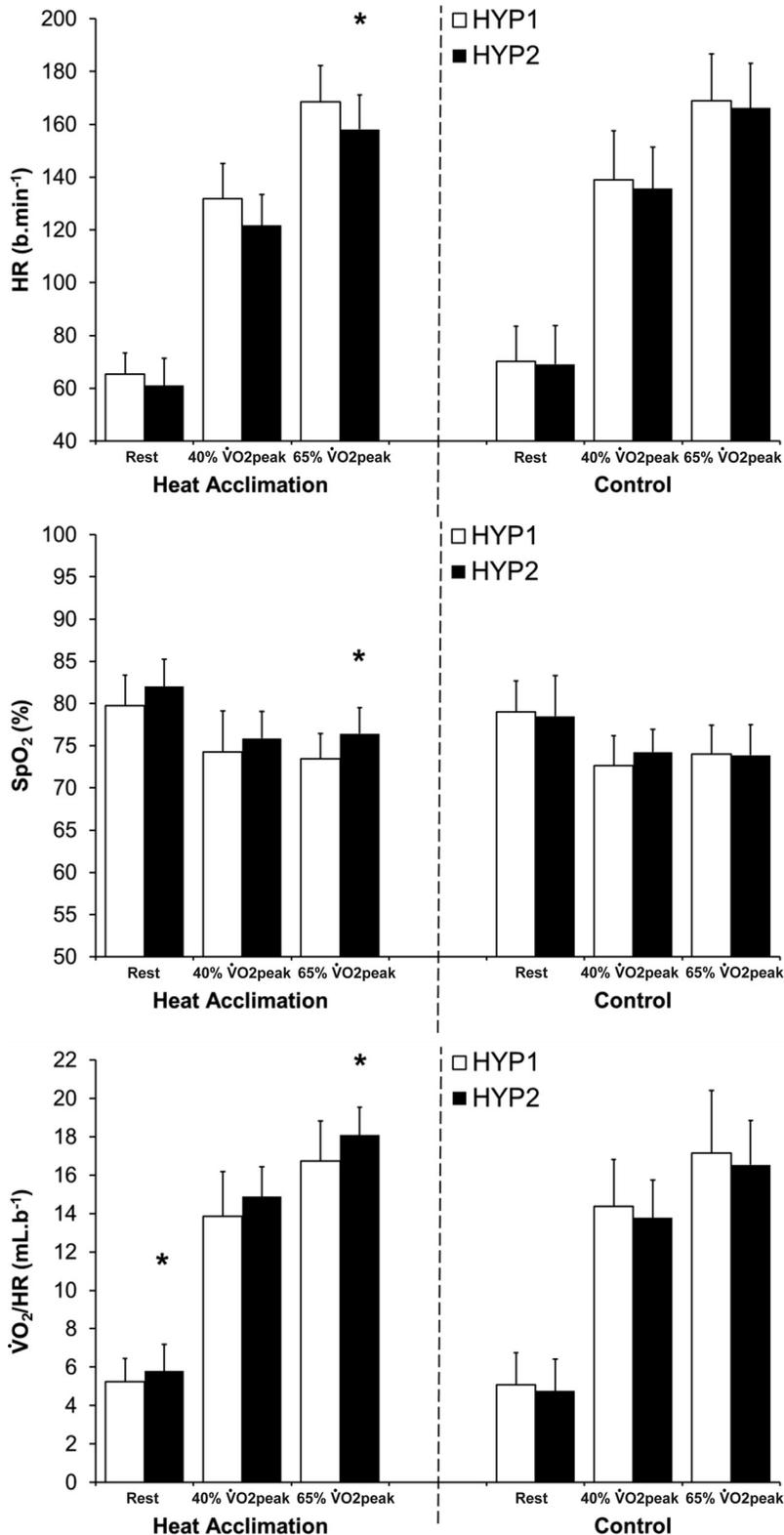


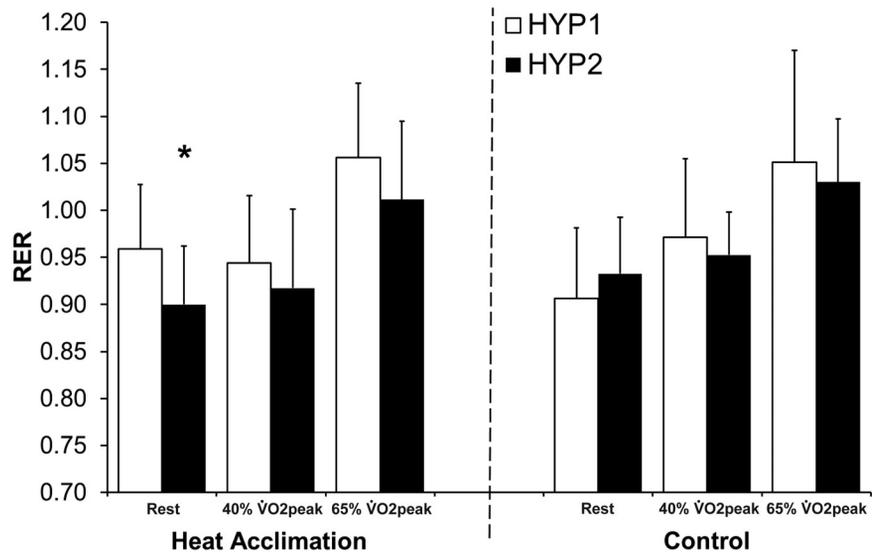
Fig. 2. Heart rate (HR; *top*), oxygen saturation (SpO₂; *middle*), and oxygen pulse [O₂ uptake ($\dot{V}O_2$)/HR; *bottom*] during rest and while exercising at 40% normoxic peak O₂ uptake ($\dot{V}O_{2peak}$) and 65% $\dot{V}O_{2peak}$ in hypoxia [inspired fraction of O₂ (fio₂) = 0.12] before (HYP1; open bars) and after (HYP2; solid bars) HA (*left*; n = 8) or CON (*right*; n = 8). Values are means \pm SD. *Significant difference from HYP1 within condition ($P < 0.05$).

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

HSP72 mRNA and HSP90 α mRNA during hypoxic tolerance tests. HSP72 mRNA increased during HYP1 ($f = 17.005$; $P = 0.001$, $np^2 = 0.567$). In the HA group, an increase in

HSP72 mRNA was observed following HYP1 ($P = 0.006$), but not HYP2 ($P = 0.440$). This was supported by the observation that HSP72 mRNA was greater post-HYP1 compared with HYP2 ($P = 0.021$). No changes in the pattern of HSP72 mRNA expression were observed in CON. HSP90 α mRNA increased before to after HYP1 and HYP2

Fig. 3. Respiratory exchange ratio (RER) during rest and while exercising at 40% normoxic $\dot{V}_{O_2\text{peak}}$ and 65% $\dot{V}_{O_2\text{peak}}$ in hypoxia ($F_{I_{O_2}} = 0.12$) before (HYP1; open bars) and after (HYP2; solid bars) HA (left; $n = 8$) or CON (right; $n = 8$). Values are means \pm SD. *Significant difference from HYP1 within condition ($P < 0.05$).



($f = 17.110$; $P = 0.001$, $np^2 = 0.568$). However, no differences were observed between HYP1 and HYP2, or between HA and CON at any time.

DISCUSSION

This experiment observed that HA reduced physiological strain and the HSP72 mRNA response to an acute hypoxic exposure combining rest and exercise. The adaptation pathway was likely mediated in part by PV expansion, which improved \dot{V}_{O_2} /HR at rest and exercise in hypoxia, as well as attenuating HR responses and preservation of Sp_{O_2} during exercise in hypoxia. Resting RER was reduced after HA, an observation not true of CON, suggesting greater fat oxidation at rest in hypoxia postintervention. At a cellular level, HA mitigated the group-specific HSP72 mRNA increase, but not the HSP90 α mRNA response to hypoxia. The HSP90 α mRNA response also increased comparably to HA before and after CON; however, no increase in HSP72 mRNA was observed in either trial in this group.

HA and CON were successfully matched for the prescribed training parameters (duration, absolute intensity, and work done; Table 1) with the equality of these training parameters giving confidence that adaptations were induced by the increased physiological/thermal strain of the hot environment of HA, compared with the temperate conditions of CON (Table 1). The elegant experimental design of Lorenzo et al. (39) is most closely representative of ours. In agreement with previous data (26), the magnitude of adaptation induced by our isothermic HA regimen is at least equal to that observed by their fixed-

intensity HA regimen (39), which improved physiological responses and exercise performance in hot and cool conditions. The authors (39) reported similar absolute changes in T_{rec} (-0.5°C ; our data = -0.49°C), HR (-15 beats/min; our data = -18 beats/min), and SR ($+0.4$ l/h; our data = $+0.4$ l/h). Additionally, over the same number of HA sessions, we observed a larger expansion of PV ($+6.5\%$; our data = $+15\%$). Despite a cascade of mechanisms well attributed to PV expansion, including increased vascular filling to support cardiovascular stability, increased specific heat capacity of blood, and attenuated skin blood flow responses, the observable magnitude of these adaptive responses may be finite, or demonstrate an exponential decay beyond moderate levels of hypervolemia (58). These responses are agreeable with the consensus that isothermic protocols controlling hyperthermia to a core temperature of at least 38.5°C should be implemented to optimize adaptations (53), due to maintenance of the endogenous thermal stimuli for adaptation (50). Increased core temperature (Fig. 1), leading to elevated and sustained sweating, is the fundamental potentiating stimulus initiating phenotypic responses known as HA (54). Consequently, in HA, increased mean T_{rec} ($+0.8^\circ\text{C}$), and the duration that T_{rec} exceeded the isothermic threshold of 38.5°C (47 min) (18), induced greater adaptation than the normothermic training of CON (Table 1). Greater heat dissipation through evaporation in hot conditions was evidenced by threefold elevation in SRs in HA compared with CON (51). Increased heat storage in HA is the stimuli for observed increases in HR, RPE, and TSS for the same exercise prescription as CON (21). HA increased BV ($+500$ ml) com-

Table 3. Comparison of physiological and perceptual data at rest and while exercising at 40% normoxic $\dot{V}_{O_2\text{peak}}$ and 65% $\dot{V}_{O_2\text{peak}}$ in hypoxia ($F_{I_{O_2}} = 0.12$) before (HYP1) and after (HYP2) heat acclimation or normothermic exercise

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

Values are means \pm SD; $n = 8$ subjects for HA and CON groups. \dot{V}_{O_2} , O_2 uptake; \dot{V}_E , ventilation; Bf, breathing frequency; LLQ, Lake Louise Questionnaire.

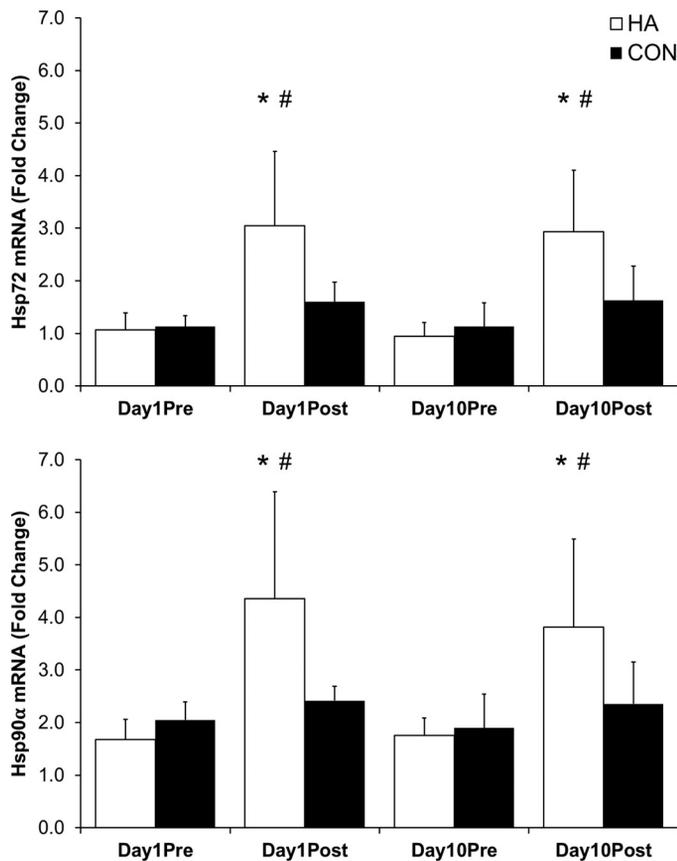


Fig. 4. Heat shock protein (HSP) 72 (*top*) and HSP90 α (*bottom*) mRNA before and after *day 1* (Day1Pre and Day1Post, respectively; *left*) and *day 10* (Day10Pre and Day10Post, respectively; *right*) of HA (open bars; $n = 7$) and CON (solid bars; $n = 8$). Values are means \pm SD. *Significant difference from Pre within day and intervention ($P < 0.05$). #Significant difference from CON within time and day ($P < 0.05$).

pared with CON (Table 2). No change in Hb_{mass} indicates HA-induced hypervolemia was a response to increases in extracellular fluid, with increases in PV (+446 ml) approximate to the absolute change in BV, reaffirming this as a primary adaptation to heat (61), and an established mechanism for the reduction in HR during exercise. Implementation of isothermic methods (50) for HA are the most probable causes for greater PV expansion (+15%) compared with others utilizing similar participants, protocol length, and environmental conditions [6.5% (39), 9.0% (50), and 11.1% (7)]. It remains to be experimentally elucidated whether maintaining lower intensity exercise, which matches heat production to evaporative heat loss, thus closely controlling T_{rec} at 38.5°C, rather than implementing passive rest following T_{rec} exceeding the target of 38.5°C, would augment even more favorable adaptations resulting from higher SRs and elevated cardiovascular response. As such, despite a large magnitude of adaptation observed within this experiment, this is a potential limitation of the implemented experimental design. With no change in gross efficiency as indicated by similar $\dot{V}O_2$, the $\dot{V}O_2/HR$ ratio becomes more efficient after HA (Fig. 2). Hypohydration from increased sweating (HA = 2.9% NBM/session, CON = 1.0% NBM/session) and the sustained endogenous stimuli (increased T_{rec}) of isothermic HA (26) increases PV expansion via increased plasma albumin and the renin-angiotensin-aldosterone

system (50). Large PV has been proposed as maladaptive due to hemodilution (14), where maintenance of cardiac output may be potentially confounded by a reduced relative O_2 -carrying capacity of blood. Improved Sp_{O_2} (+3%) following HA suggests that a 15% increase in PV is beneficial in hypoxia, even if optimal PV expansion is currently unknown. Maintenance of Sp_{O_2} following HA (Fig. 2) occurs as a reduction in HR and blood viscosity affords a greater erythrocyte alveolar transit time, facilitating more complete resaturation within the pulmonary system (11). This is important in hypoxia, and for more well-trained individuals, due to a greater reduction in Sp_{O_2} resulting from a typically larger cardiac output and reduced pulmonary gas exchange at higher exercise intensities (52).

The reduction in T_{rec} and increased SR (Table 2) following HA has a dual role in facilitating enhanced heat balance. Reduced T_{rec} mediates a greater spectrum for temperature increase, while increased SR is facilitated by an earlier sweat onset, even when accounting for decreased T_{rec} (55). Within HYP1/HYP2, the heat stress was moderate (10, 39) and would appear compensable (8), thus reduced T_{rec} following HA as a mechanism for prolonging permissible physiological strain and exercise performance in temperate hypoxia is not fundamental. Instead, reduced T_{rec} during exercise in hypoxia causes a leftward shift in the oxyhemoglobin dissociation curve, signifying the potential for enhanced O_2 saturation (73). This thermoregulatory adaptation is relevant in hypoxia vs. normoxia as O_2 utilization is more greatly compromised. Preservation of Sp_{O_2} observed at 65% $\dot{V}O_{2 peak}$ alone is likely a result of the increased demand for O_2 at the muscle at this higher intensity (63). Interestingly, improved physiological response to matched exercise did not augment a reduction in the RPE or LLQ in hypoxia (Table 3), as previously observed regarding TSS in the heat (26). It should be noted that there is potential for the reduction in physiological strain in HYP2 to be a reflection of a reduced relative exercise intensity, as HA has been shown to increase maximum $\dot{V}O_2$ in both cool and hot conditions (39). No data exist stating HA improves $\dot{V}O_{2 peak}$ or maximum $\dot{V}O_2$ in hypoxia; however, a post-HA $\dot{V}O_{2 peak}$ test in the present study would have been able to determine that this is likely to have occurred. On the basis of this notion, it should be observed that cross-acclimation was effective using a model testing an absolute workload equal before and after intervention, which may accurately reflect occupational or military populations completing a fixed task. However, it is unknown whether the reduction in physiological strain would have also been observed if workload were derived from the relative exercise intensity of a postexercise $\dot{V}O_{2 peak}$ test. This perspective is analogous to exercise performance within a given intensity domain.

Despite no change in our data, hypothetically a sufficient dose of HA could increase Hb_{mass} (60), via the induction of hypoxia-inducible factor (HIF)-1 α (42), as is well established of altitude exposure (27). Trends for increases in erythrocyte volume have been observed following 5-day interventions similar to HA ($4.1 \pm 0.9\%$) (22). Conversely, and in agreement with our data, training for 10 days in 30°C at 610 m elicited no change in erythrocyte volume ($+0.4 \pm 0.6$ ml/kg), whereas training at the same temperature at 2,000 m elicited significant gain ($+1.9 \pm 0.4$ ml/kg). This suggests long established nonthermal, O_2 -sensing pathways are most important for in-

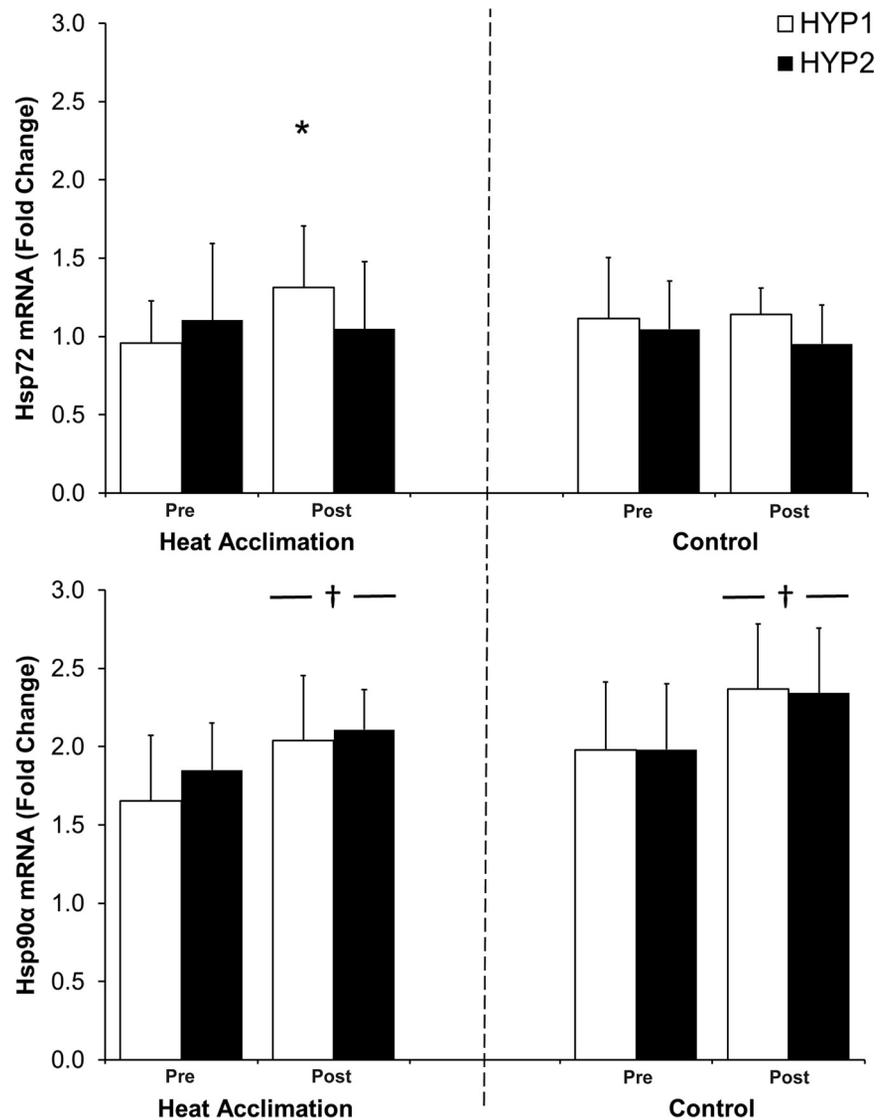


Fig. 5. HSP72 (top) and HSP90 α (bottom) mRNA in hypoxia ($F_{I_{O_2}} = 0.12$) before (HYP1; open bars) and after (HYP2; solid bars) HA (left; $n = 7$) or CON (right; $n = 8$). Values are means \pm SD. *Significant difference from Pre within condition and HYP ($P < 0.05$). †Significant difference from Pre overall ($P < 0.05$).

creasing Hb_{mass} (27, 60). Our interventions did not increase Hb_{mass} ; thus they did not, or cannot, induce sufficient heat strain and/or training load to stimulate erythropoiesis (27). This disparity from comparable research suggests more data are required to elucidate whether HA can effectively induce changes in Hb_{mass} .

The metabolic response to altitude is a preferential shift toward glycolysis (48); as such, the reduction in RER following HA, an indicator of substrate utilization, was an unexpected observation in hypoxia, although a reduction in metabolism has been observed in response to heat (30). Absent of changes in \dot{V}_E and breathing frequency (Table 3), the RER reduction at rest during HYP2 (following HA; Fig. 3) appears to be a metabolic response, rather than an artifact of hyperventilation between trials (5). The present data cannot determine whether hypoxia-induced hyperventilation from normoxia occurred or was reduced following HA. HIF-1 alters metabolism at altitude (48), the typical response being an initial increase in glycolysis on acute exposure, followed by a reduction with acclimatization/acclimation. HIF-1 is known to increase following HA (42, 62); thus increases may accelerate the desensitization, or

inhibit the immediate shift in substrate metabolism (49). Another theory implicates HSP72 as having a therapeutic role in glycogen regulation among other metabolic disorders, e.g., type 2 diabetes and obesity (29). Transgenic mice overexpressing HSP72 evidence increased fatty acid oxidation and reduced mitochondrial dysfunction, alongside increased \dot{V}_{O_2} and exercise capacity (29). At present, these mechanisms are speculative; however, responses within our data warrant further investigation to authenticate or refute this observation.

Observations that HSP72 mRNA increases (+2.5-fold) are sustained during isothermic HA are supported by our data (+2.0 \pm 1.0-fold), which also support similar sustained increases in HSP90 α mRNA (+2.4 \pm 1.5-fold) (25). It is notable that these data are supportive of others observing no daily change in resting HSP72 or HSP90 α mRNA (25, 44, 70); thus, following an initial stress response, the removal of the stress stimuli is sufficient to remove the necessity for transcription back to basal quantities within 24 h. It is this observation that reaffirms that the HSP72 mRNA response to exercise is a potential marker of acclimation; however, resting levels do not provide sufficient discrimination (44), as basal intracellular

HSP72 protein do (44, 45). These data highlight that isothermic HA could provide a stimulus for increases in HSP72 and HSP90 α protein at both the onset and culmination of the regimen (Fig. 4). Endogenous physiological and cellular strain induced by CON was insufficient to induce the respective gene transcripts for HSP72 ($+0.5 \pm 0.2$ -fold) and HSP90 α ($+0.4 \pm 0.1$ -fold), likely due to the failure to induce sufficient changes in T_{rec} or oxidative stress. Thus this exercise prescription is unlikely to increase basal protein within the cell, a requirement of cross-tolerance.

Increased HSP72 and HSP90 α mRNA in hypoxia highlights the sensitivity of each gene to both exercise and endogenous environmental stimuli (temperature and oxidative stress induced by hypoxia). Increased gene expression in HYP1 suggests our protocol was effective at providing potentiating stimuli for a heat shock protein response, albeit with smaller expression than the HA sessions, suggesting inferior endogenous stimuli for transcription. Reductions in HSP72 mRNA in HYP2 following HA evidences either reduced physiological strain during HYP2 (cross-acclimation) or an HA-induced increase in intracellular HSP72 (41) (cross-tolerance), mitigating requirements for further gene transcription. Neither of these mechanistic pathways is true of HSP90 α mRNA, which shares a similar response between HYP1 ($+0.3 \pm 0.4$ -fold) and HYP2 ($+0.4 \pm 0.4$ -fold) (Fig. 5). A longer hypoxic exposure may have elicited a greater magnitude of heat shock protein mRNA responses, especially for participants in CON, who demonstrated no increase in HSP72 mRNA. This prolonged protocol may have also further enhanced observable differences in HSP72 mRNA before and after HA. Maintenance of increased HSP90 α in HYP2 may relate to a specific role of the protein within recovery and adaptation to cellular stress, i.e., control of cellular signaling cascades (64), recovery of global protein synthesis (13), and coordination of cellular repair (15). Hence continued gene transcription may be required. Relative exercise intensity, which is known to change under different environmental conditions, affects the metabolic strain and molecular responses (4, 36). Accumulation of HSP72 and HSP90 protein occurs with HA (45); however, basal HSP90 α has been demonstrated as lower than HSP72 (45). Within HYP1/HYP2, the metabolic strain likely induced protein denaturation, activating the heat shock protein response via heat shock factor-1. However, it is plausible that basal HSP72 was sufficient to cope with the hypoxic stress post-HA (4); thus transcription was mitigated. However, basal HSP90 α protein remained lower than necessary; thus HYP2 induced further mRNA transcription to a similar extent as observed in HYP1. As previously observed (70), the present study cannot suggest that our intervention can translate the mRNA signal into HSP72- and HSP90 α -mediated thermotolerance or hypoxic cross-tolerance within leukocytes (31), because increased mRNA expression is not necessarily reflective of functional steady-state basal protein content, which may or may not be the most important component for observing cross-tolerance (71). Although it is unknown whether HA induced HSP72 and HSP90 α protein accumulation, it has previously been stated that observed mRNA increases provide an indication that the heat shock response has been activated, potentiating protein translation (70).

The present data suggest the existence of pathways for transferring adaptations to HA to other environments (varying

temperature/oxygen availability), although future experiments should determine whether the attenuated responses are specific to thermal stimuli alone, or the combined exercise-heat stress training stimuli that our intervention applied. With likely increases in aerobic capacity (39), the absolute workload model implemented may have also elicited different responses than a relative intensity model based on postacclimation aerobic capacity; thus this is a limitation of the experiment, which should be considered relevant to future research in the area. Data pertaining to cross-acclimation within this experiment are clear, particularly when cardiovascular stress compromises the individual in a particular environment (14, 73). The true thermal adaptation might be of greater relevance with regard to the heat shock protein response, which is known as responsive to different relative exercise intensities (4, 36). Benefits of increased intracellular HSP72 and HSP90 α have been recently reviewed (14, 56, 73); however, cross-tolerance pathways cannot be fully confirmed given the lack of protein data presently available. Implications for HA-induced changes in HSP72 and HSP90 α could be confirmed by measuring HSP72 and HSP90 α protein pre- and postintervention and then applying hypoxic shock/stress within ex vivo and in vivo models. Further research is required to determine the benefits of cross-acclimation and cross-tolerance across a spectrum of simulated and actual altitudes and workloads within those environments.

Conclusion. HA is an effective intervention for reducing physiological strain associated with acute normobaric hypoxia, primarily through HA-derived PV expansion improving cardiovascular efficiency, which reaffirms a cross-acclimation mechanism. Normothermic training failed to reduce physiological strain or alter the HSP72 and HSP90 α mRNA response to hypoxia. The HSP72 mRNA increase pre-HA was attenuated following acute normobaric hypoxia following HA, giving efficacy to cross-tolerance pathways at a cellular/molecular level; however, no changes in HSP90 α mRNA were observed. These data suggest hyperthermia as a viable potentiating stimulus for the cross-adaptive mechanisms.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: O.R.G., P.W.W., and N.S.M. conception and design of research; O.R.G. and G.T. performed experiments; O.R.G. and J.A.T. analyzed data; O.R.G., G.T., J.A.T., L.T., P.W.W., and N.S.M. interpreted results of experiments; O.R.G. prepared figures; O.R.G. and L.T. drafted manuscript; O.R.G., G.T., J.A.T., L.T., P.W.W., and N.S.M. edited and revised manuscript; O.R.G., G.T., J.A.T., L.T., P.W.W., and N.S.M. approved final version of manuscript.

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