Title

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

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Abstract

Heat acclimation 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 Hsp72 and Hsp90α mRNA responses in acute normobaric hypoxia.

Sixteen male participants completed ten 90 min sessions of isothermic heat acclimation (HA; 40°C/40%RH) or exercise training (CON; 20°C/40% RH). HA or CON were preceded (HYP1) and proceeded (HYP2) by a 30min normobaric hypoxic exposure (FiO_2=0.12; 10min rest, 10min cycling at 40%$\dot{V}O_{2peak}$, 10min cycling at 65%$\dot{V}O_{2peak}$).

HA induced greater rectal temperatures (T_{rec}), sweat rate (SR) and heart rates (HR) than CON during the training sessions. HA, but not CON, reduced resting T_{rec}, resting HR and increased SR and plasma volume. Haemoglobin mass did not change following HA nor CON. Hsp72 and Hsp90α mRNA increased in response to each HA session, but did not change with CON.

HR during HYP2 was lower and O_2 saturation higher at 65%$\dot{V}O_{2peak}$ following HA, but not CON. $\dot{V}O_2$/HR was greater at rest and 65% $\dot{V}O_{2peak}$ in HYP2 following HA, but was unchanged after CON. At rest, the respiratory exchange ratio 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 Hsp90α mRNA during HYP1 were maintained in HYP2.

HA reduces physiological strain, and the transcription of Hsp72, but not Hsp90α mRNA in acute normobaric hypoxia.

Keywords

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

Introduction

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 summarised 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, 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 heat acclimation may reduce physiological strain in hypoxia (14, 56, 73). Mechanistic pathways can be subdivided into cross acclimation, whereby heat acclimation attenuates physiological strain (73) and cross tolerance, whereby cellular responses to heat acclimation provide cytoprotection during hypoxia (14). Acute physiological responses to hypoxia (2) can be used as criteria for validating heat induced cross acclimation. Heat acclimation reduces glycolysis and metabolic rates during exercise (34), with plasma volume expansion (39, 50) and improved myocardial efficiency (38) preserving cardiac output and skeletal muscle blood flow. Muscle oxygenation is also sustained by heat acclimation induced maintenance of central blood volume via reductions in the core/skin temperature gradient (58) and enhanced evaporative heat loss (51). Improved temperature and haematological regulation facilitate a leftward shift in the oxyhaemoglobin saturation curve (37). Heat acclimation induces expedient and beneficial adaptations within five to fourteen daily sessions demonstrating a greater efficiency of adaptation when compared to altitude/hypoxic interventions (23).

Crosstolerance has been defined as single or repeated sub-lethal 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 heat acclimation (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 HSPA1A (Hsp72) and HSPC1 (Hsp90α) mitigate pathophysiological responses to endogenously stressful stimuli. Both Hsp72 and Hsp90α augment proportionally to increased cellular stress (increased cellular temperature) in response to *ex vivo* heat shock (45), and have been implicated as important for modulating the adaptive cellular/molecular response to hypoxia (56, 65, 66); this suggests a shared signalling pathway. Both Hsp72 and Hsp90α 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 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 know increases with acclimation (45). The delayed responsiveness of the protein response (16, 17), in comparison to the within session heat shock protein mRNA response (25, 44) emphasises 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 Hsp90α 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.

Heat acclimation has been evidenced in improving oxygen saturation and heart rates during hypoxic exercise performance (28) with heat acclimation 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 heat acclimation would reduce physiological strain and the Hsp72 and Hsp90α mRNA responses to an acute hypoxic exposure (at rest and at various exercise intensities) in comparison to exercise training matched for intensity and duration in temperate conditions. It was hypothesised that heat acclimation would reduce physiological strain in hypoxia via cardiovascular and thermoregulatory adaptations, and that the heat shock protein response to hypoxia would be reduced following heat acclimation.

Materials and Methods

Participants

Sixteen healthy males, who completed various forms of exercise training between three and six times per week, were assigned to matched groups to perform ten days of isothermic heat acclimation (HA; age 22.5±3.5 yrs., nude body mass (NBM) 74.6±7.9 kg, body surface area 1.95±0.13 m^2, peak oxygen uptake ($\dot{V}O_{2peak}$) 4.32±0.68 $L.min^{-1}$, 58.5 $mL.kg^{-1}.min^{-1}$), or act as a normothermic exercise control (CON; age 26.0±5.0 yrs., NBM 74.6±4.8kg, body surface area 1.93±0.13 m^2, $\dot{V}O_{2peak}$ 4.22±0.62 $L.min^{-1}$ 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 prior to each experimental/training session (<700 $mOsm \cdot Kg^{-1}$ H_2O (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

Prior to assessment of $\dot{V}O_{2peak}$, anthropometric data was collected with NBM measured using digital scales, precise to 0.01 kg (GFK 150, Adam Equipment Inc, Danbury, CT, USA). $\dot{V}O_{2peak}$ ($L.min^{-1}$) 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 80W, increasing by 24 $W.min^{-1}$, in temperate laboratory conditions (20°C, 40% relative humidity (RH)) at sea level (FiO_2 = 0.2093). $\dot{V}O_{2peak}$ was defined as the highest average $\dot{V}O_2$ obtained in any 30 s period with $\dot{V}O_{2peak}$ 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 $\dot{V}O_{2peak}$ was made via the attainment of a heart rate (HR) within 10 $b.min^{-1}$ of age predicted maximum, and 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 continually during all experimental/training sessions by telemetry (Polar Electro Oyo, Kempele, Finland).

Haematological Measures

Twenty four hours prior to hypoxic exposures haemoglobin mass (Hb_{mass}; g) was measured. Hb_{mass} , blood volume (BV; mL) and plasma volume (PV; mL) were determined in accordance with the oCOR-method (59). Participants were seated for 20 min, before being connected to a closed glass spirometer allowing inspiration of a CO (carbon monoxide) bolus of 1.0 $mL.kg^{-1}$ (68), followed by 2 min rebreathing of a 3.5 L O_2 bolus. Before and 4 min after CO-rebreathing, participants completely exhaled to residual volume into a CO gas meter (Pac 7000, Drager; Pittsburgh, PA, USA). CO volume not remaining within the body was calculated from the remainder CO in the spirometer and exhaled CO measured immediately after disconnecting the spirometer from the participant (68). Fingertip capillary samples, for determination of carboxyhaemoglobin 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 (RadiometerTM; Copenhagen, Denmark). Hb_{mass} was calculated from the mean change in %HbCO before and after rebreathing CO (68). At the relevant intervals within the oCOR method, haemoglobin concentration (Hb; $g.dL^{-1}$) was collected from fingertips in duplicate using a microcuvette and analysed using a B-Haemoglobin Photometer (Hemocue Limited, Ängelholm, Sweden) and haematocrit (Hct; %) was collected in triplicate (~50 µL) with glass capillary tubes and analysed following centrifugation at 14,000 rpm for 3 min (Haemotospin 1300 Centrifuge, Hawksley & Sons Ltd, West Sussex, UK) (69). The experimenter typical error of measurement for total Hbmass prior to commencing this experiment was ±1.98% (±17.0 g).

Hypoxic Exposures

Hypoxic exposures were performed 24 hours prior to commencing the first session of HA or CON (HYP1) and 24 hours 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 (FiO_2 = 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 $\dot{V}O_{2peak}$ 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 $\dot{V}O_{2peak}$ (HA= 201±41W, CON=192±37W) for a further 10 min. During rest and exercise, HR, oxygen uptake ($\dot{V}O_2$; $L.min^{-1}$), carbon dioxide production, ($\dot{V}CO_2$; $L.min^{-1}$), ventilation (V_E; $L.min^{-1}$), respiratory exchange ratio (RER) and peripheral arterial oxygen saturation (SpO_2; %) estimated using a fingertip pulse oximeter (Nonin 2500, Nonin Medical Inc, Minnesota, USA) were recorded continuously, with the final 5 min of measures used for analysis. Prior to entry, and following every ten 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.

Heat Acclimation/Exercise Protocols

Each HA or CON training session was conducted at the same time of day (07:00-10:00 h) to control for effects of daily variation in exercise performance (12) and heat shock protein expression (67) inside a purpose built environmental chamber (WatFlow control system; TISS, Hampshire, UK). Temperature and humidity were controlled using automated computer feedback (WatFlow control system; TISS, Hampshire, UK). On arrival to the laboratory, participants provided a mid-flow 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; $L.hr^{-1}$), was estimated using the change in NBM from the pre- to post- exercise periods. Participants inserted a rectal thermistor (Henleys Medical Supplies Ltd, Welwyn Garden City, UK, Meter logger Model 401, Yellow Springs Instruments, Yellow Springs, Missouri, USA) 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 stabilisation 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°C±0.4°C, 41.0±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% $\dot{V}O_{2peak}$ until the isothermic target T_{rec} of ≥38.5°C had been achieved, and, upon the attainment of a T_{rec} ≥38.5°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°C±0.2°C, 28.5±2.7% RH). CON participants initially cycled at an intensity corresponding to 65% $\dot{V}O_{2peak}$ 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 figure 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 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 is contained in Table 1.

Blood Sampling and RNA extraction

Venous blood samples were taken immediately pre- and post- HYP1 and HYP2, and pre- and post- the first (Day1) and tenth (Day10) 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 (10X Red Blood Cell Lysis Solution, Miltenyi Biotech, Bisley, UK). Samples were incubated for 15 min at room temperature before isolation via 5 min centrifugation at 400G then washed twice in 2 mL PBS and centrifugation at 400G for 5 min. Due to 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 instructions (Invitrogen, Life Technologies, Carlsbad, USA). 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, USA).

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

Hsp72 and Hsp90α relative mRNA expression was quantified using RT-QPCR. Primers β2-Microglobulin (β2-M; NCBI Accession number:NM_004048; Forward:CCGTGTGAACCATGTGACT, Reverse:TGCGGCATCTTCAAACCT), Hsp72 (NCBI Accession number:NM_005345; Forward:CGCAACGTGCTCATCTTTGA, Reverse:TCGCTTGTTCTGGCTGATGT), and Hsp90α (NCBI Accession numbers:NM_001017963 (variant 1) & NM_005348 (variant 2); Forward:AAACTGCGCTCCTGTCTTCT, Reverse:TGCGTGATGTGTCGTCATCT) were designed using primer design software (Primer Quest and Oligoanalyzer - Integrated DNA technologies, Coralville, IA, USA) (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 β2-M CT values. Fold change in relative mRNA expression was calculated using the 2-ΔΔCT method.

Statistical Analysis

A priori power analysis for key heat acclimation dependent variables selecting conventional α (0.05) and β (0.20) levels, observed eight participants were required in each experimental group. Prior to statistical analysis, all outcome variables were checked for normality using Kolmogorov-Smirnov and sphericity using the Greenhouse Geisser method prior to 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 Day1/Pre and Day10/post. Two-way mixed-design ANOVA was performed to determine differences between HA and CON, as well as HYP1 with HYP2; thus rest, 40%$\dot{V}O_{2peak}$ and 65%$\dot{V}O_{2peak}$ conditions within each HYP were analysed independently from one another. Three-way mixed-design ANOVA was performed on the Hsp72 and Hsp90α mRNA data to determine differences between pre- and post- value (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 (*d;* small = 0.2, medium = 0.5, large = 0.8) or partial eta squared (*np^2;* small = 0.01, medium = 0.06, large = 0.13) were calculated to analyse the magnitude and trends with data. All data are reported as mean±SD. For all analysis two-tailed significance was accepted at $p<0.05$.

Results

Heat Acclimation/ 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_{rec}≥38.5°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; *np^2*=0.799) 30 and 90 min (figure 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 to CON interventions (Table 1).

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Adaptation to Heat Acclimation

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

INSERT TABLE 2 APPROXIMATELY HERE

Hsp72 mRNA and Hsp90α mRNA during HA/CON

An interaction effect was observed for Hsp72 mRNA (f=20.428; p=0.001; np^2=0.611) and Hsp90α mRNA (f=10.282; p=0.007; np^2=0.422). No difference was observed between HA or CON at pre Day1 or Day10 (p=0.396 and p=0.180), but a difference was observed post HA in comparison to CON (p=0.004 and p=0.012). Hsp72 mRNA and Hsp90α 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 $\dot{V}O_2$/HR (f=6.852; p=0.020; np^2=0.329) and RER (f=5.078; p=0.041; np^2=0.266). In HYP2, at rest differences occurred following HA for $\dot{V}O_2$/HR (p=0.039; Figure 2) and RER (p=0.045; Figure 3), but not CON (p>0.05). No difference was observed in HR (f=0.820; p=0.381; np^2=0.055) nor SpO_2 (f=2.123; p=0.167; np^2=0.132) at rest in hypoxia (Figure 2).

When exercising at 40%$\dot{V}O_{2peak}$ in hypoxia, no differences were observed in the within the group*HYP comparison for HR (f=1.575; p=0.230; np^2=0.101), SpO_2 (f=0.000; p=1.000; np^2=0.000), $\dot{V}O_2$/HR (f=2.651; p=0.126; np^2=0.126) or RER (f=0.047; p=0.831; np^2=0.003) (Figure 2). When exercising at 65%$\dot{V}O_{2peak}$ in hypoxia, differences were observed for HR (f=4.751; p=0.047; np^2=0.253), SpO_2 (f=5.616; p=0.033; np^2=0.286) and $\dot{V}O_2$/HR (f=10.584; p=0.006; np^2=0.431) within the group*HYP comparison. In HYP2, at 65%$\dot{V}O_{2peak}$ differences occurred following HA for HR (p=0.001), SpO_2 (p=0.006) and $\dot{V}O_2$/HR (p=0.006), but not CON (p>0.05) see Figure 2. No difference was observed in RER (f=0.248; p=0.626; np^2=0.017) when exercising at 65%$\dot{V}O_{2peak}$ (Figure 2).

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 $\dot{V}O_2$, $\dot{V}_E$, B_f, RPE or LLQ (Table 3).

INSERT TABLE 3 APPROXIMATELY HERE

Hsp72 mRNA and Hsp90α 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 in comparison to HYP2 (p=0.021). No changes in the pattern of Hsp72 mRNA expression were observed in CON. Hsp90α mRNA increased pre to post HYP1 and HYP2 (f=17.110; p=0.001 np^2=0.568). However, no differences were observed between HYP1 and HYP2, nor between HA and CON at any time.

Discussion

This experiment observed that heat acclimation 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 SpO_2 during exercise in hypoxia. Resting RER reduced after HA, an observation not true of CON, suggesting greater fat oxidation at rest in hypoxia post intervention. At a cellular level, HA, mitigated the group specific Hsp72 mRNA increase, but not the Hsp90α mRNA response to hypoxia. The Hsp90α mRNA response also increased comparably to HA before and after CON, however no increase in Hsp72 mRNA was observed in either trials 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, in comparison to the temperate conditions of CON (Table 1). The eloquent 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 heat acclimation 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 $b.min^{-1}$; our data =-18 $b.min^{-1}$) and SR (+0.4$L.hr^{-1}$; our data = +0.4$L.hr^{-1}$). Additionally, over the same number of heat acclimation 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 (Figure 1), leading to elevated and sustained sweating, are the fundamental potentiating stimuli initiating phenotypic responses known as heat acclimation (54), consequently, in HA, increased mean T_{rec} (+0.8°C), and the duration where 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 three fold elevation in sweat rates in HA compared to 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) compared to 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 utilising 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 favourable adaptations resulting from higher sweat rates 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 (Figure 2). Hypohydration from increased sweating (HA=2.9%NBM.session^{-1}, CON=1.0%NBM.session^{-1}), and the sustained endogenous stimuli (increased T_{rec}) of isothermic heat acclimation (26) increases PV expansion via increased plasma albumin and the Renin-Angiotensin-Aldosterone system (50). Large PV has been proposed as maladaptive due to haemodilution (14), where maintenance of cardiac output may be potentially confounded by a reduced relative O_2 carrying capacity of blood. Improved SpO_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 SpO_2 following HA (Figure 2) occurs as a reduction in HR and blood viscosity affords a greater erythrocyte alveolar transit time, facilitating more complete re-saturation within the pulmonary system (11). This is important in hypoxia, and for more well-trained individuals, due to a greater reduction in SpO_2 resulting from a typically larger cardiac output, and reduced pulmonary gas exchange at higher exercise intensities (52).

INSERT FIGURE 2 APPROXIMATELY HERE

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, whilst 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 oxyhaemoglobin dissociation curve, signifying the potential for enhanced O_2 saturation (73). This thermoregulatory adaptation is relevant in hypoxia vs. normoxia as O_2 utilisation is more greatly compromised. Preservation of SpO_2 observed at 65%$\dot{V}O_{2peak}$ 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 heat acclimation has been shown to increase $\dot{V}O_{2max}$ in both cool and hot conditions (39). No data exists stating heat acclimation improves $\dot{V}O_{2peak}$ or $\dot{V}O_{2max}$ in hypoxia, however a post HA $\dot{V}O_{2peak}$ test in the present study would've been able to determine that this is likely to have occured. Based on this notion, it should be observed that cross acclimation was effective using a model testing an absolute workload equal before and after intervention,

406 which may accurately reflect occupational or military populations completing a fixed task, however it is
407 unknown whether the reduction in physiological strain would have also been observed if workload was
408 derived from the relative exercise intensity of on a post exercise $\dot{V}O_{2peak}$ test. This perspective being
409 analogous to exercise performance within a given intensity domain.
410
411 Despite no change in our data, hypothetically a sufficient dose of heat acclimation could increase Hb_{mass}
412 (60), via the induction of HIF-1α (42), as is well established of altitude exposure (27). Trends for increases
413 in erythrocyte volume have been observed following 5 d interventions similar to HA (4.1±0.9%) (22).
414 Conversely, and in agreement with our data, training for 10 d in 30°C at 610 m elicited no change in
415 erythrocyte volume (+0.4±0.6 $mL.kg^{-1}$), whereas training at the same temperature at 2,000 m elicited
416 significant gain (+1.9±0.4 $mL.kg^{-1}$). This suggests long established non-thermal, O_2 sensing pathways are
417 most important for increasing Hb_{mass} (27, 60). Our interventions did not increase Hb_{mass}, thus they did not,
418 or cannot induce sufficient heat strain and/or training load to induct erythropoietin (27). This disparity
419 from comparable research suggests more data are required to elucidate whether heat acclimation can
420 effectively induce changes in Hb_{mass}.
421
422 The metabolic response to altitude is a preferential shift towards glycolysis (48), as such the reduction in
423 RER following HA, an indicator of substrate utilisation was an unexpected observation in hypoxia, though
424 a reduction in metabolism has been observed in response to heat (30). Absent of changes in $\dot{V}_E$ and Bf (Table
425 3), the RER reduction at rest during HYP2 (following HA; Figure 3) appears to be a metabolic response ,
426 rather than an artefact of hyperventilation between trials (5). The present data cannot determine whether
427 hypoxia induced hyperventilation from normoxia occurred, or was reduced following HA. HIF-1 alters
428 metabolism at altitude (48), the typical response being an initial increase in glycolysis upon acute exposure,
429 followed by a reduction with acclimatization/acclimation. HIF-1 is known to increase following heat
430 acclimation (42, 62), thus increases may accelerate the desensitisation, or inhibit the immediate shift in
431 substrate metabolism (49). Another theory implicates Hsp72 as having a therapeutic role in glycogen
432 regulation amongst other metabolic disorders e.g. type II diabetes, obesity (29). Transgenic mice
433 overexpressing Hsp72 evidence increased fatty acid oxidation and reduced mitochondrial dysfunction,
434 alongside increased $\dot{V}O_2$ and exercise capacity (29). At present these mechanisms are speculative, however
435 responses within our data warrant further investigation to authenticate or refute this observation.
436
437 ***INSERT FIGURE 3 APPROXIMATELY HERE***
438
439 Observations, that Hsp72 mRNA increases (+2.5 fold) are sustained during isothermic heat acclimation are
440 supported by our data (+2.0±1.0 fold), which also supports similar sustained increases in Hsp90α mRNA
441 (+2.4±1.5 fold) (25). It is notable that this data is supportive of others observing no daily change in resting
442 Hsp72 or Hsp90α mRNA (25, 44, 70), thus following an initial stress response, the removal of the stress
443 stimuli is sufficient to remove the necessity for transcription back to basal quantities within 24 hours. It is

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 heat acclimation could provide a stimuli for increases in Hsp72 and Hsp90α protein at both the onset and culmination of the regimen (Figure 4). Endogenous physiological and cellular strain induced by CON was insufficient to induce the respective gene transcripts for Hsp72 (+0.5±0.2 fold) and Hsp90α (+0.4±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.

INSERT FIGURE 4 APPROXIMATELY HERE

Increased Hsp72 and Hsp90α 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 are true of Hsp90α mRNA which shares a similar response between HYP1 (+0.3±0.4 fold) and HYP2 (+0.4±0.4 fold) (Figure 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 Hsp90α in HYP2 may relate to the a specific role of the protein within recovery and adaptation to cellular stress i.e. control of cellular signalling 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, effects the metabolic strain and molecular responses (4, 36). Accumulation of Hsp72 and Hsp90 protein occurs with HA (45) however basal Hsp90α 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 Hsp90α protein remained lower than necessary thus HYP2 induced further mRNA transcription to a similar extent as observed in HYP1. As previously observed (70), the current study cannot suggest that our intervention can translate the mRNA signal into Hsp72 and Hsp90α 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). Though it is unknown whether HA induced Hsp72 and Hsp90α protein accumulation, it has previously been stated that observed mRNA increases provides an indication that the heat shock response has been activated, potentiating protein translation (70).

INSERT FIGURE 5 APPROXIMATELY HERE

The present data suggest the existence of pathways for transferring adaptations to heat acclimation 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 post acclimation 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 regards to the heat shock protein response which is known as responsive to different relative exercise intensities (4, 36). Benefits of increased intracellular Hsp72 and Hsp90α 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 heat acclimation induced changes in Hsp72 and Hsp90α could be confirmed by measuring Hsp72 and Hsp90α protein pre and post intervention, 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

Heat acclimation is an effective intervention for reducing physiological strain associated with acute normobaric hypoxia, primarily through heat acclimation derived PV expansion improving cardiovascular efficiency which reaffirms a cross acclimation mechanism. Normothermic training failed to reduce physiological strain or alter the Hsp72 and Hsp90α mRNA response to hypoxia The Hsp72 mRNA increase pre HA was attenuated following acute normobaric hypoxia following heat acclimation, giving efficacy to cross tolerance pathways at a cellular/molecular level, however no changes in Hsp90α mRNA were observed. These data suggest hyperthermia as viable potentiating stimuli for the cross adaptive mechanisms.

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

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Figure and Table legends

Figure 1. Mean ± SD Rectal Temperature (°C) during 10 days of heat acclimation (HA, n =8) and normothermic exercise (EX, n = 8).

Figure 2. Mean ± SD Heart rate (HR; top) Oxygen saturation (SpO_2; middle) and oxygen pulse ($\dot{V}O_2$/HR; bottom) during rest and whilst exercising at 40% normoxic $\dot{V}O_{2peak}$ and 65% $\dot{V}O_{2peak}$ in hypoxia (FiO_2 = 0.12) before (HYP1; clear bars) and after (HYP2; filled bars) heat acclimation (HA; left, n = 8) or normothermic exercise (CON; right, n = 8). * denotes significant difference from Hyp1 within condition (p<0.05).

Figure 3. Mean ± SD Respiratory exchange ratio (RER) during rest and whilst exercising at 40% normoxic $\dot{V}O_{2peak}$ and 65% $\dot{V}O_{2peak}$ in hypoxia (FiO_2 = 0.12) before (HYP1; clear bars) and after (HYP2; filled bars) heat acclimation (HA; left, n = 8) or normothermic exercise (CON; right, n = 8). * denotes significant difference from Hyp1 within condition (p<0.05).

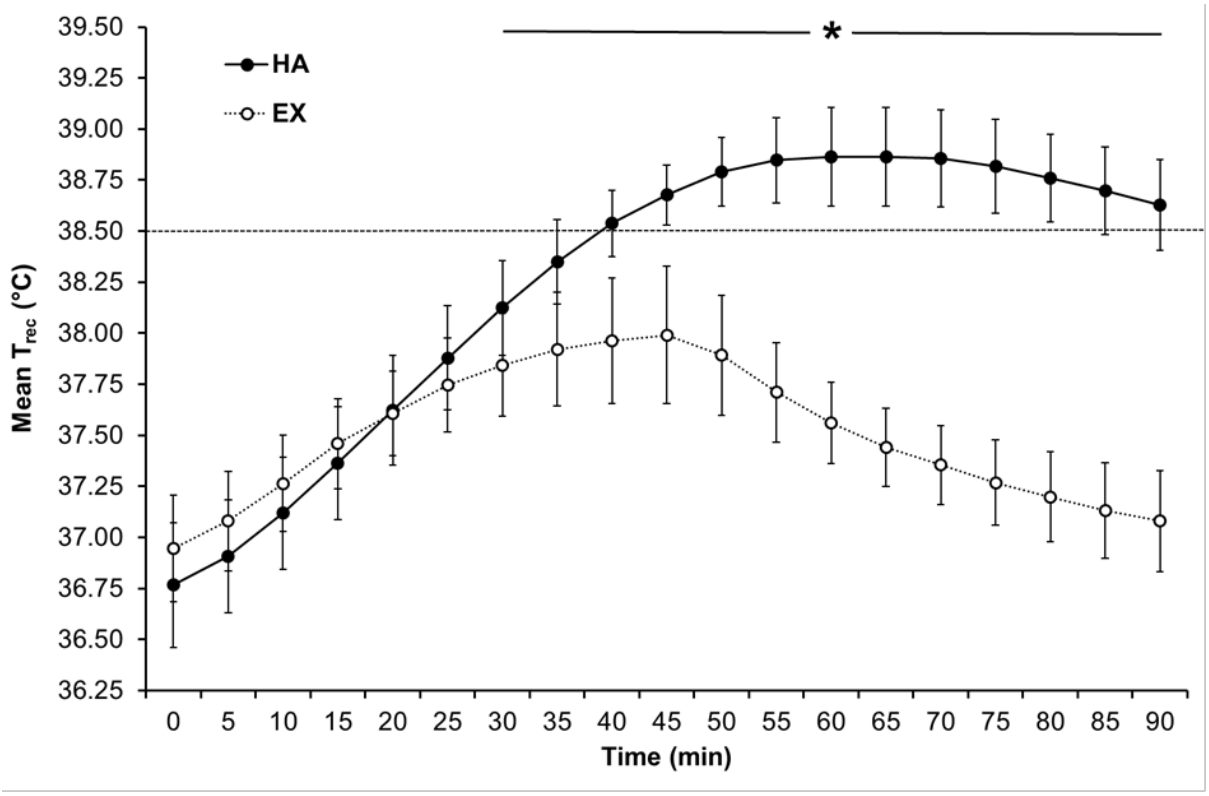
Figure 4. Mean ± SD Hsp72 (top) and Hsp90α (bottom) mRNA pre and post Day 1 (left) and Day 10 (right) of heat acclimation (HA; clear bars, n = 7) and normothermic exercise controls (CON; filled bars, n = 8). * denotes significant difference from pre within Day and Intervention (p<0.05). # denotes significant difference from CON within Time and Day (p<0.05).

Figure 5. Mean ± SD Hsp72 (top) and Hsp90α (bottom) mRNA in hypoxia (FiO2 = 0.12) before (HYP1; clear bars) and after (HYP2; filled bars) heat acclimation (HA; left, n = 7) or normothermic exercise (CON; right, n = 8). * denotes significant difference from Pre within condition and HYP (p<0.05). † denotes significant difference from Pre overall (p<0.05).

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

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

Table 3. Mean ± SD Comparison of physiological and perceptual data at rest and whilst exercising at 40% normoxic $\dot{V}O_{2peak}$ and 65% $\dot{V}O_{2peak}$ in hypoxia (FiO_2 = 0.12) before (Hyp1) and after (Hyp2) heat acclimation (HA, n = 8) or normothermic exercise (CON, n = 8). * denotes significant difference from HYP1 within condition (p<0.05).



752

753 Figure 1.

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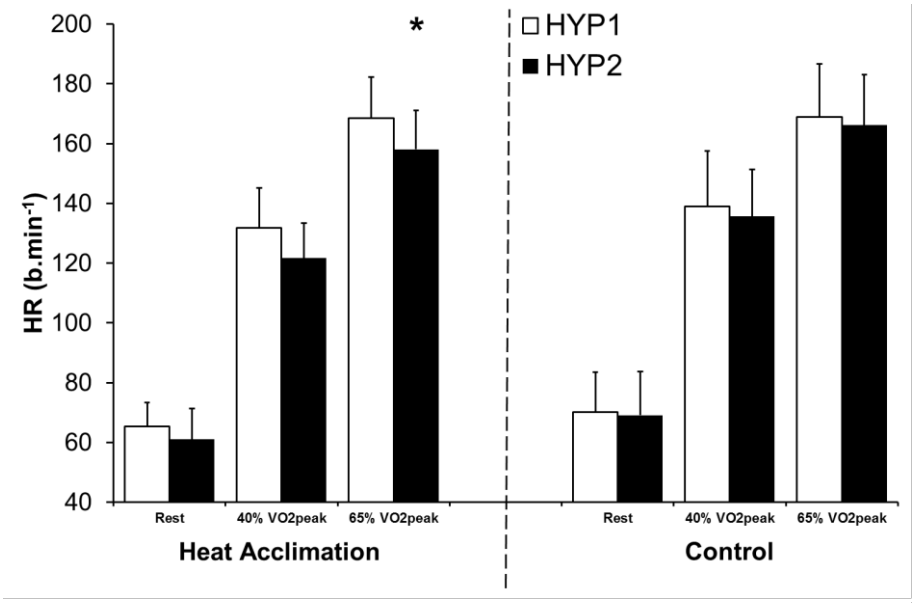

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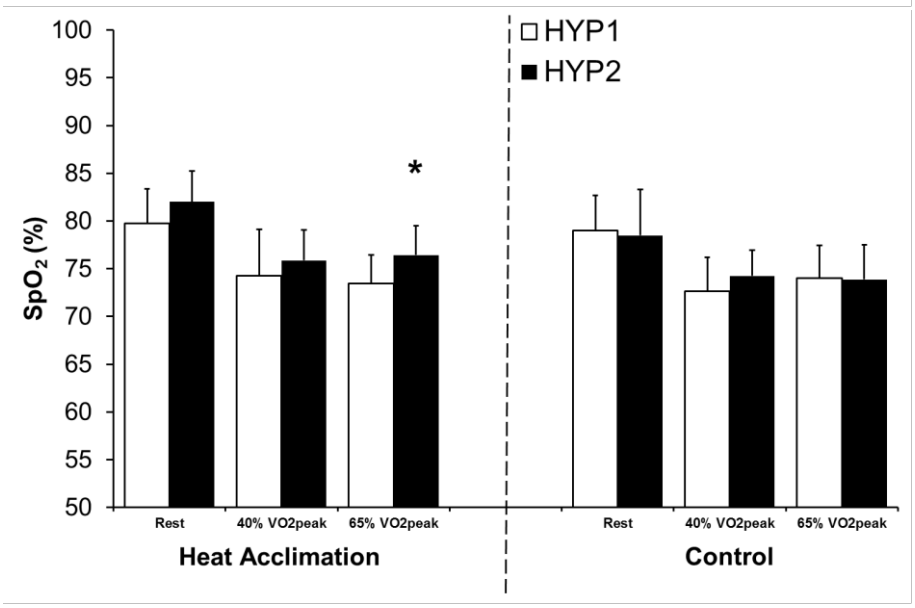

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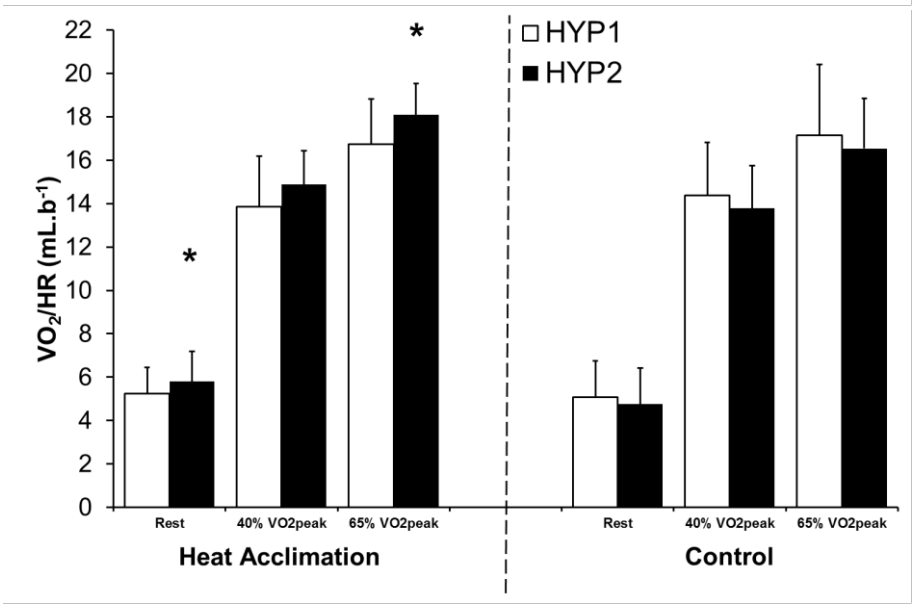

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758 Figure 2.

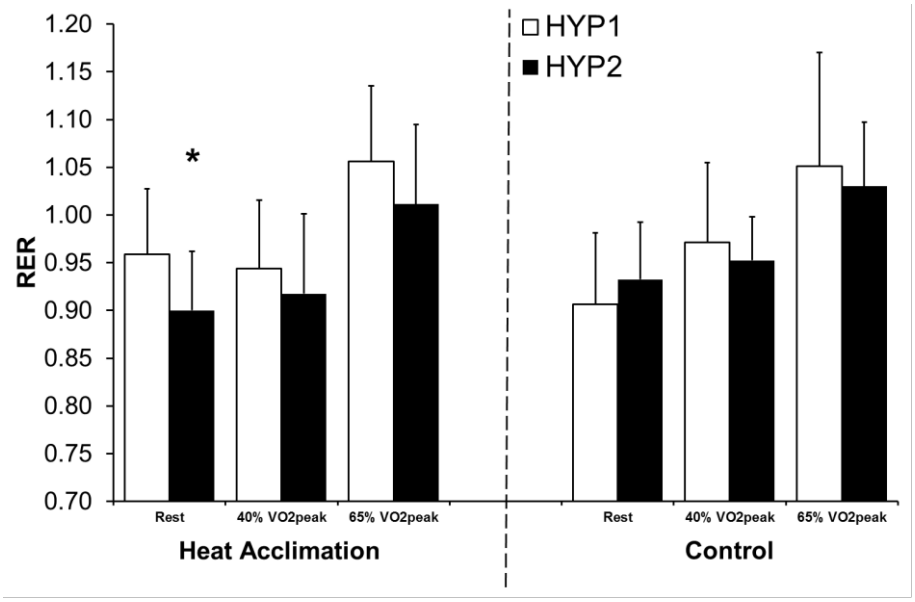
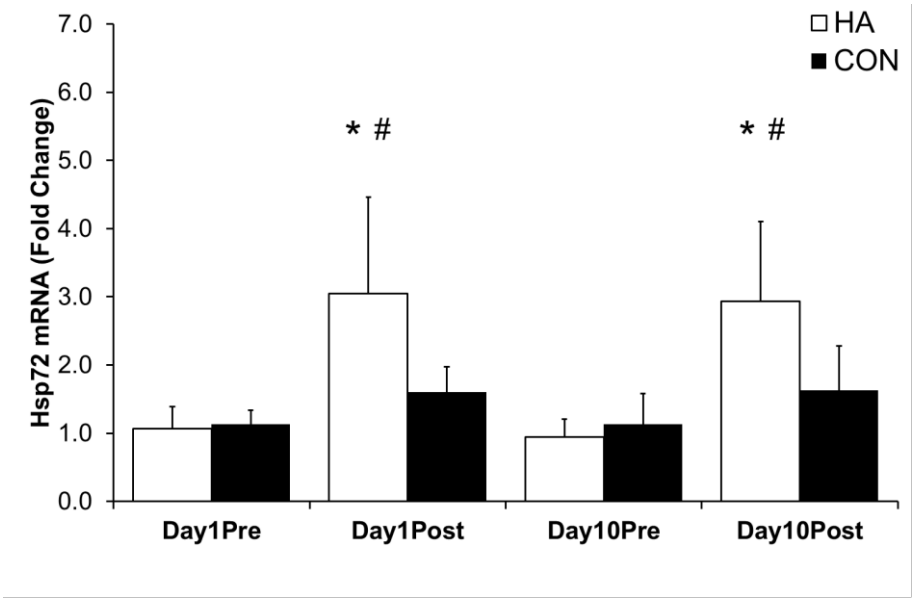

HYP1
HYP2
RER
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
*
Rest
40% VO2peak
65% VO2peak
Heat Acclimation
Rest
40% VO2peak
65% VO2peak
Control

Figure 3.



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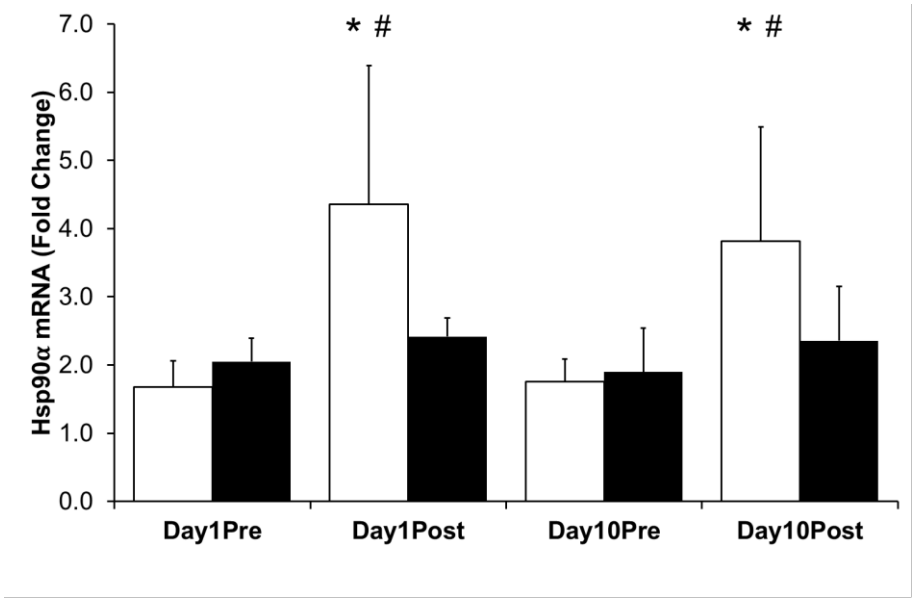

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765 Figure 4.

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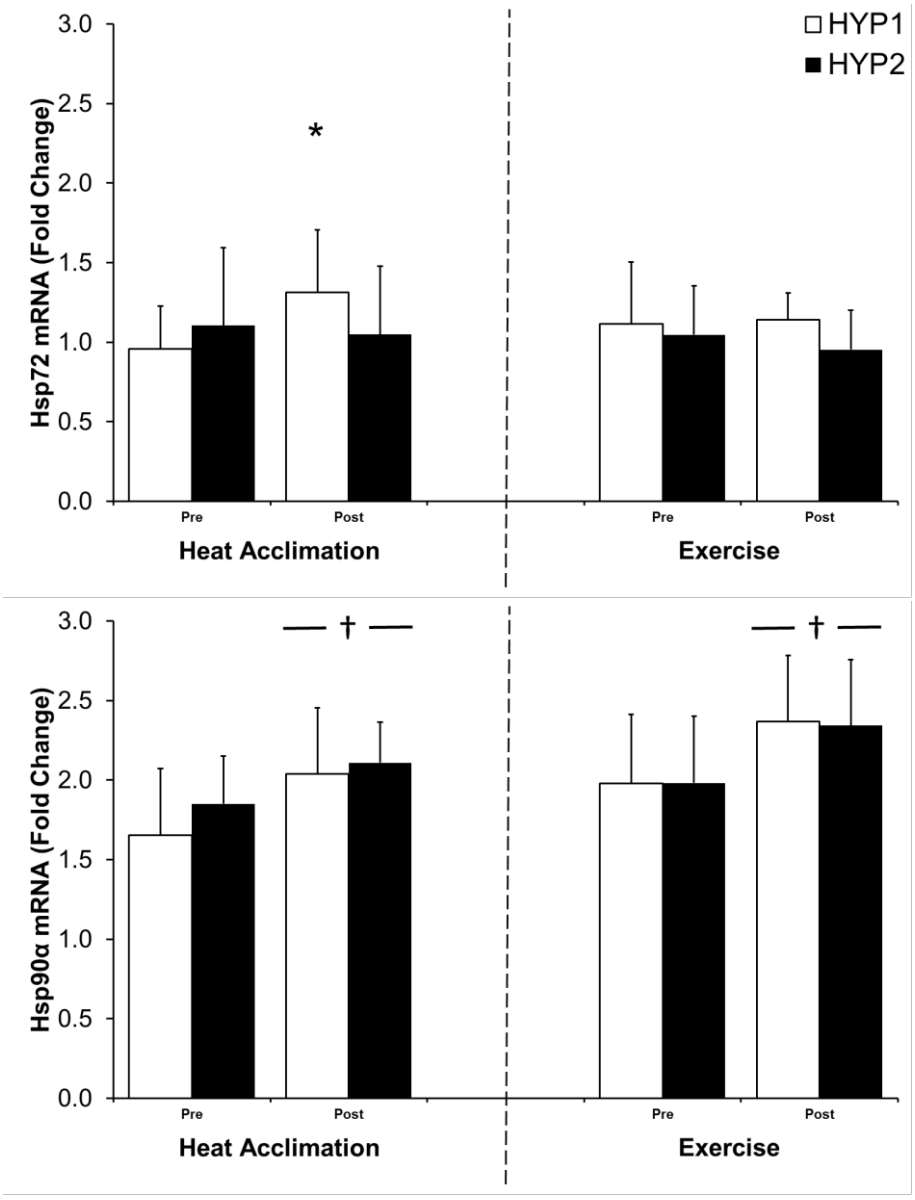

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769 Figure 5.

770 Table 1.

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

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772 Table 2.

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

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774 Table 3.

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

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