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Skeletal muscle angiogenic, regulatory and heat shock protein responses to prolonged passive hyperthermia of the human lower limb

Short Title:
Skeletal muscle responses to passive limb hyperthermia

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Abstract

Passive hyperthermia induces a range of physiological responses including augmenting skeletal muscle mRNA expression. This experiment aimed to examine gene and protein responses to prolonged passive leg hyperthermia. Seven young participants underwent 3 h of resting unilateral leg heating (HEAT) followed by a further 3 h of rest, with the contralateral leg serving as an unheated control (CONT). Muscle biopsies were taken at baseline (0 h), and 1.5, 3, 4, and 6 h in HEAT and 0 and 6 h in CONT to assess changes in selected mRNA expression via qRT-PCR, and HSP72 and VEGFα concentration via ELISA. Muscle temperature (T_m) increased in HEAT plateauing from 1.5 to 3 h (+3.5±1.5°C from 34.2±1.2°C baseline value; $p<0.001$), returning to baseline at 6 h. No change occurred in CONT. eNOS, FOXO-1, Hsp72, and VEGFα mRNA increased in HEAT ($p<0.05$) however post-hoc analysis identified that only Hsp72 mRNA statistically increased (at 4 h *vs.* baseline). When peak change during HEAT was calculated ANGPT-2 decreased (-0.4±0.2-fold), and CCL2 (+2.9±1.6-fold), FOXO-1 (+6.2±4.4-fold), Hsp27 (+2.9±1.7-fold), Hsp72 (+8.5±3.5-fold), Hsp90α (+4.6±3.7-fold), and VEGFα (+5.9±3.1-fold) increased from baseline (all $p<0.05$). At 6 h T_m were not different between limbs ($p=0.582$; CONT=32.5±1.6°C, HEAT=34.3±1.2°C), and only ANGPT-2 ($p=0.031$;-1.3±1.4-fold) and VEGFα ($p=0.030$;1.1±1.2-fold) differed between HEAT and CONT. No change in VEGFα or HSP72 protein concentration were observed over time, however, peak change in VEGFα did increase ($p<0.05$) in HEAT (+140±184 $pg.mL^{-1}$) vs CONT (+7±86 $pg.mL^{-1}$). Passive hyperthermia transiently augmented ANGPT-2, CCL2, eNOS, FOXO-1, Hsp27, Hsp72, Hsp90α and VEGFα mRNA, and VEGFα protein.

Introduction

Physiological responses to passive heating have been subject to experimental interest to further understand applications and mechanisms associated with therapeutic hyperthermia (also described as thermal therapy or heat therapy)(1, 2). Several beneficial responses, apparently similar to exercise training(3) have been reported in a range of relevant health and disease contexts e.g., ameliorated metabolic status(4–8), improved cardiovascular risk factors(3, 9–16), and enhanced muscle function(8, 17–21). Given these positive outcomes, heat therapy has been proposed as an alternative or precursor to exercise training in those unwilling or unable to engage in physical activity(2, 22, 23). Different physiological responses occur in response to whole body vs regional heating(24–28). However, elevations in local or systemic temperature increase blood flow and shear stress facilitating micro- and macrovascular adaptation(1) and increased circulation of microvesicles(29). Concurrent with these adaptations are molecular responses.

To date passive heating has been demonstrated as a modifier of an abundance of direct angiogenic and metabolic regulatory genes(27, 28, 30), and heat shock proteins [HSP (protein), Hsp (gene)] which further facilitate or contribute to adaptation(1). HSPs are a family of multifunctional proteins classified by molecular weight which support correct protein function and are therefore considered important contributors for inducing desirable adaptations(2, 31). HSPs are located across multiple tissue sites targeted by heat therapy, including skeletal, cardiac, vascular smooth muscle, and the central nervous system(32). It has been shown that inducing hyperthermia in both limbs, or the thigh alone, for a period of 90 min, promotes the expression of angiogenic regulatory and Hsp genes in human skeletal muscle(28). In that study gene expression relative to the baseline sample was augmented 30 min post lower limb heating [VEGFα, CCL2, ANGPT2, Hsp27, Hsp72, and Hsp90α, see table 1 for further gene details](28). Although most changes had acquiesced 2 h post heating (except FOXO1 and CX3L1), these data support the efficacy of local passive heating as a tool for augmenting skeletal muscle gene expression. Using a similar experimental design, it has also been demonstrated that 1 h of whole-body, but not single leg hyperthermia, augments anabolic (Akt/mTOR), mitochondrial, and Hsp signalling(27). The lack of change in the single leg heating model opposes the previously discussed work(28), suggesting that a signalling threshold had not been surpassed. Physiological data collected in that study(27) identified that whole body heating increased muscle, skin, and core temperature, but single leg heating only increased local skin and muscle temperature. This points to a role of one of those factors in augmenting the magnitude of expression in a direct or indirect manner.

Interestingly from a methodological viewpoint, with serial timepoint sampling the peak change in mRNA can be calculated on an individual participant basis across an intervention(27). This highlights that individual variability in the time course of gene expression exists and emphasises that the time-

course of heating-induced gene response is incompletely understood. Further, though data are consistent in demonstrating a significant increase in Hsp mRNA expression, and protein accumulation in response to combined exercise-heat stress(33–39), the magnitude of Hsp response to passive heating (a more feasible clinical intervention) has also not been adequately described. Further to this, whilst acute and repeated passive hyperthermia of <90 min can increase angiogenic and/or HSP protein concentrations(17, 18, 40), whether prolonging local hyperthermia elicits a greater change has not been examined. Collectively these data point towards a need to further characterise the skeletal muscle gene expression response, and subsequent alterations in protein concentration following local hyperthermia with a view to further enhancing the understanding of the therapeutic potential of heat therapy interventions. Specifically, to determine whether a longer heating duration i.e., 3 h vs 1.5 h augments the timecourse and magnitude of transcriptional response, and whether the calculation of change differs depending on the timepoints utilised.

The aims of this experiment were to examine the time course of selected gene and protein responses to prolonged (3 h) local passive leg hyperthermia, and subsequent recovery (3 h) in comparison to an unheated leg. It was hypothesised that limb heating-induced local skeletal muscle hyperthermia, would activate the expression of angiogenic and regulatory genes, and increase heat shock protein expression, with minimal alterations in systemic or contralateral limb responses. Further to this it was hypothesised that a single bout of prolonged local skeletal muscle hyperthermia would increase VEGFα and HSP72 skeletal muscle protein concentration.

Method

Participants

Seven healthy participants (two females) participated in the study (age 23 ± 2 yrs., height 172.6 ± 9.9 cm, mass 76.2 ± 13.3 kg, BMI 25.5 ± 3.6 kg.m^2, whole limb volume 9882 ± 1373 mL, lean limb volume 8768 ± 920 mL). All participants were non-smokers and free from known cardiorespiratory, metabolic, and neurological diseases. Participants arrived at the laboratory postprandial and euhydrated (urine osmolality <700 mOsmol·kgH_2O^{-1}(41)). They were required to have abstained from strenuous exercise and alcohol intake for >48 h and caffeine consumption for >12 h. *A priori* power analysis using data from a similar study [protocol 3,(28)] and established statistical conventions (α=0.05, β=0.8) had identified that six participants would be required to determine pre-post heating differences between heating and control limbs (the primary research question). Written informed consent was obtained from the participants prior to the study. All procedures were approved by the Brunel University London Research Ethics Committee (7692-A-Feb/2018-11768-1) and conformed to the guidelines of the Declaration of Helsinki.

Experimental design

Participants attended one experimental visit which commenced at 08.00±01.00 a.m. and following instrumentation and a period of supine rest (~0.5 h), baseline measurements preceded a protocol involving 3 h of unilateral whole leg passive heating (HEAT) whilst the contralateral control leg remained unheated (CONT). The passive limb heating device has been described previously(26, 42, 43), briefly a custom-built water-perfused trouser covered the entire leg, before being wrapped in foil blankets sealed with medical tape. The trouser was connected to a thermostatically controlled circulator (Julabo F-34; Seelbach) to allow a constant perfusion of 50°C water. Following removal of the passive heating stimulus, participants rested supine for a further 3 h. All measurements were taken every 0.5 h with a mean of a 60 s period recorded unless otherwise stated.

Experimental protocols

Upon arrival at the laboratory, participants voided, and stature and nude body mass were recorded whilst wearing shorts and a t-shirt (SECA model 798, Hamburg, Germany). To determine whole and lean limb volume the circumference of the heated limb was measured via an anthropometric tape measure at descending anatomical markers, with anterior and posterior skinfold thickness at the thigh, and medial and lateral skinfold thickness at the calf recorded using callipers (Harpenden, Burgess Hill, UK)(44). Following anthropometric measures, participants inserted a rectal thermistor to a marked depth of 15 cm (RET-1 Physitemp, USA) to measure core temperature (T_{core}) and entered the environmental chamber (Procema, UK; maintained at 21.6 ± 1.4°C) positioning themselves supine on a custom bed for instrumentation. A 3-lead ECG (PowerLab 26T and LabChart 7, ADI Instruments, UK) was affixed to the participant and an infrared photoplethysmography arterial blood pressure

device cuff positioned on the arm and on the middle finger of the right hand (Finometer; FMS, Netherlands). Stroke volume (SV) was estimated using the ModelFlow method included with the Beatscope computer software package (Beatscope; FMS, Netherlands), with cardiac output ($\dot{Q}$) calculated following corrections for age, height, and mass (45). For measurement of intramuscular temperatures (T_m), sterile implantable thermocouples (T-204f, PhysiTemp, USA) were inserted into the mid-portion of the vastus lateralis muscle of the heated and control leg using a 22-gauge catheter (BD Venflon; Becton-Dickson) at a depth of 2.5 cm. Skin surface temperature (T_{sk}) of the heated and control limb was measured via thermocouples (IT-18, PhysiTemp, USA) affixed to the skin over the belly of the thigh (T_{thigh}) and calf (T_{calf}). Additionally, T_{sk} was measured at forehead (T_{head}), over the belly of the right pectoralis major (T_{chest}) and left triceps brachii (T_{arm}) and on the dorsal surface of the left and right foot (T_{FOOT}) using surface temperature loggers (Hygrochron iButton, USA) set to record data at 60 s intervals. Torso temperature (T_{torso}) was calculated from an unweighted average of T_{head}, T_{chest}, and T_{arm}, leg skin temperature (T_{leg}) was calculated from an unweighted average of T_{thigh}, T_{calf}, and T_{foot}. Thermocouples were connected to a thermocouple meter (TC-2000, Sable Systems, NV, USA) and collected at 1000 Hz using commercially available data acquisition and analysis systems (PowerLab 26T, AD Instruments, LabChart 7, AD Instruments). Breath by breath (Vyntus, Carefusion) collection of expired metabolic gases occurred for 5 min to quantify whole body oxygen uptake ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), minute ventilation ($\dot{V}_E$), breathing frequency (F_b), tidal volume ($\dot{V}_T$) and to facilitate the calculation of respiratory exchange ratios (RER). Perceptual responses included whole body thermal comfort (TCOMF) and thermal sensation (TSENS) determined on a five (from 1, comfortable, to 5, very uncomfortable) and seventeen (from 0.0, unbearably cold, to 8.0, unbearably hot) point scale respectively (46), and the rating of perceived exertion (RPE) measured using a 15-point Borg scale (from 6, very very light, to 20, very very hard) (47).

Tissue and blood sampling, and analysis

A total of seven muscle biopsy samples were taken during the experiment, with five samples obtained from the heated limb (at 0, 1.5, 3, 4, and 6 h timepoints) and two samples obtained from the control limb (at 0 and 6 h). A contralateral comparison model was implemented to increase statistical power by reducing the amount of between-person variability and reduce the time, cost and discomfort associated with this invasive study (48). Skeletal muscle tissue was sampled from the vastus lateralis adjacent to the T_m site in sterile conditions under local anaesthetic (Xylocaine, 1%) using a 7 mm Bergström biopsy needle and manual suction (49). Following each biopsy, the incision was closed with steristrips and covered with a sterile dressing. For the 1.5 h sample the biopsy procedure was conducted through a custom opening in the water perfused trouser to minimize heat loss. Serial samples were obtained ~2 cm distally or proximally from one another. Following sampling, tissue was rinsed immediately in an ice cold 0.9% NaCl solution, and then immediately transferred to a 1.5 mL microtube and snap frozen in liquid nitrogen. Samples were then stored at −86°C for later analysis.

For determination of selected gene transcript responses (table 1; ThermoFisher Scientific, UK), a ~30 mg portion of the muscle sample was homogenized in 10 µL of β-Mercaptoethanol and 1 mL of Buffer RLT Plus. The homogenate was subsequently analysed via qRT-PCR in duplicate with responses characterized against the chosen housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by a commercial analytical laboratory (NMI Natural and Medical Sciences at the University of Tübingen, Reutlingen, Germany). Total RNA was isolated using the RNeasy fibrous tissue Mini Kit (#74704; Qiagen) according to the manufacturer's recommendations. The samples were digested using the DNase provided prior to synthesis of cDNA. cDNA was synthesised in the presence of hexanucleotides in a reaction volume of 15 µL using MMuLV reverse transcriptase (New England Biolabs #M0203L). To check for contamination by genomic DNA, for each sample a mock reaction lacking reverse transcriptase (-RT) was carried out. Samples then underwent a pre-amplification step (TaqMan™ PreAmpMaster Mix Kit). For the real-time PCR reaction, 1.25 µL of cDNA were employed. HSP72 (CV=2.5%) and VEGFα (CV=13.2%) protein concentrations were determined at all timepoints using commercially available ELISA kits (ThermoFisher Scientific) and normalized per mg of total protein via the Bradford method (ThermoFisher Scientific). Due to insufficient tissue sample, the 4 h timepoint (all participants) and participant #7 was excluded from the VEGFα analysis. For this reason, other genes demonstrating a change over time e.g. eNOS and FOXO1 were not analysed with HSP72 and VEGFα chosen given greater prominence in thermal and exercise literature pertaining to heat therapy(1) and angiogenesis (75).

INSERT TABLE 1 NEAR HERE PLEASE

Venous blood samples were taken at 0.5 h intervals via an 18-gauge cannula (BD Venflon; Becton-Dickson) inserted into a superficial antecubital vein of the arm. Blood draws of ~7 mL occurred at timepoints corresponding with muscle biopsy sampling (0, 1.5, 3, 4, 6 h), and ~2 mL in volume for all other time points. An equivalent volume 0.9% NaCl solution (BD PosiFlush; Becton-Dickson) was flushed through the cannula to maintain patency following each blood draw. Whole blood was immediately aliquoted to determine hematocrit (Hct), hemoglobin (Hb), and whole blood glucose [Glu] concentrations. Hct was determined by the packed cell volume method under a microscope after standard centrifugation of sodium-heparinized capillary tubes (microhematocrit tubes, HaematoSpin 1400 centrifuge; Hawksley), a mean of quadruplicate samples was recorded. Blood [Hb] was obtained by photometric analysis (HemoCue Hb 201+ System; HemoCue), and [Glu] was determined at only 0, 1.5, 3, 4 and 6 h (HemoCue Glucose 201+ System; HemoCue), for both [Hb] and [Glu] a mean of triplicate samples was recorded.

Statistical analysis

Data are presented as mean±SD unless otherwise indicated. All statistical analyses were carried out using SPSS software (Version 26) with significance for all analyses was set at $p<0.05$. All outcome variables were first checked for normality and sphericity. The Greenhouse Geisser correction for the *F* statistic and related degrees of freedom was used when data violated sphericity. One-way ANOVA was used to compare changes over time with a main effect followed up with Bonferroni adjusted post hoc comparisons. For non-parametric data (e.g., RPE, TCOMF, TSENS) a Friedman test was used to compare variables over time, with significance followed up with Wilcoxon signed rank tests comparing each timepoint to baseline. Two-way ANOVA was used to compare changes over time (0 vs. 6 h) and between control and heated limbs, with main and interaction effects followed up with Bonferroni adjusted post hoc comparisons. Peak change in mRNA and protein concentration was compared between control and heated limbs using paired sample T-tests. Pearson's correlation was performed between each of the peak and change of T_m, T_{leg}, and the peak change of all genes of interest which demonstrated a significant difference.

Results

Physiological responses

The T_m displayed a main effect for group (f=35.7, p=0.001), time (f=88.1, p<0.001), and an interaction effect (f=82.9, p<0.001). No difference in T_m at was observed at baseline but as per design differences occurred between HEAT and CONT from 0.5 h until 4 h, as displayed in Figure 1. The T_m increased (from baseline 34.7±0.9°C) in HEAT from 0.5 h to 3 h but was unchanged in CONT (baseline 33.8±1.5°C). Specifically, in HEAT T_m increased from baseline to 37.6±0.1°C (after 1.5 h, p=0.007), and 38.1±0.6°C (3 h, p<0.001), this equating to a change from baseline of 3.0±1.0°C and 3.5±1.5°C respectively. Compared to CONT this was a +3.9±1.2°C and +5.2±1.7°C difference at 1.5 and 3 h (p<0.001). The post intervention phase subsequently saw a reduction in temperature relative to the heating phase after 4 h whereby T_m at 6 h had returned to baseline and was not different between limbs (p=0.582; CONT 32.5±1.6°C, HEAT 34.3±1.2°C).

INSERT FIGURE 1 NEAR HERE PLEASE

The T_{leg} displayed a main effect for group (t=106.4, p<0.001), time (t=126.7, p<0.001), and an interaction effect (t=100.8, p<0.001). T_{leg} was greater in HEAT, from 0.5 h until 3 h (peak temperature 39.5±1.0 °C), and greater than CONT from 0.5 h onwards. Figure 1. A main effect of time was observed for T_{core} (t=4.8, p=0.032), and HR (t=6.3, p=0.003). Post hoc analysis did not identify a difference in T_{core} from baseline (37.3±0.2°C) (Figure 1). Similarly, HR was unchanged from baseline (Table 2). TSENS reported a main effect for time ($\chi 2$=61.0, p<0.001) with post hoc differences from 1 h until 3 h vs baseline (Table 2). In contrast, no statistical differences (p>0.05) were observed in T_{torso} , $\dot{Q}$, MAP, $\dot{V}O_2$, $\dot{V}CO_2$, RER, $\dot{V}_E$, [Glu], [Hb], Hct, RPE, TCOMF (p>0.05). All physiological and perceptual data are presented in Table 2.

INSERT TABLE 2 NEAR HERE PLEASE

Gene responses over time in the heated limb

Increased gene expression was observed over the course of the HEAT intervention for eNOS (f=3.9, p=0.014), Hsp72 (f=7.3, p<0.001), and VEGFα (f=3.7, p=0.018). Post hoc analyses identified that Hsp72 was higher at 4 h than baseline (p=0.048; +5.2±2.6-fold) (Figure 2). Post hoc differences could not be identified for eNOS and VEGFα (Figure 3). No effect (p>0.05) was observed for ANGPT2, CCL2, FOXO1, HIF-1α, Hsp27, Hsp60, Hsp90α, VASH-1, VEGFα.

INSERT FIGURE 2 NEAR HERE PLEASE

INSERT FIGURE 3 NEAR HERE PLEASE

At 6 h, ANGPT-2 (f=7.8, p=0.031) was lower in HEAT than CONT, and VEGFα (f=8.0, p=0.030) was higher in HEAT than CONT, with no difference observed in any other genes at this end point of the protocol (p>0.05).

Gene responses between heated and control limb

When peak change during HEAT was calculated, ANGPT-2 reduced (-0.4± 0.2; t=7.3, p<0.001), and CCL2 (2.9±1.6 fold; t=3.2, p=0.019), FOXO-1 (+6.2±4.4 fold; t=3.1, p=0.021), Hsp27 (+2.9±1.7 fold; t=2.9, p=0.029), Hsp72 (+8.5±3.5 fold; t=5.7, p=0.001), Hsp90α (+4.6±3.7 fold; t=2.5, p=0.044), and VEGFα (+5.9±3.1 fold; t=4.3, p=0.005) increased in comparison to baseline (Figure 4).

INSERT FIGURE 4 NEAR HERE PLEASE

Table 3 demonstrates the percentage of participants reporting a peak increase at each timepoint within HEAT and the average time to peak expression. Of the genes that demonstrated a change from baseline, 4 h and 6 h demonstrated the greatest number of instances where peak expression occurred. The ANGPT-2 peaked at 2.1 ± 0.8 h, Hsp27 peaked at 3.9 ± 1.8 h, Hsp72 peaked at 4.8 ± 1.7 h, and Hsp90α peaked at 4.5 ± 1.9 h. CCL2 (peak at 3.9 ± 1.6 h), FOXO-1 (peak at 3.3 ± 1.6 h) and VEGF (peak at 3.7 ± 1.9 h) did not demonstrate a clear temporal response with individual participants reporting peaks across the full range of timepoints.

INSERT TABLE 3 NEAR HERE PLEASE

Peak change in HEAT was greater than the change in CONT for ANGPT-2 (t=3.9, p=0.008), FOXO-1 (t=2.9, p=0.029), Hsp72 (t=5.5, p=0.002), Hsp90α (t=2.6, p=0.042), and VEGFα (t=4.1, p=0.006). There was no difference (p>0.05) in CCL2, eNOS, HIF1α, Hsp27, Hsp60, or VASH-1.

ANGPT2 demonstrated a relationship with change in T_{LEG} (r=0.839, p=0.018), and the peak change in Hsp72 demonstrated a relationship with FOXO1 (r=0.765, p=0.045), whilst VASH was related to Hsp27 (r=0.907, p=0.005). On the other hand, VEGFα demonstrated a relationship with eNOS (r=0.772, p=0.042), Hsp27 (r=0.821, p=0.023), and VASH (r=0.803, p=0.030).

Protein responses

No change was observed in HSP72 concentration within the experimental limb during the five timepoints measured in HEAT (f=0.8, p=0.522). Additionally, when examining protein concentration at baseline and 6 h between CONT and HEAT, no main effect of group (f=0.5, p=0.498), time (f=0.3, p=0.594), or interaction (f=0.0, p=0.867) was observed. The peak change in HSP72 protein following HEAT was not difference to CONT (t=2.3, p=0.064). Like HSP72 (Figure 5), no change was observed

in VEGFα concentration (n=6) during HEAT (f=4.7, p=0.072) or at baseline and 6 h between CONT and HEAT (group, f=0.8, p=0.409; time, f=3.6, p=0.115; interaction, f=4.8, p=0.081). The peak change in VEGFα protein following HEAT was different to CONT (t=3.5, p=0.018). Hsp72 mRNA demonstrated a relationship with HSP72 protein concentration at the 6 h timepoint (r=0.781, p=0.038).

INSERT FIGURE 5 NEAR HERE PLEASE

Discussion

The 3 h passive leg heating rapidly increased intramuscular (*vastus lateralis*) temperature, T_m being elevated by 3.0-3.5°C (+5°C vs contralateral limb) between 1.5 to 3 h and then declining progressively to baseline values by 6 h, with no or minimal systemic and contralateral leg physiological response. Peak skeletal muscle gene transcription was favourably altered from baseline in the heated limb for heat shock proteins (Hsp27, Hsp72, and Hsp90α) and regulatory genes (ANGPT-2, CCL2, FOXO-1, and VEGFα). Examination of the time course of gene change eNOS, Hsp72, and VEGFα also demonstrated a change overall, however a timepoint specific change only occurred in Hsp72 at 4 h. Generally, the regulatory genes response which reported a change (i.e., ANGPT-2, CCL2, FOXO-1, and VEGFα) peaked during or at the end of the 3 h heating period (peak expression at 3.3 ± 1.6 h from baseline), with heat shock proteins (Hsp27, Hsp72, and Hsp90α) typically peaking during recovery (peak expression at 4.4 ± 1.8 h from baseline). Only ANGPT-2 and VEGFα differed between CONT and HEAT at the end of the study (6 h) highlighting a general return to baseline of augmented genes following the cessation of local hyperthermia. Together these transcriptional responses highlight that interindividual differences exist in response to local passive hyperthermia, with an augmentation of gene response albeit at different timepoints (Table 3). The inconsistency in gene response, the implementation of acute thermal stimuli, and/or relatively short window of observation may explain the lack of change in HSP72 and VEGFα protein concentrations over the full timecourse of the protocol. As with peak gene responses, the calculation of peak change in VEGFα led to difference between limbs, this points to inter-individual protein responses to the same intervention.

Heat shock protein gene responses

The skeletal muscle heat shock protein gene response to passive heating has not been extensively considered with published data presenting conflicting findings. Early data by Morton et al. (50) did not demonstrate any change in mRNA expression of Hsp27, Hsp60, or Hsp70(50) by passive hyperthermia increasing core (+1.5°C to 38.9±0.2°C) and muscle temperatures (+3.6°C to 39.5±0.2°C) via 1 h of one-legged hot water immersion. These data are therefore in conflict with our findings. On closer inspection of those experimental methods, the post biopsy sample was taken ~48 h following heating, a time point when our Hsp time course data, and others(28), now indicate post-transcriptional concentrations would have returned to baseline. Following 90 min of local limb heating via water-perfused garments (without core temperature change), a 1.1-1.5-fold increase in Hsp27, Hsp60, Hsp72, and Hsp90 mRNA has been reported 30 min post heating(28). These data agree with the direction our findings (Figures 2 & 4). Within that experiment the comparison between only thigh heating, and lower body heating trials revealed no difference in the magnitude of change in gene response suggesting equivalent responses could be observed despite heating different tissue masses (and presumably eliciting subtly different muscle temperatures(26)). The absence of muscle temperature measurement does not allow a rigorous analysis of this hypothesis, however. A

comparison between single leg and whole-body heating (muscle temperatures=38.1±0.6°C vs 38.8±0.5°C, core temperature=37.1±0.1°C vs 39.1±0.3°C) saw increased Hsp25 (+50%), Hsp72 (+362%) and Hsp90 (+64%) mRNA expression in whole body heating, but not in single leg heating(27). In that study authors concluded localized heating may be insufficient to increase Hsp gene expression when the duration and magnitude of the heat treatment is inadequate. Based on our correlation analysis timing of peak mRNA expression and subsequent gene degradation also poorly correlate with thermal response (T_m) thus other factors appear relevant. Our data add to this conversation by demonstrating that for the same muscle temperature (~38.1°C) an extended heating duration (rather than magnitude), causes similar increases in Hsp concentrations following single leg heating compared to whole body heating protocols. Our data, combined with animal studies (51), are indicative that absolute muscle temperature may not be critical in regulating the magnitude of Hsp mRNA response, however temperature range/increase in muscle temperature are potential factors in Hsp response once a ~37.6-38.1°C muscle temperature threshold has been surpassed (for a sufficient duration). The time course of human HSP responses to interactions of heat and/or exercise not been well examined either. Using downhill running in the heat as a model for creating maximal skeletal muscle stress, it has been observed that both Hsp72 and Hsp90α mRNA peak 30 min following exercise, with elevations persisting at 3 h post, but not 24 h post(52). Thirty minutes of exercise at the anaerobic threshold elicits equal increases in Hsp72 expression 30 min and 3 h following exercise(53), yet the timepoint of final decay was not characterised. Further, Hsp27 (+4-8-fold) and Hsp72 (+15-20-fold) increases are typically greatest and most prolonged when eccentric exercise is undertaken with significant peaking between 4 and 8 h following contractions with maintained mRNA expression 24 h later(54). Similar observations have been made in a strength training paradigm(55) though the timing of the myogenic gene induction is variable, peaking 4–8 h postexercise, with all gene expression returning to baseline after 24 h.

Angiogenic and regulatory gene responses

In addition to changes in Hsp expression our data also highlight that local hyperthermia is effective in positively modifying the expression of angiogenic regulatory genes, specifically ANGPT-2 which reduced, and CCL2, eNOS, and VEGFα which increased. This is in contrast to previous work by other groups, which suggested that single leg models were ineffective at inducing changes in gene expression(27). However, in agreement with our study, Kuhlenhoelter et al.,(28), demonstrated that leg hyperthermia increased VEGFα by ~1.5 fold. Whilst CCL2 reduced in that experiment the reduction was attenuated relative to controls which may be considered analogous to our increase vs null control limb response(28). Regrettably in that study individual muscle temperatures and gene responses were not reported to confirm or refute the role this thermoregulatory variable has on individual responses a factor which limits interpretation of the magnitude and inter-individual range of response against our data. Understanding as to whether hyperthermia directly e.g., via temperature

sensing, or indirectly e.g., via shear stress(10, 56), induces mRNA signalling in heat therapy models remains equivocal and may be best assessed *ex vivo*. Given the central role that VEGFα plays in angiogenesis, including the influence on associated markers such as eNOS(57), examination of the time course of VEGFα expression is pertinent from a regulatory gene perspective. Sixty minutes of moderate intensity exercise elicits peak VEGFα increases (~4.5 fold) at 2 and 4 h post intervention(58), with a similar outcome when the duration (59) or intensity is increased (60). Prolonged two-legged knee extension exercise elicited increases in VEGFα mRNA 1.5 and 3 h after exercise onset, with the 9-fold increase peaking 1 h into recovery before returning to baseline after 20 h. In comparison to these exercise protocols, our data demonstrate that passive heating can also induce increases in VEGFα albeit to a lesser degree than exercise of a similar duration(60). The difference in observed magnitude of response between passive heating and exercise models is perhaps unsurprising, given the difference in intensity of stimulus with heating delivering a lower 'intensity' for the same duration. Nevertheless, passive heating induced changes in regulatory gene response are significant and have been shown to be sufficient to promote angiogenesis(17, 61). The observed relationships between genes e.g., the change in VEGFα and eNOS supports previous observations that VEGF receptor-2 upregulates eNOS and iNOS protein(57). The relationship between Hsp27 mRNA and VEGFα mRNA appears novel, however this is likely related to the VEGF-mediated cell migration and angiogenesis facilitated by HSP27, as demonstrated by work increasing extracellular concentrations via recombinant HSP27(62). Recently a study was undertaken to characterise the timecourse of changes in skeletal muscle regulatory gene expression in response to a session of high-intensity interval training(63) with the temporal pattern across the 23 genes of interest was highly variable (63). Both VEGFα and Hsp72 were included in the aforementioned analysis and demonstrated a peak change 9 h following exercise (Hsp72=+2.9-fold; VEGFα +1.3-fold), albeit with the change in VEGFα falling short of statistical significance. Hsp72 changes returned to baseline after 48 h. Exercise protocols therefore also show variable timeframes for peak gene expression and our data demonstrates that is now also evident in passive heating protocols. The variable peaking of expression also seems to align with the functional role of the gene(s). It is important to acknowledge that the variability in gene response during exercise protocols is expected given the various modes, durations, and intensity and the subsequent impact on cardiovascular, metabolic and temperature responses. It might be considered more unexpected that variability exists during passive heating given the relatively homogenous stimuli, with this having implications for how such intervention or treatment might be delivered. Taken together these acute increases in regulatory gene responses provide further support for vascular responses and adaptations to heat therapy. Our data are therefore supportive of the potential for heat therapy to serve as an alternative, precursor or complement to exercise training in those unwilling or unable to engage in physical activity, yet would benefit from vascular adaptation(2, 22, 23).

Protein responses

Though the HSP72 protein response to passive and exercise hyperthermia has been characterised in acutely extracellular fluid(4, 5, 37, 64–66), and at an intracellular level within circulating cells(34, 67–74) few studies have examined the timecourse skeletal muscle response to hyperthermia. In support of our observation and previously discussed gene responses within skeletal muscle, Morton et al.,(50) reported no change in HSP72 48 h following passive heating. Further to this Kim et al.,(40) observing that passive heating following eccentric exercise did not alter HSP72 (and HSP90) protein concentration 24 h following treatment. Heating via shortwave diathermy has also elicited a null response in HSP72(18). These studies, and our data point towards a null response in HSP72 intramuscular protein concentration following acute hyperthermia. A statistically significant, 45% increases in HSP72 following 2 h daily heating for 6 consecutive days(18), and +25% in response to 2 hr daily heating during 10 days of limb immobilisation(20) highlighting the merits of chronic vs acute interventions. Our absence of changes over time in VEGFα protein concentration contrasts that of others who observed elevated VEGFα after one and five days of heat treatment following eccentric exercise(40). Interestingly the same group had previously observed that heating alone did not change VEGFα concentrations after four and eight weeks of treatment perhaps revealing context specific responses(17). Ambiguity in the acute VEGFα response in skeletal muscle has also been observed in exercise models(75), with some exercise studies observing increased concentrations(76–81) and others reporting null responses or decreases(58, 82). It is notable that despite our peak VEGFα protein responses differing statistically between HEAT and CONT (irrespective of timepoint), intra-individual patterns of response point exist (Figure 5). This replicates the mRNA data within our study and the work of others(27), and published work examining protein content following acute exercise.

Methodological considerations and limitations

Given some previous work had created an equivocal picture of the relevance of a passive limb hyperthermia model, these data give reinforced credence to this method of local heat therapy and point to benefits arising from local applications that do not need to elicit systemic responses e.g., elevated core temperature. It is unlikely that the duration of exposure utilised in this study would be well tolerated in using a whole-body heating protocol such as sauna or water immersion, therefore local heating provides an opportunity for the intervention to be applied for prolonged periods. The lack of distinction in gene response between 1.5 h and 3 h timepoints suggests that prolonging hyperthermia will not lead to a greater magnitude of gene response however there could be added benefit to having gene expression elevated for a longer duration per session. The homogenous intramuscular temperature responses across timepoints during the heating phase (Figure 1) but differing timepoints associated with a peak gene response, and interindividual range of gene responses (see Figure 2 and 3) suggest that heat therapy interventions might be more effectively implemented when prescribed at an individual level. The caveats to that statement being that at the

current time the optimal individualisation variable remains unknown and should be further investigated. It remains to be fully determined whether an equivalent intervention reporting a null response in young healthy individuals would elicit a positive outcome in patients e.g., those with vascular disease. Accordingly, future experimental consideration should also be given to the population studied i.e., like our data most experimental work to date has examined responses in young, healthy participants, rather than the prospective clinical population requiring treatment. Given that the clinical populations at which this therapy might be targeted are unlikely to be able to tolerate significant exercise protocols, passive heating is perhaps a more accessible treatment regimen, and one that causes negligible observed systemic perturbation even when implemented for prolonged durations (Table 2). Finally, to fully elucidate the role of muscle temperature on the magnitude of change in gene expression and protein concentration, further experimental work examining interactions between clamping muscle temperature at increasing magnitudes of local (skeletal muscle) hyperthermia (51, 83), across differing heating durations, is warranted.

A limitation of this study is the lack of serial sampling of the control limb for changes in gene expression to confirm a null response throughout the intervention. With the exception of ANGPT-2 and VEGFα which demonstrated a continued response following heating, the lack of difference between 0 h and 6 h measures in CONT for all other genes does however support the experimental design and highlights that the variability of measured gene expression does not explain the apparent differences among individuals. The absence of serial biopsy sampling in CONT also means that quantification of the potential effects of repeated biopsies during HEAT is not possible. Had these data been available in CONT, biological and methodological variability across the 3 h of heating and 3 h of recovery could be quantified by subtracting the ΔCONT from ΔHEAT across timepoints. Nonetheless the delta between HEAT and CONT (Figure 4 and Figure 5) point to meaningful differences between interventions thus in spite of serial biopsy sampling in HEAT, a hyperthermi induced change above CONT was observed. Further to this, we acknowledge that whilst sufficient statistical power was observed for between leg comparisons and for some analyses (eNOS, Hsp72, VEGFα, ηp2 = 0.4-0.6), the observed power (0.55 – 0.76, ηp2 = 0.3-0.4) for ANGPT-2, Hsp27, Hsp90α indicates the study was underpowered for these genes. At a mechanistic level, circulating or systemic angiogenic mediators cannot be excluded from consideration as to beneficial factors arising from hyperthermia(11, 28, 84), nor can their influence on ANGPT-2 be discounted(85).

Conclusion

Prolonged (3 h) passive limb hyperthermia altered skeletal muscle ANGPT-2, CCL2, eNOS, FOXO-1, Hsp27, Hsp72, Hsp90α, and VEGFα mRNA expression and increased the individual peak change in VEGFα protein concentration in healthy human participants, without modifying HSP72 protein concentration. Future applied and mechanistic work should acknowledge that angiogenic and heat

shock protein mRNA responses peak at different times between individuals undertaking passive limb hyperthermia protocols. It is unclear whether these differences impact the magnitude of adaptation if hyperthermia were to be repeated.

Perspectives and Significance

Prolonged passive leg hyperthermia transiently increases angiogenic mediators and heat shock proteins with minimal alterations in systemic or contralateral leg responses. These data point to the relevance of local mechanisms in augmenting ANGPT-2, CCL2, eNOS, FOXO-1, Hsp27, Hsp72, Hsp90α, and VEGFα mRNA expression. Differing temporal patterns in gene response, and null protein responses point to a need to further understand relevant mRNA and protein kinetics during prolonged passive leg hyperthermia interventions.

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

Author contributions

ORG and JG-A conceived and design the experiment. ORG, RA, ZP, FNEG, JG-A performed the experiments. ORG analysed and illustrated the data. ORG, FNEG, and JG-A interpreted the results. ORG drafted the manuscript. ORG, RA, ZP, FNEG, JG-A edited and approved the final manuscript.

References

1. **Brunt VE**, **Minson CT**. Heat therapy: mechanistic underpinnings and applications to cardiovascular health. *J Appl Physiol* 130: 1684–1704, 2021. doi: 10.1152/japplphysiol.00141.2020.
2. **Cheng JL**, **MacDonald MJ**. Effect of Heat Stress on Vascular Outcomes in Humans. *J Appl Physiol* 126: 771–781, 2019. doi: 10.1152/japplphysiol.00682.2018.
3. **Hesketh K**, **Shepherd SO**, **Strauss JA**, **Low DA**, **Cooper RG**, **Wagenmakers AJM**, **Cocks M**. Passive Heat Therapy in Sedentary Humans Increases Skeletal Muscle Capillarisation and eNOS Content but Not Mitochondrial Density or GLUT4 Content. *American Journal of Physiology-Heart and Circulatory Physiology* 317: H114–H123, 2019. doi: 10.1152/ajpheart.00816.2018.
4. **Hoekstra SP**, **Bishop NC**, **Faulkner SH**, **Bailey SJ**, **Leicht CA**. Acute and chronic effects of hot water immersion on inflammation and metabolism in sedentary, overweight adults. *J Appl Physiol* 125: 2008–2018, 2018. doi: 10.1152/japplphysiol.00407.2018.
5. **Hoekstra SP**, **Wright AKA**, **Bishop NC**, **Leicht CA**. The effect of temperature and heat shock protein 72 on the ex vivo acute inflammatory response in monocytes. *Cell Stress Chaperones* 24: 461–467, 2019. doi: 10.1007/s12192-019-00972-6.
6. **Leicht CA**, **James LJ**, **Briscoe JHB**, **Hoekstra SP**. Hot water immersion acutely increases postprandial glucose concentrations. *Physiol Rep* 7, 2019. doi: 10.14814/phy2.14223.
7. **Ely BR**, **Clayton ZS**, **McCurdy CE**, **Pfeiffer J**, **Needham KW**, **Comrada LN**, **Minson CT**. Heat therapy improves glucose tolerance and adipose tissue insulin signaling in obese women with polycystic ovary syndrome. *American Journal of Physiology-Endocrinology and Metabolism* 317: E172–E182, 2019. doi: 10.1152/ajpendo.00549.2018.
8. **Ely BR**, **Francisco MA**, **Halliwill JR**, **Bryan SD**, **Comrada LN**, **Larson EA**, **Brunt VE**, **Minson CT**. Heat therapy reduces sympathetic activity and improves cardiovascular risk profile in obese women with polycystic ovary syndrome. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 317: R630–R640, 2019. doi: 10.1152/ajpregu.00078.2019.
9. **Brunt VE**, **Eymann TM**, **Francisco MA**, **Howard MJ**, **Minson CT**. Passive heat therapy improves cutaneous microvascular function in sedentary humans via improved nitric oxide-dependent dilation. *J Appl Physiol* 121: 716–723, 2016. doi: 10.1152/japplphysiol.00424.2016.
10. **Brunt VE**, **Howard MJ**, **Francisco MA**, **Ely BR**, **Minson CT**. Passive heat therapy improves endothelial function, arterial stiffness, and blood pressure in sedentary humans. *J Physiol* 0: 1–14, 2016. doi: 10.1113/JP272453.
11. **Brunt VE**, **Weidenfeld-Needham KM**, **Comrada LN**, **Francisco MA**, **Eymann TM**, **Minson CT**. Serum from young, sedentary adults who underwent passive heat therapy improves endothelial cell angiogenesis via improved nitric oxide bioavailability. *Temperature* 16: 23328940.2019.1614851, 2019. doi: 10.1080/23328940.2019.1614851.
12. **Park S-Y**, **Kwak Y-S**, **Pekas EJ**. Impacts of aquatic walking on arterial stiffness, exercise tolerance, and physical function in patients with peripheral artery disease: a randomized clinical trial. *J Appl Physiol* 127: 940–949, 2019. doi: 10.1152/japplphysiol.00209.2019.
13. **Imamura M**, **Biro S**, **Kihara T**, **Yoshifuku S**, **Takasaki K**, **Otsuji Y**, **Minagoe S**, **Toyama Y**, **Tei C**. Repeated thermal therapy improves impaired vascular endothelial function in patients with coronary risk factors. *J Am Coll Cardiol* 38: 1083–8, 2001.
14. **Neff D**, **Kuhlenhoelter AM**, **Lin C**, **Wong BJ**, **Motaganahalli RL**, **Roseguini BT**. Thermotherapy reduces blood pressure and circulating endothelin-1 concentration and enhances leg blood flow in patients with symptomatic peripheral artery disease. *American*

Journal of Physiology-Regulatory, Integrative and Comparative Physiology 311: R392–R400, 2016. doi: 10.1152/ajpregu.00147.2016.

15. **Pizzey FK**, **Smith EC**, **Ruediger SL**, **Keating SE**, **Askew CD**, **Coombes JS**, **Bailey TG**. The effect of heat therapy on blood pressure and peripheral vascular function: A systematic review and meta-analysis. *Exp Physiol* 106: 1317–1334, 2021. doi: 10.1113/EP089424.
16. **Akerman AP**, **Thomas KN**, **van Rij AM**, **Body ED**, **Alfadhel M**, **Cotter JD**. Heat therapy vs. supervised exercise therapy for peripheral arterial disease: a 12-week randomized, controlled trial. *American Journal of Physiology-Heart and Circulatory Physiology* 316: H1495–H1506, 2019. doi: 10.1152/ajpheart.00151.2019.
17. **Kim K**, **Reid BA**, **Casey CA**, **Bender BE**, **Ro B**, **Song Q**, **Trewin AJ**, **Petersen AC**, **Kuang S**, **Gavin TP**, **Roseguini BT**. Effects of repeated local heat therapy on skeletal muscle structure and function in humans. *J Appl Physiol* 128: 483–492, 2020. doi: 10.1152/japplphysiol.00701.2019.
18. **Hafen PS**, **Preece CN**, **Sorensen JR**, **Hancock CR**, **Hyldahl RD**. Repeated exposure to heat stress induces mitochondrial adaptation in human skeletal muscle. *J Appl Physiol* 125: 1447–1455, 2018. doi: 10.1152/japplphysiol.00383.2018.
19. **Pellinger TK**, **Neighbors CB**, **Simmons GH**. Acute Lower Leg Heating Increases Exercise Capacity in Patients With Peripheral Artery Disease. *Journal of Cardiovascular Nursing* 34: 130–133, 2019. doi: 10.1097/JCN.0000000000000510.
20. **Hafen PS**, **Abbott K**, **Bowden J**, **Lopiano R**, **Hancock CR**, **Hyldahl RD**. Daily heat treatment maintains mitochondrial function and attenuates atrophy in human skeletal muscle subjected to immobilization. *J Appl Physiol* 127: 47–57, 2019. doi: 10.1152/japplphysiol.01098.2018.
21. **Racinais S**, **Wilson MG**, **Périard JD**. Passive heat acclimation improves skeletal muscle contractility in humans. *Am J Physiol Regul Integr Comp Physiol* 312: R101–R107, 2017. doi: 10.1152/ajpregu.00431.2016.
22. **Hunt AP**, **Minett GM**, **Gibson OR**, **Kerr GK**, **Stewart IB**. Could Heat Therapy Be an Effective Treatment for Alzheimer's and Parkinson's Diseases? A Narrative Review. *Front Physiol* 10: 1556, 2020. doi: 10.3389/fphys.2019.01556.
23. **Ely BR**, **Clayton ZS**, **McCurdy CE**, **Pfeiffer J**, **Minson CT**. Meta-inflammation and cardiometabolic disease in obesity: Can heat therapy help? *Temperature* 5: 9–21, 2018. doi: 10.1080/23328940.2017.1384089.
24. **Chiesa ST**, **Trangmar SJ**, **Kalsi KK**, **Rakobowchuk M**, **Banker DS**, **Lotlikar MD**, **Ali L**, **González-Alonso J**. Local temperature-sensitive mechanisms are important mediators of limb tissue hyperemia in the heat-stressed human at rest and during small muscle mass exercise. *American Journal of Physiology-Heart and Circulatory Physiology* 309: H369–H380, 2015. doi: 10.1152/ajpheart.00078.2015.
25. **Cheng JL**, **Williams JS**, **Hoekstra SP**, **MacDonald MJ**. Improvements in vascular function in response to acute lower limb heating in young healthy males and females. *J Appl Physiol* 131: 277–289, 2021. doi: 10.1152/JAPPLPHYSIOL.00630.2020.
26. **Koch Esteves N**, **Gibson OliverR**, **Khir A**, **González-Alonso J**. Regional thermal hyperemia in the human leg: Evidence of the importance of thermosensitive mechanisms in the control of the peripheral circulation. *Physiol Rep* 9: e14953, 2021. doi: 10.14814/PHY2.14953.
27. **Ihsan M**, **Deldicque L**, **Molphy J**, **Britto F**, **Cherif A**, **Racinais S**. Skeletal Muscle Signaling Following Whole-Body and Localized Heat Exposure in Humans. *Front Physiol* 11: 839, 2020. doi: 10.3389/fphys.2020.00839.
28. **Kuhlenhoelter AM**, **Kim K**, **Neff D**, **Nie Y**, **Blaize AN**, **Wong BJ**, **Kuang S**, **Stout J**, **Song Q**, **Gavin TP**, **Roseguini BT**. Heat therapy promotes the expression of angiogenic regulators in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 311: R377-91, 2016. doi: 10.1152/ajpregu.00134.2016.

29. **Wilhelm EN**, **González-Alonso J**, **Chiesa ST**, **Trangmar SJ**, **Kalsi KK**, **Rakobowchuk M**. Whole-body heat stress and exercise stimulate the appearance of platelet microvesicles in plasma with limited influence of vascular shear stress. *Physiol Rep* 5: e13496, 2017. doi: 10.14814/phy2.13496.
30. **Goto K**, **Oda H**, **Kondo H**, **Igaki M**, **Suzuki A**, **Tsuchiya S**, **Murase T**, **Hase T**, **Fujiya H**, **Matsumoto I**, **Naito H**, **Sugiura T**, **Ohira Y**, **Yoshioka T**. Responses of muscle mass, strength and gene transcripts to long-term heat stress in healthy human subjects. *Eur J Appl Physiol* 111: 17–27, 2011. doi: 10.1007/s00421-010-1617-1.
31. **Cullen T**, **Clarke ND**, **Hill M**, **Menzies C**, **Pugh CJA**, **Steward CJ**, **Thake CD**. The health benefits of passive heating and aerobic exercise: To what extent do the mechanisms overlap? *J Appl Physiol* 129: 1304–1309, 2020. doi: 10.1152/JAPPLPHYSIOL.00608.2020.
32. **Henstridge DC**, **Febbraio MA**, **Hargreaves M**. Heat shock proteins and exercise adaptations. Our knowledge thus far and the road still ahead. *J Appl Physiol* 120: 683–691, 2016. doi: 10.1152/japplphysiol.00811.2015.
33. **Gibson OR**, **Turner Gareth**, **Tuttle JAlexander**, **Taylor Lee**, **Watt PW**, **Maxwell NS**. Heat acclimation attenuates physiological strain and the HSP72, but not HSP90α, mRNA response to acute normobaric hypoxia. *J Appl Physiol (1985)* 119: 889–99, 2015. doi: 10.1152/japplphysiol.00332.2015.
34. **Magalhães FDC**, **Amorim FT**, **Passos RLF**, **Fonseca MA**, **Oliveira KPM**, **Lima MRM**, **Guimarães JB**, **Ferreira-Júnior JB**, **Martini ARP**, **Lima NR v**, **Soares DD**, **Oliveira EM**, **Rodrigues LOC**. Heat and exercise acclimation increases intracellular levels of Hsp72 and inhibits exercise-induced increase in intracellular and plasma Hsp72 in humans. *Cell Stress Chaperones* 15: 885–95, 2010. doi: 10.1007/s12192-010-0197-7.
35. **Liu Y**, **Mayr S**, **Opitz-Gress A**, **Zeller C**, **Lormes W**, **Baur S**, **Lehmann M**, **Steinacker JM**. Human skeletal muscle HSP70 response to training in highly trained rowers. [Online]. *J Appl Physiol* 86: 101–4, 1999. http://www.ncbi.nlm.nih.gov/pubmed/9887119.
36. **Morton JP**, **Maclaren DPM**, **Cable NT**, **Campbell IT**, **Evans L**, **Kayani AC**, **McArdle A**, **Drust B**. Trained men display increased basal heat shock protein content of skeletal muscle. *Med Sci Sports Exerc* 40: 1255–62, 2008. doi: 10.1249/MSS.0b013e31816a7171.
37. **Gibson OR**, **Dennis A**, **Parfitt T**, **Taylor L**, **Watt PW**, **Maxwell NS**. Extracellular Hsp72 concentration relates to a minimum endogenous criteria during acute exercise-heat exposure. *Cell Stress Chaperones* 19: 389–400, 2014. doi: 10.1007/s12192-013-0468-1.
38. **Mee JA**, **Gibson OR**, **Tuttle JA**, **Taylor L**, **Watt PW**, **Doust J**, **Maxwell NS**. Leukocyte Hsp72 mRNA transcription does not differ between males and females during heat acclimation. *Temperature* 3: 549–556, 2016.
39. **Gibson OR**, **Mee JA**, **Taylor L**, **Tuttle JA**, **Watt PW**, **Maxwell NS**. Isothermic and fixed-intensity heat acclimation methods elicit equal increases in Hsp72 mRNA. *Scand J Med Sci Sports* 25: 259–268, 2015. doi: 10.1111/sms.12430.
40. **Kim K**, **Kuang S**, **Song Q**, **Gavin TP**, **Roseguini BT**. Impact of heat therapy on recovery following eccentric exercise in humans. .
41. **Sawka MN**, **Burke LM**, **Eichner ER**, **Maughan RJ**, **Montain SJ**, **Stachenfeld NS**. American College of Sports Medicine position stand. Exercise and fluid replacement. *Medicine and Science in Sport and Exercise* 39: 377–90, 2007. doi: 10.1249/mss.0b013e31802ca597.
42. **Chiesa ST**, **Trangmar SJ**, **González-Alonso J**. Temperature and blood flow distribution in the human leg during passive heat stress. *J Appl Physiol* 120: 1047–58, 2016. doi: 10.1152/japplphysiol.00965.2015.
43. **Pearson J**, **Kalsi KK**, **Stöhr EJ**, **Low DA**, **Barker H**, **Ali L**, **González-Alonso J**. Haemodynamic responses to dehydration in the resting and exercising human leg. *Eur J Appl Physiol* 113: 1499–1509, 2013. doi: 10.1007/s00421-012-2579-2.

44. **Jones PR**, **Pearson J**. Anthropometric determination of leg fat and muscle plus bone volumes in young male and female adults. [Online]. *Journal of Physiology* 204: 63P-66P, 1969. http://www.ncbi.nlm.nih.gov/pubmed/5824654 [23 May 2018].
45. **Wesseling KH**, **Jansen JR**, **Settels JJ**, **Schreuder JJ**. Computation of aortic flow from pressure in humans using a nonlinear, three-element model. *J Appl Physiol* 74: 2566–2573, 1993. doi: 10.1152/jappl.1993.74.5.2566.
46. **Toner MM**, **Drolet LL**, **Pandolf KB**. Perceptual and physiological responses during exercise in cool and cold water. [Online]. *Percept Mot Skills* 62: 211–20, 1986. http://www.ncbi.nlm.nih.gov/pubmed/3960662 [11 Feb. 2014].
47. **Borg GA**. Psychophysical bases of perceived exertion. [Online]. *Medicine and Science in Sport and Exercise* 14: 377–81, 1982. http://www.ncbi.nlm.nih.gov/pubmed/7154893 [1 Jul. 2016].
48. **MacInnis MJ**, **McGlory C**, **Gibala MJ**, **Phillips SM**. Investigating human skeletal muscle physiology with unilateral exercise models: when one limb is more powerful than two. *Applied Physiology, Nutrition, and Metabolism* 42: 563–570, 2017. doi: 10.1139/apnm-2016-0645.
49. **Tarnopolsky MA**, **Pearce E**, **Smith K**, **Lach B**. Suction-modified Bergström muscle biopsy technique: experience with 13,500 procedures. *Muscle Nerve* 43: 717–25, 2011. doi: 10.1002/mus.21945.
50. **Morton JP**, **Maclaren DPM**, **Cable NT**, **Campbell IT**, **Evans L**, **Bongers T**, **Griffiths RD**, **Kayani a C**, **McArdle a**, **Drust B**. Elevated core and muscle temperature to levels comparable to exercise do not increase heat shock protein content of skeletal muscle of physically active men. *Acta Physiol (Oxf)* 190: 319–27, 2007. doi: 10.1111/j.1748-1716.2007.01711.x.
51. **Kim K**, **Reid BA**, **Ro B**, **Casey CA**, **Song Q**, **Kuang S**, **Roseguini BT**. Heat therapy improves soleus muscle force in a model of ischemia-induced muscle damage. *J Appl Physiol* 127: 215–228, 2019. doi: 10.1152/japplphysiol.00115.2019.
52. **Tuttle JA**, **Chrismas BCR**, **Gibson OR**, **Barrington JH**, **Hughes DC**, **Castle PC**, **Metcalfe AJ**, **Midgley AW**, **Pearce O**, **Kabir C**, **Rayanmarakar F**, **Al-Ali S**, **Lewis MP**, **Taylor L**. The Hsp72 and Hsp90a mRNA responses to hot downhill running are reduced following a prior bout of hot downhill running, and occur concurrently within leukocytes and the vastus lateralis. *Front Physiol* 8, 2017. doi: 10.3389/fphys.2017.00473.
53. **Puntschart A**, **Vogt M**, **Widmer HR**, **Hoppeler H**, **Billeter R**. Hsp70 expression in human skeletal muscle after exercise. *Acta Physiol Scand* 157: 411–7, 1996. doi: 10.1046/j.1365-201X.1996.512270000.x.
54. **Paulsen G**, **Vissing K**, **Magne Kalhovde J**, **Ugelstad I**, **Lucia Bayer M**, **Kadi F**, **Schjerling P**, **Hallén J**, **Raastad T**. Maximal eccentric exercise induces a rapid accumulation of small heat shock proteins on myofibrils and a delayed HSP70 response in humans. *Am J Physiol Regul Integr Comp Physiol* 293: 844–853, 2007. doi: 10.1152/ajpregu.00677.2006.-In.
55. **Yang Y**, **Creer A**, **Jemiolo B**, **Trappe S**. Time course of myogenic and metabolic gene expression in response to acute exercise in human skeletal muscle. *J Appl Physiol* 98: 1745–1752, 2005. doi: doi: 10.1152/japplphysiol.01185.2004.
56. **Caldwell AR**, **Robinson FB**, **Tucker MA**, **Arcement CH**, **Butts CL**, **McDermott BP**, **Ganio MS**. Effect of passive heat stress and exercise in the heat on arterial stiffness. .
57. **Kroll J**, **Waltenberger J**. VEGF-A induces expression of eNOS and iNOS in endothelial cells via VEGF receptor-2 (KDR). *Biochem Biophys Res Commun* 252: 743–746, 1998. doi: 10.1006/bbrc.1998.9719.
58. **Gavin TP**, **Robinson CB**, **Yeager RC**, **England JA**, **Nifong LW**, **Hickner RC**. Angiogenic growth factor response to acute systemic exercise in human skeletal muscle. *J Appl Physiol* 96: 19–24, 2004. doi: doi: 10.1152/japplphysiol.00748.2003.

59. **Hoier B**, **Prats C**, **Qvortrup K**, **Pilegaard H**, **Bangsbo J**, **Hellsten Y**. Subcellular localization and mechanism of secretion of vascular endothelial growth factor in human skeletal muscle. *The FASEB Journal* 27: 3496–3504, 2013. doi: 10.1096/FJ.12-224618.
60. **Hiscock N**, **Fischer CP**, **Pilegaard H**, **Pedersen BK**. Vascular endothelial growth factor mRNA expression and arteriovenous balance in response to prolonged, submaximal exercise in humans. *Am J Physiol Heart Circ Physiol* 285: H1759-63, 2003. doi: 10.1152/AJPHEART.00150.2003.
61. **Hesketh K**, **Shepherd SO**, **Strauss JA**, **Low DA**, **Cooper RG**, **Wagenmakers AJM**, **Cocks M**. Passive Heat Therapy in Sedentary Humans Increases Skeletal Muscle Capillarisation and eNOS Content but Not Mitochondrial Density or GLUT4 Content. .
62. **Thuringer D**, **Jego G**, **Wettstein G**, **Terrier O**, **Cronier L**, **Yousfi N**, **Hébrard S**, **Bouchot A**, **Hazoumé A**, **Joly A-L**, **Gleave M**, **Rosa-Calatrava M**, **Solary E**, **Garrido C**. Extracellular HSP27 mediates angiogenesis through Toll-like receptor 3. *FASEB J* 27: 4169–4183, 2013. doi: 10.1096/fj.12-226977.
63. **Kuang J**, **McGinley C**, **Lee MJ-C**, **Saner NJ**, **Garnham A**, **Bishop DJ**. Interpretation of exercise-induced changes in human skeletal muscle mRNA expression depends on the timing of the post-exercise biopsies. .
64. **Périard JD**, **Ruell P**, **Caillaud C**, **Thompson MW**. Plasma Hsp72 (HSPA1A) and Hsp27 (HSPB1) expression under heat stress: influence of exercise intensity. *Cell Stress Chaperones* 17: 375–83, 2012. doi: 10.1007/s12192-011-0313-3.
65. **Faulkner SH**, **Jackson S**, **Fatania G**, **Leicht CA**. The effect of passive heating on heat shock protein 70 and interleukin-6: A possible treatment tool for metabolic diseases? .
66. **Taylor L**, **Lee BJ**, **Gibson OR**, **Midgley AW**, **Watt P**, **Mauger A**, **Castle P**. Effective microorganism – X attenuates circulating superoxide dismutase following an acute bout of intermittent running in hot, humid conditions. .
67. **Behzadi P**, **Ravanelli N**, **Gravel H**, **Barry H**, **Debray A**, **Chaseling GK**, **Jacquemet V**, **Neagoe P-E**, **Nigam A**, **Carpentier AC**, **Sirois MG**, **Gagnon D**. Acute effect of passive heat exposure on markers of cardiometabolic function in adults with type 2 diabetes mellitus. *J Appl Physiol* 132: 1154–1166, 2022. doi: 10.1152/japplphysiol.00800.2021.
68. **Amorim F**, **Yamada P**, **Robergs R**, **Schneider S**, **Moseley P**. Effects of whole-body heat acclimation on cell injury and cytokine responses in peripheral blood mononuclear cells. *Eur J Appl Physiol* 111: 1609–18, 2011. doi: 10.1007/s00421-010-1780-4.
69. **Hom LL**, **Lee EC-H**, **Apicella JM**, **Wallace SD**, **Emmanuel H**, **Klau JF**, **Poh PYS**, **Marzano S**, **Armstrong LE**, **Casa DJ**, **Maresh CM**. Eleven days of moderate exercise and heat exposure induces acclimation without significant HSP70 and apoptosis responses of lymphocytes in college-aged males. *Cell Stress Chaperones* 17: 29–39, 2012. doi: 10.1007/s12192-011-0283-5.
70. **Kuennen M**, **Gillum T**, **Dokladny K**, **Bedrick E**, **Schneider S**, **Moseley P**. Thermotolerance and heat acclimation may share a common mechanism in humans. *Am J Physiol Regul Integr Comp Physiol* 301: R524-33, 2011. doi: 10.1152/ajpregu.00039.2011.
71. **Lee BJ**, **Miller A**, **James RS**, **Thake CD**. Cross acclimation between heat and hypoxia: Heat acclimation improves cellular tolerance and exercise performance in acute normobaric hypoxia. *Front Physiol* 7:78, 2016. doi: 10.3389/fphys.2016.00078.
72. **Marshall HC**, **Campbell SA**, **Roberts CW**, **Nimmo MA**. Human physiological and heat shock protein 72 adaptations during the initial phase of humid-heat acclimation. *J Therm Biol* 32: 341–348, 2007. doi: 10.1016/j.jtherbio.2007.04.003.
73. **McClung JP**, **Hasday JD**, **He J-RR**, **Montain SJ**, **Cheuvront SN**, **Sawka MN**, **Singh IS**. Exercise-heat acclimation in humans alters baseline levels and ex vivo heat inducibility of HSP72 and HSP90 in peripheral blood mononuclear cells. *Am J Physiol Regul Integr Comp Physiol* 294: R185-91, 2008. doi: 10.1152/ajpregu.00532.2007.

74. **Maloyan A**, **Palmon A**, **Horowitz M**, **Tetievsky A**, **Cohen O**, **Eli-berchoer L**, **Gerstenblith G**, **Stern MD**, **Mcclung JP**, **Hasday JD**, **He J**, **Montain SJ**, **Cheuvront SN**, **Sawka N**, **Singh IS**, **Melling CWJ**, **Thorp DB**, **Milne KJ**, **Krause MP**, **Noble EG**, **Horo- M**. Heat acclimation increases the basal HSP72 level and alters its production dynamics during heat stress memory. .
75. **Hoier B**, **Hellsten Y**. Exercise-Induced Capillary Growth in Human Skeletal Muscle and the Dynamics of VEGF. *Microcirculation* 21: 301–314, 2014. doi: 10.1111/MICC.12117.
76. **Gavin TP**, **Drew JL**, **Kubik CJ**, **Pofahl WE**, **Hickner RC**. Acute resistance exercise increases skeletal muscle angiogenic growth factor expression. *Acta Physiologica* 191: 139–146, 2007. doi: https://doi.org/10.1111/j.1748-1716.2007.01723.x.
77. **Hoier B**, **Nordsborg N**, **Andersen S**, **Jensen L**, **Nybo L**, **Bangsbo J**, **Hellsten Y**. Pro- and anti-angiogenic factors in human skeletal muscle in response to acute exercise and training. *J Physiol* 590: 595–606, 2012. doi: https://doi.org/10.1113/jphysiol.2011.216135.
78. **Gavin TP**, **Ruster RS**, **Carrithers JA**, **Zwetsloot KA**, **Kraus RM**, **Evans CA**, **Knapp DJ**, **Drew JL**, **McCartney JS**, **Garry JP**, **Hickner RC**. No difference in the skeletal muscle angiogenic response to aerobic exercise training between young and aged men. *J Physiol* 585: 231–239, 2007. doi: https://doi.org/10.1113/jphysiol.2007.143198.
79. **Hoier B**, **Passos M**, **Bangsbo J**, **Hellsten Y**. Intense intermittent exercise provides weak stimulus for vascular endothelial growth factor secretion and capillary growth in skeletal muscle. *Exp Physiol* 98: 585–597, 2013. doi: https://doi.org/10.1113/expphysiol.2012.067967.
80. **Hoier B**, **Walker M**, **Passos M**, **Walker PJ**, **Green A**, **Bangsbo J**, **Askew CD**, **Hellsten Y**. Angiogenic response to passive movement and active exercise in individuals with peripheral arterial disease. *J Appl Physiol* 115: 1777–1787, 2013. doi: 10.1152/japplphysiol.00979.2013.
81. **Rullman E**, **Rundqvist H**, **Wågsäter D**, **Fischer H**, **Eriksson P**, **Sundberg CJ**, **Jansson E**, **Gustafsson T**. A single bout of exercise activates matrix metalloproteinase in human skeletal muscle. *J Appl Physiol* 102: 2346–2351, 2007. doi: 10.1152/japplphysiol.00822.2006.
82. **Hoier B**, **Prats C**, **Qvortrup K**, **Pilegaard H**, **Bangsbo J**, **Hellsten Y**. Subcellular localization and mechanism of secretion of vascular endothelial growth factor in human skeletal muscle. *The FASEB Journal* 27: 3496–3504, 2013. doi: https://doi.org/10.1096/fj.12-224618.
83. **Blake MJ**, **Gershon D**, **Fargnoli J**, **Holbrook NJ**. Discordant expression of heat shock protein mRNAs in tissues of heat-stressed rats. *Journal of Biological Chemistry* 265: 15275–15279, 1990. doi: 10.1016/S0021-9258(18)77252-9.
84. **Neff D**, **Kuhlenhoelter AM**, **Lin C**, **Wong BJ**, **Motaganahalli RL**, **Roseguini BT**. Thermotherapy reduces blood pressure and circulating endothelin-1 concentration and enhances leg blood flow in patients with symptomatic peripheral artery disease. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 311: R392–R400, 2016. doi: 10.1152/ajpregu.00147.2016.
85. **Akwii RG**, **Sajib MS**, **Zahra FT**, **Mikelis CM**. Role of Angiopoietin-2 in Vascular Physiology and Pathophysiology. *Cells* 8: 471, 2019. doi: 10.3390/cells8050471.

836 **Figure legends**
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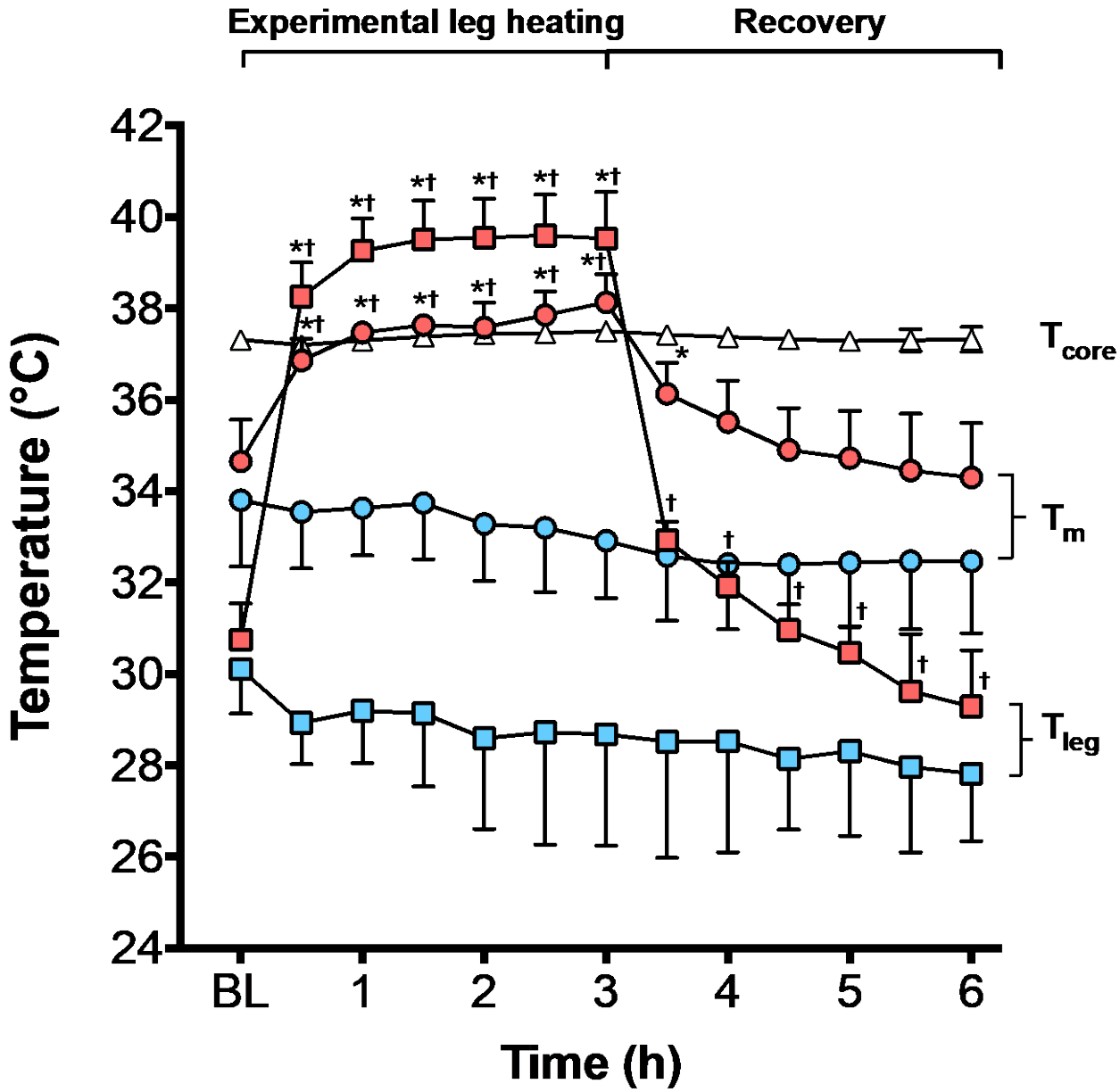

838
839 **Figure 1.** Muscle temperature ($T_{m,}$ circles), leg skin temperature (T_{leg}, squares), and core temperature
840 (T_{core}, triangles, $p > 0.05$ from baseline). Data are presented as Mean±SD (n =7). BL=baseline. *
841 denotes difference from BL, † denotes difference from CONT. Blue represents CONT, red represents
842 HEAT.
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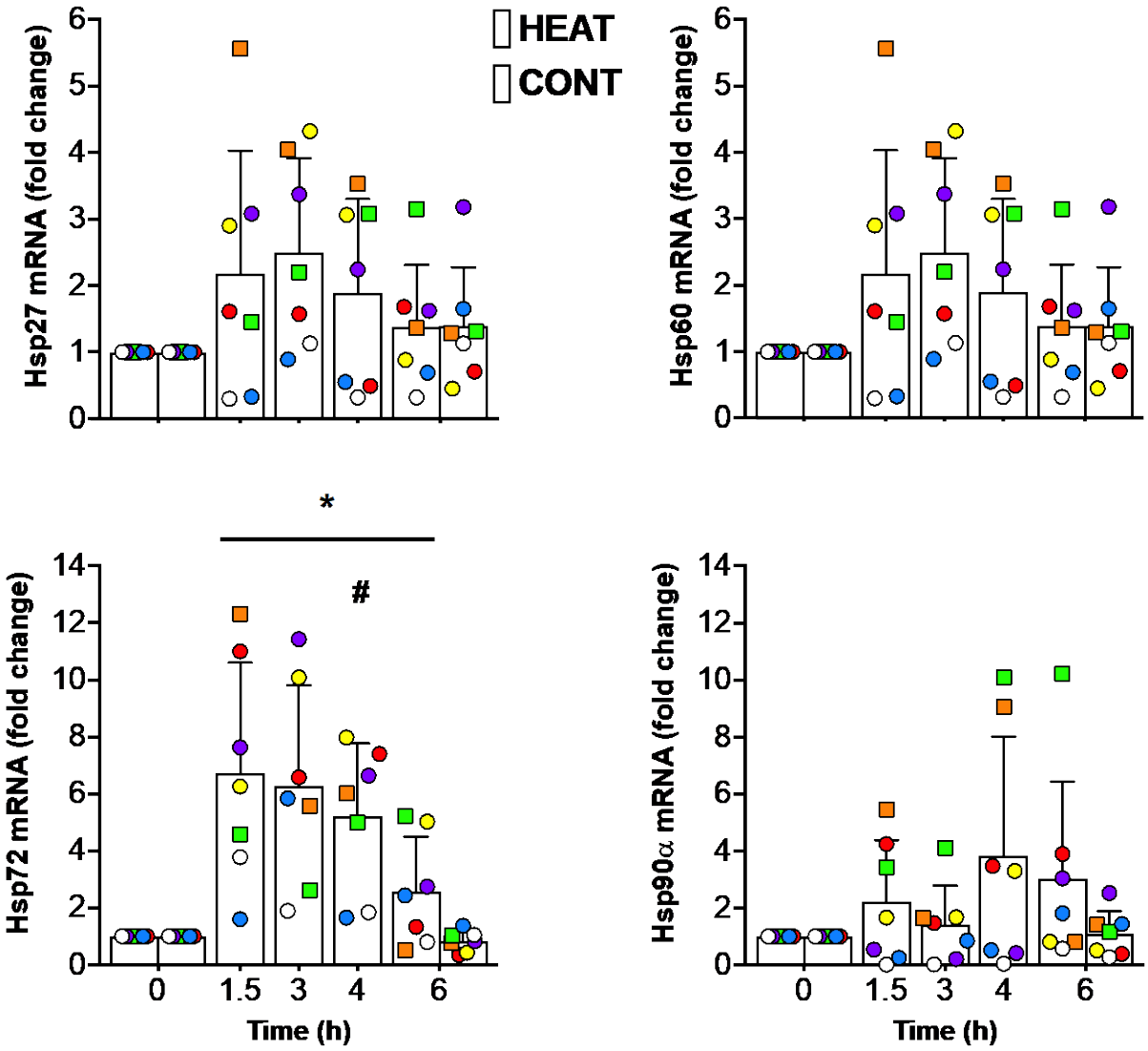

Figure 2. Heat shock protein gene responses over time for HEAT (red bars) and CONT (blue bars) limbs. Individual data points are colour coded for each participant with squares representing female participants. Data are presented as Mean±SD (n=7). * denotes a main effect for time within gene ($p<0.05$). # denotes identified post hoc difference within HEAT ($p<0.05$).

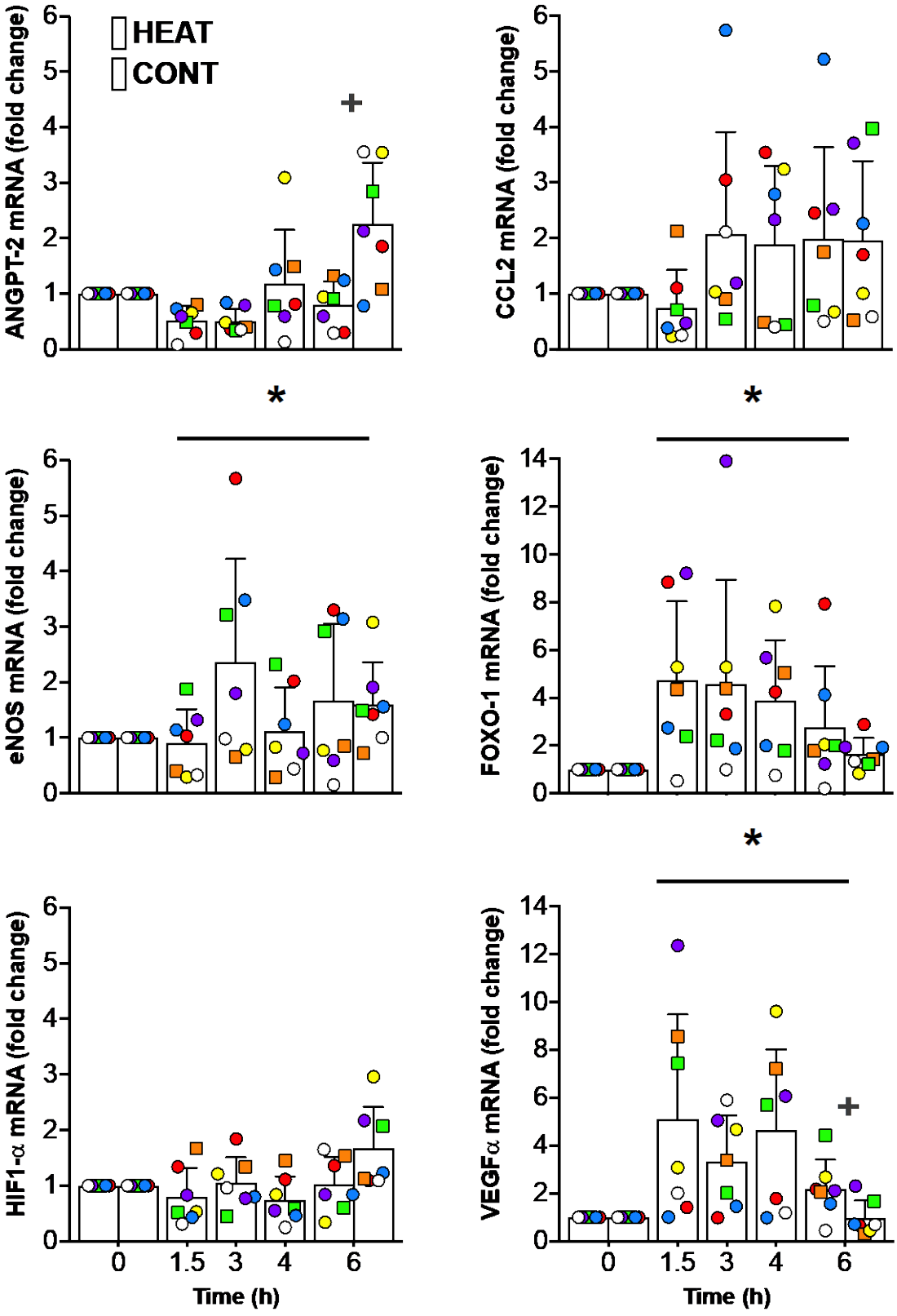

Figure 3. Regulatory gene responses over time for HEAT (red bars) and CONT (blue bars). Individual data points are colour coded for each participant with squares representing female participants. Data are presented as Mean±SD (n=7).* denotes a main effect for time within gene ($p<0.05$). + denotes difference between HEAT and CONT at 6 h timepoint.

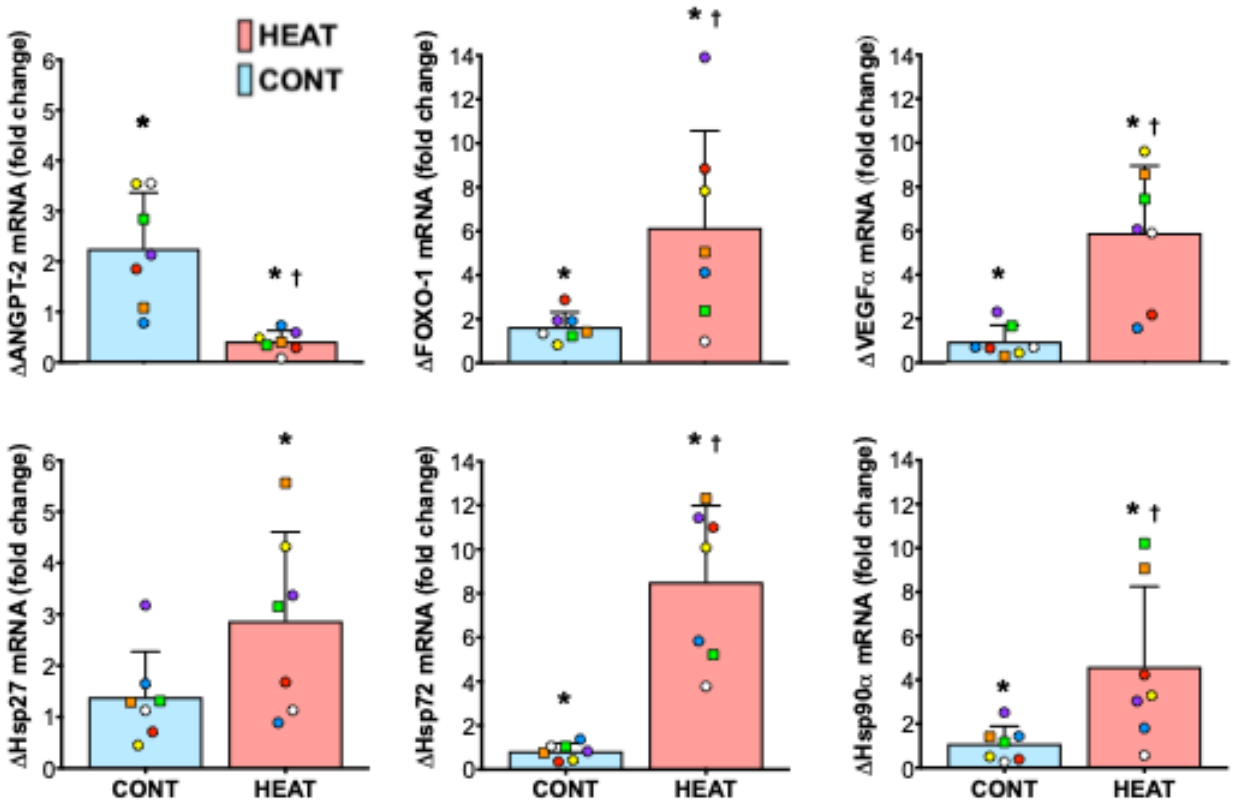

Figure 4. Peak change in selected gene responses over time for HEAT (red bars) and CONT (blue bars) limbs. Individual data points are colour coded for each participant with squares representing female participants. Data are presented as Mean±SD (n=7). * denotes a difference from baseline within trial ($p<0.05$). † denotes a difference from CONT within HEAT ($p<0.05$).

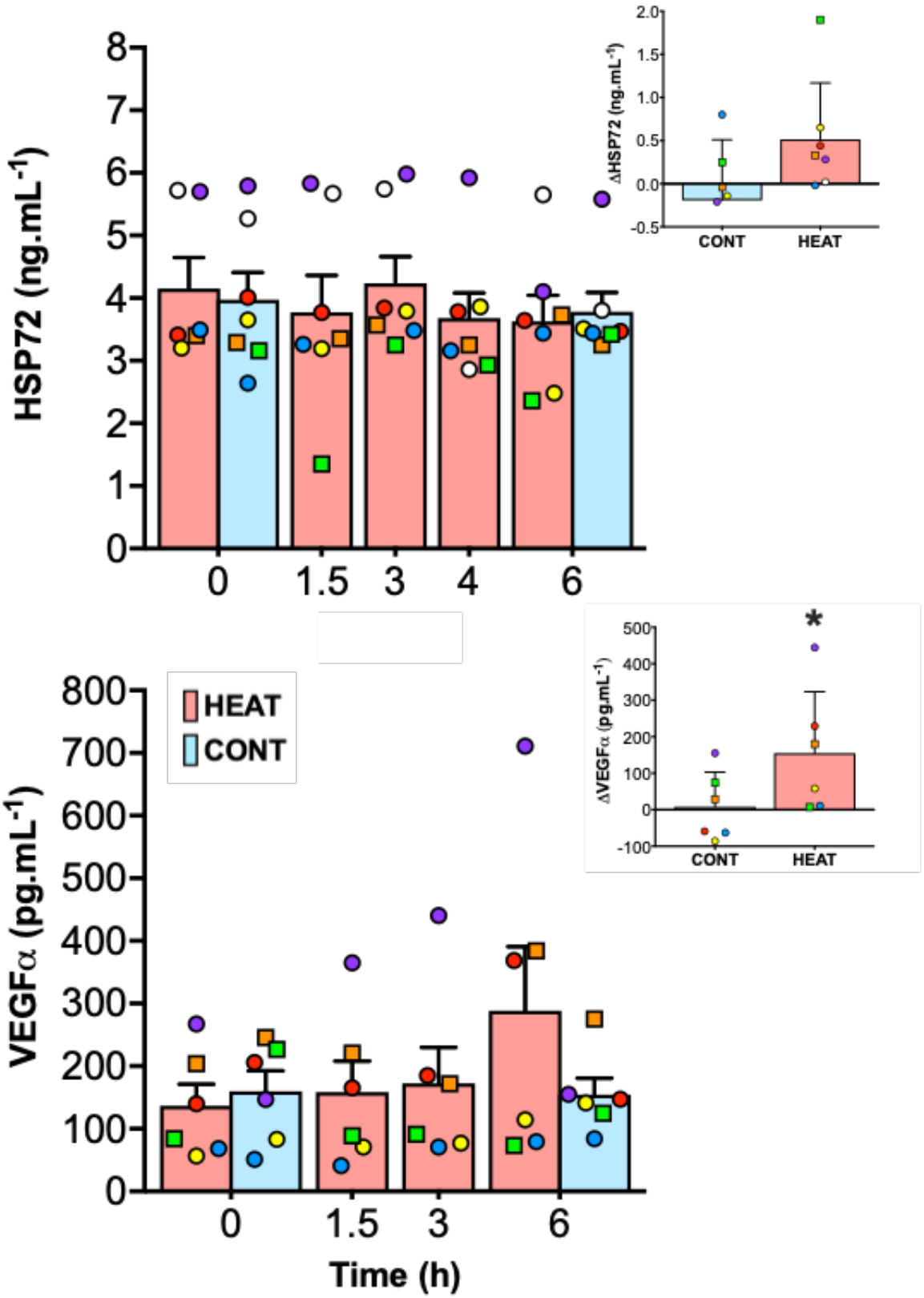

Figure 5. HSP72 (n = 7) and VEGFα (n = 6) protein concentration over time for HEAT (red bars) and CONT (blue bars). Insert figure represents peak change. Individual data points are colour coded for each participant with squares representing female participants. Data are presented as Mean±SD for 6-7 participants. * denotes a difference from CONT within HEAT ($p<0.05$)

Tables and legends

Table 1. List of genes included in the analysis

Full name	Gene abbreviation	Gene symbol	Aliases	Primers [Forward (F) & Reverse (R)]	Role(s)
Heat shock protein genes					
Heat shock protein family B (small) member 1	Hsp27	HSPB1	CMT2F, HEL-S-102, HMN2B, HS.76067, HSP27, HSP28, Hsp25, SRP27	F: CACGAGGAGCGGCAGGACGAG R:CAGTGGCGGCAGCAGGGGTGG	Chaperone activity, inhibition of apoptosis, regulation of cell and differentiation.
Heat shock protein family D (Hsp60) member 1	Hsp60	HSPD1	CPN60, GROEL, HLD4, HSP-60, HSP60, HSP65, HuCHA60, SPG13	F: GATGTCCTGGGCTGTTTCAT R: GCCTCGATCAAACTTCATGC	Prevention of protein misfolding
Heat shock protein family A (Hsp70) member 1A	Hsp72	HSPA1A	HEL-S-103, HSP70-1, HSP70-1A, HSP70.1, HSP70I, HSP72, HSPA1	F: GGTGCTGACCAAGATGAAG R: CTGCGAGTCGTTGAAGTAG	Correct folding of new or misfolded proteins. DNA repair. Induction of pro-inflammatory cytokines
Heat shock protein 90 alpha family class A member 1	Hsp90α	HSP90AA1	EL52, HEL-S-65p, HSP86, HSP89A, HSP90A, HSP90N, HSPC1, HSPCA, HSPCAL1, HSPCAL4, HSPN, Hsp89, Hsp90, LAP-2, LAP2	F: ATCAAACTTGGTCTGGGTATT R: GATGTGTCGTCATCTCCTTC	Protein folding, maintenance, and degradation. Intracellular transport. Cell signaling.
Regulatory genes					
Angiopoietin 2	ANGPT-2	ANGPT2	AGPT2, ANG2	F: TGGACAATTATTCAGCGACGTG R: GCTGGTCGGATCATCATGGTTG	A member of the angiopoietin family of growth factors, an antagonist of angiopoietin-1 in blood vasculature.
C-C motif chemokine ligand 2	CCL2	CCL2	GDCF-2, HC11, HSMCR30, MCAF, MCP-1, MCP1, SCYA2, SMC-CF	F: AGAATCACCAGCAGCAAGTGTCC R: TCCTGAACCCACTTCTGCTTGG	Myokine with role in skeletal muscle remodelling including angiogenesis
Nitric oxide synthase 3	eNOS	NOS3	ECNOS, eNOS	F: ACCCTCACCGCTACAACATC R: CTGGCCTTCTGCTCATTCTC	Maintenance of endothelial homeostasis via generation of nitric oxide in the vascular endothelium
Forkhead box O1	FOXO-1	FOXO1	FKH1, FKHR, FOXO1A	F: CTACGAGTGGATGGTCAAGAGC R: CCAGTTCCTTCATTCTGCACACG	Regulates angiostatic factors, restraining angiogenesis.
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	GAPDH	G3PD, GAPD, HEL-S-162eP	F: AATCCCATCACCATCTTCCA R:TGGACTCCACGACGTACTCA	Control for this experiment. Functional role in energy metabolism and the production of ATP and pyruvate through anaerobic glycolysis in the cytoplasm
Hypoxia inducible factor 1 alpha subunit	HIF-1α	HIF1A	HIF-1-alpha, HIF-1A, HIF-1alpha, HIF1, HIF1-ALPHA, MOP1, PASD8, bHLHe78	F: CTAGCCGGAGGAAGAACTATGAAC R: CCCACACTGAGGTTGGTTACTGT	Master regulator of vascular responses, driving transcriptional activation of genes involved in vascular reactivity and angiogenesis.
Vasohibin 1	VASH1	VASH1	KIAA1036	F: ATGGACCTGGCCAAGGAAAT R: CATCCTTCTTCCGGTCCTTG	Angiogenesis inhibitor expressed in endothelial cells via induction by pro-angiogenesis factors

Vascular endothelial growth factor A	VEGFα	VEGFA	MVCD1, VEGF, VPF	F: TTTCTGCTGTCTTGGGTGCATTGG R: ACCACTTCGTGATGATTCTGCCCT	Regulates angiogenesis by inducing proliferation, migration, and permeability of endothelial cells.

Table 2. Physiological and perceptual responses during the protocol.

	Time (h)						
	Baseline	**1**	**2**	**3**	**4**	**5**	**6**
HR ($b.min^{-1}$)	64 ± 15	72 ± 15	71 ± 13	75 ± 18	65 ± 13	63 ± 13	68 ± 17
$\dot{Q}$ ($L \cdot min^{-1}$)	6.1 ± 1.6	6.8 ± 1.1	6.6 ± 1.2	7.0 ± 1.3	6.3 ± 1.7	6.1 ± 1.8	6.8 ± 1.5
MAP (mmHg)	87 ± 9	79 ± 6	84 ± 8	86 ± 9	89 ± 15	88 ± 19	89 ± 12
$\dot{V}O_2$ ($L \cdot min^{-1}$)	0.29 ± 0.07	0.33 ± 0.08	0.35 ± 0.06	0.35 ± 0.05	0.35 ± 0.05	0.34 ± 0.04	0.34 ± 0.04
$\dot{V}_E$ ($L \cdot min^{-1}$)	8 ± 2	9 ± 2	8 ± 2	9 ± 1	8 ± 1	8 ± 2	9 ± 1
T_{torso} (°C)	32.9 ± 1.3	33.4 ± 1.3	32.6 ± 1.8	32.7 ± 1.7	33.0 ± 1.5	33.4 ± 1.1	33.1 ± 1.6
[Glu] ($mmol \cdot L^{-1}$)	5.5 ± 0.5	-	-	5.3 ± 0.4	5.5 ± 0.8	-	5.3 ± 0.6
[Hb] ($g \cdot L^{-1}$)	148 ± 11	143 ± 13	144 ± 11	147 ± 14	146 ± 12	145 ± 12	146 ± 10
Hct (%)	45.4 ± 3.1	44.5 ± 3.2	44.9 ± 3.4	44.9 ± 3.3	45.4 ± 3.1	45.2 ± 3.2	46.1 ± 2.6
RPE	6 ± 0	6 ± 0	6 ± 0	6 ± 1	7 ± 1	7 ± 2	7 ± 2
TSENS	3.8 ± 0.4	5.1 ± 0.6 *	5.4 ± 0.9 *	5.5 ± 1.1 *	3.7 ± 0.4	3.6 ± 0.4	3.7 ± 0.4
TCOMF	1 ± 1	1 ± 1	2 ± 1	1 ± 1	1 ± 0	1 ± 0	1 ± 0

Data are mean±SD (n=7). * denotes difference vs 0 h (Baseline) ($p<0.05$). HR: heart rate, $\dot{Q}$: cardiac output, MAP: mean arterial pressure, $\dot{V}O_2$: oxygen consumption, $\dot{V}_E$: minute ventilation, T_{torso}: Torso temperature, [Glu]: blood glucose concentration, [Hb]: haemoglobin concentration, Hct: haematocrit, RPE: rating of perceived exertion, TSENS: thermal sensation, TCOMF: thermal comfort.

Table 3. Frequency of peak gene response for each sample timepoint during the 3 h leg heating protocol and subsequent 3 h recovery.

	1.5 h	3 h	4 h	6 h	Time to peak (h)
Heat shock protein genes					
Hsp27 *	29%	0%	**43%**	29%	**3.9 ± 1.8**
Hsp60	**43%**	**43%**	0%	14%	2.8 ± 1.6
Hsp72 *	14%	0%	29%	**57%**	**4.8 ± 1.7**
Hsp90α *	14%	29%	0%	**57%**	**4.5 ± 1.9**
Regulatory genes					
ANGPT-2 *	**57%**	43%	0%	0%	**2.1 ± 0.8**
CCL2 *	14%	**29%**	**29%**	**29%**	**3.9 ± 1.6**
eNOS	0%	**71%**	14%	14%	3.1 ± 1.8
FOXO-1 *	**29%**	**29%**	**29%**	14%	**3.3 ± 1.6**
HIF-1a	14%	**57%**	0%	29%	3.6 ± 1.7
VASH-1	14%	**29%**	**29%**	**29%**	3.9 ± 1.6
VEGFα *	**29%**	14%	**29%**	**29%**	**3.7 ± 1.9**

* denotes a significant peak change vs baseline ($p<0.05$). Time to peak reported at mean ± SD. Bold text in cell denotes timepoint(s) corresponding to greatest proportion of peak gene changes