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ORIGINAL PAPER

Hsp72 and Hsp90α mRNA transcription is characterised by large, sustained changes in core temperature during heat acclimation

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Abstract Increased intracellular heat shock protein-72 (Hsp72) and heat shock protein-90α (Hsp90α) have been implicated as important components of acquired thermotolerance, providing cytoprotection during stress. This experiment determined the physiological responses characterising increases in Hsp72 and Hsp90α mRNA on the first and tenth day of 90-min heat acclimation (in 40.2 °C, 41.0 % relative humidity (RH)) or equivalent normothermic training (in 20 °C, 29 % RH). Pearson's product-moment correlation and stepwise multiple regression were performed to determine relationships between physiological [e.g. (T_{rec}, sweat rate (SR) and heart rate (HR)] and training variables (exercise duration, exercise intensity, work done), and the leukocyte Hsp72 and Hsp90α mRNA responses via reverse transcription quantitative polymerase chain reaction (RT-QPCR) ($n = 15$). Significant ($p < 0.05$) correlations existed between increased Hsp72 and Hsp90α mRNA ($r = 0.879$). Increased core temperature was the most important criteria for gene transcription with ΔT_{rec} ($r = 0.714$), SR ($r = 0.709$), $T_{recfinal45}$ ($r = 0.682$), area under the curve where $T_{rec} \geq 38.5$ °C (AUC38.5 °C; $r = 0.678$), peak T_{rec} ($r = 0.661$), duration $T_{rec} \geq 38.5$ °C ($r = 0.650$) and ΔHR ($r = 0.511$) each demonstrating a significant ($p < 0.05$) correlation with the increase in Hsp72 mRNA. The T_{rec} AUC38.5 °C ($r = 0.729$), ΔT_{rec} ($r = 0.691$), peak T_{rec} ($r = 0.680$), $T_{recfinal45}$ ($r = 0.678$), SR ($r = 0.660$), duration $T_{rec} \geq 38.5$ °C ($r = 0.629$), the rate of change in T_{rec} ($r = 0.600$) and ΔHR ($r = 0.531$) were the strongest correlate with the increase in Hsp90α mRNA. Multiple regression improved the model for Hsp90α mRNA only, when T_{rec} AUC38.5 °C and SR were combined. Training variables showed insignificant ($p > 0.05$) weak ($r < 0.300$) relationships with Hsp72 and Hsp90α mRNA. Hsp72 and Hsp90α mRNA correlates were comparable on the first and tenth day. When transcription of the related Hsp72 and Hsp90α mRNA is important, protocols should rapidly induce large, prolonged changes in core temperature.

Keywords Heat shock proteins · Hyperthermia · Core temperature · Heat acclimation · Thermotolerance

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Introduction

Thermotolerance is an acquired cellular adaptation to heat stress (Kuennen et al. 2011) conferring cytoprotection to subsequent thermal (McClung et al. 2008) and non-thermal (Gibson et al. 2015c) stress in vitro (McClung et al. 2008) and in vivo (Lee et al. 2016). Acquired thermotolerance is reliant upon sufficient heat shock protein (HSP) gene transcription (Moran et al. 2006) and subsequent protein translation (Silver and Noble 2012). Functionally, HSPs facilitate maintenance of cellular and protein homeostasis, with regulatory roles

in mitigating apoptosis, and facilitating recovery from and adaptation to stress [including exercise training (Liu et al. 1999) and/or thermal stress (Kuennen et al. 2011)] at a cellular, organ and whole body level (Henstridge et al. 2016).

The HSP70 family is present in two predominant isoforms: a constitutively expressed protein that demonstrates little basal change, HSC70 (HSP73), and a highly inducible 'chaperone' isoform HSP72 (HSPA1A/HSPA1B) central to cytoprotection (Kampinga et al. 2009). HSP90 also presents with a constitutively expressed isoform (HSP90β) and an inducible isoform, HSPC1 (HSP90α) (Subbarao Sreedhar et al. 2004). HSP72 provides cellular protection, notably maintaining intestinal epithelial tight junction barriers, increasing resistance to gut-associated endotoxin translocation and reducing inflammatory responses to stress (Moseley 2000; Amorim et al. 2015; Dokladny et al. 2016). In addition, HSP72 may be important in facilitating positive heat (Kuennen et al. 2011) and heat-independent adaptations (Henstridge et al. 2016). HSP90α is cytoprotective, similar to HSP72, whilst also implicit in recovery and adaptation to cellular stress, particularly the control of cellular signalling cascades (Taipale et al. 2010), recovery of global protein synthesis (Duncan 2005) and coordination of cellular repair (Erlejman et al. 2014). Increases in extracellular HSP72 (eHSP72) have been widely observed in response to acute exercise (Whitham et al. 2007; Périard et al. 2012; Gibson et al. 2014), with endogenous criteria, notably increased core temperature most important for eliciting large increases (Périard et al. 2012; Gibson et al. 2014). These eHSP72 increases are transient (Périard et al. 2012; Gibson et al. 2014) and have a proposed immunological role (Asea 2003) rather than initiating chronically beneficial (i.e. cytoprotective) HSP72 protein translation that is retained beyond the initial stressor (Marshall et al. 2007; Périard et al. 2015). Therefore, the usefulness of extracellular HSPs to characterise acquired thermotolerance (Moseley 1997; Kregel 2002), to identify cessation of the cellular stress response following adaptation in vivo (McClung et al. 2008; Kuennen et al. 2011) and ex vivo (McClung et al. 2008), or to identify functional roles in disease states (Henstridge et al. 2014a; Krause et al. 2015a) is inferior to that of the HSP gene transcript or translated protein (Lee et al. 2015). At present, the precise physiological signals for increasing Hsp72 mRNA and Hsp90α mRNA are unknown, as is whether these genes transcribe to similar stimuli and similar magnitudes during exercise/exercise-heat stress. Accordingly, similar characterisation of Hsp72 and Hsp90α gene transcription to that of eHSP72 is required given their direct relationship with thermotolerance (Lee et al. 2015).

Exercise elicits numerous cellular and molecular stressors that in isolation or combination behave as inductive stimuli for increases in HSPs (Henstridge et al. 2016). Stimuli characterising changes include, but are not limited to, whole body and local hyperthermia (Fehrenbach et al. 2001), oxidative stress/free radical formation (Khassaf et al. 2001; Taylor et al. 2010a), substrate depletion (Febbraio et al. 2002), hypoxia/ischaemia (Taylor et al. 2011), altered pH (Peart et al. 2011) and increased calcium concentration (Stary and Hogan 2016). Elevated expressions of both intracellular HSP72 (iHSP72) and intracellular HSP90α (iHSP90α) are largely dictated by their transcription factor heat shock factor 1 (HSF1), which is translocated to the nucleus where it binds to the heat shock elements (HSEs), resulting in relevant mRNA (Hsp) transcription. HSP72 and HSP90α demonstrate large changes in the net intracellular protein following acute and chronic exercise that initiates their respective gene transcripts (McClung et al. 2008; Tuttle et al. 2015). It has been demonstrated that HSP72 increases in response to thermal stress (Magalhães et al. 2010), though others have observed HSP72 protein translation as being independent of increased core and/or muscle temperature (Morton et al. 2007). At present, changes in Hsp72 mRNA, and particularly Hsp90α mRNA following heat acclimation, have not been reported relative to specific physiological stimuli either experimentally or retrospectively. As such, a dose response or minimum stimuli characterising significant transcription-translation has yet to be determined. Ambiguity in HSP response to thermal and exercise stimuli, notably during comparable heat acclimation (HA) regimes (Magalhães et al. 2010; Hom et al. 2012), suggests that a combination of/or minimum threshold for elevated endogenous stressors may be required to increase HSP protein content in vivo; such responses may well be individualised and determined by genetic, epigenetic and phenotypical factors (Horowitz 2014; Horowitz 2016). Consequently, preliminary data relative to such characterisation is required in vivo from a homogenous sample. Additionally, given the potential for epigenetic modifications in Hsp transcription (Horowitz 2016), it remains unknown whether the signals characterising increased gene expression would demonstrate equality at the onset and culmination of a HA protocol.

Inhibition of HSF1 has been proposed to increase susceptibility to acute in vivo thermal stress [i.e. heat stroke (Moran et al. 2006)], and similarly preclude procurement of optimal physiological adaptation to chronic thermal stress [i.e. heat-acclimated phenotype (Maloyan and Horowitz 2002; Kuennen et al. 2011)]. Induction of HSPs, particularly HSP72, is central to not only to the aforementioned heat adaptation (Kuennen et al. 2011), but are increasingly implicated within other positive adaptive responses to stress [i.e. promotion of mitochondrial biogenesis (Henstridge et al. 2014a)] and various disease states [e.g. type 2 diabetes mellitus (Hooper et al. 2014), cardiovascular disease (Noble and Shen 2012), and Parkinson's disease (Erekat et al. 2014)]. Reduced iHSPs are observed in disease states such as type 2 diabetes mellitus in response to insulin-sensitive HSF1 inhibition (Kurucz et al. 2002), with heat stress-induced increases

in HSP72 proving therapeutic (Gupte et al. 2011). Whilst understanding of the important role of heat shock proteins is growing, less is known of the physiological signals which facilitate the optimal transcription of the mRNA prior to protein translation (Anckar and Sistonen 2011). Characterising the signal or signals that predict Hsp72 mRNA and Hsp90α mRNA increases (and thus likely increased HSP) may enhance the efficacy of Henstridge et al. (2014b).

The aim of this experiment was to characterise the physiological stimuli (core temperature, heart rate, sweat rates) and/or training prescription markers (exercise duration, exercise intensity, exercise power and work done) that correlate most strongly with the increase in Hsp72 mRNA and Hsp90α mRNA during a 10-day HA regime or a comparable normothermic training intervention (Gibson et al. 2015c). Additionally, we sought to determine whether in a homogenous sample, experiencing equality of stress, whether the predictive criteria for Hsp72 mRNA and Hsp90α mRNA transcription would change pre- to post-HA or normothermic training. It was hypothesised that markers of thermal strain and heat storage, i.e. core temperature, would most closely predict the change in Hsp72 mRNA and Hsp90α mRNA, and these markers would demonstrate equality in predictive capacity at the beginning and end of HA/training.

Materials and methods

Participants

The analysis of Hsp72 mRNA and Hsp90α mRNA was performed on data collected from 15 participants who had performed ten 90-min isothermic HA sessions (n = 7; age = 23 ± 4 years, height = 183 ± 6 cm, mass = 76.4 ± 6.7 kg, body surface area = 1.98 ± 0.11 m^2, body mass index = 22.9 ± 1.6 kg m^2, body fat = 14.0 ± 3.1 %, $\dot{V}O_{2peak}$ = 4.16 ± 0.56 L min^{-1}) or performed normothermic exercise training in a temperate environment (n = 8 age = 26 ± 5 years, height = 179 ± 7 cm, mass = 74.6 ± 4.8, body surface area = 1.93 ± 0.10 m^2, body mass index = 23.2 ± 0.9 kg m^2, body fat = 14.5 ± 2.6 %, $\dot{V}O_{2peak}$ = 4.22 ± 0.62) from one previously published experiment (Gibson et al. 2015c) (pooled descriptive characteristics in Table 1, schematic overview in Fig. 1). Given equality of training prescription [as detailed elsewhere (Gibson et al. 2015c)], both the isothermic HA and normothermic exercise training groups were pooled into one data set for each time point to increase the heterogeneity of the physiological responses and Hsp mRNA transcription. Confounding environmental (prolonged hyperthermic and/or hypoxic stress) and pharmacological variables were all controlled in line with previous work in the field (Gibson et al. 2014; Gibson et al. 2015a). Participants commenced all trials in a euhydrated state [<700 mOsm kg^{-1} H_2O (Sawka et al. 2007)]. All protocols, procedures and methods were approved by the institutional ethics committee. Participants completed medical questionnaires and written informed consent following the principles outlined by the Declaration of Helsinki as revised in 2013 prior to commencing any preliminary or experimental sessions. In compliance with ethical approval, a testing/intervention session was terminated if a subject attained a core temperature [measured at the rectum (T_{rec})] of 39.7 °C (zero incidences).

Experimental design

Preliminary testing commenced with anthropometric assessment of participants, whom subsequently performed an incremental (24 W min^{-1}) cycle test commencing at 80 W, in temperate laboratory conditions [20 °C, 40 % relative humidity (RH)] to determine peak oxygen uptake ($\dot{V}O_{2peak}$) (Gibson et al. 2015c). Expired metabolic gas was measured at a breath by breath frequency (Metalyser 3B, Cortex, Leipzig, Germany) with $\dot{V}O_{2peak}$ defined as the highest average $\dot{V}O_2$ obtained in any 30-s period. 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 respiratory exchange ratio (RER) >1.1 in all participants (Taylor et al. 1955). The data obtained during the $\dot{V}O_{2peak}$ test was subsequently used to prescribe the HA/normothermic training intervention.

Isothermic, also known as controlled hyperthermic, heat acclimation was implemented to optimise stress and adaptation throughout the regime (Taylor and Cotter 2006; Racinais et al. 2015). Each of the ten, 90-min HA sessions was performed in hot conditions (40.2 ± 0.4 °C, 41.0 ± 6.4 % RH), with participants initially exercising, at a workload corresponding to 65 % $\dot{V}O_{2peak}$ until the isothermic target T_{rec} of ≥38.5 °C has been achieved. Upon the attainment of a T_{rec} ≥ 38.5 °C, participants rested in a seated position on the cycle ergometer within the environmental chamber resuming exercise at a low intensity (<50 % $\dot{V}O_{2peak}$) when their T_{rec} fell below 38.5 °C, and continued cycling until the target T_{rec} was re-attained (~10 min). Normothermic exercise training involved ten, 90-min sessions performed in temperate conditions (19.8 ± 0.2 °C, 28.5 ± 2.7 % RH). The normothermic exercise training 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 isothermic HA group. Both groups exercised inside a purpose-built environmental chamber (WatFlow control system; TISS, Hampshire, UK) with temperature and humidity controlled using automated computer feedback (WatFlow control system; TISS, Hampshire, UK). Sessions were conducted at the same time of day (07:00–10:00 h) to mitigate effects of daily variation in heat shock protein expression

Table 1 Mean ± SD participant descriptive characteristics and correlations with Hsp72 mRNA (ALL) and Hsp90α mRNA (ALL) datasets

Variable	Mean ± SD	Hsp72 mRNA	Hsp90α mRNA
Age (years)	24.6 ± 4.4	$r = -0.066$	$r = -0.222$
Height (cm)	181 ± 6	$r = 0.084$	$r = 0.419$*
Mass (kg)	75.4 ± 5.5	$r = -0.112$	$r = 0.356$
Body surface area (m^2)	1.95 ± 0.10	$r = -0.066$	$r = 0.412$*
BMI (kg m^2)	23.1 ± 1.2	$r = -0.149$	$r = -0.077$
Body fat (%)	14.3 ± 2.7	$r = -0.102$	$r = -0.085$
$\dot{V}O_{2peak}$ (L min^{-1})	4.20 ± 0.56	$r = 0.079$	$r = 0.316$
$\dot{V}O_{2peak}$ (mL kg min^{-1})	55.6 ± 6.0	$r = 0.134$	$r = 0.133$

* denotes $p < 0.05$

(Taylor et al. 2010b). During each session, sweat rate (SR; L h^{-1}) was estimated using the change in nude body mass (NBM) from the pre- to post-exercise periods (Detecto Physicians Scales; Cranlea & Co., Birmingham, UK), T_{rec} was recorded using a thermistor (Henleys Medical Supplies Ltd., Welwyn Garden City, UK, Meter logger Model 401, Yellow Springs Instruments, Yellow Springs, MO, USA) inserted 10 cm past the anal sphincter and HR was recorded by telemetry (Polar Electro Oyo, Kempele, Finland). During each session, HR, T_{rec} and power output (W) were recorded after 10 min of seated rest in temperate laboratory conditions, and thereafter every 5 min upon commencing exercise.

Quantification of physiological and training variables

Exercise duration (min) was the total time spent exercising during the intervention. Mean session intensity (%$\dot{V}O_{2peak}$ and W kg^{-1}) was calculated from the relative exercise intensity during each 5-min period throughout the 90-min intervention sessions. This contrasted the mean exercise intensity (%$\dot{V}O_{2peak}$ and W kg^{-1}), which reflected the mean relative exercise intensity only (power output >1 W), thus excluding periods of rest. Total work done (kJ) reflected the cumulative volume of worked performed on the cycle ergometer over the 90-min period.

Absolute sweat loss (L h^{-1}) was calculated from the change in towel-dried NBM pre- to post-sessions. Mean T_{rec} and mean HR reflected the average T_{rec} or HR recorded throughout each intervention. Peak T_{rec} and peak HR reflect the maximum T_{rec} or HR recorded throughout each intervention. Change (Δ) in T_{rec} (°C) and ΔHR (b min^{-1}) reflects the difference between resting, and peak T_{rec} and HR, respectively. Mean $T_{recfinal45}$ (°C) quantifies the mean T_{rec} between the 45th and 90th minutes of the intervention session to reflect the average temperature following the initial rate of increase. $T_{rec} \geq 38.5$ °C (min) represented the total number of minutes where T_{rec} exceeded 38.5 °C during the session. The T_{rec} area under the curve (AUC) at >38.5 °C was calculated when as the duration and magnitude where measured T_{rec} exceeded 38.5 °C (Périard et al. 2012; Gibson et al. 2014; Périard et al. 2015), using a modification to the trapezium rule (Hubbard et al. 1977). A T_{rec} of 38.5 °C was selected as an approximate minimum for intolerance during compensable heat stress (Sawka et al. 2001) and possible threshold for Hsp72 translation/transcription (Amorim et al. 2008). AUC for $T_{rec} > 38.5$ °C was calculated as follows:

$$\text{AUC } T_{rec} \geq 38.5^{\circ}\text{C}\left(^{\circ}\text{C.min}^{-1}\right) = \sum \text{time interval(min)} \times 0.5 \left[^{\circ}\text{C} > 38.5^{\circ}\text{C at the start of the intervention}\right]\text{day} + ^{\circ}\text{C} > 38.5^{\circ}\text{C at the end of the intervention day.}$$

The rate of ΔT_{rec} (°C) rate of core temperature change, typically an increase, was calculated as follows:

$$\text{Rate } \Delta\text{Trec}\left(^{\circ}\text{C.h}^{-1}\right) = \left(T_{rec}2 - T_{rec}1 \Big/ \text{time2} - \text{time1}\right) * 60$$

Note: *$T_{rec}2$ and time2 are simultaneous measurements taken at any time during the intervention*; *and $T_{rec}1$ and time1 are resting value.*

Blood sampling and RNA extraction

Venous blood samples were drawn from the antecubital vein into 6 mL EDTA tubes immediately pre- and post-first (day1) and tenth (day10) session. Using a previously validated method (Taylor et al. 2010b), venous blood (1 mL) was pipetted into 10 mL of one in ten red blood cell lysis solution (10× 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 400 G and then washed twice in 2 mL PBS, prior to further centrifugation at 400 G for 5 min. The acid guanidinium thiocyanate-phenol-chloroform extraction (TRIzol) method (Chomczynski and Sacchi 1987) was then used to extract RNA from the leukocytes in accordance with the manufacturer instructions (Sigma-Aldrich, UK; Invitrogen, Life Technologies, Carlsbad, USA). Quantity was determined at an optical density of 260 nm, whilst 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

Hsp72 and Hsp90α relative mRNA expression was quantified using reverse transcription quantitative polymerase

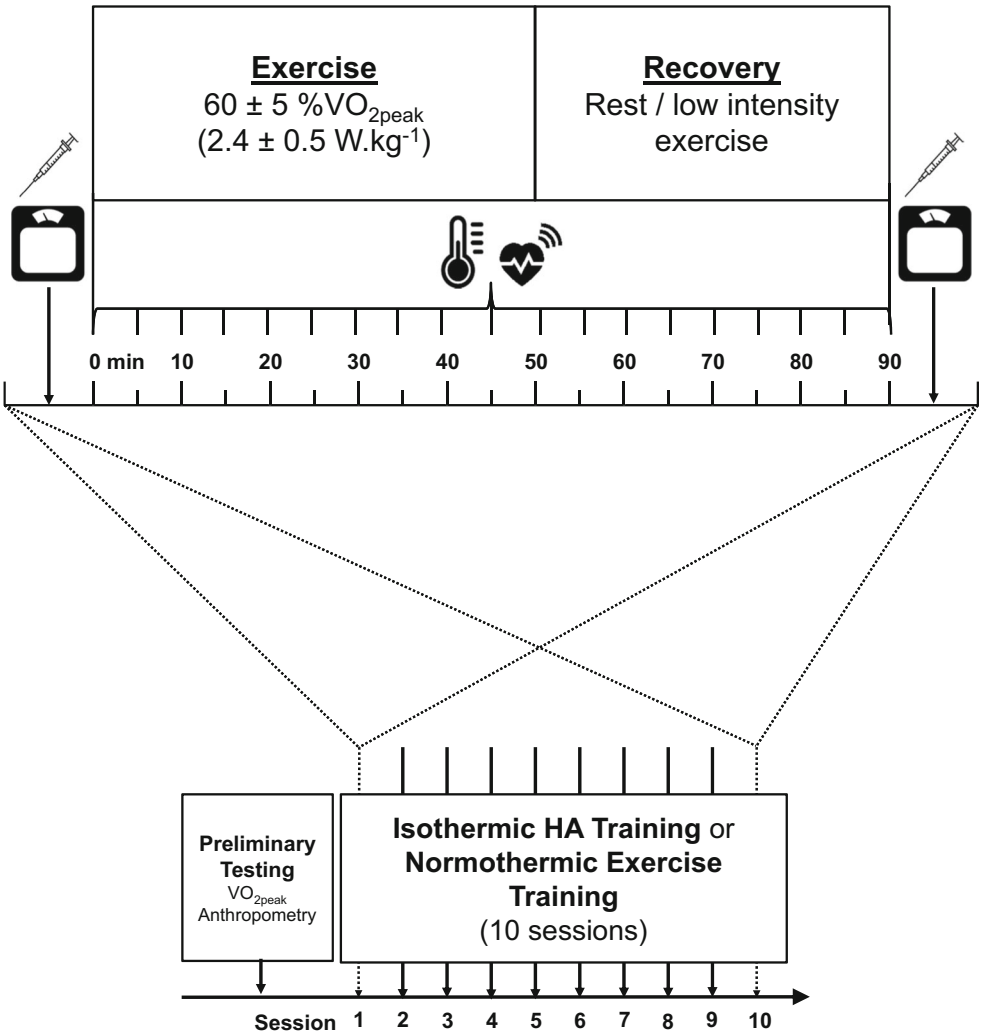

Fig. 1 Schematic overview of the study

chain reaction (RT-QPCR). Primers β2-microglobulin, Hsp72 and Hsp90α (presented in Table 2) were designed using primer design software (Primer Quest and Oligoanalyzer - Integrated DNA Technologies, Coralville, IA, USA) (Tuttle et al. 2015). During primer design, sequence homology searches were performed against the GenBank database to ensure the primers matched the gene of interest. Primers were designed to span exon-intron boundaries and avoided three or more guanine-cytosine bases within the last five bases at the 3′ end of primer to avoid non-specific binding. Further searches were performed to ensure primers did not contain secondary structures and intermolecular or intramolecular interactions (hairpins, self-dimer and cross dimers), which can inhibit product amplification. Relative Hsp mRNA expression was then quantified using RT-QPCR with reagent concentrations implemented in accordance with the manufacturer recommendations. Reactions (20.0 μL) containing 10.0 μL of SYBR Green RT-PCR Mastermix (Quantifast SYBR Green kit; Qiagen, Manchester, UK), 0.15 μL of forward primer, 0.15 μL of reverse primer, 0.20 μL of reverse transcription mix (Quantifast RT Mix, Qiagen) and 9.50 μL sample (70.0 ng RNA μL^{-1}) were prepared using the Qiagility automated pipetting system (Qiagen). Each reaction was amplified in a thermal cycler (Rotorgene Q, Qiagen) and involved reverse transcription lasting 10 min at 50 °C and a transcriptase inactivation and initial denaturation phase lasting 5 min at 95 °C. The PCR reaction then followed with a denaturation step lasting 10 s at 95 °C and a primer annealing and extension stage lasting 30 s at 60 °C repeated for 40 cycles. Fluorescence was measured following each cycle as a result of the incorporation of SYBR Green dye into the amplified PCR product. Melt curves (50 to 95 °C; Ramp protocol, 5-s stages) were analysed for each reaction to ensure only the single gene of interest was amplified. 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 $2^{-\Delta\Delta}$CT method.

Statistical analysis

Analysis was performed on data obtained on the first (day1; $n = 15$) and last (day10; $n = 15$), and the combined dataset (ALL). All data are reported as mean ± SD with two-tailed significance accepted at $p < 0.05$. 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, and each was deemed plausible unless otherwise stated. Paired sample *t* tests (SPSS, Chicago IL, USA) were implemented to determine differences between day1 and day10 for training data and the physiological responses to training. Paired sample *t* tests were also used to determine differences between the magnitude of change (i.e. pre–post) in Hsp72 mRNA and the magnitude of change in Hsp90α mRNA on day1, day10 and ALL. Effect sizes [Cohen's *d* (*d*; small = 0.20, medium = 0.50, large = 0.80)] were calculated to analyse the magnitude and trends with data. Pearson's product correlation was performed between the physiological responses and training prescription markers (see "Quantification of physiological and training variables"), and the magnitude of change (pre–post) in Hsp72 mRNA and Hsp90α mRNA, on day1, day10 and ALL, respectively. Stepwise multiple regression was performed to determine whether a combination of markers strengthened the prediction equation for Hsp72 mRNA and Hsp90α mRNA.

Results

Physiological and Hsp72 mRNA, and Hsp90α mRNA responses

No differences in the pre–post change in Hsp72 mRNA and pre–post change in Hsp90α mRNA were observed between day1 and day10 (Hsp72 mRNA: $t = 0.067$; $p = 0.948$; $d = 0.02$; Hsp90α mRNA: $t = 0.748$; $p = 0.467$; $d = 0.19$) (Fig. 2). On day10 vs day1, participants were observed as performing exercise for longer duration ($t = 5.206$; $p < 0.001$; $d < 0.01$), at a greater mean intensity ($t = 7.724$; $p < 0.001$; $d = 2.03$) and mean power ($t = 5.855$; $p < 0.001$; $d = 1.63$) and producing a greater volume of work done ($t = 6.424$; $p < 0.001$; $d = 1.66$). Reductions in the rate of T_{rec} increase were observed ($t = 2.188$; $p = 0.046$; $d = 0.58$) between day10 and day1, alongside increased SR ($t = 4.123$; $p = 0.001$; $d = 0.89$) (Table 3).

Correlates of Hsp72 mRNA, and Hsp90α mRNA increases

The strongest correlates of the pre–post session change in Hsp72 mRNA were the ΔT_{rec} (ALL $r = 0.714$; day1 $r = 0.721$; day10 $r = 0.709$), SR (ALL $r = 0.709$; day1 $r = 0.738$; day10 $r = 0.762$), the mean $T_{recfinal45}$ (ALL $r = 0.682$; day1 $r = 0.730$; day10 $r = 0.632$), AUC at 38.5 °C (ALL $r = 0.678$; day1 $r = 0.761$; day10 $r = 0.687$), the peak T_{rec} (ALL $r = 0.661$; day1 $r = 0.688$; day10 $r = 0.650$) and the duration $T_{rec} \geq 38.5$ °C (ALL $r = 0.650$; day1 $r = 0.650$; day10 $r = 0.659$) (Table 4, Fig. 3). Multiple regressions observed no improvements to the Hsp72 mRNA model with the addition of further variables for day1, day10 or ALL analyses. No participant characteristics demonstrated a significant ($p > 0.05$) relationship with change in Hsp72 mRNA (Table 1).

The strongest correlates of Hsp90α mRNA increase were the AUC at 38.5 °C (ALL $r = 0.729$; day1 $r = 0.729$; day10 $r = 0.813$), ΔT_{rec} (ALL $r = 0.691$; day1 $r = 0.715$; day10 $r = 0.690$), peak T_{rec} (ALL $r = 0.680$; day1 $r = 0.698$; day10 $r = 0.645$), mean $T_{recfinal45}$ (ALL $r = 0.678$; day1 $r = 0.714$; day10 $r = 0.617$), SR (ALL $r = 0.660$; day1 $r = 0.760$; day10 $r = 0.733$), duration $T_{rec} \geq 38.5$ °C (ALL $r = 0.629$; day1 $r = 0.670$; day10 $r = 0.563$) and rate of change in T_{rec} (ALL $r = 0.600$; day1 $r = 0.567$; day10 $r = 0.674$) relating to the largest gene transcription (Table 5, Fig. 4). Multiple regression observed that the Hsp90α mRNA model for day1 and day10 was not improved by adding further variables. The entire Hsp90α mRNA dataset (ALL) was improved ($r = 0.792$) when AUC at 38.5 °C and SR were combined. Height (ALL $r = 0.419$) and BSA (ALL $r = 0.412$), but no other participant characteristics, demonstrated a significant ($p > 0.05$) relationship with change in Hsp90α mRNA (Table 1).

Relationship between Hsp72 and Hsp90α mRNA

A significant relationship was observed between Hsp72 mRNA and Hsp90α mRNA for ALL ($r = 0.879$; $p < 0.001$) (Fig. 5) with a significant relationship also observed on day1 ($r = 0.924$; $p < 0.001$) and day10 ($r = 0.838$; $p < 0.001$) (Fig. 5). Accordingly, no significant difference was observed between the pre- to post-session change in Hsp72 mRNA and Hsp90α mRNA on day1 ($t = 1.200$; $p = 0.250$; $d = 0.32$; Hsp72 mRNA = 1.2 ± 1.2-fold change; Hsp90α mRNA = 1.4 ± 1.8-fold change), day10 ($t = -0.032$; $p = 0.975$; $d = 0.01$; Hsp72 mRNA = 1.2 ± 1.1-fold change; Hsp90α mRNA = 1.2 ± 1.5-fold change) or ALL ($t = -0.914$, $p = 0.368$; $d = 0.28$; Hsp72 mRNA = 1.2 ± 1.1-fold change, Hsp90α mRNA = 1.3 ± 1.6-fold change).

Discussion

In our experiment, ΔT_{rec}, SR, the mean $T_{recfinal45}$, the T_{rec} AUC at 38.5 °C, peak T_{rec} and the duration $T_{rec} \geq 38.5$ °C were identified as significant predictors ($R^2 \approx 0.36$–0.51; $p < 0.05$) of the change in Hsp72 mRNA (Table 4, Fig. 3). This is in agreement with previous observations regarding eHsp72

Table 2 Primer sequences

Target gene	Primer sequence (5′-3′-)	Reference sequence number	Amplicon length
B_2 microglobulin	Forward: CCGTGTGAACCATGTGACT Reverse: TGCGGCATCTTCAAACCT	NM_004048	91
Hsp72	Forward: CGCAACGTGCTCATCTTTGA Reverse: TCGCTTGTTCTGGCTGATGT	NM_005345	198
Hsp90α (variant 1 and variant 2)	Forward: AAACTGCGCTCCTGTCTTCT Reverse: TGCGTGATGTGTCGTCATCT	NM_001017963 and NM_005348	180

(Gibson et al. 2014) and is in agreement with the experimental hypothesis that endogenous markers of thermal strain and heat storage, i.e. core temperature, would most closely predict, if not directly cause, the change in Hsp72 mRNA. The experimental hypothesis also extended this observation to Hsp90α mRNA which similarly demonstrated that the AUC at 38.5 °C, ΔT_{rec}, peak T_{rec}, Mean $T_{recfinal45}$, SR, the duration $T_{rec} \geq 38.5$ °C and rate of change in T_{rec} were identified as the strongest predictors ($R^2 \approx 0.42$–0.53) of the increased gene expression (Table 5, Fig. 4). Similar characteristics (both physiological predictors and observed magnitude of prediction) were observed on day1 and day10 for both Hsp72 and Hsp90α mRNA; thus, the signal for transcription demonstrates equality at the onset and culmination of a 10-day HA or normothermic training intervention (Tables 4 and 5). The similarity of variables characterising increases in Hsp72 and Hsp90α mRNA was supported by the observation that a strong relationship ($R^2 = 0.77$) existed between the increase in Hsp72 mRNA and the increase in Hsp90α mRNA. The absolute mRNA increase was also of a comparable magnitude for both Hsp72 mRNA (~1.2 ± 1.1-fold change; +103 %) and Hsp90α mRNA (~1.3 ± 1.6-fold change; +60 %) with the lower relative increase in Hsp90α mRNA reflecting a higher basal expression (Subbarao Sreedhar et al. 2004). It is yet to be elucidated whether these variables would demonstrate a greater/lesser contribution to the magnitude of gene transcription in other tissues, i.e. skeletal muscle appears more oxidative stress dependent rather than temperature dependent compared to leukocytes, as discussed previously (Tuttle et al. 2015).

The failure for training prescription markers (exercise duration, exercise intensity, exercise power and work done) to predict the change of leukocyte Hsp72 and Hsp90α mRNA highlights the importance of designing interventions necessitating increased HSP around endogenous responses (e.g. increased core temperature), rather than exogenous variables (e.g. training prescription). SR as a predictor of increased Hsp90α mRNA is a novel observation; it is however likely that this is a secondary response, thus not causal, stemming from the required increased heat dissipation via evaporation in an attempt to acquiesce increasing heat storage (core temperature) rather than being a primary response. Manipulation of heat storage to further enhance Hsp90α mRNA transcription may be of experimental benefit within some designs and could, at least theoretically, be enhanced by facilitating greater sweating with higher humidity (e.g. >60 % RH) than those utilised within the present design (~40 % RH), thus further inhibiting evaporative heat loss (Maughan et al. 2012). Though some differences in HA adaptation have been observed by imposing dehydration (an analogue for increased sweat losses and the ensuing increases in thermal strain) (Garrett et al. 2014), the impact of this manipulation on Hsp transcription during HA remains unknown. When dehydration is imposed vs euhydration, no differences in the increases in monocyte or skeletal HSP72 have been observed during ~90 min of acute exercise-heat stress (Hillman et al. 2011) and normothermic exercise (Logan-Sprenger et al. 2015), respectively. These data reduce the appeal of increasing sweat losses as a direct method for improving the magnitude of adaptation.

A novel finding in the present study was the observation that the increases in Hsp72 and Hsp90α mRNA demonstrate a significant relationship. This provides further evidence for a common pathway towards equality of increases in HSP72 and HSP90α protein concentrations in response to ex vivo cellular stress (McClung et al. 2008). Hsp72 mRNA (Marshall et al. 2007) and Hsp90α mRNA (Tuttle et al. 2015) are responsive to thermal and physiological stimuli as supported by our data. Whilst data reporting changes in human Hsp72 mRNA is

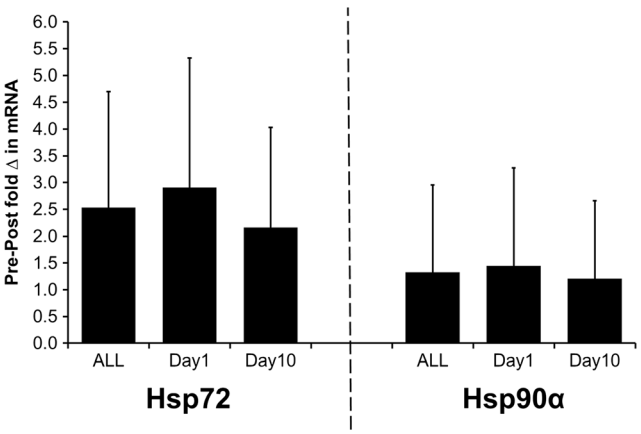

Fig. 2 Mean ± SD pre- to post-session fold change in Hsp72 mRNA (*left*) and Hsp90α mRNA (*right*) for the entire dataset (ALL), and on day1 and day10 of the intervention

Table 3 Mean ± SD training data and physiological responses for Hsp72 mRNA and Hsp90α mRNA datasets displayed for the entire data set (ALL), and day1 and day10, respectively

Measure	ALL	Day1	Day10
Exercise duration (min)	50 ± 9	43 ± 7	57 ± 5[a]
Mean intensity (%$\dot{V}O_{2peak}$)	33 ± 7	28 ± 5	38 ± 3[a]
Mean power (W kg^{-1})	1.3 ± 0.3	1.1 ± 0.3	1.5 ± 0.3[a]
Mean exercise intensity (%$\dot{V}O_{2peak}$)	60 ± 5	60 ± 6	61 ± 3
Mean exercise power (W kg^{-1})	2.4 ± 0.5	2.4 ± 0.5	2.4 ± 0.5
Total work done (kJ)	526 ± 149	447 ± 111	606 ± 142[a]
SR (L h^{-1})	0.9 ± 0.5	0.8 ± 0.4	1.0 ± 0.6[a]
Mean T_{rec} (°C)	37.92 ± 0.46	38.02 ± 0.54	37.83 ± 0.34
Mean $T_{recfinal45}$ (°C)	38.14 ± 0.73	38.19 ± 0.82	38.08 ± 0.65
ΔT_{rec} (°C)	1.68 ± 0.70	1.64 ± 0.71	1.72 ± 0.71
Rate of ΔT_{rec} (°C h^{-1})	2.06 ± 0.89	2.24 ± 1.06	1.87 ± 0.66[a]
Peak T_{rec} (°C)	38.52 ± 0.58	38.59 ± 0.65	38.44 ± 0.50
$T_{rec} \geq 38.5$ °C (min)	27 ± 28	29 ± 31	26 ± 26
AUC 38.5 °C (°C min^{-1})	8.0 ± 11.2	10.7 ± 14.0	5.3 ± 6.9
ΔHR (b min^{-1})	96 ± 24	93 ± 26	98 ± 22
Mean HR (b min^{-1})	129 ± 21	131 ± 23	127 ± 20
Peak HR (b min^{-1})	162 ± 25	164 ± 29	160 ± 21

[a] Denotes difference from day1 within gene transcript

available (Febbraio and Koukoulas 2000; Marshall et al. 2007; Stary et al. 2008; Atamaniuk et al. 2008; Tuttle et al. 2015; Gibson et al. 2015a; Gibson et al. 2015c), equivalent data characterising increases in Hsp90α mRNA is limited. Our data demonstrates a novel finding and is the first in vivo experiment demonstrating that Hsp90α mRNA responds similarly to Hsp72 mRNA when core temperature is increased during exercise in the heat. It is known that exercise (Connolly et al. 2004), exercise-heat stress (Moran et al. 2006) and exercise-induced muscle damage and heat stress (Tuttle et al. 2015) provide the stimuli for Hsp72 and Hsp90α gene induction with the stimuli to increase transcription described as proportional to the change in core temperature (38.5 ± 0.2 to 39.0 ± 0.4 °C) (Tuttle et al. 2015), a finding our data supports. Our data is congruous with findings that consistent pre–post increases in Hsp72 mRNA occur at the beginning and end of heat acclimation (Gibson et al. 2015a), should the physiological signal (T_{rec} > 38.5 °C) demonstrate equality between measurement points (Gibson et al. 2015a). In addition, we have now demonstrated that this dose response is true of Hsp90α mRNA. This observation further reinforces isothermic HA methods as optimal for ensuring signalling for HSP via the heat shock response (a key regulator of thermotolerance) and the magnitude of phenotypic heat adaptation (Kuennen et al. 2011). During the initial adaptation to heat acclimation (~3 days), Hsp72 mRNA can diminish in conjunction with reductions in mean core temperature (−0.2 to 38.7 ± 0.2 °C) and increased basal HSP72 (Marshall et al. 2007) should be the end of activity core temperature decrease. Our data now extends this signal-dependent response to

Table 4 Ranked correlations of the pre- to post-session fold change in Hsp72 mRNA for ALL, day1 and day10

ALL		Day1		Day10	
Variable	*R* =	Variable	*R* =	Variable	*R* =
ΔT_{rec} (°C)	0.714**	AUC 38.5 °C (°C min^{-1})	0.761**	SR (L h^{-1})	0.762**
SR (L h^{-1})	0.709**	SR (L h^{-1})	0.738**	ΔT_{rec} (°C)	0.709**
Mean $T_{recfinal45}$ (°C)	0.682**	Mean $T_{recfinal45}$ (°C)	0.730**	AUC 38.5 °C (°C min^{-1})	0.687**
AUC 38.5 °C (°C min^{-1})	0.678**	ΔT_{rec} (°C)	0.721**	ΔHR (b min^{-1})	0.665**
Peak T_{rec} (°C)	0.661**	Peak T_{rec} (°C)	0.688**	Rate of ΔT_{rec} (°C h^{-1})	0.661**
$T_{rec} \geq 38.5$ °C (min)	0.650**	Mean T_{rec} (°C)	0.675**	$T_{rec} \geq 38.5$ °C (min)	0.659**
Mean T_{rec} (°C)	0.565**	$T_{rec} \geq 38.5$ °C (min)	0.650*	Peak T_{rec} (°C)	0.650**
Rate of ΔT_{rec} (°C h^{-1})	0.560**	Rate of ΔT_{rec} (°C h^{-1})	0.540	Mean $T_{recfinal45}$ (°C)	0.632*
ΔHR (b min^{-1})	0.511**	ΔHR (b min^{-1})	0.391	Mean HR (b min^{-1})	0.546*
Mean HR (b min^{-1})	0.431*	Mean HR (b min^{-1})	0.341	Mean T_{rec} (°C)	0.454
Peak HR (b min^{-1})	0.374*	Peak HR (b min^{-1})	0.333	Peak HR (b min^{-1})	0.448
Mean exercise power (W kg^{-1})	0.257	Mean exercise power (W kg^{-1})	0.231	Mean power (W kg^{-1})	0.388
Total work done (kJ)	0.209	Exercise duration (min)	0.223	Total work done (kJ)	0.371
Mean power (W kg^{-1})	0.173	Mean exercise intensity (%$\dot{V}O_{2peak}$)	0.210	Mean intensity (%$\dot{V}O_{2peak}$)	0.301
Exercise duration (min)	0.165	Total work done (kJ)	0.091	Mean exercise power (W kg^{-1})	0.286
Mean exercise intensity (%$\dot{V}O_{2peak}$)	0.153	Mean intensity (%$\dot{V}O_{2peak}$)	−0.072	Exercise duration (min)	0.262
Mean intensity (%$\dot{V}O_{2peak}$)	0.053	Mean power (W kg^{-1})	−0.013	Mean exercise intensity (%$\dot{V}O_{2peak}$)	0.056

* denotes $p < 0.05$; ** denotes $p < 0.01$

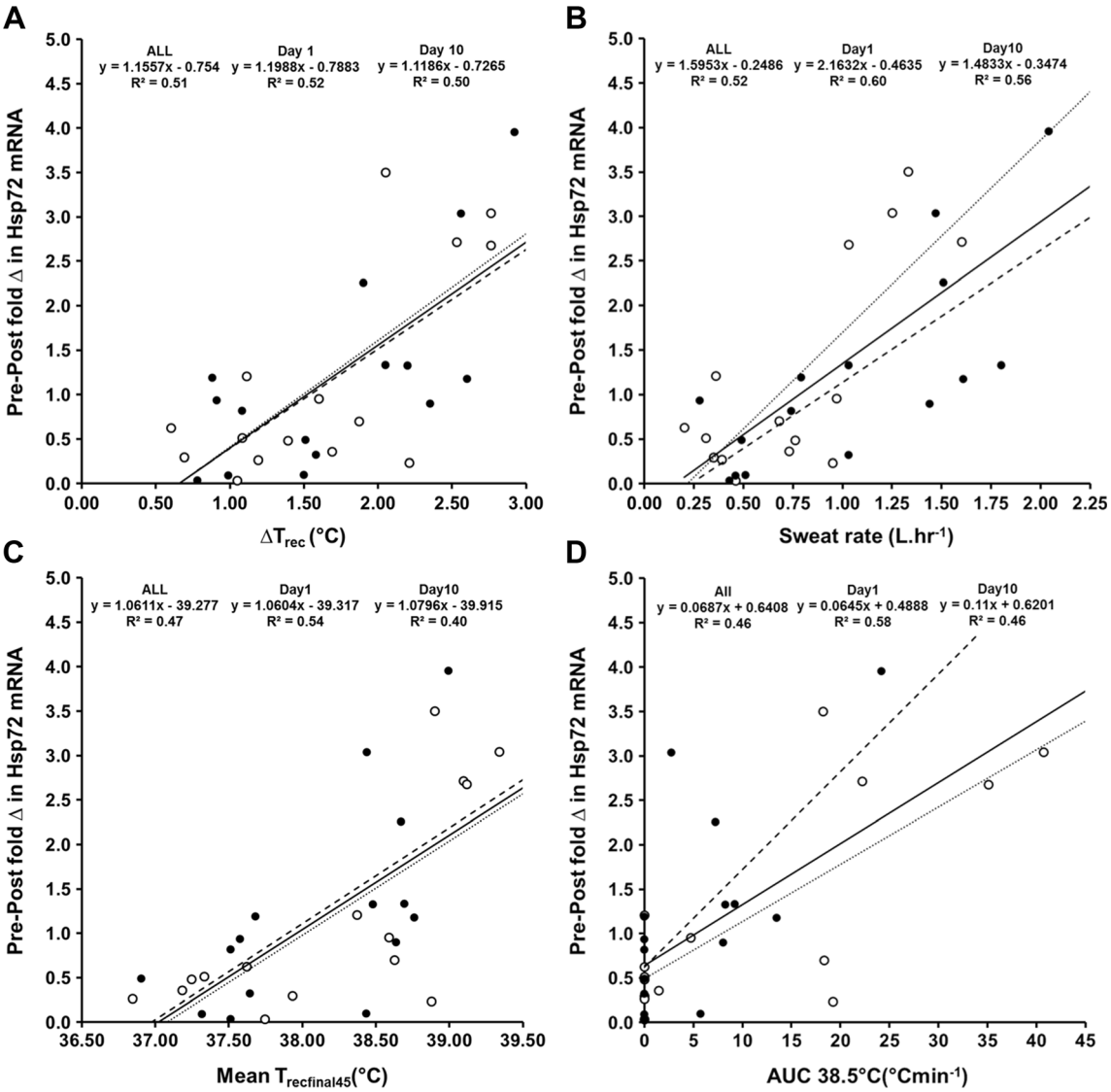

Fig. 3 Relationship between the fold change in Hsp72 mRNA and the ΔT_{rec} (**a**), SR (**b**), mean $T_{recfinal45}$ (**c**) and the AUC38.5 °C (**d**). Figures describe data for the entire dataset (ALL, *solid line*), and on day1 (*open circles*, *dotted line*) and day10 (*closed circles*, *dashed line*) of the intervention

Hsp90α mRNA. Our non-damaging exercise protocol (cycling) also supports the previously proposed observation that the leukocyte stress response is core temperature rather than exercise dependent (Tuttle et al. 2015).

Maintenance of increases in both Hsp72 and Hsp90α mRNA between day1 and day10 is unsurprising as no difference existed between the strongest correlates of the pre–post session change in gene expression (core temperature). This finding is concurrent with proposals that elevated core temperature as being an important component of HSF1 regulation (Gibson et al. 2015a; Gibson et al. 2015c), particularly in leukocytes. To ensure HSF1 activation in experiments such as those designed to achieve the heat-acclimated phenotype (Kuennen et al. 2011), mitigate heat stroke (Moran et al. 2006), facilitate positive adaptive responses to stress [i.e. promotion of mitochondrial biogenesis (Henstridge et al. 2014a)] or act as therapy for disease states [e.g. type 2 diabetes mellitus (Hooper et al. 2014), cardiovascular disease (Noble and Shen 2012) and Parkinson's disease (Erekat et al. 2014)] then eliciting an increased core temperature of +1.7 °C and ensuring a minimum core temperature of 38.5 °C is maintained for ≥27 min is necessary (Table 3). However, this prescription is from a small homogenous apparently healthy population. Some of the previously detailed disease states have marked impairments regarding their ability to thermoregulate [e.g. diabetes (Kenny et al. 2016) and multiple sclerosis (Romberg et al. 2012)]. Therefore, characterisation of the Hsp mRNA response is required within such populations—particularly—whether they have a similar core temperature-mediated dose-response relationship with Hsp mRNA increases, in light of reduced basal HSP (Bruce et al. 2003). Should the disease state not directly inhibit HSP translation, it may be that HSP accumulation is more efficient in these populations due to greater increases in core temperature

Table 5 Ranked correlations of the pre- to post-session fold change in Hsp90α mRNA for ALL, day1 and day10

ALL		Day1		Day10	
Variable	*R* =	Variable	*R* =	Variable	*R* =
AUC 38.5 °C (°C min^{-1})	0.729**	SR (L h^{-1})	0.760**	AUC 38.5 °C (°C min^{-1})	0.813**
ΔT_{rec} (°C)	0.691**	AUC 38.5 °C (°C min^{-1})	0.729**	SR (L h^{-1})	0.733**
Peak T_{rec} (°C)	0.680**	ΔT_{rec} (°C)	0.715**	ΔT_{rec} (°C)	0.690**
Mean $T_{recfinal45}$ (°C)	0.678**	Mean $T_{recfinal45}$ (°C)	0.714**	Rate of ΔT_{rec} (°C h^{-1})	0.674**
SR (L h^{-1})	0.660**	Peak T_{rec} (°C)	0.698**	ΔHR (b min^{-1})	0.655**
$T_{rec} \geq 38.5$ °C (min)	0.629**	Mean T_{rec} (°C)	0.695**	Peak T_{rec} (°C)	0.645**
Mean T_{rec} (°C)	0.601**	$T_{rec} \geq 38.5$ °C (min)	0.670**	Mean HR (b min^{-1})	0.629*
Rate of ΔT_{rec} (°C h^{-1})	0.600**	Rate of ΔT_{rec} (°C h^{-1})	0.567*	Mean $T_{recfinal45}$ (°C)	0.617*
ΔHR (b min^{-1})	0.531**	ΔHR (b min^{-1})	0.469	$T_{rec} \geq 38.5$ °C (min)	0.563*
Mean HR (b min^{-1})	0.521**	Peak HR (b min^{-1})	0.458	Peak HR (b min^{-1})	0.540*
Peak HR (b min^{-1})	0.491**	Mean HR (b min^{-1})	0.441	Total work done (kJ)	0.515*
Mean exercise power (W kg^{-1})	0.289	Mean exercise power (W kg^{-1})	0.318	Mean T_{rec} (°C)	0.419
Total work done (kJ)	0.223	Mean exercise intensity (%$\dot{V}O_{2peak}$)	0.277	Mean power (W kg^{-1})	0.383
Mean exercise intensity (%$\dot{V}O_{2peak}$)	0.183	Total work done (kJ)	0.132	Exercise duration (min)	0.323
Mean power (W kg^{-1})	0.098	Mean intensity (%$\dot{V}O_{2peak}$)	−0.132	Mean intensity (%$\dot{V}O_{2peak}$)	0.316
Exercise duration (min)	0.075	Exercise duration (min)	0.127	Mean exercise power (W kg^{-1})	0.256
Mean intensity (%$\dot{V}O_{2peak}$)	−0.045	Mean power (W kg^{-1})	−0.022	Mean exercise intensity (%$\dot{V}O_{2peak}$)	−0.025

* denotes $p < 0.05$; ** denotes $p < 0.01$

resulting from inhibited heat dissipation mechanisms (Davis et al. 2010; Carter et al. 2014; Kenny et al. 2016), a notion also true of those with a spinal cord injury (Price 2006). Previous work has identified that HSF1 is temperature dependent supporting our downstream observation regarding increased gene expression (Sonna et al. 2010). Similar endogenous signals to increase Hsp72 mRNA have been shown to increase eHSP72 i.e. elevated temperature (Gibson et al. 2014). The rate of rise in core temperature is also important to increase plasma concentrations (Périard et al. 2012). More relevant to acquiring thermotolerance (Kregel 2002) is the change in iHSP72 and iHSP90α (Lee et al. 2015). The accumulation of greater iHSP72 is more closely linked to the absolute (final) and change (delta) in core temperature (Magalhães et al. 2010) during HA. Mechanistically, it has been observed that the magnitude of iHSP72 expression at exhaustion or 24 h post-exhaustion is a result of the absolute temperature attained (>39.0 °C), rather than the rate of heat storage (Périard et al. 2015). During acute exercise-heat stress, equality of post-exhaustion iHSP72 did not differ in response to the rate of core temperature increase corresponding to different exercise intensities, and presumably different metabolic stressors, eliciting similar end temperatures of ~39.5 °C [(60 % $\dot{V}O_{2peak}$ = 2.2 ± 1.4 °C h^{-1}; 75 % $\dot{V}O_{2peak}$ = 5.1 ± 1.7 °C h^{-1} (Périard et al. 2015)]. The present study highlights the transcription of Hsp72 mRNA and Hsp90α mRNA as having similar criteria (T_{rec} > 38.5 °C for >27 min, Table 3) to that which predicts elevated eHSP72 and iHSP72 (Périard et al. 2012; Gibson et al. 2014; Périard et al. 2015). Given that the predictive capacity following this analysis is incomplete (Hsp72 mRNA = 47–58 % between variables, and Hsp90α mRNA = 46–66 % between variables), the present experiment does suggest that a large proportion of the signalling for gene transcription is unaccounted for by the core temperature alone. Accordingly, other endogenous signals responsible for the initial HSF1 activation should be sought (Vihervaara and Sistonen 2014); as previously discussed, this may be more important in skeletal muscle vs leukocytes. These signals likely include markers of metabolic stress/physiological strain such as oxidative stress (Sureda et al. 2005; Mestre-Alfaro et al. 2012), changes in catecholamine concentration (Tintinger et al. 2001; Giraldo et al. 2010) and increased cortisol (Ortega 2003). These signals also provide a rationale for the difference between increased Hsp72 mRNA, and HSP72 protein translation observed in heat acclimation (Marshall et al. 2007), and notable increases in monocyte HSP72 in hypoxia (Taylor et al. 2011), and following matched passive vs active hyperthermia (Morton et al. 2007) which collectively highlight the multifactorial signal cascade. Irrespective of the precise, causal signal(s), these data evidence that large, prolonged changes in core temperature are a predictor of the greatest change in Hsp72 mRNA and Hsp90α mRNA (from those endogenous and exogenous variables monitored), with this an important characteristic potentiating increased basal protein to mitigate the potential threat for subsequent thermal-mediated protein denaturation. The

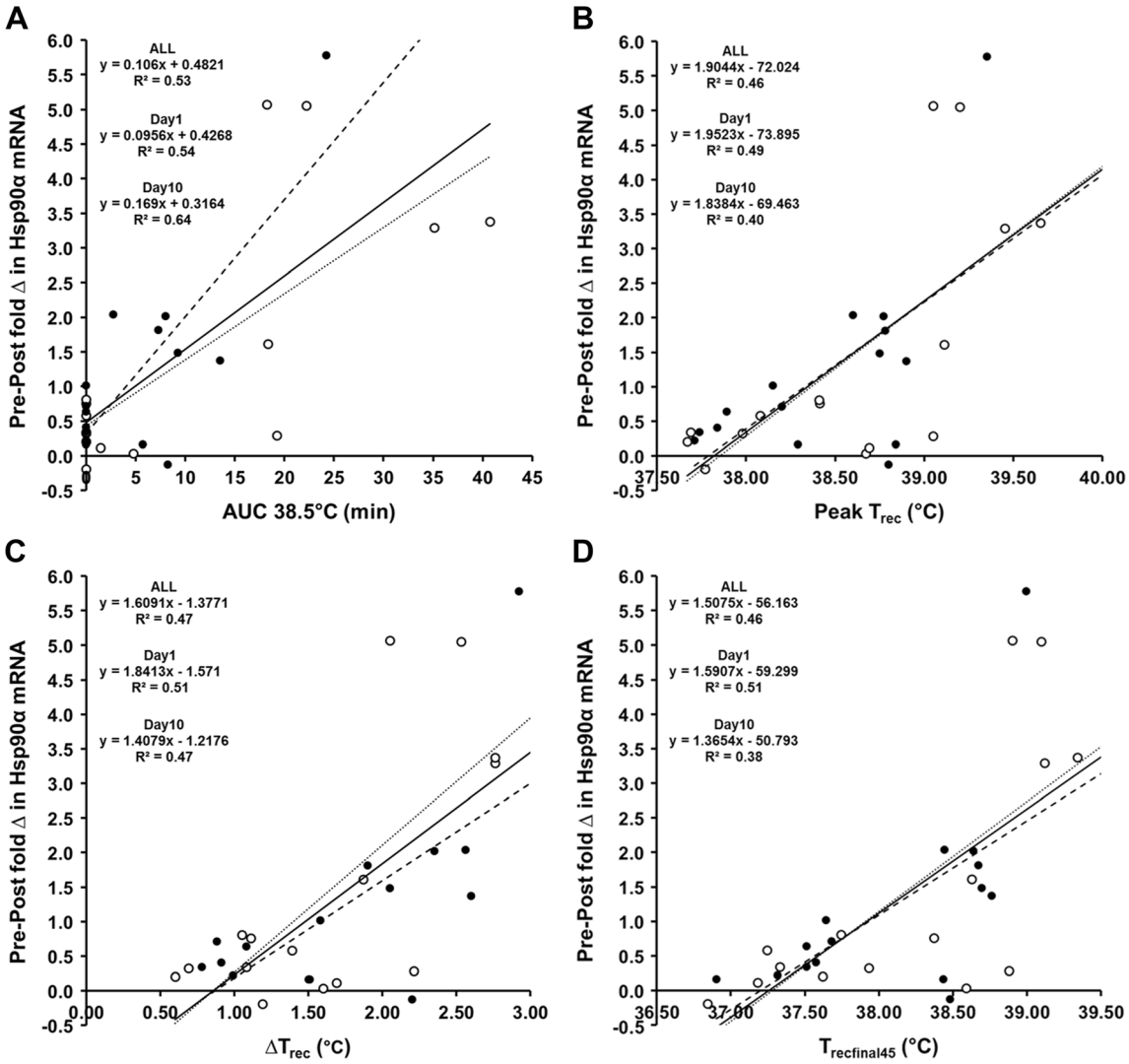

Fig. 4 Relationship between the fold change in Hsp90α mRNA and the AUC38.5 °C (**a**), peak T_{rec} (**b**), ΔT_{rec} (**c**) and the mean $T_{recfinal45}$ (**d**). Figures describe data for the entire dataset (ALL, *solid line*), and on day1 (*open circles*, *dotted line*) and day10 (*closed circles*, *dashed line*) of the intervention

"absent" predictive criteria might be also explained by other unmeasured variables, and these could include factors allied to individual differences in HSP transcription perhaps due to a responder–non responder paradigm due to innate (i.e. genetic) or acquired (previous heat exposure, HA, subsequent decay, etc) phenotypical inter- and intraindividual variation (Lyashko et al. 1994).

Despite the presence of a highly homogenous sample (Table 1) and advantageous experimental protocol whereby participants did not possess the HA phenotype at the onset of the experiment and were exposed to consistent endogenous signal being obtained throughout the experiment (Table 3), ~40 % of the variability in Hsp mRNA transcription is unaccounted for. It is difficult to determine individual responses within this experiment, largely due to the differing training parameters and exercise-heat stress variables (Table 3) observed between day1 and day10 as part of the initial experimental objectives (Gibson et al. 2015c). Accordingly, bespoke experiments utilising deductive mechanistic evidence obtained in vivo and in vitro should ensure further equality of all potentiating stimuli (thermal and cellular/molecular) to facilitate a more definitive analysis of the individual response and account for the remaining variability in transcription. In spite of some absent predictive criteria, the present experiment further evidences the observation that in experiments where iHSP72 has not increased (Watkins et al. 2007; Hom et al. 2012), the mechanism for the absent protein translation is likely due to only modest and brief increases in core temperature with lower mean core temperatures reported [<38.5 °C (Amorim et al. 2008)] than others demonstrating increased iHsp72 [>39.0 °C (Périard et al. 2015)]. Whilst exercise alone increases core temperature providing a sufficient pathway to elevate iHSP72 (Fehrenbach et al. 2000; Shin et al. 2004) and Hsp72 mRNA, and HSP90α mRNA in temperate conditions (Tuttle et al. 2015), the greater heat storage when performing equivalent exercise in hot environmental conditions provides a more

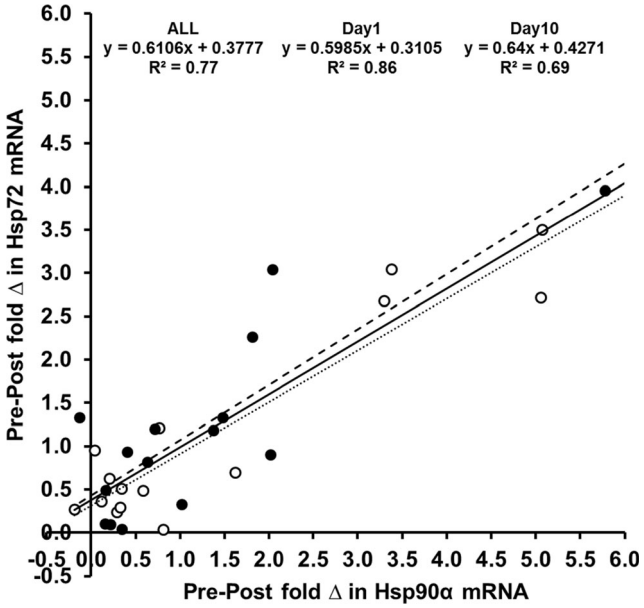

Fig. 5 Mean ± SD relationship between the fold change in Hsp72 mRNA and Hsp90α mRNA entire dataset (ALL, *solid line*), and on day1 (*open circles*, *dotted line*) and day10 (*closed circles*, *dashed line*) of the intervention

potent stimuli for increasing iHSP72 (Fehrenbach et al. 2001; Fehrenbach et al. 2003; Selkirk et al. 2009; Magalhães et al. 2010) via greater increases in Hsp72 mRNA. It has been observed that no difference in Hsp72 mRNA increases occurs within HA sessions performed in very hot environments when comparing isothermic methods (Gibson et al. 2015a) (whereby the magnitude of hyperthermia is controlled) with fixed intensity methods [where core temperature responds according to the individual capacity to dissipate heat (Gibson et al. 2015b)]. The present data demonstrates that in cooler conditions, it would be pertinent to implement isothermic methods with high initial workloads (and metabolic heat production) to provide the requisite stimuli (increased core temperature) for acquiring thermotolerance in addition to physiological heat adaptation (Racinais et al. 2015). The isothermic method of HA facilitates a targeted increase in core temperature (>38.5 °C) which can subsequently be prolonged (for ~30–60 min), to initiate Hsp72 mRNA and Hsp90α mRNA transcription (Gibson et al. 2015c). These guidelines are supported by data demonstrating that initial adaptations to HA (notably reduced core temperature and increased iHSP72) mitigate the increase in Hsp72 mRNA to subsequent fixed intensity work in equal environmental conditions (Marshall et al. 2007).

Future work in the field should address some of the limitations of this experiment, which are discussed within the present discussion section. To resolve the unknown threshold for Hsp72 and Hsp90α mRNA signalling, and dependence of temperature in isolation or coupled with exercise and concurrent responses, measurements of gene transcription should be made following stepwise increases in core temperature whilst using passive and exercise-heat stress models and multi-tissue analysis. This multi-tissue analysis should also be performed where experimental manipulations prolong the degree of hyperthermia for extended periods (beyond the 30–60 min of the present data) and be performed using passive heating models, e.g. hot water immersion or sauna (Krause et al. 2015b), in addition to the active exercise-induced heating implemented in the present experiment. A paucity of transcription/translation data exists for extreme core temperatures (in excess of 39.5 °C), despite attainment of these temperatures absent of pathophysiological complications within endurance runners (Byrne et al. 2006) and other competitive athletes, e.g. elite footballers (Mohr et al. 2012). Hsp72 and Hsp90α mRNA analysis should be paired with measurement of iHSP72 and iHSP90α protein concentrations across tissues to facilitate a more complete interpretation of the transcription and translational kinetics. This would subsequently facilitate more precise interventions allied to prescribing HA for increasing thermotolerance (Kuennen et al. 2011), reducing the severity of heat illness (Ruell et al. 2014) or facilitate a cellular test for heat stroke susceptibility (Amorim et al. 2008), and provide optimal prescriptions of therapeutic thermal interventions (Henstridge et al. 2014b; Henstridge et al. 2016). The time course of the mRNA transcription and subsequent HSP translation also both require further research to determine within group and interindividual responses of a heterogeneous cohort, including clinical populations. Finally, given the apparent translational differences between sexes in response to equivalent training (Morton et al. 2009), it remains to be determined whether this is a result of inhibited gene transcription or translational events attenuating changes in basal protein in females.

Conclusion

In experiments or interventions where the correlated transcription of Hsp72 and Hsp90α mRNA is important, such as those involving heat acclimation and heat illness, or therapeutic heat stress, the protocol should be designed to rapidly induce large changes (≥1.7 °C) in core temperature (>38.5 °C), which are maintained for prolonged periods (≥27 min). This may be achieved by initially implementing high intensity work, eliciting uncompensable heat stress and increased heat storage, followed by the maintenance of core temperature via lower intensity exercise or passive heat stress to elicit heat balance at the elevated temperature.

Compliance with ethical standards All protocols, procedures and methods were approved by the institutional ethics committee. Participants completed medical questionnaires and written informed consent following the principles outlined by the Declaration of Helsinki as revised in 2013 prior to commencing any preliminary or experimental sessions.

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