Title:

Isothermic and fixed intensity heat acclimation methods elicit equal increases in Hsp72 mRNA.

2. Submission Type:

Original Investigation

3. Names of Authors:
[1] Oliver R. Gibson, University of Brighton
[1] Jessica A. Mee, University of Brighton
[2] Lee Taylor, University of Bedfordshire
[2] James A. Tuttle, University of Bedfordshire
[1] Peter W. Watt, University of Brighton
[1] Neil S. Maxwell, University of Brighton

4. Contact Details:

[1] Oliver Gibson, o.r.gibson@brighton.ac.uk Centre for Sport and Exercise Science and Medicine (SESAME), University of Brighton, Welkin Human Performance Laboratories, Denton Road, Eastbourne, UK

[2] Muscle Cellular and Molecular Physiology (MCMP) and Applied Sport and Exercise Science (ASEP) Research Groups, Department of Sport Science and Physical Activity, Institute of Sport and Physical Activity Research (ISPAR), University of Bedfordshire, Bedford Campus, Polhill Avenue, Bedfordshire, UK

5. Preferred Running Head

Hsp72 responses to Heat Acclimation methods.

6. Abstract Word Count

214

7. Text Word Count

4,777

8. Number of Figures and Table

Three figures. Two tables.

9. Keywords:

Thermoregulation, Heat Stress, Cellular Stress Response, Hyperthermia, Thermotolerance, Heat Shock Protein 72, Heat Illness.

Abstract

Thermotolerance, to which Heat shock protein-72 (Hsp72) contributes, is an acquired state achieved following heat acclimation (HA), eliciting cellular adaption and protection against thermal stress. Optimal HA methods achieving the greatest heat shock response (HSR) are equivocal; therefore investigation of methods provoking the greatest sustained HSR is required to optimise cellular adaptation.

Twenty four males performed short term HA (STHA; five sessions) and long term HA (LTHA; STHA plus further five sessions) utilising fixed intensity (FIXED; workload = 50%$\dot{V}O_{2peak}$), continuous isothermic HA (ISO_{CONT}; target rectal temperature (T_{rec}) = 38.5°C) or progressive isothermic HA (ISO_{PROG}; target T_{rec} = 38.5°C for STHA then target T_{rec} = 39.0°C for LTHA). Leukocyte Hsp72 mRNA was measured pre and post day 1, day 5 and day 10 of HA via qRT-PCR to determine the HSR.

Hsp72 mRNA increased ($p < 0.05$) pre to post day 1, pre to post day 5, and pre to post day 10 in FIXED, ISO_{CONT} and $ISO_{PROG,}$ but no differences were observed between methods ($p > 0.05$). The equal Hsp72 mRNA increases occurring from consistent, reduced or increased endogenous strain following STHA and LTHA suggest that transcription occurs following attainment of sufficient endogenous criteria. These data give confidence that all reported HA methods increase Hsp72 mRNA and are capable of eliciting adaptations towards thermotolerance.

Introduction

Repeated exposure to stressful thermal environments initiates a phenotypic heat adaptation known as heat acclimation (HA) (Garrett et al. 2011), an element of which has been identified as thermotolerance (Moseley 1997). Thermotolerance (Moseley 1997), or acquired cellular thermotolerance (McClung et al. 2008), describes the cellular adaptation accompanying systemic changes (Magalhães et al. 2010b; Sawka et al. 2011; Hom et al. 2012) induced by successful HA. Acquired cellular thermotolerance confers cytoprotection against subsequent thermal exposure, translating to complimentary reductions in endogenous physiological and systemic strain (Yamada et al. 2007; McClung et al. 2008). An established element of acquired cellular thermotolerance involves changes in heat shock proteins (HSP) (Moseley 1997); in particular increases in the inducible, and thermosensitive protein heat shock protein HSPA1A (HSP72) (McClung et al. 2008; Beckham et al. 2008; Kampinga et al. 2009) following transcription of its gene (Hsp72 mRNA) as part of the heat shock response (HSR).

Increased basal HSP72 is commonly reported following repeated exercise-heat stress, as is the inducibility of the protein (Maloyan et al. 1999; McClung et al. 2008; Selkirk et al. 2009; Magalhães et al. 2010b; Amorim et al. 2011). Previously, extracellular HSP72 (eHSP72) has been used as a marker of the stress response. In spite of an established eHSP72 response to sufficient exercise-heat stress (Marshall et al. 2006; Yamada et al. 2007; Ogura et al. 2008; Magalhães et al. 2010b; Périard et al. 2012; Gibson et al. 2014), the mechanisms leading to an increase in circulating concentration are equivocal (Fleshner and Johnson 2005; Lancaster and Febbraio 2005b; Lancaster and Febbraio 2005a). Additionally, the biological role of eHSP72 appears more closely linked to an immunological response, rather than a process favourably augmenting thermotolerance, and the associated cytoprotective adaptations (Asea 2006). The measurement of intracellular HSP72 is optimal for determining cellular responses to HA (Magalhães et al. 2010b). HA increases basal HSP72, improving the cellular defence of heat stress, and also leading to augmented translation during heat stress (Maloyan et al. 1999). The measurement of HSP72 gene expression (Hsp72 mRNA) therefore offers an alternative marker of the magnitude of the cellular stress response, and subsequent initiation of protein transcription required for increased thermotolerance (Maloyan and Horowitz 2002). Based upon previous data (Maloyan et al. 1999) HA should increase the measured Hsp72 mRNA transcription, a process primarily regulated by Heat shock factor protein 1 (HSF-1) as part of the HSR (Kregel 2002).

HSF1 activation involves a complex series of regulatory events, including nuclear localization, oligomerisation and acquisition of HSE–DNA binding, ultimately resulting in the transcription of Hsp72 mRNA (Sarge et al. 1993), this in response to the magnitude of thermal and physiological challenge (Maloyan et al. 1999; McClung et al. 2008).

Fixed intensity HA methods (Houmard et al. 1990; Nielsen et al. 1993; Nielsen et al. 1997; Cheung and McLellan 1998; Kresfelder et al. 2006; Marshall et al. 2007; Yamada et al. 2007; Watkins et al. 2008; Sandström et al. 2008; Lorenzo et al. 2010; Lorenzo and Minson 2010; Amorim et al. 2011; Castle et al. 2011) derive exercise intensity from pre acclimation baseline testing with the workload and exogenous environment consistent day to day. Whilst thermal stress may be sufficient for the initial sessions of HA, with ongoing adaptation, the relative potentiating stimuli may diminish along with the rate of adaptation, even to the extent that the latter stage of HA are analogous to a reduction in stress (Taylor and Cotter 2006; Taylor 2014). Isothermic HA, also known as controlled hyperthermia, (Patterson et al. 2004; Magalhães et al. 2006; Garrett et al. 2009; Magalhães et al. 2010a; Magalhães et al. 2010b; Hom et al. 2012; Garrett et al. 2012; Castle et al. 2012; Garrett et al. 2014; Patterson et al. 2014) imposes session-by-session workloads based upon targeted endogenous criteria (core temperature ≥ 38.5°C), thus sustaining potentiating stimuli throughout the intervention via a combination of active then passive heat exposure (Fox et al. 1963).

The aim of the present study was to identify differences in Hsp72 mRNA response to exogenously controlled, fixed intensity HA, an endogenously controlled isothermic HA method, and a progressive endogenous isothermic HA method. We hypothesised that Hsp72 mRNA would increase following completion of an acute HA session, irrespective of the method used; however isothermic methods would sustain the magnitude of increase throughout acclimation due to sustained elevations in core temperature, with an increase in target core temperature progressively increasing transcription.

Methods

Participants

Twenty-four healthy males were assigned into fixed intensity HA (FIXED) (n = 8) continuous isothermic HA (ISO_{CONT}) (n = 8) or progressive isothermic HA (ISO_{PROG}) (n = 8), see Table 1 for descriptive characteristics. Confounding variables of smoking, caffeine, glutamine, alcohol, generic supplementation, prior thermal, hypoxic, and hyperbaric exposures were all controlled in line with previous work in the field (Taylor et al. 2011;

Gibson et al. 2014). Following full description of experimental procedures, the methods were approved by the institutional ethics committee. All participants completed medical questionnaires and provided written informed consent following the principles outlined by the Declaration of Helsinki of 1975, as revised in 2013.

Preliminary Testing

Participants consumed 500 mL of water 2 h before all preliminary and experimental exercise sessions (Sawka et al. 2007). A urine osmometer (Alago Vitech Scientific, Pocket PAL-OSMO, UK) ensured consistent hydration prior to each experimental session (Garrett et al. 2014) in accordance with established urine osmolality (<700 $mOsm \cdot Kg^{-1}$ H_2O (Sawka et al. 2007)), if this criterion was not met participants consumed 500 mL of water and rested until hydration criteria was achieved. Prior to the $\dot{V}O_{2peak}$ determination, height (cm) was measured using a fixed stadiometer (Detecto Physicians Scales; Cranlea & Co., Birmingham, UK) and NBM recorded to 0.01 kg from digital scales (ADAM GFK 150, USA). Body fat (%) was calculated (Siri 1956) from body density, derived from a four site skin fold calculation (Durnin and Womersley, 1974) using skin fold calipers (Harpenden, Burgess Hill, UK) with body surface area also calculated later (Du Bois and Du Bois 1916).

$\dot{V}O_{2peak}$ ($L.min^{-1}$) was determined from an incremental test on a cycle ergometer (Monark e724, Vansbro, Sweden) in temperate conditions (20°C, 40% relative humidity (RH). Saddle position was adjusted by the participant to their preferred cycling position and remained unchanged for all experimental trials. Starting intensity was set at 80 W with resistance applied to the flywheel eliciting 24 $W.min^{-1}$ increases at the constant cadence of 80 rpm. Heart rate (HR; $b.min^{-1}$) was monitored continually during all exercise tests by telemetry (Polar Electro Oyo, Kempele, Finland). Expired metabolic gas was measured using an online system (Metamax 3X, Cortex, Germany). $\dot{V}O_{2peak}$ was considered the highest $\dot{V}O_2$ obtained in any 10 s period.

Heat Acclimation Protocol

Each HA testing session was conducted in the morning (08:00 ± 01:00 h) to minimise daily variation in performance (Drust et al. 2005). Following provision of a urine sample and measurement of NBM, each participant was equipped with a rectal thermistor (Henleys Medical, UK, Meter logger Model 401, Yellow Springs Instruments, Yellow Springs, Missouri, USA) and a HR monitor. Resting measures, including pre- and post-session venous blood samples, were taken whilst participants were seated in temperate laboratory conditions. Following resting measures, participants mounted a cycle ergometer (Monark, e724, Vansbro, Sweden) located inside an environmental chamber and commenced exercising (40.2 ± 0.4°C, 39.0 ± 7.8% RH; WatFlow control system; TISS, Hampshire, UK). FIXED participants performed all ten 90 min sessions cycling continuously at a workload corresponding to 50% $\dot{V}O_{2peak}$ (80 rpm; 50% $\dot{V}O_{2peak}$ = 1.90 ± 0.30 $L.min^{-1}$, power at 50% $\dot{V}O_{2peak}$ = 125 ± 30 W). ISO_{CONT} (65% $\dot{V}O_{2peak}$ = 2.19 ± 0.34 $L.min^{-1}$, 175 ± 27 W) and ISO_{PROG} (65% $\dot{V}O_{2peak}$ = 2.46 ± 0.46 $L.min^{-1}$, 197 ± 36 W) participants began exercising at a workload corresponding to 65% of $\dot{V}O_{2peak}$ until a target T_{rec} of 38.5°C or 39.0°C was achieved, respectively. ISO_{CONT} targeted a T_{rec} of 38.5°C for all ten sessions, whereas ISO_{PROG} targeted a T_{rec} of 38.5°C for the first five sessions progressing to a T_{rec} of 39.0°C for the final five sessions. In both ISO_{CONT} and $ISO_{PROG,}$ once target T_{rec} had been reached, power was adjusted every 5 min, first by a 25% $\dot{V}O_{2peak}$ reduction, and then adjusted (± 5% $\dot{V}O_{2peak,}$ or seated rest) to maintain the experimental T_{rec} for a total session duration of 90 min, exercising duration was calculated based upon the duration of cycling required to reach, and then maintain the target T_{rec} in ISO_{CONT} and ISO_{PROG}. All participants in ISO_{CONT} and ISO_{PROG} were required to rest during both STHA (ISO_{CONT} = 23 ± 9 $min.session^{-1}$; ISO_{PROG} = 37 ± 9 $min.session^{-1}$) and LTHA (ISO_{CONT} = 19 ± 10 $min.session^{-1}$; ISO_{PROG} = 30 ± 9 $min.session^{-1}$), exercise was resumed once core temperature reduced below 38.5°C. During each testing session HR, T_{rec} and power output, were recorded every 5 min, a visual representation of the exercise intensities and T_{rec} responses to STHA and LTHA are presented in Figure 1.

Blood Sampling, RNA extraction and One step reverse transcription quantitative polymerase chain reaction (RT-QPCR)

Venous blood samples were taken immediately pre- and post- exercise-heat exposure on the first, fifth and tenth experimental sessions for FIXED, ISO_{CONT} and $ISO_{PROG.}$ All blood samples were drawn from the antecubital vein into 6 mL EDTA Vacuette tubes (Grenier BIO-one, UK). 1 mL of venous blood was pipetted into 10 mL of 1 in 10 red blood cell lysis solution (10X red blood Cell Lysis Solution, Miltenyi Biotech, UK). Samples were incubated for 15 min at room temperature then isolated via centrifugation at 400G for 5 min and washed twice in 2 mL PBS at 400G for 5 min to isolate all leukocytes. RNA was then extracted via the previously validated acid guanidium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi 1987). Quantity was determined at an optical density of 260 nm while quality was determined via the 260/ 280 and 260/ 230 ratios using a nanodrop spectrophotometer (Nanodrop 2000c Thermo Scientific).

Hsp72 relative mRNA expression (Hsp72 mRNA) was quantified using RT-QPCR. Primers β2-Microglobulin (β2-M; NCBI Accession number: NM_004048; Forward CCGTGTGAACCATGTGACT, Reverse, TGCGGCATCTTCAAACCT) and Hsp72 (NCBI Accession number: NM_005345; Forward CGCAACGTGCTCATCTTTGA, Reverse TCGCTTGTTCTGGCTGATGT) were designed using primer design software (Primer Quest and Oligoanalyzer - Integrated DNA technologies). 20 µL reactions containing 10 µL SYBR-Green RT-PCR Mastermix (Quantifast SYBRgreen Kit, Qiagen), 0.15 µL forward primer, 0.15 µL reverse primer, 0.2 µL reverse transcription mix (Quantifast RT Mix, Qiagen) and 9.5 µL sample (70 ng RNA/µL) were prepared in separate tubes. Each PCR reaction (Rotorgene Q, Qiagen, Manchester, UK) was then performed as follows: 10 min, 50°C (reverse transcription), 5 min 95°C (transcriptase inactivation and initial denaturation); followed by: 10 s, 95°C (denaturation), 30 s, 60°C (annealing and extension) 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 5s stages) were analysed for each reaction to ensure only the single gene of interest was amplified. A comparative critical threshold (*C*T) method was used to quantify Hsp72 mRNA in comparison with β2-M (Schmittgen and Livak 2008).

Statistical Analysis

All outcome variables were first checked for normality using Kolmogorov-Smirnov and sphericity using the Greenhouse Geisser method prior to further analysis. Two way mixed design ANOVA were performed to determine differences in dependent variables between HA methods for STHA and LTHA timescales (between HA methods and Day 1, Day 5 and Day 10). A three way mixed design ANOVA was performed on the Hsp72 mRNA data to determine differences between pre and post value (repeated measures – within subjects) on different days (repeated measures – within subjects) from independent HA methods (between subjects). Adjusted Bonferroni comparisons were used as post hoc analyses, determining where differences existed within ANOVA when a time or interaction was found. Data are reported as mean ± SD, with two-tailed significance was accepted at $p < 0.05$.

Results

Participant Characteristics

No differences ($p > 0.05$) existed between groups for descriptive variables height, NBM, BSA, body fat % or $\dot{V}O_{2peak.}$ A difference ($p < 0.05$) was observed for age whereby ISO_{PROG} was older than FIXED (+6.5 years).

Evidence of Heat Acclimation

Resting T_{rec} was reduced ($p = 0.002$), and sweat loss increased ($p = 0.002$) overall, with a significant reduction between Day 1 and Day 10 ($p = 0.003$ and $p = 0.002$ respectively), no interaction effects were observed for resting T_{rec} (; $p = 0.592$) or sweat loss ($p = 0.281$), figure 2. Resting HR demonstrated a significant overall effect ($p < 0.001$) and interaction effect ($p = 0.009$), with significant differences observed between Day 1 and Day 5 ($p < 0.001$) and Day 1 and Day 10 ($p = 0.001$) in ISO_{CONT}, and a difference between ISO_{PROG} and FIXED ($p = 0.043$), and ISO_{PROG} and ISO_{CONT} ($p = 0.015$) on Day 1, and between FIXED and ISO_{CONT} ($p = 0.038$), and FIXED and ISO_{PROG} ($p = 0.023$) on Day 10, figure 2.

Session Specific Data

Exercising duration ($p = 0.001$), mean session intensity ($p = 0.002$), total work done ($p < 0.001$), mean T_{rec} ($p = 0.002$), duration T_{rec} ≥38.5°C ($p = 0.011$), mean HR ($p = 0.019$), and peak HR ($p < 0.001$) all demonstrated overall differences between days, no between day difference was observed for peak T_{rec} ($p = 0.226$) or duration T_{rec} ≥39.0°C ($p = 0.245$).

Exercising duration ($p = 0.004$), mean session intensity ($p = 0.000$), total work done ($p = 0.004$), mean T_{rec} ($p = 0.010$), peak T_{rec} ($p = 0.004$), duration T_{rec} ≥38.5°C ($p = 0.008$), duration T_{rec} ≥39.0°C ($p = 0.005$) all demonstrated interaction effects, no interaction effect was observed for mean HR ($p = 0.077$) or peak HR ($p = 0.588$). See Table 2 for full post hoc analysis.

No differences between days or the interaction effect were observed for mean exercising intensity ($p = 0.124$; $p = 0.061$), change T_{rec} ($p = 0.227$; $p = 0.109$).

Hsp72 mRNA responses

No differences in Hsp72 mRNA were observed between days ($p = 0.236$) or across HA methods between days ($p = 0.167$). Hsp72 mRNA did increase Pre to Post overall ($p < 0.001$), and Pre to Post over time ($p = 0.034$); Day 1 ($p < 0.001$), Day 5 ($p < 0.001$) and Day 10 ($p < 0.001$). No Pre to Post difference occurred between HA methods ($p = 0.069$) or for the Pre to Post, between day, between HA methods interaction ($p = 0.217$); on Day 1 (FIXED; 2.3 ± 1.0 to 6.4 ± 2.8, ISO_{CONT}; 1.9 ± 0.6 to 4.4 ± 1.1 and ISO_{PROG}; 1.9 ± 0.8 to 7.1 ± 2.9), Day 5 (FIXED; 2.3 ± 0.8 to 4.2 ± 2.2, ISO_{CONT}; 2.3 ± 0.8 to 5.3 ± 2.5 and ISO_{PROG}; 2.2 ± 0.5 to 6.3 ± 2.2) and Day 10 (FIXED; 2.3 ± 0.7 to 4.3 ± 2.0, ISO_{CONT}; 2.1 ± 0.7 to 4.3 ± 1.3 and ISO_{PROG}; 2.0 ± 0.5 to 6.1 ± 1.7).

Discussion

The aim of this experiment was to determine whether there was a difference in the change in leukocyte Hsp72 mRNA expression between fixed intensity, continuous isothermic, and progressive isothermic methods during STHA and LTHA. Participants were successfully matched for anthropometric descriptive data and $\dot{V}O_{2peak,}$ ISO_{PROG} participants were observed as older than FIXED although the magnitude of difference is not physiologically relevant with regards to heat stress responses (Kenny et al. 2010). An anticipated increase in Hsp72 mRNA expression was observed pre to post each session of exercise-heat stress across all groups overall. No statistical difference in Hsp72 mRNA existed between HA methods, either pre or post acclimation on day 1, day 5 or day 10.

In spite of diminished endogenous stress in FIXED due to the ongoing HA adaptations the reduction was not to the extent that mRNA was statistically reduced on day 5 or day 10. Consequently equal signals for the attainment of thermotolerance are present in FIXED (active heat acclimation) as ISO_{CONT} and ISO_{PROG} methods (active and passive acclimation). This is an important observation which suggests that exercise per se is not as significant as hyperthermia. No significant pre to post increase in Hsp72 mRNA was observed by implementing a progressive increase in core temperature/hyperthermia (38.5°C to 39.0°C) suggesting targeting a T_{rec} of 38.5°C is sufficient. The reduced endogenous thermal strain (mean T_{rec}, peak $T_{rec,}$ and duration T_{rec} ≥38.5°C) did not attenuate Hsp72 mRNA responses observed following FIXED between day 1 and day 5 (following STHA) and day 10 (following LTHA) (Table 2). Previous data from our laboratory has shown FIXED day 1 presents equivalent endogenous strain to that elicited at 50% $\dot{V}O_{2peak}$ in 40°C, whereas day 10 presents strain equivalent to working at the same intensity in just 30°C (Gibson et al. 2014). This reduction in strain due to the ongoing adaptive process of HA. The attenuated endogenous criteria were not apparent within isothermic methods demonstrating the effectiveness of these methods at targeting core temperatures. Correspondingly Hsp72 increases were also maintained each day as previously within the field (Magalhães et al. 2010b). Our data further implicates these endogenous thermoregulatory markers as the most relevant signals for manipulating Hsp72 mRNA (Magalhães et al. 2010b) with all the methods tested providing sufficient endogenous stimuli for Hsp72 mRNA transcription. Different duration exercising and workload intensity across day 1 and day 5 and day 10 do not appear relevant contributors to the Hsp72 mRNA response within our experimental design, and are in accordance with previous suggestions (Hom et al. 2012). These observations, that hyperthermia rather than exercise is an important signal for Hsp72 transcription is supported by the equal post exercise expression using active then passive acclimation in ISO_{CONT} and $ISO_{PROG,}$ as active only in FIXED. This is in agreement with other passive heating data (Maloyan et al. 1999). It is not known if this is true of the mean exercise intensity required of each method which, despite not being significantly different between methods, may influence the magnitude of the mRNA response during heat acclimation (e.g. if the FIXED intensity group exercised at an intensity >50% $\dot{V}O_{2peak}$). Increased relative exercise intensity proportionally increases metabolic heat production, thus increasing core temperature (Mora-Rodriguez et al. 2008) which is associated with increased HSP72 (Mestre-Alfaro et al. 2012). This exogenous parameter of exercise-heat stress therefore cannot be disassociated from changes in Hsp72 mRNA in spite of a secondary rather than causal role (Liu et al. 2000; Milne and Noble 2002; Liu et al. 2004).

Reduced thermal endogenous strain, particularly the attenuated magnitude and rate of core temperature increase, may be most pertinent to the observed reductions in Hsp72 mRNA transcription in this study. These endogenous criteria have been considered as important in other measures of HSP responses to acclimation (Magalhães et al. 2010b). Post acclimation day increases in Hsp72 mRNA indicated that the stress presented at the start of HA, and after STHA and LTHA all surpassed the minimum required endogenous strain to elicit increased transcription of Hsp72 mRNA in leukocytes across HA methods. The Hsp72 mRNA response provides further evidence of the importance of providing a consistent stressor for adaptation, via the facilitation of consistent or elevations in core temperature throughout STHA and LTHA. Sustained Hsp72 mRNA increases demonstrate the continued stimulation of the pathway responsible for thermotolerance - the cellular stress response to heat. As Hsp72 mRNA continued to elevate throughout the HA period, complete HSP72 protein mediated acclimation benefits had not been achieved in any method, despite adaptive phenotypic HA responses following both STHA and LTHA (Horowitz and Kodesh 2010). It is currently unknown whether an upper adaptive limit to HA or thermotolerance exists at a cellular level. HA increases baseline HSP72 and blunts inducibility of HSP72 *ex vivo* heat shock (McClung et al. 2008). Theoretically, once stress is presented to a cell, thermotolerance through optimised HSP72 affords sufficient cytoprotection and therefore, normal cell function and homeostasis is maintained without further transcription (Kregel 2002). Implementation of isothermic methods give the greatest efficacy towards continual and consistent magnitudes of Hsp72 mRNA transcription and concurrent increases in HSP72 which are associated with thermotolerance *in vitro* (Kregel 2002), *in vivo* (Maloyan et al. 1999), and HA improvements in heat tolerance (Patterson et al. 2004). Augmented HSP72, enhances cell tolerance to subsequent heat insults translating to enhanced organ, systemic and whole body tolerance (Beckham et al. 2008) and when considering the heat shock response (HSR) to the stress stimuli, a repressed HSF-1 activity. HA and thermotolerance are associated, with greater physiological HA adaptation blunting HSP72 induction to heat shock *ex vivo*, with HA accompanied by elevated baseline and improved regulation of HSP72 (Yamada et al. 2007; McClung et al. 2008). It is known that HSR inhibition impairs cellular and systemic adaptations associated with thermotolerance and HA in exercising humans via reductions in circulating cytokines and cellular and systemic markers of heat strain (Kuennen et al. 2011). Phenotypic adaptations occurring throughout STHA and LTHA do not delay or mitigate the HSR requirement of the tested HA methods, with sufficient if not consistent core temperature increases (Hom et al. 2012) augmenting synergistic cellular thermotolerance (Maloyan et al. 1999; Horowitz et al. 2004) alongside systemic HA phenotype adaptations (Moseley 1997).

Both final/peak, and absolute change in T_{rec} appear to have an effect on HSP72 changes during HA (Magalhães et al. 2010b), this has been previously shown by extracellular HSP72 release (Périard et al. 2012; Gibson et al. 2014), and now Hsp72 mRNA, indicating elevated thermal stress. Mechanistically, failure for ISO_{PROG} to elicit significant differences in Hsp72 mRNA in spite of differential mean, peak, and change in T_{rec} in comparison with ISO_{CONT} suggest progressively increasing the endogenous thermal strain through isothermic HA may not augment additional phenotypic HA or acquired cellular thermotolerance. A required “threshold” for the transcription of Hsp72 mRNA appears to be surpassed by ISO_{CONT} over both STHA and LTHA time scales irrespective of a 0.5°C increase in the target temperature suggesting the rate of transcription may be maximal following attainment of an internal temperature of 38.5°C. Maximal mean T_{rec} ≥38.5°C were higher in this study and others showing increased HSP72 (McClung et al. 2008; Magalhães et al. 2010b) compared with others where mean T_{rec} <38.5°C (Yamada et al. 2007; Hom et al. 2012), no data is available for the duration spent at this T_{rec}. A “threshold” for HA appears to be surpassed by ISO_{CONT} and ISO_{PROG} over LTHA with no further benefit of a 38.5°C to 39.0°C progression in the “threshold”. We observed no difference in Hsp72 mRNA transcription between 38.5°C and 39.0°C T_{rec}, suggesting mean temperature alone may not be the most important signal for increase or that an optimal Hsp72 mRNA transcription rate may occur once a suggested threshold of 38.5°C (T_{rec}) has been surpassed (Morton et al. 2009; Amorim et al. 2011).

It appears that despite achieving consistent core temperatures, isothermic methods contain some degree of variability in the acute sessional, and adaptive responses. This variability in the response to the isothermic should be acknowledged as a potential limitation of the method. Figure 1 demonstrates that the resting temperature of ISO_{PROG} was lower than the other groups, most notably when compared with ISO_{CONT} during

STHA. Additionally ISO_{PROG} required a lower final exercise intensity in than $ISO_{CONT,}$ this despite similar temperature during STHA and higher temperature during LTHA. The variability in isothermic methods is most identifiable from exercise/rest durations between ISO_{CONT} and ISO_{PROG}, and following the progression from STHA to LTHA. Additional duration at rest in LTHA is counter intuitive with heat gain decreasing with adaptation thus greater work is required to achieve the target temperature. This appears true of the initial bout of exercise where attainment of the target temperature is delayed in LTHA compared to STHA (figure 1). Mechanistically, the additional duration at rest in LTHA, compared to STHA is facilitated by the requirement for exercise to be maintained longer during the initial bout of exercise to achieve the target temperature. The result of this is a reduced requirement for participants to resume exercise following rest as the 90 minute session ends before temperature reduces below the target threshold. During STHA, the time to target core temperature is achieved earlier in the session than in LTHA. A greater duration then remains for heat dissipation and temperature reduction, consequently initiating a resumption of exercise in accordance of the requirements of the protocol. The extended first exercise bout in LTHA reduces the time remaining in the session for resuming exercise and thus participants demonstrate less work/lower average intensity of work later in the session. The greater duration of the initial bout of exercise prior to cessation also rationalises some of the differences between ISO_{CONT} and ISO_{PROG} during LTHA. The requirement for a greater change in core temperature in ISO_{PROG}, requires participants to exercise for longer initially to attain the higher temperature as such they again perform less work later in the session. These limitations demonstrate the importance of future research optimising isothermic methods so that a greater consistency of protocol administration, and potentially consistency of Hsp72 mRNA transcription is achieved. A larger sample size may reduce the variability in the protocol administration, and may strengthen the observations of the Hsp72 mRNA particularly trends towards reductions in FIXED which may become statistically different given prolonged acclimation (i.e. +10 days) or a greater sample size. It was observed that Hsp72 mRNA Post day 5 (p = 0.100) and post day 10 (p = 0.082) reduced non significantly in comparison to day 1, an observation not true of ISOCONT (Post day 1 vs. Post day 5 p = 0.998; Post day 1 vs. Post day 10 p = 1.000) or ISOPROG (Post day 1 vs. Post day 5 p = 1.000; Post day 1 vs. Post day 10 p = 0.677). An explanation for this may relate to the variability in the change in FIXED, physiologically this might be rationalised by individual differences in acclimation rate, and thus endogenous criteria using this protocol; an element that might be further clarified by a larger sample size.

Future work could involve tissue viability/*ex vivo* experiments to quantify the increased thermotolerance induced between HA methods alongside the measurement of the HSP72 protein, the absence of which is a limitation of the present experiment. Analysis of the acute Hsp72 mRNA response to the first session of progressive isothermic HA would allow analysis of increased hyperthermia from 38.5°C to 39°C to be quantified, although the measurement of mRNA presents a limitation in itself as no data is available to confirm intracellular HSP72 increases, with differential HA methods eliciting different gains in total protein which may in itself augment a changing mRNA/protein ratio. Cellular thermotolerance is unlikely to be explicit to HSP72 alone, with a number of genes associated with the cellular stress response to hyperthermia. Therefore a wider genomic and molecular analysis would facilitate further insight into the adaptive mechanisms (Sonna et al. 2002). Data suggests an endogenous threshold/minimum criteria may exist for Hsp72 mRNA or HSP72 protein increases as proposed by others (Amorim et al. 2008; Morton et al. 2009; Magalhães et al. 2010b; Périard et al. 2012; Gibson et al. 2014). Further investigation of precise endogenous signals leading to greatest intracellular Hsp72 mRNA and HSP72 increases in leukocytes and muscle is warranted to enable links between HA and thermotolerance, to be further examined. This could be facilitated by extended HA durations beyond ten sessions to determine whether in FIXED further diminished endogenous strain would see a continued attenuation of the post session mRNA transcription, or via an experiment where either lower isothermic temperatures are targeted, or changes from baseline implemented to elicit graded minimum thresholds. Individual variability associated with metabolic heat production and retention and the respective effects they may have on Hsp72 mRNA expression could be eliminated by modifying the isothermic method to administer the exercise based upon a fixed relative rate of heat production (Cramer and Jay 2014), further optimising acquired cellular thermotolerance through repeated exercise-heat stress at an optimised asymptote of core temperature increase.

Perspectives

Continuous and progressive isothermic HA elicit and sustain similar endogenous systemic strain. This is in contrast to fixed intensity HA which elicits less varied, but diminishing thermoregulatory strain following the procurement of STHA and LTHA adaptations. Hsp72 mRNA transcription, a marker of the cellular stress response to hyperthermia and an important component of thermotolerance, demonstrated equal sessional increases utilising all HA methods. The equal Hsp72 mRNA increases occurring after equal, reduced or increased core temperature following STHA and LTHA suggest that as long as a minimum endogenous criteria is surpassed, additional endogenous thermoregulatory strain is not of further benefit, nor is continual exercise load crucial so long as hyperthermia is present. These data give confidence that all reported HA methods increase Hsp72 mRNA and are capable of eliciting adaptations towards thermotolerance.

Acknowledgements

The authors would like to thank the volunteers for their participation in this investigation. Funding and support was obtained from the University of Brighton and the University of Bedfordshire.

Conflict of Interest

The authors declare that they have no competing interests such as funding or personal financial interest.

References

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. 2011;111:1609–18.

Amorim FT, Yamada PM, Robergs R a, Schneider SM, Moseley PL. The effect of the rate of heat storage on serum heat shock protein 72 in humans. Eur J Appl Physiol. 2008;104:965–72.

Asea A. Initiation of the Immune Response by Extracellular Hsp72: Chaperokine Activity of Hsp72. Curr Immunol Rev. 2006;2:209–215.

Beckham JT, Wilmink GJ, Mackanos MA, Takahashi K, Contag CH, Takahashi T, Jansen ED. Role of HSP70 in cellular thermotolerance. Lasers Surg Med. 2008;40:704–15.

Castle P, Mackenzie RW, Maxwell N, Webborn ADJ, Watt PW. Heat acclimation improves intermittent sprinting in the heat but additional pre-cooling offers no further ergogenic effect. J Sports Sci. 2011;29:1125–34.

Castle PC, Kularatne BP, Brewer J, Mauger AR, Austen RA, Tuttle JA, Sculthorpe N, Mackenzie RW, Maxwell NS, Webborn ADJ. Partial heat acclimation of athletes with spinal cord lesion. Eur J Appl Physiol. 2012;:109–115.

Cheung SS, McLellan TM. Heat acclimation, aerobic fitness, and hydration effects on tolerance during uncompensable heat stress. J Appl Physiol. 1998;84:1731–1739.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987;162:156–9.

Cramer MN, Jay O. Selecting the correct exercise intensity for unbiased comparisons of thermoregulatory responses between groups of different mass and surface area. J Appl Physiol. 2014;116:1123–1132.

Drust B, Waterhouse J, Atkinson G, Edwards B, Reilly T. Circadian rhythms in sports performance--an update. Chronobiol Int. 2005;22:21–44.

Du Bois D, Du Bois EF. A formula to estimate the approximate surface area if height and weight be known. Arch Intern Med. 1916;17:863–871.

Durnin J V, Womersley J Body fat assessed from total body density and its estimation from skinfold thickness: measurements on 481 men and women aged from 16 to 72 years. British Journal of Nutrition. 1974; 32:77–97.

Fleshner M, Johnson JD. Endogenous extra-cellular heat shock protein 72: releasing signal(s) and function. Int J Hyperth. 2005;21:457–471.

Fox RH, Goldsmith R, Kidd DJ, Lewis HE. Acclimatization to heat in man by controlled elevation of body temperature. J Physiol. 1963;166:530–47.

Garrett AT, Creasy R, Rehrer NJ, Patterson MJ, Cotter JD. Effectiveness of short-term heat acclimation for highly trained athletes. Eur J Appl Physiol. 2012;112:1827–37.

Garrett AT, Goosens NG, Rehrer NJ, Patterson MJ, Harrison J, Sammut I, Cotter JD. Short-term heat acclimation is effective and may be enhanced rather than impaired by dehydration. Am J Hum Biol. 2014;26:311–320.

Garrett AT, Goosens NG, Rehrer NJ, Rehrer NG, Patterson MJ, Cotter JD. Induction and decay of short-term heat acclimation. Eur J Appl Physiol. 2009;107:659–70.

Garrett AT, Rehrer NJ, Patterson MJ. Induction and decay of short-term heat acclimation in moderately and highly trained athletes. Sport Med. 2011;41:757–71.

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. 2014;19:389–400.

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. 2012;17:29–39.

Horowitz M, Eli-Berchoer L, Wapinski I, Friedman N, Kodesh E. Stress-related genomic responses during the course of heat acclimation and its association with ischemic-reperfusion cross-tolerance. J Appl Physiol. 2004;97:1496–507.

Horowitz M, Kodesh E. Molecular signals that shape the integrative responses of the heat-acclimated phenotype. Med Sci Sports Exerc. 2010;42:2164–72.

Houmard JA, Costill DL, Davis JA, Mitchell JB, Pascoe DD, Robergs RA. The influence of exercise intensity on heat acclimation in trained subjects. Med Sci Sport Exerc. 1990;22:615–620.

Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, Bruford E a, Cheetham ME, Chen B, Hightower LE. Guidelines for the nomenclature of the human heat shock proteins. Cell Stress Chaperones. 2009;14:105–11.

Kenny GP, Gagnon D, Dorman LE, Hardcastle SG, Jay O. Heat balance and cumulative heat storage during exercise performed in the heat in physically active younger and middle-aged men. Eur J Appl Physiol. 2010;109:81–92.

Kregel KC. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol. 2002;92:2177–86.

Kresfelder TL, Claassen N, Cronjé MJ. Hsp70 Induction and hsp70 Gene polymorphisms as Indicators of acclimatization under hyperthermic conditions. J Therm Biol. 2006;31:406–415.

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. 2011;301:R524–33.

Lancaster GI, Febbraio M a. Mechanisms of stress-induced cellular HSP72 release: implications for exercise-induced increases in extracellular HSP72. Exerc Immunol Rev. 2005;11:46–52.

Lancaster GI, Febbraio MA. Exosome-dependent trafficking of HSP70: a novel secretory pathway for cellular stress proteins. J Biol Chem. 2005;280:23349–23355.

Liu Y, Lormes W, Baur C, Opitz-Gress A, Altenburg D, Lehmann M, Steinacker JM. Human skeletal muscle HSP70 response to physical training depends on exercise intensity. Int J Sports Med. 2000;21:351–5.

Liu Y, Lormes W, Wang L, Reissnecker S, Steinacker JM. Different skeletal muscle HSP70 responses to high-intensity strength training and low-intensity endurance training. Eur J Appl Physiol. 2004;91:330–5.

Lorenzo S, Halliwill JR, Sawka MN, Minson CT. Heat acclimation improves exercise performance. J Appl Physiol. 2010;109:1140–7.

Lorenzo S, Minson CT. Heat acclimation improves cutaneous vascular function and sweating in trained cyclists. J Appl Physiol. 2010;109:1736–43.

Magalhães FC, Passos RLF, Fonseca MA, Oliveira KPM, Ferreira-Júnior JB, Martini ARP, Lima MRM, Guimarães JB, Baraúna VG, Silami-Garcia E, Rodrigues LOC. Thermoregulatory efficiency is increased after heat acclimation in tropical natives. J Physiol Anthropol. 2010;29:1–12.

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. 2010;15:885–95.

Magalhães FDC, Machado-Moreira CA, Vimieiro-Gomes AC, Silami-Garcia E, Lima NRV, Rodrigues LOC. Possible Biphasic Sweating Response during Short-term Heat Acclimation Protocol for Tropical Natives. J Physiol Anthropol. 2006;25:215–219.

Maloyan A, Horowitz M. beta-Adrenergic signaling and thyroid hormones affect HSP72 expression during heat acclimation. J Appl Physiol. 2002;93:107–15.

Maloyan A, Palmon A, Horowitz M. Heat acclimation increases the basal HSP72 level and alters its production dynamics during heat stress. Am J Physiol. 1999;276:R1506–15.

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. 2007;32:341–348.

Marshall HC, Ferguson RA, Nimmo MA. Human resting extracellular heat shock protein 72 concentration decreases during the initial adaptation to exercise in a hot, humid environment. Cell Stress Chaperones. 2006;11:129–134.

McClung JP, Hasday JD, He JR, 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. 2008;294:R185–91.

Mestre-Alfaro A, Ferrer MD, Banquells M, Riera J, Drobnic F, Sureda A, Tur JA, Pons A. Body temperature modulates the antioxidant and acute immune responses to exercise. Free Radic Res. 2012;46:799–808.

Milne KJ, Noble EG. Exercise-induced elevation of HSP70 is intensity dependent. J Appl Physiol. 2002;93:561–8.

Mora-Rodriguez R, Del Coso J, Estevez E. Thermoregulatory responses to constant versus variable-intensity exercise in the heat. Med Sci Sports Exerc. 2008;40:1945–52.

Morton JP, Kayani AC, McArdle A, Drust B. The Exercise-Induced Stress Response of Skeletal Muscle, with Specific Emphasis on Humans. Sport Med. 2009;39:643–662.

Moseley PL. Heat shock proteins and heat adaptation of the whole organism. J Appl Physiol. 1997;83:1413–1417.

Nielsen B, Hales JR, Strange S, Christensen NJ, Warberg J, Saltin B. Human circulatory and thermoregulatory adaptations with heat acclimation and exercise in a hot, dry environment. J Physiol. 1993;460:467–485.

Nielsen B, Strange S, Christensen NJ, Warberg J, Saltin B. Acute and adaptive responses in humans to exercise in a warm, humid environment. Pflugers Arch. 1997;434:49–56.

Ogura Y, Naito H, Akin S, Ichinoseki-Sekine N, Kurosaka M, Kakigi R, Sugiura T, Powers SK, Katamoto S, Demirel H a. Elevation of body temperature is an essential factor for exercise-increased extracellular heat shock protein 72 level in rat plasma. Am J Physiol Regul Integr Comp Physiol. 2008;294:R1600–7.

Patterson M. Sustained and generalized extracellular fluid expansion following heat acclimation. J Physiol. 2004;559:327–34.

Patterson MJ, Stocks JM, Taylor N a S. Whole-body fluid distribution in humans during dehydration and recovery, before and after humid-heat acclimation induced using controlled hyperthermia. Acta Physiol (Oxf). 2014;210:899–912.

Patterson MJ, Stocks JM, Taylor NAS. Humid heat acclimation does not elicit a preferential sweat redistribution toward the limbs. Am J Physiol Regul Integr Comp Physiol. 2004;286:R512–8.

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. 2012;17:375–83.

Sandström ME, Siegler JC, Lovell RJ, Madden L a, McNaughton L. The effect of 15 consecutive days of heat-exercise acclimation on heat shock protein 70. Cell Stress Chaperones. 2008;13:169–75.

Sarge KD, Murphy SP, Morimoto RI. Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. Mol Cell Biol. 1993;13:1392–407.

Sawka MN, Burke LM, Eichner ER, Maughan RJ, Montain SJ, Stachenfeld NS. American College of Sports Medicine position stand. Exercise and fluid replacement. Med Sci Sports Exerc. 2007;39:377–90.

Sawka MN, Leon LR, Montain SJ, Sonna LA. Integrated physiological mechanisms of exercise performance, adaptation, and maladaptation to heat stress. Compr Physiol. 2011;1:1883–928.

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc. 2008;3:1101–1108.

Selkirk GA, McLellan TM, Wright HE, Rhind SG. Expression of intracellular cytokines, HSP72, and apoptosis in monocyte subsets during exertional heat stress in trained and untrained individuals. Am J Physiol Regul Integr Comp Physiol. 2009;296:R575–86.

Siri WE. The gross composition of the body. Adv Biol Med Phys. 1956;4:239–280.

Sonna LA, Fujita J, Gaffin SL, Lilly CM. Invited review: Effects of heat and cold stress on mammalian gene expression. J Appl Physiol. 2002;92:1725–42.

Taylor L, Midgley AW, Chrismas B, Hilman AR, Madden L a, Vince R V, McNaughton LR. Daily hypoxia increases basal monocyte HSP72 expression in healthy human subjects. Amino Acids. 2011;40:393–401.

Taylor N, Cotter J. Heat adaptation: guidelines for the optimisation of human performance. Int Sport Med J. 2006;7:33–57.

Taylor NAS. Human Heat Adaptation. Compr Physiol. 2014;4:325–365.

Watkins AM, Cheek DJ, Harvey AE, Blair KE, Mitchell JB. Heat Acclimation and HSP-72 Expression in Exercising Humans. Int J Sports Med. 2008;29:269–276.

Yamada PM, Amorim FT, Moseley P, Robergs R, Schneider SM. Effect of heat acclimation on heat shock protein 72 and interleukin-10 in humans. J Appl Physiol. 2007;103:1196–204.

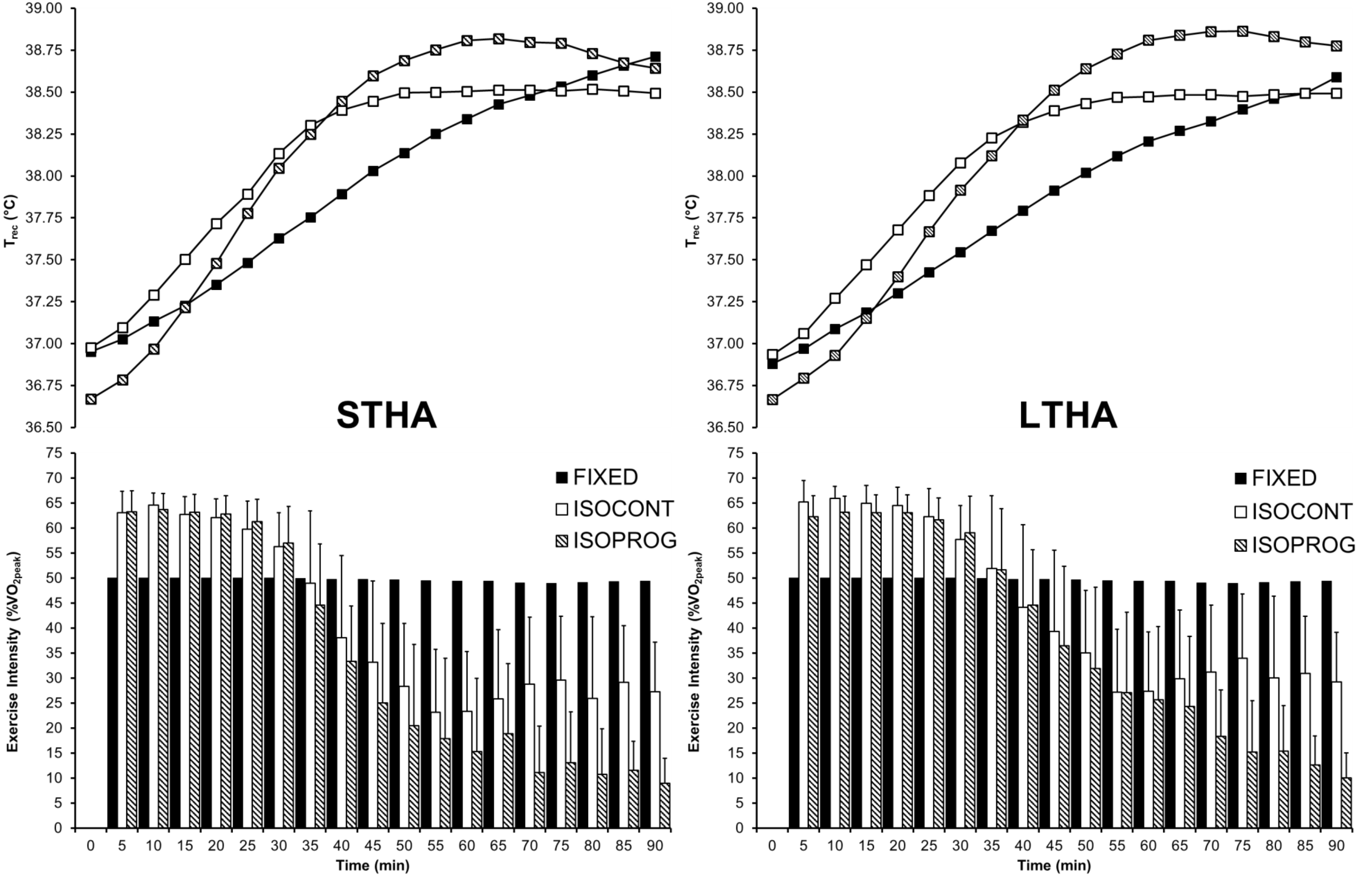
STHA
LTHA
T_{rec} (°C)
39.00
38.75
38.50
38.25
38.00
37.75
37.50
37.25
37.00
36.75
36.50
Exercise Intensity (%VO_{2peak})
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
■ FIXED
□ ISOCONT
▨ ISOPROG
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
Time (min)

Figure 1. Mean ± SD T_{rec} (top; °C) and exercise intensity (bottom; $\%\dot{V}O_{2peak}$) for the first five sessions (STHA: left) and all ten sessions (LTHA: right) of fixed intensity (FIXED, n = 8), continuous isothermic (ISO_{CONT}, n = 8), and progressive isothermic (ISO_{PROG}, n = 8) heat acclimation methods. Error bars have been removed from T_{rec} data for clarity.

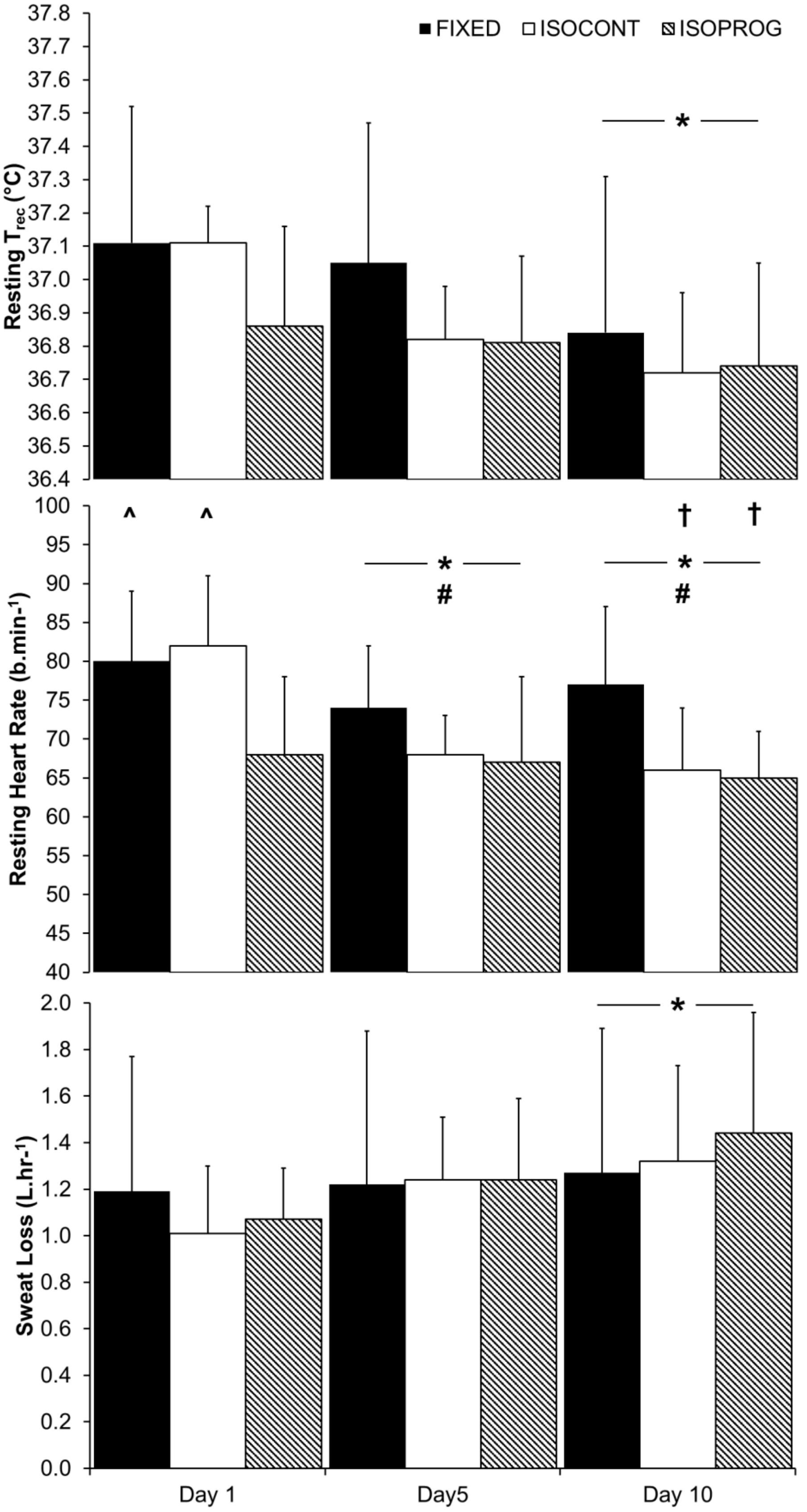

Figure 2 Mean ± SD Changes in resting T_{rec}, resting heart rate and sweat rate following STHA (Day 1 to 5) utilising fixed intensity (FIXED), continuous isothermic (ISO_{CONT}), and progressive isothermic (ISO_{PROG}) methods.* denotes significant difference overall from Day 1 ($p < 0.05$). # denotes significant difference within group and Day ($p < 0.05$). ^ denotes significant difference from ISO_{PROG} within group and Day ($p < 0.05$). † denotes significant difference from FIXED within group and Day 1 ($p < 0.05$).

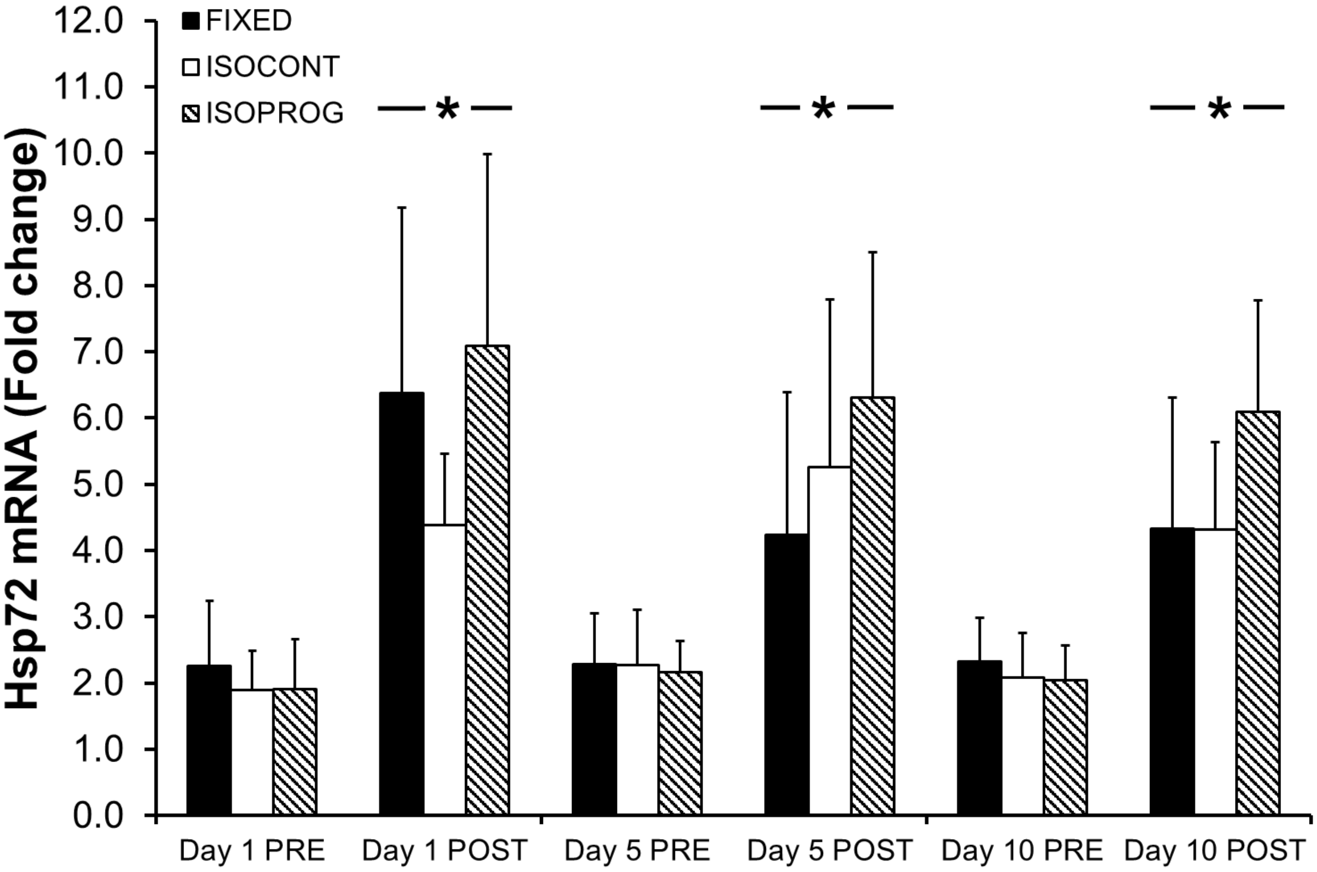

Figure 3 Mean ± SD Hsp72 mRNA pre and post sessions on Day 1, Day 5 and Day 10 of fixed intensity (FIXED) continuous isothermic (ISO_{CONT}), and progressive isothermic (ISO_{PROG}) methods. * denotes significant Pre to Post difference within session ($p < 0.05$).

Table 1. Mean ± SD Participant characteristics for fixed intensity (FIXED), continuous isothermic (ISO_{CONT}), and progressive isothermic (ISO_{PROG}) heat acclimation methods.

	FIXED	ISO_{CONT}	ISO_{PROG}
Age (years)	19.9 ± 1.0	22.6 ± 5.5	26.1 ± 4.9*
Height (cm)	179.3 ± 5.8	177.9 ± 5.8	179.5 ± 6.6
Body Mass (kg)	79.2 ± 18.3	74.2 ± 6.9	75.1 ± 8.8
BSA (m^2)	1.97 ± 0.21	1.92 ± 0.11	1.94 ± 0.11
Body fat (%)	14.9 ± 7.7	14.8 ± 2.2	14.1 ± 3.5
$\dot{V}O_{2peak}$ ($L.min^{-1}$)	3.61 ± 0.90	3.62 ± 0.69	3.79 ± 0.55

*denotes significantly difference from FIXED ($p < 0.05$)

Table 2. Mean ± SD Protocol, thermoregulatory and physiological data characterising exercise – heat stress on day one, day five and day ten of fixed intensity (FIXED), continuous isothermic (ISO$_{CONT}$), and progressive isothermic (ISO$_{PROG}$) methods.

	Day 1			Day 5			Day 10		
	FIXED	ISO$_{CONT}$	ISO$_{PROG}$	FIXED	ISO$_{CONT}$	ISO$_{PROG}$	FIXED	ISO$_{CONT}$	ISO$_{PROG}$
Exercising Duration (min)	90.0 ± 0.0	61.9 ± 10.7†	56.3 ± 16.6†	90.0 ± 0.0	76.3 ± 15.5*	53.1 ± 10.3†^	90.0 ± 0.0	78.8 ± 15.8*	70.0 ± 9.3* #
Mean Session Intensity (%$\dot{V}O_{2peak}$)	49.7 ± 0.6	36. 6 ± 5.3†	36.7 ± 11.2†	50.0 ± 0.0	47.0 ± 8.3*	32.3 ± 8.6†^	50.0 ± 0.0	50.5 ± 9.5*	45.8 ± 8.0*#
Mean Exercising Intensity (%$\dot{V}O_{2peak}$)	49.7 ± 0.6	52.6 ± 8.2	58.8 ± 5.1	50.0 ± 0.0	57.4 ± 4.9	56.8 ± 5.9	50.0 ± 0.0	58.7 ± 7.0	58.9 ± 6.2
Total Work Done (kJ)	656 ± 166	498 ± 81	554 ± 102	673 ± 165	657 ± 100*	500 ± 152	684 ± 164	719 ± 126*	708 ± 176*#
Mean T$_{rec}$ (°C)	38.17 ± 0.17	38.15 ± 0.23	38.21 ± 0.25	37.85 ± 0.22*	38.10 ± 0.19	38.27 ± 0.24†	37.74 ± 0.19*	38.04 ± 0.23†	38.18 ± 0.21†
Peak T$_{rec}$ (°C)	38.92 ± 0.26	38.65 ± 0.32	38.87 ± 0.18	38.52 ± 0.43*	38.66 ± 0.25	38.91 ± 0.24	38.40 ± 0.33*	38.67 ± 0.23	39.06 ± 0.37†
Δ T$_{rec}$ (°C)	1.81 ± 0.60	1.53 ± 0.37	2.01 ± 0.33	1.47 ± 0.74	1.74 ± 0.20	2.10 ± 0.42	1.56 ± 0.72	1.95 ± 0.32	2.32 ± 0.61†
Duration T$_{rec}$ ≥38.5°C (min)	32.5 ± 8.5	28.8 ± 15.1	44.4 ± 21.3	13.1 ± 16.0*	22.5 ± 20.7	51.3 ± 18.5†^	5.0 ± 8.0*	29.4 ± 23.5†	35.6 ± 18.6†
Duration T$_{rec}$ ≥39.0°C (min)	5.6 ± 12.1	0.0 ± 0.0	1.9 ± 3.7	1.3 ± 3.5	2.5 ± 7.1	6.9 ± 14.4	0.0 ± 0.0	0.0 ± 0.0	20.0 ± 16.0#†^
Mean HR (b.min^{-1})	159 ± 12	151 ± 13	144 ± 9	149 ± 21	148 ± 9	140 ± 8	146 ± 14	151 ± 8	144 ± 14
Peak HR (b.min^{-1})	176 ± 12	183 ± 9	182 ± 11	171 ± 26	172 ± 12	174 ± 8	164 ± 13	174 ± 11	171 ± 13

Notes: Exercising duration is cumulative time spent exercising during each of the 90 min sessions. Mean session intensity is calculated from each participant's relative exercise intensity during each five min period including rest periods during the given session. Mean exercise intensity is calculated from each participant's relative exercise intensity during each five min period excluding rest periods during the given session.

* denotes difference from Day 1 within respective method ($p < 0.05$). # denotes difference from Day 5 within respective method ($p < 0.05$).
† denotes difference from FIXED within respective day ($p < 0.05$). ^ denotes difference from ISO_{CONT} within respective day ($p < 0.05$).