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Article *in* Scandinavian Journal of Medicine and Science in Sports · May 2015

Impact Factor: 2.9 · DOI: 10.1111/sms.12430

Scand J Med Sci Sports 2015: 25 (Suppl. 1): 259–268
doi: 10.1111/sms.12430

SCANDINAVIAN JOURNAL OF
MEDICINE & SCIENCE
IN SPORTS

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

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Accepted for publication 19 January 2015

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 optimize cellular adaptation. Twenty-four males performed short-term HA (STHA; five sessions) and long-term HA (LTHA; STHA plus further five sessions) utilizing 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 reverse transcription quantitative polymerase chain reaction 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 toward thermotolerance.

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., 2010a; 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; Beckham et al., 2008; McClung 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., 2010a; 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., 2010a; Périard et al., 2012; Gibson et al., 2014), the mechanisms leading to an increase in circulating concentration are equivocal (Fleshner & Johnson, 2005; Lancaster & Febbraio, 2005a, b). Additionally, the biological role of eHSP72 appears more closely linked to an immunological response, rather than a process favorably 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., 2010a). HA increases basal HSP72, improving the cellular defense 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 & 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).

HSF-1 activation involves a complex series of regulatory events, including nuclear localization, oligomerization, 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, 1997; Cheung & McLellan, 1998; Kresfelder et al., 2006; Marshall et al., 2007; Yamada et al., 2007; Sandström et al., 2008; Watkins et al., 2008; Lorenzo et al., 2010; Lorenzo & 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. While 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 & Cotter, 2006; Taylor, 2014). Isothermic HA, also known as controlled hyperthermia (Patterson et al., 2004b, 2014; Magalhães et al., 2006, 2010a, b; Garrett et al., 2009, 2012, 2014; Castle et al., 2013; Hom et al., 2012), 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 hypothesized 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 because of 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.

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)	3.61 ± 0.90	3.63 ± 0.69	3.80 ± 0.55

*Denotes significantly difference from FIXED ($P < 0.05$).
BSA, body surface area.

Preliminary testing

Participants consumed 500 mL of water 2 h before all preliminary and experimental exercise sessions (Sawka et al., 2007). A urine osmometer (Pocket PAL-OSMO; Alago Vitech Scientific, Horsham, UK) ensured consistent hydration prior to each experimental session (Garrett et al., 2014) in accordance with established urine osmolality [< 700 mOsm/Kg 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 Nude body mass (NBM) recorded to 0.01 kg from digital scales (GFK 150, Adam Equipment Inc, Danbury, CT, USA). Body fat (%) was calculated (Siri, 1956) from body density, derived from a four site skinfold calculation (Durnin & Womersley, 1974) using skin fold calipers (Harpenden, Burgess Hill, UK) with body surface area also calculated later (Du Bois & Du Bois, 1916).

$\dot{V}O_{2peak}$ (L/min) was determined from an incremental test on a cycle ergometer (Monark e724, Monark AB, Varberg, 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 increases at the constant cadence of 80 rpm. Heart rate (HR; b/min) 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, Leipzig, Germany). $\dot{V}O_{2peak}$ was considered the highest $\dot{V}O_2$ obtained in any 10-s period.

HA protocol

Each HA testing session was conducted in the morning (08:00 ± 01:00 h) to minimize 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 Supplies Ltd, Welwyn Garden City, UK) and a HR monitor. Resting measures, including pre- and post-session venous blood samples, were taken while participants were seated in temperate laboratory conditions. Following resting measures, participants mounted a cycle ergometer (Monark, e724; Vansbro) 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

10 90-min sessions cycling continuously at a workload corresponding to 50% $\dot{V}O_{2peak}$ (80 rpm; 50% $\dot{V}O_{2peak} = 1.90 \pm 0.30$ L/min, power at 50% $\dot{V}O_{2peak} = 125 \pm 30$ W). ISO_{CONT} (65% $\dot{V}O_{2peak} = 2.19 \pm 0.34$ L/min, 175 ± 27 W) and ISO_{PROG} (65% $\dot{V}O_{2peak} = 2.46 \pm 0.46$ L/min, 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 or 39.0 °C was achieved, respectively. ISO_{CONT} targeted a T_{rec} of 38.5 °C for all 10 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} has 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; ISO_{PROG} = 37 ± 9 min/session) and LTHA (ISO_{CONT} = 19 ± 10 min/session; ISO_{PROG} = 30 ± 9 min/session), 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 Fig. 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 10th 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, Stonehouse, 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, Bisley, UK). Samples were incubated for 15 min at room temperature then isolated via centrifugation at 400 *g* for 5 min and washed twice in 2 mL phosphate-buffered saline at 400 *g* for 5 min to isolate all leukocytes. RNA was then extracted via the previously validated acid guanidium thiocyanate–phenol–chloroform extraction method (Chomczynski & 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, Waltham, MA, USA).

Hsp72-relative mRNA expression (Hsp72 mRNA) was quantified using RT-qPCR. Primers β2-Microglobulin (β2-M; NCBI accession number: NM_004048; forward: CCGTGTGAACC ATGTGACT; reverse: TGCGGCATCTTCAAACCT) and Hsp72 (NCBI accession number: NM_005345; forward: CGCAACGTG CTCATCTTTGA; reverse: TCGCTTGTTCTGGCTGATGT) were designed using primer design software (Primer Quest and Oligoanalyzer; Integrated DNA Technologies, Coralville, IA, USA). 20 µL reactions containing 10 µL SYBR-Green RT-PCR Mastermix (Quantifast SYBRgreen Kit; Qiagen, Manchester, UK), 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) 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 5-s stages) were analyzed for each reaction to ensure only the single gene of interest was amplified. A comparative critical threshold method was used to quantify Hsp72 mRNA in comparison with β2-M (Schmittgen & Livak, 2008).

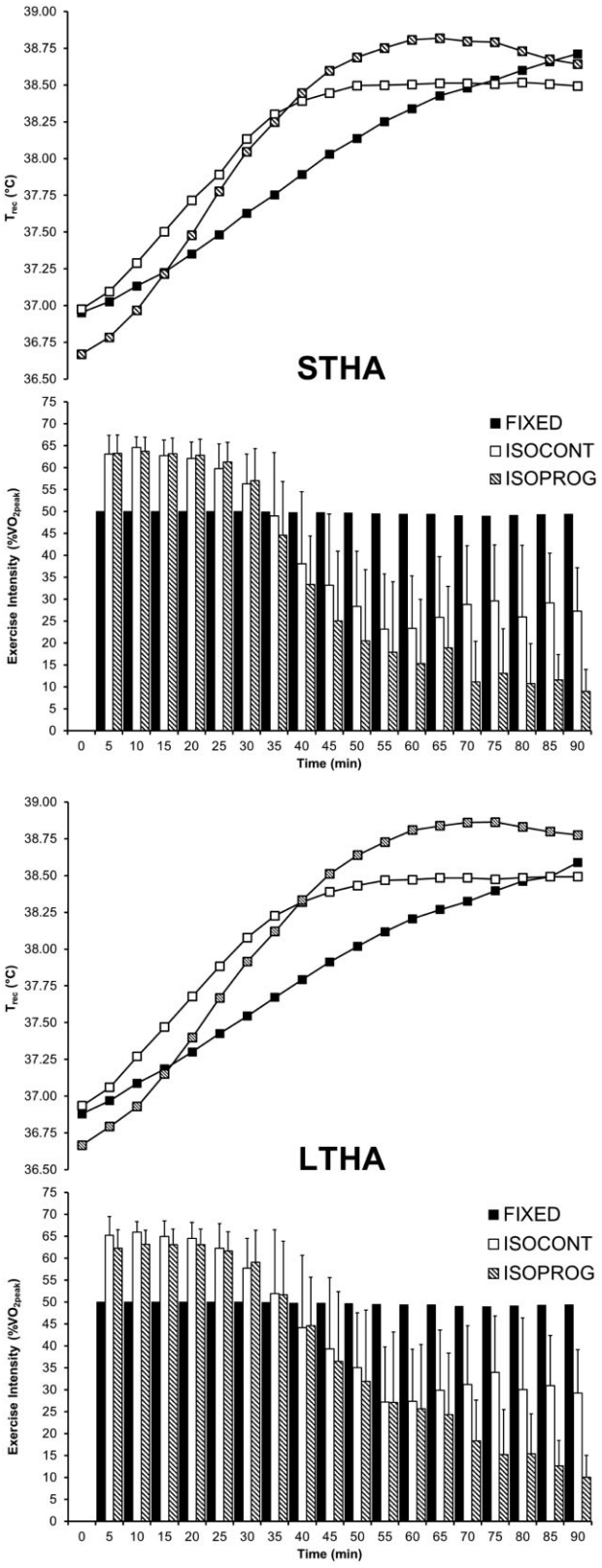

Fig. 1. Mean ± SD T_{rec} (top; °C) and exercise intensity (bottom; % $\dot{V}O_{2peak}$) for the first five sessions (STHA: left) and all 10 sessions (LTHA: right) of fixed-intensity (FIXED, $n = 8$), continuous isothermic (ISO_{CONT}, $n = 8$), and progressive isothermic (ISO_{PROG}, $n = 8$) heat acclimation methods. Error bars have been removed from T_{rec} data for clarity.

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 analysis of variance (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 HA

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 ($P = 0.281$), Fig. 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, Fig. 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 $T_{rec} \geq 38.5$ °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 $T_{rec} \geq 39.0$ °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 $T_{rec} \geq 38.5$ °C ($P = 0.008$), duration $T_{rec} \geq 39.0$ °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.

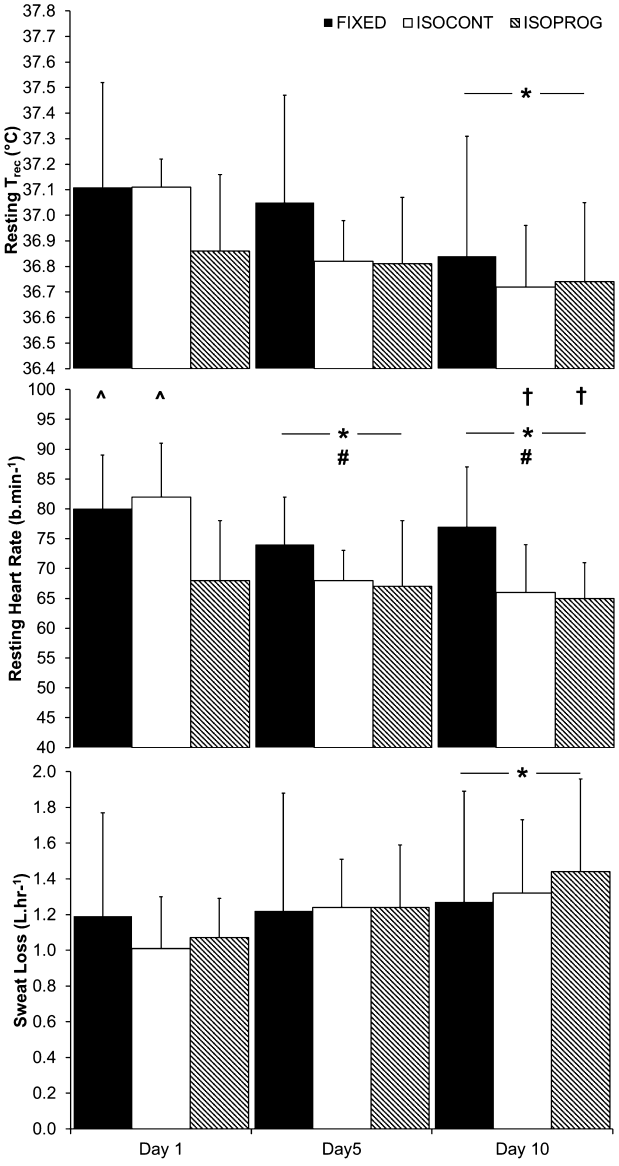

Fig. 2. Mean ± SD changes in resting T_{rec}, resting heart rate and sweat rate following STHA (days 1 to 5) utilizing fixed-intensity (FIXED), continuous isothermic (ISO_{CONT}), and progressive isothermic (ISO_{PROG}) methods.
*Denotes significant difference overall from day 1 ($P < 0.05$).
#Denotes 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 ($P < 0.05$).

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

Table 2. Mean ± SD protocol, thermoregulatory, and physiological data characterizing exercise heat stress on day 1, day 5, and day 10 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 $T_{rec} \geq 38.5$ °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 $T_{rec} \geq 39.0$ °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)	159 ± 12	151 ± 13	144 ± 9	149 ± 21	148 ± 9	140 ± 8	146 ± 14	151 ± 8	144 ± 14
Peak HR (b/min)	176 ± 12	183 ± 9	182 ± 11	171 ± 26	172 ± 12	174 ± 8	164 ± 13	174 ± 11	171 ± 13

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 5-min period including rest periods during the given session. Mean exercise intensity is calculated from each participant's relative exercise intensity during each 5-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 ($P < 0.05$).

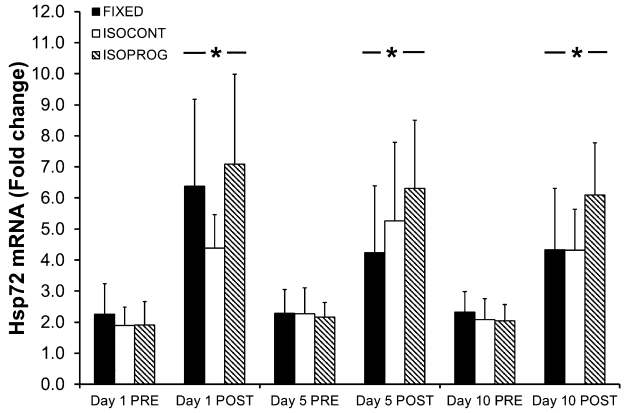

Fig. 3. Mean ± SD Hsp72 mRNA pre- and post-sessions on day 1, day 5, and day 10 of fixed-intensity (FIXED) continuous isothermic (ISO_{CONT}), and progressive isothermic (ISO_{PROG}) methods.
*Denotes significant pre to post difference within session ($P < 0.05$).

($P = 0.034$); 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) (Fig. 3).

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 (Fig. 3).

No statistical difference in Hsp72 mRNA existed between HA methods, either pre- or post-acclimation on day 1, day 5, or day 10 (Fig. 3).

In spite of diminished endogenous stress in FIXED because of 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 HA) 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 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 $T_{rec} \geq 38.5$ °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 is because of 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., 2010a). Our data further implicates these endogenous thermoregulatory markers as the most relevant signals for manipulating Hsp72 mRNA (Magalhães et al., 2010a) with all the methods tested providing sufficient endogenous stimuli for Hsp72 mRNA transcription. Different duration exercising and workload intensity across day 1, 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 HA (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, 2004; Milne & Noble, 2002).

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., 2010a). 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 & 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 optimized 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 toward 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., 2004a). 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 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., 2010a); 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 timescales 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 $T_{rec} \geq 38.5$ °C were higher in this study and others showing increased HSP72 (McClung et al., 2008; Magalhães et al., 2010a) 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 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 counterintuitive 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 with STHA (Fig. 1). Mechanistically, the additional duration at rest in LTHA, compared with 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-min 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 rationalizes 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 optimizing 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 toward 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 nonsignificantly 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 rationalized 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 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., 2010a; 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 10 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 & Jay, 2014), further optimizing acquired cellular thermotolerance through repeated exercise-heat stress at an optimized 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 utilizing 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 criterion 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 toward thermotolerance.

Key words: Thermoregulation, heat stress, cellular stress response, hyperthermia, thermotolerance, heat shock protein 72, heat illness.

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.

Conflicts of interest: The authors of this study declare that they have no conflicts of interest.

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