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Extracellular Hsp72 concentration relates to a minimum endogenous criteria during acute exercise-heat exposure

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Abstract

Extracellular heat-shock protein 72 (eHsp72) concentration increases during exercise-heat stress when conditions elicit physiological strain. Differences in severity of environmental and exercise stimuli have elicited varied response to stress. The present study aimed to quantify the extent of increased eHsp72 with increased exogenous heat stress, and determine related endogenous markers of strain in an exercise-heat model. Ten males cycled for 90 min at 50% $\dot{V}O_{2peak}$ in three conditions (TEMP, 20°C/63% RH; HOT, 30.2°C/51%RH; VHOT, 40.0°C/37%RH). Plasma was analysed for eHsp72 pre, immediately post and 24-h post each trial utilising a commercially available ELISA. Increased eHsp72 concentration was observed post VHOT trial (+172.4%) ($P<0.05$), but not TEMP (-1.9%) or HOT (+25.7%) conditions. eHsp72 returned to baseline values within 24hrs in all conditions. Changes were observed in rectal temperature (T_{rec}), rate of T_{rec} increase, area under the curve for T_{rec} of 38.5°C and 39.0°C, duration $T_{rec} \geq 38.5$°C and ≥ 39.0°C, and change in muscle temperature, between VHOT, and TEMP and HOT, but not between TEMP and HOT. Each condition also elicited significantly increasing physiological strain, described by sweat rate, heart rate, physiological strain index, rating of perceived exertion and thermal sensation. Stepwise multiple regression reported rate of T_{rec} increase and change in T_{rec} to be predictors of increased eHsp72 concentration. Data suggests eHsp72 concentration increases once systemic temperature and sympathetic activity exceeds a minimum endogenous criteria elicited during VHOT conditions and is likely to be modulated by large, rapid changes in core temperature.

Key words: Heat stress, Heat strain, Heat-shock protein, Hyperthermia, Core temperature

Introduction

The human 72kDa heat shock protein (Hsp72), HSPA1A (Kampinga et al., 2009) is the highly inducible isoform of a large family of proteins with an important role as a molecular chaperone maintaining cellular homeostasis, particularly in response to thermal stimuli (Mizzen & Welch, 1988). Research has identified extracellular changes in Hsp72 concentration within whole blood (Marshall et al. 2006; Yamada et al. 2007; Ogura et al. 2008; Magalhães et al. 2010; Périard et al. 2012), and intracellular changes in total protein expression and/or gene transcription in monocytes and systemic tissue (McClung et al. 2008; Selkirk et al. 2009; Magalhães et al. 2010; Amorim et al. 2011) in response to thermal and exercise stress. Hsp72 binds with high affinity to the plasma membrane (Asea et al., 2000) and up-regulates expression of pro-inflammatory cytokines, tumour necrosis factor-α, interleukin-1β and interleukin-6 in human monocytes. Circulating extracellular heat

shock protein 72 (eHsp72) acts as an inflammatory molecule and induces cytokine production in immune cells (A Asea, 2006). The precise biological role of eHsp72 in response to exercise-heat stress has not been fully elucidated; it is believed to contribute to the exercise-related inflammatory reaction (A Asea, 2003). Acknowledgements have been made by Ogura et al., (2008) that body temperature elevation, and increased circulating catecholamines by supplementation (M Whitham, Walker, & Bishop, 2006) or exercise response (Martin Whitham, Laing, Jackson, Maassen, & Walsh, 2007), in addition to thermal change increase eHsp72. Acute exercise-heat stress presents both thermal and sympathetic challenge and as such, changes in concentration might be used to describe the magnitude of stress presented to an individual or system exercising in different environments.

eHsp72 has been detected in peripheral circulation of healthy individuals (Pockley, Shepherd, & Corton, 1998) and is known to increase in response to single bouts of exercise (Walsh et al. 2001; Febbraio et al. 2002a; Fehrenbach et al. 2005). Thermal, oxidative, metabolic and chemical stresses are well reported stimuli for increased concentrations of intracellular (iHsp72), and eHsp72 (Welch 1992; Morimoto, 1994). Exercise in hot and humid environments increases physiological strain on the body in comparison with temperate conditions (Galloway and Maughan 1997). Combined with exercise (exercise-heat stress), environmental manipulation to induce hyperthermia (Fehrenbach et al. 2001; Oishi et al. 2002; Moran et al. 2006; Whitham et al. 2007; Sandström et al. 2008; Iguchi et al. 2012) have been reported as stimuli for further increasing eHsp72 compared to exercise alone. Indeed a strong relationship exists between plasma eHsp72 and core temperature (Ruell, Thompson, Hoffman, Brotherhood, & Richards, 2006; Sandström et al., 2009).

Repeated daily exposure to exercise and/or environmental stress results in sequential (i.e. day-on-day) increases in eHsp72 expression (Sandström et al., 2008). In vivo, such a paradigm is utilised in the attainment of a heat acclimated (HA) phenotype (Magalhães et al. 2010; Lorenzo et al. 2010; Lorenzo et al. 2011; Hom et al. 2012), with increases in iHsp72 expression accompanied by “classic” physiological adaptations (e.g. cardiovascular stability; reduced core temperature at rest and during exercise; more rapid sudomotor onset and efficiency; etc.) (Garrett et al., 2011). The response of eHsp72 to environmental factors has not been uniform, with significant increases (Whitham et al. 2007; Yamada et al. 2007; McClung et al. 2008; Magalhães et al. 2010; Périard et al. 2012), or no change (Marshall et al. 2006; Watkins et al. 2007; Hom et al. 2012) from rested basal values reported.

Exercise-heat stress research has largely implemented experimental designs where exogenous (external) factors of exercise intensity and exercise-heat stress conditions are controlled to elicit and measure changes in endogenous (internal) response. It is likely that endogenous factors are more relevant signals for stress response than exogenous variables; eHsp72 accumulation being one indicator of stress (Ruell et al., 2006). Establishment of appropriate endogenous markers and apparent minimum endogenous criteria for eHsp72 release could facilitate economical prescription of repeated exercise-heat sessions with intent of inducing the HA phenotype, a similar notion has been proposed by Gagnon et al,. (2013), with regards to investigating heat balance. More efficient procurement of HA typically achieved through exercise-heat stress exposures (≥30°C) of ≥60 min and repeated for 5 – 14 sessions (Garrett et al., 2011) would allow researchers and practitioners to prepare individuals most effectively for subsequent work in conditions presenting thermal challenge. At present the magnitude of expression has not yet been reported directly comparing changes in human eHsp72 following identical exercise in graded exogenous environments with description of changes eHsp72 compared with established endogenous physiological and thermal markers (peak and mean heart rate, core, and muscle temperature). The introduction of novel markers (rate of increase and change in core temperature, area under the curve (AUC) for core temperatures of 38.5°C and 39.0°C, duration spent exercising with core temperature ≥ 38.5°C and ≥ 39.0°C) may identify additional criteria for the prescription of exercise-heat stress based upon analysis of the acute response to stress.

The aim of this study was to determine whether increased concentration of eHsp72 were correlated to endogenous markers of heat strain, and to identify the most appropriate markers for exercise-heat administration in humans. It was hypothesised that a minimum endogenous criteria exists for the appearance of eHsp72 into extracellular spaces during acute exercise-heat stress, and that only exercising in very hot conditions would provide sufficient internal systemic strain for such appearance.

Methods

Volunteers

Ten healthy males (mean ± SD age 21.0 ± 0.5 years, height 172.1 ± 13.9 cm, nude body mass 71.1 ± 8.0 kg, body fat 14.7 ± 4.1%, peak oxygen uptake ($\dot{V}O_{2peak}$) 3.81 ± 0.60 L.min^{-1}) volunteered to participate in the study.

The confounding variables of smoking (Anbarasi, Kathirvel, Vani, Jayaraman, & Shyamala Devi, 2006), caffeine (Lu, Lai, & Chan, 2008), glutamine (Singleton, KD. 2004), generic supplementation (Hillman et al. 2011), thermal exposures (Selkirk et al., 2009), hypoxic exposures (Taylor, Midgley, & Chrismas, 2010), hyperbaric exposures (Taylor, Midgley, Sandstrom, Chrismas, & McNaughton, 2012) and alcohol (Taylor, Midgley, Chrismas, et al., 2010) were all controlled in line with previous work in the field (Taylor et al., 2011). Each volunteer was given instructions for dietary requirements in accordance with published guidelines and requested to maintain identical diets in the immediate 48hrs prior to each experimental session (Canada, 2009).

Participants were instructed to drink at least 500 ml of water 2 h before all exercise bouts (Sawka et al., 2007). A urine refractometer (Alago Vitech Scientific, Pocket PAL-OSMO, UK) was used to measure the hydration levels of the participants prior to commencement of each trial. A participant was deemed to be euhydrated if urine osmolality was <600 $mOsm \cdot Kg^{-1}$ H_2O. This experimental control was not violated for any participant for any of the experimental procedures.

After a full description of experimental procedures the protocol was approved by the institutional ethics committee and all subjects completed medical questionnaires and provided signed informed consent following the principles outlined by the Declaration of Helsinki of 1975, as revised in 2008.

Preliminary Testing

Prior to undertaking the experimental trials of the study, volunteers attended the laboratories whereby their anthropometric data was collected for height (cm) using a fixed stadiometer (Detecto Physicians Scales; Cranlea & Co., Birmingham, UK), and body density using calipers (Harpenden, Burgess Hill, UK) and a four site skin fold calculation (Durnin & Womersley, 1974). Following determination of body density, % body fat was calculated according to the method described by Siri (1956). Nude body mass (NBM) was recorded to 0.01 kg from digital scales (ADAM GFK 150, USA).

$\dot{V}O_{2peak}$ was determined as a means for estimating pre testing aerobic capacity and exercise intensity for the subsequent testing protocols. Volunteers performed an incremental $\dot{V}O_{2peak}$ test on a cycle ergometer (Monark e724, Vansbro, Sweden) at a starting intensity of 80W in temperate laboratory conditions (20°C, 40% relative humidity (RH)). Resistance was applied to the flywheel to elicit an increase of 24 $W.min^{-1}$ whilst the volunteer was informed to maintain a constant cadence of 80 rpm. The $\dot{V}O_{2peak}$ was considered as the highest $\dot{V}O_2$

obtained in any 10 s period and in line with the end-point criteria guidelines of the British Association of Sport and Exercise Sciences (Winter, 2007). Expired metabolic gas was measured using online gas analysis (Metamax 3X, Cortex, Germany). All preliminary testing was performed on the same ergometer (Monark, e724, Vansbro, Sweden). Heart rate (HR) was recorded during all exercise tests by telemetry (Polar Electro Oyo, Temple, Finland). Power outputs corresponding to 50% $\dot{V}O_{2peak}$ were calculated from the $\dot{V}O_2$: power output relationship. Saddle position was adjusted by the volunteer to their preferred cycling position and remained unchanged for all trials. During all trials volunteers wore shorts, socks, and shoes.

Experimental Protocol

Volunteers presented to the laboratories 60 min prior to testing. Time of day for testing was held constant (10:00 ± 01:00 h) to control for the effects of daily variation in performance (Drust, Waterhouse, Atkinson, Edwards, & Reilly, 2005) and HSP expression (Sandström et al., 2009; Taylor, Midgley, Chrismas, et al., 2010).

Following determination of NBM and hydration status the volunteer then inserted a disposable rectal thermistor (Henleys Medical, UK, Meter logger Model 401, Yellow Springs Instruments, Yellow Springs, Missouri, USA; accuracy ±0.20°C) 10 cm past the anal sphincter for measurement of rectal temperature (T_{rec}). Intramuscular temperature (T_{mu}) was recorded using a muscle temperature probe (Ellab Medical Precision Thermometer, Copenhagen). A 2-g sample of an anaesthetic cream (EMLAi Cream 5%; AstraZeneca Ltd., Bedfordshire, UK) was applied to the right vastus lateralis muscle 30 min before measurement of resting muscle temperature. With participants seated with the lower leg supported at 90°, a needle (18 G 1.5 inches; BD Microlance 3, Drogheda, Ireland) and a sterile, flexible muscle temperature probe (medical precision thermometer; Ellab, Copenhagen, Denmark) were inserted 4 cm into the belly of the vastus lateralis until a constant temperature was recorded. After removal of the needle, pressure and small adhesive bandage were applied to the entry site to prevent bleeding in accordance with methods described by Duffield et al. (2010).

Volunteers mounted the cycle ergometer located inside a purpose built environmental chamber with temperature and humidity controlled using automated computer feedback (WatFlow control system; TISS, Hampshire, UK), and were instructed to perform 90 min of continuous cycling exercise at 50% $\dot{V}O_{2peak}$ (50% $\dot{V}O_{2peak}$ = 1.90±0.30 L.min^{-1}, Power at 50% $\dot{V}O_{2peak}$ = 120 ± 26 W) in either temperate (TEMP; 20.3°C ± 0.4°C, 51.9 ± 14.0% RH; wet globe bulb temperature (WGBT) 15.8°C), hot (HOT; 30.2°C ± 0.1°C, 52.7 ± 3.0% RH; WGBT

24.5°C) and very hot (VHOT; 40.2°C ± 0.4°C, 39.0 ± 7.8% RH; WGBT 31.6°C) conditions. The sequence was decided by latin square design.

During each testing session HR, rating of perceived exertion (RPE, (Borg, Ljunggren, & Ceci, 1985)), thermal sensation (TSS, (Gagge, Stolwijk, & Saltin, 1969)) and T_{rec} were recorded. T_{mu} was measured immediately before and after the cessation of each trial. Later, sweat rate was calculated, derived from a change in NBM. Heat strain was calculated using Physiological Strain Index (PSI) (D S Moran, Shitzer, & Pandolf, 1998) as follows:

$PSI = (5 * (T_{rec1} - T_{rec0})/ ((39.5 - T_{rec0})) + (5 * (HR_1 - HR_o) * (180-HR_0))$. Where $_O$ indicates basal values and $_1$ indicates experimental values.

The T_{rec} area under the curve (AUC) was calculated using a modification to the trapezium rule (Hubbard et al., 1977) when T_{rec} exceeded 38.5°C (Cheuvront et al., 2008) and 39.0°C. AUC for T_{rec} >38.5°C or AUC for T_{rec} >39.0°C was calculated as:

AUC T_{rec} ≥38.5°C (°C.min^{-1}) = ∑ time interval (min) x 0.5 [°C > 38.5°C at the start of exercise-heat stress + °C > 38.5°C at the end of exercise-heat stress].

AUC T_{rec} ≥39.0°C (°C.min^{-1}) = ∑ time interval (min) x 0.5 [°C > 39.0°C at the start of exercise-heat stress + °C > 39.0°C at the end of exercise-heat stress].

In compliance with ethical approval, exercise was terminated if a subject attained a T_{rec} of 39.7°C.

Blood Sampling and Analysis

Venous blood samples were taken immediately pre- and post- and 24 hr post-test TEMP, HOT and VHOT exercise. A 10 ml whole blood sample was drawn from the antecubital fossa. Each sample was divided equally into 5 ml tubes (Starstedt, Germany) containing EDTA as anticoagulant. Whole blood samples were centrifuged (Eppendorf 5804 R Centrifuge) at 4,500 rpm for a period of 15 min to separate plasma. Plasma was pipetted (Eppendorf Research/Research Pro) into 1.5 ml microtubes (Eppendorf) and stored at -86°C (Sanyo Ultra Low, VIP Series) until analysis which utilised a commercially available HSP70 high sensitivity enzyme immunometric assay kit (Enzo Life Sciences, Michigan, USA). Quantitative determination of the inducible

Hsp72 was performed according to manufacturers' guidelines. Incubation of the 96 well kit, including the required quality control standards was performed on an orbital shaker (Heidolph Titramax 1000) at 600 rpm, and read by a platereader using absorption at 450 nm (El_x 800 Universal Microplate reader, Bio-Tek Instruments). Plasma Hsp72 concentrations were corrected for changes in venous plasma volume (Dill & Costill, 1974) with haemoglobin collected in duplicate using a microcuvette and analysed using a B-Haemoglobin Photometer (Hemocue Limited, Ängelholm, Sweden) and haematocrit collected in triplicate (~50 µl) with glass capillary tubes and analysed following centrifugation at 12-14000 rpm for 3 min (Haemotospin 1300 Centrifuge, Hawksley & Sons Ltd, West Sussex, UK).

Accuracy of the sample data was ensured by plotting a graph for linearity between known sample concentrations and optical density. A linear trendline and equation was used to translate raw plate reader result into Hsp72 units ($ng.mL^{-1}$). The intra/inter-assay variability was 10.5/17.36%, respectively. The assay sensitivity is described by the manufacturer as 0.09 $ng.ml^{-1}$ and the detection range of the assays were 0.20-12.5 $ng.ml^{-1}$ for Hsp72.

Statistical Analysis

All statistical calculations were performed using PASW software version 18.0 (SPSS, Chicago, IL, US). All outcome variables were assessed for normality of distribution and sphericity prior to further analysis and deemed plausible in all instances unless otherwise stated. A two-way (time x trial) repeated-measures Analysis of Variance (ANOVA) was performed to test significance between and within trials. One-way ANOVA with repeated measures was used to compare physiological, perceptual and thermal data between exogenous environments, bonferroni pairwise comparisons compared between separate exogenous temperature conditions.

Stepwise multiple regression analysis was performed for the six dependent variables which yielded the strongest relationship to the increase in eHsp72 concentration (rate of change in T_{rec} ($°C.hr^{-1}$), peak T_{rec} (°C), mean T_{rec} for the final 60 min (°C), duration T_{rec} ≥39.0°C (min), change in T_{rec} (°C), duration T_{rec} ≥38.5°C (min)). Nine volunteers' data were used for the model as no eHsp72 was detected for one volunteer. Data was reported as mean ± SD, with two tailed significance was accepted at $p < 0.05$.

Results

Physiological and Perceptual Measures

Mean duration for VHOT trial lasted only 86.5 ± 7.5 min in comparison to TEMP and HOT owing to two participants terminating early as Trec reached 39.7°C. No difference (f = 2.194, p = 0.140) was reported for the duration exercising in each exogenous temperature condition see table 1.

Peak (f = 28.650, p < 0.001) and mean (f = 19.951, p < 0.001) HR were significantly higher in HOT than TEMP conditions (141 ± 16 and 132 ± 13 b.min^{-1}; p < 0.001), whilst VHOT was significantly higher than TEMP (p = 0.001 and p = 0.001) and HOT (p = 0.018 and p = 0.045) see figure 1.

Calculated sweat rate was significantly different between conditions (f = 4.204, p = 0.032). VHOT (15.8 ± 4.3) was significantly greater (p = 0.042) than TEMP and HOT conditions, no difference existed between TEMP and HOT (p = 0.153), see table 1.

Perceptual measures RPE and TSS demonstrated significant difference between conditions (RPE f = 103.360, p < 0.001) (TSS f = 71.602, p < 0.001) (table 1), with peak scores significantly increasing from TEMP to HOT (p = 0.021 and p < 0.001); VHOT was significantly higher (p = 0.008 and p < 0.001) from TEMP and HOT trials. Mean RPE was significantly different between conditions (f = 22.946, p < 0.001), but only significantly greater between VHOT and TEMP and HOT conditions (p = 0.003). Mean TSS was significantly different between all conditions (f = 76.518, p < 0.001), TEMP was significantly lower than HOT (p = 0.000) and VHOT was significantly greater from TEMP (p < 0.001) and HOT (p = 0.001).

Temperature Measures

Table 2 reports the values for peak T_{rec}, statistically different between all conditions (f = 59.838, p < 0.001). TEMP was significantly lower than HOT (p = 0.002); VHOT was significantly higher than TEMP (p < 0.001) and HOT (p < 0.001). Mean T_{rec} for the time between 30 and 90 min was significantly different (f = 35.906, p < 0.001) with HOT significantly higher than TEMP (p = 0.028) and VHOT significantly higher than TEMP (p < 0.001) and HOT (p < 0.001).

The change in T_{rec} was significantly different between conditions (f = 33.621, p < 0.001), but post hoc analysis only observed significantly greater differences between VHOT, and TEMP and HOT (p < 0.001). This was also true of the rate of T_{rec} increase (f = 37.475, p < 0.001), where VHOT elicited a significantly greater rate compared to TEMP and HOT (p < 0.001).

Area Under Curve for T_{rec} of 38.5°C (f = 4.045, p = 0.035) and 39.0°C (f = 7.163, p = 0.005) (°C.min^{-1}) were significantly different between conditions overall, VHOT was significantly greater compared with TEMP and HOT (p = 0.003 and p = 0.013), but no difference was observed between TEMP and HOT.

Duration spent with rectal temperatures of ≥38.5°C (f = 18.475, p < 0.001) and ≥39.0°C (f = 9.631, p = 0.001) (min) displayed significant main effect difference but was not different between TEMP and HOT, however VHOT was significantly longer than TEMP and HOT (p = 0.014 and p = 0.06).

Main effect for end T_{mu} was observed as significant (f = 36.381, p < 0.001). Significant difference was also found between TEMP and HOT (p = 0.001); VHOT was significantly higher from TEMP (p < 0.001) and HOT (p = 0.003). The change in T_{mu} was only significantly greater between VHOT, and TEMP and HOT (p = 0.003) despite overall difference (f = 26.836, p < 0.001). Thermal data for each trial is presented in table 2.

Overall difference was observed for peak (f = 76.949, p = 0.000) and mean PSI (f = 21.278, p < 0.001) with significantly higher values observed between VHOT, and both TEMP and HOT conditions (p < 0.001 and p = 0.005, respectively), see table 1. Peak PSI was also significantly lower in TEMP compared to HOT (p = 0.003), no significant difference was observed for mean PSI (p >0.05). Figure 1 details the change in HR, T_{rec} and PSI for each condition over time.

Extracellular HSP70 expression

No difference (f = 1.677, p = 0.218) was reported in eHsp72 expression (ng.mL^{-1}) for pre testing expression during TEMP, HOT and VHOT experimental sessions. eHsp72 expression (ng.mL^{-1}) was observed as significantly different for the main effect (f = 5.928, p = 0.012) with the significant difference observed as an increase from pre to post VHOT (0.266 ± 0.094 to 0.724 ± 0.444). Following post hoc analysis no difference was found for the effect of temperature or condition in TEMP (p = 1.000) and HOT (p = 0.766) (0.349 ± 0.135 to 0.342 ± 0.165, and 0.299 ± 0.122 to 0.376 ± 0.226 respectively). No significant difference (p > 0.05) was observed between pre and 24hrs post in any exercise-heat condition. eHsp72 data presented as a percentage change from baseline, in line with previous work, for post (TEMP -1.9%; HOT +25.7%; VHOT +172.4%) and 24hrs post (TEMP -8.6%; HOT 2.6%; VHOT 17.1%) are presented in figure 2.

Relationship between eHSP70, Temperature and Physiological measures

Rate of change in T_{rec} (r = 0.702), peak rectal temperature (r = 0.655), mean T_{rec} for the final 60 min (r = 0.651), duration T_{rec} ≥39.0°C (r = 0.635), change in T_{rec} (r = 0.632), peak PSI (r = 0.603), duration T_{rec} ≥38.5°C (r = 0.559), and peak HR (r = 0.327), were submitted to a stepwise multiple regression to predict post exercise-heat exposure. The first predictor variable to enter the model was rate of change in T_{rec}; the second and final predictor variable to enter the model was change in T_{rec}. The adjusted R^2-value for this model was 0.473 and standard error of the estimate 0.228.

Discussion

The aim of this study was to determine the endogenous effects of exercise matched for power output and duration in three exogenous thermal environments on the plasma eHsp72 concentration responses. Significant changes in concentration occurred only pre to post in the VHOT group, supporting the hypothesis that endogenous thermal and physiological strain elicited only in VHOT conditions provided sufficient stimuli for eHsp72 response during exercise-heat stress. This is in line with other authors with similar experimental designs to the present study (McClung et al. 2008; Magalhães et al. 2010; Périard et al. 2012). Established endogenous physiological and thermoregulatory parameters, particularly those less commonly reported in literature determining eHsp72 changes (rate of T_{rec} increase, area under the curve (AUC) for T_{rec} of 38.5°C and 39.0°C, duration T_{rec} ≥ 38.5°C and ≥ 39.0°C), taken during each condition were analysed to determine whether they could be used to describe more effectively internal heat strain leading to increased eHsp72 concentration.

The physiological and thermoregulatory responses to each exercise-heat stress condition were as expected for matched exercise in increasing thermal environments (Galloway & Maughan, 1997; Maughan et al., 2012). Data observed three levels of strain between TEMP, HOT and VHOT conditions for peak HR, T_{rec}, PSI, and end T_{mu} suggesting that each exogenous condition was placing independent magnitudes of strain. Other thermoregulatory data (change in T_{rec}, rate of T_{rec} increase, AUC for T_{rec} of 38.5°C and 39.0°C, duration T_{rec} ≥ 38.5°C and ≥ 39.0°C, and change in T_{mu}) however were in agreement with the experimental rationale, describing two levels, where VHOT was different from TEMP and HOT, but no difference was observed between TEMP and HOT. The thermal and physiological data suggests that VHOT was of greater exercise-heat stress than TEMP and HOT; an observation paralleled by the increased concentration of eHsp72 being only reported in VHOT pre to post exercise. Regrettably, no data was collected that measured skin temperature, this addition in future research studies would allow for the calculation of whole body temperature (Burton, 1935) and the inclusion of this descriptor of endogenous strain. The observation from regression analysis that the rate

of increase, and the delta change in T_{rec} are important factors in changing eHsp72 expression is in line with the observations of Périard et al. (2012) for whom exercising at 75% $\dot{V}O_{2peak}$, revealed a relationship emerged between eHsp72 and the rate of increase in T_{rec}. The authors surmised that this was possibly due to a greater metabolic demand and energy conversion increasing T_{rec} (i.e., intensity dependent). In the present study it appears despite a lower intensity of work the exogenous conditions were sufficient to elicit different endogenous responses and eHsp72 concentrations. As only two (rate of change in T_{rec}, and change in T_{rec}) of seventeen initial dependent variables (table 1 and 2) were accepted into the regression model, it remains that changes in eHsp72 concentration is multi-factorial and that whilst ensuring endogenous thermal strain is of sufficient onset and magnitude, these determinants are only elements determining the change in concentration. These observations do however, give greater insight into means for facilitating the most economical prescription of thermal and exercise intensity components of repeated exercise-heat sessions.

The present study reported eHsp72 as only increasing immediately following the VHOT trial, with values returning to baseline within 24 hrs (Figure 2). Increased systemic eHsp72 has been shown to be exercise intensity and duration dependant in temperate conditions (Fehrenbach et al., 2005), with the addition of thermal stress (evidenced by increase T_{rec}) further increasing the magnitude of response (Marshall et al. 2006). Consequently, a heat storage independent threshold of 38.5°C (T_{rec}) has been postulated (F. T. Amorim, Yamada, Robergs, Schneider, & Moseley, 2008) and demonstrated central to the magnifying influence of thermal stress on eHsp72 concentrations (F. T. Amorim et al., 2008), compared to moderate intensity matched exercise. Data from present study supports this "minimum endogenous criteria" notion (table 2). VHOT elicited a greater internal temperature, rate of internal temperature rise and a greater duration at critical T_{rec} than TEMP and HOT, which is, supportive of the existence of minimum endogenous criteria for the induction of eHsp72 into the circulation during exercise heat stress as suggested by Amorin et al., (2008). Supporting the absence of eHsp72 increases in TEMP and HOT, T_{rec} of 37.90 ± 0.29°C and 38.35 ± 0.52°C, respectively in the present study, parallel exercise induced changes in T_{rec} data (mean maximum T_{rec} 38.48°C) resulting in no change in basal eHsp72 reported by others (Hom et al, 2012), during treadmill walking at 33°C, 30-50%RH. The present study supports the notion (Amorin et al 2008) that mean T_{rec} must exceed >38.5°C to initiate increases in eHsp72, with increases in T_{rec}, even within a thermally challenging environment insufficient to induce such elevations without T_{rec} >38.5°C.

Mechanistically, temperatures >38.5°C at the hepatosplanchnic viscera are perhaps the most important, with duration and magnitude or eHsp72 release dependant on the magnitude and duration above this element of the "threshold" (Rhind 2004; Selkirk et al, 2008, 2009). However, recent evidence (Périard et al., 2012) suggests that the same eHsp72 expression is yielded by short (27.2 min) and longer duration (58.9 min) trials by increasing exercise intensity (from 60% to 75% of $\dot{V}O_{2peak}$) with this similarity in eHsp72 expression despite differences in peak and mean T_{rec} (39.0°C and 39.7°C respectively). The data from Periard et al (2012), at least superficially, indicates that both magnitude and duration above >38.5°C is irrelevant within normal physiological boundaries (i.e. non-life threatening physical and occupational pursuits) and that it is exceeding T_{rec} of >38.5°C that is the most potent stimuli of increases in eHsp72 when combined with exercise stress.

Attenuation of release may likely occur once T_{rec} returns below "minimum endogenous criteria", although the precise duration taken for full cessation of Hsp72 release requires further elucidation – the presented data suggest this occurs sometime between immediately and 24 hr post exercise (figure 2). This pattern of elevation and return to baseline in VHOT, as observed during the first tolerance test by Magalhães et al. (2010) or, observed reduction following elevation (Marshall et al. 2006; Périard et al. 2012) from baseline, highlights the transient eHsp72 response to stress followed by removal from the circulation. However, caution must be exercised when inferences to a critical endogenous criteria model is made across a broad demographic of exercise capacities (i.e. untrained through to highly trained) as such differences are known to influence eHsp72 release kinetics and magnitudes within thermally challenging environments (Selkirk et al 2008, 2009). Therefore, future work should tightly control this potentially confounding variable.

Hepatosplanchnic and brain tissue, and peripheral blood mononuclear cells appear the principle sources of Hsp72 release into the systemic circulation (Febbraio et al., 2002; Johnson & Fleshner, 2006; G I Lancaster & Febbraio, 2005; G I Lancaster et al., 2004). Concise reviews of the proposed active and passive mechanisms of eHsp72 release are presented by Lancaster and Febbraio (2005), Fleshner and Johnson (2005) and Asea (2007). Briefly, it is proposed (Multhoff & Hightower, 1996) that exosomes secreted following the fusion of multivesicular bodies with the plasma membrane, provide the secretory pathway for cells to actively release Hsp72 (Lancaster and Febbraio 2005). It has also been proposed (Ogawa et al., 2011) that eHsp72 is triggered by circulating ATP during exercise. Further to this, it has been reported (Johnson & Fleshner, 2006) that hormone receptor mediated pathways exist allowing elevation of eHsp72 during stress. Authors demonstrated that norepinephrine may stimulate a receptor-mediated exocytotic pathway of eHsp72 release. An indirect

consequence of exercising at an elevated temperature is that of elevated cardiovascular demand and associated α-adrenergic stimulation as a means for maintaining work rate and required demands to exercising muscle, whilst attempting thermoregulation. VHOT elicited the greatest heart rate response to the exercise presented, as such this indirect measure of sympathetic activity occurring through physiological and thermal strain, supports this release mechanism. This mechanism is further evidenced by the work of Whitham et al., (2006) whom observed caffeine supplementation and increase plasma catecholamines as elevating eHsp72. Périard et al. (2012) commented that the release of eHsp72 into extracellular locations is likely to originate from varied tissues and cell types, each potentially affected by specific mechanisms of release and various inducing factors. The significance of a post-exercise increase in eHsp72 remains unclear, proposed immunological functions (Campisi, Leem, & Fleshner, 2003) as a signal for cytokine and inflammatory pathways in response to unaccustomed systemic or whole body stress (a Asea et al., 2000). Appear most relevant whereby VHOT exercise-heat stress in that trial was of a magnitude sufficient to induce an immunological response which the TEMP and HOT trials were not (figure 2).

The degree of hyperthermia during exercise-heat stress, be it induced by exogenous environment or prescribed workload, has so far been proposed central to whether Hsp72 is expressed/released, or not. It has been demonstrated that participants exposed to temperatures similar to that of VHOT (Magalhães et al., 2010; McClung et al., 2008; Yamada et al., 2007) where mean calculated heat stress was 32.46°C (WGBT), elicited largest increases in Hsp72. Marshall et al. (2006) used a greater calculated exogenous heat stress than VHOT (33.1°C WGBT) combined with lower (38% and 42.5% $\dot{V}O_{2peak}$) exercise intensity, eliciting core temperatures of 38.2°C. No change in eHsp72 was observed, suggesting that the exercise intensity/workload was insufficient in their experiment to elicit the desired thermal response, and is not presenting sufficient exercise-heat stress.

In a matched thermal environment, exercise intensity contributes to the rate of temperature increase and the degree of hyperthermia (Mora-Rodriguez et al. 2008). Whilst exercise intensity alone has been associated with increased iHsp72 (Milne and Noble 2002; Liu et al. 1999), and eHsp72 (Whitham et al. 2007; Périard et al. 2012) responses to hyperthermia and the sympathetic adrenergic stimulation of exercise offers a further insight into eliciting the greatest response based upon endogenous criteria. Whitham et al. (2006) demonstrated increased eHsp72 was associated with higher plasma levels of catecholamines and heart rate, whilst it has also been observed that following passive heating, neither epinephrine nor norepinephrine were solely responsible for eHsp72 release (Whitham et al. 2007).

The most recent, and most explicit evidence from exercise-heat stress (Periard et al. 2012) suggests that the same eHsp72 expression is yielded by short (27.2 min) and longer duration (58.9 min) trials by increasing intensity (from 60 % to 75 % of $\dot{V}O_{2peak}$). This similarity was despite differences in core temperature (39.0°C and 39.7°C respectively) albeit with both groups passing the proposed 38.5°C threshold (F. T. Amorim et al., 2008). Potential explanation could be reflected by the difference in AUC in the 60% trial, from the 75% trial, or that eHSP72 increases at a maximal rate after an exercise intensity threshold has been achieved, either alongside, or in the absence of thermal strain. Johnson and Fleshner (2006) identified α-adrenergic stimulation as responsible for Hsp72 release into the circulation, this alongside the work of Whitham et al. (2006, 2007) suggest a requirement for individuals to be presented with sustained physiological challenge during exercise – heat stress (Johnson et al. 2005). Exercise intensity, or α-adrenergic stimulation is potenitally required to be above an intensity threshold to elicit significant eHsp72 response with the greater exercise intensity data from Periard et al. (2012) leading to data contrasting that of Marshall et al. (2006). The extent to which the adrenergic contribution is required is difficult to determine precisely,

from the present study it appears with only the VHOT trial eliciting changes in eHsp72 that a mean HR, an indirect measure of sympathetic activation, of 153 ± 14 $b.min^{-1}$ is required from the intensity 50% of $\dot{V}O_{2peak.}$ The intensity of this trial may however be of greater physiological strain as a result of the increased thermoregulatory requirements which are known to increase proportionally to the ambient conditions (Galloway & Maughan, 1997; Maughan et al., 2012). Periard et al. (2012) reported HR values greater than the present study reflecting the elevated work intensity. As with the analysis of Periard et al. (2012), our regression analysis deemed HR responses insufficient predictor elements of change in eHsp72 concentration. The significant difference in HR between VHOT and, TEMP and HOT alongside elevated eHSP72 in only VHOT despite matched power, is therefore explained by the elevated cardiovascular consequence of increased thermal strain whilst maintaining power output, rather than the thermal strain being a the primary mediator of eHsp72 response.

Magalhães et al. (2010) observed only the first of two heat stress tests separated by 10 days of HA as reporting increases in eHsp72. Authors speculated that the higher iHsp72 observed following translocation of heat shock factor-1 and trimeric activation of the heat shock element promoter region of HSPA1A after HA, may have elicited increased cellular tolerance, which in combination with reduced T_{rec} and HR adaptations made through

HA, are likely to have protected participants from the same degree of cardiovascular instability and thermal strain during the second heat stress test exercise bout, and, thus, a mechanism involving release of eHsp72 to induce an inflammatory response was inhibited.

Present data fails to elucidate the precise minimum requirement for sympathetic contribution to Hsp72 transcription or translocation as identified by other research (Johnson & Fleshner, 2006) through exercise or supplementary pathways. Analysis of plasma catecholamine response would've contributed towards this known mediator regulating the release of Hsp72 in the present study. It is well reported that elevated temperature, derived from external environment, passively or through active means, leads to elevated cardiac strain (HR) and as such these two fundamental variables cannot be divided when considering the whole body response to exercise heat stress. As regression analysis has failed to accept HR as a predictor of eHsp72 in favour of thermal markers as such we cannot ignore the identification of previous discussed endogenous thermal markers despite early research demonstrating increases in eHsp72 independent of changes in core temperature as a consequence of increased plasma catecholamines. Our data acknowledges the role of HR, and more specifically the elevated cardiac contribution to exercise in the VHOT condition in comparison to HOT and TEMP conditions. It is therefore proposed that sympathetic activity, most rudimentarily measured from exercising HR is an important component of the minimum endogenous criteria for increasing eHsp72 during exercise-heat stress alongside the thermal criteria. Rather than the heat directly modulating elevated eHsp72 expression, it appears to be indirectly modulating it through via increased HR, a simple marker of adrenergic/catecholamine contribution to exercise-heat stress.

It has been reported recently that core temperature (Ruell et al. 2006; Periard et al. 2012), rate of core temperature increase (Periard et al. 2012), and interestingly, aerobic capacity (Périard et al. 2012) are endogenous factors relating to Hsp72 increases in line with the data presented within this study. In light of this, further work appears warranted to determine the role parasympathetic/sympathetic drive has in determining eHsp72 release during exercise-heat stress in individuals not acclimated to the strain presented.

It is known that training status influences the basal and eHsp72 stress response to exercise-heat stress. In addition, prior HA, or progress towards the phenotype via endurance training may elevate the immune response threshold for inducement of eHsp72 via exercise-heat stress. Njemini et al., (2004) also observed that inflammatory status, and it's variable nature is also linked to eHsp72. Selkirk et al., (2008, 2009) acknowledged

454 that the threshold for enhanced iHsp72 response, endotoxin leakage and inflammatory activation during
455 exertional heat stress, in similar exogenous conditions to the present study, occurs at a lower temperature in
456 untrained compared with trained subjects and support the endotoxin translocation hypothesis of exertional heat
457 stroke, linking endotoxin tolerance and heat tolerance.

458 This individual and changing threshold along a continuum modulated by thermotolerance, inflammatory, and
459 training status, suggests that prescription of exercise-heat stress exposure, administered controlling only simple
460 parameters such as exogenous environment and work rate, may ultimately fail to stress sufficiently some
461 individuals. The present data can therefore be used as a guide towards acute exercise heat stress prescription. It
462 is also important to consider that parameters appropriate for acute interventions shift with repeated exposures, as
463 the HA phenotype and concurrent acquired cellular thermotolerance is enhanced (Sandström et al. 2008;
464 Magalhães et al. 2010; Hom et al. 2012). Based upon these comments and the observation from the regression
465 analysis that the rate of increase in T_{rec} (VHOT 1.56 ± 0.53 °C.hr^{-1}) and the delta change in T_{rec} (VHOT 2.22 ±
466 0.65°C), it may be more appropriate to implement an isothermic (controlled hyperthermia) model of exercise-
467 heat exposure (Garrett et al., 2012, 2011) where the rate of heat production can be accelerated (F. T. Amorim et
468 al., 2008) and proposed minimum endogenous temperatures targeted (F. Amorim et al., 2011). This model
469 requires greater exercise intensity during the early stages of the exposure, thus ensuring a more rapid increase in
470 T_{rec} and consequently greater change in T_{rec}, followed by a reduction in workload once a desired temperature has
471 been achieved. The benefit of the isothermic model of exercise-heat stress is that specific endogenous
472 temperatures can be targeted, rather than being an uncontrolled response varying on an individual basis, with the
473 potential for more individualised prescription. This model of clamping at a set core temperature is an effective
474 means for mediating increases of circulating stress hormones, which subsequently contribute to induction of
475 circulating cytokine release (Rhind et al., 2004).

476 The duration in which individuals are in a state of hyperthermia may also be a contributing factor towards
477 increasing eHsp72 concentrations and as such be reflective of a greater overall "dose" of endogenous strain in
478 comparison to a short exposure to extremes of either variable. The more rapid increase in core temperature
479 during the isothermic model could be implemented to ensure a greater percentage of the total exposure time is at
480 or above the desired endogenous threshold for eHsp72 release. Whilst eHsp72 is a useful marker for describing
481 stress it should be noted that no direct role exists between secreted eHsp72 and attainment of HA. Future work
482 should consider the iHsp72 response to exercise-heat stress which might provide greater insight into acquired

cellular thermotolerance and the acquirement of HA. Within these experimental designs the confounding variable of training status and its influence on the prescription of the stress should be controlled to assess the most effective means for increasing iHsp72 gene expression and total protein in tandem with measures of eHsp72. Such data should be used to assess the global HSP response in line with the proposed eHsp72 centric minimum endogenous criteria.

In summary, it appears likely that a minimum endogenous criteria contributes to the multifactorial release of eHsp72 into the circulation during acute exercise-heat stress, a pathway that may differ from pathological stress resulting in systemic inflammation. Our data observed the endogenous requirement for release as being a minimum core temperature peak of 39.2°C, a change of 2.2°C from baseline, or achieving a mean of 38.6°C for a period of 56.5 min following a rate of increase of 1.6°C.hr^{-1} alongside heart rate requirements of 153 ± 14b.min^{-1}.

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