

1 **Title**

2 Hsp72, and Hsp90 α mRNA transcription is characterised by large, sustained changes in core temperature during heat
3 acclimation.

4

5 **Short Title**

6 Characterising Hsp72, and Hsp90 α mRNA transcription

7

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34 **Abstract**

35 Increased intracellular heat shock protein-72 (Hsp72), and -90 α (Hsp90 α) have been implicated as important components
36 of acquired thermotolerance, providing cytoprotection during stress. This experiment determined the physiological
37 responses characterising increases in Hsp72 and Hsp90 α mRNA on the first and tenth day of 90 min heat acclimation (in
38 40.2°C, 41.0% RH) or equivalent normothermic training (in 20°C, 29% RH.). Pearson's product-moment correlation and
39 stepwise multiple regression were performed to determine relationships between physiological [e.g. (T_{rec} , sweat rate (SR)
40 and heart rate (HR)] and training variables (exercise duration, exercise intensity, work done), and the leukocyte Hsp72
41 and Hsp90 α mRNA responses via RT-QPCR (n=15). Significant ($p<0.05$) correlations existed between increased Hsp72
42 and Hsp90 α mRNA ($r=0.879$). Increased core temperature was the most important criteria for gene transcription with
43 ΔT_{rec} ($r=0.714$), SR ($r=0.709$), $T_{rec\text{final}45}$ ($r=0.682$), area under the curve where $T_{rec}\geq 38.5^\circ\text{C}$ (AUC $_{38.5^\circ\text{C}}$; $r=0.678$), peak
44 T_{rec} ($r=0.661$), duration $T_{rec}\geq 38.5^\circ\text{C}$ ($r=0.650$) and ΔHR ($r=0.511$) each demonstrating a significant ($p<0.05$) correlation
45 with the increase in Hsp72 mRNA. The T_{rec} AUC $_{38.5^\circ\text{C}}$ ($r=0.729$), ΔT_{rec} ($r=0.691$), peak T_{rec} ($r=0.680$), $T_{rec\text{final}45}$
46 ($r=0.678$), SR ($r=0.660$), duration $T_{rec}\geq 38.5^\circ\text{C}$ ($r=0.629$), the rate of change in T_{rec} ($r=0.600$) and ΔHR ($r=0.531$) were the
47 strongest correlate with the increase in Hsp90 α mRNA. Multiple regression improved the model for Hsp90 α mRNA
48 only, when T_{rec} AUC $_{38.5^\circ\text{C}}$ and SR were combined. Training variables showed insignificant ($p>0.05$) weak ($r<0.300$)
49 relationships with Hsp72 and Hsp90 α mRNA. Hsp72 and Hsp90 α mRNA correlates were comparable on the first and
50 tenth day. When transcription of the related Hsp72 and Hsp90 α mRNA is important, protocols should rapidly induce
51 large, prolonged changes in core temperature.

52 **Introduction**

53 Thermotolerance is an acquired cellular adaptation to heat stress (Kuennen et al. 2011) conferring cytoprotection to
54 subsequent thermal (McClung et al. 2008) and non-thermal (Gibson et al. 2015c) stress *in vitro* (McClung et al. 2008)
55 and *in vivo* (Lee et al. 2016). Acquired thermotolerance is reliant upon sufficient heat shock protein (HSP) gene
56 transcription (Moran et al. 2006) and subsequent protein translation (Silver and Noble 2012). Functionally, HSPs
57 facilitate maintenance of cellular and protein homeostasis, with regulatory roles in mitigating apoptosis, and facilitating
58 recovery from and adaptation to stress [including exercise training (Liu et al. 1999) and/or thermal stress (Kuennen et al.
59 2011)] at a cellular, organ, and whole-body level (Henstridge et al. 2016).

60

61 The HSP70 family present in two predominant isoforms; a constitutively expressed protein that demonstrates little basal
62 change, HSC70 (HSP73), and a highly inducible ‘chaperone’ isoform HSP72 (HSPA1A / HSPA1B) central to
63 cytoprotection (Kampinga et al. 2009). HSP90 also presents with a constitutively expressed isoform (HSP90 β) and an
64 inducible isoform, HSPC1 (HSP90 α) (Subbarao Sreedhar et al. 2004). HSP72 provides cellular protection, notably
65 maintaining intestinal epithelial tight junction barriers, increasing resistance to gut-associated endotoxin translocation,
66 and reducing inflammatory responses to stress (Moseley 2000; Amorim et al. 2015; Dokladny et al. 2016). In addition
67 HSP72 may be important in facilitating positive heat (Kuennen et al. 2011), and heat independent adaptations
68 (Henstridge et al. 2016). HSP90 α is cytoprotective, similar to HSP72, whilst also implicit in recovery and adaptation to
69 cellular stress, particularly control of cellular signalling cascades (Taipale et al. 2010), recovery of global protein
70 synthesis (Duncan 2005), and coordination of cellular repair (Erleijman et al. 2014). Increases in extracellular HSP72
71 (eHSP72) have been widely observed in response to acute exercise (Whitham et al. 2007; Périard et al. 2012; Gibson et
72 al. 2014), with endogenous criteria, notably increased core temperature most important for eliciting large increases
73 (Périard et al. 2012; Gibson et al. 2014). These eHSP72 increases are transient (Périard et al. 2012; Gibson et al. 2014),
74 and have a proposed immunological role (Asea 2003) rather than initiating chronically beneficial (i.e. cytoprotective)
75 HSP72 protein translation that is retained beyond the initial stressor (Marshall et al. 2007; Périard et al. 2015). Therefore,
76 the usefulness of extracellular HSPs to characterise acquired thermotolerance (Moseley 1997; Kregel 2002), identify
77 cessation of the cellular stress response following adaptation *in vivo* (McClung et al. 2008; Kuennen et al. 2011), and *ex*
78 *vivo* (McClung et al. 2008), or to identify functional roles in disease states (Henstridge et al. 2014a; Krause et al. 2015a),
79 is inferior to that of the HSP gene transcript or translated protein (Lee et al. 2015). At present, the precise physiological
80 signals for increasing Hsp72 mRNA and Hsp90 α mRNA are unknown, as is whether these genes transcribe to similar
81 stimuli, and similar magnitudes during exercise/exercise-heat stress. Accordingly, similar characterisation of Hsp72 and
82 Hsp90 α gene transcription to that of eHSP72 is required given their direct relationship with thermotolerance (Lee et al.
83 2015).

84

85 Exercise elicits numerous cellular and molecular stressors that in isolation, or combination behave as inductive stimuli
86 for increases in HSPs (Henstridge et al. 2016). Stimuli characterising changes include, but are not limited to, whole body
87 and local hyperthermia (Fehrenbach et al. 2001), oxidative stress/free radical formation (Khassaf et al. 2001; Taylor et al.
88 2010a), substrate depletion (Febbraio et al. 2002), hypoxia/ischemia (Taylor et al. 2011), altered pH (Peart et al. 2011)
89 and increased calcium concentration (Stary and Hogan 2016). Elevated expressions of both intracellular HSP72 (iHSP72)
90 and intracellular HSP90 α (iHSP90 α) are largely dictated by their transcription factor heat shock factor 1 (HSF1), which
91 is translocated to the nucleus where it binds to the heat shock elements (HSEs), resulting in relevant mRNA (Hsp)

92 transcription. HSP72 and HSP90 α demonstrate large changes in the net intracellular protein following acute and chronic
93 exercise that initiates their respective gene transcripts (McClung et al. 2008; Tuttle et al. 2015). It has been demonstrated
94 that HSP72 increases in response to thermal stress (Magalhães et al. 2010), though others have observed HSP72 protein
95 translation as being independent of increased core and/or muscle temperature (Morton et al. 2007). At present changes in
96 Hsp72 mRNA, and particularly Hsp90 α mRNA following heat acclimation, have not been reported relative to specific
97 physiological stimuli either experimentally or retrospectively. As such a dose response, or minimum stimuli
98 characterising significant transcription-translation has yet to be determined. Ambiguity in HSP response to thermal and
99 exercise stimuli, notably during comparable heat acclimation (HA) regimes (Magalhães et al. 2010; Hom et al. 2012),
100 suggests that a combination of/ or minimum threshold for elevated endogenous stressors may be required to increase
101 HSP protein content *in vivo*; such responses may well be individualised and determined by genetic, epigenetic and
102 phenotypical factors (Horowitz 2014; Horowitz 2016). Consequently, preliminary data relative to such characterisation is
103 required *in vivo* from a homogenous sample. Additionally given the potential for epigenetic modifications in Hsp
104 transcription (Horowitz 2016), it remains unknown whether the signals characterising increased gene expression would
105 demonstrate equality at the onset and culmination of a HA protocol.

106

107 Inhibition of HSF1 has been proposed to increase susceptibility to acute *in vivo* thermal stress [i.e. heat stroke (Moran et
108 al. 2006)], and similarly preclude procurement of optimal physiological adaptation to chronic thermal stress [i.e. heat
109 acclimated phenotype (Maloyan and Horowitz 2002; Kuennen et al. 2011)]. Induction of HSPs, particularly HSP72, are
110 central to not only to the aforementioned heat adaptation (Kuennen et al. 2011), but are increasingly implicated within
111 other positive adaptive responses to stress [i.e. promotion of mitochondrial biogenesis (Henstridge et al. 2014a)] and
112 various disease states [e.g. type 2 diabetes mellitus (Hooper et al. 2014), cardiovascular disease (Noble and Shen 2012),
113 and Parkinson's disease (Erekat et al. 2014)]. Reduced iHSPs are observed in disease states such as type 2 diabetes
114 mellitus in response to insulin sensitive HSF1 inhibition (Kurucz et al. 2002), with heat stress induced increases in
115 HSP72 proving therapeutic (Gupte et al. 2011). Whilst understanding of the important role of heat shock proteins is
116 growing, less is known of the physiological signals which facilitate the optimal transcription of the mRNA prior to
117 protein translation (Anckar and Sistonen 2011). Characterising the signal, or signals, that predict Hsp72 mRNA and
118 Hsp90 α mRNA increases (and thus likely increased HSP) may enhance the efficacy of (Henstridge et al. 2014b).

119

120 The aim of this experiment was to characterise the physiological stimuli (core temperature, heart rate, sweat rates) and/or
121 training prescription markers (exercise -duration, -intensity, -power, and work done) that correlate most strongly with the
122 increase in Hsp72 mRNA and Hsp90 α mRNA during a ten day HA regime or a comparable normothermic training
123 intervention (Gibson et al. 2015c). Additionally we sought to determine whether in a homogenous sample, experiencing
124 equality of stress, whether the predictive criteria for Hsp72 mRNA and Hsp90 α mRNA transcription would change pre-
125 to-post HA or normothermic training. It was hypothesised that markers of thermal strain and heat storage i.e. core
126 temperature, would most closely predict the change in Hsp72 mRNA, and Hsp90 α mRNA, and these markers would
127 demonstrate equality in predictive capacity at the beginning and end of HA/training.

128

129 **Materials and Methods**

130 *Participants*

131 The analysis of Hsp72 mRNA and Hsp90 α mRNA was performed on data collected from fifteen participants who had
132 performed ten 90 min isothermic HA sessions (n=7; age = 23 \pm 4 years, height = 183 \pm 6 cm, mass = 76.4 \pm 6.7 kg, body
200
225 $\dot{V}_{O_{2peak}} = 4.16 \pm 0.56$ L.min⁻¹), or performed normothermic exercise training in a temperate
226 environment (n=8 age = 26 \pm 5 years, height = 179 \pm 7 cm, mass = 74.6 \pm 4.8, body surface area = 1.93 \pm 0.10 m², body
280
284 $\dot{V}_{O_{2peak}} = 4.22 \pm 0.62$) from one previously published experiment (Gibson et al. 2015c) (pooled
285 descriptive characteristics in Table 1, schematic overview in Figure 1). Given equality of training prescription [as
286 detailed elsewhere (Gibson et al. 2015c)], both the isothermic HA and normothermic exercise training groups were
287 pooled into one data set for each time point to increase the heterogeneity of the physiological responses and Hsp mRNA
288 transcription. Confounding environmental (prolonged hyperthermic and/or hypoxic stress) and pharmacological variables
289 were all controlled in line with previous work in the field (Gibson et al. 2014; Gibson et al. 2015a). Participants
290 commenced all trials in a euhydrated state [<700 mOsm \cdot Kg⁻¹ H₂O (Sawka et al. 2007)]. All protocols, procedures and
291 methods were approved by the institutional ethics committee. Participants completed medical questionnaires and written
292 informed consent following the principles outlined by the Declaration of Helsinki as revised in 2013 prior to
293 commencing any preliminary or experimental sessions. In compliance with ethical approval, a testing/intervention
294 session was terminated if a subject attained a core temperature [measured at the rectum (T_{rec})] of 39.7°C (zero
295 incidences).

296

297 ***Insert Table 1 near here please***

298 ***Insert Figure 1 near here please***

299

300 *Experimental Design*

301 Preliminary testing commenced with anthropometric assessment of participants, whom subsequently performed an
302 incremental (24 W.min⁻¹) cycle test commencing at 80 W, in temperate laboratory conditions [20°C, 40% relative

303 EQ $\dot{V}_{O_{2peak}}$ (Gibson et al. 2015c). Expired metabolic gas was measured at a breath by breath frequency
304 EQ $\dot{V}_{O_{2peak}}$ was made via the attainment of a heart rate (HR) within 10 b.min⁻¹ of age predicted

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315 "https://github.com/citation-style-language/schema/raw/master/csl-citation.json" }(Taylor et al. 1955). The data obtained
316 EQ $\dot{V}_{O_{2peak}}$ test was subsequently used to prescribe the HA/normothermic training intervention.

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636 Isothermic, also known as controlled hyperthermic, heat acclimation was implemented to optimize stress and adaptation
637 throughout the regime (Taylor and Cotter 2006; Racinais et al. 2015). Each of the ten, 90 min HA sessions were
638 performed in hot conditions ($40.2^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$, $41.0 \pm 6.4\%$ RH), with participants initially exercising, at a workload
659
661 $\dot{V}_{\text{O}_{2\text{peak}}}$ until the isothermic target T_{rec} of $\geq 38.5^{\circ}\text{C}$ was been achieved. Upon the attainment of a T_{rec}
662 $\geq 38.5^{\circ}\text{C}$, participants rested in a seated position on the cycle ergometer within the environmental chamber resuming
699 $\dot{V}_{\text{O}_{2\text{peak}}}$ when their T_{rec} fell below 38.5°C , and continued cycling until the target T_{rec} was re-attained
700 (~10 min). Normothermic exercise training involved ten, 90 min sessions performed in temperate conditions ($19.8^{\circ}\text{C} \pm$
800
828 $\dot{V}_{\text{O}_{2\text{peak}}}$, with the workload adjusted to match the total work, and exercise intensity and duration of
829 the isothermic HA group. Both groups exercised inside a purpose built environmental chamber (WatFlow control system;
830 TISS, Hampshire, UK) with temperature and humidity controlled using automated computer feedback (WatFlow control
831 system; TISS, Hampshire, UK). Sessions were conducted at the same time of day (07:00-10:00 h) to mitigate effects of
832 daily variation in heat shock protein expression (Taylor et al. 2010b). During each session sweat rate (SR; $\text{L}\cdot\text{hr}^{-1}$) was
833 estimated using the change in nude body mass (NBM) from the pre- to post- exercise periods (Detecto Physicians Scales;
834 Cranlea & Co., Birmingham, UK), T_{rec} was recorded using a thermistor (Henleys Medical Supplies Ltd, Welwyn Garden
835 City, UK, Meter logger Model 401, Yellow Springs Instruments, Yellow Springs, Missouri, USA) inserted 10 cm past
836 the anal sphincter, and heart rate (HR) recorded by telemetry (Polar Electro Oyo, Kempele, Finland). During each session
837 HR, T_{rec} , and power output (W) were recorded after 10 min of seated rest in temperate laboratory conditions, and
838 thereafter every 5 min upon commencing exercise.

839

840 *Quantification of physiological and training variables*

841 Exercise duration (min) was the total time spent exercising during the intervention. Mean session intensity ($\% \dot{V}O_{2peak}$ and
842 $W \cdot kg^{-1}$) was calculated from the relative exercise intensity during each 5 min period throughout the 90 min intervention
843 sessions. This contrasted the Mean exercise intensity ($\% \dot{V}O_{2peak}$ and $W \cdot kg^{-1}$), which reflected the mean relative exercise
844 intensity only (power output >1 W), thus excluding periods of rest. Total work done (kJ) reflected the cumulative volume
845 of worked performed on the cycle ergometer over the 90 min period.

846

847 Absolute sweat loss ($L \cdot h^{-1}$) was calculated from the change in towel-dried NBM pre-to-post sessions. Mean T_{rec} and
848 mean HR reflected the average T_{rec} or HR recorded throughout each intervention. Peak T_{rec} and peak HR reflect the
849 maximum T_{rec} or HR recorded throughout each intervention. Change (Δ) in T_{rec} ($^{\circ}C$) and ΔHR ($b \cdot min^{-1}$) reflects the
850 difference between resting, and peak T_{rec} and HR respectively. Mean $T_{recfinal45}$ ($^{\circ}C$) quantifies the mean T_{rec} between the
851 45th and 90th min of the intervention session to reflect the average temperature following the initial rate of increase.
852 $T_{rec \geq 38.5^{\circ}C}$ (min) represented the total number of minutes where T_{rec} exceeded $38.5^{\circ}C$ during the session. The T_{rec} area
853 under the curve (AUC) at $>38.5^{\circ}C$ was calculated when as the duration and magnitude where measured T_{rec} exceeded
854 $38.5^{\circ}C$ (Périard et al. 2012; Gibson et al. 2014; Périard et al. 2015), using a modification to the trapezium rule (Hubbard
855 et al. 1977). A T_{rec} of $38.5^{\circ}C$ was selected as an approximate minimum for intolerance during compensable heat stress
856 (Sawka et al. 2001) and possible threshold for Hsp72 translation/transcription (Amorim et al. 2008). AUC for T_{rec}
857 $>38.5^{\circ}C$ was calculated as:

858

859 $AUC_{T_{rec} \geq 38.5^{\circ}C} (^{\circ}C \cdot min^{-1}) = \sum \text{time interval (min)} \times 0.5 [^{\circ}C > 38.5^{\circ}C \text{ at the start of the intervention day} + ^{\circ}C > 38.5^{\circ}C$
860 $\text{at the end of the intervention day}]$.

861

862 The rate of ΔT_{rec} ($^{\circ}C$) rate of core temperature change, typically an increase, was calculated as follows:

863

864 $\text{Rate } \Delta T_{rec} (^{\circ}C \cdot hr^{-1}) = (T_{rec2} - T_{rec1} / \text{time2} - \text{time1}) * 60$

865 *Note: T_{rec2} and time2 are simultaneous measurements taken at any time during the intervention; and T_{rec1} and time1 are*
866 *resting value.*

867

868 *Blood Sampling and RNA extraction*

869 Venous blood samples were drawn from the antecubital vein into 6 mL EDTA tubes immediately pre- and post- the first
870 (Day1) and tenth (Day10) session. Using a previously validated method (Taylor et al. 2010b), venous blood (1 mL) was
871 pipetted into 10 mL of 1 in 10 red blood cell lysis solution (10X Red Blood Cell Lysis Solution, Miltenyi Biotech,
872 Bisley, UK). Samples were incubated for 15 min at room temperature before isolation via 5 min centrifugation at 400G,
873 then washed twice in 2 mL PBS, prior to further centrifugation at 400G for 5 min. The acid guanidinium thiocyanate-
874 phenol-chloroform extraction (TRIzol) method (Chomczynski and Sacchi 1987), was then used to extract RNA from the
875 leukocytes in accordance with manufacturer instructions (Sigma-Aldrich, UK; Invitrogen, Life Technologies, Carlsbad,
876 USA). Quantity was determined at an optical density of 260 nm, while quality was determined via the 260/ 280 and 260/
877 230 ratios using a nanodrop spectrophotometer (Nanodrop 2000c Thermo Scientific, Waltham, MA, USA).

878

879 *One step reverse transcription quantitative polymerase chain reaction (RT-QPCR)*
880 Hsp72 and Hsp90 α relative mRNA expression was quantified using RT-QPCR. Primers β 2-Microglobulin, Hsp72, and
881 Hsp90 α (presented in Table 2) were designed using primer design software (Primer Quest and Oligoanalyzer - Integrated
882 DNA technologies, Coralville, IA, USA) (Tuttle et al. 2015). During primer design, sequence homology searches were
883 performed against the GenBank database to ensure the primers matched the gene of interest. Primers were designed to
884 span exon-intron boundaries and avoided three or more guanine-cytosine bases within the last five bases at the 3' end of
885 primer to avoid nonspecific binding. Further searches were performed to ensure primers did not contain secondary
886 structures and intermolecular or intramolecular interactions (hairpins, self-dimer, and cross dimers), which can inhibit
887 product amplification. Relative Hsp mRNA expression was then quantified using RT-QPCR with reagent concentrations
888 implemented in accordance with manufacturer recommendations. Reactions (20.0 μ L) containing 10.0 μ L of SYBR
889 Green RT-PCR Mastermix (Quantifast SYBR Green kit; Qiagen, Manchester, UK), 0.15 μ L of forward primer, 0.15 μ L
890 of reverse primer, 0.20 μ L of reverse transcription mix (Quantifast RT Mix, Qiagen), and 9.50 μ L sample (70.0 ng
891 RNA. μ L⁻¹) were prepared using the Qiagility automated pipetting system (Qiagen). Each reaction was amplified in a
892 thermal cycler (Rotorgene Q, Qiagen) and involved reverse transcription lasting 10 min at 50°C and a transcriptase
893 inactivation and initial denaturation phase lasting 5 min at 95°C. The PCR reaction then followed with a denaturation
894 step lasting 10 s at 95°C and a primer annealing and extension stage lasting 30 s at 60°C repeated for 40 cycles.
895 Fluorescence was measured following each cycle as a result of the incorporation of SYBR Green dye into the amplified
896 PCR product. Melt curves (50 to 95°C; Ramp protocol, 5-s stages) were analyzed for each reaction to ensure only the
897 single gene of interest was amplified. Relative quantification of mRNA expression for each sample was assessed by
898 determining the ratio between the cycling threshold (CT) value of the target mRNA and β 2-M CT values. Fold change in
899 relative mRNA expression was calculated using the 2^{- $\Delta\Delta$ CT} method.

900

901 ***Insert Table 2 near here please***

902

903 *Statistical Analysis*

904 Analysis was performed on data obtained on the first (Day1; n=15), and last (Day10; n=15), and the combined dataset
905 (ALL). All data are reported as mean \pm SD with two-tailed significance accepted at p<0.05. Prior to statistical analysis,
906 all outcome variables were checked for normality using Kolmogorov-Smirnov and sphericity using the Greenhouse
907 Geisser method prior to further analysis, each was deemed plausible unless otherwise stated. Paired sample T-Tests
908 (SPSS, Chicago IL, USA) were implemented to determine differences between Day1 and Day10 for training data and the
909 physiological responses to training. Paired sample T-Tests were also used to determine differences between the
910 magnitude of change (i.e. Pre-Post) in Hsp72 mRNA and the magnitude of change in Hsp90 α mRNA on Day1, Day10
911 and ALL. Effect sizes [Cohen's d (*d*; small =0.20, medium =0.50, large =0.80)] were calculated to analyse the magnitude
912 and trends with data. Pearson's product correlation was performed between the physiological responses and training
913 prescription markers (see *Quantification of physiological and training variables*), and the magnitude of change (Pre-
914 Post) in Hsp72 mRNA, and Hsp90 α mRNA, on Day1, Day10 and ALL respectively. Stepwise multiple regression was
915 performed to determine whether a combination of markers strengthened the prediction equation for Hsp72 mRNA, and
916 Hsp90 α mRNA.

917

918 **Results**

919 *Physiological and Hsp72 mRNA, and Hsp90α mRNA responses*

920 No difference in the Pre-Post change in Hsp72 mRNA, and Pre-Post change in Hsp90α mRNA were observed between
921 Day1 and Day10 (Hsp72 mRNA: $t=0.067$; $p=0.948$; $d=0.02$, Hsp90α mRNA: $t=0.748$; $p=0.467$; $d=0.19$) (Figure 2). On
922 Day10 vs Day1 participants were observed as performing exercise for longer duration ($t=5.206$; $p<0.001$; $d<0.01$), at a
923 greater mean intensity ($t=7.724$; $p<0.001$; $d=2.03$) and mean power ($t=5.855$; $p<0.001$; $d=1.63$) and producing a greater
924 volume of work done ($t=6.424$; $p<0.001$; $d=1.66$). Reductions in the rate of T_{rec} increase were observed ($t=2.188$;
925 $p=0.046$; $d=0.58$) between Day10 and Day1, alongside increased SR ($t=4.123$; $p=0.001$; $d=0.89$) (Table 3).

926

927 ***Insert Table 3 near here please***

928 ***Insert Figure 2 near here please***

929

930 *Correlates of Hsp72 mRNA, and Hsp90α mRNA increases*

931 The strongest correlates of the Pre-Post session change in Hsp72 mRNA were the ΔT_{rec} (ALL $r=0.714$; Day1 $r=0.721$;
932 Day10 $r=0.709$), SR (ALL $r=0.709$; Day1 $r=0.738$; Day10 $r=0.762$), the Mean $T_{rec\text{final}45}$ (ALL $r=0.682$; Day1 $r=0.730$;
933 Day10 $r=0.632$), AUC at 38.5°C (ALL $r=0.678$; Day1 $r=0.761$; Day10 $r=0.687$), the peak T_{rec} (ALL $r=0.661$; Day1
934 $r=0.688$; Day10 $r=0.650$), and the duration $T_{rec\geq 38.5^\circ\text{C}}$ (ALL $r=0.650$; Day1 $r=0.650$; Day10 $r=0.659$) (Table 4, Figure
935 3). Multiple regressions observed no improvements to the Hsp72 mRNA model with the addition of further variables for
936 Day1, Day10 or ALL analyses. No participant characteristics demonstrated a significant ($p>0.05$) relationship with
937 change in Hsp72 mRNA (Table 1).

938

939 ***Insert Table 4 near here please***

940 ***Insert Figure 3 near here please***

941

942 The strongest correlates of Hsp90α mRNA increase were the AUC at 38.5°C (ALL $r=0.729$; Day1 $r=0.729$; Day10
943 $r=0.813$), the ΔT_{rec} (ALL $r=0.691$; Day1 $r=0.715$; Day10 $r=0.690$), the peak T_{rec} (ALL $r=0.680$; Day1 $r=0.698$; Day10
944 $r=0.645$), Mean $T_{rec\text{final}45}$ (ALL $r=0.678$; Day1 $r=0.714$; Day10 $r=0.617$), SR (ALL $r=0.660$; Day1 $r=0.760$; Day10
945 $r=0.733$), the duration $T_{rec\geq 38.5^\circ\text{C}}$ (ALL $r=0.629$; Day1 $r=0.670$; Day10 $r=0.563$), rate of change in T_{rec} (ALL $r=0.600$;
946 Day1 $r=0.567$; Day10 $r=0.674$) relating to the largest gene transcription (Table 5, Figure 4). Multiple regression observed
947 that the Hsp90α mRNA model for Day1 and Day10 was not improved by adding further variables. The entire Hsp90α
948 mRNA dataset (ALL) was improved ($r=0.792$) when AUC at 38.5°C and SR were combined. Height (ALL $r=0.419$) and
949 BSA (ALL $r=0.412$), but no other participant characteristics demonstrated a significant ($p>0.05$) relationship with change
950 in Hsp90α mRNA (Table 1).

951

952 ***Insert Table 5 near here please***

953 ***Insert Figure 4 near here please***

954

955 *Relationship between Hsp72 vs. Hsp90α mRNA*

956 A significant relationship was observed between Hsp72 mRNA and Hsp90α mRNA for ALL ($r=0.879$; $p<0.001$) (Figure
957 5) with a significant relationship also observed on Day1 ($r=0.924$; $p<0.001$) and Day10 ($r=0.838$; $p<0.001$) (Figure 5).
958 Accordingly, no significant difference was observed between the pre to post session change in Hsp72 mRNA, and

959 Hsp90 α mRNA on Day1 ($t=1.200$; $p=0.250$; $d=0.32$; Hsp72 mRNA=1.2 \pm 1.2 fold change; Hsp90 α mRNA=1.4 \pm 1.8
960 fold change), Day10 ($t=-0.032$; $p=0.975$; $d=0.01$; Hsp72 mRNA=1.2 \pm 1.1 fold change; Hsp90 α mRNA=1.2 \pm 1.5 fold
961 change), or ALL ($t=-0.914$, $p=0.368$; $d=0.28$; Hsp72 mRNA=1.2 \pm 1.1 fold change, Hsp90 α mRNA=1.3 \pm 1.6 fold
962 change).

963

964 ***Insert Figure 5 near here please***

965

966 Discussion

967 In our experiment ΔT_{rec} , SR, the Mean $T_{recfinal45}$, the T_{rec} AUC at 38.5°C, peak T_{rec} , and the duration $T_{rec}\geq 38.5^\circ\text{C}$ were
968 identified as significant predictors ($R^2 \approx 0.36-0.51$; $p < 0.05$) of the change in Hsp72 mRNA (Table 4, Figure 3). This is
969 in agreement with previous observations regarding eHsp72 (Gibson et al. 2014) and is in agreement with the
970 experimental hypothesis that endogenous markers of thermal strain and heat storage i.e. core temperature, would most
971 closely predict, if not directly cause, the change in Hsp72 mRNA. The experimental hypothesis also extended this
972 observation to Hsp90 α mRNA which similarly demonstrated that the AUC at 38.5°C, the ΔT_{rec} , the peak T_{rec} , Mean
973 $T_{recfinal45}$, SR, the duration $T_{rec}\geq 38.5^\circ\text{C}$, rate of change in T_{rec} were identified as the strongest predictors ($R^2 \approx 0.42-0.53$)
974 of the increased gene expression (Table 5, Figure 4). Similar characteristics (both physiological predictors and observed
975 magnitude of prediction) were observed on Day1 and Day10 for both Hsp72 and Hsp90 α mRNA, thus the signal for
976 transcription demonstrates equality at the onset and culmination of a 10 day HA or normothermic training intervention
977 (Table 4 and Table 5). The similarity of variables characterising increases in Hsp72 and Hsp90 α mRNA was supported
978 by the observation that a strong relationship ($R^2=0.77$) existed between the increase in Hsp72 mRNA and the increase in
979 Hsp90 α mRNA. The absolute mRNA increase was also of a comparable magnitude for both Hsp72 mRNA ($\sim 1.2 \pm 1.1$
980 fold change; +103%), and Hsp90 α mRNA ($\sim 1.3 \pm 1.6$ fold change; +60%) with the lower relative increase in Hsp90 α
981 mRNA reflecting a higher basal expression (Subbarao Sreedhar et al. 2004). It is yet to be elucidated whether these
982 variables would demonstrate a greater/lesser contribution to the magnitude of gene transcription in other tissue, i.e.
983 skeletal muscle appears more oxidative stress dependent rather than temperature dependent compared to leukocytes, as
984 discussed previously (Tuttle et al. 2015).

985

986 The failure for training prescription markers (exercise -duration, -intensity, -power, and work done) to predict the change
987 of leukocyte Hsp72 and Hsp90 α mRNA highlight the importance of designing interventions necessitating increased HSP
988 around endogenous responses (e.g. increased core temperature), rather than exogenous variables (e.g. training
989 prescription). SR as a predictor of increased Hsp90 α mRNA is a novel observation; it is however likely that this is a
990 secondary response, thus not causal, stemming from the required increased heat dissipation via evaporation in an attempt
991 to acquiesce increasing heat storage (core temperature) rather than being a primary response. Manipulation of heat
992 storage to further enhance Hsp90 α mRNA transcription may be of experimental benefit within some designs and could,
993 at least theoretically, be enhanced by facilitating greater sweating with higher humidity's (e.g. >60% R.H.) than those
994 utilised within the present design ($\sim 40\%$ R.H.), thus further inhibiting evaporative heat loss (Maughan et al. 2012).
995 Though some differences in HA adaptation have been observed by imposing dehydration (an analogue for increased
996 sweat losses and the ensuing increases in thermal strain) (Garrett et al. 2014), the impact of this manipulation on Hsp
997 transcription during HA remains unknown. When dehydration is imposed vs. euhydration, no differences in the increases
998 in monocyte, or skeletal HSP72 have been observed during ~ 90 min of acute exercise-heat stress (Hillman et al. 2011),

999 and normothermic exercise (Logan-Sprenger et al. 2015), respectively. These data reduce the appeal of increasing sweat
1000 losses as a direct method for improving the magnitude of adaptation.

1001

1002 A novel finding in the present study was the observation that the increases in Hsp72 and Hsp90 α mRNA demonstrate a
1003 significant relationship. This provides further evidence for a common pathway towards equality of increases in HSP72
1004 and HSP90 α protein concentrations in response to *ex vivo* cellular stress (McClung et al. 2008). Hsp72 mRNA (Marshall
1005 et al. 2007), and Hsp90 α mRNA (Tuttle et al. 2015) are responsive to thermal and physiological stimuli as supported by
1006 our data. Whilst data reporting changes in human Hsp72 mRNA is available (Febbraio and Koukoulas 2000; Marshall et
1007 al. 2007; Stary et al. 2008; Atamaniuk et al. 2008; Tuttle et al. 2015; Gibson et al. 2015a; Gibson et al. 2015c), equivalent
1008 data characterising increases in Hsp90 α mRNA is limited. Our data demonstrates a novel finding and is the first *in vivo*
1009 experiment demonstrating that Hsp90 α mRNA responds similarly to Hsp72 mRNA when core temperature is increased
1010 during exercise in the heat. It is known that exercise (Connolly et al. 2004), exercise-heat stress (Moran et al. 2006), and
1011 exercise induced muscle damage and heat stress (Tuttle et al. 2015), provide the stimuli for Hsp72, and Hsp90 α gene
1012 induction with the stimuli to increase transcription described as proportional to the change in core temperature
1013 ($38.5\pm 0.2^{\circ}\text{C}$ to $39.0\pm 0.4^{\circ}\text{C}$) (Tuttle et al. 2015), a finding our data supports. Our data is congruous with findings that
1014 consistent Pre-Post increases in Hsp72 mRNA occur at the beginning and end of heat acclimation (Gibson et al. 2015a),
1015 should the physiological signal ($T_{\text{rec}} > 38.5^{\circ}\text{C}$) demonstrate equality between measurement points (Gibson et al. 2015a).
1016 In addition, we have now demonstrated that this dose response is true of Hsp90 α mRNA. This observation further
1017 reinforces isothermic HA methods as optimal for ensuring signalling for HSP via the heat shock response (a key
1018 regulator of thermotolerance) and the magnitude of phenotypic heat adaptation (Kuennen et al. 2011). During the initial
1019 adaptation to heat acclimation (~ 3 days), Hsp72 mRNA can diminish in conjunction with reductions in mean core
1020 temperature (-0.2°C to $38.7\pm 0.2^{\circ}\text{C}$) and increased basal HSP72 (Marshall et al. 2007) should the end of activity core
1021 temperature decrease. Our data now extends this signal dependent response to Hsp90 α mRNA. Our non-damaging
1022 exercise protocol (cycling) also supports the previously proposed observation that the leukocyte stress response is core
1023 temperature rather than exercise dependent (Tuttle et al. 2015).

1024

1025 Maintenance of increases in both Hsp72, and Hsp90 α mRNA between Day1 and Day10 is unsurprising as no difference
1026 existed between the strongest correlates of the Pre-Post session change in gene expression (core temperature). This
1027 finding is concurrent with proposals that elevated core temperature as being an important component of HSF1 regulation
1028 (Gibson et al. 2015a; Gibson et al. 2015c), particularly in leukocytes. To ensure HSF1 activation in experiments such as
1029 those designed to achieve the heat acclimated phenotype (Kuennen et al. 2011), mitigate heat stroke (Moran et al. 2006),
1030 facilitate positive adaptive responses to stress [i.e. promotion of mitochondrial biogenesis (Henstridge et al. 2014a)] or
1031 act as therapy for disease states [e.g. type 2 diabetes mellitus (Hooper et al. 2014), cardiovascular disease (Noble and
1032 Shen 2012), and Parkinson's disease (Erekat et al. 2014)] then eliciting an increased core temperature of $+1.7^{\circ}\text{C}$, and
1033 ensuring a minimum core temperature of 38.5°C is maintained for ≥ 27 minutes is necessary (Table 3). However, this
1034 prescription is from a small homogenous apparently healthy population. Some of the previously detailed disease states
1035 have marked impairments regarding their ability to thermoregulate [e.g. diabetics (Kenny et al. 2016) and multiple
1036 sclerosis (Romberg et al. 2012)]. Therefore, characterisation of the Hsp mRNA response is required within such
1037 populations – particularly – whether they have a similar core temperature mediated dose response relationship with Hsp
1038 mRNA increases, in light of reduced basal HSP (Bruce et al. 2003). Should the disease state not directly inhibit HSP

1039 translation, it may be that HSP accumulation is more efficient in these populations due to greater increases in core
1040 temperature resulting from inhibited heat dissipation mechanisms (Davis et al. 2010; Carter et al. 2014; Kenny et al.
1041 2016), a notion also true of those with a spinal cord injury (Price 2006). Previous work has identified HSF1 is
1042 temperature dependent supporting our downstream observation regarding increased gene expression (Sonna et al. 2010).
1043 Similar endogenous signals to increase Hsp72 mRNA have been shown to increase eHSP72 i.e. elevated temperature
1044 (Gibson et al. 2014). The rate of rise in core temperature is also important to increase plasma concentrations (Périard et
1045 al. 2012). More relevant to acquiring thermotolerance (Kregel 2002), is the change in iHSP72 and iHSP90 α (Lee et al.
1046 2015). The accumulation of greater iHSP72 is more closely linked to the absolute (final), and change (Δ) in core
1047 temperature (Magalhães et al. 2010) during HA. Mechanistically, it has been observed that the magnitude of iHSP72
1048 expression at exhaustion, or 24hr post exhaustion is a result of the absolute temperature attained ($>39.0^{\circ}\text{C}$), rather than
1049 the rate of heat storage (Périard et al. 2015). During acute exercise-heat stress, equality of post exhaustion iHSP72 did
1050 not differ in response to the rate of core temperature increase corresponding to different exercise intensities, and
1051 $EQ \ \Delta(V, \Delta T)_{\text{peak}} = 5.1 \pm 1.7^{\circ}\text{C}\cdot\text{hr}^{-1}$ (Périard et al. 2015)]. The present study highlights the transcription of
1200 Hsp72 mRNA, and Hsp90 α mRNA as having similar criteria ($T_{\text{rec}} >38.5^{\circ}\text{C}$ for >27 min, Table 3) to that which predicts
1201 elevated eHSP72 and iHSP72 (Périard et al. 2012; Gibson et al. 2014; Périard et al. 2015). Given that the predictive
1202 capacity following this analysis is incomplete (Hsp72 mRNA= 47-58% between variables, and Hsp90 α mRNA= 46-66%
1203 between variables) the present experiment does suggest that a large proportion of the signalling for gene transcription is
1204 unaccounted for by the core temperature alone. Accordingly, other endogenous signals responsible for the initial HSF1
1205 activation should be sought (Vihervaara and Sistonen 2014); as previously discussed this may be more important in
1206 skeletal muscle vs leukocytes. These signals likely include markers of metabolic stress/physiological strain such as
1207 oxidative stress (Sureda et al. 2005; Mestre-Alfaro et al. 2012), changes in catecholamine concentration (Tintinger et al.
1208 2001; Giraldo et al. 2010), and increased cortisol (Ortega 2003). These signals also provide a rationale for the difference
1209 between increased Hsp72 mRNA, and HSP72 protein translation observed in heat acclimation (Marshall et al. 2007), and
1210 notable increases in monocyte HSP72 in hypoxia (Taylor et al. 2011), and following matched passive vs active
1211 hyperthermia (Morton et al. 2007) which collectively highlight the multifactorial signal cascade. Irrespective of the
1212 precise, causal signal(s), these data evidence that large, prolonged changes in core temperature are a predictor of the
1213 greatest change in Hsp72 mRNA, and Hsp90 α mRNA (from those endogenous and exogenous variables monitored), with
1214 this an important characteristic potentiating increased basal protein to mitigate the potential threat for subsequent thermal
1215 mediated protein denaturation. The “absent” predictive criteria might be also explained by other unmeasured variables,
1216 these could include factors allied to individual differences in HSP transcription perhaps due to a responder-non responder
1217 paradigm due to innate (ie genetic) or acquired (previous heat exposure, HA, subsequent decay, etc) phenotypical inter-
1218 intra-individual variation (Lyashko et al. 1994).

1220

1221 Despite the presence of a highly homogenous sample (Table 1), and advantageous experimental protocol whereby
1222 participants did not possess the HA phenotype at the onset of the experiment, and were exposed to consistent endogenous
1223 signal being obtained throughout the experiment (Table 3), ~40% of the variability in Hsp mRNA transcription is
1224 unaccounted for. It is difficult to determine individual responses within this experiment, largely due to the differing
1225 training parameters and exercise-heat stress variables (Table 3) observed between Day1 and Day10 as part of the initial
1226 experimental objectives (Gibson et al. 2015c). Accordingly, bespoke experiments utilising deductive mechanistic
1227 evidence obtained *in vivo* and *in vitro* should ensure further equality of all potentiating stimuli (thermal and
1228 cellular/molecular) to facilitate a more definitive analysis of the individual response and account for the remaining
1229 variability in transcription. In spite of some absent predictive criteria, the present experiment further evidences the
1230 observation that in experiments where iHSP72 has not increased (Watkins et al. 2007; Hom et al. 2012), the mechanism
1231 for the absent protein translation is likely due to only modest and brief increases in core temperature with lower mean
1232 core temperatures reported [$<38.5^{\circ}\text{C}$ (Amorim et al. 2008)] than others demonstrating increased iHsp72 [$>39.0^{\circ}\text{C}$
1233 (Périard et al. 2015)]. Whilst exercise alone increases core temperature providing a sufficient pathway to elevate iHSP72
1234 (Fehrenbach et al. 2000; Shin et al. 2004) and Hsp72 mRNA, and HSP90 α mRNA in temperate conditions (Tuttle et al.
1235 2015), the greater heat storage when performing equivalent exercise in hot environmental conditions provides a more
1236 potent stimuli for increasing iHSP72 (Fehrenbach et al. 2001; Fehrenbach et al. 2003; Selkirk et al. 2009; Magalhães et
1237 al. 2010) via greater increases in Hsp72 mRNA. It has been observed that no difference in Hsp72 mRNA increases
1238 occurs within HA sessions performed in very hot environments when comparing isothermic methods (Gibson et al.
1239 2015a) (whereby the magnitude of hyperthermia is controlled) with fixed intensity methods [where core temperature
1240 responds according to the individual capacity to dissipate heat (Gibson et al. 2015b)]. The present data demonstrates that
1241 in cooler conditions, it would be pertinent to implement isothermic methods with high initial workloads (and metabolic
1242 heat production) to provide the requisite stimuli (increased core temperature) for acquiring thermotolerance in addition to
1243 physiological heat adaptation (Racinais et al. 2015). The isothermic method of HA facilitates a targeted increase in core
1244 temperature ($>38.5^{\circ}\text{C}$) which can subsequently be prolonged (for ~30-60 min), to initiate Hsp72 mRNA and Hsp90 α
1245 mRNA transcription (Gibson et al. 2015c). These guidelines are supported by data demonstrating that initial adaptations
1246 to HA (notably reduced core temperature and increased iHSP72) mitigate the increase in Hsp72 mRNA to subsequent
1247 fixed intensity work in equal environmental conditions (Marshall et al. 2007).

1248

1249 Future work in the field should address some of the limitations of this experiment, which are discussed within the present
1250 discussion section. To resolve the unknown threshold for Hsp72 and Hsp90 α mRNA signalling, and dependence of
1251 temperature in isolation or coupled with exercise and concurrent responses, measurements of gene transcription should
1252 be made following stepwise increases in core temperature whilst using passive and exercise-heat stress models and multi
1253 tissue analysis. This multi tissue analysis should also be performed where experimental manipulations prolong the degree
1254 of hyperthermia for extended periods (beyond the 30–60 minutes of the present data), and be performed using passive
1255 heating models e.g. hot water immersion or sauna (Krause et al. 2015b), in addition to the active exercise induced heating
1256 implemented in the present experiment. A paucity of transcription/translation data exists for extreme core temperatures
1257 (in excess of 39.5°C), despite attainment of these temperatures absent of pathophysiological complications within
1258 endurance runners (Byrne et al. 2006), and other competitive athletes e.g. elite footballers (Mohr et al. 2012). Hsp72 and
1259 Hsp90 α mRNA analysis should be paired with measurement of iHSP72 and iHSP90 α protein concentrations across

1260 tissues to facilitate a more complete interpretation of the transcription and translational kinetics. This would subsequently
1261 facilitate more precise interventions allied to prescribing HA for increasing thermotolerance (Kuennen et al. 2011),
1262 reducing the severity of heat illness (Ruell et al. 2014) or facilitate a cellular test for heat stroke susceptibility (Amorim et
1263 al. 2008), and provide optimal prescriptions of therapeutic thermal interventions (Henstridge et al. 2014b; Henstridge et
1264 al. 2016). The time course of the mRNA transcription and subsequent HSP translation also both require further research
1265 to determine within group, and inter-individual responses of a heterogeneous cohort, including clinical populations.
1266 Finally, given the apparent translational differences between sexes in response to equivalent training (Morton et al.
1267 2009), it remains to be determined whether this is a result of inhibited gene transcription, or translational events
1268 attenuating changes in basal protein in females.

1269

1270 **Conclusion**

1271 In experiments or interventions where the correlated transcription of Hsp72 and Hsp90 α mRNA is important, such as
1272 those involving heat acclimation and heat illness, or therapeutic heat stress, the protocol should be designed to rapidly
1273 induce large changes ($\geq 1.7^{\circ}\text{C}$) in core temperature ($>38.5^{\circ}\text{C}$), which are maintained for prolonged periods (≥ 27 minutes).
1274 This may be achieved by initially implementing high intensity work, eliciting uncompensable heat stress and increased
1275 heat storage, followed by the maintenance of core temperature via lower intensity exercise, or passive heat stress to elicit
1276 heat balance at the elevated temperature.

1277

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Table 1. Mean \pm SD Participant descriptive characteristics and correlations with Hsp72 mRNA (ALL), and Hsp90 α mRNA (ALL) datasets. * denotes $p < 0.05$.

Variable	Mean \pm SD	Hsp72 mRNA	Hsp90 α mRNA
Age (years)	24.6 \pm 4.4	$r = -0.0622$	$r = -0.0222$
Height (cm)	181 \pm 6	$r = 0.084$	$r = 0.419^*$
Mass (kg)	75.4 \pm 5.5	$r = -0.112$	$r = 0.356$
Body Surface Area (m ²)	1.95 \pm 0.10	$r = -0.066$	$r = 0.412^*$
BMI (kg.m ²)	23.1 \pm 1.2	$r = -0.149$	$r = -0.077$
Body fat (%)	14.3 \pm 2.2	$r = -0.012$	$r = -0.085$

	7		
$EQ \ \o\ac(V,\sup8(.))O_{2peak}$ $(L.min^{-1})$	4 . 2 0 ± 0 . 5 6	r = 0. 0 7 9	r = 0. 3 1 6
$EQ \ \o\ac(V,\sup8(.))O_{2peak}$ $(mL.kg.min^{-1})$	5 5 . 6 ± 6 . 0	r = 0. 1 3 4	r = 0. 1 3 3

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1480 Table 2. Primer Sequences

Target Gene	Primer Sequence (5'-3'-)	Reference Sequence Number	Amplicon Length
B ₂ microglobulin	Forward: CCGTGTGAACCATGTGACT Reverse: TGCGGCATCTCAAACCT	NM_004048	91
Hsp72	Forward: CGCAACGTGCTCATCTTTGA Reverse: TCGCTTGTTCTGGCTGATGT	NM_005345	198
Hsp90 α (variant 1 and variant 2)	Forward: AAACTGCGCTCCTGTCTTCT Reverse: TGCGTGATGTGTCGTCATCT	NM_001017963 & NM_005348	180

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1482 Table 3. Mean \pm SD Training data and physiological responses for Hsp72 mRNA, and Hsp90 α mRNA datasets
 1483 displayed for the entire data set (ALL), and Day1 and Day10 respectively. * denotes difference from Day1 within gene
 1484 transcript.

Measure	AL L	Da y1	Da y10
Exercise Duration (min)	50 \pm 9	43 \pm 7	57 \pm 5 *
Mean Intensity EQ \o\ac(V,\s\up8(.))O _{2peak})	33 \pm 7	28 \pm 5	38 \pm 3 *
Mean Power (W.kg ⁻¹)	1.3 \pm 0.3	1.1 \pm 0.3	1.5 \pm 0.3 *
Mean Exercise Intensity EQ \o\ac(V,\s\up8(.))O _{2peak})	60 \pm 5	60 \pm 6	61 \pm 3
Mean Exercise Power (W.kg ⁻¹)	2.4 \pm 0.5	2.4 \pm 0.5	2.4 \pm 0.5
Total Work Done (kJ)	52 \pm 14 9	44 \pm 11 1	606 \pm 142 *
SR (L.hr ⁻¹)	0.9 \pm 0.5	0.8 \pm 0.4	1.0 \pm 0.6 *
Mean T _{rec} (°C)	37. 92 \pm 0.4 6	38. 02 \pm 0.5 4	37. 83 \pm 0.3 4
Mean T _{recfinal45} (°C)	38. 14 \pm 0.7 3	38. 19 \pm 0.8 2	38. 08 \pm 0.6 5
Δ T _{rec} (°C)	1.6 8 \pm 0.7 0	1.6 4 \pm 0.7 1	1.7 2 \pm 0.7 1
Rate of Δ T _{rec} (°C.hr ⁻¹)	2.0 6 \pm 0.8 9	2.2 4 \pm 1.0 6	1.8 7 \pm 0.6 6 *
Peak T _{rec} (°C)	38. 52 \pm 0.5 8	38. 59 \pm 0.6 5	38. 44 \pm 0.5 0
T _{rec} \geq 38.5°C (min)	27 \pm 28	29 \pm 31	26 \pm 26

AUC 38.5°C (°C.min ⁻¹)	8.0 ± 11. 2	10. 7 ± 14. 0	5.3 ± 6.9
Δ HR (b.min ⁻¹)	96 ± 24	93 ± 26	98 ± 22
Mean HR (b.min ⁻¹)	12 9 ± 21	13 1 ± 23	127 ± 20
Peak HR (b.min ⁻¹)	16 2 ± 25	16 4 ± 29	160 ± 21

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Table 4. Ranked correlations of the pre to post session fold change in Hsp72 mRNA for ALL, Day1 and Day10. * denotes p <0.05, ** denotes p<0.01

ALL		Day1		Day10	
Variable	R	Variable	R	Variable	R
ΔT_{rec} (°C)	0.714**	AUC 38.5°C (°C.min ⁻¹)	0.761**	SR (L.hr ⁻¹)	0.762**
SR (L.hr ⁻¹)	0.709**	SR (L.hr ⁻¹)	0.738**	ΔT_{rec} (°C)	0.709**
Mean $T_{rec\text{final}45}$ (°C)	0.682**	Mean $T_{rec\text{final}45}$ (°C)	0.730**	AUC 38.5°C (°C.min ⁻¹)	0.687**
AUC 38.5°C (°C.min ⁻¹)	0.678**	ΔT_{rec} (°C)	0.721**	ΔHR (b.min ⁻¹)	0.665**
Peak T_{rec} (°C)	0.661**	Peak T_{rec} (°C)	0.688**	Rate of ΔT_{rec} (°C.hr ⁻¹)	0.661**

$T_{rec} \geq 38.5^{\circ}\text{C}$ (min)	0 . 6 5 0 * *	Mean T_{rec} ($^{\circ}\text{C}$)	0 . 6 7 5 * *	$T_{rec} \geq 38.5^{\circ}\text{C}$ (min)	0 . 6 5 9 * *
Mean T_{rec} ($^{\circ}\text{C}$)	0 . 5 6 5 * *	$T_{rec} \geq 38.5^{\circ}\text{C}$ (min)	0 . 6 5 0 *	Peak T_{rec} ($^{\circ}\text{C}$)	0 . 6 5 0 * *
Rate of ΔT_{rec} ($^{\circ}\text{C} \cdot \text{hr}^{-1}$)	0 . 5 6 0 * *	Rate of ΔT_{rec} ($^{\circ}\text{C} \cdot \text{hr}^{-1}$)	0 . 5 4 0	Mean $T_{rec\text{final}45}$ ($^{\circ}\text{C}$)	0 . 6 3 2 *
ΔHR ($\text{b} \cdot \text{min}^{-1}$)	0 . 5 1 1 * *	ΔHR ($\text{b} \cdot \text{min}^{-1}$)	0 . 3 9 1	Mean HR ($\text{b} \cdot \text{min}^{-1}$)	0 . 5 4 6 *
Mean HR ($\text{b} \cdot \text{min}^{-1}$)	0 . 4 3 1 *	Mean HR ($\text{b} \cdot \text{min}^{-1}$)	0 . 3 4 1	Mean T_{rec} ($^{\circ}\text{C}$)	0 . 4 5 4
Peak HR ($\text{b} \cdot \text{min}^{-1}$)	0 . 3 7 4 *	Peak HR ($\text{b} \cdot \text{min}^{-1}$)	0 . 3 3 3	Peak HR ($\text{b} \cdot \text{min}^{-1}$)	0 . 4 4 8

Mean Exercise Power (W.kg ⁻¹)	0 . 2 5 7	Mean Exercise Power (W.kg ⁻¹)	0 . 2 3 1	Mean Power (W.kg ⁻¹)	0 . 3 8 8
Total Work Done (kJ)	0 . 2 0 9	Exercise Duration (min)	0 . 2 2 3	Total Work Done (kJ)	0 . 3 7 1
Mean Power (W.kg ⁻¹)	0 . 1 7 3	Mean Exercise Intensity EQ \o\ac(V,\sup8(.))O _{2peak}	0 . 2 1 0	Mean Intensity EQ \o\ac(V,\sup8(.))O _{2peak}	0 . 3 0 1
Exercise Duration (min)	0 . 1 6 5	Total Work Done (kJ)	0 . 0 9 1	Mean Exercise Power (W.kg ⁻¹)	0 . 2 8 6
Mean Exercise Intensity EQ \o\ac(V,\sup8(.))O _{2peak}	0 . 1 5 3	Mean Intensity EQ \o\ac(V,\sup8(.))O _{2peak}	- 0 . 0 7 2	Exercise Duration (min)	0 . 2 6 2
Mean Intensity EQ \o\ac(V,\sup8(.))O _{2peak}	0 . 0 5 3	Mean Power (W.kg ⁻¹)	- 0 . 0 1 3	Mean Exercise Intensity EQ \o\ac(V,\sup8(.))O _{2peak}	0 . 0 5 6

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1490 Table 5. Ranked correlations of the pre to post session fold change in Hsp90 α mRNA for ALL, Day1 and Day10. *
 1491 denotes p <0.05, ** denotes p<0.01

ALL	
Variable	R =
AUC 38.5°C (°C.min ⁻¹)	0.729**
ΔT_{rec} (°C)	0.691**
Peak T_{rec} (°C)	0.680**
Mean $T_{rec\text{final}45}$ (°C)	0.678**
SR (L.hr ⁻¹)	0.660**
$T_{rec} \geq 38.5^\circ\text{C}$ (min)	0.629**
Mean T_{rec} (°C)	0.601**
Rate of ΔT_{rec} (°C.hr ⁻¹)	0.600**
ΔHR (b.min ⁻¹)	0.531**
Mean HR (b.min ⁻¹)	0.521**
Peak HR (b.min ⁻¹)	0.491**
Mean Exercise Power (W.kg ⁻¹)	0.289
Total Work Done (kJ)	0.223

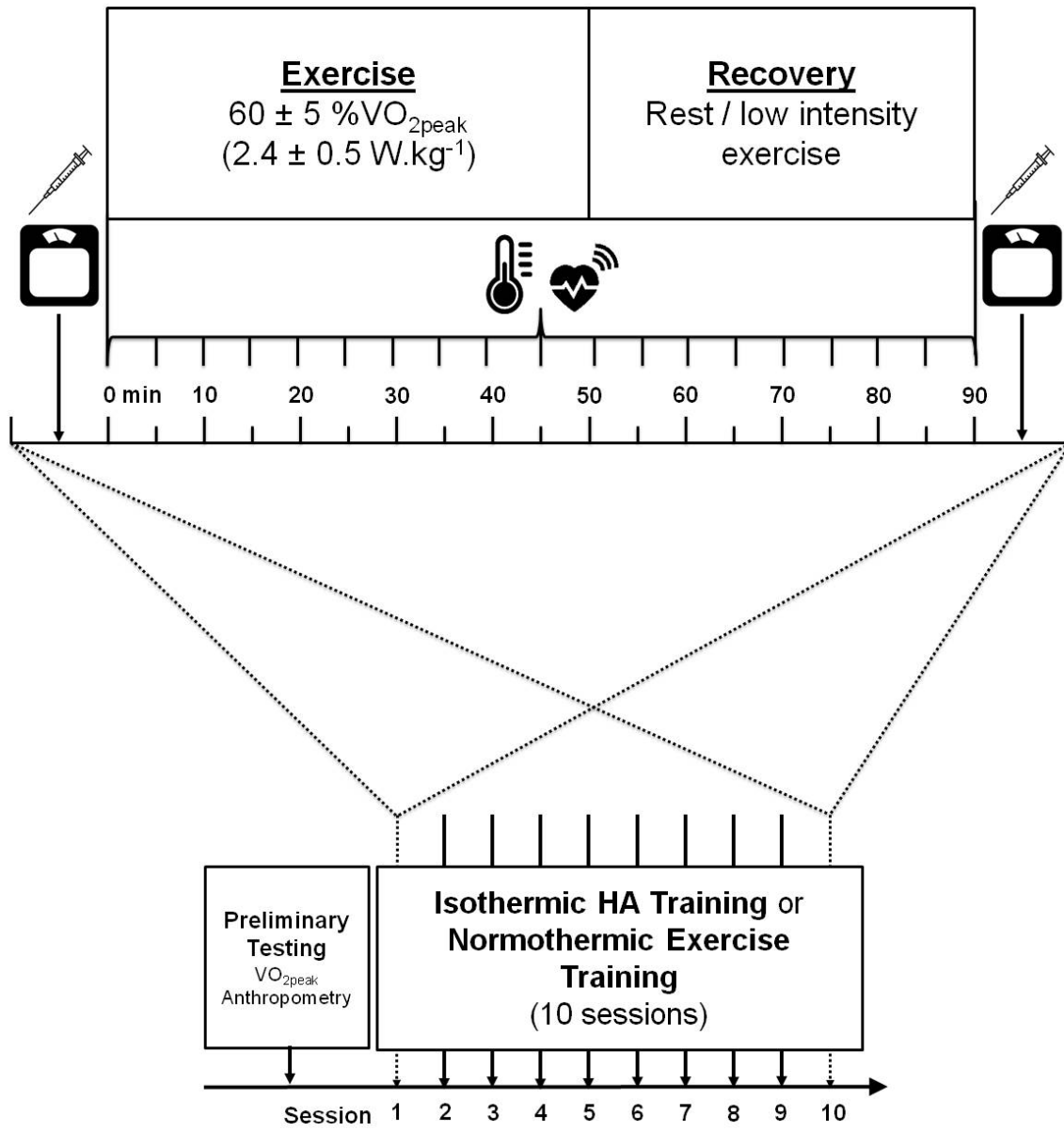
Mean Exercise Intensity $\dot{V}O_{2peak}$	0.183
Mean Power (W.kg ⁻¹)	0.098
Exercise Duration (min)	0.075
Mean Intensity $\dot{V}O_{2peak}$	-0.045

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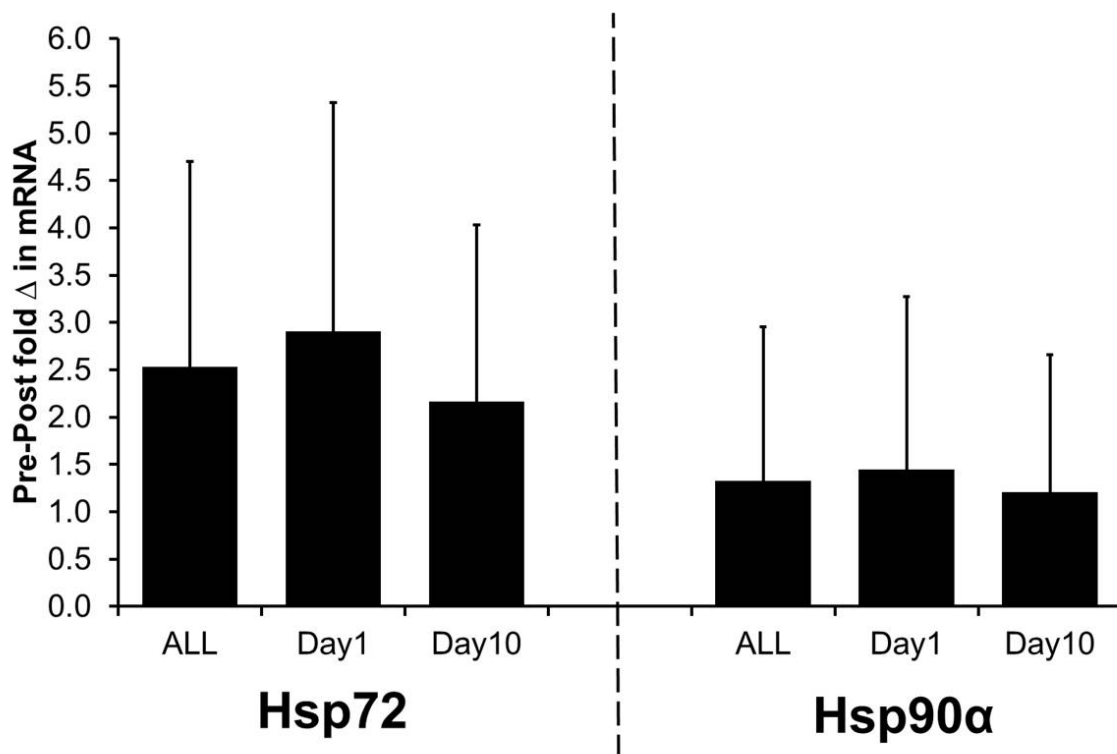
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Figure Legends



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Figure 1 Schematic overview of study.



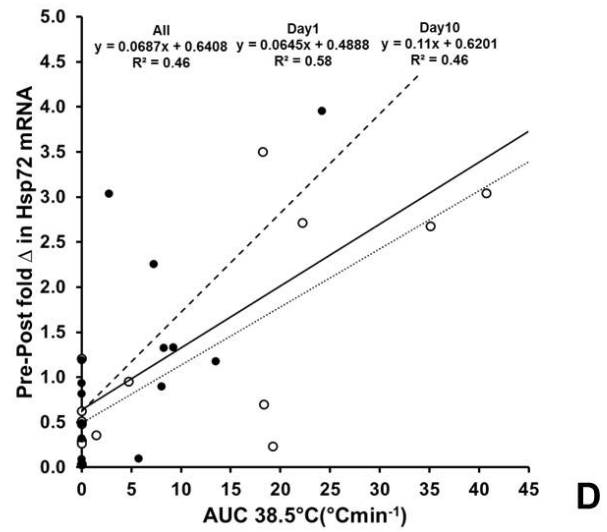
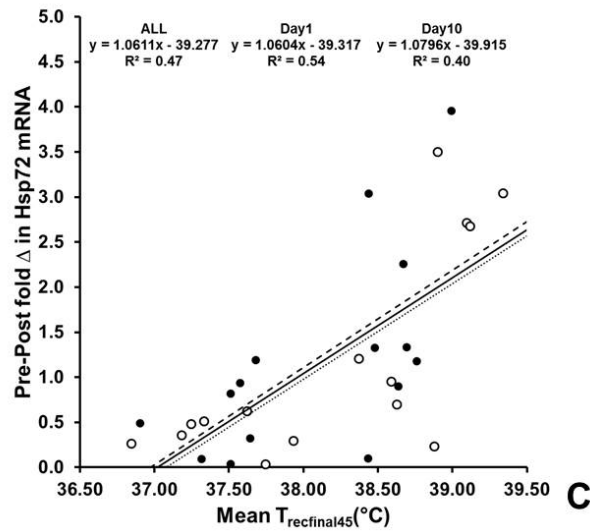
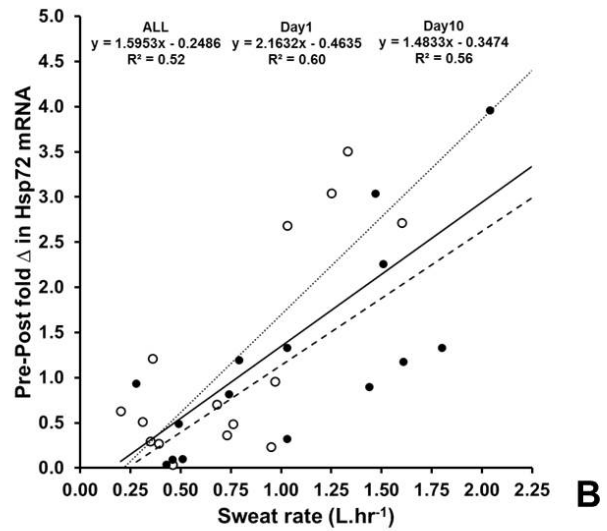
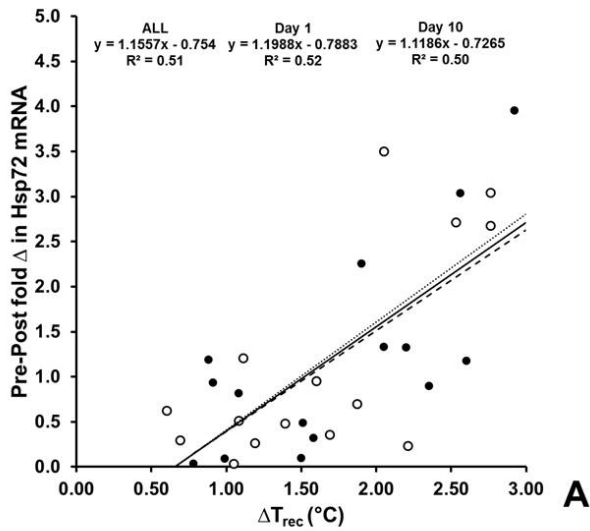
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1502 Figure 2. Mean \pm SD Pre to post session fold change in Hsp72 mRNA (left) and Hsp90 α mRNA (right) for the entire

1503 dataset (ALL), and on Day1 and Day10 of the intervention. * denotes a difference from Day1 within gene transcript

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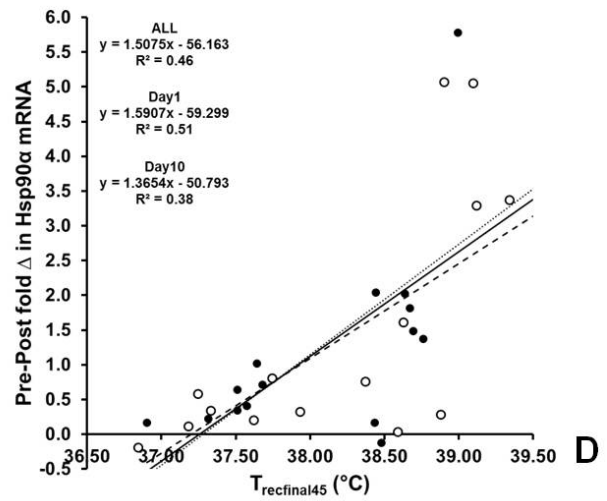
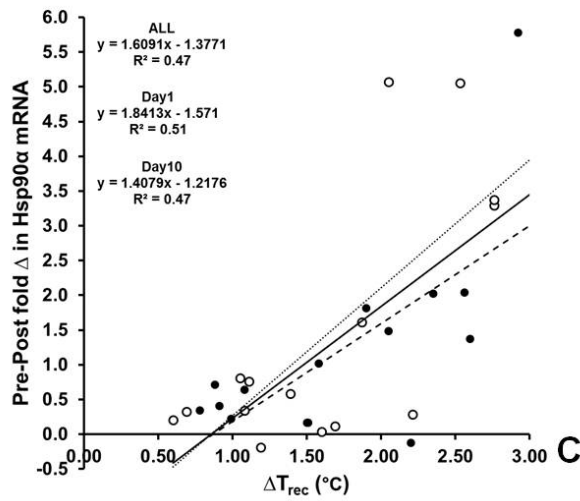
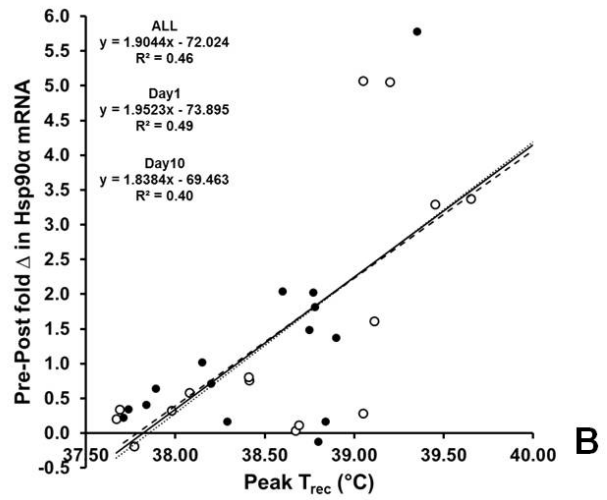
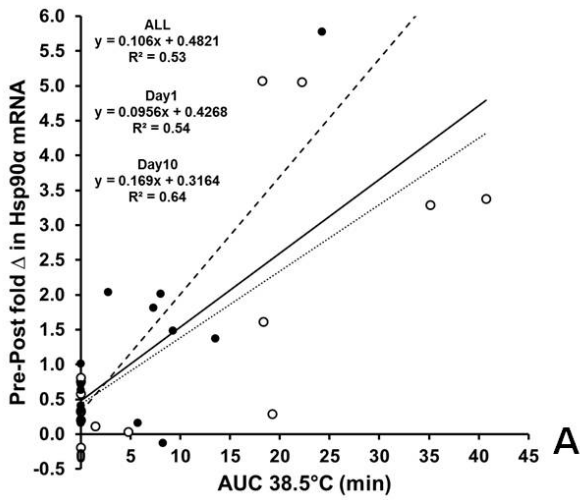


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1506 Figure 3. Relationship between the fold change in Hsp72 mRNA and the ΔT_{rec} [A], SR [B], Mean $T_{recfinal45}$ [C], and the
 1507 AUC_{38.5 $^{\circ}C$} [D]. Figures describe data for the entire dataset (ALL, solid line), and on Day1 (open circles, dotted line)

1508 and Day10 (closed circles, dashed line) of the intervention.

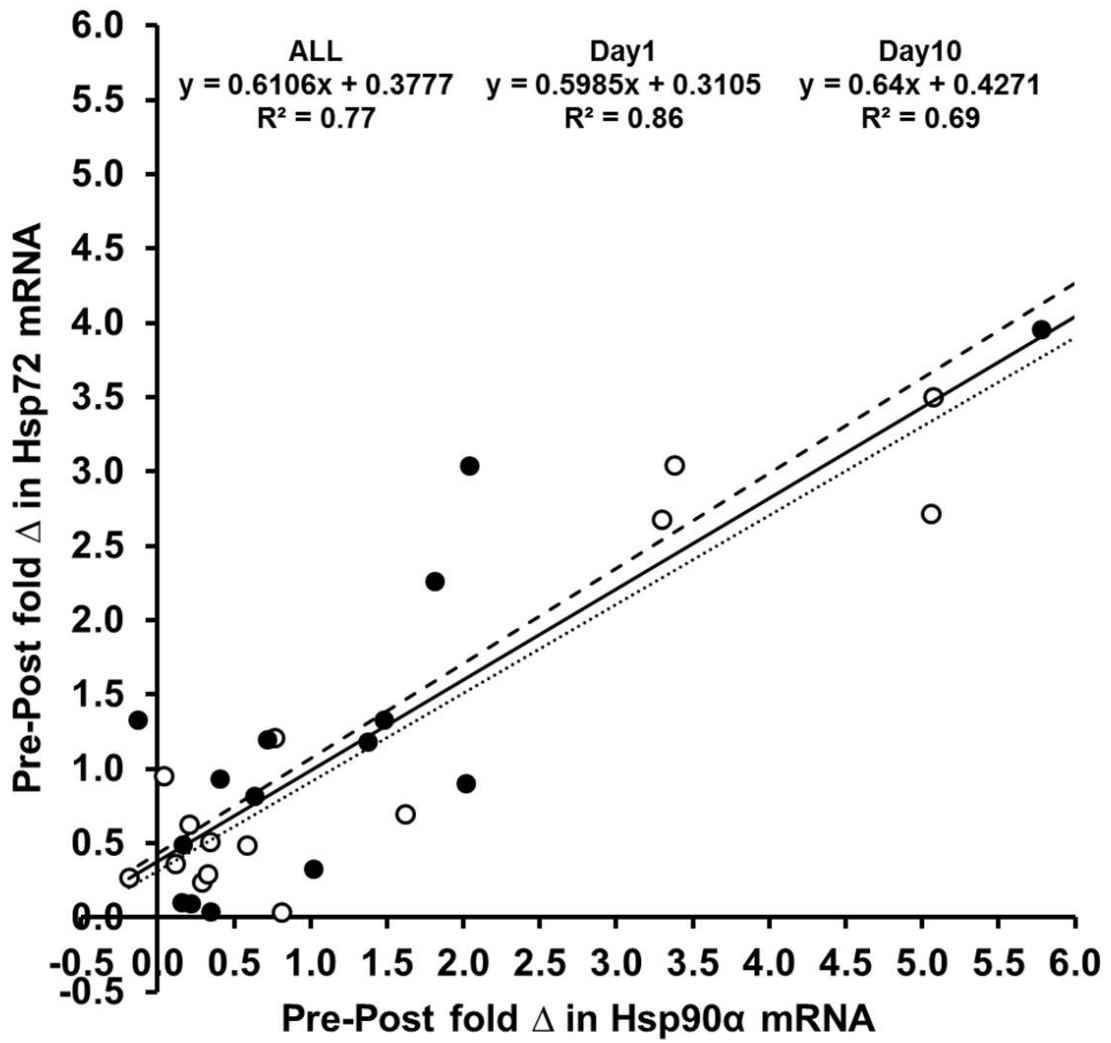
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1511 Figure 4. Relationship between the fold change in Hsp90α mRNA and the AUC38.5°C [A], peak T_{rec} [B], ΔT_{rec} [C], and
 1512 the Mean T_{recfinal45} [D]. Figures describe data for the entire dataset (ALL, solid line), and on Day1 (open circles, dotted
 1513 line) and Day10 (closed circles, dashed line) of the intervention.

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1516 Figure 5. Mean \pm SD Relationship between the fold change in Hsp72 mRNA and Hsp90 α mRNA entire dataset (ALL,
 1517 solid line), and on Day1 (open circles, dotted line) and Day10 (closed circles, dashed line) of the intervention.

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