

No endogenous circadian rhythm in resting plasma Hsp72 concentration in humans

Matthew B. Fortes · Martin Whitham

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Abstract Extra-cellular (e) heat shock protein (Hsp)72 has been shown to be elevated in a number of clinical conditions and has been proposed as a potential diagnostic marker. From a methodological and diagnostic perspective, it is important to investigate if concentrations of eHsp72 fluctuate throughout the day; hence, the purpose of the study was to measure resting concentrations of plasma eHsp72 throughout a 24-h period. Blood samples were taken every hour from 1200–2100 hours and from 0700–1200 hours the following day from seven healthy recreationally active males. Participants remained in the laboratory throughout the trial, performed light sedentary activities and were provided with standardised meals and fluids. Physical activity was quantified throughout by the use of an accelerometer. Ethylenediaminetetraacetic acid blood samples were analysed for eHsp72 concentration using a commercially available high-sensitivity enzyme-linked immunosorbent assay (intra-assay coefficient of variation=1.4%). One-way repeated measures analysis of variance revealed that measures of physiological stress such as heart rate, systolic and diastolic blood pressure remained stable throughout the trial and subjects remained sedentary throughout (mean activity energy expenditure above resting metabolic rate— $35.7 \pm 10.0 \text{ kcal} \cdot \text{h}^{-1}$). Plasma Hsp72 concentration did not fluctuate significantly throughout the day and showed no apparent endogenous circadian rhythm in absolute ($P=0.367$) or plasma volume change corrected data ($P=0.380$). Individual coefficients of variation ranged from 3.8–7.7% (mean 5.4%). Mean Hsp72 concentration across all subjects and time points was $1.49 \pm 0.08 \text{ ng} \cdot \text{ml}^{-1}$. These data show that in

a rested state, plasma eHsp72 concentration shows no apparent endogenous circadian rhythm.

Keywords Circadian variation · Heat shock protein · Stress · Endogenous rhythm

Introduction

Heat shock protein 72 (Hsp72), also termed HSPA1A (Kampinga et al. 2008), is a highly stress inducible *intracellular* protein concerned with maintaining cellular homeostasis. Despite its usual cytosolic location, Hsp72 is also present in the peripheral circulation of healthy humans (Pockley et al. 1998), with basal concentrations of *extracellular* (e)Hsp72 significantly increased in a number of stressful conditions such as exercise (Walsh et al. 2001; Febbraio et al. 2002; Lancaster et al. 2004), hyperthermia (Whitham et al. 2007) and trauma (Pittet et al. 2002). As such, much work has focused on the systemic roles of eHsp72, particularly in light of Matzinger's proposal that a number of endogenous molecules may act as danger signals during times of stress to initiate an immune response (Matzinger 2002; Fleshner and Johnson 2005). A number of recent review articles have summarised eHsp72's involvement in immunity (Pockley et al. 2008; Whitham and Fortes 2008; Johnson and Fleshner 2006).

It is well characterised that eHsp72 is elevated in a number of pathological disorders. For example, resting eHsp72 concentration is significantly higher in patients with peripheral vascular disease (Wright et al. 2000), trauma (Pittet et al. 2002), sudden sensorineural hearing loss (Park et al. 2006), septic shock (Wheeler et al. 2005), infection (Njemini et al. 2003) and prostate cancer (Abe et al. 2004) when compared to normal control subjects. As such, researchers have

M. B. Fortes · M. Whitham (✉)
School of Sport, Health and Exercise Sciences,
Bangor University,
George Building, Holyhead Road,
Bangor, Gwynedd LL57 2PZ, UK
e-mail: m.whitham@bangor.ac.uk

proposed that circulating levels of eHsp72 may be a useful marker in the diagnosis of such conditions when used in addition to established methods. Additionally, since high circulating levels of eHsp72 correlates with survival in trauma patients (Pittet et al. 2002) and is associated with protection against the development of atherosclerosis and coronary artery disease (Zhu et al. 2003; Pockley et al. 2003), low concentrations of eHsp72 in the peripheral circulation may also serve as a clinical risk factor. Furthermore, concentrations of eHsp72 decline with advancing age (Rea et al. 2001), which may be indicative of an age-related reduced ability to respond to stress that may partially account for the increased morbidity and mortality seen with ageing. Clearly, to be of value as a diagnostic marker or risk factor, it is essential that measured resting values reflect clinical pathology and are not purely down to unrelated extraneous variation, be it time of day effects, psychological or physical stress. To our knowledge, only one known study has investigated the time course of eHsp72 in human subjects in a rested non-stressed state (Fehrenbach et al. 2005). Despite variation existing between time points, Fehrenbach et al. failed to show a significant circadian variation in eHsp72. However, this study was comparatively insensitive to detecting changes in eHsp72 due to long periods (4 h) between the relatively few sampling points. Furthermore, no demographical, methodological or statistical information was presented thus making it difficult to ascertain whether a variation in resting eHsp72 exists.

Establishing (and controlling for) time of day effects is of importance in any longitudinal designed study whereby desired changes can be attributed to an intervention strategy and not just a consequence of an endogenous rhythm component in the variable of interest. It is also important to determine whether such a response is stable over time. Recent research into the effects of exercise-heat acclimation (HA) has highlighted this importance where resting levels of eHsp72 appear to decrease during HA (Kresfelder et al. 2006; Marshall et al. 2006). As such, a well-controlled investigation to establish whether resting concentrations of eHsp72 are subject to a circadian rhythm was warranted. Therefore, the purpose of this study was to measure the concentration of eHsp72 in healthy males throughout a 24-h period.

Materials and methods

Sample size calculation

A sample size calculation was performed using a freely available web-based sample size calculator (<http://www.maths.surrey.ac.uk/cgi-bin/stats/sample/singlemean.cgi>). Mean and standard deviation values of plasma Hsp72 derived from the only known study to investigate

circadian changes in eHsp72 were used for the calculation (Fehrenbach et al. 2005) resulting in a required n of 7 ($\alpha=0.05$, $\beta=0.8$).

Participants

With informed consent, seven healthy recreationally active males were recruited for the study (age— 23.3 ± 3.7 year, height— 1.83 ± 0.08 m, body mass— 77.6 ± 9.7 kg). All participants were free from infection, medication and any form of supplementation that may have interfered with the concentration of plasma Hsp72 (e.g. vitamins or protein shakes). Furthermore, participants had not recently visited a hot country, taken a sauna or any activity that may have resulted in temporary heat acclimation. Participants who 'napped' regularly or demonstrated unstable sleep/wake cycles (e.g. shift workers) were also excluded from the study. Having been made aware of procedures and risks involved in the study, participants gave written informed consent to participate in the investigation. The study was approved by the North West Wales NHS Trust ethics committee.

Experimental design

All procedures took place during March/April 2007. Participants were asked to refrain from strenuous physical activity, smoking and alcohol consumption in the 48 h prior to the start of the test. They were provided with water to consume the day before the trial ($35\text{ ml}\cdot\text{kg body weight}^{-1}$) in order to ensure euhydration. Upon arrival, a urine sample was collected and assessed for osmolality using a urine refractometer (Atago Co Ltd, Japan), and, in addition, nude body mass was taken at the start and end of the trial following voiding. A standardised breakfast was also provided to eat on the morning of the trial. Participants arrived in the laboratory at 1000 hours, and a 22-gauge cannula (Greiner Bio-one, Stonehouse, UK) was inserted into a forearm vein which was kept patent by a small infusion of 0.9% saline after each blood draw. A 2-h delay before the first blood sample at 1200 hours allowed any stress induced elevations in eHsp72 as a result of the cannulation procedure to subside. Venous blood samples were collected at hourly intervals until 2100 hours and then resumed at 0700 hours the following day until 1200 hours to allow determination of 24-h rhythms of plasma Hsp72. Unfortunately, blockages in the cannula resulted in a number of samples from three of the subjects having to be taken by venepuncture of an antecubital vein (23-gauge butterfly needle, Venisystems, Abbot Ireland, Republic of Ireland). Whilst it may be argued that repeated venepuncture may induce a stress response, unpublished pilot data from our laboratory has demonstrated no differences in eHsp72 concentration between samples derived concurrently by

cannulation and venepuncture in the same subjects. At these same time points, resting heart rate and blood pressure were assessed following a seated 10-min period, as these both provide a measure of physiological stress. As passive heating resulting in an elevation in core temperature increases the concentration of circulating Hsp72 (Whitham et al. 2007), tympanic temperature (in ear) was also assessed at these time points. Circadian rhythm is defined as a body rhythm demonstrating a periodicity of around 24 h and which may be mediated by either endogenous or exogenous factors (Shephard and Shek 1997). Exogenous factors include environmental temperature, physical activity, timing of meals and disturbances to the normal sleep/wake cycle. As such, to control for these factors, the trial took place in a laboratory that was kept at a comfortable temperature (20–24°C) and humidity (35–45% relative humidity) with a standardised light to dark cycle (constant light of 330–370 Lux from 0645–2300 hours), during which time the participants could perform light, sedentary activities such as reading, working on a computer and watching TV. The participants slept in a purpose built pleasant living area, were instructed to go to bed at 2300 hours and were woken at 0645 hours the following morning.

Tests and procedures

Participants were seated 15 min prior to each sampling time. After 10-min resting, tympanic temperature was assessed using an in-ear thermometer which detects infrared heat emissions from the tympanic membrane (ThermoScan IRT 4520, Braun GmbH, Germany). Blood pressure and heart rate was also assessed using an automated sphygmomanometer (Omron automatic oscillometric digital blood pressure monitor, Japan). Blood samples of 2×5 ml were drawn from the cannula and directed into 1× K₂ ethylenediaminetetraacetic acid (EDTA) and 1× lithium heparin-coated plasma tube (Becton Dickenson, UK). Heparinised blood was analysed in duplicate for haemoglobin concentration (Hemocue, UK) and in triplicate for haematocrit (capillary tube method) with plasma volume (PV) changes calculated using the Dill and Costill method (Dill and Costill 1974). Blood from the remaining lithium heparin tube and the K₂EDTA tube were then centrifuged immediately at 1500×g and 4°C for 10 min, aspirated into labelled Eppendorfs and frozen at –80°C for later analysis.

Control measures

Throughout the study, participants wore an accelerometer (Actigraph, USA) to quantify general activity levels and ensure participants remained sedentary. The accelerometer was worn on the non-dominant wrist for the entire duration of the study, with epoch intervals of 60 s. Counts per

minute were converted into kcal·h⁻¹ using the Actigraph Freedson kcal equation (Freedson et al. 1998) and adjusted for the fact that this equation tends to overestimate actual energy expenditure during sedentary activities by approximately 35% (Crouter et al. 2006). In order to avoid caloric restriction and acute dehydration, participants were provided with balanced meals and sufficient water throughout the duration of the study. Individual energy requirements in calories were estimated using the factor 1.3 (for sedentary activities) × resting energy expenditure (REE). REE was estimated using body weight and a formula based on males aged 18–30 ([15.3×body mass]+679; WHO 1985). Meals were provided at 1315–1345, 1815–1845, 2115 (day 1) and 0715–07:45 hours (day 2), with macro-nutrient breakdowns of 68% carbohydrate, 14% fat and 18% protein. Individual calorie intakes ranged from 2,235 to 2,672 kcal·d⁻¹. Fluids were provided over the 24 h (35 ml·kg body weight⁻¹) to maintain hydration, and participants were allowed to consume fluids over this quantity ad libidum.

Hsp72 analysis

Plasma Hsp72 was analysed in duplicate using a commercially available high-sensitivity sandwich enzyme-linked immunosorbent assay (ELISA) for use with serum and plasma samples (Assay Designs EKS-715, MI, USA). Briefly, samples and standards were added to wells coated with a mouse monoclonal antibody. Hsp72 was captured by this antibody and detected by adding a rabbit polyclonal detection antibody. Both antibodies are specific for inducible Hsp72 and do not react with other members of the HSP70 family such as Hsc73, Grp78, Dnak or Hsp71. A horseradish peroxidase conjugate bound the detection antibody and colour development was accomplished by the addition of tetramethylbenzidine substrate and stopped with an acid stop solution. The optical density of samples was read at 450 nm by a plate reader (Opsys MK, Dynex Technologies Inc, Chantilly, VA, USA) and were compared to a standard curve generated from known concentrations of recombinant Hsp72 ranging from 0.1–12.5 ng·ml⁻¹ ($r^2=0.989$). The assays sensitivity is described by the manufacturer as 0.09 ng·ml⁻¹, whilst the mean inter- and intra-assay coefficient of variation (CV) in the current study was 7.1% and 1.4%, respectively.

Statistical analysis

Differences in pre- to post-body mass and urine osmolality were investigated by means of paired *t* tests. eHsp72 concentration, heart rate, systolic and diastolic blood pressure, tympanic temperature and physical activity data were all analysed using one-way repeated measures analysis of variance with significant differences between time points assessed using Tukey's honestly significant differences

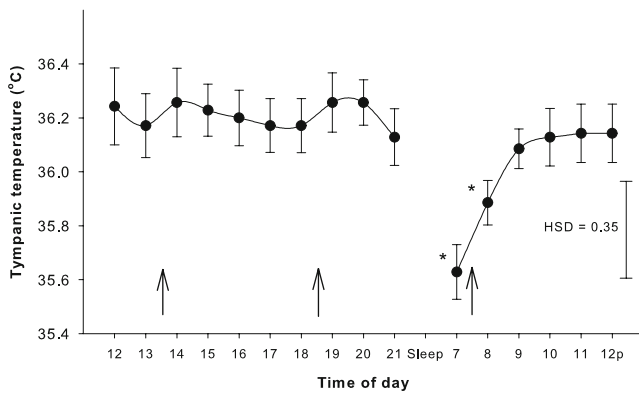


Fig. 1 Tympanic temperature measured throughout the 24-h period. HSD indicates Tukey's honestly significant difference value ($*P < 0.05$). Arrows represent main meal times. Values are mean \pm standard error of the mean (SEM), $n=7$

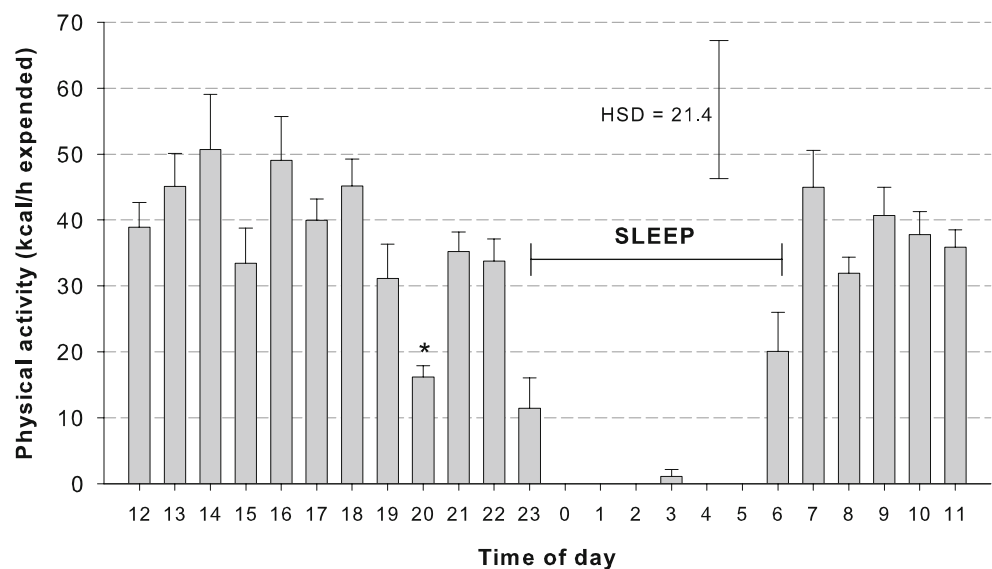
(HSD) post hoc test. Both absolute and plasma volume change corrected eHsp72 data were analysed. Coefficient of variation and box-and-whisker plot analysis were calculated for individual and mean eHsp72 data. Pearson's correlation coefficient was calculated to determine the stability of the eHsp72 response and to assess within-subject relationships between eHsp72, tympanic temperature and physical activity. Data were analysed using the Statistical Packages for the Social Sciences computer software (version 12.0). Values are mean \pm standard deviation unless otherwise stated, and significance was accepted at $P < 0.05$ in all cases.

Results

Control measures

There was no significant change in either body mass (pre— 78.1 ± 9.1 , post— 77.8 ± 9.4 kg, $P=0.370$) or urine osmolality

Fig. 2 Physical activity expenditure during the 24-h study. Values are $\text{kcal} \cdot \text{h}^{-1}$ derived from the accelerometer expended as physical activity, not including resting metabolic rate. Data during the sleeping period (from 2300 to 0700 hours) were not included in the statistical analysis. HSD indicates Tukey's honestly significant difference value ($*P < 0.05$). Values are mean \pm SEM, $n=7$



(pre— 405 ± 186 , post— 249 ± 56 $\text{mOsm} \cdot \text{kg}^{-1}$ H_2O , $P=0.052$) from pre- to post-24-h period. Heart rate ($P=0.083$), systolic ($P=0.664$) and diastolic ($P=0.254$) blood pressure remained stable throughout the trial with no significant differences between any time points.

Tympanic temperature demonstrated the extensively documented circadian rhythm that has been reported in previous literature ($P=0.008$), with lower values first thing in the morning (Fig. 1). Only the 0700 and 0800 hours samples were significantly different from any other time point. Despite inter-subject variation, physical activity remained sedentary throughout the trial (mean activity energy expenditure above resting metabolic rate— 35.7 ± 10.0 $\text{kcal} \cdot \text{h}^{-1}$). There was a significant main effect for time ($P=0.026$) with physical activity significantly lower between 2000 and 2100 hours (Fig. 2).

Plasma Hsp72

Plasma Hsp72 concentration did not change significantly throughout the 24-h period in either absolute concentration ($P=0.367$) or PV change corrected concentration ($P=0.380$; Fig. 3). Individual CVs ranged from 3.8–7.7% (mean 5.4%). Box-and-whisker plot analysis revealed no significant statistical outliers in four subjects or in the group as a whole, whilst two of the subjects had one elevated sample and one participant had one elevated and one low sample (Fig. 4). Mean eHsp72 concentration across all subjects and time points was 1.49 ± 0.08 and 1.51 ± 0.05 $\text{ng} \cdot \text{ml}^{-1}$ for absolute and PV change corrected data, respectively. Plasma Hsp72 concentration was related to tympanic temperature in one subject and related to prior physical activity in another (Table 1). However, across the whole group and in the remaining six participants, eHsp72 concentration was not related to tympanic temperature or

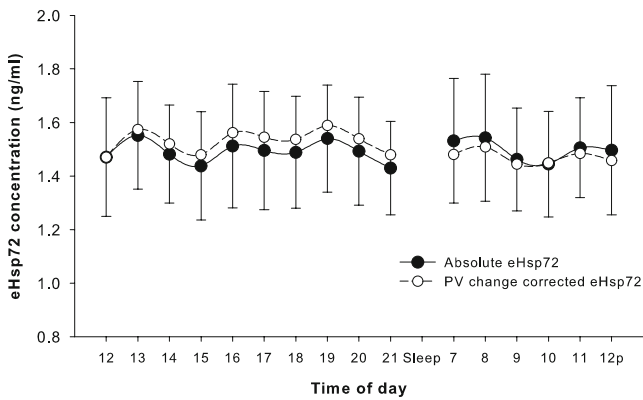


Fig. 3 Mean plasma Hsp72 concentration throughout the 24-h period of the study. Both absolute and plasma volume change corrected data are displayed. No significant differences between any of the time points. Values are mean \pm SEM, $n=7$

prior physical activity. To assess the stability of the eHsp72 response, blood samples taken at 1200 hours on both days 1 and 2 of the study were analysed and compared for eHsp72. No significant differences in either absolute or PV change corrected eHsp72 were observed between the two mid-day samples ($P=0.423$ and 0.164 , respectively). Furthermore, very small CVs of 2.9% and 2.3% and highly significant correlations ($r=0.993$, $P<0.001$ and $r=0.998$, $P<0.001$; Fig. 5) for absolute and PV change corrected eHsp72 demonstrates the stability of eHsp72 on a day-to-day basis.

Discussion

The aim of the current study was to assess whether a circadian rhythm exists in plasma Hsp72 concentration. Results from data collected from seven healthy males confirms the findings of Fehrenbach et al. (2005) that eHsp72 concentration does not fluctuate significantly throughout the course of a 24-h period. Furthermore, we have shown that the eHsp72 response is remarkably stable when measured at the same time on different days.

Visual analysis of individual rhythms of plasma Hsp72 did not show any apparent within-subject circadian rhythm although there was some inter-subject variation (Fig. 4).

The preferred method for detecting circadian rhythms is the Cosinor method, whereby a cosine curve is fitted to the data, detailing the mesor (mean), amplitude and acrophase (time of peak). However, an important assumption of this method is that a cosine pattern is apparent, and since this was not the case in the current study, this form of analysis was not performed. The single greatest fluctuation in eHsp72 concentration within the same subject was $\sim 0.5 \text{ ng}\cdot\text{ml}^{-1}$. It is difficult to say whether an absolute change of this magnitude is clinically relevant given that values reported in the literature for eHsp72 in a number of different clinical conditions vary greatly depending on the type of assay performed (i.e. differences between in-house and commercially available ELISAs). However, expressed as a percentage change compared to healthy controls, Hsp72 concentrations are typically between $\sim 20\%$ and 300% higher across a number of clinical conditions (Abe et al. 2004; Hunter-Lavin et al. 2004; Martin et al. 2003; Njemini et al. 2003; Oglesbee et al. 2005). In light of the fact that the mean coefficient of variation in the present study was 5.4% (with individual CVs ranging from 3.8–7.7%) and the single greatest within-subject difference was 18%, we are therefore confident that time-of-day effects are unlikely to affect the use of plasma Hsp72 as a clinical marker.

It is currently unknown what mediates the release of Hsp72 into the circulation under basal conditions. Whether Hsp72 is released from intra-cellular pools of Hsp72 or as a result of de novo synthesis of Hsp72 via activation of heat shock transcription factor-1 remains to be determined. Whilst every effort was made to ensure participants remained sedentary, acute non-clinical stressors such as thermal discomfort and exercise have been previously shown to affect eHsp72; thus, we investigated the possibility that these may be related to eHsp72.

Tympanic temperature was significantly lower first thing in the morning compared to all other sampling points. This was not surprising given the well-known circadian rhythm that exists in core body temperature (Waterhouse et al. 2005). Interestingly, there were slight (but non-significant) increases in tympanic temperature following the main meals, an occurrence that likely reflects diet-induced

Table 1 Within-subject and whole-sample correlations between concentration of eHsp72, tympanic temperature and physical activity

		Participant number							Sample
		1	2	3	4	5	6	7	
eHsp72 and tympanic temperature	<i>r</i>	0.14	0.27	-0.43	-0.42	-0.17	0.19	-0.76	-0.14
	<i>P</i>	0.60	0.32	0.09	0.10	0.54	0.49	0.04	0.59
eHsp72 and physical activity	<i>r</i>	0.25	-0.19	0.25	-0.21	0.10	0.79	0.40	0.16
	<i>P</i>	0.37	0.49	0.36	0.45	0.72	0.01	0.43	0.56

Significant relationships are depicted in bold

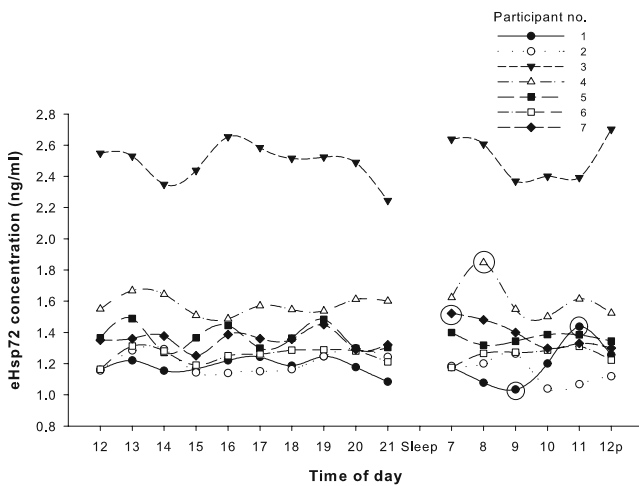


Fig. 4 Individual rhythms in absolute plasma Hsp72 concentration. Data points circled represent within-subject outliers derived from the box-and-whisker plot analysis, $n=7$

thermogenesis. It may be argued that since thermal stress is one factor that is linked with Hsp72 expression, this may have affected eHsp72 release into the circulation. Within-subject changes in tympanic temperature did not exceed 0.5°C , and since severe thermal stress resulting in large increases in rectal temperature ($\sim 2.3^{\circ}\text{C}$) by passive whole body heating showed only modest ($\sim 20\%$) increases in eHsp72 concentration in a thermal clamping study (Whitham et al. 2007), it is unlikely that normal everyday fluctuations in body temperature contribute to significant changes in eHsp72 concentration. Furthermore, this is corroborated by our results that failed to show a significant positive relationship between resting eHsp72 concentration and changes in tympanic temperature. Accelerometers were worn by the participants in order to assess physical activity levels as physical exercise has been shown to augment the eHsp72 response (Walsh et al. 2001; Febbraio et al. 2002; Whitham et al. 2007). The participants were instructed to remain sedentary throughout the study, and this is reflected by the low energy expenditure throughout the course of the study. It is therefore unlikely that physical activity was associated with eHsp72 concentration particularly since cycling for 2 h at $45\% \text{VO}_{2\text{max}}$ in 38°C heat elicited only a moderate ($\sim 30\%$) increase in eHsp72 concentration (Marshall et al. 2006). It is interesting to note that very little information is available on the intensity, duration, mode of exercise or type of everyday physical activities that are required to elicit an eHsp72 response. This is an interesting avenue of research. Establishing time of day effects are clearly important in longitudinal research and much research in eHsp72 physiology has focused on exercise stress with tests conducted over a period of hours and on repeat visits. In general, exercise results in an increase in plasma Hsp72 concentrations of $\sim 30\text{--}2,100\%$ (for a review see Whitham and Fortes 2008); hence, it is

unlikely that the typical fluctuations shown are likely to affect the results of previous or future exercise studies where time of day effects have not been controlled for. We acknowledge that different intervention strategies may elicit much smaller changes in eHsp72 concentration, though researchers should also consider the physiological relevance of such small changes. Furthermore, we have demonstrated that eHsp72 concentrations remain remarkably stable on a day-to-day basis with the small CV of $\sim 2.5\%$ between repeat sampling highlighting this. Circadian rhythms may be mediated by endogenous or exogenous factors (Shephard and Shek 1997). We controlled for external factors such as meal times, environmental temperature, light to dark cycle and physical activity, and since there was no synchronicity between these factors and eHsp72, we have demonstrated that under resting conditions, eHsp72 concentration is

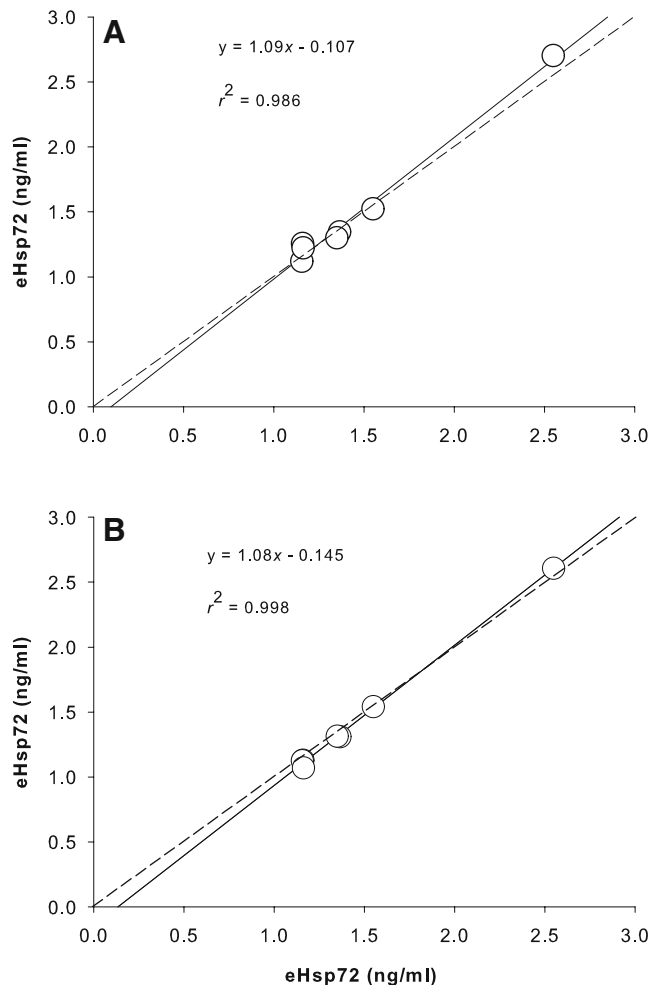


Fig. 5 Stability of the eHsp72 response. Scatter plots of eHsp72 concentration derived from samples taken at 12 noon on day 1 (x -axis) and day 2 (y -axis) of the study. Both absolute (a) and plasma volume change corrected (b) data are displayed. Both correlations are highly significant ($P < 0.001$). Solid line represents the regression line; dashed line represents the line of identity, $n=7$

subject to neither an endogenous nor an exogenous circadian rhythm.

Hsp72 is present in the circulation under normal resting conditions (Pockley et al. 1998) and is augmented with stress and in clinical conditions with research centering on the immunomodulatory aspect of eHsp72 expression. From an evolutionary standpoint, during times of stress, an enhanced immune response may prime the organism for potential pathogenic threat, whilst enhanced *intra-cellular* Hsp72 expression enables the cell to cope with physiological threats to cellular homeostasis. This is significant since *extra-cellular* Hsp72 has been shown to penetrate the cellular membrane and enter cells where it performs its usual cytoprotective *intra-cellular* roles (Guzhova et al. 1998; Novoselova et al. 2005). For example, increased uptake of extra-cellular Hsp72 increased motor neuron survival (Guzhova et al. 2001; Robinson et al. 2005), which is pertinent as these cells are incapable of synthesising Hsp72 under stressful insult. As such, this attribute of eHsp72 may be of clinical relevance in terms of the treatment of a number of neurodegenerative diseases that are associated with destabilisation of *intra-cellular* protein structure. Additionally, the decrease in resting plasma Hsp72 concentrations seen during the initial adaptation to heat acclimation (Marshall et al. 2006) may be a consequence of increased cellular uptake of eHsp72 in order to aid heat stress tolerance. From a clinical perspective, the lack of a circadian rhythm denotes that eHsp72 may be used as a diagnostic marker in a number of clinical conditions for which concentrations have been shown to be elevated with disease progression.

In conclusion, we have shown in a well-controlled and sensitive study that in a rested state, eHsp72 concentration shows no apparent endogenous circadian rhythm, remains stable when tested on a day-to-day basis and is not related to tympanic temperature or prior sedentary physical activity.

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