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Methodological considerations for the assessment of extracellular heat shock protein 72

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METHODOLOGICAL CONSIDERATIONS FOR THE ASSESSMENT OF EXTRACELLULAR HEAT SHOCK PROTEIN 72

by

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Summary

In light of the increasing amount of *in vivo* human research being conducted into the expression and potential roles of extracellular heat shock protein 72 (eHsp72), the aims of this thesis were to address a number of unanswered important methodological questions that researchers should be aware of when planning such investigations. The specific aims were; 1) to examine the effect of different blood handling procedures upon eHsp72 concentration, 2) to investigate the stability of plasma eHsp72 concentration over a 24-hour period, 3) to explore the effect of acute psychological stress upon eHsp72 concentration, and 4) to investigate the utility of salivary eHsp72 as a marker of plasma Hsp72 during exercise and to speculate upon the role of sympathetic/parasympathetic stimulated release of salivary Hsp72.

The assayed concentration of eHsp72 is affected by the matrix in which it is tested. Blood samples drawn in the presence of an anticoagulant (plasma) returned considerably higher concentrations of eHsp72 than samples that were allowed to clot (serum). Whilst this has obvious benefits in maximising eHsp72 detection, it has also prompted speculation that eHsp72 may have biological roles during the clotting process, since the majority of Hsp72 was unrecovered in serum samples.

Acute psychological stress did not alter the concentration of eHsp72. Therefore, acute anxiety associated with participation in research studies is unlikely to affect baseline or intervention data. Additionally, it has been demonstrated that resting eHsp72 concentrations do not show a circadian variation and remain remarkably stable on a day-to-day basis.

Finally, the effects of acute exercise upon salivary and plasma eHsp72 concentration was investigated. Exercise increased plasma eHsp72, but did not significantly alter salivary eHsp72 concentration or secretion rate. Furthermore, salivary eHsp72 concentration did not track plasma eHsp72. By utilising a caffeine-evoked sympathetic stimulation model, and measurement of salivary α-amylase and total protein, we have suggested that salivary eHsp72 is released independently of adrenergic-stimulation.

To conclude, it is apparent that time of day effects and acute psychological stress do not alter eHsp72 concentration, so should not be a primary concern for future investigations into the biological roles of eHsp72. Furthermore, whilst Hsp72 in present in saliva, concentrations derived from this matrix should not be used as a proxy measure of circulating Hsp72 concentration. Finally, to maximise detection and Hsp72 recovery, blood samples should be collected in the presence of an anticoagulant.

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Publications

I was fully involved with all aspects of study design, data collection, data analysis and manuscript preparation for all of the chapters presented and in the following publications that have arisen from this thesis:

Invited review

Whitham, M. and Fortes, M.B. (2008). Heat shock protein 72: release and biological significance during exercise. *Frontiers in Biosciences*, **13**, 1328-1339.

Full papers

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Abstracts/conference proceedings

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Contents

List of figures presented throughout the thesis:

List of tables presented throughout the thesis:

List of abbreviations

Abbreviated words are described in full at the first instance they are encountered during the text. However, for ease of reading a list of *commonly* used abbreviations used throughout the text is provided:

ACTH	-	Adrenocorticotropic hormone
ANOVA	-	Analysis of variance
Ca^{2+}	-	Calcium
CAF	-	Caffeine
CK	-	Creatine kinase
CV	-	Coefficient of variation
EDTA	-	Ethylenediamine-tetraacetic acid
eHsp72	-	Extracellular heat shock protein 72
Grp	-	Glucose regulated protein
HA	-	Heat acclimation
Hb	-	Haemoglobin
HPA	-	Hypothalamic-pituitary-adrenal axis
HR	-	Heart rate
HSD	-	Tukey's honestly significant difference value
HSP	-	Heat shock protein (pertaining to the family)
Hsc	-	Heat shock cognate
Hsp**	-	Heat shock protein (individual member of family, e.g. Hsp72)
Ig	-	Immunoglobulin
kCal	-	Kilocalorie
kDa	-	Kilodalton
NBM	-	Nude body mass
n	-	Number of participants in sample
PBMC	-	Peripheral blood mononuclear cell
PLA	-	Placebo
REE	-	Resting energy expenditure
RH	-	Relative humidity
rHsp72	-	Recombinant Hsp72
SAM	-	Sympatho-adrenal-medullary axis
SEM	-	Standard error of the mean
SD	-	Standard deviation
T_{re}	-	Core rectal temperature
T_{tymp}	-	Tympanic temperature
VAS	-	Visual analogue scale
VO_{2max}	-	Maximal oxygen uptake
W	-	Watts

Chapter 1

Literature Review – Extracellular heat shock protein 72

An adapted version of this literature review has been published as an invited review in "Frontiers in Biosciences":

Whitham, M. and Fortes, M.B. (2008). Heat shock protein 72: release and biological significance during exercise. *Frontiers in Biosciences*, **13**, 1328-1339.

1.1 Heat shock proteins (HSP's)

Vital to the survival of any living organism is the ability to adapt to stimuli or stressors that pose a threat to cellular integrity. When cells are exposed to stressors such as heat, oxidative stress, hypoxia etc, a group of proteins are synthesized within the cell that restores the function and structure of denatured proteins. These heat shock proteins (HSP's) have strong cytoprotective effects, acting as molecular chaperones to maintain cellular homeostasis (Mayer & Bukau, 2005).

The discovery of heat shock proteins occurred when a strange puffing pattern and an unusual profile of gene expression in the polytene chromosomes in the salivary cells of *Drosophila Melanogaster* larvae were identified when inadvertently subjected to heat stress (Ritossa, 1962). It was not until 1974 that the proteins associated with this gene expression were identified, and the term 'heat shock protein' was coined (Tissieres *et al.*, 1974). The name 'heat shock' is derived simply from the fact that at that time, increased temperature was the only stress known to evoke this response. Subsequently, other stressors such as oxidative stress, viral infections, glucose deprivation, hypoxia, exposure to ultraviolet radiation and certain chemicals have all been identified as being able to induce the intracellular expression of HSP's (Pockley, 2003;Benjamin & McMillan, 1998;Locke, 1997). The terms 'cellular stress response' and 'stress proteins' have also been introduced to reflect the universal nature of these proteins (Locke, 1997). HSP's are the most phylogenetically[1] conserved proteins, present in all prokaryotes and eukaryotes

[1] Evolutionary development of the molecule has been similar across all species of organisms (Fink, 1999)

across all species including plants. They are constitutively expressed within the cell (accounting for 5 – 10% of the total protein content of the cell in healthy growth conditions (Pockley, 2003), thus the term 'stress protein' may be viewed as a bit of a misnomer due to the involvement of this protein in normal cellular processes under healthy conditions (Locke, 1997).

1.2 Nomenclature

HSP's are typically classified into families based on their molecular weight as determined by migration on gels or from nucleic acid sequence analysis. The most common molecular masses are 60, 70, 90, 100 and 110 kilodaltons (kDa) with a group whose masses range from 15-45kDa termed the small heat shock proteins, with many isoforms present within each family. Typically, family names are written in upper case (e.g. HSP70), whereas individual members of a family are written in conventional form e.g. Hsp72 in accordance with the Cold Spring Harbor Meeting of 1996 (Hightower & Hendershot, 1997). However, due to the ever increasing discovery of new members of individual families and the inconsistencies in their nomenclature within the literature, a new nomenclature has been adopted whereby families are assigned an HSP designation, i.e. HSPA for the HSP70 family, HSPC for HSP90 etc (Kampinga *et al.*, 2008). Individual genes within each family are then given a numerical designation. For example, the most highly inducible HSP within the cytoplasm is Hsp72, which under the new nomenclature is termed HSPA1A. However, throughout this thesis except where stated, the original nomenclature will be used, since to date, the majority of studies referenced in this thesis adopted this nomenclature.

1.3 Hsp72

The most highly conserved and studied family is the 70kDa group of which 13 genes have been identified (Kampinga *et al.*, 2008). The most studied members include; the constitutive (present under normal conditions) heat shock cognate – Hsc70 (HSPA8), the stress inducible form, Hsp72 (HSPA1A) which both reside in the cytoplasm and nucleus, Grp75 or Mortalin (HSPA9) which is the mitochondrial family member, and Grp78 or BIP (HSPA5) which resides within the endoplasmic reticulum. Hsp72 is the most studied and stress inducible of all the HSP's and as such, will form the focus of this thesis.

The induction of Hsp72 depends on the heat shock element (HSE) in the promoter region of the HSP gene binding heat shock transcription factor (HSF) -1 (Wu, 1995). Hsp72 mRNA is then transcribed, followed by Hsp72 protein synthesis. Many signals have been identified that result in the expression of intracellular Hsp72; for example, adrenocorticotropin hormone (ACTH) (Blake *et al.*, 1991), corticosterone (Valen *et al.*, 2000), catecholamines (Matz *et al.*, 1996;Udelsman *et al.*, 1994), glycogen deprivation (Febbraio *et al.*, 2002b), oxidative stress (Marini *et al.*, 1996) and heat/hyperthermia (Kregel & Moseley, 1996). Hsp72 is capable of binding ATP and polypetides, with the N-terminal responsible for binding ATP, whilst the C-terminal binds polypeptides (Mayer & Bukau, 2005).

1.4 Intracellular roles of Hsp72

Abundant research has detailed the *intracellular* functions of Hsp72. Since *extracellular* (e) Hsp72 provides the focus of this thesis, the intracellular function of Hsp72 will only be briefly reviewed here.

Hsp72 is present within the cell under basal conditions where it performs essential 'housekeeping' roles such as assisting in the assembly of and chaperoning newly synthesised proteins across membranes to various cellular compartments. Under a stressful condition such as heat exposure and subsequent protein denaturation, Hsp72 is induced in large quantities. This enables Hsp72 to preserve cellular homeostasis and maintain the cytoskeletal structure by binding to denatured and aberrant proteins, facilitating re-folding or breaking down these proteins, thus limiting protein aggregation (Moseley, 1997). In addition to heat exposure, other stressors that induce the intracellular expression of Hsp72 involve physiological stressors such as exercise, hormonal stimulation and cell differentiation, environmental stressors such as hypoxia, antibiotics, ultra-violet radiation and heavy metals, and pathological stressors such as viral infections, bacterial infections, inflammation, fever, ischemia, toxins, lipopolysaccaride (LPS) and autoimmune diseases (Prohaszka & Fust, 2004;Asea, 2007). As such, it is well established that induction of intracellular Hsp72 protects a number of different tissues and organs, such as the heart, intestine, kidney and liver from a wide range of stressors, underlining the therapeutic potential of increased Hsp72 expression. Exercise-induced increases in intracellular Hsp72 have also been well documented in muscle, liver, heart, brain, adrenal, pituitary, spleen and immune cells in rats (Campisi *et al.*, 2003b;Salo *et al.*, 1991;Locke *et al.*, 1990), and in skeletal muscle and immune cells in humans (Fehrenbach *et al.*, 2000;Morton *et al.*, 2006). An interesting aspect of intracellular Hsp72 induction, is that prior stress-evoked elevations in Hsp72 may confer cytoprotection to subsequent, otherwise lethal stressors (Maloyan *et al.*, 1999), and this attribute is of particular relevance to cardioprotection and thermotolerance (Kregel, 2002;Powers *et al.*, 2001). Furthermore, induction of Hsp72 within the cell has large anti-inflammatory and anti-apoptotic effects (Jaattela *et al.*, 1998;Ianaro *et al.*, 2001).

1.5 Endogenous extracellular Hsp72.

Whilst primarily known as *intracellular* proteins, HSP's have been detected in the extra-cellular environment, promoting much interest within stress protein physiology. Heat shock proteins were first detected in the serum of humans by Pockley and colleagues in 1998. Soluble Hsp70 and Hsp60 and their respective antibodies were found under basal concentrations in normal healthy humans with concentrations twice as high in females compared to males (Pockley *et al.*, 1998;Pockley *et al.*, 1999). This group subsequently demonstrated that patients with elevated disease states such as atherosclerosis (Pockley *et al.*, 2003), and peripheral and renal vascular disease (Wright *et al.*, 2000) showed chronically elevated basal concentrations of extracellular (e)Hsp72 within their circulation compared to healthy aged matched controls, suggesting that eHsp72 may be informative of disease pathology. In addition to the earlier studies by the Pockley group, concentrations of eHsp72 have been shown to increase significantly in the circulation of *healthy* humans in response to exercise (Febbraio *et al.*, 2002a;Fehrenbach *et al.*, 2005;Walsh *et al.*, 2001;Whitham *et al.*, 2006), severe trauma (Pittet *et al.*, 2002) and passive heating (Whitham *et al.*, 2007). Furthermore, rodents exposed to tailshock stress (Johnson *et al.*, 2005) and predatory fear (Fleshner *et al.*, 2004) also express eHsp72 within their circulation. Taken together, these studies demonstrate the ubiquity of eHsp72 release in response to a variety of stressors and also across species, and that it may be until recently, an unknown factor associated with the typical stress response (Johnson & Fleshner, 2006). Hsp72 has also been detected in other extracellular compartments. In healthy disease free humans, eHsp72 is present in the cerebrospinal fluid (CSF) under basal and exercise conditions (Steensberg *et al.*, 2006), and in resting saliva samples (Fabian *et al.*, 2003). eHsp72 has also been detected in the pulmonary oedema fluid of patients with acute lung injury (Ganter *et al.*, 2006), in the synovial fluid of patients with rheumatoid arthritis

(Martin *et al.*, 2003), in postoperative wound fluid (Becker *et al.*, 2007;Flohe *et al.*, 2007) and in the cerebrospinal fluid of paediatric patients (Tang *et al.*, 2008). Whilst the significance of the presence of Hsp72 in these extracellular environments is yet to be fully determined, the focus of this review will be based around serum and plasma Hsp72 which has received much attention within the literature. Since the majority of investigations into the stress mediated effects upon eHsp72 have utilised exercise stress, it seems pertinent at this point to review the aspects of exercise that induce eHsp72.

1.6 Effect of exercise on eHsp72 concentration

Previous evidence had shown that stressors characteristic of exercising muscle such as acidosis, increased temperature, free radical formation, ischemia and glucose deprivation all induce the *intracellular* expression of Hsp72. Furthermore, exercise itself induces the expression of Hsp72 in a number of tissues such as the heart (Salo *et al.*, 1991;Skidmore *et al.*, 1995), liver (Salo *et al.*, 1991), brain (Walters *et al.*, 1998) and skeletal muscle (Salo *et al.*, 1991;Skidmore *et al.*, 1995). As Hsp72 was demonstrated *in vitro* to have potential immunostimulatory capabilities (Asea *et al.*, 2000) and that Hsp72 had been detected in the circulation of humans, Walsh *et al.* (2001) hypothesized that an acute bout of exercise would increase Hsp72 protein expression in contracting skeletal muscle which would then be released into the extracellular environment increasing the concentration of serum Hsp72. Following 60 minutes of treadmill running at 70% VO_{2max}, serum eHsp72 was indeed elevated, but the increase preceded the increase in Hsp72 mRNA and protein synthesis within the muscle tissue, prompting the authors to assert that muscle cells are not responsible for the extracellular release of eHsp72. This study was the first to document that exercise stress results in an increase in Hsp72 concentration in the circulation of

humans. Subsequent studies by the Febbraio group (Febbraio *et al.*, 2004;Febbraio *et al.*, 2002a;Lancaster *et al.*, 2004) have shown that semi-recumbent cycling at intensities of 60-65% VO_{2max} also induces a significant extracellular Hsp72 response. Therefore exercise modes that involve either predominantly eccentric (as in treadmill running) or concentric (cycling) contractions both elevate eHsp72 concentration.

Only one study has investigated the effect of different duration and intensities on the eHsp72 response to exercise (Fehrenbach *et al.*, 2005). In a cross sectional design participants conducted a marathon (~260 mins at ~65% VO_{2max}), a long run on a treadmill (120 mins at 60% VO_{2max}), a continuous run on a treadmill (60 mins at 75% VO_{2max}) or a high-intensity interval session on a 400m running track (10 x 1000m, ~35 mins at ~88% VO_{2max}). Whilst all conditions elevated eHsp72 immediately post exercise, the marathon run induced a greater concentration than all other conditions. There was also a tendency for a duration dependent eHsp72 expression, and levels had returned to baseline values within 24-hours across all conditions. Additionally, running at 80% VO_{2max} for approximately 24 minutes resulted in a greater post exercise eHsp72 concentration than running at 60% VO_{2max} (for the same duration). Taken together, these data suggest that the exercise eHsp72 response is both intensity and duration dependent. A number of studies have corroborated the finding that exercise results in an increased expression of eHsp72. A review of these exercise studies is summarised in Table 1.1

Table 1.1 Summary of studies investigating the effects of acute exercise on the extracellular Hsp72 response in humans.

Study	Mode of exercise	Intensity	Duration or distance	Serum or plasma	Effect on eHsp72. % change from pre-ex baseline to immediately post ex	Notes
Walsh *et al.* (2001)	Treadmill running	70% VO_{2max}	60 mins	S	↑ 685%	*n*=6.
Febbraio *et al.* (2002a)	Semi-recumbent cycling	~62% VO_{2max}	120 mins	S	0 – 0.88 ng/ml[a]	*n*=7.
Febbraio *et al.* (2002b)	Two-legged knee extensor exercise	40% of leg peak power output	4 – 5 hrs	S	None	*n*=7. Unable to detect eHsp72 in any samples.
Febbraio *et al.* (2004)	Semi-recumbent cycling	~65% VO_{2max}	120 mins	S	0 – 0.9 ng/ml[a]	*n*=6.
Lancaster *et al.* (2004)	Cycling	60% VO_{2max}	180 mins	S	↑ ~150%	*n*=6. Carbohydrate ingested throughout trial[b].
Peake *et al.* (2005)	Flat treadmill run	60% VO_{2max}	60 mins	P	↑ 47%	*n*=9.
	"	80% VO_{2max}	60 mins	P	↑ 88%	
	Downhill (-10%) treadmill run	60% VO_{2max}	45 mins	P	↑ 147%	
Fehrenbach *et al.* (2005)	Competitive marathon	~65% VO_{2max}	42.2km 260 mins	P	↑ ~725%	*n*=17
	Interval training	~88% VO_{2max}	10x1000m 35 mins	P	↑ ~150%	*n*=10
	Flat treadmill run	75% VO_{2max}	60 mins	P	↑ ~170%	*n*=7
	"	60% VO_{2max}	120 mins	P	↑ ~140%	*n*=7
	"	80% VO_{2max}	24 mins	S	↑ ~1100%	*n*=10
	"	60% VO_{2max}	24 mins	S	↑ ~250%	*n*=10.
Fischer *et al.* (2006)	Two-legged knee extensor exercise	50% of leg peak power output	180 mins	S	↑ ~ 300%	*n*=7.

Suzuki *et al.* (2006)	Competitive ironman triathlon: Swimming Cycling Running	Variable	3.8km ~57mins 180km ~311mins 42.2km ~231mins	P	↑ ~2100%	*n*=9. Muscle damage evident.
Whitham *et al.* (2006)	Cycling	74% VO_{2max}	90 mins	P	↑ 146%	*n*=10.
Ruell *et al.* (2006)	Competitive running	Variable	14km ~58 mins ~64 mins	P	Control ↑ ~850% EHI[c] ↑ ~2900%	*n*=7. *n*=22.
Marshall *et al.* (2006)	Cycling	43% VO_{2max}	120 mins	S	↑ ~32%	*n*=7. Hot and humid conditions (38°C, 60%RH).
Whitham *et al.* (2007)	Underwater running Core temp clamped Un clamped	59% VO_{2max} "	120 mins 120 mins	P P	↑ 131% ↑ 233%	*n*=9. No change in T_{re}[d] T_{re} ↑ 2.2°C
Yamada *et al.*, (2007)	Treadmill running/walking	56% VO_{2max}	100 mins	S	↑ 17%	*n*=12, Hot conditions (42.5°C, 28%RH).
Horn *et al.*, (2007)	Treadmill running	70% VO_{2max}	50 mins	P	↑ 167%	*n*=18
Amorim *et al.*, (2008)	Treadmill walking	~50% VO_{2max}	45 mins 83 mins	S S	↑ 100% ↑ 76%	HS[e] *n*=9 LS[e]

Note: Where exact eHsp72 values have not been reported in the published journal article, approximate changes from baseline have been calculated. Serum derived eHsp72 concentrations tend to be lower than that derived from plasma. Hence, with very low baseline eHsp72 levels, % change tends to be very high compared with plasma.

[a] eHsp72 was virtually undetectable at rest thus unable to calculate exact % change.

[b] Carbohydrate has been shown to blunt the eHsp72 response to exercise (Febbraio *et al.* 2004).

[c] EHI: exertional heat illness, those competitors who collapsed during the race with core temperatures exceeding 39°C (mean T_{re} ~41°C).

[d] T_{re}: Rectal core temperature

[e] HS: high rate of heat storage, LS: low rate of heat storage

The cumulative stressors associated with exercise result in an increase in eHsp72 within the blood. This has initiated much interest in heat shock protein physiology to determine not only the mechanism, releasing signal and origin of extracellular Hsp72 release, but perhaps more importantly, the biological significance of the presence of Hsp72 in the extracellular environment.

1.7 Releasing mechanism of eHsp72

For Hsp72 to be present in the peripheral circulation then it must be released from a cell/organ. There are two potential mechanisms for the cellular release of Hsp72. Firstly, Hsp72 is passively released from an intracellular pool following cellular lysis or death. Alternatively, or perhaps additionally, Hsp72 is actively released due to a receptor-mediated exocytotic pathway (Fleshner & Johnson, 2005;Asea, 2007). One further plausible explanation related to this is that Hsp72 is chaperoning other molecules that have been excreted from the cell (Prohaszka & Fust, 2004).

1.8 eHsp72 release and cell death

It was first suggested by Galluci *et al.* (1999) that Hsp72 is released only under pathological circumstances resulting in necrotic death and not programmed or apoptotic death. This was substantiated by two *in vitro* studies whereby induced cellular necrosis (through repeated freeze/thaw exposures, hypotonic lysis or virally induced lysis) released HSP's into the culture supernantant, whereas simulated apoptosis via exposure to ultraviolet radiation did not (Basu *et al.*, 2000;Berwin *et al.*, 2001). Tissue/cellular necrosis releases Hsp72 into the extracellular milieu via lysis of the cellular membrane

with the protein contents of the cell 'spilling' into the surrounding space. Exercise that results in damage to the sarcolemma reflects necrosis and there is some *in vivo* supporting evidence linking elevated eHsp72 with markers of muscle damage. Running a marathon (~4 hours) resulted in a greater eHsp72 response than shorter continuous runs at 60 and 75% VO_{2max} or interval training, which was accompanied by a greater concentration of post-exercise plasma creatine kinase concentration (CK – a crude marker of muscle damage) (Fehrenbach *et al.*, 2005). Following an ironman triathlon, Suzuki *et al.*(2006) showed a 22-fold increase in eHsp72 concentration that was accompanied by changes in classical markers of muscle damage such as decreases in muscle strength and squat jump height, increases in delayed onset of muscle soreness (DOMS) and CK, although no correlations were reported. Interestingly, total bilirubin and alkaline phosphotase were also elevated post race demonstrating partial evidence for haemolysis. Damage to erythrocytes (which contain Hsp72, (Gromov & Celis, 1991)) through repeated footstrikes may partially account for the elevated eHsp72 concentrations, particularly as exercise duration appears to be a main function of increased eHsp72 (Fehrenbach *et al.*, 2005). Whilst damage to muscle tissue from eccentric exercise may result in elevated eHsp72 concentrations through passive release from skeletal muscle cells, the majority of evidence to date suggests that any effect of this is only marginal. No known studies have reported a direct correlation between eHsp72 levels and markers of muscle damage. Additionally, downhill running failed to induce a greater eHsp72 response than running on a flat gradient (Peake *et al.*, 2005), and repeated eccentric elbow flexor contractions failed to elevate eHsp72 concentrations above baseline levels (Hirose *et al.*, 2004). Perhaps importantly, exercise modalities that involve no eccentric component and at relatively low intensities such as semi recumbent cycling and underwater running result in an elevation in circulating Hsp72 (Febbraio *et al.*, 2002a;Whitham *et al.*, 2006). Hsp72 is released into

the peripheral circulation within 10-30 minutes of stressor onset which is too quick for the classical protein induction/necrosis pathway and since increases in the blood can be considerable (Table 1.1) it is unlikely that such a large number of cells would simultaneously die a necrotic death (Fleshner & Johnson, 2005). It therefore seems unlikely that tissue/cellular necrosis accounts for a large proportion of the increase in eHsp72 seen with exercise.

It has been suggested that cellular necrosis is highly unregulated and only likely during trauma cases (Asea, 2005). Indeed, trauma has been shown to elicit very large concentrations of eHsp72 (da Rocha *et al.*, 2005;Pittet *et al.*, 2002). In addition, pathological conditions such as atherosclerosis (Pockley *et al.*, 2003), peripheral and renal vascular disease (Wright *et al.*, 2000), septic shock (Wheeler *et al.*, 2005) and prostate cancer (Abe *et al.*, 2004) all elevate circulating Hsp72. Whilst a passive release through necrosis may be an important mechanism in pathological circumstances and trauma, the fact that psychological stressors such as predatory fear and electric shock evoke a stress induced eHsp72 release in rodents (Fleshner *et al.*, 2004;Fleshner *et al.*, 2003;Johnson *et al.*, 2005) add further evidence to the suggestion that a pathway other than necrosis is also evident.

1.9 Active release of eHsp72

The classical protein transport pathway involves targeting of newly synthesised proteins to the endoplasmic reticulum, followed by transfer to the golgi apparatus where the protein is 'packaged' into secretory vesicles which fuse with the plasma membrane allowing the

contents to be transported into the extracellular environment (Nickel, 2005). An active release mechanism for HSP's in the absence of lysis was first demonstrated by Hightower and Guidon Jr (1989) where stimulated cultured rat embryo cells released a number of heat shock proteins. Interestingly, this release was not blocked by either monensin[2] or colchicines[3] (inhibitors of the classical protein transport pathway), leading the authors to suggest the involvement of a non-classical secretory pathway. More recently, glia-like cells have been shown to actively release Hsp70 upon heat shock *in vitro* (Guzhova *et al.*, 2001), prompting recent investigations into the mechanism of this selective exocytotic release.

It is documented that a number of proteins (e.g. IL-1β, macrophage inhibitory factor and fibroclast growth factor-2) are secreted by non-classical secretory pathways by one of a number of different processes, e.g. within secretory lysosomes, secretory exosomes and/or through plasma membrane resident transporters (Nickel, 2005). Broquet *et al.* (2003) demonstrated the presence of Hsp72 within plasma membrane detergent-resistant microdomains (also known as lipid rafts) of epithelial cells which increased significantly when exposed to heat shock. Furthermore, treatment with Brefeldin A (which inhibits the classical transport pathway) had no effect on this response, whereas treating the cells with the cholesterol[4] depleting agent methyl-β cyclodextrin (MBC) blunted the elevation in Hsp72 under heat shock conditions. This demonstrated a potential involvement of lipid

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[2] Monensin – a sodium ionophore that disrupts the structure of the golgi apparatus, thus preventing vesicle transport.

[3] Colchicine – an alkaloid that blocks microtubule assembly.

[4] Cholesterol is a crucial component of lipid rafts, thus depleting cholesterol results in a disruption in raft integrity.

rafts in Hsp72 release from cells. This same finding has been shown in peripheral blood mononuclear cells (PBMC's) in culture, with ~70% of release of Hsp72 from B-lymphocytes and ~30% from T-lymphocytes via a non-classical pathway possibly involving lysosomal lipid rafts (Hunter-Lavin *et al.*, 2004a). It should be noted however that controversy surrounds the use of cholesterol-depleting agents to identify lipid raft functions, as these agents may interfere with the integrity of structures/processes within the cell, such that the loss of a particular cellular process after cholesterol depletion cannot be contributed solely to lipid raft dysfunction (Pike & Miller, 1998). The uncertainty about the role of lipid rafts in Hsp72 release was heightened by the fact that in PBMC's, treatment with MBC did not blunt the increase in Hsp72 concentration in the culture medium (Lancaster & Febbraio, 2005). However, it is plausible that cellular specificity exists with regard to the mechanism of Hsp72 release.

Recent research has revolved around the role of exosomes in the selective release of Hsp72. Much of this interest is based on findings that exosomes contain many immuno-stimulatory molecules such as major histocompatability complex (MHC) I and II, co-stimulating molecules and adhesion molecules (Clayton *et al.*, 2001), suggesting that exosomes could provide an exocytotic pathway for other immunostimulatory molecules such as Hsp72. Exosomes are small membrane-bound vesicles (60 to 100nm) that are secreted by a number of eukaryocytes as a consequence of fusion of multivesicular bodies with the plasma membrane (Denzer *et al.*, 2000;Mou *et al.*, 2005). Several haematopoetic cells have been shown to secrete exosomes, e.g. dendritic cells, macrophages, T and B-lymphocytes, and platelets (Denzer *et al.*, 2000), and they are excreted under the basal state as a result of changes in intracellular calcium levels (Savina *et al.*, 2003). Hsp72 has been detected within exosomes in a variety of cell types, although it appears that cellular

specificity exists with regards to exosomal Hsp72 release/expression. For example, Clayton *et al.* (2005) demonstrated that exosomes derived from B-lymphocytes were positive for Hsp70 under normal conditions which increased after 3-hours of heat shock, and that this increase was as a result of an increase in the number of exosomes secreted. This is in contrast to findings of Lancaster and Febbraio (2005), who showed that the temperature dependent increase of Hsp72 in PBMC's when exposed to heat shock was due to an increase in Hsp72 concentration within each exosome and not due to an increase in the number of exosomes secreted. Furthermore, Hsp72 was detected in the lumen and on the cell surface of tumor derived exosomes (Bausero *et al.*, 2005), but only within the lumen of B-cells (Clayton *et al.*, 2005). Hsp70 has also been shown to be released from human prostate carcinoma cells by a non-classical pathway involving lysosomal endosomes that was independent of Hsp70 synthesis or cell death (Mambula & Calderwood, 2006). Whilst the supporting evidence to date suggests that the majority of Hsp72 is actively released via an exocytotic pathway into the circulation from cells under times of stress, and it is likely that exosomes, lysosomes or lipid rafts are involved, further research is needed to clarify the factors involved in Hsp72 release.

1.10 Stimulation and releasing signals of Hsp72

Exercise results in a multitude of physiological changes which individually and collectively impose stress on the body's regulatory system, for example, increases in core/local muscle temperature, reduction in blood glucose, progressive dehydration, oxidative stress etc. A number of these stressors associated with exercise are known to alter the concentration of eHsp72, thus may provide answers as to what triggers the specific release of eHsp72.

1.11 Effect of heat upon eHsp72 release

The term 'heat shock protein' clearly suggests that heat is a major stimulator of Hsp72 expression. Indeed, data suggests that the regulator of *intracellular* Hsp72 synthesis, HSF-1 is directly activated by temperature (Staib *et al.*, 2007;Zhong *et al.*, 1998). However, it is currently unclear as to the source of Hsp72 that is released from cells (i.e. does secreted eHsp72 originate from newly translated proteins or from an existing intracellular pool). Only recently has the role of heat in eHsp72 release from cells been addressed.

Amorim *et al.* (2008) investigated the rate of heat storage on eHsp72 concentration. Subjects exercised at ~50%VO_{2max} in hot environmental conditions until their core temperature had reached ~38.5°C under two conditions – high and low rate of heat storage. Both conditions increased eHsp72, but despite exercising for almost twice as long under the low heat storage condition, no changes in eHsp72 concentrations were observed between trials. Whilst the authors concluded that eHsp72 concentrations following exercise in the heat are a function of core temperature they failed to present any data specifically excluding the role of exercise alone on eHsp72 accumulation. To investigate the independent and combined effects of exercise and rises in core temperature on eHsp72, Whitham *et al.* (2007) adopted a thermal clamp technique (whereby core temperature is prevented from rising by exercising in cold water). Unsurprisingly, combined exercise and increases in core temperature generated the greatest eHsp72 response (↑233%), whilst exercise alone (↑131%) and passive heating alone (↑72%) both induced significant eHsp72 responses. Thus, increases in eHsp72 during exercise cannot solely be contributed to increases in core temperature. Furthermore, Ruell *et al.* (2006) demonstrated that only 42% of the variance in eHsp72 was explained by increases in core temperature in runners

who had suffered from exertional heat illness during a 14km race, suggesting that other factors are involved in the eHsp72 response.

1.12 Hepatic stress

Prolonged exercise results in a decrease in muscle glycogen and blood glucose if glucose is not replaced by exogenous sources (Coyle *et al.*, 1986). In order to maintain blood glucose levels, increased glycogenolysis in the liver occurs, and since the liver has been shown to release a number of acute phase proteins it was suggested that HSP's may originate from this organ. Indeed, arterial-venous difference cannulation techniques have highlighted a significant release of Hsp72 from the hepatosplanchic viscera during semi-recumbent cycling (Febbraio *et al.*, 2002a), and that this release is attenuated (but not completely eliminated) with glucose ingestion (Febbraio *et al.*, 2004). The authors concluded that ingestion of glucose reduced hepatic stress since hepatic glucose production during exercise is reduced to basal levels when glucose is ingested (Jeukendrup *et al.*, 1999).

1.13 Oxidative stress

Exercise results in increased levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that challenge cellular integrity. ROS has been shown to induce the expression of HSP's within lymphocytes (Marini *et al.*, 1996). Supplementation with combined vitamin C and the vitamin E isoform γ-tocopherol blunted the circulating Hsp72 response to exercise (Fischer *et al.*, 2006), an affect that may be due to a reduction in ROS or RNS production during exercise. Additionally, administration of the antioxidant, folic acid in type II diabetes patients (who are subjected to increased oxidative stress (Sampson

et al., 2002)) significantly reduced baseline serum Hsp72 concentration (Hunter-Lavin *et al.*, 2004b).

Taken together, these finding suggest that passive heating, blood glucose and oxidative stress all play a role in the generation of an eHsp72 response to exercise. However, these studies do not imply cause and effect, and any association may be mediated through a different signal, particularly if an active exocytotic pathway is the likely release mechanism from the cell.

1.14 eHsp72 and hormonal releasing signals

It has been suggested that eHsp72 is part of the normal stress response (Johnson & Fleshner, 2006). As such, it seems justifiable that its release may be mediated by one (or more) of the signals associated with the hypothalamic-pituitary-adrenal (HPA) or sympatho-adrenal medullary (SAM) axis (Figure 1.1), particularly as a number of stress-induced signals originating from the anterior pituitary and adrenal glands are known to increase the *intracellular* concentration of Hsp72, e.g. ACTH (Blake *et al.*, 1991), corticosterone (Valen *et al.*, 2000) and the catecholamines adrenaline and noradrenaline (Matz *et al.*, 1996;Udelsman *et al.*, 1994). By utilising adrenalectomy, hypophosectomy, adrenergic blockade and adrenergic stimulation in predatory fear and electrical shock stress paradigms in rodent models, Fleshner *et al.* have identified a probable role for catecholamines, but not ACTH nor the glucocorticoids in eHsp72 release (Fleshner *et al.*, 2004;Johnson *et al.*, 2005). More specifically, blocking of α_1-receptors by administering the α_1-receptor antagonist Prazozin and the non-selective adrenergic receptor antagonist Labetalol completely attenuated the Hsp72 response to stress. This effect was substantiated as stimulation of α_1-receptors using Phenylephrine augmented the Hsp72

release, whereas stimulation of β-receptors did not (Johnson *et al.*, 2005). Whilst this suggests a prominent role for the catecholamines, the authors suggested that noradrenaline was the likely mediator due to its greater affinity for α_1-adrenergic receptors, and that adrenalectomy would likely deplete 95-99% of available adrenaline. α_1-adrenergic receptor stimulation has been shown to increase intracellular Hsp72 expression in a variety of tissue/cell types such as brown adipose tissue (Matz *et al.*, 1996), myocardium (Meng *et al.*, 1996) and immune cells (Lacoste *et al.*, 2001). Additionally, α_1-adrenergic receptor stimulation also results in an intracellular calcium flux (Guarino *et al.*, 1996). Recall that changes in intracellular calcium can affect the release of exosomes (Savina *et al.*, 2003), thus one current hypothesis is that under times of stress increases in noradrenaline acting upon α_1-adrenergic receptors results in a calcium flux within the cell and a subsequent release of Hsp72 within exosomes (Johnson & Fleshner, 2006) (see Figure 1.1).

Whilst it appears that the release of eHsp72 in rodents is mediated by noradrenaline stimulation of α_1-adrenergic receptors, to date there has been little investigation into the releasing signals of eHsp72 in humans. Whitham *et al.* (2006) investigated the effect of supplemented caffeine upon the eHsp72 response to prolonged exercise. Caffeine is a known stimulator of sympathetic activity, and results in an elevated circulating adrenaline and to a lesser extent noradrenaline response to exercise. This attribute of caffeine provided a useful *in vivo* model to investigate the role of catecholamines in eHsp72 release. Using a randomized counterbalanced design, 10 trained males performed two trials cycling at a constant work rate (74% VO_{2max} for 90mins), separated by one week – one with caffeine supplementation (CAF - 6ml/kg body mass) and the other with placebo. Exercise in the CAF trial not surprisingly resulted in greater serum caffeine, which was

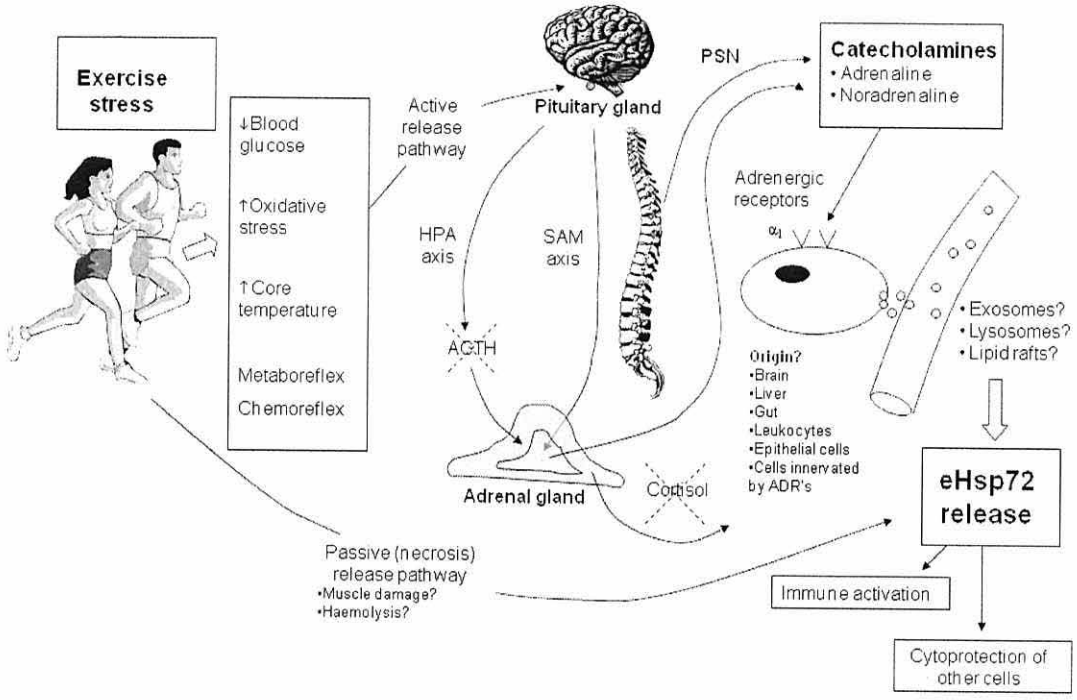

Figure 1.1 Model for the *exercise* induced release of eHsp72. Depending on the mode, intensity and/or duration of exercise, eHsp72 may be released by one of two methods: through cellular necrosis (muscle damage or haemolysis), or through an active exocytosis mechanism. Stressful stimuli associated with exercise such as a reduction in blood glucose or increases in oxidative stress and core temperature or the metabo- and chemoreflex activates a stress response via the hypothalamic-pituitary-adrenal (HPA) axis or sympatho-adrenal medullary (SAM) axis. Evidence from rodent models suggests that neither adrenocorticotropic hormone (ACTH) nor cortisol is responsible for eHsp72 release. Catecholamine stimulation of adrenergic receptors from either the adrenal medulla or from direct innervation with postganglionic sympathetic neurons (PSN) has been associated with the accumulation of Hsp72 in the circulation, possibly via an α_1-adrenergic dependent pathway. It is currently unclear as to the exact origin of eHsp72 but the mechanism for release likely involves exosomes, lysosomes or lipid rafts. Upon release into the extracellular environment, eHsp72 may act as an endogenous danger signal, serving to enhance immune responses, or aid other cells that are incapable of synthesizing or inducing *intracellular* Hsp72 themselves.

accompanied by a significantly greater eHsp72 response compared to placebo. Circulating adrenaline was significantly greater in CAF, whilst the greater concentration of noradrenaline that was observed did not reach statistical significance. Whilst this data supports animal models in that catecholamines are involved in the eHsp72 release, it

suggests a more prominent role for adrenaline under the conditions encountered in this study, which may reflect different patterns of sympathetic activation both across species and between different stressors. Additionally, that Hsp72 may be released via sympathetic stimulation lends itself to the possibility that any stressor capable of evoking a sympathetic response (possibly via the metabo or chemoreflex) may stimulate Hsp72 release (Figure 1.1).

1.15 Origin of eHsp72

As previously mentioned, using arterial-venous direct cannulation across tissue beds, Hsp72 has been demonstrated to be released in significant quantities from the hepatosplanchic viscera during exercise (Febbraio *et al.*, 2002a) and it has been suggested that eHsp72 may be chaperoning LPS released from endogenous gut bacterial flora (Johnson & Fleshner, 2006). Additionally, the same cannulation technique has identified that Hsp72 can be released from the brain following 180 minutes of semi-recumbent cycling at 60%VO_{2max} (Lancaster *et al.*, 2004), although significant individual variation was apparent. There is also clear evidence that Hsp72 is released from both immune and epithelial cells (Broquet *et al.*, 2003;Hunter-Lavin *et al.*, 2004a;Lancaster & Febbraio, 2005), although owing to the potential for sympathetic stimulated release (Johnson *et al.*, 2005;Whitham *et al.*, 2006), it is tangible that any cell innervated with sympathetic neurons or possessing adrenergic receptors may release Hsp72.

1.16 Biological significance of extracellular Hsp72: Potential roles

Perhaps the question that interests most researchers is what is the biological significance of a predominantly intracellular protein in the extracellular environment? Due to the seemingly systemic nature of the release of eHsp72, culminating evidence suggests that eHsp72 may be involved in the functioning of the immune system and in increasing cellular tolerance to stress in those cells incapable of inducing Hsp72.

1.17 Aiding cellular protection

It is well established that intracellular induction of Hsp72 results in stress tolerance and protection against otherwise lethal stressors (Maloyan *et al.*, 1999). In support of this, there is evidence that Hsp72 may play a role in cellular protection of immune cells during the cellularly stressful condition of inflammation. Intracellular Hsp72 responses to heat shock (Oehler *et al.*, 2001) and exercise (Fehrenbach *et al.*, 2000) were greater in phagocytes compared to other immune cells types. Both authors attributed this finding to the fact that phagocytes are activated very early in the inflammatory process and are the main producers of reactive oxygen species. Therefore, high levels of Hsp72 within phagocytes may help to protect the cell from their own noxious molecules (Fehrenbach *et al.*, 2000).

There is emerging evidence that *extracellular* Hsp72 may have a role in cytoprotection during stressful conditions. Marshall *et al.* (2006) have demonstrated that resting and post exercise eHsp72 concentrations decrease during the initial stages of exercise-heat acclimation (HA). Although little supporting evidence was included, it was suggested that

this occurrence may be a consequence of increased uptake of eHsp72 by distal cells in order to aid their own thermal tolerance. This hypothesis is attractive and plausible since HA increases the intracellular expression of Hsp72 (Maloyan *et al.*, 1999;McClung *et al.*, 2008;Yamada *et al.*, 2007), the circulatory system provides an ideal means by which to transport eHsp72 to cells/tissues at risk from hyperthermia, and *in vitro* studies have shown that certain cells are capable of internalizing eHsp72 (Arnold-Schild *et al.*, 1999;Novoselova *et al.*, 2005;Robinson *et al.*, 2005). Furthermore, decreased eHsp72 concentrations were found in response to HA in participants able to acclimatise, whereas those subjects unable to acclimatize to exercise in hot conditions had no change in baseline eHsp72 expression (Kresfelder *et al.*, 2006).

The proposal that eHsp72 may be taken up by distal cells is not confined to HA and may have clinical applications. Many cell types often express very little Hsp72 under basal conditions, or may be incapable of inducing Hsp72 intracellulary during stressful stimuli. For example, motor neurons contain basal Hsp72 but are incapable of synthesizing this protein when exposed to heat shock. However, exogenous application of Hsp72 to the cell culture promotes motor neuron survival (Robinson *et al.*, 2005). Glial cells also release Hsp72, and due to their close proximity may provide a means by which neurons can enhance their stress tolerance (Guzhova *et al.*, 2001). In addition, Novoselova *et al* (2005) transfected human neuroblastoma SK-N-SH cells with the apoptosis-inducing agent, *staurosporine*. When Hsp72 and its constitutive form Hsc70 were applied to the culture medium, Hsp70 was internalized by 80% of the cells, dramatically increasing the Hsp72 content (these cells usually express very little Hsp72), which disturbed protein aggregation within the cell and reduced apoptosis by 40-50%. *In vivo* evidence from murine models also suggest that intraperitoneally recombinant Hsp72 injection 3 times daily into mice

with the neurodegenerative disorder amyotropic lateral sclerosis prolonged motor neuron survival, preserved motor function, delayed symptom onset and increased lifespan (Gifondorwa *et al.*, 2007). Taken together, these data suggest a clinical role for eHsp72 as preparations based on this protein may have potential as a therapy for neurodegenerative pathologies that are associated with destabilisation of protein structure.

1.18 Immunological aspects of eHsp72

The traditional view of immunity is that the immune system is activated upon recognition of non-self peptides i.e. the self/non-self distinction. This occurs through pathogen associated molecular patterns (PAMP) being recognized by pattern recognition receptors (PRR's) on antigen presenting cells (APC). However, this view alone does not explain why transplanted organs are rejected (where there are no PAMP's to recognize) or the occurrence of autoimmune diseases (where the body's immune system responds to self antigens). A complementary view of immunity has been proposed by Matzinger, the danger model of immunity (Matzinger, 2002). In addition to the self/ non-self distinction, the danger model is concerned with damage/non-damage recognition schemas whereby the immune system is activated by *endogenous* alarm signals released from stressed, damaged, infected or dying cells. It has been suggested that in order to be a successful endogenous signal, the candidate should be essential and abundant, located intracellularly, and strongly upregulated under times of stress (Vabulas & Wagner, 2005). Heat shock proteins appear to be perfect candidates, with much evidence providing a role for Hsp72.

1.19 Heat shock proteins and immunity *in vitro*

Considering the chaperoning roles of intracellular Hsp72, it is not surprising that eHsp72 has been shown to bind antigenic peptides. Indeed, Hsp72 binds immunogenic peptides and assists in the presentation of these peptides by major histocompatibility complex (MHC) class 1 molecules (Arnold-Schild *et al.*, 1999;Basu *et al.*, 2001;Sondermann *et al.*, 2000). Much interest into the immune effects of eHsp72 has revolved around antigen-presentation in cancer immunotherapy. Following receptor mediated endocytosis, Hsp72-chaperoned peptides derived from tumors are cross-presented on MHC class molecules, initiating a tumor specific $CD8^+$ T-cell response (Multhoff, 2006;Suto & Srivastava, 1995). This mechanism forms the basis for Hsp72 anti-cancer vaccines (Calderwood *et al.*, 2007;Srivastava & Amato, 2001). Additionally, secreted Hsp72 from tumour cells can bind to adjacent cell surfaces in a paracrine or autocrine fashion outlining a potential role in the recognition of tumour cells by the immune system (Mambula & Calderwood, 2006). Indeed, Hsp72 expressed on the surface of stressed cells and tumor cells act as surveillance targets for natural killer cells (Multhoff, 2002;Multhoff *et al.*, 1999). In addition to acquired immunity effects, eHsp72 is also thought to stimulate innate immunity (Asea, 2003). Whilst a number of cell types have been shown to release Hsp72 (see earlier), eHsp72 must bind to a receptor thereby setting in motion a cascade of events resulting in the initiation of an immune response. A number of leukocytes have been shown to bind eHsp72, for example, natural killer (NK) cells (Gross *et al.*, 2003), monocytes (Asea *et al.*, 2000), macrophages (Sondermann *et al.*, 2000) and dendritic cells (Asea *et al.*, 2002). Also, a number of receptors have been shown to bind eHsp72 e.g. toll like receptors (TLR) 2, 4 and 7, CD14, CD40, CD36, CD91, LOX-1 and SR-A (Asea, 2006). Asea *et al.* (2000) demonstrated for the first time a cytokine like action of eHsp72. Hsp72 bound with high affinity to the plasma membrane of human monocytes resulting in an intracellular calcium

flux, the activation of nuclear factor kappa B (NF-κB) and upregulation of inflammatory cytokines TNF-α, IL-1β and IL-6. The term "chaperokine" has been coined, reflecting the unique chaperone and cytokine function of this protein (Asea, 2003;Asea *et al.*, 2000). More recently, binding of Hsp72 to the surface of monocytes increased matrix metalloproteinase-9 (MMP-9) expression also through the NF-κB pathway and enhanced cell motility (Lee *et al.*, 2006). This is pertinent as MMP-9 plays a crucial role in extravasation of monocytes during inflammation. A number of studies have now demonstrated *in vitro* that various sources of eHsp72 can stimulate immune effectors from APC's, for example, cytokines such as TNF-α, IL-1β, IL-6 and IL-12 (Asea *et al.*, 2000;Asea *et al.*, 2002;Campisi & Fleshner, 2003), a number of chemokines such as RANTES, macrophage inflammatory protein 1α and 1β (Lehner *et al.*, 2000), and nitric oxide (Campisi & Fleshner, 2003;Panjwani *et al.*, 2002). In addition, Hsp72 is known to activate the complement cascade independent of antibody (Prohaszka *et al.*, 2002).

1.20 Endotoxin contamination controversy

Hsp72 and the bacterial stimulant lipopolysaccharide (LPS) appear to have similar effects upon cytokine release (Asea *et al.*, 2000). As such, there is much debate within this field as to whether the cytokine-inducing actions of eHsp72 are due to 'naked' Hsp72, or bound endotoxin contamination of the recombinant preparations used. A series of experiments have shown that low LPS containing recombinant Hsp72 preparations failed to stimulate inflammatory cytokine release from immune cells *in vitro* (Johnson & Fleshner, 2006;Tsan & Gao, 2004). Furthermore, the activation of T-cells by recombinant Hsp72 has been ascribed to flagellin contamination (Ye & Gan, 2007). However, studies have been conducted that have purified and isolated Hsp72, showing that the immune activation of

eHsp72 is via a different pathway to LPS (Asea *et al.*, 2000;Panjwani *et al.*, 2002). For example, the calcium flux that was induced within 10 seconds of eHsp72 binding to the surface of monocytes does not occur with LPS stimulation and is an important signaling step that separates these two pathways (Asea *et al.*, 2000). It has since been suggested that due to the complex forming ability of Hsp72, 'naked' Hsp72 *in vivo* is unlikely (Johnson & Fleshner, 2006;Prohaszka & Fust, 2004). Exercise (Jeukendrup *et al.*, 2000), physiological relevant increases in temperature (Dokladny *et al.*, 2006) and combined exercise-heat stress (Lambert, 2004) have all been shown to increase gastrointestinal permeability. Since physical and psychological stressors have been shown to stimulate bacterial translocation from the gut, Johnson and Fleshner (2006) have suggested that under times of stress, eHsp72 may be chaperoning LPS released from endogenous gut bacterial flora, thereby stimulating both innate and acquired immunity (Quintana & Cohen, 2005). Whilst there is still a debate over endotoxin contamination in recombinant preparations, evidence exists that eHsp72 can stimulate aspects of the immune system. Furthermore, this debate does not invalidate much of the *in vivo* evidence linking stress induced increases in eHsp72 with improved immune function.

1.21 eHsp72 and immunity *in vivo*

Much of the *in vivo* work has been conducted in rodent models. For example, elevations in eHsp72 concentration in response to tail-shock were associated with reduced inflammation and quicker time to recovery following a subcutaneous *e-coli* injection, whilst physically active rats recovered quicker than sedentary rats (Campisi *et al.*, 2002;Campisi & Fleshner, 2003;Campisi & Fleshner, 2003). In addition, *in vivo* delivery of Hsp72 into mice accelerated wound closure by 60% compared to control-treated mice (Kovalchin *et al.*,

2006) which was likely due to enhanced macrophage phagocytosis of wound debris, a finding that has been confirmed by a recent study (Wang *et al.*, 2006b). Conclusive evidence in humans is currently lacking. Only one known study has investigated the effects of exercise stress linking eHsp72 and improved immune function. eHsp72 from blood samples taken before and after exercise in a hot environment was expressed on the surface on natural killer (NK) cells and monocytes. Whilst surface expression of eHsp72 on monocytes did not alter after exercise, the number of NK cells positive for eHsp72 expression doubled (Horn *et al.*, 2007). As *in vitro* evidence suggests Hsp72 stimulates the proliferation and cytolytic activity of NK cells (Multhoff *et al.*, 1999) this study proposes an *in vivo* link between increases in eHsp72 and improved immune function. Studies have also demonstrated relationships between elevated eHsp72 and improved prognosis/ outcome. Pittet *et al* (2002) found that high levels of eHsp72 derived from patients admitted to hospital with trauma were associated with improved survival regardless of the severity of injury, whereas all of the patients who died of their injuries had low serum Hsp72 concentrations. Furthermore, it is known that 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.

1.22 Perspective

This review has summarised the current literature upon eHsp72. Whilst the biological significance is yet to be fully determined, the evidence presented in this review seems to suggest that acute stress-induced increases in eHsp72 may act as "danger" signals alerting the immune system during times of stress, or may aid other cells that may be incapable of

inducing Hsp72 themselves. These attributes of the eHsp72 response have stimulated research into the effects of Hsp72 upon the ability or inability of individuals to acclimatize to heat (and susceptibility of exertional heat illness), and have placed this protein as a focus of interest in immunotherapy whereby immunoenhancement or immunosuppression is desired.

1.23 Aims of this thesis

Since extracellular Hsp72 physiology is a relatively new area, and more and more *in vivo* studies are being conducted into the biological roles of eHsp72, there are a number of important methodological questions which are yet to be answered. For example, upon review of the literature there appears to be a large difference in the reported values of eHsp72, particularly between samples collected from serum and those collected in the presence of an anticoagulant (Table 1). Additionally, there is an absence of well controlled research detailing if eHsp72 concentrations are subject to a circadian variation similar to that of major stress hormones such as cortisol and the catecholamines. This may have implications as to the timing of blood sampling. Since Hsp72 is a highly inducible stress protein, then it stands to reason that stressors other than exercise or clinical pathology may induce its expression. Of importance is the finding that acute psychological stress increases eHsp72 concentration in rodents, a finding that is currently unknown in humans. This is an important consideration since many studies involve potentially stressful procedures that may cause anxiety to participants, therefore impacting upon true baseline data. A final interesting finding is the presence of eHsp72 in other extracellular compartments such as the CSF, synovial fluid and saliva. Of these, saliva is easily obtainable and non-invasive, and as such may serve as a proxy measure for blood Hsp72.

It is currently unknown how acute stressors affect the concentration of eHsp72 within saliva.

With these points in mind the aims of this thesis are;

1. To investigate the effect of different blood handling procedures upon eHsp72 concentration.
2. To document whether eHsp72 concentration is subject to a significant circadian variation.
3. To investigate the effect of acute psychological stress upon eHsp72 concentration.
4. To determine the effects of exercise stress upon salivary Hsp72 expression and whether salivary Hsp72 concentrations track concentrations of eHsp72 within the plasma. Additionally, to investigate potential secretory mechanisms of salivary Hsp72.

Chapter 2

General Methods

2.1 Ethical approval and participant recruitment

In all investigations, approval for experimental procedures was obtained from the local ethics committee of the School of Sport, Health and Exercise Sciences of Bangor University, U.K. Additional ethical approval was required and obtained for Chapter 4 from the North West Wales NHS Trust ethics committee. Participants were made fully aware of all procedures and risks associated with involvement in each study by verbal explanation and by administering a fully informed information sheet (examples of which are detailed in Appendix A). Participants were informed that they were free to withdraw from the study at any time. Written informed consent was obtained prior to commencement of any experimental procedures (Appendix B).

2.2 Standard inclusion/exclusion criteria

All participants recruited were non-smokers and free from infection, known diseases, medication, or any form of supplementation that may have interfered with the eHsp72, thermoregulatory or exercise responses (e.g. vitamins or protein shakes). Furthermore, participants were excluded from any study had they recently (within 2 months) visited a hot country, taken a sauna or any activity that may have resulted in temporary heat acclimation.

2.3 Hsp72 determination within serum, plasma and saliva samples.

Sample preparation

Upon collection, all blood samples were pre-treated in the same manner; plasma tubes were centrifuged immediately at 1500*g* at 4°C for 10 minutes, whilst serum tubes were allowed to clot for 1-hour before centrifugation. The plasma/serum was then aspirated into 3 × labelled eppendorfs and immediately frozen at -80°C until analysis. On the day of assay, samples were thawed, and centrifuged for 2 minutes at 2000*g* in a micro-centrifuge ready for the assay.

Assay overview

For analysis of extracellular Hsp72 concentration a commercially available quantative sandwich enzyme-linked immuno-sorbent assay (ELISA) was used (EKS-700, StressGen Biotechnologies, Victoria, Canada). Hsp72 within plasma samples is captured by wells coated in a (capture) antibody specific for Hsp72. Captured Hsp72 protein is then detected by a different (primary) antibody specific for Hsp72 but one which recognizes a different epitope of the protein to the capture antibody. In this respect, the protein is "sandwiched" between the two antibodies. A secondary antibody conjugated to an enzyme is then added which binds to the primary antibody. When a substrate is added, an enzymatically catalysed reaction occurs resulting in colour generation, with the amount of colour development directly proportional to the amount of protein in the sample. The colour development is stopped by adding an acid stop solution and then read by a plate reader, with concentrations of eHsp72 quantified.

Assay procedures

Wells from the provided 96-well micro plate were pre-coated with a mouse-monoclonal antibody specific for endogenous and recombinant inducible Hsp72, which has no reactivity with other isoforms of the HSP70 family e.g. Hsc73, DnaK, Grp78 or Hsp71. Samples were diluted (1:5) in the assay diluent (details not supplied) and 100µl were added in duplicate to each well and allowed to incubate at room temperature for 2-hours under gentle agitation. The wells were washed 6 times (details not supplied) leaving only bound Hsp72 on the plate. Following this, 100µl of a biotin-conjugated anti-Hsp72 rabbit polyclonal antibody was added to each well. After 1-hour incubation at room temperature and 6 washes to remove excess antibody, 100µl avidin-horseradish peroxidase (HRP) conjugate was added to each well and incubated for 1-hour at room temperature. Following washing to remove excess HRP-conjugate, the assay was developed by adding 100µl of tetramethylbenzidine (TMB) substrate for 10 minutes. The reaction was stopped by adding 50µl of a hydrochloric acid stop solution and the optical density of each well was read at 450nm with background correction at 540nm using a plate reader (Opsys MK, Dynex Technologies Inc, Chantilly, VA, USA). A standard curve was generated by using serial dilutions of purified recombinant human Hsp72 ranging in concentration from 0.78 – 50 ng/ml. Concentrations of eHsp72 within samples were attained by interpolation of the sample OD with the standard curve.

Development of an in-house Hsp72 ELISA

The StressGen assay (EKS-700) was used for the serum and plasma analysis in Chapter 3. However, due to a supply shortage of the detection antibody (rabbit polyclonal), this assay was removed from the market in November 2006. In this intervening period, no

commercially available assay was available, so from May 2007-December 2007 a number of pilot studies in our laboratory were conducted to develop an in-house ELISA based upon previously used and documented in-house Hsp72 ELISA's (Njemini *et al.*, 2003;Rea *et al.*, 2001;Pockley *et al.*, 1998). On the first attempt, we ran into a number of problems with very variable data and a lack of signal (lack of detection of Hsp72). Therefore, to optimize the assay, a number of grid experiments were performed whereby different dilutions of the coating, primary and secondary antibodies were used in order to derive the optimal antibody pair concentrations. By using different known concentrations of rHsp72 in the experiments and expressing the OD from these samples compared to that of a blank well, a signal: noise ratio was derived. However, although a signal was obtained throughout the grid experiments we were always hampered with high background (far greater values for the blank OD than one would expect) and no distinction between the blank and positive control wells (i.e. the signal: noise ratio rarely got above 1.2, even at high concentrations of rHsp72). As a consequence of this, a number of pilots were performed to reduce the background and increase the signal. One potential reason for a lack of signal is that the recombinant preparation of Hsp72 may be denatured. However, this was unlikely since the protein used was recently purchased and hadn't undergone any repeated freeze-thaw cycles. Other techniques attempted in order to increase the signal included;

- Using a high-binding plate specifically for ELISA (Nunc Maxisorp Immunoplate, Thermo Fisher Scientific, Denmark) since the coating antibody may not have bound to the plate.

- Manual washing, since the pressure in our plate washer may have been too high resulting in stripping of the antibodies (particularly if a strong detergent is used).

- Adopting both the horseradish peroxidase and the Biotin-streptavidin detection systems.
- Using a greater concentration of rHsp72, and greater concentrations of antibodies.

However, by attempting these procedures, background interference was always affected (increased) which may have contributed to masking the detection of Hsp72. Therefore a number of techniques were adopted in order to reduce the background;

- Increasing the number of washes between steps and increasing the amount of detergent in the wash buffer in order to remove antibody residue.
- Adopting different blocking buffers and increasing the concentration of blocking agents in order to block non-specific binding sites on the assay plate. We used and tested commercially available synthetic blockers in addition to bovine serum albumin and non-fat dried milk at various concentrations.
- Reduced the concentrations of primary, secondary and coating antibodies in order to reduce non-specific binding.

Whilst these techniques all helped to reduce background the OD for the blank well was always considerably above acceptable limits (>0.4 OD units whereby <0.1 is desirable), thus it appeared that non-specific binding was occurring. To investigate this, the assay was performed with one step taken out at a time. This confirmed that the primary detection antibody seemed to be a major problem as inclusion of this always resulted in high OD's, whereby removal of this resulted in a low background. An obvious approach would have

been to try a different primary antibody, but by this time StressGen had been taken over by Assay Designs who had developed a new high-sensitivity Hsp72 ELISA (EKS-715). We were asked to test this ELISA and it immediately provided high sensitivity in detecting eHsp72. This new ELISA was released for purchase in February 2008, and as such, this assay was used for all subsequent analyses.

Assay Designs Hsp72 ELISA (EKS-715)

The procedures for this assay are identical to the original StressGen ELISA described previously except for the following alterations:

- Wells were only washed 4 times (6 washes in StressGen ELISA) after each step in a phosphate buffered saline containing bovine serum albumin and detergent (concentrations not supplied).
- The sample diluents consisted of Tris buffered saline containing detergent (concentrations not supplied, StressGen ELISA used a phosphate buffered saline diluent).
- The primary and secondary antibodies did not utilise the biotin-avidin detection system, and the assay relied solely upon the HRP- TMB reaction.
- The reaction was stopped by adding 100 µl of acid stop solution (as opposed to 50 µl in the StressGen ELISA).
- The standard curve was generated at lower concentrations (from 0.2 – 12.5 ng/ml compared with 0.78-50 ng/ml).

The reported sensitivity of this new assay (0.09 ng/ml) is far greater than that described for the previous assay (0.5 ng/ml). Throughout this thesis, the precision (intra-assay coefficient of variation - CV) of the assay is described and is calculated as the CV of duplicate standards and samples, averaged for each plate. Inter-assay CV was calculated as the CV of the standard OD's from all plates in that analysis.

2.4 Analysis of α-amylase concentration

Saliva samples were assessed for concentration of α-amylase using a kinetic assay (Cat no 1-1902, Salimetrics, USA). The assay was performed at room temperature and works on the principle of an enzymatic reaction between α-amylase and the chromagenic substrate 2-chloro-p-nitrophenol linked with maltotriose which can be spectrophotometrically measured at 405nm. Briefly, on the day of assay saliva samples were thawed and centrifuged in a microcentrifuge at 2000*g* for 2 minutes. Ten micro litres of saliva was aspirated and diluted into the assay sample diluent (phosphate buffered saline) for a final dilution of 1:200. Eight micro litres of this mix was then added to uncoated wells of an assay plate into which 320 µl of substrate was added. The microplate was placed on a shaker and the optical density was read at 405nm at exactly 1 and 3 minutes of incubation. For accuracy, only 1 strip (8 wells – 4 samples in duplicate) was tested at a time. The α-amylase concentration was determined by multiplying the change in absorbance of the 1 and 3 minute readings by 328.

2.5 Statistical Analyses

All studies presented in this thesis are fully-within subject longitudinal designs, and all contain parametric data. As such, the majority of primary analyses are fully within repeated-measures analysis of variance (ANOVA). Data were analysed for sphericity using Mauchley's sphericity test, and if significant, the degrees of freedom were adjusted using the Greenhouse-Geisser correction. In the event of a significant F test (interaction or main effect) follow-up *post-hoc* tests were performed to investigate where significant differences occurred. *Post-hoc* tests were carried out using Tukey's honestly significant difference (HSD) procedure when epsilon >0.7. However, when the assumption of sphericity was violated and epsilon was <0.7, Bonferroni adjusted t-tests were used to probe a significant main effect or interaction as recommended by Stevens pp.509 (2002). When differences between two data points were compared, paired t-tests were performed, and relationships between variables were assessed using Pearson's product moment correlation coefficient (r). Additional statistical analyses presented in the thesis include co-efficient of variation (CV) to assess the degree of variability in a measurement (Chapters 3-6), and box-and-whisker plot analysis to investigate statistical outliers (Chapter 4). All analyses were performed using the Statistical Package for the Social Sciences for windows software (version 12.0, SPSS Inc. Chicago, IL, USA). Values are reported as means ± standard error of the mean (SEM) throughout the text and in figures. For all statistical analyses, significance was accepted as $P \leq 0.05$.

Chapter 3

Effects of blood handling on extracellular Hsp72 concentration following high-intensity exercise

This study has been published as a short communication in Cell Stress Chaperones:

Whitham, M. and Fortes, M.B. (2006). Effect of blood handling on extracellular Hsp72 concentration after high-intensity exercise in humans. *Cell Stress and Chaperones*, **11**, 304-308.

3.1 Summary

Heat shock protein 72 (Hsp72) has been detected in the peripheral circulation of humans. Since intra-cellular Hsp72 binds to aggregated proteins we hypothesised that plasma-derived Hsp72 concentrations would be greater than serum-derived Hsp72 because of Hsp72 binding to aggregated clotting proteins in serum. Nine recreationally active males conducted a peak-power output test and a 40-minute time-trial cycling at 80% PPO in a hot environment (34.6°C). Immediately post exercise, venous blood was collected into four different specimen tubes: two serum tubes (one left to clot on ice for one-hour – (SI), the other at room temperature - SR), and two plasma tubes, one coated in lithium heparin (LH) and one coated with ethylenediaminetetraacetic acid (EDTA). Diluted (1:5) samples were analysed for Hsp72 in duplicate using an enzyme linked immunosorbent assay. EDTA treated blood was significantly higher in Hsp72 concentration than all other treatments ($P \leq 0.001$), whilst LH was significantly higher than SI and SR ($P < 0.05$) (EDTA: 6.46 ± 0.26, LH: 2.73 ± 0.76, SR: 0.20 ± 0.11, SI: 0.13 ± 0.09 ng/ml). Blood treated with an anti-coagulant provides significantly higher concentrations of eHsp72 than serum-derived Hsp72. Since previous research has tended to report serum values below the sensitivity of the assay, it is recommended that EDTA-coated specimen tubes be used in future investigations.

3.2 Introduction

In the relatively few human studies to date that have measured eHsp72 in response to a variety of stressors, there is a disparity in the reported concentrations of Hsp72, which appears to be linked to the method in which the blood sample is treated. For example, whilst investigating the effect of exercise duration and intensity on eHsp72 concentration, Fehrenbach *et al.* (2005) reported differences between plasma-derived and serum-derived Hsp72 despite similar exercise loads and subject characteristics between trials. For example, the assayed concentrations of plasma Hsp72 ranged from resting values of ~1.5 ng/ml to 17 ng/ml immediately post exercise, whilst serum concentrations were much lower (~0.1 ng/ml to maximum values of only 1.3 ng/ml). In addition, a review of human exercise studies obtaining eHsp72 measurements show typical eHsp72 concentrations (rest – post exercise maximum) of 0.1 - 1.3 ng/ml in serum samples (Febbraio *et al.*, 2002a;Febbraio *et al.*, 2004;Walsh *et al.*, 2001;Lancaster *et al.*, 2004), compared with ranges of 1.5 - 42 ng/ml for EDTA plasma (Fehrenbach *et al.*, 2005;Peake *et al.*, 2005). Although it is acknowledged that the nature of the exercise may have an affect on the assayed concentration, the subject characteristics and protocol used in the studies by Walsh *et al.* (2001) and Fehrenbach *et al.* (2005) were very similar yet reported post exercise eHsp72 concentrations were markedly different (serum mean 1.02 ng/ml and plasma mean 4.2 ng/ml respectively). Furthermore, it is worth noting that all of these early[5] exercise studies used the same enzyme-linked immunosorbent assay to determine eHsp72 (EKS-700, Stressgen Biotechnologies, Victoria, BC, Canada). This difference between plasma and serum Hsp72 concentration clearly poses a problem when comparisons between studies are made to assess the degree to which an independent variable affects the eHsp72

[5] This ELISA was removed from production in 2007 and was superseded by a new high-sensitivity ELISA in 2008.

concentration. Additionally, the ELISA used in the majority of these early eHsp72 analyses used a comparison with a standard curve that ranged from 0.78 to 50 ng/ml, with a sensitivity of ~0.5 ng/ml (personal communication with the manufacturer). Importantly, when serum is used (as was the case in the majority of these eHsp72 human studies) the reported concentration of eHsp72 irrespective of condition, lies at or below the sensitivity of this assay, increasing the likelihood of attaining undetectable samples.

Many assays for a number of different analytes consider the use of serum or plasma as equivalent (Miles *et al.*, 2004), but they are clearly different. When whole blood is drawn in the presence of an anti-coagulant (e.g. lithium heparin, ethylenediaminetetraacetic acid (EDTA) or sodium citrate) and centrifuged immediately to remove the cellular elements, plasma is obtained. If the whole blood is allowed to clot, centrifuging removes the fibrin clot in addition to the cellular elements, leaving blood serum. Since serum is produced *ex vivo* it is likely that the bio-chemical difference compared with plasma may affect the assayed eHsp72 concentration. Significant differences have been found between EDTA-plasma and serum for interleukins 2, 4 and 6 (Quek *et al.*, 2005), whilst Ladenson *et al.* (1974) reported significant differences between heparinised plasma and serum for a number of different chemical analytes (e.g. albumin, calcium, chloride, CK, glucose, lactate dehydrogenase, inorganic phosphate, potassium and total protein). The total protein concentration of serum is typically less than in plasma. Ladenson *et al.* (1974) reported mean concentrations of 7.29 g/dl for serum versus 7.58 g/dl for heparinized plasma, similar to values obtained by Lum and Gambino (1974) 7.21 and 7.45 g/dl respectively. This reduction has been attributed to the removal of the bulk of fibrinogen and other clotting factors into the formation of the fibrin clot, in addition to platelets that have become physically bound within the blood clot (Lundblad, 2005).

As a key *intracellular* role of Hsp72 involves binding to and re-folding aggregated proteins, we hypothesised that *extracellular* Hsp72 from exercising subjects binds to proteins involved in the clotting process, thereby decreasing the assayed concentration from serum-derived samples compared to plasma-derived blood samples. The primary aim of this study was to investigate the effect of commonly used specimen tubes and handling procedures on post-exercise eHsp72 concentration. As erythrocytes contain Hsp72 (Gromov and Celis, 1991), it is plausible that haemolysis as a consequence of venepuncture might alter the assayed concentrations of eHsp72. Therefore, as a secondary aim, we examined the relationship between eHsp72 and free haemoglobin in all samples.

3.3 Methods

Sample Size Calculation

A sample-size calculation was performed using a freely available internet sample size calculator for one-sample studies (http://www.danielsoper.com/statcalc/calc47.aspx). Mean and standard deviation values for serum and plasma Hsp72 obtained from the studies by Walsh *et al.* (2001) and Fehrenbach *et al.* (2005) respectively, were used as they adopted similar subject characteristics, exercise intensity and duration. With a two-tailed significant value of 0.05 and a power of 0.9, four subjects were required to detect a significant difference between serum and ethylenediaminetetraacetic acid (EDTA) plasma Hsp72. However, more subjects than this were recruited as pilot testing had revealed that the difference between serum and heparinised plasma-treated blood concentration of eHsp72 was approaching significance.

Participants

Nine recreationally active males volunteered to participate in the study (age: 27.6 ± 0.9 yr, height: 1.78 ± 0.02m, body mass: 75.4 ± 2.7 kg, estimated VO_{2max}: 54.3 ± 2.1 ml/kg/min).

Experimental Design

Determination of Peak Power Output (W_{max})

Having arrived at the lab after a 4-hour fast, each participant performed a maximal incremental test to volitional exhaustion on a cycle ergometer (Lode Excalibur, Gronigen, The Netherlands) to determine their peak power output (288.2 ± 13.7W). After a 5-minute

warm-up at 60 watts, the test was begun, starting at 95W with 35W increments every 3-minutes. The ergometer is electromagnetically braked which can be set in either the pedalling cadence independent mode (hyperbolic mode), or a pedalling cadence-dependent mode (linear mode). For the determination of W_{max}, the ergometer was set in hyperbolic mode in order that the participants could cycle at any desired cadence without any change in workload. W_{max} was calculated using the equation:

$$W_{max} = W_{fin} + ((t/180) \times 35)$$

Where W_{fin} is the workload of the final complete stage, and t is the time (in secs) completed of the last stage. Heart rate was recorded during the final minute of each stage (Polar Electro, Finland) and was used alongside power output in the Åstrand-Ryhming Nomogram (1954) to determine an estimation of peak oxygen consumption (VO_{2max}), which was used for demographic purposes only.

Time-trial

Following a 15-minute rest, participants then conducted a time-trial where they were instructed to complete a set amount of work (415.2 ± 19.7 kJ) as quickly as possible based on their W_{max} (Jeukendrup *et al.*, 1996). The aim of the time trial was to elicit a total energy expenditure equivalent to cycling at 80% W_{max} for 30-minutes (1800 seconds). This time-trial has been shown to have a co-efficient of variation of 3.4% when used with trained athletes (Jeukendrup *et al.*, 1996). The equation used to calculate the target amount of work was adapted from Jeukendrup *et al.* (1996) and is shown below:

$$\text{Target amount of work (kJ)} = (0.80 \times W_{max} \times 1800) / 1000$$

To measure the amount of work, the cycle ergometer was switched to linear mode, whereby increases in cadence resulted in an increase in work load. Individually specific linear factors were entered into the ergometer to provide the appropriate resistance. Participants had no contact other than to be shown when they had completed 25, 50, 75, 90 and 100% of the time-trial, and they were allowed to drink water *ad libitum* throughout the trial. To maximise the stress conditions, the time-trial was performed in a hot environment (34.6 ± 0.1 °C, 42.4 ± 1.9 % relative humidity) in an environmental chamber (Delta Environmental Inc, U.K). Core body temperature (T_{re}) was monitored throughout using a rectal thermister inserted 10cm beyond the anal sphincter which was connected to a digital thermometer (YSI Precision, Ohio, USA).

Tests and Procedures

Blood collection

Immediately on completion of the time-trial, participants were seated with blood samples collected by venepuncture (22 gauge needle: Greiner bio-one, UK) from an antecubital vein into 4 different polypropylene specimen tubes in the following standardised draw order to prevent contamination; 2 × plain tubes, 1 × lithium heparin and finally 1 × EDTA tube (Becton Dickinson vacutainer, Oxford, UK). A tourniquet was used, but was removed once blood flow had begun to prevent haemostasis. Six millilitres of blood was collected into each of two plain serum tubes and inverted 5 times. One tube was left to clot

at room temperature (SR ~21°C), whilst the other was stored on ice (SI ~4°C). After one hour, both tubes were centrifuged at 1500*g* at 4°C for 10 minutes, with the serum aspirated and aliquoted into 3 eppendorfs. A further 6ml of blood was collected into a lithium heparin coated tube (LH), whilst 4ml was collected into an EDTA coated tube (EDTA). Both plasma tubes were inverted 8 times and immediately centrifuged at 1500*g* at 4°C for 10 minutes with the resultant plasma aspirated and aliquoted into 3 eppendorfs. All samples were immediately frozen and stored at -80°C until later analysis.

Hsp72 analysis

Hsp72 was analysed using a commercially available ELISA specific for inducible eHsp72 (StressGen Biotechnologies, Victoria, BC, Canada – see general methods section for details on procedure). The intra-assay precision of the assay (CV) across all samples was calculated as 8.6%. All statistical analysis was conducted as detailed in Chapter 2.

Analysis of free haemoglobin

To determine the extent of haemolysis in all assayed samples, free haemoglobin was assessed using a spectrophotometric scanning technique (Blakney & Dinwoodie, 1975). Serum and plasma samples were read at 562, 577 and 602 nm on a spectrophotometer (U2800 UV, Hitachi, Japan), and the resulting data were used in conjunction with the absorption coefficient of oxyhaemoglobin to relate absorption to haemoglobin concentration.

3.4 Results

Performance data

The time-trial was completed in a mean time of 40.5 ± 1.7 mins, during which heart rate averaged 154 ± 6 beats per minute (80.3 ± 2.9% of age predicted maximum heart rate). Performing the time-trial in a hot environment increased core temperature ($F_{(10,80)} = 147.9$, $P < 0.001$) which had increased significantly after 20% of the time-trial completed, with a total increase of 1.2°C above pre-time trial temperature (final core temperature: 38.8 ± 0.1°C). See Figure 3.1.

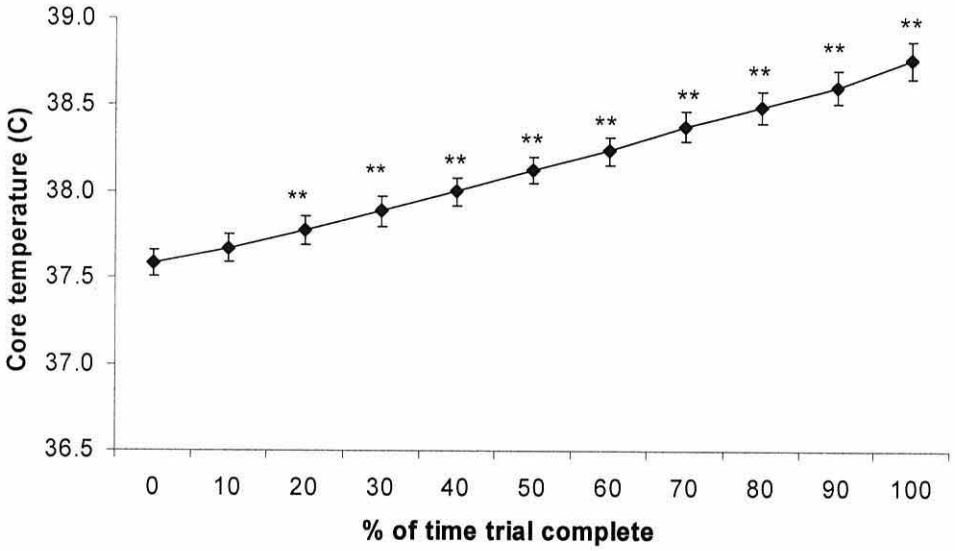

Figure 3.1 Mean (±SEM) core temperature responses during a cycling time-trial in a hot environment (note: subjects had completed an incremental test to exhaustion immediately prior to the time-trial. Therefore, core temperature at time = 0 is not a resting value), $n = 9$. Significant increase from time zero (**$P < 0.01$).

Effect of blood collection tube on post-exercise eHsp72 concentration

ANOVA revealed a main effect of blood collection tube on post-exercise eHsp72 concentration ($F_{(1.1,8.8)} = 76.9$, $P < 0.001$, $\eta^2 = 0.91$) with a large effect size (based on

established reference values by Cohen (1988) where >0.8 is defined as a large effect). *Post-hoc* tests indicated that EDTA treated blood had a significantly ($P \leq 0.001$) higher concentration of Hsp72 than all other treatments (EDTA: 6.46 ± 0.26, LH: 2.72 ± 0.76, SR: 0.16 ± 0.11, SI: 0.09 ± 0.09 ng/ml), whilst lithium heparin treated blood had a significantly higher concentration of Hsp72 than both serum clotted on ice (SI) and serum clotted at room temperature (SR) ($P < 0.05$). There was no difference between serum clotted at room temperature and serum clotted on ice (Figure 3.2). Extracellular Hsp72 was detected by ELISA in only one serum-ice sample, in two serum-room samples, in seven heparinised-plasma samples and in all nine EDTA samples. The mean intra-assay CV of the nine duplicate EDTA samples (4.8%) was lower than all other specimen types (SI: 7.0%, SR: 19.5%, LH: 17.4%).

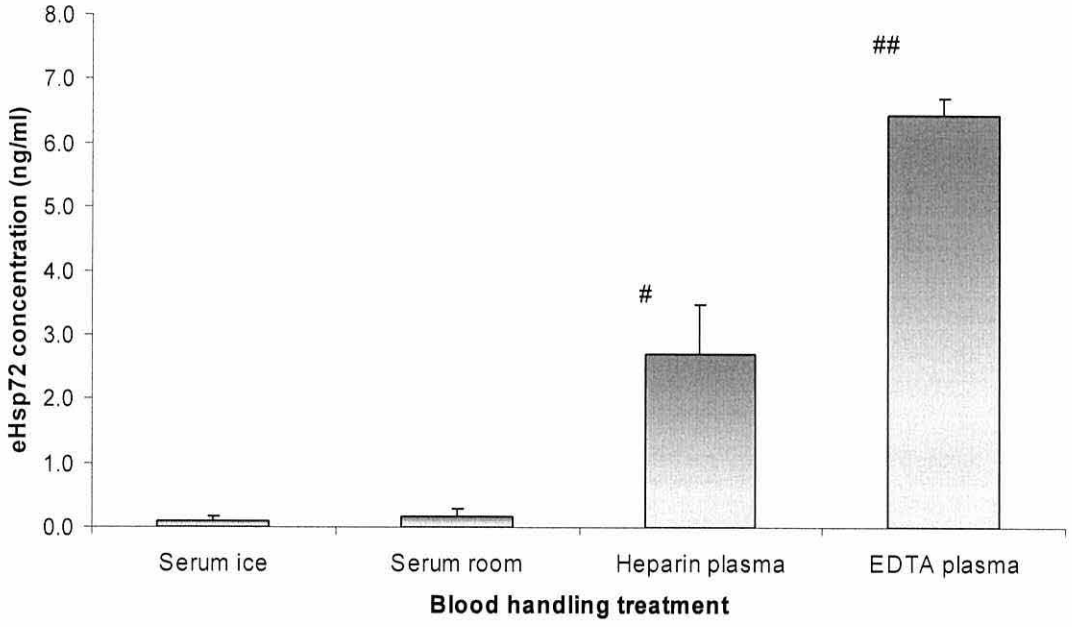

Figure 3.2. Effect of blood collection tube on post-exercise extracellular Hsp72 concentration in nine males. # $P < 0.05$ vs serum ice and serum room, ## $P \leq 0.001$ vs all other specimen tubes. Values are means, error bars represent SEM. $n = 9$.

Effect of haemolysis on eHsp72 concentration

Two samples showed clear visual evidence of extensive haemolysis. The concentration of free haemoglobin and eHsp72 in these two samples (2433 and 22750 mg/ml and 14.2 and 194.2 ng/ml for free Hb and eHsp72 respectively) were far greater than all other samples. These two samples were subsequently removed from all statistical analysis. The correlation between free haemoglobin and eHsp72 in all remaining samples (for which eHsp72 was detected) was $r = 0.36$, $P = 0.130$. Furthermore, there were no systematic trends in free haemoglobin between the four collection methods ($F_{(1.4, 8.4)} = 0.917$, $P = 0.401$).

3.5 Discussion

This study investigated the effects of different blood handling procedures on post-exercise eHsp72 concentration in humans. In line with our hypothesis, extracellular Hsp72 derived from plasma provided significantly higher concentrations than that derived from serum. Furthermore, EDTA plasma provided 2.5-fold greater eHsp72 concentration compared with lithium heparin. Based on previous literature employing different blood handling techniques, differences in eHsp72 concentration between serum and plasma can be expected. However, this is to our knowledge the first study to demonstrate a clear difference in eHsp72 concentration with the use of serum and plasma specimen tubes in a controlled within-subjects design.

In serum, the bulk of fibrin and fibrinogen have been removed by the fibrillar clot, as well as platelets that have become enmeshed within the clot (Lundblad, 2005). Because the fluid compartment remains in contact with the blood cells for a longer period of time compared with plasma, the likelihood of artifactual changes in serum may be greater (Hrubec *et al.*, 2002). Indeed, difference in assayed concentrations of proteins such as albumin, creatine kinase and total protein between serum and plasma have been reported (Ladenson *et al.*, 1974). Because of the intracellular roles of Hsp72 in chaperoning aggregated proteins, it is possible that extracellular Hsp72 may bind to proteins involved in the clotting process such as fibrin or fibrinogen, thus decreasing the assayed concentration of eHsp72. Indeed, Hsp72 peptide complexes have been shown to bind to the CD91 receptor on antigen presenting cells (Basu *et al.*, 2001) whilst other high molecular weight chaperones HSP's (Grp96) bind to CD91 on human platelets (Hilf *et al.*, 2002). Furthermore, low molecular mass HSP's (Hsp20) have been shown to bind specifically to

platelets after endothelial injury in hamsters (Kozawa *et al.*, 2002). Therefore, it could be that platelets may also be a potential destination for eHsp72 during clotting. The fibrillar clot is an important aspect of the innate immune system due to its ability to entrap invading bacteria and prevent its spread throughout the body (Dunn & Simmons, 1982). In the horseshoe crab, a number of immune effector proteins are known to bind to the fibrils of formed blood clots, and it has been suggested that rather than just a passive entrapment device, the blood clot is a delivery mechanism for proteins and cells that are capable of inducing an immune response (Armstrong & Armstrong, 2003). Whilst this is yet to be shown in humans, the immunostimulatory role of Hsp72 places this as a further possible explanation for Hsp72 binding to the fibrillar clot.

Aside from the clear differences in eHsp72 concentration between serum and plasma, the current data suggests that plasma eHsp72 concentrations are dependent on the anticoagulant used since EDTA plasma provided 2.5-fold higher eHsp72 concentrations than samples treated with lithium heparin. Lithium heparin activates anti-thrombin, which prevents thrombin from converting fibrinogen to fibrin. In contrast, EDTA is a metal ion chelator, and in the case of whole blood it binds with calcium which is crucial in thrombin formation and thus blood clotting. Substrate protein binding of Hsp72 is adenosine triphoshate (ATP) dependent (Mayer & Bukau, 2005), and because magnesium (Mg^{2+}) forms part of the ATPase domain of Hsp72 (Mayer & Bukau, 2005), it is possible that EDTA binds with Mg^{2+}. Hsp72 might therefore be prevented from binding to substrate proteins involved in the clotting process. Although it could be argued that the use of anti-coagulant chemicals such as heparin and EDTA may interfere with analyte detection, the small concentration of EDTA within a single vacutainer was unlikely to inhibit the detection of eHsp72 concentration in this study, particularly considering the magnitude of

the observed effect. A further advantage of using EDTA is the increased confidence in the precision of derived concentrations, as demonstrated by the lower co-efficient of variation between the duplicate samples compared with the other blood collection tubes.

One of the problems highlighted by the literature is that serum-derived Hsp72 concentrations lie at or below the detectable range of the assay (standard curve 0.78-50 ng/ml, sensitivity of the assay ~0.5 ng/ml). Therefore, this may increase the chance of returning undetectable samples or relying on extrapolated data from the standard curve. Moreover, if Hsp72 does indeed bind to the blood clot, this raises questions as to whether the extracellular concentration reported in studies accurately reflects the concentrations of circulating Hsp72 *in vivo*. Whilst this should not detract from studies reporting large changes in Hsp72 concentration in response to a certain stressor, it does cause problems in cross-sectional studies, in particular, where direct comparisons are made with the Hsp72 concentration of other fluids, or when correlations are performed with certain variables of interest. Additionally, because it is presently unclear as to the source of eHsp72, release from organs or cells could be wrongly discounted if Hsp72 is undetectable in serum. These points stress the need for stringent control procedures when serum is used, as it stands to reason that changes in clotting time are likely to affect the assayed concentration of Hsp72. Furthermore, the use of a glass or plastic container can markedly alter the quality of blood clotting and subsequent serum (Lundblad, 2005).

Because erythrocytes contain Hsp72 (Gromov & Celis, 1991) and haemolysis is associated with necrosis and damage to the cellular membrane, it is plausible that haemolysis could contribute to eHsp72 concentration assayed in blood samples. Indeed, two serum samples

showing the pink colour characteristic of haemolysis produced very high concentrations of eHsp72 and free haemoglobin. Inclusion of these data in the statistical analysis provides a very high correlation between eHsp72 and haemolysis. However, when removed as outliers, no significant correlation was evident. Exercise-induced haemolysis has been reported for more than 60 years (Gilligan *et al.*, 1943) and whilst it has traditionally been attributed to mechanical damage of erythrocytes as they pass through the capillaries of the foot during running (Telford *et al.*, 2003), cycling and other non-footstrike sports are also known to contribute to haemolysis during exercise (Beneke *et al.*, 2005;Selby & Eichner, 1986). As we have failed to show a relationship between eHsp72 and haemolysis, it is unlikely that eHsp72 present within the serum/plasma sources from within damaged erythrocytes under the exercise conditions encountered in this study. However, haemolysis may contribute to eHsp72 concentration during load-bearing exercise of greater duration. Indeed, increases in eHsp72 derived from heparinised plasma following an ironman triathlon were accompanied by increases in serum bilirubin (an additional marker of haemolysis) (Suzuki *et al.*, 2006). It is likely that the two samples with extensive haemolysis resulted from poor venepuncture technique which is a known cause of haemolysis (Grant, 2003).

In conclusion, the results demonstrate that blood treated with an anti-coagulant provides significantly higher concentrations of Hsp72 than serum-derived Hsp72, and that blood treated with an anti-coagulant is more likely to represent circulating *in vivo* concentrations. Since previous research has tended to report serum data at the lowest point of the detectable range of the assay, it is recommended that EDTA-coated specimen tubes be used in future investigations into the *in vivo* eHsp72 stress response.

Chapter 4

No endogenous circadian rhythm in resting plasma Hsp72 concentration in humans

This study has been accepted for publication as an original article in Cell Stress and Chaperones:

Fortes, M.B. and Whitham, M. (2009). No endogenous circadian rhythm in resting plasma Hsp72 concentration in humans. *Cell Stress and Chaperones*. ***In Press***. DOI: 10.1007/s12192-008-0082-9.

This study was funded by a grant from the North West Wales NHS Trust grants committee.

4.1 Summary

Extracellular (e) Hsp72 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-hour period. Blood samples were taken every hour from 1200h – 2100h and from 0700h – 1200h 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. EDTA blood samples were analysed for eHsp72 concentration using a commercially available high-sensitivity ELISA (intra-assay CV = 1.4%). One-way repeated measures ANOVA 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 ± 3.8 kcal/hr). 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 co-efficients of variation ranged from 3.8-7.7% (mean 5.4%). Mean eHsp72 concentration across all subjects and time points was 1.49 ± 0.08 ng/ml. These data show that in a rested state, plasma eHsp72 concentration shows no apparent endogenous circadian variation.

4.2 Introduction

Hsp72 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 eHsp72 significantly increased in a number of stressful conditions such as exercise (Walsh *et al.*, 2001;Febbraio *et al.*, 2002a;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 Matzingers proposal that a number of endogenous molecules may act as danger signals during times of stress to initiate an immune response (Matzinger, 2002;Fleshner & Johnson, 2005). A number of recent review articles have summarised eHsp72's involvement in immunity (see Chapter 1 and (Johnson & Fleshner, 2006;Pockley *et al.*, 2008).

It is well characterized 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 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 hours) between the relatively few sampling points. Furthermore, no demographical, methodological or statistical information was presented thus making it difficult to ascertain whether variation in resting eHsp72 exists and indeed the external validity of the findings.

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 the resting concentration of eHsp72 is 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 twenty-four hour period.

4.3 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 ± 1.4 yr, height: 1.83 ± 0.08 m, body mass: 77.6 ± 3.7 kg). In addition to standardised recruitment criteria (Chapter 2) participants who 'napped' regularly, or demonstrated unstable sleep/wake cycles (e.g. shift workers) were also excluded.

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 hours prior to the start of the test. They were provided with water to consume the day before the trial (35ml/kg body weigh) 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 urine osmolality <700 mOsm/l was taken as euhydrated (Casa *et al.*, 2005). A

standardised breakfast was also provided to eat on the morning of the trial. Participants arrived in the laboratory at 1000h, 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 two-hour delay before the first blood sample at 1200h 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 2100h, and then resumed at 0700h the following day until 1200h to allow determination of 24-hour 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 minute 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-hours and which may be mediated by either endogenous or exogenous factors (Shephard & 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%RH) with a standardised light/dark cycle (constant light of 330 – 370Lux from 0645h – 2300h), 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 2300h and were woken at 0645h the following morning.

Tests and procedures

Participants were seated 15 minutes prior to each sampling time. After 10 minutes resting, tympanic temperature was assessed using an in-ear thermometer which detects infra-red heat emissions from the tympanic membrane (ThermoScan IRT 4520, Braun GmbH, Germany). Blood pressure and heart rate were also assessed using an automated sphygmomanometer (Omron automatic oscillometric digital blood pressure monitor, Japan). Two × 5 ml blood samples were drawn from the cannula and directed into 1 × K_2EDTA and 1 × lithium heparin coated plasma tube (Becton Dickinson, 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 (1974). Blood samples from the remaining lithium heparin tube and the K_2EDTA tube were processed as described in the general methods section (Chapter 2).

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 seconds. Counts per minute were converted into kcal/hr 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 x body mass] + 679) (WHO, 1985). Meals were provided at 1315h-1345h, 1815h-1845h, 2115h (day one) and 0715h-0745h (day two), with macronutrient breakdowns of 68 % carbohydrate, 14% fat and 18 % protein. Individual calorie intakes ranged from 2235 to 2672 kcal/d. Fluids were provided over the 24-hours (35ml/kg body weight) 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 ELISA for use with serum and plasma samples (Assay Designs EKS-715, Michigan, USA – see general methods for procedure, Chapter 2). The assays sensitivity is described by the manufacturer as 0.09 ng/ml, whilst the mean inter and intra-assay CV in the current study was 7.1 and 1.4% respectively. Statistical analysis was performed as outlined in Chapter 2. The majority of data were analysed using one-way repeated measures ANOVA.

4.4 Results

Control measures

There was no significant change in either body mass (pre: 78.1 ± 3.7, post: 77.8 ± 3.9 kg, P = 0.370) or urine osmolality (pre: 405 ± 74, post: 249 ± 23 mOsm/l H_2O, P = 0.052) from pre to post 24-hour 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.

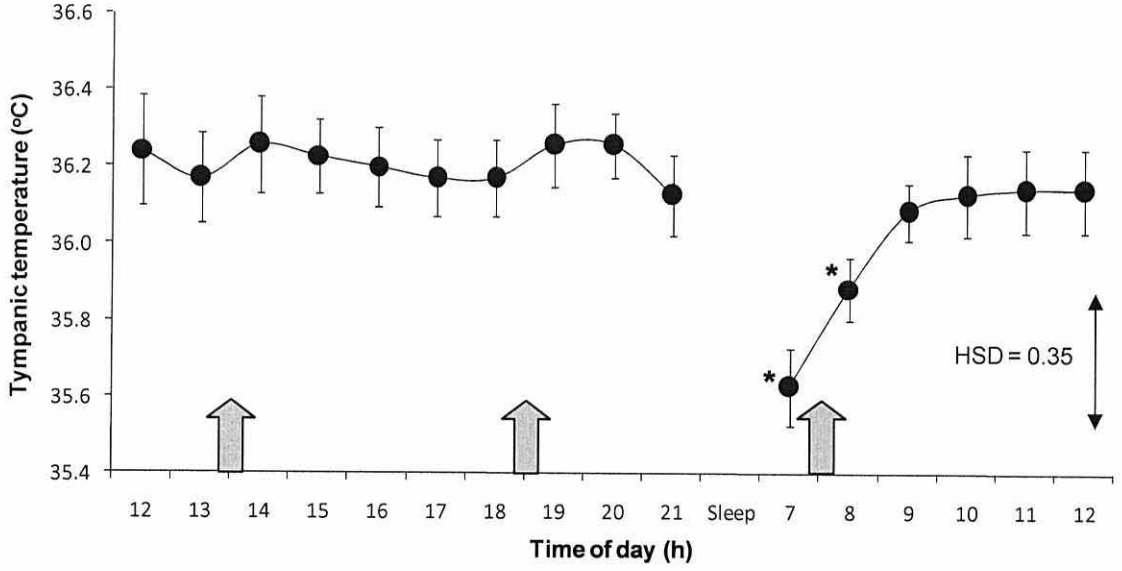

Figure 4.1. Tympanic temperature measured throughout the 24-hour period. HSD indicates Tukey's honestly significant difference value (*P < 0.05). Arrows represent main meal times. Values are mean ± SEM, n = 7

Tympanic temperature demonstrated the extensively documented circadian variation that has been reported in previous literature (P = 0.008), with lower values first thing in the morning (Figure 4.1). Only the 0700h and 0800h 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 ± 3.8 kcal/hr). There was a significant main effect for time ($P = 0.026$) with physical activity significantly lower between 2000h and 2100h (Figure 4.2).

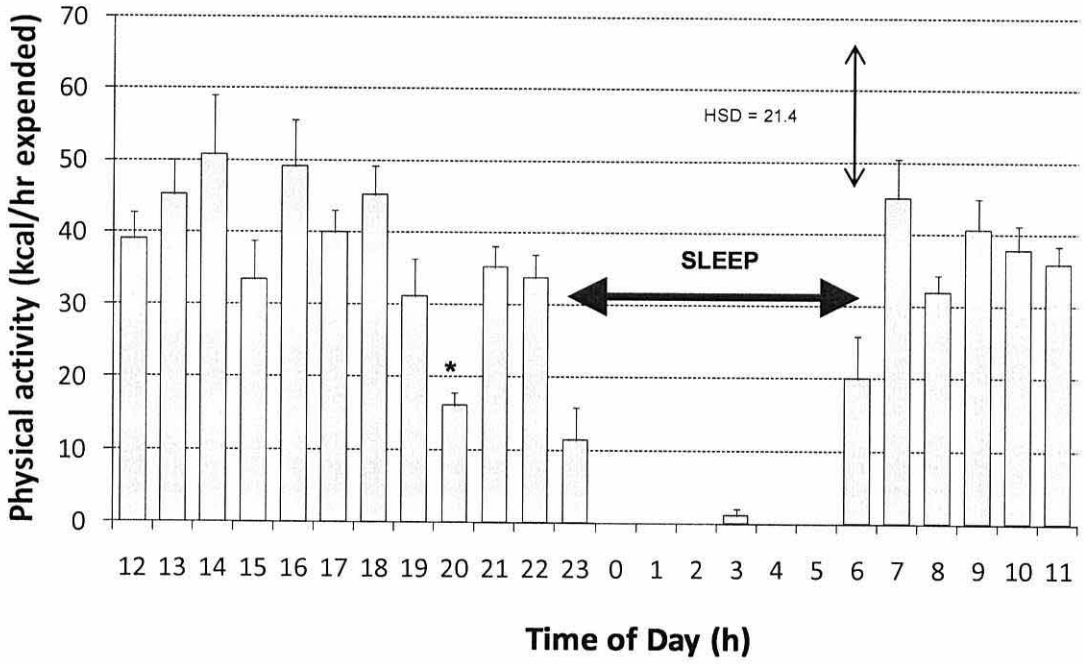

Figure 4.2. Physical activity expenditure during the 24-hour study. Values are kcal/hr derived from the accelerometer expended as physical activity, not including resting metabolic rate. Data during the sleeping period (from 2300h to 0700h) were not included in the statistical analysis. HSD indicates Tukeys honestly significant difference value (*$P < 0.05$). Values are mean ± SEM, $n = 7$.

Plasma Hsp72

Plasma Hsp72 concentration did not change significantly throughout the 24-hour period in either absolute concentration ($P = 0.367$), or PV change corrected concentration ($P = 0.380$) (Figure 4.3). Individual CV's ranged from 3.8-7.7% (mean 5.4%). Box-and-whisker plot analysis revealed no significant statistical outliers in 4 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 (Figures 4.4 and 4.5). Mean eHsp72 concentration across all

subjects and time points was 1.49 ± 0.08 ng/ml and 1.51 ± 0.05 ng/ml 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 4.1). However, across the whole group and in the remaining 6 participants eHsp72 concentration was not related to either tympanic temperature or prior physical activity.

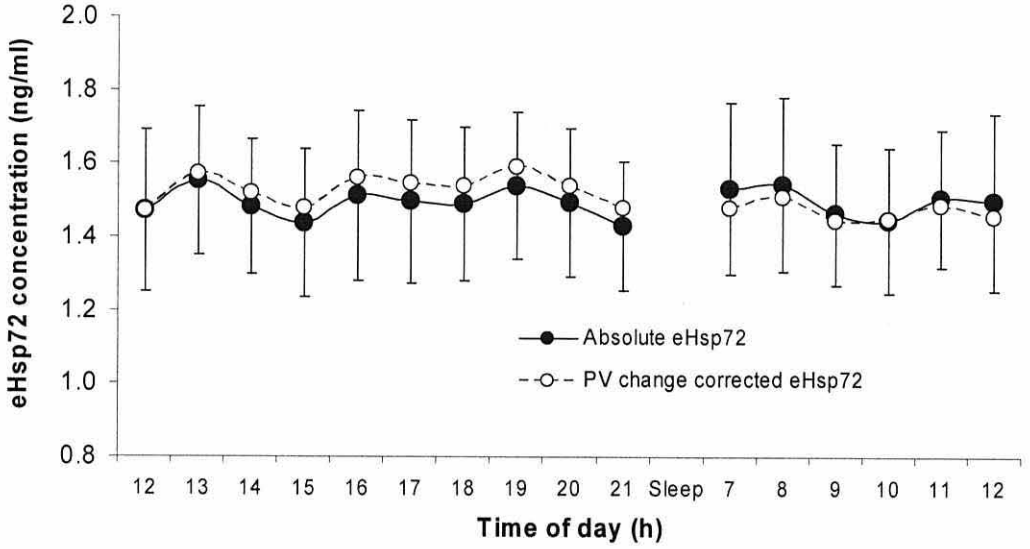

Figure 4.3. Mean plasma Hsp72 concentration throughout the 24-hour period of the study. Both absolute and plasma volume change corrected data are displayed. No significant differences between any of the time points. Values are mean ± SEM, n =7.

Table 4.1. Within subject and whole sample correlations between concentration of eHsp72, tympanic temperature and physical activity.

		Participant number							
		1	*2*	*3*	*4*	*5*	*6*	*7*	*Sample*
eHsp72 & tympanic temperature	*r*	0.14	0.27	-0.43	-0.42	-0.17	0.19	**-0.76**	-0.14
	P	0.60	0.32	0.09	0.10	0.54	0.49	**0.04**	*0.59*
eHsp72 & physical activity	*r*	0.25	-0.19	0.25	-0.21	0.10	**0.79**	0.40	0.16
	P	0.37	0.49	0.36	0.45	0.72	**0.00**	0.43	*0.56*

Significant relationships are depicted in bold

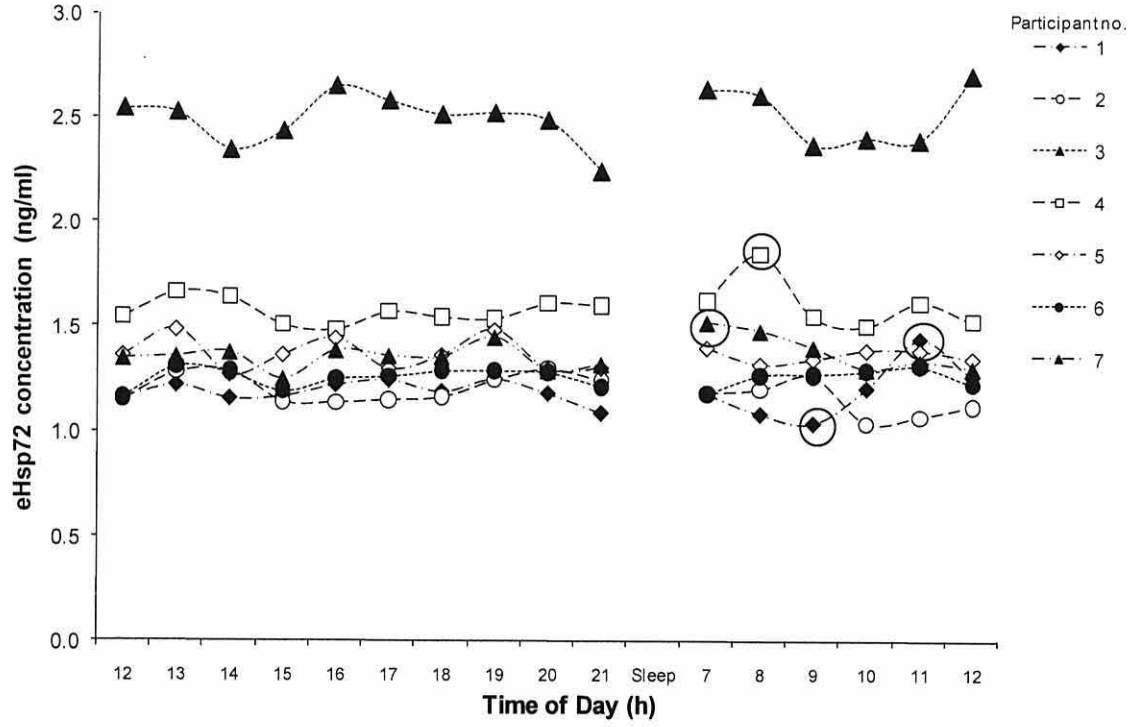

Figure 4.4. Individual rhythms in absolute plasma Hsp72 concentration. Data points circled represent within-subject outliers derived from the box-and-whisker plot analysis. $n = 7$.

To assess the stability of the eHsp72 response, absolute and PV change corrected eHsp72 samples taken at 1200h on both days 1 and 2 of the study were analysed and compared. No significant differences in either absolute or PV change corrected eHsp72 were observed between the two 1200h samples ($P = 0.423$ and 0.164 respectively). Furthermore, very small CV's of 2.9 and 2.3% and highly significant correlations ($r = 0.993$, $P < 0.001$, and $r = 0.998$, $P < 0.001$, Figure 4.6) for absolute and PV change corrected eHsp72 demonstrates the apparent stability of eHsp72 on a day-to-day basis.

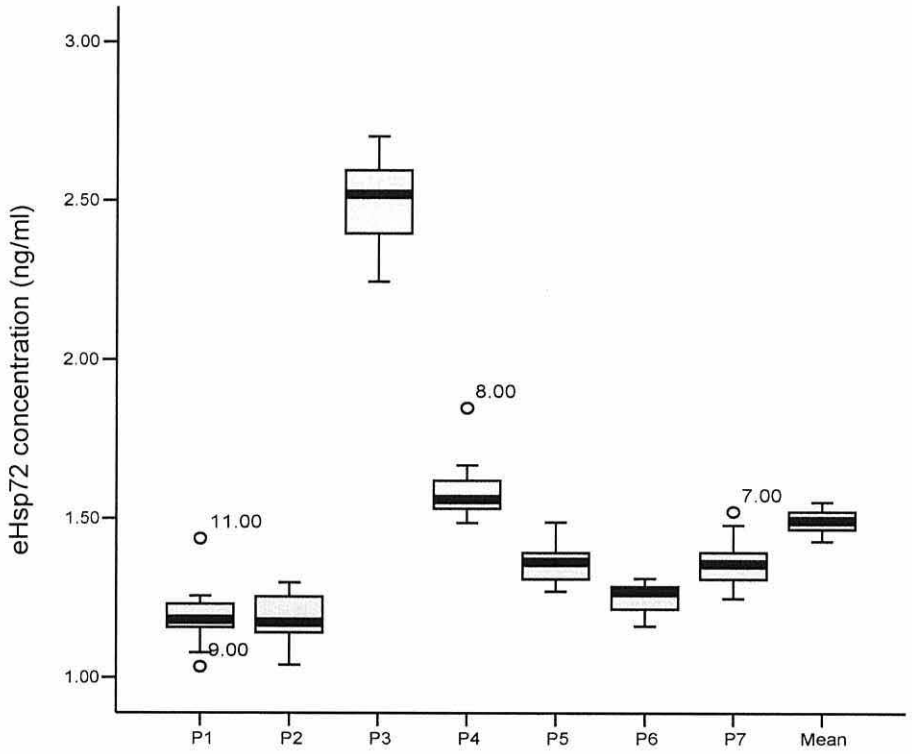

Figure 4.5. Box-and whisker plots of individual and mean eHsp72 data. The ends of the box represents the interquartile range (25th – 75th percentile), solid horizontal bar within the box represents the median, ends of the whiskers represent largest range of values not classed as a statistical outlier. Outliers are depicted by circles with corresponding sampling time and are defined as being more than 1.5 box lengths outside the box. $n = 7$.

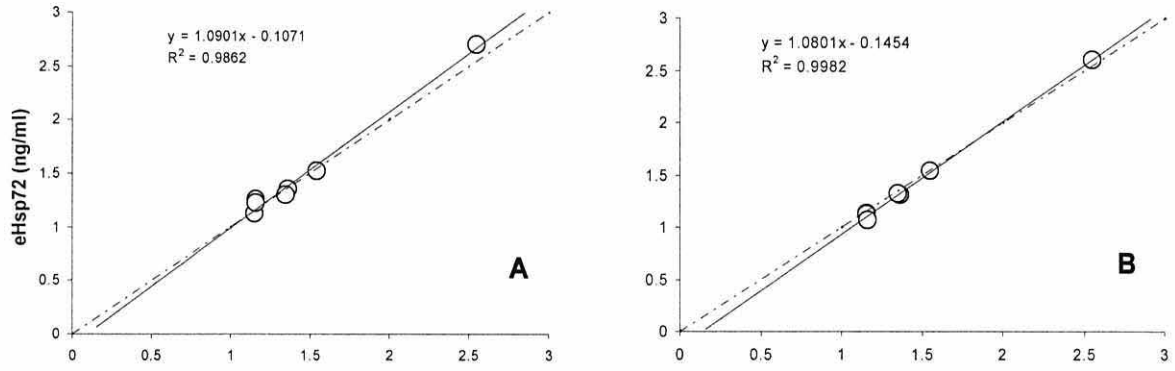

Figure 4.6. Stability of the eHsp72 response. Scatterplots of eHp72 concentration derived from samples taken at 12:00h on day 1 (x-axis) and day 2 (y-axis) of the study. Both absolute (**A**) and plasma volume change corrected (**B**) data are displayed. Both correlations are highly significant ($P < 0.001$). Solid line represents the regression line, dashed line represents the line of identity. $n = 7$.

4.5 Discussion

The aim of the current study was to assess whether a circadian variation exists in plasma Hsp72 concentration. Results from data collected from seven healthy males confirms the findings of Fehrenbach *et al* (2005), that Hsp72 concentration does not fluctuate significantly throughout the course of a 24-hour period. Furthermore, we have shown that the eHsp72 response appears to be 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 (Figure 4.3). 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 ~0.4 ng/ml. 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 ELISA's). However, expressed as a percentage change compared to healthy controls, Hsp72 concentrations are typically between ~20 – 300% higher across a number of clinical conditions (Abe *et al.*, 2004;Hunter-Lavin *et al.*, 2004b;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 CV's 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 intracellular 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 wasn't surprising given the well-known circadian variation 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 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 0.4°C and since severe thermal stress resulting in large increases in rectal temperature (~2.3°C) by passive whole body heating showed only modest (~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.*, 2006). 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-hours at 45 $\%VO_{2max}$ in 38°C heat elicited only a moderate (~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 ~30 – 2100% (refer to Table 1.1, Chapter 1), 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 ~2.5 % between repeat sampling highlighting this. Circadian rhythms in body functions may be mediated by endogenous or exogenous factors (Shephard & Shek, 1997). By controlling for external factors such as meal times, environmental temperature and physical activity, we have demonstrated that under resting conditions, eHsp72 concentration is 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 focusing 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 *intracellular* Hsp72 expression enables the cell to cope with physiological threats to cellular homeostasis. This is significant since *extracellular* Hsp72 has been shown to penetrate the cellular membrane and enter cells where it performs its usual cytoprotective intracellular roles (Guzhova *et al.*, 1998;Novoselova *et al.*, 2005). For example, increased uptake of extracellular Hsp72 increased motor neuron survival (Guzhova *et al.*, 2001;Robinson *et al.*, 2005), which is pertinent as these cells are incapable of synthesizing 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 destabilization of intracellular 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 variation 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 and that resting eHsp72 concentration is not related to tympanic temperature or prior sedentary physical activity.

Chapter 5

Effects of acute psychological and mental stress on the extracellular Hsp72 response

5.1 Summary

Whilst Hsp72 has been detected in the peripheral circulations of humans in response to a number of physiological stressors, evidence from rodent models suggests that acute psychological stress can also elevate eHsp72 concentration. Therefore, the aim of this study was to investigate whether an acute psychological stress induces the release of heat shock protein 72 into the circulation of healthy humans. Blood samples were collected at baseline and immediately after two different forms of acute psychological stress. Firstly, participants (n = 8) performed a difficult mental counting task whereby incorrect responses were penalised with a short uncomfortable electrical shock to the hand (Part 1). The second stress task involved participants (n = 7) taking part in an assessed academic presentation (Part 2). For Part 1, the stress task significantly increased heart rate and cognitive anxiety (as measured by a visual analogue scale) ($P < 0.01$), but did not alter the expression of salivary α-amylase (used as a measure of endogenous sympathetic activity, P = 0.752). In both Parts 1 and 2, acute psychological stress failed to alter eHsp72 concentrations within the plasma compared to resting baseline values (pre: 1.28 ± 0.30, post: 1.26 ± 0.22, 2h post: 1.25 ± 0.31 ng/ml, P = 0.856 for Part 1; presentation stress: 1.53 ± 0.22, baseline: 1.25 ± 0.16 ng/ml, P = 0.229, for Part 2). Acute psychological stress of the type in the current study fails to induce an eHsp72 response in the plasma of healthy humans. As such, acute psychological distress associated with participation in research studies (such as fear of venepuncture or anxiety associated with maximal exercise etc) should not be a primary concern for investigations into eHsp72.

5.2 Introduction

A number of previous studies have confirmed that Hsp72 is present in the circulation and concentrations increase as a result of a wide variety of both clinical and non-clinical stressors. For example, Hsp72 is elevated in a number of pathological conditions such as peripheral vascular disease (Wright *et al.*, 2000), 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). Non-clinical acute stressors that elicit an eHsp72 response in humans include exercise (Walsh *et al.*, 2001;Febbraio *et al.*, 2002a;Lancaster *et al.*, 2004;Whitham *et al.*, 2006), hyperthermia (Whitham *et al.*, 2007) and trauma (Pittet *et al.*, 2002).

Interestingly, eHsp72 concentrations are considerably elevated in rodents in response to acute psychological stressors. For example, predatory fear (rodents exposed to the sights and sounds of a cat but with no actual contact) and inescapable tailshock stress both significantly increased eHsp72 concentration (Fleshner *et al.*, 2004;Johnson *et al.*, 2005;Campisi *et al.*, 2003a). It is currently unknown whether acute psychological stress results in an eHsp72 response in humans. However, psychological stress evoked Hsp72 release is plausible since the proposed signals mediating eHsp72 release into the circulation are the stress hormones, noradrenaline and/or adrenaline (Johnson *et al.*, 2005;Whitham *et al.*, 2006). Plasma concentrations of catecholamines have been shown to increase in response to several different forms of psychological stress such as public speaking (Dimsdale & Moss, 1980), painful stimuli to the hand (Christou *et al.*, 2004), dental treatment (Brand *et al.*, 1995) and tandem parachute jumping (Richter *et al.*, 1996). The fact that psychological stress may potentially augment eHsp72 is interesting from a

researchers and methodological perspective, especially considering the well documented white coat effect (Tsai, 2002) and the perceived stress that participating in research studies may involve. It is currently unclear as to the sensitivity of eHsp72 release to acute stressors.

The aim of this study was to investigate whether an acute psychological stress induces the release of eHsp72 into the circulation in healthy humans. The study was separated into two parts, part one utilised a mental counting task with negative reinforcement (electrical shock to the hand) as a stressor, whilst psychological stress associated with public speaking/presentation was investigated in part two.

5.3 Methodology

Sample size

Given that no previous published studies have investigated the effect of psychological stress on the Hsp72 response in humans, no formal sample size calculation was performed. However, in rodent studies, predatory fear (Fleshner *et al.*, 2004) and electrical tail shock stress (Johnson *et al.*, 2005) was shown to induce a significant Hsp72 response with sample sizes as small as 4. Therefore, recruitment of 7-8 subjects was deemed sufficient to determine if such a response was present.

Participants

With full informed written consent, 8 healthy recreationally active males were recruited for Part 1 of the study (age: 21.8 ± 1.1 yr, body mass: 73.8 ± 1.9 kg, height: 1.79 ± 0.01 m). For Part 2 a different cohort of participants were recruited consisting of 5 healthy males (age: 21.4 ± 0.3 yr, body mass: 79.2 ± 3.9 kg, height: 1.79 ± 0.03 m) and 2 healthy females (age: 21.5 ± 0.3 yr, body mass: 66.5 ± 1.0 kg, height: 1.67 ± 0.03 m). Participants were included/excluded as detailed in Chapter 2. In addition, participants with any history of cardiac illness, anxiety attacks or epilepsy were also excluded.

Experimental Design, Tests and Procedures

Part 1.

The experimental studies took place in the School of Sport, Health and Exercise Sciences of Bangor University during January and February 2008. Participants were asked to arrive at the laboratory in a euhydrated state having avoided strenuous exercise and any caffeine

containing products in the previous 24h, and avoided tobacco, alcohol or any form of diuretic in the previous 48h. The combined mental and psychological stress test consisted of a challenging counting task with negative reinforcement of an electrical shock to the hand. Participants made two visits, a familiarisation trial, and the experimental trial.

Familiarisation

It was originally intended that the strength of the electrical shock would be standardised across all participants at a level consistent with previous research. However, pilot testing highlighted a significant individual variation in how participants perceived the strength of the shock; it was almost undetectable in some subjects whilst others found it very uncomfortable. Therefore, in order to standardise the electrical shock so that all participants perceived the same level of discomfort, participants were given a number of electric shocks that got progressively stronger until the participant rated the shock as "uncomfortable" on a likert scale. This likert scale had headings such as "I cannot feel the impulse", "the impulse is just noticeable", " the impulse is uncomfortable" and "the impulse is painful". The voltage was kept constant (200V) whilst the current was progressively increased. The strength of shock used in the experimental trial was set at 10mA higher than the level that elicited a rating of "uncomfortable". The current used in the experimental trial ranged from 75 to 180mA with a mean of 129 ± 15mA. The intensity of this shock is in line with previous research detailing a similar model of stress induction (Christou *et al.*, 2004;Donadio *et al.*, 2002;Noteboom *et al.*, 2001).

Experimental trial

We hypothesised that acute psychological and mental stress would induce an increase in Hsp72 in the circulation. In order to test this hypothesis, blood samples were taken before and after a number of stress inducing trials. These trials involved the participant counting the number of either high or low tones from a random set of high and low tones emitted from a computer programme. After each trial, participants had to respond with their answer by pressing a button the number of times they counted the respective tone. If the participant answered correctly they proceeded onto the next series of tones. If answered incorrectly, they were given an immediate uncomfortable but non-dangerous electric shock to the hand before continuing to the next trial. There were 21 trials in total of varying (increasing) difficulty (i.e. the number and frequency of high and low tones increased with progressive trials – see Figure 5.1). The whole task lasted approximately 20 minutes. The electric shock was generated from an electrical stimulation device devised for human use (Digitimer stimulator model D57A, Digitimer Ltd, Welwyn Garden City, England) and consisted of a 200V, 75 -180mA impulse delivered in a square-wave pattern over 200 mS.

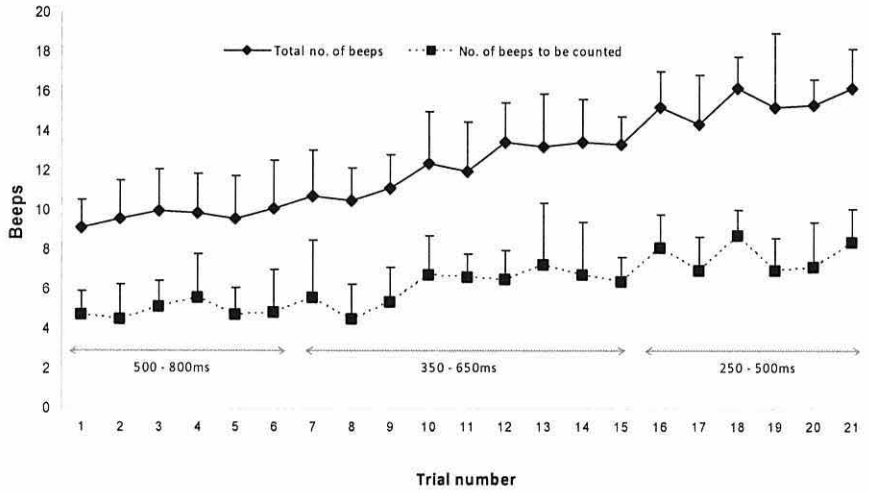

Figure 5.1. Stress test protocol displaying the number of beeps heard by participants with progressive trials (—) and the correct number of beeps (•••). Numbers beneath the arrows represent the range of inter-beep intervals for that block of trials, i.e. as the task progressed the time between beeps decreased.

Measures of stress

Upon attendance to the laboratory, participants rested quietly for 30 minutes prior to the task. To assess the degree to which participants were stressed, heart rate was continuously monitored throughout the trial using telemetry (Polar Electro, Finland) whilst blood pressure (Omron Automatic Oscillometric Digital Blood Pressure Monitor, Japan) was also assessed at various points throughout the trial. For the purposes of statistics, the heart rate data was averaged from trials 1-7, 8-14 and 15-21. Since salivary α-amylase concentration provides a useful measure of sympathetic activity and thus stress, saliva samples were collected using a pre-weighed cotton salivette placed under the tongue for 3-minutes. This was weighed on removal to calculate flow rate, and saliva removed by centrifugation at 1500*g* for 10 minutes. Saliva was frozen at -80°C for later analysis of salivary α-amylase concentration using a kinetic assay (refer to general methods, Chapter 2). Cognitive state anxiety was gauged throughout the trial at various points using a visual analogue scale (VAS). This comprised of a 100mm line anchored at either end with the phrases "not at all anxious" on the left hand side and "very anxious" on the right hand side. The participant placed a vertical line that bisected the 100mm line therefore indicating the perceived level of anxiety. Refer to Figure 5.2 for a schematic of procedures.

Blood sampling

Blood samples were taken by a qualified phlebotomist immediately prior to, immediately post and 2 hours after the stress task. One × 5ml blood sample was drawn from an antecubital vein via venepuncture using a 23-gauge butterfly needle (Venisystems, Abbott Ireland, Rep of Ireland) and collected into a K_2EDTA coated vacutainer. All samples were taken with the participant seated.

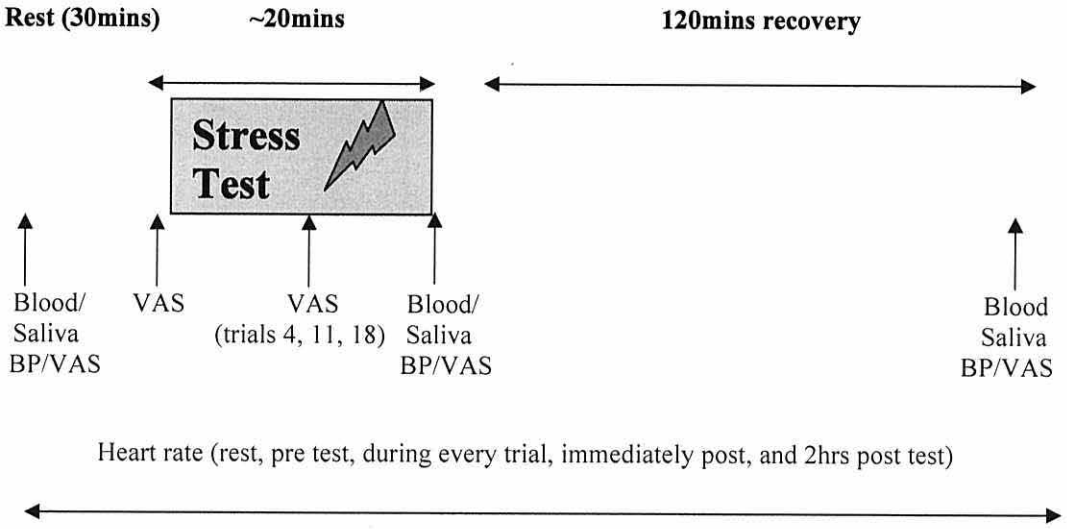

Figure 5.2. Time line of procedures for part one. BP – blood pressure, VAS – visual analogue scale (state anxiety).

Part 2

As an additional means to investigate the effect of psychological stress on eHsp72 concentration, we utilised an academic assessed presentation task. Participants recruited were all 3rd year sport science students from Bangor University taking part in their annual third year conference. As a compulsory part of their final year dissertation, all students must present results from their final research project at this conference which forms a large part of their overall degree classification.

Procedures

A large proportion (n = 55) of the total 3rd year intake (n =70) were briefed one week prior to the conference on the potential for participating in the study and informed that participation was entirely voluntary. It was decided that blood samples would be taken immediately upon completion of the participant's presentations. The presentation

consisted of a 15 minute period whereby the students present the findings from their research project in the form of a poster presentation. The student gave a 5-minute synopsis of their 3rd year study followed by 10 minutes of questions and discussion by two staff examiners. Blood samples were taken by venepuncture of an antecubital vein into a K_2EDTA coated vacutainer with the participant seated and treated as normal. Participants were asked to return to the laboratory for a further blood sample 7 days later at the same time of day, serving as a resting control sample. All students had finished exams and coursework at least 2 days prior to this control sample.

Hsp72 analysis

Thawed EDTA plasma samples were analysed for Hsp72 using a commercially available high-sensitivity sandwich ELISA (EKS-715, Assay Designs, Michigan, USA). See general methods section for details (Chapter 2). Intra-assay co-efficient of variations for eHsp72 were 5.6% and 6.5% for part 1 and 2 respectively and for α-amylase was 3.0%. Statistical analysis was conducted as outlined in Chapter 2, with the main data analysed using one-way repeated measures ANOVA.

5.4 Results

Part 1 - Measures of stress

The ANOVA revealed a main effect for heart rate ($F_{(2.49,17.4)} = 34.8$, $P < 0.001$) such that the stress inducing task significantly elevated heart rate above normal resting values by approximately 14 beats per minute (Figure 5.3). In addition, there was an anticipatory rise in heart rate immediately prior to the task which remained elevated until immediately post test. Heart rate had returned to resting levels 2 hours post stress-test. Cognitive state anxiety as measured by a visual analogue scale increased significantly as the stress-inducing task progressed ($F_{(2.13,14.9)} = 11.6$, $P < 0.01$), however *post hoc* tests revealed that the stress-induced trial values were elevated significantly only above the 2-hour post value, and not above the resting pre-stress values (Figure 5.3). There was a trend for an increase in systolic blood pressure at post test ($F_{(2,14)} = 3.59$, $P = 0.055$), whilst diastolic blood pressure did not change throughout the trial ($F_{(1.17, 8.16)} = 0.71$, $P = 0.447$). Blood pressure values were 117/71, 121/73 and 116/71 mmHg for the pre test, post test and 2-hour post test sample respectively. The stress test did not alter saliva flow rate ($F_{(2,12)} = 0.259$, $P = 0.776$) or salivary α-amylase concentration ($F_{(2)} = 0.298$, $P = 0.752$). Mean (± SD) values for α-amylase concentration were 52.9 ± 18.4, 60.9 ± 20.6 and 59.2 ± 13.9 $U \cdot ml^{-1}$ for the pre test, post test and 2-hour post test sample respectively.

eHsp72 concentration

eHsp72 was detectable in all participants. There was no difference in the concentration of plasma Hsp72 between any of the time points ($F_{(2,12)} = 0.158$, $P = 0.856$), therefore no *post-hoc* tests were performed (Figure 5.4).

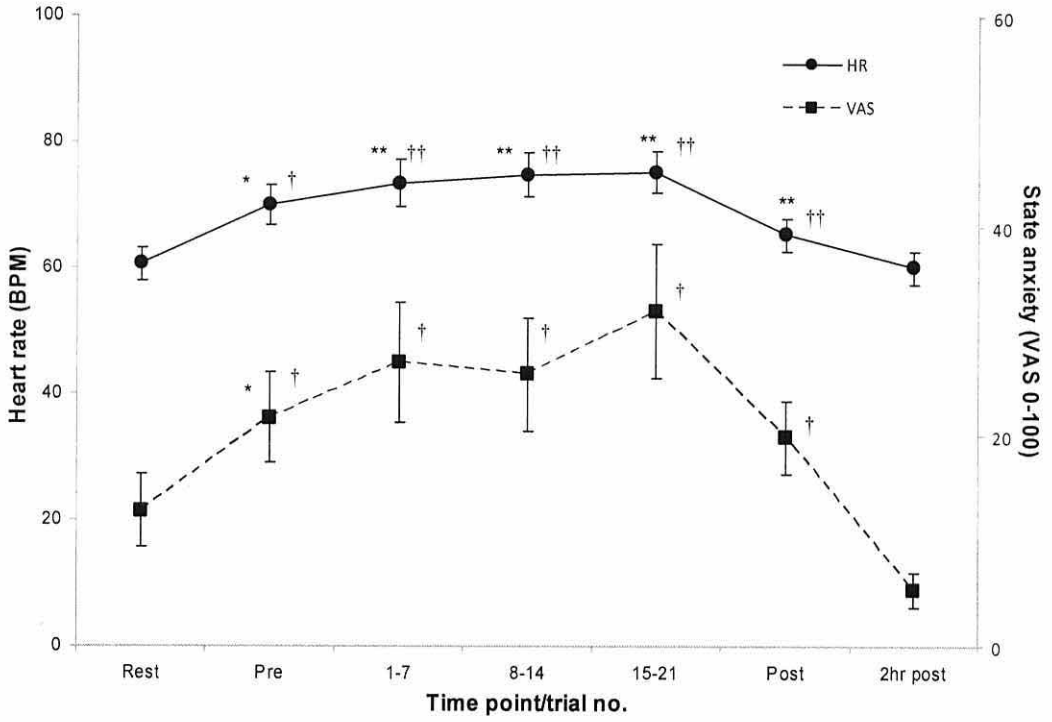

Figure 5.3. The time course of changes in heart rate (solid line) and cognitive anxiety (measured by a VAS – dashed line) in response to a combined acute psychological and mental stress task. Significant difference above rest are denoted by asterisks (* $P < 0.05$, ** $P < 0.01$) and differences from the 2-hour post sample are denoted by daggers († $P < 0.05$, †† $P < 0.01$). Values are means ± SEM, $n = 8$.

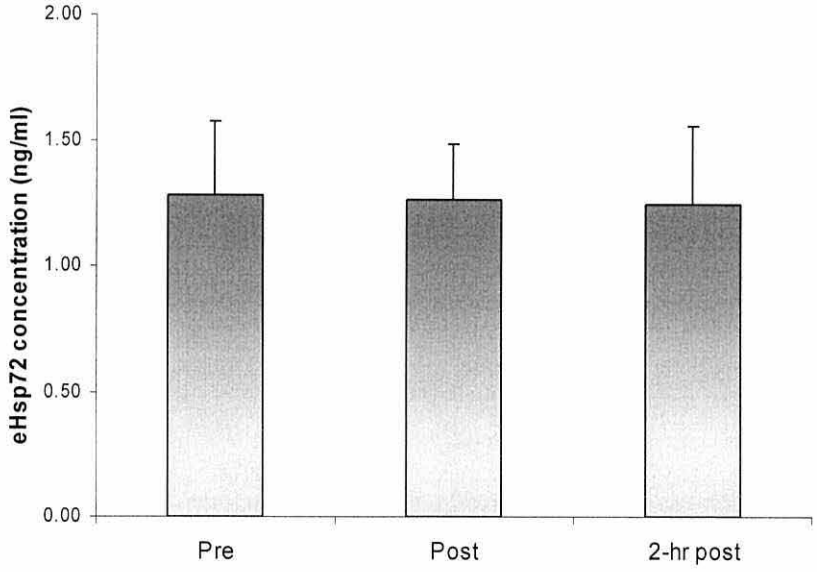

Figure 5.4. Plasma concentration of Hsp72 immediately pre, post and 2-hours after a combined psychological and mental stress inducing task. No significant difference between any time point. Values are mean ± SEM, $n = 8$.

Part 2.

eHsp72 concentration

eHsp72 was detectable in all subjects. Despite a visual increase in eHsp72 immediately after the presentation compared to resting control (Figure 5.5) there was no significant difference between conditions (t = -1.338, P = 0.229). As can be seen by the individual data presented in Figure 5.5, data from one subject (1) is skewing the mean data, and when the t-test is re-run with this subject excluded, the difference between the means becomes less significant (P = 0.353).

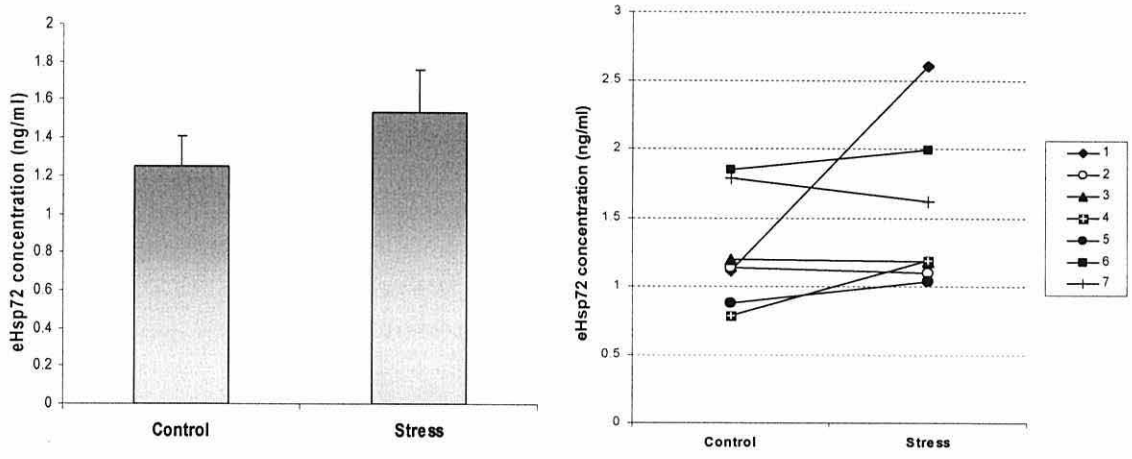

Figure 5.5. eHsp72 responses to a stressful presentation. Both mean (± SEM) (left), and individual responses are presented (right). n = 7.

5.5 Discussion

The aim of this study was to investigate if an acute psychological or mental stress could elicit an eHsp72 response in humans. To test this, eHsp72 concentrations were compared before and after two independent stressors; a mental counting task with negative reinforcement (electrical shock), and a stressful presentation. Neither of these acute psychological stressors altered eHsp72 concentration within the plasma.

In humans, a large number of investigations have demonstrated that eHsp72 concentrations are elevated in a variety of clinical pathologies. For example, eHsp72 concentration is significantly elevated in patients with peripheral vascular disease (Wright *et al.*, 2000), sudden sensorineural hearing loss (Park *et al.*, 2006), septic shock (Wheeler *et al.*, 2005), infection (Njemini *et al.*, 2003) prostate cancer (Abe *et al.*, 2004) and myeloid leukaemia (Nylandsted, 2008) . However, other non-clinical stressors have also shown to elevate eHsp72, for example exercise (Walsh *et al.*, 2001;Febbraio *et al.*, 2002a;Lancaster *et al.*, 2004;Whitham *et al.*, 2006), hyperthermia (Whitham *et al.*, 2007) and trauma (Pittet *et al.*, 2002). A feature of these forms of stress is that they all involve a degree of alteration to physiological homeostasis (i.e. increase in temperature, alteration in plasma glucose, increased free radical formation). Since Hsp72 may be passively released from cells via necrosis (as is the case of trauma) or by damage to the cellular membrane (as is the case in exercise-induced haemolysis) it is possible that a proportion of eHsp72 is derived from this mechanism of release. However, the fact that non-muscle damaging exercise such as moderate intensity semi-recumbent cycling (Febbraio *et al.*, 2002a) and underwater running (Whitham *et al.*, 2007) result in elevated concentrations of eHsp72 suggests that a further means of eHsp72 trafficking is evident. Indeed, *in vitro* studies have shown that

Hsp72 is also released via an active exocytotic process involving exosomes (Clayton *et al.*, 2005;Lancaster & Febbraio, 2005) or lipid rafts (Broquet *et al.*, 2003;Hunter-Lavin *et al.*, 2004a), whilst *in vivo* studies utilising adrenergic blockade (Johnson *et al.*, 2005) and caffeine evoked sympathetic stimulation (Whitham *et al.*, 2006) have identified a role for the stress hormones noradrenaline and adrenaline in this active eHsp72 release. Further evidence supporting an active release mechanism were gleaned from rodent models whereby acute psychological stress (which presumably does not result in cellular damage or necrosis) induced significant increases in plasma eHsp72 (Johnson *et al.*, 2005;Fleshner *et al.*, 2004).

The fact that we have failed to show an increase in eHsp72 in response to psychological stress could be due to a number of reasons. Firstly, the psychological stress models simply were not stressful enough. This is entirely plausible since it is ethically challenging in humans to evoke a psychological stressor comparable to that of predatory stress in animals in which a real threat to well being is perceived. Additionally, it is likely that whilst the electrical shock intensity was uncomfortable for the participants, relatively speaking, the intensity of the shock was probably much less than that administered to rodents where ethical impositions are far less stringent. However, from a researchers and methodological point of view, the failure of eHsp72 to increase under the types of stressor involved in this study is encouraging since it is unlikely that cognitive anxiety that may be associated with participation in research studies (such as fear of needles, or experimental protocols) is greater than those imposed in the current study. Hence, it is unlikely that acute psychological stress will impact upon baseline eHsp72 concentration. Secondly, whilst the type of stress increased cognitive arousal, the effects upon physiological arousal may not have been significant enough to induce and eHsp72 response. There is currently very little

research detailing the type or intensity of physical stressor required to increase eHsp72 expression within the circulation. Since there is reasonably strong evidence for the role of adrenaline and noradrenaline mediated sympathetic stimulation of eHsp72 (Johnson *et al.*, 2005;Whitham *et al.*, 2006), it is logical to suggest that the lack of an eHsp72 response may coincide with a lack of adrenergic-mediated stimulation. We did not measure the concentration of stress hormones in the current study. However, although heart rate was increased (in Part 1), albeit by only 14 bpm, saliva flow rate and concentrations of salivary α-amylase (which serve as a marker for endogenous adrenergic activity (Chatterton, Jr. *et al.*, 1996)) were not altered by stress. A similar psychological stressor in the form of a public speaking task to Part 2 showed a comparable heart rate profile to that of the current study (mean increase of 13 bpm), but plasma concentrations of adrenaline increased only marginally (12%) and noradrenaline remained unaltered with stress (Redwine *et al.*, 2003). Additionally, electrical hand shock stress (comparable in intensity and duration to that in Part 1) resulted in significant but very small increases in catecholamine concentration (14 and 7% for adrenaline and noradrenaline respectively). This is in contrast to a different, more extreme form of psychological stress, parachute jumping during which the concentration of catecholamines increased considerably (~700 and 75% for adrenaline and noradrenaline respectively). Furthermore, the only study known to compare concentrations of eHsp72 and catecholamines (Whitham *et al.*, 2006) showed that the 130% increase in plasma Hsp72 following 90 minutes cycling at 70%VO_{2max} was accompanied by increases in adrenaline and noradrenaline of 750 and 260% respectively. It is currently unknown as to the degree of sympathetic stimulation required to elevate eHsp72 above baseline levels (either via direct innervation of postganglionic sympathetic neurons, or increases in plasma concentrations of catecholamines) and this is a much needed future direction of research. However, it is likely that the type of psychological stressor involved in the current study

failed to evoke a significant increase in sympathetic activity, and thus failed to stimulate Hsp72 secretion into the plasma.

Whilst it appears that *acute* psychological stress fails to alter eHsp72 concentration, the effects of *chronic* psychological stress may show a different profile of expression. For example, the mitochondrial chaperone, Hsp60 has also been discovered in the extracellular environment (Pockley *et al.*, 1999). In a large scale analysis from 229 civil servants, high plasma concentrations of Hsp60 were associated with social isolation and psychological distress (Lewthwaite *et al.*, 2002). Additionally, high levels correlated positively with the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) which is thought to play a major role in atherogenesis and the development of coronary heart disease (Libby, 2001). Due to the inflammatory effects of eHsp72 (Asea *et al.*, 2000), it is plausible that chronically high levels within the blood may play in the pathogenesis of a number of clinical pathologies associated with chronic low-grade inflammation (for example diabetes, atherosclerosis, autoimmune diseases etc). Therefore, future studies might wish to investigate if eHsp72 concentrations within the blood are elevated with long-term psychological stress.

In conclusion, we have demonstrated that acute psychological stress in the form of a mental counting task with electrical hand shock, and a stressful presentation did not alter eHsp72 concentrations within the blood of healthy humans. As such, acute anxiety associated with participation in research studies should not be a concern for researchers measuring eHsp72.

Chapter 6

Salivary and plasma eHsp72 responses to exercise and caffeine supplementation

6.1 Summary

Hsp72 has recently been detected within the saliva compartment, and it has been speculated that its presence may contribute to oral defence. It is currently unknown how stress affects salivary Hsp72. Therefore, utilising a caffeine induced sympathetic stimulation model the effects of exercise upon salivary eHsp72 expression were explored. Additionally, the relationship between salivary and plasma eHsp72 concentration and the mechanisms behind salivary Hsp72 release were investigated. Six healthy males performed two exercise bouts (30mins running at 50%VO_{2max} followed immediately by 30mins running at 70%VO_{2max}) in hot conditions (30°C) separated by one week; one with caffeine supplementation (CAF – 6ml/kg body mass) the other with placebo (PLA). Plasma and saliva samples were collected and assayed for eHsp72 concentration by ELISA. Saliva samples were also assessed for α-amylase and total protein concentration. Mean salivary Hsp72 concentration (13.2 ± 0.9 ng/ml) was significantly greater than plasma Hsp72 concentration (1.8 ± 0.1 ng/ml) and concentrations of saliva and plasma Hsp72 were unrelated ($r = 0.132$, $P = 0.369$). Exercise resulted in a significant increase in plasma Hsp72 but not in salivary Hsp72 concentration. Caffeine supplementation and exercise increased the concentrations of α-amylase and total protein, whilst the salivary Hsp72: α-amylase ratio was decreased in CAF. Salivary Hsp72 was not altered by exercise and did not track plasma Hsp72 concentration. Salivary Hsp72 secretion appears to be released independent of α-amylase, via a non-adrenergic mechanism.

6.2 Introduction

The detection of the 70kDa heat shock protein (Hsp72) in the peripheral circulation of humans has prompted much research into the potential systemic roles for this predominantly "intracellular" protein. An immuno-modulatory role has become apparent in addition to increasing interest in Hsp72's involvement in exertional heat illness and the ability and/or inability of individuals to acclimatise to exercise in the heat (Kresfelder *et al.*, 2006;Marshall *et al.*, 2006;McClung *et al.*, 2008;Yamada *et al.*, 2007). In addition to being present in the blood, Hsp72 has been detected in other extracellular fluids such as the synovial fluid (Martin *et al.*, 2003), the cerebrospinal fluid (Steensberg *et al.*, 2006;Tang *et al.*, 2008), pulmonary oedema fluid (Ganter *et al.*, 2006) and also in saliva (Fabian *et al.*, 2003). The presence of eHsp72 in saliva is of interest from a methodological perspective since saliva collection is a simple non-invasive method which may be employed when blood samples cannot be taken.

Hsp72 was first detected in saliva by the Western blot method (Fabian *et al.*, 2003). Compared to resting values, concentrations of salivary Hsp72 decreased in response to chewing invoked stimulation, but when expressed relative to flow rate (which increases considerably when stimulated (Dawes, 2004)) Hsp72 output increased. Additionally, photo-acoustic stimulation also increases Hsp72 output (Fabian *et al.*, 2003;Fabian *et al.*, 2004). Owing to the cytoprotective and immune activating roles of eHsp72, Fabian *et al* allude to the potential immunological function of salivary eHsp72 in preventing bacterial infection and maintaining periodontal and mucosal health by way of a non-specific continuous immune surveillance (Fabian *et al.*, 2007). In support of this, Hsp72 recognises and binds to sulphoglycolipids (surface proteins of mucosal cells) (Boulanger *et*

al., 1995). Thus, Hsp72 may occupy sulphoglycolipid binding sites, thereby inhibiting bacterial attachment to mucosal surfaces within the oral cavity (Fabian *et al.*, 2007). Furthermore, Hsp72 has been shown to activate the complement cascade independent of antibody (Prohaszka *et al.*, 2002) which may be significant since complement activation is one of the first periodontal defence mechanisms against bacterial contamination (Fabian *et al.*, 2007).

A robust feature of eHsp72 in the circulation is the increase in concentration that is seen with exercise (Table 1.1, Chapter 1). Evidence from rodent and human studies suggests that this increase in concentration may result from adrenergic stimulation (Johnson *et al.*, 2005;Whitham *et al.*, 2006), and a subsequent specific exocytosis from the cell which may serve to prime the immune system or enhance cellular protection via increased eHsp72 uptake from other cells during this stressful period (Chapter 1). It is currently unknown as to the origin of salivary Hsp72 or whether concentrations within the saliva are reflective of the concentration within the plasma. Whether a relationship exists is likely dependent upon the mechanism of salivary Hsp72 secretion, i.e. is there a specific exocytosis from salivary glands or mucous cells upon stressful stimuli or is Hsp72 simply passively released as exudate from blood serum. Initial evidence suggests that Hsp72 release into saliva is independent of the actively secreted enzyme α-amylase (Fabian *et al.*, 2003;Fabian *et al.*, 2004) suggesting that Hsp72 release is not via an active exocytosis from salivary glands, although a research model that can test this further is warranted.

Saliva secretion lies under autonomic control and all salivary glands are innervated by both cholinergic parasympathetic and adrenergic sympathetic nerve fibres (Chicharro *et al.*,

1998). Saliva flow at rest is primarily under parasympathetic control which evokes most of the fluid secretion into saliva. Sympathetic stimulation does not inhibit saliva secretion but does evoke protein secretion through exocytosis of protein storage granules from acinar cells within salivary glands (Proctor & Carpenter, 2007). Exercise alone increases sympathetic activity and increases the α-amylase and total protein concentration of saliva (Walsh *et al.*, 1999), whilst caffeine supplementation during exercise has been shown to augment salivary α-amylase and IgA concentration and secretion rates further suggesting that increased sympathetic activity may enhance mucosal immune function (Bishop *et al.*, 2006). Caffeine supplementation is routinely used in exercise physiology as a model to enhance sympathetic activity, and it has been established that caffeine increases the concentration of eHsp72 within the plasma of healthy humans during exercise compared to administration of a placebo (Whitham *et al.*, 2006). It is currently unknown how a physically stressful stimuli such as exercise affects *unstimulated* salivary Hsp72. Furthermore, caffeine supplementation during exercise provides a useful *in vivo* model in which to explore the role of sympathetic/parasympathetic stimulation upon salivary Hsp72 release, allowing further speculation upon the role of this protein within the salivary compartment.

The purpose of this study was therefore, to explore the effects of exercise upon salivary eHsp72 expression, to investigate the relationship between salivary and plasma eHsp72 concentrations, and to gain further insight as to salivary Hsp72 release.

6.3 Methods

Sample size calculation

In order to recruit a sample size sufficient to detect a significant increase in the concentration of plasma eHsp72 following exercise (from which salivary Hsp72 concentration could be compared), mean and standard deviation data were taken from a previous study (Whitham *et al.*, 2006) and entered into a web-based sample size calculator (http://www.danielsoper.com/statcalc/calc47.aspx). Recruiting 3 Participants was required to detect changes in plasma eHsp72 with an alpha level of 0.05 and beta level of 0.8. Therefore, recruiting 6 participants was deemed to provide sufficient statistical power.

Participants

Six healthy endurance-trained males gave written informed consent to participate in the study having been fully informed of all procedures and risks involved in the experimental trials. Mean (± SD) demographical data for the participants were: age 21.8 ± 1.9 yr; height 1.79 ± 0.04m; body mass 71.4 ± 3.1kg; maximal oxygen uptake (VO_{2max}) 60.7 ± 2.8ml/kg/min and habitual caffeine intake 171 ± 37 mg/day calculated from their responses to a questionnaire.

Preliminary testing

Approximately one week prior to the experimental trials, each participant performed continuous incremental exercise on a motor driven treadmill (HP cosmos) to volitional exhaustion to determine their VO_{2max}. The participant began running at 6 km/h and 1%

grade with speed increased by 2 km/h every 2 minutes until the speed reached 14 km/h. Upon reaching this velocity, the treadmill grade was increased by 2.5% every 2 minutes until the subject reached volitional exhaustion or a plateau in the VO_2- work rate relationship was observed. Heart rate was measured throughout using radio telemetry (Polar Electro Ltd, Oy, Finland), and expired gases were collected and analysed for volume, O_2 and CO_2 concentrations using an online breath-by-breath automated gas analyser (Cortex metalyser, USA). Using interpolation of the VO_2-work rate relationship, the running speed equivalent to 50 and 70% VO_{2max} was calculated. A post-exercise saliva sample was also collected in order to ascertain individual flow rates so that adequate saliva volume (~1.5-2ml) could be collected during the experimental trials.

Saliva collection

Unstimulated saliva samples were collected from participants using a passive drool technique into a pre-weighed saliva collection container (Greiner Bio-one, GmbH, Germany). Participants were not allowed to eat or drink anything other than water throughout each trial so that the natural oral environment was maintained. Participants were seated quietly and were instructed to rinse their mouth with water before swallowing in order to standardise the residual amount of saliva prior to collection. A saliva collection container was placed to the lips, and whilst leaning forwards and with their head down, saliva was allowed to passively collect (without spitting) into the container for approximately 3-6 minutes depending on individual flow rates. Each collection was accurately timed and performed with minimal oral-facial movement. Saliva volume was measured by weighing the sample tube immediately after collection and correcting for pre-

weight. By assuming the density of saliva to be 1.00g/ml, saliva flow rate was calculated by dividing the volume collected by the time of collection.

Experimental trials

On two occasions, separated by at least one week and at the same time of day to control for circadian fluctuations in saliva flow rate (Dawes, 2004), participants performed 2 × 30 minute running bouts in an environmental chamber (Delta Environmental Inc, UK) where ambient conditions were maintained at 30°C and 40% relative humidity with either caffeine (CAF) or placebo (PLA) supplementation in a single-blind randomised crossover design. Participants were given a detailed list of common caffeine-containing products to abstain from in the 48 hours prior to each subsequent visit. They were also instructed to refrain from exercise, alcohol, and diuretic intake in the 24-hours prior to testing. In addition, participants were asked to consume a high carbohydrate containing meal the night before the first visit, and breakfast on the morning of the trial, to replicate these before the second visit, and to ensure they consumed plenty of fluids over this same period. To ensure participants began exercise in a euhydrated state a urine sample was collected upon arrival and assessed for osmolality using a urine refractometer (Atago URC-Osmo, Japan) with values <700 mOsm/l taken as euhydrated (Casa *et al.*, 2005). After the urine collection, the participant was administered either the CAF or PLA solution. In the caffeine trial, participants were instructed to consume 6mg/kg body mass of caffeine (Sigma-Aldrich, W22 240-2) dissolved in 3ml/kg body mass of artificially sweetened grapefruit-flavoured water. In the placebo trial, participants were given the same volume of grapefruit-flavoured water but with no caffeine. After 45 minutes of sitting quietly in the laboratory, nude body mass was assessed and a resting saliva and blood sample were

taken (PRE). Blood samples were collected by venepuncture of an antecubital vein using a 23-gauge butterfly needle (Venisystems, Abbott Ireland, Rep of Ireland). Subjects then entered the environmental chamber and ran on a motor driven treadmill at a constant speed equivalent to 50%VO_{2max} for 30 minutes. Participants were allowed to drink water *ad libitum* throughout the exercise bout. Upon completion, a blood and saliva sample was taken before the second exercise bout was conducted at 70%VO_{2max} for 30 minutes (MID). Further blood and saliva samples were taken immediately upon cessation of exercise (POST) and 20 minutes following this second exercise bout (20 min POST) (see Figure 6.1 for a schematic of procedures). Core body temperature (T_{re}) was monitored throughout through the use of a rectal thermistor inserted 10cm beyond the anal sphincter (YSI precision, Ohio, USA), and heart rate (Polar electro, Oy, Finland) was assessed at 5 minute intervals throughout exercise. A physiological strain index (PSI) was calculated every 5 minutes based on core temperature and heart rate data using the equation developed by Moran *et al* (Moran *et al.*, 1998):

$$PSI = 5 \times \frac{(T_{re,t} - T_{re,0})}{(39.5 - T_{re,0})} + 5 \times \frac{(HR_t - HR_0)}{(180 - HR_0)}$$

Where $T_{re,t}$ and HR_t are measurements taken at any time point and $T_{re,0}$ and HR_0 are the pre-test resting values. The index ranges from 0 (no physiological strain) to 10 (very high). A final, post-exercise nude body mass was recorded, and sweat rates were calculated correcting for *ad libitum* fluid intake.

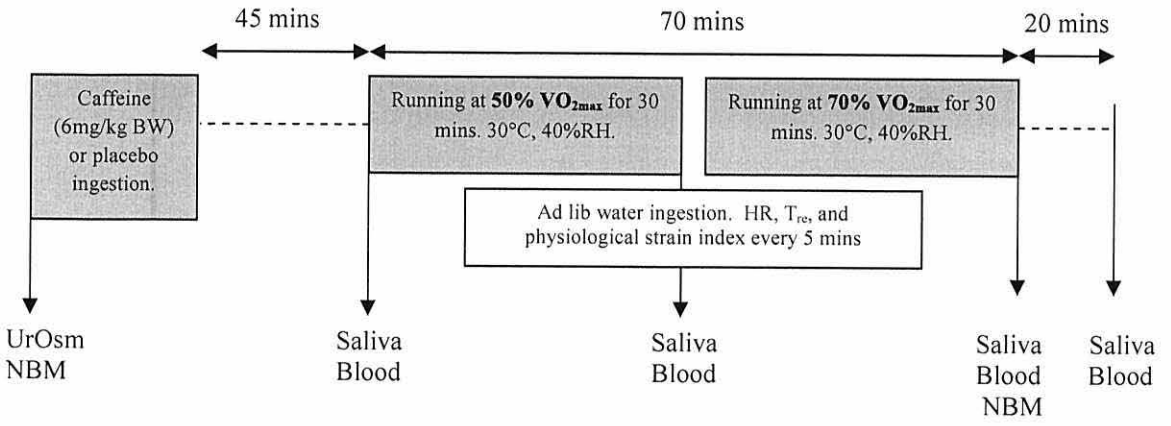

Figure 6.1. Schematic depicting measures and procedures during all experimental exercise sessions. UrOsm – urine osmolality, NBM – nude body mass, HR – heart rate, T_{re} – core (rectal) temperature.

Plasma and saliva analysis

Blood samples were collected into 2 x 4ml ethylenediaminetetraaceticacid (EDTA) coated vacutainers (Becton Dickinson & Co, UK). Blood samples were analysed for haemoglobin (Hemocue, UK) and haematocrit (capillary tube method) with plasma volume change calculated using the method of Dill and Costill (1974). Saliva samples were immediately aliquoted into eppendorfs and frozen at -80°C. Plasma (EDTA) and whole saliva samples were assessed for concentrations of eHsp72 using the same high-sensitivity sandwich ELISA (EKS-715, Assay Designs Inc, USA- see general methods for protocol). Individual participant's plasma and saliva samples for both the caffeine and placebo trials were always assayed on the same plate. Saliva and plasma samples were thawed and centrifuged prior to analysis and diluted 1:5 in the assay diluent in order to overcome matrix interference between the samples and standard diluent. Salivary eHsp72 is reported both as an absolute concentration (ng/ml) and as a secretory rate (ng/min) by multiplying the concentration by the flow rate. To investigate potential secretory routes of saliva eHsp72, saliva samples were also assessed for total protein concentration (BCA protein assay kit

Cat no. 23227, Thermo Scientific, USA), and α-amylase activity using a kinetic assay (see general methods, Chapter 2). All biochemical analyses were performed in duplicate. Intra assay co-efficient of variations were as follows; 3.8% for salivary α-amylase, 2.9% for salivary total protein, 4.2% for salivary eHsp72 and 2.7% for plasma eHsp72. Inter-assay CV for eHsp72 was 5.6%.

Statistical analysis

All data were analysed using two-way fully repeated measures ANOVA following the procedures outlined in the general methods (Chapter 2).

6.4 Results

Subjects arrived in the laboratory for both trials in a euhydrated state with no individual urine osmolality value >700 mOsm/l, or difference between trials (mean, 408 ± 98 and 437 ± 84 mOsm/l for CAF and PLA respectively, t = -0.395, P = 0.709).

Cardiovascular and thermoregulatory responses to exercise

Heart rate increased with exercise and was significantly greater in the CAF trial than PLA during exercise at 70%VO_{2max} (Mean HR, 174 ± 4 and 168 ± 3 bpm for CAF and PLA respectively, time × trial interaction, $F_{(12,60)} = 3.55$, $P < 0.001$). No difference was observed between trials in heart rate whilst running at 50%VO_{2max}. Core temperature increased with exercise in both trials (main effect for time, $F_{(12,60)} = 52.4$, $P < 0.001$). Core temperature had increased by 0.79 ± 0.13 and 0.76 ± 0.14°C for CAF and PLA respectively during exercise at 50%VO_{2max}, and upon cessation of exercise after running at 70%VO_{2max} for 30min had increased above baseline by 1.92 ± 0.15 for CAF and 1.90 ± 0.16°C for PLA. There were no between trial differences. Additionally, mean sweat rate (as calculated from changes in nude body mass from pre to post-exercise correcting for *ad libitum* water intake) did not differ between trials (CAF: 14.6 ± 2.0, PLA: 18.0 ± 1.9 ml/min, t = -1.12, P = 0.314). The effect of CAF and exercise upon the combined contribution of cardiovascular and thermoregulatory stress was assessed by calculating a physiological strain index (PSI - Moran *et al*, 1998). PSI increased significantly in both trials and was significantly greater during CAF than PLA for the last 25min of exercise (time × trial interaction, $F_{(12,60)} = 114.3$, $P < 0.001$, Figure 6.2).

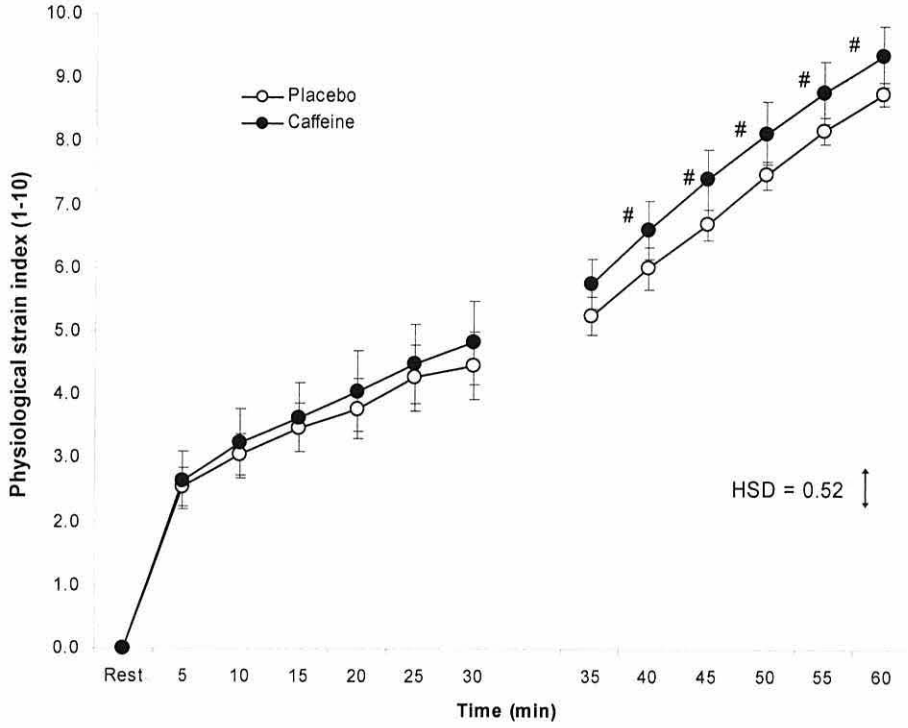

Figure 6.2. Effect of caffeine supplementation (●) and placebo (○) upon physiological strain index during exercise at 50% (time 0-30min) and 70%VO_{2max} (time 30-60min) in hot (30°C) conditions. Values are means ± SEM. HSD, Tukey's honestly significant difference. # Significant between trial difference ($P < 0.05$). n = 6.

Plasma and salivary Hsp72 responses to exercise

Plasma eHsp72 concentration data were corrected for changes in plasma volume. ANOVA revealed a main effect of time ($F_{(3,15)} = 23.4$, $P < 0.001$) and trial ($F_{(1,5)} = 6.75$, $P < 0.05$) but no time × trial interaction was observed ($F_{(1.7,.7)} = 0.83$, $P = 0.49$). *Post-hoc* analysis confirmed that in both CAF and PLA trials, exercise induced a significant increase in eHsp72 concentration within the plasma immediately following the exercise protocol which remained elevated 20-mins post exercise in the CAF trial alone (Figure 6.3). Despite the main effect for trial, and visual analysis that the CAF trial appeared to induce a greater eHsp72 response than PLA, at no time point was this difference statistically significant.

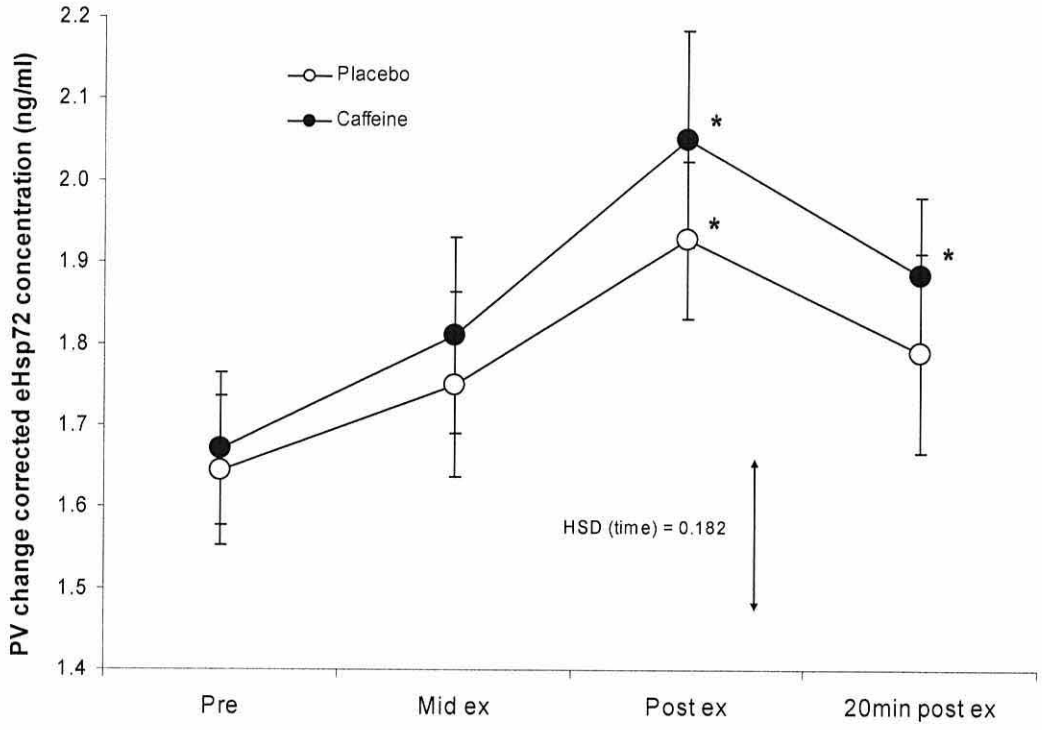

Figure 6.3. Effect of caffeine supplementation (●) and placebo (○) upon plasma volume change corrected eHsp72 concentration during exercise at 50% (time 0-30min) and 70%VO_{2max} (time 30-60min) in hot (30°C) conditions. Values are means ± SEM. HSD = Tukey's honestly significant difference value. * Significantly different from pre-exercise value ($P < 0.05$). $n = 6$.

Hsp72 was detected in all saliva samples. Across all samples, the concentration of Hsp72 in saliva was significantly greater than in EDTA plasma (mean concentration 13.2 ± 0.9 and 1.8 ± 0.1 ng/ml for saliva and plasma Hsp72 respectively, $t = 13.6$, $P < 0.001$). As can be seen from Figure 6.4, saliva Hsp72 concentration remained unaffected by exercise alone, or exercise with caffeine supplementation (no time × trial interaction, ($F_{(3,15)} = 2.13$, $P = 0.140$). Additionally, no effect was observed for salivary Hsp72 secretion rate ($F_{(3,15)} = 1.83$, $P = 0.184$, Figure 6.5).

Pearsons correlational analysis failed to show a significant positive relationship between individual saliva and plasma Hsp72 concentrations (Table 6.1) or in the sample as a whole ($r = 0.132$, $P = 0.369$).

Table 6.1. Within subject and whole sample correlations between plasma and salivary Hsp72 concentration during exercise at 50% (time 0-30min) and 70%VO_{2max} (time 30-60min) in hot conditions.

	Participant number						
	1	*2*	*3*	*4*	*5*	*6*	*Sample*
r	0.47	-0.06	-0.60	-0.03	-0.07	-0.66	0.13
P	0.170	0.868	0.066	0.945	0.845	0.036	0.369

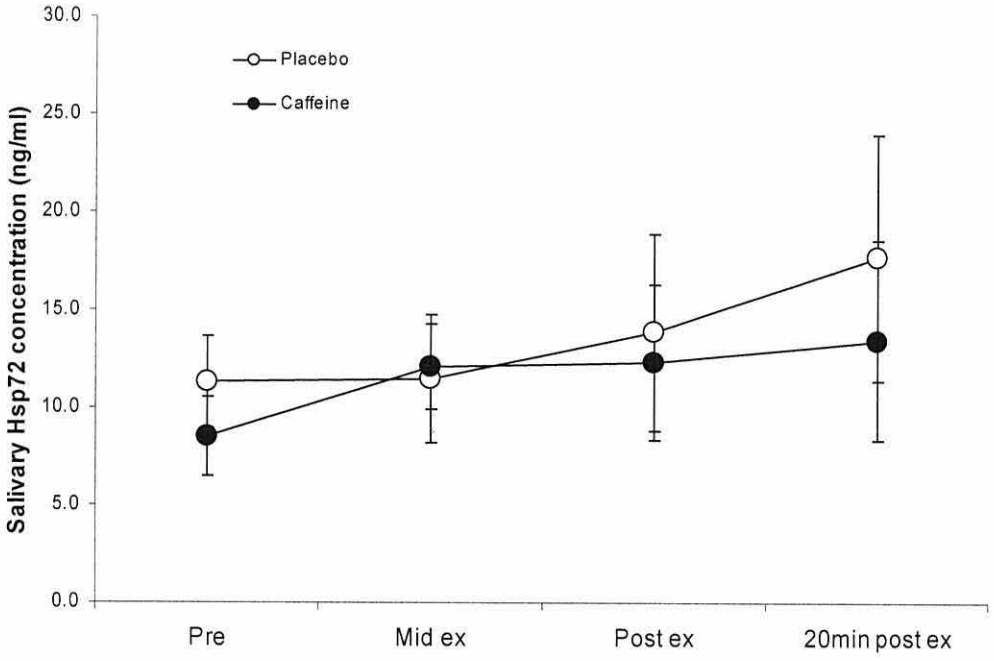

Figure 6.4. Effect of caffeine supplementation (●) and placebo (○) upon salivary Hsp72 concentration during exercise at 50% (time 0-30min) and 70%VO_{2max} (time 30-60min) in hot (30°C) conditions. Values are means ± SEM. No significant difference between any time points or between trials. $n = 6$.

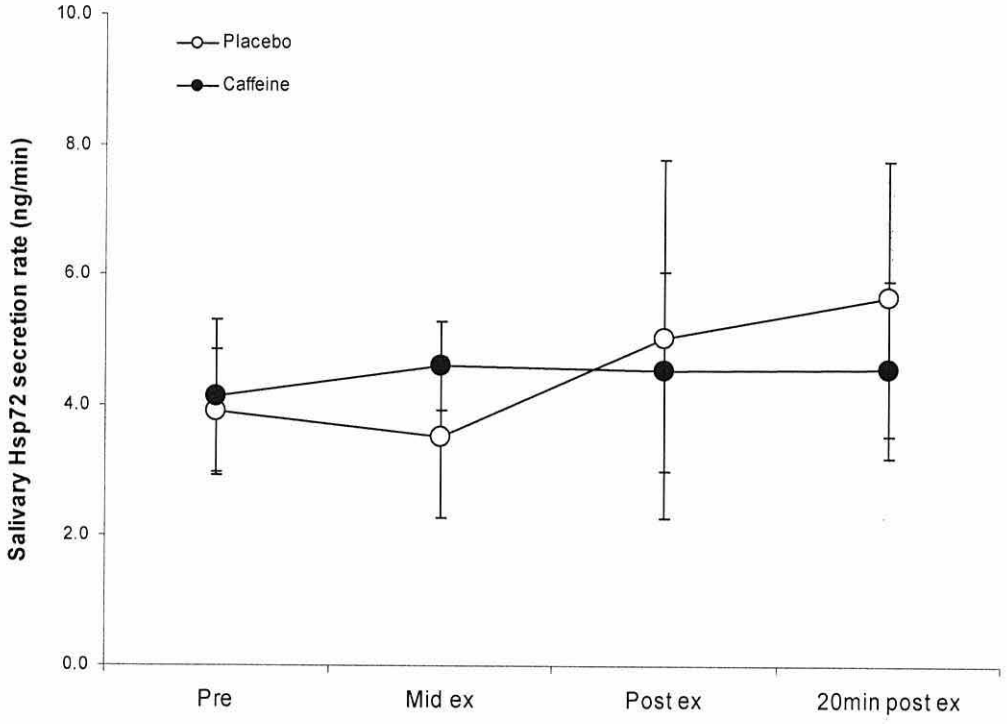

Figure 6.5. Effect of caffeine supplementation (●) and placebo (○) upon salivary Hsp72 secretion rate during exercise at 50% (time 0-30min) and 70%VO_{2max} (time 30-60min) in hot (30°C) conditions. Values are means ± SEM. No significant difference between any time point or between trials. $n = 6$.

Effect of caffeine supplementation and exercise upon saliva flow rate, saliva total protein and salivary α-amylase concentration

Saliva flow rate remained unchanged with exercise in both trials (no time × trial interaction, $F_{(3,15)} = 0.24$, $P = 0.868$, Table 6.2). Total protein concentration demonstrated a significant time × trial interaction ($F_{(3,15)} = 4.56$, $P < 0.05$). *Post-hoc* analysis revealed that total protein concentration had increased significantly above pre-exercise values immediately post exercise in CAF and remained elevated 20 min later. Furthermore, total

protein was significantly greater than PLA immediately upon cessation of exercise. Total protein concentration during PLA did not change significantly (Table 6.2). When expressed as a secretion rate, total protein output immediately post exercise in CAF had increased significantly above pre-exercise and was significantly greater than PLA (main effect for time, $F_{(3,15)} = 4.01$, $P < 0.05$, main effect for trial, $F_{(1,5)} = 8.82$, $P < 0.05$).

Table 6.2. Effect of caffeine supplementation (CAF) and placebo (PLA) before, during and after progressive exercise upon saliva flow rate, total protein concentration and total protein output. Values are means (± SEM), $n = 6$.

	Pre-ex	*Mid-ex*	*Post-ex*	*20min post-ex*
Saliva flow rate (ml/min)				
CAF	0.48 (0.07)	0.40 (0.05)	0.38 (0.06)	0.41 (0.06)
PLA	0.37 (0.04)	0.32 (0.07)	0.31 (0.07)	0.34 (0.07)
Total protein concentration (μg/ml)				
CAF	969 (85)	1450 (200)	2476 (341)* #	1779 (303)*
PLA	1175 (128)	1060 (48)	1561 (202)	1730 (383)
Total protein output (μg/min)				
CAF	451 (64)	556 (52)	967 (214)* #	665 (67)
PLA	405 (51)	329 (58)	482 (121)	557 (121)

* Significantly different from pre-exercise value ($P < 0.05$). # CAF significantly greater than PLA ($P < 0.05$)

Salivary α-amylase concentration was significantly higher in CAF than PLA immediately post exercise (time × trial interaction, ($F_{(3,15)} = 4.45$, $P < 0.05$), and was significantly greater immediately post and 20min post-exercise compared to pre-exercise values (Figure 6.6). Exercise also resulted in an increase in saliva α-amylase concentration immediately post exercise in PLA. Salivary α-amylase secretion rate during CAF immediately post exercise was significantly greater than PLA (main effect for trial, $F_{(3,15)} = 15.0$, $P < 0.05$).

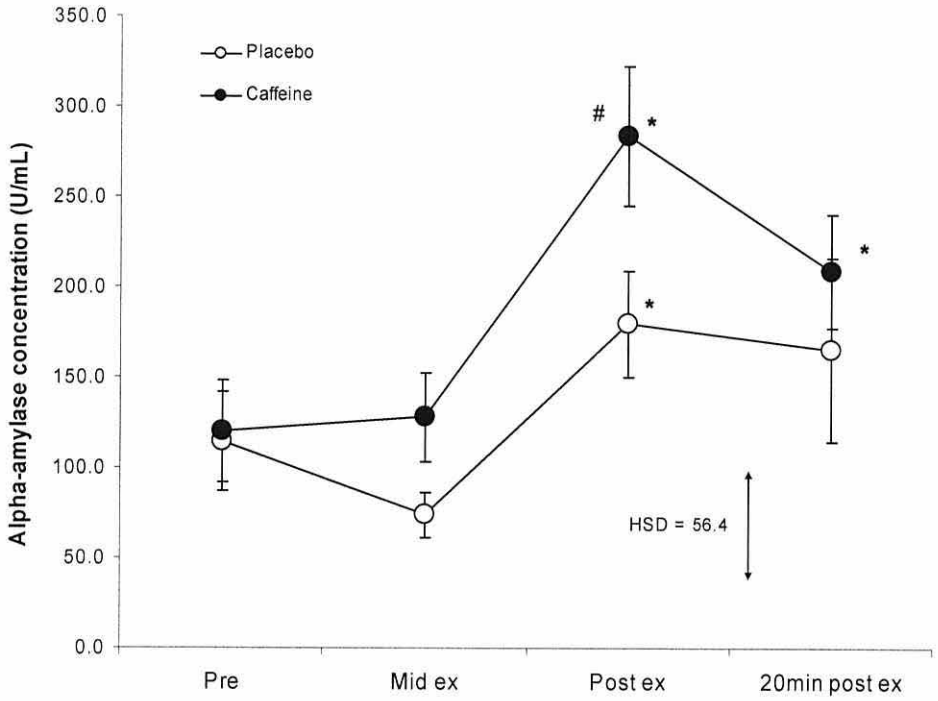

Figure 6.6. Effect of caffeine supplementation (●) and placebo (○) upon salivary α-amylase concentration during exercise at 50% (time 0-30min) and 70%VO_{2max} (time 30-60min) in hot (30°C) conditions. Values are means ± SEM. HSD, Tukey's honestly significant difference value. * Significantly different from pre-exercise value ($P < 0.05$). # Significant between trial difference ($P < 0.05$). $n = 6$.

Role of sympathetic stimulation upon salivary Hsp72 release

To investigate the role of sympathetic stimulation upon salivary Hsp72 secretion, salivary Hsp72 concentration was expressed relative to α-amylase and total protein concentration. The salivary Hsp72: α-amylase ratio was significantly lower in CAF than PLA (main effect for trial, $F_{(3,15)} = 9.62$, $P < 0.05$, Figure 6.7A), suggesting that salivary Hsp72 release occurs via an independent mechanism than salivary α-amylase secretion. Whilst there was a similar trend in the salivary Hsp72: total protein ratio (Figure 6.7B) this did not reach statistical significance ($P = 0.105$). The α-amylase: total protein ratio remained unaffected by exercise or caffeine supplementation (no time × trial interaction, $F_{(3,15)} = 0.436$, $P = 0.730$).

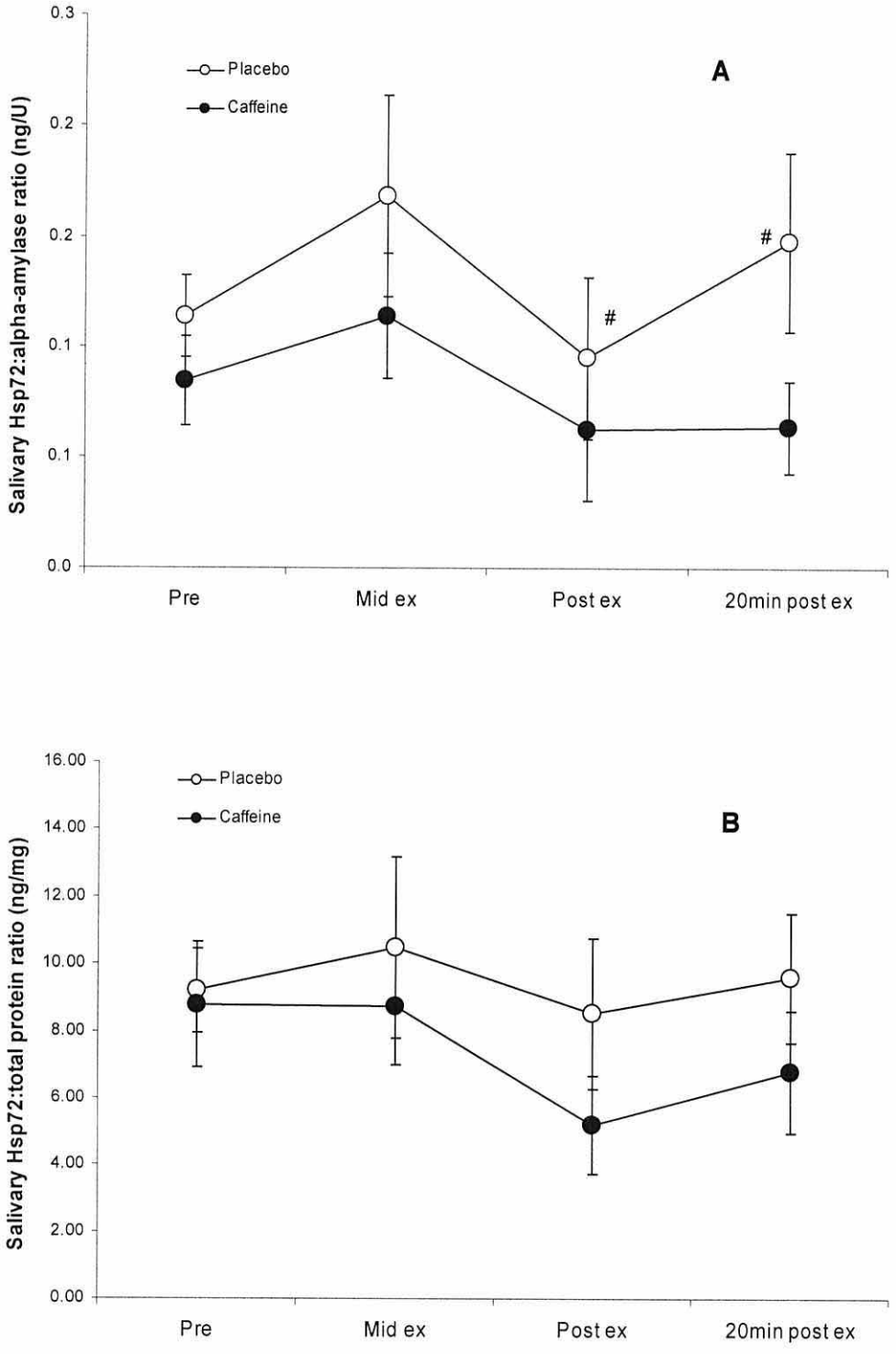

Figure 6.7. Effect of caffeine supplementation (●) and placebo (○) upon the salivary Hsp72: α-amylase ratio (A) and on the salivary Hsp72: total protein ratio (B) during exercise at 50% (time 0-30min) and 70%VO_{2max} (time 30-60min) in hot (30°C) conditions. Values are means ± SEM. # Main effect of time, PLA > CAF ($P < 0.05$). $n = 6$

6.5 Discussion

The purpose of this study was to explore the effects of exercise upon salivary eHsp72 expression, to investigate the relationship between salivary and plasma eHsp72 concentrations, and to gain further insight as to the role of autonomic stimulation of salivary Hsp72. Whilst Hsp72 is present in the saliva at far greater concentrations than in plasma, we have demonstrated that salivary Hsp72 concentration and secretion rate remains unaltered by exercise, and as such is not related to the concentration of plasma eHsp72 (which shows a robust increase with strenuous exercise). Furthermore, by utilising a caffeine-evoked sympathetic stimulation model during exercise, we are able to speculate that Hsp72 secretion within saliva occurs independent of a sympathetic stimulated active release mechanism.

Consistent with previous research (Whitham *et al.*, 2006), exercise resulted in a significant increase in plasma concentration of eHsp72 under both CAF and PLA conditions. However, plasma eHsp72 concentration failed to increase significantly after 30 minutes of exercise at 50%VO_{2max}. Very little information exists on the intensity, duration or mode of exercise required to elicit an eHsp72 response. Since research suggests eHsp72 release into plasma may be via sympathetic stimulation of adrenergic receptors (Johnson *et al.*, 2005) it is plausible that exercise at such a relatively low intensity, with only a modest increase in core temperature (~0.8°) may not generate afferent feedback (i.e the metaboreflex and chemoreflex) at a level sufficient to activate adrenergic- mediated eHsp72 release. Conversely, the additive effect of both exercise bouts (50 and 70%VO_{2max} for 30 mins each in hot (30°C) environmental conditions) and the large increase in core temperature (~1.9°C) are liable to evoke a significant sympathetic response and eHsp72

release, particularly since it appears that eHsp72 concentration during exercise is both duration and intensity dependent (Fehrenbach *et al.*, 2005) and related to heat storage (Whitham *et al.*, 2007). The lack of a difference between PLA and CAF post exercise in eHsp72 concentration is likely due to the small sample size since a previous study within our laboratory has shown exercise with CAF supplementation to augment eHsp72 concentration (Whitham *et al.*, 2006). However, as the primary aim of this study was to investigate salivary Hsp72, we used caffeine supplementation purely as an *in vivo* model to increase and investigate the role of sympathetic activity upon salivary Hsp72. Unfortunately, we do not presently have the catecholamine data (plasma concentration of adrenaline and noradrenaline). However proxy measures of sympathetic activity such as heart rate and salivary α-amylase were significantly greater during CAF than PLA. Salivary α-amylase has been shown to be a correlate of plasma noradrenaline and endogenous sympathetic activity during exercise ($r = 0.64$) (Anderson *et al.*, 1984;Chatterton, Jr. *et al.*, 1996), thus we are confident that caffeine supplementation in the quantity administered in the current study (6ml/kg body mass) was sufficient to enhance sympathetic activity during exercise and provide a useful model with which to investigate the potential sources/mechanisms of salivary Hsp72 release. The finding that salivary Hsp72 secretion rate in the current study did not change after exercise, that caffeine supplementation failed to increase salivary Hsp72, and that salivary Hsp72 concentration showed no relationship to plasma eHsp72 concentration suggests an unlikely role for adrenergic-receptor stimulated salivary Hsp72 release.

This is the first known study to investigate the effect of a physical stressor on salivary Hsp72. The failure of salivary Hsp72 concentration to change during a stressor such as exercise is surprising since we and others (Febbraio *et al.*, 2002a;Fehrenbach *et al.*,

2005;Walsh *et al.*, 2001) have demonstrated a robust increase in plasma Hsp72 concentration within blood plasma following exercise, especially considering that it had been speculated that saliva Hsp72 may originate as a passive filtrate from blood plasma (Fabian *et al.*, 2007). Additionally, photo-acoustic and chewing evoked stimulation have both previously shown to alter salivary Hsp72 expression (Fabian *et al.*, 2003;Fabian *et al.*, 2004). The differential salivary Hsp72 response to exercise (as in the current study) and chewing (Fabian *et al.*, 2003) likely relates to the clear difference in the composition of stimulated and unstimulated saliva. Stimulated saliva is evoked by either masticatory or gustatory stimulation, which significantly increase flow rate (Dawes, 2004). Clearly, a large increase in flow rate is likely to result in a diluting effect on the protein of interest, particularly if protein secretion occurs via an independent non-active mechanism. The flow rates in response to chewing invoked stimulation in the study by Fabian *et al.* (2003) of ~3.8ml/min are far in excess (10-fold) of the unstimulated flow rates in the current study (mean flow rate ~0.35ml/min), thus is it probable that the decrease in the concentration of salivary Hsp72 in response to chewing evoked stimulation is primarily a consequence of a diluting artifact. By expressing salivary Hsp72 as a secretion rate, alterations in flow rate can be accounted for.

It has been suggested that salivary Hsp72 may originate from one of five sources: 1) salivary glands, 2) mucosal glands, 3) periodontal tissues, 4) mucosal lesions (both of these as exudated blood serum or as direct blood contamination) and 5) from bacteria (Fabian *et al.*, 2007). To date, only the first of these has been investigated. Utilising photo-acoustic and chewing evoked stimulation methods, Fabian *et al.* (2003 and 2004) suggested that salivary Hsp72 release occurred via a mechanism independent of the traditional secretory route of salivary proteins. Data from the current study is in support of this hypothesis for

several reasons; 1) Salivary Hsp72 concentration and secretion rate in addition to saliva flow rate did not alter after exercise (Figures 6.4 and 6.5), whereas the main secretory protein of saliva, α-amylase along with total protein concentration (both of which are released in large quantities after exercise (Walsh *et al.*, 1999) increased significantly (Figure 6.6 and Table 6.2). 2) The additive effect of caffeine supplementation with exercise failed to augment salivary Hsp72 (Figures 6.4 and 6.5), in contrast to α-amylase and total protein concentration which were increased significantly above placebo (Figures 6.6 and Table 6.2), and 3) The salivary Hsp72: α-amylase ratio was significantly lower during CAF than PLA (Figure 6.7A), suggesting that salivary Hsp72 is not released in direct proportion to α-amylase. The salivary Hsp72: total protein ratio showed a similar trend (Figure 6.7B). Conversely, sympathetic stimulation had no effect on the α-amylase: total protein ratio. Taken together, these data reinforce previous findings that salivary Hsp72 is released via an independent mechanism to that of α-amylase which is released from acinar cells upon (predominantly) sympathetic stimulation (Fabian *et al.*, 2007;Nater *et al.*, 2006)

Salivary glands receive neural input from both parasympathetic and sympathetic nerves (Proctor & Carpenter, 2007). Generally, salivary secretion lies under parasympathetic control, and increases in parasympathetic activity can give rise to substantial protein secretion (Asking & Emmelin, 1987). However, protein secretion from salivary glands tends to be modulated by sympathetic nerve impulses. For example, previous *in vivo* research in exercising humans has shown that caffeine induced sympathetic stimulation increases IgA and α-amylase concentration (Bishop *et al.*, 2006) whilst graded electrical stimulation of sympathetic nerves on top of parasympathetic background stimulation considerably increases the secretion of a number of proteins such as peroxidase, IgA, α-

amylase, and total protein from rodent parotid and submandibular glands (which together account for approximately 85-90% of all secretions into saliva) (Anderson *et al.*, 1995;Asking, 1985;Carpenter *et al.*, 2000;Skov *et al.*, 1988). Importantly, neural control of salivary secretion is not identical in all salivary glands. For example, in humans, whereas parotid and submandibular gland secretion is evoked primarily from adrenergic (noradrenaline) innervation, the sublingual and minor mucous glands receive sparse adrenergic innervation and secretion from these glands is initiated via cholinergic (acetylcholine) stimulation (Chicharro *et al.*, 1998;Proctor & Carpenter, 2007). Cholinergic stimulation evokes the majority of fluid and mucin secretion into saliva (Garrett, 1987). Acetylcholine released from parasympathetic nerves innervating the salivary glands acts upon the muscarinic cholinergic receptors, M1 and M3 (Gautam *et al.*, 2004) and subsequent fluid and electrolyte secretion results from an increase in the intra-cellular calcium concentration (Melvin *et al.*, 2005). Hsp72 is present in and released from epithelial cells (Broquet *et al.*, 2003) and also released from a number of haematopoetic cells via exosomes (Clayton *et al.*, 2005;Lancaster & Febbraio, 2005). Since exosome release appears to be related to the degree of rise in intracellular calcium concentrations (Savina *et al.*, 2003), this provides a potential rationale for the parasympathetically stimulated continual release of Hsp72 into saliva. Therefore, it is tangible that salivary Hsp72 might originate via parasympathetic innervation of the minor mucous or sublingual glands. Future research might wish to focus on the effect of anticholinergic agents on salivary Hsp72 in human volunteers, or specifically of atropine administration in rodent models since atropine is a competitive antagonist of muscarinic cholinergic receptors.

A possible source of salivary eHsp72 which is worth consideration is bacterial derived eHsp72. Saliva is sterile when it enters the mouth, but becomes contaminated by bacteria

attached to the oral surfaces (Tenovuo, 2004). Since, the oral cavity provides a perfect environment for bacterial growth, it is feasible that eHsp72 released from bacteria may contribute to salivary eHsp72. However, the cross-reactivity of the ELISA to the bacterial (*E-coli)* homologue of Hsp72 (DnaK) is reported by the manufacturer (Assay Designs) as less than 0.5%. However, to minimize this occurrence, a small amount of saliva collected from participants was sterile filtered (using Millex-GV 0.22 μm pore size filters, Millipore) and frozen immediately upon collection. Analysis of this data showed the same results as in the untreated saliva, i.e. no effect of exercise or caffeine intake on salivary Hsp72 concentration ($P = 0.359$) or secretion rate ($P = 0.432$). Thus, it appears unlikely that bacterial derived eHsp72 contributes to salivary eHsp72 expression.

One of the most striking findings to come from the current study was the seven-fold greater concentration of Hsp72 within the saliva compared to that in plasma, an effect confirmed by the fact that salivary Hsp72 was greater than plasma eHsp72 in all participants at every time point. This finding cannot be attributable to differences between ELISA's or in the methodological protocols since both saliva and plasma samples for the same participant were always assayed on the same plate using the same reagents. This would seem to suggest that the majority of salivary Hsp72 does not originate as passive filtrate from blood or as direct blood contamination from mucosal legions or the periodontal tissues. Saliva acts as a first line of defence against pathogenic threat and contains numerous proteins with antibacterial and antiviral functions such as peroxidases, lysozymes, agglutinins, lactoferrin, and immunoglobulins (Tenovuo, 2004). Since Hsp72 may prevent bacterial attachment to mucosal surfaces (Boulanger *et al.*, 1995), Hsp72's involvement in complement activation (Prohaszka *et al.*, 2002) and owing to its roles in cellular protection, the continual presence of Hsp72 within the saliva at such high

concentrations might serve to enhance oral defence (Fabian *et al.*, 2007). Chewing evoked stimulation increased saliva flow rate and salivary Hsp72 output (Fabian *et al.*, 2003). Thus, parasympathetic evoked increases in flow rate (and potentially salivary Hsp72) may serve to enhance oral defence during food ingestion, when presumably the oral cavity is at its greatest threat from microbial challenge. Clearly, future studies to investigate the immune functions of salivary Hsp72 are warranted.

In summary, we have demonstrated that Hsp72 concentrations within the saliva are significantly greater than but not reflective of plasma concentrations, and that a physical stressor such as exercise does not alter the salivary Hsp72 concentration. Furthermore, it is likely that the appearance of Hsp72 within the saliva occurs independent of α-amylase via a non-adrenergic mechanism. We have speculated that salivary Hsp72 may be released via cholinergic stimulation, although further research is required to clarify this.

Chapter 7

General discussion

7.1 Background

Investigations into the biology of eHsp72 have identified a number of immunological roles (Pockley *et al.*, 2008). The majority of this work has been performed *in vitro*, and initial *in vivo* research seems to suggest that increased eHsp72 may enhance certain aspects of immune function (Horn *et al.*, 2007;Ortega *et al.*, 2008). Clearly, more *in vivo* research is required to confirm the immunological potential of eHsp72. *In vitro* research has also identified a potential mechanism by which eHsp72 may enter distal cells via endocytosis and perform its usual intracellular chaperoning and cellular protection roles during times of stress (Arnold-Schild *et al.*, 1999;Novoselova *et al.*, 2005;Robinson *et al.*, 2005). Numerous studies have investigated the effects of exercise upon eHsp72 concentration, and a number of recent studies have focused upon the interplay between Hsp72 and the ability or inability of individuals to acclimatise to exercise in the heat (Kresfelder *et al.*, 2006; Yamada *et al.*, 2007). Additionally, across a large range of clinical conditions eHsp72 concentrations are elevated and may serve as an informant of disease pathology. In light of the increasing amount of *in vivo* human research being conducted on eHsp72, the aims of this thesis were to examine a number of currently unanswered important methodological questions that researchers should be aware of when planning *in vivo* studies. The specific aims were; 1) to examine the effect of different blood handling procedures upon eHsp72 concentration, 2) to investigate the stability of plasma eHsp72 concentration over a 24-hour period, 3) to explore the effect of acute psychological stress upon eHsp72 concentration, and 4) to investigate the utility of salivary eHsp72 as a marker of plasma Hsp72 during exercise and to speculate upon the role of sympathetic/parasympathetic stimulated release of salivary Hsp72.

7.2 Summary of findings

It has been demonstrated that the resting extracellular Hsp72 response is robust to time of day effects and psychological stress, i.e. concentrations of eHsp72 in healthy humans did not change when measured throughout a 24-hour period (Chapter 4) nor were altered by different acute psychological stressors (Chapter 5). Whilst Hsp72 is detectable in saliva and present in significantly higher concentrations than in the plasma, evidence presented suggests that salivary Hsp72 concentrations do not track the concentration of eHsp72 within the blood (Chapter 6). Furthermore, in order to maximise sample recovery and derive concentrations that may more accurately reflect the *in vivo* physiological concentration, blood samples should be drawn in the presence of an anticoagulant since this matrix provided significantly higher concentrations than serum derived eHsp72 (Chapter 3).

7.3 Significance of findings

The finding that plasma derived samples and in particular EDTA returns higher concentrations of eHsp72 than serum clearly has important implications for research, both past and present. Early reported studies into eHsp72, and indeed studies performed in our own laboratory were characterised by very low concentrations and a large number of undetectable samples. Whilst this could have purely been down to an absence of eHsp72 in the blood, research presented by Fehrenbach *et al.* (2005) in which both plasma and serum samples were collected in independent cohorts but which returned two vastly different ranges of concentration led to the suggestion that the matrix in which blood is collected may alter eHsp72 expression within the sample. Whilst findings from Chapter 3 have the obvious implication that blood samples collected in EDTA will maximise Hsp72

recovery and may relate more closely to the *in vivo* physiological concentration within the blood, it also asks the fascinating question of why Hsp72 concentrations are significantly lower in serum. We have speculated that this may be due to Hsp72's ability to bind aggregated proteins (and therefore bind to the clot), but it is unclear as to the biological relevance of such an action. Following the finding that low molecular weight HSP's bind specifically to platelets after endothelial injury in hamsters (Kozawa *et al.*, 2002) more recent evidence points to a potential function of Hsp72 in wound repair. Following injury, one of the first mechanisms towards repair is an inflammatory response with an extravasation of leukocytes to the site of injury. Interestingly, incubation of neutrophils with Hsp72 at physiological relevant concentrations has been shown to stimulate neutrophil chemotaxis in a dose dependent manner (Ortega *et al.*, 2008). Since phagocytosis is an early event in wound healing, it is plausible that Hsp72 binds to the blood clot and due to its chemoattractant ability may upregulate neutrophil chemotaxis. Of further relevance to this speculation is that in murine models of wound repair, exogenous application of Hsp72 accelerates wound repair compared with control, an attribute that occurs through enhanced phagocytosis by macrophages of wound debris (Kovalchin *et al.*, 2006). Thus, it has been suggested that Hsp72 may "jump start" the tissue repair process following injury (Wang *et al.*, 2006a). Taken together, these findings support a role for Hsp72 binding to the blood clot *in vivo*, where Hsp72 may have functions in the chemotaxis of leukocytes to the site of injury, and during early tissue repair. In support of a systemic role for increased Hsp72 following acute exercise stress, chemotaxis of neutrophils was greater when incubated with physiological relevant post-exercise concentrations of Hsp72 compared with those incubated with baseline concentrations of Hsp72 (Ortega *et al.*, 2008). These recent findings fit nicely with one commonly held view that from an evolutionary standing, stress induced Hsp72 concentrations within the blood

may serve to enhance immune responses should a pathogenic threat ensue (Fleshner *et al.*, 2003;Johnson & Fleshner, 2006). Indeed, survival outcome is higher in trauma patients with high concentration of plasma Hsp72 (Pittet *et al.*, 2002). This is a fascinating aspect of extracellular Hsp72 biology that warrants further research.

Since it appears that the majority of Hsp72 within serum may be bound to the clot, this may question the findings of previous studies that have drawn conclusions based on measured values in serum. For example, conclusions based upon the finding that under resting conditions, eHsp72 concentrations are 3 times greater in the cerebrospinal fluid (CSF) than that collected in serum (Steensberg *et al.*, 2006) are in doubt since it was shown in Chapter 3 that plasma concentrations (which may more closely reflect *in vivo* physiological concentrations) are considerably greater than serum (far greater than a factor of 3). It has also been postulated that Hsp72 is not released from skeletal muscle since the venous-arterial difference concentrations in serum eHsp72 was unchanged during exercise (Febbraio *et al.*, 2002a). However, since arterial Hsp72 was only detected at rest in one of the seven subjects and due to the poor recovery of eHsp72 in serum, it is clear that further research using cannulation methods across skeletal muscle are performed using EDTA treated Hsp72 in order to fully discount Hsp72 release from non-damaged skeletal muscle.

Whilst this thesis aimed to resolve a number of methodological issues with regard to eHsp72 sampling, a number of insights into the mechanisms and releasing signals that mediate eHsp72 release have been made. Severe haemolysis due to poor venepuncture (as assessed by free haemoglobin concentration within the plasma) was associated with extremely high values of eHsp72 (Chapter 3). This lends support to the passive release

mechanism of Hsp72, i.e that Hsp72 can be released from damaged cells (Basu *et al.*, 2000), and a number of recent studies have demonstrated this mechanism *in vivo*. Whilst eHsp72 levels were not correlated to free haemoglobin during the type of exercise encountered in Chapter 3, haemolysis as a result of ultra-endurance exercise is associated with elevated plasma Hsp72 (Suzuki *et al.*, 2006). In clinical settings, Hsp72 is present within wound fluid drained from the thorax of patients after open heart surgery and major lung resection (Becker *et al.*, 2007), and is associated with free haemoglobin and CK concentration, indicating lysis of erythrocytes and myocardial damage may contribute to eHsp72. More recently, it has been demonstrated that serum Hsp70 levels are elevated in the syndrome of haemolytic anaemia, elevated liver-enzymes and low platelet count (HELLP syndrome – a severe variant of pre-eclampsia[6]), and that serum Hsp70 concentrations are strongly related to total bilirubin and free haemoglobin ($r = 0.77$) (Madach *et al.*, 2008). Thus, it appears that under certain conditions, cellular damage or necrosis may account for a large proportion of eHsp72 within the extracellular environment, although evidence suggests that under healthy conditions or during times of non-damaging stress, eHsp72 release may be mediated by an active process (Johnson *et al.*, 2005;Whitham *et al.*, 2006).

Considering the potential sensitivity of a stress protein to acute stress, an investigation into the effect of psychological stress upon eHsp72 was clearly an important avenue of research. This was considered even more pertinent considering the well documented white coat effect, whereby measured blood pressure values are higher during clinical visits than

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[6] A pathological condition that may occur during late pregnancy which is characterised by hypertension and proteinuria

during 24-hour ambulatory measures (Tsai, 2002). This effect may be attributable to the increased anxiety associated with clinical visits and the coupled increase in sympathetic-neural activity (Smith *et al.*, 2002). It is feasible that acute psychological stress might elevate eHsp72 concentrations, particularly in light of this finding in rodents (Campisi *et al.*, 2003a;Fleshner *et al.*, 2004;Johnson *et al.*, 2005). However, we failed to find any effect of mental counting and electric shock stress, or an assessed academic presentation on eHsp72 responses (Chapter 5). This should not be construed as an argument against sympathetic stimulation and eHsp72 release since it is very likely that neither of these models were stressful enough to elicit significant sympathetic responses. Whilst analysis of the α-amylase data in Part 1 tends to support this, it is acknowledged that we are unable to comment on either the perceived amount of stress (cognitive) or amount of physiological stress incurred in Part 2 since we were unable to measure any of these parameters due to ethical impositions placed upon us (i.e. interfering with 3rd year student preparations before an important academically assessed piece of work). However, anecdotal reports from students suggest that this oral presentation is more stressful than any other activity encountered during their degree. Hence, from an external validity point of view, the failure of eHsp72 to respond to the acute psychological stressors adopted in this study is encouraging. Researchers can be confident of establishing true baseline data without the concern that anxiety (be it fear of venepuncture, anxiety associated with maximal exercise, etc) is going to impact upon eHsp72 data collected.

Also encouraging from a methodological perspective is the finding that eHsp72 concentrations did not vary significantly throughout a 24-hour period and remained remarkably stable on a day-to-day basis (Chapter 4). Although this advocates that the timing of blood sampling is not an important consideration with regards to longitudinal

studies (where participants are re-tested on different days), it is always good scientific practice to standardise the timing of repeat visits since circadian variations may be present in other tested variables. The stress hormones cortisol, adrenaline and noradrenaline have been documented to follow a circadian variation in concentration (Nicolau *et al.*, 1991;Schofl *et al.*, 1997;Young *et al.*, 2004). Whilst eHsp72 concentrations appear to be independent of cortisol (Fleshner *et al.*, 2004;Johnson *et al.*, 2005;Whitham *et al.*, 2006), evidence has suggested that eHsp72 release may be mediated via the catecholamines, adrenaline and/or noradrenaline (Johnson *et al.*, 2005;Whitham *et al.*, 2006). The absence of a circadian variation could be interpreted that eHsp72 release occurs independent of catecholamine stimulation. However, the peak in plasma catecholamine concentration that occurs during the late afternoon appears to be related to physical activity and rises in core temperature (Shephard & Shek, 1997), hence by controlling for these factors, it is not surprising that eHsp72 did not fluctuate significantly throughout the day. Moreover, it is likely that a certain level of sympathetic stimulation may be required before Hsp72 is released via this active mechanism. Indeed, the typical fluctuations in eHsp72 throughout the day of ± 5.4%, and results from Chapter 5 seem to suggest this may be the case. Thus, an important avenue for future research is to address the level of stress, be it exercise intensity, psychological stress or direct sympathetic stimulation that is required to elevate eHsp72 above basal levels.

That resting eHsp72 levels do not change (Chapter 4) or respond to acute psychological stress (Chapter 5) may have important clinical implications. A number of studies have suggested that eHsp72 is considerably elevated (20-300% versus healthy controls) in a number of clinical diseases and may serve as a biomarker for disease pathology (Abe *et al.*, 2004;Njemini *et al.*, 2003;Park *et al.*, 2006;Wheeler *et al.*, 2005;Wright *et al.*, 2000;Yeh *et*

al., 2008). Therefore, an assayed high concentration of eHsp72 may likely reflect clinical pathology rather than being a purely time of day effect. Thus eHsp72 measurement may be used as an adjunct marker of disease pathology. Since eHsp72 remains stable on a day-to-day basis, once baseline data is collected from individual patient, disease progression may be monitored by alterations in eHsp72 expression. However, it is acknowledged that the current cohort were healthy males, and clearly before any clinical recommendations are made upon the utility of eHsp72 as a biomarker of *disease progression*, time of day effects and day-to-day variations in eHsp72 expression should also be investigated within specific patient groups. For example, eHsp72 concentration is elevated during diabetic ketoacidosis (Oglesbee *et al.*, 2005)(a condition characterised by elevated blood glucose). When blood glucose is returned to normal levels, eHsp72 concentrations also return to within normal limits, thus systemic daily variations in blood glucose in this condition may affect measured eHsp72.

The finding that eHsp72 concentrations decrease during the initial adaptation to exercise heat acclimation (HA) (Marshall *et al.*, 2006;Kresfelder *et al.*, 2006) has led to speculation that eHsp72 may enhance the cytoprotection of distal cells. Indeed, exercise in the heat imposes one of the greatest threats to cellular homeostasis, thus increased cellular uptake of eHsp72 may confer cytoprotection during this stressful time. At the time the studies by Marshall *et al.* and Kresfelder *et al.* were published, it was unknown as to the intra-subject variability associated with eHsp72 expression. Due the robustness of the eHsp72 response (Chapter 4), the fact that eHsp72 concentrations decrease during the first 2-5 days of HA suggests that this is a genuine finding and not merely the result of endogenous day-to-day variation. However, whilst an attractive hypothesis, that decreased levels of eHsp72 are a result of increased cellular uptake by other cells is purely conjecture as no evidence has

been presented showing cellular uptake of eHsp72 during HA. Indeed, since intracellular Hsp72 induction is a cellular adaptation of HA (McClung *et al.*, 2008;Yamada *et al.*, 2007), and that intracellular Hsp72 acts as a negative feedback regulator of Hsp72 transcription via interaction with HSF1 (Boshoff *et al.*, 2000;Locke, 1997), reduced plasma Hsp72 may be a result of a downregulation of Hsp72 transcription and cellular release. It is apparent that further research is required to investigate the cross talk between intracellular and extracellular Hsp72 during heat acclimation. Additionally, an investigation into the interplay between intra and extracellular Hsp72 is perhaps even more pertinent considering the potential therapeutic role for Hsp72 in insulin resistance that is characteristic of Type-II diabetes (Chung *et al.*, 2008). Using rodent models of obesity, decreased expression of intracellular Hsp72 within skeletal muscle was associated with insulin resistance, and that regardless of the means used to elevate Hsp72 (i.e. heat treatment, muscle-specific transgenic overexpression or by pharmacological administration), insulin resistance was improved. Thus it remains to be seen what role if any extracellular Hsp72 may have in insulin resistance.

In contrast to plasma Hsp72 which appears to be mediated in part by adrenergic stimulation (Johnson *et al.*, 2005;Whitham *et al.*, 2006) and which increased significantly with exercise, salivary Hsp72 was unchanged by exercise or by the utilization of caffeine evoked sympathetic stimulation (Chapter 6). The obvious implication of this finding is that salivary Hsp72 cannot be used as a proxy measure of plasma Hsp72 concentration. This independence of salivary Hsp72 from the release of Hsp72 into plasma is not unique to saliva. Exercise also failed to alter the concentration of Hsp72 within the cerebrospinal fluid of healthy males, suggesting that the CSF Hsp72 pool is also segregated from the blood (Steensberg *et al.*, 2006). As such, it appears that salivary Hsp72 is not released via

an adrenergic mechanism for which the majority of protein secretion into saliva is (Chicharro *et al.*, 1998). We have speculated that salivary Hsp72 release may lie under parasympathetic control. Since saliva flow rate (which was unaffected by exercise) is also regulated by parasympathetic impulses, the potential for cholinergic stimulated release may have benefits during eating when saliva flow rates are high and thus increased secretion of salivary Hsp72 may prevent bacterial adhesion to mucosal surfaces (Boulanger *et al.*, 1995;Fabian *et al.*, 2007). Indeed, salivary Hsp72 output is increased during chewing invoked stimulation (Fabian *et al.*, 2003). The presence of Hsp72 at high concentrations in the saliva may be beneficial in light of its potential to stimulate both humoral and cellular immune responses. Additionally, owing to the speculation that Hsp72 may bind to blood clots (Chapter 3), its chemoattractant ability (Ortega *et al.*, 2008) and its functions in facilitating wound repair (Kovalchin *et al.*, 2006), salivary Hsp72 may have a significant role in mucosal and periodontal defence mechanisms particularly whilst eating and during dental brushing when small lesions may occur (Fabian *et al.*, 2007). However, as research into the *in vivo* immune function of Hsp72 is currently in its infancy, it would be inappropriate at this time to suggest that an increase in flow rate may confer enhanced oral defence via increased output of salivary Hsp72. Clearly, more research is required to clarify the immune functions of salivary Hsp72.

7.4 Conclusions and future directions

This thesis aimed to answer a number of methodological issues associated with eHsp72 collection. However, it is hoped that speculation derived from Chapters 3 and 6 in particular will stimulate further research into the functions of eHsp72 in blood clotting,

wound repair and in oral mucosal defence. The major conclusions and future research avenues drawn from this thesis are:

1. Blood samples drawn in the presence of an anticoagulant (e.g. EDTA or lithium heparin) provide significantly greater concentrations of eHsp72 than when blood is allowed to clot and the serum removed. EDTA in particular is recommended since concentrations were greatest in this matrix and eHsp72 was detected in 100% of samples. It is tangible that eHsp72 may bind to clotting proteins during the clotting process, thus future research should investigate this occurrence adopting both *in vitro* and *in vivo* models.

2. Resting plasma concentrations of eHsp72 are not subject to a circadian variation in healthy males (mean CV throughout the 24-hour period – 5.4%) and remain stable on a day-to-day basis (mean CV – 2.9%).

3. Resting concentrations of plasma eHsp72 were not affected by acute psychological stressors such as mental counting with electrical hand shock or assessed academic presentations.

4. Salivary eHsp72 concentration and secretion rate was not affected by exercise and did not correlate with the concentrations of eHsp72 within the plasma. Therefore, salivary eHsp72 should not be used as a non-invasive proxy measure of plasma eHsp72.

5. The release of salivary eHsp72 shows a significant independence of the release of the secretory enzyme α-amylase and a moderate independence from total protein. This suggests that salivary Hsp72 release may occur via a non-adrenergic mechanism. Further research should investigate the function of cholinergic stimulation of muscarinic receptors upon salivary Hsp72 release and indeed the (immune) functions of eHsp72 within the oral environment.

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APPENDIX A

Bangor University

School of Sport, Health and Exercise Sciences

Effect of blood handing on post exercise extracellular Hsp72 concentration

Subject Information sheet

Project Investigators

Matthew Fortes	01248 ******	******@bangor.ac.uk
Dr Martin Whitham	01248 ******	******@bangor.ac.uk

Invitation to take part
We are looking for moderately fit male adults to take part in a study involving exercise in hot ambient conditions. Before you decide to take part it is important that you understand why the research is being conducted and what it will involve. Please take time to read the following information carefully and discuss it with the investigator. Ask if there is anything that is not clear or if you would like more information. This study has been approved by the ethical committee of The School of Sport, Health, and Exercise Sciences.

Do I have to take part?
This is entirely your decision. If you decide to take part you will be asked to sign a consent form. You will be given a copy of this form to keep for your information. You will be free to withdraw at any time without giving a reason. If you decide not to participate, it will not affect your relationship with the School of Sport, Health and Exercise Sciences. All the information collected will be treated confidentially.

BACKGROUND

Heat shock proteins (HSPs) are very small proteins that reside in cells and protect the cell from injury. Recently, it has been discovered that these proteins may have important roles in the functioning of the immune system, and therefore may be involved in the resistance to infection (Asea, 2003). While it seems apparent that exercise is capable of causing a release of HSPs into the general circulation, there is inconsistency in the literature as to the magnitude of this response. This may be due to the handling of the blood after the sample has been taken. This study aims to assess the effect of different blood handling procedures on blood HSP concentrations. Since some handling procedures might cause an underestimation of HSP concentrations, the blood in which HSP is measured needs to have a high concentration of HSP. In order to achieve this, you will be asked to carry out strenuous exercise in hot ambient conditions.

The study will consist of two exercise trials separated by around 15minutes.

Peak Power Output determination
This exercise trial will be carried out on a cycle ergometer and you will be asked to exercise until you feel so tired that you do not want to go on. This will allow us to measure how much power you can generate at your maximum effort. We shall measure your heart rate by simple means.

Time trial
Also on the cycle ergometer, you will then be asked to carry out a time trial in a climatic chamber in hot conditions. The time trial will last around 30 – 40 minutes and is individually specific based on your performance in the peak power output determination test. You will not be distracted in any way during the time trial, except to tell you how much of the time trial you have completed (25, 50, 75, 90, or 100%). For your safety your core temperature will be monitored by a rectal thermister. These are commonly used in thermoregulation research and most people do not notice them once they have started exercising. Immediately after the time trial has been completed, a blood sample will be taken from a forearm vein by a trained phlebotomist.

Advantages and disadvantages of taking part
Taking part in this study will enable you to receive comprehensive feedback, with full explanations of your fitness level. In addition, performing exercise under controlled environmental condition will allow you to see how you perform in hot conditions. The feedback you will receive regarding your fitness level is similar to that which many fitness testing centres provide as a fee-paying service.

The disadvantages of taking part in this study are the discomfort associated with the use of temperature thermisters, venepuncture and exercising in a hot environment. Any discomfort you experience will be short lived. There are standard risks associated with intense exercise. Accordingly, persons trained in CPR and AED will be readily available or present at all exercise trials.

INFORMATION FOR PARTICIPANTS

Study Title: Twenty-four hour rhythms of plasma Hsp72

You are being invited to take part in a research study. Before you decide whether to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, family and your GP if you wish. If there is anything that is not clear or if you would like more information, please do not hesitate to ask us. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled "Medical Research and You". This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW. Thank you for reading this.

What is the purpose of this study?

Heat shock proteins (Hsp's) are proteins that are released into the circulation during times of stress. This stress may be in the form of anxiety, exercise or infection. Previous research has suggested that by measuring these proteins in the blood, one might be able to diagnose certain medical conditions. Little is known about how the concentrations of heat shock proteins in blood might be affected by the time of day, or how much sleep you have. If these things do affect the concentration of these proteins, then this has implications on when you should take a blood sample and the correct diagnosis of medical conditions. Therefore, the purpose of this study is to measure the concentration of heat shock protein 72 (Hsp72 - a certain type of heat shock protein) throughout a thirty-six hour cycle.

Why have I been chosen?

You have been chosen because you are a male aged between 18 and 35 years of age, free from infection, medication or supplements (eg Vitamin C), and have a regular nocturnal sleeping pattern (approx 6.5 – 9 h per night with no habitual napping).

Do I have to take part?

No. You are under no pressure to take part in the study. Taking part in the research is entirely up to you. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to drop out at any time without giving a reason. This will not affect your relationship with any of the research staff or with the School of Sport, Health and Exercise Sciences.

What will happen to me if I take part?

The study will require one visit to the School of Sport, Health and Exercise Sciences (SSHES), which will involve you staying in the living quarters for a period of 24 hours. During this time, you will have scheduled blood samples taken from a cannula which will remain in the arm throughout the duration of the study. This procedure is similar to that carried out during a hospital stay. Two 5ml blood samples will be taken at 1-hour intervals during two normal, non-active days. Heart rate, blood pressure, and oral temperature will also be taken by simple means at these time points.

Throughout the study period, you will remain inactive, but will be permitted to read, watch television, use the internet etc. Activity levels will be monitored using an accelerometer, a light, matchbox sized device that is worn around your waist. You will sleep in the living quarters, allowing blood samples to be taken just prior to going to bed, and immediately following awakening. You will also be fed sufficient, balanced meals at set time points, and will also be provided with sufficient fluids. The study will begin at 10am, and finish at 12noon the following day.

What do I have to do?

In the 24 hours prior to your visit, we ask you to drink plenty of fluids, avoid exercise or strenuous physical activity and to try to get a good nights sleep (approximately 6-8 hours). We also ask that you refrain from consuming any tobacco or alcohol in the 2 days before your visit and throughout the trial. We also ask that you provide us with information on how many hours you have slept each night in the 7 days prior to your visit.

What are the possible disadvantages and risks of taking part?

The disadvantage of taking part in this study is the discomfort associated with the collection of venous blood samples. Any discomfort you experience will be short lived. You may also encounter bruising of the area around the cannula, and there is also a very small risk of infection associated with cannulation. All procedures will be put in place to ensure sterility of the cannulation and blood letting procedure.

What are the possible benefits of taking part?

There will be no clinical benefit to you taking part in this study. However, since plasma Hsp72 has diagnostic potential, the research may provide guidelines on the time of day controls required for the diagnostic use of this measure. You will be paid £20 for participating in the study.

What if something goes wrong?

If you are harmed whilst taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study you may contact the Head of the School of Sport, Health and Exercise Sciences, Professor Mike Khan.

Will my taking part in this study be kept confidential?

All information collected about you during the course of the study will be kept strictly confidential. Any information about you which leaves the university will have your name and address removed so that you cannot be recognised from it.
The following people will have access to your records:

Dr Martin Whitham (University lecturer) and Matthew Fortes (University researcher).

What will happen to the results of the research study?
The results from this study will be presented at the 2007 International Conference of Stress and published in a leading international physiology journal. Furthermore, this data may be used by future studies investigating the diagnostic potential of Hsp72. Your blood samples will be stored for a period of six years and may be used in future investigations in this area of research. Again, you will not be able to be identified from this. A written report will be given to any participants who are interested in the results of the study.

Who is organizing and funding the research?
This research is organized by the University of Wales, Bangor. The research is funded by a grant from the North West Wales NHS Trust.

Who has reviewed the study?
The study has been approved by the School of Sport, Health and Exercise Sciences ethics committee of the University of Wales, Bangor, and the North West Wales Local Research Ethics Committee.

Contact for further information
If you require any further information about the study please contact:

Matthew Fortes 01248 ****** (office) or ***** ****** (mobile) Office 210, George building
Dr. Martin Whitham 01248 ****** (office)

Thank you very much for taking the time to read this information sheet.

If you choose to participate in this study you will be given a copy of the information sheet and a signed consent form to keep.

• PRIFYSGOL CYMRU •
UNIVERSITY OF WALES
BANGOR

School of Sport, Health and Exercise Sciences,
Bangor University,
George Building,
Bangor,
Gwynedd, LL57 2PX

Effect of psychological/mental stress on the extracellular heat shock protein response

Participant Information sheet

Project Investigators

Dr Martin Whitham	01248 ******	******@bangor.ac.uk
Matthew Fortes	07766 ******	******@bangor.ac.uk

Invitation to take part
You are being invited to take part in a research study. Before you decide whether to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, family and your GP if you wish. If there is anything that is not clear or if you would like more information, please do not hesitate to ask us. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled "Medical Research and You". This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW. Thank you for reading this.

What is the purpose of this study?
Heat shock proteins (HSPs) are very small proteins that reside in cells and protect the cell from injury. Recently, it has been discovered that these proteins are released into the circulation during times of stress and may have important roles in the functioning of the immune system.
Previous studies on humans have demonstrated that stressors such as exercise, trauma and infection result in an increase in HSP concentration in the blood. In addition, psychological stressors such as predatory fear and electric shock have been shown to induce an HSP response in rodents.
Therefore, the purpose of this study is to investigate whether a combined psychological/mental stress increases the concentration of HSP's in the blood of humans.

Why have I been chosen?
You have been chosen because you are a male aged between 18 and 35 years of age, free from infection, medication or supplements (e.g. Vitamin C, protein shakes). In addition, you will not have a history of cardiac illness, anxiety attacks or epilepsy.

Do I have to take part?
This is entirely your decision. If you decide to take part you will be asked to sign a consent form. You will be given a copy of this form to keep for your information. You will be free to withdraw at any time without giving a reason. If you decide not to participate, it will not affect your relationship with the School of Sport, Health and Exercise Sciences or any of the researchers. All the information collected will be treated confidentially.

What will happen to me if I take part?
The study will involve you making two visits to the School of Sport, Health and Exercise Sciences. Both visits will be separated by at least 3 days. On arrival, a small blood sample will be taken from a forearm vein by a qualified phlebotomist. This procedure is similar to that carried out during a normal hospital stay. You will then be asked to fill out a number of questionnaires that relate to your anxiety. 30 minutes after the first blood sample, further blood samples will be taken before and after a number of stress inducing trials. The trials will involve you counting the number of either high or low tones emitted randomly from a computer. If you answer incorrectly, you will be given an immediate uncomfortable but non-dangerous shock to the hand before continuing to the next trial. Trials will be of varying (increasing) difficulty and the whole task will last around 15 - 20 minutes. The intensity of the shock is in line with previous research detailing a similar model of stress induction in humans. A final blood sample will be taken 2-hours after the completion of the stress test resulting in a total of 4 blood samples per visit. The second visit will involve exactly the same procedures as the first.

What do I have to do?
In the 24 hours prior to your visit, we ask you to refrain from consuming any caffeine containing products (e.g. coffee, coke), tobacco or alcohol.

What are the possible disadvantages and risks of taking part?
The disadvantage of taking part in this study is the discomfort associated with venepuncture (taking of blood) and the electrical shock to the hand. Although the intensity of the shock is intended to be uncomfortable and to induce acute stress, the level of stress experienced is not likely to be larger than that experienced in everyday life situations (e.g. anxiety surrounding public speaking, an interview or exam stress). Any discomfort you experience will be short lived. All procedures will be put in place to ensure sterility of all blood letting procedures. A first aider trained in CPR/AED will also be present throughout both trials.

What are the possible benefits of taking part?
There will be no clinical benefit to you taking part in this study. However, this study will allow future studies to utilize this stress model to explore the significance of heat shock protein release under times of stress. There will be a £20 prize for the best performance on the reaction task.

What if something goes wrong?
If you are harmed whilst taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study you may contact the Head of the School of Sport, Health and Exercise Sciences, Dr. Mike Khan.

Will my taking part in this study be kept confidential?
All information collected about you during the course of the study will be kept strictly confidential. The following people will have access to your records:

Dr Martin Whitham (University lecturer) and Matthew Fortes (University researcher).

What will happen to the results of the research study?
The results from this study may be published in a leading international physiology journal. Furthermore, results obtained may allow this psychological stress model to be used in future investigations into the heat shock protein response to stress or allow further insight into the negative effects of chronic psychological stress upon immunity. Your blood samples will be stored for a period of six years and may be used in future investigations in this area of research. Again,

you will not be able to be identified from this. A written report will be given to any participants who are interested in the results of the study.

Who is organizing and funding the research?
This research is organized by the University of Wales, Bangor.

Who has reviewed the study?
The study has been approved by the School of Sport, Health and Exercise Sciences ethics committee of Bangor University.

Contact for further information
If you require any further information about the study please contact either of the project investigators whose contact details are displayed at the start of this document.

Thank you very much for taking the time to read this information sheet.

If you choose to participate in this study you will be given a copy of the information sheet and a signed consent form to keep.

PRIFYSGOL
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UNIVERSITY

School of Sport, Health and Exercise Sciences,
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Effect of caffeine supplementation and exercise upon salivary and plasma heat shock protein 72 concentration

Participant Information sheet

Project Investigators

Matthew Fortes	01248 ******	******@bangor.ac.uk
Steve O'Hara		******@bangor.ac.uk
Dr Martin Whitham	01248 ******	******@bangor.ac.uk

Invitation to take part
You are being invited to take part in a research study. Before you decide whether to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, family and your GP if you wish. If there is anything that is not clear or if you would like more information, please do not hesitate to ask us. Take time to decide whether or not you wish to take part.

What is the purpose of this study?
Heat shock proteins (HSPs) are very small proteins that reside in cells and protect the cell from injury. Recently, it has been discovered that these proteins are released into the blood during times of stress and may have important roles in the functioning of the immune system. It has also recently been shown that Hsp72 (the heat shock protein that responds the greatest to stress) is present within the saliva of resting healthy humans. It is currently unknown whether these heat shock proteins increase in concentration within the saliva in response to exercise, whether saliva Hsp72 is an accurate marker of plasma (blood) Hsp72 and whether caffeine supplementation has any additional effect on salivary Hsp72 concentration. The purpose of this study is to answer these questions.

Why have I been chosen?
You have been chosen because you are a male aged between 18 and 35 years of age, free from infection, medication or supplements (e.g. Vitamin C, protein shakes).

Do I have to take part?
This is entirely your decision. If you decide to take part you will be asked to sign a consent form. You will be given a copy of this form to keep for your information. You will be free to withdraw at any time without giving a reason. If you decide not to participate, it will not affect your relationship with the School of Sport, Health and Exercise Sciences or any of the researchers. All the information collected will be treated confidentially.

What will happen to me if I take part?
The study will involve you making three visits to the School of Sport, Health and Exercise Sciences. During the first visit (approximately 40 minutes) you will be asked to perform an incremental exercise test on a treadmill. This test starts off easy and gets progressively harder until you cannot continue. This will allow us to calculate your maximal oxygen uptake (a good indicator of your overall fitness) and to calculate the intensity at which you will exercise during the remaining two visits.

The subsequent two trials (each approximately 2 hours), require you to perform 60 minutes of running (2 x 30 minute bouts during each session) on a motor driven treadmill in a hot environment (30°C) with either caffeine or placebo supplementation. Throughout each trial a number of blood and saliva samples will be collected (see Figure 1 below for a flow chart of the procedures). The blood sample will be taken from a forearm vein by a qualified phlebotomist. This procedure is similar to that carried out during a normal hospital stay. The saliva will be collected by a passive drool technique which will be demonstrated during the first visit. For your safety, your core temperature will be monitored by a rectal thermister. These are commonly used in thermoregulation research and most people do not notice them once they have started exercising.

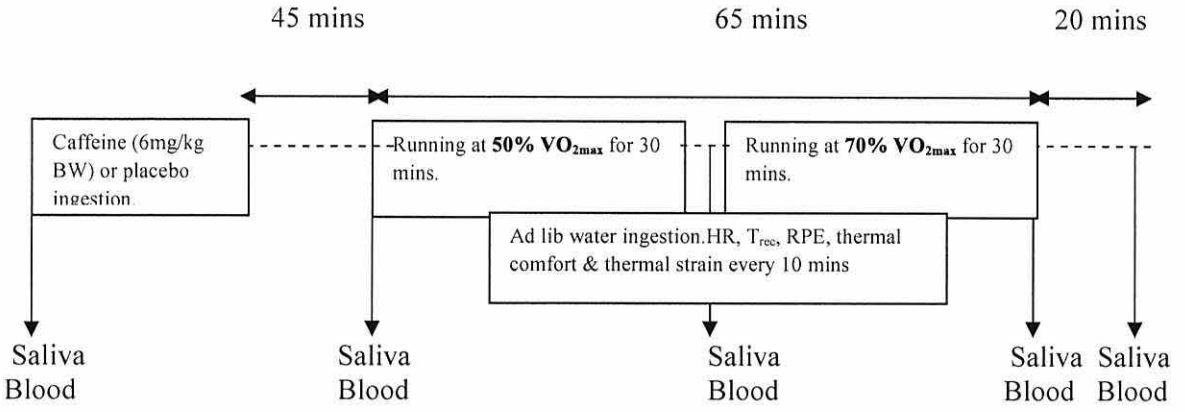

Figure 1. Schematic depicting measures and procedures during trials 2 and 3. HR – heart rate, T_{rec} – core (rectal) temperature, RPE – rating of perceived exertion.

What do I have to do?
In the 48 hours prior to your visit, we ask you to refrain from consuming any caffeine containing products (e.g. coffee, tea, red bull, pro-plus, coke, chocolate etc). We also ask that in the 24 hours prior to each test you do not perform any unaccustomed exercise, drink alcohol or consume any form of diuretics or tobacco. We also ask that you consume plenty of fluids on the day before each exercise trial and to consume a meal high in carbohydrates the night before exercise (such as pasta, rice or potatoes). We also ask that you record what you eat on the day before the second trial and replicate this meal on the night before the third trial.

What are the possible disadvantages and risks of taking part?
The disadvantages of taking part in this study are the discomfort associated with the use of temperature thermisters, venepuncture and exercising in a hot environment. Any discomfort you experience will be short lived. There are standard risks associated with intense exercise. Accordingly, persons trained in CPR and AED will be readily available or present at all exercise trials

What are the possible benefits of taking part?
Taking part in this study will enable you to receive comprehensive feedback, with full explanations of your fitness level. In addition, performing exercise under controlled environmental condition will allow you to see how you perform in hot conditions. The feedback you will receive regarding your fitness level is similar to that which many fitness testing centres provide as a fee-paying service

What if something goes wrong?
If you are harmed whilst taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study you may contact the Head of the School of Sport, Health and Exercise Sciences, Professor. Michael Khan.

Will my taking part in this study be kept confidential?
All information collected about you during the course of the study will be kept strictly confidential. It will be anonymized so that only the following people will have access to your records:

Dr Martin Whitham (University lecturer), Matthew Fortes and Steve O'Hara (University researcher).

What will happen to the results of the research study?
The results from this study may be published in a leading international physiology journal. Your blood samples will be stored for a period of six years and may be used in future investigations in this area of research. Again, you will not be able to be identified from this. A written report will be given to any participants who are interested i
n the results of the study.

Who is organizing and funding the research?
This research is organized by the University of Wales, Bangor.

Who has reviewed the study?
The study has been approved by the School of Sport, Health and Exercise Sciences ethics committee of Bangor University.

Contact for further information
If you require any further information about the study please contact any of the project investigators whose contact details are displayed below:

Matthew Fortes	01248 ******	******@bangor.ac.uk
Steve O'Hara		******@bangor.ac.uk
Dr Martin Whitham	01248 ******	******@bangor.ac.uk

Thank you very much for taking the time to read this information sheet.

If you choose to participate in this study you will be given a copy of the information sheet and a signed consent form to keep.

APPENDIX B

INFORMED CONSENT TO PARTICIPATE
IN A RESEARCH PROJECT OR EXPERIMENT

Title of Research Project:

The researcher conducting this project subscribes to the ethics conduct of research and to the protection at all times of the interests, comfort, and safety of participants. This form and the information sheet have been given to you for your own protection and full understanding of the procedures. Your signature on this form will signify that you have received information which describes the procedures, possible risks, and benefits of this research project, that you have received an adequate opportunity to consider the information, and that you voluntarily agree to participate in the project.

Having been asked by Matthew Fortes of the School of Sport, Health and Exercise Sciences at Bangor University to participate in a research project experiment, I have received information regarding the procedures of the experiment.

I understand the procedures to be used in this experiment and any possible personal risks to me in taking part.

I understand that I may withdraw my participation in this experiment at any time.

I also understand that I may register any complaint I might have about this experiment to Professor Michael Khan, Head of the School of Sport Health and Exercise Sciences, and that I will be offered the opportunity of providing feedback on the experiment using standard report forms.

I may obtain copies of the results of this study, upon its completion, by contacting:

Matthew Fortes, ****@bangor.ac.uk**

I confirm that I have been given adequate opportunity to ask any questions and that these have been answered to my satisfaction. ☐

I have been informed that the research material will be held confidential by the researcher. ☐

I agree to participate in the study ☐

NAME (please type or print legibly): _____

ADDRESS: (Optional)_____

_____

PARTICIPANT'S SIGNATURE: _____ **DATE:** _____

RESEARCHER'S SIGNATURE: _____ **DATE:** _____

Two sheets should be completed - one for the participant and one for the researcher