THE UNIVERSITY OF HULL

Monocyte heat shock protein 72 at rest and in response to environmental and exercise stress – implications for cross tolerance *in vivo*.

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Lee Taylor BSc (H)

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List of Abbreviations, Acronyms and Symbols

^{o}C	degrees centigrade
ATA	atmosphere absolute
AT	anaerobic threshold
B1	bout one
B2	bout two
CSR	cellular stress response
DBD	domain binding domain
DCI	decompression illness
EDL	extensor digitorum longus
ELISA	enzyme linked immunoassay
EPO	erythropoietin
eHSP72	extracellular heat shock protein 72
EXB1	exercise bout one
EXB2	exercise bout two
HA	hyperbaric air
HBO	hyperbaric oxygen
HO-1	inducible heme oxygenase 1
HR_{max}	maximal heart rate
g	gravity
G	granulocyte
GSH	reduced glutathione
GSSG	oxidised glutathione
GXT	graded exercise test
H_2O_2	hydrogen peroxide

HO-2	heme oxygenase 2
HRR	heart rate reserve
HR	repatic repeats
HSE	heat shock element
HSP	heat shock protein
HSP27	heat shock protein 27
HSP32	inducible heme oxygenase
HSP40	heat shock protein 40
HSP60	heat shock protein 60
HSP70	heat shock protein family 70
HSP72	inducible heat shock protein 72
HSP73	constitutive heat shock cognate 73
HSP78	heat shock protein 78
HSP90	heat shock protein 90
HSR	heat shock response
iHSP72	intracellular heat shock protein 72
IHT	intermittent hypoxic training
kDa	kilo Dalton
L	lymphocyte
LT	lactate threshold
M	monocyte
MCP-1	monocyte chemoattractant protein 1
MDA	malondialdehyde
MFI	mean fluorescence intensity
mM	milimole
mmHg	millimetres of mercury

MVC	maximal voluntary contraction
mmol·L	milimole per litre
NA	normobaric air
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
ng/ml	nanograms millilitre
O_2	oxygen
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffering solution
PHE_R	prolonged hypoxic exposure at rest
PO	power output
RPE	rate of perceived exertion
RT-PCR	real-time polymerase chain reaction
RONS	reactive oxygen and nitrogen species
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBARS	thiobarbituric Acid Reactive Substances
T_c	core temperature
$T_{c\,max}$	core temperature maximum
$T_{c\,min}$	core temperature minimum
TGSH	total glutathione
µL	micromole
µg/ml	microgram per millilitre
VCAM-1	vascular cellular adhesion molecule 1
$\dot{V}O_{2\,max}$	maximal oxygen consumption
W	watts

Abstract

The human body endures stress on a daily basis, with many occupational and recreational activities beset with such challenges to homeostasis. These challenges include that of exercise and exposure to challenging environments (hypoxia and hyperbaria). A group of specialised proteins, termed heat shock proteins (HSP) provide protection to such stressors at a cellular level. This cellular defence mechanism protects and oversees whole body protein homeostasis, which is vital to all cellular processes.

One such protective HSP, is HSP72, which is present in almost all cellular compartments and has received extensive and widespread research interest – with elevations in HSP72 indicatively linked to augmented cellular and whole body resistance to various exercise and environmental stressors. Despite this extensive research interest, several fundamental areas of concern with regard to HSP72 have not been satisfactorily addressed or delineated. In general, the experimental chapters of this thesis were designed to investigate several broad research questions related to those areas that have not been sufficiently addressed, as highlighted by the Literature Review. These areas include the reliance on thermal and/or mechanical stress to induce elevations in HSP72, both *in vitro* and *in vivo*, to initiate conveyed cellular protection. No *in vivo* attempts have been made to use a non-thermal and/or non-mechanical based stimulus, such as a hypoxic or hyperbaric exposure, to induce elevations in basal HSP72 in an attempt to confer cellular tolerance to future episodes of stress. Additionally, any potential relationships between changes in redox balance and stress induced changes in HSP72 expression have not been investigated *in vivo*, this potential interplay could be important when discussing any likely mechanisms for HSP72 dependent conferred cellular tolerance. In order to investigate such hypoxic or hyperbaric mediated changes in basal HSP72 expression securely, basal expression of monocyte expressed HSP72

(*mHSP72*) warrants investigation (diurnal and/or circadian variation), as, at present, this has not be conducted securely or adequately.

The first experimental chapter investigated basal expression of *mHSP72* over a 24 h period. Seventeen recreationally active (mean ± SD: 5.9±2.2 $h \cdot wk^{-1}$) male subjects (19.8±4.3 yr, 177±6.4 cm, 75.7±10.9 kg) had blood samples taken every 4 h from 0900 until 0900 the next day, at rest, within a temperature regulated laboratory. Core temperature, as assessed by ingestible telemetric temperature sensor pill, was obtained at 5 min intervals. Basal *mHSP72* expression was found to follow a circadian rhythm, which was correlated to core temperature (r_s=0.41, $p<0.001$). Notably, during "waking" hours (0900 – 2100), this circadian rhythm was shown to follow a quadratic trend in expression ($F = 21.2$, $p < 0.001$).

The second experimental chapter investigated the repeatability of the quadratic trend in basal *mHSP72* expression demonstrated within the previous experimental chapter. Twelve healthy recreationally active (mean ± SD: 5.2±1.9 $h \cdot wk^{-1}$) male subjects (20.2±1.9 yr, 178.7±5.6 cm, 75.1±6.0 kg) had blood samples taken on three separate days (separated by three days) over a 9 h period (0800, 1100, 1400) at rest within a temperature regulated laboratory. Results supported those from the previous chapter, whereby, the quadratic trend in basal *mHSP72* expression was evident on three separate days ($F = 26.0$; $p = 0.001$; partial $\eta^2 = 0.74$), where *mHSP72* decreased between 0800 and 1100 (mean difference = -17%; 95% CI = -24%, -10%; $p < 0.001$) and then increased between 1100 and 1400 (mean difference = 8%; 95% CI = 2%, 14%; $p = 0.015$). In conjunction with the first experimental chapter, these results demonstrate the importance in controlling the time of day interventions are administered *in vivo*, as differential responses may be seen due to differences in basal HSP72 expression. Furthermore, when regular blood samples are required post intervention, the timetabling of such collections needs to be stringently adhered to, due to within-day variation in

basal *mHSP72*. Differing basal values of *mHSP72* are known to determine the magnitude of post stressor *mHSP72* expression and thus any variation (even minimal) in basal mHSP72 is important.

The third experimental chapter investigated the potential of an environmental stressor to disrupt the quadratic trend in basal *mHSP72* and explored whether any such changes in *mHSP72* may have a relationship with alterations in redox balance. Six healthy recreationally active (mean ± SD: 5.9±2.3 $h.wk^{-1}$) male subjects (mean ± SD: 21.3±7.2 yr, 179.2±4.8 cm, 79.3±9.9 kg) participated within the study. Control values (NA) for *mHSP72* were obtained one week before the first hyperbaric air (HA) exposure with the hyperbaric oxygen (HBO) exposure following a week later (i.e. 3 study days NA, HA and HBO each separated by one week). These exposures commenced at 1500 and involved a simulated dive consisting of HA (2.8 ATA) or HBO (20 min O_2, 5 min HA cycle) within a hyperbaric chamber constituting 78 min bottom time. Within each study day blood samples were taken at 0900, 1300, 1700 and 2100. The administration of HBO and HA were sufficient to disrupt the quadratic trend shown within the NA condition ($F = 27.6$, $p < 0.001$). The model demonstrated significant main effects for condition ($F = 24.7$, $p < 0.001$) and time ($F = 9.6$, $p < 0.001$), and a condition x time interaction effect was also observed ($F = 7.1$, $p < 0.001$). Decomposition of this interaction effect revealed a reduction in *mHSP72* was evident post hyperbaric exposures, whereby, *mHSP72* expression at 1700 was significantly higher in NA than in HA ($p = 0.016$) and HBO ($p < 0.001$), this reduction was still evident in both HA and HBO compared to NA at 2100 ($p < 0.001$). In addition to quantification of *mHSP72*, a measure of oxidative stress, thiobarbituric acid reactive substances (plasma TBARS), was also retrospectively assessed from the isolated plasma of these blood samples. There were no significant main effects observed for condition ($F = 0.7$; $p = 0.50$) or time ($F = 0.06$; $p = 0.81$), and no significant condition x time interaction effect ($F = 0.5$;

p = 0.62) for plasma TBARS. Despite the failure of the hyperbaric environments to elicit increases in basal *mHSP72*, one important physiological contribution may be contrived of this reduction in *mHSP72*, as *in vitro* and *in vivo* low basal *mHSP72* content is indicatively correlated to enhanced post stressor HSP72 expression. Such hyperbaric mediated reductions in basal content may allow enhanced HSP72 expression post stressor, an intervention which may be of benefit to hyperthermic exercise acclimation protocols which seek elevated *mHSP72* as part of the *in vivo* heat acclimation process.

The fourth experimental chapter employed an acute hypoxic exposure (75 min, 2980 m) at rest in an attempt to disrupt the previously demonstrated quadratic trend in basal *mHSP72* expression and explored whether any such changes in *mHSP72* may have a relationship with alterations in redox balance. Twelve healthy recreationally active (mean ± SD: 5.1±1.5 $h.wk^{-1}$) male subjects (19.8±3.5 yr, 175.5±10.8 cm, 73.1±8.0 kg) participated in the study. Testing was conducted on consecutive days, with all subjects providing control samples on this first day with the hypoxic exposure administered on the second day. This exposure commenced and ceased at 0930 and 1045 respectively. Blood samples were taken at 0800, 1100, 1400, 1700 and 2000. In addition to quantification of *mHSP72* a measure of oxidative stress, plasma TBARS, was also retrospectively assessed from the isolated plasma of these blood samples. There was a significant quadratic trend in *mHSP72* for the control condition (F = 23.5; p = 0.002; partial η^2 = 0.77) with no such trend evident for the hypoxic condition (largest F ratio was for a quadratic trend: F = 3.9; p = 0.087). The condition-by-time interaction was significant for *mHSP72* (F = 19.5; p = 0.003; partial η^2 = 0.74), where the difference between the control and hypoxic conditions were significantly different at 1100 (p = 0.002), 1400 (p < 0.001), 1700 (p = 0.034) and 2000 (p = 0.041). No significant trend was observed for plasma TBARS in the control condition (F = 0.8; p = 0.41), but a

significant quadratic trend was evident for the hypoxia condition ($F = 36.1$; $p = 0.001$; $\eta^2 = 0.84$). Plasma TBARS increased by 98% (95% CI 30%, 166%) from pre-intervention to immediately post-intervention and decreased thereafter until pre-intervention levels were reached at 2000. This difference in trends within the plasma TBARS data between conditions showed up as a linear-by-quadratic interaction in the two-way analysis ($F = 41.5$; $p < 0.001$; $\eta^2 = 0.86$), where significant differences between conditions were observed only at 1100 ($p = 0.006$) and 1400 ($p = 0.032$). The results demonstrate that an acute hypoxic exposure (75 min, 2980 m) was sufficient to induce significant increases in *mHSP72* post intervention. This increase in *mHSP72* may be linked to the significant increase in oxidative stress (plasma lipid peroxidation - TBARS) but a cause and effect relationship cannot be claimed. Caution is required when interpreting any change in lipid based oxidative stress and its interplay with increases in a protein (*mHSP72*) whose predominate role is to respond to stress induced changes in protein conformation.

The fifth experimental chapter employed ten consecutive days of once daily hypoxia (75 min, 2980 m) in an attempt to increase basal levels of *mHSP72* in excess of that shown in the previous chapter, which utilised a single acute hypoxic exposure. Furthermore, a measures of oxidative stress (plasma TBARS) and EPO secretion were obtained at various time points within the experimental design. Additionally, in line with recent conflicting research investigating the use of intermittent hypoxic training at rest, any changes in maximal oxygen consumption post hypoxic acclimation period were also investigated. Eight healthy recreationally active (mean ± SD: 5.3±1.8 $h.wk^{-1}$) male subjects (20.2±4.4 yr, 172.1±13.9 cm, 71.1±8.0 kg) volunteered to participate in the study. The hypoxic exposure (75 min, 2980 m) was administered daily for 10 consecutive days, with the exposure commencing and ceasing at 0930 and 1045 respectively. Blood samples were taken immediately pre and post hypoxic exposures on

days 1, 2, 3, 4, 5 and 10 for analysis of *mHSP72* and plasma TBARS, with EPO specific blood samples taken pre and post hypoxia on days 1, 2, 3, and 10. The maximal oxygen consumption tests were conducted (at sea level) 8 days before and 48 h after the 10 day exposure period. Hypoxic exposure in this manner was sufficient to induce significant ($F = 73.2$, $p < 0.001$, partial $\eta^2 = 0.92$) day-on-day increases in *mHSP72* which was proportional to the basal content of the prior day. This increase had a distinct fast (days 1 – 5, 30% increase) and slow (days 5 – 10, 16% increase) phase in accumulation which was seen in tandem with significant (main effects for day ($F = 9.0$, $p = 0.024$, partial $\eta^2 = 0.60$) and time (157.4, $p < 0.001$, partial $\eta^2 = 0.96$)) daily transient increases in oxidative stress (plasma TBARS). Within each day, *mHSP72* expression was consistently higher after hypoxic exposure ($F = 6.2$, $p = 0.047$, partial $\eta^2 = 0.51$). The plasma TBARS concentration increased significantly ($p < 0.05$) by around 20% in response to each hypoxic exposure. A significant 5th order polynomial trend was observed for EPO concentration over the 10 days ($F = 34.5$, $p = 0.001$, partial $\eta^2 = 0.85$), characterised by a dramatic 39% increase in EPO concentration the day after the first hypoxic exposure ($p = 0.001$), followed by a relative plateau over the rest of the hypoxic exposure period, and then a dramatic reduction immediately post-intervention. There were no significant changes observed over the 10 day hypoxic exposure intervention period for absolute maximal oxygen consumption ($t = 2.3$, $p = 0.065$) or max heart rate ($t = 1.6$, $p = 0.16$), however, there was a small significant increase in time to exhaustion for the incremental test ($t = 3.9$, $p = 0.008$, $\acute{\omega}^2 = 0.50$), though this is likely physiologically negligible. The results within this experimental chapter demonstrated the ability of once daily hypoxia at rest to elicit increases in *mHSP72*, without the reliance on thermal or mechanical stressors, relied upon previously within the literature to evoke such increases, both *in vivo* and *in vitro*. This increase in *mHSP72* may be linked to the significant daily increases in oxidative stress (lipid peroxidation – plasma

TBARS) but a cause and effect relationship cannot be claimed. Caution is required when interpreting any change in lipid based oxidative stress and its interplay with increases in a protein (*mHSP72*) whose predominate role is to respond to stress induced changes in protein conformation. The efficacy of increasing basal *mHSP72* expression to confer *in vivo* cellular tolerance to further stressors (exercise, hypoxia, hyperbaria, etc) has not been directly investigated within this chapter and requires further, possibly *ex vivo* and/or *in vivo* research.

The sixth and final experimental chapter, utilised five days of once daily hypoxic exposures (the fast phase of accumulation highlighted in the previous chapter) to increase basal *mHSP32* and *mHSP72*. Eight healthy recreationally active (mean ± SD: 6.8±1.8 $h.wk^{-1}$) male subjects (20.8±3.2 yr, 1.77±15.7 cm, 72.1±11.0 kg, power output at lactate threshold 184±37 W) volunteered to participate in the study. Initial lactate threshold testing on an SRM cycle ergometer allowed lactated threshold to be calculated using the Dmax method with subjects then completing 30 mins of cycling (familiarisation) at 90% of their power output at LT. The hypoxic exposure (75 min, 2980 m) was administered daily for five consecutive days at rest, with the exposure commencing and ceasing at 0930 and 1045 respectively. Blood samples were taken immediately prior to the first hypoxic exposure (hypoxic day 1) and 30 min post final hypoxic exposure (hypoxic day 5). Seven days prior to the hypoxic acclimation period, subjects performed 60 min cycling on a SRM cycle ergometer at 90% of their power output at LT (exercise bout 1 – EXB1), this exercise bout was repeated 1 day post cessation of the hypoxic period (exercise bout 2 – EXB2). Physiological measures of exercise performance (heart rate, ratings of perceived exertion, blood lactate) in EXB1 and EXB2 were recorded throughout to ensure any changes in the biochemical measures of stress protein expression (*mHSP72* and *mHSP32*) and alterations in redox balance (plasma TBARS and glutathione) were not associated with variations in performance

intensity between bouts. Blood samples were taken immediately pre and post exercise and 1, 4 and 8 h post exercise for *mHSP72* and immediately pre, post and 1 h post exercise for *mHSP32*, plasma TBARS and glutathione. The five day hypoxic acclimation period was sufficient to reduce the disturbance to redox balance of 60 min prolonged aerobic exercise at 90% of LT. This reduction was evident by the significant increase (32.5%; 95% CI = 19.0% to 45.9%; $p < 0.001$) in GSSG post EXB1 being absent post EXB2 ($p = 0.26$). Such a reduction in disturbance to redox balance post exercise is likely attributable to the prior induction (increased content pre EXB2 compared to pre EXB1) and thus bio availability of the potently antioxidant stress protein *mHSP32* ($p = 0.024$) and the highly stress inducible *mHSP72* ($p < 0.001$), in addition to favourable alterations in glutathione ratios. The GSSG was 16.5% lower pre-exercise (95% CI = 3.5% to 29.5%; $p = 0.018$) and 39.9% lower immediately post-exercise (95% CI = 27.2% to 52.6%; $p < 0.001$) in EXB2 compared to EXB1. Furthermore, a significant 32.5% increase in GSSG was observed from pre-exercise to immediately post-exercise for EXB1 (95% CI = 19.0% to 45.9%; $p < 0.001$), whereas no significant change was observed in response to EXB2 ($p = 0.26$). The hypoxic acclimation period induced significant increases of 2.6 malondialdehyde equivalents in plasma TBARS (95% CI = 1.5 to 3.6 malondialdehyde equivalents; $t = 5.6$, $p = 0.001$). Plasma TBARS increased significantly from pre- to post-exercise ($p = 0.001$) in both EXB1 and EXB2. EXB1 demonstrated an increase of approximately 100% (95% CI = 54.2% to 156.9%; $p < 0.001$) in *mHSP72* immediately post exercise, with values remaining elevated by approximately 50% 1, 4, and 8 h post exercise. The expression kinetics in EXB2, compared to EXB1, did not demonstrate a significant increase ($p \geq 0.79$) in *mHSP72* immediately post exercise. A similar significant ($p = 0.003$) EXB1 mediated increase was also observed for *mHSP32* immediately post exercise, with the response absent ($p \geq 0.99$) immediately post EXB2. The hypoxia mediated increases

(approximately 60% (95% CI = 52.9% to 81.4%; $t = 11.1$, $p < 0.001$)) in basal *mHSP72* remained elevated (approximately 50% higher compared to control and pre EXB1) before the commencement of EXB2. *mHSP32* exhibited a similar hypoxia mediated response with increases of approximately 26% (95% CI = 3.2% to 47.6%; $t = 2.7$, $p = 0.03$) post hypoxic acclimation period, these elevations where enduring until the commencement of EXB2. The combination of increased bio available *mHSP32* and *mHSP72* prior to exercise commencing in EXB2 compared to EXB1 may acquiesce the disturbance to redox balance during the second, physiologically identical exercise bout. Furthermore, the favourable alterations in whole blood glutathione redox balance, before commencement of EXB2 compared to EXB1 (i.e. a reduction GSSG and increase in GSH), may, in tandem with elevated basal stress protein levels (*mHSP32* and *mHSP72*), or independently, potentially augment the body's ability to deal with exercise induced disturbances to redox balance.

The novel findings within this thesis include the establishment of the quadratic trend in basal *mHSP72* expression, which has been shown to be consistently repeatable. This diurnal variation was moderately correlated to core temperature. These findings illustrate the importance of controlling the time of day both interventions and blood samples are administered and collected respectively, specifically, due to basal values being indicatively linked to the magnitude of post stressor *mHSP72* expression. Furthermore, the successful use of hypoxia to induce *in vivo* elevations in basal *mHSP72* can be considered novel, as can the demonstration of day on day increases in basal *mHSP72* in response to once daily hypoxia (for ten consecutive days). This increase in *mHSP72* is the first demonstrated *in vivo* increase in basal *mHSP72* without the reliance on a thermal and/or mechanical stressor. However, objective evidence that such increases in basal *mHSP72* have conveyed any tolerance to further stressors has not been obtained. Some support in the final experimental chapter does indicate that a

protective adaptation may have occurred, although, the efficacy of increasing basal *mHSP72* expression to confer *in vivo* cellular tolerance to further stressors (exercise, hypoxia, hyperbaria, etc) has not been thoroughly investigated within this thesis and requires further, possibly *ex vivo* and/or *in vivo* research. Additionally, there appears to be some evidence that oxidative stress may be a stimulus for increases in *mHSP72*, as significant increases in TBARS are seen in conjunction with significant elevations in *mHSP72* in experimental chapters 4, 5 and 6. This increase in *mHSP72* may be linked to the significant daily increases in oxidative stress (lipid peroxidation – plasma TBARS) but a cause and effect relationship cannot be claimed. Caution is required when interpreting any change in lipid based oxidative stress and its interplay with increases in a protein (*mHSP72*) whose predominate role is to respond to stress induced changes in protein conformation. In further support of a change in redox balance providing a stimulus for increased *mHSP72* expression, whole blood glutathione ratios indicate a shift towards a pro-oxidant state have occurred in tandem with increases in *mHSP72 and mHSP32* within the final experimental chapter. The absence of such a shift in redox balance (post hypoxic acclimation period) coincided with an absence of significance increases in *mHSP72 and mHSP32* expression post exercise. Again, although a cause and effect relationship cannot be claimed to have been established this does provide some evidence that oxidative stress may be a stimulus for hypoxia and exercise induced increases in HSP72. Additionally experimental limitations and lack of prior relevant HSP72 data rendered the use of power/sample calculations not feasible; this is a limitation of the presented thesis. However, the data presented can be used to inform such calculations for future experimental designs. Furthermore, the thesis lacks the use of sham/control groups for experimental chapters 3, 4, 5, and 6 due to economic and logistical restrictions, such groups should be included in future experimental designs. Some chapters may have benefited from use of condition randomisation, but logistical

and economic restrictions rendered this unfeasible. Future research of a similar manner is recommended to include such randomisation if possible.

The novel findings presented in this thesis may have a part to play in hypoxia mediated cross tolerance *in vivo*. Specifically, the potential of the hypoxic protocol utilised to convey tolerance to the oxidative stress associated with sub-maximal aerobic exercise. Such cross tolerance has been postulated within this thesis to originate from hypoxia mediated increased bio available *mHSP32* and *mHSP72*, and favourable glutathione changes within the blood. However, future research should investigate such changes within the blood in tandem to those within the muscle, and expand upon the limited subject numbers used within the experimental chapters of this thesis (data presented can be used for future power/sample calculations). Additionally, attention must be paid to the expanding volume of literature which highlights oxidative stress as a key signalling molecule in the procurement of physiological adaptation (within skeletal muscle and blood). Therefore, it would be likely that a protocol such as the one developed here would be of greater benefit during a period of repeat athletic performance, i.e. within a competitive sporting season, whereby, a reduction in the disturbance to redox balance experienced by an athlete can minimise the recovery time required to return to optimal performance.

Chapter 1. General Introduction

1.1. General Introduction

Hans Seyle, a Canadian Physician, pioneered research (1950) into the events and environments humans endure on a daily basis and investigated how the body coped and/or adapted to ensure survival. Selye's research began in the 1950's, focusing upon the body's response to high (hyperthermic) and low (hypothermic) ambient temperatures, strenuous exercise and several other damaging stimuli, he in turn named these challenges stressors (Selye, 1950). Stressors are events or substances that disrupt and compromise homeostasis and can be internal (e.g. altered blood pH) or external (e.g. hyperthermic temperatures) in origin (Weinert, 1992).

Many occupational and recreational activities are beset with stressors, with commercial divers experiencing hyperbaria (Barratt et al., 2002) and underground miners enduring high ambient temperatures and humidity in locations such as South Africa and Australia (Stewart, 1982). Recently, the high altitude football controversy arose in May 2007 with international matches played at an altitude in excess of 2,750 m banned by FIFA, due to concerns over players' health and unfair advantage to altitude acclimated teams.

Sporting competition, training and recreational pursuits all place stress upon the human body. These exercise induced challenges include, but are not limited to, mechanical heat production and acquisition from the environment (Hargreaves, 2008), lactate accumulation (Davison et al., 2007) and increased reactive oxygen species (ROS) production (Albina et al., 2007). Increasingly, athletes utilise heat acclimation protocols (Sandstrom et al., 2008) to augment their performance during hyperthermic exercise (Madden et al., 2008a). Altitude/hypoxic training (Wilber, 2007), particularly the "live high" and "train low" strategy (Levine and Stray-Gundersen, 1997) is used frequently for its reputed benefits on exercise performance. This demonstrates humans have not

only overcome stressors to live their daily lives, but, have in fact, utilised them to their advantage.

The ability to overcome stressors, such as those mentioned previously serves testament to the adaptability of the human body, and is reliant upon multiple biological systems working in harmony. One such system is a group of highly specialised stress proteins, frequently termed heat shock proteins (HSP), which exert their protective influence upon numerous cellular compartments throughout the body (Kregel, 2002; Katschinski, 2004). It is not surprising considering the diverse and important roles stress proteins play in a number of biological functions (Bukau and Horwich, 1998), including cell survival (Garrido et al., 2001) and maintaining pertinent protein functions and structures (Hartl, 1996), that there has been a significant increase in scientific research in this area, with particular focus on exercise (Yamada et al., 2008) and disease (Soti and Csermely, 2007). Chronic disease (Kim et al., 2007), pharmacological interventions (Macario and de Macario, 2000) and carcinogenic tumour proliferation (Bolhassani and Rafati, 2008) are cited as inducers of increased stress protein expression.

Exercise, in comparison to homeostatically toxic stressors (pharmacological interventions, diseased states, etc), is unique, and is acknowledged as a non-toxic inducer of stress protein expression (Locke and Noble, 1995; Locke and Noble, 1997). Tissieres (1974) and colleagues, identified several subsets of these proteins following Ritossa's (1962) initial serendipitous discovery of chromosomal "puffing" under hyperthermic stress. These stress proteins are members of a multi gene family and are found in all major cellular compartments, with convention dictating that they are named according to their molecular weight (Benjamin and McMillan, 1998). Family members range in size (molecular weight) from 10 kiloDalton (kDa) to 170 kDa with differing roles within the cell dependent upon their location and isoform being expressed and occupy both prokaryotes and eukaryotes (Knowlton, 1997).

Exercise as a stimulus for stress protein expression elicits induction and synthesis of several distinct HSP family members (Morton et al., 2009c). The most frequently cited for their induction with exercise are, Ubiquitin (8 kDa) (Thompson and Scordilis, 1994), αB-crystallin (22 kDa), HSP27 (Thompson et al., 2001; Thompson et al., 2002; Thompson et al., 2003), HSP32 (Fehrenbach et al., 2003; Thompson et al., 2005), HSP60 (Khassaf et al., 2001; Morton et al., 2006; Morton et al., 2008) and HSP72 (Liu et al., 1999). Of these proteins, HSP72 has received greatest interest within the literature, particularly with regards to its stress inducible characteristics (Morimoto and Santoro, 1998) and its involvement with conveyed cellular tolerance (Kalmar and Greensmith, 2009). This stress induced increase in expression forms part of the heat shock response (HSR), commonly termed the cellular stress response (CSR) (Lindquist, 1986). The CSR has been utilised in clinical and applied exercise science settings for therapeutic treatments (Sherman and Multhoff, 2007) and augmentation of hyperthermic exercise performance (Sandstrom et al., 2008). *In vitro* cell cultures (Li et al., 2007), rodent exercise protocols (Shima et al., 2008) and human *in vivo* models (Yogaratnam et al., 2007c) have demonstrated and/or postulated elevated stress protein expression conveying tolerance to various ischemic/oxidative stressors (e.g. elevated ROS production) (Kalmar and Greensmith, 2009), including exercise (Morton et al., 2009c). This elevation in basal stress protein expression, *in vivo*, has typically involved a thermal (McClung et al., 2008) and/or mechanical stimulus (Paulsen et al., 2009). Therefore, an alternative method to induce such elevations would be beneficial, given the cost of the equipment and expertise required to generate a sufficient safe and reliable thermal stimulus, and the reduction in various facets of exercise performance that are attributable to exercise induced muscle damage (mechanical stimulus) (Ascensao et al., 2008).

In vitro evidence within human (Patel et al., 1995) and animal (Wang et al., 2006) cell lines and cultures suggest that hypoxia (Fei et al., 2007) and hyperbaria (Yogaratnam et al., 2007b) may be such an alternative method to induce increased basal expression in HSP72 *in vivo*. Additionally, any potential relationships between changes in redox balance and stress induced changes in HSP72 expression have not been investigated *in vivo*. This potential interplay could be important when discussing any likely mechanisms for conferred cellular tolerance or hormesis due to elevated HSP72 expression and alterations in redox balance – specifically as *in vivo* exposure to alterations in redox balance is increasingly cited as stimulus for hormesis to occur (Radak et al., 2008; Fisher-Wellman and Bloomer, 2009; Ji et al., 2010; Powers et al., 2010a).

In order to investigate this question securely a current gap within the literature will need to be addressed first. Currently, the basal expression of HSP72 has not been securely investigated, although attempts have been made (Fehrenbach et al., 2005; Fortes and Whitham, 2009) issues regarding control and the type of HSP72 assessed render the interpretation of these studies problematic (discussed in detail within the literature review and experimental chapters). Evidently, to investigate any stressor mediated changes in HSP72 the basal expression (any circadian and/or diurnal variation) of said protein must be established. Establishment of any such variation will allow any stressor mediated changes in *mHSP72* (hypoxia or hyperbaria) to be attributed to the interventions employed rather than any potential circadian and/or diurnal variation. Given the vital role HSP72 plays in whole body homeostasis (governor of protein homeostasis) and the importance of circadian/diurnal variations in human physiology (immunological functioning, sleep/wake cycles, blood pressure, etc) in general, it is not unfeasible that such circadian and/or diurnal variations may be present in *mHSP72* expression. This thesis will directly address these afore mentioned research questions.

These thesis aims (research questions) can be summarised as experimental objectives:

Experiment 1

i) Investigate the basal expression of HSP72 during a 24 h period at 4 h intervals.

ii) Influence and/or relationship of core temperature on basal HSP72 expression to be assessed.

Experiment 2

i) Investigate the repeatability of any circadian/diurnal trend in basal HSP72 expression.

ii) Compare intracellular and extracellular HSP72 expression sensitivity.

Experiment 3

i) Investigate the effect of hyperbaric air and hyperbaric oxygen on basal HSP72 expression.

ii) Explore whether any such hyperbaric stress mediated changes in HSP72 may have a relationship with alterations in redox balance.

Experiment 4

i) Investigate the effect of acute hypoxia on basal HSP72 expression.

ii) Explore whether any such acute hypoxia mediated changes in HSP72 may have a relationship with alterations in redox balance.

Experiment 5

i) Investigate the effect of once daily hypoxia for ten consecutive days on basal HSP72 expression.

ii) Explore whether any such daily hypoxia mediated changes in HSP72 may have a relationship with alterations in redox balance.

iii) Examine whether once daily hypoxia for ten consecutive days effects maximal oxygen consumption.

iv) Examine the EPO response to once daily hypoxia for ten consecutive days.

Experiment 6

i) Investigate the effect of once daily hypoxia for five consecutive days on basal HSP32 expression.

ii) Investigate the effect of prior induction of HSP32 and HSP72 on the exercise induced stress protein response.

iii) Investigate the effect of prior induction of HSP32 and HSP72 on exercise induced disturbances in redox balance.

Elucidation of these experimental objectives may allow greater efficacy in their application to exercise performance in challenging environments and may provide a strategy in conveying protection to the bio-chemical rigours of exercise.

Chapter 2. Literature Review

2.1. The heat shock protein family

The important role stress proteins play in various biological systems and cascades has ensured a significant increase in the scientific research in the area, particularly exercise performance (Whitham and Fortes, 2008; Yamada et al., 2008; Morton et al., 2009c) and their role in heat acclimation strategies (McClung et al., 2008; Sandstrom et al., 2008; Amorim et al., 2010; Magalhães et al., 2010). This literature review will introduce two key members of the stress protein families (HSP72 and HSP32), whilst, detailing at depth the regulation, expression and structure of HSP72 during homeostasis (circadian and diurnal expression) and under stress (exercise and environmental).

HSP70

The HSP70 family of proteins has four major members with HSP75 (mitochondrial HSP70) and HSP78 (Grp78, BiP) performing chaperone functions in the mitochondria and endoplasmic reticulum respectively. The remaining family members, and most widely studied proteins, are the constitutive heat shock cognate 70 (known as HSC70 or HSP73) and the highly inducible isoform HSP70 (HSP72). Specifically, the highly conserved and inducible HSP72 has a plethora of functions, predominantly overseeing protein homeostasis (Goldberg, 2003; Arslan et al., 2006). Protein specific functions include *de novo* folding (Fink, 1999), refolding (Hartl, 1996) and degradation (Garrido et al., 2001), in addition to postulated extracellular pro-inflammatory (Pockley, 2003; Hickman-Miller and Hildebrand, 2004; Asea, 2005) and intracellular anti-inflammatory responses (Ianaro et al., 2001). In brief, these specific functions enable HSP72 to provide acquired thermotolerance (Sandstrom et al., 2008), cytoprotection (Garramone et al., 1994; Lepore et al., 2000; Suzuki et al., 2000; Maglara et al., 2003; McArdle et al., 2004) and cross tolerance to non-related stressors (Levi et al., 1993; Horowitz, 2002; Arieli et al., 2003; Shein et al., 2005; Shein et al., 2007), with elevated levels postulated

to provide tolerance to the biochemical stresses that accompany exercise (Locke and Noble, 1995; Morton et al., 2009c).

Heme oxygenase-1 (HO-1 or HSP32)

Heme oxygenase, or HSP32 exists as two isozymes, inducible heme oxygenase-1 (HO-1) and a constitutively expressed heme oxygenase-2 (HO-2) (Yachie et al., 1999). Faulty HO-1 gene coding is implicitly linked to the pathophysiology of many human diseases, with pharmaceutical induction of HO-1 exerting both therapeutic and protective effects in experimental conditions of cardiovascular and neuromuscular disease (Exner et al., 2004; Soares and Bach, 2009; Gozzelino et al., 2010). Under homeostatic conditions HO-1 resides within the heme pockets of hemoproteins. Under stress, such as exercise induced oxidative stress, this stored heme becomes bio-available (free) to directly catalyse oxygen free radicals (Pamplona et al., 2007).

It has been shown that HO-1 not only provides cytoprotection to acute oxidative stress (Vile et al., 1994; Reeve and Tyrrell, 1999), but also mediates adaptive cytoprotective responses to oxidative stress that are associated with increased cellular survival in light of future oxidative insults (Balla et al., 1992). Thus, the role of HO-1 as a protective, anti-inflammatory and anti-apoptotic enzyme (Otterbein et al., 2000; Sikorski et al., 2004) is well established, as is its induction with exercise induced oxidative stress (Niess et al., 1999b; Thompson et al., 2005).

2.1.1. The HSP70 family of stress proteins

A serendipitous discovery falsely pigeonholed HSPs as solely heat sensitive (Ritossa, 1962; 1963b; 1963a; Tissiere et al., 1974), but modern research has demonstrated a plethora of stressors such as hypoxia (Patel et al., 1995; Weinstein et al., 2004), ischemia (Richard et al., 1996), oxidative stress (Kukreja et al., 1994a), exercise (Locke and Noble, 1995), hyperbaria (Yogaratnam et al., 2007c), hypothermia (Yang et al.,

1996) and hyperthermia (Ritossa, 1963b; Cairo et al., 1985) that can provoke increased expression of HSPs. Other diverse cellular insults such as oxidative/ischemic stress (Benjamin et al., 1990), substrate depletion (Febbraio and Koukoulas, 2000), immunological disorders (Hickman-Miller and Hildebrand, 2004) and acidosis (Gapen and Moseley, 1995) are known to induce elevated HSP expression also. Specifically, the cited inducers of HSP72 are classed as environmental, pathological and physiological in nature, see Figure 2.1 (Morimoto, 1998). Consequently, HSPs are more commonly termed stress proteins within the modern literature, which better reflects the diverse stressors which can induce their expression. Of these proteins, HSP72 and HSP73 have been investigated extensively (Tavaria et al., 1996). The highly stress inducible member of the 70 kDa stress protein family (HSP72) is expressed rapidly under cellular insult (Fehrenbach et al., 2000a; Fehrenbach et al., 2005; Morton et al., 2006), however, under homeostatic conditions its expression is low (Locke et al., 1991). Conversely, HSP73, the non-inducible member of the HSP70 family, performs vital roles within the cell during homeostasis (Kiang and Tsokos, 1998), whilst not being stress inducible (Locke and Noble, 1995; Locke and Noble, 2002). Inducible HSP72 predominately resides in the cytoplasmic or nuclear region, whilst, cognate HSP73 is found within all the major cellular compartments (Tavaria et al., 1996).

Housekeeping duties related to protein homeostasis, such as *de novo* folding of newly synthesised peptides, are serviced by HSP73. Conversely, in response to any stress induced changes in protein conformation, for example, hyperthermia induced peptide distortion, HSP72 is rapidly synthesised to assist in the refolding of damaged proteins (Hartl, 1996).

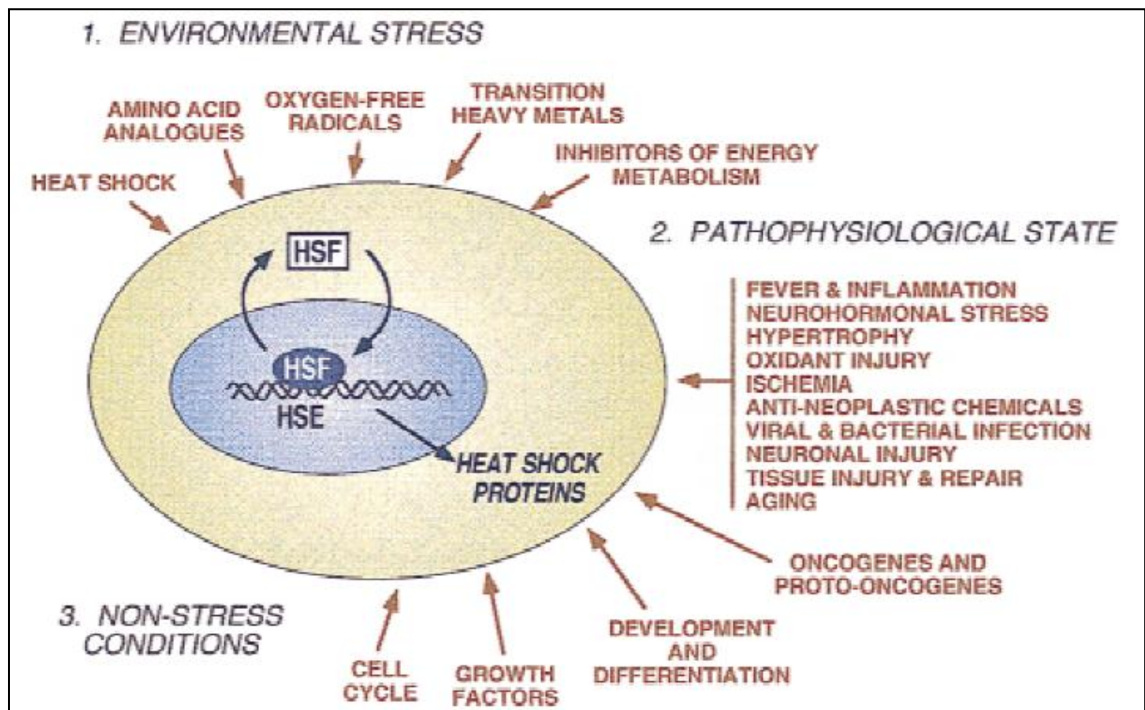

Figure 2.1. Conditions that induce the heat shock response. Heat shock gene expression represented here by the activation of heat shock factor and resulting binding to the heat shock element, with successful binding inducing HSP72 expression. The regulatory conditions are represented by environmental and physiological stress, taken from Morimoto (1998).

2.1.2. HSP72 and HSP73

Novel work from Locke and colleagues (1990) demonstrated the physiological differences in function and expression of HSP72 and HSP73. Rats were run on a motorised treadmill at 24 m/min for 20, 40, or 60 min, or to exhaustion (86 ± 41 min). Soleus muscles, peripheral lymphocytes and spleen cells were removed (Locke et al., 1990). Time specific differences were seen in HSP72 expression, with elevations only evident close to or at exhaustion within lymphocytes and spleen cells. However, soleous muscle tissue demonstrated increases in HSP72 after 20 min of running (Locke et al., 1990). During analysis two distinct isoforms of the HSP70 family (HSP72, HSP73) were observed, an acidic isoform (HSP73) synthesised at low levels in all tissues under homeostatic conditions and a basic highly inducible isoform (HSP72) which was not evident in any tissues under homeostatic conditions. This basic inducible isoform

(HSP72) demonstrated increased mobility and upregulation under stress in comparison to HSP73 (Locke et al., 1990). In summary, no changes in HSP73 expression were seen between control and fatigued tissues, whereas rats run for >20 min showed greatly enhanced HSP72 synthesis in all tissues in comparison to control. This early pioneering work clearly demonstrates the stress inducible characteristics of HSP72 and the presence of HSP73 under homeostatic conditions, yet lack of upregulation post stressor (Locke et al., 1990).

Further to this initial experiment (Locke et al., 1990), the fibre and muscle specific expression of HSP72 and HSP73 was explored, both in homeostasis and in response to exercise (Locke et al., 1991). The predominantly oxidative (red) soleous muscle demonstrated constitutive HSP72 expression, whereas, the glycolytic (white) portion of the gastrocnemius muscle expressed no basal HSP72 (Locke et al., 1991). Muscles providing postural support or ambulation are under constant albeit low levels of stress and possess a high distribution of oxidative fibres to service these minimal but enduring energy requirements. Low basal expression of HSP72 was seen in muscles predominately constituted of slow oxidative fibres (Locke et al., 1991), presumably to rectify any biochemical induced changes in protein conformation due to continuous oxidative metabolism. This basal expression of HSP72 has been commonly cited to perform "house keeping" protein duties (Locke and Tanguay, 1996; Pockley et al., 1998). Further to these animal models, within humans, various stressors have been shown to be efficacious in elevating HSP72 expression in peripheral blood mononuclear cells (PBMC), such as granulocytes and monocytes, with no change in HSP73 (Fehrenbach et al., 2000b). Similar findings have been found within human skeletal muscle (Thompson et al., 2003; Morton et al., 2008). The original animal physiology detailed by Locke and colleagues (1990 and 1991) is evidently seen in humans with regards to the stress inducible characteristics of HSP72 and HSP73 (Fehrenbach et al.,

2000b; Thompson et al., 2003; Morton et al., 2008). Despite these differences in function and expression, both isoforms share comparable biochemical characteristics with high sequence homology of 95 % (Welch, 1992).

2.1.3. HSP72 and HSP73 molecular structure

Both HSP72 and HSP73 are highly conserved members of the stress protein family and demonstrate a 60-78% amino acid base identity among eukaryotic cells and a 40-60% identity in comparison to eukaryotic HSP72 (Lindquist, 1986). Three distinct and functionally discrete domains (44, 18 and 10 kDa in weight) are evident when the molecular structure of HSP70 (HSP72 and HSP73) is scrutinised (Figure 2.2a) (Kiang and Tsokos, 1998). Both isoforms share identical 44 and 18 kDa domains, with molecular differences within the C-terminal, whereby, HSP72 differs by 26 amino acid residues and is 6 amino acids shorter than HSP73 (Leung, 1997). Recombinant DNA analysis has characterised and mapped the previously mentioned domains (see Figure 2.2b) (Denisenko and Yarchuk, 1990; Wang et al., 1993; Leung, 1997). The ATPase domain is contained within the 44 kDa fragment, whilst, the peptide binding domain that binds denatured and/or orchestrates *de novo* protein folding is located within the internal 18 kDa fragment (Wang et al., 1993). The peptide binding domain is where newly synthesised or denatured proteins are passed through and folded into their virgin form or sequestered and refolded to attain their native state (Hartl, 1996; Katschinski, 2004). As illustrated by Figure 2.2, both HSP72 and HSP73 possess a highly conserved terminal sequence motif (known as the EEVD terminal sequence) (Hightower, 1994). This sequence directly effects the magnitude of mRNA translation during heat shock (or other stressor) and is present in all eukaryotic HSP70s (Leung, 1997). Interestingly both HSP72 and HSP73 possess the same EEVD terminal and since mRNA expression is directly proportional to HSP72 synthesis (Mizzen and Welch, 1988) it is an oddity that HSP72 and HSP73 demonstrate such differences in upregulation in response to a

stressor. This elucidates towards a rate limiting mechanism, yet undiscovered, which exerts control on HSP72 expression during and post stress (Asea, 2007b; 2007a).

2.1.4. HSP72 regulation and mechanism of expression

The mechanisms underpinning transcriptional and translational control of HSP72 expression and regulation experienced a surge of research interest in the 1990's (Sadis and Hightower, 1992; Palleros et al., 1993; Szabo et al., 1994; McCarty et al., 1995; Banecki and Zylicz, 1996; Buchberger et al., 1996; Gamer et al., 1996; Hartl, 1996; Minami et al., 1996; Pierpaoli et al., 1997). These advancements (Tomanek and Somero, 2002) have clearly detailed four components of a conserved mechanism in the regulation of HSP72 expression and associated signalling pathways. Firstly, an accumulation of denatured proteins within the intracellular environment is required; this accumulation represents a potent stimulus for HSP72 induction and synthesis (Palleros et al., 1991; Sonna et al., 2002a; Sonna et al., 2002b). Secondly, the presence and physiological requirement of other HSPs (co-chaperones) or cellular factors (Minami et al., 1996; Tomanek and Somero, 2002). Thirdly, involvement of ATP hydrolysis and/or binding initiated activation (Sadis and Hightower, 1992; Wang et al., 1993; Szabo et al., 1994). Finally, differential recognition of target polypeptides and modulation of their conformation and/or assembly (Palleros et al., 1994; Gabai and Sherman, 2002). It can be seen that the transcription, translation, translocation and synthesis of human HSP72 are complex and multifaceted, and is subject to numerous intracellular and extracellular collaborations, including influence from higher centres (Asea, 2007b). A growing volume of research has implicated other members of the stress protein family (e.g. HSP40 and HSP90) to perform vital voles within HSP72 regulation (Minami et al., 1996; Tomanek and Somero, 2002).

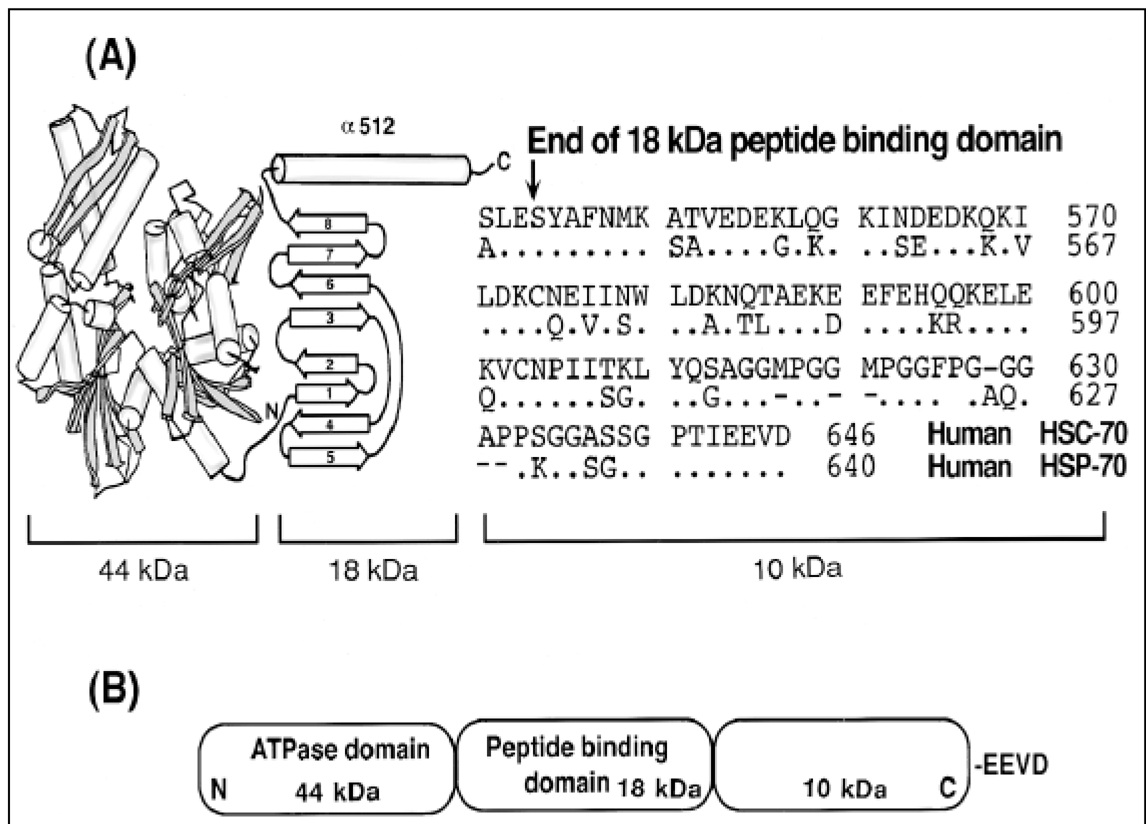

Figure 2.2a/b. Molecular structure of HSP73 (HSC70 above) and HSP72 (HSP70 above). (A) 44 kDa fragment (amino acid residues 1-386) at N-terminus contains 4 domains forming 2 lobes with a deep cleft between. 18 kDa fragment (amino acid residues 384-543) contains two 4-stranded antiparallel β-sheets and single α-helix. 10 kDa fragment (amino acids residues 542-646 for HSP73 and 542-640 HSP72) at c-terminus conserves EEVD terminal sequence. (B) N-terminal 44 kDa domain is ATPase domain; 18 kDa is peptide binding domain; c-terminal 10 kDa fragment carries highly EEVD terminal sequence, which is present in all eukaryotic HSP70 and HSP90, adapted from Kiang and Tsokos, (1998).

Those HSPs that aid or support other stress proteins to perform their function, aid translocation, or contribute to regulatory pathways are named co-chaperones (Fink, 1999). Cellular temperature sensing, and therefore HSP72 regulation, is dependent on free HSPs binding to amino acid analogues or HSF1. Establishment of this equilibrium is pivotal in maintaining protein homeostasis and/or coping with a cellular insult (Katschinski, 2004). This mechanism of HSF1/HSP72 activation is termed the cellular thermometer model and is widely accepted within the current literature (Tomanek and Somero, 2002; Katschinski, 2004). A schematic representation of this model is shown in Figure 2.5. Due to experimental advances in equipment and methods, and the sheer

volume and diversity of research conducted on the HSR, this model still has limitations and has scope to be elucidated further.

As discussed previously, a plethora of environmental, pathological and physiological stressors induce a marked increase in HSP72 (Lindquist and Craig, 1988). This upregulation in HSP72 expression is controlled at a transcriptional level via the synthesis of mRNA (Sonna et al., 2002a; Sonna et al., 2002b). Transcriptionally, HSP72 expression is regulated by heat shock transcription factors (HSFs), which act as an activator protein (Pirkkala et al., 2001; Akerfelt et al., 2007; Voellmy and Boellmann, 2007). The HSFs (see Figure 2.3 for molecular structure of HSFs) bind specifically with a positive control element, constructed of short nucleotide sequences, known as the heat shock element (HSE) (Akerfelt et al., 2007). The expression of HSP72 is reliant upon the presence of the HSE, which is located upstream in the promoter region of heat shock responsive genes and is constructed of five nGAAn amino acid sequences (Kiang and Tsokos, 1998; Morimoto, 1998). It is the HSE that represents the binding domain for constitutively expressed HSFs. There are four distinct HSFs, with HSF1, 2 and 4 found within the human gene pool and HSF3 found exclusively within avian biology (Pirkkala et al., 2001). With reference to HSF1 and Figure 2.3, the amino-terminal helix-turn-helix DNA binding domain (DBD) is the most conserved functional domain within HSF1 (Morimoto, 1998). The DBD is where an active HSF1 protein and the HSE of a target HSP gene (HSP72) bind via the leucine zipper (coiled-coil trimerization domain),this binding elicits HSP72 synthesis (Pirkkala et al., 2001). Upon successful complex formation and transcription, translation is initiated resulting in newly synthesised HSPs (Hartl, 1996).

Adjacent to the DBD, on HSF1, are three hydrophobic heptad repeats (HR-A/B) which mediate trimer formation (Sorger and Nelson, 1989; Clos et al., 1990; Peteranderl and Nelson, 1992). The homotrimer is formed when three HSF1 HR A/B repeats bind

together (see Figure 2.3) (Morimoto, 1998). The resulting monomer (repressed) to trimer (active) transition fully exposes the DBD (leucine zipper) allowing high affinity binding of HSF1 to the HSE of the gene promoter (i.e. HSP72) (Lu et al., 2007). Additionally, hyperphoshorylation of the HSF1 protein bound to the HSE is required to make HSF1 fully transcriptionally competent (Holmberg et al., 2001). The activation of HSF1 in this manner is essential as HSF1 in its monomeric state lacks transcriptional activity and DNA binding (Rabindran et al., 1993; Rabindran et al., 1994). Interactions

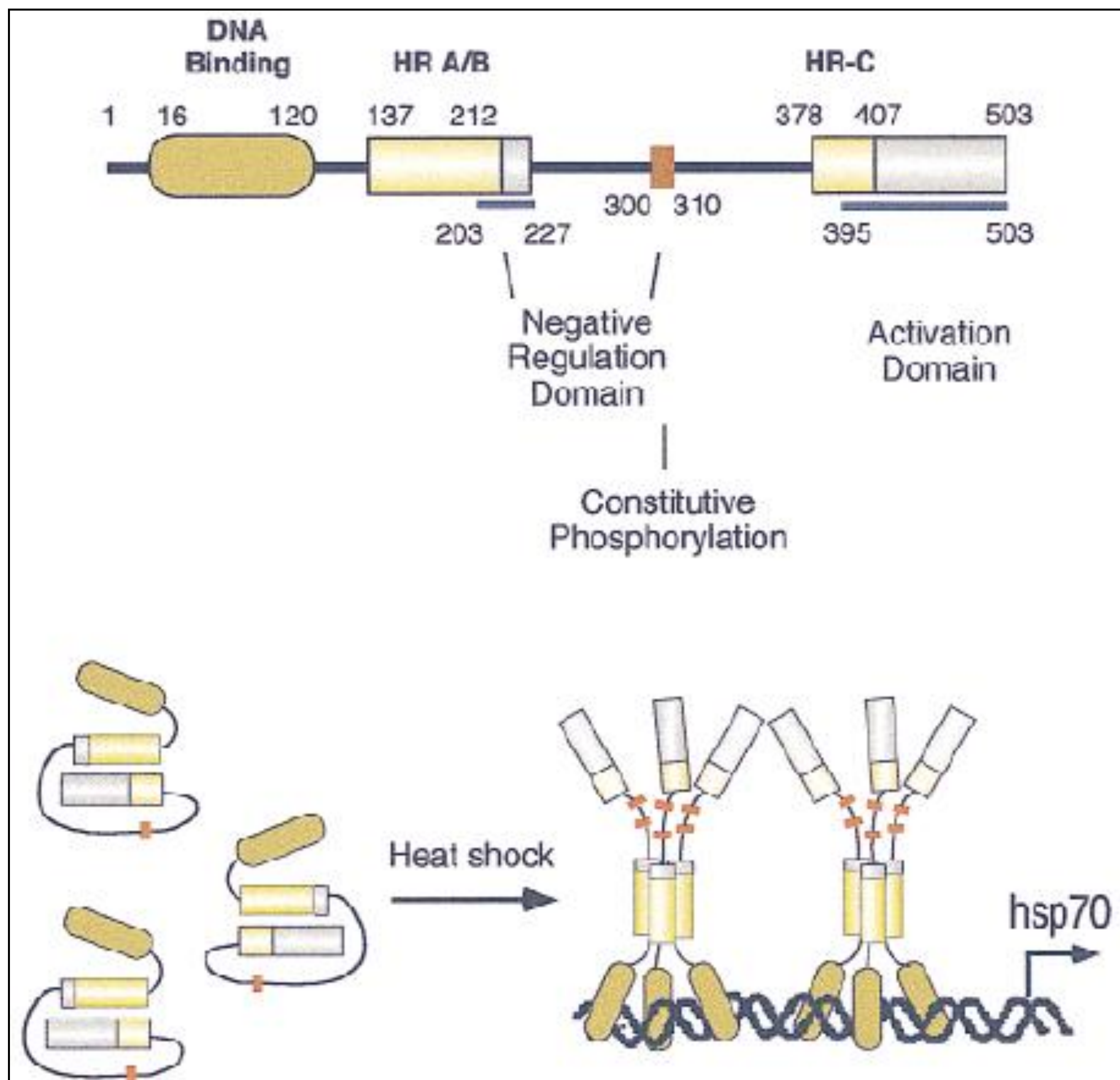

Figure 2.3. A schematic of the intermolecular negatively regulated HSF monomer that, upon stress exposure, is activated to from homotrimers with DNA binding activity, taken from Morimoto (1998).

between HSFs and other HSPs maintain HSF1 in its monomeric state, i.e. multi-chaperone complex, see Figure 2.5 (Tomanek and Somero, 2002). Therefore, the

construction of the HSF1 homotrimer is vital, if this did not occur, HSP72 would not dissociate from its multi-chaperone complex and acquiesce endogenous and exogenous stressors, i.e. hyperthermia induced peptide distortion.

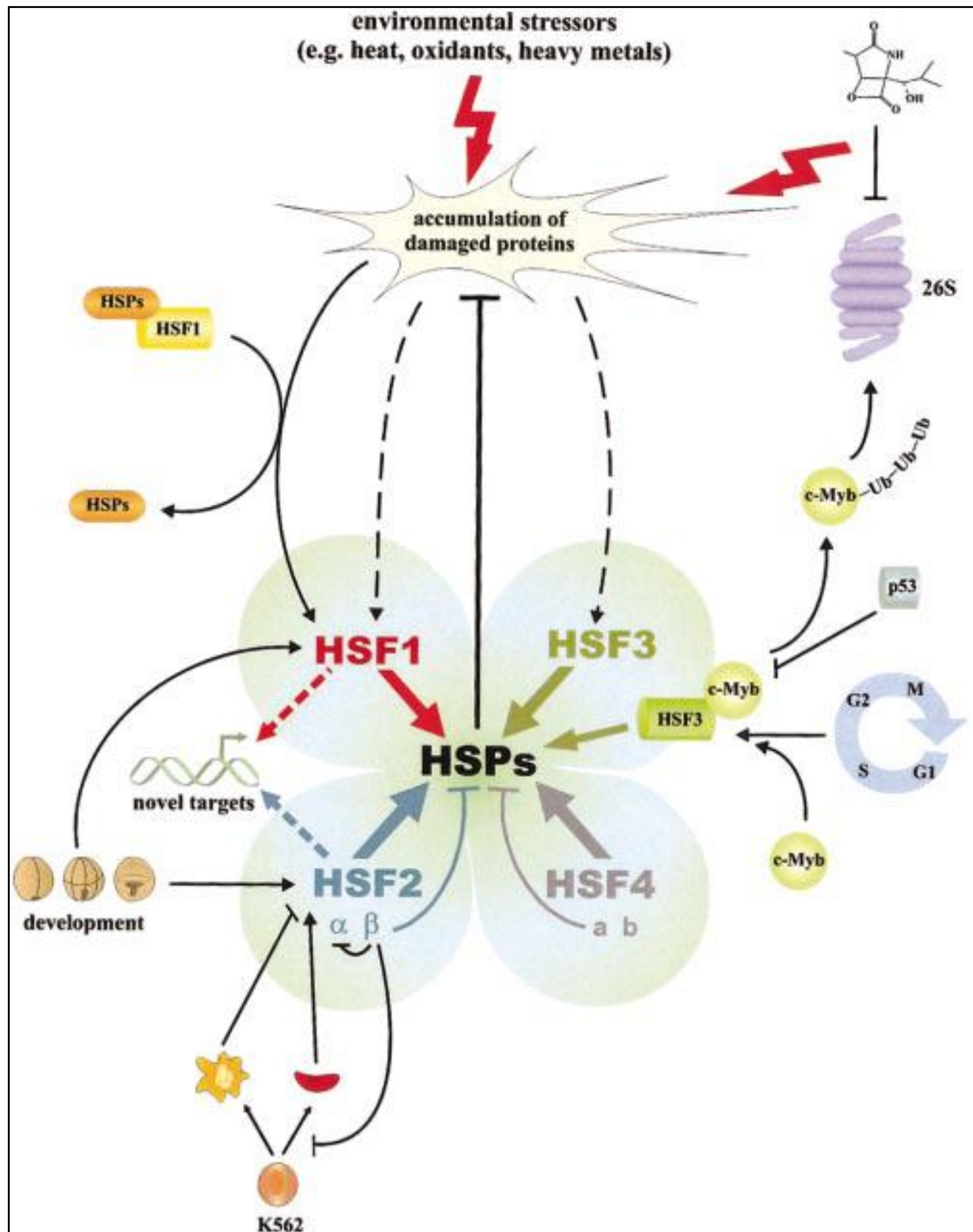

Figure 2.4. Model of differential HSF functions. Environmental stressors and dysfunctions in the ubiquitin-proteasome pathway induce accumulation of aberrant and short-lived proteins, which in turn leads to dissociation of HSF1/HSP complexes and activation of HSF1. Activation of the HSR ultimately leads to synthesis of HSPs, thereby initiating the attenuation of the stress response, taken from Pirkkala et al., (2001).

During cellular homeostasis HSF1 monomers co-exists with HSP72 (Rabindran et al., 1993; Rabindran et al., 1994) HSP90 (Zou et al., 1998; Bharadwaj et al., 1999) and HSP40 (Duina et al., 1998; Shi et al., 1998; Marchler and Wu, 2001) in a multi-chaperone complex (Pirkkala et al., 2001; Tomanek and Somero, 2002) . The formation of this complex holds the DBD in a foldedstate and thus prevents HSF1/HSE binding occurring i.e. locking HSF1 within the complex (Tomanek and Somero, 2002). Adjacent to the transactivation domain (carboxyl terminal) is another set of hydrophobic heptad repeats (HR-C) which exert negative control (attenuation) and suppression of the

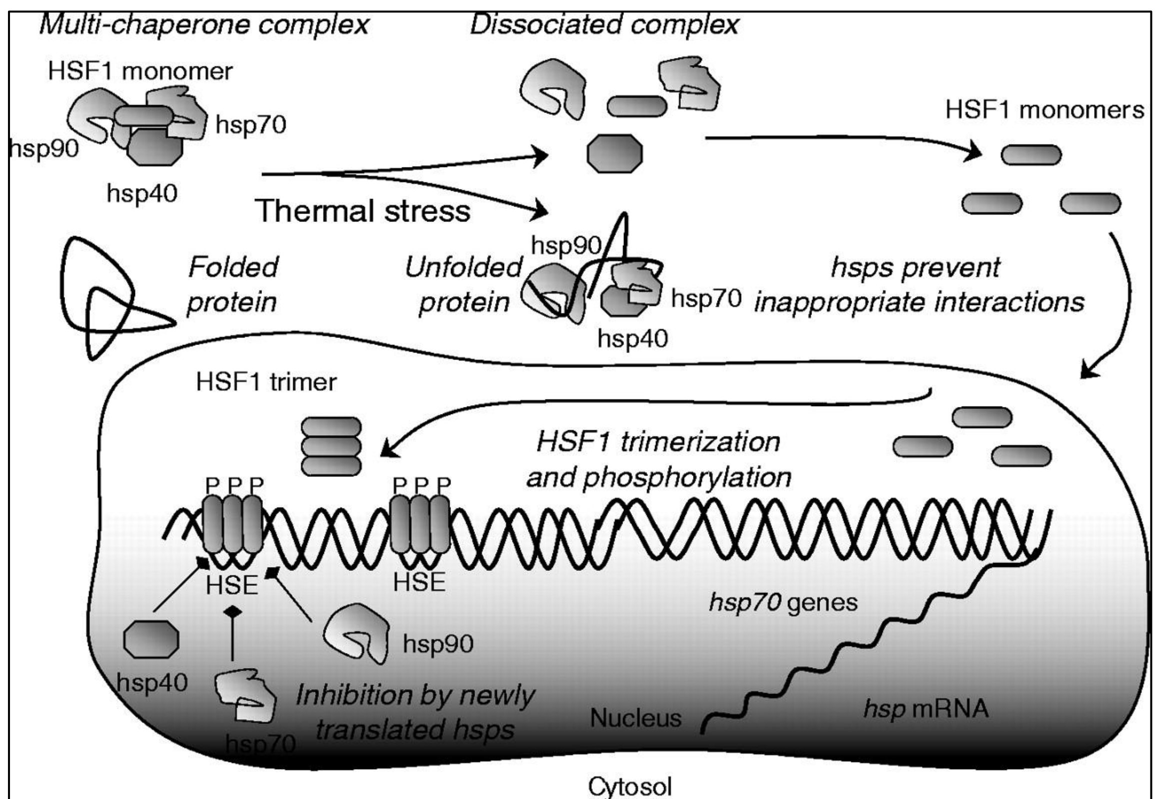

Figure 2.5. The regulatory model of transcriptional activation leading to the *de novo* synthesis of HSPs. Under non-stressful conditions, HSF1 monomers are associated with a chaperone complex that consists at of HSP72, HSP90 and HSP40. During stress, the chaperones dissociate from the complex and bind to unfolded proteins. Dissociation of the complex thus frees HSF1 monomers, which are then able to move into the nucleus and bind to the HSE. HSF1 trimers bound to the HSE become hyper-phosphorylated before they are transcriptionally competent. As HSP levels increase, their binding to HSF1 triggers its dissociation from the HSE, leading to a decrease in HSP gene transcription (diamond-shaped ends indicate this inhibitory effect), taken from Tomanek and Somero (2002).

HSR via interaction with the HR A/B repeats, i.e. prevents HSF1 trimerization (Nakai and Morimoto, 1993; Rabindran et al., 1993). Housed between the HR A/B and HR-C repeats are further sequences which negatively regulate transcriptional activation and DNA binding, additionally constitutive phosporylation also occurs within this region (McMillan et al., 1998). It is the interaction of the HR repeats which inhibit excessive or continued HSP72 expression. Disturbances in this process of inhibition have been identified as a source of chronic disease (Brodsky and Chiosis, 2006; Kim et al., 2007; Soti and Csermely, 2007).

2.1.5. Function of HSP72

The stress protein HSP72 is synthesised in cells across various compartments of the body in response to various stimuli, these include heat (Powers et al., 2001), hypoxia (Benjamin et al., 1990; Wang et al., 2006) and hyperbaria (Matsuo et al., 2000). The predominant function of HSP72 is to behave as a molecular chaperone (Arrigo, 2005; Voellmy and Boellmann, 2007). On a basic level a molecular chaperone can be summarised as a protein which binds to an unstable protein (aggregated amino acid sequence) thereby stabilising it, however, the function of HSP72 is not limited to this role (Gabai and Sherman, 2002). Binding and release of this targeted protein substrate allows regulation and continuity to ensure the correct *in vivo* changes, i.e. refolding of the protein, oligomeric assembly, or translocation and transport of the protein to a specific sub cellular compartment, can occur (Hendrick and Hartl, 1993). Additionally, this regulated binding and release of the target peptide allows functional interplay in various cellular signalling pathways by controlled switching between active and inactive conformations of the protein concerned (Buchberger et al., 1995). The protein folding action of HSP72 is well documented within the literature and is performed during homeostasis and under the influence of stressors. During homeostasis *de novo* protein synthesis (folding) is overseen and orchestrated by HSP73 whilst during cellular

insult HSP72 refolds denatured or aggregated proteins to their native state. Additionally, during episodes of stress HSP72 exerts an anti-apoptotic influence by protecting and preventing protein aggregation occurring in the first instance (Garrido et al., 2001). Finally, under stress if the protein cannot be refolded due to excess denaturing it can be recycled by HSP72 (degraded) (Kiang and Tsokos, 1998; Kregel, 2002).

2.1.6. Circadian and/or diurnal variation in HSP7

Rhythm is acknowledged as the most ubiquitous feature of nature and evolution (Lemmer, 2009). Living organisms, whether that is simple unicellular or complex multi-cellular organisms, are continually influenced by external stimuli (Hastings et al., 2008). It is the cyclic nature of such stimuli (principally daily and seasonal patterns of light resulting from the earth’s rotation around its central axis, food availability and climatic changes) that provide the stopwatch for many human, animal and plant biological rhythms (Reddy and O'Neill, 2010). Within human biology two major rhythms have been documented in biochemical, physiological and behavioural processes; circadian rhythms which follow an approximate 24 h cycle and diurnal variations which are within day fluctuations in amplitude (over 24 h period) (Reilly and Waterhouse, 2009). Key gross human *in vivo* circadian rhythms are known to include blood pressure, heart rate and body temperature (Maywood et al., 2006; Hastings et al., 2007; White, 2007; Lemmer, 2009), amongst many intricate cellular processes including gene expression and *de novo* protein synthesis (Hastings et al., 2008; Hofstra and de Weerd, 2008). The amalgamation of these cyclic processes is vital for optimal health (von Wichert, 2004; Maywood et al., 2006; Chrusciel et al., 2009; Montagnana et al., 2009) and performance (Reilly, 1990; Drust et al., 2005; Reilly and Waterhouse, 2009) of the organism in question, with the human body being no exception. Disruption to these human *in vivo* circadian rhythms reduce temporal homeostasis with concomitant health consequences, conversely those organisms whose internal clocks display synchrony are healthier and

have increased tolerance to stressors both internal (infection) and external (environmental) in origin (Reddy and O'Neill, 2010).

The past two decades have elucidated the effect of these cyclic patterns (often coined chronobiology) on *in vivo* human exercise performance (Reilly, 1990; Eichner, 1994; Drust et al., 2005; Hastings et al., 2008; Reilly and Waterhouse, 2009). For example, the circadian fluctuations in body temperature are known to produce differential ratings of perceived exertion (RPE), for identical exercise bouts, dependent on whether body temperature is at its lowest or highest temperature within the established diurnal variation (Waterhouse et al., 2004; Waterhouse et al., 2005). Perceived exertion was shown to increase markedly after 5 min of exercise at 0500 (lowest body temperature within circadian rhythm) compared to 1100 and 1700 (highest body temperature within circadian rhythm) (Waterhouse et al., 2004). Furthermore, the same study (Waterhouse et al., 2004) reported that after 30 min of exercise, the lowest RPE was seen when exercise began at 1100. Athletes whose internal clocks have been de-synchronised (crossing international time zones – jet lag) may suffer from various connotations of a similar ilk to those mentioned with regards to body temperature (Waterhouse et al., 2004; Waterhouse et al., 2005), with for example, psychomotor performance (Edwards et al., 2005; Edwards et al., 2007; Reilly et al., 2007), flexibility (Gifford., 1987) and muscle strength (Coldwells et al., 1994; Martin et al., 1999; Callard et al., 2000) all known to demonstrate a diurnal variation within a given circadian period.

Given the diverse roles HSP72 plays during homeostasis (governor of protein homeostasis (Hartl, 1996)), in diseased states (carcinogenic tumour proliferation (Bolhassani and Rafati, 2008)) and under exercise induced stress (molecular chaperone (Fehrenbach and Niess, 1999; Yamada et al., 2008; Morton et al., 2009b)) it is surprising that a well controlled project has not investigated its basal circadian rhythm. Postulations such as these have been tentatively explored within the literature; however,

they have not procured physiologically relevant findings and have suffered from low experimental numbers (Fehrenbach et al., 2005; Fortes and Whitham, 2009). Fortes and Whitham (2009) performed a power calculation based on the work by Fehrenbach et al. (2005) and concluded a required n of 7 (α=0.05, β=0.8) to investigate the presence of a circadian rhythm in extracellular HSP72 (eHSP72). However, this calculation failed to recognise the variance of eHSP72 across other previous research with regards to basal or control values (as reviewed by (Yamada et al., 2008)). Therefore, it is likely that a n of 7 is inadequate for such a experimental design, given the modest nature of the variation within the data used by Fehrenbach et al. (2005) (for their power calculation) compared to other basal/control eHSP72 data (Yamada et al., 2008). These rationalisations make the sample sizes used previously (Fehrenbach et al., 2005; Fortes and Whitham, 2009) unlikely to be adequate or accurate.

The attempts made by Fehrenbach et al. (2005) and Fortes and Whitham (2009) both utilised measures of eHSP72 via the problematic enzyme linked immunoassay (ELISA) kit/assay which lacks the sensitivity of iHSP72 analysis via flow cytometry (discussed in detail in section 2.6. and 3.5.) (Bachelet et al., 1998; Ireland et al., 2007). In brief, eHSP72 has limitations with regards to the bio-origin and cellular function of its presence (Yamada et al., 2008). Contradictory postulations have been made to the role of eHSP72 within the extracellular milieu, with eHSP72 being credited with both a pro- and anti-inflammatory (Pockley et al., 2008) role, with further insinuations as a player in both immunostimulatory and immunosuppressant cascades also made (Pockley, 2003). Evidently, it is unlikely that eHSP72 can function in biologically polar opposite roles within the immune (Pockley et al., 2008) and inflammatory (Pockley, 2003) responses *in vivo* (Pockley et al., 1998). Further compounding this lack of consensus to function of eHSP72 within the literature, equivocal evidence demonstrates that increases in eHS72 improve survivability in trauma patients (Pittet et al., 2002), yet, low

serum levels are indicative of decreased morbidity within other critically injured patients (Da Rocha et al., 2005).

Additionally problematic to the problems regarding eHSP72 function, is the bio-origin of eHSP72, which has not been clearly delineated *in vivo* or *in vitro*. Exercise mediated release of eHSP72 has been demonstrated from the brain (Lancaster et al., 2004) and liver (Febbraio et al., 2002a), though, concrete evidence of the bio origin of eHSP72 remains elusive *in vivo* (Yamada et al., 2008). *In vitro* evidence suggests the release of eHSP72 may originate from cell necrosis (Gallucci et al., 1999), lipid rafts (Broquet et al., 2003), exosomes (Clayton et al., 2005; Lancaster and Febbraio, 2005), B cells (Lancaster and Febbraio, 2005), PBMCs (Lancaster and Febbraio, 2005) or by exocytosis by glial cells (Guzhova et al., 2001), all of these may contribute in some way to the exercise or stress mediated *in vivo* release of eHSP72 (Yamada et al., 2007; Amorim et al., 2008; Whitham and Fortes, 2008; Yamada et al., 2008). Therefore, insufficient knowledge is present with regards to the source and function of eHSP72, which, makes postulations as to the worth of any potential physiological cyclic rhythms present limited.

Additionally, large eHSP72 inter- and intra-plate ELISA assay variance is evident within the literature (Yamada et al., 2008), which due to the amplitude of such variations across a circadian period makes identification of any circadian or diurnal variation in eHSP72 difficult to pinpoint. Therefore, issues regarding the bio-origin, biological function and the experimental error present make the assessment of eHSP72 with regard to a circadian variation flawed. It is this rationale which may underpin why previous research in this regard has found no circadian rhythm to be present (Fehrenbach et al., 2005; Fortes and Whitham, 2009).

Conversely, the bio-origin and function of intracellular HSP72 (iHSP72) is well cited within the literature (please see sections 2.1.4, 2.2 and 2.5) and thus examination of whether iHSP72 adheres to an *in vivo* circadian rhythm is both needed and more warranted than eHSP72. Currently, there is a lack of research specifically addressing any circadian and/or diurnal variation in iHSP72 *in vivo*, which is important, as varying levels (i.e. high or low) of basal iHSP72 are implicitly linked to an organisms, including the human body, ability to survive cellular and whole body stress (Paulsen et al., 2009). Given that basal values are indicatively linked *in vitro* (McClung et al., 2008) and *in vivo* (Vince et al., 2010) to stressor mediated expression, this lack of research into true biologically relevant *in vivo* basal values of iHSP72 are something this thesis will attempt to address directly.

2.2. HSP72, thermal preconditioning and cytoprotection.

Extended or chronic exposure to extreme stress is evidently harmful, yet, it is well reported that mild (preconditioning) stress, typically hyperthermia, can limit the negative outcome of subsequent stressful insults (Kalmar and Greensmith, 2009). As such transient exposure to most environmental stressors can convey protection to subsequent related and non-related stressors, with this protective mechanism underpinned by changes in concentration of the molecular chaperone HSP72 (Morimoto and Santoro, 1998). The utilisation of preconditioning stresses in this manner has been shown to protect skeletal muscle (Lepore et al., 2001), cardiac muscle (Benjamin and McMillan, 1998) and neurons (Brown, 2007) from further related (i.e. repeated exercise) and non-related stressors (heat shock preconditioning for oxidative challenges). In fact, a negative correlation has been shown between HSP72 levels and the degree of myocardial injury post infarct (Hutter et al., 1994).

Challenges to homeostasis, whether that is exercise or high ambient temperatures, are met with rapid synthesis of HSP72 mRNA. This stress induced transcription is rapid and is detectable within one minute post stressor (Perdrizet, 1997). Typically, a lag in transcription of HSP72 mRNA until HSP72 protein expression is seen, such as the 20 h delay demonstrated by others (Paulsen et al., 2007). This delay is an important factor when attempts are made to confer cellular tolerance, as it is the presence of the fully functioning protein and not the gene which exerts cytoprotection intracellularly (Guisasola et al., 2006). In general, muscle HSP72 protein is significantly increased 24 h post exercise, (Thompson et al., 2001; Thompson et al., 2002; Thompson et al., 2003; Morton et al., 2006) despite the almost immediate transcription of HSP72 mRNA (Puntschart et al., 1996; Perdrizet, 1997; Walsh et al., 2001). This period immediately after exercise, until protein expression is typically seen (24 h post exercise), has not been investigated extensively, likely due to subject discomfort from repeated muscle biopsy sampling, and as such, the precise time taken for protein induction post stressor requires further investigation. The presence of elevated HSP72 protein is evident up to seven days post a single bout of exercise (Khassaf et al., 2001; Morton et al., 2006). It is this prolonged presence of HSP72 within various cellular compartments both *in vitro* (Garramone et al., 1994; McArdle et al., 2004) and *in vivo* (Yamada et al., 2007; Amorim et al., 2008; Magalhães et al., 2010), which conveys cellular tolerance to related (Yamada et al., 2007; Amorim et al., 2008; Sandstrom et al., 2008; Magalhães et al., 2010) or non-related (Shima et al., 2008) sub-lethal stressors. This phenomena is widely regarded in the literature as conferred cellular tolerance (McArdle et al., 2004; Hung et al., 2005; Hammerer-Lercher et al., 2006; Madden et al., 2008a; Shima et al., 2008).

Classically, *in vitro*, cells are heat shocked with a sub lethal thermal challenge which induces HSP72 synthesis (Hammerer-Lercher et al., 2006). This increased bio-

availability of HSP72 enables subsequent stressful exposures to be counteracted more effectively than naïve cells (non heat shocked cells) (Zhang et al., 2002). This phenomenon was demonstrated within rodents who were heat shocked (15 min at 41-42 $^{\circ}C$) and then exercised at high environmental temperatures 48 hrs later. Control animals (~63 min) were unable to exercise for as long as preconditioned rodents (~89 min) within a hyperthermic (36.5^{o}C) environment (Chen, 1999). The authors attributed this augmented exercise capacity to significant elevations of HSP72 in the muscle and leukocytes, in addition to increased mitochondrial enzymes (Chen, 1999).

In addition to the classic heat shocked mediated acquired thermotolerance, recent work has focused on heat shock conveying tolerance to the oxidative rigours of various activities and for other non-related stressors. This recent work was stimulated by some classic findings within mice. These rodents, via transgenic upregulation of HSP72, demonstrated resistance to the biochemical rigours of ischemic reperfusion injury, specifically, a favourable return to optimal myocardial contractile function and reduced myocardial damage compared to those animals with lower HSP72 expression (Marber et al., 1995; Plumier et al., 1995). More recent work has utilised heat shock rather than transgenic manipulations to increase basal HSP72 content. A rodent model demonstrated that those animals that had undergone heat shock and thus had elevated HSP72 expression, displayed augmented reactive oxygen species (ROS) scavenging activity and reduced muscle injury post downhill running compared to control animals (Shima et al., 2008). Additionally, heat acclimation and the associated elevations in basal HSP72 have been shown to confer protection to brain injury within rodents (Shein et al., 2005; Shein et al., 2007).

Heat shocked cells when exposed to a subsequent stressor *in vitro* demonstrate a blunted HSP72 response in comparison to their initial response to the primary stressor (Li et al., 1983; Meyer et al., 1983; Ryan et al., 1991). Post exercise, *in vitro* heat shock of

PBMCs obtained post *in vivo* exercise demonstrates similarly blunted HSP72 responses (Fehrenbach et al., 2000b). In fact, more recent *in vivo* (Gjovaag and Dahl, 2006) and *in vitro* (Vince et al., 2010) work has demonstrated that the increases in HSP72 are directly proportional to the basal content of the tissues examined. Similarly, within exercising humans prior exercise blunts both the mRNA and protein HSP72 response to a repeated identical exercise bout (Vissing et al., 2009). It was postulated by others, that further translation of HSP72, in response to the subsequent stressor, is suppressed via negative feedback due to already high HSP72 expression (Madden et al., 2008a). These postulations draw support from the cellular thermometer model of HSP72 regulation discussed previously (Tomanek and Somero, 2002; Katschinski, 2004).

The predominant mechanism for this conveyed tolerance is the anti-apoptotic role HSP72 exerts in inhibiting or even preventing programmed cell death (Buzzard et al., 1998; Garrido et al., 2001; Suzuki et al., 2001; Quindry et al., 2007; Bienemann et al., 2008; Selkirk et al., 2009). Garrido et al (2001) hypothesised that HSP72 can interrupt the apoptotic cascade for damaged cells at various locations dependent upon the cellular insult in question. By doing this HSP72 provides the machinery and transportation for amino acid analogues (denatured proteins) to be refolded and/or transported to a homeostatically safe location respectively, allowing their correct structure to be reconstituted (Garrido et al., 2001).

In summary, it can be seen that heat shocking cells with a sub-lethal thermal stress confers a rapid cytoprotective influence via an upregulation in HSP72, which in light of a further cellular insult, e.g. hyperthermia, hypoxia or hyperbaria, can exert an anti-apoptotic influence (Horowitz, 2002; Biamonti, 2004). Despite the CSR being blunted (reduced HSP72 transcription), in light of a subsequent stressor, the previously elevated HSP72 content confers cellular protection and increased cellular survival (Madden et al., 2008a). This cytoprotective mechanism is referred to as conferred cellular tolerance.

2.3. Hypoxia and the HSP72 response

It has been postulated that the ubiquitous and highly inducible HSP72 serves as a useful marker of the cellular response to hypoxic insult (Fei et al., 2007). Hypoxia has been shown to induce HSP72 expression in animal (Wang et al., 2006) and human tissues (Patel et al., 1995) *in vitro*, but the magnitude of hypoxia utilised, often pharmacologically, is not comparable to the hypoxic load experienced by humans travelling to elevated altitudes. Human thyroid FRTL-5 cells *in vitro* pre-conditioned with heat shock to induce HSP72 expression display protection from the necrotic effect of hypoxia (Kiang et al., 1996). Kiang et al, (1996) hypothesised HSP72 may play a vital role in the defence of a hypoxic injury. However, the same response within human *in vivo* studies remains elusive, despite the well cited response *in vitro* within animal and human tissues/cells (Patel et al., 1995; Kiang et al., 1996; Wang et al., 2006).

Several animal model studies have confirmed HSP72 is up-regulated in response to hypoxia (Das et al., 1995; Bruemmer-Smith et al., 2001; Weinstein et al., 2004). Within a hypoxic environment the lung is the primary organ directly exposed to the low levels of oxygen (O_2). Mice exposed to hypoxic conditions for 3 wks displayed no change in HSP72 levels within total lung tissue. Interestingly, bronchiolar epithelial cells demonstrated a significant reduction in HSP72 expression, with an initial upregulation in expression seen in response to the hypoxic environment, however, this gradually fell during the course of exposure to levels ultimately lower than basal values (Kim et al., 2006). It has been shown that a dose response relationship exists in HSP72 expression within the cardiac muscle of Yaks, with elevations above 6000 m failing to invoke HSP72 upregulation, with peak expression seen at 5000m and gradually decreasing in magnitude to 3300m, whilst a plateau in expression was observed in elevations below 3300m (Wang et al., 2006). This plateau below 3300 m is likely attributable to the animals investigated being native highland animals (i.e. evolutionary residents of high

altitude domains) (Wang et al., 2006). With the afore mentioned *in vitro* and *in vivo* animal model work focusing upon a diverse array of animals any similarity of the response in human tissues *in vitro* and *in vivo* requires elucidation.

Shinkai et al. (2004) used human lymphocytes *in vitro* and exposed them to varying concentrations of hydrogen peroxide (H_2O_2). It was shown that mild to moderate O_2 stress (10–100 mmol.L^{-1} H_2O_2) induced a HSP72 response, with 100 mmol.L^{-1} H_2O_2 producing the largest response, with lower concentrations eliciting progressively smaller responses in a dose response manner. However, concentrations in excess of 100 mmol·L^{-1} H_2O_2 produced a reduced HSP72 response in comparison to the 100 mmol·L^{-1} H_2O_2 concentration. Additionally, the optimum response concentration protected cells from post stressor lethal damages, whereas severe oxidative stress in excess of 100 mmol·L^{-1} (i.e. 500 mmol·L^{-1} H_2O_2,), induced irreversible cell damage and induced significant apoptosis in human lymphocytes (Shinkai et al., 2004). This *in vitro* work (Shinkai et al., 2004) demonstrates a dose response relationship between HSP72 and hypoxia and provides evidence that hypoxic preconditioning mirror previous heat shock preconditioning protocols designed to convey oxidative protection (Lepore et al., 2000).

Research into the cardiac architectures stress protein response has shown human bypass grafts to demonstrate elevated HSP72 expression after 6 h hypoxia (Hammerer-Lercher et al., 2001). Furthermore, it has been shown that chronic exposure to hypoxia in infant human hearts leads to adaptation via increased HSP72 mRNA transcription and redistribution of HSP72 protein from the particulate to the cytosolic fraction. This response is important, as when viewed in tandem, increased mRNA transcription and sub cellular redistribution of HSP72 are fundamental in the mammalian HSR (Rafiee et al., 2003), and ultimately in the conveyance of protection to a subsequent cellular insult.

One human study (Vogt et al., 2001) has investigated the muscle HSP72 response to exercise under conditions of hypoxia, however, training conducted under the influence of hypoxia compared to intensity matched exercise under normoxic conditions did not demonstrate any significant differences between conditions. Furthermore, the influence of hypoxia alone was not investigated (Vogt et al., 2001). At present well controlled and designed human *in vivo* investigations into the HSP72 and HSP32 response, to both chronic and acute hypoxia, are lacking and require further elucidation. Specifically, disturbances to redox balance have been postulated to provide a stimulus for adaptation and increased resistance to cellular stress (Jackson et al., 2007; Powers et al., 2008; Kalmar and Greensmith, 2009; Powers et al., 2010a), similarly, stressor mediated increases in HSP72 is known to provide conveyed cellular tolerance *in vivo* (McClung et al., 2008; Paulsen et al., 2009). Hypoxia is known to disturb redox balance *in vivo* (Bailey et al., 2000; 2001), and has been shown in animal tissues to increase HSP72 expression (Das et al., 1995; Bruemmer-Smith et al., 2001; Weinstein et al., 2004), therefore the interplay of oxidative stress and HSP72 may be an important relationship when attempts are made to confer cellular tolerance *in vivo* to the bio-chemical rigours of exercise (Kalmar and Greensmith, 2009). Further elucidation of this relationship, would provide evidence to postulations made elsewhere that hypoxia/HSP mediated cross tolerance is initiated by oxidative stress and that oxidative stress (disturbed redox balance) itself may be a stimulus for increases HSP72 expression *in vivo* (Kalmar and Greensmith, 2009). Furthermore, none of the previous work detailed above has utilised a sensitive measure of intracellular HSP72 (iHSP72), such as flow cytometry, and have generally relied upon measures of extracellular HSP72 (eHSP72) via ELISA (discussed in detail in section 2.6.4.1).

2.4. Hyperbaria and the HSP72 response

Hyperbaric O_2 (HBO) is a common tool used for many clinical applications such as augmented healing of diabetic wounds (Zamboni et al., 1997), neuro-rehabilitation (Collet et al., 2001), the treatment of autism (Rossignol, 2007) and its traditional use in decompression illness (DCI) (Brubakk and Neuman, 2002).

Hyperbaria is considered a stressor to homeostasis and thus in line with other stressors such as heat (Lovell et al., 2008) and hypoxia (Fei et al., 2007) it would be postulated that exposure to hyperbaria would elicit increased HSP72 expression. Shyu et al. (2004) demonstrated HBO exposure (100% O_2, 3 atmospheres absolute (ATA), 1 h) elicited an elevated HSP72 expression within a mouse cell line. It became evident from this work that a dose response relationship exists between HBO and HSP72 expression *in vitro*. This relationship was proven to be both time and dose dependant at both an mRNA and total protein level (Shyu et al., 2004). Significant induction of HSP72 has also been reported *in vivo* 24 h post HBO exposure (100% O_2, 2.5 ATA, 3 x 20 min) in human lymphocytes (Dennog et al., 1999) and in response to a hyperbaric saturation dive (41 ATA 8 days, 32 day decompression) within the PBMCs (Matsuo et al., 2000). However HSP72 response to a traditional compressed air dive or wet dive remains undocumented.

Recent research has focused upon HBO pre-treatment increasing basal HSP72 expression in patients facing cardiac surgery (Yogaratnam et al., 2007a; Yogaratnam et al., 2007b; Yogaratnam et al., 2007c), however, the HSR to hyperbaria within a healthy subject population *in vivo* remains undocumented. Furthermore, none of the previous work detailed above has utilised a sensitive measure of iHSP72, such as flow cytometry, and have generally relied upon measures of eHSP72 via ELISA (discussed in detail in section 2.6.4.1).

2.5. HSP72 in response to exercise

Inducible HSP72 has been investigated extensively within the literature in response to exercise. Early work focused upon animal exercise models, or cell and tissue isolates/cultures, which mimicked a specific facet of the biochemical consequences of exercise. Such exercise induced or associated permutations in homeostasis, i.e., acidosis, hyperthermia, glucose deprivation and oxidative stress, where studied in isolation and in cohort with respect to HSP72 expression. More current research has moved away from cell and tissue isolates/cultures and focused on animal models with the very latest research now utilising human *in vivo* exercise studies. This section of the literature review will focus predominately on human *in vivo* exercise studies, but will first review early pioneering animal exercise research.

2.5.1. Pioneering animal HSP72 response to exercise

The first evidence of HSP72 expression being examined *in vivo*, in relation to exercise, was seen in rats that were swum to exhaustion (~59 min), exposed to hyperthermia (41 min, 42°C,) or hypothermia (87 min, 20°C), or had their descending aorta banded to induce ischemia (Hammond et al., 1982). Hearts extracted from those animals demonstrated no exercise or hyperthermia induced synthesis of proteins in the 70 kDa range. However, those exposed to hyperthermia and aortic banding had obvious increases in HSP72 post stressor (Hammond et al., 1982). Conversely, increases in HSP72 mRNA during and post exercise in both skeletal and cardiac muscle in rodents has been seen, with maximal elevations seen 30 – 60 min post exercise within skeletal muscle and 6 h post exercise in cardiac tissues (Salo et al., 1991). It was postulated by Salo et al. (1991) that the induction of HSP72 mRNA was the physiological response to the combination of the oxidative and hyperthermic stress of exercise. Exercised rats (treadmill running) demonstrated time dependant elevations in HSP72 protein

expression, both during and after exercise in muscle tissue, PBMCs and spleen cells compared to pre exercise controls (Locke et al., 1990; 1991). This expression was shown to be fibre type specific, with constitutive HSP72 expression only seen in muscles predominately oxidative in nature, with stress induced HSP72 expression seen almost exclusively in non-aerobic fibres (Locke et al., 1990). Conversely, findings elsewhere (Hernando and Manso, 1997), demonstrated a single exercise bout, induced HSP72 expression in both oxidative (soleus) and predominantly glycolytic extensor digitorum longus (EDL) muscle in comparison to control tissues. Hernando and Manso (1997) noted that HSP72 demonstrated a rapid yet transient increase in expression within the EDL, whilst, elevations in the soleus were stable and enduring. Again constitutive expression of HSP72 was predominately seen in the principally oxidative soleus muscle (Hernando and Manso, 1997). Further to these initial investigations, elevated HSP72 expression was seen in both soleus and EDL muscle 24 h post contractile activity (McArdle et al., 2001).

This early animal model work (Hammond et al., 1982; Locke et al., 1990; 1991; Salo et al., 1991; Hernando and Manso, 1997; McArdle et al., 2001) contributed significantly to further understanding of the *in vivo* stress protein response to exercise. However, despite investigation of the exercise induced HSP72 expression in animal muscle fibres (Hammond et al., 1982; Locke et al., 1990; 1991; Salo et al., 1991; Hernando and Manso, 1997; McArdle et al., 2001), the effect of exercise without associated increases in core/muscle temperature was not delineated. Some animal studies did investigate such temperature related issues with respect to exercise and HSP72 expression. Normothermic treadmill running in rats was shown sufficient to induce elevations in HSP72 within the locomotor muscles (soleous and gastrocnemius), however, non locomotive tissues (EDL) demonstrated no increase in comparison to control tissues (Skidmore et al., 1995). In line with previous findings (Locke et al., 1991), a fibre type

specific pattern of exercise induced HSP72 expression was seen in the muscles analysed (Skidmore et al., 1995). The findings above demonstrate that exercise alone can induce significant HSP72 expression within animal exercise models. However, although termed thermoneutral, the exercise in question (Skidmore et al., 1995) would produce heat storage within the skeletal muscle and as such there would be an obvious thermal stimulus to the accumulation of HSP72 despite no increases in core temperature (T_c). Notably, hyperthermic compared to thermoneutral exercise performance significantly increased HSP72 expression compared to exercise or hyperthermia alone by up to ~200% in a tissue dependant manner (Skidmore et al., 1995). Further animal model work consolidated these findings with acute exercise being shown to elevate HSP72 levels within organs such as the heart, liver, kidney and contracting skeletal muscle (Samelman, 2000; Samelman et al., 2000).

Prior to work by Demirel et al. (1999), duration and intensity of exercise stress had not been elucidated with respect to the magnitude of the HSP72 response. It was shown that adrenal HSP72 expression increases proportionally with increasing duration and intensity of daily exercise stress (Demirel et al., 1999). Additionally, as shown previously (Gonzalez et al., 2000), the increase in adrenal HSP72 was inversely related to basal values, i.e. pre stressor values indicatively linked to post stressor expression (Demirel et al., 1999). This dichotomy of pre exercise basal values and their influence on the magnitude of expression post stressor is consistently reported within the literature, *in vitro* (Vince et al., 2010) and *in vivo* (Gjovaag et al., 2006).

2.5.2. HSP72 response to exercise – human studies

The animal exercise models detailed in the previous section (2.5.1) provide insight into the exercise mediated HSP72 response. However, evidently, these projects do not address the exercise mediated HSP72 responses within humans. The following section

of the literature review will address such exercise mediated changes in HSP72 expression. Three principle tissues of expression are investigated with regards to exercise mediated changes in basal HSP72 expression. These tissues are skeletal muscle, PBMCs and the serum/plasma of blood. Given the multitude of published human *in vivo* articles on exercise induced changes in HSP72, summary tables (Tables 2.1, 2.2, and 2.3) for each tissue (muscle, PBMC and serum/plasma) have been provided. These tables provide information regarding the exercise type, intensity and duration whilst detailing the sample tissue, method of analysis and results gained. The reader is directed to such tables (Tables 2.1, 2.2 and 2.3) when reading sections 2.5.2, 2.5.3 and 2.5.4 (including all sub sections).

2.5.2.1. Human HSP72 skeletal muscle response to exercise

The response of skeletal muscle to acute and chronic exercise is of interest to physiologists, particularly those from the realm of exercise science. One such response, that of HSP72, is part of the broader protective stress protein response. Increases in HSP72 concentration service to acquiesce the biochemical and mechanical stressors associated with human *in vivo* exercise (Morton et al., 2009c). However, the plethora of exercise protocols (intensity, duration, mode, etc), subject characteristics (gender, age, training status, etc) and measurement techniques employed (western blotting, northern blotting, RT-PCR, fixed cycle PCR, SDS page) make inter and intra study comparisons problematic when examining previous literature.

Investigations of the HSP72 response within skeletal muscle generally examine the mRNA (gene) and total HSP72 (protein) response of vastus lateralis muscle and less frequently the biceps brachii. Muscle samples are obtained by muscle biopsy. This procedure has innate methodological problems due to its invasive nature and the

specialist skills and qualifications required to conduct the process. Unless otherwise stated, data presented were obtained by biopsy from the vastus lateralis muscle.

Aerobic exercise

Treadmill running has been shown to induce HSP72 gene expression (mRNA) between 0.5 and 3 h after exercise, with no increase in HSP72 protein (Puntschart et al., 1996; Walsh et al., 2001). This lack of increase in protein expression, to accompany increased gene expression, was due to insufficient sampling post exercise, i.e. in excess of 3 h (discussed later).

Active but untrained males, demonstrated increases in HSP72 protein 2 (~179%) and 7 days (~178%) post treadmill running (Morton et al., 2006) with the response absent in trained males (Morton et al., 2008). This lack of response was attributed to elevated basal HSP72 protein content within the trained subjects compared to untrained controls (Morton et al., 2008). This increased bioavailability of HSP72 protein was postulated to more readily service the biochemical challenges associated with aerobic activity compared to the lower basal values seen in untrained individuals (Morton et al., 2008; Morton et al., 2009c).

Exercise training induced differences between genders has been shown in HSP72 protein expression (Morton et al., 2009b). Continuous or interval training (6 wks) produced greater mean increases in men (~30.5%) than women (~3.5%) (Morton et al., 2009b). This project clearly details exercise induced gender differences in HSP72 protein expression. Mechanistically, Morton et al. (2009a) attributed these gender based differences to the protective effect of estrogen, principally its temperature regulating and antioxidant properties. It was postulated that these protective functions limited exercise mediated disruptions to homeostasis, thereby, reducing the stress stimulus for elevated HSP72 expression within the female subjects compared to the males (Morton

et al., 2009b). These postulations draw support from rodent exercise models whereby reduced exercise mediated muscle HSP72 induction is seen with exogenous administration of estrogen (Paroo et al., 1999; Paroo et al., 2002).

The HSP72 protein response to various acute and chronic treadmill protocols is evidently, gender (Morton et al., 2009b) and training status (Morton et al., 2008; Morton et al., 2009c) dependant. Other investigators have examined the HSP72 protein response to rowing exercise and training (Liu et al., 1999; Liu et al., 2000). A 4 wk training program within competitive rowers induced ~181%, ~405%, ~456% and ~363% week by week increases (from baseline) in HSP72 protein (Liu et al., 1999). The authors speculate (Liu et al., 1999) that the HSP72 response appears to be related to the total exercise amount (wk 1: 210, wk 2: 230, wk 3: 150, wk :4 130 min/day^{-1}), but this appears counterintuitive given the increases in HSP72 from baseline discussed previously do not correlate with these suggestions. This total exercise amount was composed of rowing (endurance training (mean blood lactate 0.45 mmol/l) and high intensity endurance training (mean blood lactate 6.4mmol/l)), unspecified training (gymnastics, etc) and resistance training with no information on intensity given for the latter two components (Liu et al., 1999). Evidently, due to the experimental limitations with regards to exercise intensity for the non rowing components, and the different ratios of the exercise components within each individual training week, the study does not address the question of whether the HSP72 response depends more on form, intensity or volume of exercise (Liu et al., 1999).

The limitations (exercise intensity and training block composition) discussed above (Liu et al., 1999) were addressed in a further publication from the same authors (Liu et al., 2000). Exercise intensity mediated differences were clearly shown in well trained rowers during a 3 wk training program (Liu et al., 2000), with prescribed training intensity peaks in group A (end of wk 1) and group B (end of wk 2) displaying the

greatest increases in HSP72 protein expression compared to all other time points during the training intervention (Liu et al., 2000).

In addition to running and rowing exercise, but to a lesser extent, cycling exercise induced changes in HSP72 mRNA and protein has been investigated. Exhaustive cycling has been shown to elicit increases in HSP72 mRNA (Febbraio and Koukoulas, 2000), whilst, 45 min sub-maximal cycling induced large increases in HSP72 protein content (Khassaf et al., 2001). Although examined independently previously (Febbraio and Koukoulas, 2000; Khassaf et al., 2001), it would be logical to assume, as detailed in responses to treadmill exercise (Morton et al., 2006), that if sufficient post exercise sampling was conducted, increased HSP72 gene expression would be indicative of increased HSP72 protein accumulation in response to cycling exercise. The influence of ambient temperature on cycling exercise has been investigated, with no change in HSP72 protein evident under hypothermic (9°C) or hyperthermic (39°C) conditions (Watkins et al., 2007). This lack of change can be attributed to the high basal pre exercise HSP72 values, due to inadequate exercise restriction before exercise, such restrictions are required as exercise induced increases in HSP72 are evident 7 days post exercise (Morton et al., 2006). When adequate pre exercise controls are in place, cycling exercise performed at a high or low intensity, demonstrated high intensity activity was required to induce elevated HSP72 protein expression (Vogt et al., 2001), in line with increases shown within trained rowers (Liu et al., 1999; Liu et al., 2000). When this high intensity cycling activity was performed under hypoxia or normoxia, no significant differences were seen between conditions (Vogt et al., 2001). However, at present, cycling exercise mediated HSP72 mRNA and protein responses are under investigated within the literature and would benefit from greater exploration, specifically, the co-investigation of gene and protein HSP72 expression in response to cycling exercise.

It can be seen that running (Morton et al., 2006), rowing (Liu et al., 1999) and cycling (Khassaf et al., 2001) exercise increase both HSP72 mRNA and protein content within the vastus lateralis muscle *in vivo*. These increases can be dependent on gender (Morton et al., 2009b), exercise intensity (Vogt et al., 2001) and training status (Morton et al., 2006; Morton et al., 2008). Additionally, basal values are also affected by training status (Morton et al., 2006; Morton et al., 2008) and gender (Morton et al., 2009b).

Static and resistance exercise

The nature of whole body exercise, such as running, manifests a multifaceted cascade of mechanical, biochemical and hyperthermic disturbances to whole body homeostasis. Consequently, exercise induced occurrences such as these are difficult to isolate and delineate from the general stress protein response to exercise. To alleviate some of these aspects, tightly controlled resistance exercise training protocols or repeated one limb movements have been employed.

Increases in both HSP72 gene and protein expression were seen 4-5 h post exhaustive two-legged knee extensor exercise (Febbraio et al., 2002b), whilst, eccentric preacher curl activity demonstrated increases in total HSP70 protein and gene expression 48 h post exercise (Thompson et al., 2001; Thompson et al., 2003). A further project, by this cohort (Thompson et al., 2002), utilised the same identical exercise bout separated by 4 wks, with no exercise training between bouts. Increases were seen 48 h post bout one (B1) and two (B2) respectively. Interestingly, pre exercise basal HSP72 protein content was reduced by a third in B2 compared to B1 (Thompson et al., 2002). However, this series of experiments (Thompson et al., 2001; Thompson et al., 2002; Thompson et al., 2003) has some limitations due to utilisation of mixed gender subjects, whose ratios have also been different within each experimental project. This flaw is evidenced due to the gender specific differences in the HSP72 response to exercise, i.e. males, on average,

demonstrating a much greater response (Morton et al., 2009b). This may underpin the large peak difference (837%) in HSP72 response between projects despite the identical exercise stimulus (Thompson et al., 2001; Thompson et al., 2002; Thompson et al., 2003). Similarly, 5 to 8 wks of resistance training of the elbow flexors in untrained subjects induced increases in HSP72 protein (Gjovaag and Dahl, 2006). Additionally, trained individuals exposed to 12 wks of high intensity concentric or eccentric training demonstrated no changes within the eccentric condition and a decrease of 46.1% in the concentric condition, with respect to HSP72 protein expression (Gjovaag et al., 2006). This lack of response (Gjovaag and Dahl, 2006), in contrast to that previously shown (Gjovaag et al., 2006), may be due to elevated basal values within trained compared to untrained subjects, as shown elsewhere (Morton et al., 2008). However, both these studies (Gjovaag and Dahl, 2006; Gjovaag et al., 2006), as do others (Thompson et al., 2001; Thompson et al., 2002; Thompson et al., 2003) lack stringent controls (training status and gender). Additionally, different durations and frequency of training interventions within conditions were used by Gjovagg and colleagues (2006). Therefore, interpretation of these results (Thompson et al., 2001; Thompson et al., 2002; Thompson et al., 2003; Gjovaag and Dahl, 2006; Gjovaag et al., 2006) must be done with caution.

A recent project, utilising a similar experimental protocol to Thompson et al. (2002), demonstrated ~315% (96 h post) and ~200% (48 h) increases in HSP72 protein content 48 h post exercise in bout 1 (B1) and bout 2 (B2) respectively (Paulsen et al., 2009). B1 and B2 were separated by 3 wks with no exercise training taking place between bouts. Interestingly, in contrast to Thompson et al. (2002), who demonstrated a decrease in basal HSP72 protein from baseline to immediately before B2, basal values remained elevated (by up to 50%) before the commencement of B2 compared to B1 (Paulsen et al., 2009). Peak post exercise expression was attenuated by approximately ~100% in B2,

compared to B1 (Paulsen et al., 2009). These kinetics, resemble those seen in *in vitro* models of cellular preconditioning, i.e. stressor mediated increases in basal HSP72 to confer cellular tolerance to a subsequent non-lethal stressor (McArdle et al., 2003). The increases in basal values before exposure to a second stressor, i.e. exercise, reduce the magnitude of HSP72 response to the second exposure. These *in vivo* findings (Paulsen et al., 2009) draw support from the cellular thermometer model of HSP72 regulation discussed previously in section 2.2.4. (Tomanek and Somero, 2002; Katschinski, 2004). However, this project (Paulsen et al., 2009) again suffered from the same limitations of mixed gender subjects, whose differential HSP responses to exercise (Morton et al., 2009b) ensure the observed results need careful interpretation. Despite these limitations, results (Paulsen et al., 2009) do support those shown elsewhere (Morton et al., 2008), whereby exercise training induces elevations in basal HSP72 protein.

With respect to prior activity blunting exercise induced elevations in HSP72 and increasing basal content, one all male resistance exercise project has been completed (Vissing et al., 2009). Two bouts of 30 min bench stepping (60 steps per min) performed 6 wks apart (no further exercise training between bouts) demonstrated no change in cytosolic HSP72 protein at any time. However, a ~240% increase post B1 was seen in cytoskeletal HSP72, yet post B2 no significant changes were seen. Additionally, attenuated HSP72 mRNA expression was also seen with a reduction of ~44% between bouts from ~890% (B1) to ~490% (B2) (Vissing et al., 2009). This increase in basal values due to a prior stressor again supports the notion of conferred cellular tolerance in light of a future cellular insult (McArdle et al., 2004). Furthermore, this attenuation in expression post subsequent stressor has been shown elsewhere (Thompson et al., 2002), although subject demographics, specifically, gender and training status (as discussed above) make these previous findings (Thompson et al.,

2002) less secure compared to the well controlled (gender and training status) project of Vissing et al. (2009).

Research with well controlled subject demographics elsewhere (Tupling et al., 2007), demonstrated some interesting findings in response to 30 min single knee extension exercise (30% MVC), with increases in HSP72 protein seen in a muscle fibre type dependant manner. Mean increases of ~396%, ~236% and ~214% within type I, IIa and IIx muscle fibres respectively, were seen in HSP72 protein content. The authors postulated that the differential responses were linked to the phonotypical redox balance and oxidative capacity of the specific fibre types in question (Tupling et al., 2007). Results (Tupling et al., 2007) may support postulations detailed elsewhere (Morton et al., 2009b), whereby, the absence of exercised induced elevations in female subjects was hypothesised to be due to the capacity of estrogen to exert protection to oxidative stress.

It can be seen that resistance or static exercises can induce both HSP72 gene and protein expression with prior activity evidently increasing basal content. This elevation reduces the magnitude of post stressor HSP72 expression in light of further exercise. These kinetics resemble those seen *in vitro* in instances of conferred cellular tolerance. However, due to some methodological limitations, particularly gender and training status of subjects recruited, research of this nature must be carefully critiqued during inter-study comparison.

2.5.2.2. Summary - human HSP72 skeletal muscle response to exercise

It can be seen that the exercise mediated, skeletal muscle HSP72 response is multifaceted and dependant on the mode (Thompson et al., 2003; Morton et al., 2009b), intensity and duration (Morton et al., 2009b) of the physical activity employed. Additionally, the gender and training status of the subjects examined contribute to the

HSP72 expression in response to exercise (Morton et al., 2009b). Even methodologically similar projects produce very different peak HSP72 protein responses (Thompson et al., 2001; Thompson et al., 2002; Thompson et al., 2003; Gjovaag and Dahl, 2006; Gjovaag et al., 2006). These differences in peak HSP72 response may partly be due to inadequate experimental controls (Thompson et al., 2001; Thompson et al., 2002; Thompson et al., 2003; Gjovaag and Dahl, 2006; Gjovaag et al., 2006), as it is known gender (Morton et al., 2009b) and training status (Morton et al., 2006; Morton et al., 2008; Morton et al., 2009b) of subjects recruited provoke differential HSP72 responses. Furthermore, there will be subtle methodological and laboratory based differences during sample analysis between laboratories, which in addition to afore mentioned control issues, may contribute to the differential HSP72 responses seen to similar exercise stimuli (Thompson et al., 2001; Thompson et al., 2002; Thompson et al., 2003; Gjovaag and Dahl, 2006; Gjovaag et al., 2006).

It can be seen that exercise of a sufficient intensity and duration initiates elevated HSP72 mRNA transcription, which when translated, results in elevated HSP72 protein both within the muscoskeletal architecture (myofibril) and cytosol (Paulsen et al., 2007). Further physical activity and exercise training can attenuate such protein expression, elevating basal values and servicing exercise stress more readily, thus reducing peak protein expression after similar exercise stress (Vissing et al., 2009). See Table 2.1 for a summary of all human exercise studies investigating the muscle HSP72 response.

Table 2.1. Summary table of human exercise studies investigating skeletal muscle HSP72

Authors	Exercise type	Tissue	Measurement	Results (peak significant increase in iHSP72 from baseline)
<u>Acute Exercise</u>				
(Puntschart et al., 1996)	Treadmill running 30 min at AT	Vastus Lateralis	Western Blot Northern Blot	No significant ↑ HSP72 protein any time point ↑~400% mRNA at all time points post exercise
(Febbraio and Koukoulas, 2000)	Cycling to exhaustion at ~65% VO_{2max}	Vastus Lateralis	Fixed Cycle PCR	↑~220% HSP72 mRNA 40 min into exercise ↑~260% HSP72 mRNA 40 min at fatigue Did not measure HSP72 protein
(Walsh et al., 2001)	Treadmill running 60 mins 70% VO_{2max}	Vastus Lateralis	RT-PCR	↑~650% mRNA 2 h post No change total protein
(Khassaf et al., 2001)	Cycling 45 min 70% VO_{2max}	Vastus Lateralis	Western Blot	↑~3200% HSP72 post 6 days post exercise
(Thompson et al., 2001)	Eccentric bicep curl MVC 2 x 25 reps	Biceps Brachii	Western Blot	↑~1064% 48h post exercise total HSP70
(Thompson et al., 2002)	1st bout 1st bout 2 x 25 MVC arm extensions 2nd bout 2 wk later 2 x 25 MVC arm extensions Eccentric preacher curl action	Biceps Brachii	SDS Page and Western Blot	↑~73% 48 h post exercise total HSP70 ↑~80% 48h post exercise total HSP70
(Febbraio et al., 2002b)	Exhaustive cycling (4-5 h 40% max power output)	Vastus Lateralis	Fixed Cycle PCR	↑~200% HSP72 protein and mRNA
(Thompson et al., 2003)	50 x MVC 50 x MVC Treadmill running 30 min self paced Treadmill running 30 min self paced	Biceps Brachii	PCR PCR	↑ ~227% 48 h post exercise total protein ↑ ~320% 48 h post exercise mRNA No significant changes total protein ↑ ~200% 48 h post exercise mRNA

(Morton et al., 2006)	Treadmill running 45 min at LT	Vastus Lateralis	Western Blot	↑ ~179% HSP72 protein 48 h post exercise ↑ ~178% HSP72 protein 7 d post exercise
(Tupling et al., 2007)	30 % MVC 30 min knee single knee extension	Vastus Lateralis	Western Blot I-histochemistry	↑ ~396% HSP72 in Fibre Type I ↑ ~236% HSP72 in Fibre Type IIa ↑ ~214% HSP72 in Fibre Type IIx ↑ ~200%/~1000% at 0.5/96h post (myofibril)
(Paulsen et al., 2007)	300 max eccentric quadriceps contractions	Vastus Lateralis	Western Blot I-histochemistry Northern Blot	↑ ~200% 24, 96, 168 h post (cytosolic) ↑ ~1500%/~2000%/~1000% at 4/8/24 h post (mRNA)
(Watkins et al., 2007)	Cycling 60 min 75% VO_{2max} (39°C) Cycling 60 min 75% VO_{2max} (9°C)	Vastus Lateralis	Western Blot	No significant ↑ HSP72 protein any time point No significant ↑ HSP72 protein any time point
(Morton et al., 2008)	Treadmill running 45 min 75% VO_{2max} (trained subjects – ~7.7 h/wk physical activity	Vastus Lateralis	Western Blot	No significant ↑ 48 h or 7 day post exercise
(Vissing et al., 2009)	1st bout 30 min bench stepping at 60 steps min^{-1} 2nd bout 8 wk later repeat of above	Vastus Lateralis	Western Blot	Cytosolic HSP72 protein expression no change ↑240% Cytoskeletal HSP72 7 d post bout 1 ↑ 890% increase HSP72 mRNA 3h post 1st bout ↑ 490% increase HSP72 mRNA 3h post 2nd bout
(Paulsen et al., 2009)	1st bout x70 MVC arm extension 2nd bout 3wk later x70 MVC arm extensions	Vastus Lateralis	Western Blot	↑ ~315% 96 h post ↑ ~200% 48 h post
<u>Exercise Training</u>				
(Liu et al., 1999)	Rowing 4 wk ET	Bicep Brachii	Western Blot	↑ ~181, ~405, ~456 and ~363 each respective week – total HSP72 protein.
(Liu et al., 2000)	Group A Rowing 3 wk ET Intensity Peak Wk 1 Group B Rowing 3 wk ET Intensity Peak Wk 2	Vastus Lateralis	SDS PAGE	↑ ~64% (control to wk 1) HSP72 protein ↑ ~67% (control to wk 2) HSP72 protein

(Vogt et al., 2001)	*High Group 30 min 5 x wk 65.6% VO_{2max} High Group 30 min 5 x wk 67 % VO_{2max} *Low Group 30 min 5 x wk 52.4% VO_{2max} Low Group 30 min 5 x wk 57.8% VO_{2max} *= hypoxia (3850m) / relative VO_{2max} stated NB all groups cycle ergoemter	Vastus Lateralis	PCR	High – Normoxia Peak ↑ 146.5% High – Hypoxia Peak ↑ 137.7% Low – Normoxia Peak No significant ↑ Low – Hypoxia Peak No significant ↑
(Gjovaag et al., 2006)	Tricep extensions 12 wks of 2-3 trainign sessions p.wk of 8-4 reps high/low intesity 1RM	Biceps Brachii	Western Blot	No significant change eccentric group ↓~33% concentric group
(Gjovaag and Dahl, 2006)	Tricep Extensions various intensities and volumes ~6 wk training program	Biceps Brachii	Western Blot	No significant changes between conditions ↑~111% mean response across all conditions
(Morton et al., 2009b)	Continous Training 50% VO_{2max} (26 to 32 min) Interval Training 50% VO_{2max} 6 x 4 min - interspaced with 100% VO_{2max} 5 x 1 min N.B All conditions treadmill running	Vastus Lateralis	SDS-PAGE and Western Blot	↑~38% ↑~23%

↑ (increase), ↓ (decrease) ~ (mean), MVC (maximum voluntary contraction), AT (anaerobic threshold), LT (lactate threshold)
N.B. Peak significant percentage increase calculated from control or resting sample were possible.

2.5.2.3.Human HSP72 response in peripheral blood mononuclear cells (PBMC)

Blood derived leukocytes were the first tissue to undergo *in vivo* investigation of exercise induced permutations in their iHSP72 expression utilising western blot analysis (Ryan et al., 1991). Recently, flow cytometry and its use to evaluate exercised induced changes in PBMC iHSP72 expression has grown in popularity. Principally, due to the highly specific identification of HSP72 expression (unlike eHSP72 via ELISA), whilst, not requiring a medically trained practitioner for sample collection (i.e. muscle biopsy collection). Furthermore, specifically within monocytes, it is recognised as a rapid and reliable method of evaluating HSP72 expression (Bachelet et al., 1998). As such, its use within the field of exercise science is growing rapidly. These and other methods of HSP72 analysis *in vivo* have been discussed and reviewed further in section 3.5. The interpretation of blood and muscle HSP72 data has not been sufficiently investigated in unison *in vivo*. Therefore, inter-study comparisons must be done with caution, as changes in blood and muscle HSP72 are at present, not known to be reflective or indicative of one another and as such their expression may in fact be differential or opposing. These facts must be considered when comparing and contrasting the muscle and blood (PBMC) HSP72 response to exercise.

Early work utilising western blot analysis of PBMCs lacked the specificity and sensitivity of flow cytometry (Bachelet et al., 1998; Nolan and Sklar, 1998; Vignali, 2000; Fehrenbach, 2005; Herzenberg et al., 2006; Ireland et al., 2007). This likely accounts for early work demonstrating no change in leukocyte expression post treadmill running (Ryan et al., 1991; Shastry et al., 2002) or cycle ergometer exercise (Chang et al., 1998). Similarly insensitive, RT-PCR analysis has shown no change in PBMC iHSP72 post hyperthermic cycling (Marshall et al., 2007).

Analysis by flow cytometry has demonstrated, post half marathon completion (~90.34 finish time), increases in HSP72 positive granulocytes (G) and monocytes (M), 24 h after exercise, with no change seen in lymphocytes (L), although, increases in leukocyte mRNA were evident (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b), These projects also demonstrated, conversely to findings within the muscle (Morton et al., 2008), that trained subjects had lower basal M HSP72 (*mHSP72*) and G HSP72 (*gHSP72*) (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b). However, *ex vivo* heat shock of basal PBMC cells (untrained and trained) demonstrated significantly accelerated HSP72 mRNA synthesis in trained compared to untrained samples (Fehrenbach et al., 2000a). This demonstrates that despite lower basal HSP72, trained individuals are better able to acquiesce non-lethal thermal stress via an accelerated HSP72 response, which is also of a greater magnitude (Fehrenbach et al., 2000a). Regular aerobic exercise can therefore be assumed to down regulate basal *mHSP72* and *gHSP72* expression, yet under a challenge to homeostasis, this protective cellular mechanism is more rapidly available to protect cellular function. Ambient temperature dependant differences have also been shown in response to treadmill running with a prior hyperthermic run, in comparison to a normothermic run, increasing basal HSP72 content and thus conferring tolerance to a subsequent hyperthermic exercise bout (Fehrenbach et al., 2001). This increase in bio-available HSP72 is now regarded as a classic indicator of acquired thermotolerance, with this project being the first to demonstrate such findings within exercising humans (Fehrenbach et al., 2001). This paradigm is something that had been extensively demonstrated *in vitro* (Garramone et al., 1994; Lepore et al., 2000; Suzuki et al., 2000; Maglara et al., 2003; McArdle et al., 2004). These studies (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Fehrenbach et al., 2001), and one other (Whitham et al., 2004) have consistently demonstrated no

change in L HSP72 expression post exercise, and, as such modern literature focuses upon only *mHSP72* and *gHSP72* expression in response to *in vivo* exercise stress.

A recent well controlled project investigated the effect of training status on *m*HSP72 expression during uncompensable heat stress. In response to hyperthermic treadmill walking (40°C), trained subjects demonstrated significant increases in *mHSP72* (range 50% to 100%) at T_c in excess of 38.5 ^{o}C; a response which was absent within untrained controls (Selkirk et al., 2009). These findings are in line with previous work where trained subjects, despite lower basal values, display larger and more rapid increases in HSP72 response to unaccustomed heat stress compared to untrained subjects (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Fehrenbach et al., 2001). This rapid increase in PBMC HSP72 is likely to resist the negative bio-chemical rigours of hyperthermic exercise as it has been shown that high basal PBMC HSP72 content is indicative of improved thermotolerance (Fehrenbach et al., 2001), with pronounced increases in basal PBMC HSP72 content seen after exercise heat acclimation protocols (Yamada et al., 2007; McClung et al., 2008; Magalhães et al., 2010). However, some of these heat acclimation projects (McClung et al., 2008) suffer from the control issues stated previously (mixed gender subjects), and therefore, must be examined carefully.

2.5.2.4. Summary – human HSP72 response in peripheral blood mononuclear cells

It can be seen that flow cytometry is a more sensitive measure of PBMC expressed HSP72 at rest and in response to exercise in comparison to western blot and RT-PCR techniques. In general, exercise increases both M and G HSP72, with no change evident after exercise in L (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Fehrenbach et al., 2001). Training status effects basal HSP72, with trained compared to untrained populations displaying decreased basal expression. Despite this, HSP72 is more rapidly unregulated and attenuated in response to exercise and hyperthermic exercise within

trained subjects (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Whitham et al., 2004; Magalhães et al., 2010). See Table 2.2 for a summary of all human exercise studies investigating the PBMC HSP72 exercise response.

Table 2.2. Summary table of human exercise studies investigating the PBMC HSP72 response

Authors	Exercise type	Tissue	Measurement	Results (peak significant increase in iHSP72)
<u>Acute Exercise</u>				
(Ryan et al., 1991)	Treadmill walk/run 2 h total exercise 4-6 kph 30-45 min and 75-90 min at 2-10% grade	L	Western Blot	Unchanged expression in L any time point
(Chang et al., 1998)	Cycling 20 min 60% VO_{2max}	L	Western Blot	Unchanged expression in L any time point
(Fehrenbach et al., 2000a)	Running half marathon ~90.34 min	G L M	Flow Cytometry Flow Cytometry Flow Cytometry	↑~125% in $HSP72^+$ cells 24 h post Unchanged $HSP72^+$ cells any time point ↑~300% in $HSP72^+$ cells 24 h post
(Fehrenbach et al., 2000b)	Running half marathon ~90.34 min	G L M	Flow Cytometry Flow Cytometry Flow Cytometry	↑~100% in $HSP72^+$ cells 24 h post Unchanged $HSP72^+$ cells any time point ↑~200% in $HSP72^+$ cells 24 h post
(Fehrenbach et al., 2001)	Normothermic and hyperthermic condition Bout 1 treadmill running 60 min 90% AT (18°C) Bout 2 treadmill running 60 min 90% AT (28°C) Hyperthermic condition Bout 1 treadmill running 60 min 90% AT (28°C) Bout 2 treadmill running 60 min 90% AT (28°C)	G L M G L M	Flow Cytometry and RT-PCR Flow Cytometry and RT-PCR	↑~67% in $HSP72^+$ cells 24h h post mRNA reported only no sig ↑ ↑~67 % in $HSP72^+$ cells 24 h post ↑~67% in $HSP72^+$ cells 24h h post mRNA reported only no sig ↑ ↑~67 % in $HSP72^+$ cells 24 h post
(Shastry et al., 2002)	Treadmill Running 60 min 70% VO_{2max}	L	Western Blot	Non significant ↑~0.4ng% 15 h post
(Schneider et al., 2002)	Not detailed – simply referred to as exercise induced.	G L M	Flow Cytometry Flow Cytometry Flow Cytometry	↑~45% in $HSP72^+$ cells 24 h post Unchanged $HSP72^+$ cells any time point ↑~55% in $HSP72^+$ cells 24 h post

(Shin et al., 2004)	Trained - Treadmill running 60 min 70% HRR Untrained - Treadmill running 60 min 70% HRR	PBMC	Western Blot	↑ ~141% 30 min post ↑ ~139% 30 min post
(Selkirk et al., 2009)	Treadmill walking to exhaustion 4.5 kph 2% incline 40°C clothing ensemble = un-compensable heat stress	M	Flow Cytometry	↑~10% in HSP72+ cells at T_c 39.5°C in trained subjects No significant ↑ any T_c untrained subjects
Exercise Training				
(Whitham et al., 2004)	Cycling 73% increase in training load for 6 days Pre = before intensified training Post = after intensified training period Endurance trained cyclists	Pre G Pre L Pre M Post G Post L Post M	Flow Cytometry Flow Cytometry Flow Cytometry Flow Cytometry Flow Cytometry Flow Cytometry	↑~30% in HSP72+ cells 24h post VO_{2max} Unchanged HSP72+ cells any time point Unchanged HSP72+ cells any time point Unchanged HSP72+ cells any time point Unchanged HSP72+ cells any time point ↑~25% in HSP72+ cells 24h post VO_{2max}
(Yamada et al., 2007)	10 days - walk/run 100 min 53% VO_{2max} (~42.5°C)	PBMC	SDS-PAGE	↑~100% days 6-10 compared to control
(Marshall et al., 2007)	Cycling 120 min 38% VO_{2max} (~38.0 °C) 3 consecutive days	PBMC	RT-PCR	No significant ↑ HSP72 protein any time point
(McClung et al., 2008)	Treadmill walking max 100 min per day1.56 m/s 4 % gradient 10 consecutive days (49°C)	PBMC	Western Blot	↑200~% mRNA post exercise day 1 ↑~18% HSP72 protein
(Magalhães et al., 2010)	90 min treadmill running 50% VO_{2max} (40°C) pre (HST1) / post (HST2) 11 day acclimation period(*) *30 min treadmill running 1% grade ~2.20 m.s.$^{-1}$ + *30 min treadmill running 1% grade ~1.69 m.s.$^{-1}$ * performed consecutively	PBMC	Western Blot	Pre to post HA ↑~300% HST 1 ↑~200% (post), ↑~350% (1h post) HST 2 ↑~300% pre, post and 1h post compared to control

G (granulocytes), L (lymphocytes), M (monocytes), AT (anaerobic threshold), HRR (heart rate reserve), HST (heat stress test), HA (heat acclimation)
N.B. Peak significant percentage increase calculated from control or resting sample where possible.

2.5.2.5. Human eHSP72 serum and plasma response to exercise

Exercised induced changes in blood soluble HSP72, or extra cellular HSP72 (eHSP72), have been extensively reported within the literature in both the serum and plasma of whole blood. The origin of eHSP72, i.e. tissue of release, is not known with certainty and as such postulations about the physiological effect of changes in eHSP72 are problematic. Inter and intra ELISA plate variability and extensive use of both "in house" and commercially available ELISA kits introduce variability in the data reported. Additionally, an improvement in sensitivity from 0.8 ng/mL to 0.2 ng/mL with the introduction of a new high sensitivity ELISA kit further exasperates the problems with data variability.

In general, basal serum derived eHSP72 values tend to be lower than those obtained from plasma (Whitham and Fortes, 2008). Consequently, when percentage changes are calculated, serum values are higher compared with those found within the plasma. Further complicating differences between sample type, is the recently demonstrated differences in plasma eHSP72 sample yield when using various anti-coagulation blood tubes, with EDTA coated tubes yielding approximately 137% greater eHSP72 values compared to their heparin counterparts (Whitham and Fortes, 2006). These relative differences are important to consider when comparing exercised induced permutations in eHSP72 within humans.

<u>Aerobic exercise</u>

Serum and plasma eHSP72 have shown to be increased after treadmill running by 685% (Walsh et al., 2001) and 170% (Fehrenbach et al., 2005) respectively, despite similar activity load. This demonstrates the large differences sample choice, i.e. serum (Walsh et al., 2001) or plasma (Fehrenbach et al., 2005), can make when quantifying exercise mediated changes in eHSP72.

It can be seen that running, whether that be on the track, within a laboratory on a treadmill, or during competitive road running, induces elevations of eHSP72, which are intensity and duration dependant (Walsh et al., 2001; Fehrenbach et al., 2005; Gomez-Merino et al., 2006; Horn et al., 2007). One project (Fehrenbach et al., 2005) measured the eHSP72 response to an array of running loads, with, for example, a ~725% increase in plasma eHSP72 seen after half marathon completion, compared to 170% increases post 60 min treadmill running (75% $\dot{V}O_{2\max}$) (Fehrenbach et al., 2005). Individuals who completed 100 km of road running (~539 min completion time) (Gomez-Merino et al., 2006) or 90 min of treadmill running (Horn et al., 2007) have also shown plasma increases of 1600% and 270% respectively – both studies (Gomez-Merino et al., 2006; Horn et al., 2007) utilised matched (age, VO2max, weight and height) groups of sub-elite runners.

This demonstrates the influence of load on exercise induced changes in eHSP72. The influence of running gradient has been shown to produce differential eHSP72 responses within the plasma, with downhill compared to neutral gradient running resulting in approximately 50% higher peak increases (Peake et al., 2005). These elevations were correlated to increases in bio-chemical muscle damage within the downhill compared to neutral gradient condition (Peake et al., 2005). Peake et al. (2005) did not provide a mechanistic answer for this increased eHSP72 release within the downhill condition, the origin of exercise mediated eHSP72 has still not been sufficiently addressed within the literature (Whitham and Fortes, 2006) at present and thus mechanism of release is not known (discussed later in this section).

At this point the eHSP72 response to running exercise, within both the serum and plasma, is evidently intensity, duration and exercise modality (damaging and non-damaging) dependant (Walsh et al., 2001; Fehrenbach et al., 2005; Peake et al., 2005; Gomez-Merino et al., 2006). However, the environment in which the exercise had been

performed had been tightly controlled to remain thermoneutral or was unreported, which has previously been shown to be an important factor in the HSP72 response to exercise within the PBMCs (Fehrenbach et al., 2001). Runners performing a 14 km road race, who finished (~58 min completions time) with serious symptoms of exertional heat illness displayed up to ~2900% increases in plasma eHSP72 compared to those finishers with minor symptoms of exertional heat illness and control subjects (~68 min completion time) who had peak increases of ~850% (Ruell et al., 2006). Furthermore, clearly delineating the contribution of elevated T_c in differences in eHSP72 expression within the plasma, it was shown that 120 min of underwater running with T_c clamped (approximately 36.25^{o}C), or unclamped (approximately 38.5^{o}C), produced increases of ~129% and ~212% respectively in plasma eHSP72 (Whitham et al., 2007). Furthermore, a 10 day heat acclimation period, demonstrated ~110% increases from pre exercise day 1 to post exercise day 10 in serum eHSP72 (Yamada et al., 2007; Amorim et al., 2008). This demonstrates that exercise mediated increases in eHSP72 can be influenced by changes in ambient temperature. Furthermore, aerobically trained individuals appear better equipped to deal with such challenges in hyperthermic environments (40°C). Trained individuals display elevated basal plasma eHSP72 values and a more speedy response to the increases in T_c associated with uncompensable heat stress during treadmill exercise, compared to their untrained counterparts (Selkirk et al., 2009).

Cycling compared to running is fundamentally less damaging to the muscle architecture (Starkie et al., 2001; Clarkson and Hubal, 2002), even when identical intensities are utilised. As such the sum exercise stress from these bouts is lower than those of a similar intensity when running. These elementary differences are seen within the literature when comparing peak increases in eHSP72 within the serum or plasma in response to running or cycling/non weight bearing fixed leg exercise. In general, cycling induced elevations in eHSP72 are much lower.

Contradictory evidence exists with zero (Febbraio et al., 2002b) or ~300% (Fischer et al., 2006) increases in serum HSP72 seen after prolonged two legged leg extensor activity. Similarly low increases (Febbraio et al., 2002b), just above the limits of detection of the assay utilised, have been shown post cycle ergometer exercise (Febbraio et al., 2002a; Febbraio et al., 2004; Lancaster et al., 2004). Exercise mediated increases of ~0.88ng/ml (Febbraio et al., 2002a), ~0.9ng/ml (Febbraio et al., 2004) and ~1.0ng/ml (Lancaster et al., 2004) are only 0.10ng/ml above the limit of detection for the assay used at this period of time, and explain why basal values were undetectable in the aforementioned projects. This inability to detect low basal and exercise induced values in serum eHSP72 led to many studies utilising plasma measures of eHSP72. On this note, a project demonstrated clearly the increased sample yield of eHSP72 from plasma compared to serum, with the former yielding up to a 32 fold exercise mediated increase compared to the latter (Whitham and Fortes, 2006). Additionally, this project also detailed the need to use EDTA anti-coagulation blood collection tubes, opposed to heparinised tubes, with the former yielding a 2.4 fold greater sample yield than the latter in plasma samples (Whitham and Fortes, 2006). Despite empirically backed data supporting a switch to measures of plasma eHSP72, several projects have continued to use serum derived measures of eHSP72. Such mistakes are evident in projects investigating acclimation to hyperthermic exercise, whereby, although increases have been shown during and post protocol, the peak increases of approximately 90% (Marshall et al., 2006; Yamada et al., 2007; Amorim et al., 2008; Sandstrom et al., 2008) are small compared to thermoneutral acute exercise bouts (increases of 150 – 180%), investigating plasma eHSP72 (Whitham et al., 2006).

Origin of eHSP72 in response to exercise

The physiological importance of post exercise changes in eHSP72 remain elusive, with equivocal evidence demonstrating that increases improve survivability in trauma

patients (Pittet et al., 2002), yet, low serum levels are indicative of decreased morbidity within other critically injured patients (Da Rocha et al., 2005). Furthermore, despite demonstrated exercise mediated release of eHSP72 from the brain (Lancaster et al., 2004) and liver (Febbraio et al., 2002a), concrete evidence of the bio origin of eHSP72 remains elusive. *In vitro* evidence suggests the release of eHSP72 may originate from cell necrosis (Gallucci et al., 1999), lipid rafts (Broquet et al., 2003), exosomes (Clayton et al., 2005; Lancaster and Febbraio, 2005), B cells (Lancaster and Febbraio, 2005), PBMCs (Lancaster and Febbraio, 2005) or by exocytosis by glial cells (Guzhova et al., 2001), all of these may contribute in some way to the exercise mediated *in vivo* release of eHSP72 (Yamada et al., 2007; Amorim et al., 2008; Whitham and Fortes, 2008; Yamada et al., 2008). More recently, the role of eHSP72 as an immunostimulatory or immunosuppressive agent has been postulated, however, this immunomodulatory function is likely dependant on the nature and context that eHSP72 is present and requires further extensive *in vivo* investigation (Pockley, 2003; Pockley et al., 2008).

2.5.2.6. Summary - human eHSP72 serum and plasma response to exercise

It can be seen that exercise mediated changes in eHSP72 are intensity (Fehrenbach et al., 2005), duration (Fehrenbach et al., 2005) and mode dependant (Febbraio et al., 2002a; Febbraio et al., 2002b; Peake et al., 2005). The use of plasma derived eHSP72 is recommended for increased assay efficacy with basal and exercise induced values higher within plasma compared to serum (Whitham and Fortes, 2006). Furthermore, it can be seen that hyperthermia and exercise alone can induce elevated eHSP72 compared to pre exercise values, however, the combination of both results in increases which are elevated in excess of their combined singular sum responses (Whitham et al., 2007). Attempts to investigate the eHSP72 response to extended periods of exercise training have not been well controlled and riddled with methodological oversights, such as insufficient exercise abstinence before basal HSP72 values are obtained (Banfi et al.,

2004). Despite the extensive research conducted into plasma and serum exercise mediated changes in eHSP72, results still require careful interpretation as the bio-origin of eHPS72 remains elusive and as such the physiological consequences of changes in eHSP72 necessitate further elucidation. See Table 2.3 for a summary of all human exercise studies investigating the eHSP72 serum/plasma response.

Table 2.3. Summary table of human exercise studies investigating the serum and plasma eHSP72 response to exercise

Authors	Exercise type (subjects)	Tissue	Measurement	Results (peak significant increase eHSP72)
Acute Exercise				
(Walsh et al., 2001)	Treadmill running 70% VO_{2max}	S	ELISA	↑~685%
(Febbraio et al., 2002a)	120mins cycling 62% of VO_{2max}	S	ELISA	↑ ~0.88 ng/ml[1] arterial
(Febbraio et al., 2002b)	4-5 h exhaustive two legged knee extensor exercise 40% of leg peak power output	S	ELISA	No eHSP72 in any samples pre or post
(Febbraio et al., 2004)	Cycling 120mins at ~65% VO_{2max} with regular glucose intake	S	ELISA	↑ ~0 – 0.9 ng/ml[1] arterial
(Lancaster et al., 2004)	Cycling 180mins 60% VO2 max (6 endurance trained males)	S	ELISA	↑ ~1.0 ng/mL arterial
(Hirose et al., 2004)	Bout 1 -6*5 reps MVC arm extensions	P	ELISA	↑~140%
	Bout 2 - 6*5 reps 4 wk separation MVC arm extensions			↑~120%
(Fehrenbach et al., 2005)	Running 260mins ~65% VO_{2max} (42.2 km)	P	ELISA	↑~725%
	Running 35mins ~88% VO_{2max} (10x1000 m)	P	"	↑~150%
	Running 60mins 75% VO_{2max}	P	"	↑~170%
	Running 120 mins 60% VO_{2max}	P	"	↑~140%
	Running 24 mins 80% VO_{2max}	S	"	↑~1100%
	Running 24 mins 60% VO_{2max}	S	"	↑~250%

(Peake et al., 2005)	Treadmill running 60 mins flat 60% VO_{2max} Treadmill running 60 min flat 85% VO_{2max} Treadmill running 45 min downhill 60% VO_{2max}	P P P	ELISA " "	↑47% ↑147% ↑88%
(Suzuki et al., 2006)	Competitive ironman	P	ELISA	↑~2100%
(Whitham et al., 2006)	Cycling 90 min 74% VO_{2max} With caffeine supplementation	P	ELISA "	↑146% ↑180%
(Whitham and Fortes, 2006)	Cycling VO_{2max} test followed by 40 min	S P P	ELISA " "	0.2 ng/ml[4] 2.7 ng/ml (heparin)[4] 6.4 ng/ml (EDTA)[4]
(Ruell et al., 2006)	Running thermoneutral 14km ~58min " hyperthermic 14km ~64min	P P	ELISA "	↑ ~850% ↑ ~2900%
(Fischer et al., 2006)	3 h exhaustive two legged knee extensor exercise 50% of leg peak power output.	S	ELISA	↑~300%
(Marshall et al., 2006)	2 days cycling 120 min 42.5% VO_{2max} (~38°C)	S	ELISA	↑~160% day 1
(Gomez-Merino et al., 2006)	Running 100km ~539min	P	ELISA	↑~1600%
(Horn et al., 2007)	Treadmill Running 90 min 70% VO_{2max}	P	ELISA	↑~270%
(Whitham et al., 2007)	Underwater running 120 min 59% VO_{2max} T_c clamped Underwater running 120 min 59% VO_{2max} T_c unclamped	P P	ELISA "	↑~129% ↑~212%
(Amorim et al., 2008)	50% VO_{2max} treadmill running until 38.5^{o}C or 95% HR_{max} Low heat storage (LS) ~0.54 W m $^{-2}$ min $^{-1}$ High heat storage (HS) ~1.04 W m $^{-2}$ min $^{-1}$	S	ELISA	↑~78% ↑~94% No significant difference between conditions

(Selkirk et al., 2009)	Treadmill walking to exhaustion 4.5 kph 2% incline 40°C clothing ensemble = un-compensable heat stress	P	ELISA	↑~38% at 39.5^{o}C T_c in trained subjects No significant change at any T_c in untrained subjects
Exercise Training				
(Banfi et al., 2006)	Elite soccer training 1 year.	P	ELISA	↑~180 compared to sedentary controls
(Yamada et al., 2007)	10 days - walk/run 100 min 53% VO_{2max} (~42.5^{o}C)	S	ELISA	↑~110%
(Sandstrom et al., 2008)	Cycling 90 min 50% VO_{2max} 15 consecutive days (~32°C)	S	ELISA	↑ 110% post 15 days
(Magalhães et al., 2010)	90 min treadmill running 50% VO_{2ma} (40°C) pre (HST1) / post (HST2) 11 day acclimation period (*) *30 min treadmill running 1% grade ~2.20 $m.s.^{-1}$ + *30 min treadmill running 1% grade ~1.69 $m.s.^{-1}$ * performed consecutively	P	ELISA	No change pre to post HA ↑~40% immediately post HST1 No change post HST2

↑ (increase), ~ (mean), T_c (core temperature), P (plasma), S (serum), HST (heat stress test), MVC (maximal voluntary contraction)
N.B. Peak significant percentage increase calculated from control or resting sample were possible.

2.6. Human HSP32 response to exercise

Inducible HSP32 is involved with the degradation of heme to biliverdin, carbon monoxide and iron (Belcher et al., 2010). This ability of HSP32 to catabolise free heme stops the sensitisation of cells and their induction to programmed cell death (apoptosis) (Gozzelino et al., 2010) and similarly to HSP72 (Garrido et al., 2001), HSP32 induction with stress can convey an anti-apoptotic influence (Soares and Bach, 2009). During homeostasis, the pro-oxidant effect of heme is regulated by the insertion of heme into the pockets of hemoproteins (Belcher et al., 2010). However, during stress, such as exercise induced oxidative stress, some hemoproteins can release their prosthetic heme groups, with this free heme now capable of generating pro-oxidants (Gozzelino et al., 2010). The cellular influence of HSP32 during exercise is important as free heme can catalyse the production of free radicals, which are known to disrupt exercise efficiency (Niess et al., 1999b; Thompson et al., 2005).

Limited well controlled *in vivo* human exercise studies have been conducted with respect to exercise induced changes in HSP32 in comparison to the plethora of studies published on exercise and HSP72 expression. The first study to demonstrate exercised induced increases in PBMC HSP32 via flow cytometry detailed a ~700% increase immediately after half marathon completion in L from virtually undetectable basal values (Niess et al., 1999b). The upregulation of HSP32 in response to stress has been shown to directly protect L against oxidative stress (Speit et al., 2000). This rapid accumulation of HSP32 in L is due to the pro-oxidant stimulus of exercise, which is counteracted by the anti-oxidative cytoprotective influence of HSP32 (Gozzelino et al., 2010). Notably, exercise mediated increases in HSP72 are not evident in response to any exercise load within L HSP72 (*lHSP72*), conversely, exercise mediated HSP32 expression is greatest within L (~700%) compared to increases of ~35 % and ~300% in

M and G respectively (Niess et al., 1999b). These smaller increases in M and G are probably due to higher basal values within *lHSP32* (70%) and *gHSP32* (25%) compared to negligible basal expression within L (2%). These higher basal values in *mHSP32* and *gHSP32* are likely attributable to the ability of M and G to generate greater levels of ROS under homeostasis and thus require greater HSP32 expression at rest to acquiesce such challenges to redox balance (Niess et al., 1999a; Niess et al., 1999b; Niess et al., 2000). Additionally, it can be postulated, as seen with HSP72 (Gjovaag and Dahl, 2006; McClung et al., 2008; Vince et al., 2010), increased basal values attenuate the post stressor magnitude in stress protein expression compared to that seen from low basal values. This postulation warrants further investigation.

To detail exercise intensity specific differences in HSP32 expression the same cohort utilised three exercise conditions (Fehrenbach et al., 2003). The half marathon condition produced the largest increases in L (~70%), M (~25%) and G (~55%), compared to the exhaustive treadmill run group (110% of AT until exhaustion) L (~10%), M (~0%) and G (~10%) and eccentric exercise subjects (6 x 10 quadriceps eccentric contractions) L (~15%), M (~0%) and G (~0%) (Fehrenbach et al., 2003). Complimentary to these results, 75 min treadmill running (70% $\dot{V}O_{2\max}$) was shown to induce significant increases in HSP32 mRNA (~260%) and protein (~200%) within leukocytes 2 h post exercise (Thompson et al., 2005). These differential stress protein responses within leukocyte sub-sets may be attributable to the high basal HSP32 values within G and M (Niess et al., 1999a; Niess et al., 1999b; Niess et al., 2000), negatively regulating post stressor expression, similarly to those kinetics seen for HSP72 (Gjovaag and Dahl, 2006; Vince et al., 2010). These postulations regarding PBMC HSP32 stress mediated changes in expression, and their correlation to basal values, are under investigated in the literature at present, and would benefit from investigation in a manner similar to that

which Vince et al. (2010) conducted *in vitro*, with regard to HSP72 stressor mediated expression.

Ex vivo induction of HSP32 is modulated by previous endurance activity, with a reduced up-regulation seen within harvested lymphocytes upon exposure to H_2O_2 compared to controls (Markovitch et al., 2007). However, in opposition to this groups previous findings (Thompson et al., 2005), no significant increases were seen in lymphocyte HSP32 mRNA or protein at any time point post exercise (100 min treadmill running) compared to control (Markovitch et al., 2007). This lack of well controlled human studies requires exercise permutations in HSP32 to be explored in greater detail.

Interestingly, this human *in vivo* work has not suffered with the problems associated with similar work investigating HSP72. Specifically, the extensive examination of eHSP72 utilising the flawed ELISA method (discussed in chapter 4) has not been replicated within research investigating HSP32. In general, well designed projects have utilised flow cytometry which others (Fehrenbach et al., 2003; McClung et al., 2008; Selkirk et al., 2009; Vince et al., 2010) have adopted and considered being reliable, sensitive and accurate in the assessment of iHSP72 and specifically *mHSP72* expression.

2.7. Oxidative stress

A poignant dichotomy exists between the need for O_2 as an essential molecule to life and the homeostatically toxic products of its use in oxidative metabolism (Taylor and Pouyssegur, 2007). Exercise is known to disrupt the delicate balance between free radicals (pro-oxidant) and their amelioration via the body's antioxidant defence system, with this disruption slanting redox balance in favour of free radical production (Fisher-Wellman and Bloomer, 2009). Molecular O_2 consumption is increased at the onset of aerobic exercise with complimentary increases in the formation and production of free radicals (Steinberg et al., 2007). In general, these pro-oxidants are referred to as reactive

oxygen and or nitrogen species (RONS), which, for simplicity, these species will be collectively known as (RONS) form this point forward (Fisher-Wellman and Bloomer, 2009). These O_2 derived pro-oxidants can damage lipids, proteins and DNA (Bloomer and Goldfarb, 2004) with denaturing of the latter two a potent stimulus for increases in HSP72 concentration (Kulkarni et al., 2007; Bienemann et al., 2008).

Regular exercise is well known to have a plethora of positive physiological and psychological health benefits, including a lowered threat of all-cause mortality along with a reduced risk of cardiovascular disease, cancer and diabetes (Blair et al., 2001; Crespo et al., 2002; Oguma et al., 2002). Therefore, the notion that exercise and the well cited repeated generation and exposure to oxidative stress (RONS) representing a wholly harmful event to health and muscle function may not be totally correct (Powers and Jackson, 2008; Fisher-Wellman and Bloomer, 2009). For example, low levels of RONS can perform positive physiological functions and are required for optimal muscle contractile force (Coombes et al., 2001; Jackson, 2008; Powers and Jackson, 2008; Powers et al., 2010a; Little and Cochran, 2011). Additionally, the necessity of RONS to procure exercise training induced skeletal muscle adaptation has recently been elucidated (Powers et al., 2010a; Little and Cochran, 2011). Specifically, many signalling molecules are manipulated by RONS, including redox-sensitive kinases, phosphatises and the transcription factor NF-κB. These redox-sensitive molecules act as downstream effectors of RONS and serve as critical signalling events leading to skeletal muscle remodelling in response to increased contractile activity (i.e. physical exercise) (Powers et al., 2010a; Little and Cochran, 2011). However, despite the obvious need for RONS to procure maximal skeletal muscle adaptation (Powers and Jackson, 2008; Powers et al., 2010a; Little and Cochran, 2011), chronic (disease or immobilisation) versus acute (exercise induced) presence of such reactive species within both the intra- and extra-cellular milieu does initiate negative cellular events – such as necrosis,

apoptosis and proteolysis (Powers et al., 2005; Powers et al., 2007). Therefore, it is evident that contradictory signalling functions of RONS are present *in vivo*, which are related to the level and duration of the disturbance to redox balance (Powers et al., 2010a). Simplistically, this can be summarised as chronic increased RONS presence can lead to negative cellular events (e.g. proteolysis) where as acute increased RONS presence (e.g. from exercise) can promote cell adaptation and survival (Powers et al., 2010a). Despite these positive cellular contributions (Powers and Jackson, 2008; Powers et al., 2010a; Little and Cochran, 2011), excess RONS production is implicitly linked to the causation of oxidative stress and ultimately results in severe damage to biological tissue if left unchecked (Powers et al., 2005; Clanton, 2007; Powers et al., 2007; Fisher-Wellman and Bloomer, 2009).

2.7.1 Radicals, non-radicals and oxidative stress.

The production of RONS is a normal product of aerobic metabolism (Sies, 1997) with the majority of intracellular RONS originating from the mitochondria (Finkel and Holbrook, 2000; Powers and Jackson, 2008; Nikolaidis and Jamurtas, 2009). Such pro-oxidants are extremely short lived (i.e. short half life) and are classed as either radicals (e.g. superoxide) or non-radicals (e.g. H_2O_2) (Kohen and Nyska, 2002). For example, superoxide radicals formed in the mitochondrial membrane, as a result of O_2 reduction, trigger a cascade of events in the fatty acids of phospholipids resulting in membrane lipid peroxidation (Evans, 2000). The body's antioxidant defence system serves to protect the cells from excess RONS production and is comprised of both endogenous (bilirubin, uric acid, superoxide dismutases, catalase, glutathione peroxidise, etc.) and exogenous (cartoenoids, tocopherols, ascorbate, bioflavonoids, etc.) compounds (Bloomer and Goldfarb, 2004; Halliwell and Gutteridge, 2007; Powers and Jackson, 2008; Fisher-Wellman and Bloomer, 2009; Nikolaidis and Jamurtas, 2009). It is the interaction of free radical production (RONS) and antioxidant defence that serves to

maintain intracellular redox balance within a homeostatically acceptable margin. This process, basal RONS production and removal by antioxidants, is constantly occurring within the body and as such the positive (Powers and Jackson, 2008; Powers et al., 2010a; Little and Cochran, 2011) and negative effects (Powers et al., 2005; Powers et al., 2007; Powers and Jackson, 2008) on physiological function are a constant presence *in vivo* (Bloomer and Goldfarb, 2004; Fisher-Wellman and Bloomer, 2009). The process (mechanism of action) of antioxidant defence is discussed later within this section.

The contradictory *in vivo* cellular consequences of RONS (Powers et al., 2005; Powers et al., 2007; Powers and Jackson, 2008; Powers et al., 2010a) have ensured exercise induced permutations to redox balance *in vivo* have received extensive interest within the literature, and, as such, have been recently reviewed (Bloomer and Goldfarb, 2004; Jackson et al., 2007; Powers and Jackson, 2008; Fisher-Wellman and Bloomer, 2009; Powers et al., 2010a). This high volume of *in vivo* research will be the focus of this section of the review, as it is directly relevant to the studies conducted within this thesis unlike the majority of previous *in vitro* and animal model research.

2.7.2 The source and functions of oxidant production during exercise.

Exercise disturbs whole body homeostasis, with a multitude of physiological responses seen, including several key events within skeletal muscle and blood (Jackson et al., 2007; Powers and Jackson, 2008; Nikolaidis and Jamurtas, 2009; Powers et al., 2010a). Both skeletal muscle and blood are central to energy provision, metabolism and waste product removal during exercise. Atypical responses within the blood include, but are not limited to, increases in the concentration of blood lactate (Stringer et al., 1994) and blood temperature (Nybo et al., 2002), and, decreases in arterial blood O_2 (Stringer et al., 1994) and pH (Hermansen and Osnes, 1972). In addition to disturbances to blood

homeostasis (Nikolaidis and Jamurtas, 2009), exercise induces alterations within skeletal muscle homeostasis (Powers and Jackson, 2008; Powers et al., 2010a; Little and Cochran, 2011). Atypical skeletal muscle responses include, but are not limited to, increases in muscle lactate concentration and temperature, and, decreases in muscle O_2 and pH (Hermansen and Osnes, 1972; Stringer et al., 1994; Kilgore et al., 1998; Majerczak et al., 2010; Miyazaki, 2010). These homeostatic disturbances, both within the skeletal muscle (Powers and Jackson, 2008; Powers et al., 2010a) and blood (Nikolaidis and Jamurtas, 2009), are able to modify redox balance within the tissue in question. Therefore, the source of exercise induced increases in oxidative stress are principally attributed to skeletal muscle generating elevated oxidant production, which, in turn generates RONS (Powers and Jackson, 2008; Powers et al., 2010a). However, recently, the contribution of blood as a RONS generator and redox balance regulator during exercise has also received growing support (Jackson et al., 2007; Powers and Jackson, 2008; Nikolaidis and Jamurtas, 2009) in addition to the muscle-centric explanations previously relied upon (Powers et al., 1999).

In general, oxidant production (sources discussed later in this section) leads to the formation of some or all of the following radicals; superoxide, H_2O_2, hydroxyl radicals, singlet oxygen, nitric oxide, peroxynitrite, hyperchlorite and secondary radical species (Powers and Jackson, 2008; Powers et al., 2010a) to varying degrees both within the muscle (Powers and Jackson, 2008) and blood (Powers and Jackson, 2008; Nikolaidis and Jamurtas, 2009). Of these numerous radicals it is the formation of nitric oxide and superoxide, the latter of which dismutates rapidly to from H_2O_2, which are regarded as the primary radicals generated by skeletal muscle and blood at rest and during exercise (Jackson et al., 2007; Powers and Jackson, 2008; Powers et al., 2010a). These radicals (nitric oxide, superoxide and H_2O_2) provide the precursors for generation of other more

highly reactive radicals such peroxynitrite and hydroxyl radicals, amongst others (Halliwell and Gutteridge, 2007; Jackson et al., 2007).

Therefore, this section will detail the source of oxidant production, focusing upon nitric oxide and superoxide, at rest and during exercise in both skeletal muscle and blood.

Skeletal muscle

Superoxide and nitric oxide are the principle radicals generated at rest and during exercise by skeletal muscle (Halliwell and Gutteridge, 2007). Through the addition of one electron to ground-state oxygen superoxide is formed, which occurs by either several enzymatic systems located within the cells or within the mitochondria via one electron transfer (Halliwell and Gutteridge, 2007; Powers et al., 2010a). Many subcellular sites are capable of producing superoxide within skeletal muscle at rest and during exercise. Several of these sites demonstrate increased activity during muscular contraction (i.e. physical activity and/or exercise). These endogenous sites include the mitochondrion, sarcoplasmic reticulum, transverse tubules, sarcolemma and the cytosol (Powers and Jackson, 2008; Powers et al., 2010a).

The electron transport chain, particularly complexes I and III, represent the predominant site of superoxide generation within the mitochondria (Barja, 1999). Interestingly, mitochondria residing within type II, as opposed to type I muscle fibres, have been shown to promote higher levels of ROS and thus superoxide formation (Anderson and Neufer, 2006). Contributing to mitochondria mediated superoxide generation (Barja, 1999; Anderson and Neufer, 2006; Powers and Jackson, 2008; Powers et al., 2010a), NADPH oxidases which reside within the sarcolemma, transverse tubules, plasma membrane and sarcoplasmic reticulum are known to produce superoxide within both resting end exercising muscle fibres (Halliwell and Gutteridge, 2007; Powers and Jackson, 2008; Powers et al., 2010a). This production of superoxide is a continuous

process at rest which is increased during exercise, with exercise induced increases evidently increasing the spontaneous and superoxide dismutase mediated dismutation of superoxide into H_2O_2 (Halliwell and Gutteridge, 2007). The exercise induced increases in superoxide generation and thus formation of H_2O_2 compared to at rest is important with regards to redox balance. The long half life of H_2O_2 facilitates its diffusion with cells and across membranes, thus, disturbing redox balance (Halliwell and Gutteridge, 2007). This increase in diffusion of H_2O_2 across the plasma membrane of skeletal muscle into the extra cellular environment, coupled with the ability of skeletal muscle cells to directly release superoxide into the extracellular environment evidently has an impact of redox balance, slanting such balance in favour of a pro-oxidant environment (Halliwell and Gutteridge, 2007; Powers and Jackson, 2008; Powers et al., 2010a). Additionally, the presence of the radicals H_2O_2 and superoxide, as previously mentioned, are known precursors for the formation of other highly reactive radicals such as peroxynitrite and hydroxyl radicals, amongst others (Halliwell and Gutteridge, 2007; Jackson et al., 2007).

Three isoforms of nitric oxide synthase (NOS1, NOS2 and NOS3) synthesise nitric oxide from the amino acid L-arginine (Halliwell and Gutteridge, 2007; Powers and Jackson, 2008; Powers et al., 2010a). Continuously expressed at rest, with augmented generation seen with increased muscular contractions (i.e. during exercise), nitric oxide is generated within the sarcolemma and mitochondria. Nitric oxide can react with superoxide to form the potent oxidising agent peryoxynitrite which can result in thiol group depletion within cells (Halliwell and Gutteridge, 2007; Powers and Jackson, 2008; Powers et al., 2010a). Similarly to superoxide and H_2O_2, nitric oxide can diffuse across the plasma membrane into the extracellular space thus slanting such redox balance in favour of a pro-oxidant environment (Halliwell and Gutteridge, 2007; Powers and Jackson, 2008; Powers et al., 2010a).

Blood

The exact origin of oxidative damage and RONS detected in the blood is largely unknown (Halliwell and Gutteridge, 2007; Nikolaidis and Jamurtas, 2009). Blood interacts with all organs and tissues and therefore many potential sources of RONS are likely (Halliwell and Gutteridge, 2007; Nikolaidis and Jamurtas, 2009). As discussed above a skeletal muscle-centric approach is often used to explain free radical and RONS generation at rest and during exercise (Powers et al., 1999; Powers and Jackson, 2008; Powers et al., 2010a; Little and Cochran, 2011). However, such a muscle biased approach precludes the potential for other organs and tissues contributing to the exercise induced disturbances to redox balance (Jenkins, 2000; Lamprecht et al., 2004; Nikolaidis and Jamurtas, 2009). The potential for tissues other than skeletal muscle to contribute to such exercise induced alterations in redox balance has been recognised in more recent muscle-centric reviews, with the white blood cells, lungs and heart all credibly postulated as potential sources of free radical production (Jackson et al., 2007; Powers and Jackson, 2008). Of these potential sources, white blood cells and the blood in general has received increasing interest as a tissue that generates reactive species and regulates redox balance during exercise (Nikolaidis and Jamurtas, 2009). Specifically within the blood, the serum or plasma (termed plasma for simplicity from here on in), erythrocytes and leukocytes are capable of autonomously generating substantial amounts of oxidants and thus RONS at rest and during exercise (Halliwell and Gutteridge, 2007; Nikolaidis and Jamurtas, 2009).

RONS are principally formed within the plasma via reactions between oxidants and metals. For example, a low level of H_2O_2 is constantly present within the plasma due to the ubiquitous nature of H_2O_2 generation from virtually all cells and the solubility of H_2O_2 (Halliwell and Gutteridge, 2007). In the presence of any unbound tissue iron (as released by injured/pro-inflammatory cells), H_2O_2, via a Fenton reaction, can become a

highly reactive hydroxyl radical (Halliwell and Gutteridge, 2007; Nikolaidis and Jamurtas, 2009).

Erythrocytes, in contrast to plasma, are minimally permeable and thus their likely sources of oxidant production are easier to postulate. Principally, such radical production is from bound haemoglobin (oxyhaemoglobin) occasionally releasing a superoxide oxidant (Cimen, 2008). This autoxidation, given the high concentration of oxyhaemoglobin within the erythrocytes (5mM), can produce substantial quantities of superoxide, even with relatively low levels of autoxidation within erythrocytes (Cimen, 2008; Nikolaidis and Jamurtas, 2009). This release of superoxide occurs when deoxyhaemoglobin, specifically the heme element, is loaded with O_2, with the resulting oxyhaemoglobin occasionally releasing a superoxide radical (Cimen, 2008; Nikolaidis and Jamurtas, 2009). This superoxide radical, in cohorts with the omnipresent H_2O_2, catalyse the highly reactive hydroxyl radical, via the Haber-Weiss reaction (Halliwell and Gutteridge, 2007; Cimen, 2008; Nikolaidis and Jamurtas, 2009).

The phagocytotic killing mechanism of the leukocytes is well cited (Malm, 2002; Stenmark et al., 2005; Halliwell, 2006; Halliwell and Gutteridge, 2007). During and post exercise, the neutrophils (which represent 50 – 70% of total leukocyte number) increase in number (Fehrenbach et al., 2000b; Malm, 2002; Whitham et al., 2004). The function of neutrophils is phagocytotic at sites of inflammation or damage, digesting any such related cellular debris (Malm, 2002). In response to such cellular damage (as induced by both damaging and non-damaging exercise), to service their digestive function, a notable increase in O2 consumption is seen within neutrophils, this increase is known as the oxidative burst (Halliwell, 2006; Halliwell and Gutteridge, 2007). This oxidative burst is mediated by activation of NADPH, which when oxidised, produces two superoxide radicals which can be readily converted to two highly reactive hydroxyl radicals (Halliwell, 2006; Halliwell and Gutteridge, 2007). This conversion of

superoxide to hydroxyl radical is achieved either via dismutation in cohort with H_2O_2 or via a Fenton reaction (Halliwell, 2006; Halliwell and Gutteridge, 2007). The generation of superoxide and subsequent other radicals is an ongoing process *in vivo* at rest within neutrophils, however, this production has been shown to be increased immediately or a few hours post non-muscle-damaging exercise (Ookawara et al., 2003) and 1-5 days post muscle-damaging exercise (Cannon et al., 1990).

Summary

At rest and during/post exercise both skeletal muscle (Jackson et al., 2007; Powers and Jackson, 2008; Little and Cochran, 2011) and blood (Nikolaidis and Jamurtas, 2009) are able to produce oxidants. Skeletal muscle contractile function generates the relatively nonreactive species nitric oxide and superoxide (the latter rapidly dismutates into H_2O_2); however, these radicals (superoxide, nitric oxide and H_2O_2) are precursors for the highly reactive oxidants peroxynitrite and the hydroxyl radical (Powers and Jackson, 2008; Powers et al., 2010a). Evidently, with exercise, contractile function is increased and thus so is oxidant production, with the consensus amongst the literature that reactive species production is elevated 2 to 4 fold within contracting skeletal muscle (Jackson et al., 2007). Recent evidence suggests that such intracellular muscle originated reactive species incrementally increases in efflux across the cell membrane into the blood during increasing contractions (Bailey et al., 2003; Bailey et al., 2004). Additionally, similarly to the muscle, within the blood superoxide and H_2O_2 are present, originating from the plasma, erythrocytes and leukocytes, which in turn are precursors for the highly reactive hydroxyl radical to be formed (Nikolaidis and Jamurtas, 2009). Therefore, it can be seen, that both the blood and muscle contribute to the generation of "precursor" radicals (nitric oxide, superoxide and H_2O_2), which, dependent upon their tissue of origin and co-complexes present can be converted into highly reactive radicals such as the hydroxyl radical and peroxynitrite (Halliwell and Gutteridge, 2007; Nikolaidis and

Jamurtas, 2009). The presence of such radicals evidently disturbs redox balance. These O_2 derived pro-oxidants can damage lipids, proteins and DNA (Bloomer and Goldfarb, 2004) with denaturing of the latter two a potent stimulus for increases in HSP72 concentration (Kulkarni et al., 2007; Bienemann et al., 2008). However, despite the negative cellular consequences of chronic oxidative stress, acute oxidative stress, such as that seen in response to exercise, can provide the stimulus for positive adaptations to occur, such as skeletal muscle remodelling and resistance to future challenges to redox balance (Powers and Jackson, 2008; Powers et al., 2010a; Little and Cochran, 2011).

2.7.3 Measurement of oxidative stress in humans.

The measurement of exercise mediated changes in oxidative stress is not without problems, especially direct measurement. Such measures are difficult due to the high reactivity and short half lives of the pro-oxidants to be assessed (e.g. 10^{-5} for the superoxide radical and 10^{-9} for the hydroxyl radical, respectively). Technological advancements have alleviated some of these problems, but the cost and high degree of labour associated with spin resonance spectroscopy, radiolysis and flash photolysis have ensured that exercised induced changes in oxidative stress are generally measured by indirect measures (Dalle-Donne et al., 2006; Fisher-Wellman and Bloomer, 2009).

The reaction of RONS with certain biomolecules produces some stable molecular products of oxidation. Typically in response to exercise, indirect end products of lipid peroxidation, such as malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS), are investigated (Dalle-Donne et al., 2006). Alternatively, changes in the antioxidant defence system can be measured, principally the major endogenous antioxidant glutathione (Dalle-Donne et al., 2006; Fisher-Wellman and Bloomer, 2009). The experimental research conducted within this thesis will examine these measures of oxidative stress (TBARS, glutathione) enabling direct comparison

with previous literature within the field, as discussed in recent reviews (Dalle-Donne et al., 2006; Fisher-Wellman and Bloomer, 2009). Therefore, this section of the review will focus solely on oxidative data generated *in vivo* in response to exercise.

2.7.4. Circadian and/or diurnal variation in TBARS and glutathione.

It has been shown that no circadian or diurnal variation is present in TBARS or MDA (Kanabrocki et al., 2002) and glutathione within healthy male subjects (Blanco et al., 2007; Wu et al., 2008).

2.7.5. Exercise induced changes in oxidative stress.

Elevations in various oxidative stress biomarkers provide evidence for increases in RONS production following both acute anaerobic (Bloomer and Goldfarb, 2004) and aerobic exercise (Vollaard et al., 2005; Fisher-Wellman and Bloomer, 2009). However, interpretation of the data generated, as with that of HSP72, is problematic. Exercise induced RONS production has been demonstrated to be duration (Bloomer et al., 2007) and intensity (Goto et al., 2003) dependant with the dietary intake (Watson et al., 2005), age (Sacheck et al., 2006) and training status (Fatouros et al., 2004) of the participants also impacting upon any changes post activity. Further compounding these problems, the timing of sample collection and the specificity and sensitivity of the biomarker chosen are all influencing factors (Dalle-Donne et al., 2006). Therefore, the *in vivo* data available must be interpreted with caution as even similar protocols can produce markedly different results (Bloomer and Goldfarb, 2004; Fisher-Wellman and Bloomer, 2009).

2.7.5.1. Exercise mediated changes in TBARS

The most routinely employed indirect measure of exercise induced changes in oxidative stress is that of TBARS. However, despite its widespread use it is has several limitations (Powers et al., 2010b). Specifically, the assay employed, in addition to the

targeted detection of aldehydes, may also react with biological molecules such as salic acids, prostaglandins and carbohydrates (Powers et al., 2010b). These targeted aldehydes, including the three carbon chain aldehyde MDA are formed during the composition of lipid hydroxides. This lack of specificity is demonstrated when MDA is specifically measured post exercise and reports no change in values, whereas, investigations of TBARS generally show increases (Bloomer and Goldfarb, 2004; Dalle-Donne et al., 2006; Fisher-Wellman and Bloomer, 2009). Despite these limitations, measures of TBARS are still routinely employed within *in vivo* exercise studies and is considered a useful measure of exercise induced oxidative stress, particularly when complementary glutathione redox data are generated (Bloomer and Goldfarb, 2004; Dalle-Donne et al., 2006; Fisher-Wellman and Bloomer, 2009; Powers et al., 2010b). However, although recognised as an inexpensive and simple bio-marker of oxidative stress, TBARS, as discussed above, does have some technical problems and these must be considered when interpreting the literature and any potential experimental findings (Powers et al., 2010b).

Sub-maximal aerobic exercise has been shown to increase TBARS post exercise (Sen et al., 1994b; Laaksonen et al., 1996; Alessio et al., 1997; Borsheim et al., 1999; Laaksonen et al., 1999; Meijer et al., 2002; Vincent et al., 2004; Nikolaidis et al., 2007). It has been shown that 30 min ergometer cycling at the aerobic or anaerobic threshold produces peak increases in TBARS of ~80% and ~105% respectively, with the completion of a $\dot{V}O_{2\max}$ test failing to induce significant changes (Sen et al., 1994b). Exercise of a similar mode performed at a lower intensity (60% $\dot{V}O_{2\max}$) for 40 min demonstrated peak increases of 50% in type I diabetic participants and their experimental controls (Laaksonen et al., 1996; Laaksonen et al., 1999). Other projects have found similar findings in response to cycle ergometer exercise of a similar intensity and duration (Meijer et al., 2002; Vincent et al., 2004) and in longer duration

activity of comparable intensity (Borsheim et al., 1999). Treadmill running (30 min 80% $\dot{V}O_{2max}$) has shown similar increases in TBARS in control subjects of 48%, with vitamin C supplementation for 1 day and 14 days reducing this response to 12.6% and 33% respectively (Alessio et al., 1997). Additionally, swimming (12 x 50 m sprints at 70-75 max velocity) induced increases in TBARS in children of approximately 30% (Nikolaidis et al., 2007).

Maximal aerobic exercise has also been shown to increase TBARS post exercise (Sumida et al., 1989; Szczesniak et al., 1998; Miyazaki et al., 2001; Nikolaidis et al., 2006; Steinberg et al., 2006; Lwow et al., 2007; Michailidis et al., 2007; Steinberg et al., 2007). Graded maximal exercise tests (GXT) on a cycle ergometer have shown peak increases of ~25% (Miyazaki et al., 2001), ~75% (Steinberg et al., 2007) and ~120% (Steinberg et al., 2006) in TBARS, with significant increases shown elsewhere (Sumida et al., 1989; Lwow et al., 2007). The importance of timing of sample collection was eloquently shown with immediately, 5 and 20 min post GXT completion TBARS values fluctuating between increases of ~20%, ~120% and ~80% respectively (Steinberg et al., 2006). These time sensitive variations in TBARS values post exercise may explain the variation in TBARS across methodologically similar protocols (Bloomer and Goldfarb, 2004; Steinberg et al., 2006; Steinberg et al., 2007; Fisher-Wellman and Bloomer, 2009). Similar findings have been found to biphasic treadmill running (45 min 72.5% $\dot{V}O_{2max}$ followed by 90% $\dot{V}O_{2max}$ until exhaustion) with peak increases in TBARS of 75% (Nikolaidis et al., 2006) and 133% (Michailidis et al., 2007). These differential responses (75% compared to 133%) to an identical protocol with similar participant characteristics illustrates the variability that can be seen when using the TBARS assay. Post sub-maximal and maximal aerobic protocols, exercise induced elevations in TBARS typically and consistently increase post exercise and return to baseline within one hour post exercise (Szczesniak et al., 1998; Bloomer et al., 2005; Steinberg et al.,

2006; Steinberg et al., 2007; Fisher-Wellman and Bloomer, 2009). Despite these consistent findings, as discussed previously, the TBARS assay does have some technical problems and these must be considered when interpreting the literature and any potential experimental findings (Powers et al., 2010b).

2.7.5.2. Exercise mediated changes in glutathione redox

The major non-enzymatic endogenous anti-oxidant glutathione has been routinely employed as a measure of exercised induced permutations in redox balance. Glutathione exists in a reduced (GSH) and oxidised form (GSSG). When present as GSH within a cell or tissue that has been compromised redox balance, GSH is able to donate one electron to other unstable molecules such as RONS (neutralising the pro-oxidant affect of RONS). This process transiently leaves GSH reactive (pro-oxidant), however, this pro-oxidant form of GSH quickly reacts with another reactive glutathione complex to form glutathione disulfide (GSSG). GSH can be regenerated from GSSG by the enzyme glutathione reductase. In non-compromised cells, i.e. those experiencing homeostasis GSH represents 90% of total glutathione (TGSH) with GSSG contributing <10%. Simply, an increased GSSG to GSH ratio is considered indicative of a move towards a pro-oxidant state. Typically, increases in GSSG and reductions in GSH are accompanied by no change in TGSH following a variety of non-eccentric aerobic exercise protocols, see Table 2.4 for summary of such changes.

It is important to note that when assessing changes in GSSG:GSH ratios, within the blood, that whole blood is used to provide a more global representation of redox balance within the blood (Powers et al., 2010b). Using whole blood ensures mitochondrial, nuclear, and cytosolic compartments of all blood cells, as well as the plasma and serum are assessed (Powers et al., 2010b). Additionally, it is important to ensure that artifactual GSH oxidation does not occur during blood sample procurement and thus

compromising GSSG:GSH ratios upon analysis (Powers et al., 2010b). Methodological consideration of artifactual GSH oxidation is discussed and addressed in section 3.4.2.

Despite this plethora of research (Bloomer and Goldfarb, 2004; Fisher-Wellman and Bloomer, 2009), few studies have reported complete indices of glutathione (GSH, GSSG and TGSH) in response to exercise, which is important to accurately gauge changes in glutathione ratios and thus the antioxidant capacity of the blood or muscle. Therefore, this examination of human exercise based studies will focus solely on those which present data on all the indices of the glutathione redox system. Early work demonstrated that three days of cycle ergometer aerobic exercise training (90 min at 65% $\dot{V}O_{2\max}$) was insufficient to alter exercise-induced changes in glutathione redox in moderately trained volunteers (Viguie et al., 1993). Measures taken 15 min post exercise completion on day 1 demonstrated decreases in GSH (~45%), increases in GSSG (~30%), with no change in TGSH. Samples collected on day 3 demonstrated similar changes in glutathione redox, however, GSSG increased significantly more (~60%) (Viguie et al., 1993). Similarly, a shorter duration of 40 min cycle ergometer exercise at a slightly lower intensity (60% $\dot{V}O_{2\max}$) elicited decreases in GSH (~13%), increases in GSSG (~50%) with no change in TGSH (Laaksonen et al., 1999). These two studies (Viguie et al., 1993; Laaksonen et al., 1999), supported by other cycle ergometer (Marin et al., 1990; Sastre et al., 1992) and treadmill research (Goldfarb et al., 2007) elucidated the redox kinetics of blood glutathione in response to sub-maximal exercise. Medved et al., (2004) furthered this work by examining the glutathione response within the blood and muscle in tandem. Additionally they examined the anti-oxidant efficacy of N-acetylcysteine infusion pre exercise. They utilised a biphasic cycle ergometer exercise protocol (45 min at 71% $\dot{V}O_{2\max}$ followed by 90% $\dot{V}O_{2\max}$ until exhaustion). Similar decreases in blood GSH (~7%), increases in GSSG (~54%) and no change in TGSH have been shown by others (Viguie et al., 1993; Laaksonen et al., 1999)

in response to the first phase of the protocol, with completion on the second phase eliciting larger changes in GSH (~14%) and GSSG (~72%) (Medved et al., 2004). Conversely, within the muscle, decreases in GSH (~23%), GSSG (~43%) and TGSH (~37%) were seen post phase one completion, with values remaining relatively unchanged post phase two (Medved et al., 2004). This was the first well controlled study to demonstrate differential responses of muscle and blood glutathione to an identical exercise stressor within humans *in vivo*. Mechanistically, these differential responses in the muscle compared to the blood, i.e. a decrease and increase in GSSG respectively, are likely attributable to muscle GSSG being exported to the blood where there is a higher GSH concentration to acquiesce to the pro-oxidant effect of GSSG (Fisher-Wellman and Bloomer, 2009). Furthermore, the infusion of N-acetylcysteine 20 min prior to exercise attenuated all the exercise induced changes in glutathione redox, within both the muscle and blood (Medved et al., 2004). There have been no subsequent human studies examining muscle and blood glutathione in unison, with one recent rodent model demonstrating similar findings (Veskoukis et al., 2009). This is an area of research which would benefit from greater exploration *in vivo*.

2.7.5.3. Hypoxia and oxidative stress

Hypoxia and the associated reduction in the partial pressure of O_2 reduces the arterial blood O_2 content and thus leads to tissue hypoxia. Additionally, this hypoxic state can compromise cells metabolically which can progress, if unchecked, to cellular apoptosis and necrosis (Taylor and Pouyssegur, 2007). Clearly, due to the O_2 paradox, the oxidative stress cannot be due to increases in tissue oxygenation as shown elsewhere (Clanton, 2005). Mechanistically, with respect to oxidative stress and the generation of RONS in response to hypoxia, the precise cellular occurrences which provoke such increases in production rate and concentration are still largely unknown (Subudhi et al., 2004; Wilber et al., 2004; Clanton, 2005). One theory is that RONS production itself is

not increased, but antioxidant utilisation is elevated and thus depleted due to systemic hypoxia, and as such, previously acquiesced RONS are no longer dealt with by the body's antioxidant defence system and thus accumulate (Moller et al., 2001). However, in direct opposition to this theory, Wing et al. (2000) observed increases in lipid production following 60 min of hypoxia. Therefore, it is likely that the hypoxia induced disturbance to redox balance stems from the reoxygenation of hypoxic tissue (Clanton and Klawitter, 2001; Clanton, 2007). The onset of hypoxia has been shown to induce a rapid increase in RONS formation, which upon reperfusion of hypoxic tissues is greatly increased compared to the initial RONS accumulation (Clanton and Klawitter, 2001; Clanton, 2007). Specifically, the accumulation of hypoxanthine and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), which are highly pro-oxidant molecules and known precursors for increases in the toxic anion superoxide (Askew, 2002; Behn et al., 2007). Therefore, the need for further research to elucidate the precise mechanism responsible for increases in oxidative stress post hypoxia is required.

Table 2.4. Summary table of non-eccentric aerobic exercise studies investigating the exercise mediated changes in glutathione redox.

Reference	GSSG	GSH	TGSH
Complete index of exercise induced changes in glutathione			
(Marin et al., 1990)	↑	↓	↔
(Sastre et al., 1992)	↑	↓	↔
(Viguie et al., 1993)	↑	↓	↔
(Laaksonen et al., 1999)	↑	↓	↔
(Medved et al., 2004)	↑	↓	↔
(Goldfarb et al., 2007)	↑	↓	↔
Non-complete index of exercise induced changes in glutathione			
(Gohil et al., 1988)	↑	↓	
(Kretzschmar et al., 1991)		↓	

(Sen et al., 1994b)	↑		↔
(Laaksonen et al., 1996)	↑		↔
(Szczesniak et al., 1998)		↓	
(Chung et al., 1999)	↑	↓	
(Hellsten et al., 2001)			↔
(Svensson et al., 2002)		↓	↔
(Wilber et al., 2004)		↓	
(Bloomer et al., 2005)	↑	↓	
(Goldfarb et al., 2005)	↑	↓	
(Watson et al., 2005)	↑	↓	
(Nikolaidis et al., 2006)	↑	↓	
(Steinberg et al., 2006)		↓	
(Michailidis et al., 2007)	↑	↓	
(Nikolaidis et al., 2007)	↑	↓	
(Steinberg et al., 2007)		↓	

Despite the need for further mechanistic research, there is a growing body of evidence demonstrating that an increase in RONS formation is evident during, and most pertinently, post hypoxia. Increases such as these have been seen within animals (Chang et al., 1989; Radak et al., 1994; Hoshikawa et al., 2001; Sarada et al., 2002; Magalhaes et al., 2004; Magalhaes et al., 2007) and humans (Bailey et al., 2000; 2001; Joanny et al., 2001; Moller et al., 2001). These increases in RONS within humans have been elucidated further, with well controlled studies investigating acute hypoxia mediated changes in glutathione redox now available. Similar to exercise induced changes in glutathione ratios (Marin et al., 1990; Sastre et al., 1992; Viguie et al., 1993; Laaksonen et al., 1999; Medved et al., 2004; Goldfarb et al., 2007), acute hypoxia has been shown to decrease GSH, increase GSSG with no change in TGSH (Vats et al., 2008; Sinha et

al., 2010). The administration of vitamin E or N-acetylcysteine ameliorated these negative hypoxia induced changes in glutathione redox (Vats et al., 2008). However, the TBARS or MDA response to a bout of acute hypoxia *in vivo* remains undocumented.

2.8. Overall Summary

Assessing changes in intracellular HSP72 (iHSP72) by flow cytometry, particularly whether any circadian or diurnal variation is present in their expression, is lacking *in vivo*. The first phase of this thesis explores such permutations. Establishment of these trends will enable the secure investigation of various environmental exposures and their efficacy in disrupting such patterns of expression.

The post exercise response of iHSP72 is well versed *in vivo* within skeletal muscle (Morton et al., 2009c) and the PBMCS (Yamada et al., 2008), with the eHSP72 response equally well reported within the serum and plasma of whole blood (Whitham and Fortes, 2008). However, *in vivo*, limited research has focused upon correlating basal values to exercise performance and the efficacy of such values in resisting the bio-chemical rigours of aerobic exercise. Such changes in basal expression (increases) may enable the transfer of cross tolerance to further non-lethal non-related stressors *in vivo*. This conferred cellular tolerance is something which has been demonstrated extensively *in vitro* (Lepore et al., 2000; Lepore et al., 2001; Shein et al., 2005; Shein et al., 2007; Shima et al., 2008).

The protein damaging effect of increased cellular concentrations of RONS and their negative effect on cellular redox *in vitro* is clearly evident (Celedon et al., 1998; Gonzalez et al., 2002; Clanton, 2007). This increase in oxidative stress *in vivo*, from hypoxia and HBO, in response to exercise or hypoxic exercise requires further investigation. Specifically, whether hypoxia or HBO can elicit changes in iHSP72 *in vivo* when assessed by the sensitive measurement technique of flow cytometry

(Bachelet et al., 1998). Damaged proteins and oxidative stress are efficacious in the pathophysiology of each other and as such both represent a potent stimulus for induction of iHSP72 (Beckmann et al., 1990; Palleros et al., 1991; Baler et al., 1992; Gething and Sambrook, 1992; Sadis and Hightower, 1992; Hightower, 1994; Buchberger et al., 1996; Cox and Walter, 1996; Hartl, 1996; Szabo et al., 1996; Strickland et al., 1997; Celedon et al., 1998; Fink, 1999; Gonzalez et al., 2002; Goldberg, 2003). Therefore, such changes via a non thermal, non-mechanical stressor, such as hypoxia or hyperbaria may enable increases in basal iHSP72 to be elicited. Establishment of a protocol which increases basal iHSP72, may enable iHSP72 mediated conferred cellular tolerance to aerobic exercise induced permutations of redox balance. These *in vivo* postulations have been demonstrated within rodents and *in vitro* (Lepore et al., 2000; Lepore et al., 2001; Shein et al., 2005; Shein et al., 2007; Shima et al., 2008).

2.9. Aims and purposes

The experiments within this thesis aim to initially establish any circadian and/or diurnal variation in iHSP72 expression. Establishment of such fluctuations across a given period of time, i.e. 24 h, are important as decreased or increased basal iHSP72 expression is correlated to an organism's cellular resistance to challenges in homeostasis. As such, it may be evident that time of day variation in iHSP72 may leave an organism, or for example an exercising human, more susceptible to the bio-chemical rigours of exercise.

It is well cited that both thermal and exercise stress increases both iHSP72 and eHSP72, however, the use of a non-thermal non-exercise based protocol to increase basal HSP72 has not been attempted *in vivo*. The confirmation of findings, such as those regarding cyclic trends in basal expression, would enable secure elucidation of the effect of

various non-lethal *in vivo* environmental stressors (hypoxia and hyperbaria) on basal iHSP72 expression. Of specific interest would be the ability of such exposures to increase basal iHSP72 expression, similar to the kinetics seen in response to hyperthermic exercise acclimation protocols. The goal of such increases would be in initiating conferred tolerance to the bio-chemical rigours of aerobic exercise, such as disruptions to redox balance. Additionally, the oxidative and stress protein response to such environmental stressors would be accessed to both acute and repeated acute exposures, as it is known that repeated exposure to stressors such as exercise, modulate both the basal and stress inducible characteristics of iHSP72 and the magnitude of disturbances to redox balance.

These thesis aims (research questions) can be summarised as experimental objectives:

<u>Experiment 1</u>

i) Investigate the basal expression of HSP72 during a 24 h period at 4 h intervals.

ii) Influence and/or relationship of core temperature on basal HSP72 expression to be assessed.

<u>Experiment 2</u>

i) Investigate the reproducibility of any circadian/diurnal trend in basal HSP72 expression.

ii) Compare intracellular and extracellular HSP72 expression sensitivity.

<u>Experiment 3</u>

i) Investigate the effect of hyperbaric air and hyperbaric oxygen on basal HSP72 expression.

ii) Explore whether any such hyperbaric stress mediated changes in HSP72 may have a relationship with alterations in redox balance.

Experiment 4

i) Investigate the effect of hypoxia on basal HSP72 expression.

ii) Explore whether any such hypoxia mediated changes in HSP72 may have a relationship with alterations in redox balance.

Experiment 5

i) Investigate the effect of once daily hypoxia for ten consecutive days on basal HSP72 expression.

ii) Explore whether any such daily hypoxia mediated changes in HSP72 may have a relationship with alterations in redox balance.

iii) Examine whether once daily hypoxia for ten consecutive days effects maximal oxygen consumption.

iv) Examine the EPO response to once daily hypoxia for ten consecutive days.

Experiment 6

i) Investigate the effect of once daily hypoxia for five consecutive days on basal HSP32 expression.

ii) Investigate the effect of prior induction of HSP32 and HSP72 on the exercise induced stress protein response.

iii) Investigate the effect of prior induction of HSP32 and HSP72 on exercise induced disturbances in redox balance.

Elucidation of these experimental objectives may allow greater efficacy in their application to exercise performance in challenging environments and may provide a strategy in conveying protection to the bio-chemical rigours of exercise.

Chapter 3. General Methodologies

3. General Methodologies

This chapter describes the general procedures that were performed in the studies outlined in the following experimental chapters. The specific rationale and methodologies for each individual experiment are presented in their respective chapters.

3.1. Participants

The participants for each experiment performed in this thesis were healthy male subjects, aged between 18 and 25. Participants were considered recreationally active and were all non-smokers due to the impact smoking has upon HSP72 expression (acute and chronic increases) (Anbarasi et al., 2006). Before undertaking the various experimental procedures each subject was informed of the procedures and risks involved in participation and subsequently all gave informed consent in written and verbal format. Each subject was screened medically and ethical approval for each of the experimental procedures contained within this thesis was obtained from the Department of Sport, Health and Exercise Science Ethics Committee at the University of Hull. Subjects were required to be free of any musculoskeletal injury or any acute or chronic illness and were not taking any medication that would affect the variables measured in this thesis.

3.2. Anthropometric data

Body mass (kg) and height (cm) were used as subject descriptive data and were measured using SECA digital scales (Holtian Ltd, Crymych, Dyfed) and Holtain Stationmaster (Holtain Ltd, Crymych, Dyfed) respectively.

3.3. Body Temperature Measurement

Body T_c was measured using a small ingestible temperature sensor pill (CorTemp™, HQ Inc, Palmetto, FL). Ingestion 2 h prior to the first reading was incorporated into experimental designs, as this has been recommended to allow sufficient time for

motility into the small intestine and to minimise the effects of cold liquids on temperature readings (O'Brien et al., 1998). In this thesis the pill was always administered 2 h prior to readings to ensure it was properly located in the small intestine at the onset of the study. This time of pill ingestion, coupled with the restrictions of food and fluid intake prior to arrival at the laboratory, ensured that the temperature recorded was not influenced by these factors. These pills have been validated in humans in comparison to the gold standard rectal probe method, with only a 0.19 °C mean bias evident in comparison to the gold standard method (Casa et al., 2007). Throughout the experimental studies T_c was measured at 5 min intervals. The temperature pill consists of a temperature sensitive quartz crystal oscillator with a silver oxide battery, covered with silicone rubber. The sensor transmits a continuous, low-frequency radio wave, which varies with temperature. The signal was received by a data logger placed level with the small of the back (CorTemp™ Data Recorder HT150016, HQ Inc, Palmetto, FL). A linear correlation that exceeds 0.999 exists between signal frequency and temperature and thus represents minimal error of measurement from the pill to the data logger (O'Brien et al., 1998). The pills are individually calibrated by the manufacturer with accuracy of the temperature sensor pill being ± 0.1 ºC. The temperature pill passes through the upper portion of the gastrointestinal tract, into the small and large intestine and is capable of tracking body T_c for 72 h following ingestion. The magnitude of temperature fluctuation attributed to movement of the pill through the gastrointestinal tract has been estimated at 0.2 - 0.3 ºC (Kolka et al., 1993).

3.4. Blood Collection

Venous blood samples were drawn by standard venepuncture technique from an antecubital vein, after 10 min in a supine position, into potassium EDTA (iHSP72) or sodium citrate (Glutathione) vacuette tubes (Vacuette®, Greiner Bio-one, UK). The blood collection timings i.e. time points are highlighted in their respective chapters.

This consistent timing of blood sample collection was important to account for diurnal and circadian variations in basal *mHSP72* (chapters 4 and 5), especially as basal values can dictate the magnitude of stressor mediated changes in HSP72, both *in vivo* (Gjovaag and Dahl, 2006) and *in vitro* (Vince et al., 2010).

3.4.1. HSP72 specific blood collection considerations

Fortes and Whitham (2009) demonstrated that repeated venepuncture demonstrated no stress induced changes in HSP72 in comparison to repeated cannulation blood draws. Therefore, rather than regularly flushing cannulas, which may dilute/alter sample concentration repeated venepuncture blood draws were utilised. Additionally, it is important to note that EDTA coated tubes were utilised as these have been shown to yield higher HSP72 values than other anti coagulation tubes, which for low basal HSP72 values is an important factor (Whitham and Fortes, 2006).

3.4.2. Glutathione specific blood collection considerations

It is important to note that sodium citrate treated blood was collected for analysis of glutathione. Immediately after collection a 2 ml aliquot was mixed with 8 ml of freshly prepared 5% meta-phosphoric acid (Sigma-Aldrich Company Ltd., Dorset, England). This mixture was transferred to 1.5 ml eppendorf tubes, stored on ice for 15 min, then centrifuged at 13,000*g and 4˚C for 15 min. The supernatant was collected and stored at -80°C for later analysis for TGSH and GSSG glutathione. This immediate preparation of glutathione specific blood samples is important to ensure glutathione reductase activity is limited to an absolute minimum and thus glutathione ratios are a true reflection of the exercise and hypoxic intervention. These considerations are line with recent guidelines to protect against artifactual GSH oxidation (Powers et al., 2010b). Whole blood glutathione ratios were ascertained throughout the experimental chapters within this thesis.

3.5. Method development for HSP72 quantification

There exists numerous experimental methods and techniques to measure both iHSP72 and eHSP72. These include microplate (MTS assay), microscopy, flow cytometry, ELISA, BIAcore and bead assay based methods (Ireland et al., 2007). Innate difficulties (i.e. arbitrary units) with quantification are seen in microplate, microscopy, BIAcore and bead assay based methods (arbitrary units used) with flow cytometry remaining only semi-quantitative (Ireland et al., 2007). ELISA based methods for quantification of serum and plasma HSP72 provide quantitative values (Ireland et al., 2007; Yamada et al., 2008). However, this method (ELISA) is not without limitations, which have been discussed in section 2.6.2. Furthermore, published work has discussed the variations in values obtained with differing ELISA kits upon the same samples (Njemini et al., 2003; Yamada et al., 2008). The precise mechanism and cellular mechanics that underpin these failures for ELISA based kits are discussed in chapter 2.6.4 and 5. These failures led to the re-evaluation of flow cytometry as a method to gain quantitative values. The literature acknowledges that flow cytometric detection of iHSP72 (Duffy et al., 1993; Chant et al., 1995; Bachelet et al., 1998; Fehrenbach, 2005; Ireland et al., 2007) is a rapid, easy, accurate and quantitative method (Nolan and Sklar, 1998; Vignali, 2000; Herzenberg et al., 2006), particularly suited for the determination of protein levels in individual cells from a heterogeneous population such as PBMCs (Bachelet et al., 1998; Fehrenbach, 2005; Ireland et al., 2007), and is applicable to cohort studies (Duffy et al., 1993; Chant et al., 1995; Bachelet et al., 1998; Fehrenbach, 2005; Ireland et al., 2007). Bachelet et al (1998) performed comparisons between biometabolic labelling, western blotting, immunofluorescence and immunoperoxidase microscopic analysis and demonstrated a high level of agreement between these different methods; however, cytometry was more sensitive for iHSP72 detection than western blotting. It is for these reasons that flow cytometry has been used to ascertain HSP72 values. Additionally,

iHSP72 measures were investigated due to lack of knowledge regarding the bio-origin, physiological effects and debates regarding the accuracy of the ELISA kit based methods used to obtain values of eHSP72 (Yamada et al., 2008).

Of the PBMCs, L HSP72 expression was shown to be negligible at rest with no increase post exercise stress (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Whitham et al., 2004). Specifically, M in comparison to G, are the most stress sensitive of the PBMCs and express the greatest amount of HSP72 in response to heat stress (Bachelet et al., 1998). Recent experimental research investigating the PBMC response to exercise in various environmental conditions have reported data within M only (McClung et al., 2008; Selkirk et al., 2009), and reflect the importance of M specific HSP72 responses. This is why M expressed HSP72 analysed via flow cytometry has been chosen to quantify HSP72 expression within this thesis (rationale discussed above). Further method development has incorporated CD14 into the *mHSP72* assay, which ensured *mHSP72* was gated during analysis. The protocols employed within all experimental chapters include extended periods of data collection, every 3-4 h, with the potential for samples in future studies needing to be obtained at altitude and consequently frozen for retrospective analysis. Therefore, method development was conducted into the impact of freezing strategies upon the accuracy of the assay utilised. Three tubes of blood were taken from subjects and either processed live, flash frozen with liquid nitrogen or slow frozen via a -80 ^{o}C freezer. The results demonstrated that flash freezing compromised results beyond the accuracy dictated in the manufacturer’s specifications whilst the slow freezing protocol increased the standard deviation between triplicate tubes and different subjects beyond acceptable levels. Therefore, the decision to process all samples live was taken.

3.6. Monocyte HSP72 and HSP32 assay

It is known that *mHSP72* produces the greatest amount of PBMC iHSP72 in response to heat, therefore it was postulated that these cells would be most sensitive to changes in T_c which was of specific interest within chapter 4 (Bachelet et al., 1998). Furthermore no increase in iHSP72 in either neutrophils or L was found in our laboratory post thermal stress (data not shown). Information regarding oxidative stress induced changes in PBMC iHSP72 *in vivo* has not been reported within the literature and this thesis will directly address this lack of data.

Whole blood (100 μL) from EDTA tubes was transferred into a 2mL red blood cell lysing buffer (Erythrolyse, AbD Serotec, UK). Cells obtained after red cell lysis were fixed and permeablised (AbD Serotec, UK) and a negative control (FITC, AbD Serotec, UK) or anti-HSP72 antibody (SPA-810, Assay Designs, USA) or anti HSP32 antibody (OSA-111, Assay Designs, USA) was added to a final concentration of 100ug/ml, this was used to label 1 x 10^6 cells according to the manufacturer's instructions and then incubated for 30 min in the dark. An IgG1 (HSP72) or IgG2 (HSP32) isotype and concentration matched FITC conjugated negative control was used to asses non-specific binding. Samples were then washed with phosphate buffered saline (PBS) before analysis on a BDFACSCalibur (BD Biosciences) by flow cytometry with M gated by forward/side scatter properties and further discriminated by CD14 expression. Mean fluorescence intensity (MFI) was then calculated using CELLQuest software (BD Biosciences) with a total of 50,000 cells counted.

This accuracy and sensitivity of this technique has been discussed in section 3.5 with information regarding the performance of the antibodies used discussed in section 4.4.

3.7. Blood measure of oxidative stress

3.7.1. Glutathione

Both TGSH and GSSG were analyzed using a commercially available kit (Total Glutathione Detection Kit, Assay Designs, Ann Arbor, MI) with values ascertained from whole blood. For determination of TGSH previously prepared blood (described previously in section 3.4.2.2.) was diluted to 1:40 in a 1X assay buffer solution and transferred to a 96 well plate. A standard curve was produced from serially diluting 50 µl 1X assay buffer and 50 µl GSSG. A reaction mixture containing 20 µl of glutathione reductase (GRx) was added to all wells on the plate, which was subsequently read at 405 nm every minute for 10 min in a micro plate reader. All standards and samples were analyzed in triplicate with the mean reported.

For determination of GSSG 1 µl of a 2M 4-vinylpyridine (Sigma-Aldrich Company Ltd., Dorset, England) solution was added to 50 µl previously prepared blood samples (described previously). At the same time 3 µl of 2M 4-vinylpyridine solution was added to 150 µl GSSG for standard curve determination. Samples were incubated at room temperature for 1 h after which 5 µl of 4-vinylpyridine-treated blood samples was added to 45 µl of assay buffer in a 96 well plate. To create a standard curve, 50 µl of the standard curve solution was added to 50 µl assay buffer and serially diluted. All standards and samples were analyzed in triplicate.

3.7.2. TBARS

An EDTA Vacuette tube filled with venous blood was mixed and then centrifuged at 1,500 x g for 10 min, with the resulting EDTA plasma removed and stored at -80°C. This plasma was, at a later date, analysed for lipid peroxidation using a commercially available kit following manufacturer's instructions (ZeptoMetrix, USA) as used

previously elsewhere (Vince et al, 2009). Previously frozen plasma samples, obtained via centrifugation on their first thaw were brought to room temperature. An aliquot (100 µl) was added to 100 µl of sodium dodecyl sulfate (SDS) solution and 500 µl of thiobarbituric acid solution. Samples were incubated for 1 h at 95˚C after which time they were cooled to room temperature and centrifuged at 3000*g for 15 min. Samples were added to a 96 well plate and read at 532 nm in a microplate reader (Biotek Synergy HT-R, Biotek Instruments, Vermont, USA). Results are expressed in malondialdehyde (MDA) equivalents. All standards and samples were analyzed in triplicate.

3.8. General experimental restrictions and controls

Smoking may trigger HSP72 expression through oxidative stress and hence why only non-smokers were recruited for all projects (Anbarasi et al., 2006). Due to their ability to increase HSP72 expression, caffeine (Whitham et al., 2006; Lu et al., 2008) and glutamine (Singleton et al., 2004) consumption were barred from all meals and beverages for 48 h prior to and during all experimental conditions. This control was achieved by the implementation of a standardised diet given to all subjects. The content of this diet was developed upon investigation of a two week food diary recorded by volunteers prior to any experimental procedures taking place. Upon investigation of this data, and in cohort with volunteers, a food intake and standardised diet was developed, which, subjects followed when away from the laboratory and was adhered to when providing meals under experimental conditions. Compliance was monitored via a questionnaire, administered before and during the intervention periods with adherence at 100% for all projects.

Subjects abstained from alcohol and exercise for seven days prior to testing. Furthermore, subjects refrained from all supplementation (i.e., vitamins, ergogenic aids,

etc.) throughout the various study periods and abstained from such supplementation for 30 days prior to experimental procedures commencing. Additionally, volunteers were requested to abstain from prolonged thermal exposures (baths, saunas, steam rooms, tanning devices, etc) for seven days prior to and during all experimental conditions. Furthermore, subjects who had visited or resided at altitudes in excess of 1000 m or climates with ambient temperatures in excess of 30°C, or had experienced high pressure environments, i.e. hyperbaria, three months prior to study commencement, were excluded during recruitment. Compliance was monitored via a questionnaire administered before and during the intervention period and was at 100% in all studies. Additionally subjects who habitually took supplements (vitamins, minerals, etc) were excluded during recruitment.

All laboratory based experiments and trials were conducted and/or administered within an externally regulated temperature controlled laboratory (from here after referred to as a temperature controlled laboratory). This laboratory although not fully temperature controlled is centrally controlled with regards to air temperature. Measures were taken to ensure air temperature was consistent and unaltered throughout all laboratory based testing procedures. This is reflected by the temperatures (mean ± SD) reported for the durations of each experimental chapter; 18.1ºC ± 0.9ºC, 20.9ºC ± 0.3ºC, 20.8ºC ± 0.3ºC, 21.2ºC ± 0.2ºC, 20.9ºC ± 0.5ºC and 20.9ºC ± 0.5ºC experimental chapters 1-6 respectively.

Experimental chapter specific controls in terms of familiarisation, randomisation, sample/power calculations, etc are commented upon within individual experimental chapters.

3.9. Generation of hypoxia and physiological monitoring during exposures

All hypoxic exposures were generated and administered within an externally regulated temperature controlled laboratory (from here after referred to as a temperature controlled laboratory). The hypoxic exposure consisted of 75 min at a simulated altitude of 2,980 m which equates to an O_2 concentration of 14.5 % at a barometric pressure of 775 mmHg, with the intervention commencing and ceasing at 0930 and 1045 respectively. The timing of the hypoxic exposure was consistent across chapters 7, 8 and 9 to ensure stressor mediated increases in *mHSP72* were not unduly affected by differences in basal *mHSP72* content, which were shown to follow circadian and diurnal rhythms in chapters 4 and 5 respectively. Furthermore, the elevation utilised is known to be well tolerated within humans *in vivo* and had been given ethical approval by the Department of Sport, Health and Exercise Science Ethics Committee at the University of Hull. Hypoxia was delivered via a hypoxicator (Hypoxico HYP123 hypoxicator, New York, USA). The hypoxicator utilises O_2 filtration to generate the necessary hypoxic load. During the hypoxic exposure(s), heart rate and O_2 saturation were recorded every 5 min via a finger pulse oximeter (Nonin 9550 Onyx II Finger Pulse Oximeter, Nonin Medical Inc, Plymouth, USA).

Alterations in T_c have been shown to impact upon *mHSP72* expression (chapter 4), however, the hypoxic exposure detailed above, demonstrated no significant changes in the T_c of subjects throughout the hypoxic exposure during pilot testing within a temperature controlled laboratory. Furthermore, as shown in chapter 4 and during pilot testing, T_c was unchanged when residing within the same laboratory during “waking” hours (0800 – 2100).

3.10. Statistics

Statistical analysis was performed using SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL) and Minitab® version 14.2 (Minitab Inc., State College, PA). Data are presented as mean ± standard deviation (SD). The specific statistical procedures employed in each individual experiment are presented in their respective chapters.

Chapter 4.Experiment 1: Variation in basal heat shock protein 72 is correlated to core temperature in human subjects.

This experimental chapter has formed the basis of the publication detailed below:

Sandstrom, M. E., Madden, L. A., Taylor, L., Siegler, J. C., Lovell, R. J., Midgley, A. W. & McNaughton, L. 2009. Variation in basal heat shock protein 70 is correlated to core temperature in human subjects. Amino Acids, 37, 279.

NB: Although not first author on the publication detailed above, I contributed significantly to the recruitment, design, sample acquisition, sample processing and writing up of this novel piece of scientific work. However, due to this being my first project (and peer reviewed co-authored publication) within my PhD studies it was decided by my supervisors that Dr Sandstrom should oversee the writing up process of this manuscript and as such she is evidently listed as first author. Additionally, this experimental chapter formed part of a larger completed project, which produced the publication below:

Madden, L. A., Vince, R. V., Sandstrom, M. E., Taylor, L., McNaughton, L. & Laden, G. 2008. Microparticle-associated vascular adhesion molecule-i and tissue factor follow a circadian rhythm in healthy human subjects. Thrombosis and Haemostasis, 99, 909-915.

4.1. Introduction

The functions of HSP72 indicate that there is a need for a presence of this molecular chaperone across various cellular compartments, albeit a low level at rest. Its induction *in vitro* (Ritossa, 1962; Vince et al., 2010) and *in vivo* (McClung et al., 2008; Selkirk et al., 2009; Magalhães et al., 2010) is implicitly linked to thermal stress, with exercise in addition to thermal stress augmenting the response in excess of their sum singular responses (Whitham et al., 2007). However, *in vitro* research lacks relevance in comparison to the thermal loads experienced by humans at rest, during exercise and in extreme hyperthermic environments. For example, *in vitro* (Oehler et al., 2001; Sonna et al., 2002b) cells were heat shocked to (39-45°C), a magnitude rarely seen within humans for prolonged periods.

These high temperatures fail to elucidate the sensitivity of HSP72 regulation *in vivo* under homeostasis or stressful conditions, such as those experienced by the body during hyperthermic exercise, with ethical constraints generally terminating such laboratory based exercise at a T_c of 39.5^{o}C (Selkirk et al., 2009), however, during un-monitored competition (matches and events) this ethical guideline is known to be exceeded (Casa et al., 2007; Sonna et al., 2007; Noakes, 2008). Furthermore, in the absence of thermal stress *in vivo*, i.e. thermoneutral environments, T_c has been shown to follow a tightly regulated circadian rhythm (Krauchi and Wirzjustice, 1994; Krauchi and Wirz-Justice, 2005; Reilly et al., 2007) with variation in T_c shown to be small at 0.6^{o}C over a 24 h period, from 36.4 ^{o}C to 37.5^{o}C (Krauchi and Wirzjustice, 1994). Diurnal variations and circadian rhythms play a vital role in whole body homeostasis (Maywood et al., 2006; Hastings et al., 2007; Hastings et al., 2008; Montagnana et al., 2009; Reddy and O'Neill, 2010) and sporting performance (Edwards et al., 2005; Edwards et al., 2007; Reilly et al., 2007; Reilly and Waterhouse, 2009).

Given the diverse roles HSP72 plays during homeostasis and under stress (Benjamin and McMillan, 1998) and the importance of circadian rhythms to whole body homeostasis (Montagnana et al., 2009) it is surprising that basal circadian HSP72 expression has not been elucidated securely. Two flawed attempts have been made (Fehrenbach et al., 2005; Fortes and Whitham, 2009), both of which have used measures of eHSP72 via the problematic ELISA kit which lacks the sensitivity of flow cytometry (discussed in section 3.5). Furthermore, small subject numbers of six (Fehrenbach et al., 2005) and seven (Fortes and Whitham, 2009) were utilised which lack statistical power upon subsequent analysis of the data obtained (discussed in detail in section 2.1.6). Due to the highly heat sensitive nature of *mHSP72* and the sensitivity of flow cytometry to detect such changes (Duffy et al., 1993; Bachelet et al., 1998; Fehrenbach, 2005; Ireland et al., 2007), their combination may make an ideal tool to investigate subtle changes in *mHSP72* during the tightly regulated circadian variation in T_c (Krauchi and Wirzjustice, 1994). Therefore, this approach may provide a more secure vessel to explore any circadian variation in iHSP72 than previous attempts with regards to eHSP72 (Fehrenbach et al., 2005; Fortes and Whitham, 2009).

It is likely given that exercise has been shown to increase iHSP72 within skeletal muscle for seven days post exercise (Morton et al., 2006); many previous projects are unlikely to have yielded true baseline values in iHSP72, which is important as basal values can dictate the magnitude of expression post stressor (McClung et al., 2008). Therefore, the time of day basal variations in iHSP72, in truly rested subjects, are unanswered. Additionally, it is obvious that few studies to date have obtained a valid resting basal value for iHSP72. This is pertinent as both *in vitro* (Vince et al., 2010) and *in vivo* (Gjovaag and Dahl, 2006; McClung et al., 2008) magnitude of expression in HSP72 post stressor is dependent on basal values.

With the well cited functions of HSP72 in both stressed and unstressed cells (Benjamin and McMillan, 1998) the notion of HSP72 following a circadian rhythm, which may be linked to T_c, is an interesting one. The implications of such findings could have a multifaceted impact on several scientific disciplines, including the biomedical (Brodsky and Chiosis, 2006) and sports science related fields (Magalhães et al., 2010). Therefore, the aims of this experimental chapter were to investigate; 1) the basal expression of iHSP72 during a 24 h circadian period at 4 h intervals and 2) the influence and/or relationship of core temperature on basal HSP72 expression.

4.2. Methods

Subjects

Seventeen recreationally active (mean ± SD: 5.9±2.2 $h \cdot wk^{-1}$) male subjects (177±6.4 cm, 75.7±10.9 kg, 19.8±4.3 years) volunteered to participate in the study. Please see section 3.8 for general experimental restrictions and controls which were adhered to within this experimental chapter.

Experimental Design

Each subject was given a temperature sensor pill (CorTemp™, HQ Inc, Palmetto, FL) to swallow at 0700 the morning of the testing to allow adequate time for motility into the small intestine and to minimize the effects of the ingestion of cold liquids on temperature readings (O'Brien et al., 1998). On the day of testing, the subjects reported to a temperature-controlled laboratory (mean ± SD: WBGT 18.1ºC ± 0.9ºC throughout the experimental time period – 24 h) at 0800 for acclimatisation. All procedures took place within a controlled environment, including blood sampling, non-exertional recreational activities (e.g. watching DVD’s) and sleeping. Furthermore, subjects were instructed to go to bed at 2300 and stay in bed until 0700 the morning after.

Dietary intake was equivalent between subjects and food was provided at 0930, 1330 and 1800. Blood samples were drawn from participants in the same order every 4 h from 0900 to 0900 the following day. Please see section 3.4 for HSP72 specific blood collection controls and considerations.

Heart rate was sampled and recorded every 5 s throughout the 24 h period on eight randomly selected subjects (Team System, Polar Electro, Finland). T_c was recorded at 5 min intervals during the 24 h period (CorTemp™ Data Recorder HT150016, HQ Inc, Palmetto, FL). In line with previous heart rate data (Greenleaf and Castle, 1971; Gonzalez-Alonso et al., 2000; Kay and Marino, 2003; Kenefick et al., 2009), the heart rate data in the present study were time-averaged over 5 min intervals, with the mean heart rate for each hour calculated from these 5 min intervals.

Due to the lack of previous data utilising the same assay to monitor basal values in iHSP72 a power calculation was not possible.

<u>Monocyte HSP72 assay</u>

See general methods chapter, section 3.7. The assay was run live every 4 h, at all time points during the 24 h period.

<u>Statistical analyses</u>

Statistical assumptions were checked using conventional graphic methods with T_c, *mHSP72* and heart rate not displaying normal distribution. Central tendency and dispersion are reported as the mean (SD). Differences in *mHSP72* concentration over time were analysed with a one-way repeated measures analysis of variance using Minitab® version 14.2 (Minitab Inc., State College, PA). Tukey tests were subsequently used to compare the *mHSP72* concentration at the first time point (0900) with each of the other time points. The relationships between *mHSP72* expression and T_c and

between *mHSP72* expression and heart rate were determined using Spearman rank correlation coefficient (due to absence of normality within this data) using SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL). Several outliers were observed and since these were checked and verified as valid, the correlations were reported with and without the outliers. A probability significance less than 0.05 was considered statistically significant. Polynomial contrasts were used to identify linear or quadratic trends in basal *mHSP72* expression over the 24 h period.

4.3. Results

HSP72

There was a statistically significant time-of-day effect for *mHSP72* concentration ($F = 7.4$; $p < 0.001$, Table 4.1 and Figure 4.1). Table 4.1 shows that paired differences between the *mHSP72* concentrations at 0900 were statistically significant from all other time points except 21:00. Furthermore, there was a significant positive correlation between T_c and *mHSP72* expression (r_s=0.44, p<0.001, Figure 4.4. r_s=0.41, p<0.001 after three outliers were removed). Additionally there was an obvious quadratic trend in *mHSP72* expression during "waking" hours (0900 – 2100) ($F = 21.2$, $p < 0.001$)

Core Temperature

The mean T_c for the subjects at the time of blood sampling is shown in Figure 4.1. One temperature pill was lost due to secretion before the 2100 recording. T_c demonstrated a decrease in the evening with the lowest values observed during the early morning hours. T_c, HSP72, and to a lesser extent heart rate, did not increase to the same values as that seen at 0900 on the previous day. This is probably due to the fact that the subjects had no physical activity the second morning of the trial, being at rest, because they were already at the study location.

Heart Rate

Heart rate data monitored over 24 h are shown in Figure 4.2 The findings correlate with previous studies (Millarcraig et al., 1978; White, 2007). In line with previous heart rate data (Greenleaf and Castle, 1971; Gonzalez-Alonso et al., 2000; Kay and Marino, 2003; Kenefick et al., 2009), the heart rate data in the present study were time-averaged over 5 min intervals, with the mean heart rate for each hour calculated from these 5 min intervals. A correlation between heart rate and *mHSP72* was also observed (r_s=0.30, p=0.03, Figure 4.3; r_s = 0.23; p = 0.11 after the two outliers were removed).

Table 4.1. Paired comparisons of the mean *mHSP72* concentration for seven time points during a 24 h period.

Comparison (times)	Mean difference (MFI)	95% confidence interval (MFI)	Adjusted P value
09:00 - 13:00	-1.57	-2.81, -0.34	0.004
09:00 - 17:00	-1.45	-2.70, -0.19	0.01
09:00 - 21:00	-0.04	-1.28, 1.19	1.0
09:00 - 01:00	-1.54	-2.85, -2.23	0.01
09:00 - 05:00	-2.01	-3.30, -0.73	0.0002
09:00 - 09:00	-0.56	-2.93, -0.19	0.02

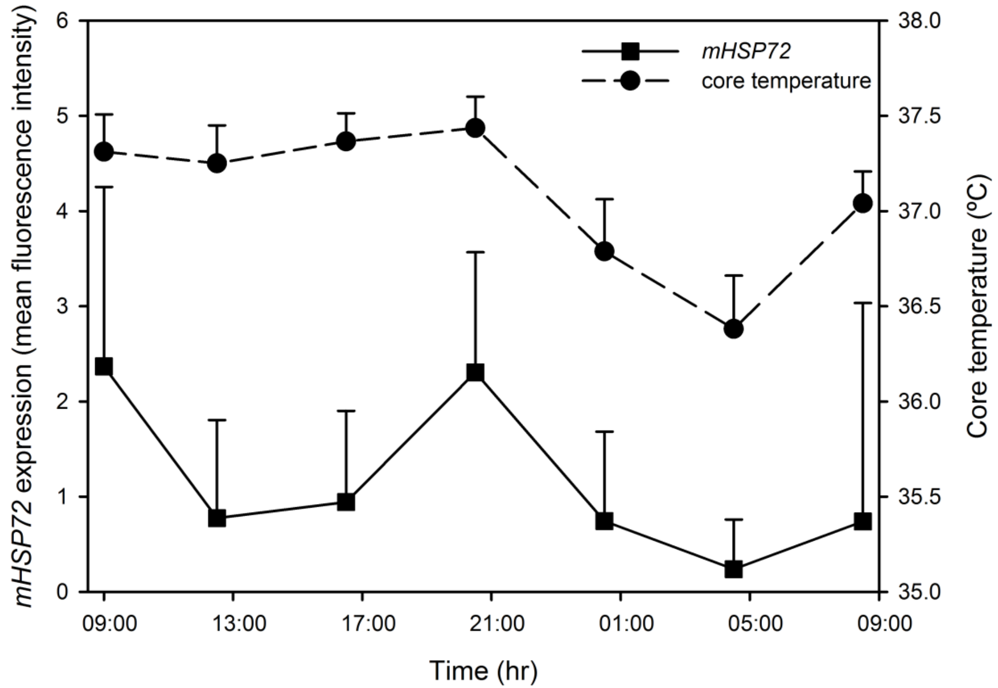

Figure 4.1. Mean *mHSP72* expression and mean T_c over the 24 h experimental period (n = 17). Error bars represent the standard deviation of the mean.

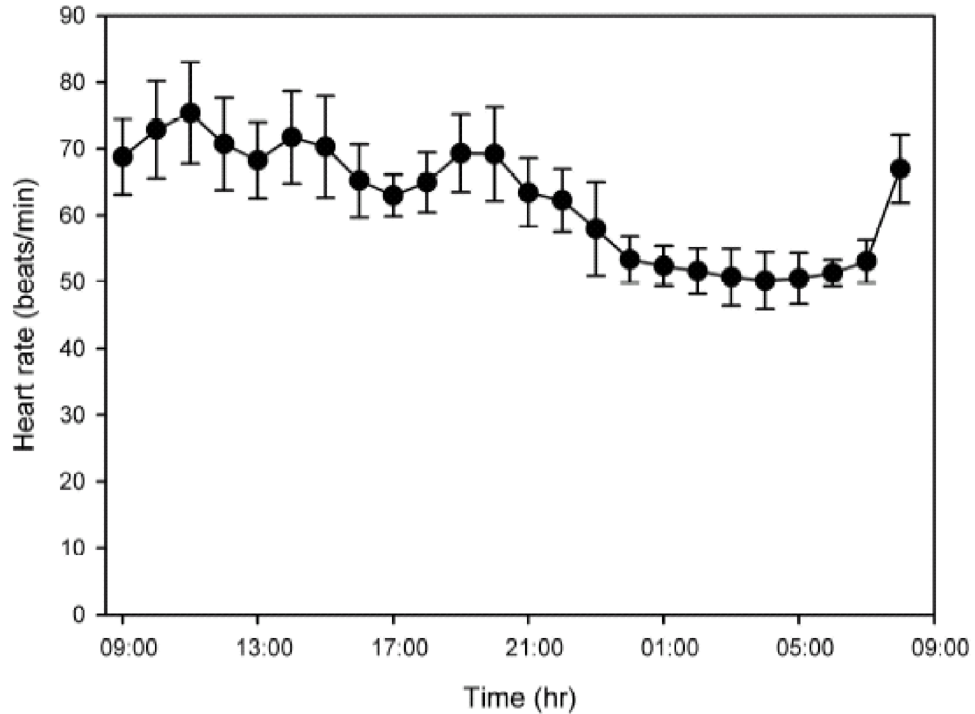

Figure 4.2. Mean heart rate over the 24 h experimental period (n = 8). Error bars represent the standard deviation of the mean.

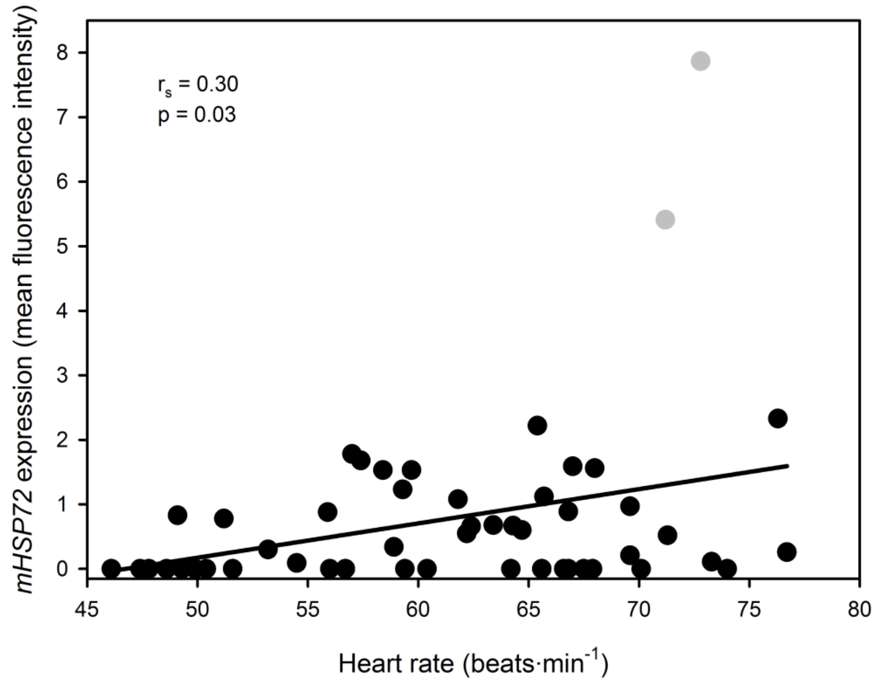

Figure 4.3. Scatterplot of *mHSP72* expression against heart rate at times 0900, 1300, 1700, 2100, 0100, 0500 and 0900 (n = 8). The solid black diagonal line is the least squares line of best fit. rs = Spearman rank correlation coefficient.

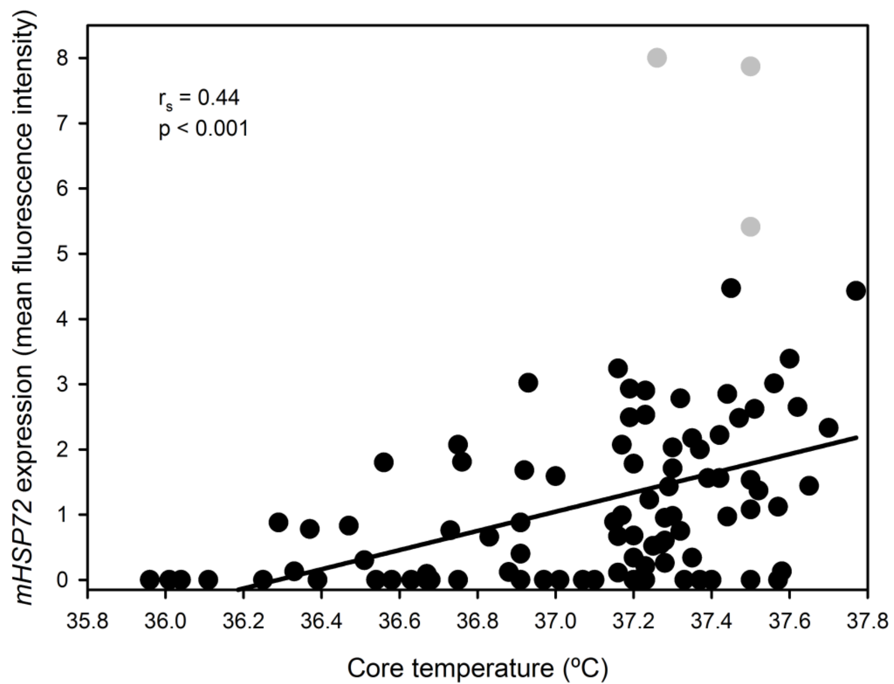

Figure 4.4. Scatterplot of *mHSP72* expression against T_c at times 0900, 1300, 1700, 2100, 0100, 0500 and 0900 (n = 17). The solid black diagonal line is the least squares line of best fit. rs = Spearman rank correlation coefficient.

4.4. Discussion

This experimental chapter investigated the diurnal variation in *mHSP72* expression and its relationship with T_c. It was evident that *mHSP72* expression followed a statistically significant ($p<0.001$) time-of-day variation which was significantly correlated (albeit with a moderate effect size $r_s=0.44$) to T_c. The T_c values here were in agreement with data reported previously, with a range across the 24 h experimental period of 0.6^{o}C (Krauchi and Wirzjustice, 1994; Reilly et al., 2007). Furthermore, during waking hours (0900 – 2100) there was a significant ($F = 21.2$, $p < 0.001$) quadratic trend in *mHSP72* expression.

Within the present study *mHSP72* expression was assessed over 24 h on male subjects at rest, to investigate its basal variation and its correlation to T_c. Evidently, during resting conditions, there is a diurnal variation in *mHSP72* which correlates moderately with T_c (Fig. 4.4), suggesting a potential role in homeostasis. Given that HSP72 is the most inducible (exercise, environmental stress and disease) and abundant of the HSPs (Kregel, 2002; Katschinski, 2004) and that high endogenous levels of HSP72 provide cellular protection to habitual *in vivo* (McClung et al., 2008; Amorim et al., 2010; Magalhães et al., 2010) and *in vitro* (Maloyan et al., 1999) stress, whilst, being increasingly recognised to offer protection to further non-lethal non-related stressors, e.g. heat shock conferring tolerance to ischemic reperfusion injury *in vitro* (Shein et al., 2005; Shein et al., 2007; Shima et al., 2008). This potential variable role of HSP72 in maintaining homeostasis and moderate correlation to T_c is a logical one, given the key role HSP72 has in many biological functions (Benjamin and McMillan, 1998) and the key role variation in T_c (T_c moderately correlated to *mHSP72* in the present chapter) is known to play in homeostasis (Krauchi and Wirz-Justice, 2005; Waterhouse et al., 2005). Despite the important cellular functions of HSP72, investigation into basal iHSP72 expression has not been previously conducted and thus the findings within this

experimental chapter (moderate correlation of T_c and *mHSP72*) can be considered novel. It is likely that the sensitivity of the iHSP72 method (flow cytometry) utilised (Duffy et al., 1993; Chant et al., 1995; Bachelet et al., 1998), in light of other typical eHSP72 (Fehrenbach et al., 2005; Fortes and Whitham, 2009) techniques and approaches previously employed (discussed earlier in sections 2.6 and 3.5), was able to detect the small changes in *mHSP72* and thus demonstrate the circadian variation in basal *mHSP72*, which was moderately correlated to changes in T_c (Figure 4.4), and detected the quadratic trend in expression during waking hours (Figure 4.1).

Given the minimal variation in air temperature throughout the experimental period (WBGT 18.1ºC ± 0.9ºC) and the circadian variation in T_c of 0.6^{o}C within the same period (which is line with previous data 0.6^{o}C (Krauchi and Wirzjustice, 1994; Reilly et al., 2007)) it is unlikely that variation in air temperature was a confounding factor for *mHSP72* expression or $T_{c.}$ Issues regarding laboratory temperature control have been discussed in section 3.8. However, from the presented results a clear cause and effect relationship between T_c and *mHSP72* cannot be claimed. Future work could clamp T_c, via passive heat control strategies within an experimental group, and compare such an experimental group to the presented data and a control condition.

Cellular stress disrupts homeostasis and can lead to rapid HSP72 expression. This has been widely studied within thermal and exercise preconditioning (Madden et al., 2008a). Regular exercise has been shown to lead to decreased basal HSP72 concentrations within M and L (Fehrenbach et al., 2000b). Human leukocytes isolated following an increase in T_c to 40ºC or above, have also been shown to produce a lower amount of HSP72 in response to an immediate subsequent thermal challenge (Ryan et al., 1991), suggesting acquisition of thermal tolerance. Furthermore, it has been demonstrated *in vitro* that basal HSP72 expression post stressor is correlated to its basal value (Vince et al., 2010). Therefore, the data presented here may have implications for athletes in a

thermal/exercise preconditioning training regime. Theoretically, training at a time when basal *mHSP72* concentration is lowest may cause the maximum increase in *mHSP72* response. This could be important in conferring increased cellular protection to subsequent stress via increased basal *mHSP72*. However, given that the two lowest nadirs in *mHSP72* expression are 0500 and 1300 respectively (Figure 4.1), prescription of exercise at 0500 is unlikely and thus 1300 is a more likely time of exercise prescription.

When exercise is performed at times when T_c is highest (T_c max) and lowest (T_c min), within the circadian rhythm, the further increase due to exercise has been shown to be reduced at T_c max compared to T_c min (Waterhouse et al., 2004). Thermoregulatory changes brought about by exercise may depend upon T_c prior to the period of exercise and therefore time-of-day may be of importance to athletes. Athletes crossing time zones will also be affected by circadian rhythms, as they may be competing when T_c is reduced. The reduction in T_c tends to increase rate of perceived exertion (RPE) during short exercise periods, which may then demand longer recovery times between training sessions (Waterhouse et al., 2004). RPE has been shown to increase markedly after 5 min of exercise at 0500 compared to 1100, 1700 and 2300 (Waterhouse et al., 2004). Furthermore, the same study reported that after 30 min, the lowest RPE was seen when exercise began at 1100. Exercise undertaken at 0500 (at T_c min), resulted in the most rapid increase in T_c, albeit to levels still ultimately lower than exercise performed at other times when resting T_c was higher (Waterhouse et al., 2004). This indicates that thermoregulation may be most effective at T_c max and T_c min to protect from hyperthermia and hypothermia, respectively. As shown, *mHSP72* is moderately correlated to T_c and therefore the *mHSP72* minimum observed at 0500 may have an effect on exercise RPE and the coinciding rapid increase in T_c following exercise at this time (Waterhouse et al., 2004). The rapid rise in T_c could lead to problems in protein

synthesis (Selkirk et al., 2009; Amorim et al., 2010) and be responsible for the noted higher rate of RPE at this time (Waterhouse et al., 2004; Waterhouse et al., 2005). It may be suggested, from the data presented here, to exercise at times of the day when basal *mHSP72* is lowest, for example 1300 (rather than the unpractical 0500 minimum, see Figure 4.1), which may induce increased *mHSP72* expression that could be valuable in conferring tolerance to subsequent bouts of exercise (McClung et al., 2008; Amorim et al., 2010; Magalhães et al., 2010).

Quantification of HSP72 is not without difficulties, as discussed in the general methodologies chapter. During the course of this study it became evident that both the commercial ELISA kit and the antibody clone used in this and many other studies only recognises so called 'bio-available' HSP72 (Assay Designs, personal communication). That is HSP72 that is unbound, i.e. not performing chaperonic function. Binding of HSP72 to denatured or nascent proteins thus renders the antibody-binding site blocked and unrecognisable. The antibody clone used (SPA-810) is directed against an amino acid sequence (aa 437-504, worldwide patent WO 01/42423) that is within the peptide-binding domain of HSP72 (aa 383-508). This has implications in all measurements of HSP72 in live cells, specifically in those projects utilising a stressful intervention, whereby, if the stress response is initiated HSP72 may be undetectable if performing chaperonic functions. Therefore, accurate values of stress induced increases in HSP72 may be unobtainable.

In regards to future studies looking at increases in *mHSP72* with any intervention it might be important to control for the subjects T_c pre-testing. Several studies have found that the increase in HSP72 is correlated to basal levels *in vitro* (Maloyan et al., 1999; Vince et al., 2010) and *in vivo* (Gjovaag and Dahl, 2006; McClung et al., 2008). High basal levels could be one reason why very small changes in HSP72 have been observed in previous studies (Shastry et al., 2002; Liu et al., 2004; Watkins et al., 2007). The

mechanism behind the blunted increase in *mHSP72* with higher basal levels has previously been discussed and attributed to increased basal levels negatively regulating the transcriptional competency of HSF1 (Sandstrom et al., 2008), i.e. in line with the cellular thermometer model of HSP72 regulation, discussed in section 2.2.4. However, there are many factors other than T_c that affects the up-regulation of HSP72, which further adds to the difficulty controlling human studies.

The data derived in the current experimental chapter has some important implications to consider when designing and conducting future research in general and specifically to this thesis. Evidently, as *mHSP72* values fluctuate in accordance to the circadian rhythm shown in Figure 4.1, projects employing an intervention *in vivo*, may evoke differential *mHSP72* responses dependant on the time of day they are administered. This postulation is supported by specific data regarding *mHSP72* obtained *in vivo* (McClung et al., 2008) and *in vitro* (Vince et al., 2010), whereby, basal *mHSP72* is indicatively linked to the magnitude of post stressor *mHSP72* expression. Therefore, administration of any intervention must be conducted at the same time of day to allow intra and inter study comparison to be done securely. Furthermore, serial blood collection conducted on different days must be done at identical time periods to account for the circadian variation shown in Figure 4.1. These suggestions may seem obvious, however, previous work investigating exercise induced changes in *mHSP72* (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Fehrenbach et al., 2001; Selkirk et al., 2009) have not controlled for such circadian variations, which are important with regards to stressor mediated increases in iHSP72 as discussed above. It is plausible such variation in iHSP72 may need to be accounted for within skeletal muscle, although this has not been investigated within the presence study.

These factors will be incorporated into all future experimental procedures within this thesis. Additionally, despite the evidence presented here, notably the quadratic trend

during “awake” hours, this phenomenon has only been shown on one occasion and requires further evidence to confirm its repeatability, as well as, investigation in other tissues *in vivo*, such as skeletal muscle.

Chapter 5. Experiment 2: Daily quadratic trend in basal monocyte expressed HSP72 in healthy human subjects.

This experimental chapter has formed the basis of the publication detailed below:

Taylor, L., Midgley, A., Chrismas, B., Madden, L., Vince, R. V. & McNaughton, L. 2010. Daily quadratic trend in basal monocyte expressed HSP72 in healthy human subjects. Amino Acids, 38, 1483-1488.

5.1. Introduction

Previously, in chapter 4, *mHSP72* was shown under homeostatic conditions to follow a circadian variation in expression. Most notably, within the first 12 h of the 24 h project, during the "waking hours" (0900 to 2100), this circadian variation was shown to follow a quadratic trend ($F = 21.2$, $p < 0.001$). This expression was moderately correlated ($r_s=0.44$, $p<0.001$, Figure 4.4.) to T_c which may indicate that *mHSP72* is linked to homeostatic regulation at rest. It is unsurprising that *mHSP72* may well be linked to such cellular regulation, as HSP72 is known to be a governor of protein homeostasis (Benjamin and McMillan, 1998) and as such permutations in protein homeostasis ultimately impinge on all facets of successful functioning within the human body, i.e. mRNA synthesis (Bukau and Horwich, 1998).

Inter-subject variation in basal HSP72 values has been demonstrated within the muscle (Khassaf et al., 2001; Morton et al., 2006; Morton et al., 2009b) and in the PBMCs as shown in chapter 4 and elsewhere (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Fehrenbach et al., 2001; McClung et al., 2008; Selkirk et al., 2009). Both tissues have shown inter-subject variation in expression at numerous time points with basal/resting values demonstrating the most notable inter-subject variation within muscle (Khassaf et al., 2001; Morton et al., 2006) and PBMCs in this thesis (chapter 4) and elsewhere (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Fehrenbach et al., 2001; McClung et al., 2008; Selkirk et al., 2009). Additionally, further complicating this issue, there are basal gender and training status differences within the muscle (Morton et al., 2006; Morton et al., 2008; Morton et al., 2009b) and PBMC (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Selkirk et al., 2009), which have been shown to influence the magnitude of response post exercise stress *in vivo* (Morton et al., 2009b; Selkirk et al., 2009; Amorim et al., 2010; Magalhães et al., 2010). Therefore, in light of this evidence, it is plausible that despite the quadratic trend in circadian variation demonstrated in

chapter 4, inter-day variation in expression could be present and potentially underpin the quadratic and circadian trend illustrated previously. Disclosure of such fluctuations may strengthen/weaken those novel findings (i.e. circadian rhythm and quadric trend in basal *mHSP72*) and contribute to future work within the area. Specifically, as it is known differences in basal HSP72, i.e. daily basal fluctuations or training status differences, are implicitly linked to post stressor expression *in vitro* (Maloyan et al., 1999; Vince et al., 2010) and *in vivo* (Gjovaag and Dahl, 2006; McClung et al., 2008). Any such variation may be relevant to sporting performance, training schedules, heat acclimation strategies and the methodologies employed to measure HSP72 *in vivo*.

The aims of this chapter were to investigate; 1) the repeatability of the quadratic trend in basal *mHSP72* expression shown in chapter, and 2) compare iHSP72 (*mHSP72*) and eHSP72 expression sensitivity within the quadratic trend time period.

5.2. Methods

Subjects

Twelve healthy recreationally active non-smoking male subjects (mean ± SD: 20.2±1.9 years, 178.7±5.6 cm, 75.1±6.0 kg, PA (physical activity/h.week^{-1}) 5.2±1.9) volunteered to participate in the study. Please see section 3.8 for general experimental restrictions and controls which were adhered to within this experimental chapter.

Blood sampling and experimental protocol

Subjects consumed identical meals for each of the three experimental days, which were provided at 0830 and 1200. Subjects remained within the temperature controlled laboratory (average WBGT 20.9±0.3 ^{o}C, humidity 48±3 %) throughout the course of the study and slept within a nearby University owned accommodation block throughout the eight day extended study period. Study days were separated by three days (Monday,

Friday, Tuesday). On each of the study day's subjects spent their time under resting conditions within the temperature controlled laboratory. Blood samples were taken at 0800, 1100 and 1400. Please see section 3.4 for *mHSP72* specific blood collection controls and considerations.

Methods, procedures and restrictions replicated those used previously to gain serial data for *mHSP72* (chapter 4) and other physiological markers of homeostasis (Madden et al., 2008b; Vince et al., 2009) with sample frequency being increased to 3 h increments compared to the 4 h collections used previously. This increase in sampling frequency was designed to achieve greater insight into the expression kinetics of *mHSP72* over the targeted (9 h) period. Additionally, with greater experience of the assay employed and reduction in subject numbers in chapter 4 from seventeen to twelve in the present chapter, the minimum turnaround time required for assay completion and sample acquisition on the Flow Cytometer was reduced. Therefore, it was hoped that 3 h sample timings could be employed in all subsequent experimental chapters where longitudinal measures of *mHSP72* were required, and thus, greater insight into expression kinetics of *mHSP72* could be gained over a specific time period.

Monocyte HSP72 assay

See general methods chapter, section 3.7. The assay was run live every 3 h, at all time points on all experimental days.

eHSP72 ELISA

Plasma eHSP72 was analysed in triplicate using a commercially available high-sensitivity ELISA according to the manufacturers' protocol (Assay Designs EKS-715, MI, USA). EDTA plasma was obtained via centrifugation of the EDTA blood tube (10 min, 1500x g), stored at -80°*C* and analysed at a later date for plasma eHSP72. Due to the methodological and physiological concerns regarding the value of eHSP72

(discussed in sections 2.6 and 3.5), measures of eHSP72 were utilised to directly compare the sensitivity of eHSP72 ELISA to iHSP72 flow cytometry in assessing the presence of the previously disclosed quadratic trend in expression (chapter 4). Due to the absence of such a quadratic trend in eHSP72, in line with previous work (Fortes and Whitham, 2009), retrospective analysis was only conducted on one day of the protocol.

Statistical analysis

Statistical analyses were performed using PASW statistics 17 (SPSS Inc., Chicago, IL). Statistical assumptions were checked using conventional graphic methods with distribution deemed plausible for all variables. Central tendency and dispersion are reported as the mean (SD).

Polynomial contrasts were used to identify linear or quadratic trends in basal *mHSP72* expression at the same three times (0800, 1100, 1400) on three different days. The *mHSP72* expression at 1100 and 1400 was expressed as a percentage change from 0800 for each day (Morton et al., 2006; Morton et al., 2008; Morton et al., 2009a; Morton et al., 2009b). Polynomial contrasts were also used to identify linear or quadratic trends in plasma *mHSP72* at the same three times on a single day. In the event of a significant F statistic, Fisher's least significance test was used to locate significant paired differences. The relationship between eHSP72 and *mHSP72* was assessed using Pearson Correlation. Two-sided statistical significance was accepted as $p < 0.05$.

The statistical approach in this experimental chapter has developed from that used previously, i.e. a switch from Minitab® to PASW statistics 17, due to the *mHSP72* data being normalised (i.e. presented as a percentage of the first value obtained in line with previous work in the area), and as such, polynomial contrasts were a more suitable statistical method of analysis compared to the one-way repeated measures of analysis adopted in chapter 4. Furthermore, the adoption of PASW statistics (SPSS) would

provide sufficient statistical operating software to perform liner mixed models in subsequent experimental chapters. This change in software, specifically, the potential to conduct statistical analysis using linear mixed models may be important for future experimental chapters, whereby, due to the nature of *in vivo* interventions missing data may be a problem. The use of linear mixed models accommodates such missing data into its covariant structure and thus addresses the problem of losing a subject's whole data set, due to one missing value. Therefore, linear mixed models do not assume homogeneity of variance which is a problem during one way analysis of variance; also, they fit different covariate structures unlike on way analyses of variance. In summary, this switch in operating software and statistical methods would provide greater statistical power of analysis in the current and future experimental chapters.

5.3. Results

Figure 5.1 shows the mean ± SD basal percentage change in *mHSP72* expression for each time and for each day. A significant quadratic trend was observed for time ($F = 26.0$; $p = 0.001$; partial $\eta^2 = 0.74$), where *mHSP72* decreased between 0800 and 1100 (mean difference = -17%; 95% CI = -24%, -10%; $p < 0.001$) and then increased between 1100 and 1400 (mean difference = 8%; 95% CI = 2%, 14%; $p = 0.015$). The main effect for day ($F = 2.6$, $p = 0.14$) and the day*time interaction effect ($F = 3.9$, $p = 0.08$) were not significant. Figure 5.2 shows the mean ± SD plasma eHSP72 for each time point on day 2. No significant effect of time was observed ($F = 2.0$, $p = 0.21$). There was no relationship between plasma eHSP72 and *mHSP72* ($p = 0.56$).

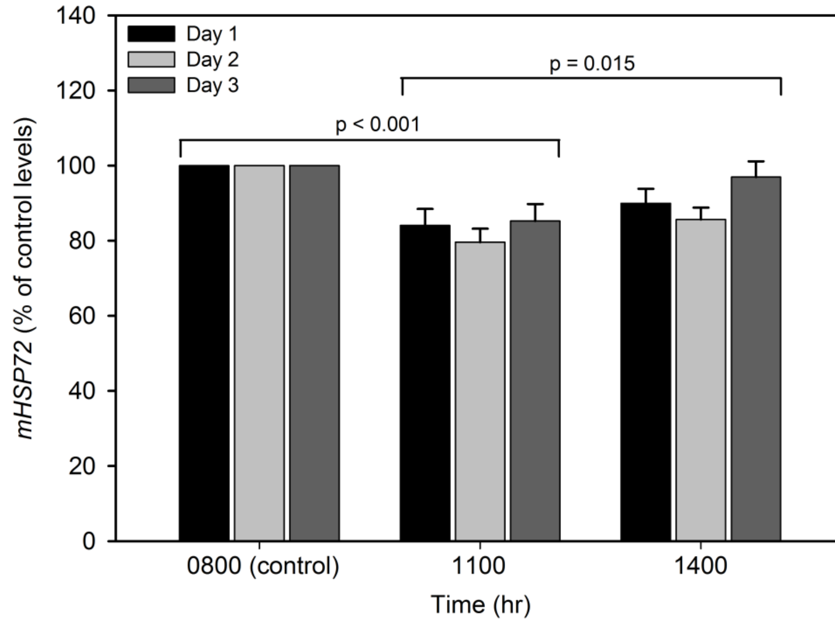

Figure 5.1. Mean *mHSP72* expression at 0800, 1100 and 1400 on three separate days. The error bars represent the SD of the mean. Data at 1100 and 1400 are represented as the percentage change from 0800 (control). The p values represent significant differences in basal *mHSP72* between times (n = 12).

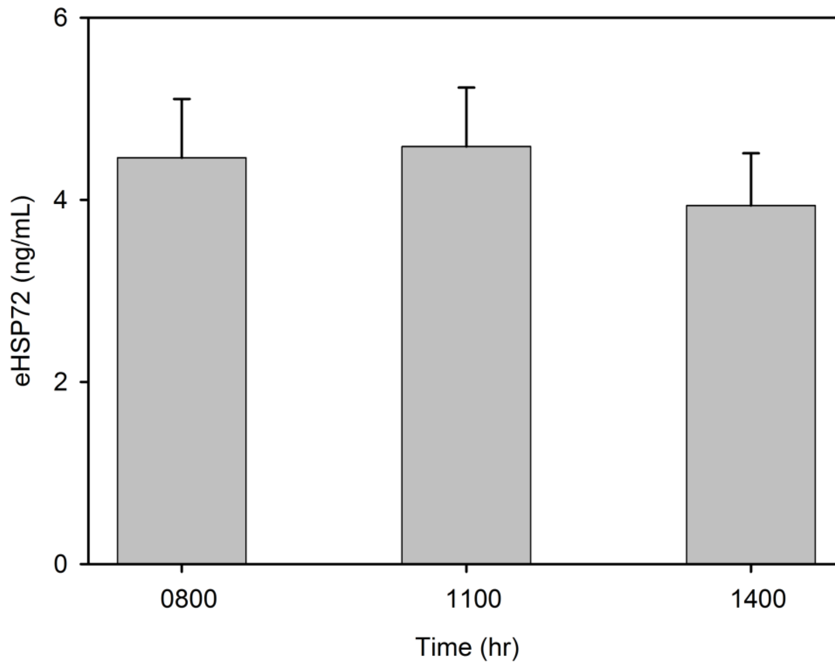

Figure 5.2. Mean plasma eHSP72 at 0800, 1100 and 1400 on a single day. The error bars represent the SD of the mean (n = 12).

5.4. Discussion

This study was conducted to ascertain whether an intra/inter-day variation existed in *mHSP72* and found that there was no significant difference in expression between days. However, a significant quadratic trend in basal *mHSP72* values was seen within each experimental day. The significant quadratic trend for time (a decrease followed by an increase) is consistent for all days and all subjects bar one.

Previously, in chapter 4, it was shown over a 24 h period basal *mHSP72* expression followed a circadian rhythm when analysed via flow cytometry, which was composed of two, 12 h components, with the "awake" component demonstrating a quadratic trend in expression. Utilising the high sensitivity ELISA is the most popular method of measuring eHSP72, however, basal values often fall at or below the 90 pg / mL sensitivity with inter and intra assay variation seen (Whitham and Fortes, 2006; Whitham et al., 2006; Yamada et al., 2008), therefore protocols utilising ELISA potentially miss subtle basal variations in expression. This may account for the lack of a diurnal variation in basal eHSP72 values shown by others (Fehrenbach et al., 2005; Fortes and Whitham, 2009). Additionally, differences between commercially available and in-house ELISAs are often large. Analysis of eHSP72 via ELISA is less time consuming compared to flow cytometry, however, its cost is greater (Yamada et al., 2008). Additionally, it can be seen that plasma ELISA measurements are a relatively insensitive measure of basal eHSP72 expression, with large inter-subject variation, in comparison to flow cytometry (Figure 5.1 and Figure 5.2). This lack of sensitivity was despite using plasma as opposed to serum derived eHSP72 and the utilisation of EDTA rather than heparinised blood collection tubes, which have in combination been shown to maximise eHSP72 yield (Whitham and Fortes, 2006). No relationship was evident between eHSP72 and *mHSP72* expression ($p = 0.56$). Additionally, any potential future elucidation of cyclic rhythms in eHSP72 lack biological importance until the bio-

origin and function of eHSP72 is indentified with certainty (as discussed in section 2.1.6).

Western blotting has proved a popular analytical technique and has previously shown inter-subject variations in basal iHSP72 values within muscle (Khassaf et al., 2001), potentially due to a lenient exclusion protocol (physical activity) and experimental restrictions pre, during and post study. Recently, stronger research methods were utilised by Morton et al., (2006), with exclusion criteria, subject restrictions and specifically physical activity tightly controlled. Despite more rigorous control methods, this inter-subject variation in basal muscle iHSP72 was still evident (Morton et al., 2006). However, due to western blotting being less sensitive than ELISA to changes in PBMC iHSP72 (Yamada et al., 2008) its ability to detect subtle changes i.e. daily variation in basal iHSP72 expression may be questionable. It is important to gain an accurate true basal value as pre exercise values have been correlated to the magnitude of HSP72 response post stressor *in vitro* (Maloyan et al., 1999; Vince et al., 2010) and *in vivo* (Gjovaag and Dahl, 2006; McClung et al., 2008; Vissing et al., 2009).

Notably on three separate days, the quadratic trend demonstrated previously in chapter 4, in this instance, is shown to be consistent and repeatable. The presence of such a quadratic trend impacts upon any study that measures HSP72 expression in human subjects, especially those which look to take serial measurement across different days, as differing basal values due to the quadratic trend need controlling for. It is particularly relevant for studies that explore HSP72 expression in response to a stressor (various interventions), for example, exercise or hyperthermia, where the quadratic trend in basal HSP72 expression could ultimately affect the speed and magnitude of the stress protein response and consequently the duration and maximal expression of HSP72 seen. Elevated muscle HSP72 is present seven days post exercise (Morton et al., 2006; Paulsen et al., 2007; Tupling et al., 2007), therefore any physical activity seven days

prior to HSP72 sampling may illicit undue effects on basal values potentially producing erroneous values, which may also be evident within the blood, i.e. PBMCs, but this postulation requires further investigation *in vivo*. Contrary to much of the literature some research (Puntschart et al., 1996; Shastry et al., 2002; Liu et al., 2004; Watkins et al., 2007) has demonstrated minimal or no increase in HSP72 in response to stress, whereas similar protocols have demonstrated a conflicting significant increase in stress protein expression post stressor (Walsh et al., 2001; Thompson et al., 2002). These methodological flaws in establishing an accurate basal HSP72 value could account for previous research demonstrating no elevation in HSP72 expression in response to exercise (Puntschart et al., 1996; Shastry et al., 2002; Liu et al., 2004; Watkins et al., 2007). In the present study physical activity was tightly controlled for seven days prior to study commencement and thus previous inter-subject variation in expression may be due to inadequate controls for physical exercise being utilised.

Every effort was made by the research team in an attempt to exclude all relevant factors which could theoretically induce a HSP72 response and hence impact basal HSP72 expression. Psychological stress has been shown to induce HSP72 expression (Hoekstra et al., 1996; Isosaki and Nakashima, 1998), however, subjects reported no impending deadlines, personal issues or other psychological stressors in recruitment or throughout the study.

Basal expression is indicatively linked to the magnitude of the HSP72 response to a stressor *in vivo* (McClung et al., 2008). Specifically within M, basal values were recently shown to determine post stressor expression of *mHSP72 in vitro* (Vince et al., 2010). Therefore the quadratic trend in basal *mHSP72* expression should be considered when designing studies. It requires careful postulation when protocols utilise separate days of testing where the stress response may differ to an identical stressor, on a separate day, due to the timing of the intervention with respect to the quadratic trend

shown here. This quadratic trend in basal *mHSP72* expression could impact upon performance, whereby training or performance may be augmented or hindered dependant on the basal level of endogenous HSP72. This paradigm could be of particular concern during heat acclimation strategies or hyperthermic exercise performance/competition as high levels of endogenous HSP72 are implicitly linked to increased cellular survival (McMillan et al., 1998) and contribute to augmented hyperthermic athletic performance *in vivo* (Marshall et al., 2007; Yamada et al., 2007; Amorim et al., 2008; McClung et al., 2008; Sandstrom et al., 2008; Selkirk et al., 2009; Amorim et al., 2010; Magalhães et al., 2010). Whether the quadratic trend associated changes in *mHSP72* expression could manifest a significant impact upon exercise performance needs further elucidation and such a cause and effect relationship cannot be claimed from the data within the present chapter. The notion of low basal levels of HSP72 as a marker of training status or acclimation status also requires further investigation. It could potentially be used if an athlete was having regular basal HSP72 values taken longitudinally and an accurate non trained/non acclimated range of values could be ascertained.

The data derived in the current experimental chapter has some important implications to consider when designing and conducting future research in general and specifically to this thesis. As discussed in chapter 4, the conformation of the quadratic trend in *mHSP72* expression supports previous suggestions within that chapter, that blood sample collection and the scheduling of interventions require careful planning. This attention ensures that stressor mediated changes in HSP72 are not differentially effected by differences in basal values, a known influence on stressor mediated increases in *mHSP72 in vitro* (Vince et al., 2010) and *in vivo* (McClung et al., 2008). Furthermore, particularly when attempting to increase basal *mHSP72 in vivo*, via an environmental stressor, differential interventions must be administered at identical time's within the

experimental design, to ensure that any changes in *mHSP72* are due to the interventions and not the circadian variations in basal *mHSP72* shown here and in chapter 4, particularly, their potential effects on the stress response. Future work should look to examine the link between muscle and PBMC expressed HSP72 and whether the two demonstrate comparable or opposing quadratic trends in basal expression. These findings and postulations will be incorporated and examined in experimental designs and investigations respectively, within subsequent experimental chapters.

Chapter 6. Experiment 3: The effect of the hyperbaric environment on HSP72 expression *in vivo*

This experimental chapter has formed the basis of the publication detailed below:

Taylor, L., Midgley, A., Sandstrom, M., Chrismas., B, & McNaughton, L. (2011) The effect of the hyperbaric environment on heat shock protein 72 expression *in vivo*. Research in Sports Medicine – In press.

NB: Although first author on the publication detailed above, others contributed significantly to the sample acquisition and processing of this novel piece of scientific work. Additionally, this experimental chapter formed part of a larger completed project, which produced the publication below:

Vince, R. V., McNaughton, L., Taylor, L., Midgley, A. W., Laden, G. & Madden, L. A. (2009). Release of vcam-1 associated endothelial microparticles following simulated scuba dives. European Journal of Applied Physiology, 105, 507 -513.

6.1. Introduction

Low basal expression of *mHSP72* during homeostasis has been shown to follow a circadian variation in expression which is correlated to heart rate and T_c (chapter 4). Furthermore, such expression during "waking" hours has been shown on four occasions to follow a quadratic trend in expression (chapters 4 and 5).

It is known that high cellular concentrations of HSP72 exert a protective influence in light of cellular stress in comparison to those cells which have a lower concentration *in vitro* (Kiang et al., 1998). These elevations are typically achieved via heat shocking cells and then exposing them to a further non-lethal thermal stress (Samali et al., 1999) or a non-related non-lethal stressor (oxidative stress) (Kiang et al., 1998). This thermal preconditioning has been utilised *in vivo*, typically within hyperthermic exercise acclimation protocols (Yamada et al., 2007; Amorim et al., 2008; McClung et al., 2008; Sandstrom et al., 2008; Amorim et al., 2010; Magalhães et al., 2010). For instance, *in vivo*, increased eHSP72 and iHSP72 has been correlated to augmented hyperthermic exercise performance, when compared to pre heat acclimation values (Selkirk et al., 2009; Amorim et al., 2010; Magalhães et al., 2010).

In light of previous findings, it would be interesting if a non-thermal non-mechanical stressor could be used to disrupt the previously shown circadian variation (chapter 4) and particularly the quadratic trend (chapters 4 and 5) in *mHSP72* expression. Specifically, if such a stressor or exposure, *in vivo*, could elevate basal *mHSP72*. Therefore, an alternative method (non thermal or mechanical) to induce such elevations would be beneficial, given the cost of the equipment and expertise required to generate a sufficient safe and reliable thermal stimulus, and the reduction in various facets of exercise performance that are attributable to exercise induced muscle damage (mechanical stimulus) (Ascensao et al., 2008). Accomplishment of such a phenomenon

may provide a tool in subsequent chapters to elicit conferred *in vivo* cellular tolerance to further non-related stressors (e.g. exercise induced disturbances to redox balance) via elevated basal HSP72 expression.

The physiological and biochemical effects of administrating hyperbaric air (HA) are largely unexplored within the literature. Limited early research focused on HA breathing being efficacious in inducing narcotic cognitive effects (Atkins, 1968). Recent literature examines potential hazards for commercial and recreational divers, i.e. faulty equipment (steel cylinder corrosion), and contaminated air supplies (carbon monoxide poisoning) (Millar and Mouldey, 2007). Little research is available on the bio-chemical response of HA breathing *in vivo*. In comparison, the effects of HBO have been extensively explored. Therapeutic applications include augmented healing of diabetic wounds, treatment of decompression illness (DCI), neuro-rehabilitation and as a complementary therapy for autism, amongst others (Barratt et al., 2002).

It is known that elevated ROS production is seen in subjects exposed to HBO compared with those breathing normobaric atmospheric air (NA) (Groger et al., 2008). Frequently, HBO has been used within clinical populations (i.e. cardiac surgery patients) with markers of stress, such as eHSP72 and oxidative stress often measured pre and post operatively (Alex et al., 2005). Consequently, any elevation in HSP72 and/or oxidative stress is likely attributable to the trauma of surgery (Alex et al., 2005; Yogaratnam et al., 2007b). Previous studies have utilised commercially available ELISA kits to examine eHSP72 within the serum and plasma of subjects post various hyperbaric interventions and/or surgery (Dennog et al., 1999; Matsuo et al., 2000; Alex et al., 2005; Yogaratnam et al., 2007a; Yogaratnam et al., 2007b; Yogaratnam et al., 2007c). However, this method (ELSIA) has recently been shown to have methodological limitations, to be insensitive compared to measurements of *mHSP72* (chapter 5) and the bio-origin and physiological effects of eHSP72 remain unsolved (all discussed in sections 2.6.1 and

2.5.2.5) within the literature (Yamada et al., 2008). The rationale for investigation of both HBO and HA is in an attempt to see if increased O_2 partial pressure (HBO) and/or the hyperbaric environment *per se* can induce changes in *mHSP72* and/or oxidative stress.

HBO has been demonstrated to increase ROS generation (Conconi et al., 2003; Benedetti et al., 2004) which given the limitations above (diseased subjects and use of ELISA) has provided some evidence that ROS may be a stimulus for HBO induced increases in eHSP72 expression (Dennog et al., 1999; Rothfuss et al., 2001). Although not directly proven *in vivo*, it is likely that skeletal muscle (Jackson et al., 2007; Jackson, 2008; Powers and Jackson, 2008; Powers et al., 2010a) as well as blood (Nikolaidis and Jamurtas, 2009) contributes to the HBO induced disturbances in redox balance. Therefore, given the ability of both the blood (Nikolaidis and Jamurtas, 2009) and skeletal muscle (Jackson et al., 2007; Jackson, 2008) to produce radicals and thus disrupt redox balance, both tissues likely contribute to HBO mediated disruption of redox balance (as discussed in section 2.7.2). It has been suggested, with some strong *in vitro* evidence that iHSP72 expression is correlated to antioxidant activity and thus sensitive to changes in ROS (i.e. increases) (Currie et al., 1988; Mocanu et al., 1993). Oxidative stress is known to induce protein damage and apoptosis *in vivo* and *in vitro*, both responses are known inducers of HSP72 (Kalmar and Greensmith, 2009). Therefore, it is likely that any such HBO induced increases in ROS and oxidative stress (disturbances to redox balance) are theoretically acquiesced by stress induced HSP72 induction; in order to potentially reduce any ROS mediated tissue and cell damage (Kalmar and Greensmith, 2009; Vince et al., 2011). However, such postulations lack *in vivo* evidence, with no data present within healthy human subjects exploring HBO and HA induced iHSP72 expression (using Flow Cytometry) and any potential link to changes in redox balance.

The aim of this chapter was to investigate; 1) the effect of HA and HBO on basal *mHSP72* expression in light of the previous disclosed quadratic trend in basal expression, and 2) explore whether any such hyperbaric stress mediated changes in *mHSP72* may have a relationship with alterations in redox balance.

6.2. Methods

Subjects

Six healthy, recreationally active, non-smoking male subjects (mean ± SD: 21.3±7.2 yr, 179.2±4.8 cm, 79.3±9.9 kg, 5.9±2.3 $h.wk^{-1}$physical activity and 7.4±0.7 h sleep per night) volunteered to participate in the study. Please see section 3.8 for general experimental restrictions and controls which were adhered to within this experimental chapter.

A sample size of six was not dictated by a sample/power calculation but by the limitations of the hyperbaric chamber to be used (capacity and availability).

Blood sampling and experimental protocol

Telemetric temperature sensor pills (CortTemp™, HQ Inc., Palemetto, FL) were consumed by all subjects at 0700 to monitor T_c wirelessly, with T_c sampled at 5 min intervals throughout the 12 h testing period (CortTemp™ Data Recorder HT150016, HQ Inc., Palemetto, FL). Consumption of pills allowed sufficient time for ingestion of fluids and meals to not exert any undue influence on T_c readings (O'Brien et al., 1998). Heart rate was recorded every 5 s throughout the 12 h period (Team System, Polar Electro, Finland). In line with previous heart rate data (Greenleaf and Castle, 1971; Gonzalez-Alonso et al., 2000; Kay and Marino, 2003; Kenefick et al., 2009), the heart rate data in the present study were time-averaged over 5 min intervals, with the mean heart rate for each hour calculated from these 5 min intervals. Subjects consumed

equivalent meals for each experimental condition, which were provided at 0930, 1330 and 1730. On each of the study day's subjects spent their time under resting conditions within the temperature controlled laboratory (20.8±0.5 ^{o}C, humidity 48±3 %) except for the hyperbaric exposures and slept within a nearby University owned accommodation block the night prior to each experimental day. See Table 6.1 for timeline of experimental procedures.

Subjects remained within the temperature controlled laboratory (average WBGT 19.9 ± 0.4 ^{o}C) throughout the study days (12 h) under resting conditions. Control values (NA) were obtained one week before the first HA exposure with the HBO exposure following a week later (i.e. 3 study days NA, HA and HBO each separated by one week – all conditions including NA conducted within the hyperbaric chamber). Subjects were familiarised with the chamber two weeks prior to experimental testing commencing, of note subjects were exposed to the noise of the chamber operating (no hyperbaric stress generated) and the "medical type" environment of the chambers situ. The experimental conditions were not randomised as previous work has shown that the stress response requires six or more hyperbaric exposures for the hyperbaria mediated stress response to begin showing signs of adaptation (Obad et al., 2010). Even in the absence of this rationale (Obad et al., 2010) randomisation would not have been possible due to the limited availability (only available for three consecutive weeks) of the chamber and its ability to produce only HA, NA or HBO exclusively. The temperature and humidity within the chamber mirrored (set and maintained by the chamber) those values which were seen within the laboratory on each experimental day (mean ± SD: 20.8±0.5 ^{o}C, humidity 48±3 %).

The various exposures commenced at 1500 and involved a simulated dive consisting of HA (2.8 ATA) or HBO (20 min O_2, 5 min HA cycle) within a hyperbaric chamber constituting 78 min bottom time (total time (mean ± SD) spent within the chamber 98 ±

12 min), variation due to time required for set up of hoods and safety procedures for each experimental condition. The timing of hyperbaric exposure was dictated by the availability of the facilities to be used and was not adopted for a particular methodological rationale. However, it is important to note that both the HBO and HA exposures followed identical timelines to account for the circadian and daily variations shown in basal *mHSP72*, in previous experimental chapters. Decompression was conducted in accordance with the United States Navy standard decompression tables (Brubakk and Neuman, 2002).

Blood samples were taken at 0900, 1300, 1700 and 2100. Please see section 3.4 for *mHSP72* specific blood collection controls and considerations. Methods, procedures and restrictions replicated those used previously to gain serial data for *mHSP72* (chapter 4 and 5) and other physiological markers of homeostasis (Madden et al., 2008b; Vince et al., 2009). Due to the logistical (capacity and availability) and methodological (booking times of Flow Cytometer) constraints with use of the hyperbaric chamber and sample acquisition, sample frequency reverted back to 4 h intervals as used in chapter 4. This was unfortunate, given the want to use 3 h sample timings as discussed in chapter 5, however, this was unavoidable.

<u>Monocyte HSP72 assay</u>

See general methods chapter, section 3.7. The assay was run live every 4 h, at all time points on all experimental days.

<u>Measurement of oxidative stress</u>

See general methods chapter, section 3.8.2. Analysis of plasma TBARS was conducted pre (1300) and post (1700) HBO and HA exposures and at the corresponding times on the control day.

Table 6.1. Timeline of experimental design and procedure

Time	Experimental Event
0700	Subjects consume temperature pills and rest
0800	Subjects report to laboratory and rest
0900	Pre-exposure blood sample
1300	Pre-exposure blood sample
1500	Exposure initiated
1618	Exposure completed
1700	Post-exposure blood sample
2100	Post-exposure blood sample

Statisical analysis

All statistical analyses were conducted using IBM SPSS Statistics 18 (SPSS Inc., Chicago, IL). Statistical assumptions were checked using conventional graphic methods with distribution deemed plausible for all variables except T_c. Central tendency and dispersion are reported as the mean (SD).

The effect of condition (NA, HA, HBO) on *mHSP72* expression, *Tc*, and plasma TBARS over time was determined using two-way linear mixed models for repeated measures. One observed case in the *mHSP72* model had a standardised residual of 5.6, so the results are reported with this extreme outlier omitted (please note that whether or not the results were statistically significant was not altered by the removal of the outlier). A quadratic term was included in a one-way model for time for *mHSP72* expression in the NA condition in an attempt to corroborate previous observations that basal *mHSP72* expression follows a diurnal quadratic trend (chapters 4 and 5). In the event of a

significant F ratio in any omnibus F test, post hoc comparisons with Sidak-adjusted p values were used to identify significant differences between paired means. The relationship between *mHSP72* and T_c was investigated using a Spearman rank correlation coefficient. Two-tailed statistical significance was accepted as $p < 0.05$.

6.3. Results

Figure 6.1 shows mean *mHSP72* expression for each time point during each experimental condition. In the two-way model, significant main effects for condition ($F = 24.7$, $p < 0.001$) and time ($F = 9.6$, $p < 0.001$) were observed for *mHSP72* expression. As a significant condition x time interaction effect was also observed ($F = 7.1$, $p < 0.001$), interpretation will focus on decomposition of the interaction effect. There were no significant differences in *mHSP72* expression between conditions at 0900 and 1300, however, at 1700 *mHSP72* expression was significantly higher in NA than in HA ($p = 0.016$) and HBO ($p < 0.001$) and remained so at 2100 ($p < 0.001$). In the one-way model for the NA condition a significant quadratic trend was observed ($F = 27.6$, $p < 0.001$).

No significant main effect for time was observed for T_c ($F = 0.08$, $p = 0.29$). The main effect for condition ($F = 1.2$, $p = 0.35$) and the condition x time interaction ($F = 2.0$, $p = 0.12$) for T_c were not significant. Similarly, no significant effect for time was observed for heart rate ($F = 1.0$, $p = 0.37$). The main effect for condition ($F = 1.8$, $p = 0.34$) and the condition time interaction ($F = 1.2$, $p = 0.41$) for heart rate were also not significant. No relationship was observed between *mHSP72* expression and T_c ($r_s = 0.04$, $p = 0.79$).

Figure 6.2 shows plasma TBARS pre (1300) and post (1700) intervention for each experimental condition. There were no significant main effects observed for condition ($F = 0.7$; $p = 0.50$) or time ($F = 0.06$; $p = 0.81$), and no significant condition x time interaction effect ($F = 0.5$; $p = 0.62$) for plasma TBARS.

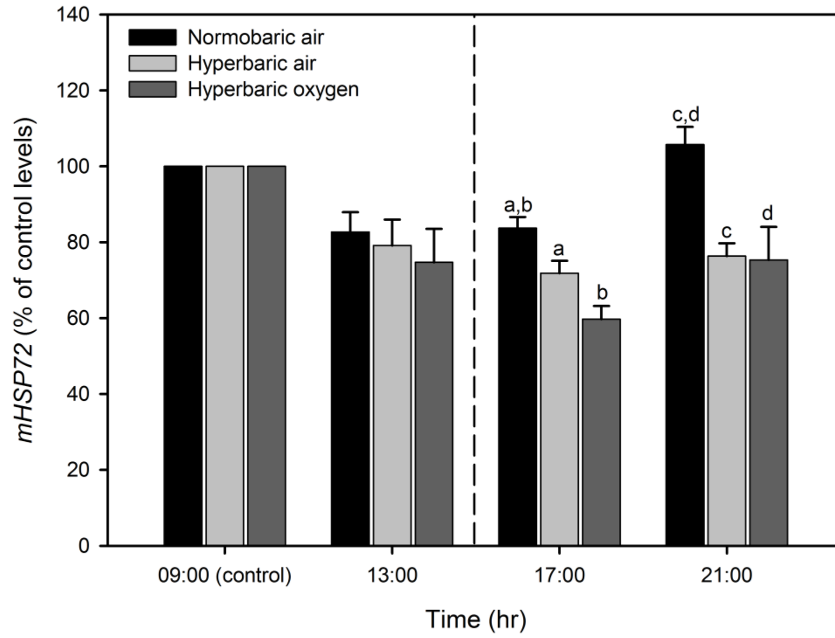

Figure 6.1. Mean *mHSP72* expression over time for each of the three experimental conditions. Error bars represent the standard deviations of the mean. The dashed vertical line represents the time of the simulated dive. Like letters represent significant differences between means ($p < 0.05$).

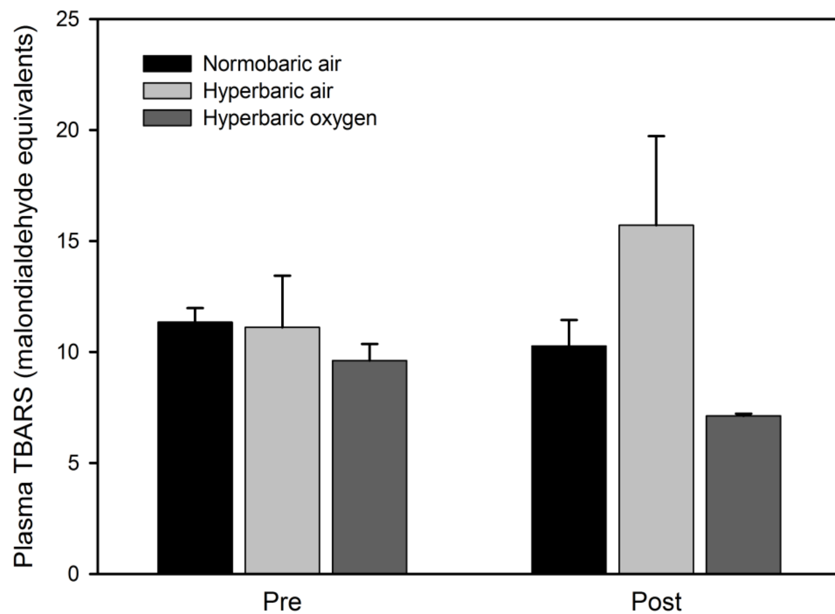

Figure 6.2. Plasma TBARS pre (1300) and post (1700) intervention for the three experimental conditions. Error bars represent the standard deviation of the mean.

6.4. Discussion

It can be seen that control values for *mHSP72* demonstrate the quadratic trend in expression as previously reported in chapter 4 and 5, further confirming the repeatability of *mHSP72* expression in this manner during waking hours. The hyperbaric conditions showed significantly decreased *mHSP72* expression following exposure in comparison to the control values (Figure 6.1). It has been previously shown that decreased levels of *mHSP72* result in a significant induction of HSP72 when exposed to a subsequent (heat) stress (Vince et al., 2010) and that increased *mHSP72* observed at different times of the circadian cycle provide cellular protection against stress. The HA and HBO conditions resulted in an impairment of *mHSP72* expression compared to control data and as such could result in the cells being less equipped to deal with stress (Vince et al., 2010; Vince et al., 2011). The data presented here for the HA condition is allied to a simulated dive breathing air, and as such does not take into account the vigorous nature of an open water dive, which itself may induce *mHSP72* synthesis, but rather reflects on the effect of breathing air under pressure (Madden and Laden, 2009; Obad et al., 2010). However, the data (changes in *mHSP72*) does show a disruption to homeostasis following breathing air at pressure and is in agreement with studies on decompression sickness (Barratt et al., 2002).

Work elsewhere, conducted *in vitro*, has shown HSP72 to be increased 6 and 24 h post HBO exposure (Shinkai et al., 2004). Other cell lines, *in vitro*, have also shown similar increases of HSP72 after HBO exposure (Oh et al., 2005), however these were *in vitro* studies on endothelial cell lines and as such may not accurately reflect changes within the blood cells of the circulation *in vivo*. Contradictory evidence exists *in vivo*, demonstrating that during the initial stages of a saturation dive, HSP72 values are decreased in comparison to pre dive values (Matsuo et al., 2000). This is in line with the data presented within the current study (Figure 6.1).

Substantial work exists demonstrating the *in vitro* effects of hyperoxia and its impact upon markers of oxidative stress, such as plasma TBARS (Gille et al., 1994). Repeated exposures to HBO results in accumulation of lipid peroxides (TBARS) in diseased (Benedetti et al., 2004) and healthy subjects (Bader et al., 2007; Obad et al., 2010). Previously, oxidative stress has been implicated as a key transduction signal for various cytokines and hormones (Bader et al., 2007; Fisher-Wellman and Bloomer, 2009; Kalmar and Greensmith, 2009). Data such as this demonstrates that a single exposure to HBO results in elevated lipid peroxides (Bader et al., 2007). However, this present study demonstrates no significant differences in plasma TBARS post exposure, although an increase post HA was observed (Figure 6.2). An increase in subject numbers may address this lack of significance, as due to experimental constraints of the chamber utilised, six subjects were the maximum permitted during the project. Additionally, an individual's sensitivity (training, anti-oxidant status, habitual hyperbaric pressure exposure, etc) to O_2 saturation (i.e. hyperoxia) (Fehrenbach and Northoff, 2001) and the different subject populations used in previous research (diseased and healthy), make it difficult to say with certainty, whether no change or an increase/decrease in markers such as plasma TBARS, is accurate in response to HBO or HA.

Previous research (Dennog et al., 1999; Yogaratnam et al., 2007b) has repeatedly relied upon suspect measures of eHSP72 via ELISA to quantify the HSP72 response to various HBO exposures and as such the sensitive technique and assay used here may in fact be a truer reflection of HSP72 expression in response to such changes in environment. In support of this, the antioxidant defence systems of PBMCs is augmented in habitual scuba divers (Ferrer et al., 2007) although such divers do demonstrate cumulative disturbances to redox balance (Obad et al., 2010). Therefore, the lowering in basal *mHSP72* shown in the present study may represent the first step in

a positive adaptation within the PBMCs within pressure naïve subjects to counteract the increase in oxidative stress shown elsewhere (Bader et al., 2007); habitual divers are known to accumulate sustained disruptions in redox balance (Obad et al., 2010). Postulations such as this are supported by previous work demonstrating that reduced basal *mHSP72* is indicative of a training induced augmentation of the HSR in light of further stress, whereby, despite lower basal *mHSP72*, synthesis is increased in light of future challenges to homeostasis in comparison to those subjects with high basal *mHSP72* (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Fehrenbach et al., 2001; Vince et al., 2010; Vince et al., 2011).

Hyperbaric and/or oxidative stress initiates alterations in shear stress within the tissues and vasculature, which is further exasperated by decompression (Barratt et al., 2002; Vince et al., 2009). These permutations have been shown to activate monocyte chemotatic protein-1 (MCP-1) (Shyy et al., 1993; Mojsilvis-Petrovic et al., 2007). Recently, MCP-1 has been shown to play a pivotal role in the inflammatory chemokine cascade (Mojsilvis-Petrovic et al., 2007) and has been shown to influence the transmigration of monocytes to the subendothelial space (Navab et al., 1991). Additionally, shear stress has been shown to induce monocyte recruitment to endothelial cells (Shyy et al., 1993) or transmigration (Navab et al., 1991) of monocytes preferentially expressing HSP72 to the endothelium or subendothelial space. Such interplay, between MCP-1 and *mHSP72* may underpin the decrease in *mHSP72* due to the transmigration and preferential binding of *mHSP72* to the endothelium and thus a reduction of *mHSP72* within the circulation. However, such postulations as this require extensive *in vivo* examination. Additionally, significant increases in both vascular cell adhesion molecule-1 (VCAM-1) and CD105 cellular microparticles has been demonstrated post HA exposure compared to HBO, accompanied by a reduction in endothelial function, utilising a similar protocol (Vince et al., 2009), suggesting that

HBO is efficacious in reducing the vascular stress encountered from the hyperbaric environments.

Experimental limitations are seen by lack of randomisation (though as discussed this is unlikely to be a confounding factor (Obad et al., 2010)). However, measures of perceived stress were not taken throughout the experimental procedures (within the laboratory or chamber itself) and should be done in future studies of a similar nature. Despite this lack of measures with regards to perceived stress, heart rate and T_c were unchanged (see section 6.3) throughout the experimental period (within the laboratory and chamber itself). Additionally, subjects were familiarised with the chambers situ and operating procedures (noise, medical environment, etc) and thus it is unlikely that the stress of the chamber environment and its function was a confounding factor. However, it is recommended that future work should employ randomisation and appropriate measures of perceived stress.

In conclusion, breathing air at pressure disrupts the quadratic trend in *mHSP72* and therefore homeostasis. Despite the exact mechanism for the impairment of *mHSP72* expression post hyperbaric exposure being unclear, its implication for investigation is interesting as the response may be due to pressure *per se*. In the present study, alterations in *mHSP72* did not occur in tandem with significant changes in redox balance (plasma TBARS). Additionally, any possible link with markers of endothelial function, and the impaired *mHSP72* expression in hyperbaric environments warrants further investigation.

The data derived in the current experimental chapter has some important implications to consider when designing and conducting future research in general and specifically to this thesis. Attempts to increase basal *mHSP72* evidently failed within the current chapter, with reductions in such values actually seen post exposures. Therefore, the

stressor utilised (hyperbaria) needs to be reconsidered in future attempts to elicit increased basal *mHSP72* expression. Such considerations could include the use of an acute hypoxic exposure, which in contrast to HBO, HA and NA would reduce the partial pressure of O_2 within the circulation *in vivo* and potentially represent a greater challenge to homeostasis than the hyperbaric stressors used within the current chapter.

Chapter 7. Experiment 4: The effect of acute hypoxia on HSP72 expression and oxidative stress *in vivo*.

This experimental chapter has formed the basis of the publication detailed below:

Taylor, L., Midgley, A., Chrismas, B., Madden, L., Vince, R. V. & McNaughton, L. (2010). The effect of acute hypoxia on heat shock protein 72 expression and oxidative stress *in vivo*. European Journal of Applied Physiology, 109, 849-855.

7.1. Introduction

In the previous chapter HBO and HA were shown effective in disrupting a previously demonstrated diurnal quadratic trend in basal *mHSP72* expression (chapter 4, 5 and 6). However, these interventions (chapter 6) did not elevate basal *mHSP72 in vivo*, in fact a reduction was seen post intervention compared to control. Therefore, another strategy was required to increase basal *mHSP72* without the stimulus of exercise or hyperthermia. The importance of eliciting such potential increases without the use of exercise or hyperthermia would be the first such instance *in vivo*.

It has been postulated that the ubiquitous and highly inducible HSP72 serves as a useful marker of the cellular response to hypoxic insult (Fei et al., 2007). Hypoxia has been shown to induce HSP72 expression in animal (Wang et al., 2006) and human tissues (Patel et al., 1995) *in vitro*, but, the magnitude of hypoxia utilised, often pharmacologically, is not comparable to the hypoxic load experienced by humans travelling to elevated altitudes. Human thyroid FRTL-5 cells *in vitro* pre-conditioned with heat shock to induce HSP72 expression display protection from the necrotic effect of hypoxia (Kiang et al., 1996). Kiang et al, (1996) hypothesised HSP72 may play a vital role in the defence of a hypoxic injury. However, the same response within human *in vivo* studies remains elusive, despite the well cited response *in vitro* within animal and human tissues/cells (Patel et al., 1995; Kiang et al., 1996; Wang et al., 2006).

Oxygen's reactive chemical nature and critical underpinning of eukaryotic life ensures a dichotomy exists between O_2 supply to an organism, tissue or cell and the harmful (cytotoxic products) or beneficial outcome (O_2 delivery to tissues) (Taylor and Pouyssegur, 2007). Long term O_2 deficiency (hypoxia) at a cellular level can result in functional impairment and a shortening of lifespan (Brahimi-Horn and Pouyssegur, 2007). A state of low cellular O_2 saturation is fundamental in the pathophysiology of

chronic diseases (for example, chronic obstructive pulmonary disease, coronary artery disease), which are characterised by insufficient respiration and reduced delivery of O_2 to the tissues (Sieck, 2000). Hypoxia within an organism compromises cells metabolically, often resulting in apoptosis and elevated production of cytotoxic substrates and compounds (Kiang and Tsen, 2006). The onset of hypoxia within the tissues initiates a rapid burst of ROS formation, which upon reperfusion, is greatly increased compared to the initial ROS accumulation (Zuo and Clanton, 2005).

Hypoxia has been demonstrated to increase ROS generation (Bailey et al., 2000). Although not directly proven *in vivo*, it is likely that skeletal muscle (Jackson et al., 2007; Jackson, 2008; Powers and Jackson, 2008; Powers et al., 2010a) as well as blood (Nikolaidis and Jamurtas, 2009) contributes to hypoxia mediated disturbances in redox balance (Bailey et al., 2000; Zuo and Clanton, 2005). Therefore, given the ability of both the blood (Nikolaidis and Jamurtas, 2009) and skeletal muscle (Jackson et al., 2007; Jackson, 2008) to produce radicals and thus disrupt redox balance, both tissues likely contribute to the hypoxia mediated disruption of redox balance (as discussed in section 2.7.2). It has been suggested, with some strong *in vitro* evidence that iHSP72 expression is correlated to antioxidant activity and thus sensitive to changes in ROS (i.e. increases) (Currie et al., 1988; Mocanu et al., 1993). Oxidative stress is known to induce protein damage and apoptosis *in vivo* and *in vitro*, both responses are known inducers of HSP72 (Kalmar and Greensmith, 2009). Therefore, it is likely that any such hypoxia induced increases in ROS and oxidative stress (disruption to redox balance) are theoretically acquiesced by stress induced HSP72 induction; in order to potentially reduce any ROS mediated tissue and cell damage (Kalmar and Greensmith, 2009; Vince et al., 2011). However, such postulations lack *in vivo* evidence, with no data present within healthy human subjects exploring hypoxia induced iHSP72 expression (using Flow Cytometry) and any potential link to changes in redox balance.

The aims of this study were to investigate: 1) the effect of acute hypoxia on basal *mHSP72* expression, and 2) whether any such acute hypoxia mediated changes in *mHSP72* may have a relationship with alterations in redox balance.

7.2. Methods

Subjects

Twelve healthy recreationally active non-smoking male subjects (mean ± SD age: 19.8±3.5 years, height 175.5±10.8 cm, body mass 73.1±8.0 kg and physical activity 5.1±1.5 $h.week^{-1}$) volunteered to participate in the study. Please see section 3.8 for general experimental restrictions and controls which were adhered to within this experimental chapter.

A sample size of twelve was not dictated by a sample/power calculation but by the logistical and technical limitations of the specialist hypoxicators utilised. These machines were only available for a one week period, with the altitude utilised (equivalent to 2980 m) limiting gas flow to four subjects per machine.

Blood sampling and experimental protocol

Testing was conducted on consecutive days, with all subjects providing control samples on this first day and the hypoxic exposure (75 min, 2980 m) administered on the second day (the control condition was conducted using an identical experimental setup, i.e. masks worn, hypoxicator running, etc – though sea level air was provided rather than the low oxygen air used in the hypoxic condition). Subjects were familiarised with the experimental equipment three days prior to experimental testing commencing, of note subjects were exposed to the noise of the hypoxicator running, the novel feel of wearing the masks used to deliver the gas (no hypoxic air generated during familiarisation) and the “medical type” environment of the laboratory where all experimental testing took

place. Both exposures (control and hypoxic) commenced and ceased at 0930 and 1045 respectively. Randomisation of conditions was not feasible for the present study due to the restrictions placed upon equipment availability and the inability of the hypoxicators to generate differential concentrations of O_2 (i.e. only sea level or hypoxic gas could be generated, not a combination of both). Furthermore, the availability of the Flow Cytometer was limited to the two specific days used within the experimental design. Please see section 3.9 for information regarding the rationale for the intensity, duration and timing of the hypoxic exposure, the generation of hypoxic air and the monitoring of the physiological response to such exposures.

Blood samples were taken at 0800, 1100, 1400, 1700 and 2000. Sample frequency was able to revert back to the 3 h sample intervals employed in chapter 5, which would enable greater insight into the expression kinetics of *mHSP72* during the 12 h experimental period. Please see section 3.4 for HSP72 specific blood collection controls and considerations. Methods, procedures and restrictions replicated those used previously to gain serial data for *mHSP72* (chapters 4, 5 and 6) and other physiological markers of homeostasis (Madden et al., 2008b; Vince et al., 2009). Subjects consumed identical meals for each of the study days, which were provided at 0830, 1330 and 1830. Subjects remained within the temperature-controlled laboratory (mean ± SD: WBGT 21.2±0.2 ^{o}C, humidity 47±4 %) throughout the course of each study day (12 h). Subjects did not sleep within the laboratory, but within a nearby University owned accommodation block for the night prior to and during the course of the study. Subjects gained 10±0.6 h sleep prior to study commencement and repeated this sleep cycle on the night separating control and hypoxic sampling (10±1.1 h).

Monocyte HSP72 assay

See general methods chapter, section 3.7. The assay was run live every 4 h, at all time points on all experimental days.

Measurement of oxidative stress

See general methods chapter, section 3.8.2. Analysis of plasma TBARS was conducted at all time points on both experimental days.

Statistical analysis

Statistical analyses were performed using SPSS for Windows software, version 16.0 (SPSS Inc., Chicago, IL). Statistical assumptions were checked using conventional graphic methods with distribution deemed plausible for all variables. Central tendency and dispersion are reported as the mean (SD).

Separate polynomial contrasts were used to identify trends over time for the *mHSP72* and plasma TBARS data for the control and hypoxic conditions. Two-way analyses were used to establish any condition-by-time interactions. The *mHSP72* expression at 1100, 1400, 1700 and 2000 was expressed as a percentage of the 0800 value for each condition as used elsewhere (Morton et al., 2007) and in previous experimental chapters 5 and 6. One-way repeated measures analysis of variance was used to identify differences over time for the mean heart rate and oxyhaemoglobin saturation during the hypoxic intervention period. The sphericity assumption was violated for the heart rate data (Mauchly's W = 0.0002; $p < 0.001$) and therefore a Huynh-Feldt correction was applied to the degrees of freedom of the F ratio. In the event of a significant F ratio, Fisher's least significance difference tests were used to locate significant paired differences. Two-tailed statistical significance was as accepted as $p < 0.05$.

7.3. Results

Figure 7.1 shows the mean basal *mHSP72* expression at 0800, 1100, 1400, 1700 and 2000 for the control and hypoxic conditions. There was a significant quadratic trend for the control condition ($F = 23.5$; $p = 0.002$; partial $\eta^2 = 0.77$), where basal *mHSP72* expression decreased between 0800 and 1100 and then increased thereafter. This quadratic trend in control *mHSP72* expression has been shown to be repeatable and consistent on several occasions in chapters 4, 5 and 6. There was no significant polynomial trend for the hypoxic condition (largest F ratio was for a quadratic trend: $F = 3.9$; $p = 0.087$), which was almost a 'mirror image' of the control condition response over the 12 h period (Figure 7.1). The quadratic term for the condition-by-time interaction was significant ($F = 19.5$; $p = 0.003$; partial $\eta^2 = 0.74$), where the difference between the control and hypoxic conditions were significantly different at 1100 ($p = 0.002$), 1400 ($p < 0.001$), 1700 ($p = 0.034$) and 2000 ($p = 0.041$).

Figure 7.2 shows the mean plasma TBARS concentration at 0800, 1100, 1400, 1700 and 2000 for the control and hypoxic conditions. No significant trend was observed for plasma TBARS in the control condition ($F = 0.8$; $p = 0.41$), but a significant quadratic trend was evident for the hypoxia condition ($F = 36.1$; $p = 0.001$; $\eta^2 = 0.84$). Plasma TBARS increased by 98% (95% CI 30%, 166%) from pre-intervention to immediately post-intervention and decreased thereafter until pre-intervention levels were reached at 2000. This difference in the trends in the plasma TBARS data between conditions showed up as linear-by-quadratic interaction in the two-way analysis ($F = 41.5$; $p < 0.001$; $\eta^2 = 0.86$), where significant differences between conditions were observed only at 1100 ($p = 0.006$) and 1400 ($p = 0.032$).

Figure 7.3 shows heart rate and oxyhaemoglobin saturation during the intervention period in the hypoxic condition. There was a significant effect of time on heart rate

during the intervention period ($F = 27.7$; $p < 0.001$; partial $\eta^2 = 0.77$), characterised by a large significant increase in heart rate at the start of the intervention ($p < 0.001$), whereas the heart rate remained relatively stable during the rest of the intervention period. There was also a significant effect of time for oxyhemoglobin saturation ($F = 78.6$; $p < 0.001$; partial $\eta^2 = 0.91$), where oxyhemoglobin saturation exhibited a considerable decrease between 0 and 5 min ($p < 0.001$) and between 5 and 15 min ($p < 0.001$) of the intervention period and remained relatively stable thereafter.

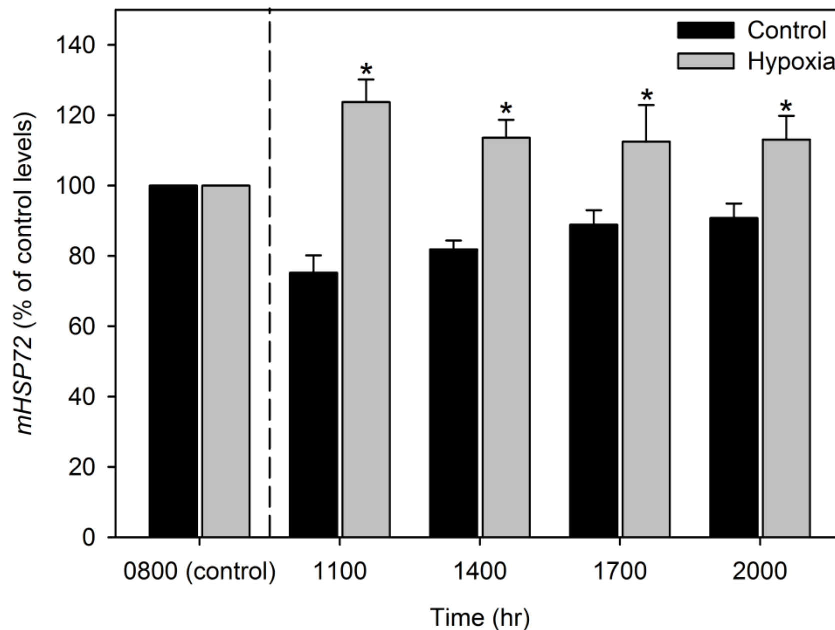

Figure 7.1. Mean (SD) *mHSP72* over the 12 h data collection period for the control and hypoxic conditions. The values are given as a percentage of the 0800 expression for each condition. The vertical dashed line represents the intervention period in the hypoxic condition. * Significant difference between conditions for a given time point.

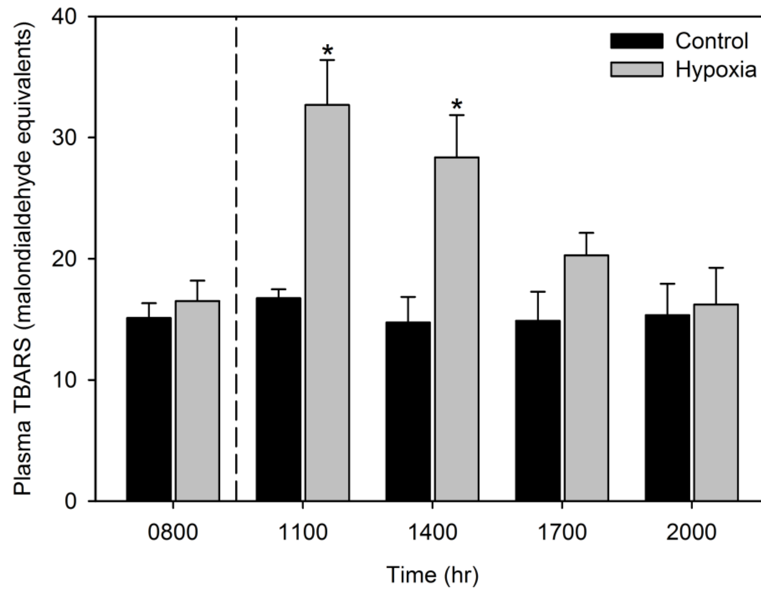

Figure 7.2. Mean (SD) plasma thiobarbituric acid reactive substances (TBARS) over the 12 h data collection period for the control and hypoxic conditions. The vertical dashed line represents the intervention period in the hypoxic condition. * Significant difference between conditions for a given time point.

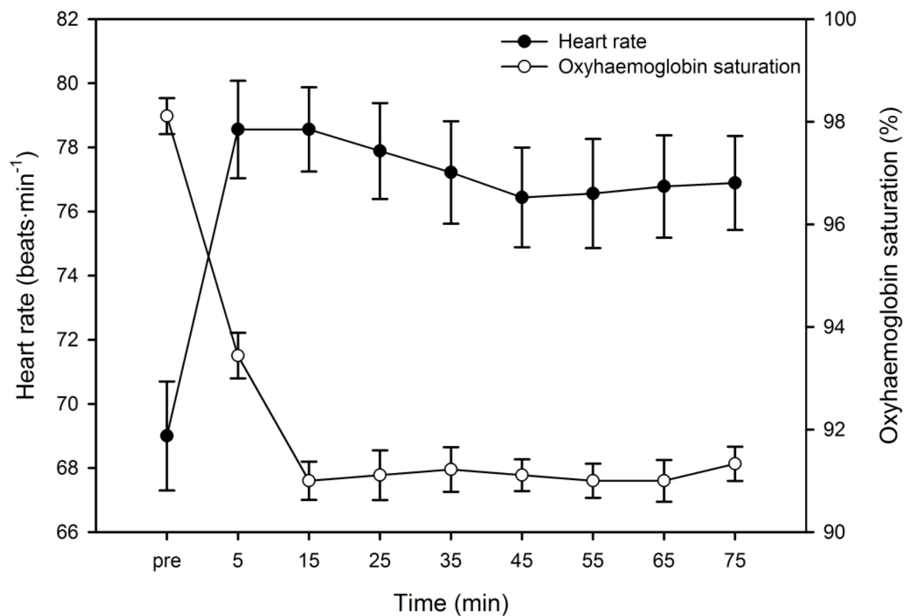

Figure 7.3. Mean (SD) heart rate and oxyhaemoglobin saturation during the hypoxic intervention period.

7.4. Discussion

This study was conducted to determine whether an acute 75 min hypoxic exposure at a simulated altitude of 2980m was sufficient to induce *mHSP72* and disrupt the previously disclosed quadratic trend in basal *mHSP72* expression (chapters 4 and 5). The intervention proved a suitable stimulus to induce significantly elevated *mHSP72* in comparison to the control condition, at all time points post intervention. Additionally, no quadratic trend in *mHSP72* expression was evident within the hypoxic condition, yet, was evident within the control condition.

The significant increases in *mHSP72* expression (1100, 1400, 1700 and 2000), suggest, as postulated by others, that HSP72 may serve as a useful indicator of the cellular response to hypoxia (Fei et al., 2007). Currently, the hypoxia induced *mHSP72* expression *in vivo* is absent from the literature, as is its link to disruptions in redox balance. *In vitro* animal and human cell/tissue isolates, and animal models have clearly shown increased HSP72 expression in response to hypoxia (Das et al., 1995; Bruemmer-Smith et al., 2001; Weinstein et al., 2004), as this *in vivo* research also demonstrates (Figure 7.1). However, these *in vitro* models employ a severely hypoxic micro-environment (physical blood flow restriction or pharmacological methods), which is extreme in comparison to the *in vivo* hypoxic load experienced on ascent/descent from altitude. Elevations of 3300 m to 6000 m for three weeks have demonstrated increased HSP72 expression in cardiac and brain tissues within animals, with the response absent at lower elevations (Wang et al., 2006). The results presented here suggest an acute elevation of 2980 m for 75 min is sufficient to induce significant *in vivo* human *mHSP72* expression, which is enduring for the 10 h post exposure in comparison to control (Figure 7.1). The acute hypoxic insult and significant increase in *mHSP72* expression demonstrate previous increases post hypoxia within chronic animal

models and human cell isolates (Das et al., 1995; Bruemmer-Smith et al., 2001) are present in this instance within humans (Figure 7.1).

One human study (Vogt et al., 2001) has investigated the muscle HSP72 response to exercise under conditions of hypoxia, however, training conducted under the influence of hypoxia compared to intensity matched exercise under normoxic conditions did not demonstrate any significant differences between conditions. Furthermore, the influence of hypoxia alone was not investigated (Vogt et al., 2001). At present well controlled and designed human *in vivo* investigations into the HSP72 response to both chronic and acute hypoxia are lacking and require further elucidation.

In vitro hypoxic environments and their ability to elicit elevated ROS production from organs, tissues and cells is established within the literature (Clanton, 2005; Zuo and Clanton, 2005; Clanton, 2007). Hypoxic exposure here elicited significant increases in plasma TBARS compared to control at 1100 and 1400 (Figure 7.3). It can be postulated that as oxidative stress (TBARS) and *mHSP72* are both significantly elevated immediately post exposure compared to control, their interaction may contribute to the pathophysiology of *in vivo* acute hypoxia. These postulations (disruptions in redox balance providing a stimulus for increases in *mHSP72* expression) are supported by *in vitro* experimental evidence (Currie et al., 1988; Mocanu et al., 1993; Kukreja et al., 1994b; Ahn and Thiele, 2003) and have been discussed in-depth in a recent review (Kalmar and Greensmith, 2009).

However, as discussed in section 2.7.5.1, the TBARS assay has several fundamental issues regarding specificity and although recognised as an inexpensive and simple bio-marker of oxidative stress, TBARS, as discussed above, does have some technical problems and these must be considered when interpreting the literature and any potential experimental findings (Powers et al., 2010b). It is a limitation of the present

chapter that lipid peroxidation (plasma TBARS) was solely used as a marker of disruption to redox balance when stress induced protein expression (*mHSP72*) was examined. Future work should employ measures of protein oxidation (e.g. protein carbonyls), in order to more securely investigate the relationship between hypoxia mediated disruptions in redox balance and *mHSP72* expression. Such measures where beyond the budget for the present study. Other limitations include the lack of randomisation for experimental conditions, although the use of a familiarisation trial likely excludes lack of randomisation as a confounding factor within the experimental design. However, randomisation is recommended for future studies.

The hypoxia mediated increases in plasma TBARS post intervention are in line with those shown in response to exercise (Fisher-Wellman and Bloomer, 2009). Whereby, post acute stressor a transient elevation in oxidative stress, specifically plasma TBARS is seen, which returns to values approximating baseline within one hour post exercise (Steinberg et al., 2006; Steinberg et al., 2007). However, within the present study plasma TBARS values were elevated in excess of the exercise mediated elevations seen elsewhere (approximately 1 h) (Steinberg et al., 2006; Steinberg et al., 2007) and stay elevated for approximately three hours post hypoxic exposure. This prolonged increase in plasma TBARS in response to hypoxia compared to exercise is likely attributable to the hypoxic stimulus being a greater challenge to O_2 homeostasis. Experimental evidence exists in support of this hypothesis, with 40 min cycle ergometer exercise (60% $\dot{V}O_{2\max}$) known to elicit negligible decreases in oxyhemoglobin saturation accompanied by 50% increases in plasma TBARS post exercise. However, the hypoxic condition within the present study reduces oxyhemoglobin by approximately 10% (Figure 7.3) with 113% peak increases in plasma TBARS seen post exposure. This greater acute stress within hypoxia (2980 m) compared to exercise (60% $\dot{V}O_{2\max}$), likely exerts a greater disruption in redox balance and thus requires a longer period to return to

baseline values, i.e. in excess of three hours post hypoxia compared to within one hour post exercise. Similarly, the lack of disturbance in redox balance within chapter 6 may be a contributing factor to why HBO and HA failed to induce elevated *mHSP72* expression. This failure of HBO and HA to induce oxidative stress may fail to induce disturbances in protein conformation as discussed in the next paragraph, which are known to be potent inducers of HSP72.

Oxidative stress may be a key factor in elevated HSP72 expression, with its presence exerting negative (genotoxic) or positive (therapeutic modalities) effects upon an organism dependant on its intracellular location and concentration (Thom, 2009). Oxidative stress has been highlighted as a key signalling molecule (Ushio-Fukai and Alexander, 2004; Calabrese et al., 2007) in pathways and transduction cascades for a variety of hormones, cytokines and growth factors (Allen and Balin, 1989; Maulik, 2002). High cellular levels of oxidative stress are known to damage membrane structures and proteins and activate pathways of apoptosis (Semenza, 2000; Kulkarni et al., 2007), which represent potent stimuli for HSP72 induction (Baler et al., 1992; Garrido et al., 2001; Bienemann et al., 2008). Elevations in oxidative stress, as measured by plasma TBARS and their cellular effects immediately post intervention could account for the post exposure elevations in *mHSP72* and potentially their continued elevation in comparison to control. Whether this significant increase in oxidative stress originates from the onset and/or during the transition to a hypoxic state, or upon reperfusion of hypoxic tissues is not known. However, it has been shown that a rapid initial burst of ROS formation occurs at the onset/transition to a hypoxic state, although, upon reperfusion ROS generation is far greater than during the transitory phase (Zuo and Clanton, 2005).

Increased bio-available HPS72 within an organism is implicitly linked to increased cellular protection and survival to a subsequent stressor (Garramone et al., 1994; Lepore

et al., 2000; Suzuki et al., 2000; Maglara et al., 2003; McArdle et al., 2004). Heat stressed cells displaying elevated HSP72 expression, demonstrate HSP72 dependant tolerance to the necrotic effects of hypoxia, in comparison to their non-heat shocked controls (Kiang et al., 1996). Additionally, heat acclimated animals have been shown to possess conferred cellular tolerance to the hypoxic rigours or various surgical procedures, attributed in part to elevated HSP72 expression (Levi et al., 1993; Horowitz, 2002; Arieli et al., 2003). Cell lines exposed to chronic hypoxia display an initial upregulation of HSP72 which gradually falls during the course of exposure to levels ultimately lower than basal values (Kim et al., 2006). These expression kinetics resemble those seen during hyperthermic exercise acclimation (McClung et al., 2008; Sandstrom et al., 2008; Amorim et al., 2010; Magalhães et al., 2010). The hypothetical reversal of this paradigm, i.e. hypoxia conferring tolerance to hyperthermic stress, is an interesting notion, particularly when viewed as a potential aid to gaining heat acclimation for hyperthermic exercise. Hyperthermic exercise acclimation results in increases of baseline levels of HSP72 (Yamada et al., 2007; McClung et al., 2008; Amorim et al., 2010; Magalhães et al., 2010). It is not proposed that the elevations in *mHSP72* seen in the current study represent the initiation of cytoprotection, tissue-level protection or other protective acclimation processes. However, this small novel human study, demonstrating a hypoxia mediated increase in *mHSP72*, requires further investigation. Potentially, whether the increase in *mHSP72* seen in the present study, may through further research, provide any conveyed cellular tolerance to a future stressor (e.g. exercise induced permutations to redox balance). However, all of these postulations must be explored carefully, with any theoretical claims on athletic performance or clinical relevance viewed with scepticism until fully elucidated.

In conclusion, a 75 min hypoxic exposure at 2980 m was sufficient to induce significant increases in *mHSP72* (1100, 1400, 1700 and 2000) and plasma TBARS (1100, 1400)

post exposure. Future work should explore whether these permutations are enduring for repeated intermittent or prolonged hypoxic exposures *in vivo*. Additionally, this hypoxic induced stress protein response requires exploration in the various tissues/cells previously examined by others, i.e. muscle, serum, plasma and other PBMCs, to ascertain if their trend in expression is tissue specific. Finally, it is highly recommended that future studies use several bio-markers of cellular oxidative damage (disruptions to redox balance), including protein carbonyls, to more securely investigate the relationship between hypoxia mediated disruptions to redox balance and increases in protein (*mHSP72*) concentration.

Chapter 8. Experiment 5: Daily hypoxia increases basal monocyte HSP72 expression in healthy human subjects.

This experimental chapter has formed the basis of the publication detailed below:

Taylor, L., Midgley, A., Chrismas, B., Hillman, A., Vince, R. V., Madden, L. & & McNaughton, L. (2011). Daily hypoxia increases basal monocyte hsp72 expression in healthy human subjects. Amino Acids, 40, 393 – 401.

8.1. Introduction

The quadratic trend in basal *mHSP72* during "waking" hours (chapters 4, 5, 6 and 7) in homeostatic conditions was disrupted by acute exposure to hyperbaria (decreases) and hypoxia (increases) (chapters 6 and 7 respectively). The acute hypoxia (75 min 2,980 m) mediated increases were still evident nine hours post exposure when data collection ceased. This lack of *mHSP72* data nine hours post intervention raises several key physiological questions. Firstly, how enduring are these hypoxia mediated increases in *mHSP72*, i.e. are they still evident in excess of 9 h post exposure? Secondly, would a repeat hypoxic exposure further increase the elevations in *mHSP72*? Exploration of these postulations may further enhance the potential of a hypoxic protocol to convey *mHSP72* mediated tolerance to further non-lethal stressors *in vivo*, in line with previous *in vitro* evidence (McArdle et al., 2004).

Modern altitude training (live high train low) has been shown to improve sea level athletic performance in a carefully controlled study (Levine and Stray-Gundersen, 1997). Current hypoxic training practises have sought to use intermittent hypoxic training (*IHT*), often utilising hypoxicator machines, to generate the necessary hypoxic load without the cost of commuting to and living at elevated altitudes (Hamlin and Hellemans, 2007). Specific to athletic training and performance, administration of intermittent hypoxia during physical activity and at rest has been utilised in attempts to augment athletic performance, with varying efficacy (Levine, 2002). The terminology used for *IHT* was often confusing, and has recently been addressed (Bartsch et al., 2007). In its truest form, intermittent hypoxic exposures last for approximately 60 to 90 min and involves repeated switches between normoxic and hypoxic air (Bartsch et al., 2007). Continuous hypoxic exposures of 1 to 4 h, at various elevations, ensure altitude sickness does not present and are referred to as prolonged hypoxic exposures, with hypoxic periods in excess of 4 h representing chronic hypoxic exposures (Levine, 2002).

Researchers have utilised intermittent hypoxic exposures in the treatment of clinical disorders (hypertension, Parkinson's disease, etc), altitude pre-acclimation and for sporting performance (Serebrovskaya, 2002).

Ultimately, the use of both acute and chronic *IHT* are designed to mimic the reputed benefits of more traditional chronic altitude training practises of the live high train low protocol (Levine and Stray-Gundersen, 1997). The acute physiological and bio-chemical responses of altitude exposure are an increase in minute ventilation to boost partial O_2 tension, to counteract the reduction in the partial pressure of O_2 seen with ascent to altitude. This results in an increase in blood pH due to increased excretion of carbon dioxide via hyperventilation. The use of *IHT*, can utilise such changes in homeostasis as a stimulus for physiological adaptation, which coincidentally, can augment sea level athletic performance. This improved performance stems from increases in muscle buffering capacity (2,3-bisphosphoglycerate mediated) and red blood cell mass (erythropoietin (EPO) mediated). EPO is a glycoprotein hormone that controls erythropoiesis, or red blood cell production.

High endogenous levels of HSP72 have been postulated to provide protection against the biochemical rigours of aerobic exercise (exercise induced permutations in redox balance) (Locke and Noble, 1995; Morton et al., 2009c), with daily PHE_R known to elicit no (Katayama et al., 2004), marginal (Katayama et al., 2003) or mixed (Rodriguez et al., 2007) augmentation of sea level exercise performance. Therefore, any observed changes in *mHSP72*, oxidative stress and EPO expression were to be viewed in light of potential alterations in physiological measures of maximal exercise performance ($\dot{V}O_{2\max}$). Any potential changes in maximal oxygen uptake are not hypothesised to stem from alterations in basal *mHSP72* expression but from the contentious (Katayama et al., 2003; Katayama et al., 2004; Rodriguez et al., 2007) efficacy of PHE_R in augmenting sea level performance (as discussed previously above).

Despite extensive previous research, at present, repeated daily prolonged hypoxic exposures at rest (*PHE_R*), and the impact of such exposures on *mHSP72* expression and markers of oxidative stress *in vivo*, have not been documented, in healthy human subjects. The importance of delivering hypoxia at rest is to pursue a non-exercise and non-thermal protocol *in vivo* to increase basal *mHSP72* expression, in line with previous experimental objectives.

The aims of this study were to investigate; 1) the effect of once daily *PHE_R* for ten consecutive days on basal *mHSP72* expression, 2) whether any such daily hypoxia mediated changes in *mHSP72* may have a relationship with alterations in redox balance, 3) whether once daily *PHE_R* for ten consecutive days effects maximal oxygen consumption, and 4) the EPO response to once daily *PHE_R* for ten consecutive days.

8.2. Methods

Subjects

Eight healthy recreationally active male subjects (mean ± SD age 20.2±4.4 years, height 172.1±13.9 cm, body mass 71.1±8.0 kg and physical activity 5.3±1.8 $h.wk^{-1}$) volunteered to participate in the study. Please see section 3.8 for general experimental restrictions and controls which were adhered to within this experimental chapter.

A sample size of eight was not dictated by a sample/power calculation but by the logistical and technical limitations of the specialist hypoxicators utilised. These machines were only available for a two week period, with the altitude utilised (equivalent to 2980 m) limiting gas flow to four subjects per machine.

Blood sampling and experimental protocol

The hypoxic exposure (75 min, 2980 m) was administered daily for 10 consecutive days, with the exposure commencing and ceasing at 0930 and 1045 respectively. Subjects

were familiarised with the experimental equipment three days prior to experimental testing commencing, of note subjects were exposed to the noise of the hypoxicator running, the novel feel of wearing the masks used to deliver the gas (no hypoxic air generated during familiarisation) and the "medical type" environment of the laboratory where all experimental testing took place. This exposure was used in chapter 7 and its use within the current chapter will facilitate inter chapter comparison. Please see section 3.9 for information regarding the generation of hypoxic air and the monitoring of the physiological response to such exposures. Subjects remained within the temperature-controlled laboratory (mean ± SD: WBGT 20.9 ± 0.5 ^{o}C, humidity 47 ± 4 %) during the exposures. Subjects did not sleep within the laboratory, but within a nearby University owned accommodation block for the night prior to and during all days of the study. Subjects gained 10 ± 1.8 h sleep prior to study commencement and repeated this sleep cycle on all study nights (10.5 ± 1.8 h).

Blood samples were taken immediately pre and post hypoxic exposures on days 1, 2, 3, 4, 5 and 10 for analysis of *mHSP72* and plasma TBARS with EPO specific blood samples taken on days 1, 2, 3, and 10. Sampling was limited to specific days as blood collection on all experimental days would have involved 24 separate venepunctures with pilot testing demonstrating significant volunteer discomfort under this regime. Additionally, pilot work demonstrated that EPO expression peaked after 2/3 days of hypoxic exposures and remained relatively unchanged until cessation of the hypoxic protocol. Hypoxia mediated *mHSP72* expression demonstrated a biphasic response, with plasma TBARS illustrating a consistent diurnal transitory response. In light of this pilot work (*mHSP72*, plasma TBARS and *EPO*) sampling frequency was designed to limit subject discomfort whilst gaining maximum insight into the expression kinetics of the parameters investigated (*mHSP72*, EPO and plasma TBARS). Blood samples were also drawn 24 h pre (baseline levels) and 48 h post 10 day intervention period. Blood

samples were drawn immediately prior to (0925 - 0930) and post (1045 - 1050) hypoxic exposure on each hypoxic day, for control sampling and 48 h post hypoxic intervention period. Post hypoxic blood sampling was conducted immediately post intervention, unlike previous work (chapter 7), to ascertain if the *mHSP72* response is instantaneous post hypoxia. Please see section 3.4 for HSP72 specific blood collection controls and considerations.

It is an experimental limitation that a control group was not utilised. However, due to the longitudinal nature of the experimental period, the limitation of the hypoxicators used (four participants per unit) and the short availability of the units (one week period) the use of a control group was not possible. Furthermore, the economic cost of using a control group of a similar design to experimental group used would have cost a further £3,126 in antibody and consumables. Therefore, within the economic constraints of the present chapter the use of a control group would not have been possible. The lack of red blood cell mass measurements is again attributable to economic limitations. Simple measures of haematocrit have proven variable in the assessment of hypoxia/EPO mediated increases in red blood cell mass, therefore, a modern approach is to use a Flow Cytometer based method (see (Lippi et al., 2006; Piagnerelli et al., 2007) for discussion) for quantification and identification of increased red blood cell mass (Koistinen et al., 2000; Lippi et al., 2006). Other reliable approaches are seen via the use of ELISA based methods of red blood cell mass concentrations (Koistinen et al., 2000; Lippi et al., 2006), though these are expensive. Therefore, economic restrictions and the time taken for HSP related sample acquisition on the Flow Cytometer made measures of red blood cell mass impossible for the present study.

Additionally, the efforts made in the previous experimental chapters were repeated to ensure *mHSP72* values were not unduly affected by factors aside from the hypoxic exposures. Methods, procedures and restrictions replicated those used in the previous

experiments to gain serial data for *mHSP72* and other physiological markers of homeostasis (Madden et al., 2008b; Vince et al., 2009).

The $\dot{V}O_{2\max}$ tests were conducted (at sea level) 8 days before and 48 h after the 10 day exposure period, to ensure the undue influence of physical activity (Morton et al., 2006) was not seen in *mHSP72* pre, during and post hypoxic programme. Additionally, subjects had been familiarised with the $\dot{V}O_{2\max}$ protocol and equipment to negate possible test order effects. The continuous incremental treadmill $\dot{V}O_{2\max}$ protocol utilised started at a speed of 8 kph which increased by 1 kph per min until volitional fatigue of the subject. The treadmill incline was maintained a 1% throughout the protocol. Before study commencement subjects completed a food diary, with a standardised daily food regime agreed upon between subjects and research organisers based on this. Compliance was monitored via this food diary and was at 100%.

<u>Monocyte HSP72 assay</u>

See general methods chapter, section 3.7. The assay was run live.

<u>Measurement of oxidative stress</u>

See general methods chapter, section 3.8.2. Analysis of plasma TBARS was conducted retrospectively.

<u>Measurement of EPO</u>

Measurement of EPO was obtained by chemiluminescent immunoassay (Immulite 1000 EPO Kit and Immulite 1000 analyser; Siemens DPC, USA) as per manufacturer's instructions retrospectively.

<u>Statistical analysis</u>

Statistical analyses were performed using PASW statistics 17 software (SPSS Inc., Chicago, IL). Normality was checked using quantile-quantile (Q-Q) plots and deemed plausible in each instance. Polynomial contrasts were used to identify trends in *mHSP72*, EPO and plasma TBARS concentrations, oxyhaemoglobin saturation and heart rate across time, both within days (pre-to-post intervention) and across the 10 day intervention period. In the event of a significant F statistic, Fisher's least significance test was used to locate significant paired differences. The *mHSP72* expression was expressed as a percentage of the first measurement, which is in accordance with previous literature (Morton et al., 2007) and experimental chapters 5, 6, and 7. Paired t tests were used to compare the $\dot{V}O_{2\max}$ values, maximal heart rate (HR_{max}) and time to exhaustion before and after the 10 day intervention period. Two-tailed statistical significance was accepted as $p < 0.05$.

8.3. Results

There were no significant changes observed over the 10 day hypoxic exposure intervention period for absolute $\dot{V}O_{2\max}$ ($t = 2.3$, $p = 0.065$) or HR_{max} ($t = 1.6$, $p = 0.16$), however, there was a small significant increase in time to exhaustion for the incremental test ($t = 3.9$, $p = 0.008$, $\acute{\omega}^2 = 0.50$) (Table 8.1), though this is likely physiologically negligible.

A significant 5th order polynomial trend was observed for EPO concentration over the 10 days ($F = 34.5$, $p = 0.001$, partial $\eta^2 = 0.85$), characterised by a dramatic 39% increase in EPO concentration the day after the first hypoxic exposure ($p = 0.001$), followed by a relative plateau over the rest of the hypoxic exposure period, and then a dramatic reduction immediately post-intervention (Figure 8.1). There was no significant within-day effect ($F = 0.01$, $p = 0.92$) and no significant within-day, between-day interaction ($F = 3.2$, $p = 0.12$).

A significant linear trend was observed for *mHSP72* expression, where *mHSP72* expression increased throughout the 10 day experimental period ($F = 73.2$, $p < 0.001$, partial $\eta^2 = 0.92$) (Figure 8.2). The *mHSP72* expression increased, on average, by 30% per day between days 0 and 5, and by 16% per day between days 5 and 10. Within each day, *mHSP72* expression was consistently higher after hypoxic exposure ($F = 6.2$, $p = 0.047$, partial $\eta^2 = 0.51$). However, no significant within-day, between-day interaction was observed ($F = 0.4$, $p = 0.53$).

There were significant main effects for day ($F = 9.0$, $p = 0.024$, partial $\eta^2 = 0.60$) and time (157.4, $p < 0.001$, partial $\eta^2 = 0.96$) for plasma TBARS concentration, however, the within-day, between-day interaction displayed a significant quadratic-by-linear trend ($F = 11.2$, $p = 0.015$, partial $\eta^2 = 0.65$). The plasma TBARS concentration increased significantly by around 20% in response to each hypoxic exposure (Figure 8.3), whereas no differences were observed in the control days, pre and post hypoxic intervention.

The change in oxhaeomoglobin saturation within each 75 min hypoxic exposure period exhibited a significant quadratic trend ($F = 3678.4$, $p < 0.001$, partial $\eta^2 = 0.998$). Oxyhaeomoglobin saturation dropped from 99% to around 90-91% 10 min after hypoxic exposure, remained depressed for the remainder of the hypoxic exposure, and returned to baseline 10 min after exposure was terminated (Figure 8.4). A within-day, between-day linear-by-quartic interaction effect also was observed for oxyhaemoglobin saturation ($F = 33.1$, $p = 0.001$, partial $\eta^2 = 0.83$).

Heart rate exhibited no significant trend over the 10 day intervention period ($F = 2.3$, $p = 0.18$), however, a significant quartic trend was observed across time within each day ($F = 397.4$, $p < 0.001$, partial $\eta^2 = 0.98$) (Figure 8.5). The mean heart rate was 70 beats/min 10 min before the hypoxic exposure, significantly increased to 83 beats/min within 5 min of exposure ($p < 0.001$), significantly decreased to 80 beats/min 5 min

later ($p = 0.013$), and remained relatively constant for the remainder of the exposure, before returning to baseline 10 min after exposure ($p < 0.001$). A within-day, between-day linear-by-quartic interaction effect also was observed for heart rate ($F = 8.6$, $p = 0.022$, partial $\eta^2 = 0.55$).

Table 8.1. Mean (SD) VO_{2max}, maximal heart rate (HR_{max}) and time to exhaustion observed during the incremental test before and after the 10 day hypoxic exposure intervention period.

Variable	Before	After
VO_{2max} (mL/kg/min)	50.1 (6.5)	50.8 (6.6)
VO_{2max} (mL/min)	3930 (769)	3948 (776)
HR_{max} (beats/min)	191 (10)	195 (6)
Time to exhaustion (min)	11.8 (1.5)	12.1 (1.6)*

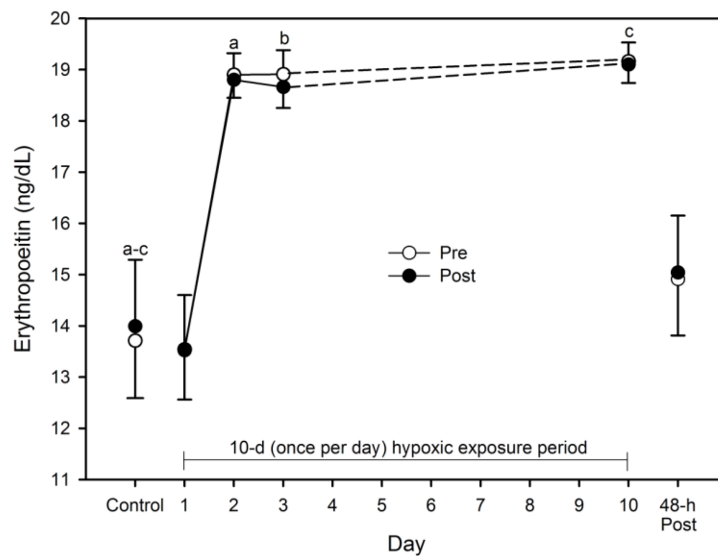

Figure 8.1. Mean (SD) erythropoietin concentration immediately before (pre) and after (post) hypoxic exposure over the 10-d intervention period. Control values, where no hypoxic exposure occurred, are also shown for before (control) and after (48 h post) the intervention period. Like letters above error bars represent significant differences in mean erythropoietin concentration across time ($p < 0.05$).

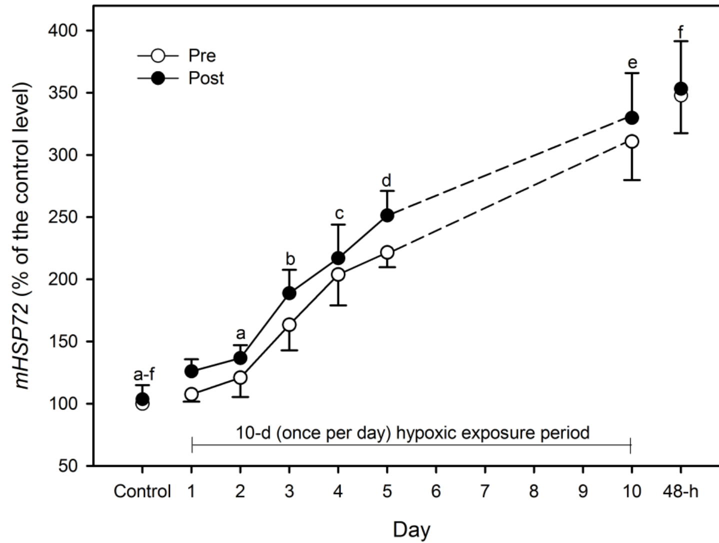

Figure 8.2. Mean (SD) *mHSP72* expression immediately before (pre) and after (post) hypoxic exposure over the 10 day intervention period. Control values, where no hypoxic exposure occurred, are also shown for before (control) and after (48 h post) the intervention period. The *mHSP72* is expressed as a percentage of the first measurement (control level). Like letters above error bars represent significant differences in mean HSP72 across time ($p < 0.05$).

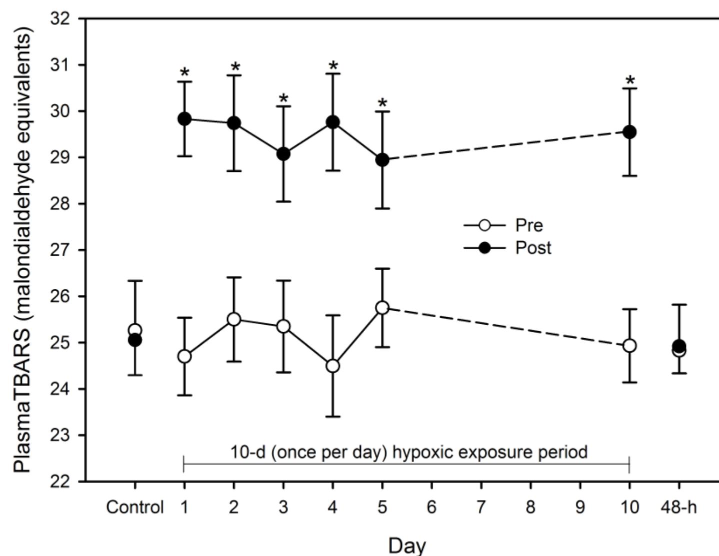

Figure 8.3. Mean (SD) plasma TBARS concentration immediately before (pre) and after (post) hypoxic exposure over the 10 day intervention period. Control values, where no hypoxic exposure occurred, are also shown for before (control) and after (48 h post) the intervention period. * Significantly different from pre ($p < 0.05$).

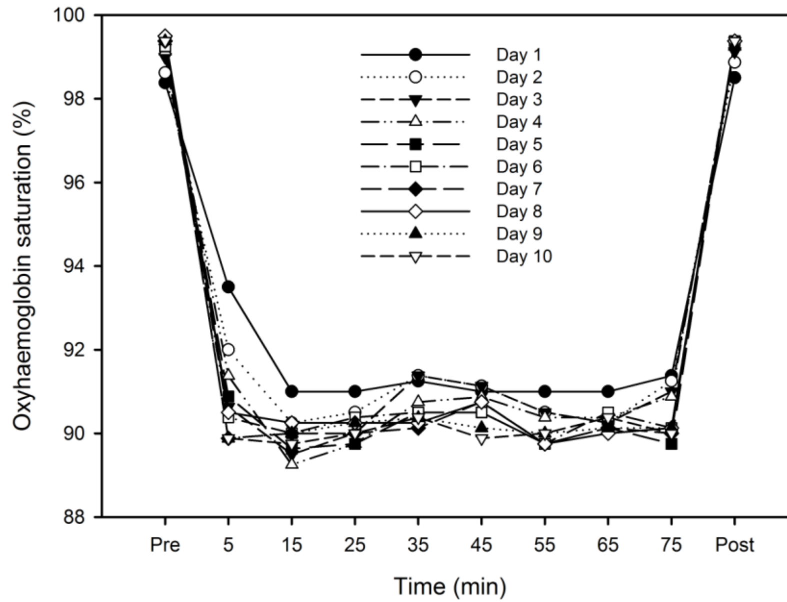

Figure 8.4. Mean oxyhaemoglobin saturation before (pre), during, and after (post) 75 min of hypoxic exposure for each day of the 10-d intervention period. Error bars have been omitted to aid clarity.

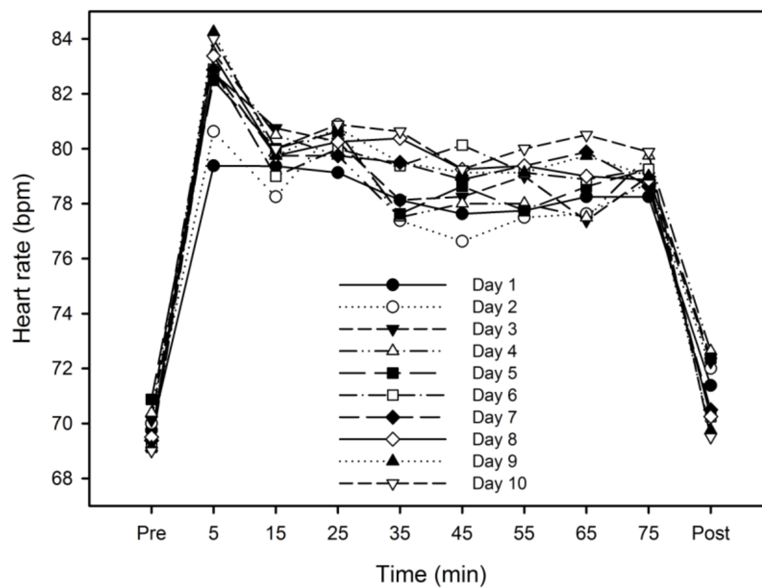

Figure 8.5. Mean heart rate before (pre), during, and after (post) 75 min of hypoxic exposure for each day of the 10 day intervention period. Error bars have been omitted to aid clarity.

8.4. Discussion

The same daily oxidative stress, as determined by plasma TBARS reaching approximately the same value after each hypoxic exposure (Figure 8.3) is caused by hypoxic air breathing (Bailey et al., 2001). This transient increase in oxidative stress appears to be a trigger for *mHSP72* synthesis, as shown elsewhere *in vitro* (Kukreja et al., 1994b; Ahn and Thiele, 2003), resulting in an increase in *mHSP72* proportional to the basal content pre exposure and the prior day (Gjovaag and Dahl, 2006; Vince et al., 2010).

It can be seen that 10 consecutive days of daily PHE_R are sufficient to induce significant basal elevations in *mHSP72* (Figure 8.2). Previously (chapter 7), it was shown that an identical hypoxic stimulus elicits a prolonged (9 h) significant elevation in concentrations of *mHSP72* compared to control, however, these findings were not examined in excess of 9 h post exposure (chapter 7). The present study, demonstrates 24 h post first exposure, *mHSP72* is significantly elevated, with increases compared to control evident on each subsequent day and 48 h post final exposure (Figure 8.2). Immediately, post first hypoxic exposure, no significant increase is evident in *mHSP72*, this is likely attributable to the immediate nature of post hypoxia blood sampling, i.e. within 5 min of hypoxia ceasing. Previously (chapter 7), approximately 30 min post hypoxia, significant increases in *mHSP72* were seen. Future work could examine this period immediately post hypoxia to elucidate the threshold in time required, post hypoxia, for significantly elevated *mHSP72* expression to occur. Interestingly, the elevations in *mHSP72* demonstrated an initial rapid rise, 30% day-on-day up to day 5, whereas average increases between days 5 and 10 are approximately 50% lower at 16% day-to-day (Figure 8.2). For example, extrapolating data from day 5 to day 7 (30% per day) would demonstrate a notable plateauing in *mHSP72*, with minimal *mHSP72* increase from this point, despite further hypoxic exposure. This initial phase (days 0 – 7)

of hypoxia mediated *mHSP72* expression, suggest that as a pre-conditioning strategy, the first week of PHE_R may be sufficient to induce close to maximum at-rest (baseline) *mHSP72* levels.

The maximum increase of ~200% seen in *mHSP72* within the present study is line with the immediate post exercise increases seen in *mHSP72* in response to hyperthermic treadmill exercise (~200%) (Fehrenbach et al., 2001), normothermic half marathon completion (~200%) (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b) and uncompenstable heat stress (Selkirk et al., 2009). There is no data available to compare *mHSP72* expression in response to mechanical stress. It is not suggested that whole body cytoprotection has been initiated or conferred by these hypoxia mediated elevations in *mHSP72* within the present study. Assumptions regarding the skeletal muscle and eHSP72 content in response to an intervention such as the one utilised here requires further elucidation in tandem with measures of *mHSP72*. This would allow a greater understanding of the *in vivo* contribution of both eHSP72 and iHSP72 to hypoxia and any potential conferred cellular tolerance to further non-lethal non-related stressors. Although as previously discussed, questions of the physiological origin and effect of eHSP72 still remain.

Endurance trained individuals expose themselves to repeated periods of oxidative stress as a consequence of regular aerobic training (Fisher-Wellman and Bloomer, 2009), resulting in decreased basal *mHSP72* expression (Fehrenbach et al., 2000a). However, the present study demonstrates no reduction in basal *mHSP72* in response to daily elevations in oxidative stress, as measured by plasma TBARS. Although endurance training has been shown to decrease basal levels of *mHSP72* (Fehrenbach et al., 2000a), in comparison to untrained controls, individuals who are heat acclimated and thus acquire some degree of acquired thermotolerance display elevated basal values in *mHSP72* compared to non heat acclimated controls (McClung et al., 2008). This

elevation in iHSP72 within heat acclimated individuals is present in PBMCs (Fehrenbach et al., 2001) aside from M and within skeletal muscle (Marshall et al., 2007). The HSP72 expression kinetics seen for acquired thermotolerance whereby daily hyperthermic exercise stress elicits sustained increases in *mHSP72*, compared to baseline levels (Yamada et al., 2007; Amorim et al., 2008; McClung et al., 2008; Magalhães et al., 2010), mirror those seen here for PHE_R (Figure 8.2).

Increased bio-available HSP72 within cell cultures and animal models is implicitly linked to augmented cellular protection and survival to subsequent stressors (Garramone et al., 1994; McArdle et al., 2004). Heat stressed cells have been shown to display HSP72 dependant resistance to the necrotic effects of hypoxia (Kiang et al., 1996) with heat acclimated animals displaying resistance to the hypoxic rigours of various surgical procedures (Horowitz, 2002; Arieli et al., 2003). Continued elevations in *mHSP72* (Figure 8.2) may provide protection for subsequent hypoxic exposures, in the same way elevated HSP72 expression in response to hyperthermic exercise protocols provide protection to exercise in high ambient temperatures (Yamada et al., 2007; Amorim et al., 2008; Magalhães et al., 2010). Significantly, decreased apoptosis is seen in animals that have attained heat acclimation upon exposure to further non-lethal non-related stressors (Horowitz et al., 2004). This ability of heat acclimation to confer tolerance to subsequent diverse stressors has led to common protective pathways being suggested as the underlining mechanism in heat acclimation induced preconditioning (Shein et al., 2007). It has been shown that heat shock and ischemia induce similar changes in expression of various genes (Horowitz et al., 2004), including increases in HSP72 and EPO (Shein et al., 2005; Shein et al., 2007). This cross tolerance is hypothesised to stem from increases in oxidative stress providing the stimulus for hormesis to occur (Fisher-Wellman and Bloomer, 2009).

Endurance training leads to physiological and haematological improvements in $\dot{V}O_{2max}$ (McNicol et al., 2009). This augmentation has been linked to biologically derived ROS acting in a hormetic manner, thus potentially conveying tolerance to further stressors (Fisher-Wellman and Bloomer, 2009). The present study does not support this hypothesis (Fisher-Wellman and Bloomer, 2009); here we demonstrate daily transient hypoxia mediated increases in plasma TBARS (note not exercise training induced disturbances to redox balance), yet augmentation of any of the parameters associated with increased maximal exercise performance are either unchanged, or in the case of time to exhaustion, physiology negligible (Table 8.1). These results are aligned with findings elsewhere (Katayama et al., 2003; Katayama et al., 2004). This study does not address any hormetic influence of repeated PHE_R induced changes in oxidative stress on sub-maximal aerobic exercise performance; this avenue of research may warrant further investigation. Specifically as elevations in HSP72, as seen within M in the current study, have been postulated to provide protection to the bio-chemical rigours of aerobic exercise (exercise induced permutations to redox balance) (Locke and Noble, 1995; Morton et al., 2009c).

Research specifically utilising PHE_R have employed a vast array of methodologies (elevations, delivery systems, subject training status etc) and consequently no consensus within the literature has been reached regarding EPO expression in response to PHE_R or *IHT* in general (Levine, 2002). A protocol similar to the one employed here (14 day, 2 h daily exposures at 4100 m, at rest) demonstrated no significant changes in haemoglobin, EPO and reticulocytes (Lundby et al., 2005), with findings by Katayama et al. (2003) supporting these observations. However, in accordance with the data presented in the current study, though to a greater magnitude (55% versus 39% increase from control in the current study), Rodriguez et al. (2000) demonstrated that a single hypoxic exposure of 90 min (5000 m) was sufficient to induce a significant increase in serum EPO

concentration. Additionally, in agreement with the current study's results (Figure 8.1), immediately post exposure, no significant change in EPO expression was demonstrated (Rodriguez et al., 2000). Rodriguez et al. (2000) only monitored EPO expression for 5 h post exposure, where results in this current study demonstrate that elevated EPO expression in response to PHE_R is prevalent for up to 48 h post exposure. The elevations in expression seen here mirror those seen previously for chronic exposures, whereby EPO production peaks 24 h post hypoxic exposure (Abbrecht and Kittell, 1972). Under the influence of chronic hypoxia, this zenith (24 h post exposure) in expression dwindles to a nadir, which is still significantly elevated compared to control (Berglund et al., 2002). Results presented here (Figure 8.1), utilising PHE_R rather than chronic exposures, demonstrate no reduction in this initial zenith in expression (24 h post exposure), with no apparent nadir throughout the 10 day period. Daily PHE_R likely provides a greater stimulus for EPO response due to repeated periods of oxidative stress (Figure 8.3), specifically daily reperfusion of hypoxic tissues and a resulting transient elevation in oxidative stress (Clanton, 2007), in comparison to chronic exposures (Berglund et al., 2002).

Oxidative stress has been highlighted as a key signalling factor in pathways and transduction cascades for a variety of hormones, genes, cytokines and growth factors (Allen and Balin, 1989; Maulik, 2002). Therefore, increases in oxidative stress could be a stimulus (signalling molecule) for the elevated HSP72 expression seen during and post the 10 day PHE_R period (Allen and Balin, 1989; Maulik, 2002). High cellular levels of oxidative stress are known to damage membrane structures and proteins and activate pathways of apoptosis (Kulkarni et al., 2007), which are known to represent a powerful stimuli for HSP72 induction (Bienemann et al., 2008).

However, as discussed in section 2.7.5.1, the TBARS assay has several fundamental issues regarding specificity and although recognised as an inexpensive and simple bio-

marker of oxidative stress, TBARS, as discussed above, does have some technical problems and these must be considered when interpreting the literature and any potential experimental findings (Powers et al., 2010b). It is a limitation of the present chapter that lipid peroxidation (TBARS) was solely used as a marker of disruption to redox balance when stress induced protein expression (*mHSP72*) was examined. Future work should employ measures of protein oxidation (e.g. protein carbonyls); in order to more securely investigate the relationship between hypoxia mediated disruptions in redox balance and *mHSP72* expression. Such measures where beyond the budget for the present study. Other limitations include the lack of control condition (for reasons discussed in section 8.1) and addition of such a condition is recommended for future studies.

In summary, it can be seen that daily administration of PHE_R is sufficient to induce sustained increases in *mHSP72* and EPO, with transient increases in oxidative stress also seen. These elevations in *mHSP72* display an initial fast phase in response to hypoxia (day 1 – 5) and a slower phase (day 5 – 10). It can be postulated that 7 days PHE_R in this manner would be sufficient to induce close to maximum at rest hypoxia mediated *mHSP72* expression, with respect to a 10 day exposure period. The observed hypoxia induced *mHSP72* response may benefit from investigation in other tissues and cells, for example, other PBMCs, serum, plasma and muscle tissue. Additionally, whether the hypoxia mediated increases in *mHSP72* may provide some resistance or conferred cellular tolerance to the bio-chemical rigours of sub-maximal exercise may warrant further investigation. It is not proposed that the elevations in *mHSP72* seen in the current study represent the initiation of cytoprotection, tissue-level protection or other protective acclimation processes. However, this small novel human study, demonstrating hypoxia mediated increases in basal *mHSP72* for ten consecutive days (in line with increases seen to hyperthermic exercise (McClung et al., 2008; Magalhães

et al., 2010)), requires further investigation. Potentially, whether the increase in *mHSP72* seen in the present study, may through further research, provide any conferred cellular tolerance to a further *in vivo* stressor (e.g. sub maximal exercise mediated disturbances to redox balance). However, all of these postulations must be explored carefully, with any theoretical claims on athletic performance or clinical relevance viewed with scepticism until fully elucidated.

The data derived in the current experimental chapter has some important implications to consider when designing and conducting future research in general and specifically to this thesis. Most pertinently, as stated in the experimental rationale for this thesis and in previous experimental chapters, a major research goal of this project was to develop a non-thermal non-exercise protocol to increase basal *mHSP72*. The data presented in this chapter (Figure 8.2) suggests that 10 days PHE_R may be a suitable protocol to elicit such increases (which are in line with other exercise and/or hyperthermia induced elevations in *mHSP72*). Therefore, previous postulations that elevated *mHSP72* may confer tolerance to the exercise induced disruptions to stress protein expression (Yamada et al., 2008) and redox balance (Fisher-Wellman and Bloomer, 2009), as associated with aerobic exercise, can now be tested within experimental designs incorporating an exercise stimulus, before and after a period of daily PHE_R acclimation.

Chapter 9. Experiment 6: Hypoxia mediated prior induction of monocyte expressed HSP72 and HSP32 provides protection to the disturbances to redox balance of sub-maximal exercise.

This experimental chapter has formed the basis of the publication detailed below:

Taylor, L., Hillman, A. Midgley, A. Peart, D. Chrismas., B & McNaughton, L. (2011) Hypoxia mediated prior induction of monocyte expressed HSP72 and HSP32 provides protection to the sub-maximal exercise induced disturbances to redox balance. Medicine and Science in Sports and Exercise – In Review.

9.1. Introduction

The ability of once daily PHE_R to induce day on day increases in basal *mHSP72* has been shown previously in chapter 8. These increases in basal *mHSP72* content are interesting when viewed in light of *in vitro* findings which correlate high basal HSP72 values with increased cellular survival in light of related and non-related cellular insults (Lepore et al., 2000; Lepore et al., 2001; Shein et al., 2005; Shein et al., 2007; Shima et al., 2008). It is likely such elevations *in vivo* will not augment maximal or submaximal aerobic normothermic exercise performance; however, due to the extended challenge to redox balance associated with prolonged aerobic exercise compared to maximal graded incremental exercise testing (Bloomer and Goldfarb, 2004; Fisher-Wellman and Bloomer, 2009), increased basal HSP72 may confer cellular tolerance to the biochemical rigours (disturbances to redox balance) seen during prolonged sub-maximal exercise (Medved et al., 2004), as demonstrated *in vitro* (Shima et al., 2008). This increased disturbance to redox balance in prolonged aerobic exercise compared to maximal graded incremental exercise has been discussed in sections 2.7.5, 2.7.5.1, and 2.7.5.2.

It is not surprising given the pivotal roles HSP72 plays in essential biological cascades and functions, that its involvement within the pathophysiology of exercise has been extensively reviewed (Noble et al., 2008; Whitham and Fortes, 2008; Yamada et al., 2008; Morton et al., 2009c). However, despite this well documented response, the correlation between different basal stress protein expressions pre exercise and their effects on exercised induced disruptions to *in vivo* redox balance are absent from the literature. The previous chapter (8), demonstrated that hypoxia mediated elevations in HSP72 are not sufficient in providing any resistance to the oxidative stress encountered during that exercise bout (chapter 8). However, there is no empirical data suggesting that preconditioning strategies that increase basal HSP72 expression prior to exercise

are/are not successful in providing protection to the redox balance disturbances associated with prolonged aerobic exercise performance (Medved et al., 2004), or alleviating the stress protein response seen (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Fehrenbach et al., 2001; Selkirk et al., 2009). Although acute *in vivo* hypoxia (2980 m, 75 min) has proved a suitable stimulus for elevating basal *mHSP72* (chapter 7 and 8), the elevation in this stress protein alone, may not confer tolerance to the bio-chemical rigours (disturbances to redox, increases in stress protein expression) of aerobic exercise.

Haem oxygenase-1 (HSP32) is known to be inducible under conditions of oxidative stress (Rothfuss et al., 2001). It has been postulated elsewhere, that exercised-induced oxidative stress may be counteracted by HSP32 expression, which may augment the capacity of antioxidant pathways (Rothfuss et al., 2001; Fehrenbach et al., 2003; Kalmar and Greensmith, 2009). The increased production and concentration of RONS during and post exercise (up to 1 h) are negatively related to cellular function and homeostasis (Bloomer and Goldfarb, 2004; Fisher-Wellman and Bloomer, 2009), particularly during recovery from exercise when high levels can inhibit the onset of recovery (Ascensao et al., 2008; Ascensao et al., 2011). However, as discussed in section 2.7.2, the presence and function of exercise mediated radicals within the tissues (muscle and blood originated) evidently have both positive (Jackson, 2008; Powers and Jackson, 2008; Powers et al., 2010a; Little and Cochran, 2011) and negative (Adrie et al., 2000; Powers et al., 2005; Ascensao et al., 2007; Powers et al., 2007; Ascensao et al., 2008; Ascensao et al., 2011) cellular effects, dependent on type, duration and location of origin (as discussed in section 2.7.2). The rationale for measuring HSP32 in this final experiment chapter was principally due to its inherent involvement with the oxidative stress response *in vivo* (Rothfuss et al., 2001; Fehrenbach et al., 2003), and as such, it

was postulated that in tandem with HSP72, a more global representation of the exercise and PHE_R mediated disturbance to redox balance could be ascertained.

Any imbalance between the provisions of antioxidant systems to render RONS inactive reduces exercise performance and efficiency, increases severity of delayed onset muscle soreness and ultimately negatively affects the speed of post-exercise recovery (Ascensao et al., 2007; Ascensao et al., 2008; Ascensao et al., 2011). However, when maximum exercise adaptation is sought within a tissue (muscle or blood) it is acknowledged that oxidative stress is likely central to mediate such remodelling of the tissue in question, and thus provides the stimulus for enhanced cellular tolerance to develop (Nikolaidis and Jamurtas, 2009; Powers et al., 2010a). Therefore, a preconditioning strategy, which could reduce such exercised induced disturbances to redox balance, may be advantageous in an applied exercise setting whereby recovery and return to optimal performance is sought (Ascensao et al., 2007; Ascensao et al., 2008; Ascensao et al., 2011) rather than adaptation (Nikolaidis and Jamurtas, 2009; Powers et al., 2010a).

The foremost endogenous non-enzymatic antioxidant in the body is glutathione. During exercise GSH protects against RONS, actively detoxifying components of the various RONS cascades such as hydroxyl radicals, H_2O_2 and lipid peroxides (Alin et al., 1985). Simply, an increased GSSG to GSH ratio is indicative of a move towards a pro-oxidant state. This cyclic process is constantly occurring during homeostasis and is accelerated in response to disturbances in redox balance, such as that which occurs with exercise (Medved et al., 2003; Medved et al., 2004). Additionally, it has been postulated that prior induction of HSPs (HSP32, HSP72, etc) may provide protection against oxidative stress (Kalmar and Greensmith, 2009). Therefore, a preconditioning strategy that elevates basal values of HSP32, HSP72 and GSH may augment the body’s antioxidant capacity and wider defence network, and thus, may be beneficial for recovery from

prolonged submaximal exercise induced disturbances to redox balance. The rationale for assessing glutathione in this final experimental chapter is to provide further data on the hypoxia and exercise induced disturbances to redox balance in addition to plasma TBARS, specifically as glutathione data is viewed as superior to plasma TBARS data in response to exercise (Bloomer and Goldfarb, 2004; Steinberg et al., 2006; Fisher-Wellman and Bloomer, 2009). Furthermore, the use of two or more biomarkers of oxidative damage to tissues and cells is line with recent guidelines on assessing exercise induced disturbances to redox balance (Powers et al., 2010b).

Therefore, the aims of this chapter were to investigate; 1) the effect of once daily hypoxia for five consecutive days on basal HSP32 expression, 2) the effect of prior induction of HSP32 and HSP72 on the exercise induced stress protein response, and 3) the effect of prior induction of HSP32 and HSP72 on exercise induced disturbances in redox balance.

9.2. Methods

Subjects

Eight healthy recreationally active male subjects (mean ± SD age 20.8±3.2 years, height 1.77±15.7 cm, body mass 72.1±11.0 kg, physical activity 6.8±1.8 $h.wk^{-1}$ and power output (PO) at lactate threshold (LT) 184±37 Watts (W)) volunteered to participate in the study. Please see section 3.8 for general experimental restrictions and controls which were adhered to within this experimental chapter.

A sample size of eight was not dictated by a sample/power calculation but by the logistical and technical limitations of the specialist hypoxicators utilised. These machines were only available for a one week period, with the altitude utilised (equivalent to 2980 m) limiting gas flow to four subjects per machine.

It is an experimental limitation that a control group was not utilised. However, due to the longitudinal nature of the experimental period, the limitation of the hypoxicators used (four participants per unit) and the short availability of the units (one week period) the use of a control group was not possible. Furthermore, the economic cost of using a control group, of a similar design to the experimental group used, would have cost a further £3,126 in antibody and consumables. Therefore, within the economic and equipment constraints of the present chapter the use of a control group was not possible.

Preliminary measurements

All subjects underwent initial lactate threshold testing on an SRM cycle ergometer (Schoberer Rad Mebtechnik, Konigskamp, Germany) using an incremental protocol starting at 125 W, increasing 20 W every 4 min until exhaustion. Capillary blood samples were collected every 2 and 4 min into lithium heparinized microvettes (Microvette CB300, Sarstedt, Numbrecht, Germany) and analyzed using a blood lactate analyzer (YSI 2300 STAT, YSI Inc, Yellow Springs, OH). LT was calculated using the Dmax method (Cheng et al., 1992). All tests were conducted at sea level within a temperature controlled laboratory. Upon completion of the LT test, subjects completed 30 mins of cycling (familiarisation) at 90% of their LT, on the cycle ergometer to be used for both exercise bouts. Subjects were also familiarised to the physiological data collection battery to be used (RPE, finger prick blood sampling, etc) and the “medical type” environment both exercise bouts would take place within.

Blood sampling and experimental protocol

Hypoxic protocol and blood sampling

The hypoxic exposure (75 min, 2980 m) was administered daily for five consecutive days at rest, with the exposure commencing and ceasing at 0930 and 1045 respectively. Subjects were familiarised with the experimental hypoxic equipment two days prior to

experimental testing commencing, of note subjects were exposed to the noise of the hypoxicator running, the novel feel of wearing the masks used to deliver the gas (no hypoxic air generated during familiarisation) and the "medical type" environment of the laboratory where all experimental testing took place. Please see section 3.9 for information regarding the generation of hypoxic air and the monitoring of the physiological response to such exposures. In light of previous work (chapter 8) utilising a 10 day acclimation period, the current project employed only 5 days of exposures due to the previous project demonstrating an initial rapid phase of *mHSP72* accumulation in response to hypoxia within the first 5 days of exposures. Blood samples were taken immediately prior to the first hypoxic exposure (hypoxic day 1) and 30 min post final hypoxic exposure (hypoxic day 5). Please see section 3.4 for HSP72 specific blood collection controls and considerations. The day to day changes in hypoxia induced *mHSP72* expression and other haematological markers during repeated daily hypoxic exposures have been demonstrated elsewhere in detail (chapter 8).

Exercise protocol and blood sampling

Seven days prior to the hypoxic acclimation period, subjects performed 60 min cycling on a SRM cycle ergometer at 90% of their power output at LT (exercise bout 1 – EXB1), this exercise bout was repeated 1 day post cessation of the hypoxic period (exercise bout 2 – EXB2). LT determined exercise intensities are recognised as a more rigorously controlled exercise stress, than for example percentages of $\dot{V}O_{2\max}$, with this approach used previously in other HSP related exercise studies (Morton et al., 2006; Morton et al., 2008; Morton et al., 2009b). Exercise was conducted at sea level and within a temperature controlled laboratory. Exercise commenced at 0930 on each exercise day, this consistent timing of blood sample collection was important to account for diurnal and circadian variations in basal *mHSP72* (chapters 4 and 5), especially as basal values can dictate the magnitude of stressor mediated changes in HSP72, both *in vivo* (Gjovaag

and Dahl, 2006) and *in vitro* (Vince et al., 2010). Physiological measures of exercise performance (heart rate, ratings of perceived exertion, blood lactate) in EXB1 and EXB2 were recorded throughout to ensure any changes in the biochemical measures of stress protein expression (*mHSP72* and *mHSP32*) and alterations in redox balance (plasma TBARS and glutathione) were not associated with variations in performance intensity between bouts. Blood samples were taken immediately pre and post exercise and 1, 4 and 8 h post exercise for *mHSP72* and immediately pre, post and 1 h post exercise for *mHSP32*, plasma TBARS and glutathione. The sampling was conducted in this time dependant manner due to previous literature demonstrating HSP32, plasma TBARS and glutathione returning to values approximate to control between 30 min and 1 h post exercise (Bloomer and Goldfarb, 2004; Fisher-Wellman and Bloomer, 2009).

Subjects remained within the temperature-controlled laboratory during the hypoxic exposures and EXB1 and EXB2 (mean ± SD: WBGT 20.1 ± 0.7 ^{o}C, humidity 47 ± 4 %). Subjects did not sleep within the laboratory, but within a nearby University owned accommodation block for the night prior to and during all days of the study. Subjects gained 9.8 ± 2.2 h sleep prior to study commencement and repeated this sleep cycle on all study nights (10.7 ± 1.5 h). Methods, procedures and restrictions replicated those used previously to gain serial data for *mHSP72* (previous experimental chapters) and other physiological markers of homeostasis (Madden et al., 2008b; Vince et al., 2009).

Measurement of oxidative stress

See general methods chapter, section 3.8.1 and 3.8.2. Analysis of plasma TBARS was conducted retrospectively. Glutathione specific blood samples were prepared as stated in section 3.4 and analysed retrospectively.

Statistical analyses

All statistical analyses were completed using IBM SPSS Statistics 18 (SPSS Inc., Chicago, IL). Statistical assumptions were checked using conventional graphic methods and were deemed plausible unless stated otherwise. Central tendency and dispersion are reported as the mean (SD). The effect of exercise bout and time on *mHSP72*, *mHSP32*, GSSG, GSH, TGSH, plasma TBARS, lactate, and heart rate were investigated using linear mixed models. The effect of the hypoxic intervention on *mHSP72*, *mHSP72*, GSSG, GSH, TGSH, and plasma TBARS was investigated using paired t tests. The *mHSP72* and *mHSP32* expression were expressed as a percentage of the first measurement, which is in accordance with previous literature (Morton et al., 2007) and previous experimental chapters (chapters 5, 6, 7 and 8). The GSSG, GSH and TGSH data were also expressed as a percentage of the first measurement. The *mHSP32*, plasma TBARS, GSH, and TGSH data used in linear mixed models were log-transformed to correct positively skewed residuals. Relationships between the changes in *mHSP32* and *mHSP72* and markers of oxidative stress (GSH, GSSG and plasma TBARS) were investigated using Pearson's correlations. Two-tailed statistical significance was accepted as $p < 0.05$.

9.3. Results

There were significant mean increases of 67.2% in *mHSP72* (95% CI = 52.9% to 81.4%; $t = 11.1$, $p < 0.001$), 25.4% in *mHSP32* (95% CI = 3.2% to 47.6%; $t = 2.7$, $p = 0.03$), 7.1% in GSSG (95% CI = 5.1% to 9.1%; $t = 9.5$, $p < 0.001$), and 2.6 malondialdehyde equivalents in plasma TBARS (95% CI = 1.5 to 3.6 malondialdehyde equivalents; $t = 5.6$, $p = 0.001$) in response to the 5 day hypoxic intervention, whereas no significant changes were observed for GSH ($t = 1.3$, $p = 0.22$) and TGSH ($t = 1.2$, $p = 0.25$).

There were significant exercise bout x time interaction effects for *mHSP72* ($F = 10.4$, $p < 0.001$) and *mHSP32* ($F = 3.9$, $p = 0.034$) (Figure 9.1 and 9.2). For the first exercise

bout a pronounced 105.5% increase in *mHSP72* was observed from pre- to immediately post-exercise (95% CI = 54.2% to 156.9%; $p < 0.001$), thereafter dropping to about 150% of the pre-exercise value and remaining relatively stable for the remainder of the post-exercise period. A similar response also was observed for *mHSP32* ($p = 0.003$). In contrast, no significant changes were observed in response to the second exercise bout for *mHSP72* ($p \geq 0.79$) or *mHSP32* ($p \geq 0.99$). When comparing across exercise bouts, only the pre-exercise values were significantly different for *mHSP72* ($p < 0.001$) and *mHSP32* ($p = 0.024$), with higher values in EXB2 compared to EXB1.

Significant main effects for exercise bout ($F = 23.4$, $p = 0.001$) and time ($F = 12.9$, $p = 0.001$), and a significant exercise bout x time interaction ($F = 21.5$, $p < 0.001$) were observed for GSSG (Figure 9.3). The GSSG was 16.5% lower pre-exercise (95% CI = 3.5% to 29.5%; $p = 0.018$) and 39.9% lower immediately post-exercise (95% CI = 27.2% to 52.6%; $p < 0.001$) for the second exercise bout compared to the first. Furthermore, a significant 32.5% increase in GSSG was observed from pre-exercise to immediately post-exercise for the first exercise bout (95% CI = 19.0% to 45.9%; $p < 0.001$), whereas no significant change was observed for the second ($p = 0.26$).

There was a significant main effect for time ($F = 7.0$, $p = 0.005$) and a significant exercise bout x time interaction ($F = 4.9$, $p = 0.02$) for GSH (Figure 9.4), but the main effects for exercise bout failed marginally to reach statistical significance ($F = 4.0$, $p = 0.064$). The GSH was significantly higher for the second exercise bout compared to the first, only immediately post-exercise. This was because GSH decreased from pre-exercise to post-exercise and then significantly increased from immediately post-exercise to 1 h post-exercise for the first exercise bout, but no significant changes were observed for the second.

No statistically significant main effects of exercise bout ($F = 0.02$, $p = 0.89$) and time ($F = 0.1$, $p = 0.90$), or exercise bout x time interaction ($F = 0.01$, $p = 0.99$) were observed for TGSH.

There was a significant main effect for time for plasma TBARS ($F = 10.3$, $p < 0.001$), where plasma TBARS increased significantly from pre- to post-exercise ($p = 0.001$) and then decreased from immediately post-exercise to 1 h post exercise ($p = 0.002$) (Figure 9.5). However, the main effect for exercise bout ($F = 0.5$, $p = 0.50$) and the exercise bout x time interaction ($F = 1.2$, $p = 0.32$) were not statistically significant.

No significant relationships were observed between the change in HSP32 and the changes in GSH ($r = 0.06$, $p = 0.88$), GSSG ($r = 0.03$, $p = 0.94$), or plasma TBARS ($r = 0.46$, $p = 0.25$). Similarly, no significant relationships were observed between the change in HSP72 and the changes in GSH ($r = 0.09$, $p = 0.83$), GSSG ($r = 0.28$, $p = 0.50$), or plasma TBARS ($r = -0.37$, $p = 0.36$).

There were significant differences across time for blood lactate (Figure 9.9) during the 60 min exercise periods ($F = 79.1$, $p < 0.001$), rising from 1.1 mM at rest to 2.6 mM after 30 min (mean difference = 1.5 mM; 95% CI = 1.2 mM to 1.8 mM; $p < 0.001$), and remaining stable for the last 30 min. No significant main effect for exercise bout ($F = 0.01$, $p = 0.91$) and no significant exercise bout x time interaction ($F = 0.003$, $p = 1.0$) were observed . No main effects for exercise bout ($F = 0.02$, $p = 0.88$) and time ($F = 0.2$, $p = 0.90$), and no exercise bout x time interaction ($F = 0.06$, $p = 0.98$), were observed for heart rate (Figure 9.11). Due to the small range in RPE (Figure 9.10) data (2-3 units) it was not appropriate to report formal statistics on the data. However, it was clear by visually scrutinising the data that there were negligible differences between exercise bouts.

The change in oxyhaemoglobin saturation (Figure 9.7) and heart rate (Figure 9.8) during the 75 min of hypoxic exposure, for each of the 5 days of the hypoxic exposure period, are shown in Figures 9.6 and 9.7, respectively. Oxyhaeomoglobin saturation dropped from 99% before each hypoxic exposure to around 90-91% 15 min into hypoxic exposure, remained depressed for the remainder of the hypoxic exposure, before returning to baseline 10 min after exposure. Heart rate increased from around 70 beats/min 10 min before hypoxic exposure to around 81 beats/min during the first 5 min of exposure. Apart from a small initial decrease, heart rate remained relatively constant for the remainder of the hypoxic exposure, almost returning to baseline within 10 min after exposure.

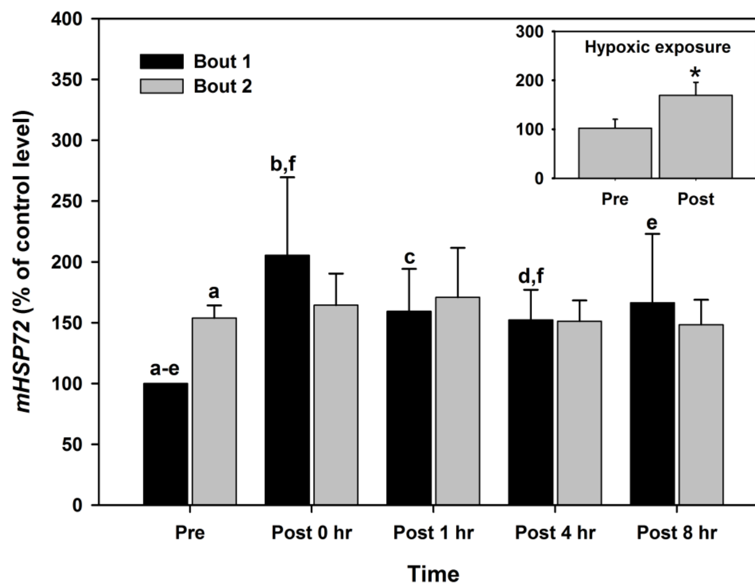

Figure 9.1. Mean (SD) *mHSP72* expression immediately pre and post, and 1 h, 4 h, and 8 h post exercise. Bout 1 and Bout 2 refer to two bouts of 60 min of exercise at 90% of lactate threshold, separated by 5 day hypoxic intervention period. The inset graph shows the mean (SD) *mHSP72* expression before and after the 5 day hypoxic intervention period. The *mHSP72* is expressed as a percentage of the first measurement. Like letters represent significant differences between means. * Post significantly higher than pre ($p < 0.05$).

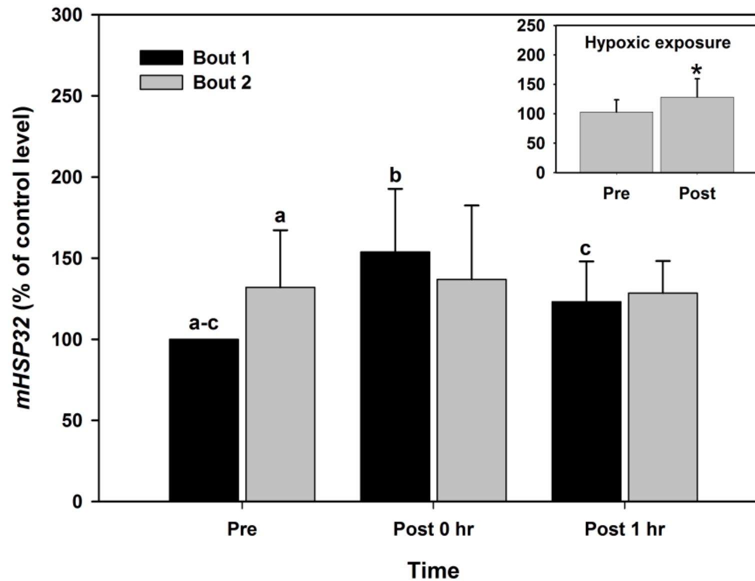

Figure 9.2. Mean (SD) *mHSP32* expression immediately pre and post, and 1 h, 4 h, and 8 h post exercise. Bout 1 and Bout 2 refer to two bouts of 60 min of exercise at 90% of lactate threshold, separated by the 5 day hypoxic intervention period. The inset graph shows the mean (SD) *mHSP32* expression before and after the 5 day hypoxic intervention period. The *mHSP32* is expressed as a percentage of the first measurement. Like letters represent significant differences between means. * Post significantly higher than pre ($p < 0.05$).

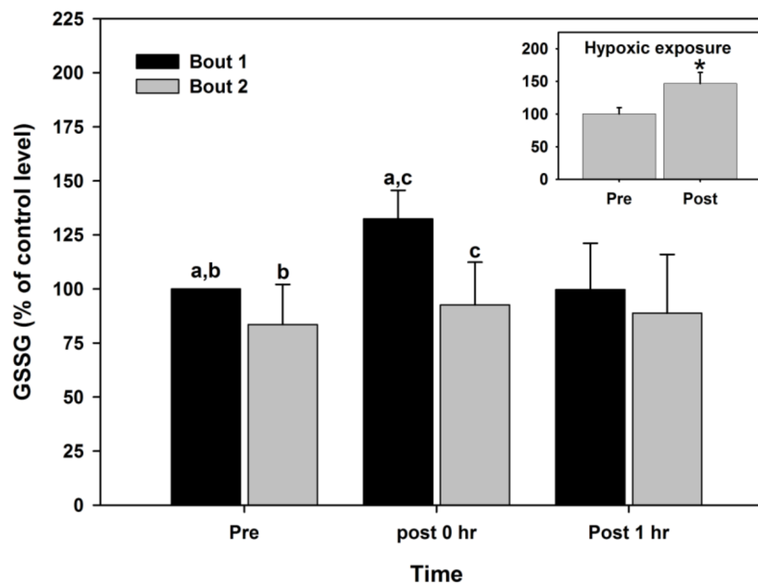

Figure 9.3. Mean (SD) whole blood GSSG expression immediately pre, post and 1 h post exercise. Bout 1 and Bout 2 refer to two bouts of 60 min of exercise at 90% of lactate threshold, separated by the 5 day hypoxic intervention. The inset graph shows the mean (SD) GSSG before and after the 5 day hypoxic intervention period. Like letters represent significant differences between means. * Post significantly higher than pre ($p < 0.05$).

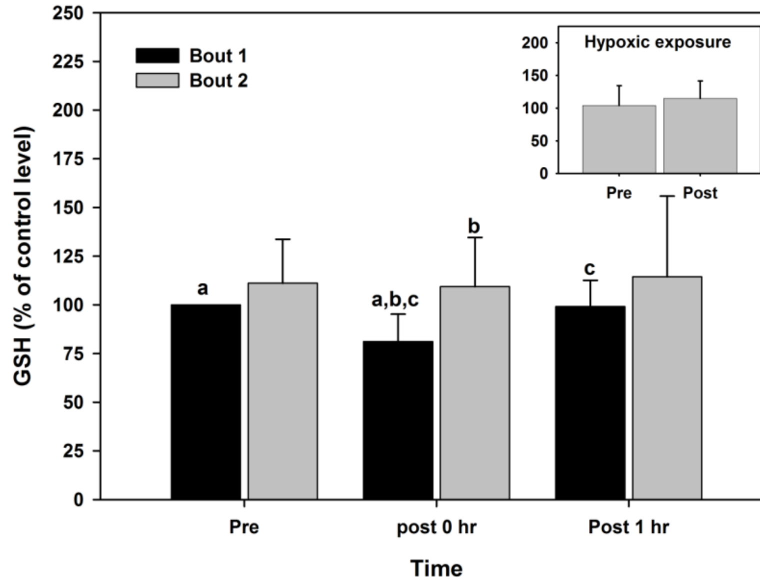

Figure 9.4. Mean (SD) whole blood GSH expression immediately pre, post and 1 h post exercise. Bout 1 and Bout 2 refer to two bouts of 60 min exercise at 90% of lactate threshold, separated by the 5 day hypoxic intervention period. The inset graph shows the mean (SD) GSSG before and after the 5 day hypoxic intervention period. Like letters represent significant differences between means.

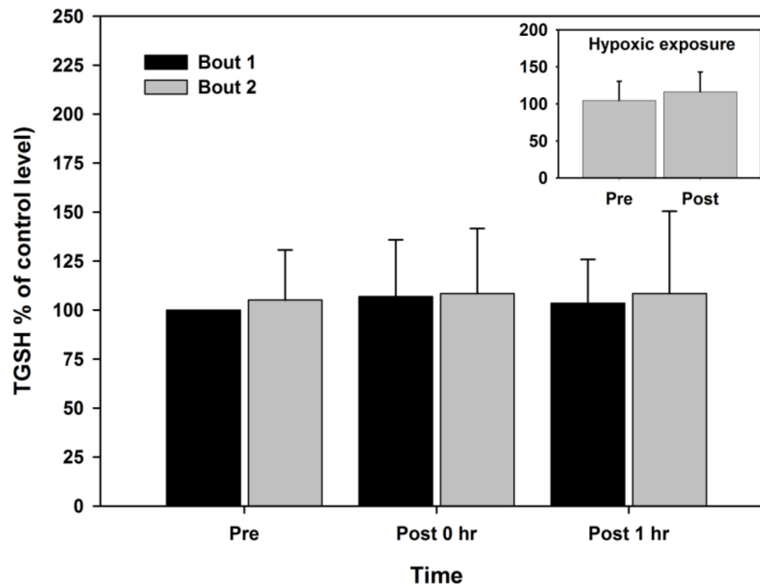

Figure 9.5. Mean (SD) whole blood TGSH expression immediately pre, post and 1 h post exercise. Bout 1 and Bout 2 refer to two bouts of 60 min of exercise at 90% of lactate threshold, separated by the 5 day hypoxic intervention. The inset graph shows the mean (SD) TGSH before and after the 5 day hypoxic intervention period.

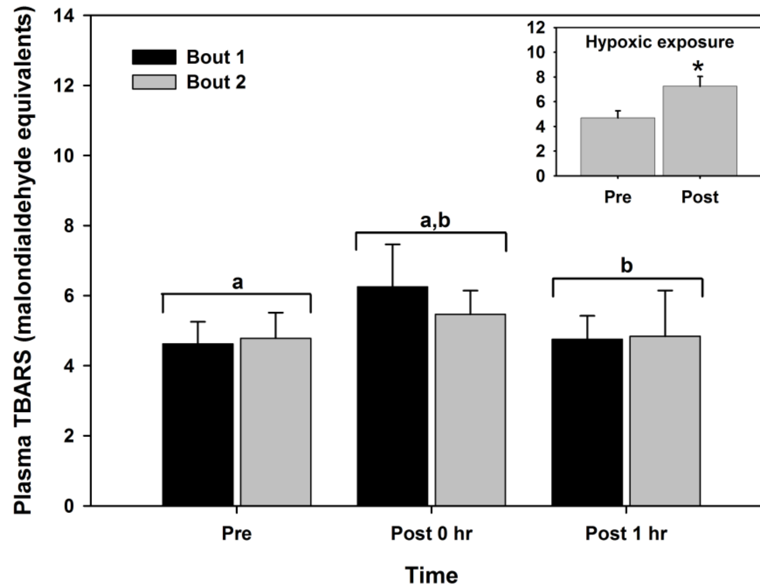

Figure 9.6. Mean (SD) plasma TBARS expression immediately pre, post and 1 h post exercise. Bout 1 and Bout 2 refer to two bouts of 60 min exercise at 90% of lactate threshold, separated by the 5 day hypoxic intervention period. The inset graph shows the mean (SD) plasma TBARS before and after the 5 day hypoxic intervention period. Like letters represent significant differences across time (main effect). * Post significantly higher than pre ($p < 0.05$).

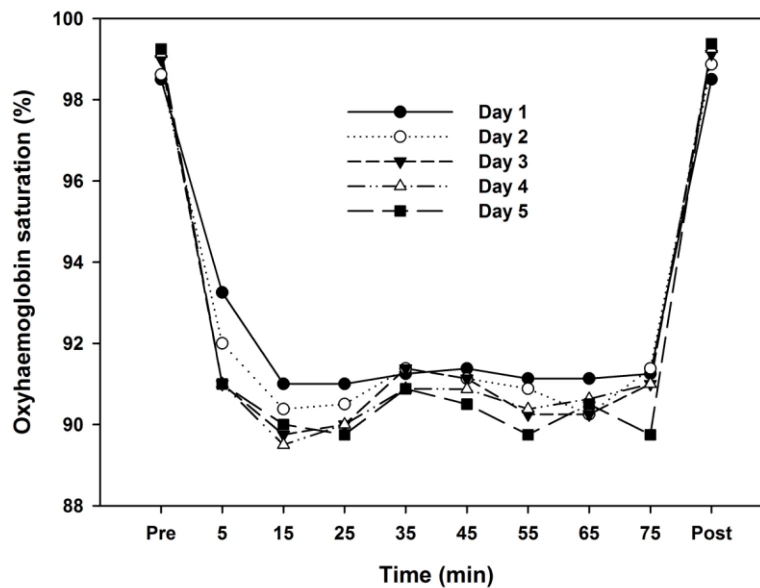

Figure 9.7. Mean oxyhaemoglobin saturation pre, during and post 75 min of hypoxic exposure for each day of the 5 day intervention period. Error bars have been omitted to aid clarity.

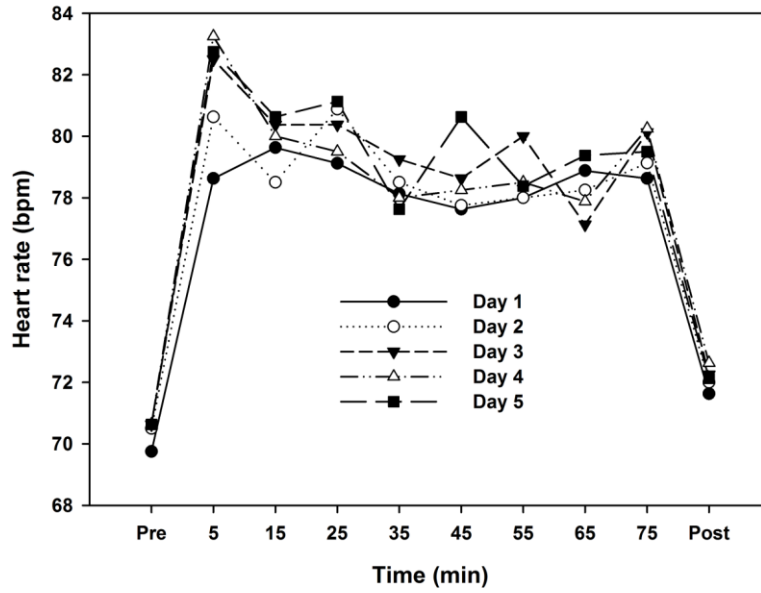

Figure 9.8. Mean heart rate pre, during and post 75 min of hypoxic exposure for each day of the 5 day intervention period. Error bars have been omitted to aid clarity.

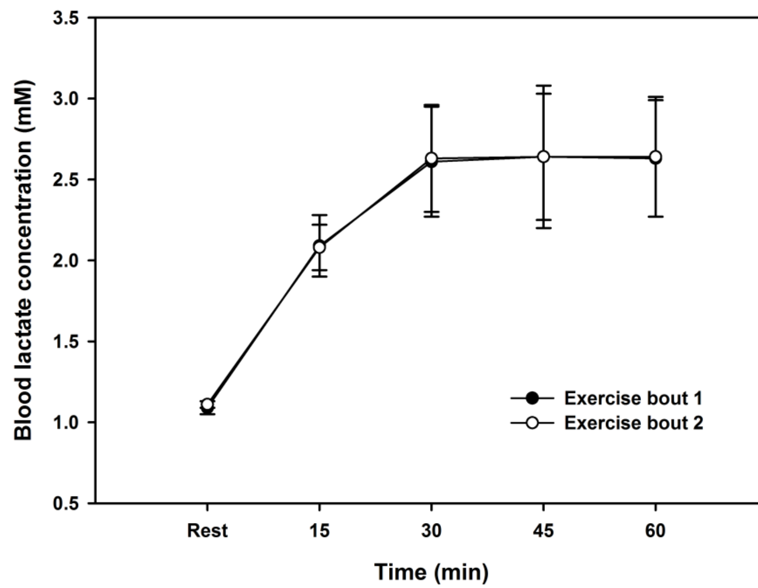

Figure 9.9. Mean (SD) blood lactate concentration at rest and during the two, 60-min exercise bouts.

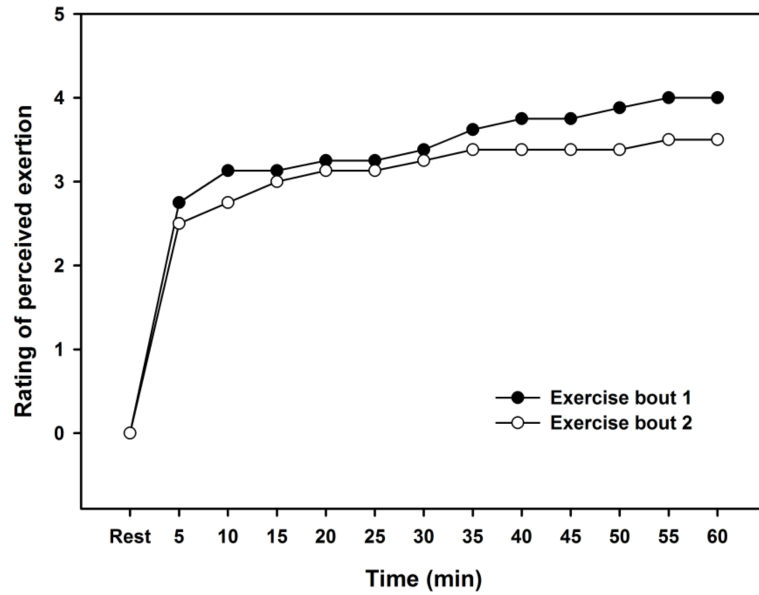

Figure 9.10. Mean rating of perceived exertion at rest and during the two, 60-min exercise bouts. Error bars have been omitted because the data exhibited extremely little variability across subjects.

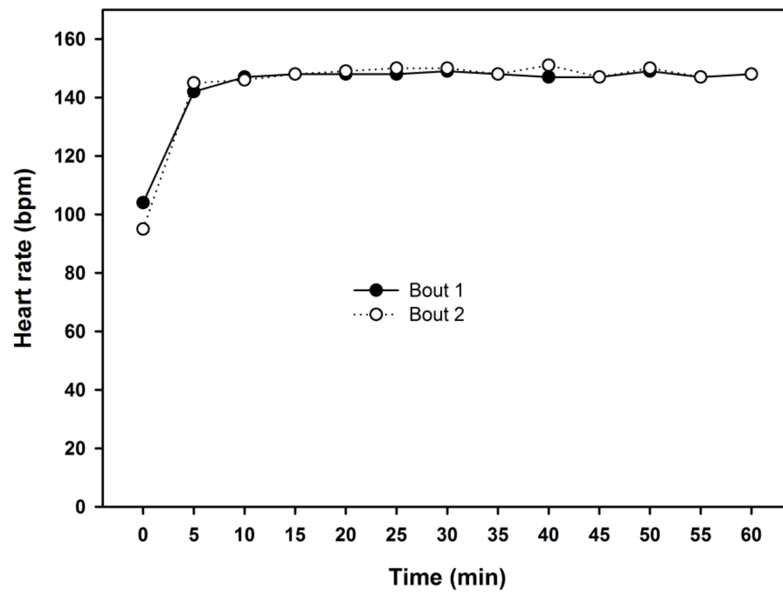

Figure 9.11. Mean heart rate prior to exercise commencement and during the two, 60-min exercise bouts. Error bars have been omitted to aid clarity.

9.4. Discussion

The principal finding of the present study was that a five day hypoxic acclimation period was sufficient to reduce the disturbance to redox balance of 60 min prolonged aerobic exercise at 90% of LT. This reduction was evident by the significant increase (32.5%; 95% CI = 19.0% to 45.9%; $p < 0.001$) in GSSG post EXB1 being absent post EXB2 ($p = 0.26$). Such a reduction in disturbance to redox balance post exercise is likely attributable to the prior induction (increased content pre EXB2 compared to pre EXB1) and thus bio availability of the potently antioxidant stress protein *mHSP32* ($p = 0.024$) and the highly stress inducible *mHSP72* ($p < 0.001$), in addition to favourable alterations in glutathione ratios.

It is likely that the increased co-bioavailability of *mHSP72* (Kalmar and Greensmith, 2009), *mHSP32* (Rothfuss et al., 2001) and GSH (Nikolaidis and Jamurtas, 2009) may augment the antioxidant capacity of the blood. This augmentation may allow exercise mediated increases in oxidant production (skeletal muscle and blood originated, see section 2.7.2), and thus disturbances to redox balance, to be more readily dealt with post hypoxic acclimation period. Despite these observations, establishment of cause and effect cannot be claimed, with such evidence requiring further, likely *in vitro*, experimentation (discussed and suggested in chapter 10). Additionally, no relationships were evident between changes in stress protein and bio-markers of redox balance (see section 9.3). It is important to note attempts have been made to control changes in exercise intensity between EXB1 and EXB2 (see Figures 9.9, 9.10 and 9.11) accounting for alterations in any of the biochemical variables investigated. Physiological data from the two exercise bouts demonstrated no significant differences between bout and bout x time for heart rate, blood lactate and RPE. Therefore, the two exercise bouts were as near to as identical as physiologically possible with similar environmental conditions also experienced (mean ± SD for both exercise bouts: WBGT 21.2 ± 0.2 ^{o}C, humidity

47 ± 4 %). As such the differences in the various biochemical markers assessed are likely attributable to the hypoxic intervention.

HSP72

Within the present study EXB1 demonstrated an increase of approximately 100% in *mHSP72* immediately post exercise, with values remaining elevated by approximately 50% 1, 4, and 8 h post exercise (Figure 9.1), in line with previous increases seen in iHSP72 post exercise (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Fehrenbach et al., 2001; Morton et al., 2006). The literature investigating cycling induced increases in PBMC expressed HSP72, analysed by flow cytometry is limited. One study has investigated such occurrences, although reporting significant increases pre to post intensified training in M and G, the increases are likely physiologically negligible (Whitham et al., 2004), as such inferences from their data are incomparable to the data presented within the present study. Additionally, their example gating strategy appears incorrect to that used in each experimental chapter in the present thesis and by others (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Fehrenbach et al., 2001; Selkirk et al., 2009), with, for example, no CD14 specific gating performed to distinguish M from other cell populations (Whitham et al., 2004). Furthermore, flow cytometry analysis was not performed live with cells left up to 48 h before acquisition, which is known to negatively affect cell acquisition and subsequent data analysis (Bachelet et al., 1998; Ireland et al., 2007). Elsewhere, western blotting and RT-PCR techniques have been used with no increases evident post cycling exercise in thermoneutral (Chang et al., 1998) and hyperthermic environments (Marshall et al., 2007). However, similar findings to the data in the present study, specifically within M, have been found elsewhere when analysed by flow cytometry post running exercise in thermoneutral (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Fehrenbach et al., 2001; Schneider

et al., 2002) and hyperthermic environments (Fehrenbach et al., 2001; Selkirk et al., 2009).

Seven days post EXB1, before the commencement of the hypoxic acclimation period, basal *mHSP72* values returned to concentrations approximate to control (pre EXB1 values). This return to baseline is pertinent within the study design, as basal values are known to be indicative of the magnitude of stress mediated HSP72 response both *in vivo* (Gjovaag and Dahl, 2006; McClung et al., 2008; Sandstrom et al., 2008) and *in vitro* (Vince et al., 2010). The hypoxia mediated increases (approximately 60%) in basal *mHSP72* remain elevated (approximately 50% higher compared to control and pre EXB1) before the commencement of EXB2 (inset of Figure 9.1). The expression kinetics in EXB2, compared to EXB1, did not demonstrate a significant increase in *mHSP72* immediately or 1, 4, or 8 h post exercise. This difference is likely attributable to the prior induction and thus bio availability of *mHSP72* conferring protection to the subsequent non lethal stressor, as shown *in vitro* (Garramone et al., 1994; Lepore et al., 2000; Suzuki et al., 2000; Maglara et al., 2003; McArdle et al., 2004). However, *in vivo*, this is the first demonstration of prior induction of HSP72 via a non-thermal non-mechanical stressor conveying protection to one of the bio-chemical rigours (challenges to redox balance) associated with aerobic exercise.

Experimental data exists in support of HSP72 mediated tolerance to oxidative and ischemic stress (Shima et al., 2008). It has been established that functional impairment, whether that be in regulation or efficacy in HSP synthesis, is present in various diseases and pathological conditions, such as those affiliated with chronic oxidative stress pathology i.e. in neurodegeneration and cardiovascular disease (Kalmar and Greensmith, 2009). Mouse models with transgenic upregulation of HSP72 have demonstrated resistance to the biochemical rigours of oxidative/ischemic stress compared to control animals (Marber et al., 1995; Plumier et al., 1995). Furthermore, within exercising

rodents, animals demonstrating enhanced HSP72 expression also display augmented ROS scavenging capacity and reduced muscle injury post downhill running compared to those animals with low HSP72 expression (Shima et al., 2008). The authors (Shima et al., 2008), postulated elevations of HSP72 may protect the antioxidant defence system in skeletal muscle by enhancing the adaptive HSP72 mRNA response. Mechanistically, *in vitro*, evidence exists that elevations in oxidative stress are a trigger for increases in HSP72 concentration (Currie et al., 1988; Mocanu et al., 1993; Kukreja et al., 1994b; Ahn and Thiele, 2003), with similar findings recently shown *in vivo* to acute (chapter 7) and repeated daily hypoxic exposures (chapter 8). Therefore, it is likely the repeated disturbance to redox balance from the daily hypoxic exposure (chapter 8) may be acting as a stimulus for elevated HSP72 expression and as a potential stimulus for hormesis (conveyed cellular protection to exercise mediated disturbances to redox balance) to occur in preparation for EXB2 (Radak et al., 2005; Ji et al., 2006; Gomez-Pinilla, 2008; Radak et al., 2008; Fisher-Wellman and Bloomer, 2009; Goto and Radak, 2010; Ji et al., 2010). However, caution is required when making such postulations based on plasma TBARS data, without supporting data specifically reporting measures of protein oxidation (protein carbonyls), such limitations of plasma TBARS are subsequently discussed in the glutathione and plasma TBARS section of the present discussion.

<u>HSP32</u>

Similarly to HSP72, the inducible isoform of HSP32 has been shown to increase in concentration under *in vitro* experimental conditions of ischemia, hypoxia and inflammation within cell and tissue lines (Abraham et al., 1987; Motterlini et al., 1996; Doi et al., 1999; Motterlini et al., 2000; Foresti et al., 2001; Rothfuss et al., 2001). A rodent exercise model demonstrated 700% and 400% increases in HSP32 mRNA 1 and 3 h post exercise respectively (Essig et al., 1997). However, these conditions lack

specificity to those experienced during *in vivo* exercise or sojourn to altitude within humans.

Within humans, post half marathon completion, significant elevations in HSP32 have been shown in L, M and G, as measured by flow cytometry (Niess et al., 1999b). This initial study (Niess et al., 1999b) was expanded in attempts to delineate exercise mode, duration and intensity with regards to HSP32 expression (Fehrenbach et al., 2003). Findings demonstrated that increases in HSP32 expression were seen post half marathon completion in L, M and G, as shown previously (Niess et al., 1999b), but not post eccentric or exhaustive aerobic exercise (Fehrenbach et al., 2003). Findings within the current study demonstrate comparable findings (Niess et al., 1999b; Fehrenbach et al., 2003), with significant increases in *mHSP32* seen immediately post (approx 50%) and 1 h post (approx 30%) EXB1. Interestingly, this increase in EXB1 (pre to post and 1h post) is absent from EXB2. This absence of an increase (EXB1 compared to EXB2) post exercise is likely attributable to the hypoxic intervention significantly increasing (approx 30%) basal *mHSP32* content, with these elevations sustained (significantly increased pre EXB2 compared to pre EXB1) at comparable levels prior to commencement of EXB2 compared to pre EXB1 (Figure 8.2). This increase in bio-available *mHSP32* may likely ameliorate the disturbance in redox balance experienced during the exercise bout via the potent antioxidant capacity of HSP32 (Gozzelino et al., 2010).

<u>Glutathione and plasma TBARS</u>

Measures of oxidative stress through indirect quantification of lipid peroxidation via plasma TBARS have been extensively used within the literature. Most research has demonstrated elevations in plasma TBARS post maximal (Bloomer and Goldfarb, 2004; Steinberg et al., 2007) and sub-maximal exercise (Laaksonen et al., 1999; Fisher-

Wellman and Bloomer, 2009), which return to baseline values within one hour post exercise (Bloomer and Goldfarb, 2004; Steinberg et al., 2006; Steinberg et al., 2007; Fisher-Wellman and Bloomer, 2009). However, the utilisation of plasma TBARS to measure exercise induced changes in oxidative stress is not without limitations. The literature has reported concerns about the specificity of the TBARS assay and these considerations have been discussed in recent reviews (Fisher-Wellman and Bloomer, 2009; Powers et al., 2010b). The present study demonstrated a significant increase in plasma TBARS post EXB1 and EXB2, which returns to values approximate to baseline 1 h post exercise. These results are in line with previous research for sub-maximal exercise (Laaksonen et al., 1999; Steinberg et al., 2006; Steinberg et al., 2007; Fisher-Wellman and Bloomer, 2009). However, no significant differences were evident in plasma TBARS data between the two exercise bouts within the present study. This may be attributable to the lack of specificity of the plasma TBARS assay in assessment of the exercise mediated changes in oxidative stress. This has been discussed in section 2.8. The author has attempted to alleviate the problems associated with plasma TBARS analysis, via utilisation of measures of whole blood glutathione redox balance, which have been shown to be a more sensitive and specific measure of disturbances to redox balance in response to exercise, in comparison to plasma TBARS (Jammes et al., 2004; Steinberg et al., 2006; Steinberg et al., 2007; Fisher-Wellman and Bloomer, 2009).

Within EXB1 post exercise; there was a significant increase in GSSG, a decrease in GSH and no change in TGSH, with these exercised induced permutations in GSSG returning to baseline 1 h post exercise in both EXB1 and EXB2. These findings are in line with previous literature, whereby a multitude of non-eccentric aerobic exercise protocols elicit decreases in GSH, increases in GSSG and no change in TGSH (Fisher-Wellman and Bloomer, 2009). Interestingly, within EXB2, despite comparable physiological performance data, the significant increase in GSSG is absent compared to

EXB1. This may likely be attributable to the hypoxia mediated changes in basal GSSG, whereby, pre exercise values are significantly lower within EXB2 compared to EXB1. This reduction in GSSG is seen in tandem with an elevation in basal GSH values pre exercise in EXB1 compared to EXB2 (Figure 9.3). Such alteration in GSH:GSSG are indicative of a more favourable basal antioxidant capacity (Ilhan et al., 2004). Therefore, the increase in bio available GSH (failed marginally to reach significance) may present augmented resistance to exercise induced disturbances to redox balance during EXB2. Previously, it has been shown within animals (Leeuwenburgh and Ji, 1995) and humans (Sen et al., 1994a; Sen et al., 1994b) the importance of GSH in protecting against resting and exercise induced oxidative stress, with reduced availability of GSH correlated to the decreased ability of an organism to survive an oxidative cellular insult. The hypoxia mediated changes in glutathione ratios, i.e. decreased GSSG and increased GSH, may provide a more favourable blood glutathione environment for the acquiesce of exercised induced oxidative stress (Ilhan et al., 2004).

However, as discussed previously (sections 2.7.3, 2.7.5 and 2.7.5.1), the TBARS assay has several fundamental issues regarding specificity and although recognised as an inexpensive and simple bio-marker of oxidative stress, TBARS, as discussed, does have some technical problems and these must be considered when interpreting the literature and any experimental findings (Powers et al., 2010b). The technical limitations of TBARS were sort to be minimised via the inclusion of whole blood glutathione measures, which, is in line with recent recommendations to assess more than one bio-measure of oxidative stress when examining exercise induced changes in redox balance (Powers et al., 2010b). Despite these efforts, future work should employ measures of protein oxidation (e.g. protein carbonyls); in order to more securely investigate the relationship between hypoxia mediated disruptions in redox balance and *mHSP72* expression. Such measures where beyond the budget for the present study. Other

limitations include the lack of a control condition (for reasons discussed in section 8.1) and addition of such a condition is recommended for future studies.

Summary HSP32, HSP72 and glutathione

The combination of increased bio available *mHSP32* and *mHSP72* prior to exercise commencing in EXB2 compared to EXB1 may acquiesce the disturbance to redox balance during the second, physiologically identical exercise bout. Furthermore, the favourable alterations in whole blood glutathione redox balance, before commencement of EXB2 compared to EXB1 (i.e. a reduction GSSG and increase in GSH), may, in tandem with elevated basal stress protein levels (*mHSP32* and *mHSP72*) or independently potentially augment the body's ability to deal with exercise induced disturbances to redox balance. This highlights a current topic of interest within the literature, whereby, maximising oxidative stress is viewed as a positive physiological cascade to procure maximal physiological adaptation (Powers et al., 2010a; Little and Cochran, 2011). However, using a practical example, within a competitive season, i.e. a soccer season, limitation of the disturbance to redox balance from a match may be desirable, as this exercise induced disturbance in redox balance is known to limit the time taken for full recovery (Ascensao et al., 2007; Ascensao et al., 2008; Ascensao et al., 2011), and thus, reduction in such disturbances may ensure players return to optimal performance with the lowest decrement in repeat performance. This dichotomy in the physiological *in vivo* effects of oxidative stress is a point of interest within the current literature and has been addressed in recent reviews (Powers et al., 2005; Jackson et al., 2007; Powers et al., 2007; Jackson, 2008; Powers and Jackson, 2008; Nikolaidis and Jamurtas, 2009; Powers et al., 2010a; Little and Cochran, 2011).

The present study brings to fruition a series of experimental studies (chapters 4 to 9) investigating the potential of hypoxia mediated changes in basal stress protein

expression to convey tolerance of sub-maximal aerobic exercise induced disturbances to redox balance. To my knowledge, this is the first human *in vivo* study to demonstrate prior hypoxia mediated elevation/induction of basal *mHSP32* and *mHSP72* and favourable adaptations in whole blood glutathione redox conferring tolerance to sub-maximal aerobic exercise induced disturbances to redox balance. However, the studies presented do not establish a clear cause and effect relationship between hypoxia mediated stress protein expression and protection to exercise induced disturbances to redox balance. Future work should investigate the blood and muscle response in cohort and examine a wider range of oxidative (protein carbonyls) and stress protein parameters both *in vivo* and *in vitro*, in an attempt to establish clear cause effect evidence for the novel findings presented within this thesis.

Chapter 10. General Discussion and Conclusions

10.1 General Discussion

To draw this thesis to a close it is pertinent to revisit the experimental goals set in the general introduction section. These objectives are reiterated below with a statement regarding how such goals have, or have not, been satisfied.

Experiment 1

i) Investigate the basal expression of *mHSP72* during a 24 h period at 4 h intervals.

ii) Influence and/or relationship of core temperature on basal *mHSP72* expression to be assessed.

- *Basal mHSP72 expression was shown follow a diurnal variation which moderately correlates to* T_c *over a 24 h period, additionally, during waking hours (0900 – 2100) a quadratic trend in expression was evident.*

Experiment 2

i) Investigate the repeatability of any circadian/diurnal trend in basal *mHSP72* expression.

- *The quadratic trend in basal mHSP72 expression was shown to be reproducible on three separate days.*

ii) Compare iHSP72 and eHSP72 expression sensitivity.

- *Assessment of eHSP72 expression (ELISA method) was shown to be a relatively insensitive measure of basal HSP72 expression with large inter-subject variation, in comparison to mHSP72 (iHSP72) assessed by flow cytometry.*

Experiment 3

i) Investigate the effect of hyperbaric HA and HBO on basal *mHSP72* expression.

- *Administration of HA and HBO elicited decreases in basal mHSP72 expression in comparison to the control condition (control condition demonstrated the quadratic trend in mHSP72 expression).*

ii) Explore whether any such hyperbaric stress mediated changes in *mHSP72* may have a relationship with alterations in redox balance.

- *No significant differences in oxidative stress (plasma TBARS) were seen post hyperbaric exposures and thus may not influence hyperbaria mediated changes in mHSP72 expression.*

Experiment 4

i) Investigate the effect of acute hypoxia on basal *mHSP72* expression.

- *Acute hypoxia (75 min, 2980 m) induced significantly elevated mHSP72 expression in comparison to the control condition, at all time points post intervention. Additionally, no quadratic trend in mHSP72 expression was evident within the hypoxic condition, yet, was evident within the control condition.*

ii) Explore whether any such acute hypoxia mediated changes in *mHSP72* may have a relationship with alterations in redox balance.

- *The acute hypoxic exposure (75 min, 2980 m) elicited significant increases in plasma TBARS compared to control immediately and 3 h post intervention. The disturbance to redox balance, was postulated to represent a stimulus for the elevations in mHSP72 seen at the same time points.*

Experiment 5

i) Investigate the effect of once daily hypoxia for ten consecutive days on basal *mHSP72* expression.

- *Once daily hypoxia (75 min, 2980 m) elicited day on day increases in mHSP72 expression which was proportional to the basal content pre exposure. A biphasic response was seen, with 30% day-on-day increases up to day 5, whereas, average increases between days 5 and 10 are approximately 50% lower at 16% day-to-day.*

ii) Explore whether any such daily hypoxia mediated changes in *mHSP72* may have a relationship with alterations in redox balance.

- *The same significant increase in daily oxidative stress, as determined by plasma TBARS, reaching approximately the same value after each hypoxic exposure was caused by administration of once daily acute hypoxia. The daily disturbances to redox balance were postulated as a stimulus for the biphasic day on day increases in mHSP72.*

iii) Examine whether once daily hypoxia for ten consecutive days effects maximal oxygen consumption.

- *The hypoxic acclimation period failed to significantly augment any of the parameters associated with maximal oxygen consumption.*

iv) Examine the EPO response to once daily hypoxia for ten consecutive days.

- *EPO expression was significantly elevated compared to baseline values from 24 h onwards within the 10 day acclimation period. This zenith in expression was maintained across the experimental period, with EPO values returning to those approximate to baseline upon cessation of the acclimation period.*

<u>Experiment 6</u>

i) Investigate the effect of once daily hypoxia for five consecutive days on basal *mHSP32* expression.

- *Basal mHSP32 expression was shown to be significantly increased post hypoxic acclimation period compared to baseline values, with this increase still evident prior to commencement of EXB2.*

ii) Investigate the effect of prior induction of *mHSP32* and *mHSP72* on the exercise induced stress protein response.

- *The well cited submaximal aerobic exercise induced stress protein response (as demonstrated in EXB1) was absent within EXB2, likely due to the hypoxia mediated increases in basal mHSP32 and mHSP72. This prior induction likely conveys conferred cellular tolerance to the stress of EXB2 compared to the identical exercise stress experienced in EXB1.*

iii) Investigate the effect of prior induction of *mHSP32* and *mHSP72* on exercise induced disturbances in redox balance.

- *The typical submaximal aerobic exercise induced disturbances to redox balance (increases in GSSG, decreases in GSH and no change in TGSH) are ameliorated post hypoxic acclimation, with, no changes seen in glutathione ratios in response to EXB2 compared to those typical responses seen in EXB1. This is likely due to a decrease in basal GSSG and increase in GSH post acclimation period compared to control values. Additionally, the prior induction of the potently antioxidant mHSP32 and highly stress responsive mHSP72 may contribute to increased cellular tolerance to exercise mediated disruptions to redox balance.*

The aim of this thesis was to investigate and establish any *in vivo* circadian or diurnal variation in *mHSP72* expression and whether such trends could be disrupted by a non-lethal environmental exposure (hypoxia or hyperbaria). Such disruption, specifically an increase in basal *mHSP72*, was to be refined into a novel *in vivo* protocol to induce such elevations without the well cited use of a thermal (McClung et al., 2008) or mechanical (Vissing et al., 2009) stimulus. Establishment of such a protocol was to then be used in an attempt to confer tolerance to exercise stress, specifically the oxidative stress associated with sub-maximal aerobic exercise (Fisher-Wellman and Bloomer, 2009). Therefore, the purpose of this final chapter was to integrate findings from the experimental studies in the thesis to form an overall conclusion of the research conducted, as well as discussing potential limitations and directions for future work.

Limited flawed investigation had been conducted into the circadian and diurnal variation in eHSP72 expression *in vivo* (Fehrenbach et al., 2005; Fortes and Whitham, 2009), with no such attempts made in regard to iHSP72. Both previous attempts, with regards to eHSP72, incorporated limited subject numbers (n=10) compared to those used in experimental chapter 1 (n=18), and utilised the problematic eHSP72 ELISA method (Fehrenbach et al., 2005; Fortes and Whitham, 2009), with these experimental design and technical limitations discussed in sections 2.1.6, 2.5.2.5, 2.5.2.6 and 3.5. With respect to experimental chapter 1, it was demonstrated that basal *mHSP72* expression was moderately correlated to T_c over a 24 h experimental period. The minimal fluctuations of T_c (0.6^{o}C) throughout the 24 h experimental period demonstrate that *mHSP72* is highly sensitive to changes in T_c, as demonstrated by the moderate correlation of *mHSP72* to T_c. Notably, during "waking" hours (0900 – 2100) basal *mHSP72* demonstrated a quadratic trend in expression.

This quadratic trend in basal *mHSP72* expression was then demonstrated to be repeatable on three separate occasions in experimental chapter 2, and was evident in the

control values in experimental chapters 3 and 4. This sensitivity of *mHSP72* to changes in T_c, and during the course of a 24 h period at rest, has implications for all *in vivo* experimental research examining *mHSP72* in response to a stressor. Specifically, due to the influence basal values (which are shown to fluctuate over a 24 h period at rest) have on the magnitude of post stressor *mHSP72* expression *in vivo* (McClung et al., 2008) and *in vitro* (Vince et al., 2010). Previous work within the area of exercise mediated changes in HSP72 expression, including that of *mHSP72*, has not sufficiently controlled for changes in basal *mHSP72* attributable to the circadian variation in basal expression shown in experimental chapter 1. Therefore, to improve the methodological integrity of future research, one recommendation which could be made from the results of the present thesis would be to standardise the time an intervention is administered during experimental procedures across conditions. It is likely there is no "best" time for administrating an intervention, the pertinent point is that a consistent time of administration of an intervention should be adhered to, to aid inter- and intra-study comparisons with regards to *in vivo* stressor mediated changes in iHSP72. Furthermore, the importance of controlling T_c before *in vivo* investigation of stressor mediated changes in *mHSP72*, for example before exercise, would also be important, as *mHSP72* is sensitive to small changes in T_c at rest (experimental chapter 1). It could be said that individual differences in T_c before commencement of an exercise bout may alter basal *mHSP72* levels, with different basal values in *mHSP72* known to influence the magnitude of post stressor *mHSP72* response *in vitro* and *in vivo*. Considerations of these factors (basal values, circadian variation in *mHSP72* and sensitivity of *mHSP72* to changes in T_c) would improve the reliability of the research methods employed to measure *mHSP72 in vivo* in response to various stressors. Such considerations would ensure that differential *mHSP72* responses to various stressors are not attributable to the

variations in basal values due to the circadian rhythm (incorporating the quadratic trend) shown in experimental chapters 1 and 2, or due to intra-subject differences in T_c.

With the repeatability of this quadratic trend during "waking" hours (experimental chapters 1 and 2) now shown and the basal expression of *mHSP72* better understood, the question of whether a non-lethal environmental stressor could disrupt such basal expression *in vivo* could now be investigated, specifically, whether such an environmental exposure could increase the basal content of *mHSP72*. The first attempt to achieve such elevations with HBO and HA administration actually elicited a decrease in basal *mHSP72* compared to control conditions (experimental chapter 3). Previously, hyperbaria had been shown to increase eHSP72 expression post exposure (Dennog et al., 1999), however, this project did not use healthy subjects and utilised the questionable eHSP72 ELISA method. Therefore, in future, when investigating the HSP72 response to hyperbaria it could be recommended to investigate iHSP72, specifically *mHSP72*, as it may better represent the stress protein response to *in vivo* hyperbaria compared to eHSP72 (as discussed in sections 2.5 and 3.5). Despite the failure of hyperbaria to increases basal *mHSP72*, the observed decreases may be of benefit for *in vivo* models of exercise heat acclimation. Specifically, as lower basal values are correlated to increased post stressor expression of *mHSP72* both *in vitro* (Vince et al., 2010) and *in vivo* (McClung et al., 2008), and as exercise heat acclimation increases iHSP72 (McClung et al., 2008; Amorim et al., 2010; Magalhães et al., 2010), the use of a prior exposure to hyperbaria may augment the *mHSP72* response seen to the hyperthermic exercise bouts employed by lowering basal *mHSP72* and consequently increasing the magnitude of post stressor *mHSP72* expression. Postulations such as these require further extensive *in vivo* examination.

Due to the failure of HBO and HA to increase basal *mHSP72*, the stimulus of hypoxia was sought as a surrogate to the thermal (McClung et al., 2008; Amorim et al., 2010;

Magalhães et al., 2010) and mechanical (Paulsen et al., 2007; Paulsen et al., 2009; Vissing et al., 2009) stressors relied upon previously by others to increase basal HSP72 expression. Increases in HSP72 concentration have been shown *in vivo* in response to hypoxia (Das et al., 1995), with *in vitro* evidence of oxidative stress being the stimulus for such responses (Currie et al., 1988; Mocanu et al., 1993; Chirico et al., 1998; Ahn and Thiele, 2003). Experimental chapter 4 demonstrated an acute hypoxic exposure (2980 m, 75 min) was successful in elevating basal *mHSP72* compared to control, with the elevations seen in tandem with significant increases in oxidative stress (plasma TBARS) post exposure. This increase in both oxidative stress and *mHSP72* supports *in vitro* evidence that oxidative stress may be a stimulus for HSP72 expression (Currie et al., 1988; Mocanu et al., 1993; Chirico et al., 1998; Ahn and Thiele, 2003). These findings provide evidence that hypoxia may be a stimulus to increase basal *mHSP72 in vivo*, in line with other stressors such as thermal (McClung et al., 2008) and mechanical stress (Vissing et al., 2009). Recent interest in the literature has focused upon exercise induced oxidative stress being a conveyer of hormesis for augmented athletic performance (Radak et al., 2008; Fisher-Wellman and Bloomer, 2009), in addition to its involvement in successful ageing (Radak et al., 2005; Ji et al., 2006; Ji et al., 2010) and mental well being (Gomez-Pinilla, 2008). The acute hypoxic protocol used here may be a suitable substitute for exercise, with respect to inducing *in vivo* oxidative stress. This application would be particularly suitable for those populations, i.e. the infirm and/or elderly etc, who cannot perform exercise. Additionally, as shown extensively *in vitro*, high cellular levels of HSP72 provide cytoprotection to further sub-lethal stressors (Garramone et al., 1994; McArdle et al., 2004), including exercise and thermal stress (Garramone et al., 1994; Shima et al., 2008). Furthermore, similar *in vivo* findings have shown increased iHSP72 to represent acquired thermal tolerance (McClung et al., 2008; Amorim et al., 2010; Magalhães et al., 2010) in exercising humans. This hypoxic

exposure here may provide a novel *in vivo* mechanism for increasing basal *mHSP72* without the prior reliance on thermal (McClung et al., 2008) or mechanical stress (Vissing et al., 2009). However, these postulations require extensive *in vivo* investigation before claims of cytoprotection, tissue-level protection or other protective acclimation process can be attributed to the *in vivo* administration of hypoxia.

The success of acute hypoxia to elicit such elevations in *mHSP72* (experimental chapter 4) was then examined under the implementation of ten consecutive days of once daily hypoxia (experimental chapter 5). Exposures administered in this manner produced day-on-day increases in *mHSP72*. Most notably, there was a rapid phase of *mHSP72* accumulation (0 – 5 days), followed by minimal increases in the remaining experimental period (5 – 10 days). The HSP72 expression kinetics seen for acquired thermotolerance, whereby, daily hyperthermic exercise stress elicits sustained increases in *mHSP72*, compared to baseline levels (McClung et al., 2008), mirror those seen here for PHE_R. The similar expression kinetics, may, with further *in vivo* investigation, provide a surrogate stressor (hypoxia) compared to those previously used (mechanical and thermal) to increase basal concentrations of *mHSP72*. The magnitude of such hypoxia mediated increases in *mHSP72* is comparable to that of hyperthermic running exercise (Fehrenbach et al., 2000a; Fehrenbach et al., 2000b; Fehrenbach et al., 2001), though due to the measurement techniques employed by mechanical stimulus projects (northern and western blotting (Vissing et al., 2009), electron and confocal microscopy (Paulsen et al., 2009)) comparisons (hypoxia compared to mechanical) in magnitude of stressor mediated changes in iHSP72 cannot me made. Such increases in *mHSP72* have been shown to be integral to the process of acquired thermal tolerance *in vivo* (Yamada et al., 2007; McClung et al., 2008; Amorim et al., 2010; Magalhães et al., 2010), with *in vitro* increases conveying cytoprotection to related and non-related stressors (Kiang et al., 1996; Shein et al., 2007; Shima et al., 2008). Whether such hypoxia mediated

mHSP72 increases *in vivo* can provide such cytoprotection requires further *in vivo* investigation. These increases in *mHSP72* were correlated to transient increases in plasma TBARS, supporting findings in experimental chapter 4, whereby, hypoxia mediated increases in oxidative stress may provide the stimulus for increases in *mHSP72*, as shown *in vitro*. Furthermore, EPO was significantly elevated from the second day of the exposure throughout the remaining experimental period. Despite these elevations in EPO and *mHSP72* the hypoxic intervention period failed to augment maximal aerobic exercise performance or reduce the disturbance to redox balance of such exercise. This lack of protection to disturbances to redox balance is likely due to the nature of the exercise used, i.e. maximal aerobic exercise. The increased disturbance to redox balance during prolonged sub-maximal aerobic exercise compared to maximal aerobic exercise are well reported (Bloomer and Goldfarb, 2004; Fisher-Wellman and Bloomer, 2009). Therefore, it is likely that any *in vivo mHSP72* mediated resistance to oxidative stress may be seen during prolonged sub-maximal aerobic exercise compared to maximal aerobic exercise. The protocol utilised was successful in increasing basal *mHSP72* expression, without the previous reliance on a thermal (McClung et al., 2008) or mechanical stimulus (Vissing et al., 2009).

Experimental chapter six utilised the initial rapid accumulation of *mHSP72* during the first five days of once daily hypoxic acclimation (demonstrated in experimental chapter 5). Hypoxic exposure in this manner increased basal *mHSP72* and *mHSP32*, which may have contributed towards a reduction in the disturbance to redox balance associated with 60 min cycle ergometery exercise post acclimation period, compared to a physiologically identical exercise bout conducted prior to the hypoxic acclimation period. The combination of increased basal *mHSP72* (Shima et al., 2008) and *mHSP32* (Gozzelino et al., 2010) both exert protective influence to the submaximal exercise mediated disturbances to redox balance, with both known to be inducible under

conditions of elevated oxidative stress (Ahn and Thiele, 2003; Gozzelino et al., 2010). Their ability to negate oxidative stress induced peptide distortion is well cited *in vitro*, with this ability reducing cellular apoptosis and necrosis (Kiang et al., 1998; Gozzelino et al., 2010). Therefore, it is plausible; that the hypoxia mediated prior induction of these stress proteins (*mHSP32* and *mHSP72*) has conferred cellular tolerance to a further non-lethal non related stressor, i.e. prolonged submaximal exercise induced disturbances to redox balance. This notion of conferred cellular tolerance has been demonstrated extensively *in vitro* (Garramone et al., 1994; McArdle et al., 2004). In addition to the findings regarding stress protein expression, the hypoxic exposures elicited favourable adaptations in GSH:GSSG ratios. This increase in basal GSH and decrease in GSSG represents an increase in the antioxidant capacity of the blood (Bloomer and Goldfarb, 2004; Fisher-Wellman and Bloomer, 2009), and as such, may more readily acquiesce the exercise induced disturbances to redox balance of EXB2 compared to EXB1, due to this alteration in glutathione ratios. The findings within experimental chapter six highlight a current topic of interest within the literature with oxidative stress viewed as both a positive physiological occurrence for adaptation (Powers et al., 2010a; Little and Cochran, 2011), yet, also acts negatively when optimal (shortest duration) time to recovery is required post exercise (Alessio et al., 1997; Ascensao et al., 2007; Ascensao et al., 2008; Ascensao et al., 2011). Therefore, any real world athletic application of this protocol requires extensive future *in vivo* testing, particularly in light of the differential roles oxidative stress can play in sporting performance, training and recovery (Powers et al., 2005; Ascensao et al., 2007; Powers et al., 2007; Ascensao et al., 2008; Jackson, 2008; Powers and Jackson, 2008; Powers et al., 2010a; Ascensao et al., 2011; Little and Cochran, 2011).

Experimental chapter six represents the fruition of a series of experimental chapters that have gone some way in establishing a non-thermal non-mechanical hypoxic protocol to

increase basal *mHSP72*. These increases in *mHSP72* and *mHSP32*, in tandem with favourable GSH:GSSG changes, may have serviced the reduction in oxidative stress post exercise within EXB2 compared to EXB1, post hypoxic acclimation period.

10.2 Experimental limitations and future recommendations.

The experimental findings presented within this thesis are novel, however, they have been observed in relatively small sample size groups. Furthermore, within chapters 6, 7, 8 and 9 no sham/control condition was utilised due to logistical and economic experimental constraints (as discussed in respective chapter method sections). This dictates that these novel findings require further and extensive investigation *in vivo*, with expanded subject numbers and the incorporation of a sham condition where applicable. The use of a control/sham condition is highly recommended to be included within future experimental designs akin to those discussed within this thesis.

There is a notable absence of power/sample size calculations within the experimental chapters. This absence was initially (chapters 4 and 5) due to a lack of data/research which had employed the same method of HSP analysis (specific flow cytomertic assay). Subsequent chapters (chapters 6, 7, 8 and 9) again did not employ power/sample calculations due to logistical and economic restrictions (as discussed in respective chapter method sections) dictating the sample sizes employed. The inclusion of retrospective calculations has been considered, but given the widespread criticisms at this approach (Hoenig and Heisey, 2001; Lenth, 2001; Kraemer et al., 2006; Walters, 2009), such calculations have not been included. In line with guidelines for prospective power calculations, these should only be done with specific research questions in place (Biau et al., 2008; Bacchetti, 2010; Dorey, 2011). Therefore, the data presented within the present thesis could be used by other researchers to inform their power/sample calculations, when employing the same assay as used in the experimental chapters

presented here. The use of power/sample calculations is highly recommended to be included within future experimental designs akin to those discussed within this thesis.

Specific experimental limitations are seen by the oxidative measurements (biomarkers of oxidative damage to tissues and cells) employed within the present thesis. As discussed previously (sections 2.7.3, 2.7.5, 2.7.5.1), the TBARS assay has several fundamental issues regarding specificity and although recognised as an inexpensive and simple bio-marker of oxidative stress, TBARS, as discussed, does have some technical problems and these must be considered when interpreting the literature and any experimental findings (Powers et al., 2010b). The technical limitations of TBARS were sort to be minimised via the inclusion of whole blood glutathione measures, which, is in line with recent recommendations to assess more than one bio-measure of oxidative stress when examining exercise induced changes in redox balance (Powers et al., 2010b). Despite these efforts, future work should employ measures of protein oxidation (e.g. protein carbonyls and DNA damage); in order to more securely investigate the relationship between hypoxia/exercise mediated disruptions in redox balance and *mHSP72* expression. Such measures where beyond the budget for the present study and thesis in general. The use of other (e.g. protein carbonyls and DNA damage) biomarkers of oxidative stress, in addition to, or as a substitute to plasma TBARS and complementary to whole blood glutathione measures, is highly recommended to be included within future experimental designs akin to those discussed within this thesis.

Furthermore, the investigation of further members of the stress protein family in addition to HSP32 and HSP72 would provide greater insight into the broader *in vivo* stress protein response to environmental stressors. Additionally, the PBMC, muscle and eHSP72 response require secure investigation in cohort in response to environmental and exercise stress. Completion of projects such as these would allow a more global

perspective of whole body physiology at rest, and in response to environmental and exercise stress, to be gained, *in vivo*.

Despite addressing the experimental objectives set (see section 10.1 for summary) and providing some evidence for mechanistic stimuli for environmental/exercise induced permutations to redox balance, stress protein homeostasis and conveyed cellular tolerance. Throughout the thesis it cannot be said that cause and effect has been established. For instance in experimental chapter 1, it cannot be said with certainty that the change in T_c caused the variation seen in *mHSP72*. To establish such cause and effect in this instance, T_c would require clamping (i.e. maintaining a consistent temperature throughout the experimental period) to see if such manipulation would remove the variation seen in *mHSP72*. Additionally, the limitations of the bio-markers of oxidative stress used make cause and effect statements regarding conveyed cellular tolerance, redox balance originating stimuli for elevated HSP expression and hypoxia mediated alterations to redox balance difficult to make with absolute certainty. Therefore, future work (experimental objectives) should focus on providing such evidence for cause and effect.

10.3 Future experimental objectives

There is evidently a plethora of potential research studies, experimental designs and experimental objectives that are warranted in response to the novel findings presented within this thesis. This section will pose some exemplar research studies that are viewed as a priority given the data presented in the present thesis.

1) A well controlled (in line with recommendations provided throughout this thesis and section 10.2, e.g. power calculations, control groups, etc) *in vivo* project analysing PBMC, muscle and serum/plasma based HSP responses to exercise (running exercise recommended) which utilises a variety (whole blood glutathione, protein carbonyls,

DNA damage, etc) of bio-markers of oxidative damage to tissues and cells (i.e. disturbances to redox balance). Incorporated into this experimental design (similarly to experimental chapter 6 in the present thesis) could be a five day hypoxic acclimation period, with exercise pre and post acclimation period. Such a research study would further inform the novel data presented within experimental chapter 6.

2) Following *in vivo* hypoxic acclimation, PBMCs could be isolated by density gradient centrifugation (in line with (Vince et al., 2011)). Monocytes could then be exposed to *in vitro* oxidative and thermal stressors. The hypoxia preconditioned cells response could then be compared to control PBMC (no hypoxic acclimation period before isolation) and the HSP and redox balances disturbances could be assessed in line with recommendations made in example one. Such a research study could provide cause and effect evidence to support the novel findings presented within experimental chapters 4, 5 and 6, principally those disturbances to redox balance are or are not a stimulus for increases stress protein expression. Additionally, this would also provide cause and effect evidence whether hypoxic acclimation does provide conveyed cellular tolerance to disturbances in redox balance.

10.4 Conclusions

In conclusion, it is evident that basal *mHSP72* follows a diurnal circadian trend in expression, with a quadratic trend in expression observable during waking hours. The quadratic trend in expression can be interrupted by acute hyperbaria (decreased) and hypoxia (increased). Once daily hypoxic exposures (ten consecutive days) produced day-on-day increases in *mHSP72*, which did not convey any protection to disturbances to redox balance from maximal aerobic exercise, nor did the hypoxic stimulus itself elicit any performance benefits to maximal aerobic exercise. However, when a similar five day acclimation period was utilised in light of sub-maximal exercise, a significant

reduction in the disturbance to redox balance, from the exercise bout, was evident. These findings may have applications during a competitive sport season, for example soccer season, whereby, limiting the disturbance to redox balance from physical activity may ultimately reduce time taken to return to optimal performance. Such findings do however highlight the current paradox of exercise induced permutations to redox balance and their role in both procurement of maximal physiological adaptation to exercise stress (Powers et al., 2010a; Little and Cochran, 2011) and their negative effect on exercise recovery (Ascensao et al., 2008; Ascensao et al., 2011).

Chapter 11 References

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