THE UNIVERSITY OF HULL

The effects of hydration status on markers of oxidative and cellular stress during prolonged exercise in hyperthermic environments

being a Thesis submitted for the Degree of Doctor of Philosophy

in the University of Hull

by

Angela R. Hillman, MSc

July 2011

Table of Contents

Acknowledgements

The existence of this doctoral thesis is owed in part to many people, both professionally & personally. Professionally I owe the utmost gratitude to my supervisor, Dr. Jason Siegler whose assistance, feedback, support, and expertise have been invaluable. Thank you for putting up with all of my bad luck and for helping motivate me through all of it. Many thanks to Mr. Nigel Mitchell of British Cycling/Team Sky for the thesis concept. Additionally, thanks also to Dr. Rob Robergs and Dr. Lars McNaughton, without whom I would never have ventured to England to embark on this journey. I must also acknowledge the biochemical assistance of Dr. Rebecca Vince, the statistical guidance and just general loveliness of Dr. Adrian Midgley, and the assistance and friendship of the technical staff ("Team Tech") from the Department of Sport, Health and Exercise Science.

Personally, I would like to thank Miss Amanda Reichert whose steadfast friendship across all the miles & proofing of this thesis was a godsend. Also, I owe the utmost gratitude to Miss Bryna Chrismas, Dr. Lee Taylor, Mr. Mark Turner, and Mr. Dan Peart whose advice, assistance, and friendship have been a true blessing. I could never imagine better friends & colleagues!

Most importantly, I'd like to thank my parents, Laurie and Jay for being so supportive and understanding of me and the pursuit of my education, though it has taken me far from home for many years. Your love and support through the many years of academic work have been unwavering and deeply appreciated. I hope that I've made you proud!

Published Work and Work Under Review

Hillman AR, Vince R, Taylor L, McNaughton L, Mitchell N and Siegler J. (2011) Progressive dehydration during prolonged exercise with and without environmental heat stress results in increased oxidative stress. *Appl Physiol, Nutr and Metab*, 36, 698-706.

Hillman AR, Taylor L, Turner, MC, McNaughton L, and Siegler J. (2011) Effects of hydration status on oxidative and cellular stress expression in untrained subjects during normothermic and hyperthermic aerobic exercise. *Journal of Science and Medicine in Sport*, In Review

Hillman AR, Turner MC, Peart DJ, Bray JW, Taylor L, and Siegler J. (2011) A comparison of hyperhydration vs. *ad libitum* fluid intake strategies on measures of thermoregulation and endurance performance. *Applied Physiology, Nutrition and Metabolism*, In Review

List of Figures

List of Tables

List of Abbreviations, Acronyms, and Symbols

˚C	degrees centigrade
BML	body mass loss
BM	body mass
FIP	F_2-Isoprostane
g	gravity
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidised glutathione
GXT	graded exercise test
H_2O_2	hydrogen peroxide
Hb	haemoglobin
Hct	haematocrit
HO-1	inducible heme oxygenase 1(HSP32)
HO-2	heme oxygenase 2
HR	heart rate
HS	heat storage
HSP	heat shock protein
HSP32	heat shock protein 32 (inducible heme oxygenase 1)
HSP70	heat shock protein family 70
HSP72	inducible heat shock protein 72
kg	kilogram
LOOH	lipid hydroperoxide

LT	lactate threshold
MDA	malondialdehyde
mg/dl	milligram per decilitre
ml	millilitre
mM	millimole
mmol/l	millimole per litre
nmol	nanomole
·OH	hydroxyl radical
PBMC	peripheral blood mononuclear cell
PC	protein carbonyl
pmol	picomole
PO	power output
PSI	physiological strain index
PV	plasma volume
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPE	rating of perceived exertion
$O_2^{-}\cdot$	superoxide
SOD	superoxide dismutase
SOsm	serum osmolality
Tb	mean body temperature
TBARS	thiobarbituric acid reactive substances
Tc	core temperature
TFC	total fluid consumed

TGSH	total glutathione (GSSG + GSH)
Ts	skin temperature
TT	time trial
UV	urine volume
UOsm	urine osmolality
µl	microlitre
µg/ml	microgram per millilitre
µmol/l	micromole per litre
VO_{2max}	maximal oxygen consumption
VO_{2peak}	peak oxygen consumption
W	watts
8-OHdG	8-hydroxydeoxyguanosine

Abstract

The relationships between hyperthermia, dehydration and oxidative stress have been thoroughly studied separately within the literature both *in vitro* and *in vivo*. However, no *in vivo* attempts have been made to manipulate the hydration status of individuals to investigate the resulting changes in oxidative and cellular stress during and after exercise in hyperthermic conditions and what effect these changes may have on exercise performance.

The purpose of the first experiment was to investigate the effects of exercise-induced dehydration with and without hyperthermia on oxidative stress. Seven healthy male trained cyclists (mean ± SD) age: 36 ± 6 yrs, height: 177.4 ± 6.5 cm, weight: 72.8 ± 7.0 kg, and power output (PO) at lactate threshold (LT): 199.3 ± 19.0 Watts (W) completed 90 min cycling exercise at 95% LT followed by a 5 km time trial (TT) in four conditions: 1) euhydration in a warm environment (EU-W, control), 2) dehydration in a warm environment (DE-W), 3) euhydration in a thermoneutral environment (EU-T), and 4) dehydration in a thermoneutral environment (DE-T) (W: 33.9 ± 0.9°C; T: 23.0 ± 1.0°C). Whole blood oxidised glutathione (GSSG) increased significantly post exercise in dehydration trials only (DE-W: $p < 0.01$, DE-T: $p = 0.03$), and while not significant, whole blood total glutathione (TGSH) and plasma thiobarbituric acid reactive substances (TBARS) tended to increase post exercise in dehydration trials ($p = 0.08$ for both). Intracellular monocyte heat shock protein 72 (HSP72) concentration was increased ($p = 0.01$) while intracellular lymphocyte HSP32 concentration was decreased for all trials ($p = 0.02$). Exercise-induced dehydration led to an increase in GSSG concentration while maintenance of euhydration attenuated these increases regardless of environmental condition. Additionally, evidence of increased cellular stress (measured via HSP) was

found during all trials independent of body mass loss and environment. Finally, total distance covered during 90 min and PO during both 90 min and 5 km TT performance were reduced during only the DE-W trial, likely a result of combined cellular stress, hyperthermia and dehydration. These findings highlight the importance of fluid consumption during exercise to attenuate thermal and oxidative stress during prolonged exercise in the heat.

The purpose of the second experiment was to investigate the effect of prolonged exercise-induced dehydration with and without hyperthermia on cellular and oxidative stress markers in untrained individuals, to serve as a comparison to the results of the first experimental chapter. Seven untrained male university students (mean ± SD) age: 21 ± 3 yrs, height: 181.1 ± 9.2 cm, weight: 76.8 ± 8.8 kg, and PO at LT 100.0 ± 13.0 W, who were unacclimatised to heat, participated in this study. Subjects completed the same experimental protocol as outlined in experimental chapter one, in warm (33.9 ± 1.0°C) and thermoneutral (22.9 ± 1.0°C) environments. Whole blood GSSG increased an average of 32% ($p < 0.01$) as a result of prolonged exercise, however unlike the trained subjects of experiment one, there was no effect of body mass loss on GSSG ($p = 0.63$). Similarly, intracellular monocyte HSP72 concentration increased 14% ($p < 0.01$) as a result of prolonged cycling regardless of body mass loss and environmental heat stress, analogous to subjects in experiment one. While there were no significant changes as a result of hydration or environment, a relationship was found between GSSG concentration and body mass loss ($r^2 = 0.5$, $p = 0.05$), while HSP72 was correlated with body temperature and levels of heat storage ($r^2 = 0.5$, $p = 0.01$). Similar to the trained individuals in experiment one, PO during the 90 min (7%, $p < 0.01$) and TT (14%, $p < 0.01$) were decreased while thermoregulation

was impaired during DE-W only. These results demonstrate the increased level of stress in untrained subjects as a result of exercise and highlight the importance of participation in recommended physical activity to aid in positive cellular adaptations leading to superior antioxidant defences to aid in disease prevention.

In light of the findings from the first experimental chapter that dehydration can significantly influence oxidative stress in trained subjects, the purpose of the third experimental chapter was to compare pre-exercise hyperhydration with plain water (W) or water with glycerol (G) to no hyperhydration (C) on markers of oxidative stress prior to and after a 90 min TT. Seven trained male cyclists and triathletes (age: 28 ± 8 yrs, height: 178.4 ± 7.8 cm, and mass: 73.2 ± 9.6 kg) covered as much distance as possible during a 90 min cycle after G, W or C. Blood was collected pre ingestion (PRE), post ingestion/pre exercise (PI), immediately post exercise (PE) and 1 hour post exercise (1HR) and analysed for whole blood TGSH, GSSG, and plasma levels of lipid hydroperoxides (LOOH) and protein carbonyls (PC). TGSH concentration increased post exercise in W and C ($p < 0.01$) while PC concentration increased post exercise during C only ($p = 0.03$). Additionally, GSSG concentration was greater PI and PE in C compared to G ($p = 0.05$, and $p < 0.01$, respectively), likely due to the inferior amount of fluid retained during C compared to the G and W trials. Therefore, it appears that both pre exercise hyperhydration with *ad libitum* fluid ingestion during exercise is sufficient to attenuate rises in exercise-induced oxidative stress.

The novel findings presented in this thesis indicate fluid ingestion plays a vital role in providing cellular protection from oxidative stress. These results suggest that individuals

participating in prolonged exercise should consume adequate fluid during exercise to avoid dehydration, matching fluid intake with body mass loss. Additionally, individuals who wish to hyperhydrate prior to exercise may enhance their ability to delay dehydration and thus enhance their cellular protection from oxidative stress.

Chapter 1. General Introduction

General Introduction

Exercising for prolonged periods can lead to dehydration (Sawka et al., 2007) and oxidative stress (Steinberg et al., 2006), both of which are believed to have a negative impact upon performance. Many researchers have reported that as little as a 1-2% reduction in body mass can negatively impact exercise performance (Cheuvront et al., 2003) and cognitive function (Maughan, 2003), however others have found as much as 7% can be tolerated (Sharwood et al., 2004). Dehydration leads to a decrease in plasma (PV) and total blood volume (Coyle and Gonzalez-Alonso, 2001), a decrease in stroke volume and an increase in heart rate (HR) (Gonzalez-Alonso et al., 2000) as well as increased core temperature (Tc) (Montain and Coyle, 1992) and decreased time to fatigue (Montain et al., 1998).

On a cellular level, dehydration causes hyperosmolality and cellular shrinkage which can lead to insulin resistance, catabolism, and increased cellular susceptibility to oxidative damage and apoptosis (Schliess and Haussinger, 2002). Oxidative stress, resulting from the production of free radicals can also lead to apoptosis (Mattson, 2006) as well as oxidation of lipids, proteins, and DNA (Powers and Jackson, 2008). Accumulation of oxidised proteins and DNA leads to failure of normal cell function (Fisher-Wellman and Bloomer, 2009) which in turn causes an inflammatory response and cell death (Mattson, 2006). While dehydration impairs cellular function, hyperhydration and hypoosmotic swelling has been shown to protect cells from heat and oxidative stress (Schliess and Haussinger, 2002) and attenuate DNA damage (Martins and Meneghini, 1994). Newly emerging research has shown that fluid intake during recovery from dehydration can attenuate oxidative stress (Paik et al., 2009). The authors found a significant increase in malondialdehyde (MDA), a by-product of lipid peroxidation during dehydration trials that was significantly attenuated

with rehydration, demonstrating that adequate hydration can counteract oxidative damage. This may be important with regards to pre-exercise hydration status and the ability of euhydration to attenuate oxidative stress.

Cellular protection from free radicals comes from endogenous and exogenous antioxidant systems. The foremost endogenous non-enzymatic antioxidant in the body is glutathione. During normal resting conditions glutathione is found in the reduced (GSH) state. During exercise, in order to combat peroxidation, GSH is oxidised by glutathione peroxidase (GPx) to GSSG and then reduced back by glutathione reductase (GR). This cycle is continuous, with the goal of maintaining the levels of GSH in order to counter the oxidative stress. During times of high oxidative stress, increased amounts of GSSG and decreased amounts of GSH will be found with no change in total glutathione (TGSH) (Laaksonen et al., 1999, Sen et al., 1994b). Further cellular protection is provided by heat shock proteins (HSP), ubiquitous stress molecules expressed both extra- and intra-cellularly to aid in response to heat stress, oxidative stress, or other forms of cellular damage (Kalmar and Greensmith, 2009). There are numerous families of HSP; of particular interest are HSP 70 and HSP 32. HSP 70 aids in the clearance of damaged proteins and refolding of denatured proteins, as well as saving cells from activated death pathways (Kalmar and Greensmith, 2009). The effects of hydration status on HSP expression have not been thoroughly studied. However, in human hepatocytes it has been shown that moderate hyperosmolality, as a result of dehydration, blocks the heat induced expression of HSP 70, causing impaired protein synthesis and thermotolerance (Kurz et al., 1998). Additionally, while dehydration can block HSP 70 induction, hyperhydration and the ensuing hypoosmotic swelling has been

shown to increase HSP 70 induction (Kurz et al., 1998). Therefore it is clear how important hydration status can be to thermotolerance and cellular protection via HSP 70 expression.

HSP 32 is expressed in a wide range of tissues as a result of oxidative stress (Otterbein and Choi, 2000) and provides anti-inflammatory and anti-apoptic cellular protection (Kalmar and Greensmith, 2009). HSP 32 has not been studied widely in exercising subjects and no data exist linking HSP 32 expression to hydration status. Since HSP 32 is induced as a result of oxidative stress, it is inferred that dehydration would result in increased expression of HSP 32.

With the emerging evidence that dehydration and hyperthermia can result in cellular damage via oxidative stress, and the body's ability to maintain cellular function during states of euhydration, it is important to understand the relationship of hydration status on oxidative stress. The findings of the proposed research will aid in our understanding of the potential effect of hydration and training status on oxidative stress during prolonged exercise in extreme environmental conditions.

Chapter 2. Review of Literature

Chapter 2: Review of Literature

Because the topic of the current thesis is rather novel and no research exists linking all aspects of the work herein, it was difficult to decide how to lay out the literature review. However, because the main focus of this thesis is oxidative stress, this topic will be thoroughly reviewed first, including the mechanisms of oxidative stress and resulting damage during varying durations of exercise. This will be followed by a review of heat shock proteins, which are intrinsically linked with both oxidative stress and hyperthermia. Next the physiological changes that occur as a result of exercise in hyperthermic environments, including the resulting dehydration and prevention of dehydration and hyperthermia will be discussed. Finally, the last section of this review will focus on detailing the limited research to date linking oxidative stress, dehydration and hyperthermia, demonstrating the rationale for the following research.

2.1. Oxidative Stress

Oxidative stress results from an increased production of pro-oxidant radicals (ROS) and a concomitant failure of the body's antioxidant defences to neutralise these threats (Halliwell and Gutteridge, 2007). During resting conditions there is a delicate balance between pro- and anti-oxidants that aid in the body's maintenance of homeostasis (Halliwell and Gutteridge, 2007). Exercise tips the cellular environment towards the pro-oxidant as a result of increased oxygen uptake and cellular metabolism, resulting in greater ROS production (Halliwell and Gutteridge, 2007). If not neutralised, the ROS produced can lead to lipid and protein peroxidation as well as DNA oxidation.

Oxidative stress is highly correlated with many human conditions, which in turn is highly correlated with lifestyle characteristics, including aerobic fitness. Many of these conditions result from failures in the antioxidant defence system. For example, basal levels of oxidative stress are greater in the sedentary population compared to their trained counterparts (higher production of ROS and a decreased ability to neutralise the radicals) due to a sedentary lifestyle (Alessio, 1993, Miyazaki et al., 2001). Similarly, during exercise in sedentary individuals, markers of lipid peroxidation, protein oxidation (Falone et al., 2010), and DNA damage (Niess et al., 1996) are increased, while antioxidant activity (such as the glutathione system) is decreased (Douris et al., 2009, Falone et al., 2010) compared to trained individuals. This increase in ROS production and oxidative stress puts sedentary and untrained individuals at a higher risk for disease development (Ceriello and Motz, 2004).

While exercise-induced oxidative stress has long been regarded as detrimental to exercise performance and health, current understanding of the paradigm has revealed a hormetic effect of exercise-induced oxidative stress. It is believed that the stress incurred as a result of a mild bout of exercise infers favourable adaptations and protection for subsequent, more severe bouts of exercise or other stress (Ji et al., 2010). Additionally, the ROS produced as a result of exercise and metabolism potentially serve as important cellular signalling mechanisms (Powers et al., 2010) and neutralising these ROS with exogenous antioxidants can potentially cause as much damage as the radicals themselves (Powers and Jackson, 2008).

It is well documented that ROS are continuously produced as a result of cellular metabolism. The most biologically active ROS produced by muscles are superoxide ($O_2^{-}\cdot$) and hydrogen peroxide (H_2O_2), while other major reactive species in cells include the hydroxyl radical (·OH), nitric oxide (NO) and peroxynitrite ($ONOO^-$). The production of these radicals and their ability to cause damage can be attenuated by various endogenous and exogenous antioxidant defence systems, as well as exercise training. A brief description of these radicals is detailed below, indicating where the radical is produced and how it reacts with cellular components, resulting in further radical production and possibly cellular damage (*Figure 2.1*).

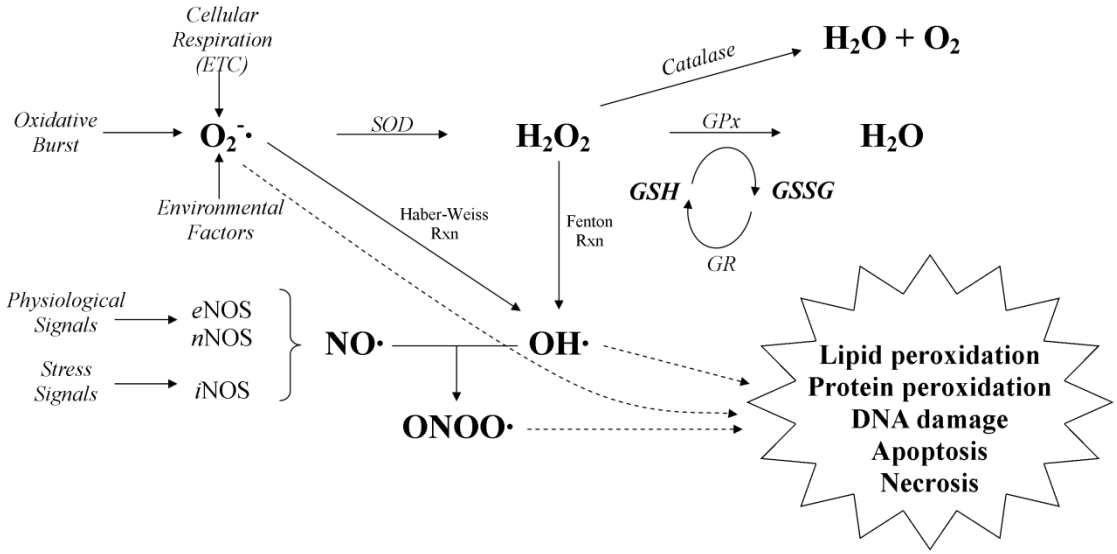

Figure 2.1. Production of reactive oxygen (ROS) and nitrogen (RONS) species and the role of endogenous antioxidants. Superoxide ($O_2^{-}\cdot$) which is formed under conditions of increased oxygen demand and metabolism is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) within mitochondria. H_2O_2 can then be rapidly removed by glutathione peroxidase (GPx), which is most commonly found in red blood cells or by catalase, which is found exclusively in peroxisomes. If not neutralised by antioxidants, $O_2^{-}\cdot$ and H_2O_2 can form the hydroxyl radical (OH·) via the Haber-Weiss and Fenton reactions, respectively. OH· is highly reactive and will combine with un-neutralised nitric oxide radicals (NO·) to form the powerful oxidant peroxynitrite (ONOO·). If left un-quenched, OH·, NO·, and ONOO· can result in lipid peroxidation, protein peroxidation, DNA damage, and cell death via apoptosis and necrosis. Adapted from Newsholme et al. (2011).

Superoxide ($O_2^{-}\cdot$)

Superoxide ($O_2^{-}\cdot$) is an anion that is formed as an intermediate in biochemical reactions. It is relatively membrane impermeable, but has a relatively long half life, which allows for diffusion within the cell (Powers and Jackson, 2008). Although superoxide is generally considered unreactive compared with other radicals, it can react with nitrous oxide and may possibly become protonated to produce hydroperoxyl radical (HO_2), which may allow for the transfer of superoxide across membranes (Powers & Jackson, 2008). Superoxide can act to reduce biomaterials such as cytochrome C, or oxidise others such as ascorbate and still yet, can be dismutated by superoxide dismutase (SOD) which provides a major source of another ROS, hydrogen peroxide, in the cells (Powers and Jackson, 2008).

Mitochondria have been widely believed to be the dominant source of superoxide, with the major sites of generation mainly due to a "leak" between complexes I and II of the electron transport chain (ETC) (Muller et al., 2004), specifically in type II fibres, which leak two to three times more ROS than type I fibres (Anderson and Neufer, 2006). Early researchers concluded that superoxide is produced at rates greater than 50 or 100 fold during aerobic exercise and that 2-5% of oxygen consumed was reduced to form superoxide (Boveris and Chance, 1973). However, recent research suggests mitochondria may not be the dominant source of ROS during exercise (Jackson et al., 2007) and the estimated amount of 2-5% oxygen consumption to superoxide formation is actually only increased 2 to 4 fold during exercise (St-Pierre et al., 2002), much lower than originally estimated.

Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is formed from the dismutation of superoxide by SOD as well as a number of enzyme systems (Powers and Jackson, 2008), by microsomes and peroxisomes (Droge, 2002), and xanthine oxidase (Sachdev and Davies, 2008). There is also good evidence for the generation of H_2O_2 directly by the mitochondria (Sachdev and Davies, 2008), but this has yet to be substantiated. H_2O_2 is stable and has a relatively long half life (Powers and Jackson, 2008). The cytotoxicity of H_2O_2 is mainly in its ability to form the hydroxyl radical. While H_2O_2 is unable to oxidise DNA or lipids directly (Powers and Jackson, 2008), it can inactivate some enzymes, usually by oxidation of essential thiol groups (Halliwell and Gutteridge, 2007). Exposure of cells to large doses of H_2O_2 can cause inhibition of glycolysis by oxidising glyceraldehyde-3-phosphate dehydrogenase resulting in ATP depletion (Halliwell and Gutteridge, 2007).

Hydroxyl Radicals (OH·)

Hydroxyl radicals (OH·) are highly reactive with a strong oxidising potential (Powers and Jackson, 2008) and will react with almost every type of molecule found in living cells including sugars, amino acids, phospholipids, nucleotides, and organic acids (Halliwell and Gutteridge, 2007). Reactions involving OH· illustrate an important principle in radical chemistry: reactions of a free radical with a non-radical species produces a different free radical, which may be more or less reactive than the original radical (Halliwell and Gutteridge, 2007). Radicals produced by reactions with OH· are usually less reactive because OH· is such an aggressive species (Halliwell and Gutteridge, 2007). OH· radicals damage molecules close to their site of generation (Powers and Jackson, 2008) and their reactivity is such that it is virtually impossible to prove their existence other than by

detecting the presence of products of their reactions. Because they are so highly reactive, OH· radicals are not membrane permeable (Powers and Jackson, 2008). Many of the hydroxyl molecules that are produced come from the metal ion-dependent break-down of hydrogen peroxide (H_2O_2), also known as the Fenton reaction (Cheng et al., 2002). The ·OH has also been postulated to be formed by the interaction between superoxide and nitric oxide (Cheng et al., 2002) and has also been implicated as a mediator of ischaemia/reperfusion injury, as well as playing a direct or indirect role in diseases such as hepatitis, stroke, Parkinson's disease, aging, and Alzheimer's disease (Cheng et al., 2002).

Nitric Oxide (NO)

Nitric oxide (NO) is a major signalling molecule found in neurons and the immune system. It is a small molecule and diffuses rapidly through both water and membranes, allowing it to easily pass from one cell to the next (Brown and Borutaite, 2002). NO is continuously produced by skeletal muscle by nitric oxide synthetases and this production increases with muscular contraction (Balon and Nadler, 1994).

NO itself, at physiological concentrations, is relatively unreactive (Ignarro, 2000), however, it may be converted to a number of reactive nitrogen species. The most reactive of these species being peroxynitrite ($ONOO^-$), which can oxidise/nitrate other molecules or decay to produce other damaging species (Brown and Borutaite, 2002). NO causes cell death by a variety of mechanisms that are still not clearly defined, but include energy-depletion necrosis and oxidant-induced apoptosis (Brown and Borutaite, 2002) as well as DNA damage (Nguyen et al., 1992).

Peroxynitrite ($ONOO^-$)

Peroxynitrite is a powerful oxidant formed by the inevitable reaction of NO and SO (Pacher et al., 2007) and is the primary reaction when both are present (Powers and Jackson, 2008). Although peroxynitrite is a strong oxidant, it reacts at a relatively slow rate and can diffuse through cell membranes (Pacher et al., 2007). Peroxynitrite's limited reactivity makes it a selective oxidant, which increases its influence over biological processes (Pacher et al., 2007). Peroxynitrite mediates the oxidative modifications of lipid cell membranes and lipoproteins (Rubbo et al., 2009). Many biomolecules are oxidised and/or nitrated by $ONOO^-$ including protein tyrosine residues, thiols and unsaturated fatty acids. One very important aspect of peroxynitrite-mediated lipid peroxidation is that during the process, OH and NO radicals are formed which can further initiate lipid peroxidation (Rubbo et al., 2009).

As can be seen from the review of free radicals, the primary target of many radical attacks is on the cellular wall, leading to lipid peroxidation. If the by-products of lipid peroxidation are not neutralised they can lead to further damage of cellular components, including proteins and DNA. Accordingly, the following section will detail the mechanisms and consequences of lipid, protein and DNA damage.

2.1.2. Lipid Peroxidation

Lipid peroxidation causes impairment of membrane functioning and changes in fluidity, which allows proteins within the membrane to be more directly attacked (Halliwell and Chirico, 1993). This attack impairs essential membrane functions through inactivation of membrane-bound receptors and enzymes (Powers and Jackson, 2008) as well as increasing

nonspecific permeability to ions such as calcium (Halliwell and Chirico, 1993) which can lead to cell death (Orrenius et al., 1989). Lipid peroxidation can also contribute to the development of atherosclerotic lesions in blood vessels (Halliwell and Chirico, 1993).

Lipid peroxidation has three major stages including initiation, propagation and termination. Lipid peroxidation is initiated by an attack on a fatty acid or fatty acyl side chain resulting in the abstraction of a hydrogen proton (H^+) (Halliwell and Chirico, 1993). The greater the number of double bonds in the fatty acid, the easier it is to remove an H^+. This is why polyunsaturated fatty acids are more susceptible to free radical attack than monounsaturated fatty acids or saturated fatty acids (Halliwell and Chirico, 1993). Propagation of lipid peroxidation can continue for the conversion of hundreds of fatty acid side chains into lipid hydroperoxides (LOOH) (Halliwell and Chirico, 1993). The length of propagation depends upon many factors including the lipid-protein ratio in the membrane, the fatty acid composition, the oxygen concentration and the presence of a chain-breaking antioxidant (Halliwell and Chirico, 1993). The most important of such chain-breaking antioxidants is α-tocopherol (vitamin E) (Halliwell and Chirico, 1993). Other chain breaking antioxidants include vitamin C (ascorbate) and beta-carotene.

LOOH have been long believed to play a role in human disease (Esterbauer, 1993) and indeed both MDA and 4-hydroxynonenol (4-HNE) formation have been implicated in many disease states such as atherosclerosis (Leonarduzzi et al., 2005), ischaemia (Braughler and Hall, 1992), arthritis (Morquette et al., 2006), diabetes (Slatter et al., 2000) and Alzheimer's (Galasko and Montine, 2010); however the exact influence of LOOH and their decomposition products in human disease still needs further elucidation. One of the

major roles of lipid peroxidation in disease is the potential for MDA and particularly 4-HNE to modify proteins which can provoke immune responses (Dalle-Donne et al., 2006) and inhibit protein and DNA synthesis (Esterbauer et al., 1991). While the literature on the effects of exercise on lipid peroxidation is robust, the results are equivocal and require further elucidation because whilst exercise of varying intensities and durations have been shown to increase or have no effect on lipid peroxidation, there appears to be no pattern.

Measuring lipid peroxidation is usually accomplished by measuring losses of unsaturated fatty acids, amounts of primary peroxidation products and second hand products (Halliwell and Chirico, 1993) because few lipid peroxidation products can be produced in pure form (Esterbauer, 1993). LOOH are produced as the primary by-product of polyunsaturated fatty acid peroxidation (Esterbauer, 1993) and decompose to form unstable end products such as MDA and 4-HNE, conjugated dienes, and isoprostanes, while second hand products include hydrocarbon gases such as pentane or ethane (Urso and Clarkson, 2003).

MDA can be measured by high performance liquid chromatography (HPLC), spectrophotometry, spectroflurescence or the most common method, the thiobarbituric acid (TBARS) assay. Research measuring lipid peroxidation via the TBARS assay is highly equivocal with exercise of varying durations and intensities eliciting both increases and null findings (Atalay et al., 1997, Falone et al., 2010, Groussard et al., 2003, Ilhan et al., 2004, Jammes et al., 2004, Jammes et al., 2009, Laaksonen et al., 1999, Michailidis et al., 2007, Miyazaki et al., 2001, Morillas-Ruiz et al., 2005, Nikolaidis et al., 2006, Rahnama et al., 2007, Sen et al., 1994b, Steinberg et al., 2007, Steinberg et al., 2006, Tanimura et al., 2010, Vider et al., 2001). It should be noted, however, that the use of the TBARS assay has been

criticised by many researchers for being non-specific to MDA (Oh-ishi et al., 2000). Despite the mixed results received using the TBARS assay it is continually used because it is relatively cost effective and easy to conduct.

Lipid Peroxidation and Exercise

The majority of studies investigating the effects of exercise on lipid peroxidation primarily utilise the TBARS assay or direct measurements of MDA and short duration exercise of less than 30 min, such as graded exercise tests (GXT) to exhaustion. Of these studies in untrained individuals, the majority have noted increases in MDA or TBARS post exercise (Ashton et al., 1998, Jammes et al., 2009, Laaksonen et al., 1999, Michailidis et al., 2007, Sen et al., 1992, Steinberg et al., 2007, Steinberg et al., 2006), while few have found no change (Groussard et al., 2003). However, it is interesting to note that although Groussard et al. (2003) did not find significant results immediately post exercise, a significant increase was found after 20 min of recovery time. Investigations utilising trained individuals seem to be equivocal with some authors noting increases post GXT (Falone et al., 2010, Munoz Marin et al., 2010, Vider et al., 2001), while others have found no change as a result of GXT (Alessio et al., 2000, Quindry et al., 2003, Rahnama et al., 2007). Additional investigations utilising LOOH and F_2-Isoprostanes (FIP) also indicate increases post GXT (Ashton et al., 1998) and after a 50 min run (McAnulty et al., 2005).

Few studies have utilised a moderate to prolonged duration protocol (60-90 min) to investigate changes in lipid peroxidation as a result of exercise. Two investigations measuring TBARS have found opposite results, with one noting significant increases from 90 min cycling at ~60% VO_{2max} (Borsheim et al., 1999) and one finding no significant

changes from 90 min cycling at 70% VO_{2max} (Morillas-Ruiz et al., 2005). These opposing findings may result from differences in training status, as Borsheim et al. (1999) utilised untrained participants, noting increased stress, while the trained participants of Morillas-Ruiz et al. (2005) did not have an increase in oxidative stress. Additionally, Rush and Sandford (2003) found no significant changes in LOOH as a result of a 90 min cycle at 50% VO_{2max}, while Quindry et al., (2003) found a similar result when participants ran for 60 min at LT-10%. Due to the limited availability of research utilising moderate duration protocols and the equivocal results from those that do, it is difficult to discern the effects of this exercise duration on lipid peroxidation markers. The two studies that have utilised long duration protocols (>2 hrs) have found a significant increase in MDA as a result of a half or full marathon (Knez et al., 2007) and significant increases in FIP after an ultramarathon (Skenderi et al., 2008). Similarly, a study utilising trained cyclists completing a mountain cycle race also found significantly increased MDA as a result of the 178 km ride (Tauler et al., 2006), which took a mean of 283 min to complete.

Research focusing on the effects of high intensity exercise on lipid peroxidation appears to be equivocal, similar to the effects of aerobic exercise on lipid peroxidation. Bloomer et al., (2005) used cross trained men to investigate the effects of 30 min squatting at 70% one repetition maximum (1RM) on MDA finding no change in MDA content after the exercise. Similarly, these same authors conducted a study using 12 resistance trained men to investigate the effects of sprinting exercise and an extended bout of squatting (40 ± 2 reps). Neither exercise intervention significantly increased MDA concentrations, however, they noted a tendancy for MDA to decrease as a result of both exercise modes (Bloomer et al., 2006). Contrarily, 30 min cycling at LT (Sen et al., 1994b) and sustained contractions of

the thumb abductor muscle at 50% of maximal voluntary contraction have resulted in augmented TBARS and LOOH (Alessio et al., 2000, Steinberg et al., 2006). Differences in these responses may be due to the training status of the subjects, as those studies that found increases in TBARS used high intensity trained individuals, but those that had no significant increases used subjects specifically trained toward the mode of exercise used.

It may be of particular interest to athletic populations to understand how multiple days of exercise can influence lipid peroxidation; however, few studies have been conducted to elucidate this relationship. The first study by Vigue et al. (1993) used untrained subjects cycling 90 min at 65% VO_{2max} for three consecutive days, finding no significant change in LOOH and, in fact, noted a non significant decrease. Similarly, Tanimura et al. (2001) found no significant changes in TBARS concentration from three consecutive days of cycling 60 min at 75% VO_{2max}. However, Shing et al. (2007) found a significant increase in MDA as a result of a single day of interval cycling followed by a 30 km time trial for the two consecutive days after. Additionally, the authors (Shing et al., 2007) noted a significant cumulative effect of the three days of exercise.

Recently, researchers have focused on investigating the impact of exercise training on lipid peroxidation because of the implied health benefits and possible reduction in disease. Few studies have done so, but those that have, found positive benefits of the training (Goto et al., 2003, Miyazaki et al., 2001). Miyazaki et al. (2001) utilised a 12 week training program consisting of a 60 min run at 80% VO_{2max}, five days per week, in nine untrained men. They found that while exercise augmented the concentration of plasma TBARS, this increase was less after the training program. Additionally, the subjects had lower basal TBARS

levels after the training program. Similarly, Goto et al. (2003) used a 12 week training program consisting of either mild, moderate or high intensity exercise for 30 min per day, five to seven days per week, in 26 untrained men (split between the three training loads). The authors noted that mild and moderate exercise training tended to decrease the production of MDA post exercise and that interestingly, the high intensity training resulted in augmented MDA production after the training program. While it appears that training can improve the body's ability to counteract the oxidative load of exercise, high intensity training can hamper this adaptation, therefore exercise should be of a mild intensity. However, because there is little data and these investigations used only MDA rather than multiple markers, it is difficult to make definitive conclusions regarding exercise training and lipid peroxidation.

2.1.3. Protein Oxidation

Protein oxidation by ROS occurs on many amino acids in two main sites: the backbone and side chains. A wide range of different radicals can be formed from the attack of a radical on a protein because of the varied configurations of amino acid side chains which offer up many sites to be attacked (Hawkins and Davies, 2001). A major pathway of protein oxidation is the abstraction of a H^+ by a hydroxyl radical from amino acid residues forming carbon-centred radicals (protein carbonyls (PC)). In addition to proton abstraction, radicals can add electrons, transfer electrons, and rearrange structures leading to the formation of additional radicals. The primary fate of carbon-centred free radicals is to undergo further reactions with oxygen leading to the formation of alkyl, peroxyl, and alkoxyl radicals (Hawkins and Davies, 2001), which can continue the process of protein oxidation by producing more carbon-centred radicals (Stadtman, 2001), which may lead to DNA

damage (Morin et al., 1998). Protein oxidation can ultimately lead to loss of protein function and thus accumulation of oxidised proteins in human tissue, a phenomenon that has been shown to have a linear relationship with age (Stadtman and Berlett, 1997). Accumulation of oxidised protein has also been indicated in many disease states including atherosclerosis, Alzheimer's and Parkinson's diseases, muscular dystrophy, diabetes, and rheumatoid arthritis (Dalle-Donne et al., 2003, Stadtman, 2001) with augmented levels in diseased populations compared to healthy, un-diseased counterparts (Stadtman and Berlett, 1997).

The most commonly used method for assessing protein oxidation is to measure plasma PC content, using assays based on the methods of Levine et al. (1994). The data on the effects of exercise on PC formation is limited and thus the relationship between exercise and PC formation is not fully understood. When increases are found, PC values are elevated immediately post exercise (Bloomer et al., 2007a), peaking at four hours post exercise (Michailidis et al., 2007) and remaining elevated for up to 24 hours post exercise (Bloomer et al., 2005, Michailidis et al., 2007). Similar to lipid peroxidation, the literature is abundant with investigations utilising short term exercise to evaluate the resulting PC. Of the authors who have employed GXT to induce increased PC production one has found an increase (Alessio et al., 2000) and one has found no change (Falone et al., 2010). This might be due to the training status of the individuals used. While Alessio et al. (2000) the utilised untrained participants only, Falone et al. (2010) used both trained and sedentary participants. Alessio et al. (2000) found a 67% increase in PC formation after GXT in their subjects while Falone et al. (2010) found increases in the untrained subjects only. Other authors have used submaximal exercise of 30-60 min to induce changes in PC, with

equivocal results. While only one author has noted no change from 30 min of cycling at 70% VO_{2max} (Bloomer et al., 2005), this same research group found an increase from both 30 min and 60 min of cycling at 70% VO_{2max}, with greater augmentation resulting from longer duration (60 min > 30 min) (Bloomer et al., 2007a). Additionally, PC formation was increased up to 32% from 45 min steady state cycling at 70% VO_{2max} followed by a bout to exhaustion at 90% VO_{2max} (Michailidis et al., 2007, Nikolaidis et al., 2006). These discrepant findings likely result from the differences in training status of the individuals, as the individuals in Bloomer et al. (2005) were cross trained compared to the endurance (Bloomer et al., 2007a) or untrained (Michailidis et al., 2007, Nikolaidis et al., 2006) individuals of the other studies. It may be that cross training, which includes both high intensity exercise and endurance training, better prepares these individuals to face the oxidative challenge of shorter duration exercise.

Data on prolonged exercise is much the same, with two studies noting significance after 90 min (Morillas-Ruiz et al., 2005) as well as 60 and 120 min (Bloomer et al., 2007a) in trained subjects. After 90 min of cycling exercise at 70% VO_{2max}, Morillas-Ruiz et al. (2005) found a 15% increase in PC content. Similarly, Bloomer et al. (2007a) investigated the effects of 60 or 120 min of cycling at 70% VO_{2max} finding an increase in PC content after both exercise durations, however PC content was augmented to a greater extent as a result of the longer duration exercise. To the author's knowledge, only one study conducted in humans has investigated the use of a long duration protocol on PC content, utilising trained cyclists competing in a 172 km mountain stage bike ride with a mean finishing time of 238 min (Tauler et al., 2006). The authors noted a 40% increase in PC formation as a result of the cycling bout.

Aside from short duration protocols, high intensity exercise has been the most explored avenue of research with regards to PC formation as a result of exercise. One short, high intensity bout of cycling exercise has been used to induce changes in PC post exercise, with the authors noting an increase of 111% in plasma PC concentration from a Wingate test (Bloomer et al., 2007b). This same study also noted significant increases in PC formation (74%) as a result of 15 squat repetitions at 70% of 1RM. Similarly, Bloomer et al. (2005) found a significant increase in PC formation as a result of 30 min of continuous squatting at 70% 1RM. Finally, Alessio et al. (2000) found a 12% increase in PC formation as a result of 10-12 min of intermittent maximal voluntary contractions. However, not all studies have found increases in PC from high intensity exercise. Bloomer et al. (2006) using resistance trained men performing six 10 sec sprints with 3 min resting periods followed, 14 days later by 6 ± 1 sets of 40 ± 2 reps of squats, did not find a significant change in PC formation post exercise. The authors did note that while not significant, the sprint exercise increased PC more than the squatting exercise (Bloomer et al., 2006).

Finally, there appears to be no effect of training programs utilised to increase endurance capacity upon the formation of exercise-induced PC. Miyazaki et al. (2001) employed a 12 week endurance training program consisting of 60 min running at 80% VO_{2max} five days per week, while Rahnama et al. (2007) employed a less intensive 20-40 min at 70-75% VO_{2max} three days per week for eight weeks. Neither of the studies found any significant changes in PC content as a result of the GXT completed before and after training, nor did they find any changes in the formation of PC as a result of the training programs.

2.1.4. DNA Damage

It has been assumed that the most biologically significant target of oxidative damage is DNA (Halliwell, 2000) . It is estimated that for every 10^{12} oxygen molecules entering a cell in a day, 1 in 200 damages DNA (Aust and Eveleigh, 1999). Estimates suggest an average of at least a few hundred oxidative DNA lesions occur in each cell per day (Niess, 2005). Oxidation of DNA can result in damage to all four bases (guanine, thymine, adenine, and cytosine) as well as the sugar deoxyribose (Aust and Eveleigh, 1999) and is often measured through the formation of strand breaks or chromosomal alterations (Halliwell and Aruoma, 1991). DNA damage by oxidative stress does not involve direct attack of O_2 or H_2O_2 upon the DNA (Halliwell and Aruoma, 1991); however DNA can be attacked directly by other ROS, primarily the hydroxyl radical and peroxynitrite. Reactions of the hydroxyl radical with DNA leads to a wide variety of damage to all four bases as well as deoxyribose with the latter leading to the formation of carbon-centred radicals that can undergo further oxidation (Aust and Eveleigh, 1999). Peroxynitrite preferentially reacts with guanine resulting in the formation of 8-hydroxydeoxyguanosine (8OHdG) which can undergo secondary oxidation by peroxynitrite (Aust and Eveleigh, 1999). DNA damage by oxidation is considered a potential pathophysiological factor in the development of cancer (Niess, 2005, Valko et al., 2004) although no direct link between the detection of oxidative DNA damage and cancer exists to date (Aust and Eveleigh, 1999).

DNA damage is most often assessed by measuring oxidised DNA by-products, generally with gas chromatography or mass spectroscopy. These methods require hydrolysis of the samples before analysis, which has been criticised because it may lead to artifactual increases in the oxidation products (Aust and Eveleigh, 1999). Additionally, samples can

be analysed using HPLC technique, although this method also utilises hydrolysis prior to sample analysis. Another draw-back of using HPLC is that only quantification of damage and not identification of where the damage is located in the sample is possible (Aust and Eveleigh, 1999). Measurement of 8OHdG and similar by-products via enzyme linked immunosorbent assay in urine and plasma/serum is also frequently utilised to quantify DNA oxidation, however it has been suggested that this method is only semiquantitative (Dizdaroglu et al., 2002). Finally, measurement via single gel electrophoresis (the COMET assay) may provide a more comprehensive picture of oxidative damage because it can measure both damage and repair (Collins et al., 1997). A variety of modes and intensities have been used to analysed DNA oxidation as a result of exercise, however the data are limited and equivocal, therefore conclusions about exercise and DNA oxidation are difficult to make.

Short term protocols, utilising trained and untrained subjects completing GXT, both cycling and running, have shown no significant increases in DNA damage measured via the COMET assay (Niess et al., 1996) or in urine measured by enzyme linked immunosorbent assay (Sumida et al., 1997). Additionally, a 30 min bout of cycling exercise at 70% VO_{2max} in cross trained subjects did not result in a significant increase in DNA damage in serum (Bloomer et al., 2005). However, one study employing a 60 min cycle at 70% VO_{2max} in trained subjects did lead to a significant increase in the formation of 8OHdG as measured in urine (Orhan et al., 2004). Data from moderate duration exercise are limited and equivocal with one study finding a 19% increase in 8OHdG in urine post 90 min of cycling at 70% VO_{2max} (Morillas-Ruiz et al., 2005), while another found no changes after a 20 km run (Sumida et al., 1997). To the author's knowledge, only one previous study has

investigated the effects of long duration exercise on DNA oxidation (Tsai et al., 2001). The authors utilised endurance-trained men to complete a 42 km marathon finding a significant rise in 8OHdG in urine immediately post race. Additionally, this elevation in 8OHdG in urine was sustained for the subsequent 24, 48, 72 hours through one week after exercise.

Similar to other durations of exercise, the effect of multiple days of exercise on DNA oxidation is limited and appears equivocal. Viguie et al. (1993) employed three consecutive days of cycling for 90 min at 65% VO_{2max}, which resulted in no significant increases in DNA oxidation, and in fact the authors noted a non-significant decline in 8OHdG. Contrary to these findings, two other studies using multiple days of exercise have found significant increases in DNA oxidation as a result (Almar et al., 2002, Radak et al., 2000). Almar et al., (2002) used eight trained cyclists to complete both a four day and three week stage race separately, finding significant increases in the formation of 8OHdG in urine after the first day of the four day race and after the first week of the three week race period, but no significant changes after these. Similarly, Radak et al., (2000) measured urinary 8OHdG in five ultra-endurance trained males completing a four day ultramarathon consisting of 93 km, 120 km, 56 km, and 59 km on days one through four, respectively. The authors noted a significant rise in urinary 8OHdG after the completion of day one, but no further increases, and in fact found decreases for the remainder of the race with significantly lower values on day four than any other exercise day. These results tend to suggest that after the initial bout of exercise, the body is able to adapt and manage oxidative stress that would otherwise result in DNA damage.

Studies employing high intensity protocols to investigate the formation of DNA oxidation by-products appear to have similar results regardless of the actual modes of exercise. Bloomer et al. (2005) used cross-trained men performing 30 min of continuous squatting exercise at 70% 1RM to investigate DNA oxidation. While the values approached statistical significance, the authors did not find any significant increases in serum 8-OHdG. The same authors then used resistance trained men performing 15 reps of squats at 70% 1RM and a 30 sec Wingate and were unable to induce any changes in plasma 8OHdG, similar to their previous work (Bloomer et al., 2007b).

Few studies have investigated the effects of training on the formation of DNA oxidation by-products, but the few that have find similar results. Goto et al. (2003) used a 12 week training program consisting of either mild (25% VO_{2max}), moderate (50% VO_{2max}) or high intensity (75% VO_{2max}) exercise for 30 min per day, five to seven days per week in 26 untrained men (split between the three training loads). The authors noted that mild and moderate exercise training tended to decrease the production of 8OHdG post exercise and that interestingly the high intensity training resulted in a significant increase in plasma 8OHdG production after the 12 week training program. Okamura et al. (1997) used 10 well trained distance runners completing an eight day running camp that consisted of training runs of 30 ± 3 km per day for the whole eight days. The authors noted significant increases in urinary 8OHdG after the eight days of training compared to a three day control period; however they did not find a significant difference in lymphocyte DNA damage from one day post compared to pre training camp (Okamura et al., 1997). Finally, Poulsen et al. (1996) trained 23 men for 8-11 hours per day, six days per week for 30 days finding a 33% increase in urinary 8OHdG compared to pre training values, however 11 of the men were

smokers, who had a larger increase compared to the non-smokers likely skewing the data. As a whole it appears exercise training can increase the production of DNA damage markers if the exercise intensity is high compared to mild or moderate.

Summary

Taken together, all of the research linking exercise to the production of oxidative stress by-products appears to indicate that oxidative stress is augmented as a result of exercise, although the duration and intensity appears to play a key role. Similarly, the training status of the individuals may also be very important in determining the resulting stress. Because of the varying protocols, markers measured and methods to do so, further research is warranted to identify the underlying cause of the oxidative stress during exercise and to establish stronger links between the modes, durations and intensities that may cause the oxidative stress.

2.2. Antioxidant Defences

Rather than measuring the by-products of oxidative stress, some researchers choose to measure oxidative stress via quantification of redox balance (antioxidant activity). The body utilises both endogenous and exogenous antioxidant systems which quench free radicals and defend against oxidative stress and damage. Endogenous antioxidants, such as glutathione, catalase, and superoxide dismutase, among others are the body's in house defence systems, while exogenous antioxidants such as vitamins C and E are consumed in the diet and utilised by the body. Because this thesis employs the measurement of the glutathione system, this will be discussed in detail below; however the neutralisation of

radicals by the other antioxidant defences such as catalase, superoxide dismutase, and vitamins C and E are highly important and reviewed extensively elsewhere (Herrera and Barbas, 2001, Mates et al., 1999, Peake, 2003).

2.2.1. Glutathione

The foremost endogenous non-enzymatic antioxidant in the body is the glutathione system. Glutathione provides the cell with multiple lines of defence against cellular insult by scavenging for free radicals (particularly the hydroxyl radical, for which there is no known enzymatic defence (Bains and Shaw, 1997)) and reducing many of the damaging by-products of oxidative stress (Maher, 2005). During normal resting conditions glutathione is found in the reduced (GSH) state, the majority of which is found in the cytoplasm of the cell where it is synthesised (Maher, 2005). Additional pools of GSH can be found in the nuclei and the mitochondria, however the concentration of these pools are independent of that in the cytoplasm (Maher, 2005).

GSH is bio-synthesised from glutamate, cysteine, and glycine (Maher, 2005) (see *Figure 2.2*). Oxidised glutathione (GSSG) is formed by the reduction of H_2O_2 at the expense of GSH which is catalysed by glutathione peroxidase (GPx). GSSG can be reduced by glutathione reductase (GR) back to GSH where it is free to aid in further cellular protection (Maher, 2005).The distribution of glutathione enzymes throughout the body is not uniform, with greater concentrations of GPx found in the brain compared to other tissues such as the liver and heart (Maher, 2005). Because of this discovery, much current research has focused on linking GSH depletion to aging (Jones et al., 2002), ischaemia/stroke injury (Arthur et al., 2004) and Parkinson's (Halliwell, 2001).

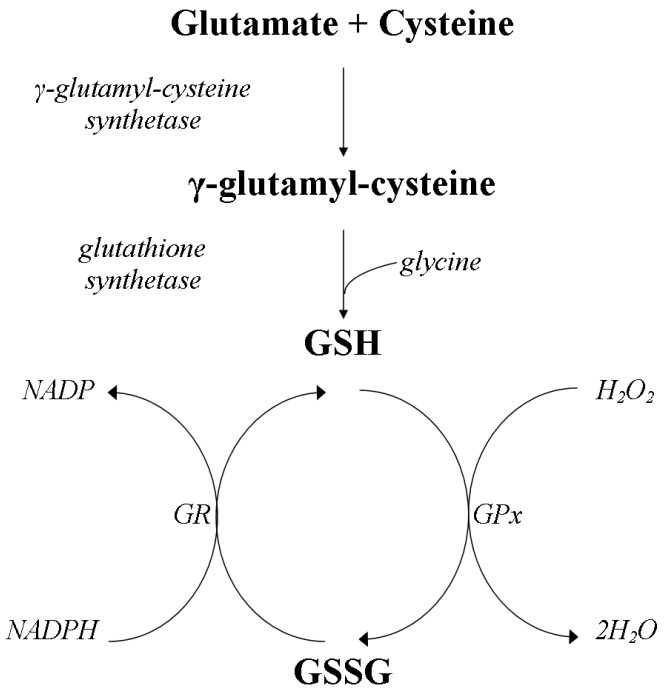

Figure 2.2. Glutathione (GSH) recycling reaction. GSH is bio-synthesised from glutamate, cysteine, and glycine. Oxidised glutathione (GSSG) is formed from the reduction of H_2O_2 at the expense of GSH which is catalysed by glutathione peroxidase (GPx). GSSG is reduced by glutathione reductase (GR) back to GSH where it is free to aid in further cellular protection.

Glutathione can be measured in blood as well as tissue. While blood measurements of glutathione are usually made in the plasma, the concentration of GSH and GSSG in plasma is negligible (0.5%) compared to the concentration in erythrocytes (99.5%) (Gohil et al., 1988, Serru et al., 2001). Changes in blood glutathione are mostly reported as decreases in GSH and increases in GSSG, with no change in TGSH (Sen, 1999). Increases in TGSH have rarely been reported post exercise, as with Sen et al. (1994), who found increases in TGSH post 30 min of cycle ergometry at 70% VO_{2max} but not during 30 min of cycling at 50% VO_{2max}. The authors concluded that increases in TGSH may be due to efflux of GSSG from other tissues besides the blood. Increases in TGSH could also be due to training status, as individuals with higher training status may be better conditioned to maintain

adequate GSH levels during exercise (Leeuwenburgh et al., 1997). Tissue (skeletal muscle, myocardium and liver) measurements of glutathione are usually measured in rats, where most researchers have found a decrease in glutathione in skeletal muscle (Duarte et al., 1993), myocardium (Sen et al., 1994a), and liver (Sen et al., 1994a) post exercise, likely due to increase in superoxide production (Duarte et al., 1993). Typical resting concentration of GSH and GSSG are ~500 µmol/l and ~70 µmol/l in the whole blood, respectively (Serru et al., 2001), while TGSH concentration is typically 3.5 µM/g Hb in red blood cells (Matsubara and Machado, 1991). Resting GSH concentrations tend to decrease with age (Matsubara and Machado, 1991, Serru et al., 2001), with less of a decline in aging and exercise in females compared to males (Ilhan et al., 2004, Wang et al., 2003).

Glutathione deficiency has been linked with many disease states including Alzheimer's and Parkinson's diseases (Vina et al., 2004, Sian et al., 1994), cancer (Balendiran et al., 2004), cardiovascular disease (Emdin et al., 2005), and both type I and type II diabetes (Yoshida et al., 1995) amongst others. The underlying aetiology of glutathione deficiency has been linked in many cases to genetic malfunctions (Dahl et al., 1997); however deficiency can also result from lifestyle choices (Leeuwenburgh and Ji, 1995, Liu et al., 1996, Madrigal et al., 2001). While genetic GSH deficiency is irreversible, models of GSH depletion via immobilisation have been utilised in rats to investigate the implications during exercise. Leeuwenburg and Ji (1995) demonstrated that GSH deficiency was associated with decreased GPx activity, which further inhibits the body's ability to recycle GSH. Similarly, Sen, Atalay, and Hanninen (1994) induced glutathione deficiency in rats, ~50% in blood, liver and lung and 80-90% in skeletal muscle in heart, to test the effects on endurance exercise. Time to exhaustion was decreased by 50% in the glutathione deficient compared

to non-deficient rats. The authors concluded that GSSG levels at rest are a strong predictor of post-exercise levels, whereby individuals with a favourable glutathione status at rest maintained a more favourable redox status following exercise-induced oxidative stress. In contrast to these findings, a recent bed rest study in humans indicated a trend for glutathione synthesis rates to increase as a result of 35 days of bed rest (Agostini et al., 2010); however because of the limited human data on glutathione depletion, further research is needed to make conclusions about the impact of sedentary lifestyle/immobilisation on glutathione levels in humans.

Endurance exercise training has been shown to improve glutathione protection against oxidative stress in rats (Kihlstrom, 1990, Leeuwenburgh et al., 1997) and humans (Douris et al., 2009, Elokda and Nielsen, 2007). Kihlstrom et al. (1990) showed endurance swim training on rats provided enhanced protection of the heart by way of elevated GSH levels and a more efficient NADPH supply system to the heart. Likewise, Leeuwenburgh et al. (1997) trained rats by running for two hours per day, five days per week, for 10 weeks. The authors found a substantial (62%) increase in GPx activity and increased GSH in the skeletal muscle, with no changes in blood GSH levels. In humans, the data on the effects of training on the glutathione system in humans is very limited. Two studies have shown enhanced glutathione protection as a result of superior aerobic fitness (Douris et al., 2009, Ortenblad et al., 1997) while others have shown directly the effects of training on the glutathione system (Elokda and Nielsen, 2007, Miyazaki et al., 2001). Elokda and Nielsen (2007) mimicked cardiac rehabilitation exercise (40 min/day three days/week for six weeks) consisting of aerobic training, circuit weight training, or a combination of both. The authors found that training increased resting levels of GSH and decreased resting levels of

GSSG and the effects were greater in the combination of aerobic and circuit weight training compared to the exercises alone. Similarly, 12 weeks of running for 60 min at 80% VO_{2max} five days per week increased GPx activity by 12% (Miyazaki et al., 2001). Assessing individuals of varying fitness levels, Douris et al. (2009) found individuals who practiced martial arts for one hour per day twice a week for at least four years had increased resting GSH and decreased resting GSSG while being better able to respond to an acute bout of oxidative stress compared to their sedentary counterparts. Finally Ortenblad et al. (1997) found increased GPx activity in the resting muscle of jump trained athletes compared to untrained subjects. These results seem to suggest that exercise training of varying intensities and modes can increase the efficiency and function of the glutathione system, likely leading to a decrease in exercise-induced oxidative stress and potential for disease development.

2.3. Oxidative Stress and Heat Shock Proteins

While heat shock proteins (HSP) are traditionally viewed as proteins which are ubiquitously expressed during times of stress such as during exercise, exposure to extreme environmental temperatures, or sickness and disease, recent work has indicated that HSP are intrinsically linked with oxidative stress (Kalmar and Greensmith, 2009), thus the author decided to measure HSP concentration in the studies described in this thesis. While there are numerous families of HSP, of particular interest to this thesis are the 70 kiloDalton (HSP70) and 30 kiloDalton (HSP30) families, thus only these will be discussed with regard to their induction and function during prolonged exercise similar in nature to that of the studies in this thesis. While not directly relevant to this thesis, other families of

HSP have been linked with the oxidative stress response and are thoroughly reviewed elsewhere (Morton et al., 2009).

2.3.1. HSP70

The inducible isoform of the HSP70 family, HSP72, is expressed at low levels during non-stress situations (Locke et al., 1991) and acts to facilitate proper protein folding (Beckmann et al., 1990), as well as saving cells from activated death pathways (Moseley, 1997). During times of stress, HSP72 manages denatured proteins, provides cytoskeleton stabilisation (Moseley, 1997), and eradicates damaged proteins (Kalmar and Greensmith, 2009). Long-term protection is provided by HSP72 to vital tissues by way of both increased thermotolerance (Landry et al., 1982) and heat acclimation (Sandstrom et al., 2008).

Traditionally, HSP's were thought to only be induced by heat shock. Early research focused on the passive induction of HSP in the fruit fly (Ritossa, 1962, Tissieres et al., 1974) while more recently only two investigations have focused on induction of HSP72 via passive hyperthermia in human skeletal muscle (Morton et al., 2007) and peripheral blood mononuclear cells (PBMC's) (Lovell et al., 2007). Through 90 min of *in vitro* heat shock of PBMC's at 37-41˚C, Lovell et al. (2007) were able to show that the concentration of HSP72 between temperatures was not different, however the induction of HSP72 appeared to be temperature-dependent. Heat shock of 37-39˚C resulted in the greatest induction 4 hours post, while heat shock of 40-41˚C resulted in an immediate increase. This may indicate more of an immediate protective response earlier in the recovery period from a higher temperature of insult (Lovell et al., 2007). Furthermore, the resulting expression of heat shock at 37˚C may indicate a lower threshold for HSP induction than previously

thought (Lovell et al., 2007). Also in 2007, Morton et al. investigated the use of passive heating similar to exercise and rationalised that because exercise can result in hyperthermia and increased HSP72 expression (Febbraio and Koukoulas, 2000, Kim et al., 2004, Morton et al., 2006), passive heating to similar levels should also induce HSP72 expression. Subjects were passively heated for 60 min in a tank of water at 45˚C resulting in an increase of 3.6˚C in muscle and 1.5˚C in core temperature, respectively. Although these values were nearly identical to those found during exercise in their previous study (Morton et al., 2006), the authors did not find any significant changes in HSP72 expression in the skeletal muscle, suggesting that the elevated T_c and muscle temperatures during exercise are not the sole stressors responsible for induction of HSP72 expression.

Expansion on the traditional view of HSP being induced only by heat shock has led to copious amounts of research into other potent stressors, such as exercise and oxidative stress (see *Figure 2.3*). Many investigations employ exercise modes involving near maximal or electrically-stimulated muscle contractions and downhill treadmill running that result in augmented HSP72 expression (Paulsen et al., 2007, Shima et al., 2008), likely due to their ability to induce muscle damage and an inflammatory response (Peake et al., 2005). Less strenuous and likely non-damaging exercise modalities have also been employed, resulting in equivocal findings (Febbraio and Koukoulas, 2000, Febbraio et al., 2002, Fehrenbach et al., 2000a, Fehrenbach et al., 2005, Fehrenbach et al., 2000b, Watkins et al., 2007).

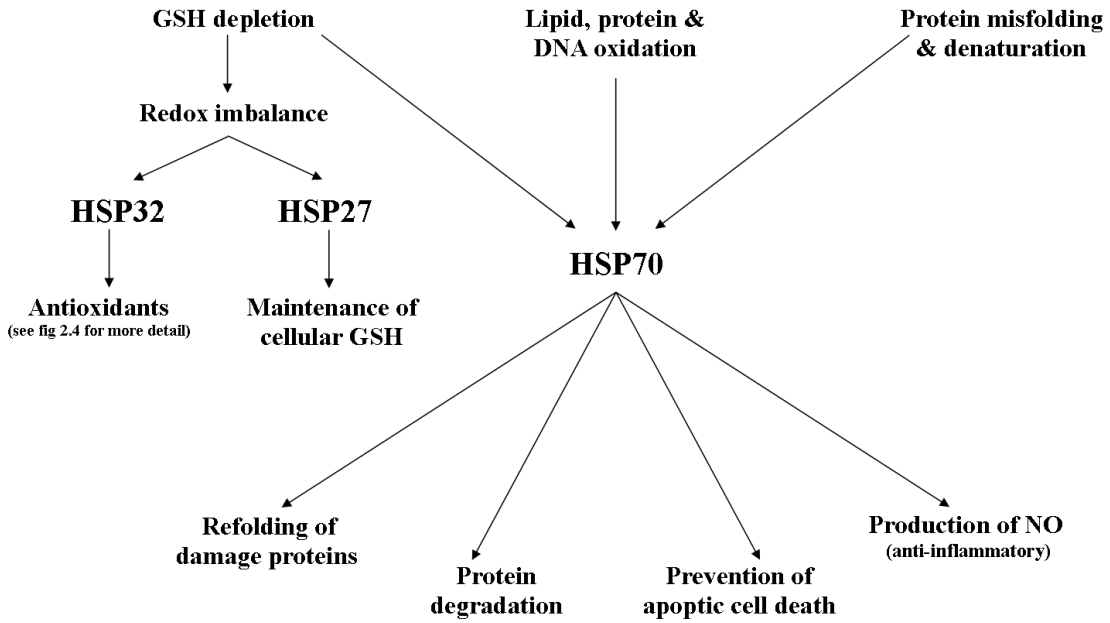

Figure 2.3. Relationship between oxidative stress and heat shock protein (HSP) expression. Glutathione (GSH) depletion results in the expression of HSP32 and 27 with HSP32 resulting in the production of antioxidants (see *Figure 2.4* for more detail) while HSP27 aids in the maintenance of the cellular GSH pool. Oxidative stress and protein misfolding and denaturation result in HSP70 expression which plays a critical role in cell survival by refolding or degradation of damaged proteins, prevention of apoptic cell death and production of nitric oxide (NO) which provides anti-inflammatory protection. Adapted from Kalmar and Greensmith (2009).

While cycling to exhaustion (~190 min) at 63% of VO_{2peak} lead to an increase in HSP72 mRNA (Febbraio and Koukoulas, 2000) and 4-5hours of cycling at 40% W_{max} resulted in a 200% increase in muscle HSP72 protein and mRNA (Febbraio et al., 2002) in normothermic environments, other authors have failed to find significant changes in HSP72 from 60 min of cycling at 75% VO_{2max} in either a normo-or hyperthermic environment (Watkins et al., 2007). These equivocal findings likely resulted from the differences in duration. Indeed, HSP72 expression has been shown to be both duration and intensity dependent (Fehrenbach et al., 2005). Fehrenbach et al. (2005) compared four exercise modalities on the induction of HSP72 expression and found that marathon running (260 $\pm$ 39 min at 65% VO_{2max}) led to the greatest HSP72 concentration when compared to a long run (120 min at 60% VO_{2max}), interval training (10 x 1000 m at 88% VO_{2max}), and

continuous running (60 min at 75% VO_{2max}). The authors (Fehrenbach et al., 2005) also investigated two differing intensities of running, 60% VO_{2max} vs. 80% VO_{2max}. Exercise times for both runs were identical to each other (23.7 ± 7 min); however the HSP72 expression was greater during the 80% VO_{2max} intensity.

Additionally, the training status and gender of subjects must be taken into account when comparing HSP72 expression. Morton et al. (2008) demonstrated elevated HSP72 in the skeletal muscle of trained vs. untrained subjects whilst Fehrenbach et al. (2000) showed lower HSP72 levels in monocytes of trained subjects. These differences may lie in the different tissues studied (muscle vs. blood) and the fact that these have yet to be correlated. The benefit to a higher training status is the heightened HSP72 response to stressors (Fehrenbach et al., 2000a, Fehrenbach et al., 2000b, Magalhaes et al., 2010) and possibly increased protection and/or survivability of muscle cells (Yamada et al., 2008).

2.3.2. Heme Oxygenase (HSP32)

Heme oxygenase or HSP32 exists in two isoforms (HO-1), which is highly inducible, and HO-2 which is constitutively expressed (Gozzelino et al., 2010). HO-1 functions to degrade free heme, which is toxic to cells because of its participation in the Fenton reaction, to carbon monoxide, biliverdin, and ferritin (Soares and Bach, 2009). Biliverdin is then converted to the cytoprotective antioxidant bilirubin (Clark et al., 2000) (see *Figure 2.4*). This process allows for prevention of programmed cell death (Seixas et al., 2009) as well as providing cytoprotection against a variety of diseases and reestablishment of homeostasis after insult (Soares and Bach, 2009).

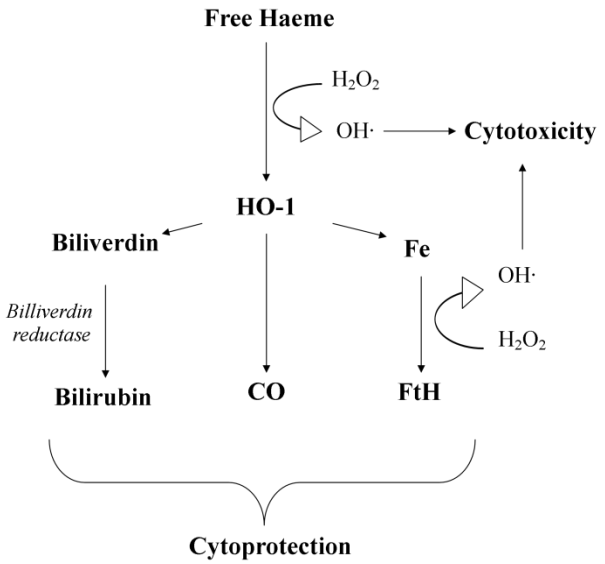

Figure 2.4. The elimination of free haeme by heme oxygenase-1 (HO-1 or HSP32) produces biliverdin, carbon monoxide (CO) and iron (Fe). Biliverdin is converted by biliverdin reductase to the antioxidant bilirubin while Fe is stored by ferritin H chain (FtH). All three products of free haeme catabolism are cytoprotective. Adapted from Gozzelino et al. (2010).

HO-1 is commonly referred to as HSP32 because of its supposed induction by heat shock, though few human studies indicate a direct link between heat shock alone and HSP32 expression (Nakabe et al., 2007). In fact, HSP32 expression has been shown to decrease following heat shock (Grasso et al., 2003). Exercise is known to be a potent inducer of HSP32 (Fehrenbach et al., 2003a, Niess et al., 1999, Thompson et al., 2005) and this appears to be duration dependent (Fehrenbach et al., 2003a). Fehrenbach et al. (2003) employed three different exercise modalities to induce an increase in HSP32 concentration: a continuous run to exhaustion at 110% LT, a half marathon (90.3 $\pm$ 12.8 min at ~105% LT), and 6 sets of 10 repetitions of eccentric quadriceps contractions. The authors found the half marathon resulted in the greatest increase in HSP32 expression compared to continuous running and eccentric exercise, which had very little to no change in HSP32 expression. Similar results were found from Niess et al. (1999) and Thompson et al. (2005). In Niess et al. (1999) nine endurance trained men completed a half marathon run. The

authors found a significant increase in HSP concentration in monocytes and lymphocytes three hours after the run and noted that trained individuals express lower basal levels of HSP32 compared to their untrained counterparts (Niess et al., 1999). Similar results can be seen from Thompson et al. (2005) whose participants completed a 75 min run at 75% VO_{2max} to induce HSP32 expression. The authors found a 2-fold increase in HSP32 mRNA expression and protein in lymphocytes two hours post exercise, confirming the results of Niess et al. (1999). Little else is known about the effects of exercise on HSP32 induction. However, Markovitch, Tyrrell and Thompson (2007) showed that a prior bout of intermittent exercise (60 min at 65-100% VO_{2max}) followed by an *ex-vivo* challenge of H_2O_2 caused a reduced upregulation of HSP32. This result could be indicative of post-exercise immuno-suppression or positive adaptive changes as a result of the prior exercise bout (Markovitch et al., 2007).

2.4. Physiological Effects of Exercise in Hyperthermic Environments

As previously discussed above, oxidative stress is believed to be augmented by hyperthermic environments, both passively and during exercise. This is likely due to an increase in metabolic rate resulting in augmented ROS production. Additionally, reductions in the efficacy of antioxidant defences as a result of heat stress may play a role (Ozturk and Gumuslu, 2004). Most importantly, hyperthermic exercise can result in dehydration, which has been indicated in augmenting oxidative stress (Paik et al., 2009). Therefore it is necessary to explore some of the physiological changes that occur during prolonged exercise in hyperthermic environments that may lead to these perturbations.

Prolonged exercise in normothermic environments can lead to mild hyperthermia with increased heart rate (HR), core temperature (Tc) and skin temperature (Ts) (Nassis and Geladas, 2002). This is exacerbated in hyperthermic environments when thermoregulation is impaired (Ely et al., 2010, Tatterson et al., 2000). Increased HR during hyperthermic exercise is widely believed to result from augmented sweat rates and blood volume redistribution which can decrease plasma and blood volume (Montain and Coyle, 1992). These changes can result in decrements in stroke volume, whereby HR must be increased in an effort to maintain cardiac output (Gonzalez-Alonso et al., 1997, Gonzalez-Alonso et al., 2000). While HR is often higher during exercise in hyperthermic compared to normothermic environments (Nybo et al., 2001, Tucker et al., 2006), this appears to be dependent on hydration status as there are often no differences in HR between these two environments when dehydration is not a factor (Altareki et al., 2009, Buono and Wall, 2000, Ely et al., 2010, Tatterson et al., 2000, Wimer et al., 1997).

Contrary to this, Tc and Ts are often augmented as a result of exercise in hyperthermic environments independent of hydration status (Altareki et al., 2009, Ely et al., 2010, Nybo et al., 2001), although others have noted no significant differences between the two environments (Buono and Wall, 2000, Tatterson et al., 2000). It is important to note that the methodology of many investigations conducted in hyperthermic environments manipulate the hydration status of their participants and because dehydration has been shown to increase Tc in a linear fashion (Montain and Coyle, 1992), the results of these investigations may be confounded, and will be addressed in the next section.

2.4.1. Dehydration and Thermoregulation

The impairment of thermoregulation during exercise in hyperthermic environments often results from lack of adequate fluid intake, and thus dehydration. Dehydration during endurance exercise causes a cascade of cardiovascular strain-inducing events (cardiovascular drift), which begins with dehydration and a loss of body water from the extracellular fluid. This loss in body water causes a decrease in plasma volume (PV) which in turn results in a decrease in total blood volume. In order to compensate for this loss in blood volume and to maintain adequate blood flow to the exercising muscles, HR is increased (Nadel et al., 1980). This increase in HR without a concomitant increase in blood volume results in a decrease in stroke volume, which will ultimately result in a decline in cardiac output if increased HR does not augment venous return (Gonzalez-Alonso et al., 2000). The decrease in blood volume due to dehydration also inhibits the body's natural heat dissipation ability by decreasing sweat rate in an effort to conserve body water, which in turn causes Tc to steadily climb until fatigue occurs (Gonzalez-Alonso et al., 2008).

Hypohydration and dehydration have been shown to cause an increase in Tc during exercise in both normothermic (Armstrong et al., 2006) and hypertherthermic environments (Barr et al., 1991, Buono and Wall, 2000, Gonzalez-Alonso et al., 1997, Ishijima et al., 2009, Montain and Coyle, 1992). This Tc increase has been shown to rise in a linear fashion with increasing amounts of dehydration (Montain and Coyle, 1992, Sawka et al., 1985) and if not corrected, this increase in Tc will continue until fatigue. The magnitude of Tc elevation ranges from 0.1˚C to .25˚C for every percent of body mass lost (Montain and Coyle, 1992, Sawka et al., 1985), augmenting the thermal strain on the exercising body, which also follows a linear pattern (Sawka et al., 1985). Heat acclimation is generally

believed to decrease the occurrence of cardiovascular strain, however even heat-acclimated subjects have demonstrated a linear relationship between dehydration and Tc that will lead to fatigue (Sawka et al. 1983, Sawka, et al. 1985). While the leading theory details that Tc is affected mostly by levels of dehydration, the research groups of Dr. Noakes are highly critical of this concept, citing results of multiple marathon races where the winning athletes rarely consume fluid matching sweat losses (becoming dehydrated), yet these individuals finish races with the fastest times and high Tc. The authors also conclude those at greatest risk for heat injury are individuals who maintain high metabolic rates during exercise, when maintaining optimum hydration may not even help (Noakes et al., 1991).

Additionally, while contemporary research indicates there is a critical level of Tc that determines when fatigue will occur during exercise, a study by Pugh et al. (1967) seems to contest this. Tc taken post race on 47 runners completing a marathon shows that attainment of a Tc of $\geq$ 40˚C does not necessarily cause fatigue. Seven of the finishing runners had Tc > 40˚C while all of those who did not complete the course had Tc $\leq$ 40˚C. This indicates that athletes who were overcome by fatigue had lower Tc than those who finished the race. Unfortunately, this appears to be one of the only studies to report Tc > 40˚C, most likely due to the limits set by many human subject review boards and ethics committees.

In uncompensable heat stress, when thermoregulation is not possible, increasing levels of dehydration and Tc leads to the attainment of a critically high Tc and ultimately fatigue. Nielsen et al. (1993) concluded that core temperature *per se*, rather than circulatory failure, is the limiting factor during endurance exercise in hyperthermic environments. Many authors suggest there is a critical level for Tc and that once this level is attained individuals

will fatigue (Cheung and McLellan, 1998, Gonzalez-Alonso et al., 1999b, Nielsen et al., 1993). Gonzalez-Alonso et al. (1999b) concluded that trained individuals in laboratory tests seem to fatigue at a relatively consistent Tc of 40°C despite differing Tc pre-exercise, while in untrained individuals the Tc at exhaustion ranges from 38-40°C. The differences between trained and untrained individuals can be attributed to the difference in their fitness levels, as individuals who are more highly trained have higher sweat rates and heat dissipation ability (Nadel et al., 1974, Shvartz et al., 1974) as well as lower resting Tc and higher Tc temperature tolerated at fatigue (McLellan, 2001, Mora-Rodriguez et al., 2010).

Maintenance of sweating during hyperthermic exercise is critical to maintenance of Tc. Strenuous exercise in hot climates increases the body's dependence on evaporative cooling which can lead to large volumes of body water lost through sweating (Sawka et al., 2001). In order to naturally dissipate heat from the body during exercise, skin blood flow and sweat rate must increase (Montain and Coyle, 1992). Several authors have found that sweat rates are reduced when body water losses are not prevented with fluid ingestion (Buono and Wall, 2000, Sawka et al., 1983, Sawka et al., 1985), while sweat rates are maintained when fluid is consumed (Hamilton et al., 1991). Similarly, skin blood flow is decreased (Buono and Wall, 2000, Sawka et al., 1985) while skin temperature is augmented (Gonzalez-Alonso et al., 1999a, Kavouras et al., 2006, Marino et al., 2004) during dehydration. However the relationship between dehydration, sweat rates, skin blood flow and skin temperature is not well understood. In an effort to better understand this relationship, Montain and Coyle (1992) studied 7 trained cyclists completing 2 hours of cycling at 65% VO_{2max} while receiving no fluids, drinking 80% of fluid loss (Gatorade), or receiving a plasma volume expanding solution (dextran) intravenously. The authors found an increase

in skin blood flow from fluid replacement, but not from increased plasma volume demonstrating that fluid ingestion attenuates hyperthermia by increasing skin blood flow and sweat rates thus preserving heat transfer from the core to the periphery during exercise.

2.4.2. The Impact of Dehydration and Hyperthermia on Exercise Performance

While the effects of dehydration and hyperthermia on the thermoregulatory system are undeniable, the effect they have on exercise performance is more contentious. While hyperthermia alone has been shown to degrade aerobic exercise performance (Abbiss et al., 2010, Altareki et al., 2009, Ely et al., 2010, Tatterson et al., 2000, Tucker et al., 2006), there appears to be no effect of hyperthermia alone on high intensity exercise performance, although the data is limited (Cheuvront et al., 2006). Investigations on hyperthermia in tandem with dehydration, however, are less equivocal and indicate these two phenomena serve to heavily degrade exercise performance (Ebert et al., 2007, Ganio et al., 2006, Kavouras et al., 2006, Nybo et al., 2001).

The investigations into hyperthermia and exercise performance have utilised time trial protocols of varying lengths and times, ranging from 4 km and 390 sec (Altareki et al., 2009) to 100 km and 181 min (Abbiss et al., 2010). It appears that duration of protocol does not influence performance as Altareki et al. (2009) showed PO and time to completion were diminished in the heat compared to cool conditions (35˚C vs. 13˚C, respectively) for a 4 km TT, while using a much longer protocol of 100 km resulted in similar decreases in PO and time to completion in the heat compared to cool conditions (34˚C vs. 11˚C, respectively) for Abbiss et al. (2010). Varying times have also resulted in performance decrements with 15 (Ely et al., 2010), 30 (Tatterson et al., 2000) and approximately 50 min

(Tucker et al., 2006) resulting in a mean decrement of 17% in total work performed, a decrease of 6.5% in PO and a decline of 2.4 W/min, respectively.

From these investigations it is clear that hyperthermia alone results in serious performance decrements without the addition of dehydration. From further research we can see that superimposition of dehydration during hyperthermic exercise results in a 72% decrease in time to exhaustion following dehydration without rehydrating (Kavouras et al., 2006), a 70% decrease in time to exhaustion in after pre-warming (Marino et al., 2004) and a 50% decline in GXT performance after dehydration/rehydration (Nybo et al., 2001). Additionally, Ebert et al. (2007), in an effort to determine if dehydration could provide a performance advantage, rationalised that a decline in weight (i.e. dehydration) would aid cyclists in performing uphill cycling trials. Subjects completed 2 hours of cycling at 53% maximum aerobic power followed by a hill climb at 88% maximum aerobic power simulated by an 8% incline on a treadmill. While subjects did indeed lose body mass (1.9 kg), this resulted in a decrease in power, which equated to a 29% decline in performance, leading the authors to conclude that dehydration does not aid in simulated hill climb performance, but degrades performance as previously found.

The common denominator in the majority of these studies is a significant augmentation in heat storage (HS) during the hyperthermic trials, which likely led to anticipatory reductions in power output in order to pace appropriately and maintain thermoregulatory homeostasis (Abbiss et al., 2010, Tucker et al., 2004). While the literature concerning dehydration and hyperthermia is vast and equivocal, many believe and conclude that both are detrimental to

performance; however more research, particularly more applicable to sporting performance is required to better understand the paradigm.

2.5. Glycerol Ingestion and Prevention of Hyperthermia and Dehydration

Because of the impairments to thermoregulation and exercise performance that dehydration causes, many researchers have utilised substances such as glycerol as the most common intervention for increasing/maintaining cellular water content. Glycerol is a naturally occurring alcohol that is used clinically to treat oedema (Frank et al., 1981), but is also used as a hyperhydrating agent because of its ability to create an osmotic gradient (Nelson and Robergs, 2007). Roughly 80% of glycerol is metabolised to glycerol 3-phosphate in the liver with the remainder occurring in the kidneys (Frank et al., 1981). Approximately 70% of glycerol 3-phosphate is oxidised to form dihydroxyacetone phosphate, which can be readily converted into glyceraldehyde 3-phosphate which can proceed through glycolysis or gluconeogenesis (Stryer, 1995). The remaining ~30% can combine with free fatty acids to form triglycerides (Robergs and Roberts, 1997) *(Figure 2.5)*.

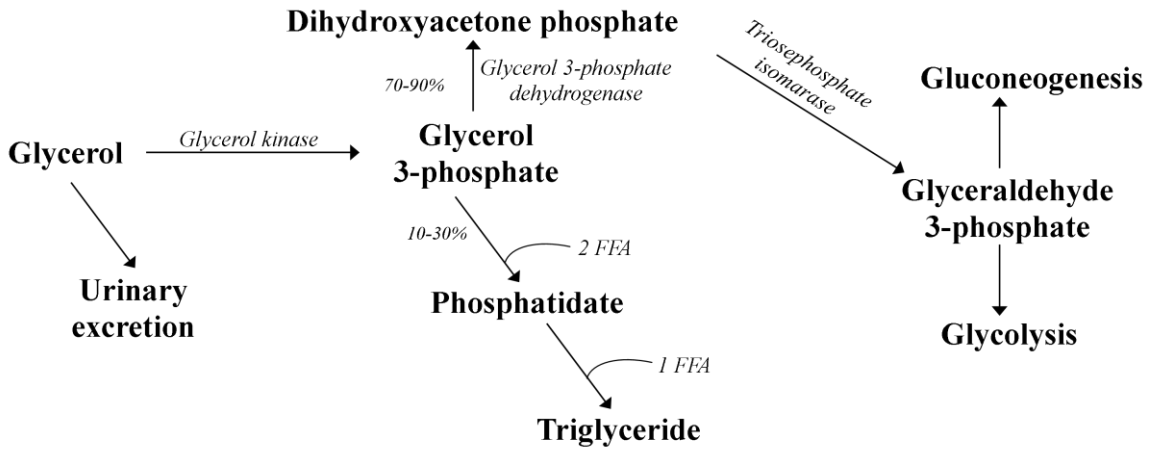

Figure 2.5. The metabolism of glycerol. Glycerol is metabolised to glycerol 3-phosphate by glycerol kinase to form glycerol 3-phosphate. Glycerol 3-phosphate is then oxidised by glycerol 3-phosphate dehydrogenase to form dihydroxyacetone phosphate, which can be readily converted into glyceraldehyde 3-phosphate (G3P) by triosphosphate isomerase. G3P can then proceed through glycolysis or gluconeogenesis. The remaining glycerol 3-phosphate can combine with free fatty acids (FFA) to form triglycerides.

Following ingestion, glycerol is rapidly absorbed via passive diffusion primarily in the intestines and is evenly distributed among the intra- and extracellular compartments (Lin, 1977). Glycerol is then absorbed intracellularly in the tubules of the kidneys (Warburton et al., 2000), creating an osmotic gradient whereby additional consumption of water will result in greater total body water, thus creating a hyperhydrated state (Nelson and Robergs, 2007). Normal plasma glycerol concentration is approximately 0.05-0.1 mmol/l (Lin, 1977), while post ingestion glycerol levels in the blood can increase up to ~16 mmol/l (Montner et al., 1996) . As the level of glycerol in the blood increases glycerol will begin to appear in the urine (Kruhoffer and Nissen, 1963). In order to maintain hyperhydration, glycerol and additional fluid must be continually ingested; failure to continue this regimen will result in a decrease in plasma glycerol concentration as a result of metabolism (Bortz et al., 1972) and urinary excretion (Nelson et al., 2010) leading to diminished ability to maintain hyperhydration.

Unfortunately, the literature is lacking data on the optimal dosage and ingestion period for glycerol hyperhydration. According to Nelson and Robergs (2007), the most commonly used glycerol dosage is 1.0 g/kg BM with ~21.4 ml/kg of additional fluid, typically water. However, the dosage of glycerol ranges from 0.5 g/kg to 1.5 g/kg (Goulet et al., 2006, Hitchins et al., 1999, Riedesel et al., 1987) with additional water ingestion of 20 ml/kg to 26 ml/kg (Anderson et al., 2001, Goulet et al., 2006, Hitchins et al., 1999, Latzka et al., 1997, Magal et al., 2003, Marino et al., 2003, Riedesel et al., 1987). While glycerol dosage is typically prescribed based on kg of body weight, some researchers choose to prescribe dosages based on amounts of lean body mass or total body water. Additionally, ingestion time periods have varied as much as the actual dose of glycerol, with exercise beginning anywhere from 30 – 210 min after ingestion (Coutts et al., 2002, Goulet et al., 2006, Goulet et al., 2008, Latzka et al., 1997, Marino et al., 2003, Riedesel et al., 1987, Wingo et al., 2004).

The primary aim of the majority of glycerol research is focused on its hyperhydration properties. The use of glycerol and water beverages prior to prolonged exercise, often in hot environments, in an effort to increase and maintain total body water through exercise has been highly investigated. Additionally, research has focused on the thermoregulatory and cardiovascular advantages provided by pre-exercise glycerol hyperhydration.

Pre-exercise Glycerol Hyperhydration

The rationale behind employing pre-exercise glycerol hyperhydration is to increase PV (Warburton et al., 2000). In doing so, blood volume and venous return (Watt et al., 2000) will be preserved, which might provide positive cardiovascular changes and aid in

thermoregulation (Nelson and Robergs, 2007). However, results of pre-exercise glycerol hyperhydration research investigating changes in fluid retention, thermoregulatory and cardiovascular changes have been equivocal.

It is quite clear that glycerol hyperhydration results in increased body mass (Goulet et al., 2008, Magal et al., 2003, Montner et al., 1999) and commonly decreases urine output (Anderson et al., 2001, Goulet et al., 2006, Hitchins et al., 1999, Lyons et al., 1990, Marino et al., 2003, Montner et al., 1999, Wingo et al., 2004) compared to placebo; however these alterations do not always translate to positive changes in renal and blood parameters. Doses of glycerol varying between 1.0-1.2 g/kg in 20-26 ml/kg of water have led to significant increases in body weight and decreases in urine output. While urine output decreases, increased fluid retention rates have only been seen in a few studies from glycerol loads of 1.0-1.2 g/kg increasing fluid retention between 500-1000 ml (Coutts et al., 2002, Freund et al., 1995, Montner et al., 1999, Riedesel et al., 1987) leading to 6-7% increases in PV (Coutts et al., 2002, Freund et al., 1995, Hitchins et al., 1999); however others have presented no changes in fluid retention rates or PV changes (Latzka et al., 1997) from pre-exercise glycerol hyperhydration. It has been suggested that non-significant findings with regard to fluid retention and glycerol hyperhydration likely result from an insufficient fluid intake or hydration period prior to exercise (Nelson and Robergs, 2007).

One of the main objectives in manipulating pre-exercise hydration values for researchers is to provide positive cardiovascular changes during exercise. While few authors have noted pre-exercise glycerol hyperhydration decreases HR during exercise in the hyperthermic (Anderson et al., 2001) and moderate (Montner et al., 1996, Montner et al., 1999)

environments, many others have failed to find a difference (Goulet et al., 2006, Goulet et al., 2008, Lyons et al., 1990, Marino et al., 2003, Scheadler et al., 2010, Wingo et al., 2004). While many studies have focused on HR changes from glycerol ingestion, few have investigated the direct impact of glycerol on cardiovascular parameters such as stroke volume and cardiac output. Latzka et al. (1998) found a decrease in stroke volume (14%) as a result of treadmill walking at 55% VO_{2max} after glycerol hyperhydration (1.2 g/kg and 29.1 ml/kg BM) while also noting no change in cardiac output even with an increase in HR. In contrast, Montner et al. (1996) found an increase in stroke volume after hyperhydration (1.2 g/kg and 26 ml/kg BM) during cycling at 61% W_{max} but only as a result of continued glycerol ingestion during exercise. These discrepancies most likely result from differing exercise protocols, variations in environmental conditions, subjects' heat acclimation status, and whether or not the participants were allowed subsequent fluid ingestion during exercise.

The primary goal of pre-exercise glycerol hyperhydration for maintenance of thermoregulation is to increase fluids in the vascular space (Convertino, 1987) thus improving skin blood flow and heat dissipation (Nelson and Robergs, 2007). Findings by Anderson et al. (2001) support this paradigm, where glycerol hyperhydration attenuated the rise in Tc during 90 min of cycle exercise at 98% LT. These results are supported by those of Montner et al. (1998) who found glycerol ingestion significantly lowered exercise Tc compared to placebo during 110 min of cycling exercise at 44% VO_{2max}. However, these results appear isolated, as the majority of the research has found no aid to thermoregulation with glycerol ingestion (Goulet et al., 2006, Goulet et al., 2008, Lyons et al., 1990, Marino et al., 2003, Scheadler et al., 2010, Wingo et al., 2004). It is likely that the equivocal

findings may result from differences in study design, subject fitness/heat-acclimation status and additional fluid intake/beverage temperature during exercise thus this area warrants further investigation.

2.6. Effects of Hyperthermia and Dehydration on Oxidative Stress

As the primary focus of this thesis was to investigate the effects of dehydration on oxidative stress during hyperthermic exercise, it is important to summarise the current literature linking hyperthermia, dehydration and oxidative stress. As has been detailed previously (section 2.4), the addition of environmental heat stress both passively and during exercise can increase the stress levels of the participants. Accordingly, a few authors have investigated the impact of hyperthermia alone on oxidative stress (Laitano et al., 2010, McAnulty et al., 2005, Ohtsuka et al., 1994). Ohtsuka et al. (1994) used a 10 min passive heating protocol in 42˚C hot water bath to induce a significant decrease in red blood cell GSH concentration and GPx activity and a significant increase in red blood cell TBARS. Similarly, Laitano et al. (2010) used a 75 min exposure to a hot water-perfused body suit to increase Tc by 2˚C to measure resting and exercise-induced changes in glutathione. The authors found an increase in whole blood GSSG concentration as a result of passive heating while the addition of exercise exacerbated these findings, whereas whole blood GSSG concentration was not affected by exercise without hyperthermia. Importantly, the authors noted these findings were independent of changes to hydration status because participants were allowed to ingest fluid *ad libitum* which maintained euhydration (as assessed by changes in BM). Similar results were found from an investigation by McAnulty et al. (2005) who had six male athletes run on a treadmill in 35˚C conditions at 50% VO_{2max} until they reached a Tc of 39.5˚C (~50 min). The authors found increased levels of plasma FIP

and LOOH as a result of exercise, however only FIP concentration was augmented by the hyperthermic exercise compared to normothermic exercise. However, it is very important to note that the authors of this study failed to take into account the hydration status of the subjects, who had become dehydrated by ~3%, which may have played a role in the significant findings.

Indeed dehydration has been shown both *in vitro* (Martins and Meneghini, 1994, Saha et al., 1992) and *in vivo* (Paik et al., 2009) to affect oxidative stress. Hypoosmolality has also been shown to attenuate DNA damage by hydrogen peroxide in Chinese hamster fibroblasts (Martins and Meneghini, 1994), while liver cell shrinkage induced by hyperosmolality creates some oxidative stress as indicated by an increasing GSSG excretions (Saha et al., 1992). To the knowledge of the author, only one study exists that has aimed to investigate the effects of dehydration on oxidative stress. Paik et al. (2009) utilised a pre-exercise dehydration protocol consisting of exercise and passive hyperthermia in 10 moderately trained men. Following dehydration, subjects either remained dehydrated or were euhydrated with water or carbohydrate beverage. The authors also included a control group who did not undergo the dehydration protocol. Following rehydration, participants ran to exhaustion at 80% VO_{2max} on a treadmill (time to exhaustion ranged from 28-42 min). Blood parameters were measured pre dehydration, post dehydration, post rehydration, and post exercise. The authors found increased MDA concentration, total antioxidant capacity and DNA damage as a result of the dehydration protocol alone. What is interesting is that the authors noted no increase in MDA concentration after rehydration prior to exercise. It must be noted, however that the authors used heat as a means for dehydrating subjects and this may have confounded their results.

In light of the findings of Paik et al. (2009), it appears that hydration status prior to and during exercise may play a role in attenuating the resulting oxidative stress. This would rationalise the use of substances such as glycerol prior to exercise to increase body water. Because glycerol is distributed evenly intra- and extracellularly, this would likely pull water into the cells, maintaining cell integrity in an effort to provide a "buffer" before stress, which fluid consumption during exercise would likely maintain.

While it is well known how hyperthermia and exercise can affect HSP expression, the effects of hydration status on HSP72 and HSP32 expression, specifically during exercise, have not been thoroughly investigated. However, to the author's knowledge no human research exists linking these topics. In fact only one study examining the effect of hydration on HSP72 expression exists. In cultured rat hepatocytes Kurz et al. (1998) showed that moderate hyperosmolality as a result of dehydration blocks the heat induced expression of HSP72, causing impaired protein synthesis and thermotolerance. Conversely, while dehydration can block HSP72 induction, hyperhydration and hypoosmotic swelling can increase HSP72 induction (Kurz et al., 1998). Because HSP32 has not been studied widely in exercising subjects, no data exist linking HSP32 expression to hydration status. As HSP32 is induced as a result of oxidative stress and hydration status can have an impact upon cellular susceptibility to oxidative damage, it is inferred that dehydration would result in augmented expression of HSP32.

In summary, while the effects of hyperthermia and dehydration have been thoroughly investigated and demonstrate a severe threat to homeostasis, and while *in vitro* work

demonstrates these two phenomena can adversely affect the body's ability to cope with oxidative stress, properly controlled *in vivo* work is lacking. Therefore this thesis aims to clarify the relationship between hyperthermia, dehydration and oxidative stress during prolonged 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 first two experiments were conducted in a greenhouse with temperature (dry bulb) and relative humidity controlled by the author. Average thermoneutral conditions were 22.2 ± 0.0˚C and 41.4 ± 3.3%, while average warm conditions were 33.4 ± 0.4˚C and 43.2 ± 2.9% for temperature and relative humidity, respectively. For experiment three, all trials were completed in an environmental chamber (Model SSR60-20H, Design Environmental Ltd, Wales, UK) controlled via computer at a temperature of 35˚C and relative humidity of 40%. Temperature sensors were calibrated before all experiments.

The protocol employed in the first two experimental chapters was designed as a fixed intensity exercise for a prolonged duration. Exercise intensity was prescribed based on each individual's lactate threshold (LT) because it has been shown to be more accurate for predicting endurance exercise performance compared to maximal oxygen uptake (VO_{2max}) (Coyle et al., 1988, Yoshida et al., 1987). Following the initial bout of exercise was a 5 km TT for additional acute stress and for assessment of performance. The protocol was designed to maximise dehydration and stress while trying to standardise the amount of work performed by each participant. During the third experimental chapter subjects were asked to cover as much distance as possible during a 90 min ride that was self-paced to reflect real cycling training and performance. The experimental protocols for all three chapters were designed to result in maximal stress and fatigue without participants becoming exhausted (where they would hypothetically be unable to continue exercising). Although the exercise protocol for the first two experiments was prescribed at a fixed

intensity, subjects were able to deviate from this intensity by slowing down/speeding back up during the conditions that were extremely taxing to their system, ultimately making the protocol self-paced; we allowed this and preferred it over the participants ceasing exercise, therefore I accept this as a possible limitation to the first two studies.

3.1. Subjects

The participants for each experiment performed in this thesis were healthy male subjects, aged between 18 and 45. Subjects for the first and third experiments were considered well trained cyclists and triathletes recruited from local clubs, while subjects for the second experiment were considered healthy, but untrained university students, partaking in no organised physical activity. 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/supplementation that would affect the variables measured in this thesis (including vitamin supplements, ergogenic aids and anti-inflammatory medications). Because smoking and caffeine consumption have the potential to affect HSP and oxidative stress markers (Krisko et al., 2005, Varma et al., 2010, Whitham et al., 2006), all participants were non-smokers and were asked to refrain from caffeine consumption for 24 h prior to and during all experimental conditions. Additionally, subjects were asked to refrain from alcohol consumption and exercise for 24 hours prior to testing.

3.2. Anthropometric Data

Body mass (kg) and height (cm) were used as subject descriptive data and were measured using digital scales accurate to ± 100 g (TANITA WB-100 Yiewsley, UK) and Holtain Stationmaster (Holtain Ltd, Crymych, Dyfed) respectively.

3.3. Preliminary Fitness Assessment

For experiments one and two, all subjects underwent initial LT testing on an SRM cycle ergometer (Schoberer Rad Mebtechnik, Konigskamp, Germany) using an incremental protocol starting. For the trained cyclists, this protocol began at 100 W, while for the untrained participants the protocol began at 50 W. The protocol increased 20 W every 4 min until exhaustion (~30 min) for all participants. Capillary blood samples were collected every 2 min into lithium heparinised microvettes (Microvette CB300, Sarstedt, Numbrecht, Germany) and analysed using a blood lactate analyser (YSI 2300 STAT, YSI Inc, Yellow Springs, OH). Lactate threshold was calculated using the Dmax method (Cheng et al., 1992). Following completion of the LT test and a rest period (10-20 min), subjects completed a 5 km time trial (TT) familiarisation where they were instructed to complete the 5 km distance as quickly as possible. Subjects were able to visualise their distance covered and PO; however they were blinded to their elapsed time. This protocol was used for the first two experiments; however the fitness status of participants in experiment three was not assessed because the protocol was self-paced rather than at a fixed power output, as were the two previous studies. Participants of all three experiments completed detailed training logs indicating their fitness status.

3.4. Hydration Status Assessment

Upon arrival to the laboratory and prior to exercise, hydration status of subjects was assessed using urine (UOsm) and serum (SOsm) osmolality (collected via venipuncture, see section 3.6) measured by freeze-point depression method (Advanced Instruments Model 3320, Advanced Instruments Inc, Massachusetts, USA). Additional time points for assessment of UOsm and SOsm are highlighted in their respective chapters.

3.5. Thermoregulation Measurement

While it was not originally an intention of this thesis to observe the effects of thermal strain on oxidative stress markers, only Tc was measured during the initial experiment. However, after further research it became clear that there may be a link between thermal stress and oxidative stress; therefore the author deemed it necessary to add measurement of skin temperature (Ts) during experiments two and three. Tc was measured using rectal thermometers (Grant Instrument, Cambridge, UK) inserted by the participants 10 cm past the anal sphincter. Ts was measured using four skin thermistors (Grant Instrument, Cambridge, UK) placed on the chest, lateral bicep, medial thigh and lateral calf on the left side of the body. Skin thermistors placement was measured individually to ensure positioning directly on muscles, avoiding placement on bone or tendon. Signals from both the rectal thermometer and skin thermistors were received by a data logger system (Squirrel 1000, Grant Instrument, Cambridge, UK). Ratings of perceived exertion (RPE) (Borg, 1970), thermal sensation (Gagge et al., 1969), and sweating sensation (Hostler et al., 2009) along with heart rate (HR), Tc and Ts were measured at 5 min intervals.

Mean Ts was calculated using the following equation (Ramanathan, 1964):

$$\text{Ts} = [0.3(T_{\text{arm}} + T_{\text{chest}})] + [0.2(T_{\text{thigh}} + T_{\text{calf}})]$$

where T_{arm} is arm temperature, T_{chest} is chest temperature, T_{thigh} is thigh temperature, and T_{calf} is calf temperature.

Mean body temperature (Tb) was calculated using the following equation (Colin et al., 1971):

$$\text{Tb} = (0.79 \times Tc) + (0.21 \times Ts)$$

Heat storage (HS) was calculated using the following equation (Adams et al., 1992):

$$\text{HS}\ (\text{W} \cdot \text{m}^{-2}) = 0.965 \times \frac{\text{BM} \times \Delta\text{Tb}}{\text{Ad}}$$

where 0.965 is the specific heat of body tissues in $Wh^{-1}°C^{-1}kg^{-1}$, BM is body mass in kg, ΔTb is change in mean body temperature, and Ad is body surface area in m^2 (Dubois and Dubois, 1989).

Physiological strain (PSI) was measured using the following equation (Moran et al., 1998):

$$\text{PSI} = 5 \times \frac{Tc_{\text{a}} \times Tc_{\text{b}}}{39.5 \times Tc_{\text{b}}} + \frac{HR_{\text{a}} \times HR_{\text{b}}}{180 \times HR_{\text{b}}}$$

where Tc_a is the post exercise Tc, Tc_b is the pre exercise Tc and HR_a is the post exercise HR and HR_b is the pre exercise HR.

3.6. Blood Collection

While the author would have preferred to cannulate participants, allowing for serial blood sampling, this was not possible due to University ethics restrictions. Therefore, venous blood samples were drawn by standard venipuncture technique from an antecubital vein into K_3EDTA (HSP72, HSP32, TBARS, glycerol, and glucose), sodium citrate (TGSH and GSSG), lithium heparin (PC, LOOH, haematocrit, and haemoglobin), and clot activator serum (SOsm) vacuette tubes (Vacuette®, Greiner Bio-one, UK). For HSP analysis, whole blood was sampled from K_3EDTA tubes prior to their centrifugation. For attainment of serum, vacuettes were stored for at least 30 min at room temperature then centrifuged at 3000 x *g* after which supernatants were collected and subsequently stored at -80˚C for later analysis. Plasma was collected following identical procedures; however, vacuettes were centrifuged after collection.

During the first two experimental studies, blood samples were collected at the following time points: pre exercise, post 90 min, and post TT; however due to collection issues, missing samples, and budgetary constraints, only samples from pre exercise and post TT were analysed for HSP72, HSP32, TBARS and whole blood glutathione (HSP32 was not analysed during the second experiment because the antibody was unavailable from any manufacturer for an undetermined length of time). During the third experimental study, blood was sampled pre ingestion, post ingestion/pre exercise, post exercise and one hour post exercise. Ideally the author would have liked to have a further blood sample at four hours post exercise as PC concentration has been shown to increase up to this point (Michailidis et al., 2007); however because of cost and because the subjects had already spent 5 hours in the lab we found it impractical to ask them to remain a further two hours.

Due to the reported lack of specificity in the TBARS marker (Oh-ishi et al., 2000), this was not analysed in the third study and in its place blood was analysed for LOOH and PC content, which are considered to be more accurate and reliable markers of oxidative stress. Additionally, because the levels of HSP did not have a remarkable response to changes in hydration or environment and because of the expense involved in the analysis, the author decided to opt for additional measures of oxidative stress instead of HSP analysis for the third experiment.

3.7. HSP72 and HSP32 Assay

Numerous experimental methods exist to measure HSP72 and HSP32. The literature acknowledges that flow cytometric detection of HSP's is a rapid, easy, accurate and quantitative method, which is more sensitive than western blotting (Bachelet et al., 1998). It is for these reasons that flow cytometry was used to determine intracellular concentrations of HSP72 & HSP32. Of the peripheral blood mononuclear cells (PBMCs), lymphocyte HSP72 expression at rest and changes during exercise have been shown to be negligible (Fehrenbach et al., 2000b). Monocytes appear to be the most sensitive of the PBMCs and express the greatest amount of HSP72 & HSP32 in response to exercise and heat stress (Fehrenbach et al., 2003a, Fehrenbach et al., 2000b), while HSP32 has been shown to protect lymphocytes from oxidative stress (Speit et al., 2000), and therefore investigation of HSP32 is necessary in these cell populations.

For analysis of intracellular HSP72 and 32, whole blood (100 µl) from EDTA tubes was transferred into 2 ml red blood cell lysing buffer (Erythrolyse, Item No: BUF04B, AbD Serotec, Oxford, UK). Cells obtained after red cell lysis were fixed and permeabilised

(Leucoperm, Item No: BUF09B, AbD Serotec, Oxford, UK) then IgG1 (HSP72 FITC, Item No: ADI-SAB-600FI-200, Enzo Life Sciences, Exeter, UK) or IgG2b (HSP32 PE, Item No: ADI-SAB-602PE-F, Enzo Life Sciences, Exeter, UK) monoclonal antibody or corresponding negative control (HSP72: SPA-810, HSP32: OSA-111, Enzo Life Sciences, Exeter, UK) was added to a final concentration of 100 µg/ml and then incubated for 30 min in the dark. Samples were then washed with phosphate buffered saline before analysis on a BDFACSCalibur (BD Biosciences) with mono- and lymphocytes gated by forward/side scatter properties. Mean fluorescence intensity (MFI) was then calculated using CELLQuest software (BD Biosciences) with a total of 25,000 cells counted. Final HSP values were calculated from the difference in pre and post exercise MFI. Gating strategies are shown in Figure 3.1.

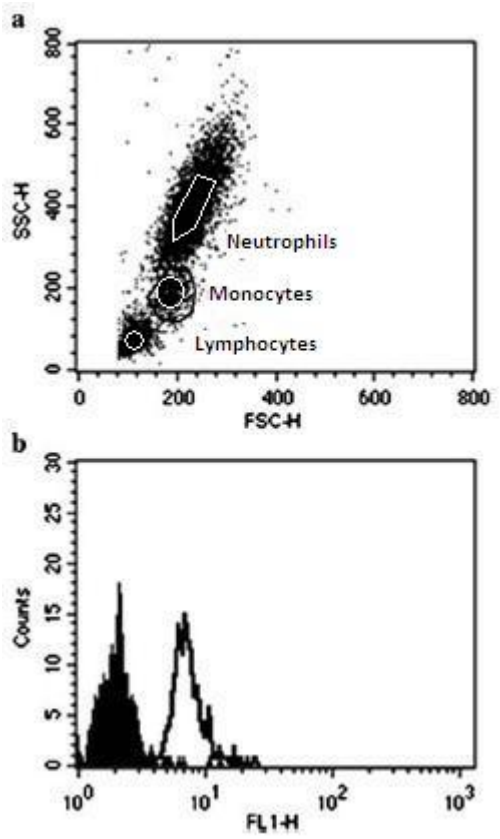

Figure 3.1. Typical flow cytometry profiles, showing **a)** forward scatter/side scatter and gated neutrophils, monocytes, and lymphocytes and **b)** fluorescence intensity of monocytes incubated with isotype matched negative control (*solid*) and anti-hsp72 (*line*) antibodies.

3.8. Blood Measure of Oxidative Stress

3.8.1. Glutathione

Blood glutathione status has been reported to accurately indicate the redox status in the body (Veskoukis et al., 2009). Immediately after collection of sodium citrate treated blood a 2 ml aliquot was mixed with 8 ml of freshly prepared 5% meta-phosphoric acid (Sigma-Aldrich Company Ltd., Dorset, England) to facilitate removal of proteins that can interfere with the reaction and to preserve the amount of GSH present when the samples are collected. This mixture was transferred to 1.5 ml eppendorf tubes, stored on ice for 15 min, then centrifuged at 13,000 x g and 4˚C for 15 min. The supernatant was collected and

stored at -80°C for later analysis of TGSH and GSSG using a commercially available kit (Total Glutathione Detection Kit, Assay Designs, Item No: ADI 900-160, Enzo Life Sciences, Exeter, UK). The average inter- and intra-assay coefficients of variation for GSSG quantification were 1.74% and 1.77%, respectively, for all three experiments.

For determination of TGSH previously prepared blood (50 µl, described above) was diluted to 1:40 in assay buffer solution and transferred to a 96 well plate. A standard curve was produced from serially diluting 50 µl assay buffer and 50 µl GSSG standard. All wells were treated with a mixture containing 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) and 10 µl of glutathione reductase to form a chromagen (5-thio-2-nitrobenzoate (TNB)), the concentration of which was measured every minute for 10 min at 405 nm in a microplate reader (Biotek Synergy HT-R, Biotek Instruments, Vermont, USA). This procedure was replicated for determination of GSSG after the samples were first treated with 1 µl of 2M 4-vinylpyridine (Sigma-Aldrich Company Ltd., Dorset, England) to block any free thiols from completing the reaction, thus overestimating the actual concentration of GSSG. At the same time 6 µl of 2M 4-vinylpyridine solution was added to 300 µl GSSG for standard curve determination. GSSG standards and samples were incubated at room temperature for 1 h and analysed identical to the TGSH samples. All standards and samples were analysed in triplicate with the mean reported. The average inter- and intra-assay coefficients of variation for TGSH quantification were 3.24% and 3.25%, respectively, for all three experiments.

3.8.2. TBARS

TBARS was measured for the first two experiments using a commercially available kit (ZeptoMetrix, Helvetica Health Care, Item No: 0801192, Geneva, Switzerland). An aliquot (100 µl) of previously prepared plasma 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 x g for 15 min. Supernatant was collected and added to a 96 well plate and read at 532 nm in a microplate reader. A malondialdehyde (MDA) standard curve was constructed and TBARS concentration was calculated by interpolation from this standard curve. Results are expressed in MDA equivalents. All standards and samples were analysed in duplicate. The average inter- and intra-assay coefficients of variation for TBARS quantification were 2.00% and 1.91%, respectively, for all three experiments.

3.8.3 Protein Carbonyls

Because of the reported lack of specificity in the TBARS assay, in the final study the author decided to replace the analysis with a more accurate measure of oxidative stress. Therefore, protein carbonyl content was measured using a commercially available kit (Protein Carbonyl Assay Kit, Item No: 10005020, Cayman Chemical, Cambridge Bioscience, Cambridge, UK). Previously prepared lithium heparinised plasma sample (200 µl, described in 3.6) was added to an eppendorf tube with 800 µl of dinitrophenylhydrazine (DNPH) serving as the sample tube while 200 µl of the same plasma was added to 800 µl of 2.5M hydrochloric acid (HCl) serving as the control tube. All tubes were incubated in the dark at room temperature for one hour, while being briefly vortexed every 15 min. An aliquot (1 ml) of 20% trichloroacetic acid (TCA) was added to each eppendorf and

vortexed. All tubes were incubated on ice for 5 min then centrifuged at 10,000 x g for 10 min at 4˚C followed by a wash in 10% TCA, 5 min incubation on ice and centrifugation at 10,000 x g for 10 min at 4˚C. The supernatant was discarded and the pellet resuspended in 1 ml of a 1:1 mixture of ethanol/ethyl acetate and vortexed thoroughly, then centrifuged at 10,000 x g for 10 min at 4˚C. This step was repeated two more times followed by resuspension of the pellet in 500 µl of guanidine hydrochloride. After vortexing, tubes were centrifuged a final time at 10,000 x g for 10 min at 4˚C. An aliquot (200 µl) of supernatant from each sample and control tube was added to a 96 well plate and read in a microplate reader at an absorbance of 360 nm. All samples and standards were analysed in duplicate.

Protein carbonyl concentration was calculated using the following equation:

$$\text{PC (nmol/ml)} = \frac{\text{CA}}{0.011\ \mu\text{M}} \times \frac{500\ \mu\text{l}}{200\ \mu\text{l}}$$

where CA is the average absorbance of the controls subtracted from the average absorbance of the samples and 0.011 μM^{-1} is the extinction coefficient of DNPH, 500 µl/200 µl gives the concentration of protein carbonyls in the original sample. The inter- and intra-assay coefficients of variation for PC quantification were 2.12% and 2.14%, respectively.

3.8.4 Lipid Hydroperoxides

Similar to above, because TBARS has been found to be non-specific to MDA, the author substituted its measurement with a more sensitive marker of lipid peroxidation. Therefore, lipid hydroperoxides concentration (LOOH) was measured using a commercially available kit (Lipid Hydroperoxide Assay, Item No: NWK-LHP01, Northwest Life Science Specialities, LLC, AMS Biotechnologies, Abingdon, UK). The assay is based on the

reaction of LOOH with ferrous iron to form ferric iron which forms a chromagen at a measurable absorbance of 560 nm. While the measurement of LOOH by assay kit has commonly been criticised because LOOH are unstable in the presence of reducing metals, this kit corrected for this, as well as the presence of any H_2O_2 by treating samples with tris(2-carboxyethyl)phosphine (TCEP) and catalase.

To measure LOOH concentration, previously prepared lithium heparinised plasma (100 µl, described in 3.6) was added to three eppendorfs, one served as a sample (X), one as blank (Y) and one as recovery (Z) while 100 µl of water was added to three vials servings as calibrator (A), reagent blank (B) and reducing reagent blank (C). An aliquot (5 µl) of catalase was added to all eppendorfs, vortexed and incubated for 10 min at room temperature. An aliquot (500 µl) of methanol-butylated hydroxytoluene (BHT) solution was added to all eppendorfs, vortexed and centrifuged at 10,000 x g for 5 min. An aliquot (10 µl) of water was added to all B and X eppendorfs while 10 µl of TCEP reagent was added to all C and Y eppendorfs and 10 µl of calibrator was added to all A and Z eppendorfs. All eppendorfs were incubated one hour at room temperature followed by the addition of 50 µl of XOF reagent (1:1 mixture of xylenol orange and iron) to all eppendorfs. Samples were vortexed and incubated one hour at room temperature. Following centrifugation at 10,000 x g for 5 min a 180 µl aliquot of supernatant from each eppendorfs was added in duplicate to a 96 well microplate and read at an absorbance of 560 nm. LOOH concentration in the reaction mix was calculated using the following equation:

$$\text{LOOH } (\mu\text{M}) = \frac{\text{Aa} - \text{Ab}}{\acute{\varepsilon}560} \times \frac{\text{Ax} - \text{Ay} - \text{Ab} - \text{C}}{\text{Az} - \text{Ax}}$$

where A_A-A_B is the absorbance of the calibrator (A) minus the absorbance of the reagent blank (B), ε560 is the measured absorbance divided by the concentration of LOOH

multiplied by the path length (1 cm), A_X is the sample (X) absorbance, A_Y is the sample blank (Y), A_Z is the calibrated sample (Z) and A_{B-C} is the absorbance of the reagent blank (B) minus the absorbance of the reduced reagent blank (C). The inter- and intra-assay coefficients of variation for LOOH quantification were 10.82% and 7.02%, respectively.

3.9. Measurement of Plasma Glycerol

Many studies that employ the use of glycerol fail to provide blood concentrations, therefore in the third experiment plasma glycerol concentration was measured using a commercially available kit (Glycerol Assay Kit, Item No: 10010755, Cayman Chemicals, Cambridge Bioscience, Cambridge, UK). A 10 µl aliquot of previously prepared K_3EDTA plasma (described in 3.6) was added to each sample well of a 96-well plate. A reaction was initiated by adding 150 µl of glycerol enzyme mixture (including glycerol kinase, glycerol phosphate oxidase, and peroxidise) to each well. The plate was incubated at room temperature for 15 min then the absorbance was read at 540 nm on a microplate reader. All samples and standards were analysed in duplicate. Glycerol concentration was calculated using the following equation:

$$\text{Glycerol (mg/L)} = \frac{CA - \text{y-intercept}}{slope}$$

where the corrected absorbance (CA) was calculated by subtracting the absorbance value of the standard with 0 mg/L of glycerol from all standards and samples and y-intercept and slope were obtained from a graph of the standard curve (glycerol concentrations of 0-1000 mg/L). The inter- and intra-assay coefficients of variation for LOOH quantification were 1.94% and 3.02%, respectively.

3.10. Plasma Volume Changes

Because changes in plasma volume can effect blood concentrations of biochemical markers (Kargotich et al., 1997), all concentrations of blood oxidative stress markers, as well as concentrations of glucose and glycerol were corrected for plasma volume changes. In the first two experimental chapters haematocrit and haemoglobin were measured by automated blood gas analyser (ABL800 Basic, Radiometer Medical, Brønshøj, Denmark), however because these machines can inaccurately quantify these markers, for the final study capillary blood samples were collected for measurement of haematocrit (Micro Haematocrit Reader, Hawksley, UK) and haemoglobin (Hemocue 201, Hemocue, Ltd, Sheffield, UK). All measurements were taken in triplicate. For all three experiments, plasma volume changes were calculated using the methods of Dill and Costill (1974) using the following equation:

$$\text{PV change} = \left[\left(100 - \frac{\text{Hb}_b}{\text{Hb}_a}\right) \times \frac{1\text{-}(\text{Hct}_a\text{-}100)}{1\text{-}(\text{Hct}_b\text{-}100)}\right] - 100$$

Oxidative stress markers, glucose and glycerol concentrations were corrected for these plasma volume changes using the following equation:

$$\text{Corrected concentration} = \frac{(uncorrected\ concentration\ \times 100)}{100 +/- PV\ change}$$

Chapter 4. Experiment 1: Exercise-induced dehydration during prolonged exercise results in increased oxidative stress.

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

Hillman AR, Vince RV, Taylor L, McNaughton L, Mitchell N and Siegler J. (2011) Exercise-induced dehydration with and without environmental heat stress results in increased oxidative stress. *Appl Physiol, Nutr and Metab*, 36, 698-706.

Abstract

While *in vitro* work has revealed dehydration and hyperthermia can elicit increased cellular and oxidative stress, *in vivo* research linking dehydration, hyperthermia and oxidative stress is limited. The purpose of this experiment was to investigate the effects of exercise-induced dehydration with and without hyperthermia on oxidative stress. Seven healthy male trained cyclists (mean ± SD) age: 36 ± 6 yrs, height: 177.4 ± 6.5 cm, weight: 72.8 ± 7.0 kg, and power output (PO) at lactate threshold (LT): 199.3 ± 19.0 Watts (W) completed 90 min cycling exercise at 95% LT followed by a 5 km time trial (TT) in four trials: euhydration in a warm environment (EU-W), dehydration in a warm environment (DE-W), euhydration in a thermoneutral environment (EU-T), and dehydration in a thermoneutral environment (DE-T) (W: 33.9 ± 0.9°C; T: 23.0 ± 1.0°C). Whole blood oxidised glutathione (GSSG) increased significantly post exercise in dehydration trials only (DE-W: $p < 0.01$, DE-T: $p = 0.03$), and while not significant total glutathione (TGSH) and thiobarbituric acid reactive substances (TBARS) tended to increase post exercise in dehydration trials ($p = 0.08$ for both). Intracellular monocyte heat shock protein 72 (HSP72) concentration was increased ($p = 0.01$) while Intracellular lymphocyte HSP32 concentration was decreased for all trials ($p = 0.02$). Exercise-induced dehydration led to an increase in GSSG concentration while maintenance of euhydration attenuated these increases regardless of environmental condition. Additionally, we found evidence of increased cellular stress (measured via HSP) during all trials independent of hydration status and environment. Finally, total distance covered and 90 min PO were decreased, while 5 km TT completion time was slower during the DE-W vs. all other trials. These findings highlight the importance of fluid consumption during exercise to attenuate thermal and oxidative stress during prolonged exercise in the heat.

4.1. Introduction

Exercising for prolonged periods in the heat without adequate fluid intake can lead to dehydration (Sawka et al., 2007) and oxidative stress (Paik et al., 2009), both of which are believed to have a negative impact upon performance. A primary cause of decreased performance as a result of dehydration is believed to culminate from a progressive increase in cardiovascular and thermal strain, which can be exacerbated when exercising in a hyperthermic environment (Cheuvront et al., 2010, Montain and Coyle, 1992). On a cellular level, dehydration has been shown to cause hyperosmolality and cellular shrinkage (Schliess and Haussinger, 2002). The extent of the dehydration, as with performance, may dictate the degree of cellular susceptibility to oxidative damage and apoptosis (Schliess and Haussinger, 2002). Additionally, altering the hyperthermic environment under such conditions may further influence the vulnerability of the cell (Flanagan et al., 1995), however this has yet to be documented in humans.

The oxidative damage occurring during exercise is derived primarily from reactive oxygen species (ROS). Although ROS are produced at low levels under normal physiological conditions and are an important component to many signalling pathways, the increase in ROS observed during exercise can lead to oxidative stress (Alessio, 1993), measurable via the formation of by-products (thiobarbituric acid reactive substances (TBARS) or the antioxidant defence systems (glutathione)). Specifically, oxidative damage often occurs to the lipid membranes of cells, the by-products of such events becoming harmful radicals that may further initiate protein and DNA damage (Powers and Jackson, 2008). If excessive ROS production continues, ultimately, normal cell function will fail (Fisher-Wellman and Bloomer, 2009), leading to activation of inflammatory and cell death pathways (Mattson,

2006), as well as decreased force production during exercise (Reid, 2001). The degree of oxidative damage, in relation to exercise, has been shown to be intensity and duration dependent (Goto et al., 2003, Knez et al., 2007). Presumably the damage may be exacerbated further if these conditions coincide with extreme environmental conditions (i.e. heat stress), however this has not been investigated *in vivo* and warrants further elucidation.

While *in vitro* research has shown increased cellular susceptibility to oxidative damage and apoptosis (Schliess and Haussinger, 2002), decreased cell-stress tolerance (evidenced by decreased HSP72 expression (Kurz et al., 1998)) and increased DNA damage (Martins and Meneghini, 1994) as a direct result of dehydration, there is limited *in vivo* research linking exercise-induced oxidative stress and hydration. To our knowledge, only one previous study has investigated the effects of dehydration on oxidative stress *in vivo* (Paik et al., 2009). Although the authors reported an increase in oxidative stress as a result of passive dehydration, methodological limitations may have confounded their results as heat stress alone has been shown to increase oxidative stress (Laitano et al., 2010). Alternatively, while there are studies that have used hyperthermia as a means of investigating changes in oxidative stress, these investigations have not accounted for the hydration status of their participants (McAnulty et al., 2005, Ohtsuka et al., 1994) even though dehydration has been shown to increase oxidative stress (Paik et al., 2009, Saha et al., 1992). Therefore, the purpose of this investigation was to determine the effect of exercise-induced dehydration with and without hyperthermia on oxidative and cellular stress. It was hypothesised that exercise-induced dehydration would increase oxidative stress and in conjunction with hyperthermia would augment the level of stress compared to exercise-induced dehydration without hyperthermia.

4.2. Methods

Subjects

Seven competitive male cyclists, unacclimatised to heat, participated in this study. Physical characteristics (mean ± SD) were age: 36 ± 6 yrs, height: 177.4 ± 6.5 cm, weight: 72.8 ± 7.0 kg, and power output (PO) at lactate threshold (LT): 199.3 ± 19.0 Watts (W). Subjects were free of any cardiac or metabolic diseases, did not smoke, and refrained from supplementation of all kinds (i.e., vitamins, ergogenic aids, etc.) during the testing period. A sample size of 7 was not dictated by power analysis but rather due to time and budgetary constraints.

Preliminary Measurements

All subjects underwent initial lactate threshold (LT) testing as described in section 3.3

Experimental Design

The remaining four visits consisted of exercise trials in either a warm environment (33.9 ± 0.9˚C) with (DE-W) or without dehydration (EU-W), or a thermoneutral environment (23.0 ± 1.0˚C) with (DE-T) or without dehydration (EU-T). All trials were completed in an environmentally controlled greenhouse and separated by at least one week. Trials were randomised by environment only (i.e., they started in either warm or thermoneutral environment and always started with a dehydration trial) as it was necessary to estimate the volume of fluid subjects would need in order to maintain euhydration during the EU-W and EU-T trials. On trial days, subjects reported to the laboratory 2 hours postprandial and having consumed 500 ml water in accordance with the ACSM position stand (Sawka et al.,

2007). Subjects were asked to keep a food diary and consume the same diet prior to and on all trial days. Additionally, they were requested to refrain from caffeine or alcohol consumption and strenuous exercise for the 24 hour period prior to each trial.

Upon arrival to the laboratory subjects rested in an inclined-supine position for 20 min prior to collection of pre blood samples (described below). To assure subjects were in a euhydrated state prior to exercise, urine (UOsm) and serum osmolality (SOsm) were measured in triplicate by freeze-point depression (Advanced Instruments Model 3320, Advanced Instruments Inc, Massachusetts, USA). Subjects self-measured their nude weight (±100 g) (TANITA WB-100 Yiewsley, UK) then placed a rectal thermometer (Grant Instrument REC 100 mm) 10 cm past the anal sphincter and positioning of a recordable heart rate monitor (S810i, Polar Electro, OY, Finland). All exercise protocols were completed on a Velotron cycle ergometer (RacerMate, Seattle, WA, USA), which has been validated for continuous exercise (Abbiss et al., 2009) and has been shown to provide highly reproducible performance data in competitive cyclists (Sporer and McKenzie, 2007). The Velotron was factory calibrated and the zero-point was reset before beginning the study. Subjects completed a 5 min warm up at 70 W and 80 rpm followed by completion of 90 min of exercise at 95% of each individual's LT, with a pause at 45 min for collection of blood. The authors designed this protocol to ensure $\geq$ 2% dehydration was achieved during both environmental conditions. The protocol also is similar to previous research investigating changes in redox status, particularly glutathione (Gohil et al., 1988, Morillas-Ruiz et al., 2005, Viguie et al., 1993). During the 90 min protocol subjects were instructed to maintain their pace by following a computer pacer set to 95% of each individual's LT. After completion of the 90 min exercise a rest period of 15 min was use to facilitate blood

draw and prepare the cyclists for the TT. During the 5 km TT subjects were asked to complete the trial as quickly as possible while being blinded to the elapsed time. The addition of the 5 km TT after the 90 min ride was to ensure subjects were adequately stressed to provide changes in redox status. Rest periods and TT were conducted in the same environment as exercise at 95% LT. A final blood collection and nude weight were taken after completion of the 5 km TT.

In line with previous investigations (Gonzalez-Alonso et al., 2000, Greenleaf and Castle, 1971, Kenefick et al., 2009), core temperature (T_c), ratings of perceived exertion (RPE, Borg scale), room temperature, and humidity were recorded every 5 min during exercise. HR and PO were recorded every 5 sec; this data was then averaged over 5 min to match the recording times of the other variables. Fluid was prohibited during dehydration trials, while during euhydration trials subjects consumed the same quantity of water as body weight lost during the dehydration trial (1 kg BM = 1 L water).

Blood Collection and Analysis

Blood was drawn from a vein in the antecubital region pre-exercise, at 45 min, post 90 min, and post TT; however only blood samples from pre-exercise and post TT were analysed due to budgetary constraints. HSP analysis was conducted live (see general methods section 3.7) while TGSH, GSSG (see general methods section 3.8.1) and plasma TBARS (see general methods section 3.8.2) were conducted retrospectively. Haematocrit and haemoglobin were measured from whole blood immediately after draw (ABL800 Basic, Radiometer Medical, Brønshøj, Denmark) and used to calculate plasma volume changes.

All oxidative stress markers were corrected for plasma volume changes (see general methods section 3.10.).

Statistical Analysis

All data are represented as mean ± SEM. Statistical analysis was completed using Linear Mixed Models for repeated measures (SPSS 18.0, Chicago, IL, USA) which allows for modelling with both fixed and random effects, as well as missing or incomplete data. Different covariance structures were assumed and the one that minimised the Hurvich and Tsai's criterion was chosen for the final model. Where a significant F ratio was observed, post hoc comparisons with Sidak-adjusted p values were used to identify which pairs of means were significantly different. Two-tailed statistical significance was accepted as $p < 0.05$. Data for glutathione and HSP changes are represented as percent change from baseline, which is in accordance with previous literature (Gohil et al., 1988, Morton et al., 2007).

4.3. Results

Thermoregulation

Table 4.1 represents the urine, haemodynamic and fluid changes for all trials. UOsm significantly increased post exercise in all trials ($p < 0.01$) while SOsm increased post exercise in dehydration trials only ($p< 0.01$). Body mass loss was significantly greater in DE-W vs. all other trials and PV change was significantly less in EU-T vs. all other trials.

Table 4.1. Presented are mean ± SEM (n = 7) data for changes observed in urine (UOsm) and serum (SOsm) osmolality (mOsm·kg^{-1}), body mass loss (BML in kg), percent BML (%BML), plasma volume (PVΔ as a %), and total fluid consumption (TFC in litres) after both 90 min of exercise and the 5 km time trial.

	Thermoneutral				Warm			
	Dehydration (DE-T)		Euhydration (EU-T)		Dehydration (DE-W)		Euhydration (EU-W)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
UOsm	226±71	481±95*	179±17	298±51*	206±21	463±72*	269±35	383±77*
SOsm	291±1	303±1*	292±1	290±1	291±1	305±1*	289±2	288±1
BML	--	2.1±0.2	--	0.1±0.2	--	2.7±0.2	--	0.2±0.4
%BML	--	3.0±0.3	--	0.1±0.2	--	3.8±0.3†	--	0.2±0.2
PVΔ	--	-7.1±1.0	--	-1.9±1.2‡	--	-6.3±1.5	--	-3.1±0.9
TFC	--	--	--	2.1±0.1	--	--	--	2.7±0.2

*Significantly increased vs. pre ($p < 0.01$)
†Significantly greater vs. DE-T ($p < 0.01$)
‡Significantly less than all other trials ($p < 0.01$)

Figure 4.1 represents the Tc and HR data for the 90 min exercise bout. HR and Tc significantly increased post exercise for all trials ($p < 0.01$). Additionally, Tc and HR were significantly higher post exercise in the warm vs. thermoneutral trials ($p < 0.01$ for both). During the TT, there was a main effect for condition ($F = 3.08$; $p = 0.05$) with lower Tc in EU-T vs. DE-T only. Additionally, while HR was increased post TT from resting conditions, there was no significant difference in average HR between trials ($F = 1.43$; $p = 0.27$).

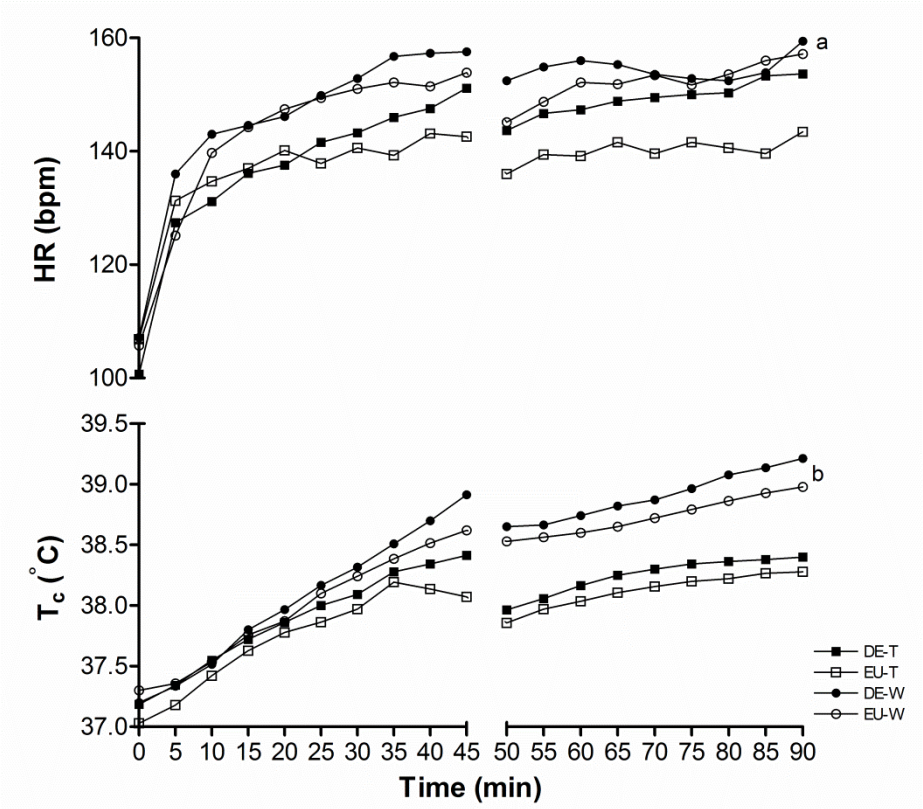

Figure 4.1. Presented are mean (*n* = 7) data for average heart rate (HR) and core temperature (Tc) during 90 min exercise in a thermoneutral environment with (DE-T) and without dehydration (EU-T), and a warm environment with (DE-W) and without (EU-W) dehydration. HR and Tc significantly increased post exercise for all trials ($p < 0.01$). [a]Significantly higher HR post exercise in warm vs. thermoneutral trials ($p < 0.01$). [b]Significantly higher Tc post exercise in warm vs. thermoneutral trials ($p < 0.01$). Error bars were omitted for clarity.

Cellular Stress

TGSH/GSSG

There was a significant interaction (condition x time) for GSSG ($F = 9.32$; $p < 0.01$) and a main effect for time ($F = 8.71$; $p = 0.02$), with an increase in GSSG post exercise for dehydration trials only (DE-W: $p < 0.01$, DE-T: $p < 0.05$). There was no main effect for condition ($F = 0.39$; $p = 0.77$) for GSSG pre to post exercise (*Figure 4.2*).

There was no significant interaction (condition x time) for TGSH ($F = 1.08$; $p = 0.37$). Similarly, there was no main effect for time ($F = 4.42$; $p = 0.08$) or condition pre to post

exercise (F = 0.05; p = 0.98). Assessing TGSH via relative changes (percent change pre to post) did not provide additional insight (F = 0.97; p = 0.43; *Figure 4.2*).

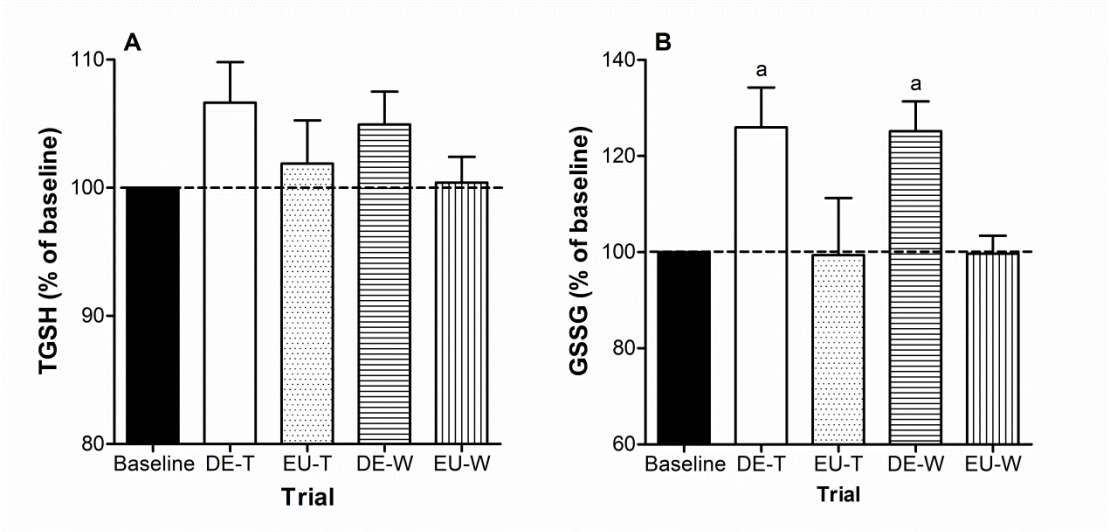

Figure 4.2. Presented are mean ± SEM (*n* = 7) data for baseline (pre) and percent change (pre to post) in total (TGSH) (**A**) & oxidised (GSSG) glutathione (**B**) pre to post exercise in a thermoneutral environment with (DE-T) and without dehydration (EU-T), and a warm environment with (DE-W) and without (EU-W) dehydration. [a]Significantly increased vs. baseline ($p < 0.01$).

TBARS

There was no significant interaction (condition x time) for TBARS (F = 0.75; p = 0.53). Similarly, there was no main effect for time (F = 2.36; p = 0.13) or condition (F = 2.38; p = 0.08; *Table 4.2*).

Table 4.2. Presented are mean ± SEM (*n = 7*) data for changes observed in oxidised (GSSG) and total (TGSH) glutathione expressed in pmol, thiobarbituric acid reactive substances (TBARS) in MDA equivalents ($nmol \cdot ml^{-1}$), monocyte HSP72 (*m*HSP72), lymphocyte HSP72 (*l*HSP72), monocyte HSP32 (*m*HSP32), and lymphocyte HSP32 (*l*HSP32) as mean fluorescence intensity after both 90 min of exercise and the 5 km time trial.

	Thermoneutral				Warm			
	Dehydration (DE-T)		Euhydration (EU-T)		Dehydration (DE-W)		Euhydration (EU-W)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
GSSG	91±9	115±13*	93±13	93±13	86±16	108±16*	96±15	96±17
TGSH	3342±181	3568±242	3432±200	3488±223	3377±193	3561±263	3425±185	3432±180
TBARS	16.4±1.7	23.7±5.4	17.0±3.6	18.1±1.9	15.6±1.1	23.6±6.4	11.9±1.2	13.4±1.3
*m*HSP72	3.6±0.6	4.6±0.6*	4.5±0.9	5.9±0.8*	3.3±0.5	4.4±0.2*	4.1±0.7	5.2±0.5*
*l*HSP72	1.9±0.5	1.9±0.3	1.8±0.6	2.1±1.2	1.6±0.3	1.9±0.2	1.7±0.2	1.9±0.3
*m*HSP32	26.8±9.0	26.2±13.1	36.2±12.4	32.8±9.8	32.4±4.6	27.0±5.2	36.7±17.2	30.6±10.2
*l*HSP32	25.8±2.6	22.5±2.5†	28.3±3.8	23.8±2.7†	26.0±1.5	24.0±3.0†	28.2±4.9	22.5±2.5†

*Significantly increased vs. pre ($p < 0.05$)
†Significantly decreased vs. pre ($p < 0.05$)

HSP

For HSP72 concentration in both monocytes and lymphocytes there was no significant interaction (condition x time) (monocytes: $F = 0.09$; $p = 0.97$; lymphocytes: $F = 0.87$; $p = 0.49$). Of the two cell types, only monocytes changed over time ($F = 15.59$; $p < 0.01$), with increased concentration post exercise but no there was difference apparent between conditions ($F = 1.56$; $p = 0.23$). For HSP32 concentration in monocytes, there was no significant interaction (condition x time) ($F = 0.82$; $p = 0.49$), nor was there any difference between time ($F = 0.60$; $p = 0.45$) or condition ($F = 1.10$; $p = 0.38$). However, for HSP32 concentration in lymphocytes, while there was no significant interaction (condition x time) ($F = 0.22$; $p = 0.88$), there was a significant main effect for time ($F = 5.10$; $p = 0.03$), with significant decreases in HSP32 concentration post exercise. Similar to monocytes, there was no main effect for condition ($F = 0.20$; $p = 0.89$; *Table 4.2* and *Figures 4.3-4.4*).

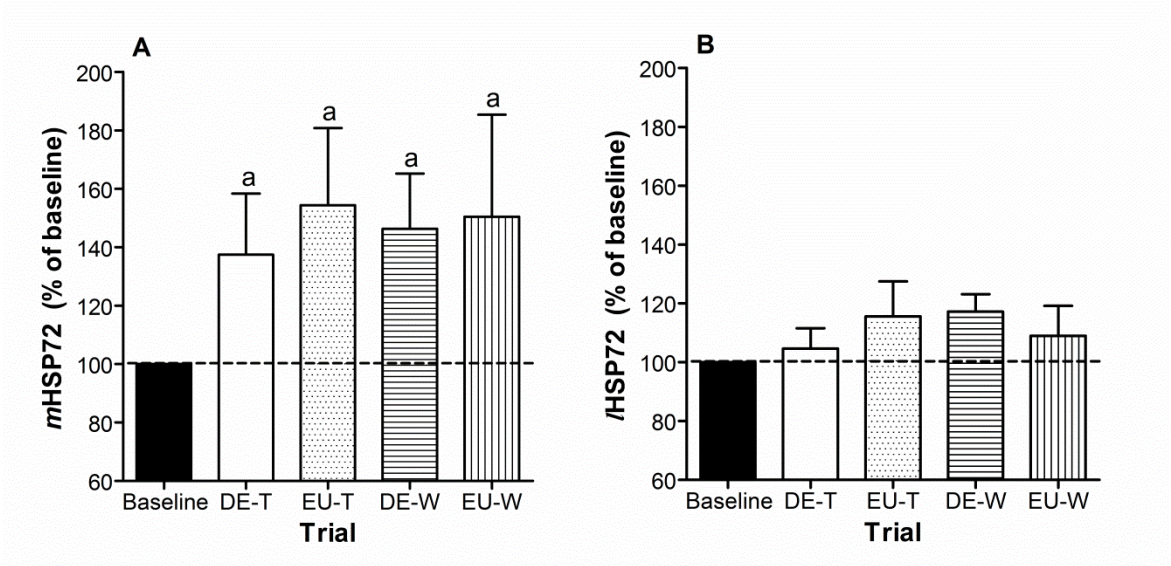

Figure 4.3. Presented are mean ± SEM (*n* = 7) data for baseline (pre) and percent change (pre to post) in monocyte (**A**), and lymphocyte HSP72 (**B**) concentration pre to post exercise in a thermoneutral environment with (DE-T) and without dehydration (EU-T), and a warm environment with (DE-W) and without (EU-W) dehydration. [a]Significantly increased from baseline.

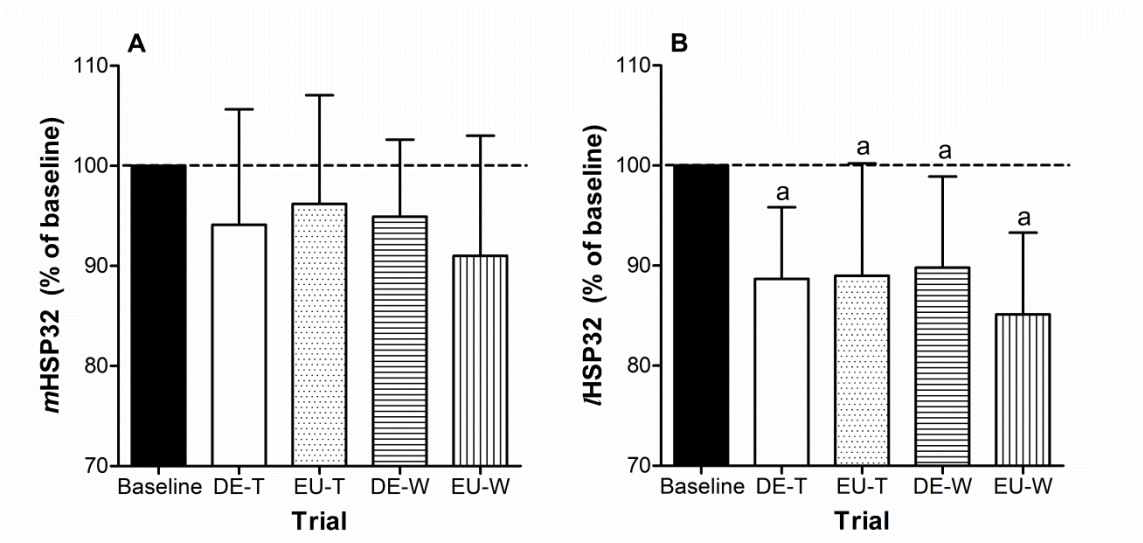

Figure 4.4. Presented are mean ± SEM (*n* = 7) data for baseline (pre) and percent change (pre to post) in monocyte (**A**), and lymphocyte HSP32 (**B**) concentration pre to post exercise in a thermoneutral environment with (DE-T) and without dehydration (EU-T), and a warm environment with (DE-W) and without (EU-W) dehydration. [a]Significantly decreased from baseline.

90 min Exercise and 5 km TT Performance

Table 4.3 represents the performance characteristics for 90 min exercise and 5 km TT. Total distance covered during 90 min was less in DE-W vs. DE-T and EU-T ($p < 0.01$). Additionally, PO during the 90 min and 5 km TT were significantly less vs. all other trials ($p < 0.02$ and $p < 0.03$, respectively). Finishing time for the TT was slower in DE-W vs. all other trials ($p < 0.01$; *Figure 4.5*).

Table 4.3. Presented are mean ± SEM ($n = 7$) data for the performance results of total distance covered (TDC) in kilometres (km), power output (PO) in watts (W), and rating of perceived exertion (RPE) after both 90 min of exercise and the 5 km time trial.

	Thermoneutral		Warm	
	Dehydration (DE-T)	Euhydration (EU-T)	Dehydration (DE-W)	Euhydration (EU-W)
90 min TDC	48.5 ± 1.3	49.6 ± 0.9	45.6 ± 1.1*	47.1 ± 1.4
90 min PO	190 ± 8	194 ± 8	172 ± 11†	177 ± 13
5 km PO	268 ± 12	282 ± 14	229 ± 12‡	262 ± 16
90 min RPE	13 ± 2	13 ± 2	15 ± 3	14 ± 2

*Significantly different from DE-T and EU-T ($p < 0.01$)
†Significantly different from all other trials ($p < 0.03$)
‡Significantly different from all other trials ($p < 0.02$)

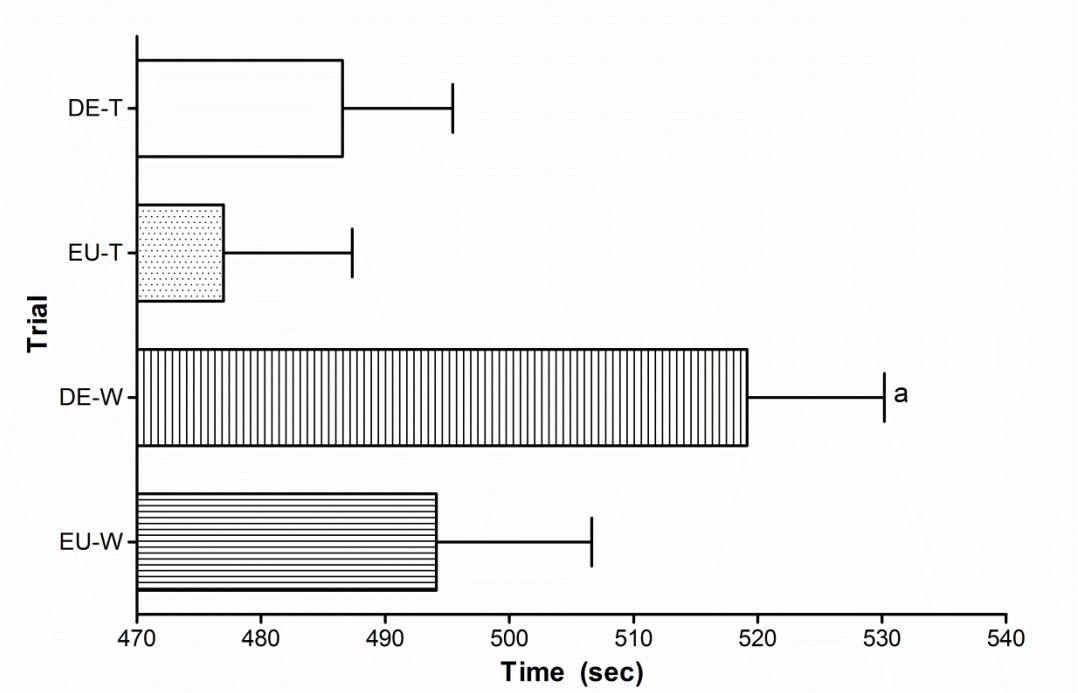

Figure 4.5. Presented are mean ± SEM (*n* = 7) data for 5 km time trial (TT) completion time (sec) in a thermoneutral environment with (DE-T) and without dehydration (EU-T), and a warm environment with (DE-W) and without (EU-W) dehydration. [a]Significantly slower vs. all other trials.

4.4. Discussion

The purpose of this investigation was to examine the effect of exercise-induced dehydration with or without environmental heat stress on oxidative and cellular stress. Firstly, we found increased whole blood GSSG concentration after exercise-induced dehydration, while maintenance of euhydration attenuated these increases regardless of environmental condition. Secondly, although cellular stress measured via monocyte HSP72 concentration was augmented after exercise, neither exercise-induced dehydration nor environmental heat stress had any additional influence on the stress response. Finally, the ability to both sustain exercise at 95% LT and perform during a 5km TT was reduced only during the heat dehydration (DE-W) trial. This reduction in performance occurred seemingly independent of increased cellular stress levels.

Endurance exercise (Gohil et al., 1988, Viguie et al., 1993), as well as passive and exercise hyperthermia (Laitano et al., 2010), are known to elicit increases in GSSG production. We observed a mean increase of 29% in GSSG concentration during dehydration trials; however by maintaining euhydration we were able to attenuate the increase in GSSG concentration irrespective of exercise or environmental heat stress (*Figure 4.2*). This finding is in contrast with those of Gohil et al. (1988) and Viguie et al. (1993), who reported an increase in whole blood GSSG concentration from prolonged cycling exercise at 65% VO_{2max}. While the authors of these investigations (Gohil et al., 1988, Viguie et al., 1993) allowed subjects to consume fluid *ad libitum* during their protocols, they did not report the total volume of fluid consumed or the thermoregulatory or body mass changes in their subjects, making it difficult to determine if the increases in GSSG were a result of the exercise alone or whether the hydration status of the subjects may have perpetuated the changes. It is also possible that GSSG concentrations may have been influenced by the haemoconcentration associated with levels of dehydration observed in the current study. However, Laitano et al. (2010) have previously reported that decreases in plasma volume could explain only less than 10% of the increase in GSSG concentration during passive and exercise hyperthermia. In the current study, GSSG concentration increased 27% and 30% while PV declined 7% and 6% during DE-T and DE-W, respectively. While we acknowledge the decrease in PV may have played a role in the increased GSSG concentration, we believe this to be minimal. If this were not the case, then we would have expected to see a rise in GSSG concentration during the euhydration trials when PV was also decreased (2% and 3% in thermoneutral and warm, respectively).

While the addition of heat to the exercise model in the current study was used to induce a greater dehydration in subjects, we acknowledge previous research has shown increased oxidative stress from passive (Laitano et al., 2010, Ohtsuka et al., 1994) and exercise hyperthermia (Laitano et al., 2010). Recently, Laitano et al. (2010) found heat stress increased whole blood GSSG levels at rest and during exercise when Tc had been passively elevated to a similar degree as the current study (~2°C). Similarly, Ohtsuka et al. (1994) used a 10 min passive heating protocol in 42˚C hot water bath to induce a significant decrease in GSH. However, the influence of heat on changes in glutathione concentration was not evident in our study. It is possible that the short duration of these protocols did not allow for the antioxidant defences to react to the insult in such a short time period (Ohtsuka et al., 1994). Alternatively, the higher training status of our subjects vs. the recreationally active subjects of the other two studies could have led to positive adaptations in our subject's antioxidant defence systems, enabling them to tolerate the exercise induced oxidative stress more effectively, as shown previously elsewhere (Falone et al., 2010, Miyazaki et al., 2001, Niess et al., 1996). While the human research investigating the effects of exercise (Gohil et al., 1988), hyperthermia (Ohtsuka et al., 1994) and dehydration on glutathione changes has been scarce, *in vitro* animal models examining the effect of dehydration on the glutathione system have shown hyperosmotic treatment to rat hepatocytes results in cellular shrinkage and increased production of GSSG (Hussinger et al., 1990, Saha et al., 1992). It has been concluded from these animal models that cellular shrinkage results in a net oxidation and loss of GSH and that cellular swelling (hypotonic treatment) attenuates the formation of GSSG (Saha et al., 1992). Therefore, coupled with our findings from the current study, it is plausible that the act of consuming fluids during

exercise at a rate matching sweat loss can decrease the oxidative stress potential via maintenance of hydration and the antioxidant defence systems.

Although not directly influencing GSSG production in the current study, the influence of hyperthermia on other markers of oxidative stress, such as lipid peroxidation measured via F_2-Isoprostanes (FIP) and malondialdehyde (MDA), have been reported (McAnulty et al., 2005, Paik et al., 2009). McAnulty et al., (2005) found increased lipid peroxidation (via FIP) from exercise that was augmented by the addition of hyperthermia (35°C). However, the authors failed to take into account the hydration status of the subjects, who had become dehydrated by ~3%. Paik et al. (2009) found dehydration of 3% induced by passive heating resulted in a significant increase in MDA. While in the current study TBARS (an indirect measure of MDA) tended to increase during dehydration ($p = 0.08$), the increases in MDA from Paik et al., (2009) were similar to GSSG increases in our own study. Our contradictory findings to the literature is most likely due to a myriad of factors including lower stress levels incurred during cycle exercise rather than running (Byrne et al., 2004), shorter duration of exposure to heat (Lovell et al., 2007), increased scavenging activity of catalase (Mena et al., 1991), or lack of specificity of the TBARS assay (Fisher-Wellman and Bloomer, 2009). However, the current study and previous work (Paik et al., 2009) implies that hydration state can influence markers of lipid peroxidation, warranting further investigation.

Another common marker used to assess the body's tolerance to stress-induced changes in whole-body homeostasis is through heat shock protein expression (HSP). Increased expression of HSP's is known to result from both exercise (Fehrenbach et al., 2000a) and

hyperthermia (Cairo et al., 1985). In the current study we chose to measure HSP concentration in monocyte and lymphocyte populations due to their intrinsic links with oxidative stress and immune system function (Kalmar and Greensmith, 2009). HSP concentration is widely accepted to be increased to a greater extent post exercise in monocytes (Fehrenbach et al., 2003b). However, investigations involving HSP32 have additionally focused on lymphocyte populations because of the protection shown by the inducible isoform heme oxygenase (HO-1) to these cells (Speit et al., 2000). We found that exercise in both a thermoneutral & warm environment with and without dehydration led to an increase in concentration of HSP72 in monocytes, while concurrently causing a decline in HSP32 concentration in lymphocytes (*Figures 4.3 and 4.3, respectively*). It appears the increased HSP72 concentration was stimulated by physical exercise regardless of exercise intensity, environment or hydration status of the subjects, likely because of the exercise-induced hyperthermia, as evidenced by an increase $\geq$ 1°C in T_c during all trials (Fehrenbach et al., 2000b, Oehler et al., 2001). The effect of hydration on HSP expression (both 72 & 32) *in vivo* is unknown. However, *in vitro* research on cultured rat hepatocytes has shown reduced HSP72 expression as a result of hyperosmotic treatment (Kurz et al., 1998), while there has been no research focused on hydration and HSP32 expression. While *in vitro* research has linked dehydration with decreased HSP72 expression (Kurz et al., 1998), we could not make this same inference. It appears the influence of increased oxidative stress (Kalmar and Greensmith, 2009) may have been larger than the influence of dehydration on increasing HSP72 concentration. Additionally, the added benefit of positive training adaptations concurrent with increased levels of hyperthermia and cellular stress may have outweighed the effects of dehydration thus leading to increased HSP72 concentration (Fehrenbach et al., 2000b).

The reason for the decrease in lymphocyte HSP32 concentration is harder to discern. Research concerning HSP32 has shown increased expression post exercise in mono- and lymphocytes from prolonged running (Fehrenbach et al., 2003a, Niess et al., 1999, Thompson et al., 2005) while intermittent treadmill running (Markovitch et al., 2007), exhaustive running and eccentric exercise (Fehrenbach et al., 2003a) have failed to induce increased HSP32 expression. In fact, while not statistically significant, decreases in HSP32 expression have been seen as a result of prolonged running (Thompson et al., 2005) and eccentric exercise (Fehrenbach et al., 2003a). Interestingly, the fluid loss reported in the Thompson study (1.7 ± 0.1 L) may have been the reason for decreased HSP32 expression, similar to the decrease observed in the current study (*Figure 4.4*). Alternatively, Markovitch et al. (2007) found decreased *ex vivo* HSP32 expression in lymphocytes treated with hydrogen peroxide two hours after a previous bout of exercise. While subjects were asked to refrain from exercise in the 24 hours prior to their trial in the current study, it is possible that the exercise training they performed in the days prior to their trials could have led to adaptive responses (Markovitch et al., 2007) or possibly delayed HSP induction (Fernandez et al., 2003). Additionally, we speculate the decrease in HSP32 concentration in our study may be as a result of the lower basal HSP32 expression in trained compared to untrained subjects (Niess et al., 1999) and that this, in combination with a high level of oxidative stress, resulted in less bio-available HSP32 (Sandstrom et al., 2009).

In conclusion, the current study found increased GSSG concentration after exercise-induced dehydration, while maintenance of euhydration attenuated these increases. Additionally, exercise-induced dehydration did not influence the increase of cellular HSP

concentration post exercise. While we found increased oxidative and cellular stress as a result of exercise and dehydration, the occurrence of these stress markers was not amplified as a result of the environmental heat stress. These findings highlight the importance of fluid consumption and maintenance of euhydration during prolonged exercise to attenuate thermal and oxidative stress during prolonged exercise in the heat.

Chapter 5. Experiment 2: Effects of hydration status on oxidative and cellular stress expression in untrained subjects during normothermic and hyperthermic aerobic exercise.

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

Hillman AR, Taylor L, Turner MC, McNaughton L, and Siegler J. (2011) Effects of hydration status on oxidative and cellular stress expression in untrained subjects during prolonged normothermic and hyperthermic aerobic exercise. *Journal of Science and Medicine in Sport*, In Review

Abstract

Oxidative stress has long been associated with varying degrees of aerobic fitness and most recently with hyperthermia and hydration status. The purpose of the current experiment was to investigate the effect of prolonged exercise-induced dehydration with and without hyperthermia on oxidative stress markers in untrained individuals, to serve as a comparison to the results of the first experimental chapter. Seven untrained males (mean ± SD) age: 21 ± 3 yrs, height: 181.1 ± 9.2 cm, weight: 76.8 ± 8.8 kg, and PO at LT 100.0 ± 13.0 W completed 90 min of exercise at 95% LT followed by a 5 km time trial (TT) in four trials: euhydration in a warm (EU-W) or thermoneutral (EU-T) environment and dehydration in a warm (DE-W) or thermoneutral (DE-T) environment (W: 33.9 ± 1.0°C; T: 22.9 ± 1.0°C). Increases in whole blood oxidised glutathione (GSSG) (32%, $p < 0.01$) and intracellular monocyte heat shock protein 72 (HSP72) (14%, $p < 0.01$) concentration were found regardless of hydration status and environmental stress. Additionally we noted a relationship between GSSG concentration and body mass loss ($r^2 = 0.5$, $p = 0.05$), and HSP72 with body temperature and heat storage ($r^2 = 0.5$, $p = 0.01$). Similar to the trained individuals in experiment one, PO during the 90 min (7%, $p < 0.01$) and TT (14%, $p < 0.01$) were decreased while thermoregulation was impaired during DE-W only. These results demonstrate the increased level of stress in untrained subjects as a result of exercise, highlighting the importance of regular physical activity to aid in positive cellular adaptations leading to superior antioxidant defences to aid in disease prevention.

5.1. Introduction

In the first experimental chapter it was observed that dehydration resulted in augmented oxidative stress (GSSG) while maintaining euhydration attenuated this stress. Additionally, the superimposition of hyperthermia did not appear to intensify the stress, which we believe was likely due to enhanced thermoregulation of trained individuals. However, this 'trained' response to the imposed exercise and environmental condition may not be the same for all individuals. It has been noted that untrained individuals who maintain sedentary lifestyles have higher basal (Miyazaki et al., 2001) and exercise-induced (Falone et al., 2010, Miyazaki et al., 2001, Niess et al., 1996) oxidative stress as well as greater potential for development of many diseases (Chen et al., 1994, Laaksonen et al., 1996, Nishiyama et al., 1998, Valko et al., 2004). Therefore the addition of dehydration and hyperthermia during endurance exercise could be exacerbated in sedentary individuals because of their inferior antioxidant defences (Alessio, 1993) and impaired thermotolerance (Greenhaff, 1989), although this has yet to be documented. Accordingly, the purpose of the second experiment was to investigate the effect of prolonged exercise-induced dehydration with and without hyperthermia on cellular and oxidative stress in sedentary individuals. It was hypothesised that exercise would result in augmented oxidative and cellular stress, which would be amplified by the addition of dehydration and hyperthermia. Additionally, it was believed that the level of oxidative stress would be amplified in the untrained vs. the trained participants.

5.2. Methods

Subjects

Seven untrained male university students, unacclimatised to heat, participated in this study. Physical characteristics (mean ± SD) were age: 21 ± 3 yrs, height: 181.1 ± 9.2 cm, weight: 76.8 ± 8.8 kg, and power output (PO) at lactate threshold (LT): 100 ± 13 Watts (W). Subjects were free of any cardiac or metabolic diseases, did not smoke, and refrained from supplementation of all kinds (i.e., vitamins, ergogenic aids, etc.) during the testing period. Before participation each subject gave written informed consent and completed a medical health questionnaire. The study was approved by the Departmental Human Ethics Committee following the principles outlined in the Declaration of Helsinki.

Preliminary Measurements

A preliminary measurement of the LT was carried out and is detailed in the general methodologies (section 3.3.).

Experimental Design

Experimental design of the study was identical to that of experiment one, with the addition of skin temperature measurement, the methods of which are detailed in section 3.5.

Statistical Analysis

All data are represented as mean ± SEM. Statistical analysis was completed using Linear Mixed Models for repeated measures (SPSS 18.0, Chicago, IL, USA). Different covariance structures were assumed and the one that minimised the Hurvich and Tsai's criterion was chosen for the final model. Where a significant F ratio was observed, post hoc comparisons

with Sidak-adjusted p values were used to identify which pairs of means were significantly different. Pearson's correlations (r^2) were conducted to evaluate the relationship between body mass loss, Tc, Tb, and oxidative stress variables. Two-tailed statistical significance was accepted as $p < 0.05$.

5.3. Results

Cellular Stress

TGSH/GSSG

While there was no significant interaction (condition x time) ($F = 2.03$; $p = 0.15$), GSSG increased pre to post exercise for all trials ($F = 12.78$; $p = 0.01$), however there was no main effect between conditions ($F = 0.59$, $p = 0.64$; *Figure 5.1*). The correlation between GSSG concentration and body mass loss was strong ($r^2 = 0.5$; $p < 0.01$) however, a relationship with GSSG was not evident for HS ($r^2 = 0.1$; $p = 0.60$) or Tb ($r^2 = 0.1$; $p = 0.63$).

Similar to GSSG, there was no significant interaction (condition x time) for TGSH ($F = 1.60$; $p = 0.22$). However unlike GSSG, TGSH did not increase over time ($F = 3.37$; $p = 0.08$) and was not significantly different between trials ($F = 1.42$; $p = 0.27$; *Figure 5.1*). Evaluating the relationship between TGSH concentration and body mass loss, we found an r^2 of 0.4 ($p < 0.01$) indicating a moderate relationship. Similar to GSSG, there was no relationship between TGSH concentration and HS ($r^2 = 0.02$; $p = 0.35$) and Tb ($r^2 = 0.01$; $p = 0.50$).

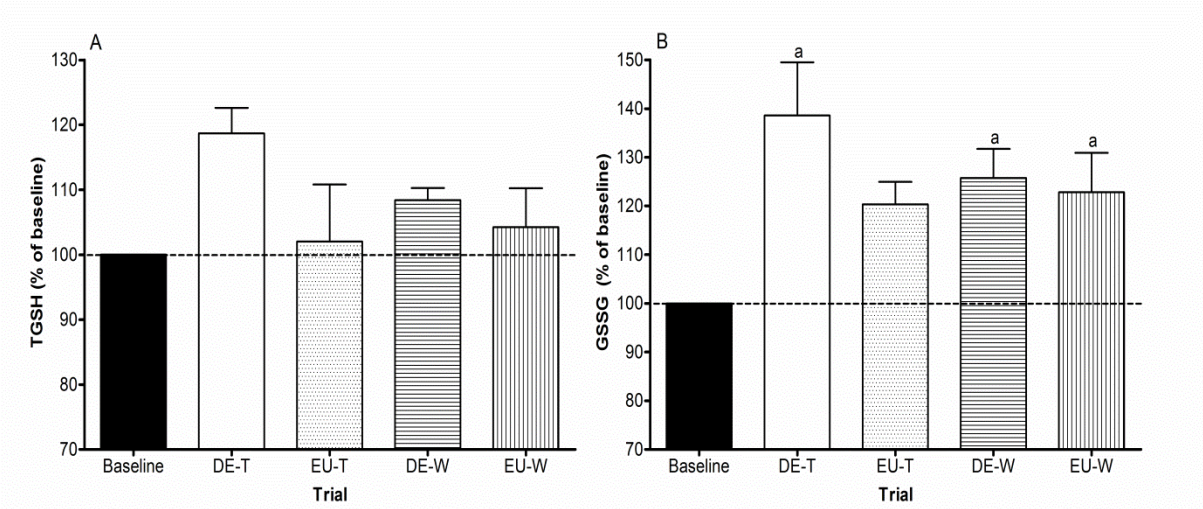

Figure 5.1. Presented are mean ± SEM (*n = 7*) data for percent change in total (TGSH) and oxidised glutathione (GSSG) pre to post exercise in a thermoneutral environment with (DE-T) and without dehydration (EU-T), and a warm environment with (DE-W) and without (EU-W) dehydration. [a]GSSG concentration was significantly increased post exercise ($p < 0.01$).

TBARS

There was no significant interaction (condition x time) for TBARS ($F = 1.12$; $p = 0.36$). Similarly, TBARS concentration did not increase with time ($F = 2.51$; $p = 0.13$) and was not different between conditions ($F = 0.38$; $p = 0.77$) pre to post exercise (% change from baseline DE-T: 91.5 ± 8.1%, EU-T: 129.0 ± 16.4%, DE-W: 133.5 ± 28.4%, EU-W: 122.1 ± 17.1%). TBARS was moderately correlated with body mass ($r^2 = 0.5$; $p < 0.01$).

HSP

In monocytes there was no significant interaction (condition x time) for HSP72 ($F = 0.16$; $p = 0.93$). However, HSP72 concentration increased pre to post exercise for all trials ($F = 8.33$; $p < 0.01$) with no differences between conditions ($F = 0.04$; $p = 0.99$). Similarly, for lymphocytes there was no significant interaction (condition x time) for HSP72 ($F = 0.36$; $p = 0.79$). There was also no significant increase in HSP72 concentration pre to post exercise

(F = 0.77; p = 0.41) and no indication of a trend between conditions (F = 0.76; p = 0.53; *Figure 5.2*). There was a strong relationship between HSP72 concentration and HS and Tb, (r^2 = 0.5, p = 0.01 for both), however there was no relationship between HSP72 concentration and body mass loss (r^2 = 0.1, p = 0.60).

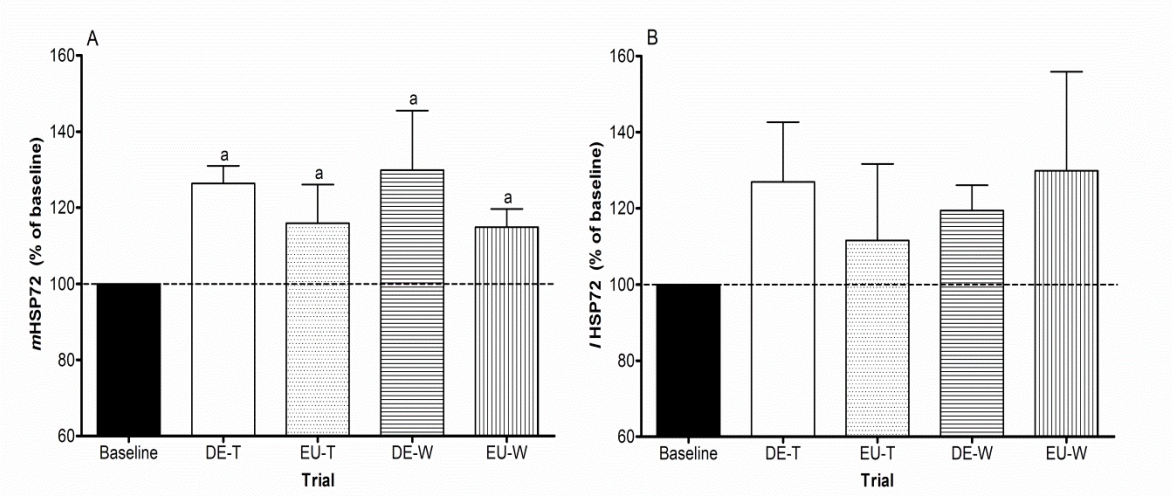

Figure 5.2. Presented are mean ± SEM (*n = 7*) data for percent change in (**A**) monocyte heat shock protein 72 (*m*HSP72) and (**B**) lymphocyte (*l*HSP72) concentration pre to post exercise in a thermoneutral environment with (DE-T) and without dehydration (EU-T), and a warm environment with (DE-W) and without (EU-W) dehydration. [a]*m*HSP72 concentration was significantly increased post exercise for all trials (p < 0.01).

Table 5.1 represents a comparison of the oxidative stress markers between trained participants from experiment one and the current experiment. Resting and post exercise GSSG, TGSH, and TBARS were all elevated in untrained vs. trained participants (p < 0.01). Additionally, post exercise GSH was lower in untrained vs. trained (p < 0.01). There was no difference in HSP72 concentration at any time between the participants.

Table 5.1. Comparison between trained participants of experiment one and the untrained participants of experiment two (mean ± SEM) for pre and post exercise concentrations of total (TGSH), oxidised (GSSG) and reduced (GSH) glutathione in pmol, heat shock protein 72 (HSP72 mean fluorescent intensity (MFI)), and thiobarbituric acid reactive substances (TBARS in malondialdehyde equivalents ($nmol \cdot ml^{-1}$)).

	Untrained		Trained	
	Pre	Post	Pre	Post
TGSH	4035 ± 364*	4163 ± 329*	3394 ± 190	3689 ±239
GSSG	1010 ± 78*	1290 ± 148*	92 ± 13	108 ± 16
GSH	3025 ± 323	2873 ± 298†	3302 ± 183	3581 ± 228
HSP72	4.2 ± 0.7	5.0 ± 0.9	3.9 ± 0.6	5.0 ± 0.5
TBARS	25.4 ± 3.8*	28.5 ± 4.5*	15.2 ± 1.9	20.7 ± 4.0

*Greater vs. corresponding time in trained subjects (all $p < 0.01$)
†Less vs. corresponding time in trained subjects ($p < 0.01$)

Thermoregulation

Table 5.2 represents the urine, haemodynamic and fluid changes for all trials. UOsm and SOsm significantly increased post exercise in dehydration trials only ($p < 0.01$). Figure 5.3 represents the Tc, Ts and HR changes pre to post 90 min exercise. HR significantly increased post exercise for all trials ($p < 0.01$) and was higher post exercise in DE-W vs. DE-T & EU-T ($p < 0.01$) and EU-W vs. EU-T ($p = 0.03$). Ts was significantly higher post exercise in warm vs. thermoneutral trials ($p < 0.01$). Tc was significantly higher post exercise in DE-W vs. DE-T & EU-T ($p < 0.01$) & dehydration vs. euhydration trials ($p < 0.01$). Thermoregulation during DE-W was impaired showing significant main effects for Tb ($F = 40.19$, $p < 0.01$), HS ($F = 10.30$, $p < 0.01$), and PSI ($F = 9.11$, $p < 0.01$) which resulted in greater Tb in warm trials vs. thermoneutral trials ($p < 0.01$ for all), significantly greater HS in DE-W vs. EU-T ($p < 0.03$) and significantly higher PSI in DE-W vs. EU-T and EU-W ($p < 0.01$ and $p = 0.02$, respectively; *Figure 5.4*).

Table 5.2. Presented are mean ± SEM (*n* = 7) data for changes in urine (UOsm) and serum (SOsm) osmolality (mOsm·kg^{-1}), total body mass loss (BML, kg), percent BML (%BML), plasma volume change (PVΔ, %), and total fluid consumed (TFC in litres) during 90 min exercise and 5 km time trial (TT).

	Thermoneutral				Warm			
	Dehydration (DE-T)		Euhydration (EU-T)		Dehydration (DE-W)		Euhydration (EU-W)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
UOsm	405±6	571±7*	406±6	408±8	418±9	717±8*	425±9	461±9
SOsm	290±1	302±1*	290±1	291±1	290±1	304±1*	290±1	291±1
BML	--	0.9±0.1	--	0.2±0.1	--	1.2±0.1	--	0.2±0.1
%BML	--	1.2±0.1	--	0.1±0.1	--	1.5±0.1	--	0.2±0.1
PVΔ	--	-4.9±1.1	--	-4.9±1.4	--	-1.2±1.7	--	-2.8±0.8
TFC	--	--	--	0.9±0.01	--	--	--	1.2±0.01

*Significantly increased vs. pre ($p < 0.01$).

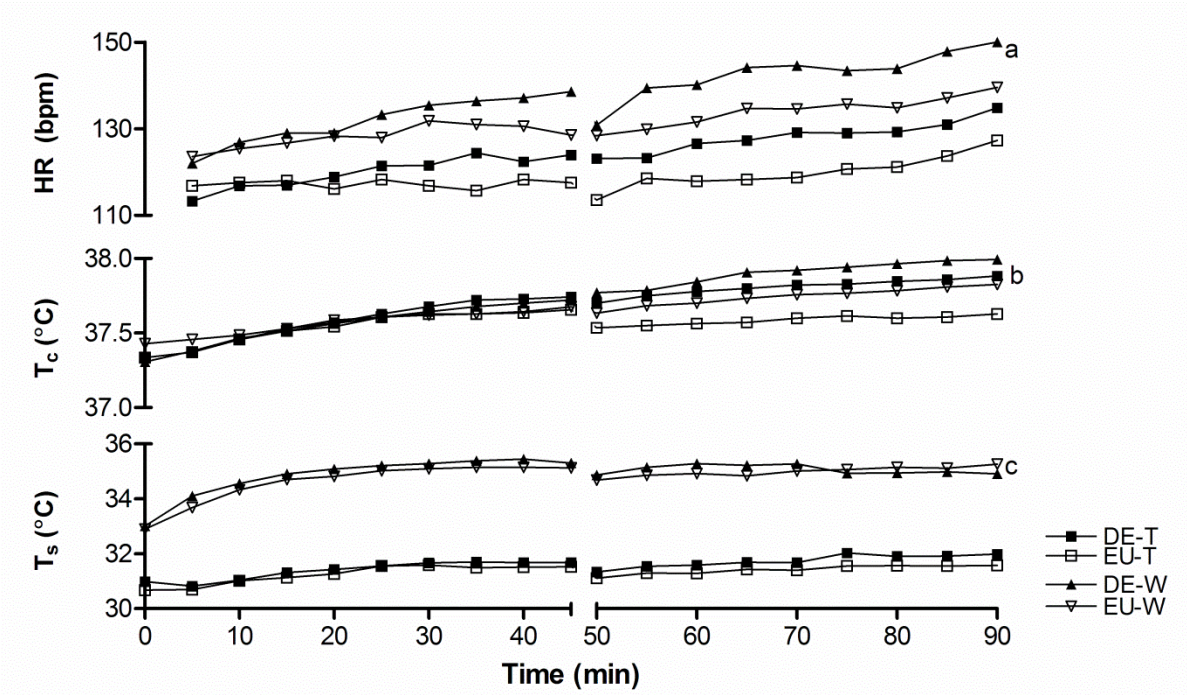

Figure 5.3. Presented are mean (*n = 7*) data for average heart rate (HR), core temperature (T_c) and skin temperature (Ts) pre to post exercise in a thermoneutral environment with (DE-T) and without dehydration (EU-T), and a warm environment with (DE-W) and without (EU-W) dehydration. [a]Significantly increased HR pre to post exercise for all trials ($p < 0.01$) and higher post exercise HR in DE-W vs. DE-T & EU-T ($p < 0.01$) and EU-W vs. EU-T ($p = 0.03$). [b]Tc significantly higher post exercise in DE-W vs. DE-T & EU-T ($p < 0.01$) & dehydration vs. euhydration trials ($p < 0.01$).[c]Significantly higher Ts post exercise in warm trials vs. thermoneutral trials ($p < 0.01$). Error bars were omitted for clarity.

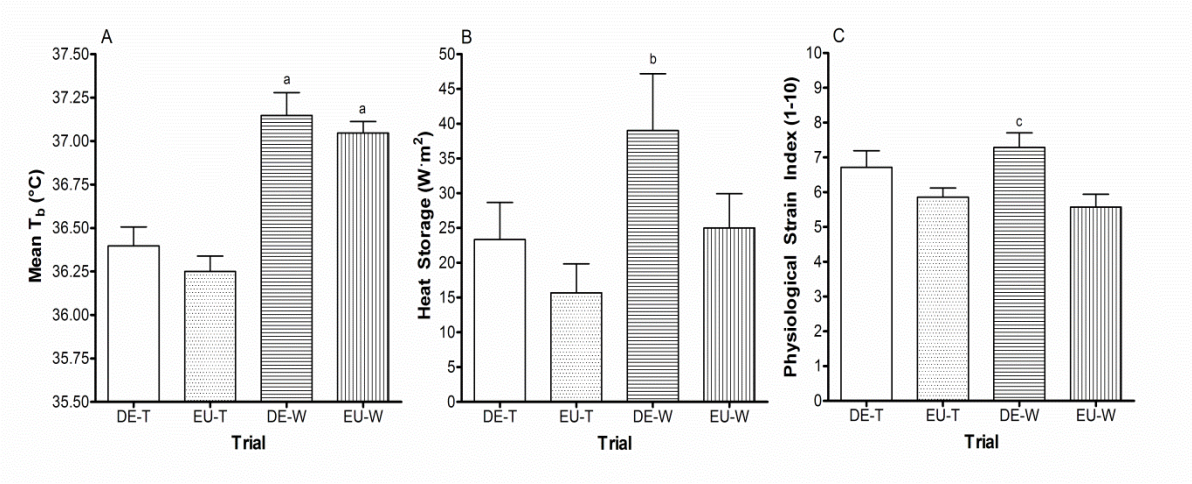

Figure 5.4. Presented are mean ± SEM (*n = 7*) data (**A**) for mean body temp (Tb), (**B**) heat storage (HS) and (**C**) physiological strain index (PSI) pre to post exercise in a thermoneutral environment with (DE-T) and without dehydration (EU-T), and a warm environment with (DE-W) and without (EU-W) dehydration. [a]Significantly higher Tb in warm trials vs. thermoneutral trials ($p < 0.01$). [b]Significantly greater HS for DE-W vs. EU-T only ($p < 0.01$). [c]Significantly higher PSI in DE-W vs. EU-T and EU-W only ($p < 0.01$ and $p = 0.02$, respectively).

90 min Exercise and 5km TT Performance

The DE-W trial resulted in a multitude of performance decrements including a tendency for decreased PO during the 90 min ($F = 2.99$; $p = 0.06$) and a significant decrease during the TT ($F = 3.58$; $p = 0.03$). Additionally, total distance covered was less ($p < 0.01$ and $p = 0.05$, vs. EU-T and EU-W, respectively) while TT completion time was slower ($p = 0.04$) in the DE-W trial vs. the EU-T and EU-W trials (*Table 5.3*).

Table 5.3. Presented are mean ± SEM (n = 7) data for the performance results of total distance covered (TDC, km), power output (PO, W), and rating of perceived exertion (RPE) after both 90 min of exercise and the 5 km time trial and TT completion time (sec).

	Thermoneutral		Warm	
	Dehydration (DE-T)	Euhydration (EU-T)	Dehydration (DE-W)	Euhydration (EU-W)
TDC (km)	36 ± 1	38 ± 1	35 ± 1*	37 ± 1
90 min PO (W)	100 ± 5	103 ± 6	96 ± 6	100 ± 5
5 km PO (W)	197 ± 7	214 ± 11	175 ± 14†	201 ± 11
90 min RPE	10 ± 2	9 ± 1	12 ± 2	10 ± 2
5 km RPE	17 ± 2	15 ± 3	18 ± 2	16 ± 3
TT time	568 ± 21	544 ± 19	595 ± 23‡	545 ± 12

*Significantly less distance covered in DE-W vs. EU-T and EU-W ($p < 0.01$ and $p = 0.05$, respectively).
†Significantly lower PO vs. EU-T ($p = 0.03$).
‡Significantly slower TT time in DE-W vs. EU-T and EU-W ($p = 0.04$).

5.4. Discussion

The purpose of this investigation was to investigate the effect of exercise-induced dehydration with or without environmental heat stress on oxidative and cellular stress in untrained participants. A secondary aim of this experiment was to compare the resting and post exercise oxidative stress of the untrained participants to the responses of the trained participants from experiment one. Firstly, exercise led to an increase in GSSG concentration independent of hydration status or environmental heat stress. Secondly, HSP72 concentration was increased as a result of exercise, seemingly independent of hydration and environmental heat stress. Finally, exercise performance during 90 min and TT were diminished during only the DE-W condition. This decrease in performance was likely as a result of the interaction between dehydration and thermal stress ($r^2 = 0.6$, $p < 0.01$), as HS and PSI were also augmented during this trial alone. Additionally, comparisons between the responses of the participants in the current experiment to those of

experiment one revealed oxidative stress markers tended to be lower pre and post exercise in trained participants (*Table 5.1*).

In the current experiment, we observed an average increase of 34% in GSSG concentration post exercise in both normo- and hyperthermic environments, while fluid consumption during exercise did not significantly attenuate cellular stress levels ((% are increases from baseline) DE-T: 38%, EU-T: 25%, DE-W: 31% and EU-W: 27%; *Figure 5.1*). While we anticipated an exaggerated stress response from our untrained subjects, previous data led the author to also expect this increase to be exacerbated when coupled with thermal stress (Laitano et al., 2010, Ohtsuka et al., 1994). However, the response was not indicative of a proliferation in oxidative stress during hyperthermic conditions and in some instances even quantifiably less than under the normothermic conditions (mean difference: 6% between warm and hyperthermic trials; *Figure 5.1*). This most likely resulted from the lower intensity maintained by the participants during the heat trials (20 W lower PO in warm vs. thermoneutral) even though HS was elevated.

The subjects' perception of exercising in the heat or under a fluid restricted condition does not appear to have influenced these cellular stress findings, as there was no meaningful difference between conditions for both the performance parameters and RPE (*Table 5.3*). This suggests that absolute exercise intensity may play a more predominant role than exercise duration in the production of oxidative stress in untrained individuals. Indeed, while it has been reported that prolonged exercise results in augmented oxidative stress compared to shorter duration bouts (Bloomer et al., 2007a), it has also been shown that oxidative stress is increased to a greater degree as a result of high-intensity compared to

lower intensity exercise (Quindry et al., 2003, Wang and Huang, 2005). Although the author acknowledges these findings may result from the self-paced nature of the exercise protocol, during pilot work it was discovered that by fixing the intensity for 90 min (coupled with the sedentary nature of our participants) a majority of our participants were unable to finish the prescribed exercise time. Ultimately, the author felt it more important to provide consistent heat exposure durations rather than have subjects exercise for different time periods. The ability to self-pace also likens the exercise protocol to a typical training session. As a result, and to provide greater ecological validity, the author decided upon the self-paced pacer protocol and accepted the different intensities as a limitation.

Although the relationship between GSSG formation and HS in the current study is tenuous ($r^2 = 0.10$), the data indicates increased GSSG concentration may be intrinsically linked with hydration status. While the level of dehydration in this study was modest (mean of 1.3% of body mass), after correcting for plasma volume changes, GSSG concentrations were 8% greater during the dehydration trials compared to the euhydration trials (*Figure 5.1*). Unfortunately, this was not a significant as was the case in the first experiment and is likely due to the sedentary nature of the subjects.

As previously addressed, sedentary individuals are known to have increased basal (Miyazaki et al., 2001) and exercise-induced (Douris et al., 2009, Niess et al., 1999) production of free radicals, as well as lower resting GSH concentration (Elokda and Nielsen, 2007) and greater resting GSSG concentration (Douris et al., 2009, Tessier et al., 1995) compared to their trained counterparts. Indeed, resting GSSG concentration of the subjects in the experiment was 10-fold greater while TGSH concentration was 16% greater

(mean difference of 918 pmol and 641 pmol, respectively) compared to the trained cyclists recruited for experiment one. This resulted in a lower basal GSH concentration in the sedentary population compared to the trained subjects (mean difference of 227 pmol *Table 5.1*).

An average increase of 14% in monocyte HSP72 concentration as a result of exercise, regardless of body mass loss, environmental temperature or exercise intensity was found in the current experiment (*Figure 5.2*); results that are in line with the first experiment and the literature (Febbraio and Koukoulas, 2000, Febbraio et al., 2002). While there was no significant influence of body mass loss on HSP72 concentration (*Figure 5.2*), there appears to be a relationship between HS and HSP72 concentration, which was greatest during DE-W ($r^2 = 0.7$). Interestingly, Tc, Tb, HS, and PSI were also significantly increased (*Figure 5.4*) while performance was significantly decreased during this trial (*Table 5.3*), demonstrating a relationship between thermoregulation and HSP72 expression during exercise. This is in contrast to the oxidative response (GSSG), and may indicate that HSP72 expression is more intrinsically linked with exercise in combination with environmental heat stress compared to exercise, heat shock or dehydration alone in sedentary individuals. Additionally, although not statistically significant, the untrained subjects of the current investigation had higher basal HSP72 expression compared to the participants of the first experiment (*Table 5.1*), in line with previous studies (Fehrenbach et al., 2000a).

Similar to the first experiment, the current study did not find any significant changes in plasma TBARS levels. This was unexpected and could be due to methodological constraints that only allowed for measurement of TBARS pre and immediately post-

exercise, as previous investigations have reported no change in TBARS levels until after 20 min (Groussard et al., 2003) and even up to an hour (Peart et al., 2011) into the recovery period. Additionally, the lack of significant findings might be a result of the reported lack of specificity of the assay (Fisher-Wellman and Bloomer, 2009) as previous studies using similar exercise modes and subjects to this have found increases in other markers of lipid peroxidation (Wang and Huang, 2005). Comparison of TBARS levels between the untrained and trained participants revealed higher resting and post exercise TBARS concentrations in the untrained participants (*Table 5.1*), findings that are in line with the literature (Miyazaki et al., 2001).

In summary, the current study found an increase in GSSG and HSP72 concentrations in untrained individuals as a result of prolonged cycling regardless of body mass loss and environmental heat stress. While there were no differences as a result of hydration or environment, the author noted a relationship between GSSG concentration and body mass loss, while HSP72 was correlated with body temperature and levels of heat storage. Additionally, basal and post exercise concentrations of oxidative stress markers (GSSG, TGSH, and TBARS) were augmented in untrained compared to trained subjects, while there was no difference in HSP72 concentration, confirming other reports that oxidative stress levels are dependent upon training status.

Chapter 6. Experiment 3: The effect of glycerol hyperhydration on markers of oxidative stress during prolonged cycling in the heat

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

Hillman AR, Turner MC, Peart DJ, Bray JW, Taylor L, and Siegler J. (2011) A comparison of hyperhydration vs. *ad libitum* fluid intake strategies on measures of thermoregulation and endurance performance – *Applied Physiology, Nutrition and Metabolism*, In Review

Abstract

In light of the findings from the first experimental chapter that dehydration can significantly influence oxidative stress in trained subjects, the purpose of the third experimental chapter was to compare pre-exercise hyperhydration with plain water (W) or water with glycerol (G) to no hyperhydration (C) on markers of oxidative stress prior to and after a 90 min TT. Seven trained male cyclists and triathletes (age: 28 ± 8 yrs, height: 178.4 ± 7.8 cm, and mass: 73.2 ± 9.6 kg) covered as much distance as possible during a 90 min cycle after G, W or C. Blood was collected pre ingestion (PRE), post ingestion/pre exercise (PI), immediately post exercise (PE) and 1 hour post exercise (1HR) and analysed for whole blood TGSH, GSSG, and plasma levels of lipid hydroperoxides (LOOH) and protein carbonyls (PC TGSH concentration increased post exercise in W and C ($p < 0.01$) while PC concentration increased post exercise during C only ($p = 0.03$). Additionally, GSSG concentration was greater PI and PE in C compared to G ($p = 0.05$, and $p < 0.01$, respectively), likely due to the inferior amount of fluid retained during C compared to the G and W trials. Therefore, it appears that both pre exercise hyperhydration with *ad libitum* fluid ingestion during exercise is sufficient to attenuate rises in exercise-induced oxidative stress.

6.1. Introduction

In the first experimental chapter it was demonstrated that dehydration increased GSSG concentrations in trained cyclists however, in the second experimental chapter while there was a similar trend, GSSG concentration was not significantly affected by changes in body mass loss. These disparate findings are likely a result of the training status of the subjects with trained participants possessing superior antioxidant defence systems that provide enhanced protection and are likely aided by euhydration. As a result of the attenuation in oxidative stress from water ingestion during exercise the author rationalised that manipulation of hydration prior to exercise (increasing total body water via glycerol hyperhydration) would likely result in augmentation of this attenuation in stress. Therefore, the third experimental chapter utilised trained participants to investigate the effects of pre exercise hyperhydration on oxidative stress.

As previously discussed, *in vitro* hyperosmolality (dehydration) creates oxidative stress (Saha et al., 1992) while hypoosmolality (hyperhydration) attenuates DNA damage (Martins and Meneghini, 1994). Glycerol is often employed prior to exercise to hyperhydrate participants by increasing plasma volume (Hitchins et al., 1999), decreasing urine volume (Goulet et al., 2006), and increasing water retention (Montner et al., 1999). Following ingestion, glycerol is evenly distributed among the intra- and extracellular compartments (Lin, 1977), which would likely result in cellular hypotonicity and presumably maintain cellular integrity providing cellular protection against oxidative stress. Additionally, glycerol ingestion has been shown to augment blood glucose concentrations (Maughan and Gleeson, 1988), which has been shown previously to attenuate the oxidative (McAnulty et al., 2007a, McAnulty et al., 2007b, McAnulty et al., 2003) and immune

(Nieman et al., 2005, Nieman et al., 2006) response to exercise; however the mechanism behind this needs further elucidation.

Therefore, it was the purpose of this experiment to compare pre-exercise hyperhydration with glycerol or water to no hyperhydration on markers of oxidative stress. We hypothesised that glycerol hyperhydration would result in superior maintenance of hydration and attenuation of oxidative stress compared to water and control.

6.2. Methods

Subjects

Seven trained male cyclists and triathletes, unacclimatised to heat, participated in this study. Physical characteristics (mean ± SD) were age: 28 ± 8 yrs, height: 178.4 ± 7.8 cm, and weight: 73.2 ± 9.6 kg. Subjects participated in an average of 15 hrs endurance training, riding 371 ± 121 km per week. Subjects were free of any cardiac or metabolic diseases, did not smoke, and refrained from supplementation of all kinds (i.e., vitamins, ergogenic aids, anti-inflammatory medicines, etc.) during the testing period. Before participation each subject gave written informed consent and completed a medical health questionnaire. The study was approved by the Departmental Human Ethics Committee following the principles outlined in the Declaration of Helsinki.

Experimental Design

Each subject visited the lab on four occasions, an initial familiarisation followed by three experimental trials. All trials were completed in an environmentally controlled chamber (Model SSR60-20H, Design Environmental, Gwent, Wales, UK; 35˚C and 40% relative

humidity), separated by at least one week, and administered in a randomised fashion. During the familiarisation trial subjects were asked to keep a food diary and consume the same diet prior to and on all trial days. Additionally, they were requested to refrain from caffeine, alcohol and anti-inflammatory consumption and strenuous exercise for the 24 hour period prior to each trial.

Subjects reported to the laboratory at 9 am in a euhydrated state and two hours postprandial. Following resting nude weight measurement, urine and blood sample collection, subjects completed either a hyperhydration protocol or a control trial (C). Hyperhydration was achieved by consuming either 1.2 g of glycerol per kg of body weight in 26 ml of water per kg of body weight (G), or an equal volume of aspartame flavoured water (W) at a temperature of 16˚C. Drinks were evenly administered every 30 min for a 120 min period prior to exercise while participants rated symptoms on a questionnaire anchored at 0 (none) to 10 (maximal) for physiological parameters (dizziness, headache, stomach fullness, and nausea). For C trial subjects came to the lab at 9 am and rested for 120 min (duration of hyperhydration period). Following the 120 min in hyperhydration and C trials, a blood sample was collected along with nude weight measurement, placement of a rectal thermometer and positioning of a recordable HR monitor and skin thermistors. After preliminary measurements were complete subjects entered the environmental chamber for completion of the exercise task.

In the first two experimental studies the exercise of the participants during the 90 min was prescribed at a fixed intensity to facilitate comparison of performance. However, because the participants were unable to maintain the prescribed exercise intensity, in this

experiment participants were asked to cycle for the same duration but were allowed to self pace in an effort to be more ecologically valid. All exercise protocols were completed on a Wattbike cycle ergometer (Wattbike Ltd, Nottingham, UK), which has been validated for continuous exercise (Hopker et al., 2010). Subjects completed a 5 min warm up at 70 W and 80 rpm followed by a 90 min cycle where they were asked to cover as much distance as possible in that time. During the 90 min cycle subjects were allowed to consume water (16˚C) *ad libitum.* A final nude weight was taken after completion of exercise along with a blood sample and one further blood sample 60 min post exercise. All urine was collected during the experimental period for measurement of total urine volume (UV).

Tc, Ts, HR, RPE, thermal sensation, and sweating sensation were recorded at 5 min intervals. Mean Ts, mean body temperature (Tb) and heat storage (HS) were calculated according to the methods in section 3.5.

Blood Collection and Analysis

Blood was drawn from a vein in the antecubital region pre-ingestion (PRE), post-ingestion/pre-exercise (PI), post exercise (PE), and one hour post exercise (1HR) into four different Vacuette tubes and analysed for glycerol concentration, SOsm, total (TGSH) and oxidised (GSSG) glutathione concentration, protein carbonyl (PC) concentration, and lipid hydroperoxide (LOOH) concentration according to manufacturer's instructions. Assay procedures are outlined in sections 3.8.1 through 3.9. Plasma blood glucose (BG) concentration was measured in duplicate from K_3EDTA plasma on the first thaw (YSI 2300 Stat, YSI Inc., Yellow Springs, OH, USA). Capillary blood samples were collected for measurement of haematocrit (Micro Hematocrit Reader, Hawksley, UK) and haemoglobin

(Hemocue 201, Hemocue, Ltd, Sheffield, UK) for calculation of plasma volume changes. All blood parameters were corrected for plasma volume changes (see section 3.10).

Statistical Analysis

Data are represented as mean ± SEM. Statistical analysis was completed using Linear Mixed Models for repeated measures (SPSS 18.0, Chicago, IL, USA). Different covariance structures were assumed and the one that minimised the Hurvich and Tsai criterion was chosen for the final model. Where a significant F ratio was observed, post hoc comparisons with Sidak-adjusted p values were used to identify which pairs of means were significantly different. Pearson's correlations (r^2) were conducted to evaluate the relationship between body mass loss, Tb, BG, and oxidative stress variables. Two-tailed statistical significance was accepted as $p < 0.05$.

6.3. Results

Cellular Stress

Figure 6.1 presents the changes in TGSH and GSSG concentration throughout the protocol. For TGSH there was a significant main effect for time ($F = 14.90$, $p < 0.01$), however there was no main effect for condition ($F = 0.29$, $p = 0.70$; *Figure 6.1*). For GSSG there was no main effect for time ($F = 3.38$, $p = 0.07$) however there was a main effect for condition ($F = 4.71$, $p < 0.05$; *Figure 6.1*). For PC there was a main effect for time ($F = 9.41$, $p < 0.01$) however there was no main effect for condition ($F = 0.70$, $p = 0.51$; *Figure 6.2*). Finally, there was no main effect for time ($F = 0.74$, $p = 0.53$) or condition ($F = 1.66$, $p = 0.24$; *Figure 6.3*) for LOOH concentration.

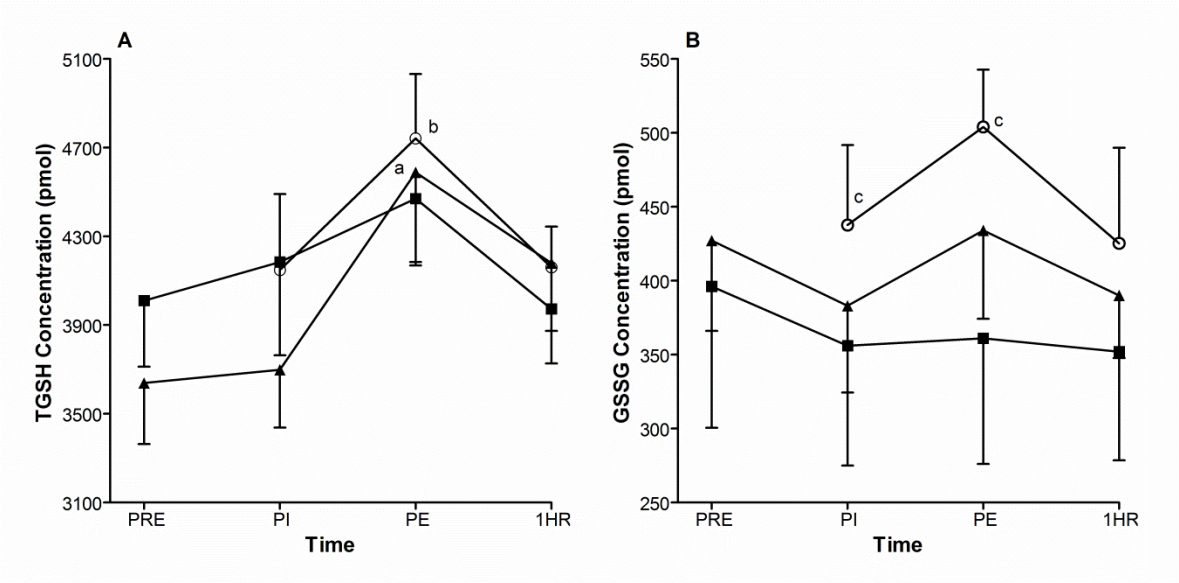

Figure 6.1. Presented are mean ± SEM (*n = 7*) data for (**A**) total (TGSH) and (**B**) oxidised (GSSG) glutathione pre ingestion (PRE), post ingestion/pre exercise (PI), post exercise (PE) and one hour post exercise (1HR) during glycerol hyperhydration (G, ■), water hyperhydration (W, ▲) or control (C, ⊖). [a]Significantly increased PE vs. PRE and PI ($p = 0.04$ and $p < 0.01$, respectively) in W trial. [b]Significantly greater in PE vs. PI and HR ($p = 0.01$ for both) in C trial. [c]Significantly greater concentration in C vs. G PI and PE ($p = 0.05$ and $p < 0.01$, respectively).

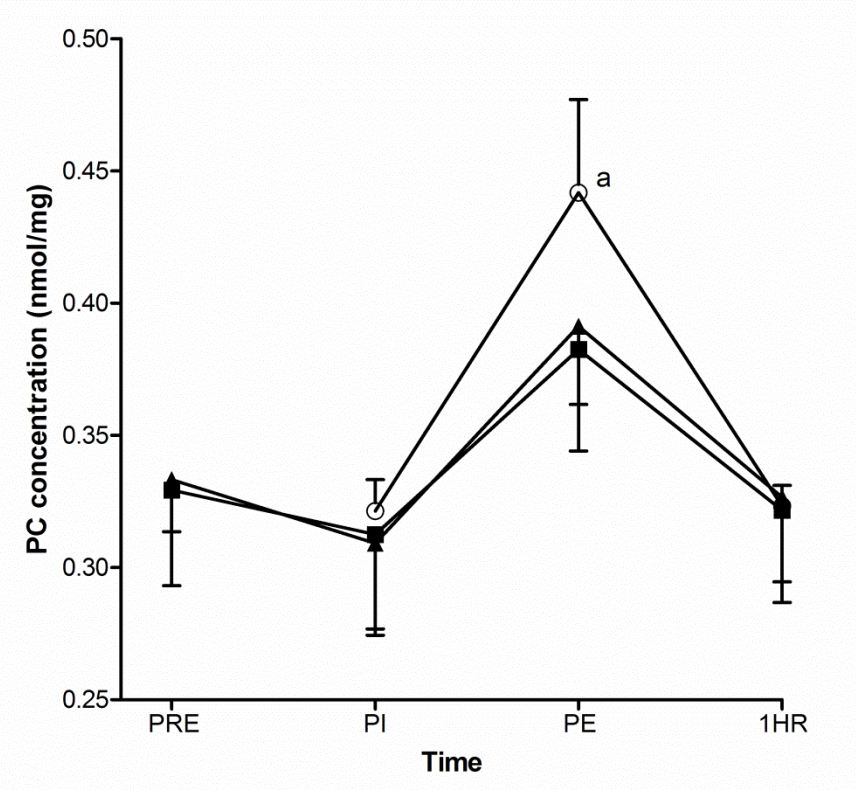

Figure 6.2. Presented are mean (*n = 7*) data for protein carbonyls (PC) pre ingestion (PRE), post ingestion/pre exercise (PI), post exercise (PE) and one hour post exercise (1HR) during glycerol hyperhydration (G, ■), water hyperhydration (W, ▲) or control (C, ⊖). [a]PE greater vs. PI and HR for C ($p < 0.01$).

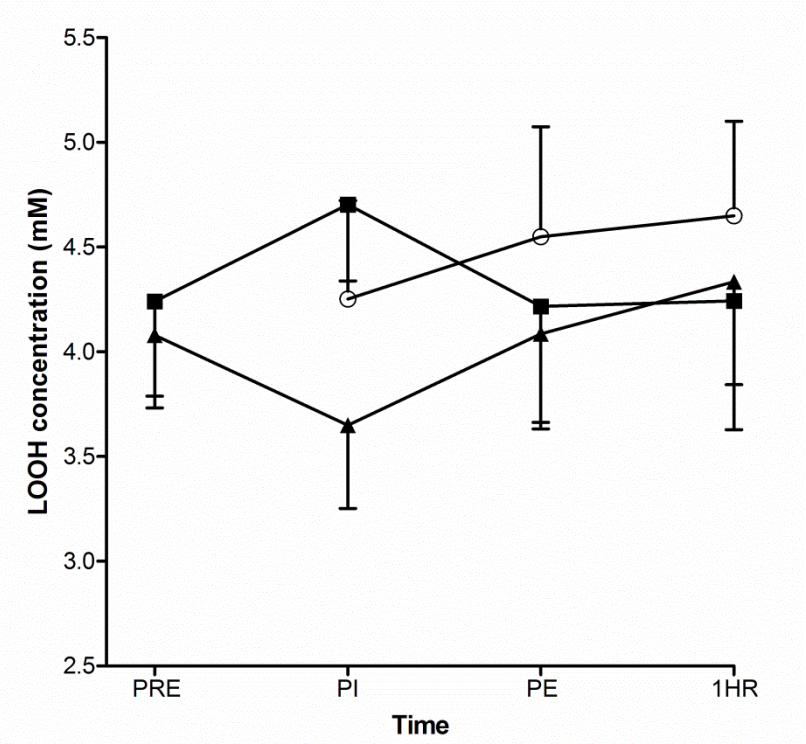

Figure 6.3. Presented are mean ± SEM (*n = 7*) data for lipid hydroperoxides (LOOH) pre ingestion (PRE), post ingestion/pre exercise (PI), post exercise (PE) and one hour post exercise (1HR) during glycerol hyperhydration (G, ■), water hyperhydration (W, ▲) or control (C, ○).

The relationship between GSSG, TGSH, LOOH, PC, body mass loss, Tb, and BG via Pearson's correlations are summarised in Table 6.1. Table 6.2 presents the data for fluid changes during hyperhydration as well as pre to post exercise. Total fluid consumed was greater in C vs. G and W ($p = 0.03$), however urine volume was greater during C vs. G and W ($p < 0.01$) which resulted in less fluid retained in C vs. G and W ($p < 0.01$). Plasma glycerol (mmol/l) increased PI in G ($p < 0.01$) and remained elevated throughout the trial ($p < 0.01$). Additionally, plasma glycerol concentration increased PE in C ($p<0.01$). Plasma glucose concentration (mg/dL) increased PI during G trial ($p = 0.04$) as well as PE during G and C trials ($p < 0.01$ for both; *Figure 6.4*).

Table 6.1. Presented are the Pearson's correlations between oxidised (GSSG) and total (TGSH) glutathione, lipid hydroperoxides (LOOH), protein carbonyls (PC), body mass loss (BM), mean body temperature (Tb), and blood glucose (BG) pre to post ingestion (PRE to PI), post ingestion to post exercise (PI to PE) and pre ingestion to post exercise (PRE to PE).

PRE to PI		PI to PE		PRE to PE	
PC & TGSH	$r^2=0.4^{*}$	PC & Tb	$r^2=0.4^{\dagger}$	PC & Tb	$r^2=0.4^{\ddagger}$
PC & LOOH	$r^2=0.5^{*}$	PC & BG	$r^2=0.6^{\partial}$	PC & BG	$r^2=0.4^{*}$
PC & BM	$r^2=0.4^{*}$	PC & BM	$r^2=0.4^{\dagger}$	GSSG & LOOH	$r^2=0.5^{\partial}$
GSSG & LOOH	$r^2=0.4^{\dagger}$	GSSG & LOOH	$r^2=0.5^{\partial}$		
GSSG & BG	$r^2=0.3^{\dagger}$	GSSG & BG	$r^2=0.3^{\dagger}$		

$^{*}p = 0.01$
$^{\dagger}p = 0.04$
$^{\ddagger}p = 0.03$
$^{\partial}p < 0.01$

Table 6.2. Presented are mean ± SEM (n = 7) data for body mass changes (BMΔ), plasma volume changes (PVΔ), total fluid ingested (TFI in litres), and total urine volume (UV in litres) during hyperhydration and post exercise, and percent dehydration (%BML) and total fluid retained (TFR in litres) post exercise during the glycerol, water and control trials.

	Glycerol (G)		Water (W)		Control (C)
	Hyperhydration	Exercise	Hyperhydration	Exercise	Exercise
BMΔ	0.67 ± 0.24	-0.06 ± 0.21	0.50 ± 0.15	-0.44 ± 0.38	-0.19 ± 0.38
PVΔ	6.75 ± 0.80	-5.16 ± 1.31	5.50 ± 0.65	-5.46 ± 1.53	-5.69 ± 0.47
TFI	1.90 ± 0.10	1.39 ± 0.10	1.90 ± 0.10	1.49 ± 0.19	$2.00 \pm 0.23^{*}$
UV	1.12 ± 0.05	0.27 ± 0.03	1.30 ± 0.07	0.38 ± 0.04	$0.84 \pm 0.02^{\dagger}$
%BML	--	-0.11 ± 0.32	--	-0.66 ± 0.52	-0.20 ± 0.23
TFR	--	1.90 ± 0.10	--	1.71 ± 0.19	$1.16 \pm 0.15^{\ddagger}$

*Significantly greater vs. G and W ($p = 0.03$)
†Significantly greater vs. G and W ($p < 0.01$)
‡Significantly less vs. G and W ($p < 0.01$ for both)

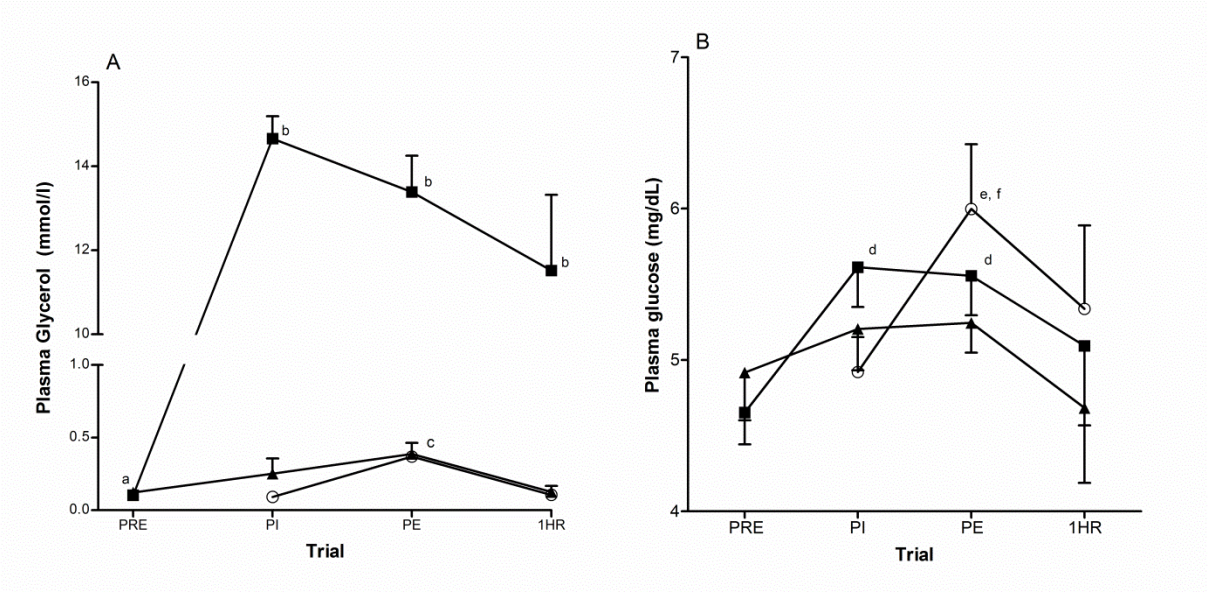

Figure 6.4. Presented are mean (*n = 7*) data for (**A**) plasma glycerol and (**B**) glucose concentrations pre ingestion (PRE), post ingestion/pre exercise (PI), post exercise (PE) and one hour post exercise (1HR) during glycerol hyperhydration (G, ■), water hyperhydration (W, ▲) or control (C, ⊖). [a]PRE lower vs. PI, PE and HR for G trial ($p < 0.01$ for all). [b]G greater than corresponding time in W and C trials ($p < 0.01$). [c]PE greater vs. PRE, PI and HR for C trial ($p < 0.01$). [d]Greater vs. PRE for G ($p = 0.04$ and $p < 0.01$ for PI and PE, respectively). [e]PE greater vs. PI for C ($p < 0.01$). [f]PE C greater vs. W ($p = 0.02$).

Thermoregulation

HR increased with exercise ($F = 272.52$, $p < 0.01$) however there was no difference between trials at any point ($F = 1.13$, $p = 0.35$). Tc ($F = 105.62$, $p < 0.01$), Ts ($F = 36.57$, $p < 0.01$), and Tb ($F = 208.14$, $p < 0.01$) increased post exercise, however there were no differences between conditions (*Figure 6.5*). While all ratings were increased pre to post exercise ($P < 0.01$), there were no significant differences between trials for RPE, thermal sensation or sweating sensation ($P > 0.05$ for all).

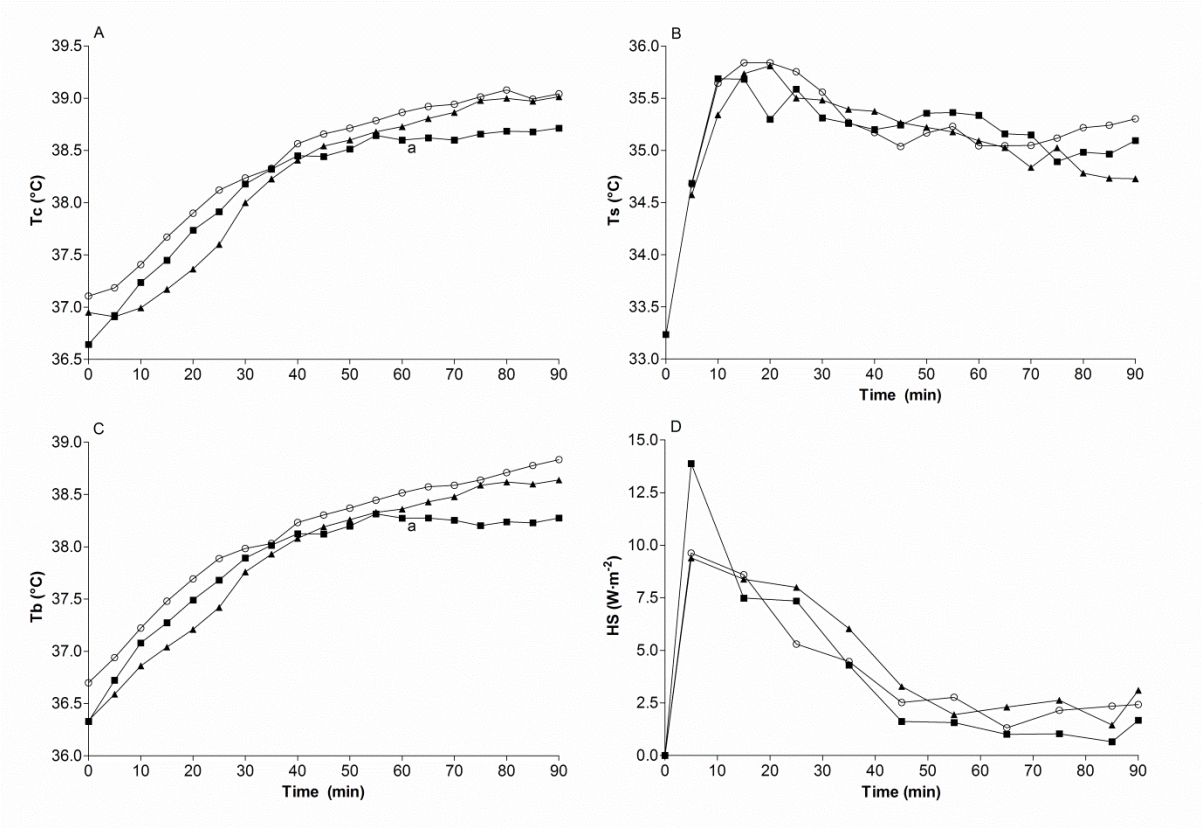

Figure 6.5. Presented are mean (*n = 7*) data for (**A**) core temperature (Tc) (**B**) mean body temperature (Tb) and (C) heat storage (HS) during glycerol hyperhydration (G, ■), water hyperhydration (W, ▲) or control (C, ⊖). [a]Tc and Tb significantly lower after 60 min in G ($p < 0.01$).

Exercise Performance

There was no significant difference between trials for either total distance covered (G: 51 ±2 km, W: 51 ±2 km, C: 50 ±1 km; F = 1.43, p = 0.28) or PO (G: 159 ±15 Watts, W: 160 ±14 Watts, C: 156 ±13 Watts; F = 0.50, p = 0.62) during the 90 min exercise.

6.4. Discussion

The major finding of the current experiment is that oxidative stress appears to have been attenuated by fluid consumption prior to and during exercise, in particular glycerol hyperhydration maintained significantly lower GSSG concentrations pre and post exercise

compared to control trial. Additionally, hyperhydration (glycerol and water) was superior to control trial for attenuating increased PC concentration post exercise.

While there were minimal difference between the pre exercise hydration interventions, there does appear to be an influence of hydration on all markers. Hyperhydration maintained significantly lower levels of GSSG and tended to maintain lower PC concentration compared to control throughout the protocol (*Figures 6.1 and 6.2, respectively*). Interestingly, glycerol hyperhydration resulted in a 10% increase in LOOH post ingestion (*Figure 6.3*), which is likely due to increased lipid formation during glycerol metabolism (Robergs and Griffin, 1998). This result is likely physiologically insignificant because additional markers of stress show a decrease (GSSG, PC) or no change (TGSH) during this same period and LOOH levels returned to below baseline PE. Additionally, GSSG, TGSH, PC, and LOOH concentrations tended to be higher PE during C compared to G and W (*Figures 6.1-6.3*). This was possibly due to the inferior amount of fluid retained during C compared to the G and W trials (*Table 6.2*). However, because only one of the oxidative stress markers was significantly increased PE (PC concentration), it is plausible that because subjects drank more fluid during exercise in C compared to G and W (*Table 6.2*) this facilitated attenuation of oxidative stress in a similar manner to pre exercise hyperhydration. Therefore, it appears that both pre exercise hyperhydration as well as fluid ingestion during exercise to match sweat losses are sufficient to blunt rises in exercise-induced oxidative stress; the mechanism of this attenuation, however, remains to be elucidated.

While *in vitro* work indicates euhydration is vital to attenuating oxidative stress (Martins and Meneghini, 1994, Rios et al., 2011, Saha et al., 1992, Zhang et al., 2004) only one previous *in vivo* investigation has directly investigated the relationship between dehydration and oxidative stress (Paik et al., 2009). In this study, the authors found a significant attenuation in oxidative stress when participants rehydrated with both water and sports drink (CHO) and that this attenuation was significantly greater in participants consuming CHO. They concluded this resulted from faster recovery of plasma volume and lower degree of dehydration with consumption of CHO compared to water, which has been previously described (Criswell et al., 1992, Maughan and Gleeson, 1988, Shi and Gisolfi, 1998). Indeed, in the current study BG levels and body mass loss were strongly correlated throughout the protocol (average $r^2 = 0.60$, $p < 0.01$). However, additional research has also noted a significant attenuation in lipid peroxidation by-products from ingestion of CHO by way of increased or maintained BG levels (McAnulty et al., 2007b, McAnulty et al., 2003). After ingestion, approximately 70-90% of glycerol is metabolised to glyceraldehyde 3-phosphate and used in gluconeogenesis (Stryer 2005; *Figure 2.5*) and should thus aid in maintaining adequate glucose levels. While the majority of investigations utilising glycerol have found no change, we found a significant rise in BG post glycerol ingestion, as well as post exercise with and without glycerol ingestion, but not during water ingestion (*Figure 6.4*). Although stress was attenuated as a result of fluid ingestion in all trials, BG was only increased in two of the three trials; therefore it is unlikely the increase in BG was the sole reason for the decreased stress in the current study. However, there was a moderate relationship between BG and PC concentration after hyperhydration and exercise ($r^2 = 0.6$, $p < 0.01$, $r^2 = 0.4$, $p = 0.01$, respectively) therefore it is likely that both increased BG

concentration as well as increased hydration played a role in attenuating the oxidative stress.

It is also important to note that these studies comparing CHO and water ingestion on oxidative stress were limited in that they did not include a control trial or a hydration measurement (i.e., blood and urine osmolality, body mass changes, etc.) making it unclear whether fluid ingestion *per se* or CHO ingestion is responsible for attenuation of oxidative stress. Similarly, in the current study the participants were allowed to drink fluid *ad libitum* during all trials, making it difficult to discern whether the hyperhydration had any effect alone, therefore future work should aim to disseminate the effects of pre exercise hyperhydration independent of additional fluid consumption on oxidative stress as well as thoroughly address with proper controls the effect of CHO ingestion on oxidative stress.

In conclusion, the current study found attenuation of oxidative stress as a result of pre exercise hyperhydration and *ad libitum* fluid consumption during exercise and may be due in part to increased BG levels.

Chapter 7: General Discussion and Conclusions

7.1. 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 effect of dehydration on oxidative and cellular stress markers.

- *Dehydration significantly increased GSSG concentration while euhydration attenuated these increases.*

ii) Investigate the effect of hyperthermia on oxidative and cellular stress markers.

- *Hyperthermia alone had no effect on any of the markers of oxidative or cellular stress.*

iii) Investigate the combined effect of dehydration and hyperthermia on oxidative and cellular stress markers.

- *The superimposition of hyperthermia during dehydration did not result in augmented levels of oxidative or cellular stress.*

Experiment 2

i) Investigate the effect of dehydration and hyperthermia individually, as well in tandem, on oxidative and cellular stress markers in untrained individuals.

- *GSSG and HSP72 concentrations were increased as a result of exercise regardless of body mass loss or environmental stress. However, there was a relationship between GSSG concentration and body mass loss as well as a relationship between HSP72 concentration and HS.*

ii) Compare the oxidative and cellular stress response in untrained participants to the trained participants in experiment 1.

- *Untrained subjects showed greater basal and post exercise GSSG, TGSH, GSH, and TBARS concentrations. There was no difference between trained and untrained subjects in resting or post exercise HSP72 concentration.*

Experiment 3

i) Investigate the effect of pre-exercise glycerol or water hyperhydration on oxidative stress markers.

- *Glycerol hyperhydration maintained significantly lower GSSG concentrations pre and post exercise compared to control trial. Additionally, hyperhydration (glycerol and water) was superior to control trial for attenuating increased PC concentration post exercise.*

ii) Investigate the effect of pre-exercise glycerol or water hyperhydration on exercise thermoregulation and performance

- *Pre-exercise glycerol hyperhydration resulted in enhanced thermoregulation via decreased Tb and Tc after 60 min of exercise, however, there was no effect of either water or glycerol hyperhydration on exercise performance.*

At the time this research was undertaken there had been no previous investigations, and since there has been one published work into the effects of altered hydration state on markers of oxidative and cellular stress during exercise (Paik et al., 2009). Additionally, while there has been limited investigation into the effect of hyperthermia on markers of

oxidative and cellular stress (Laitano et al., 2010, McAnulty et al., 2005, Ohtsuka et al., 1994), only one of these investigations has not been flawed by confounding variables such as altered hydration status, however the exercise used in this study is impractical and not applicable to daily physical activity (Laitano et al., 2010). Finally, no previous work has investigated the combined effects of exercise-induced dehydration with and without thermal stress on markers of oxidative and cellular stress, which was the aim of the present work.

In the first experimental study the author aimed to investigate the effects of dehydration on oxidative stress in trained cyclists. Previous *in vitro* investigations have demonstrated increased cellular susceptibility to damage as well as a net oxidation of GSH that occurs from dehydration-induced cellular shrinkage (Saha et al., 1992). Therefore, it was hypothesised that dehydration would result in augmented oxidative stress and that this phenomenon would be amplified when combined with hyperthermia. Indeed a significant influence of hydration on oxidative stress was found; GSSG concentration increased by a mean of 29% post exercise as a result of dehydration, while euhydration actually reduced GSSG concentration. However, there was no additive effect of hyperthermia on changes in GSSG concentration. Additionally, it was hypothesised that HSP concentrations would be diminished as a result of dehydration, in conjunction with previous *in vitro* investigations (Kurz et al., 1998). However, on the contrary HSP72 concentration was augmented while HSP32 concentration was decreased, but neither appeared to be by affected by hydration or environment. The author suspects the augmentation in HSP72 concentration was a result of the increased oxidative stress ensuing from exercise. The decrease in HSP32 concentration was at first perplexing; however, upon further examination of exercise induced changes in

HSP32 it was found that many other investigations have also noted similar decreases in HSP32 concentrations (Fehrenbach et al., 2003a, Thompson et al., 2005), making our results unremarkable; however, the mechanism for this decline is still unclear. The author attributes the attenuation in oxidative stress and HSP expression to the enhanced aerobic capacity of our subjects who were highly trained competitive cyclists.

As a result of the findings of the first experiment the author decided to pursue a follow up study using untrained participants, to compare the physiological responses to those of the trained cyclists. Interestingly, in contrast to the trained subjects of the first experiment, oxidative stress in the participants of the second study was not hydration-dependent; oxidative stress increased as a result of exercise, regardless of hydration status or environmental heat stress. However, there was no relationship between hydration status and GSSG but there was a trend for GSSG concentration to be higher during dehydration trials. These results are likely due to the inferior antioxidant defences of the untrained participants (Alessio, 1993), which was confirmed by augmented resting and post exercise concentrations of oxidative stress markers (TGSH, GSSG, and TBARS) in the untrained compared to the trained participants. Matching the findings of the first experiment, HSP72 concentration increased as a result of exercise, regardless of hydration or thermal stress. Additionally, there was a significant relationship between HS and HSP72. Unfortunately, this relationship could not be established for the first study because Ts was not measured therefore Tb and HS could not be calculated. However, the author suspects that as a result of their greater aerobic fitness levels, HSP72 expression in the trained participants would be less dependent upon HS and Tb.

Finally, because oxidative stress was attenuated by fluid ingestion during exercise in the trained cyclists, the author decided to alter the pre-exercise hydration status of participants, as many athletes do, to understand the effect this would have on oxidative stress during exercise. Accordingly, hyperhydration was attained using either glycerol or water two hours prior to exercise, while participants were allowed to consume fluid *ad libitum* during exercise trials. These trials were all completed in a hot environment because the use of glycerol hyperhydration would realistically have no impact on thermoregulation and performance in normothermic environment and the first two experimental chapters demonstrated that oxidative stress was not altered by hyperthermic exposure, contrary to other reports (Laitano et al., 2010, McAnulty et al., 2005, Ohtsuka et al., 1994). The primary finding of the third experiment was that oxidative stress appears to have been attenuated by fluid consumption prior to and during exercise. There was no difference between glycerol and water interventions for attenuating rises in PC concentration, however glycerol hyperhydration was superior to water for attenuating rises in GSSG concentration. Additionally, similar to the first experiment, fluid consumption during exercise without hyperhydration (control) attenuated oxidative stress (no significant increases in GSSG or LOOH post exercise). While there was no significant difference between the pre exercise hydration interventions, there does appear to be an influence of hydration on all markers. Additionally, while not significant, oxidative stress tended to be greater in the control trial compared to the hyperhydration trials, likely due to the inferior amount of fluid retained during the control trial. However, it is possible that because subjects drank more fluid during exercise in the control compared to the hyperhydration trials, this facilitated attenuation of oxidative stress to the same extent as pre exercise hyperhydration.

Taken together, the findings of these three experiments appear to show that both pre exercise hyperhydration as well as fluid ingestion during exercise are sufficient to blunt rises in exercise-induced oxidative stress; the mechanism of this attenuation, however, remains to be elucidated. *In vivo* research has shown an increase in cellular susceptibility to ROS attack during dehydration, however no human cellular *in vivo* experiments have investigated the relationship between cellular dehydration and oxidative stress; therefore it is difficult to say if this is the mechanism by which oxidative stress is attenuated with fluid consumption. It is possible that oxidative stress is simply attenuated by the increase in cell volume and maintenance of cell wall integrity. Additionally, it is possible that because the markers of oxidative stress were measured in blood, the concentrations were altered by plasma volume shifts and thus may not accurately depict the stress levels. However, all oxidative stress marker concentrations have been corrected for plasma volume shifts therefore the author believes the concentrations do accurately reflect the oxidative stress. Future investigations are needed to elucidate further the relationship between hydration status and oxidative stress.

7.2. Experimental Limitations

Notably lacking from this thesis are power calculations for appropriate sample sizes. This is due to a variety of reasons, most important of which was lack of data to base a power sample on. Few studies previously used flow cytometric analysis of HSP making it difficult to calculate a necessary sample size based on that data. Additionally, no previous published investigations exist using the glutathione assay kit we employed, therefore no data exists to use in a power calculation. Additionally, the particular kit used to measures glutathione

concentrations in pmol, while most published literature report their concentrations in µmol/l or nmol/l. After many attempts through Enzo Life Sciences technical support to convert our data to those of the literature failed, the author determined it would not be possible to make the conversions that would aid in not only a power calculation, but also in making our data more analogous to the literature. Finally, power calculations for the final study were not completed because the study size was dictated by the cost of sample analysis and failure to recruit more subjects. Although the author sought to utilise more participants, recruitment of trained cyclists who were in training for the upcoming cycling season and financial constraints only allowed for the use of seven participants.

The studies in this thesis are also limited by the oxidative stress markers used, particularly the use of the TBARS assay for the first two experiments. Although TBARS has been reported to lack specificity for MDA (Oh-ishi et al., 2000), the assay is a straightforward and cost effective marker for lipid peroxidation. However, as the thesis evolved it became clear that the use of more sensitive assays was necessary. Thus, in the final study the author chose to measure a marker of lipid peroxidation (LOOH), a marker of protein oxidation, (PC) and a marker of redox status (GSSG). Additional measurement of F_2-isoprostanes (FIP) and DNA damage were sought, however, they were expensive and technically complex and therefore excluded. Also excluded was the measurement of HSP72 as this did not show any relationship with body mass loss or oxidative stress in the first two experiments. To further improve upon the changes made in the final experiment, future studies involving the topics laid out in this thesis may also venture to measure HSP27, which has been linked with glutathione homeostasis (Arrigo et al., 2005) and may aid in clarifying its role in oxidative stress. Additionally, use of superior techniques, such as

HPLC for measurement of glutathione, TBARS, and FIP would make these markers more sensitive and accurate; however, this technique is also expensive.

It is important to note that the controls for the final experiment were not complete, impeding the determination of the effect pre-exercise hyperhydration and *ad libitum* fluid intake during exercise had on oxidative stress. Because the first experimental study demonstrated that fluid ingestion during exercise results in attenuated stress, the author concedes there should have been a trial(s) without fluid ingestion during exercise. However, because this is not practical and would not have real world applications, the author did not choose this scenario.

7.3. Future recommendations

As a result of the novel findings and also the limitations of the current thesis, the author believes there is a plethora of future research that could be sought to further clarify the relationship between body mass loss and oxidative stress. Most importantly the literature is lacking any data on the effects of non-exercise, non-thermal stress induced dehydration on markers of oxidative stress. Such a scenario would be common, especially in those participating in physical activity where rehydration and refuelling do not return body fluids to resting levels, which may take up to 24 hours when 2-5% of body mass is lost (Burke, 2007) or individuals who simply do not consume enough fluid throughout the day. Additionally, for the sake of maintaining real world applicability, measurement of oxidative stress markers in individuals participating in competitions would be appealing. This may reveal whether the oxidative stress is detrimental or whether it impacts performance. Finally, given the link between aging, oxidative stress and cardiac disease, it

would be relevant to investigate the effects of hydration changes in older adults, with or without cardiac disease, on oxidative stress, particularly those who are taking medicines for blood pressure, which would alter body fluid status.

7.4. Conclusions

It is clear that hydration status plays a role in the production of oxidative stress during exercise in trained individuals. Maintenance of euhydration as well as pre-exercise hyperhydration attenuated rises in oxidative stress. However, oxidative stress in untrained individuals did not appear to respond to changes in hydration status, likely because of their inferior antioxidant defences. Additionally, HSP72 concentration appears to be intrinsically linked with exercise hyperthermia and does not appear to be affected by changes in body mass loss. These findings highlight the importance of proper fluid intake prior to and during exercise, as well as participation in regular physical activity to attain systemic adaptation and hormesis.

Chapter 8: References

Abbiss, C. R., Burnett, A., Nosaka, K., Green, J. P., Foster, J. K. & Laursen, P. B. 2010. Effect of hot versus cold climates on power output, muscle activation, and perceived fatigue during a dynamic 100-km cycling trial. *J Sports Sci,* 28**,** 117-25.

Abbiss, C. R., Quod, M. J., Levin, G., Martin, D. T. & Laursen, P. B. 2009. Accuracy of the Velotron ergometer and SRM power meter. *Int J Sports Med,* 30**,** 107-12.

Adams, W. C., Mack, G. W., Langhans, G. W. & Nadel, E. R. 1992. Effects of varied air velocity on sweating and evaporative rates during exercise. *J Appl Physiol,* 73**,** 2668-74.

Agostini, F., Dalla Libera, L., Rittweger, J., Mazzucco, S., Jurdana, M., Mekjavic, I. B., Pisot, R., Gorza, L., Narici, M. & Biolo, G. 2010. Effects of inactivity on human muscle glutathione synthesis by a double-tracer and single-biopsy approach. *J Physiol,* 588**,** 5089-104.

Alessio, H. M. 1993. Exercise-induced oxidative stress. *Med Sci Sports Exerc,* 25**,** 218-24.

Alessio, H. M., Hagerman, A. E., Fulkerson, B. K., Ambrose, J., Rice, R. E. & Wiley, R. L. 2000. Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. *Med Sci Sports Exerc,* 32**,** 1576-81.

Almar, M., Villa, J. G., Cuevas, M. J., Rodriguez-Marroyo, J. A., Avila, C. & Gonzalez-Gallego, J. 2002. Urinary levels of 8-hydroxydeoxyguanosine as a marker of oxidative damage in road cycling. *Free Radic Res,* 36**,** 247-53.

Altareki, N., Drust, B., Atkinson, G., Cable, T. & Gregson, W. 2009. Effects of environmental heat stress (35 degrees C) with simulated air movement on the thermoregulatory responses during a 4-km cycling time trial. *Int J Sports Med,* 30**,** 9-15.

Anderson, E. J. & Neufer, P. D. 2006. Type II skeletal myofibers possess unique properties that potentiate mitochondrial H(2)O(2) generation. *Am J Physiol Cell Physiol,* 290**,** C844-51.

Anderson, M. J., Cotter, J. D., Garnham, A. P., Casley, D. J. & Febbraio, M. A. 2001. Effect of glycerol-induced hyperhydration on thermoregulation and metabolism during exercise in heat. *Int J Sport Nutr Exerc Metab,* 11**,** 315-33.

Armstrong, L. E., Whittlesey, M. J., Casa, D. J., Elliott, T. A., Kavouras, S. A., Keith, N. R. & Maresh, C. M. 2006. No effect of 5% hypohydration on running economy of competitive runners at 23 degrees C. *Med Sci Sports Exerc,* 38**,** 1762-9.

Arrigo, A. P., Virot, S., Chaufour, S., Firdaus, W., Kretz-Remy, C. & Diaz-Latoud, C. 2005. Hsp27 consolidates intracellular redox homeostasis by upholding glutathione in its reduced form and by decreasing iron intracellular levels. *Antioxid Redox Signal,* 7**,** 414-22.

Arthur, P. G., Lim, S. C., Meloni, B. P., Munns, S. E., Chan, A. & Knuckey, N. W. 2004. The protective effect of hypoxic preconditioning on cortical neuronal cultures is associated with increases in the activity of several antioxidant enzymes. *Brain Res,* 1017**,** 146-54.

Ashton, T., Rowlands, C. C., Jones, E., Young, I. S., Jackson, S. K., Davies, B. & Peters, J. R. 1998. Electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise. *Eur J Appl Physiol,* 77**,** 498-502.

Atalay, M., Laaksonen, D. E., Niskanen, L., Uusitupa, M., Hanninen, O. & Sen, C. K. 1997. Altered antioxidant enzyme defences in insulin-dependent diabetic men with increased resting and exercise-induced oxidative stress. *Acta Physiol Scand,* 161**,** 195-201.

Aust, A. E. & Eveleigh, J. F. 1999. Mechanisms of DNA oxidation. *Proc Soc Exp Biol Med,* 222**,** 246-52.

Bachelet, M., Mariethoz, E., Banzet, N., Souil, E., Pinot, F., Polla, C. Z., Durand, P., Bouchaert, I. & Polla, B. S. 1998. Flow cytometry is a rapid and reliable method for evaluating heat shock protein 70 expression in human monocytes. *Cell Stress Chaperones,* 3**,** 168-76.

Bains, J. S. & Shaw, C. A. 1997. Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. *Brain Res Brain Res Rev,* 25**,** 335-58.

Balendiran, G. K., Dabur, R. & Fraser, D. 2004. The role of glutathione in cancer. *Cell Biochem Funct,* 22**,** 343-52.

Balon, T. W. & Nadler, J. L. 1994. Nitric oxide release is present from incubated skeletal muscle preparations. *J Appl Physiol,* 77**,** 2519-21.

Barr, S. I., Costill, D. L. & Fink, W. J. 1991. Fluid replacement during prolonged exercise: effects of water, saline, or no fluid. *Med Sci Sports Exerc,* 23**,** 811-7.

Beckmann, R. P., Mizzen, L. E. & Welch, W. J. 1990. Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. *Science,* 248**,** 850-4.

Bloomer, R. J., Davis, P. G., Consitt, L. A. & Wideman, L. 2007a. Plasma protein carbonyl response to increasing exercise duration in aerobically trained men and women. *Int J Sports Med,* 28**,** 21-5.

Bloomer, R. J., Falvo, M. J., Fry, A. C., Schilling, B. K., Smith, W. A. & Moore, C. A. 2006. Oxidative stress response in trained men following repeated squats or sprints. *Med Sci Sports Exerc,* 38**,** 1436-42.

Bloomer, R. J., Fry, A. C., Falvo, M. J. & Moore, C. A. 2007b. Protein carbonyls are acutely elevated following single set anaerobic exercise in resistance trained men. *J Sci Med Sport,* 10**,** 411-7.

Bloomer, R. J., Goldfarb, A. H., Wideman, L., McKenzie, M. J. & Consitt, L. A. 2005. Effects of acute aerobic and anaerobic exercise on blood markers of oxidative stress. *J Strength Cond Res,* 19**,** 276-85.

Borg, G. 1970. Perceived exertion as an indicator of somatic stress. *Scand J Rehabil Med,* 2**,** 92-8.

Borsheim, E., Knardahl, S. & Hostmark, A. T. 1999. Short-term effects of exercise on plasma very low density lipoproteins (VLDL) and fatty acids. *Med Sci Sports Exerc,* 31**,** 522-30.

Bortz, W. M., Paul, P., Haff, A. C. & Holmes, W. L. 1972. Glycerol turnover and oxidation in man. *J Clin Invest,* 51**,** 1537-46.

Boveris, A. & Chance, B. 1973. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J,* 134**,** 707-16.

Braughler, J. M. & Hall, E. D. 1992. Involvement of lipid peroxidation in CNS injury. *J Neurotrauma,* 9 Suppl 1**,** S1-7.

Brown, G. C. & Borutaite, V. 2002. Nitric oxide inhibition of mitochondrial respiration and its role in cell death. *Free Radic Biol Med,* 33**,** 1440-50.

Buono, M. J. & Wall, A. J. 2000. Effect of hypohydration on core temperature during exercise in temperate and hot environments. *Pflugers Arch,* 440**,** 476-80.

Burke, L. 2007. *Sports Nutrition,* Oxford ; New York, Oxford University Press.

Byrne, C., Twist, C. & Eston, R. 2004. Neuromuscular function after exercise-induced muscle damage: theoretical and applied implications. *Sports Med,* 34**,** 49-69.

Cairo, G., Bardella, L., Schiaffonati, L. & Bernelli-Zazzera, A. 1985. Synthesis of heat shock proteins in rat liver after ischemia and hyperthermia. *Hepatology,* 5**,** 357-61.
Ceriello, A. & Motz, E. 2004. Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. *Arterioscler Thromb Vasc Biol,* 24**,** 816-23.
Chen, M. F., Hsu, H. C. & Lee, Y. T. 1994. Effects of acute exercise on the changes of lipid profiles and peroxides, prostanoids, and platelet activation in hypercholesterolemic patients before and after treatment. *Prostaglandins,* 48**,** 157-74.
Cheng, B., Kuipers, H., Snyder, A. C., Keizer, H. A., Jeukendrup, A. & Hesselink, M. 1992. A new approach for the determination of ventilatory and lactate thresholds. *Int J Sports Med,* 13**,** 518-22.
Cheng, F. C., Jen, J. F. & Tsai, T. H. 2002. Hydroxyl radical in living systems and its separation methods. *J Chromatogr B Analyt Technol Biomed Life Sci,* 781**,** 481-96.
Cheung, S. S. & McLellan, T. M. 1998. Heat acclimation, aerobic fitness, and hydration effects on tolerance during uncompensable heat stress. *J Appl Physiol,* 84**,** 1731-9.
Cheuvront, S. N., Carter, R., 3rd, Haymes, E. M. & Sawka, M. N. 2006. No effect of moderate hypohydration or hyperthermia on anaerobic exercise performance. *Med Sci Sports Exerc,* 38**,** 1093-7.
Cheuvront, S. N., Carter, R., 3rd & Sawka, M. N. 2003. Fluid balance and endurance exercise performance. *Curr Sports Med Rep,* 2**,** 202-8.
Cheuvront, S. N., Kenefick, R. W., Montain, S. J. & Sawka, M. N. 2010. Mechanisms of aerobic performance impairment with heat stress and dehydration. *J Appl Physiol,* 109**,** 1989-95.
Clark, J. E., Foresti, R., Sarathchandra, P., Kaur, H., Green, C. J. & Motterlini, R. 2000. Heme oxygenase-1-derived bilirubin ameliorates postischemic myocardial dysfunction. *Am J Physiol Heart Circ Physiol,* 278**,** H643-51.
Colin, J., Timbal, J., Houdas, Y., Boutelier, C. & Guieu, J. D. 1971. Computation of mean body temperature from rectal and skin temperatures. *J Appl Physiol,* 31**,** 484-9.
Collins, A. R., Dobson, V. L., Dusinska, M., Kennedy, G. & Stetina, R. 1997. The comet assay: what can it really tell us? *Mutat Res,* 375**,** 183-93.
Convertino, V. A. 1987. Fluid shifts and hydration state: effects of long-term exercise. *Can J Sport Sci,* 12**,** 136S-139S.
Coutts, A., Reaburn, P., Mummery, K. & Holmes, M. 2002. The effect of glycerol hyperhydration on olympic distance triathlon performance in high ambient temperatures. *Int J Sport Nutr Exerc Metab,* 12**,** 105-19.
Coyle, E. F., Coggan, A. R., Hopper, M. K. & Walters, T. J. 1988. Determinants of endurance in well-trained cyclists. *J Appl Physiol,* 64**,** 2622-30.
Coyle, E. F. & Gonzalez-Alonso, J. 2001. Cardiovascular drift during prolonged exercise: new perspectives. *Exerc Sport Sci Rev,* 29**,** 88-92.
Criswell, D., Renshler, K., Powers, S. K., Tulley, R., Cicale, M. & Wheeler, K. 1992. Fluid replacement beverages and maintenance of plasma volume during exercise: role of aldosterone and vasopressin. *Eur J Appl Physiol Occup Physiol,* 65**,** 445-51.
Dahl, N., Pigg, M., Ristoff, E., Gali, R., Carlsson, B., Mannervik, B., Larsson, A. & Board, P. 1997. Missense mutations in the human glutathione synthetase gene result in severe metabolic acidosis, 5-oxoprolinuria, hemolytic anemia and neurological dysfunction. *Hum Mol Genet,* 6**,** 1147-52.

Dalle-Donne, I., Giustarini, D., Colombo, R., Rossi, R. & Milzani, A. 2003. Protein carbonylation in human diseases. *Trends Mol Med,* 9**,** 169-76.

Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D. & Milzani, A. 2006. Biomarkers of oxidative damage in human disease. *Clin Chem,* 52**,** 601-23.

Dizdaroglu, M., Jaruga, P., Birincioglu, M. & Rodriguez, H. 2002. Free radical-induced damage to DNA: mechanisms and measurement. *Free Radic Biol Med,* 32**,** 1102-15.

Douris, P. C., Elokda, A. S., Handrakis, J. P., Principal, S., Rondo, E., Bovell, J., Coughlin, W. P., Mastroianni, C. N., Wong, M. J. & Zimmerman, T. 2009. Martial art training enhances the glutathione antioxidant system in middle-aged adults. *J Strength Cond Res,* 23**,** 1518-23.

Droge, W. 2002. Free radicals in the physiological control of cell function. *Physiol Rev,* 82**,** 47-95.

Duarte, J. A., Appell, H. J., Carvalho, F., Bastos, M. L. & Soares, J. M. 1993. Endothelium-derived oxidative stress may contribute to exercise-induced muscle damage. *Int J Sports Med,* 14**,** 440-3.

Dubois, D. & Dubois, E. F. 1989. Nutrition Metabolism Classic - a Formula to Estimate the Approximate Surface-Area If Height and Weight Be Known (Reprinted from Archives Internal Medicine, Vol 17, Pg 863, 1916). *Nutrition,* 5**,** 303-311.

Ebert, T. R., Martin, D. T., Bullock, N., Mujika, I., Quod, M. J., Farthing, L. A., Burke, L. M. & Withers, R. T. 2007. Influence of hydration status on thermoregulation and cycling hill climbing. *Med Sci Sports Exerc,* 39**,** 323-9.

Elokda, A. S. & Nielsen, D. H. 2007. Effects of exercise training on the glutathione antioxidant system. *Eur J Cardiovasc Prev Rehabil,* 14**,** 630-7.

Ely, B. R., Cheuvront, S. N., Kenefick, R. W. & Sawka, M. N. 2010. Aerobic performance is degraded, despite modest hyperthermia, in hot environments. *Med Sci Sports Exerc,* 42**,** 135-41.

Emdin, M., Pompella, A. & Paolicchi, A. 2005. Gamma-glutamyltransferase, atherosclerosis, and cardiovascular disease: triggering oxidative stress within the plaque. *Circulation,* 112**,** 2078-80.

Esterbauer, H. 1993. Cytotoxicity and genotoxicity of lipid-oxidation products. *Am J Clin Nutr,* 57**,** 779S-785S; discussion 785S-786S.

Esterbauer, H., Schaur, R. J. & Zollner, H. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med,* 11**,** 81-128.

Falone, S., Mirabilio, A., Pennelli, A., Cacchio, M., Di Baldassarre, A., Gallina, S., Passerini, A. & Amicarelli, F. 2010. Differential impact of acute bout of exercise on redox- and oxidative damage-related profiles between untrained subjects and amateur runners. *Physiol Res*.

Febbraio, M. A. & Koukoulas, I. 2000. HSP72 gene expression progressively increases in human skeletal muscle during prolonged, exhaustive exercise. *J Appl Physiol,* 89**,** 1055-60.

Febbraio, M. A., Ott, P., Nielsen, H. B., Steensberg, A., Keller, C., Krustrup, P., Secher, N. H. & Pedersen, B. K. 2002. Exercise induces hepatosplanchnic release of heat shock protein 72 in humans. *J Physiol,* 544**,** 957-62.

Fehrenbach, E., Niess, A. M., Passek, F., Sorichter, S., Schwirtz, A., Berg, A., Dickhuth, H. H. & Northoff, H. 2003a. Influence of different types of exercise on the expression of haem oxygenase-1 in leukocytes. *J Sports Sci,* 21**,** 383-9.

Fehrenbach, E., Niess, A. M., Schlotz, E., Passek, F., Dickhuth, H. H. & Northoff, H. 2000a. Transcriptional and translational regulation of heat shock proteins in leukocytes of endurance runners. *J Appl Physiol,* 89**,** 704-10.

Fehrenbach, E., Niess, A. M., Voelker, K., Northoff, H. & Mooren, F. C. 2005. Exercise intensity and duration affect blood soluble HSP72. *Int J Sports Med,* 26**,** 552-7.

Fehrenbach, E., Passek, F., Niess, A. M., Pohla, H., Weinstock, C., Dickhuth, H. H. & Northoff, H. 2000b. HSP expression in human leukocytes is modulated by endurance exercise. *Med Sci Sports Exerc,* 32**,** 592-600.

Fehrenbach, E., Veith, R., Schmid, M., Dickhuth, H. H., Northoff, H. & Niess, A. M. 2003b. Inverse response of leukocyte heat shock proteins and DNA damage to exercise and heat. *Free Radic Res,* 37**,** 975-82.

Fernandez, P., Guillen, M. I., Gomar, F. & Alcaraz, M. J. 2003. Expression of heme oxygenase-1 and regulation by cytokines in human osteoarthritic chondrocytes. *Biochem Pharmacol,* 66**,** 2049-52.

Fisher-Wellman, K. & Bloomer, R. J. 2009. Acute exercise and oxidative stress: a 30 year history. *Dyn Med,* 8**,** 1.

Flanagan, S. W., Ryan, A. J., Gisolfi, C. V. & Moseley, P. L. 1995. Tissue-specific HSP70 response in animals undergoing heat stress. *Am J Physiol,* 268**,** R28-32.

Frank, M. S., Nahata, M. C. & Hilty, M. D. 1981. Glycerol: a review of its pharmacology, pharmacokinetics, adverse reactions, and clinical use. *Pharmacotherapy,* 1**,** 147-60.

Freund, B. J., Montain, S. J., Young, A. J., Sawka, M. N., DeLuca, J. P., Pandolf, K. B. & Valeri, C. R. 1995. Glycerol hyperhydration: hormonal, renal, and vascular fluid responses. *J Appl Physiol,* 79**,** 2069-77.

Gagge, A. P., Stolwijk, J. A. & Saltin, B. 1969. Comfort and thermal sensations and associated physiological responses during exercise at various ambient temperatures. *Environ Res,* 2**,** 209-29.

Galasko, D. & Montine, T. J. 2010. Biomarkers of oxidative damage and inflammation in Alzheimer's disease. *Biomark Med,* 4**,** 27-36.

Ganio, M. S., Wingo, J. E., Carrolll, C. E., Thomas, M. K. & Cureton, K. J. 2006. Fluid ingestion attenuates the decline in VO2peak associated with cardiovascular drift. *Med Sci Sports Exerc,* 38**,** 901-9.

Gohil, K., Viguie, C., Stanley, W. C., Brooks, G. A. & Packer, L. 1988. Blood glutathione oxidation during human exercise. *J Appl Physiol,* 64**,** 115-9.

Gonzalez-Alonso, J., Calbet, J. A. & Nielsen, B. 1999a. Metabolic and thermodynamic responses to dehydration-induced reductions in muscle blood flow in exercising humans. *J Physiol,* 520 Pt 2**,** 577-89.

Gonzalez-Alonso, J., Crandall, C. G. & Johnson, J. M. 2008. The cardiovascular challenge of exercising in the heat. *J Physiol,* 586**,** 45-53.

Gonzalez-Alonso, J., Mora-Rodriguez, R., Below, P. R. & Coyle, E. F. 1997. Dehydration markedly impairs cardiovascular function in hyperthermic endurance athletes during exercise. *J Appl Physiol,* 82**,** 1229-36.

Gonzalez-Alonso, J., Mora-Rodriguez, R. & Coyle, E. F. 2000. Stroke volume during exercise: interaction of environment and hydration. *Am J Physiol Heart Circ Physiol,* 278**,** H321-30.

Gonzalez-Alonso, J., Teller, C., Andersen, S. L., Jensen, F. B., Hyldig, T. & Nielsen, B. 1999b. Influence of body temperature on the development of fatigue during prolonged exercise in the heat. *J Appl Physiol,* 86**,** 1032-9.

Goto, C., Higashi, Y., Kimura, M., Noma, K., Hara, K., Nakagawa, K., Kawamura, M., Chayama, K., Yoshizumi, M. & Nara, I. 2003. Effect of different intensities of exercise on endothelium-dependent vasodilation in humans: role of endothelium-dependent nitric oxide and oxidative stress. *Circulation,* 108**,** 530-5.

Goulet, E. D., Robergs, R. A., Labrecque, S., Royer, D. & Dionne, I. J. 2006. Effect of glycerol-induced hyperhydration on thermoregulatory and cardiovascular functions and endurance performance during prolonged cycling in a 25 degrees C environment. *Appl Physiol Nutr Metab,* 31**,** 101-9.

Goulet, E. D., Rousseau, S. F., Lamboley, C. R., Plante, G. E. & Dionne, I. J. 2008. Pre-exercise hyperhydration delays dehydration and improves endurance capacity during 2 h of cycling in a temperate climate. *J Physiol Anthropol,* 27**,** 263-71.

Gozzelino, R., Jeney, V. & Soares, M. P. 2010. Mechanisms of cell protection by heme oxygenase-1. *Annu Rev Pharmacol Toxicol,* 50**,** 323-54.

Grasso, S., Scifo, C., Cardile, V., Gulino, R. & Renis, M. 2003. Adaptive responses to the stress induced by hyperthermia or hydrogen peroxide in human fibroblasts. *Exp Biol Med (Maywood),* 228**,** 491-8.

Greenhaff, P. L. 1989. Cardiovascular fitness and thermoregulation during prolonged exercise in man. *Br J Sports Med,* 23**,** 109-14.

Greenleaf, J. E. & Castle, B. L. 1971. Exercise temperature regulation in man during hypohydration and hyperhydration. *J Appl Physiol,* 30**,** 847-53.

Groussard, C., Rannou-Bekono, F., Machefer, G., Chevanne, M., Vincent, S., Sergent, O., Cillard, J. & Gratas-Delamarche, A. 2003. Changes in blood lipid peroxidation markers and antioxidants after a single sprint anaerobic exercise. *Eur J Appl Physiol,* 89**,** 14-20.

Halliwell, B. 2000. Why and how should we measure oxidative DNA damage in nutritional studies? How far have we come? *Am J Clin Nutr,* 72**,** 1082-1087.

Halliwell, B. 2001. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. *Drugs Aging,* 18**,** 685-716.

Halliwell, B. & Aruoma, O. I. 1991. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. *FEBS Lett,* 281**,** 9-19.

Halliwell, B. & Chirico, S. 1993. Lipid peroxidation: its mechanism, measurement, and significance. *Am J Clin Nutr,* 57**,** 715S-724S; discussion 724S-725S.

Halliwell, B. & Gutteridge, J. M. C. 2007. *Free radicals in biology and medicine,* Oxford ; New York, Oxford University Press.

Hamilton, M. T., Gonzalez-Alonso, J., Montain, S. J. & Coyle, E. F. 1991. Fluid replacement and glucose infusion during exercise prevent cardiovascular drift. *J Appl Physiol,* 71**,** 871-7.

Hawkins, C. L. & Davies, M. J. 2001. Generation and propagation of radical reactions on proteins. *Biochim Biophys Acta,* 1504**,** 196-219.

Herrera, E. & Barbas, C. 2001. Vitamin E: action, metabolism and perspectives. *J Physiol Biochem,* 57**,** 43-56.

Hitchins, S., Martin, D. T., Burke, L., Yates, K., Fallon, K., Hahn, A. & Dobson, G. P. 1999. Glycerol hyperhydration improves cycle time trial performance in hot humid conditions. *Eur J Appl Physiol Occup Physiol,* 80**,** 494-501.

Hopker, J., Myers, S., Jobson, S. A., Bruce, W. & Passfield, L. 2010. Validity and reliability of the Wattbike cycle ergometer. *Int J Sports Med,* 31**,** 731-6.

Hostler, D., Gallagher, M., Jr., Goss, F. L., Seitz, J. R., Reis, S. E., Robertson, R. J., Northington, W. E. & Suyama, J. 2009. The effect of hyperhydration on

physiological and perceived strain during treadmill exercise in personal protective equipment. *Eur J Appl Physiol,* 105**,** 607-13.
Hussinger, D., Lang, F., Bauers, K. & Gerok, W. 1990. Control of hepatic nitrogen metabolism and glutathione release by cell volume regulatory mechanisms. *Eur J Biochem,* 193**,** 891-8.
Ignarro, L. J. 2000. *Nitric oxide : biology and pathobiology,* San Diego ; London, Academic Press.
Ilhan, N., Kamanli, A. & Ozmerdivenli, R. 2004. Variable effects of exercise intensity on reduced glutathione, thiobarbituric acid reactive substance levels, and glucose concentration. *Arch Med Res,* 35**,** 294-300.
Ishijima, T., Hashimoto, H., Satou, K., Muraoka, I., Suzuki, K. & Higuchi, M. 2009. The different effects of fluid with and without carbohydrate ingestion on subjective responses of untrained men during prolonged exercise in a hot environment. *J Nutr Sci Vitaminol (Tokyo),* 55**,** 506-10.
Jackson, M. J., Pye, D. & Palomero, J. 2007. The production of reactive oxygen and nitrogen species by skeletal muscle. *J Appl Physiol,* 102**,** 1664-1670.
Jammes, Y., Steinberg, J. G., Bregeon, F. & Delliaux, S. 2004. The oxidative stress in response to routine incremental cycling exercise in healthy sedentary subjects. *Respir Physiol Neurobiol,* 144**,** 81-90.
Jammes, Y., Steinberg, J. G., Delliaux, S. & Bregeon, F. 2009. Chronic fatigue syndrome combines increased exercise-induced oxidative stress and reduced cytokine and Hsp responses. *J Intern Med,* 266**,** 196-206.
Ji, L. L., Dickman, J. R., Kang, C. & Koenig, R. 2010. Exercise-induced hormesis may help healthy aging. *Dose Response,* 8**,** 73-9.
Jones, D. P., Mody, V. C., Jr., Carlson, J. L., Lynn, M. J. & Sternberg, P., Jr. 2002. Redox analysis of human plasma allows separation of pro-oxidant events of aging from decline in antioxidant defenses. *Free Radic Biol Med,* 33**,** 1290-300.
Kalmar, B. & Greensmith, L. 2009. Induction of heat shock proteins for protection against oxidative stress. *Adv Drug Deliv Rev,* 61**,** 310-8.
Kargotich, S., Goodman, C., Keast, D., Fry, R. W., Garcia-Webb, P., Crawford, P. M. & Morton, A. R. 1997. Influence of exercise-induced plasma volume changes on the interpretation of biochemical data following high-intensity exercise. *Clin J Sport Med,* 7**,** 185-91.
Kavouras, S. A., Armstrong, L. E., Maresh, C. M., Casa, D. J., Herrera-Soto, J. A., Scheett, T. P., Stoppani, J., Mack, G. W. & Kraemer, W. J. 2006. Rehydration with glycerol: endocrine, cardiovascular, and thermoregulatory responses during exercise in the heat. *J Appl Physiol,* 100**,** 442-50.
Kenefick, R. W., Ely, B. R., Cheuvront, S. N., Palombo, L. J., Goodman, D. A. & Sawka, M. N. 2009. Prior heat stress: effect on subsequent 15-min time trial performance in the heat. *Med Sci Sports Exerc,* 41**,** 1311-6.
Kihlstrom, M. 1990. Protection effect of endurance training against reoxygenation-induced injuries in rat heart. *J Appl Physiol,* 68**,** 1672-8.
Kim, K. B., Kim, M. H. & Lee, D. J. 2004. The effect of exercise in cool, control and hot environments on cardioprotective HSP70 induction. *J Physiol Anthropol Appl Human Sci,* 23**,** 225-30.
Knez, W. L., Jenkins, D. G. & Coombes, J. S. 2007. Oxidative stress in half and full Ironman triathletes. *Med Sci Sports Exerc,* 39**,** 283-8.

Krisko, A., Kveder, M. & Pifat, G. 2005. Effect of caffeine on oxidation susceptibility of human plasma low density lipoproteins. *Clin Chim Acta,* 355**,** 47-53.

Kruhoffer, P. & Nissen, O. I. 1963. Handling of Glycerol in the Kidney. *Acta Physiol Scand,* 59**,** 284-94.

Kurz, A. K., Schliess, F. & Haussinger, D. 1998. Osmotic regulation of the heat shock response in primary rat hepatocytes. *Hepatology,* 28**,** 774-81.

Laaksonen, D. E., Atalay, M., Niskanen, L., Uusitupa, M., Hanninen, O. & Sen, C. K. 1996. Increased resting and exercise-induced oxidative stress in young IDDM men. *Diabetes Care,* 19**,** 569-74.

Laaksonen, D. E., Atalay, M., Niskanen, L., Uusitupa, M., Hanninen, O. & Sen, C. K. 1999. Blood glutathione homeostasis as a determinant of resting and exercise-induced oxidative stress in young men. *Redox Rep,* 4**,** 53-9.

Laitano, O., Kalsi, K. K., Pook, M., Oliveira, A. R. & Gonzalez-Alonso, J. 2010. Separate and combined effects of heat stress and exercise on circulatory markers of oxidative stress in euhydrated humans. *Eur J Appl Physiol*.

Landry, J., Bernier, D., Chretien, P., Nicole, L. M., Tanguay, R. M. & Marceau, N. 1982. Synthesis and degradation of heat shock proteins during development and decay of thermotolerance. *Cancer Res,* 42**,** 2457-61.

Latzka, W. A., Sawka, M. N., Montain, S. J., Skrinar, G. S., Fielding, R. A., Matott, R. P. & Pandolf, K. B. 1997. Hyperhydration: thermoregulatory effects during compensable exercise-heat stress. *J Appl Physiol,* 83**,** 860-6.

Leeuwenburgh, C., Hollander, J., Leichtweis, S., Griffiths, M., Gore, M. & Ji, L. L. 1997. Adaptations of glutathione antioxidant system to endurance training are tissue and muscle fiber specific. *Am J Physiol,* 272**,** R363-9.

Leeuwenburgh, C. & Ji, L. L. 1995. Glutathione depletion in rested and exercised mice: biochemical consequence and adaptation. *Arch Biochem Biophys,* 316**,** 941-9.

Leonarduzzi, G., Chiarpotto, E., Biasi, F. & Poli, G. 2005. 4-Hydroxynonenal and cholesterol oxidation products in atherosclerosis. *Mol Nutr Food Res,* 49**,** 1044-9.

Levine, R. L., Williams, J. A., Stadtman, E. R. & Shacter, E. 1994. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol,* 233**,** 346-57.

Lin, E. C. 1977. Glycerol utilization and its regulation in mammals. *Annu Rev Biochem,* 46**,** 765-95.

Liu, J., Wang, X., Shigenaga, M. K., Yeo, H. C., Mori, A. & Ames, B. N. 1996. Immobilization stress causes oxidative damage to lipid, protein, and DNA in the brain of rats. *FASEB J,* 10**,** 1532-8.

Locke, M., Noble, E. G. & Atkinson, B. G. 1991. Inducible isoform of HSP70 is constitutively expressed in a muscle fiber type specific pattern. *Am J Physiol,* 261**,** C774-9.

Lovell, R., Madden, L., Carroll, S. & McNaughton, L. 2007. The time-profile of the PBMC HSP70 response to in vitro heat shock appears temperature-dependent. *Amino Acids,* 33**,** 137-44.

Lyons, T. P., Riedesel, M. L., Meuli, L. E. & Chick, T. W. 1990. Effects of glycerol-induced hyperhydration prior to exercise in the heat on sweating and core temperature. *Med Sci Sports Exerc,* 22**,** 477-83.

Madrigal, J. L., Olivenza, R., Moro, M. A., Lizasoain, I., Lorenzo, P., Rodrigo, J. & Leza, J. C. 2001. Glutathione depletion, lipid peroxidation and mitochondrial dysfunction are induced by chronic stress in rat brain. *Neuropsychopharmacology,* 24**,** 420-9.

Magal, M., Webster, M. J., Sistrunk, L. E., Whitehead, M. T., Evans, R. K. & Boyd, J. C. 2003. Comparison of glycerol and water hydration regimens on tennis-related performance. *Med Sci Sports Exerc,* 35**,** 150-6.

Magalhaes, F. D., Amorim, F. T., Passos, R. L., Fonseca, M. A., Oliveira, K. P., Lima, M. R., Guimaraes, J. B., Ferreira-Junior, J. B., Martini, A. R., Lima, N. R., Soares, D. D., Oliveira, E. M. & Rodrigues, L. O. 2010. Heat and exercise acclimation increases intracellular levels of Hsp72 and inhibits exercise-induced increase in intracellular and plasma Hsp72 in humans. *Cell Stress Chaperones*.

Maher, P. 2005. The effects of stress and aging on glutathione metabolism. *Ageing Res Rev,* 4**,** 288-314.

Marino, F. E., Kay, D. & Cannon, J. 2003. Glycerol hyperhydration fails to improve endurance performance and thermoregulation in humans in a warm humid environment. *Pflugers Arch,* 446**,** 455-62.

Marino, F. E., Kay, D. & Serwach, N. 2004. Exercise time to fatigue and the critical limiting temperature: effect of hydration. *J Therm Biol,* 29**,** 21-29.

Markovitch, D., Tyrrell, R. M. & Thompson, D. 2007. The effect of prior exercise on ex vivo induction of heme oxygenase-1 in human lymphocytes. *Free Radic Res,* 41**,** 1125-34.

Martins, E. A. & Meneghini, R. 1994. Cellular DNA damage by hydrogen peroxide is attenuated by hypotonicity. *Biochem J,* 299 (Pt 1)**,** 137-40.

Mates, J. M., Perez-Gomez, C. & Nunez de Castro, I. 1999. Antioxidant enzymes and human diseases. *Clin Biochem,* 32**,** 595-603.

Matsubara, L. S. & Machado, P. E. 1991. Age-related changes of glutathione content, glutathione reductase and glutathione peroxidase activity of human erythrocytes. *Braz J Med Biol Res,* 24**,** 449-54.

Mattson, M. P. 2006. Neuronal life-and-death signaling, apoptosis, and neurodegenerative disorders. *Antioxid Redox Signal,* 8**,** 1997-2006.

Maughan, R. J. 2003. Impact of mild dehydration on wellness and on exercise performance. *Eur J Clin Nutr,* 57 Suppl 2**,** S19-23.

Maughan, R. J. & Gleeson, M. 1988. Influence of a 36 h fast followed by refeeding with glucose, glycerol or placebo on metabolism and performance during prolonged exercise in man. *Eur J Appl Physiol Occup Physiol,* 57**,** 570-6.

McAnulty, S., McAnulty, L., Nieman, D., Morrow, J., Dumke, C. & Utter, A. 2007a. Carbohydrate effect: hormone and oxidative changes. *Int J Sports Med,* 28**,** 921-7.

McAnulty, S. R., McAnulty, L., Pascoe, D. D., Gropper, S. S., Keith, R. E., Morrow, J. D. & Gladden, L. B. 2005. Hyperthermia increases exercise-induced oxidative stress. *Int J Sports Med,* 26**,** 188-92.

McAnulty, S. R., McAnulty, L. S., Morrow, J. D., Nieman, D. C., Owens, J. T. & Carper, C. M. 2007b. Influence of carbohydrate, intense exercise, and rest intervals on hormonal and oxidative changes. *Int J Sport Nutr Exerc Metab,* 17**,** 478-90.

McAnulty, S. R., McAnulty, L. S., Nieman, D. C., Morrow, J. D., Utter, A. C., Henson, D. A., Dumke, C. L. & Vinci, D. M. 2003. Influence of carbohydrate ingestion on oxidative stress and plasma antioxidant potential following a 3 h run. *Free Radic Res,* 37**,** 835-40.

McLellan, T. M. 2001. The importance of aerobic fitness in determining tolerance to uncompensable heat stress. *Comp Biochem Physiol A Mol Integr Physiol,* 128**,** 691-700.

Mena, P., Maynar, M., Gutierrez, J. M., Maynar, J., Timon, J. & Campillo, J. E. 1991. Erythrocyte free radical scavenger enzymes in bicycle professional racers. Adaptation to training. *Int J Sports Med,* 12**,** 563-6.

Michailidis, Y., Jamurtas, A. Z., Nikolaidis, M. G., Fatouros, I. G., Koutedakis, Y., Papassotiriou, I. & Kouretas, D. 2007. Sampling time is crucial for measurement of aerobic exercise-induced oxidative stress. *Med Sci Sports Exerc,* 39**,** 1107-13.

Miyazaki, H., Oh-ishi, S., Ookawara, T., Kizaki, T., Toshinai, K., Ha, S., Haga, S., Ji, L. L. & Ohno, H. 2001. Strenuous endurance training in humans reduces oxidative stress following exhausting exercise. *Eur J Appl Physiol,* 84**,** 1-6.

Montain, S. J. & Coyle, E. F. 1992. Influence of graded dehydration on hyperthermia and cardiovascular drift during exercise. *J Appl Physiol,* 73**,** 1340-50.

Montain, S. J., Smith, S. A., Mattot, R. P., Zientara, G. P., Jolesz, F. A. & Sawka, M. N. 1998. Hypohydration effects on skeletal muscle performance and metabolism: a 31P-MRS study. *J Appl Physiol,* 84**,** 1889-94.

Montner, P., Stark, D. M., Riedesel, M. L., Murata, G., Robergs, R., Timms, M. & Chick, T. W. 1996. Pre-exercise glycerol hydration improves cycling endurance time. *Int J Sports Med,* 17**,** 27-33.

Montner, P., Zou, Y., Robergs, R., Murata, G., Stark, D. M., Quinn, C., Wood, S., Lium, D. & Greene, E. R. 1999. Glycerol Hyperhydration Alters Cardiovascular and Renal Function. *J Exerc Physiol,* 2.

Mora-Rodriguez, R., Del Coso, J., Hamouti, N., Estevez, E. & Ortega, J. F. 2010. Aerobically trained individuals have greater increases in rectal temperature than untrained ones during exercise in the heat at similar relative intensities. *Eur J Appl Physiol,* 109**,** 973-81.

Moran, D. S., Shitzer, A. & Pandolf, K. B. 1998. A physiological strain index to evaluate heat stress. *Am J Physiol,* 275**,** R129-34.

Morillas-Ruiz, J., Zafrilla, P., Almar, M., Cuevas, M. J., Lopez, F. J., Abellan, P., Villegas, J. A. & Gonzalez-Gallego, J. 2005. The effects of an antioxidant-supplemented beverage on exercise-induced oxidative stress: results from a placebo-controlled double-blind study in cyclists. *Eur J Appl Physiol,* 95**,** 543-9.

Morin, B., Davies, M. J. & Dean, R. T. 1998. The protein oxidation product 3,4-dihydroxyphenylalanine (DOPA) mediates oxidative DNA damage. *Biochem J,* 330 (Pt 3)**,** 1059-67.

Morquette, B., Shi, Q., Lavigne, P., Ranger, P., Fernandes, J. C. & Benderdour, M. 2006. Production of lipid peroxidation products in osteoarthritic tissues: new evidence linking 4-hydroxynonenal to cartilage degradation. *Arthritis Rheum,* 54**,** 271-81.

Morton, J. P., Kayani, A. C., McArdle, A. & Drust, B. 2009. The exercise-induced stress response of skeletal muscle, with specific emphasis on humans. *Sports Med,* 39**,** 643-62.

Morton, J. P., MacLaren, D. P., Cable, N. T., Bongers, T., Griffiths, R. D., Campbell, I. T., Evans, L., Kayani, A., McArdle, A. & Drust, B. 2006. Time course and differential responses of the major heat shock protein families in human skeletal muscle following acute nondamaging treadmill exercise. *J Appl Physiol,* 101**,** 176-82.

Morton, J. P., Maclaren, D. P., Cable, N. T., Campbell, I. T., Evans, L., Bongers, T., Griffiths, R. D., Kayani, A. C., McArdle, A. & Drust, B. 2007. Elevated core and muscle temperature to levels comparable to exercise do not increase heat shock protein content of skeletal muscle of physically active men. *Acta Physiol (Oxf),* 190**,** 319-27.

Morton, J. P., Maclaren, D. P., Cable, N. T., Campbell, I. T., Evans, L., Kayani, A. C., McArdle, A. & Drust, B. 2008. Trained men display increased basal heat shock protein content of skeletal muscle. *Med Sci Sports Exerc,* 40**,** 1255-62.

Moseley, P. L. 1997. Heat shock proteins and heat adaptation of the whole organism. *J Appl Physiol,* 83**,** 1413-7.

Muller, F. L., Liu, Y. & Van Remmen, H. 2004. Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J Biol Chem,* 279**,** 49064-73.

Munoz Marin, D., Olcina, G., Timon, R., Robles, M. C., Caballero, M. J. & Maynar, M. 2010. Effect of different exercise intensities on oxidative stress markers and antioxidant response in trained cyclists. *J Sports Med Phys Fitness,* 50**,** 93-8.

Nadel, E. R., Fortney, S. M. & Wenger, C. B. 1980. Effect of hydration state of circulatory and thermal regulations. *J Appl Physiol,* 49**,** 715-21.

Nadel, E. R., Pandolf, K. B., Roberts, M. F. & Stolwijk, J. A. 1974. Mechanisms of thermal acclimation to exercise and heat. *J Appl Physiol,* 37**,** 515-20.

Nakabe, N., Kokura, S., Shimozawa, M., Katada, K., Sakamoto, N., Ishikawa, T., Handa, O., Takagi, T., Naito, Y., Yoshida, N. & Yoshikawa, T. 2007. Hyperthermia attenuates TNF-alpha-induced up regulation of endothelial cell adhesion molecules in human arterial endothelial cells. *Int J Hyperthermia,* 23**,** 217-24.

Nassis, G. P. & Geladas, N. D. 2002. Effect of water ingestion on cardiovascular and thermal responses to prolonged cycling and running in humans: a comparison. *Eur J Appl Physiol,* 88**,** 227-34.

Nelson, J. L., Harmon, M. E. & Robergs, R. A. 2010. Identifying Serum Glycerol Concentration Associated With Urinary Glycerol Excretion. *Med Sci Sports Exerc,* 42**,** 448.

Nelson, J. L. & Robergs, R. A. 2007. Exploring the potential ergogenic effects of glycerol hyperhydration. *Sports Med,* 37**,** 981-1000.

Newsholme, P., Krause, M., Newsholme, E. A., Stear, S. J., Burke, L. M. & Castell, L. M. 2011. BJSM reviews: A to Z of nutritional supplements: dietary supplements, sports nutrition foods and ergogenic aids for health and performance--Part 18. *Br J Sports Med,* 45**,** 230-2.

Nguyen, T., Brunson, D., Crespi, C. L., Penman, B. W., Wishnok, J. S. & Tannenbaum, S. R. 1992. DNA damage and mutation in human cells exposed to nitric oxide in vitro. *Proc Natl Acad Sci U S A,* 89**,** 3030-4.

Nielsen, B., Hales, J. R., Strange, S., Christensen, N. J., Warberg, J. & Saltin, B. 1993. Human circulatory and thermoregulatory adaptations with heat acclimation and exercise in a hot, dry environment. *J Physiol,* 460**,** 467-85.

Nieman, D. C., Davis, J. M., Henson, D. A., Gross, S. J., Dumke, C. L., Utter, A. C., Vinci, D. M., Carson, J. A., Brown, A., McAnulty, S. R., McAnulty, L. S. & Triplett, N. T. 2005. Muscle cytokine mRNA changes after 2.5 h of cycling: influence of carbohydrate. *Med Sci Sports Exerc,* 37**,** 1283-90.

Nieman, D. C., Henson, D. A., Gojanovich, G., Davis, J. M., Murphy, E. A., Mayer, E. P., Pearce, S., Dumke, C. L., Utter, A. C., McAnulty, S. R. & McAnulty, L. S. 2006. Influence of carbohydrate on immune function following 2 h cycling. *Res Sports Med,* 14**,** 225-37.

Niess, A. M. 2005. *Generation and Disposal of Reactive Oxygen and Nitrogen Species,* Champaign, IL, Human Kinetics.

Niess, A. M., Hartmann, A., Grunert-Fuchs, M., Poch, B. & Speit, G. 1996. DNA damage after exhaustive treadmill running in trained and untrained men. *Int J Sports Med,* 17**,** 397-403.

Niess, A. M., Passek, F., Lorenz, I., Schneider, E. M., Dickhuth, H. H., Northoff, H. & Fehrenbach, E. 1999. Expression of the antioxidant stress protein heme oxygenase-1 (HO-1) in human leukocytes. *Free Radic Biol Med,* 26**,** 184-92.

Nikolaidis, M. G., Jamurtas, A. Z., Paschalis, V., Kostaropoulos, I. A., Kladi-Skandali, A., Balamitsi, V., Koutedakis, Y. & Kouretas, D. 2006. Exercise-induced oxidative stress in G6PD-deficient individuals. *Med Sci Sports Exerc,* 38**,** 1443-50.

Nishiyama, Y., Ikeda, H., Haramaki, N., Yoshida, N. & Imaizumi, T. 1998. Oxidative stress is related to exercise intolerance in patients with heart failure. *Am Heart J,* 135**,** 115-20.

Noakes, T. D., Myburgh, K. H., du Plessis, J., Lang, L., Lambert, M., van der Riet, C. & Schall, R. 1991. Metabolic rate, not percent dehydration, predicts rectal temperature in marathon runners. *Med Sci Sports Exerc,* 23**,** 443-9.

Nybo, L., Jensen, T., Nielsen, B. & Gonzalez-Alonso, J. 2001. Effects of marked hyperthermia with and without dehydration on VO(2) kinetics during intense exercise. *J Appl Physiol,* 90**,** 1057-64.

Oehler, R., Pusch, E., Zellner, M., Dungel, P., Hergovics, N., Homoncik, M., Eliasen, M. M., Brabec, M. & Roth, E. 2001. Cell type-specific variations in the induction of hsp70 in human leukocytes by feverlike whole body hyperthermia. *Cell Stress Chaperones,* 6**,** 306-15.

Oh-ishi, S., Heinecke, J. W., Ookawara, T., Miyazaki, H., Haga, S., Radak, Z., Kizaki, T. & Ohno, H. 2000. Role of Lipid and Lipoprotein Oxidation. *In:* RADÁK, Z. (ed.) *Free radicals in exercise and aging.* Champaign, IL: Human Kinetics.

Ohtsuka, Y., Yabunaka, N., Fujisawa, H., Watanabe, I. & Agishi, Y. 1994. Effect of thermal stress on glutathione metabolism in human erythrocytes. *Eur J Appl Physiol Occup Physiol,* 68**,** 87-91.

Okamura, K., Doi, T., Hamada, K., Sakurai, M., Yoshioka, Y., Mitsuzono, R., Migita, T., Sumida, S. & Sugawa-Katayama, Y. 1997. Effect of repeated exercise on urinary 8-hydroxy-deoxyguanosine excretion in humans. *Free Radic Res,* 26**,** 507-14.

Orhan, H., van Holland, B., Krab, B., Moeken, J., Vermeulen, N. P., Hollander, P. & Meerman, J. H. 2004. Evaluation of a multi-parameter biomarker set for oxidative damage in man: increased urinary excretion of lipid, protein and DNA oxidation products after one hour of exercise. *Free Radic Res,* 38**,** 1269-79.

Orrenius, S., McConkey, D. J., Bellomo, G. & Nicotera, P. 1989. Role of Ca2+ in toxic cell killing. *Trends Pharmacol Sci,* 10**,** 281-5.

Ortenblad, N., Madsen, K. & Djurhuus, M. S. 1997. Antioxidant status and lipid peroxidation after short-term maximal exercise in trained and untrained humans. *Am J Physiol,* 272**,** R1258-63.

Otterbein, L. E. & Choi, A. M. 2000. Heme oxygenase: colors of defense against cellular stress. *Am J Physiol Lung Cell Mol Physiol,* 279**,** L1029-37.

Ozturk, O. & Gumuslu, S. 2004. Age-related changes of antioxidant enzyme activities, glutathione status and lipid peroxidation in rat erythrocytes after heat stress. *Life Sci,* 75**,** 1551-65.

Pacher, P., Beckman, J. S. & Liaudet, L. 2007. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev,* 87**,** 315-424.

Paik, I. Y., Jeong, M. H., Jin, H. E., Kim, Y. I., Suh, A. R., Cho, S. Y., Roh, H. T., Jin, C. H. & Suh, S. H. 2009. Fluid replacement following dehydration reduces oxidative stress during recovery. *Biochem Biophys Res Commun,* 383**,** 103-7.

Paulsen, G., Vissing, K., Kalhovde, J. M., Ugelstad, I., Bayer, M. L., Kadi, F., Schjerling, P., Hallen, J. & Raastad, T. 2007. Maximal eccentric exercise induces a rapid accumulation of small heat shock proteins on myofibrils and a delayed HSP70 response in humans. *Am J Physiol Regul Integr Comp Physiol,* 293**,** R844-53.

Peake, J. M. 2003. Vitamin C: effects of exercise and requirements with training. *Int J Sport Nutr Exerc Metab,* 13**,** 125-51.

Peake, J. M., Suzuki, K., Hordern, M., Wilson, G., Nosaka, K. & Coombes, J. S. 2005. Plasma cytokine changes in relation to exercise intensity and muscle damage. *Eur J Appl Physiol,* 95**,** 514-21.

Peart, D. J., McNaughton, L. R., Midgley, A. W., Taylor, L., Towlson, C., Madden, L. A. & Vince, R. V. 2011. Pre-exercise alkalosis attenuates the heat shock protein 72 response to a single-bout of anaerobic exercise. *J Sci Med Sport*.

Powers, S. K., Duarte, J., Kavazis, A. N. & Talbert, E. E. 2010. Reactive oxygen species are signalling molecules for skeletal muscle adaptation. *Exp Physiol,* 95**,** 1-9.

Powers, S. K. & Jackson, M. J. 2008. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol Rev,* 88**,** 1243-76.

Quindry, J. C., Stone, W. L., King, J. & Broeder, C. E. 2003. The effects of acute exercise on neutrophils and plasma oxidative stress. *Med Sci Sports Exerc,* 35**,** 1139-45.

Radak, Z., Pucsuk, J., Boros, S., Josfai, L. & Taylor, A. W. 2000. Changes in urine 8-hydroxydeoxyguanosine levels of super-marathon runners during a four-day race period. *Life Sci,* 66**,** 1763-7.

Rahnama, N., Gaeini, A. A. & Hamedinia, M. R. 2007. Oxidative stress responses in physical education students during 8 weeks aerobic training. *J Sports Med Phys Fitness,* 47**,** 119-23.

Ramanathan, N. L. 1964. A New Weighting System for Mean Surface Temperature of the Human Body. *J Appl Physiol,* 19**,** 531-3.

Reid, M. B. 2001. Invited Review: redox modulation of skeletal muscle contraction: what we know and what we don't. *J Appl Physiol,* 90**,** 724-31.

Riedesel, M. L., Allen, D. Y., Peake, G. T. & Al-Qattan, K. 1987. Hyperhydration with glycerol solutions. *J Appl Physiol,* 63**,** 2262-8.

Rios, E. C., Moretti, A. S., Velasco, I. T., Souza, H. P., Abatepaulo, F. & Soriano, F. 2011. Hypertonic saline and reduced peroxynitrite formation in experimental pancreatitis. *Clinics (Sao Paulo),* 66**,** 469-76.

Ritossa, F. 1962. New puffs induced by temperature shock, dnp and salicilate in salivary chromosomes of d.Melanogaster drosophila. *Experientia,* 18**,** 571-573.

Robergs, R. A. & Griffin, S. E. 1998. Glycerol. Biochemistry, pharmacokinetics and clinical and practical applications. *Sports Med,* 26**,** 145-67.

Robergs, R. A. & Roberts, S. 1997. *Exercise physiology : exercise, performance, and clinical applications,* St. Louis, Mosby.

Rubbo, H., Trostchansky, A. & O'Donnell, V. B. 2009. Peroxynitrite-mediated lipid oxidation and nitration: mechanisms and consequences. *Arch Biochem Biophys,* 484**,** 167-72.

Rush, J. W. & Sandiford, S. D. 2003. Plasma glutathione peroxidase in healthy young adults: influence of gender and physical activity. *Clin Biochem,* 36**,** 345-51.

Sachdev, S. & Davies, K. J. 2008. Production, detection, and adaptive responses to free radicals in exercise. *Free Radic Biol Med,* 44**,** 215-23.

Saha, N., Stoll, B., Lang, F. & Haussinger, D. 1992. Effect of anisotonic cell-volume modulation on glutathione-S-conjugate release, t-butylhydroperoxide metabolism and the pentose-phosphate shunt in perfused rat liver. *Eur J Biochem,* 209**,** 437-44.

Sandstrom, M. E., Madden, L. A., Taylor, L., Siegler, J. C., Lovell, R. J., Midgley, A. & McNaughton, L. 2009. Variation in basal heat shock protein 70 is correlated to core temperature in human subjects. *Amino Acids,* 37**,** 279-84.

Sandstrom, M. E., Siegler, J. C., Lovell, R. J., Madden, L. A. & McNaughton, L. 2008. The effect of 15 consecutive days of heat-exercise acclimation on heat shock protein 70. *Cell Stress Chaperones,* 13**,** 169-75.

Sawka, M. N., Burke, L. M., Eichner, E. R., Maughan, R. J., Montain, S. J. & Stachenfeld, N. S. 2007. American College of Sports Medicine position stand. Exercise and fluid replacement. *Med Sci Sports Exerc,* 39**,** 377-90.

Sawka, M. N., Montain, S. J. & Latzka, W. A. 2001. Hydration effects on thermoregulation and performance in the heat. *Comp Biochem Physiol A Mol Integr Physiol,* 128**,** 679-90.

Sawka, M. N., Toner, M. M., Francesconi, R. P. & Pandolf, K. B. 1983. Hypohydration and exercise: effects of heat acclimation, gender, and environment. *J Appl Physiol,* 55**,** 1147-53.

Sawka, M. N., Young, A. J., Francesconi, R. P., Muza, S. R. & Pandolf, K. B. 1985. Thermoregulatory and blood responses during exercise at graded hypohydration levels. *J Appl Physiol,* 59**,** 1394-401.

Scheadler, C., Garver, M., Kirby, T. & Devor, S. 2010. Glycerol Hyperhydration and Endurance Running Performance in the Heat. *J Exerc Physiol,* 13.

Schliess, F. & Haussinger, D. 2002. The cellular hydration state: a critical determinant for cell death and survival. *Biol Chem,* 383**,** 577-83.

Seixas, E., Gozzelino, R., Chora, A., Ferreira, A., Silva, G., Larsen, R., Rebelo, S., Penido, C., Smith, N. R., Coutinho, A. & Soares, M. P. 2009. Heme oxygenase-1 affords protection against noncerebral forms of severe malaria. *Proc Natl Acad Sci U S A,* 106**,** 15837-42.

Sen, C. K. 1999. Glutathione homeostasis in response to exercise training and nutritional supplements. *Mol Cell Biochem,* 196**,** 31-42.

Sen, C. K., Atalay, M. & Hanninen, O. 1994a. Exercise-induced oxidative stress: glutathione supplementation and deficiency. *J Appl Physiol,* 77**,** 2177-87.

Sen, C. K., Marin, E., Kretzschmar, M. & Hanninen, O. 1992. Skeletal muscle and liver glutathione homeostasis in response to training, exercise, and immobilization. *J Appl Physiol,* 73**,** 1265-72.

Sen, C. K., Rankinen, T., Vaisanen, S. & Rauramaa, R. 1994b. Oxidative stress after human exercise: effect of N-acetylcysteine supplementation. *J Appl Physiol,* 76**,** 2570-7.

Serru, V., Baudin, B., Ziegler, F., David, J. P., Cals, M. J., Vaubourdolle, M. & Mario, N. 2001. Quantification of reduced and oxidized glutathione in whole blood samples by capillary electrophoresis. *Clin Chem,* 47**,** 1321-4.

Sharwood, K. A., Collins, M., Goedecke, J. H., Wilson, G. & Noakes, T. D. 2004. Weight changes, medical complications, and performance during an Ironman triathlon. *Br J Sports Med,* 38**,** 718-24.

Shi, X. & Gisolfi, C. V. 1998. Fluid and carbohydrate replacement during intermittent exercise. *Sports Med,* 25**,** 157-72.
Shima, Y., Kitaoka, K., Yoshiki, Y., Maruhashi, Y., Tsuyama, T. & Tomita, K. 2008. Effect of heat shock preconditioning on ROS scavenging activity in rat skeletal muscle after downhill running. *J Physiol Sci,* 58**,** 341-8.
Shing, C. M., Peake, J. M., Ahern, S. M., Strobel, N. A., Wilson, G., Jenkins, D. G. & Coombes, J. S. 2007. The effect of consecutive days of exercise on markers of oxidative stress. *Appl Physiol Nutr Metab,* 32**,** 677-85.
Shvartz, E., Magazanik, A. & Glick, Z. 1974. Thermal responses during training in a temperate climate. *J Appl Physiol,* 36**,** 572-6.
Sian, J., Dexter, D. T., Lees, A. J., Daniel, S., Agid, Y., Javoy-Agid, F., Jenner, P. & Marsden, C. D. 1994. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. *Ann Neurol,* 36**,** 348-55.
Skenderi, K. P., Tsironi, M., Lazaropoulou, C., Anastasiou, C. A., Matalas, A. L., Kanavaki, I., Thalmann, M., Goussetis, E., Papassotiriou, I. & Chrousos, G. P. 2008. Changes in free radical generation and antioxidant capacity during ultramarathon foot race. *Eur J Clin Invest,* 38**,** 159-65.
Slatter, D. A., Bolton, C. H. & Bailey, A. J. 2000. The importance of lipid-derived malondialdehyde in diabetes mellitus. *Diabetologia,* 43**,** 550-7.
Soares, M. P. & Bach, F. H. 2009. Heme oxygenase-1: from biology to therapeutic potential. *Trends Mol Med,* 15**,** 50-8.
Speit, G., Dennog, C., Eichhorn, U., Rothfuss, A. & Kaina, B. 2000. Induction of heme oxygenase-1 and adaptive protection against the induction of DNA damage after hyperbaric oxygen treatment. *Carcinogenesis,* 21**,** 1795-9.
Sporer, B. C. & McKenzie, D. C. 2007. Reproducibility of a laboratory based 20-km time trial evaluation in competitive cyclists using the Velotron Pro ergometer. *Int J Sports Med,* 28**,** 940-4.
St-Pierre, J., Buckingham, J. A., Roebuck, S. J. & Brand, M. D. 2002. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem,* 277**,** 44784-90.
Stadtman, E. R. 2001. Protein oxidation in aging and age-related diseases. *Ann N Y Acad Sci,* 928**,** 22-38.
Stadtman, E. R. & Berlett, B. S. 1997. Reactive oxygen-mediated protein oxidation in aging and disease. *Chem Res Toxicol,* 10**,** 485-94.
Steinberg, J. G., Ba, A., Bregeon, F., Delliaux, S. & Jammes, Y. 2007. Cytokine and oxidative responses to maximal cycling exercise in sedentary subjects. *Med Sci Sports Exerc,* 39**,** 964-8.
Steinberg, J. G., Delliaux, S. & Jammes, Y. 2006. Reliability of different blood indices to explore the oxidative stress in response to maximal cycling and static exercises. *Clin Physiol Funct Imaging,* 26**,** 106-12.
Stryer, L. 1995. *Biochemistry,* New York, W.H. Freeman.
Sumida, S., Okamura, K., Doi, T., Sakurai, M., Yoshioka, Y. & Sugawa-Katayama, Y. 1997. No influence of a single bout of exercise on urinary excretion of 8-hydroxy-deoxyguanosine in humans. *Biochem Mol Biol Int,* 42**,** 601-609.
Tanimura, Y., Shimizu, K., Tanabe, K., Kono, I. & Ajisaka, R. 2010. Effects of three consecutive days exercise on lymphocyte DNA damage in young men. *Eur J Appl Physiol*.

Tatterson, A. J., Hahn, A. G., Martin, D. T. & Febbraio, M. A. 2000. Effects of heat stress on physiological responses and exercise performance in elite cyclists. *J Sci Med Sport,* 3**,** 186-93.

Tauler, P., Sureda, A., Cases, N., Aguilo, A., Rodriguez-Marroyo, J. A., Villa, G., Tur, J. A. & Pons, A. 2006. Increased lymphocyte antioxidant defences in response to exhaustive exercise do not prevent oxidative damage. *J Nutr Biochem,* 17**,** 665-71.

Tessier, F., Margaritis, I., Richard, M. J., Moynot, C. & Marconnet, P. 1995. Selenium and Training Effects on the Glutathione System and Aerobic Performance. *Med Sci Sports Exerc,* 27**,** 390-396.

Thompson, D., Basu-Modak, S., Gordon, M., Poore, S., Markovitch, D. & Tyrrell, R. M. 2005. Exercise-induced expression of heme oxygenase-1 in human lymphocytes. *Free Radic Res,* 39**,** 63-9.

Tissieres, A., Mitchell, H. K. & Tracy, U. M. 1974. Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. *J Mol Biol,* 84**,** 389-98.

Tsai, K., Hsu, T. G., Hsu, K. M., Cheng, H., Liu, T. Y., Hsu, C. F. & Kong, C. W. 2001. Oxidative DNA damage in human peripheral leukocytes induced by massive aerobic exercise. *Free Radic Biol Med,* 31**,** 1465-72.

Tucker, R., Marle, T., Lambert, E. V. & Noakes, T. D. 2006. The rate of heat storage mediates an anticipatory reduction in exercise intensity during cycling at a fixed rating of perceived exertion. *J Physiol,* 574**,** 905-15.

Tucker, R., Rauch, L., Harley, Y. X. & Noakes, T. D. 2004. Impaired exercise performance in the heat is associated with an anticipatory reduction in skeletal muscle recruitment. *Pflugers Arch,* 448**,** 422-30.

Urso, M. L. & Clarkson, P. M. 2003. Oxidative stress, exercise, and antioxidant supplementation. *Toxicology,* 189**,** 41-54.

Valko, M., Izakovic, M., Mazur, M., Rhodes, C. J. & Telser, J. 2004. Role of oxygen radicals in DNA damage and cancer incidence. *Mol Cell Biochem,* 266**,** 37-56.

Varma, S. D., Hegde, K. R. & Kovtun, S. 2010. Oxidative stress in lens in vivo: inhibitory effect of caffeine. A preliminary report. *Mol Vis,* 16**,** 501-5.

Veskoukis, A. S., Nikolaidis, M. G., Kyparos, A. & Kouretas, D. 2009. Blood reflects tissue oxidative stress depending on biomarker and tissue studied. *Free Radic Biol Med,* 47**,** 1371-4.

Vider, J., Lehtmaa, J., Kullisaar, T., Vihalemm, T., Zilmer, K., Kairane, C., Landor, A., Karu, T. & Zilmer, M. 2001. Acute immune response in respect to exercise-induced oxidative stress. *Pathophysiology,* 7**,** 263-270.

Viguie, C. A., Frei, B., Shigenaga, M. K., Ames, B. N., Packer, L. & Brooks, G. A. 1993. Antioxidant status and indexes of oxidative stress during consecutive days of exercise. *J Appl Physiol,* 75**,** 566-72.

Vina, J., Lloret, A., Orti, R. & Alonso, D. 2004. Molecular bases of the treatment of Alzheimer's disease with antioxidants: prevention of oxidative stress. *Mol Aspects Med,* 25**,** 117-23.

Wang, H., Liu, H. & Liu, R. M. 2003. Gender difference in glutathione metabolism during aging in mice. *Exp Gerontol,* 38**,** 507-17.

Wang, J. S. & Huang, Y. H. 2005. Effects of exercise intensity on lymphocyte apoptosis induced by oxidative stress in men. *Eur J Appl Physiol,* 95**,** 290-7.

Warburton, D. E., Gledhill, N. & Quinney, H. A. 2000. Blood volume, aerobic power, and endurance performance: potential ergogenic effect of volume loading. *Clin J Sport Med,* 10**,** 59-66.

Watkins, A. M., Cheek, D. J., Harvey, A. E., Goodwin, J. D., Blair, K. E. & Mitchell, J. B. 2007. Heat shock protein (HSP-72) levels in skeletal muscle following work in heat. *Aviat Space Environ Med,* 78**,** 901-5.

Watt, M. J., Garnham, A. P., Febbraio, M. A. & Hargreaves, M. 2000. Effect of acute plasma volume expansion on thermoregulation and exercise performance in the heat. *Med Sci Sports Exerc,* 32**,** 958-62.

Whitham, M., Walker, G. J. & Bishop, N. C. 2006. Effect of caffeine supplementation on the extracellular heat shock protein 72 response to exercise. *J Appl Physiol,* 101**,** 1222-7.

Wimer, G. S., Lamb, D. R., Sherman, W. M. & Swanson, S. C. 1997. Temperature of ingested water and thermoregulation during moderate-intensity exercise. *Can J Appl Physiol,* 22**,** 479-93.

Wingo, J. E., Casa, D. J., Berger, E. M., Dellis, W. O., Knight, J. C. & McClung, J. M. 2004. Influence of a Pre-Exercise Glycerol Hydration Beverage on Performance and Physiologic Function During Mountain-Bike Races in the Heat. *J Athl Train,* 39**,** 169-175.

Yamada, P., Amorim, F., Moseley, P. & Schneider, S. 2008. Heat shock protein 72 response to exercise in humans. *Sports Med,* 38**,** 715-33.

Yoshida, K., Hirokawa, J., Tagami, S., Kawakami, Y., Urata, Y. & Kondo, T. 1995. Weakened cellular scavenging activity against oxidative stress in diabetes mellitus: regulation of glutathione synthesis and efflux. *Diabetologia,* 38**,** 201-10.

Yoshida, T., Chida, M., Ichioka, M. & Suda, Y. 1987. Blood lactate parameters related to aerobic capacity and endurance performance. *Eur J Appl Physiol Occup Physiol,* 56**,** 7-11.

Zhang, Z., Dmitrieva, N. I., Park, J. H., Levine, R. L. & Burg, M. B. 2004. High urea and NaCl carbonylate proteins in renal cells in culture and in vivo, and high urea causes 8-oxoguanine lesions in their DNA. *Proc Natl Acad Sci U S A,* 101**,** 9491-6.