Effects of protocol familiarisation on exercise-induced oxidative stress.

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Abstract

Familiarisation can be defined as the process of repeatedly carrying out a particular exercise protocol so that subjects become acquainted with the procedures. Familiarisation has been commonly carried out to remove trial order effects during intervention studies. It has been shown that familiarisation can influence performance and elicit a protective or 'repeated bout effect' on markers of muscle damage and muscle soreness in subsequent bouts. It is possible that familiarisation might show trial order effects where protocols do not involve muscle damage and it is important to assess the number of familiarisation sessions required to remove this effect. The aim of the current study was to evaluate the effects of two familiarisation protocols, consisting of a single familiarisation and three familiarisation sessions that were completed over a duration of four weeks, on markers of oxidative stress, resistance to oxidation and antioxidant defence induced by a bout of progressive maximal cycling.

Twenty six males (mass: 80.9 ± 1.8 kg; height: 176.8 ± 1.1 cm; age: 22 ± 0.4 yrs) were assigned to either a control group (n=8) or an experimental group (n=18). All subjects completed a baseline incremental cycle protocol that started at 60 W and increased 10 W every minute until exhaustion. This exercise challenge was then repeated (matched by individual exercise time to exhaustion) approximately three weeks later (T2). In addition, during this three week period the experimental group repeated this exercise challenge on two further occasions, with approximately one week between each exercise bout. Breath-by-breath respiratory data and heart rate were continuously recorded during both main exercise trials. Venous blood samples were taken prior to exercise, at 10 minutes post-exercise and 24 hours post-exercise.

These samples were used to obtain Haematocrit, Haemoglobin concentrations, lactate concentrations, glucose concentrations, lipid hydroperoxides (HPO) concentrations, low density lipoprotein (LDL) lag times and concentrations of antioxidant vitamins (vitamin C, retinol, α-tocopherol, γ-tocopherol, α-carotene, β- carotene, lycopene).

Incremental cycling increased concentrations of HPO by approximately 25% above resting values (timing effect: $P = 0.001$, *Partial Eta*$^2 = 0.461$), thereby confirming that lipid peroxidation resulted from the exercise protocol. Concentrations of α – tocopherol increased approximately 6% from baseline following exercise (timing effect: $P = 0.012$, *Partial Eta*$^2 = 0.176$). There was a tendency for HPO concentration to be reduced in the second trial when compared to the first (trial effect: $P = 0.060$, *Partial Eta*$^2 = 0.183$). This trend for an attenuated response was also apparent in low density lipoprotein (LDL) lag times (trial effect: $P = 0.058$, *Partial Eta*$^2 = 0.201$) and in vitamin C concentration (trial effect: $P = 0.061$, *Partial Eta*$^2 = 0.190$). No differences were found between the control and experimental groups (group effect: $P \geq 0.293$, *Partial Eta*$^2 = 0.065$) demonstrating that no additional benefit was gained from two additional familiarisation sessions.

The current study highlights the need for familiarisation in interaction studies that measure oxidative stress; furthermore, these data suggest that one familiarisation session is sufficient to reduce the trial order effect. Further research is needed to investigate the mechanisms responsible for this familiarisation effect. It is possible that the act of familiarisation could reduce free radical production and/or up-regulate enzymatic antioxidant defences during a subsequent bout of repeated exercise.

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1 Introduction

Free radicals are molecules that contain an unpaired electron in their outer orbit and when produced in humans include oxygen, nitrogen and chlorine species (Sen, 1995; Fang *et al.*, 2002). Free radicals generated through oxygen metabolism are referred to as reactive oxygen species (ROS). Reactive Oxygen Species are generated continuously through resting metabolism and control critical actions such as contractile function, signal transduction, gene transcription and activity within cells (Reid, 2001; Fang *et al.*, 2002).

Oxygen leaks from the electron transport chain and forms ROS such as superoxide and hydrogen peroxide (Moller *et al.*, 1996; Alessio *et al.*, 1997). These reactive molecules have the ability to damage a variety of cells, which include lipids, proteins, and DNA (Davison *et al.*, 2002). However, under resting conditions the damaging effects of ROS are quenched by an elaborate system of antioxidants within the body. A fine balance exists within the body between ROS and antioxidants; oxidative stress is said to occur when the balance shifts towards ROS and cell damage may arise (Blumberg, 2004). Consequently, oxidative stress can be caused by increased ROS production or decreased antioxidant response.

Exercise, of greater than moderate intensity, has been shown to induce oxidative stress (Moller *et al.*, 1996; Umegaki *et al.*, 2000; Vider *et al.*, 2001; Inayama *et al.*, 2002; Quindry *et al.*, 2003). For example, Moller *et al.* (1996) demonstrated that oxidative stress only occurred when exercise intensity was greater than 65% $\dot{V}O_{2\,max}$ even when the exercise was of long duration. This probably reflects the large

increase in oxygen flux associated with aerobic exercise. However, the point at which oxidative stress occurs is reliant on a number of factors: the exercise protocol (intensity (Quindry *et al.*, 2003), duration (Moller *et al.*, 1996) and type (Alessio *et al.*, 2000)), the training status of subjects (Urso and Clarkson, 2003) and the antioxidant status of the subjects (Ashton *et al.*, 1999).

As oxidative stress has been associated with damage at cellular level and pathology in a number of medical conditions such as Alzheimer's disease, Atherosclerosis and autoimmune disease's (Moller *et al.*, 1996), many studies have been undertaken that evaluate the efficacy of interventions in reducing oxidative stress. Alessio *et al.* (1997) reported a reduction in thiobarbaturic acid-reactive substances (TBARS), induced by 30 minutes of submaximal exercise, following oral supplementation with vitamin C. In a similar study by Goldfarb *et al.* (2005), vitamin C supplementation was found to have no influence on TBARS or markers of lipid peroxidation. Equivocal findings associated with intervention studies could be, in part, due to differences in the subject's experience of the exercise protocols. A trial order effect may exist in intervention studies where subjects are not habituated to exercise protocols prior to testing. It is important that any trial order effect is eliminated from these intervention studies as this may mask important findings regarding the prevention of exercise-induced oxidative stress.

Familiarisation can be defined as the process of repeatedly carrying out a particular exercise protocol so that subjects can become acquainted with the procedures. This process is frequently undertaken in order to ensure that changes in performance from one trial to the next are not a result of learning effects (Moir *et al.*, 2005). Most

intervention studies have familiarised all subjects with the protocol prior to testing so that a trial order effect does not exist between interaction or control groups. It has been shown that without familiarisation prior to testing, improved performance in subsequent bouts may occur independently of the intervention (Laursen *et al.*, 2003; Chwalbinska-Moneta *et al.*, 2005). Laursen *et al.* (2003) found that using a 40 km time trial and trained athletes, speed was lower in the first compared to the second and third trials. This demonstrated that familiarisation to the protocol was important even when highly trained cyclists use their own race bikes. In addition to improved performance in subsequent trials, familiarisation shows additional benefits which may greatly influence research findings. Eston *et al.* (2000) used a downhill running protocol and found that one familiarisation session led to a decrease in delayed onset muscle soreness (DOMS) following a subsequent bout. This protective or 'repeated bout' effect was found to last for up to 5 weeks. Additional research, primarily involving eccentric exercise and muscle damage, has shown similar findings (Clarkson *et al.*, 1992; Rowlands *et al.*, 2001); although, the mechanisms behind this protective effect remain unclear.

An important consideration for researchers undertaking intervention studies is the number of familiarisation sessions required to remove the trial order effect. Some authors have used one familiarisation session prior to testing (e.g., Morgan *et al.*, 2004), while others have favoured two or more sessions (e.g., Barker *et al.*, 2001; Mayo *et al.*, 2001). Calder and Gabriel (2006) demonstrated that one intense familiarisation session and three less intense sessions performed on consecutive days were equally effective in reducing markers of muscle damage after eccentric exercise.

Although the influence of familiarisation on performance and on muscle damage has been well documented, the mechanism(s) responsible for this phenomenon have not been clarified. Consequently, familiarisation might elicit beneficial effects in subsequent bouts where protocols do not involve muscle damage. Training studies have shown that regular aerobic training can reduce oxidative stress caused by exercise (Urso and Clarkson, 2003); however, the mechanisms behind this remain unclear. A reduction in oxidative stress may come from a decrease in ROS production or from a more efficient antioxidant defence (Finaud *et al.*, 2006). It is possible that familiarisation might show a similar effect, reducing oxidative stress in a subsequent bout of identical exercise. It is possible that familiarisation might in some way reduce ROS production, but it is more likely that it could up-regulate the defence system against ROS causing a reduction in oxidative stress in subsequent bouts of the same exercise.

In summary, it is well documented that intense aerobic exercise induces oxidative stress primarily in the form of lipid peroxidation. It is possible that the act of familiarisation might alter the balance between ROS production and antioxidant defence during subsequent bouts of identical exercise and, therefore, affect oxidative stress. However, the influence of familiarisation on oxidative stress has not been previously investigated. Therefore, the aim of the current study was to evaluate the effects of one and multiple familiarisation sessions on markers of oxidative stress, resistance to oxidation and antioxidant defence induced by a bout of progressive maximal cycling.

1.1 Null Hypotheses

HO_1 Progressive maximal cycling will not influence blood markers of oxidative stress, resistance to oxidation or the concentrations of antioxidant vitamins.

HO_2 Familiarisation will not influence blood markers of oxidative stress, resistance to oxidation or the concentrations of antioxidant vitamins.

HO_3 The number of familiarisation sessions will not affect blood markers of oxidative stress, resistance to oxidation or the concentrations of antioxidant vitamins.

2.0 Review of Literature

2.1 Free Radicals and Reactive Oxygen Species (ROS)

Free radicals are molecules that contain an unpaired electron in their outer orbit and are capable of existing independently (Sen, 1995). Electrons are more stable when paired in orbital so free radicals are generally more reactive than non-radical species; although the reactivity of radicals is variable. Free radicals produced in humans include oxygen, nitrogen and chlorine species (Fang *et al.*, 2002). Free radicals generated through oxygen metabolism are referred to as reactive oxygen species (ROS) and are produced continuously in healthy individuals through resting metabolism. Reactive oxygen species are not always radical species and other molecules and ions, such as hydrogen peroxide, can be classed as ROS. At physiological levels ROS control critical actions such as contractile function, redox signalling, gene transcription and activity within cells (Reid, 2001; Fang *et al.*, 2002; Atalay *et al.*, 2006).

During resting oxygen metabolism approximately 2-5% of oxygen fails to be reduced in the mitochondria to form water and energy, it leaks from the electron transport chain and forms ROS such as superoxide and hydrogen peroxide (Moller *et al.*, 1996; Alessio *et al.*, 1997) (Figure 2.1). During exercise, oxygen consumption increases 10-15 fold (Alessio, 1993; Brites *et al.*, 1999), this causes an oxygen flux to active muscle that may increase up to 100-200 fold (Sen, 1995; Mastaloudis *et al.*, 2001). This increased oxygen consumption leads to a concomitant increase in the production

of ROS through the electron transport chain with greater numbers of electrons leaking and forming ROS. Superoxide is one of the primary ROS resulting from electron leakage and while alone it is not the most harmful species (Suzuki *et al.*, 1996) it does give rise to other free radicals (Figure 2.1). In the presence of trace metals these include hydrogen peroxide and hydroxyl radicals which are highly reactive with low substrate specificity (Reid, 2001). In the presence of nitric oxide, superoxide can also produce peroxynitrite (Ashton *et al.*, 1999), a potent ROS. Konorev *et al.* (1998) found that when tested alongside nitric oxide and superoxide individually, peroxynitrite had irreversible inactivation effects on creatine kinase showing it to be capable of greatest damage.

Hydroxyl radical is thought to be the most reactive radical known to chemistry (Halliwell, 1991). It is formed when hydrogen peroxide reacts with oxygen in the presence of iron and copper ions (Figure 2.1) and is present in increased levels during exercise. As with other free radicals when hydroxyl radical reacts or binds with a non-radical that non-radical will become a radical species. This starts a chain of reactions often resulting in damage. The chain reactions initiated by hydroxyl radical can result in DNA and thiol damage but most commonly, under physiological conditions, in the damage to fatty acid side chains of lipids (lipid peroxidation).

$O_2 + e^- + H^+ \rightarrow HO_2^\bullet$	Hydroperoxyl Radical
$HO_2^\bullet \rightarrow H^+ + O_2\bullet^-$	Superoxide Radical
$O_2\bullet^- + 2\,H^+ + e^- \rightarrow H_2O_2$	Hydrogen Peroxide
$H_2O_2 + e^- \rightarrow OH^- + OH^\bullet$	Hydroxyl Radical

Figure 2.1 Reduction of Oxygen

Alongside the increased oxygen flux leading to increased electron leakage there are a number of other mechanisms by which ROS are generated during exercise. During intense exercise, levels of intracellular ADP are increased; this triggers catabolism of the excess ADP and conversion of Xanthine dehydrogenase to radical generating enzyme Xanthine oxidase (Moller *et al.*, 1996; Aguilo *et al.*, 2005). Exercise also brings about a hypoxia and a reoxygenation cycle where blood is redistributed to areas of greatest need. The reperfusion of tissues with blood following cessation of exercise can alter calcium homeostasis and increase the production of superoxide by xanthine oxidase within the muscle (Caillaud *et al.*, 1999; McArdle *et al.*, 2000, Sacheck and Blumberg, 2001).

While exercise generates an increase in the primary production of ROS through increased rates of metabolism, exercise also leads to increased free radical production through secondary mechanisms resulting from the damage caused by the primary free radicals (Aguilo *et al.*, 2005). Disruption of calcium homeostasis or damage to iron containing proteins resulting from exercise can increase ROS production (Masaloudis *et al.*, 2001; Sacheck and Blumberg, 2001). Macrophages and Leukocytes recruited to repair muscle damage and control inflammation also release secondary ROS during intense or exhaustive exercise which could lead to further muscle damage (Moller *et al*, 1996; Urso and Clarkson, 2003). Muscle damage post exercise such as delayed onset muscle soreness (DOMS) also causes inflammation and can result in the release of superoxide from the neutrophil NADPH oxidase (Cooper *et al.*, 2002). While exercise increases the generation of ROS by

numerous pathways, damage to cells may not always be caused, as the body has an elaborate defence system against ROS attack.

2.2 Antioxidant Defence

Even during resting conditions reactive oxygen species are continuously being produced within the body. Although ROS are vital for cell function, an increased number of ROS can cause cell damage or even death (Gilbert, 2000). Under resting conditions ROS are quenched by a number of antioxidants which prevent damage being caused. These antioxidants can be classified as endogenous or exogenous. Endogenous antioxidants are those synthesised within the body, generally being enzymes (for example, glutathione peroxidase, catalase and superoxide dismutase) and exogenous are those often found in nutrients, such as vitamin A, C and E. Antioxidants decrease ROS by various mechanisms including prevention of ROS formation, quenching ROS and reducing them to less reactive species and by providing the optimal environment for other antioxidants to operated efficiently (Sen *et al.*, 1994).

Glutathione (GSH) is one of the most important antioxidants within cells and it is a major non-protein thiol source important in retaining the redox status of cells (Moller *et al.*, 1996; Reid, 2001; Sacheck and Blumberg, 2001; Inayama *et al.*, 2002). Glutathione is both an exogenous and an endogenous antioxidant and serves numerous functions in preventing damage caused by ROS. Glutathione scavenges ROS directly and through chain reactions with other antioxidants which are GSH dependent (Sen *et al.*, 1994; Fang *et al.*, 2002). Glutathione controls the

decomposition of hydrogen peroxide and lipid peroxides into less reactive species and participates in the enzymatic decomposition of phospholipids hydroperoxide (Sen *et al.*, 1994).

Various enzymatic and dietary antioxidants also work alongside GSH to aid the defence system. Superoxide dismutase (SOD) breaks down superoxide to less reactive hydrogen peroxide while catalase reduces hydrogen to water and oxygen (Reid, 2001). Glutathione peroxidase (Gpx) breaks down GSH in its oxidised form (GSSG) into water and alcohol. The ratio of GSH to GSSG is important to the defence against free radical damage during exercise (Sacheck and Blumberg, 2001). In addition to the enzymatic antioxidants, dietary nutrients also elicit a protective effect.

Vitamin E is a key antioxidant nutrient and its primary function is to scavenge single oxygen peroxyl radicals to protect muscle membranes against damage during exercise (Mastaloudis *et al.*, 2001; Reid, 2001). Vitamin E levels have been shown to increase during and immediately following strenuous exercise but then decrease post exercise (3 hours), possibly due to consumption of vitamin E to defend cells against oxidative stress (Aguilo *et al.*, 2003; Aguilo *et al.*, 2005). A number of other dietary nutrients have also been suggested as potential antioxidants including β –carotene (Reid, 2001; Tauler *et al.*, 2002) and creatine (Lawler *et al.*, 2002). A large number of studies have investigated various nutrients and their potential antioxidant status hypothesising that supplementation may be used to protect against the increased number of ROS generated through exercise and other pathways.

Ascorbic acid (vitamin C) is possibly the most effective water-soluble antioxidant which scavenges ROS inhibiting lipid peroxidation (Ashton *et al.*, 1999; Reid, 2001). Ascorbic acid can regenerate vitamin E, but only functions in a defensive manner when transition metals are absent (Sen, 1995). Chao *et al.* (2002) used a group of male smokers to demonstrate the antioxidant nature of ascorbate. They found that when ascorbate levels were sufficient to reduce all ferric ions to ferrous ions, that ascorbate then inhibited the oxidation of lipids.

2.3 Oxidative Stress

A fine balance exists within the body between ROS and the antioxidant defence system; oxidative stress is said to occur when the balance shifts towards ROS and cell damage may arise (Blumberg, 2004; Finaud *et al.*, 2006). This can be due to increased ROS production or to a decreased antioxidant response. Oxidative stress may cause damage to proteins, lipids and DNA; as a result of this it is thought to be involved in a number of pathological conditions such as Parkinson's disease, Alzheimers disease, diabetes mellitus, cardiovascular diseases, autoimmune disease's and cancers (Moller *et al.*, 1996; Seghrouchni *et al.*, 2002; Elosua *et al.*, 2003; Peake and Suzuki, 2004). A number of factors can influence the shift towards oxidative stress including pollution, smoking, alcohol, radiation and exercise.

During resting metabolism, in a healthy individual, the antioxidant defence can adequately quench all the free radicals produced. However, during aerobic exercise

increased metabolism elevates the production of ROS to a level where the antioxidant defences may be overwhelmed and this can lead to Oxidative stress.

Lipid peroxidation occurs when membrane cholesterol and fatty acids are attacked by reactive species, producing more peroxide radicals. The cell can then become permeable to sodium and calcium ions; this results in increased water absorption to the point where the cell may rupture (Frankel, 1980). This peroxidation reaction can be caused by most ROS and causes most damage in the presence of metals as they can act as catalysts (Fang *et al.*, 2001). Lipid peroxidation is initiated by the hydroxyl radical which is produced from superoxide reacting with trace metals (Reid, 2001) and continues as a chain reaction resulting in damage. Hydroxyl radical may attack the fatty acid side chains and subtract one of the hydrogen atoms from one of the carbon atoms combining with it to form water. The hydroxyl radical is removed but a carbon radical is created. This carbon radical may then combine with oxygen to form peroxyl radical. Peroxyl radical can attack other fatty acid side chains and so the reaction continues. One hydroxyl radical can therefore convert a large number of fatty acid side chains to lipid hydroperoxides (HPO) (Halliwell, 1991). Accumulation of HPO in a membrane can cause changes in its fluidity which may disrupt its function and can cause it to collapse (Finaud *et al.*, 2006). Lipid hydroperoxides (HPO) can also form conjugated dienes and aldehydes such as malondialdehyde (MDA) resulting in further damage.

Alongside damage to lipids the chain reaction initiated by hydroxyl radical may also cause damage to proteins and to DNA (Halliwell, 1991). Hydroxyl radical may attack both the backbone and side chains of a protein and cause it to lose its structure

and function (Cabiscol *et al.*, 2000). Like lipid peroxidation, protein oxidation is a chain reaction and damage to a protein can induce further production of ROS which in turn can cause further damage. ROS damage to DNA can cause both single and double strand breaks (Cabiscol *et al.*, 2000; Barzilai and Yamamoto, 2004), most damage again being caused by the hydroxyl radical. DNA repair is continual but ROS may cause changes to this repair process which can result in mutations (Finaud *et al.*, 2006).

2.3.1 Exercise Effects on Oxidative Stress

While strong evidence exists to show that exercise increases the production of ROS, evidence as to when this increase results in oxidative stress remains unclear. The point at which oxidative stress can be found following exercise could be dependent on a number of factors including the exercise modes and intensities used in studies, difference in the training status of subjects and the measurement technique utilised for assessing oxidative stress (Moller *et al.*, 1996).

2.3.1.1 Exercise Intensity

It has been suggested that exercise intensity may have an influence on the degree of oxidative stress caused. Increased ROS occurs as a result of increased oxygen consumption during aerobic exercise. Up to a point these ROS are adequately quenched by antioxidants but at intensities above this, aerobic exercise may induce oxidative stress. Alessio (1993) showed that as exercise intensity increased so did concentrations of oxidative stress biomarkers (TBARS, electroparaamagnetic

resonance (EPR) and expired pentane). Quindry *et al.* (2003) investigated the oxidative response to differing intensity bouts of acute exercise and found that at higher intensities biomarkers of oxidative stress were greater, and that at intensities greater than lactate threshold there were increases in circulating neutrophils indicating muscle damage. Marquez *et al.* (2001) measured glutathione oxidation following exercise as a measure of oxidative stress; they found that only when exercise was exhaustive did glutathione oxidation occur. Moller *et al.*, (1996) found that oxidative stress only occurred if exercise intensity was greater than 65% $\dot{V}O_2$ max and that after this point lipid peroxidation was proportional to the intensity of exercise. However, in this study trained subjects were used so it is possible that the threshold at which oxidative stress is caused after a single bout of exercise may be higher than that which may induce stress in an untrained individual (Moller *et al.*, 1996; Quindry *et al.*, 2003). Inayama *et al.* (2002) found no significant increase in GSH concentration following 30 min of exercise at individual ventilatory threshold level suggesting exercise needs to be of greater intensity than this to induce oxidative stress.

2.3.1.2 Exercise Duration

Exercise duration could have a greater influence on exercise induced oxidative stress than intensity alone. It is possible that total energy expenditure during exercise is critical to the level of oxidative stress caused. While several studies have suggested that exercise below a certain intensity does not result in oxidative stress (Moller *et al.*, (1996, Marquez *et al.*, 2001, Inayama *et al.*, 2002) others have found increased markers following lower intensity but greater duration activities (Sanchez-Quesada *et*

al., 1995, Marzatico *et al.*, 1997, Mastaloudis *et al.*, 2001). Marzatico *et al.* (1997) compared measures of oxidative stress following a half marathon and 6 x 150-m sprints and found that both protocols induced oxidative stress in both endurance and sprint trained individuals. Mastloudis *et al.* (2001) found increased markers of lipid peroxidation following an ultramarathon (50 km) suggesting that at intensities below anaerobic threshold oxidative stress is induced when duration is sufficient enough to overwhelm antioxidant defences.

The production of ROS is increased with the elevations in oxygen flux that are associated with aerobic exercise (Sen, 1995; Mastaloudis *et al.*, 2001). Long duration aerobic exercise may cause a greater increase in oxygen flux than shorter duration exercise of a higher intensity. The overall oxygen consumption during an exercise protocol may therefore be more important than exercise intensity alone in controlling the exercise-induced oxidative stress caused.

2.3.1.3 Exercise Type

The type of exercise undertaken can also influence the degree of oxidative stress caused. Alessio *et al.* (2000) compared the magnitude of oxidative stress caused by aerobic and isometric exercise. They concluded that isometric exercise caused greater lipid peroxidation while aerobic exercise caused greater protein oxidation forming carbonyls. As aerobic exercise increases oxygen flux to a greater extent than isometric exercise (Alessio *et al.*, 2000) it is possible that aerobic exercise has a greater protective effect. In support of this hypothesis, Radak *et al.* (2000) determined that a trained heart is more resistant to ROS damage. Eccentric exercise

increases oxidative damage as it can put an increased stress on muscle fibres compared to the stress associated with muscle actions (Alessio *et al.*, 2000, Eston *et al.*, 2000). Bloomer *et al.* (2005) investigated the effects of both acute aerobic and anaerobic exercise on blood markers of oxidative stress. They found that after 30 minutes of squatting 70% of 1 repetition max and after 30 min cycling at 70% $\dot{V}O_{2\,max}$ there was no significant increase in MDA concentration when compared to resting values. There was a significant increase in glutathione oxidation following aerobic exercise but not following anaerobic

2.3.1.4 Training Status

An individual's training status can greatly influence the extent to which a bout of exercise causes oxidative stress (Alessio *et al.*, 2000). A number of studies have shown that aerobic training can decrease the oxidative stress response to an exercise protocol. Fatouros *et al.* (2004) found that 16 weeks of training at 50-80% of maximum heart rate reduced post-exercise lipid peroxidation following a maximal treadmill run. Basal levels of total antioxidant capacity (TAC) and GPX were also found, indicating an increased protection against ROS. Urso and Clarkson (2003) found similar results observing a smaller increase in MDA following a 12 week strenuous endurance training programme. Sen *et al.* (1995) stated that in diabetic young men exercise induced increases in MDA were lower in those with greater aerobic capacity, suggesting that aerobic fitness offered protection against exercise induced oxidative stress. It is generally accepted that regular physical exercise elicits a protective effect against oxidative stress (Moller *et al.*, 1996, Radak *et al.*, 2001,

Senturk *et al.*, 2001) but a number of mechanisms have been suggested as to how this effect is brought about.

Moller *et al.* (1996) suggested a number of different theories as to how exercise training brings about the protective effect against oxidative stress. Including the theory that exercise increases the number of mitochondria in muscle cells; this could mean that each mitochondria will have a lower oxidative load which could decrease the production of ROS. Moller *et al.* (1996) also suggested that trained subjects have a strengthened immune system causing lower neutrophil bacteriocidal activity than untrained counterparts following exercise. It is also possible that as training strengthens muscle fibers this causes less oxidative stress through muscle damage. Senturk *et al.* (2001) used trained and sedentary rats to look at the effects of oxidative stress on erythrocytes. They found trained rats tended to have a shorter erythrocyte life span and lower oxidative stress measures; it is possible that younger erythrocytes are more resistant to oxidative stress so that those trained rats with the younger population had an increased resistance.

Radak *et al.* (2000) showed that regular physical exercise increased the number of myocardial capillaries and up regulated the activity of cardiac antioxidant enzymes in rats. A heart trained by regular exercise is therefore more resistant to oxidative stress induced by ROS. While the extent to which each of these mechanisms protects against exercise-induced oxidative stress is not known, it is clear that regular physical activity does offer a protective effect against exercise-induced oxidative stress what is not clear is whether this protection comes as a result of reduced production of ROS or an increased antioxidant defence.

2.3.1.5 Antioxidant Status

The potential for dietary supplements to act as antioxidants or to enhance antioxidant status has been a frequent topic for research as it may have potential to decrease oxidative stress not only during exercise (Alessio *et al.*, 1997, Ashton *et al.*, 1999, Senturk *et al.*, 2001, Metin *et al.*, 2002), but also in diseased or special populations (Fang *et al.*, 2001, Chao *et al.*, 2002, Davison *et al.*, 2002). Low antioxidant status has been associated with increased death from cancer and atherosclerosis (Chao *et al*, 2002) so supplementing with antioxidant nutrients could be potentially beneficial. Alessio *et al.* (1997) showed a 13% increase in total antioxidant activity at rest following eccentric muscle contractions after three weeks of 400mg·day^{-1} vitamin C supplementation. However, significant benefits from this increase in baseline values were not seen following a 30 min run at 80% $\dot{V}O_{2\ max}$. Ashton *et al.* (1999) also investigated the effects of vitamin C supplementation on free radical production. They found a 50% decrease in post exercise electron spin resonance (ESR) signal compared to the control (non-supplement) group showing vitamin C to have some protective effects against exercise-induced oxidative stress.

α –tocopherol has also been used as a supplement to increase antioxidant protection following exercise and its deficiency has been shown to increase lipid peroxidation (Goldfarb, 1993). Metin *et al.* (2002) found that when using female rats those supplemented with α- tocopherol had lower levels of lipid peroxidation whether they were in a trained or sedentary group. Itoh *et al.* (2000) found that after a 4 week supplementation period the leakage of muscle enzymes in response to long duration high intensity exercise was significantly reduced.

Many studies have investigated the combined supplementation of vitamin C and α – tocopherol as it may have a greater protective effect than each nutrient individually; possibly due to the capacity for vitamin C to regenerate α –tocopherol (Chao *et al.*, 2002, Urso and Clarkson, 2002). Senturk *et al.* (2001) combined vitamin C and α – tocopherol and found reduced erythrocyte deterioration in relation to exercise–induced oxidative stress showing that damage could be minimised through vitamin therapy.

Antioxidant status has been shown to affect the oxidative stress caused by exercise (Alessio *et al.*, 1997, Ashton *et al.*, 1999, Senturk *et al.*, 2001). Consequently, it is not only valuable to look at supplementation to reduce oxidative stress, but it is also important to measure antioxidant status of subjects involved in non-supplementation intervention studies.

2.3.2 Measurement of Oxidative Stress

2.3.2.1 Electron Spin Resonance (ESR)

Electron spin resonance (ESR) is a direct method of determining and characterising oxygen free radicals in human blood (Davison *et al.*, 2002; McArdle *et al.*, 1999; Sen, 1995). There are two applications for using ESR in biological tissues, to directly examine the radicals using the ESR or to use spin traps producing more stable radicals which are detectable by ESR (McArdle *et al.*, 1999). α-phenyl-*N-tert-*

butylnitrone (PBN) is a commonly used spin trap as it has wide reactivity with free radicals (McArdle *et al.*, 1999); however, this may mean it has low specificity.

Very few studies have utilised this comparatively new method and an even smaller number in human models (Ashton *et al.*, 1998; Ashton *et al.*, 1999; Davison *et al.*, 2002; Urso and Clarkson, 2003). Electron spin resonance signal in a free radical concentration has been demonstrated to increase up to 3 fold following exhaustive exercise (Sen, 1995; Ashton *et al.*, 1998). Ashton *et al.* (1998) compared the ESR signal intensities with measures of lipid peroxidation and total antioxidant capacity. ESR correlated well with HPO both pre and post exercise showing ESR to be comparable with previously accepted assessments of free radical species. Urso and Clarkson (2003) also agreed with these findings and found ESR results to be parallel with those of HPO and MDA.

While several studies show that ESR is viable as a sensitive method of assessing free radical production, it may have limitations (Leeuwenbergh and Heinecke, 2001; Sacheck and Blumberg, 2001). McArdle *et al.* (1999) found that following plyometric exercise no increase in ESR signal was identifiable despite an increased concentration of oxidised glutathione for up to three days post exercise. It is possible that the protocol used did not cause a large enough increase in ROS production to result in an imbalance great enough to cause oxidative stress to occur.

While ESR is a direct measure of free radicals it is difficult to use in this direct manner in humans as ROS are at such a low concentration (Leeuwenbergh and Heinecke, 2001) and more commonly a spin trap technique is needed. Spin traps may

not be as specific as some other indirect measures of oxidative stress as multiple ROS can yield the same radical adduct (Leeuwenbergh and Heinecke, 2001).

2.3.2.2 Lipid Peroxidation

Lipid peroxidation is one of the most commonly utilised markers of exercise-induced oxidative stress (Alessio and Goldfarb, 1988, Alessio *et al.*, 1997 and 2000, Chao *et al.* 2002, Davison *et al.* 2002, Kawano *et al.* 2002, Quindry *et al.* 2003) and can be measured by a number of methods, such as malondialdehyde, TBARS,and lipid hydroperoxides..

Malondialdehyde (MDA) is a biomarker, generated as a consequence of lipid peroxidation, which has been frequently measured to determine oxidative damage, as the concentration of this biomarker may express the degree of oxidative stress caused (Alessio, 1997, Alessio *et al.*, 2000, Sacheck and Blumberg, 2001). Malondialdehyde can be measured directly by high performance liquid chromatography (HPLC) but is more commonly measured indirectly by thiobarbaturic acid-reactive substances (TBARS). A large number of studies have used TBARS to quantify levels of MDA (Alessio *et al.* 1993, Caillaud, 1999, Kawano *et al.* 2002, Metin *et al.* 2002, Quindry *et al.* 2003). Sen *et al.* (1994) found that following exhaustive exercise TBARS did not show significant changes where other measures of oxidative stress did. Alessio *et al.* (1993) found that while exercise had similar effects on TBARS and MDA, TBARS increased significantly following exercise while MDA did not. Thiobarbaturic acid-reactive substances have been reported to be accurate *in vitro*, (Urso and Clarkson, 2003) but have come under some criticism for the measurement

of oxidative stress in humans mainly due to lack of specificity (Alessio *et al.* 1993, Caillaud, 1999, Sacheck *et al.* 2003). Thiobarbaturic acid-reactive substances react with not only MDA but with saturated and unsaturated non-functional aldehydes and carbohydrates (Alessio *et al.*, 2000). Thiobarbaturic acid-reactive substances are also susceptible to artefacts due to variations in the lipid content of samples and iron contamination from the reagents (Kawano *et al.*, 2002).

In addition, MDA itself may not be an accurate measure of exercise-induced oxidative stress as not all studies report an increased MDA response to exercise. This could be due to variation in exercise protocol and intensity but may also be due to the effects of training status on this pro-oxidant biomarker. Resting MDA levels are higher in trained subjects (Urso and Clarkson, 2003) so studies using MDA to assess training effects on oxidative stress biomarkers could be flawed.

F2α-isoprotanes have been used as a marker of lipid peroxidation and they might be a more specific biomarker of lipid peroxidation than MDA (Sacheck *et al.*, 2003). F2α-isoprotanes are derived through the peroxidation of arachidonic acid and have been associated with oxidative stress (Sacheck and Blumberg, 2001, Vollaard *et al.*, 2005). Mastaloudis *et al.* (2001) used F2α-isoprotanes to assess lipid peroxidation during an ultramarathon (50 km) and after a sedentary trial. As expected plasma F2α-isoprotanes increased during the ultramarathon but not during the sedentary trial. Sacheck *et al.* (2003) compared F2α-isoprotanes and MDA as measures of lipid peroxidation and found the results to reflect each other. In a review of oxidative stress assays Dotan *et al.* (2004) found that levels of F2α-isoprotanes were directly

correlated ($r > 0.7$, $P < 0.05$) with levels of MDA, HPO, GSH, Gpx, α –tocopherol and ascorbic acid.

Lipid Hydroperoxides (HPO) are a primary oxidative product formed earlier in oxidative pathways and lead to MDA production (Urso and Clarkson, 2003); they can be measured in blood and in tissue samples. A number of studies have quantified HPO as a measure of lipid peroxidation (Alessio *et al.*, 2000; Subudhi *et al.*, 2001; Davison *et al.*, 2002; Quindry *et al.*, 2003). Davison *et al.* (2002) and Quindry *et al.* (2003) found an increase in HPO following exhaustive exercise ($\dot{V}O_{2\ max}$ protocol). Alessio (1993) suggested that HPO was not always detected following sub-maximal exercise possibly due to the protocol intensity not being sufficiently strenuous to cause oxidative stress to occur. A number of studies have found significant increases in HPO concentrations without a significant increase in levels of MDA following maximal aerobic exercise (Ashton *et al.*, 1999; Alessio *et al.*, 2000; Quindry *et al.*, 2003). Thus, indicating that HPO may be a more sensitive marker of lipid peroxidation following exercise.

Measures of the rate of oxidation of low density lipoproteins (LDL) have also been used to quantify oxidative stress. The oxidation of LDL yields products such as conjugated dienes which then break down to aldehydes such as MDA (Poirier *et al.*, 2001). Conjugated dienes have therefore been measured to indicate lipid peroxidation resulting from exercise. The concentration of conjugated dienes shows the amount of hydroperoxides formed during oxidation; however, LDL lag time to conjugated diene formation is a more commonly used marker (Ayres *et al.*, 1998; Sharman *et al.*, 2004). Conjugated dienes are an early product of lipid peroxidation

and the lag time preceding their formation reflects the resistance of lipoproteins to oxidation (Bonnefont-Rousselot *et al.*, 2000). Ayres *et al.* (1998) found a decrease in lag times following exercise in women; indicating a decreased resistance to oxidation. Wezstien *et al.* (1998) reported similarly showing decreased LDL lag times following a 30 minute submaximal treadmill run. Parathasarathy *et al.* (1998) found a significant decrease in LDL oxidation rate (lag-time) following exercise ($P \leq 0.03$) and suggested this may be due to decreased antioxidants or increased peroxides associated with the LDL and that tissue exposed to recurrent oxidative stress may generate defensive strategies to minimise consequences of such stress. Few studies have used this assay when compared with the use of MDA and HPO; however, conjugated dienes are a primary product of lipid peroxidation and as a result are a more specific biomarker than MDA or other secondary markers such as F2α-isoprotanes.

2.3.2.3 Antioxidant Defence

While pro-oxidant markers of lipid peroxidation such as HPO are commonly used to determine oxidative stress, antioxidant status can also indirectly demonstrate the exercise-induced oxidative stress caused (Sen *et al.*, 1994; Caillaud *et al.*, 1999; Marquez *et al.*, 2001; Senturk *et al.*, 2001; Metin *et al.*, 2002; Tauler *et al.*, 2002). While changes in antioxidant activity reflect an increased need for defence they do not reflect oxidative stress itself as an increased defence may have prevented oxidative stress from taking place. Measures of antioxidants provide key information regarding the need for an antioxidant defence, and the consumption of those

antioxidants, but can not be used independently of other assays measuring oxidative stress.

2.3.2.3.1 Non-Vitamin Derived Antioxidants

Glutathione (GSH) is one of the key antioxidants to defend against oxidative stress in particular lipid peroxidation and as a result is commonly used to assess its extent. The ratio between GSH and its oxidised form (GSSG) has proved most promising. Tauler *et al.* (2002) concluded intense cycling exercise led to a decreased GSH/GSSG ratio. Sen (1995) reported that oxidation of GSH in tissue may be a more consistent index of exercise-induced oxidative stress when compared to other assays. In an earlier study, Sen *et al.*, (1994) found an increase in blood concentration of GSSG indicated oxidative stress when no changes were detected using TBARS. However, Inayama *et al.* (2002) found no significant changes in GSH levels following exercise. Variations in these findings could be due to variation in the exercise protocols employed by the respective authors. Tauler *et al.* (2002) used a 171 km cycle race whereas a 30 min sub-maximal running protocol was used by Inayama *et al.* (2002). In addition, the antioxidant status of the subjects may have been different as male professional cyclists (n=9) were used in Tauler *et al.* (2002) while untrained females (n=6) were employed in Inayama *et al.* (2002). Vollaard *et al.* (2005) stated that there were too many variations in the methodology used to measure GSH/GSSG ratio and that this has had a large influence on the inconsistent findings associated with this assay.

Other antioxidant enzymes that have been measured as indicators of oxidative stress include superoxide dismutase (SOD), catalase and glutathione peroxidase (Gpx). Superoxide dismutase is one of the key antioxidant enzymes within cells and is a primary defence against superoxide anion breaking it down to less reactive hydrogen peroxide (Reid, 2001). Consequently, changes in SOD concentration could provide indirect information about superoxide production as increased production of superoxide should lead to an increase in SOD concentration (Caillaud *et al.*, 1999). In agreement with this theory, intense exercise has been reported to increase post-exercise levels of SOD (Senturk *et al.*, 2001, Tauler *et al.*, 2002). Yet, Brites *et al.*, (1999) found that when comparing trained soccer players with untrained counterparts those trained had higher resting values of SOD. This makes SOD difficult to apply in the determination of oxidative stress levels between individuals with a varied training status.

Catalase is an important antioxidant enzyme which breaks down hydrogen peroxide to water and oxygen (Reid, 2001). Several studies have measured catalase activity in response to exercise; none the less, results have been equivocal. Senturk *et al.* (2001) found no significant differences in catalase activity when comparing pre to post exercise values in either trained or sedentary groups. However, Tauler *et al.* (2002) found a 33% reduction in catalase activity following an exhaustive cycle ride (171 km, mean time 270 ± 12 min). It remains possible that catalase activity is not affected until exercise reaches exhaustion or that it responds only to endurance type activities. Dotan *et al.* (2004) evaluated 58 data sets involving oxidative stress and exercise and found that catalase correlated well with many of the more frequently

used assays for determining the degree of oxidative stress caused (catalase - Gpx $r = 0.81, P < 0.01$, catalase - MDA, $r = 0.8, P < 0.01$).

Glutathione peroxidase (Gpx) also plays an important role in the detoxification of hydrogen peroxide to water and oxygen and oxidises GSH to GSSG. Caillaud *et al.* (1999) found no significant difference in Gpx following exercise while Tauler *et al.* (2002) found the decrease in Gpx activity to be the largest change of those enzymes measured (Catalase, GR, SOD and Gpx) with a 65% decrease in activity following exhaustive exercise.

2.3.2.3.2 Antioxidant Vitamins

Antioxidant vitamins are commonly measured to assess to extent of antioxidant defence (Liu *et al.*, 1999; Davison *et al.*, 2002; Quindry *et al.*, 2003; Aguilo *et al.*, 2005). Antioxidant vitamin concentrations within plasma change as a result of oxidative stress and are therefore used as indicators of it. As vitamins C and E are considered to be the most potent antioxidant vitamins (Reid, 2001) it is these which are most frequently measured. Concentrations of α-tocopherol have been shown to increase following strenuous exercise, possibly due to increased mobilisation of the antioxidant defence. Aguilo *et al.* (2005) found significant increases in α-tocopherol following a 171 km cycle. Mastaloudis *et al.* (2001) had previously shown similar findings following a 50 km ultramarathon. Not all studies have found this increase in α-tocopherol following exercise; Davison *et al.* (2002) reported that α-tocopherol decreased following a maximal cycle protocol. These equivocal findings could be

due to a number of factors including the large differences between the exercise protocols.

While concentrations of α-tocopherol following exercise have shown equivocal results, the pattern of vitamin C concentrations are even less clear. Quindry *et al.* (2003) found a 26% decrease in vitamin C following a maximal treadmill protocol whereas following a similar protocol on a cycle ergometer Davison *et al.* (2002) showed no change in vitamin C concentration. Sacheck *et al.* (2003) found an increase in vitamin C following a downhill running protocol; however, these samples were not taken immediately post exercise so may not reflect the direct defence against exercise induced oxidative stress.

A number of other antioxidant vitamins have been measured to identify changes in response to exercise. Research suggests that with the exception of α-tocopherol and vitamin C concentrations no other antioxidant vitamins alter in concentration in response to high intensity exercise. The equivocal findings associated with α-tocopherol and vitamin C concentrations could be due to differences in protocols, subject variation or to different timings of the blood samples taken to measure the vitamin concentrations.

2.3.2.3.3 Total Antioxidant Absorbance Capacity

Another approach to using antioxidants to indicate the extent of oxidative stress caused is to measure total antioxidant absorbance capacity, with the aim of eliminating the need to measure a large number of antioxidants individually.

Typically a tissue or blood sample is added to a derived free radical *in vitro* and the ability of the tissue or blood to resist oxidative stress is measured (Urso and Clarkson, 2003). Alessio *et al.* (1997 & 2000) used an oxygen radical absorbance capacity (ORAC). In the earlier of the two studies ORAC did not increase significantly after a 30 min aerobic exercise protocol. However, Alessio *et al.* (2000) compared aerobic and isometric exercise and measured a 25% increase in ORAC following aerobic exercise and a 9% increase after isometric exercise. Total antioxidant capacity measures do not correlate well with assays used to measure lipid peroxidation as increased antioxidant defence does not necessarily reflect oxidative damage (Urso and Clarkson, 2003). Total antioxidant capacity may also be influenced by nutritional effects making interpretation of findings difficult (Finaud *et al.*, 2006)

No single measure of oxidative stress is without its flaws; therefore research tends to favour a combination of markers including pro-oxidant and antioxidant markers to determine the occurrence of exercise-induced oxidative stress (Mastaloudis *et al.*, 2001; Davison *et al.*, 2002; Quindry *et al.*, 2003).

2.4 Familiarisation

Familiarisation refers to the process of repeating an exercise protocol so that subjects become acquainted with the procedures. Familiarisation is frequently carried out to ensure a trial order effect does not exist within intervention studies and that any changes in performance are not a result of learning effects (Moir *et al.*, 2005). It is possible that this process of protocol familiarisation could elicit some protective

effect against muscle damage and potentially against oxidative stress caused by the exercise protocol itself.

2.4.1 Familiarisation to Exercise Protocols

Most intervention studies chose to familiarise all subjects with the protocol prior to testing so that a trial order effect does not exist between interaction or control groups. Morgan *et al.* (2004) familiarised all subjects to a 2hr cycle at 40% $\dot{V}O_{2\ Max}$ so that subjects could be aware of the pacing which was required before they completed the testing phase of the study. Barker *et al.* (2001) required completion of two familiarisation trails of both a treadmill and a calf ergometer protocol before entry to their study. Chwalbinska-Moneta *et al.* (2005) did not familiarise subjects to a progressive incremental cycle protocol but found a familiarisation effect in repeated trials. Laursen *et al.* (2003) investigated the reproducibility of a lab based 40 km cycle time trial using 43 highly trained cyclists. They performed three trials and found that speed was lower in the first compared to the second and third trials (correlation $r = 0.59$ and $r = 0.64$ respectively). They concluded that the trial was reproducible but that familiarisation to the protocol was important even with highly trained cyclists using their own race bikes.

If a familiarisation effect occurs when a protocol involves an exercise mode that is frequently used by subjects (e.g. cycling), then familiarisation to protocols less commonly undertaken is probably essential (Belardinelli *et al.*, 1996; Ashton *et al.*, 1998; Sonetti *et al.*, 2001; Sturdy *et al.*, 2004). Rowlands *et al.*, (2001) used isometric muscle strength measures to assess loss in muscle strength following bouts

of downhill running. Subjects were familiarised with the use of this ergometer prior to testing so that the familiarisation or learning effect did not influence results. Mayo *et al.* (2001) used a simultaneous arm cranking and leg cycling exercise. Subjects attended a minimum of two familiarisation sessions until they demonstrated satisfactory skills (as judged by primary researcher).

Familiarisation might have far reaching effects on exercise studies, as familiarisation seems to effect exercise performance by improving performance in second and third trials (Laursen *et al.*, 2003). It is possible in an intervention study that familiarisation to a protocol rather than the intervention itself will cause improved performances, something which could alter findings. It is therefore clear that prior to beginning testing it is important to carry out at least one familiarisation trail for all subjects (Laursen *et al.*, 2003). The number of familiarisation sessions required to remove the trial order effect remains unclear but is an important consideration for intervention studies.

2.4.2 Oxidative Stress and DOMS

Unaccustomed exercise, especially eccentric exercise where muscles produce force while lengthening, frequently causes inflammation and damage to muscles that result in stiffness, pain on movement, reduced flexibility and tenderness (Balnave and Thompson, 1993). This soreness is usually felt 10 - 48 hours following exercise and is termed Delayed Onset Muscle Soreness (DOMS). There has been a failure in the literature to agree on a mechanism responsible for DOMS and it is possible that this phenomenon it is mediated through a number of channels.

Exercise-induced oxidative stress and ROS have been suggested to be contributory mechanisms of muscle damage (Lee *et al.*, 2002). However, this seems to be as a secondary mediator regulating the inflammatory response (Aoi *et al.*, 2004). This inflammatory response does seem to be strongly involved in DOMS and is one of the suggested mediators (Aoi *et al.*, 2004). Several studies have investigated the possible link between ROS and DOMS (Lee *et al.*, 2002; Aoi *et al.*, 2004; Close *et al.*, 2004). Close *et al.* (2004) assessed DOMS (Creatine Kinase and visual analogue scale) and oxidative stress (ESR and MDA) following a 30 min run at elevation 0% and at -15%. They found that all measures increased significantly following the downhill run but not following the run on the flat. However, the relationship between measures of DOMS and ROS after exercise did not follow the same pattern. Measures of ROS did not peak until 72 hours following exercise, when ratings of DOMS were subsiding. This suggests that ROS are not responsible for initiating damage but possibly contribute to mediating recovery.

Lee *et al.* (2002) found that CK was not significantly related to the GSH/GSSG ratio; but, that there was a significant relationship between DOMS and oxidative stress when protein carbonyls (PC) were used as a maker of oxidative stress. They concluded that oxidative stress does occur in response to high intensity eccentric exercise in the first 48 hours after exercise. While this may be the same time frame post exercise that DOMS occurs, it is possible that the two occur independently of each other.

There is evidence that unaccustomed eccentric exercise causes DOMS and if the intensity of exercise is sufficient oxidative stress also occurs. There is limited literature on the role of oxidative stress and ROS in the aetiology of DOMS, but there could be an effect as a secondary mediator of the inflammatory response (Aoi *et al.* 2004). While the mediators of DOMS and exercise induced oxidative stress may differ, the primary cause of both is overload. DOMS occurs when muscles are damaged due to unaccustomed overload whilst exercise induced oxidative stress occurs when increased free radicals, generated by exercise, overload the defensive system. Therefore it might be possible that a protective effect that works for one system could be transferable to another.

2.4.3 Protective Effects of Familiarisation on DOMS

It has been demonstrated that a 'repeated bout' or familiarisation effect exists with DOMS that follows exercise protocols that are primarily eccentric in nature, wher the primary bout of exercise elicits a protective effect against damage in a secondary bout of similar exercise (Eston *et al.* 1996; Clarkson *et al.* 1998; Eston *et al.* 2000). Eston *et al.* (1996) found that a bout of eccentric exercise induced some protective effect on a subsequent bout of different eccentric activity by reducing the severity of DOMS. Strength loss, muscle soreness and elevated creatine kinase activity were all reduced following the second bout of eccentric exercise. Eston *et al.* (2000) showed that a prior bout of downhill running protected against further damage caused by a subsequent bout and also that this 'repeated bout effect' could last at least five weeks. Rowlands *et al.* (2001) used a similar downhill running protocol with two bouts separated by two weeks to investigate the relationship between exercise

intensity and the 'repeated bout effect'. They hypothesised that the greater the muscle damage caused by the initial bout the greater the adaptations and protective effect against the second bout would be. The study used 18 male sports science students so results may only be representative of this small population. Following both the first and second exercise bout DOMS and decreased muscle strength were apparent; however, subjects in all exercise groups perceived less pain following the secondary bout showing no significant difference in the protective effect of the familiarisation. Clarkson *et al.* (1992) used an eccentric arm exercise protocol and found decreased DOMS when the protocol was repeated 5 days later.

2.4.3.1 Potential Mechanisms Behind the Familiarisation Effect

While there are several studies demonstrating the protective effect of eccentric exercise on DOMS, the mechanism behind this remains unclear. Eston *et al.* (1996) stated that it is possible that the initial bout adapts motor-recruitment so that during the second bout the force is more evenly distributed between the muscle fibres and therefore reduces the chance of severe damage to any one motor unit. Also damage to muscles may cause remodelling to occur, damaged cells are replaced by new cells which may not be as susceptible to damage. Eston *et al.* (2000) furthered these theories, suggesting that the repeated bout effect could, in addition to neural changes, be due to connective tissue or cellular adaptations.

Muscle damage can occur when there is disruption to non-contractile connective tissue; Eston *et al.* (2000) suggested that the initial bout leads to an increase in

intramuscular connective tissue which provides protection for subsequent bouts. The cellular theory suggests that muscle damage resulting from eccentric exercise is as a result of irreversible sarcomere strain where some units may be stretched extensively leading to loss of contractile integrity. Adaptation to this damage may result in an increase in the number of sarcomeres connected in a series. This reduces the strain on sarcomeres during subsequent bouts, limiting muscle damage (Eston *et al.*, 2000). It is unclear whether any one of these mechanisms is completely responsible for this phenomenon or if an interaction of all three is responsible. These theories were hypothesised rather than investigated and more research needs to be undertaken to determine the underlying mechanism behind the familiarisation effect.

2.4.4 Familiarisation as a Protector Against Oxidative Stress

The process of familiarisation to a protocol has been shown to increase exercise performance, improving performance in second and third trials (Laursen *et al.* 2003). It also seems to offer some protective effects against DOMS in subsequent bouts of eccentric exercise (Eston *et al.*, 1996 and 2000).

The 'repeated bout effect' as demonstrated by Eston *et al.* (1996 and 2000) shows a protective effect of familiarisation against further muscle damage leading to a less severe DOMS response to subsequent exercise bouts. While the influence of familiarisation on performance and on markers of muscle damage has been well documented the mechanisms responsible for these effects remain unclear. While DOMS is not proven to be interlinked in the same mechanism as ROS, it is feasible that familiarisation could offer a similar 'repeated bout effect' in protocols not

involving muscle damage, including intervention studies measuring oxidative stress. To this date there appears to be no literature investigating familiarisation as a possible protector against exercise induced oxidative stress.

3.0 Methodology

3.1 Subjects

Thirty four apparently healthy males (age: 21.8 ± 0.3 years; mass: 80.0 ± 1.6 kg; height: 1.78 ± 0.01 m) volunteered to participate in the study, which had been granted Local Research Ethics Committee approval (Appendix A). Prior to giving informed consent to participate in the study (Appendix C) all subjects completed a health questionnaire (Appendix D) and were given a comprehensive information sheet detailing procedures (Appendix B).

Subjects were interviewed for suitability and excluded from the study if they had taken any dietary supplements (e.g., creatine, vitamins, etc.) within the eight weeks prior to the start of testing, if they undertook exercise training on cycles, or if they had completed a maximal exercise protocol in the three months prior to commencing testing. The physical characteristics of the subjects that completed all the study requirements are presented in Table 3.1.

3.2 Experimental Design

A schematic of the experimental design is presented in figure 3.1. During the first testing session the subjects completed baseline anthropometric measurements and the first main exercise trial (T1), which consisted of an incremental cycle protocol that

Table 3.1 Summary of subject's physical characteristics at baseline

	Exp group	Con group	P value
Age (years)	21.4 ± 0.4	23.0 ± 0.7	0.067
Height (m)	1.77 ± 0.01	1.77 ± 0.03	0.943
Mass (kg)	81.2 ± 2.0	80.0 ± 4.0	0.777
Body composition (% body fat)	16.2 ± 0.8	16.3 ± 0.9	0.944
Maximum oxygen uptake ($ml \cdot kg^{-1} min^{-1}$)	48.9 ± 1.3	49.5 ± 1.6	0.800

Values are mean ± SEM. P values represents grouping interaction.

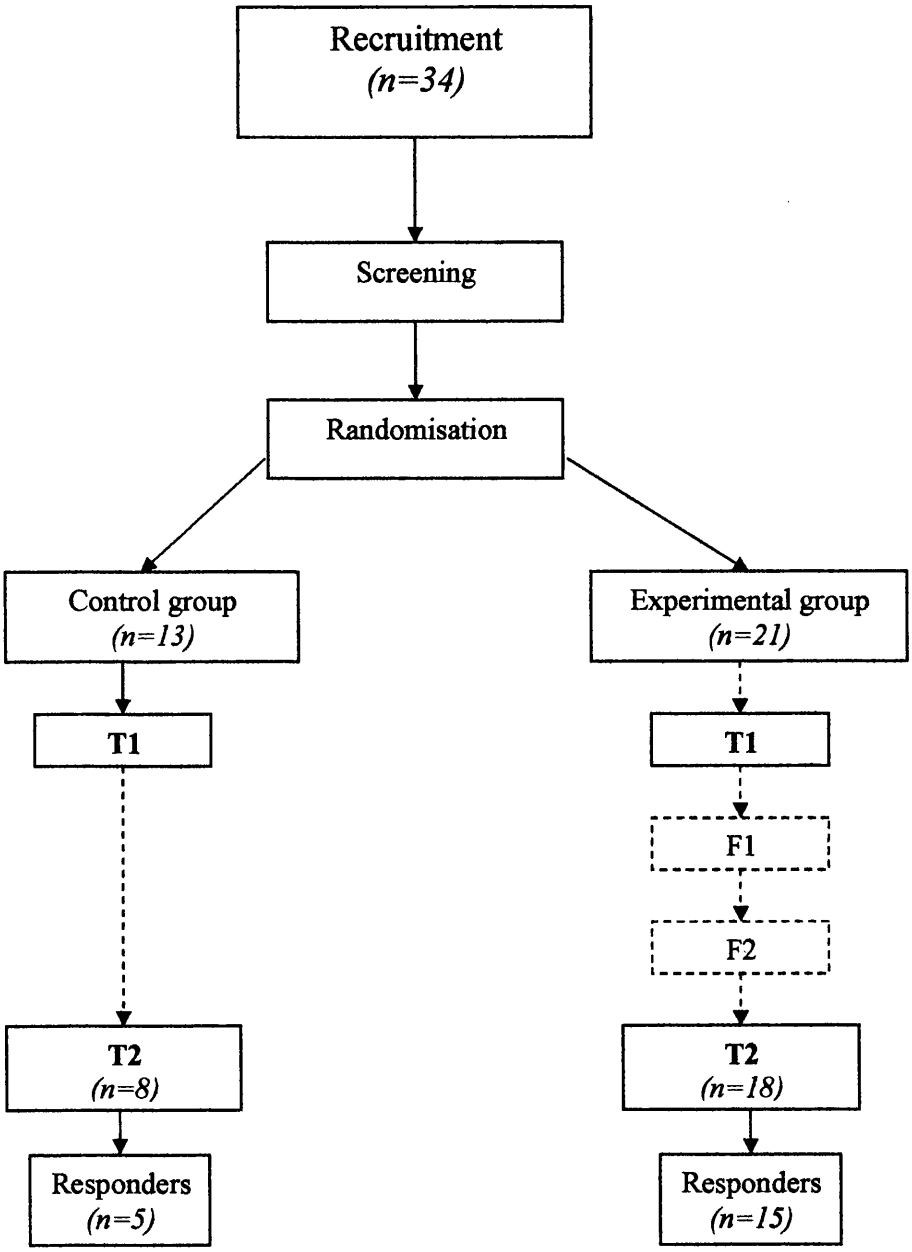

Figure 3.1 Schematic of the experimental design.

was continued until volitional exhaustion. Subsequently, the subjects were randomly assigned to either the experimental group (Exp) or the control group (Con). The Exp group repeated the incremental cycle protocol on two further occasions (5 – 9 days apart; F1 and F2). Approximately 21 days after T1 the subjects repeated the incremental cycle protocol (second main exercise trial; T2). Exercise time during T2 was matched to that completed in T1 so that all subjects completed exactly the same amount of external work during both main exercise trials. Subjects who did not demonstrate an increase in lipid hydroperoxides were classified as non-responders and separated from responders in statistical analysis.

Anthropometric measurements and body composition analysis were carried out prior to undertaking the exercise protocol on both of the main exercise trials. Venous blood samples were collected prior to and 10 min after completion of the exercise protocol and a capillary sample taken from the tip of the thumb immediately on cessation of exercise. An additional venous blood sample was taken 24 hours post-exercise in a fasted state. A schematic of the main exercise trials is presented in figure 3.2.

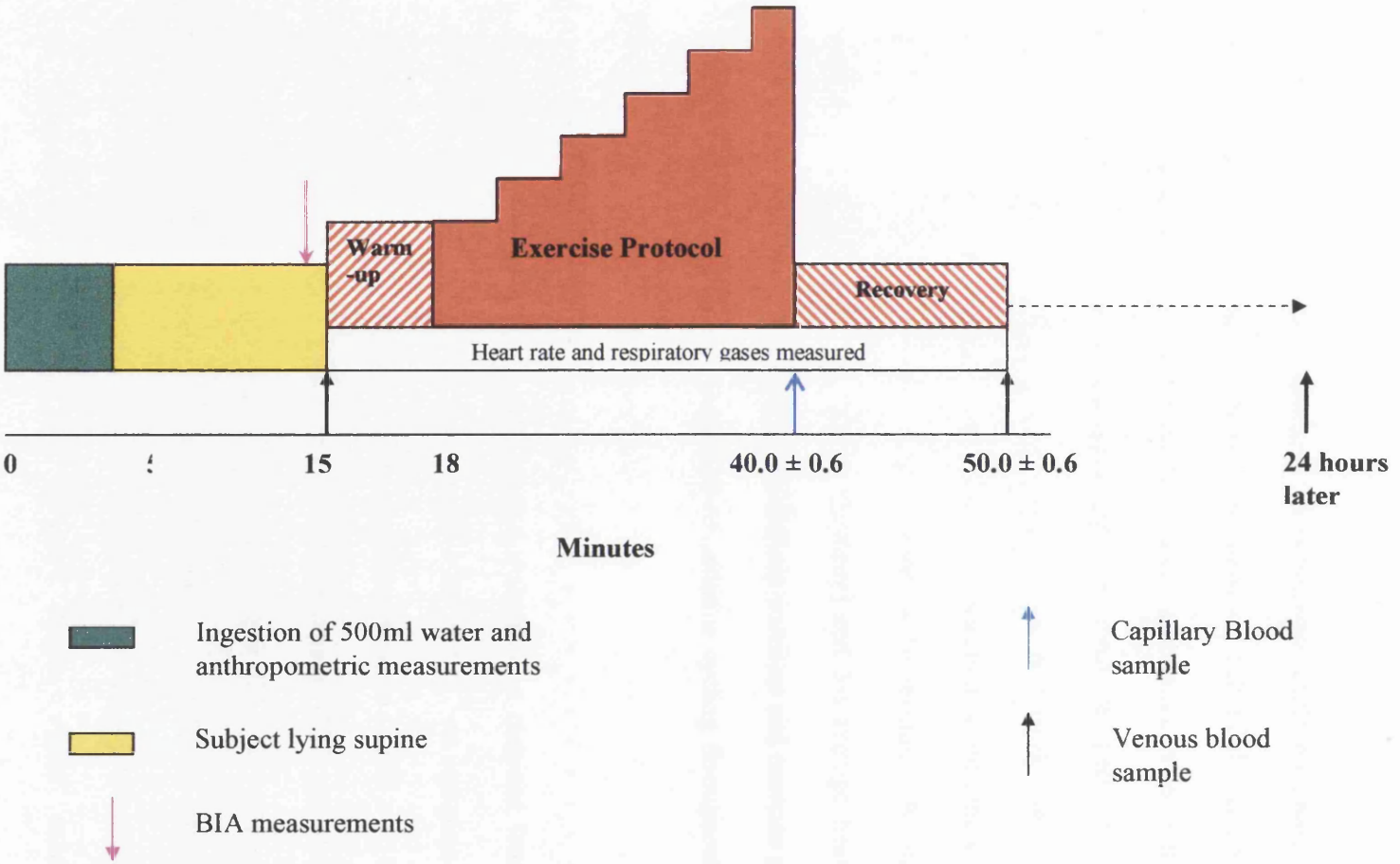

Figure 3.2 Schematic diagram of the main trial procedures.

3.3 Experimental Procedures

3.3.1 Exercise Protocol

Subjects were seated on a calibrated electromagnetic cycle ergometer (Excalibur Sport, Lode, Holland) and fitted with an electrode belt (T61 transmitter; Polar, Finland) and a face mask (Oxycon Pro; Jaeger, Germany). After a three minute warm-up at a work rate of 60 W subjects had a two minute rest period before the start of the test. The incremental cycling protocol began at a work rate of 60 W and increased by 10 W each minute until volitional exhaustion or until the subject could no longer maintain a minimum pedal cadence of 50 rev·min^{-1}. Breath-by-breath respiratory data (Oxycon Pro; Jaeger, Germany) and 5-s average heart rate data (S810; Polar, Finland) were recorded throughout warm-up and exercise periods. All Subjects received verbal encouragement to continue cycling throughout the initial exercise trial.

The measurement of $\dot{V}O_{2\ max}$ was accepted if the subject obtained four of the six following objective criteria: [1] blood lactate > 8.0 mmol·l^{-1} on cessation of exercise (Astrand and Saltin, 1967); [2] a respiratory exchange ratio (RER) > 1.10 (Farrell *et al.*, 1979); [3] heart rate > 95% of age predicted maximum (220- age of subject); [4] a plateau in oxygen consumption curve, defined as > 0.28 l·min^{-1} increase in oxygen consumption in final minute of exercise (Ashton *et al.*, 1998); [5] rate of perceived exertion (RPE) on the Borg scale of >19 (Appendix G); and [6] failure of the subject to maintain a cadence of 50 rev·min^{-1} despite verbal encouragement.

3.3.2 Anthropometry

On arrival at the laboratory subjects were asked to void before mass and stature were measured. Stature was measured (to the nearest cm) using a portable stadiometer (Harpenden stadiometer; Holtain, UK) while subjects stood erect with their feet flat and heels touching the backboard. Subjects heads were held in the Frankfurt plane whist measurements were taken. Body mass was measured using balanced beam scales (Seca 710; Seca, Germany). Subjects wore minimal clothing and were instructed to remain still. Measurements were taken to the nearest 0.1 kg.

3.3.3 Bioelectrical Impedance Analysis

Before bioelectrical impedance analysis (BIA) (Quadscan Bioimpedance analyser; Bodystat, Isle of man) was undertaken to measure body composition, subjects ingested 500 ml of water to ensure hydration and lay in a supine position on a non-conducting surface for a minimum of 10 min. Subject characteristics (age, gender, mass, height) were entered into the machine to allow adjustments to the prediction equations. Bioelectrical impedance analysis was used to estimate percentages of body fat and lean mass and volumes and percentages of body water (Total body water (TBW), extra cellular water (ECW) and intra cellular water (ICW)). Skin was cleaned with 70% alcohol prior to application of electrodes. Two current injector electrodes were placed just below the phalangeal-metacarpal joint of the third finger in the dorsal side of the hand and just below the transverse arch on the superior side of the foot midway between the second and third metatarsal. The two voltage-sensing detector electrodes were placed on the posterior side of the right wrist, at the line

bisecting the styloid processes of the ulna and radius and on the anterior side of the ankle, over the axis of the medial and lateral malleoli. An electric current was introduced to the skin via surface electrodes situated on the right wrist and ankle and whole-body resistance to the current was used to calculate body composition. Impedance at 5 kHz and 200 kHz and internal predictive equations were used to estimate ICW, ECW and TBW. Impedance at 50 kHz and a manufacturer's equation was used to give body fat, lean mass and dry lean mass values.

3.3.4 Respiratory Data

Breath-by-breath pulmonary gas exchange and minute ventilation were continuously measured during warm up and the exercise test using an on-line gas analysis system (Oxycon pro, Jaeger, Germany). Gases were continuously drawn from the mask and analysed for concentrations of oxygen and carbon dioxide. Respiratory exchange ratio (RER) was also recorded. The system was calibrated prior to each test using known gas concentrations following manufacturer's instructions. Gas analysers were calibrated with known gases (5.18% carbon dioxide in a nitrogen balance; BOC, UK) and a reference gas (ambient air). A Triple V bidirectional turbine was used to measure flow rate and was calibrated prior to each test using two reference flow rates and weekly using a 3-litre syringe (Hans Rudolph, Canada) with a range of flow rates. Oxygen consumption ($\dot{V}O_2$) was averaged to 10-s intervals and $\dot{V}O_{2\ peak}$ determined as highest value.

3.3.5 Heart Rate Data

Heart rate was continuously recorded in 5-s intervals using short range telemetry (S810 heart rate monitor; Polar, Finland) during warm-up and throughout the exercise test. Data was then exported (Polar Precision Performance SW version 3; Polar, Finland) and used to give maximum heart rate averaged over the last 30 seconds of exercise.

3.3.6 Blood Sampling

3.3.6.1 Venous Blood sampling

During all venous blood samples the method of venepuncture (Vacutainer system; Becton-Dickinson Ltd, UK) was used to obtain 28 ml of whole blood from an antecubital vein. Venous blood was collected in two 5.5 mL containers (Becton-Dickinson Ltd, UK) containing the anticoagulant ethylenediaminetetra-acid (EDTA). Several small aliquots were removed for the triplicate determination of haemoglobin concentration (Haemocue limited, UK), haematocrit (Micro haematocrit MK IV, Hawsley, England), and blood glucose and lactate concentrations (YSI 2300 Stat Plus; Yellow Springs Instruments, USA). Changes in plasma volumes were estimated as previously described by Dill and Costill (1974). The remaining blood was centrifuged at 4000 rpm for 15 min (Labofuge 200; Kendro lab products, Germany) to obtain plasma. An aliquot of plasma (100 μL) was added to 900 μL of freshly prepared 10% metaphosphoric acid, mixed and frozen at -70 °C for

subsequent vitamin C analysis. The remaining plasma was frozen and stored at -70 °C prior to subsequent analysis. Two further 10.5 ml of blood were extracted into two serum separation tubes (Becton-Dickinson Ltd, UK) left to stand for 15 min then centrifuged at 4000 rpm for 15 min to obtain serum. The serum was transferred to appropriate containers and subsequently stored at -70 °C prior to analysis.

3.3.6.2 Capillary Blood Sampling

A capillary blood sample was taken from the tip of the thumb immediately on cessation of exercise. The thumb was cleaned using a 70% alcohol swab, dried and punctured using an automated lancet and an Accu-check Softclix Pro (Roche products Ltd, UK). The initial drop of blood was removed using a clean tissue and approximately 75 μL of blood was collected in a capillary tube (Hawksley and Sons Ltd, UK). Blood was analysed for glucose and lactate concentrations (YSI 2300 Stat Plus; Yellow Springs Instruments, USA).

3.3.7 Blood Analysis

3.3.7.1 Haemoglobin and Haematocrit

Concentrations of haemoglobin were determined (in duplicate) from whole blood using an automated 2-wavelength photometer (570 nm and 880 nm) (B-Haemoglobin analyser; Hemocue, UK) following the manufacturers instructions. The photometer was calibrated daily using a microcuvette of know absorbance.

Haematocrit was determined (in duplicate) using heparinised capillary tubes filled with whole blood. Each tube was centrifuged for 5 min at 12000 rev·min^{-1} (Micro Haematocrit MK IV, Hawksley, UK). A microhaematocrit reader (Hawksley and Sons, UK) was then used to quantify the packed cell volume (Haematocrit %).

3.3.7.2 Blood Lactate and Glucose Concentrations

Approximately 25 µl of whole blood was used for the determination (in duplicate) of blood lactate and glucose concentrations using an automated electrochemical analyser (YSI 2300 stat plus; Yellow Springs Instruments, USA) as described by manufacturers instructions. Both assays are based on the principle that the substrate is oxidised to form hydrogen peroxide (H_2O_2) (reaction 1). The H_2O_2 produced is oxidised by the platinum anode which produces electrons (reaction 2); consequently, the electron flow is linearly proportional to the substrate, as follows:

Reaction 1 (Glucose): β-D-glucose + O_2 → Glucono-δ-lactone + H_2O_2

Reaction 2: $H_2O_2 \rightarrow 2H^+ + O_2 + 2e^-$

3.3.7.3 Changes in Plasma Volume

Changes in plasma volume were calculated from measured values of haematocrit and haemoglobin as previously described by Dill and Costill (1974).

3.3.7.4 Serum Lipid Hydroperoxides

Serum lipid hydroperoxides (HPO) were measured using the method of Wolff (1994) as described in McEneny *et al.* (1998). Hydroperoxides oxidise ferrous ions to ferric ions in dilute acids and the resultant ferric ions can be determined using ferric-sensitive dyes as an indirect measure of hydroperoxide concentration, using the following principle:

Reaction 1: $Fe^{2+} + ROOH \rightarrow Fe^{3+} + OH^- + RO\cdot$

Reaction 2: $RO\cdot + Sorbitol \rightarrow Ketone + HO_2\cdot$

Reaction 3: $HO_2\cdot + Fe^{2+} \rightarrow Fe^{3+} + H_2O_2$

3.3.7.5 Resistance to LDL Oxidation

Low density lipoprotein (LDL) was isolated from plasma using a density gradient method and oxidised according to the method of McDowell *et al.* (1995). The lag times were used as a measure of the resistance of the particle to oxidation and were taken as the intercept between absorbance at time zero and the point where maximum LDL oxidation occurred.

3.3.7.6 Antioxidant Vitamin Concentrations

Vitamin C concentrations were determined by means of a fluorimetric assay, using a centrifugal analyser (Cobas Fara) with florescence attachment according to the method of Vuilleumier (1993). This procedure is based on the enzymatic oxidation of ascorbic acid and subsequent quinoxaline formation to generate a flurescent derivative measured on the centrifugal analyser.

Plasma concentrations of retinol, α-tocopherol, γ-tocopherol, α-carotene, β- carotene and lycopene were measured by high-performance lipid chromatography (HPLC) with electrochemical detection according to the methods of Catignani and Bieri (1983) and Thurnham *et al.* (1988).

3.3.8 Nutritional Analysis

Prior to the testing sessions (T1 and T2) all subjects were instructed to refrain from any additional training and consuming alcohol for 24 hours, and from ingesting food or caffeine on the morning of the testing session. A questionnaire was completed by the subjects to determine whether these procedures had been followed (Appendix E). Subjects completed dietary records for two days prior to testing and the day on which the testing was completed to ensure that their nutritional status could be assessed and matched between each trial (Appendix F). A nutritional analysis programme (Comp Eat, pro version 5.8.0, UK) was used to assess the 3-d diet records completed for T1 and T2. The average daily intake of nutrients over each 3-d period was then used for statistical analysis.

3.4 Statistical Analysis

Statistical analysis was carried out using the statistical package for social sciences (version 13.0.1; SPSS, IL, USA). Group values were expressed as mean ± standard error of the mean (SEM). Significance level was set at the 5% level ($P < 0.05$), with 7.5% ($P < 0.075$) considered to show a trend. Independent samples t-tests were used to compare body composition characteristics and dietary analysis between the groups prior to testing. Shapiro-Wilks' test for normality was consulted prior to analysis. Data which contained only one time point per trial was compared using a two way mixed model ANOVA (group x trial). Mixed model analysis of variance (ANOVA) with repeated measures were used to compare groups, timing and trials (within-subject factors: trial x timing; between-subject factor: grouping). Mauchly's test was consulted and Greenhouse-Geisser correction applied if sphericity was violated. If a significant P value was identified for the interaction effect (group x trial x timing), the number of familiarisations was deemed to have a significant effect. If a significant P value was shown for timing effects, multiple pairwise comparisons were made to identify where the significance lay. Where differences existed between groups at baseline ($P < 0.05$) deltas were used to express percentage change over the exercise period.

4 Results

4.1 Dietary Intake and Body Composition

No significant differences were found in dietary intakes between trial 1 and 2 (trial effect: $P \geq 0.083$, *Partial Eta*^{2} = 0.176). A significant difference existed between groups in the mean energy consumption in the three days prior to testing (group effect: $P = 0.039$, *Partial Eta*^{2} = 0.293) and in consumption of alcohol (group effect: $P = 0.012$, *Partial Eta*^{2} = 0.557), the control group consumed significantly more alcohol and had a higher mean energy consumption (Table 4.1). No other differences were shown between the dietary intakes of the groups.

No significant differences in body composition were found between groups in either trial (group effect: $P \geq 0.348$, *Partial Eta*^{2} = 0.040). The Exp group had a higher mass than the Con group (81.2 ± 2.0 compared to 80.1 ± 4.0 kg) at T1 with little change to T2 (Table 4.2). No significant differences were found in body composition across the trials (trial effect: $P \geq 0.497$, *Partial Eta*^{2} = 0.02).

Table 4.1 Mean dietary intake (± SEM) over 3 days prior to trial 1 and trial 2 for experimental (Exp) and control (Con) groups.

	Trial 1 Exp	Trial 1 Con	Trial 2 Exp	Trial 2 Con	*P* value
Energy (KJ)	8914 ± 477	10123 ± 1477	8943 ± 297	11329 ± 2202	0.496
Protein (g)	94.1 ± 5.0	87.8 ± 24.2	86.8 ± 4.2	92.0 ± 24.1	0.790
Fat (total) (g)	75.5 ± 4.5	71.8 ± 12.6	75.2 ± 5.3	76.6 ± 14.4	0.699
Carbohydrates (g)	242.5 ± 13.2	226.4 ± 55.8	242.2 ± 12.3	252.1 ± 38.9	0.566
Sugars (total) (g)	81.8 ± 9.4	102.0 ± 23.2	78.1 ± 10.2	98.5 ± 25.2	0.746
Starch (g)	158.6 ± 7.1	112.9 ± 33.3	165.0 ± 8.6	150.8 ± 17.4	0.121
fibre (g)	11.7 ± 1.0	8.6 ± 2.6	12.4 ± 0.6	12.2 ± 1.5	0.083
calcium (mg)	924.3 ± 54.0	778.0 ± 190	864.4 ± 79.2	892.3 ± 56.2	0.758
sodium (mg)	3002 ± 298	2312 ± 815	3232 ± 255	2429 ± 595	0.590
iron (mg)	12.9 ± 1.6	9.2 ± 2.3	12.7 ± 1.5	10.6 ± 1.6	0.586
Thiamin (B1) (mg)	1.7 ± 0.2	1.4 ± 0.5	1.7 ± 0.1	1.7 ± 0.3	0.508
Riboflavin (B2) (mg)	1.7 ± 0.1	2.0 ± 0.6	1.6 ± 0.2	2.4 ± 0.3	0.331
Vit B6 (mg)	2.3 ± 0.2	2.3 ± 0.5	2.3 ± 0.2	3.0 ± 0.3	0.218
Vit B12 (µg)	5.4 ± 0.6	4.9 ± 1.2	4.6 ± 0.5	2.7 ± 0.7	0.233
Folate (µg)	240 ± 21	306 ± 40	236 ± 22	363 ± 42	0.460
Vit C (mg)	53.7 ± 9.8	33.7 ± 12.4	39.4 ± 6.4	37.9 ± 9.3	0.687
Vit A (µg)	532 ± 154	464 ± 194	525 ± 199	436 ± 58	0.980
Vit D (µg)	2.1 ± 0.7	0.7 ± 0.3	1.6 ± 0.4	1.3 ± 0.5	0.892
Vit E equivalents (mg)	6.1 ± 0.6	4.9 ± 1.6	6.2 ± 0.8	3.4 ± 0.3	0.374
Cholesterol (mg)	242 ± 30	229 ± 74	221 ± 22	260 ± 68	0.851
alcohol (g)	23.4 ± 9.3	80.4 ± 17.4	27.2 ± 9.2	99.9 ± 38.2	0.524

P value presents trial effect.

Table 4.2 Body Composition (Mean ± SEM) measures for experimental (Exp) and control (Con) groups.

	Trial 1 Exp	Trial 1 Con	Trial 2 Exp	Trial 2 Con	*P* value
Mass (kg)	81.2 ± 2.0	80.1 ± 4.0	81.2 ± 2.0	81.0 ± 5.4	0.911
Body Fat (%)	16.2 ± 0.8	16.3 ± 0.9	16.8 ± 0.7	16.7 ± 1.4	0.948
Body Fat mass (kg)	13.3 ± 0.9	13.3 ± 1.3	13.8 ± 0.8	13.9 ± 2.0	0.994
Fat free (%)	83.8 ± 0.8	83.7 ± 0.9	83.3 ± 0.7	83.3 ± 1.4	0.972
Fat free mass (kg)	67.7 ± 1.3	66.6 ± 2.7	67.6 ± 1.4	67.2 ± 3.4	0.865
Total Body Water (%)	56.8 ± 0.8	56.9 ± 1.1	56.4 ± 0.7	57.1 ± 1.6	0.651
Extra cellular Water (%)	23.4 ± 0.3	23.5 ± 0.5	23.2 ± 0.3	23.5 ± 0.7	0.668
Intracellular water (%)	33.9 ± 0.4	33.4 ± 0.5	33.4 ± 0.3	35.1 ± 1.9	0.348

P value represents group effect.

4.2 Oxygen Uptake and Heart Rate Response to Exercise

During the initial trial the Exp group cycled for 21:57 ± 0:41 min:s and reached a final load of 277 ± 8 W. The Con group cycled for 22:00 ± 57 min:s up to a maximum load of 276 ± 10 W. The individual exercise times were matched (T1 to T2) in the second trial.

No interaction effect was shown for maximal oxygen uptake (trial x group effect: P = 0.672, *Partial Eta*^{2} = 0.013). Mean $\dot{V}O_{2\ peak}$ were 48.9 ± 1.3 and 49.5 ± 1.6 ml·kg^{-1} ·min^{-1} for the Exp and Con groups respectively during trial 1. No significant difference existed between groups for performance in the exercise protocol (group effect: $P \geq 0.075$, *Partial Eta*^{2} = 0.157) (Table 4.3). All subjects met at least four of the stated criteria for determining maximal oxygen consumption. 44% of those in the exp group and 50% of those in the control group met all six of the criteria.

In T2 there was a decrease in heart rate maximum in both groups from 198 ± 2 beats·min^{-1} in T1 to 194 ± 2 beats·min^{-1} in T2 for the Exp group (trial effect: P = 0.002, *Partial Eta*^{2} = 0.630) and 192 ± 4 to 188 ± 10 beats·min^{-1} for the Con group (trial effect: P = 0.581, *Partial Eta*^{2} = 0.374). There was no significant change in mean $\dot{V}O_{2\ peak}$ between trials for either the Exp group (49.2 ± 1.3 ml·kg^{-1} min^{-1}) or for the Con group (44.9 ± 0.4 ml·kg^{-1} min^{-1}) (trial effect: P = 0.684, *Partial Eta*^{2} = 0.009).

Table 4.3 Exercise performance parameters during the initial trial.

	Trail 1 Exp	Trial 1 Con	*P* Value
Heart rate maximum (beats·min^{-1})	198.4 ± 1.8	191.6 ± 3.7	0.148
$\dot{V}O_{2\ Peak}$ (ml·kg $^{-1}$ min $^{-1}$)	48.9 ± 1.3	49.5 ± 1.6	0.347
Respiratory Exchange Ratio (RER)	1.30 ± 0.02	1.27 ± 0.04	0.498
Test duration (min)	22.0 ± 0.8	22.0 ± 1.0	0.985
Maximum Load (W)	227 ± 8	276 ± 10	0.944
Maximum Rate of Perceived Exertion (RPE)	19.3 ± 0.2	19.5 ± 0.3	0.577
Blood Lactate Concentration (mmol·l $^{-1}$)	8.59 ± 0.32	9.26 ± 0.70	0.331

Values represent mean ± SEM. P value shows group effect.

4.3 Blood Analysis

No interaction effect was identified for concentrations of Haemoglobin (Hb), Haematocrit (Hct), lactate or glucose (trial x group effect: $P \geq 0.260$, *Partial Eta*^{2} = 0.205).

Blood concentrations of Hb following the exercise protocol were increased above resting levels by an average of 6.0 ± 1.2% for the Exp group and 7.0 ± 2.1% for the Con group across both trials (timing effect: $P = 0.023$, *Partial Eta*^{2} = 0.332) and returned to baseline by 24 hours post exercise. Haemoglobin concentrations were similar for both groups and no significant differences were identified between groups (group effect: $P = 0.464$, *Partial Eta*^{2} = 0.055) (Table 4.4).

Haematocrit (Hct) followed a similar pattern to Hb (Table 4.4). Haematocrit increased significantly (timing effect: $P \leq 0.001$, *Partial Eta*^{2} = 0.715) following exercise and returned to baseline within 24 hours. No significant differences were found between the groups (group effect: $P = 0.504$, *Partial Eta*^{2} = 0.028).

Blood lactate concentrations were significantly increased following exercise in both groups (timing effect: $P = 0.000$, *Partial Eta*^{2} = 0.881), with no significant difference between the groups (group effect: $P = 0.485$, *Partial Eta*^{2} = 0.045) (Table 4.5). Blood lactate concentrations taken immediately on cessation of exercise demonstrated lactate concentrations of 8.59 ± 0.32 and 9.26 ± 0.7 mmol·L^{-1} for the Exp and Con groups during T1 and 8.01 ± 0.30 and 9.18 ± 1.34 mmol·L^{-1}, respectively during T2 (Figure 4.1).

Table 4.4 Haemoglobin and Haematocrit concentrations for Experimental (Exp) and Control (Con).

	Group	*Trial 1*			*Trial 2*			*P value*
		Pre	**Post**	**24hours post**	**Pre**	**Post**	**24hours post**	
Haemoglobin	**Exp**	145.6 ± 2.8	154.6 ± 2.5	144.1 ± 2.1	146.4 ± 1.9	154.4 ± 2.1	145.7 ± 3.0	0.023
($g \cdot dL^{-1}$)	**Con**	143.9 ± 2.8	150.9 ± 3.8	144.0 ± 5.7	141.7 ± 6.4	153.8 ± 3.9	148.0 ± 4.7	
Haematocrit	**Exp**	43.6 ± 0.6	45.9 ± 0.6	42.4 ± 0.8	43.3 ± 0.6	46.1 ± 0.6	43.7 ± 0.8	0.000
(%)	**Con**	43.2 ± 1.2	45.3 ± 0.9	42.5 ± 1.7	42.5 ± 1.9	46.0 ± 1.2	43.4 ± 1.5	

Values represent mean ± SEM. P values show timing effects.

Table 4.5 Blood lactate and glucose concentrations for both groups across both trials

	Group	Trial 1				Trial 2				P value
		Pre	Immediately post	Post	24 hours post	Pre	Immediately post	Post	24 hours post	
Lactate	Exp	1.47 ± 0.16	8.59 ± 0.32	5.73 ± 0.33	1.08 ± 0.14	1.08 ± 0.08	8.01 ± 0.3	5.05 ± 0.29	1.19 ± 0.21	0.000
($mmol \cdot L^{-1}$)	Con	1.08 ± 0.2	9.26 ± 0.7	5.56 ± 0.38	1.14 ± 0.26	1.07 ± 0.22	9.18 ± 1.34	5.15 ± 0.55	1.22 ± 0.26	
Glucose	Exp	4.07 ± 0.12	4.79 ± 0.21	4.72 ± 0.27	4.07 ± 0.07	4.18 ± 0.09	4.42 ± 0.11	4.61 ± 0.16	4.21 ± 0.09	0.001
($mmol \cdot L^{-1}$)	Con	4.15 ± 0.13	5.22 ± 0.3	5.13 ± 0.27	4.40 ± 0.16	4.01 ± 0.04	4.37 ± 0.25	4.6 ± 0.14	4.16 ± 0.08	

Values represent mean ± SEM. P values show timing effects.

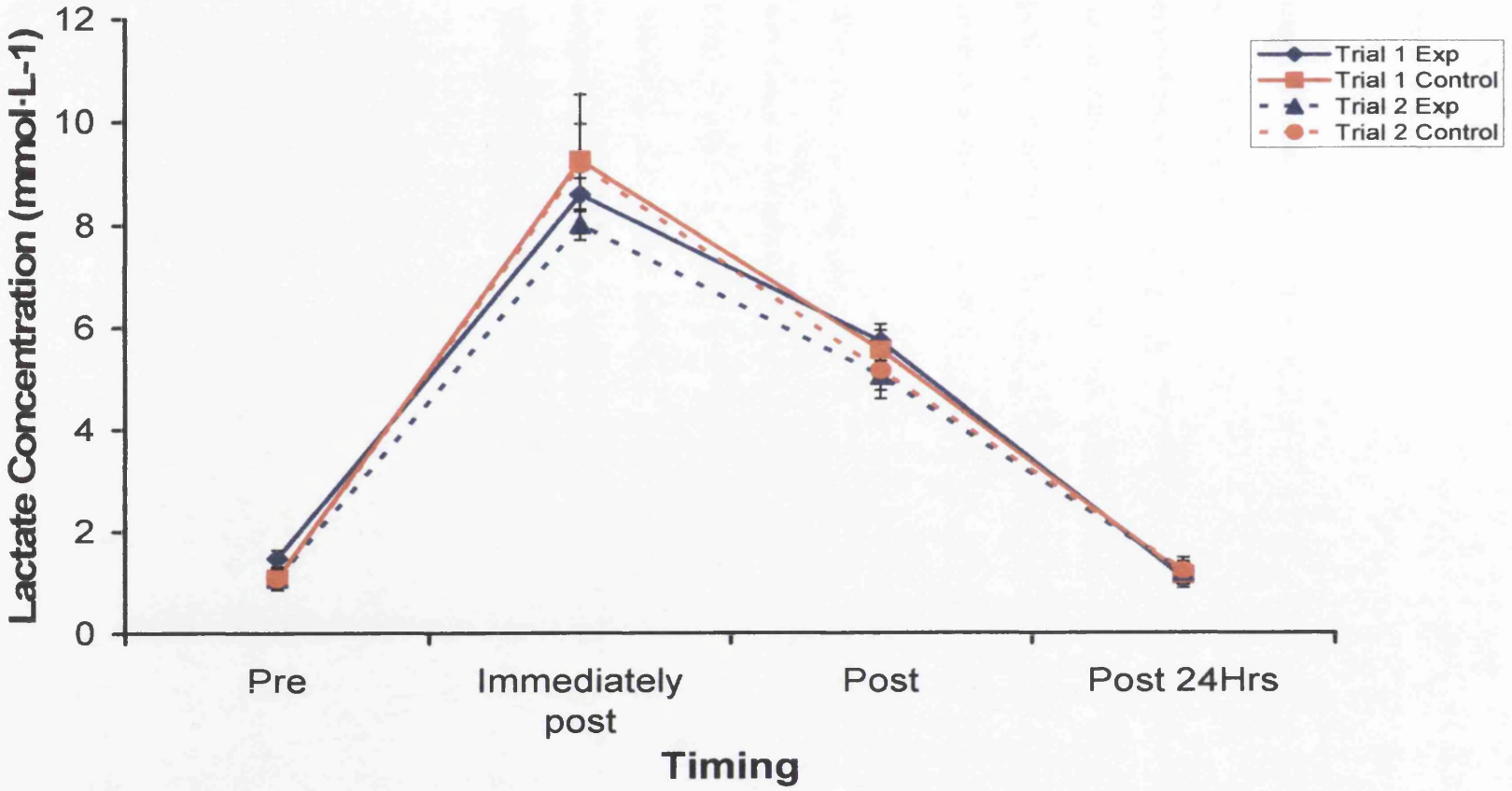

Figure 4.1 Blood lactate concentration across each time point for all trials.

Blood lactate concentrations decreased from this point and by ten minutes post exercise the Exp group had decreased by 31.9 ± 3.5% in T1 and 36.9 ± 2.5% in T2 and the Con group by 41.4 ± 2.7% and 42.3 ± 4.0% respectively. By 24 hours post - exercise concentrations had returned to pre exercise levels.

Blood Glucose concentrations were significantly elevated following exercise in both groups (timing effect: $P = 0.001$, *Partial Eta*$^2 = 0.552$). No significant difference existed between groups (group effect: $P = 0.837$, *Partial Eta*$^2 = 0.004$). Peak glucose concentration was recorded immediately post exercise for both groups in T1 whereas peak concentration was recorded ten minutes post exercise in T2. Glucose concentrations had returned to baseline within 24 hours.

When data was separated into responders and non-responders a significant difference was found in blood lactate levels between those who did and did not respond ($P = 0.041$, *Partial Eta*$^2 = 0.235$). Those who did not demonstrate an increase in HPO following exercise had lower post exercise lactate concentration than those who responded. No significant differences were found for blood glucose, haemoglobin or Haematocrit ($P \geq 0.650$, *Partial Eta*$^2 = 0.021$).

When non-responders were removed from the data set, no significant differences existed between the control and exp groups for haemoglobin, Haematocrit, glucose or lactate concentration (group effect: $P \geq 0.491$, *Partial Eta*$^2 = 0.044$). No significant interaction effects were identified when non-responders were removed from the data set (trial x group effect: $P \geq 0.065$, *Partial Eta*$^2 = 0.459$).

4.4 Lipid Hydroperoxides (HPO)

Lipid Hydroperoxides (HPO) increased from pre to post exercise (timing effect: P = 0.001, *Partial Eta*^{2} = 0.461). However, a significant difference existed between the Exp and Con groups (group effect: P = 0.017, *Partial Eta*^{2} = 0.214) (Figure 4.2); the Con group exhibited a lesser response than those in the Exp group (Exp increased 19.3% pre – post T1 compared to only a 11.7% increase in the Con group). There was no significant difference between T1 and T2 for either group (trial effect: P = 0.514, *Partial Eta*^{2} = 0.018). However, a significant difference was still found between the Exp and Con groups for HPO (group effect: P = 0.031, *Partial Eta*^{2} = 0.180).

Analysis of individual data sets revealed that three subjects in each group did not demonstrate the expected increase in HPO following exhaustive exercise and were classified as 'non-responders'.

Following removal of the non-responding data sets no significant difference existed between the Exp and Con groups for HPO (group effect: P = 0.383, *Partial Eta*^{2} = 0.043). The percentage change in HPO across T1 and T2 showed a tendency in both groups for a reduced effect in T2 compared to T1. The Exp group demonstrated a 25.9 ± 4.2% increase pre to post T1 which decreased to 17.9 ± 1.7% across T2 while the Con group decreased from 23.7 ± 4.7% change in T1 to 12.9 ± 5.0% across T2. (Figure 4.3). This trial effect showed a trend (trial effect: P = 0.060, *Partial Eta*^{2} = 0.183) for a decreased response to lipid peroxidation in T2.

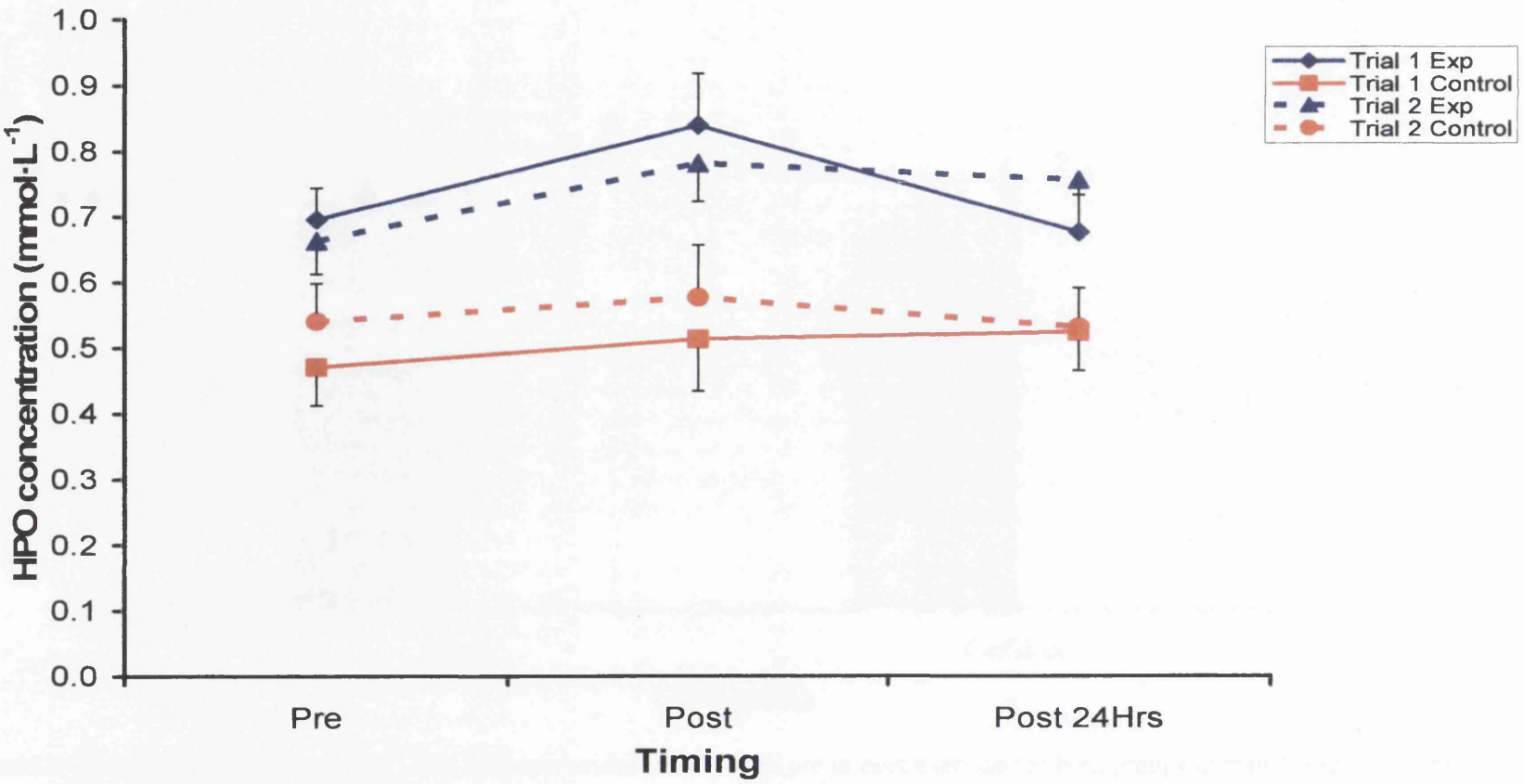

Figure 4.2 Lipid hydroperoxide concentrations across trial 1 and trial 2 for Exp and Con groups

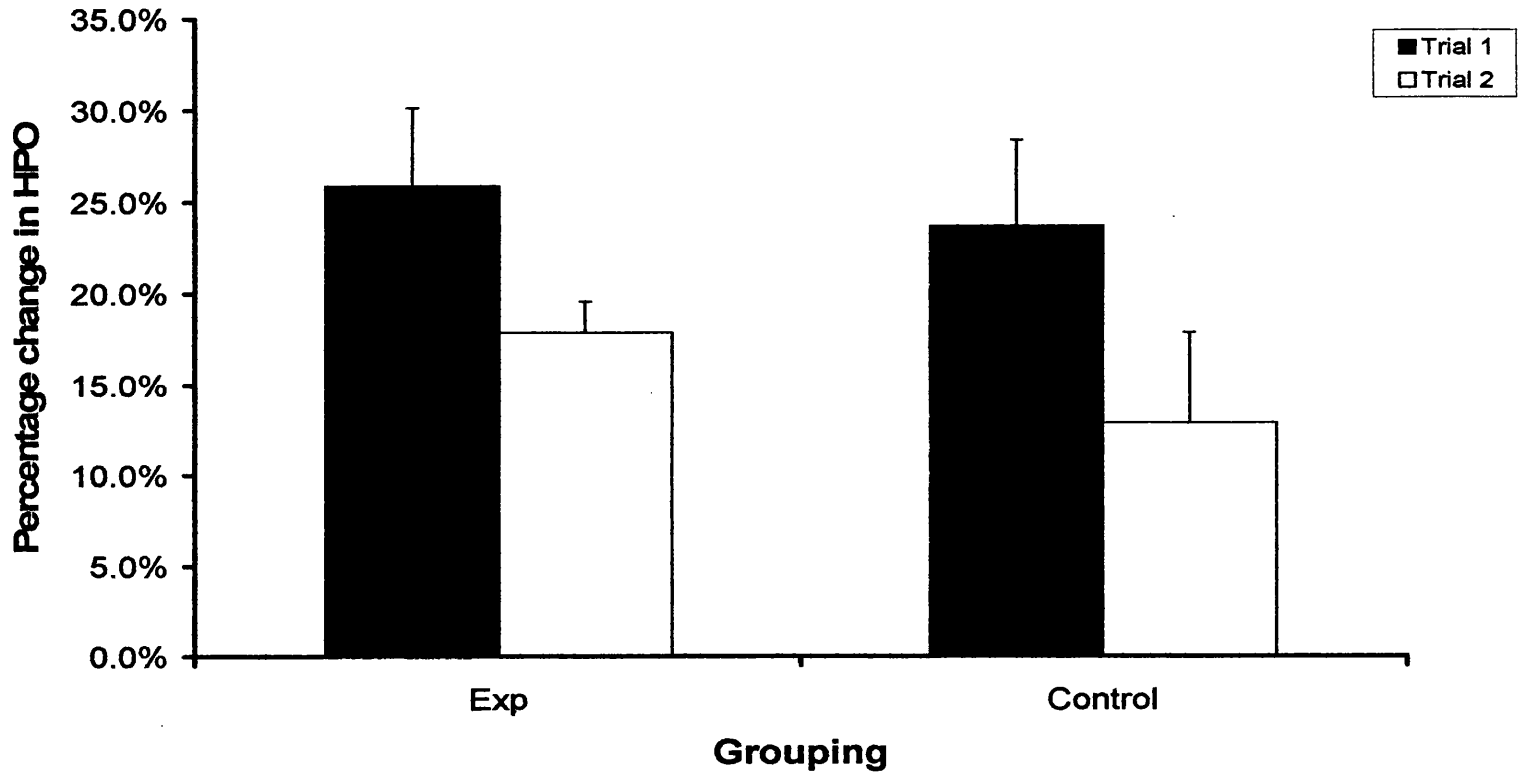

Figure 4.3 Changes in concentration of Lipid hydroperoxides (HPO) from pre to post exercise for both groups in trail 1 and trial 2 (non-responders removed).

4.5 LDL Lag Times

No interaction effect was found for LDL lag times (trial x group effect: $P = 0.350$, *Partial Eta*$^2 = 0.055$); however, significant differences were apparent between those in the Con and Exp groups (group effect: $P = 0.044$, *Partial Eta*$^2 = 0.231$) at baseline. Both the Con and the Exp groups showed an increase in LDL lag time across T1 (Exp increased 4.3% ± 3.4%, Con increased 1.8% ± 5.4%) whereas in T2 both groups decreased from pre to post exercise (Exp decreased 0.01% ± 1.4%, Con decreased 8.4% ± 6.6%). While none of the changes in LDL lag time following exercise were significantly different from baseline (timing effect: P = 0.757, *Partial Eta*$^2 = 0.017$) a trend existed for differing responses between trials (trial effect: $P = 0.058$, *Partial Eta*$^2 = 0.201$) showing a trend for an attenuated response in T2 (Figure 4.4). When expressed as deltas no significant difference was found between groups (group effect: P = 0.383, *Partial Eta*$^2 = 0.048$).

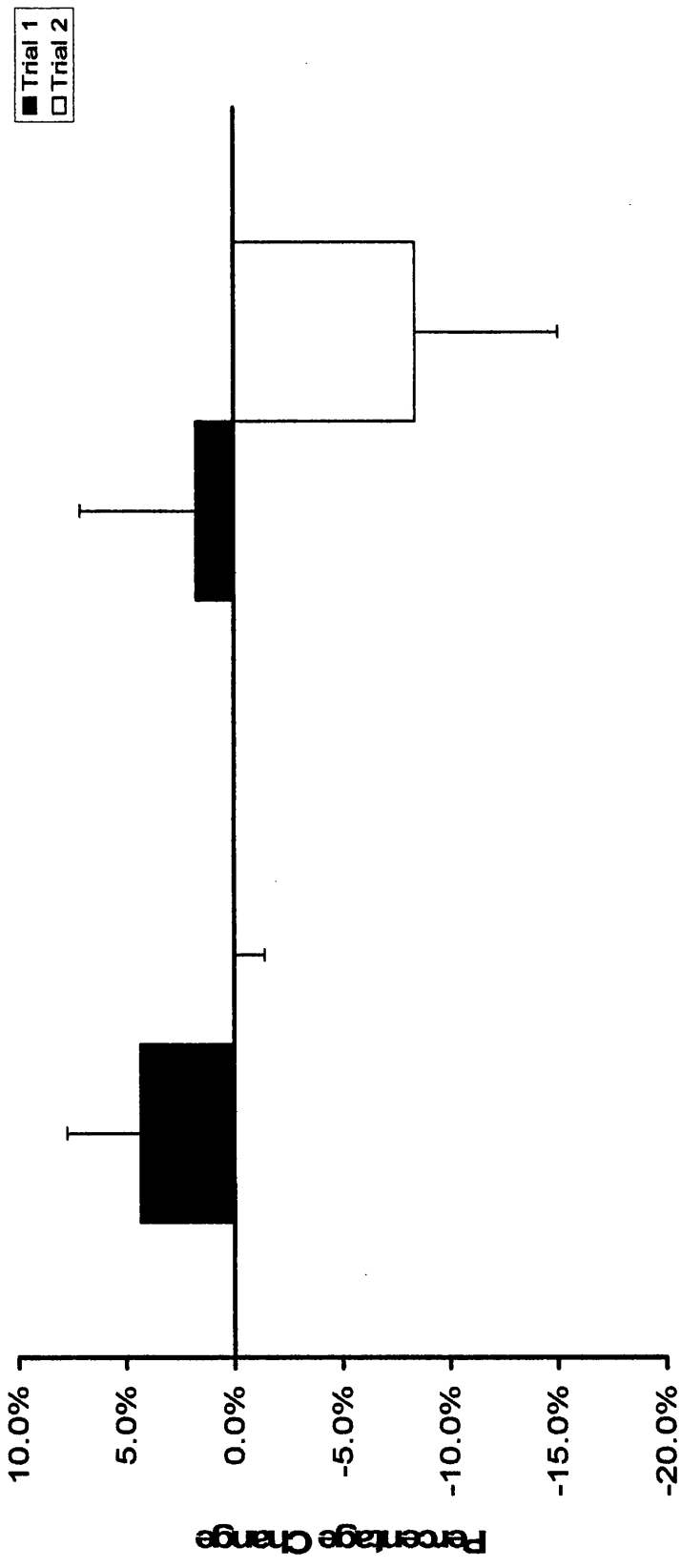

Figure 4.4 Percentage change in LDL lag times between pre and post exercise for both groups and trials (non-responders removed).

4.6 Antioxidant Vitamins

No significant interaction effects were found for any antioxidant nutrients (trial x group effect: $P \geq 0.243$, *Partial Eta*^{2} = 0.079).

No significant differences were found between the Exp and Con groups at baseline (group effect: $P \geq 0.143$, *Partial Eta*^{2} = 0.122) with the exception of retinol where a trend was shown (group effect: P = 0.051, *Partial Eta*^{2} = 0.206) (Table 4.6). Concentrations of α-tocopherol were significantly increased pre to post exercise (timing effect: P = 0.006, *Partial Eta*^{2} = 0.258) in both groups (Figure 4.5). No significant timing effects were shown in any other antioxidant vitamins (timing effect: $P \geq 0.133$, *Partial Eta*^{2} = 0.112).

Vitamin C concentration in the blood showed no significant differences between the Exp and Con groups (group effect: P = 0.369, *Partial Eta*^{2} = 0.048). A trend existed in the vitamin C response to the exercise protocol so that a lesser response was apparent in T2 compared to T1 (trial effect: P = 0.061, *Partial Eta*^{2} = 0.190). The increase in vitamin C concentration from pre to post exercise was 12.1 ± 11.6% for the Exp group during T1, decreasing to 2.1 ± 6.3% during T2. While the Con group went from and increase of 43.8 ± 23.4% during T1, down to 5.8 ± 3.9% during T2 (Figure 4.6).

Table 4.6 Plasma antioxidant vitamin levels for both groups across trial 1 and 2 (non-responders removed).

	Group	*Trial 1*			*Trial 2*			*P value*
		Pre	**Post**	**24hours post**	**Pre**	**Post**	**24hours post**	
Vitamin C	**Exp**	45.7 ± 4.1	48.8 ± 4.8	48.7 ± 3.6	45.8 ± 3.0	46.0 ± 3.5	45.0 ± 4.5	0.369
(μMol·L $^{-1}$)	**Con**	35.8 ± 5.7	48.8 ± 2.5	48.7 ± 2.4	35.8 ± 5.2	38.2 ± 5.9	45.0 ± 4.1	
Retinol	**Exp**	2.09 ± 0.10	2.25 ± 0.16	2.20 ± 0.12	2.06 ± 0.13	2.16 ± 0.14	2.19 ± 0.14	0.051
(μMol·L $^{-1}$)	**Con**	1.56 ± 0.09	1.63 ± 0.14	1.60 ± 0.07	1.71 ± 0.07	1.87 ± 0.12	1.63 ± 0.19	
α-Tocopherol	**Exp**	27.25 ± 1.25	29.22 ± 1.65	27.80 ± 1.63	27.90 ± 1.52	28.93 ± 1.77	29.90 ± 1.90	0.408
(μMol·L $^{-1}$)	**Con**	24.44 ± 1.04	25.08 ± 1.11	25.13 ± 0.95	26.13 ± 1.14	29.43 ± 2.65	23.47 ± 1.41	
γ-Tocopherol	**Exp**	1.38 ± 0.17	1.41 ± 0.15	1.37 ± 0.12	1.42 ± 0.10	1.39 ± 0.10	1.4 ± 0.13	0.905
(μMol·L $^{-1}$)	**Con**	1.28 ± 0.08	1.29 ± 0.10	1.39 ± 0.04	1.49 ± 0.09	1.67 ± 0.15	1.37 ± 0.20	
α-Carotene	**Exp**	0.17 ± 0.02	0.20 ± 0.04	0.17 ± 0.02	0.18 ± 0.03	0.19 ± 0.02	0.18 ± 0.03	0.748
(μMol·L $^{-1}$)	**Con**	0.20 ± 0.03	0.21 ± 0.03	0.21 ± 0.04	0.17 ± 0.03	0.17 ± 0.02	0.18 ± 0.02	
β- Carotene	**Exp**	0.55 ± 0.11	0.65 ± 0.12	0.56 ± 0.09	0.57 ± 0.10	0.61 ± 0.09	0.62 ± 0.13	0.143
(μMol·L $^{-1}$)	**Con**	0.86 ± 0.26	0.88 ± 0.29	1.02 ± 0.50	0.82 ± 0.43	0.89 ± 0.50	1.32 ± 0.75	
Lycopene	**Exp**	1.46 ± 0.37	1.81 ± 0.38	1.64 ± 0.39	1.36 ± 0.25	1.84 ± 0.31	1.67 ± 0.33	0.713
(μMol·L $^{-1}$)	**Con**	1.62 ± 0.12	1.77 ± 0.17	1.71 ± 0.34	1.49 ± 0.25	1.58 ± 0.23	2.13 ± 0.46	

Values represent mean ± SEM. P values shows group effect.

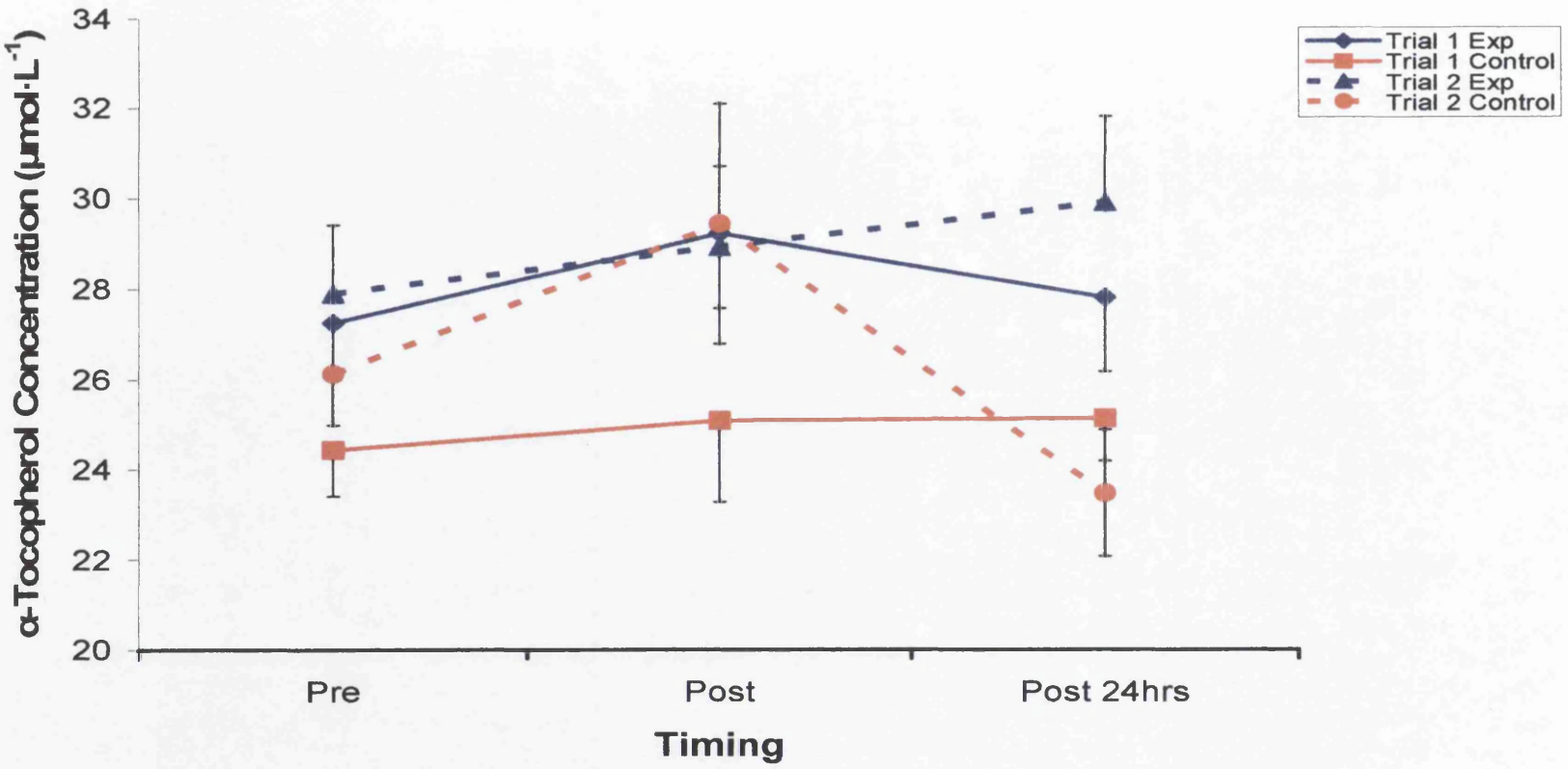

Figure 4.5. Changes in α-Tocopherol concentration (Non-responder removed).

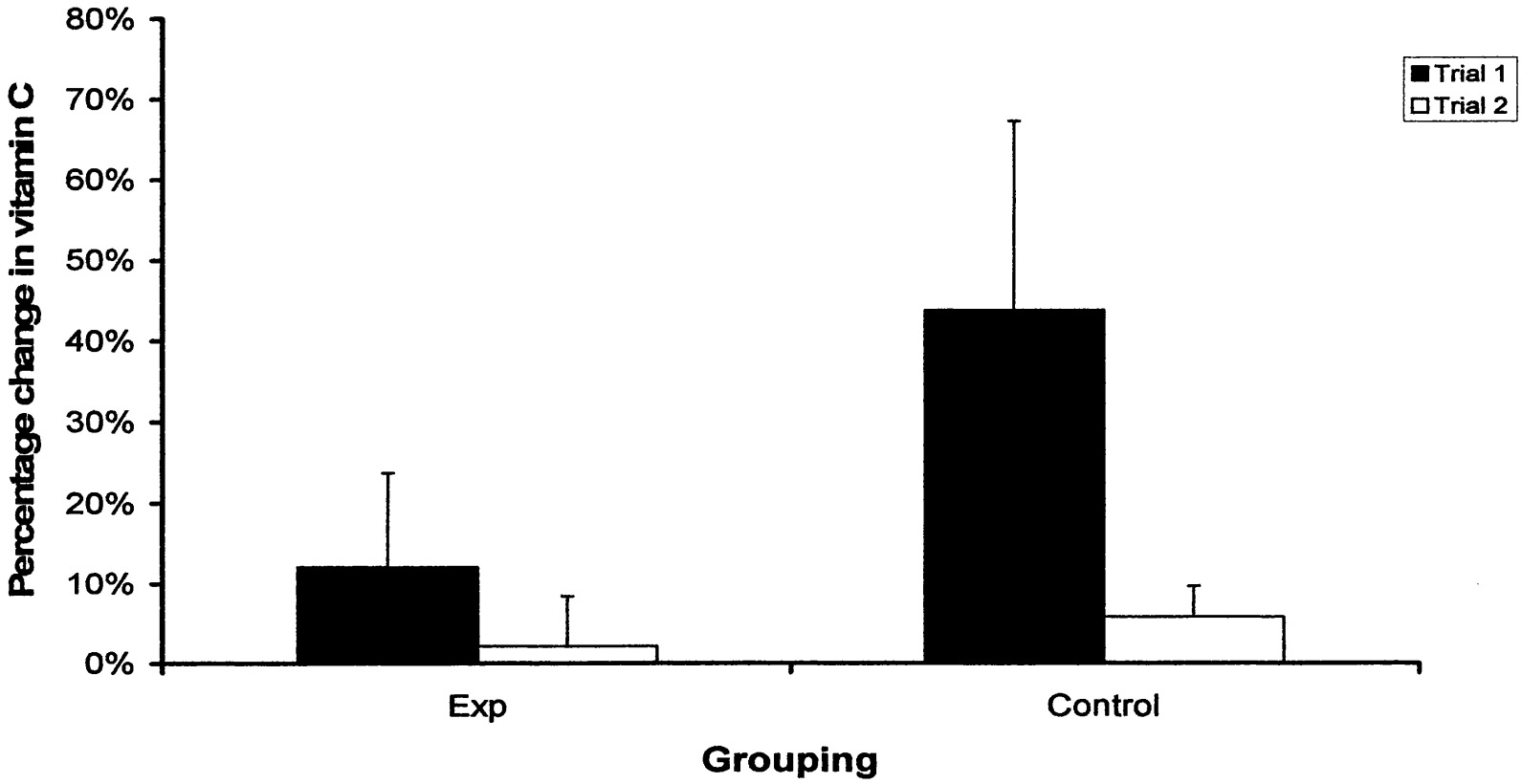

Figure 4.6 Percentage change in Vitamin C concentration pre to post exercise for both trials (non-responders removed).

5 Discussion

This study aimed to identify whether one or three familiarisation sessions would influence markers of oxidative stress, resistance to oxidation or antioxidant defence following exhaustive cycling. The main finding was that no significant interaction effects existed between trials or experimental groups for any of the markers considered (trial x group effect: $P \geq 0.210$, *Partial Eta*^{2} = 0.086). Serum lipid hydroperoxide (HPO) concentrations tended to be lower during the second exercise trial (trial effect: $P = 0.060$, *Partial Eta*^{2} = 0.183); however, no difference existed between experimental groups (group effect: $P = 0.383$, *Partial Eta*^{2} = 0.043), suggesting that repeating the bout of exercise three times provides no additional benefit than a single familiarisation session.

Serum lipid hydroperoxide (HPO) concentrations increased following exercise (timing effect: $P = 0.001$, *Partial Eta*^{2} = 0.461). However, three subjects from both groups (control group and experimental group) demonstrated decreases in HPO concentrations following exercise and were classified as non-responders. These non-responders created a significant difference between the groups (group effect: P = 0.017, *Partial Eta*^{2} = 0.214) as they made up a larger percentage of the control group. Interestingly, post-exercise lactate concentrations were lower in the non-responders when compared with those of the responders (group effect: $P = 0.041$, *Partial Eta*^{2} = 0.235). This may have been due to decreased effort on the part of the non-responders; therefore, this finding might explain why these individuals did not demonstrate oxidative stress. No significant differences were found between

responders and non-responders for any other measured exercise parameters (group effect: $P \geq 0.430$, *Partial Eta*$^2 = 0.035$).

After removing non-responders from subsequent analyses, no significant difference existed between the experimental and control groups in HPO concentration (grouping effect: $P = 0.125$, *Partial Eta*$^2 = 0.126$). The concentrations of serum HPO increased by 26 ± 4 % in the experimental group and 24 ± 5% in the control group during exercise in the initial trial. This finding is in general agreement with previous findings (Ashton *et al.*, 1999; Alessio *et al.*, 2000; Davison *et al.*, 2002; Quindry *et al.*, 2003). Davison *et al.* (2002) found HPO increased from 0.89 ± 0.12 mmol·l^{-1} at rest to 1.11 ± 0.23 mmol·l^{-1} post exercise in apparently healthy subjects, when using a maximal cycling protocol. Alessio *et al.* (2000) found that HPO increased by 24% immediately after a progressive maximal treadmill protocol.

Lipid Hydroperoxides (HPO) are a primary by-product of lipid peroxidation which occurs as a result of a chain reaction propagated by the hydroxyl radical (Reid, 2001). The increase in HPO following exercise implies that the exercise protocol resulted in lipid peroxidation (Mills *et al.*, 1997). Consequently, increases in HPO suggest that, as anticipated, oxidative stress to lipid membranes did occur following the maximal exercise protocol.

Significant increases in HPO concentrations after exercise remained apparent in trial 2 (timing effect: $P \leq 0.001$, *Partial Eta*$^2 = 0.711$); however, there was a tendency for this response to be attenuated during the second trial (trial effect: $P = 0.060$, *Partial Eta*$^2 = 0.183$) when compared with the first. Both the experimental and control

groups reacted similarly (trial x group effect: $P = 0.765$, *Partial Eta*$^2 = 0.005$), demonstrating decreases of 82 ± 2% and 87 ± 5% in the response from trial 1 to trial 2, respectively. This finding suggests that completing a single familiarisation session or three familiarisation sessions afford similar protection against the oxidative stress caused by exhaustive cycle exercise.

Concentration of α–tocopherol increased significantly (timing effect: $P = 0.006$, *Partial Eta*$^2 = 0.258$) following exercise. No significant difference existed between the groups (group effect: $P = 0.408$, *Partial Eta*$^2 = 0.041$). Plasma α –tocopherol is a key antioxidant in the defence against lipid peroxidation with the primary function of scavenging single oxygen peroxyl radicals (Mastaloudis *et al.*, 2001; Reid, 2001). This increase in α–tocopherol concentration following exercise is in agreement with previous research findings (Mastaloudis *et al.*, 2001; Aguilo *et al.*, 2003; Aguilo *et al.*, 2005). Mastaloudis *et al.* (2001) found that plasma α –tocopherol concentrations were increased immediately following a 50 km ultramarathon. However, Davison *et al.* (2002) demonstrated that α–tocopherol concentrations significantly decreased pre- to post-exercise (16.9 ± 2.5 and 15.5 ± 3.1 $\mu mol \cdot l^{-1}$ at rest and immediately post-exercise respectively) following a progressive maximal cycle.

Exercise did not influence concentrations of plasma vitamin C (timing effect: $P = 0.078$, *Partial Eta*$^2 = 0.139$) and no interaction effect was identified (trial x group effect: $P = 0.607$, *Partial Eta*$^2 = 0.016$). Vitamin C is one of the most potent dietary antioxidants which scavenges reactive oxygen species (ROS) thereby inhibiting lipid peroxidation (Ashton *et al.*, 1999; Reid, 2001). Quindry *et al.* (2003) found a 26% decrease in vitamin C immediately following a progressive maximal treadmill

protocol when compared with baseline values. Davison *et al.* (2002) found no change in vitamin C concentration following a maximal cycle protocol. Interestingly, Davison *et al.* (2002) also found a decrease in α –tocopherol following exercise, whereas Mastaloudis *et al.* (2001) and the current study found that α –tocopherol concentrations increased following exercise. It is well documented that vitamin C can regenerate α –tocopherol (Sen, 1995; Ashton *et al.*, 1999; Reid, 2001); therefore, it is possible that the lack of change in vitamin C concentrations in the current study reflects the fact that vitamin C had been used to regenerate α –tocopherol in order to protect against lipid peroxidation.

In addition, it is possible that differences in the timing of blood samples might partly explain variations in the reported responses of blood antioxidant vitamins after exercise. Both Davison *et al.* (2002) and Quindry *et al.* (2003) took blood samples immediately after the completion of exercise and found no increase in vitamin C or α –tocopherol; whereas, during the current study blood samples were taken 10 min after exercise and demonstrated increased α–tocopherol concentrations. The pattern of the mobilization, quenching and regeneration cycle for antioxidant vitamins is not yet known. However, the current data combined with previous research suggests that following intense exercise, the concentrations of plasma vitamin C and α –tocopherol remain depleted until exercise induced mobilization of these antioxidant vitamins restores and eventually enhances circulatory concentrations during the recovery period.

Another possible cause for discrepancies between studies is the lack of information supplied regarding changes in plasma volume. In the current study plasma volume

decreased by 9.8 ± 12.0% in the experimental group and 7.9 ± 1.3% in the control group during the initial trial. While many studies do state that plasma volume changes were accounted for (Mastaloudis *et al.*, 2001; Davison *et al.*, 2002; Quindry *et al.*, 2003; Sacheck *et al.*, 2003) others fail to mention if this has been considered or not (Ashton *et al.*, 1999; Alessio *et al.*, 2000; Vider *et al.*, 2001). Changes in plasma volume affect the concentration of metabolites within the plasma; therefore, changes in plasma volume could result in changes in α –tocopherol or vitamin C with exercise rather than any alteration to vitamin mobilization or quenching itself.

During the second trial the response in vitamin C to the exercise protocol was reduced when compared with trial 1 (trial effect: $P = 0.061$, *Partial Eta*$^2 = 0.190$). This trend was apparent in both groups with no significant difference between them (group effect: $P = 0.293$, *Partial Eta*$^2 = 0.065$). An attenuated response during the second trial indicates less antioxidant defence was required during this trial when compared with the initial trial. As vitamin C has been demonstrated to regenerate α – tocopherol (Sen, 1995; Ashton *et al.*, 1999; Reid, 2001), it is possible that the reduced vitamin C response to exercise during trial 2 was due to increased regeneration of α –tocopherol.

Other antioxidant vitamins (vitamin C, retinol, γ-tocopherol, α-carotene, β- carotene, lycopene) followed similar patterns for both groups during the study (trial x timing effect: $P \geq 0.297$, *Partial Eta*$^2 = 0.141$). No differences existed between the groups for any antioxidant vitamins across both trials (group effect: $P \geq 0.143$, *Partial Eta*$^2 = 0.122$). Analysis of dietary intake of antioxidant vitamins showed no significant difference in intake between the groups across trials (trial x group effect: $P \geq 0.134$,

Partial Eta^2 = 0.135). Dietary composition of the subjects was similar to the average found for British males aged 19-24 yrs old (Hoare *et al.*, 2005).

No interaction effect was apparent (trial x group effect: $P = 0.350$, *Partial Eta*^{2} = 0.055) for low density lipoprotein (LDL) oxidation lag times and the exercise protocol did not influence LDL lag times (timing effect: $P = 0.757$, *Partial Eta*^{2} = 0.017). However, previous research has shown decreases in lag times following exercise (Sanchez-Quesada *et al.*, 1995; Wetzstien *et al.*, 1998). Sanchez-Quesada *et al.* (1995) found that following a 4 hour continuous run the lag times to conjugated diene formation were decreased by 14% ($P \leq 0.05$) when compared to resting values. Wetzstein *et al.* (1998) found that a 30 min treadmill run at 70% of peak oxygen consumption resulted in a significant decrease in LDL lag time ($P = 0.03$). The exercise protocols used in each of these studies were different to that used in the current study which might in part account for the different response encountered. The current study design aimed to create as little muscle damage as possible by minimising eccentric muscle activity; whereas running protocols possibly include oxidative stress born from muscle damage in addition to increased oxygen flux.

While LDL lag times did not change significantly from baseline to post exercise in either trial, the lag times increased slightly following exercise in trial 1 (3.7 ± 2.8%) and decreased slightly (-4.2 ± 1.9%) in the second trial. However, a trend existed between trials (trial effect: $P = 0.058$, *Partial Eta*^{2} = 0.201). This response suggests that there was a decreased need for mobilization of defence against oxidation in the second trial when compared with the first. No significant difference in this response was found between the groups (group effect: $P = 0.383$, *Partial Eta*^{2} = 0.048)

showing that no additional benefit was gained from two additional familiarisation sessions.

All subjects in the current study reached the criteria for determining maximal effort and the time taken to reach this point was matched in all subsequent bouts so that workload remained the same (all subjects reached the same workload in trial 2). In the experimental group there was a significantly lower peak heart rate in trial 2 when compared to trial 1 (trial effect: $P = 0.002$, *Partial Eta*$^2 = 0.630$). No significant differences existed in any other markers of protocol performance across the trials (trial effect: $P \geq 0.199$, *Partial Eta*$^2 = 0.034$).

No significant difference existed between experimental groups at baseline for any of the exercise parameters (group effect: $P \geq 0.077$, *Partial Eta*$^2 = 0.157$). Peak oxygen uptake during the protocol was 48.9 ± 1.3 and 49.5 ± 1.6 ml·kg $^{-1}$·min^{-1} for the experimental and control groups, respectively during trial 1. This is similar to that found by Ashton *et al.* (1999) who used males aged 18-30 yrs and a progressive maximal cycle protocol. The mean heart rate maximum of 195 ± 6 beats·min^{-1} in the present study is similar to that found by Quindry *et al.* (2003) using a progressive treadmill protocol to volitional exhaustion (190.8 ± 7.3 beats·min^{-1}). Peak lactate concentrations in the current study were slightly higher than those found by Alessio *et al.* (2000) following a treadmill test to volitional exhaustion (6.77 ± 0.1 mmol·L^{-1}). The current study found an average 753% increase following exercise whereas Alessio *et al.* (2000) found only a 479% increase. This could be due to differences in exercise protocol as Alessio *et al.* (2000) used a treadmill test rather than a cycle ergometer.

The trend for an attenuated response in HPO (trial effect: $P = 0.060$, *Partial Eta*^{2} = 0.183) and vitamin C (trial effect: $P = 0.061$, *Partial Eta*^{2} = 0.190) concentrations, and the decreased lag time to LDL oxidation (trial effect: $P = 0.058$, *Partial Eta*^{2} = 0.201), during the second trial may be comparable to the 'repeated bout effect' described in Eston *et al.* (1996 and 2000), when referring to DOMS. No previously published data has investigated the potential protective effect of familiarisation on oxidative stress. Consequently, this is the first study which has demonstrated that familiarisation to an exercise protocol might reduce oxidative stress caused in a second bout of the same exercise.

The mechanisms behind this finding are unclear. The 'repeated bout effect' was apparent in both the experimental and the control groups, suggesting that the reduced response was not due to a training effect. It is possible that the lowered response in the second trial might have been the result of decreased stimulus. All subjects matched the intensity and duration of exercise achieved in the first trial during the second. However, in the experimental group there was a significantly lower peak heart rate in trial 2 when compared to trial 1 (trial effect: $P = 0.002$, *Partial Eta*^{2} = 0.630) suggesting less effort was needed to complete the second trial. Consequently, it is plausible that the exercise protocol led to lowered ROS during the second exercise trial.

Another potential mechanism that might explain this 'repeated bout effect' is a feedback response from the initial trial. It is plausible that the mobilization of antioxidant defences post exercise during trial 1 created a feedback mechanism that

improved the mobilization of antioxidant defences during the second exercise trial; thereby, improving the ability to scavenge ROS during exercise and reducing the mobilization of antioxidant defences after the cessation of exercise. Therefore, when samples were taken 10 minutes post exercise, concentrations of vitamin C and the LDL defence might have been reduced when compared to the same time point in the initial trial.

While the mechanisms behind the 'repeated bout effect' remain unclear, it is apparent from the present study that any effect that may exist can be brought about by just one prior bout of an identical exercise protocol. No additional reduction in oxidative stress or in antioxidant defence was brought about through two additional familiarisation sessions. This data suggests that in research that measures oxidative stress a trial order effect may exist without familiarisation; additionally, performing more than one familiarisation session would not be beneficial. Traditionally familiarisation has been carried out where muscle damage is expected to result from the exercise protocol (Barker *et al.*, 2001; Rowlands *et al.*, 2001); importantly, the present study suggests that familiarisation is essential when undertaking intervention studies involving free radical damage and oxidative stress.

6 Conclusions and Recommendations

6.1 Conclusions

The current study found that progressive maximal cycling increased concentrations of serum lipid hydroperoxides (HPO) (timing effect: P = 0.001, *Partial Eta*^{2} = 0.461), indicating that lipid peroxidation resulted from the exercise protocol. Concentrations of α –tocopherol were also increased post exercise (timing effect: P = 0.006, *Partial Eta*^{2} = 0.258), indicating an increased antioxidant defence following exercise. No difference was found in this response between the experimental and control groups (group effect: $P \geq$ 0.383, *Partial Eta*^{2} = 0.043). Therefore, lipid peroxidation occurred to a similar extent in both groups.

Concentrations of HPO were increased following exercise in trial 2 (timing effect: P = 0.01, *Partial Eta*^{2} = 0.461); however, there was a tendency for this response to be attenuated (trial effect: P = 0.060, *Partial Eta*^{2} = 0.183) when compared with the initial trial. This attenuated response in lipid peroxidation suggests that less oxidative stress resulted from the second trial when compared to the first. Both the experimental and control groups reacted similarly (trial x group effect: P = 0.767, *Partial Eta*^{2} = 0.005). This data demonstrated that no additional benefit was gained from two additional familiarisation sessions.

The current study highlights the need for familiarisation in intervention studies that measure oxidative stress born from increased oxygen flux; furthermore, these findings suggest that one familiarisation session is sufficient in eliminating the trial order effect in such studies. Further research is needed to investigate the mechanisms responsible for this familiarisation effect.

6.2 Limitations and Future Recommendations

The current study is the first to suggest that exercise familiarisation offers a protective effect against oxidative stress; however, these findings were not statistically significant. Statistical power was reduced as data from six subjects (3 control group and 3 experimental group) were removed prior to analysis as these individuals were classified as "non-responders". Oxidative stress represents an imbalance between ROS production and antioxidant defence. Therefore, it is likely that inter-individual differences in ROS production and antioxidant defences explains the presence of non-responders in this study. Future research, using similar exercise protocols, should account for non-responders when considering the number of subjects that are to be recruited.

In addition, all the subjects used in this study were apparently healthy males (22 ± 0.4 yrs); consequently, this limits the application of the findings. It would be interesting to identify the requirement for familiarisation in other exercising populations, such as females of a similar age or a diseased population. Vina *et al.* (2006) found that gender influenced the amount of exercise-induced oxidative stress. These authors suggested that oestrogens cause elevated expression of antioxidant

enzymes and resulted in reduced oxidative stress in females. Future research could replicate the current study using a female population to establish whether familiarisation offers similar benefits as identified in the male population. The study could also be replicated using diseased patients, as the effect on patients with a disease traditionally associated with increased oxidative stress may have a very different response.

The current study found a significant increase in HPO after exercise, which indicates lipid peroxidation had occurred during exercise. However, this reduction in lipid peroxidation might have been caused by a decrease in ROS or an increase in antioxidant defence: consequently, a measure of radical production would allow further explanation of the mechanisms responsible for this finding. Future research could directly measure free radicals via electron spin resonance (ESR) analyses of blood or muscle biopsy samples.

Previous research and the current study used concentrations of antioxidant vitamins post-exercise to demonstrate the changes in antioxidant defence during intense exercise (e.g., Caillaud *et al.*, 1999; Marquez *et al.*, 2001; Senturk *et al.*, 2001; Metin *et al.*, 2002; Tauler *et al.*, 2002, Kingsley *et al.*, 2005). The findings from studies such as these have been equivocal, primarily due to differences in the timing of blood samples and the lack of understanding regarding the recruitment of antioxidant vitamins to defend against ROS and their regeneration post exercise. Consequently, future research could collect regular blood samples during and up to 48 hours following exercise in order to gain a better understanding of this cycle.

The protective effect of familiarisation against oxidative stress was similar in both experimental groups. Subjects in the control group had an average of 21 days between trials and no differences were identified when compared with those who completed two additional familiarisation sessions within this time. However, no data are available to evaluate the maximum length of time that familiarisation continues to offer protection against oxidative stress. Future research could aim to identify the length of time that familiarisation remains effective in attenuating oxidative stress following exercise.

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Appendix A

BUSINESS SERVICES CENTRE/POWYS LOCAL HEALTH BOARD

SWANSEA LOCAL RESEARCH ETHICS COMMITTEE

Application for Ethics Committee Approval.

N.B. All questions should be answered. Answers should be typewritten. PLEASE DO NOT REMOVE ANY SECTION OF THIS APPLICATION FORM B IF THE SECTION IS NOT APPLICABLE TO YOUR APPLICATION PLEASE MARK IT ANOT APPLICABLE@.

---

1. TITLE OF PROJECT

Exercise, Oxidative stress and Creatine supplementation.

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2. NAMES OF RESEARCH TEAM LOCATION **QUALIFICATIONS** **STATUS**

Dr. Liam Kilduff,	Bsc (Hons), PhD	Lecturer
Mr. Mike Kingsley,	MSc, BPhEd, PGCE	Lecturer
Mr. Dan Cunningham,	Bsc (Hons)	Post graduate
Miss. Laura Burns,	Bsc (Hons)	Post graduate

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3. WHAT OTHER TRIALS ARE EACH MEMBER OF THE RESEARCH TEAM CURRENTLY ENGAGED IN? Please list all trials of ALL OF THOSE NAMED AS PART OF THE RESEARCH TEAM in Question 2.

Effects of Creatine supplementation on muscle strength and quality of life in patients with Chronic Obstructive Pulmonary Disease.

Effects of creatine supplementation on muscle mass, strength and walking distance in patients with intermittent claudication.

The effect of managed exercise rehabilitation on the physiological recovery of coronary care patients.

Antioxidant status of elite Greek athletes.

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4. OBJECTIVE (i.e. hypothesis which it is intended to test)

H_{O1}: Protocol familiarisation will have no effect on oxidative stress levels following maximal cycling.

H_{O2}: Creatine supplementation will not affect oxidative stress levels following maximal cycling.

5. DESIGN OF THE STUDY (describe briefly)

During the initial visit to the laboratory all subject's will complete measurements of stature (), body mass () and body composition (Quadscan Bioimpedance analyzer; Bodystat, Isle of Man). Following an initial interview in order to assess individual suitability, all subjects will be screened according to the AHA/ACSM (1998) joint position statement (Appendix C) and informed consent will be obtained. At this point a health questionnaire will be completed and an informed consent form signed.

On the morning of the first testing session (T1) subjects will complete a continuous incremental test to volitional exhaustion on a calibrated cycle ergometer (Excalibur Sport, Lode, Holland). Breath-by-breath respiratory parameters and heart rate will be continuously recorded throughout the trial. In addition, venous blood samples of less than 30 ml will be taken from the antecubital vein pre-exercise, immediately post exercise, 30 minutes post-exercise and 24 hr post-exercise.

Following T1 subjects will be assigned in a double blind fashion, to either a familiarisation group (n=20) or a control group (no familiarisation) (n=10). Subjects in the familiarisation group will then visit the lab on two further occasions 5-7 days apart to become familiarised with the exercise protocol (F1 and F2). No blood samples will be obtained during F1 and F2.

Seven days following familiarisation, all subjects will return to the lab to complete the second testing session (T2), which will follow the same procedure's as T1. Subjects will be assigned, in a double blind fashion, to either a Cr group or a placebo group. Subjects will either supplement with 20g creatine and 180g CHO per day (Cr group) or with 180g CHO per day for seven days. The placebo group will follow the same procedure as the Cr group with regard to the preparation of the supplements. A third and final post-supplementation test will be carried out following the 7-d supplementation period (T3).

The subjects will be required to avoid participating in strenuous exercise and refrain from ingesting alcohol and caffeine for 48 hours prior to and 48 hours after each trial.

6. SCIENTIFIC BACKGROUND - give a brief account, with references where relevant, or attach relevant information.

Free radicals are molecules that contain an unpaired electron and are capable of existing independently. Highly reactive free radicals called reactive oxygen species (ROS) are produced as a bi-product of oxidative metabolism (Sen, 1995). Elevated free radical production can lead to an increase in tissue damage by reacting with polyunsaturated fatty acids on the cellular membrane, nucleotides in DNA, and critical sulfhydryl bonds in proteins (Machlin & Bendich 1987). Antioxidants, produced by the body and in conjunction with exogenous antioxidants (e.g. ascorbic acid, glutathione and α-tocopherol), help provide protection from the ravages of free radical species (Sen, 2001).

Strenuous exercise causes a 10 – 20 fold increase in total body oxygen uptake and up to 200-fold increases in oxygen flux through active muscles and has been demonstrated to lead to increased ROS production when compared to resting conditions (Sen, 2001). It has been suggested that regular exercise may provide an increased protection against oxidative damage (Quindry *et al.*, 2003) possibly due to an enhanced antioxidant status developed with chronic training (Tessier *et al.*, 1995).

A single bout of eccentric exercise has consistently been demonstrated to provide protection against muscle damage and delayed onset muscle soreness (DOMS) caused by further eccentric exercise, although the mechanisms behind this phenomenon are still unclear (Eston *et al.*, 2000). While DOMS may not represent oxidative stress the question does remain as to whether familiarisation with a similar exercise type may result in a decreased level of oxidative stress. Most studies opt for familiarising all subjects in an attempt to eliminate a trial order effect (e.g. Alessio & Goldfarb, 1988; Balsom *et al.*, 1995; Eston *et al.*, 2000; Gilliam *et al.*, 2000); however, to our knowledge, no study has

investigated the potential effects of a familiarisation bout of exercise on oxidative stress. If simple familiarisation techniques have potential to enhance antioxidant status, then familiarisation could be used in a variety of populations to reduce the effects of oxidative stress associated with exercise.

In recent years, numerous studies have investigated the effects of Creatine (Cr) supplementation on exercise performance and body composition, with the balance of available evidence suggesting oral Cr loading can increase muscle Cr content, fat-free mass, (Kilduff *et al.*, 2002) improve anaerobic sprint performance (Casey *et al.*, 1996) and promote greater gains in strength (Kilduff *et al.*, 2002). Subjects will be administered with Cr and CHO or CHO depending on which group they are assigned to. Evidence suggesting that 5g of Cr administered with 93g of CHO maximises the amount of Cr retained by the body during supplementation (Green *et al.* 1996). Despite the plethora of research into the effect of Cr supplementation on body composition and exercise performance only one study to date has investigated the effects of Cr as a possible antioxidant against aqueous radical and reactive species ions (Lawler *et al.*, 2002). Lawler *et al* (2002) reported that Cr acted directly as an antioxidant, however they used a highly controlled "in vitro" experiment and therefore the results may not be applicable to the whole body "in vivo" experiment.

In order to test if Cr has an in vivo antioxidant affect it is necessary to undertake an exercise protocol that will cause oxidative stress. Continuous incremental tests to volitional exhaustion have been demonstrated to cause significant increase in markers of oxidative stress and elevate free radical production. This protocol has been found to produce significant free radical production (Aston *et al.* 1998). Therefore the aims of the present study are to examine the effects of Cr supplementation on antioxidant status and the effects of protocol familiarisation on free radical production in a group of healthy male subjects following high-intensity maximal exercise.

Alessio, H.M. and Goldfarb, A.H. (1988). Lipid peroxidation and scavenger enzymes during exercise: adaptive response to training. Journal of Applied Physiology 64(4), 1333-1336.

Balsom, P.D., Soderlund, K., Sjodim, B. and Ekblom, B. (1995). Skeletal muscle metabolism during short duration high intensity exercise: influence of creatine supplementation. ACTA Physiology Scand. 154, 303-310.

Casey, A., Constantin-Teodosiu, D., Howell, S., Hultman, E. and Greenhaff, P.L. (1996) Creatine ingestion favourably affects performance and muscle metabolism during maximal exercise in humans. American Journal of Physiology. 271 E31-E37.

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Gilliam, J.D., Hohzorn, C., Martin, D. and Trimble, M.H. (2000). Effect of oral creatine supplementation on isokinetic torque production. Med Sci Sports Ex. 32, 5, 993-996.

Green AL, Hultman E, Macdonald IA, Sewell DA, Greenhaff PL. (1996) Carbohydrate ingestion augments skeletal muscle creatine accumulation during creatine supplementation in humans. American Journal of Physiology. 271 821-6

Kilduff LP, Vidakovic P, Cooney G, Twycross-Lewis R, Amuna P, Parker M, Paul L, Pitsiladis YP. (2002). Effects of creatine on isometric bench-press performance in resistance-trained humans. Medicine and Science in Sports and Exercise; 34(7):1176-83

Lawler, J.M., Barnes, J., Wu, G., Song, W. and Demaree, S. (2002) Direct antioxidant properties of creatine. Biochemical and Biophysical Research Communications 290, 47-52.

Sen, C.K. (2001) Antioxidants in Exercise Nutrition. Sports Med 31(13) 891-908

Sen, C.K. (1995) Oxidants and Antioxidants in Exercise. The American Physiological Society.

Machlin, L.J. and Bendich, A. (1987) Free radical tissue damage: protective role of antioxidant nutrients. Federation of American Societies for Experimental Biology. 441-445.

Quintry, J.C., Stone, W.L., King, J. and Broeder, C.E. (2001). The effects of acute exercise on neutrophils and plasma oxidative stress. Med Sci Sports Ex. 35, 7, 1139-1145.

Reid, M.B (2001) Redox modulation of skeletal muscle contraction: what we know and what we don't. Journal of Applied Physiology. 90, 724-731

7. SUBJECTS

Thirty healthy male subjects between the ages of 18 and 45 will be recruited to take part in this study. Subjects will be recruited on the basis that they are physically active but have not supplemented with creatine or other dietary supplements (e.g. vitamins) for at least 8 weeks prior to the start of the study and have not completed a maximal exercise protocol for three months prior to starting testing. The subject's eligibility will be assessed by interview prior to their informed consent for participation in the study and a health questionnaire (Appendix C) completed to identify any contraindications to exercise.

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8. DURATION OF RESEARCH (over what period of time do you wish approval to be granted?

2 years

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9. LOCATION AND ADDRESS OF THE PREMISES WHERE THE RESEARCH WILL BE CONDUCTED.

Exercise Physiology Laboratory, Ground floor, Vivian Tower, University of Wales Swansea, Singleton Park, Swansea.

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10. MEDICAL AND OTHER STAFF SUPPORT AND OTHER FACILITIES AVAILABLE (please give details)

Not Applicable

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11. WHAT NATIONALLY APPROVED GUIDELINES HAVE BEEN FOLLOWED IN PREPARING THIS PROTOCOL

American College of Sport and Medicine (ACSM).

British Association of Sport and Exercise Sciences (BASES).

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12. CONSENT OF MEDICAL PRACTITIONERS

Will this need to be obtained?

a) from consultants	NO
b) from general practitioners	NO

13. ONLY TO BE COMPLETED IF DRUGS ARE BEING USED.

SUBSTANCES TO BE GIVEN TO THE SUBJECTS
(Indicate those extra to normal clinical management)

A) DRUGS
NotApplicable

B) OTHER SUBSTANCES (fluids, diets and others)

(i) State what other substances Creatine (Cr) and Glucose (CHO)

(ii) State: Both Powder

Route of Administration: Both Orally

Amount: Cr- 5g four times/day for 7 days, CHO- 180g four times daily

Frequency: Four times daily for 7 days

Desired Effects: Protective effect against oxidative stress

Possible Side Effects: Increased lean body mass, increase in athletic performance.

Precautions:

(iii) Other (eg. physical agents such as heat, radiation, electricity, other, details of amount and desired or side effects) Not Applicable

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C) RADIO ACTIVE SUBSTANCES

(If radio-isotopes are to be used you are required to obtain the approval of the Health Minister through the DOH, Administration of Radioactive Substances Advisory Committee. These formalities should be completed before your protocol is submitted to the Local Research Ethics Committee).

Are radio-isotopes to be used in this study? NO

If radio-isotopes are to be used, please provide the following information:

Has approval been obtained from the DOH Administration of Radioactive Substance?
Advisory Committee? Not Applicable

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14. INVESTIGATIVE PROCEDURES

Blood Sampling- Venous blood samples will be collected from the antecubital vein with suitable vacutainers by trained and qualified staff. Blood samples will be taken pre-exercise, at exhaustion, 30

minutes post-exercise and 24 hrs post-exercise and analysed for levels of free radical production, oxidative stress and for antioxidant status.

Progressive Exercise - Subjects will complete a continuous incremental test to volitional exhaustion in order to determine maximal oxygen uptake ($\dot{V}O_2$max), the anaerobic threshold (AT), and the maximal work rate (WRmax) on a calibrated cycle ergometer. Breath-by-breath oxygen uptake will be continuously recorded using a computerised online gas analysis system (Oxycon Pro; Jaeger). Heart rate will be continuously recorded using a portable heart rate telemetry device (Polar 810s: Polar, Finland). The tests will be incremental and progressive; all subjects will commence at a 60 W workrate and are required to keep the cadence at 70 rpm. Workload will be increased by 30 W every 3 mins until exhaustion. The objective criteria for $\dot{V}O_2$max determination will be: a respiratory exchange ratio (RER) > 1.15 at termination of the test, a plateauing of the oxygen uptake curve where observed, heart rate > 90% of age predicted maximum and failure of the subject to cycle at 70 rpm despite verbal encouragement.

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15. DISCOMFORT

Due to the blood sampling procedure required for this study there may be some discomfort caused however this will be of short duration and will be kept to a minimum. The frequency of visits to the lab may also be an inconvenience however the majority of these visits will take less than 15 minutes. Prior to each testing session you will be asked to refrain from alcohol and strenuous exercise for 48 hours and not to eat on the morning of each testing session. During supplementation you will be asked to refrain from ingesting caffeine which could be a possible disadvantage for you. There may also be some degree of discomfort associated with the maximal exercise protocol as this may result in muscle soreness following completion.

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16. PLEASE STATE THE RISK, IF ANY, TO THE PHYSICAL AND MENTAL HEALTH OF THE SUBJECT

As with all maximal exercise there will be potential health risks however risks are small and health screening prior to exercise should reduce these further.

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17. INFORMED CONSENT

The submission should be specific about the following:

Will consent be given in written or verbal form? Written
(a copy of any consent form should be attached).

How will subjects be given information about the study? Information Sheet (Appendix A)

(a copy of the information sheet, whether it is to be read out or given to the subject, should be attached).

How will the patient or volunteer be asked to participate? Written

How much time will be allowed for subjects to consider and consult others before giving consent? 24 hours

If the study includes patients unable to give informed consent, what alternative process will be used? Not Applicable

What will the patient be told about: Fully informed (See information sheet, Appendix A)

*** the project and what it is intended to show** Appendix A

*** the frequency of procedures and what they entail** Appendix A

*** possible risks and side effects** Appendix A

*** exclusion criteria with reference to medical history, etc C** See Health questionnaire, Appendix C

*** entitlement to leave study.** Appendix A

Please give below page references to the main protocol in order to cover these points. Also, please provide any additional details not adequately covered in the protocol.

An example of an information sheet and a consent form is available from the secretary on request.

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18. COMPUTERS

Are computers to be used to store data? YES

If so, is the data registered under the Data Protection Act? YES

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19. SPONSORSHIP

Is the project being sponsored? NO

If so, please give the name and address of the organisation providing the sponsorship.

Please give full details of:

(i) payments to patient / health volunteer No Financial Payment,

(ii) payments to a trust or research fund No Financial Payment,

(iii) personal payment or personal benefit to researcher No Financial Payment

Is there any cost to the Trusts NO

If yes Not Applicable

20. COMPENSATION

What compensation / insurance arrangements cover this research?

The University of Wales Swansea has legal liability in two categories covering public and clinical trials.

21. The information supplied above is to the best of my knowledge and belief accurate.

I understand that approaches to individual members of the Ethics Committee (excluding questions of procedural points) are not welcome and will be reported to the Committee.

I understand that I will be invited to explain my protocol to the Committee.

I understand that the Local Research Ethics Committee gives Ethics approval only. I have addressed permission for additional resources to the appropriate Consultant / Head of Department/Trust

SIGNATURE OF INVESTIGATOR/S * .....

.....

DATE OF SUBMISSION .....

*** PLEASE ENSURE THAT ALL THOSE NAMED IN QUESTION 2 SIGN THE APPLICATION**

LREC.APPLICATION.FORM.WPD 2000 18/01/01

Appendix B

DEPARTMENT OF SPORTS SCIENCE

SUBJECT INFORMATION SHEET

Date :

Contact Details:

Laura Burns - [redacted]

Dan Cunningham [redacted]

1. Study title

Factors affecting the formation of radical species in healthy males

2. Invitation paragraph

You have been invited to take part in a scientific study aiming to gain an increased understanding of the formation of free radical species in relation to exercise. The parameters being measured in the study are frequently used as determinants of aerobic fitness therefore a comprehensive evaluation of this aspect of fitness will be conveyed to you. We would be very grateful if you could give us your commitment to this study and we will do our best to provide a meaningful physiological profile which may help in any future training program.

3. What is the purpose of this study?
The aim of this study is to investigate the effects of familiarisation and of creatine supplementation on free radical formation.

4. Why have I been chosen?
You have been chosen because for this study we require male subjects who are not accustomed to cycling and who are not engaged in a structured weight-training program. Subjects must also have not supplemented with either creatine or dietary supplements (Vitamins etc) for 8 weeks prior to commencement of testing. We also require people who are enthusiastic and dedicated to completing the testing procedure. Participation is completely voluntary and you are free to leave the study at any point with out giving any reason.

5. What will happen to me if I take part?
You will be required to visit the exercise physiology lab a maximum of 12 times during the testing period (5 weeks). Body composition will be determined and on a maximum of 5 occasions a maximal exercise protocol will be complete. Blood samples will be taken prior to and on four occasions following each exercise bout.

6. What are the possible disadvantages of taking part?

As with all maximal exercise there are health risks however risks are small and health screening prior to exercise should reduce these further. Due to the blood sampling procedure required for this study there may be some discomfort caused however this should be of short duration and will be kept to a minimum. The frequency of visits to the lab may also be an inconvenience however the majority of these visits will take less than 15 minutes.

7. What are the possible benefits of taking part?

The nature of the testing procedure will mean that a number of physiological parameters will be measured. As a result a comprehensive picture of subject's fitness can be delivered to the subjects following testing. Creatine supplementation associated with the study may elicit some benefits in performance and increases in fat free mass.

8. Will my taking part in the study be kept confidential?

All information in the study will be kept confidential. The data produced from the testing may be published but information in the results will not indicate your involvement in the study. Your name will remain anonymous and will not be included in the study.

Appendix C

DEPARTMENT OF SPORTS SCIENCE
SUBJECT CONSENT FORM

Contact Details:
Laura Burns – [redacted]
Daniel Cunningham - [redacted]

Project Title:
Factors affecting the formation of radical species in healthy males

Please initial box

1. I confirm that I have read and understood the information sheet dated ...../...../..... (version number .....) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of data obtained may be looked at by responsible individuals from the University of Wales Swansea or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to these records. ☐
4. I agree to take part in the above study.

Name of Subject	Date	Signature
Name of Person taking consent	Date	Signature
Researcher	Date	Signature

Appendix D

AHA/ACSM Health/Fitness Facility Pre-participation Screening Questionnaire.

Name _____

Address _____

Phone number home _____ work _____

Emergency contact name _____ phone _____

Date of birth _____

History ***Assess your health needs by marking all true statements.***

You have had:
☐a heart attack
☐heart surgery
☐cardiac catheterization
☐coronary angioplasty (PTCA)
☐pacemaker/implantable cardiac defibrillator/rhythm disturbance
☐heart valve disease
☐heart failure
☐heart transplantation
☐congenital heart disease

If you marked any of the statements in this section, consult you a health provider before engaging in exercise. You may need to use a facility with a medically qualified staff.

Symptoms and other health issues:
☐You experience chest discomfort with exertion.
☐You experience unreasonable breathlessness.
☐You experience dizziness, fainting, blackouts.
☐You take heart medications.
☐You take prescription medication(s).
☐You have musculoskeletal problems.
☐You have concerns about the safety of exercise.
☐You are pregnant.

Cardiovascular risk factors
☐You are a man older than 45 years.
☐You are a woman older than 55 years or you have had a hysterectomy or you are postmenopausal.
☐You smoke.
☐Your blood pressure is greater than 140/90.
☐You don't know your blood pressure.
☐You take blood pressure medication.
☐Your blood cholesterol level is >240 mg/dL.
☐You don't know your cholesterol level.
☐You have a blood relative who had a heart attack before age 55 (father or brother) or age 65 (mother or sister).
☐You are diabetic or take medicine to control your blood sugar
☐You are physically inactive (i.e., you get less than 30 minutes of physical activity on at least 3 days per week).
☐You are more than 20 pounds overweight.

If you marked two or more of the statements in this section, you should consult your healthcare provider before engaging in exercise. You might benefit by using a faciltiy with professionally qualified exercise staff to guide your exercise program.

☐**None of the above is true.**
You should be able to exercise safely without consulting your healthcare provider in almost any facility that meets your exercise programme needs.

Appendix E

Subject Questionnaire

Name: **Test Number:**

Have you undertaken any strenuous physical activity in the last 24 hours? If yes please give details of the activity (Intensity, Type etc.)

Have you consumed any alcohol in the last 24 hours? If yes how much?

Have you eaten this morning? If so what and how much?

Have you been to the toilet this morning?

Have you consumed caffeine or caffeine containing foods/drinks in the last 24 hours? If yes when and in what form?

Do you take any supplements? (E.g. Vitamins, Protein, Cyclone, Creatine etc.) If so which?

Appendix F

DIETARY RECORD

Please read these important instructions carefully

- Please record all food and drink consumed.
- Please record the food and drink at the time eaten and not from memory at the end of the day.
- You should include all meals and snacks (including water, etc.).
- Remember to include any additions to foods (e.g. sauces, salad dressings, etc.).

DESCRIBING FOODS AND DRINKS

1. Please give cooking methods (e.g. fried, steamed, poached, etc.).
2. Please give as many details about the food as possible:
   e.g. Brand names: Miracle margarine
   Generic names: cod fillet, diet coke

RECORDING THE AMOUNTS OF FOOD YOU EAT

Weighing foods

1. Initially weigh each component of your meal or snack before you begin eating and complete the "weight of item" column on the form.
2. When you have finished eating weigh any food item remaining and complete the "weight remaining" column on the form.

Known quantities

3. Record the quantity of food identified on pre-packed food eaten and complete the "weight of item" column on the form (e.g. 220g tin of backed beans, etc.).
4. Record quantities in everyday terms (e.g. cup of infused tea, teaspoon of sugar, etc.).

It is very important that you do not adjust what you eat or drink because you are keeping this record.

We are interested in your dietary intake, NOT the perfect diet!

Name _____

Day _____ Date _____

- Please record all the food and drink you consume.
- Please record the method of cooking, type of food and quantity of food.

Morning:

	Time	Description of food/drink		Quantity of food/drink	
		Type of food	***Cooking method***	***Weight of item***	***Weight remaining***
Breakfast					
Snacks					
Lunch					

Afternoon and Evening:

	Time	Description of food/drink		Quantity of food/drink	
		Type of food	*Cooking method*	*Weight of item*	*Weight remaining*
Snacks					
Dinner					
Snacks					

Appendix G

Borg scale rate of perceived exertion (RPE)

6	
7	Very, very light
8	
9	Very light
10	
11	Fairly light
12	
13	Somewhat hard
14	
15	Hard
16	
17	Very hard
18	
19	Very, very hard
20	

Appendix H Subject characteristics and Exercise parameters raw data.

Trial 1							
Subject No	Weight (kg)	Height (m)	Age (yrs)	BF%	TBW%	ECW%	ICW %
Exp Group							
1	96.1	1.83	20	19.8	53.6	22.2	37.8
2	80.9	1.77	21	18.9	53.5	22.2	32
3	84.2	1.84	26	17.1	55.5	22.8	32.9
4	97.4	1.75	21	21.6	53.5	22.2	31.6
5	89.5	1.77	21	17.2	56.4	22.7	34.0
6	77.9		23	19.3	53.3	22.3	32.0
7	75.1	1.70	22	18.9	54.5	22.4	32.9
8	74.2	1.80	19	10.0	62.0	25.5	35.8
11	90.3			19.6	53.7	22.3	31.7
12	73.0	1.73	20	11.2	61.8	25.3	35.8
13	83.0	1.76	20	12.7	60.1	24.5	35.1
14	78.0	1.73	21	13.2	60.1	24.5	35.1
18	64.7	1.70	25	14.2	60.4	24.9	35.3
20	81.8	1.77	22	19.1	53.4	22.1	31.9
21	74.4	1.78	20	16.5	55.1	22.7	33.2
22	88.6	1.81	21	17.7	55.1	22.8	32.6
23	75.0	1.73	22	12.5	60.9	25.1	35.2
24	77.8	1.75	20	12.6	60.2	24.7	34.8
Control Group							
9	83.5		22	16.8	55.1	22.8	32.2
10	100.4	1.86	25	19.9	54.5	22.2	32.3
16	84.0	1.77	22	17.7	55.1	22.4	33.3
28	83.1	1.80	23	17.0	55.5	22.9	32.8
29	74.8			17.0	54.7	22.9	32.2
31	82.8	1.78	26	15.8	58.0	23.9	33.7
32	69.1	1.65	21	15.6	58.3	24.3	34.2
33	62.8	1.71	22	10.8	64.0	26.3	36.6

Trial 2					
Subject No	Weight (kg)	BF%	TBW%	ECW%	ICW %
Exp Group					
1	95.2	19.1	54.2	22.1	32.6
2	80.3	17.8	54.7	22.7	32.8
3	84.2	17.1	55.5	22.8	32.9
4	97.6	21.2	54.0	22.3	31.8
5	90.2	18.4	55.2	22.4	33.0
6	77.2	19.8	52.5	21.9	31.7
7	75.3	19.4	53.9	22.2	32.7
8	76.0	10.3	61.7	25.3	35.8
11	90.8	20.3	53.0	22.0	31.3
12	73.6	12.6	60.2	24.7	35.2
13	82.5	14.4	58.4	23.9	34.3
14	79.4	13.1	60.3	24.7	34.9
18	64.5	16.6	58.3	24.2	34.3
20	79.7	19.2	53.1	22.0	32.0
21	73.9	16.9	54.7	22.6	32.8
22	87.3	18.8	55.9	23.1	33.0
23	76.7	13.3	60.4	24.9	34.6
24	77.2	13.7	58.8	24	34.7
Control Group					
9					
10	100.5	21.0	53.3	21.6	31.9
16	85.4	17.6	55.5	22.7	33.1
28	84.5	19.5	53.1	22.0	31.6
29	75.0				
31	83.3	16.1	57.4	23.8	33.6
32	69.2	14.6	59.7	24.7	43.8
33	63.2	11.2	63.4	26.1	36.4

Trial 1

Subject No	RER	Plateau	RPE	Max HR (beats·min^{-1})	HR (beats·min^{-1}) (30s)	HR (beats·min^{-1}) (15s)	VO_2 (ml·kg^{-1} min^{-1}) (30s)	VO_2 (ml·kg^{-1} min^{-1}) (15s)	Test time (min)	Max Watts
Exp Group										
1	1.31	0.11	19	198.68	194.59	194.53	39.45	38.69	18.5	240
2	1.26	0.07	20	200.67	196.11	195.96	40.10	39.18	20.03	260
3	1.32	0.15	18	191.69	185.88	186.22	47.57	46.98	26.07	320
4	1.26	0.15	20	200.00	195.23	195.03	40.59	40.31	22.08	280
5	1.27	0.20	20	194.17	190.35	191.50	50.25	50.87	28.35	340
6	1.4	0.08	19	181.57	181.57	181.82	49.96	49.71	24.39	300
7	1.36	0.14	19	198.68	194.80	194.98	48.03	48.61	23.21	290
8	1.16	0.03	20	206.19	201.78	201.87	56.32	56.29	17.05	230
11	1.36	0.26	19	196.72	194.24	194.53	47.02	46.86	25.32	310
12	1.39	0.27	19	197.37	192.16	192.44	50.33	48.67	19.14	250
13	1.33	0.22	20	199.34	195.68	196.04	48.89	48.82	22.55	280
14	1.36	0.29	20	202.02	197.35	198.17	54.15	54.54	22.17	280
18	1.38	0.42	19	217.39	213.69	213.05	47.13	48.61	15.34	210
20	1.24	0.26	19	203.39	199.74	199.93	43.80	44.40	18.16	240
21	1.31	0.34	20	195.44	191.69	191.85	54.13	52.58	21.43	270
22	1.25	0.07	19	186.92	182.03	182.66	51.24	52.06	25.3	310
23	1.25	0.06	19	198.68	194.79	195.42	56.06	58.09	22.26	280
24	1.16	0.22	19	201.34	194.36	194.21	55.25	54.55	24.08	300
Control Group										
9	1.37	0.36	20	198.68	193.71	194.24	44.88	43.40	20.44	260
10	1.33	0.14	18	172.41	169.98	170.16	46.92	47.02	25.3	310
16	1.44	0.19	20	192.31	184.64	185.00	51.18	51.47	25.49	310
28	1.10	0.25	20	193.55	191.26	191.43	46.11	45.06	20.1	260
29	1.20	0.09	19	200.67	195.41	195.70	47.86	47.60	18.56	240
31	1.15	0.28	20	186.34	181.98	182.71	53.65	53.88	24.31	300
32	1.25	0.11	18	197.37	193.98	193.74	55.76	54.03	19.44	250
33	1.32	0.19	20						22.32	280

Trial 2								
Subject No	**RER**	**Plateau**	**RPE**	**Max HR (beats·min^{-1})**	**HR (beats·min^{-1}) (30s)**	**HR (beats·min^{-1}) (15s)**	**VO_2 (ml·kg^{-1} min^{-1}) (30s)**	**VO_2 (ml·kg^{-1} min^{-1}) (15s)**
Exp Group								
1	1.31	0.28	20	196.08	192.01	192.08	40.05	40.57
2	1.26	0.23	19	196.72	192.68	192.82	45.60	45.86
3	1.32	0.23	20				50.63	50.53
4	1.26	0.51	19	194.17	188.81	189.01	38.17	38.29
5	1.27	0.04	19				51.85	52.15
6	1.4	0.14	20	180.72	177.22	177.47	48.15	47.41
7	1.36	0.14	20	198.68	193.63	194.26	48.47	47.32
8	1.16	0.24	18		196.90	197.15	55.80	55.47
11	1.36	0.3	20	185.76	183.57	184.30	47.42	48.03
12	1.39	0.04	19	186.34	183.92	184.39	45.92	45.43
13	1.33	0.13	19	190.48	187.00	187.27	49.21	49.12
14	1.36	0.16	19	197.37	193.40	193.98	51.77	52.38
18	1.38	0.27	20	206.19	205.13	205.05	49.63	48.89
20	1.24	0.2	19	198.68	193.55	194.00	43.17	43.16
21	1.31	0.26	18	192.31	188.11	188.51	59.54	59.39
22	1.25	0.01	19	188.68	184.73	184.54	50.56	48.17
23	1.25	0.23	19	200.67	196.60	197.55	55.48	55.80
24	1.16	0	18		186.92	187.22	53.28	53.28
Control Group								
9	1.42	0.09	20	198.02	194.97	195.40	44.43	44.59
10	1.41	0.41	19	177.51	174.72	174.88	45.31	46.14
16	1.35	0.09	19	190.12	181.84	183.47	50.42	51.06
28	1.24	0.19	18	189.54	188.21	189.13	45.70	46.09
29	1.28	0.11	19	198.57	194.98	194.10	45.46	46.60
31	1.28	0.14	19	185.44	179.76	181.17	52.85	53.05
32	1.25	0.01	18	193.88	190.78	190.89	57.76	58.92
33	1.3	0.26	20					

Appendix I Dietary intake raw data for T1 and T2.

Trial 1

Subject No.	Energy (Kcal)	Protein (g)	Fat (total) (g)	Polyunsaturated fat (g)	Monosaturated fat (g)	Saturates (g)	Carbohydrates (g)	Sugars (total) (g)	Starch (g)	Fibre (g)	Calcium (mg)
Exp group											
2	2143	97.78	85.17	10.39	30.68	34.8	150.16	24.3	125.72	7.59	1051
3	1500	77.67	45.95	6.66	16.14	19.8	206.54	53.67	152.81	8.06	859
4	2172	115.89	88.5	14.77	28	37.75	244.17	76.48	167.69	12.86	1223
5	2993	92.36	98.25	15.05	31.75	27.16	311.89	113.25	198.25	11.18	953
6	2255	77.94	80.64	9.1	27	37.37	313.29	112.37	200.23	16.73	1178
7	1704	99.88	48.62	8.24	15.38	19.05	231.3	72.76	157.75	13.6	873
8	2043	90.69	62.6	10.2	22.22	22.73	297.92	119.7	171.83	6.42	1026
11	2020	83.02	56.64	8.88	18.11	23.39	274.55	115.43	159.07	13.57	1139
12	2174	97.54	97.84	15.39	34.83	40.94	241.19	43.61	185.8	10.25	970
13	1869	106.9	77.28	14.17	24	21.1	198.85	45.84	152.24	15.95	631
14	1654	47.22	67.22	6.82	24.46	32.65	228.94	124.63	104.3	6.85	807
18	2656	123.76	79.48	13.85	30.85	28.59	226.06	82.29	137.35	10.62	719
23	1896	104.95	87.96	6.92	22.62	35.42	182.55	42.83	139.73	14	536
24	2730	102	80.71	11.88	30.34	32.98	288.19	118.28	167.07	16.22	975
Con group											
9	1423	25.01	46.07	1.56	15.28	26.46	128.86	78.11	50.24	3.36	365
10	3079	123.16	86.17	10.12	30.39	33.37	370.12	171.07	155.63	11.2	1221
15	2673	74.39	99.7	12.59	28.92	31.67	258.74	73.15	183.62	14.66	580
16	2497	128.68	55.42	10	19.53	17.89	147.72	85.68	62.04	5.17	946

Trial 1												
Subject No.	**Phosphorus (mg)**	**Magnesium (mg)**	**Sodium (mg)**	**Potassium (mg)**	**Chloride (mg)**	**Iron (mg)**	**Zinc (mg)**	**copper (mg)**	**Selenium (µg)**	**Iodine (µg)**	**Thiamin (B1) (mg)**	**Riboflavin (B2) (mg)**
Exp group												
2	1695	298	4348	2505	6454	12.19	9.01	0.95	43.28	83.73	1.66	2.05
3	1404	245	2474	2886	3791	9.61	8.85	1.04	43.97	308.36	1.07	1.34
4	1461	240	3822	3579	5949	7.46	9.7	0.92	30.92	190.23	2.53	2.34
5	612	303	391	584	589	15.98	9.72	1.28	33.87	70.4	1.89	1.73
6	1359	323	2976	3758	4687	11.83	11.7	5.61	33.65	135.25	1.8	1.69
7	1327	251	2954	2841	5129	11.66	8.25	0.96	73.86	95.8	1.63	1.23
8	1262	210	2743	1680	4434	8.89	10.55	1.58	64.99	142.57	1.11	0.96
11	1567	349	4083	3064	6328	32.03	10.57	1.13	36.34	153.25	3.01	2.73
12	1153	239	3199	2751	4470	12.44	8.67	1.02	70.11	118.21	1.61	1.77
13	1440	281	3660	3183	5459	11.83	8.24	1.01	40.35	61.04	1.3	1.19
14	947	183	1331	2695	2060	7.31	5.37	0.65	18.65	167.29	1.06	1.97
18	1634	319	3616	3503	5604	13.26	15.94	0.78	60.45	78.08	2.32	1.86
23	1401	256	2322	3015	3303	9.54	12.06	0.66	55.72	130.67	1.37	1.2
24	1530	378	4108	4044	6016	16.22	12.14	1.61	52.11	58.09	1.12	1.38
Con group												
9	555	248	633	1853	1165	2.34	2.46	1.80	2.38	163.67	0.20	1.00
10	1617	433	3717	4498	3397	11.91	10.92	2.62	42.68	218.81	1.76	2.04
15	1243	283	3700	2942	6441	12.33	10.37	1.16	22.93	41.13	1.23	1.30
16	1977	399	1196	3672	2188	10.14	11.71	0.82	53.35	194.17	2.41	3.76

Trial 1 Subject No.	Nicotinic acid (mg)	Vit B6 (mg)	Vit B12 (µg)	Folate (µg)	Vit C (mg)	Vit A (µg)	Vit D (µg)	Vit E equivalents (mg)	Cholesterol (mg)	alcohol (g)	Water (g)
Exp group											
2	37.91	2.44	8.15	366.65	6.08	215.64	0.85	4.12	340.56	61.23	1988.6
3	32.69	1.92	4.28	193.85	42.7	494.92	0.52	4.63	232.93	0	999.52
4	50.04	2.43	5.24	167.09	26.7	268.64	0.25	8.33	328.05	0	2066.99
5	37.39	2.53	5.39	282.12	51.82	2317.17	2.91	10.95	392.41	80.29	1993.5
6	32.15	2.93	5.51	324.2	58.52	456.91	1.29	5.47	140.55	5.93	979.58
7	45.47	2.34	1.3	239.72	145.81	269.98	0.36	6.96	165.42	0	884.69
8	23.48	1.24	5.04	125.15	10.83	189.87	4.63	3.15	155.27	0	799.61
11	37.21	2.15	4.42	260.87	64.42	491.22	0.69	5.54	170.32	21.33	1711.98
12	28.51	1.59	6.83	135.34	68.5	357.77	1.54	9.98	304.38	0	784.23
13	39.6	2.21	3.33	185.94	24.74	114..86	0.63	6.5	206.56	0	734.02
14	20.07	1.72	4.56	185.34	44.94	195.43	0.7	4.92	92.55	0.19	627.77
18	55.4	3.46	8.04	339.79	97.1	484.48	2.75	5.22	407.34	84.95	2197.94
23	47.85	2.99	9.3	227.87	33.04	610.38	10.6	4.11	359.72	0	689.86
24	36.01	2.44	4.33	324.87	76.93	565.74	1.58	5.52	87.79	74.24	2700.53
Con group											
9	17.82	0.89	5.52	219.89	10.41	179.36	0.17	1.09	66.15	59.31	2197.21
10	44.98	2.87	7.82	316.52	68.84	1026.48	0.30	6.08	354.43	59.31	2814.27
15	35.32	2.54	1.93	278.48	29.67	409.18	0.79	8.63	138.28	70.87	1454.59
16	79.64	3.06	4.25	410.00	25.96	240.58	1.45	3.89	354.97	132.02	2715.3

Trial 2 Subject No.	Energy (Kcal)	Protein (g)	Fat (total) (g)	Polyunsaturated fat (g)	Monosaturated fat (g)	Saturates (g)	Carbohydrates (g)	Sugars (total) (g)	Starch (g)	Fibre (g)	Calcium (mg)
Exp group											
2	2129	93.33	74.68	13.57	24.43	27.18	232.64	29.58	202.75	10.23	1424
3	1980	84.6	63.05	9.40	23.57	24.84	230.30	45.57	182.31	14.97	560
4	2130	100.82	92.18	14.6	31.68	39.67	239.90	55.64	184.32	14.02	951
5	2244	71.69	117.76	29.98	33.06	37.64	238.29	98.42	137.10	12.08	777
6	2176	86.06	83.17	10.68	28.90	36.71	227.22	109.99	166.54	11.20	878
7	1868	43.54	37.34	7.14	13.31	13.98	203.32	86.46	114.07	13.70	549
8	2018	103.14	56.35	6.31	15.85	19.58	293.32	101.91	190.74	12.51	887
11	2518	87.99	49.26	7.70	14.29	19.26	346.31	148.58	175.80	6.76	1321
12	1921	93.38	83.21	6.81	28.24	41.15	213.22	39.22	173.83	14.56	965
13	1946	95.72	77.21	10.17	22.95	18.48	229.92	32.15	197.04	12.07	786
14	1993	70.99	83.13	5.71	27.59	36.43	257.60	98.64	158.69	13.49	1083
18	2010	90.93	85.63	18.59	35.52	24.76	163.49	32.07	130.83	11.81	321
23	2861	95.59	66.67	13.58	22.06	24.44	302.25	108.33	193.46	11.75	945
24	2105	96.88	83.14	14.58	32.45	27.81	212.30	107.18	102.29	14.76	654
Con group											
9	3567	51.16	92.28	5.87	28.78	48.48	322.43	160.61	153.52	9.22	797
10	3666	142.83	108.79	12.75	37.34	43.45	300.72	105.35	194.54	15.08	812
15	1791	50.65	57.49	5.29	16.75	30.67	235.91	89.80	145.10	14.48	921
16	1799	123.28	47.81	6.32	14.54	16.99	149.31	38.35	109.84	9.98	1039

Trial 2

Subject No.	Phosphorus (mg)	Magnesium (mg)	Sodium (mg)	Potassium (mg)	Chloride (mg)	Iron (mg)	Zinc (mg)	copper (mg)	Selenium (µg)	Iodine (µg)	Thiamin (B1) (mg)	Riboflavin (B2) (mg)
Exp group												
2	1686	261	4571	2172	5313	13.17	11.57	1.03	45.69	61.3	2.05	1.98
3	1157	281	2221	3341	3726	10.12	9.66	1.09	35.73	82.04	1.63	1.13
4	1313	216	4697	3260	6985	8.38	8.73	0.97	32.8	101.57	2.38	1.69
5	1165	228	2835	2897	4573	9.16	8.9	0.95	22.19	73.84	1.93	1.27
6	1297	247	2789	2543	4079	9.33	11.06	1.24	44.47	79.76	1.03	1.14
7	843	220	2696	2098	4347	8.23	5.75	0.5	21.23	42.41	1.1	1.01
8	1407	269	2879	3071	4503	18.99	13.95	1.43	52.68	240.81	2.76	2.32
11	1753	316	2996	2964	3511	29.95	9.07	1.64	38.3	115.67	1.92	2.71
12	1259	236	4058	2413	6484	13.71	12.91	0.91	37.57	87.00	1.32	1.20
13	1348	213	4656	2607	6136	9.45	8.00	0.91	29.61	32.57	1.55	1.29
14	1383	278	2681	3515	4285	11.78	7.72	0.97	25.15	203.81	1.51	2.81
18	1337	281	1512	3459	2434	10.76	10.21	0.72	33.17	50.01	1.12	1.07
23	1630	363	3223	3304	4673	13.41	10.6	0.87	79.54	86.67	1.5	2.06
24	1587	326	3429	2833	4915	11.72	11.9	1.38	63.6	81.82	2.13	1.32
Con group												
9	1238	378	2232	3388	3639	7.48	4.39	0.70	12.42	75.02	0.82	2.14
10	2051	465	3669	4699	5693	14.86	12.67	1.41	63.54	102.03	1.97	2.83
15	1003	229	2939	2569	4680	10.67	4.61	0.77	21.88	71.50	1.73	1.72
16	1870	347	877	3098	1615	9.50	9.00	1.46	76.76	267.79	2.08	3.06

Trial 2 Subject No.	Nicotonic acid (mg)	Vit B6 (mg)	Vit B12 (µg)	Folate (µg)	Vit C (mg)	Vit A (µg)	Vit D (µg)	Vit E equivalents (mg)	Cholesterol (mg)	alcohol (g)	Water (g)
Exp group											
2	20.68	1.83	6.89	306.68	7.61	3063.15	1.26	7.84	208	30.61	1282.23
3	28.84	2.78	2.05	294.2	70.13	156.13	0.92	5.59	206.65	30.61	1399.59
4	38.54	2.19	4.26	160.13	22.38	192.83	0.12	8.17	282.05	0	1357.85
5	30.61	1.91	4.82	149.77	34.42	474.78	1.46	13.34	363.72	0	899.92
6	31.68	1.78	3.23	163.97	37.42	201.16	0.62	5.57	189.47	5.93	908.01
7	5.99	1.74	2.11	260.16	38.15	328.49	0.66	4.28	63.8	84.58	1439.69
8	43.68	3.44	7.95	351.68	96.21	373.32	3.34	3.11	184.62	0	983.26
11	42.36	1.87	3.94	217.01	17.99	323.21	0.58	5.77	212.17	59.89	2095.31
12	29.60	1.48	5.28	152.04	17.35	360.67	1.87	2.39	256.11	0	563.89
13	25.30	1.72	3.09	157.97	20.27	241.83	1.1	9.00	336.51	0	689.08
14	30.16	2.57	4.86	270.87	47.72	227.93	0.45	3.98	103.57	0	1515.65
18	46.46	3.17	6.07	229.63	36.3	669.21	3.81	5.45	199.27	37.88	1669
23	53.23	3.34	6.07	404.87	62.28	239.04	4.13	7.22	212.43	105.62	2411.35
24	45.66	2.40	4.17	178.85	43.13	491.97	1.87	4.45	280.36	25.26	1574.5
Con group											
9	47.65	2.80	1.47	425.36	25.67	558.38	0.54	3.63	174.83	187.08	3049
10	85.79	4.01	4.4	444.95	39.6	397.77	2.80	4.04	444.42	140.02	2673.78
15	29.01	2.74	1.58	285.49	63.61	494	0.93	3.26	143.74	26.40	1004.65
16	69.42	2.47	3.25	297.38	22.89	295.07	1.02	2.52	277.43	45.92	2018.59

Appendix J Haemoglobin and Haematocrit raw data.

Trial1						
Subject No	**Haemoglobin ($g \cdot dL^{-1}$)**			**Haematocrit (%)**		
Exp Group	**Pre**	**Post**	**24h Post**	**Pre**	**Post**	**24h Post**
1	140	143	152	43.5	46	43
2	150	166	145	45	48	43
3	141	159	150	42	47	43
4	137	154	141	42	45	41
5	149	163	147	45	48	45
6	147	156	148	44	46	43
7	133	132	134	41	41	40
8	150	156	134	43	46	41.5
11	169	173		50	52	50
12	146	158	149	42	45	43.5
13	143	158	143	44	47	42
14	132	155		42.5	45	44
18	135	144	150	42	44.5	47
20	170	166	150	44.5	48	44
21	159	164		46.5	48.5	48
22	150	150	154	45	46	44.5
23	126	140	124	38	41.5	35.5
24	144	145	141	43.5	45	40.5
Control Group						
9	136	147	135	40.5	44	42
10	147	158		44	45	44
16	142	153	148	41.5	45	42.5
28	134	138	127	39	40.5	36.5
29	153	160	158	46.5	46	46.5
31	148	154	152	47	48.5	45.5
32	137	133		40	43.5	42
33	154	164		47	48.5	47

Trial 2 Subject No	Haemoglobin ($g \cdot dL^{-1}$)			Haematocrit (%)		
Exp Group	Pre	Post	24h Post	Pre	Post	24h Post
1	146	158		43	47	
2	147	149		43	44.5	
3	144	156	142	43	45	42
4	139	146		41	43	
5	150	161	151	44.5	48	45
6	150	149	131	44	45	43
7	147	153	148	43	45	41
8	152	161		45	48	
11	165	174	169	50	52	50
12	145	159		43	47	
13	139	162	138	42.5	48.5	44
14	142	144	139	39.5	43	40
18	136	144	157	42.5	45	42.5
20	160	167	147	43	46.5	41.5
21	151	157	145	45.5	48	44.5
22	145	144	147	45	46	46.5
23	131	144		38	42.5	
24	146	151	134	43	45	43
Control Group						
9						
10	151	165	155	44	48	44
16	138	145	144	40	44	43
28	125	147	136	39	43.5	38.5
29						
31	156	159	159	48	49	46
32	122	144	134	36	43	40
33	158	163	160	48	49	48.5

Appendix K Blood Lactate and Glucose raw data.

Trial1								
Subject No	Lactate ($mmol \cdot L^{-1}$)				Glucose ($mmol \cdot L^{-1}$)			
		Immediately		24 hours		Immediately		
Exp Group	Pre	post	Post	post	Pre	post	Post	24 hours post
1	1.95	6.18	4.56	1.59	2.98		3.95	4.35
2	1.88	9.15	6.86	1.13	4.39	5.01	5.01	4.37
3	2.61	10.2	8.32	1.5	4.77	5.53	5.42	4.3
4	1.71	9.36	6.11	0.91	4.34	6.56	6.5	4.06
5	2.2	7.81	4.6	1.08	3.89	4.33	4.2	4.03
6	1.21		5.89	1.19	3.81		4.82	3.77
7	3.05	9.62	4.53	1.92	4.51	3.74	4.17	3.55
8	0.59	8.71	5.92	0.65	4.23	5.16	5.56	4.42
11	1.67	9.32	6.94		4.53	4.93	5.1	
12	0.66	7.98	5.65	1.13	3.51	3.56	3.21	3.7
13	1.43	8.96	6.84	1.06	4.19	5.22	5.44	4.09
14	1.05	7.24	4.59		3.59	4.36	1.14	
18	1.97	7.82	3.8	1.98	4.05	4.12	3.53	4.19
20	1.03	8.92	6.61	0.72	3.66	5.08	5.2	4.27
21	1.02	7.52	5.31		4.86		6.14	
22	1.3	6.22	2.7	0.435	4.04		4.49	4.17
23	0.94	10.6	6.46	0.735	3.65	4.15	3.42	3.81
24	0.69	10.4	6.52	0.412	4.27	5.31	6.04	4.39
Con Group								
9	0.85	8.5	4.93	1.16	4.62	4.85	4.78	4.9
10	1.92	9.06	5.19		4.49	4.49	5.12	
16	0.41	8.16	5.38	0.52	4.23	5.49	5.58	4.18
28	1.04	13.3	7.41	1.94	4.36	5.98	5.21	4.66
29	1.89	8.08	3.91	1.11	3.75	4.76	4.72	4.09

31	1.08		6.6	0.68	3.91		4.95	4.32
32	0.52	9.53	5.2		3.61	4.45	3.98	
33	0.91	8.18	5.71		4.26	6.51	6.66	

Trial 2

Subject No	Lactate ($mmol \cdot L^{-1}$)				Glucose ($mmol \cdot L^{-1}$)			
		Immediately		24 hours		Immediately		24 hours
Exp Group	Pre	post	Post	post	Pre	post	Post	post
1	1.11	5.78	3.98		4.03	4.15	4.61	
2	0.62	7.16	4.16		4.08	4.74	4.07	
3	0.98	8.63	5.11	1.04	4.37	5.09	5.34	4.15
4	1.45	7.01	3.54		4.35	4.52	4.83	
5	1.43	7.13	4.64	1.15	3.9	3.84	4.13	3.9
6	1.29	10.1	6.34	1.32	4.69	3.94	3.85	3.88
7	1.85	8.28	6.56	3.34	4.29	4.42	5.35	4.42
8	1.34	8.1	6.25		4.02	4.04	4.59	
11	1.25	8.44	4.95	0.503	4.31	4.51	4.68	4.06
12	0.8	8.7	5.11		3.78	3.68	3.36	
13	1.25	7.9	5.55	1.27	4.55	5	5.23	4.58
14	0.97	7.88	5.01	0.57	4.53	4.54	4.81	4.43
18	1.27	6.41	2.99	0.96	4.15	3.91	3.67	4.2
20	0.83	9.44	6.99	1.38	4.43	4.95	5.19	4.96
21	0.80	7.69	6.04	0.74	3.17	4.15	5.55	3.81
22	0.77	7.06	3.47	1.08	4.21	4.79	5.08	4.03
23	0.62		4.17		4.72		4.27	
24	0.85	10.4	5.42	0.77	4.05	4.83	5.11	4.17
Con Group								
9								
10	1.66	6.49	3.24	2.13	4.04	4.25	4.61	4.02
16	0.58	8.07	5.46	0.69	4.09	4.76	4.44	4.27
28	1.74	14.3	6.6	1.43	4.13	4.36	4.31	4.48
29								
31	0.79	6.58	4.71	0.70	3.82	3.32	4.7	4.15
32	0.64	12.2	6.62	0.58	4.02	4.44	4.36	3.91
33	0.90	7.45	4.24	1.73	3.92	5.11	5.27	4.25

Appendix L Lipid hydroperoxide raw data.

Lipid Hydroperoxides ($mmol \cdot L^{-1}$)	Trial 1			Trial 2		
Exp group	Pre	Post	24 hours Post	Pre	Post	24 hours Post
1	1.18	1.69	0.89	1.01	1.21	1.04
2	0.81	1.05	0.95	0.62	0.69	0.77
3	0.86	1.41	0.70	0.97	1.07	0.60
4	0.89	1.02	0.91	1.11	1.32	0.92
5	0.73	0.92	0.73	0.65	0.86	0.74
6	0.93	0.96	1.21	0.77	0.89	0.80
7	0.76	0.73	0.58	0.88	1.01	0.85
8	0.66	0.80	0.53	0.60	0.74	0.52
11	0.58	0.73	0.52	0.60	0.72	1.50
12	0.58	0.76	1.03	0.65	0.75	0.77
13	0.38	0.40	0.40	0.38	0.45	0.34
14	0.73	0.83	0.60	0.61	0.79	0.60
18	0.60	0.71	0.66	0.61	0.71	1.57
20	0.66	0.59	0.53	0.49	0.65	0.81
21	0.74	0.97	0.72	0.59	0.66	0.51
22	0.65	0.49	0.43	0.54	0.59	0.52
23	0.50	0.73	0.51	0.55	0.63	0.48
24	0.28	0.32	0.27	0.29	0.33	0.23
Con group						
9	0.48	0.46	0.59	0.53	0.43	0.42
10	0.56	0.65	0.76	0.92	1.11	0.76
16	0.62	0.51	0.54	0.59	0.54	0.74
28	0.35	0.46	0.43	0.49	0.54	0.37
29	0.52	0.51	0.50	0.40	0.45	0.48
31	0.35	0.48	0.44	0.41	0.48	0.41
32	0.39	0.43	0.40	0.49	0.46	0.39
33	0.49	0.61	0.54	0.50	0.60	0.69

Appendix M LDL lag time raw data.

LDL lag-times (min)	Trial 1			Trial 2		
Exp group	Pre	Post	24 hours post	Pre	Post	24 hours post
1	96.9	102.7	97.2	84.3	86.7	87.6
2	96.3	92.2	107.1	102.8	106.6	102.1
3	134.5	119.3	114.1	122.5	124.5	127.5
4	135.8	138.4	132.0	123.7	120.8	120.9
5	123.9	124.9	127.7	130.8	123.4	123.8
6	83.8	105.5	85.9		78.5	100.1
7	86.5	74.0	143.0	140.2	143.9	132.9
8	90.1	110.8	107.7	110.1	110.1	99.9
11	103.6	111.5	109.3	114.0	117.0	107.9
12	92.2	96.3	91.8	102.1	90.8	87.0
13	65.7	71.0	68.8	73.8	75.4	76.1
14	111.7	85.5	82.9	73.0	66.8	67.2
18	72.4	73.1	71.1	68.3	68.7	70.1
20	90.9	99.0	93.6	83.5	96.3	95.4
21	80.9	76.0	83.8	76.5	82.9	80.6
22	80.4	82.8	78.1	77.3	87.3	76.7
23	60.2	74.4	77.0	75.3	76.6	72.9
24	62.1	67.0	61.2	67.3	69.8	68.9
Con group						
9	69.6	60.7	67.2	70.0	63.1	71.8
10	81.0	76.9	77.0	77.0	77.7	76.1
16	69.3	83.3	76.8	79.9	76.1	71.2
28	84.3	77.0	81.1	78.3	56.3	74.0
29	86.7	73.0	74.8	81.0	78.8	71.5
31	57.9	70.2	61.1	50.4	49.2	57.1
32	70.1	73.6	63.1	68.5	65.7	68.6
33	85.4	82.2	78.3		90.3	94.0

Appendix N Antioxidant Vitamin raw data.

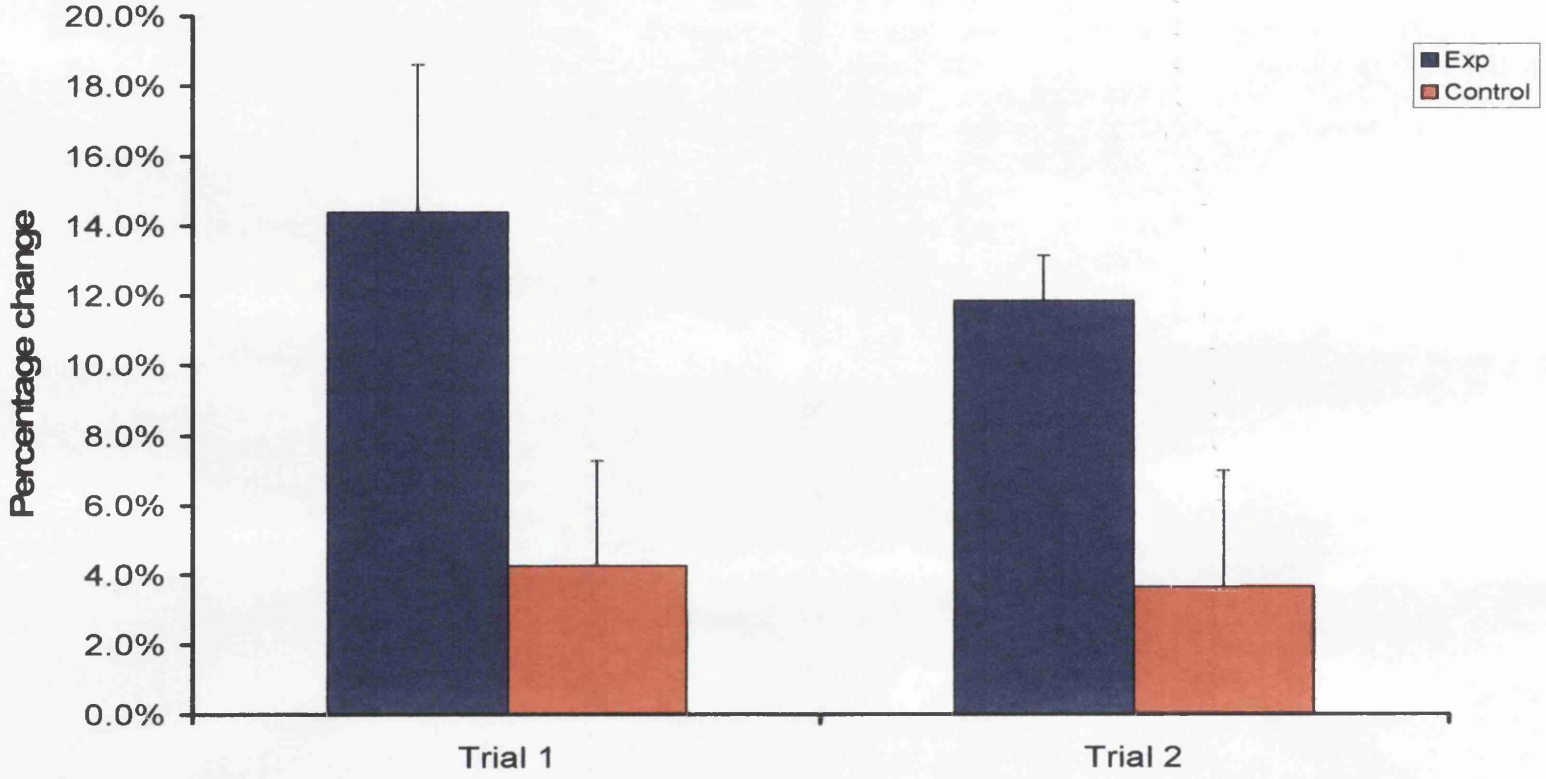
Vitamin C (µMol·L $^{-1}$)						
	Trial 1			Trial 2		
Exp group	Pre	Post	24 hours post	Pre	Post	24 hours post
1	31.1	71.9	40.7	25.4	41.9	42.0
2	47.2	44.5	47.0	38.6	35.0	34.9
3	21.2	35.8	46.3	56.3	41.8	52.5
4	38.7	64.8	55.7	44.9	35.8	45.1
5	62.7	72.2	76.1	64.1	69.6	72.5
6	37.9	32.0	39.5	28.4	21.7	17.4
7	44.3	38.0	40.5	41.7	31.3	37.6
8	66.7	43.5	61.0	51.7	59.6	9.9
11	27.7	25.6	35.1	46.0	33.0	42.0
12	37.9	24.0	41.3	38.4	43.1	52.4
13	22.6	20.9	25.0	29.4	32.7	29.0
14	62.5	63.2	59.8	55.7	52.7	45.5
18	61.6	51.2	43.4	45.1	52.7	47.0
20	47.2	44.4	35.2	44.7	33.1	36.0
21	62.1	77.6	71.6	55.3	69.8	65.8
22	62.7	61.1	51.6	41.8	43.7	40.9
23	53.6	51.0	49.5	48.8	50.0	52.2
24	52.0	53.1	39.2	59.2	51.1	67.2
Con group						
9	45.5	24.5	62.8	31.1	23.8	28.6
10	41.3	46.7	43.6	48.5	50.2	44.3
16	49.2	59.7	61.3	39.6	41.1	45.1
28		54.2	53.5	43.3	46.9	47.0
29	41.8	68.9	48.2	65.9	68.7	30.5
31	39.1	50.7	46.7	18.0	16.8	39.7
32	43.7	52.1	44.4	33.6	35.7	35.0
33	18.9	40.3	55.2	35.4	41.6	59.2

	Retinol (μMol·L $^{-1}$)						Lycopene (μMol·L $^{-1}$)					
	Trail 1			Trial 2			Trail 1			Trial 2		
Exp group	Pre	Post	24 hours post	Pre	Post	24 hours post	Pre	Post	24 hours post	Pre	Post	24 hours post
1	2.316	3.183	2.909	2.567	2.738	3.057	1.657	2.359	2.157	3.456	3.319	1.589
2	2.293	2.179	2.544	2.544	2.464	2.339	0.581	1.543	0.995	1.157	1.143	1.692
3	1.460	1.335	1.300	1.996	1.859	1.939	0.454	0.449	0.592	0.446	0.330	0.578
4	2.202	2.145	2.453	1.848	1.711	2.167	5.510	1.286	5.670	0.703	0.919	1.267
5	2.053	1.802	1.871	1.369	2.316	1.426	1.316	0.646	1.127	0.811	4.002	1.092
6	1.540	2.453	2.167	1.574	1.734	1.529	0.795	4.540	4.243	0.651	2.005	0.208
7	2.304	3.075	3.116	4.346	4.262	4.011	1.194	0.913	0.991	2.350	1.792	1.396
8	2.446	2.599	2.613	2.711	3.186	3.242	1.182	1.712	0.978	1.674	2.106	4.986
11	2.376	2.585	2.012	2.152	2.194	2.390	1.078	0.351	0.909	0.858	0.412	0.983
12	2.893	3.675	2.837	2.837	3.228	2.613	0.603	2.921	0.612	0.703	2.522	0.744
13	2.010	1.770	1.960	1.930	1.970	2.000	4.244	3.783	2.655	3.442	1.844	1.882
14	1.957	1.998	1.719	1.481	1.579	1.649	0.459	0.395	0.410	0.414	0.263	2.161
18	2.278	2.599	2.809	2.697	2.432	2.613	0.864	4.428	0.868	2.142	4.006	4.048
20	1.686	2.082	2.146	1.787	1.667	1.980	1.231	2.481	1.648	1.640	0.840	0.723
21	2.164	1.732	1.722	1.851	1.603	1.796	1.029	0.957	0.724	0.952	2.043	1.454
22	2.128	1.778	2.063	2.008	2.257	2.772	1.111	1.040	1.025	0.996	1.294	0.759
23	1.575	1.999	2.072	1.382	1.741	2.045	1.111	0.741	1.370	1.820	1.432	1.554
24	1.805	1.676	1.989	1.943	1.584	1.989	1.002	1.047	1.276	1.115	1.300	0.770
Con group												
9	1.179	1.363	1.225	1.133	1.234	1.124	1.345	1.404	1.581	1.008	1.286	1.220
10	1.289	1.299	1.501	1.695	2.257	2.110	1.301	1.464	0.910	0.770	0.802	3.025
16	2.190	1.950	1.660	1.810	1.750	1.670	1.924	2.497	1.354	1.683	1.748	0.738
28	1.650	1.430	1.720	1.890	1.760		1.419	1.368	1.074	1.145	1.538	
29	2.950	1.980	1.730	1.730	1.900	1.680	3.539	1.548	2.463	3.590	1.899	2.406
31	1.480	1.620	1.520	1.800	1.910	1.460	1.565	1.855	1.836	1.666	1.703	1.271
32	1.600	1.650	1.460	1.460	1.530	1.220	1.899	2.323	2.802	2.225	2.246	2.811
33	1.800	2.130	1.820	1.730	1.880	1.730	1.918	1.853	1.944	1.649	1.590	1.420

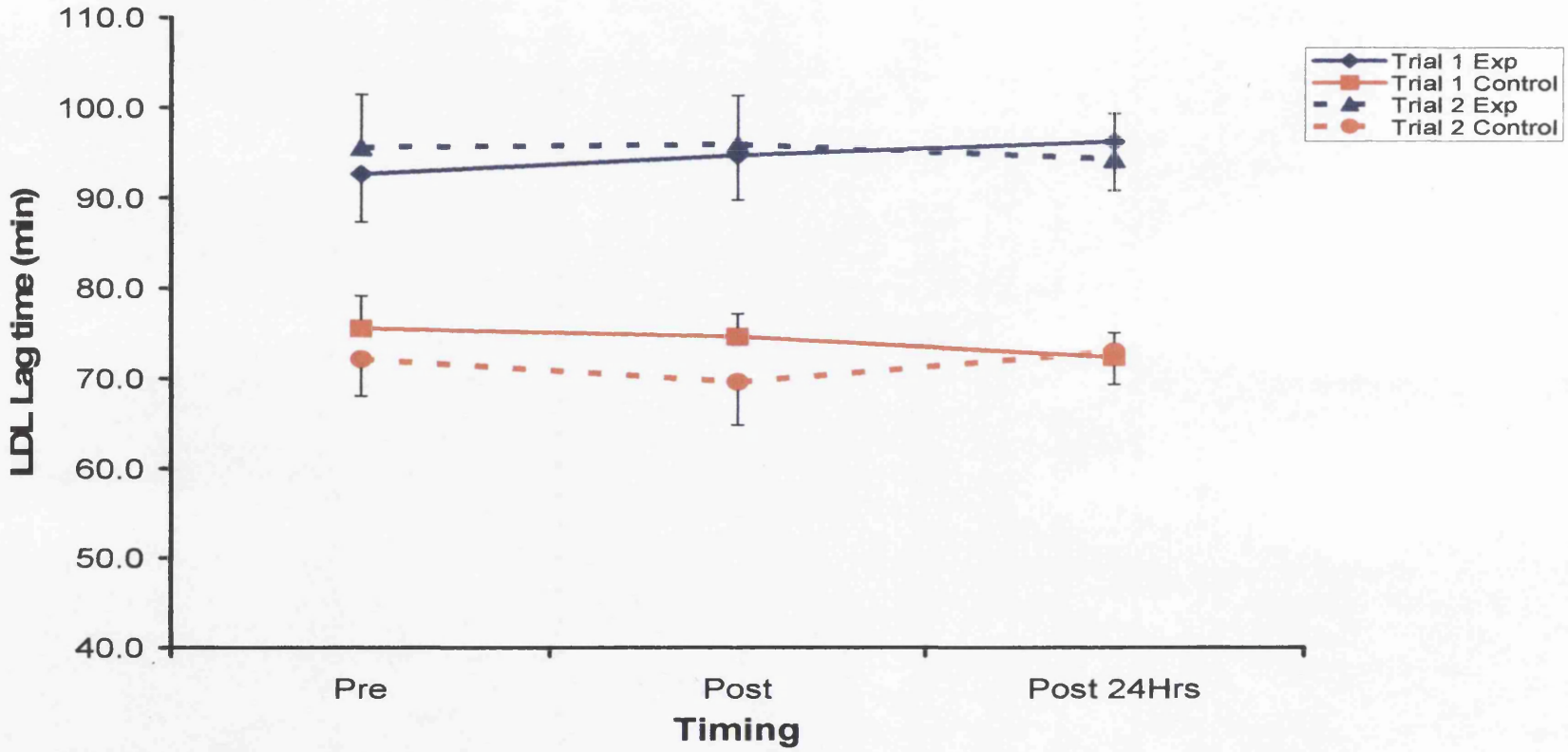
	α-tocopherol (μMol·L $^{-1}$)						μ-tocopherol (μMol·L $^{-1}$)					
	Trail 1			Trial 2			Trail 1			Trial 2		
Exp group	Pre	Post	24 hours post	Pre	Post	24 hours post	Pre	Post	24 hours post	Pre	Post	24 hours post
1	29.69	37.62	31.84	29.74	35.16	39.68	1.22	1.59	1.45	1.53	1.70	2.24
2	31.27	38.01	33.16	30.05	35.11	33.54	1.88	2.16	2.02	1.93	2.05	1.89
3	22.77	20.69	20.39	26.45	27.11	28.15	1.64	1.48	1.45	1.53	1.54	2.12
4	38.97	39.20	40.84	40.57	40.87	43.67	2.26	2.28	2.18	2.14	2.05	2.29
5	21.97	24.40	21.73	21.16	24.01	20.54	0.97	0.99	0.89	1.09	1.22	1.18
6	25.79	28.77	28.23	26.04	22.99	23.63	1.30	1.48	1.39	1.34	1.20	1.37
7	25.05	29.67	29.19	44.32	45.45	42.36	1.08	0.92	0.99	1.16	1.15	1.20
8	30.71	30.98	28.43	33.72	35.28	34.85	0.82	0.77	0.74	1.03	1.04	1.06
11	24.66	26.53	21.17	23.13	24.27	25.79	0.83	0.83	0.79	0.87	0.89	0.99
12	26.51	28.89	27.13	33.47	32.18	30.63	0.81	0.83	0.89	1.17	1.05	1.12
13	20.42	17.97	23.27	24.98	20.37	26.23	1.00	0.94	1.35	1.43	1.24	1.17
14	24.93	25.77	20.87	16.27	16.78	16.37	0.81	0.80	0.95	0.86	0.93	0.73
18	25.63	32.62	33.85	30.72	30.31	31.34	1.08	1.59	1.23	1.27	0.90	1.01
20	25.47	28.11	26.96	24.57	24.43	25.57	1.47	1.96	2.21	2.05	1.54	1.48
21	27.57	26.71	25.31	27.65	25.37	26.32	1.99	1.35	1.45	1.61	1.58	1.45
22	27.99	28.02	30.29	29.81	28.95	32.28	1.21	1.15	1.29	1.31	1.61	1.37
23	33.33	35.58	36.75	30.78	37.00	38.49	3.12	2.74	2.23	1.87	1.77	1.88
24	24.53	24.60	24.08	23.75	27.16	29.30	1.00	1.33	1.49	1.59	1.66	1.92
Con group												
9	20.48	23.85	19.80	22.66	24.22	22.14	1.37	1.51	2.20	1.31	1.27	1.28
10	28.10	31.18	28.36	28.15	38.72	21.11	1.49	1.57	1.41	1.75	2.22	1.09
16	28.92	27.32	20.21	23.74	24.29	24.14	1.24	1.27	2.05	2.26	1.47	1.16
28	24.87	20.82	25.09	26.73	25.45		1.19	1.00	1.44	1.53	1.35	
29	37.31	24.89	24.00	24.77	25.03	22.34	2.02	1.37	1.48	1.34	1.23	1.09
31	22.18	23.47	25.29	25.62	30.06	24.30	1.02	1.12	1.50	1.24	1.63	0.94
32	22.77	23.02	22.47	22.00	23.29	21.39	1.36	1.31	1.32	1.33	1.53	1.72
33	24.27	26.93	24.46	28.14	29.63	27.09	1.35	1.43	1.29	1.61	1.60	1.71

	α-carotene (μMol·L^{-1})						β-carotene (μMol·L^{-1})					
	Trail 1			Trial 2			Trail 1			Trial 2		
Exp group	Pre	Post	24 hours post	Pre	Post	24 hours post	Pre	Post	24 hours post	Pre	Post	24 hours post
1	0.158	0.136	0.119	0.143	0.177	0.138	0.374	0.419	0.372	0.496	0.594	0.435
2	0.121	0.197	0.120	0.129	0.161	0.156	0.350	0.499	0.388	0.424	0.487	0.501
3	0.107	0.114	0.113	0.074	0.084	0.108	0.160	0.147	0.184	0.100	0.117	0.157
4	0.205	0.203	0.224	0.146	0.169	0.143	0.456	0.411	0.480	0.228	0.307	0.284
5	0.170	0.193	0.218	0.243	0.182	0.215	0.347	0.391	0.429	0.514	0.551	0.462
6	0.103	0.136	0.167	0.090	0.152	0.088	0.245	0.390	0.444	0.183	0.314	0.168
7	0.105	0.202	0.122	0.113	0.180	0.166	0.278	0.517	0.435	0.394	0.432	0.406
8	0.453	0.428	0.313	0.386	0.371	0.484	1.462	1.620	1.069	1.216	1.199	1.791
11	0.212	0.154	0.221	0.203	0.182	0.243	1.233	1.020	1.277	0.879	0.904	1.393
12	0.141	0.136	0.132	0.088	0.193	0.131	0.375	0.468	0.342	0.311	1.055	0.570
13	0.189	0.162	0.115	0.134	0.088	0.084	0.926	0.806	0.651	0.787	0.494	0.300
14	0.249	0.256	0.272	0.259	0.154	0.237	1.250	1.249	1.318	1.145	0.595	1.221
18	0.105	0.609	0.266	0.510	0.407	0.323	0.212	1.490	0.651	1.213	1.142	0.926
20	0.139	0.217	0.146	0.144	0.133	0.121	0.248	0.412	0.271	0.268	0.214	0.185
21	0.112	0.126	0.115	0.144	0.249	0.176	0.390	0.408	0.355	0.449	0.883	0.575
22	0.182	0.153	0.177	0.169	0.222	0.178	0.623	0.473	0.550	0.524	0.575	0.428
23	0.126	0.115	0.123	0.143	0.156	0.163	0.302	0.263	0.306	0.377	0.384	0.402
24	0.056	0.057	0.098	0.063	0.093	0.054	0.145	0.156	0.196	0.161	0.189	0.102
Con group												
9	0.062	0.062	0.062	0.054	0.063	0.067	0.370	0.381	0.385	0.261	0.304	0.327
10	0.128	0.146	0.092	0.086	0.094	0.153	0.544	0.600	0.340	0.262	0.276	0.700
16	0.088	0.098	0.085	0.100	0.096	0.097	0.304	0.362	0.415	0.504	0.481	0.264
28	0.147	0.141	0.130	0.129	0.167		0.443	0.428	0.284	0.278	0.361	
29	0.218	0.139	0.139	0.172	0.118	0.153	1.091	0.719	0.813	0.805	0.479	0.691
31	0.205	0.227	0.254	0.180	0.180	0.157	0.737	0.739	0.938	0.571	0.483	0.531
32	0.264	0.297	0.310	0.258	0.215	0.228	1.889	2.022	2.972	2.529	2.889	3.557
33	0.258	0.254	0.244	0.188	0.173	0.190	0.693	0.593	0.584	0.472	0.440	0.475

Appendix O Figures showing analyses including non-responders



Percentage change in lipid hydroperoxides from pre to post exercise for trial 1 and trial 2.



LDL lag times across trial 1 and 2 for both Experimental and Control groups.