THE ACTIVITY AND ADAPTATION OF XANTHINE OXIDASE IN RESPONSE TO HIGH-INTENSITY SWIMMING EXERCISE

By

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ABSTRACT

Xanthine Oxidase (XO) is an enzyme that catalyses a reaction to form uric acid. Following high-intensity, hypoxic exercise it produces superoxide radicals upon oxygen reperfusion. The present study investigated the activity and adaptation of XO in response to four swimming sessions, consisting of four high-intensity 50m freestyle bouts (four-minutes rest), in two groups of young, healthy participants (n=7 competitively trained, n=7 not swimming trained). Physical fitness, VO_2max (ml/kg/min), was not significantly different between groups ($p = .121$). Swimming times (seconds) were significantly and consistently faster in the trained group ($p \leq .003$), reflecting group differences in swimming experience. Blood samples were taken pre- and post-intervention, measuring antioxidant capacity and XO protein content and during swimming sessions measuring XO activity.

XO activity was not significantly different between groups. In trained participants, XO protein content was significantly less ($P = .036$) pre-intervention and significantly ($p = .017$) increased from pre-to-post intervention. Non-trained participants showed no significant change in XO protein content pre-to-post intervention. Antioxidant capacity demonstrated an almost identical pattern to XO protein content.

Results suggest training influences XO protein expression, which affects antioxidant capacity, independent of XO activity. These findings could have beneficial application for health however, further research is required.

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BMI	Body Mass Index
CHF	Chronic Heart Failure
CO_2	Carbon Dioxide
DNA	Deoxyribonucleic Acid
EE	Energy Expenditure
eNOS	endothelial Nitric Oxide Synthase
ERK	Extracellular signal-regulated kinase (1&2)
ETC	Electron Transport Chain
FRAP	Ferric Reducing Ability of Plasma
FRS	Free Radical Specie(s)
GPX	Glutathione Peroxidase
GSH/GSSG	Glutathione oxidised/reduced
HR	Heart Rate
HSP70	Heat Shock Protein 70

iNOS	inducible Nitric Oxide Synthase
Mn-SOD	Manganese Superoxide Dismutase (mitochondrial)
mRNA	messenger Ribonucleic Acid
NAD^+ / NADH	Nicotinamide Adenine Dinucleotide (Oxidised/reduced)
NF-κB	Nuclear Factor – Kappa B
NO•	Nitric Oxide radical
NRF (1/2)	Nuclear Respiratory Factor (1/2)
ONOO	Peroxynitrite
OH•	Hydroxyl Radical
PPARα	Peroxisome Proliferator-Activated Receptor Alpha
PGC-1α	PPAR gamma coactivator – 1 alpha
P38 MAPK	P38 Mitogen-Activated Protein Kinase
RA	Rheumatooid Arthritis
ROS	Reactive Oxygen Specie(s)

RPE	Rate of Perceived Exertion
SD	Standard Deviation
SO•	Superoxide Radical
SOD	Superoxide Dismutase
TAC	Total Antioxidant Capacity
TCA	Tricarboxylic Acid
UA	Uric Acid
VCO_2	Volume of CO_2
VO_2	Volume of Oxygen
XDH	Xanthine Dehydrogenase
XO	Xanthine Oxidase
3-NT	3 – Nitrotyrosine

INTRODUCTION

HEALTH BENEFITS OF EXERCISE

Exercise is important to maintain good health (Warburton, Nicol and Bredin, 2006; Pearce, 2008), defined as the "complete state of physical, mental and social well-being" ('WHO | Constitution of WHO: principles', 2016). Exercise is linked to a reduction in the development of disease and is utilised in the treatment of numerous diseases, such as coronary heart disease (CHD) (Leitzmann *et al.*, 2015), type-II diabetes (Castaneda *et al.*, 2002) and obesity (Donahoe *et al.*, 1984; Blaak and Saris, 2002). Physical fitness is a relevant predictor of mortality (Erikssen *et al.*, 1998) and regular exercise has also been shown to improve lifespan through improved cardiovascular health (Zhang and Zhang, 2009) and an upregulation in protective proteins associated with exercise stimulus (Ristow and Schmeisser, 2014). It may therefore be suggested that regular exercise is a key lifestyle factor that can be manipulated to alter health status.

EXERCISE ADAPTATION FOR HEALTH

Exercise triggers a multitude of cellular events, which in turn can stimulate a variety of molecular signalling events; this can result in numerous changes across the systems within the human body. Perhaps those most well characterised are the changes that occur within skeletal muscle, but changes to blood vessel structure and function, metabolism of macronutrients and redox homeostasis have been well researched. Over time with repeated activation of signalling cascades through repeated exercise stimulation, physiological alterations - or adaptations - occur leading to increases in physical fitness and biological function that benefit

our health. Adaptations are dependent on the stimulus, thus in this case the type of exercise that has taken place. Generally, exercise is categorised grossly as aerobic or endurance type exercise and resistance type exercise (Howley, 2001), and both forms of exercise stimulate different adaptive process in response to specific signals that result from the stimulus.

Resistance exercise generally provokes adaptations that promote anabolism and improve muscle strength and power, and plays an additional role in the regulation and adaptation of circulating hormones, such as testosterone, insulin and growth hormones (Kraemer and Ratamess, 2005). Resistance exercise is recognised to be beneficial to our health in many ways (Kraemer, Ratamess and French, 2002) including but not limited to, reduction in age related loss of muscle mass known as sarcopenia (Seguin and Nelson, 2003), favourably changing body composition to reduce body fat and increase lean mass, improving insulin sensitivity and glycaemic control (Castaneda *et al.*, 2002).

Endurance exercise initiates adaptations that increase our capacity to exercise for sustained periods of time, often quantified by $VO_{2\ max}$, or the maximal volume of oxygen utilised per kilogram of body mass per minute. This value is used as an indication of cardiovascular fitness in research and can help monitor changes in exercise capacity (Bassett and Howley, 2000). Changes that favour our health include alterations in substrate utilisation such as increasing the proportion of fat (vs carbohydrate) that is metabolised (Talanian *et al.*, 2007), and a favourable change in blood lipid profile (reduced low-density lipoprotein cholesterol (LDL-C) and increased high-density lipoprotein cholesterol (HDL-C)) (Escalante *et al.*, 2012) (Kelley, Kelley and Franklin, 2006). An altered ratio of blood lipids can have a positive impact on cardiovascular health (Patel *et al.*, 2017).

THE ADAPTIVE PROCESS

Molecular adaptations can lead to phenotypic changes and these changes materialise through a series of stimulated transcriptional events that activate or supress gene transcription and translation to regulate the gene expression of metabolic proteins (Egan and Zierath, 2013). These transcriptional changes are reliant on cell signalling to activate transcription factors of specific genes. Typical examples of adaptations that occur as a result of endurance exercise include an increase in skeletal muscle mitochondrial content and mitochondrial enzyme content and activity (Holloszy, 1967), accompanied by alterations in substrate utilisation, and a decrease in the amount of lactic acid that is produced. These changes result in the ability of an individual to exercise for longer before the onset of fatigue (Holloszy and Coyle, 1984; Holloszy and Booth, 1976). Exercise generates signals in a variety of ways, including in the form of radical species of molecular oxygen derived from metabolic process. These signals act to provoke adaptations such as the upregulation of proteins. This often occurs via activation of co-activators such as peroxisome proliferator-activated receptor gamma coactivator – 1 alpha (PGC-1α), which trigger transcription factors such as p38 mitogen-activated protein kinase (p38 MAPK) (Akimoto *et al.*, 2005; Russell *et al.*, 2005), nuclear respiratory factor- one (NRF1) and two (NRF2) and peroxisome proliferator-activated receptor alpha (PPARα) (Baar, 2004). Transcription factors bind to target gene sites to increase protein expression, resulting in an increase in functional protein synthesis, for example mitochondrial biogenesis and enzyme adaptations (Latchman, 1997).

REDOX ALTERATIONS AND RADICAL SPECIES

It is very clear that the generation of signal is key in the process of exercise adaptation. Increased production of radical species in metabolic process changes in the 'redox' status of the cell. The term 'redox' is used as an abbreviation for reduction and oxidation reactions. Exercise initiates a host of changes that can affect redox status, in addition to the generation of radical species such as increased temperature, acidosis, and production of lactate, which all contribute to the disruption of the redox balance in the cell (Nikolaidis and Jamurtas, 2009). Redox disruption resulting from exercise is a significant stimulus for muscle cell signalling leading to muscular adaptation (Kang *et al.*, 2009; Powers *et al.*, 2010). Radical species or free radical species (FRS) is an umbrella term for molecules which contain an unpaired electron (Halliwell and Gutteridge, 1984). These highly reactive molecules are produced as a normal function of metabolism (Sies, 1997). The simplest form of radical species is the hydrogen radical ($H^{\bullet}$), a hydrogen atom with one single electron. The most common reactive species is the oxygen centred superoxide ($SO^{\bullet}$) anion (Sies, 1997) and the most reactive and therefore damaging free radical is the hydroxyl radical ($OH^{-\bullet}$). FRS and other reactive oxidants such as hydrogen peroxide (H_2O_2) are referred to as a reactive oxygen species (ROS) and the nitrogen radical nitric oxide ($NO^{\bullet}$) can be referred to as reactive nitrogen species (RNS). $NO^{\bullet}$ can also react with SO to form peroxynitrite (ONOO) - a long lived and highly reactive molecule (Sies, 1997; Khan *et al.*, 1998). In addition, research shows ROS can mediate a variety of transcriptional changes that lead to adaptation (Scheele, Nielsen and Pedersen, 2009), specifically to signal transcription of antioxidant defence enzymes to protect against oxidative damage (Gomez-Cabrera, Domenech and Viña, 2008).

FRS are very transient acting molecules because of their reactive nature and quickly interact with macromolecules. The free radical itself is therefore very hard to detect and quantify

(Sachdev and Davies, 2008), and thus research studies focus on the adducts formed from interaction with macromolecules, as an indirect measurement of their production. Adducts are formed when FRS interact to oxidise macromolecules such as lipids and proteins, which can often alter the structure and therefore function of the molecule (Slater, 1984). The product of the interactions with these molecules that can be detected and quantified in research to monitor the action of FRS. Molecules that commonly fall victim of FRS include lipids, proteins and nucleic acids, which consequentially can have a damaging effect on membrane function through interaction with lipid membranes themselves and membrane bound proteins (Slater, 1984).

OXIDATIVE STRESS AND ANTIOXIDANTS

Oxidative stress is a term widely used to describe the resulting state when radical species disrupt the homeostatic balance and overwhelm antioxidant capacity (Halliwell and Cross, 1994; Fisher-Wellman, Bloomer and Roane, 2009).

Antioxidants are either endogenous (enzymes) or exogenous (consumed naturally as part of the diet or as food supplements). Exogenous antioxidants include vitamin E and C, both successful in scavenging free radical species. These antioxidants have differing functions, as vitamin E (α-tocopherol) is lipid-soluble whereas vitamin C is water-soluble (Mccord, 2000), therefore will target different FRS. Enzymatic antioxidants include the likes of catalase and glutathione peroxidase, which act to break down peroxides formed, such as hydrogen peroxide and lipid hydroperoxide formed during radical interactions (Cheeseman and Slater, 1993).

The glutathione redox system is capable of protecting against radical damage by FRS utilising Nicotinamide Adenosine Dinucleotide (NAD^+) as it's electron acceptor in the oxidation and reduction of glutathione (GSH), therefore neutralising hydrogen peroxidase to prevent damage (Mccord, 2000) (Figure. 1). Glutathione possesses a vital role in the maintenance of cellular redox status, through its antioxidant properties (Banerjee *et al.*, 2003). In exercise, glutathione in the oxidised form have shown to increase by 72% following an exhaustive bout (Sastre *et al.*, 1992). In a rodent study, (Ji, Stratman and Lardy, 1988) it was found that exercise increased glutathione peroxidase (GPX) activity in skeletal muscle mitochondria, which was correlated to time spent exercising demonstrating the antioxidant function of glutathione. Decreases in the levels of GPX have been referred to as a down-regulation of the enzyme, however Ji, Stratman and Lardy, (1988) suggest that these decreases are indicative of changes in mitochondrial redox state through the oxidation of free glutathione. Exercise can cause adaptive response in GPX activity, shown by significant increases in GPX up to six days after exercise in mouse quadricep muscle (Salminen and Vihko, 1983) – another example of a beneficial adaptation to increase endogenous antioxidant protection against harmful oxidative stress (Ji, Stratman and Lardy, 1988).

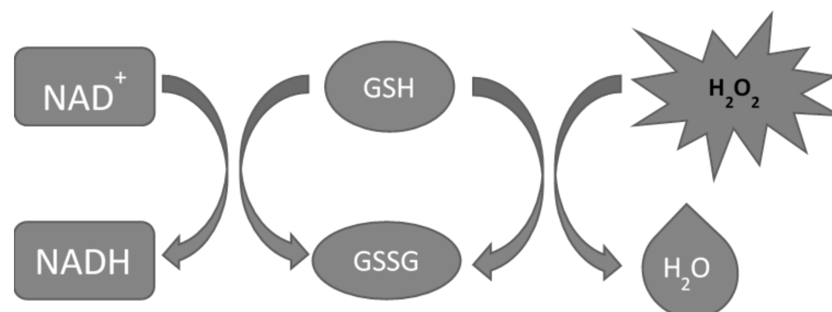


Figure 1. Showing the mechanism of glutathione. Adapted from (Kramer, Zakher and George, 1988)

Superoxide Dismutase (SOD) is the only known enzyme to utilise a free radical specie (superoxide) as its substrate, therefore preventing the accumulation of damaging superoxide concentrations (Cheeseman and Slater, 1993).

Exercise leads to adaptations in our enzymatic antioxidant defence (Li, Gomez-Cabrera and Vina, 2006). SOD and GPX have been documented in research to increase with exercise that induces oxidative stress. In humans, there are three genes that code for superoxide dismutase enzymes, which have different metals – copper/zinc, manganese and iron – at their centre ultimately dictating their location – cytosol, mitochondria and extracellular space respectively (McCord, 2000). Research has suggested SOD is increased by a single bout of exercise through activation of the transcription factor Nuclear Factor-kappa B (NF-κB) (Hollander *et al.*, 2001; JI *et al.*, 2004a). Research shows an up-regulation of SOD, amongst other enzymes, following exercise training with a number of them being up-regulated through the binding of NF-κB (Gomez-Cabrera, Domenech and Viña, 2008). This up-regulation of endogenous antioxidant capacity is another example of a beneficial adaptation of exercise to our health in the prevention of oxidative damage. In two parallel rodent studies, Ji *et al.*, (2004) studied the binding of NF-kB and the time-course of NF-kB activation, finding exercise increased NF-kB binding to a greater extent. Their findings from the time-course study demonstrated that NF-kB can be activated by redox sensitivity, followed by the assumption that this is through an increase in oxidative stress, leading to an increase in the amount of Manganese – Superoxide dismutase (Mn-SOD) – found in the mitochondria – that is expressed following exercise. Other research confirms the regulation of SOD through the same molecular cascades, induced by hormones. It has been found that females have a greater antioxidant enzyme capacity due to the activation of antioxidant enzyme genes by oestrogen (Borrás *et al.*, 2003). Oestrogen as an activator of transcription factors appears to work through p38 MAPK/ERK pathway shown

by an increase in extracellular signal-regulated kinases one and two (ERK1 and ERK2), which activates Nuclear NF-κB to increase the transcription of Mn-SOD and GPX (Borrás *et al.*, 2005). It is therefore clear from documented research that antioxidant enzymes such as SOD are signalled and regulated via MAPK and NF-κB transcription factors, and concentrations may vary between male and females due to the influence of oestrogen.

ASSESSMENT OF REDOX STATUS

Examples of adducts that are formed resulting from free radical interactions are lipid hydroperoxides, protein carbonyls and 3-nitrotyrosine. Lipids are a vital molecule for biological function, making up all cell membranes in the form of a phospholipid bilayer and further to this, they also are vital in membrane transport (Yeagle, 1989). Given their abundance, this also makes lipids highly prone to free radical interaction (Cheeseman and Slater, 1993). Once formed, lipid hydroperoxides can also break down to form versions of lipid radical species, which can then interact with other elements of the cell (Cheeseman and Slater, 1993). One of the first studies to investigate lipid peroxidation was by Dillard *et al.*, (1978), who studied lipid peroxidation by monitoring expired pentane following one hour of exercise. The results demonstrate an increase in pentane expiration, and therefore an increase in lipid peroxidation occurs during exercise. Free radical interaction with proteins is less likely to occur and also only gives reasons to cause damage if they are left to accumulate (Cheeseman and Slater, 1993). Protein/radical interaction forms protein carbonyls (Suzuki, Carini and Butterfield, 2010). Bloomer *et al.*, (2007) measured proteins carbonyls as a measure of oxidative stress elicited comparing a single set of squats and cycling sprint exercise in resistance trained men and found a significant increase compared to pre-exercising

levels, with there being no difference between squatting and cycling exercise. The increase in protein carbonyls was as much as 111% in the cycling sprint condition. Three-Nitrotyrosine (3-NT) is formed when peroxynitrite (formed from $NO^{\bullet}$ and SO as previously mentioned) interacts with the amino acid L-tyrosine or protein bound tyrosine and is also used as a biomarker of oxidative stress inflicted by peroxynitrite (Ahsan, 2013). Exercise training studies measuring 3-NT have shown significant decreases following exercise training studies (Fatouros *et al.*, 2004; Wadley *et al.*, 2014). In addition to measuring adducts and antioxidant capacity, we can also assess redox status by looking at the activity of inflammatory processes which respond to ROS through increases in the expression of inflammatory cytokines (Fubini and Hubbard, 2003), such as inflammatory events and the action of radical producing processes such as the action of xanthine oxidase (XO) (Viña *et al.*, 2000).

EXERCISE AND REACTIVE OXYGEN SPECIES

Studies undertaken in the 1980's suggested that oxidative stress arose following exercise suggesting that exercise was damaging with harmful effects on proteins and DNA. However, research into the mechanisms of exercise adaptation has allowed a greater understanding of the role of radical species. It is now known that radicals contribute to the beneficial effects of exercise on health (Nikolaidis and Jamurtas, 2009). The upregulation of antioxidant enzymes is an example of how low levels of oxidative stress can have a beneficial effect on our body and stimulate a protective mechanism against themselves. The concept of Hormesis was proposed by Radak, Chung and Goto, (2005) and states that the relationship between radical release and exercise could be drawn as a bell-shaped curve. Low concentrations of radicals are beneficial, but high concentrations may be damaging (Stebbing, 1982). In addition, the

importance of the role of FRS in exercise adaptation has emerged in research through studies investigating antioxidant supplementation. Multiple studies have investigated the effect of dietary antioxidant supplementation on the adaptive response to exercise. Despite their effectiveness in reducing harmful oxidative stress, supplementation of both vitamin C and E have shown to reduce the beneficial effects of exercise (Gomez-Cabrera *et al.*, 2008), confirming that FRS are a necessary component in the adaptive process.

Davies *et al.*, (1982) identified the production of FRS following exhaustive exercise in their rodent experiment which demonstrated a two-to-three fold increase in free radical concentrations in gastrocnemius skeletal muscle and liver tissues using Electron Paramagnetic Resonance (EPR) – a direct measure of FRS (Zweier and Kuppusamy, 1988). Davies *et al.*, (1982) also supplemented rats with vitamin E – a radical quenching vitamin (Traber and Atkinson, 2007) – and found a 40% reduction in endurance exercise capacity in those rats, drawing to a conclusion that the damage elicited from the free radical release during exercise contributes to the stimulation of mitochondrial biogenesis, increasing aerobic exercise capacity.

McArdle *et al.*, (2001) were first to establish free radical release specifically from muscle cells during contraction *in vivo*. This was achieved using a micro dialysis probe during electrical stimulation of hind-limb mouse muscle, eliciting a 0.7mmol increase in the amount of SO which declined upon cessation of contraction. To precisely determine that production of SO was from contraction of the muscle, the group also electrically stimulated cultured myotubes and found a forty-fold increase in SO release specifically from the myocytes upon stimulation. The addition of SOD to the cell culture medium had a significant effect in reducing the amount of superoxide present. McArdle *et al.*, (2001) also demonstrated transient but significant increases in muscle SOD and Heat Shock protein 70 (HSP70) with stimulated

contraction, whilst catalase activity increased slower over a longer period. These levels remained elevated up to 72 hours post contraction. These data are suggestive of an adaptive response of antioxidant mechanisms with exercise stimulation.

SOURCES OF FREE RADICAL SPECIES

There are various intracellular and extracellular sources of FRS induced by exercise that are dependent on the intensity and conditions that exercise create. Sources or sites of production of FRS that have been well documented include mitochondria, cytosol, plasma membrane, endothelium and extracellular fluid (Jackson, 2008), and are dependent on exercise intensity (Vollaard, Shearman and Cooper, 2005).

MODERATE INTENSITY EXERCISE – THE ELECTRON TRANSPORT CHAIN

Moderate intensity aerobic exercise is linked with adaptations eliciting an increase in mitochondrial content and in associated mitochondrial enzymes (Holloszy and Coyle, 1984). Mitochondria contribute to a large majority of SO production, with papers reporting 1-2% of oxygen consumed by mitochondria being converted to superoxide anion (Turrens, 2003). Production of ROS can occur from various sites within the mitochondria, which are made up of outer and inner membrane compartments.

Exercise at a low-to-moderate intensity that continues for prolonged periods of time, utilises aerobic oxidative phosphorylation to generate energy in the form of adenosine triphosphate (ATP). Aerobic energy production via oxidative phosphorylation is the major source of energy for long-term muscle contraction. The process transfers electrons carried from the

process of glycolysis and the tricarboxylic acid (TCA) cycle following the breakdown/oxidation of substrates such as glucose and fat, utilising molecular oxygen as the ultimate electron acceptor. This takes place via the electron transport chain (ETC) located in the inner membrane of mitochondria. The ETC consists of five protein complexes, of which electrons are passed from one to the next via a series of carrier molecules to the final complex ATP synthase – ultimately producing vast amounts of ATP (Hatefi, 1985; Saraste, 1999). It works by achieving an electrochemical gradient to drive the ATP synthase enzyme at the end of the chain to assemble ATP from Adenosine Diphosphate (ADP) and Phosphate (Mitchell, 1974; Saraste, 1999).

It has been established that mitochondrial production of ROS in the inner compartment occurs from reduction of molecular oxygen with a single electron due to ETC 'leakage' at two main sites: complex one – the reduced pyridine nicotinamide adenine dinucleotide (NADH) electron carrier into the ETC – and complex three – Cytochrome C electron carrier within the ETC (Liu, Fiskum and Schubert, 2002; Murphy, 2009; Circu and Aw, 2010). It has also been found that ROS can be produced in the intermembrane cytosolic space from Krebs cycle enzyme complexes (Circu and Aw, 2010), an intermediate pathway that links substrate (anaerobic) and oxidative (aerobic) phosphorylation.

HIGH-INTENSITY EXERCISE – XANTHINE OXIDASE

In addition to the mitochondrial sources of FRS, additional extracellular sources of ROS have been identified – particularly during high intensity exercise or prolonged exhaustive exercise. Xanthine Oxidoreductase enzymes are a class of enzymes that catalyse the hydroxylation reaction of hypoxanthine and xanthine during the final stages of uric acid (UA) synthesis

(figure 2) (Enroth *et al.*, 2000; Ardan, Kovačeva and Čejková, 2004). The UA synthesised from the action of xanthine oxidase (XO) has antioxidant properties and is known to be a powerful scavenger of the peroxynitrite (Ames *et al.*, 1981) formed by the interaction of SO and $NO^{\bullet}$. These enzymes exist in two forms: Xanthine dehydrogenase (XDH) and XO. Under normal, resting conditions XDH is the form that is most plentiful in cells, which converts to XO - through oxidation of sulfhydryl residues or via proteolysis (Enroth *et al.*, 2000) - when conditions in the cell change, such as during intense hypoxic natured anaerobic exercise and conditions of temporary ischaemia are created. The substrates utilised by these enzymes during catalysis include purine, pyrimidine, ptrerin and aldehyde molecules (Enroth *et al.*, 2000). During catalysis, XDH predominantly utilises the natural purine molecule nicotine adenosine dinucleotide (NAD^+) as its substrate, whereas XO utilises oxygen to transfer electrons during the catalysis of hypoxanthine and xanthine, leaving a reactive oxygen molecule as a by-product of its activity (Harris, Sanders and Massey, 1999; Enroth *et al.*, 2000; Kuzmin, Pustovit and Abramochkin, 2016). Because of this, the activity of XO could be termed pathophysiological (Hare and Johnson, 2003; Ardan, Kovačeva and Čejková, 2004). The 'burst' of SO that is produced from the activity of XO is free to interact with macromolecules in the cells or vasculature, which without sufficient antioxidant capacity can favour damaging oxidative conditions. XO has therefore been implicated in the pathologies of multiple diseases and conditions such as myocardial ischaemia (Chambers *et al.*, 1985) chronic heart failure (CHF) (Doehner *et al.*, 2002; Hare and Johnson, 2003), rheumatoid arthritis (RA) (Taysi *et al.*, 2002), and diabetes (Liu *et al.*, 2015).

Figure 2. XO catalysation of hypoxanthine to UA.

Following anaerobic exercise, when oxygen is limited, XO is thought to be a dominant source of SO. During high-intensity or exhaustive exercise, a state of temporary hypoxia is created. During exercise thus, oxygen is limited, but XO is active. Immediately upon cessation of exercise and during recovery oxygen is re-perfused via the circulation and XO produces a burst of SO (Sachdev and Davies, 2008). SO produced from XO following hypoxia can be related to the damage caused by 'the oxygen paradox' because of the nature of the enzyme's action.

Quick, (1935) discuss the oxygen deficit that occurs with high intensity anaerobic exercise happening before lactate accumulates leading to UA retention as a manifestation of the process. However, given the knowledge we now possess of the action of XO during intense exercise, this retention of UA could be due to the activity of XO during the recovery phase following intense exercise as oxygen is replenished to pay off the oxygen debt (Gaesser and Brooks, 1984). In addition, Sastre *et al.*, (1992) report a linear relationship between lactate:pyruvate ratios and oxidised-to-reduced glutathione ratios pre- and post-exhaustive exercise. As previously explained, lactic acid or lactate production increases under anaerobic exercise conditions, where the ETC is not the predominant fuel source, therefore the correlation with glutathione ratio (oxidised:reduced) and lactate suggest oxidative FRS are produced by alternative sources (Vollaard, Shearman and Cooper, 2005). Given the similar conditions needed to produce lactic acid, XO activity provides an explanation of the source of these radicals during such intense anaerobic exercise (Bloomer and Goldfarb, 2004).

The role of XO during intense exercise has been highlighted in research. Early research to suggest activity of XO looked at concentration of UA – the product of XO catalysis - following intensive exercise.

Armand J Quick, (1935) looked at the effect of exercise in the excretion of UA, based on observations of lactic acid increases causing a retention of UA. In the study, participants took part in strenuous exercise, which induced mild exhaustion – conditions we now understand to activate XO (Bloomer and Goldfarb, 2004; Vollaard, Shearman and Cooper, 2005). The results demonstrated a decrease in UA with exercise, but concentrations increased again in the hours following exercise. In conclusion, it was presumed that UA retention occurs due to excessive production of lactic acid during exercise. However later research could provide a possible explanation for the latent increase in UA concentrations by the paradoxical nature of XO as previously described. XDH is converted to XO during intense exercise and during the recovery period following exercise is actively catalysing hypoxanthine and xanthine to UA using the increase oxygen as a substrate (Sachdev and Davies, 2008). Lactate:Pyruvate and glutathione ratios have also been found to be correlated linearly (Sastre *et al.*, 1992), which could also suggest a role of the oxidative activities of XO in the research study by Quick (1935).

Further research in UA includes research by Sutton *et al.*, (1980) and (Green and Fraser, 1988). Sutton et al., (1980) found increases in UA in male participants following a 5000 metre race and a forty-two kilometre marathon. Hellsten *et al.*, (1988) suggest increases in UA explained by Sutton et al., (1980), combined with their own study investigating concentrations of hypoxanthine, xanthine and UA in venous and arterial samples during exercise, are indicative of XO activity increasing during intense exercise. Green and Fraser (1988) found an increase in serum UA concentrations with supra-maximal exercise compared

to sub-maximal exercise, also suggestive of XO activity due to the nature of the exercise eliciting increases in UA.

In a rodent study, Viña *et al.*, (2000) successfully identified an increase in XO activity following a bout of exhaustive exercise. To further confirm the role of XO in exercise-induced radical production, the group inhibited the enzyme with allopurinol. Allopurinol is a well-established inhibitor of XO used in clinical settings for the treatment of hyperuricaemia (Smalley *et al.*, 2000; Siu *et al.*, 2006). In doing so they found a reduction in post-exercise oxidation of glutathione. Their results show that XO is indeed a source of FRS production during intense exercise and that blocking its action leading to a decrease in oxidative stress and tissue damage.

Continuing research found that the activities of XO in rodents is also apparent in humans (Gomez-Cabrera *et al.*, 2003). Again, using allopurinol, they found reduced markers of muscle damage with allopurinol dosing in participants taking part in the Tour de France, specifically in the final sprint stages that are arguably the most intensive, exhaustive section of the race.

The importance of XO having a role in adaptation has emerged more recently. Kang et al., (2009) investigated the activation of PGC-1α by XO generated ROS in rats. In their experiments, they compared rested control, exercise-only and exercise-allopurinol rats. They found XO activity and ROS production was significantly increased in exercised rats, compared to only a small increase in XO activity in allopurinol and control rats. They found a significant increase in the content of PGC-1α, NF-kB and Tfam in exercise-only rats in comparison to controls by up to 200%. The content of the same proteins significantly

decreased in the allopurinol rats, providing some strong evidence that XO production of ROS plays a role in skeletal muscle adaptations.

Wadley *et al.*, (2013) looked at exercise response in rats to XO inhibition also using allopurinol. They looked at the adaptive cell signalling responses of exercise of mitochondrial enzymes and antioxidant enzymes (SOD) both acutely and over a six-week training period. Their results show the administration of allopurinol influenced a reduction in XO activity to 5% of baseline and some signalling responses with acute exercise were dampened. Training increases in PGC-1α were not affected by the inhibition and there was also no difference in the content of SOD mRNA. However, their results show glutathione is still oxidised to the same level as controls and therefore suggest that ROS production is not from XO and must be from other sources. They conclude that allopurinol itself was responsible for the changes in signalling, rather than the reduced action of XO ROS production as there were no changes to training-induced adaptations with allopurinol administration.

Sanchis-Gomar *et al.*, (2013) researched the effects of XO inhibition on markers of cardiovascular health in response to acute intense exercise in a double-blind control study amongst football players. Half of the players were given a dose of 300mg allopurinol prior to a match whilst the other half received a cellulose placebo. They found the players who were given allopurinol had lower levels of serum UA compared to control players following the football match. In relation to cardiovascular health, markers were found to be unrelated to XO activity as the inhibited group showed now differences to the control group in three out of the five markers that were measured. The other two markers increased with an exercise effect and were also insignificantly different between the groups, therefore it is suggested that the metabolism of these protein markers is altered by exercise and is also unrelated to XO activity.

ADAPTATION OF XANTHINE OXIDASE

From the available evidence in research, it seems clear that XO does in fact have an important role to play in exercise adaptation processes involving transcription factor activation, however evidence to show an adaptation of XO itself is minimal. Does XO adapt to repeated exercise stimulation, and if there is a change in XO activity and expression, is it beneficial to our health?

The previously described study of Green and Fraser, (1988) could be an indicator of an adaptive response of XO activity, as UA concentrations increased during the time-course of the exercise bouts and remained elevated for several days following exercise. Hellsten *et al.*, (1997) investigated the role of XO in relation to inflammation following intense eccentric exercise in male participants. Participants took part in five bouts of eccentric leg exercises having muscle biopsy and venous blood samples taken at intervals pre- and post-exercise to examine the time-course of XO activity. They measured plasma hypoxanthine and UA concentrations to measure plasma XO activity and found an increase in activity pre- to post-exercise, which remained elevated at twenty-four and ninety-six hours post exercise. They conclude that XO may contribute to the ROS generated in secondary inflammatory events due to the association they found with inflammatory markers.

There is a minimal amount of research that looks at the adaptation of XO protein expression, or changes in XO activity, over a training period. The study previously described by Hellsten *et al.*, (1997) provides some evidence of the elevated activity of XO following intense leg exercises, similar to the activities of other proteins which adapt to exercise stimulation such as PGC-1α. Also in the study by Green and Fraser, (1988) investigating UA which as previously explained could be used as a marker of XO activity, provides some evidence of a potential

adaptive response. From these evidences, it can only be speculated that there may be an adaptive response of XO resulting from exercise training, however no study to date and to our knowledge specifically looks at the adaptation of XO to exercise.

MODALITY OF EXERCISE – SWIMMING

From the available research, we know to activate XO, certain conditions are required to convert XDH into its oxidative XO form. Research identifies XO activity during high intensity exhaustive exercise, where conditions of temporary hypoxia are created to convert the enzyme to XO. When followed by a period of rest, allowing a significant influx of oxygen through the muscles, XO then uses molecular oxygen as a substrate to catalyse the hypoxanthine to UA reaction. This produces ROS as a by-product of the reaction.

In an ideal research study, maximal stimulation is required to assess the variable being investigated. Therefore, exercise should be a whole-body exercise, which activates most muscle groups during the exercise bouts. Taking this into account, for the present study, maximal swimming exercise was chosen as the exercise mode.

In the UK, swimming is the populations highest participation sport, despite a steady decline in participation numbers in recent years (*Active People Survey - Sport England*, 2017). Despite its popularity and the use of swimming in exercise recommendations, research is limited using swimming as the modality of exercise due to constraints with measurements and skills required to carry out the exercise (Tanaka, 2009). From a research perspective it is clear that evidence surrounding the health benefits of exercise is limited, and what little evidence is available demonstrates mixed results and interpretations as to whether swimming elicits similar beneficial results to alternative exercises such as running or cycling (Tanaka, 2009).

As a whole body rhythmic exercise that uses major muscle groups, swimming is an exercise which complies with outlined physical activity guidelines (World Health Organization, 2010). The non-weight bearing characteristics of swimming also make it suitable for a vast majority of populations including the obese, elderly and in those with arthritis (Tanaka, 2009). It is also beneficial to reduce the risk of injury and increase comfort whilst maximally exercising, as the thermo-conductivity of water reduces heat illness (Sheldahl *et al.*, 1982), on condition that the water is at an appropriate temperature for exercise.

Swimming is an example of exercise modality that alters oxygen uptake and increases the demand for anaerobic metabolism (Spriet, 2006). Competitive swimming training, which involves restricted breathing, has also demonstrated an increase in respiratory capacity in comparison to other athletic disciplines (Aspenes and Karlsen, 2012). The volume of oxygen consumed increases during post-exercise recovery, seemingly in a linear fashion with exercise intensity and duration (Borsheim and Bahr, 2003) and has been suggested to be linked to the oxygen deficit that occurs with anaerobic exercise metabolism and lactate clearance (Gaesser and Brooks, 1984). Based on these alterations in oxygen uptake (Spriet, 2006), swimming exercise – particularly maximal intensity swimming – can therefore create conditions of temporary hypoxia seemingly fit to increase XO activity, whilst maximally stimulating muscle contraction. During the recovery between bouts of exercise, the increased VO_2 consumption should be suitable to induce XO ‘oxidative burst’ that occurs with oxygen reperfusion. In addition, research has found an association of XO with endothelial cell damage during periods of hypoxia (Terada *et al.*, 1992) and also an association between XO and arterial blood pressure in vivo in hypertensive rats (Suzuki *et al.*, 1998).

There are many factors that contribute to energy cost and VO_2 consumption (Xu and Rhodes, 1999) however in swimming, research has shown stroke technique is an important factor

(Costill *et al.*, 1985) This study therefore, looked at trained competitive swimmers, in comparison to active individuals not trained in swimming to determine the effects of swimming exercise training and swimming technique on XO adaptations and activity, and the potential benefits any adaptations observed could have on our health.

AIMS

The purpose of the present study was to assess swimming as a mode of exercise to activate XO in humans to elicit a response to an acute bout and repeated bouts of swimming. The study aims to investigate both the activity and expression of XO in response to maximal swimming exercise in experienced, competitively trained swimmers and less-experienced, non-competitively trained swimmers.

The study aims to establish whether repeated exposure to hypoxic conditions created by maximal swimming exercise can elicit an acute response of XO activity and/or an adaptative response over a four-week period of repeated swimming sessions. In addition, the study aims to determine whether training status and swimming experience influences the adaptative response of XO to repeated maximal swimming. Furthermore, any changes found in XO activity and/or expression that occur will consider whether these changes due to swimming exercise could be beneficial to our health.

HYPOTHESIS

It is hypothesised that XO activity will be greater in non-trained swimmers, compared to competitively trained swimmers in response to an acute swimming bout. In addition to activity increasing, we hypothesise an adaptive response of XO activity and protein content/expression to swimming training in both participant groups. Based on the research available, adaptations occur from repeated exercise or training. By repeating bouts of high intensity swimming exercise, repeatedly creating temporary conditions of hypoxia it is therefore predicted that both groups will observe an adaptive response of XO to the intervention. In addition, the greatest adaptive response is expected to be observed in the non-trained participant group, due to increased exposure to high-intensity, hypoxic type exercise during swimming bouts and the difference in swimming technique compared to the trained group.

METHOD

PARTICIPANT RECRUITMENT

Ethical permission was obtained from the University of Birmingham Science, Technology, Engineering and Mathematics (STEM) ethics committee. Sixteen male and female adults aged 18-25 years were recruited to participate in the study (n=16). Participants were healthy, had no pre-existing health conditions and were not taking medication, determined by completion of a general health questionnaire following recruitment. All participants were provided with an information sheet detailing the study protocol upon their expression of interest to take part and were given the opportunity to ask any questions before signing a consent form prior to study commencement. All data were recorded and kept according to the General Data Protection Rules (2018). Data were pseudonymised by assigning identification numbers to each participant. Participants were required to be available for five consecutive weeks to complete the protocol and had the right to withdraw at any time point during the study. Over the course of the study, two participants (n=2) withdrew from those initially recruited due to time constraints to adhere to the protocol.

Participants were organised into two groups based on the inclusion criteria. The competitive accustomed swimmer group (n = 7) was formed of competitively trained swimmers from the University of Birmingham swimming club. To be included in the study, participants had to be training for approximately eight hours per week, have a personal best time of 33 seconds or less for 50 metres freestyle, and have a VO_{2max} between 40 – 60 ml/kg/min.

The unaccustomed swimmer group (n= 7) was formed of individuals who are physically and purposefully active, also having a VO_{2max} between 40 – 60 ml/kg/min but were not currently

training in swimming exercise and did not swim for more than three hours per month recreationally.

Table 1. Criteria for trained and non-trained groups of swimmers.

	Trained	**Non-Trained**
Condition Criteria	Training approx. 8hrs p/week Able to swim 50m in ≤ 33s VO_{2max} between 40-60ml/kg/min	Swim ≤ 3hrs p/month Physically and purposefully active VO_{2max} between 40-60ml/kg/min

EXPERIMENTAL PROTOCOL

Week 1: Baseline Testing

During this week, participants went through baseline measurements and testing. These measures included:

1. A VO_{2max} test on a lode bike using Vyntus Vmax and SentrySuite software.
2. Height (cm) and Weight (kg) (to calculate rate of oxygen utilisation for VO_{2max})
3. Resting venous whole blood sample (Pre-intervention).
4. Familiarisation session of the maximal swimming exercise intervention (non-trained participants).

Following completion of these measures, participants who met the inclusion criteria continued with the study. Those who did not meet the criteria were excluded and any data recorded was destroyed. Participants who were able to continue then went completed a familiarisation of the exercise intervention in preparation for the 'test' exercise in the weeks to come.

Week 2: Exercise test set 1

Following a warm-up, the first exercise test was conducted. Participants carried out the exercise intervention as instructed by the researcher. Pre-, mid-, and post-exercise blood samples were collected using finger prick blood sampling. The mid-exercise blood sample was collected after the second repetition of the exercise intervention set.

Week 3 & 4: 'Training sets'

During the next two weeks, participants visited the pool to carry out the exercise protocol once a week, however no blood samples were taken as previously described. These sessions also helped to keep in touch with the participants and to keep them engaged with the study.

Week 5: Exercise test set 2 and Post-intervention blood samples.

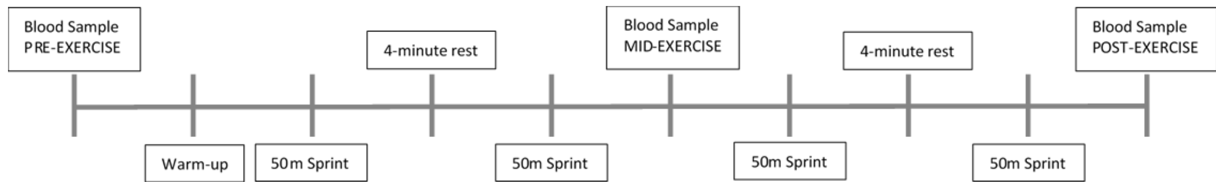
This was the final exercise bout of the study participants complete. This was completed in the same way as exercise test 1. The samples from this test were compared to the samples from the first exercise test. A second venous blood sample was collected twenty-four hours after the exercise test was carried out. This sample was compared to the venous blood sample taken pre-intervention.

EXERCISE INTERVENTION

To create the conditions to stimulate XO radical production, High Intensity Interval Training (HIIT) type exercise was used, consisting of four repetitions of 50-metres maximal swimming exercise. This type of exercise intervention was chosen as high intensity interval training in swimming has shown to induce oxidative stress (Deminice *et al.*, 2010) and should elicit conditions of temporary hypoxia to activate XO. The swimming pool was 25m in length and participants completed two lengths to complete the 50-metre distance required. Intensity was monitored using Garmin Vívosmart heart-rate monitor ("vivosmart HR | Garmin,"), that participants wore on their wrist during swimming exercise, and self-report measures of perceived exertion using the Borg Scale of Rate of Perceived Exertion (RPE). RPE was used to ensure the correct intensity was achieved amongst both groups of participants (Ueda and Kurokawa, 1995). A rest period of 4 minutes was allocated between the sprint bouts to allow for participants to recover for the next, whilst also fulfilling the XO activating conditions to cause the "oxidative burst" of ROS. Where blood samples were taken, this rest period was extended to 10 minutes to allow time for the finger-prick sample to be collected, as health and safety procedures at the swimming pool facilities required the samples to be taken in a room away from poolside.

Participants completed this set of 4 x 50m once a week for four weeks (four sets in total). The first and fourth sets were named as 'test sets', as during these sets blood samples were taken pre-, mid- and post-exercise. Details of these 'test sets' are visualised in schematic 1. The second and third sets were named 'training sets' consisting of only the described swimming exercise. This exercise intervention was used for both groups of participants. Data recorded during all exercise sets included heart rate, rate of perceived exertion (Borg Scale) and the time taken to swim the 50m bouts. Testing for all participants was undertaken at the same

time in the morning for each visit throughout the protocol in order to minimise fluctuations in diet and amount of sleep between participants.



Schematic 1. Displaying the 'test set' procedure.

VO_{2MAX} TESTING PROCEDURE

The $VO_{2\ max}$ test was performed on a cycle ergometer ("Lode - Excalibur sport,") after a baseline pre-intervention blood sample was taken. Participant's height and weight were measured to calculate $VO_{2\ max}$ and a polar chest strap was used to monitor heart rate. Gas analysis took place using Vmax Vyntus CPX ("Vmax Vyntus^{TM} CPX - Vyaire,") equipment together with SentrySuite software. The incremental test started at 50 watts and increased in increments of twenty-five watts every three minutes. Participants were instructed to keep cadence (RPM) above 60 RPM to achieve the correct load. Rate of perceived exertion (RPE), heart rate and VO_2 were recorded at the end of each increment. When the participant's RPE reached fifteen, load increments increased by twenty-five watts in one-minute stages until the participant could no longer continue to maintain the cadence above 60 RPM. A $VO_{2\ max}$ test result is achieved when two of many criteria are met, such as the plateau of oxygen consumption despite an increase in workload, RER (>1.1) and HR (+/- 15 age-predicted HRmax) (Linderman *et al.*, 2008). For this study, the measurement of $VO_{2\ max}$ was determined by the maximal rate of oxygen consumption measured in ml/kg/min despite an increased workload. At this point the test was stopped. The measurement of $VO_{2\ max}$ is a gold standard

measurement of aerobic fitness (Cumming and Friesen, 1967; Figueroa-colon *et al.*, 2000). The results from this test determined the participant's eligibility to continue to the rest of the study, outlined in the criteria.

BLOOD SAMPLING

VENOUS BLOOD SAMPLES

Venous blood samples collected from an antecubital vein in the forearm pre- and post-intervention. From each sample whole blood and plasma aliquots were prepared. Plasma was prepared by centrifugation of the sample at 1500rpm for 10 minutes at 8°C. All samples were frozen at -80°C for later analysis.

FINGER-PRICK BLOOD SAMPLES

The finger-prick blood samples collected during the exercise tests were collected pre-, mid- and post-exercise. Blood was collected in 1mg anticoagulant (K2 EDTA) lined tubes suitable for finger-prick collection ("BD Microtainer® MAP Microtube for Automated Process - BD,"). On each occasion 250µl of blood was collected. Samples were kept on ice and transported back to the laboratory where they were pipetted into aliquots and frozen at -80°C.

BLOOD MEASURES

The venous samples taken pre- and post-intervention measured XO protein content, and Total Antioxidant Capacity (TAC). The finger prick blood samples taken during the exercise

intervention tests were measured for XO activity. These measures and the methods of detection are summarised in table 3.

Table 2. Summary of blood measures and methods used for detection.

Blood Measure	Method
Xanthine Oxidase Protein Content	Western Blot
Xanthine Oxidase Activity	Xanthine Oxidase enzyme activity assay kit (Abcam)
Total Antioxidant Capacity	Ferric reducing ability of plasma (FRAP) assay.

LABORATORY ANALYSIS

PROTEIN DEPLETION

Plasma samples were depleted of albumin and IgG preparation for western blotting. Albumin and IgG was removed from plasma samples using a ProteoPrep® Blue Albumin & IgG Depletion Kit (Sigma-Aldrich) according to the manufacturer's instruction. This process removed the masking effect of larger proteins, allowing the visualisation of low abundance small proteins via western blot (Rengarajan, de Smet and Wiggert, 1996).

XANTHINE OXIDASE WESTERN BLOT

Depleted samples (30μg protein, quantified via BCA assay (Smith *et al.*, 1985) 20μl) were loaded into an 8% Acrylamide gel. Molecular weight markers (xμl) were also loaded to give context to gel running and transfer (Amersham ECL DualVue, RPN 810). Proteins were then transferred to nitrocellulose membranes (Amersham protran 0.2μm) using the Mini Trans-

Blot Cell transfer system (BioRad) for 1 hour. Membranes were blocked with 5% milk in TBST 0.5% for 1 hour and washed x 3 in 0.05% TBST. Membranes were then incubated in the monoclonal primary antibody for XO (Abcam) followed by incubation with secondary antibody (Cell Signalling Anti-rabbit IgG, HRP-linked Antibody 7074). Membranes were developed using ECL substrate (Clarity Western, Biorad) according to manufacturer instruction and imaged using C-digit scanner (Licor). Finally, bands were quantified using Image Studio (Licor).

XANTHINE OXIDASE ENZYME ACTIVITY ASSAY

The protocol for the XO enzyme assay was completed according to instructions provided by the manufacturer of the kit (Abcam – ab102522). The assay was carried out using fluorometric analysis, with a black ninety-six well plate. Fluorescence intensity is proportional to the amount of XO protein content in the sample and therefore XO activity is measured accurately.

To prepare the samples for the assay, finger prick sampled blood (20µl) was added to assay buffer (80µl) as per assay instructions, to make a four-times dilution. Background control, positive control, standards and prepared sample (50µl) were added to wells on the 96 well plates, before reaction mix (50µl) was added to each well. Reaction mix was made up of assay buffer (88%), substrate mix (4%), enzyme mix (4%), and OxiRed Probe (4%). Background control mix was made up of assay buffer (92%), enzyme mix (4%) and OxiRed Probe (4%). The positive control was supplied in the kit used for analysis (Abcam – ab102522) and H_2O_2 standards were made up in the range of 0-to-10 nmol/well. The plate was read once

immediately at 535nm excitation/590nm emission, and once following 20 minutes incubation at 23 °C (Tecan).

TOTAL ANTIOXIDANT CAPACITY – FERRIC REDUCING ABILITY OF PLASMA (FRAP)

Plasma samples were diluted 1:1 with ultrapure water (Millipore). Standards were freshly prepared using Ascorbic acid to create a 7-point standard curve ranging from 0µM to 1000µM. Sample or standard (10µl) was then pipetted into wells of a 96-well microtiter plate in duplicate. FRAP reagent was made by combining 30ml acetate buffer (300mM), 3ml TPTZ solution (10.6mM) and 3ml ferric chloride solution (20mM) before 300µl was added to each well. The plate was then incubated for 8 minutes at room temperature. Plate reading was completed at 650nm and values were calculated using linear regression. Values were expressed as µM of antioxidant power relative to ascorbic acid. The total antioxidant capacity assay used assesses all soluble antioxidants in plasma, including ascorbic acid, and UA.

CALCULATIONS

CALCULATION OF HEART RATE RELATIVE TO HEART RATE AT VO_{2MAX}

Heart rate (HR) was recorded during the VO_{2max} test undertaken as previously described during baseline measures. HR was recorded when participants reached their maximum and stopped the test. HR was also taken at each stage of the swimming exercise intervention. Using this HR data, we decided to calculate what percentage of the VO_{2max} recorded HR the participants were exercising at during the intervention exercise bouts.

$$\%HR = Exercise\ HR / VO_{2\,max}\ HR * 100$$

CALCULATION OF ENERGY EXPENDITURE

An estimation of energy expenditure was calculated using $VO_{2\,max}$ spirometry data. The values for oxygen inspiration and CO_2 expiration were taken at the point their $VO_{2\,max}$ was reached, which were then inserted into an equation to calculate their energy expenditure at $VO_{2\,max}$ (Singer, 2016). Urinary nitrogen was excluded from the equation, as this was not measured in the experiment. Research has shown a linear relationship between HR and VO_2 (Keytel *et al.*, 2005), a pattern which has shown to be shifted to the right in swimming compared to walking or cycling (McArdle, Glaser and Magel, 1971). The percentage HR calculated as described was then utilised to calculate the energy expenditure of participants during the swimming exercise bouts to monitor any differences between the two groups.

$$Energy\ Expenditure\ (Kcal) = (3.581 \times VO_2) + (1.448 \times VCO_2) - 1.773\ urinary\ nitrogen\ (g)$$

STATISTICAL ANALYSIS

To analyse the results for statistical significance, the data were analysed using IBM SPSS analytical software version 25.

Data were analysed by the means of a mixed design ANOVA – comparing within-subject repeated measures and between-subject comparison effects. All statistics are reported to a significance confidence level of 95% ($p < .05$ alpha value). Comparisons for the participant

information, such as BMI, $VO_{2\ max}$ values were analysed using an independent samples t-test, again to a confidence level of 95% ($p < .05$).

RESULTS

PARTICIPANT DATA

A summary of participant data is outlined in table 3. This shows the number of participants in each group condition split by sex for each group that completed the study. Comparison of participant baseline $VO_{2\ max}$ values (M= 47.77 ml/kg/min, SD= 6.41ml/kg/min(trained), M= 42.46 ml/kg/min, SD= 5.45ml/kg/min (non-trained); t (12) = 1.669, p= .121), and BMI measures (M= 22.59, SD= 1.87(trained), M= 24.94, SD= 2.94(non-trained); t(12) = -1.787, p= .099) are not significantly different between the two participant groups.

Table 3. Participant information.

	Trained	**Non-trained**
Sex	Male n= 6 Female n= 1	Male n= 4 Female n= 3
BMI / kg/m^2	22.58 +/- 1.87	24.94 +/- 2.94
$VO_{2\ max}$. (ml/kg/min)	47.77 +/- 6.41	42.46 +/- 5.45

EXERCISE TESTING RESULTS

SWIMMING TIME

The mean swimming time for the four 50m bouts at each stage (test 1, training session 1, training session 2, and test 2) of the exercise intervention are represented in figure 3. Statistical analysis demonstrated significant differences between the mean times of each group (M=30.98 sec SD=2.67 sec (trained), M= 46.21sec SD= 2.67 sec (non-trained); p= .002), with the trained group swimming significantly faster than the non-trained group across the four experimental visits. Analysis also revealed that the trained group of swimmers were significantly faster than the non-trained group at each stage of the exercise intervention (Test 1: M= 30.86 sec, SD= 1.61sec (trained) M= 45.36 sec, SD= 9.37 sec (non-trained), p= .002; Training 1: M= 30.93 sec, SD= 1.34 sec (trained) M= 46.17, SD= 9.58 sec (non-trained), p= .001, Training 2: M= 31.04 sec, SD= 1.79 sec (trained) M= 47.04 sec, SD=9.97 sec (non-trained), p= .001, Test 2: M= 31.04 sec, SD= 1.65 sec (trained) M= 46.25 sec, SD=10.68 sec (non-trained), p= .003). There was a significant difference in swimming times within the non-trained group of swimmers between the first experimental visit and the third visit (M= 45.36 sec, SD= 9.37 sec (test1) M= 47.04 sec, SD= 9.97 sec (training 2), p= .01), whereas those in the trained group showed no significant differences in their swimming times across all four visits (M=30.86 sec (test1), 30.93 sec (training 1), 31.04 sec (training 2), 31.04 sec (test 2); p= 1.00).

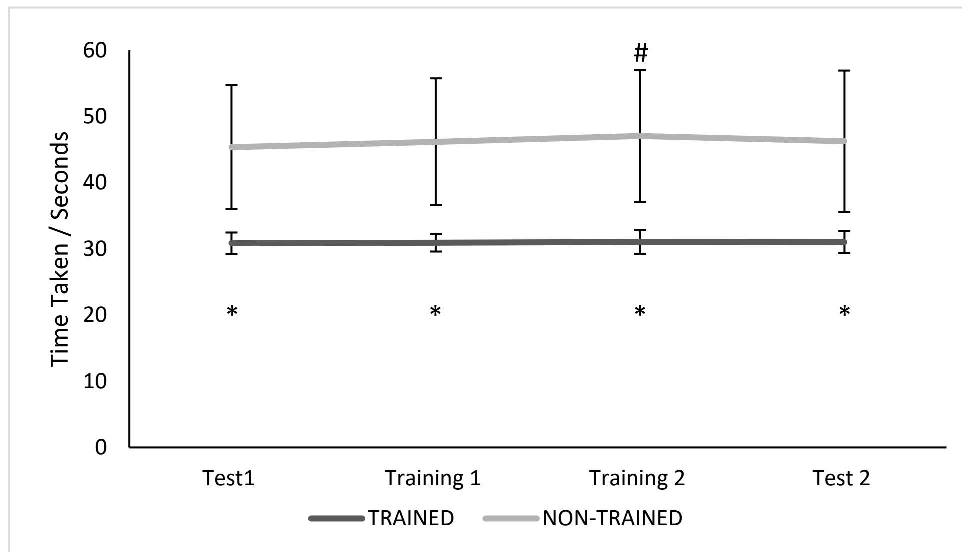


Figure 3. Shows the time data for both groups of swimmers. Significant results between the two groups are indicated by the (*), showing the trained group completed 50m significantly faster than the non-trained group at each stage of the protocol. The significant difference within the non-trained group between stage one and stage three of the protocol are indicated by the (#). Error bars show the standard deviation of the mean.

RATE OF PERCEIVED EXERTION

RPE scores measured during each experimental visit are represented in figure 4. Statistical analysis of the data showed there were no statistically significant differences between the two groups for RPE scores at any stage of the intervention (Test 1: M= 16, SD= 2 (trained), M= 16, SD= 1 (non-trained), p= .570; Training 1: M= 16, SD= 1 (trained), M= 16, SD= 2 (non-trained), p= .977 ; Training 2: M= 16, SD= 2 (trained), M= 16, SD= 1 (non-trained), p= .759; Test 2: M= 16, SD= 1 (trained), M= 16, SD= 1 (non-trained), p= .765). There were also no significant results for the within group comparison of RPE scores (p= 1.00).

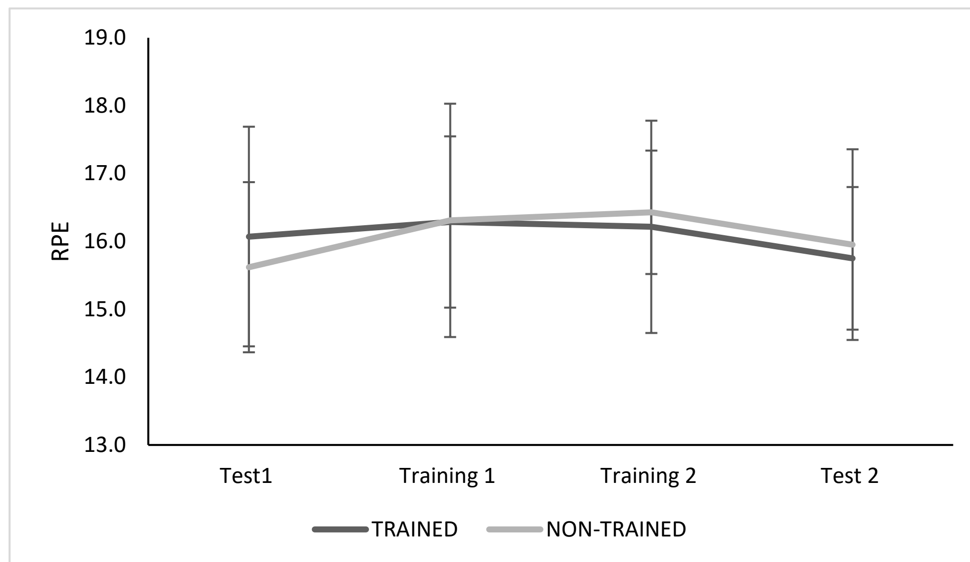


Figure 4. Average Rate of Perceived Exertion in trained and non-trained swimmers over the course of the exercise intervention. Error bars show the standard deviation of the mean.

HEART RATE DATA

The HR data taken at each experimental visit is represented in figure 5. Analysis showed there were no statistically significant differences between the two groups at any stage of the intervention (Test1: M= 128 bpm, SD= 19 bpm (trained) M= 128bpm, SD= 14 bpm (non-trained), p= .942; Training 1: M= 134 bpm, SD= 33 bpm (trained), M= 125 bpm, SD= 12 bpm (non-trained), p= .536; Training 2: M= 126 bpm, SD= 25 bpm (trained), M= 130 bpm, SD= 21 bpm (non-trained), p= .739; Test 2: M= 132 bpm, SD= 25 bpm (trained), M= 130 bpm, SD= 15 bpm (non-trained), p= .861). There were also no significant differences in HR between participants within the groups, for both groups of participants (p= 1.00 for all within group comparisons).

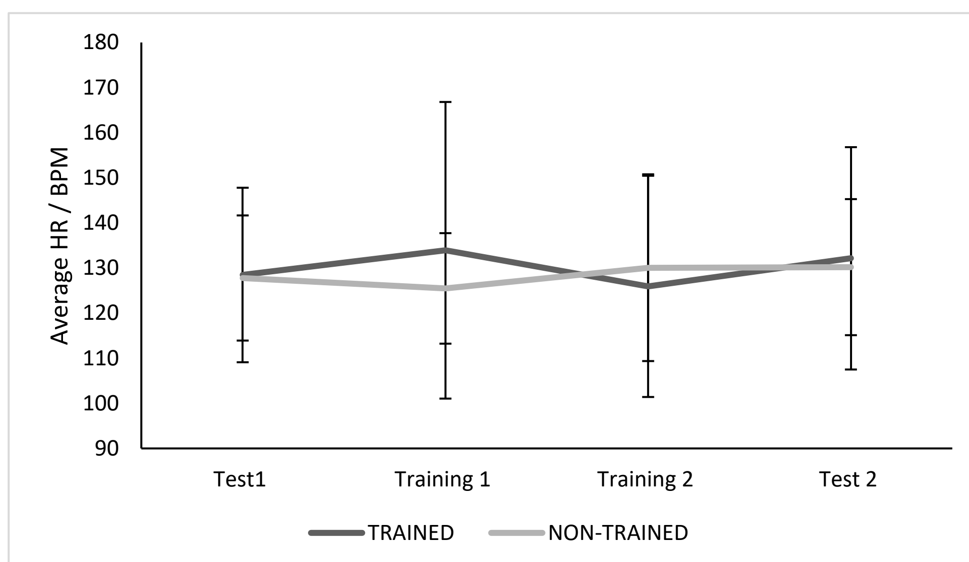


Figure 5. Shows the average HR for each group at each stage of the experiment intervention. Error bars show the standard deviation of the mean.

RELATIVE HEART RATE

When the HR data taken during swimming exercise was expressed as a percentage relative to their HR measured at $VO_{2\ max}$ during the baseline $VO_{2\ max}$ testing, the graphical data in figure 6 observes the non-trained group of swimmers working at a higher percentage of their $VO_{2\ max}$ HR during the swimming exercise in comparison to the trained group. Statistical analysis revealed the differences between the two groups for their relative HR were non-significant (Test 1: M= 71.78%, SD= 10.77% (trained) M= 78.25%, SD= 15.49% (non-trained), p= .382; Training 1: M= 75.23%, SD= 19.85% (trained) M= 77.32%, SD= 18.30% (non-trained), p= .846; Training 2: M= 70.34%, SD= 13.20% (trained) M= 78.82%, SD= 10.97% (non-trained), p= .226; Test 2: M= 74.25%, SD= 15.90% (trained) M= 79.85%, SD= 16.88% (non-trained), p= .535). No statistically significant results were found within the participant groups at each stage of the intervention (p= 1.00 for all within group comparisons).

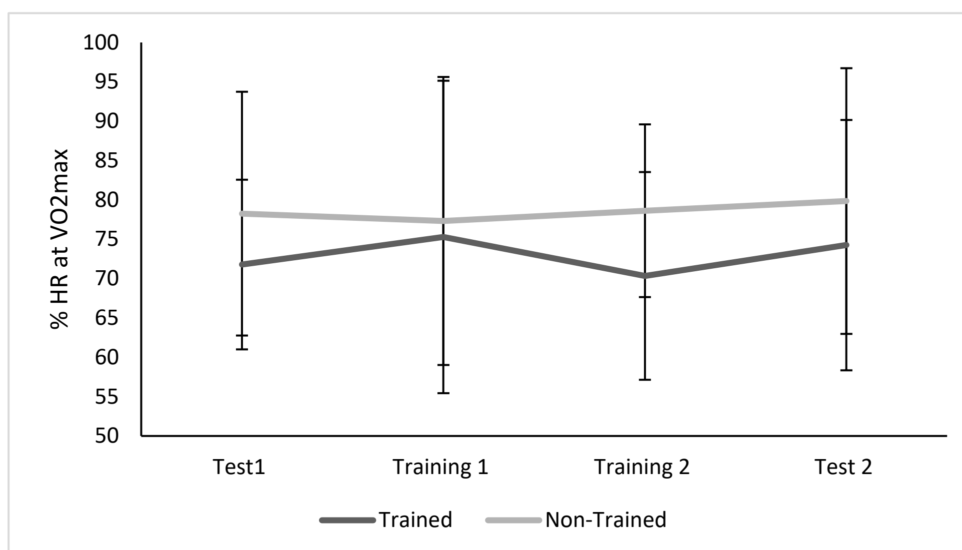


Figure 6. Shows relative HR results when the absolute HR data at each stage of the intervention was expressed as a percentage of the participants HR taken at their $VO_{2\ max}$. Error bars show the standard deviation of the mean.

ENERGY EXPENDITURE

Following the observation of the relative HR data being higher in the non-trained group, energy expenditure was explored. Explanation of how this was calculated, and the rationale are explained previously in the methods section. The data obtained from the calculations is represented in figures 7 and 8. Statistical analysis shows there is no significant difference between the two groups for energy expenditure at $VO_{2\ max}$ (M= 18.40 kcal, SD= 2.23 kcal (trained), M= 17.08 kcal, SD= 3.24 kcal; $t(12) = .884$, $p= .394$) (figure 7). There are also no statistically significant differences in energy expenditure between the two groups at each stage (Test 1: M= 13.17 kcal, SD= 2.55 kcal (trained) M= 13.13 kcal, SD= 2.24 kcal (non-trained), p= .974; Training 1: M= 13.92 kcal, SD= 3.38 kcal (trained) M= 12.96 kcal, SD= 2.69 kcal (non-trained), p= .602; Training 2: M= 12.899 kcal, SD= 2.69 kcal (trained) M= 13.19 kcal, SD= 1.57 kcal (non-trained), p= .808; Test 2: M= 13.616 kcal, SD= 3.29 kcal

(trained) M= 13.527 kcal, SD= 3.18 kcal (non-trained), SD= 1.225, p= .960), nor are there any significant differences within the groups (p= 1.00 for all within group comparisons).

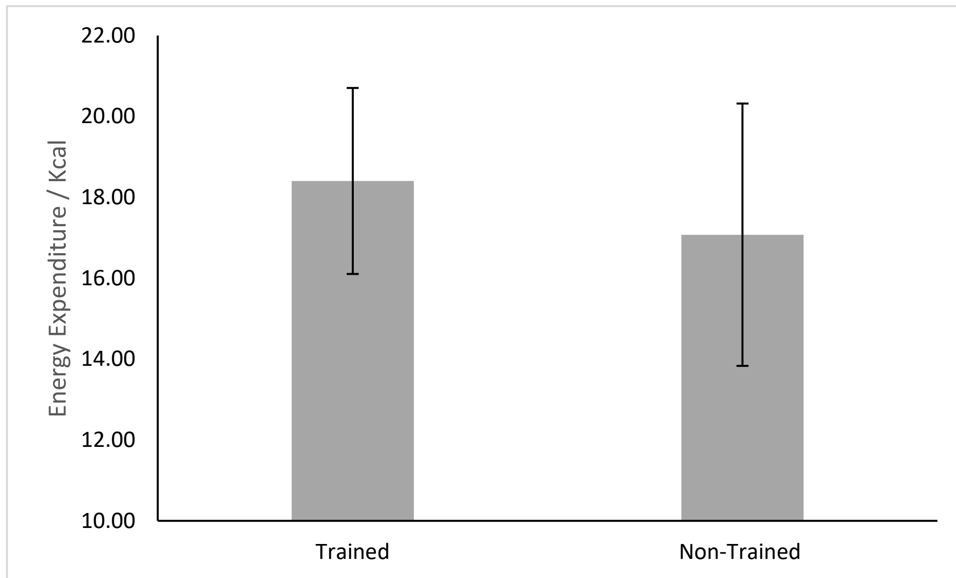


Figure 7. Shows the estimated energy expenditure calculated at $VO_{2\ max}$ for both the trained and non-trained swimmer groups. Error bars show the standard deviation of the mean.

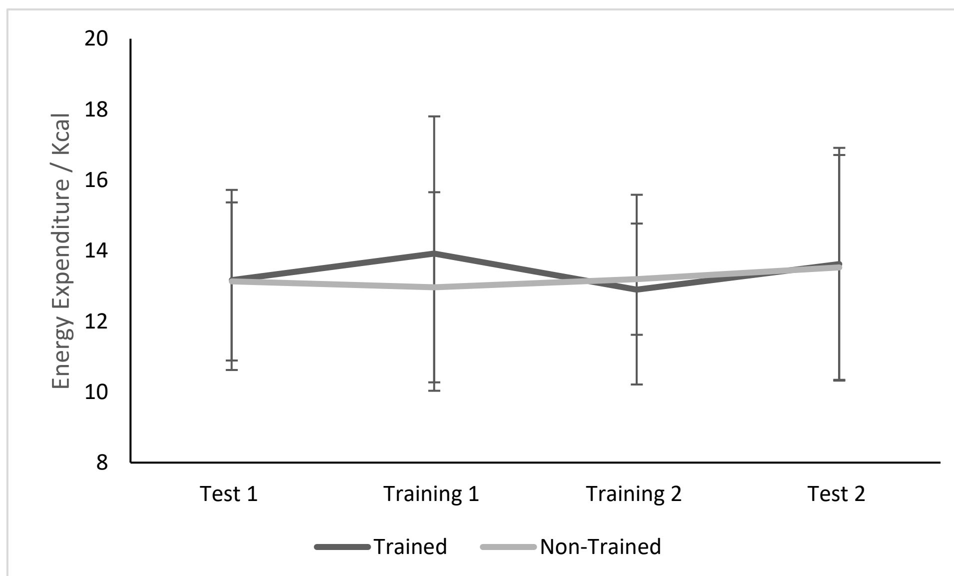


Figure 8. Shows the energy expenditure calculated for each stage of the intervention protocol. Error bars show the standard deviation of the mean.

XANTHINE OXIDASE ACTIVITY

The XO activity assay results were expressed as a percentage change from the first scan of the plate to the second scan following the twenty-minute incubation period as instructed. This provided information on the relative activity of XO before, during and after exercise. Results from this assay are presented in figure 9.

A statistically significant difference was found between the two groups for average XO activity (mean values of all activity samples taken), with the non-trained swimmer group having a significantly greater average XO activity than the trained group's total XO activity (M= 41.16%, SD= 1.61% (trained), M= 46.95%, SD= 1.82% (non-trained), P= .021) (figure 10).

However, despite a significant difference in average XO activity between the groups, no significant differences were found within the groups between the samples taken in each test and no differences were found between the samples across test one and test two (p= 1.00 for all comparisons in both groups). No significant differences were found between the two groups for all samples taken pre, mid and post exercise for both tests (Test 1/pre: M= 38.44%, SD= 7.45% (trained) M= 45.87%, SD= 6.34% (non-trained), p= .067; Test 1/mid: M= 41.11%, SD= 7.14% (trained) M= 47.23%, SD= 6.49% (non-trained), p= .119; Test 1/post: M= 40.40%, SD= 11.53% (trained) M= 46.50%, SD= 12.36% (non-trained), p= .359; Test 2/pre: M= 42.94%, SD= 5.05% (trained) M= 49.97%, SD= 8.88% (non-trained), p= .094; Test 2/ mid: M= 41.714%, SD= 6.23% (trained) M= 44.57%, SD= 9.15% (non-trained), p= .507; Test 2/post: M= 42.33%, SD= 6.01% (trained) M= 47.54%, SD= 9.91% (non-trained), p= .256).

No significant differences were found between the two groups for the percentage change between exercise test one and test two (Table 4). Increases from test one to test two were observed for most samples except for the Mid-exercise sample in the non-trained group which actually showed a slight decrease.

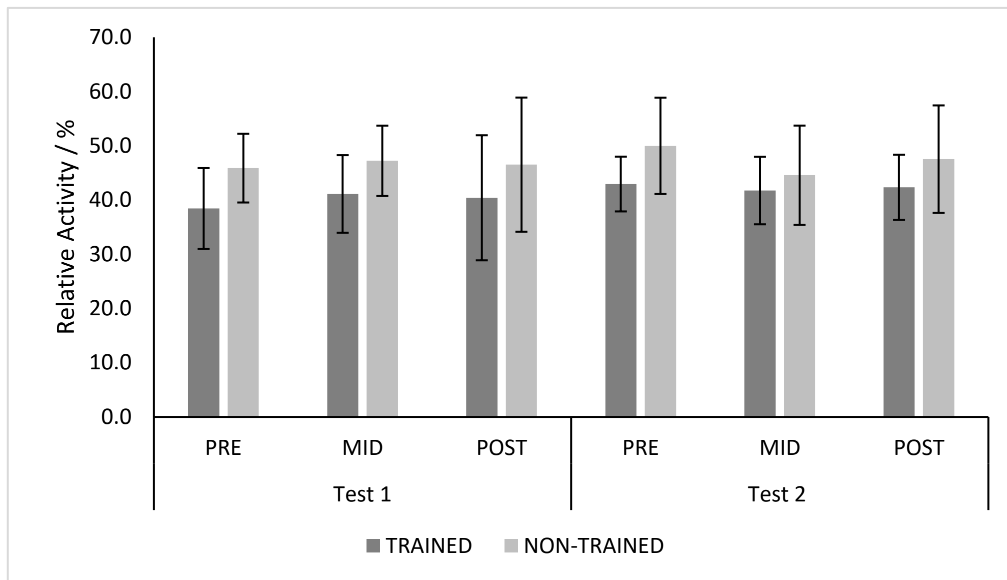


Figure 9. Graph shows the relative XO activity for samples taken pre-, mid-, and post-exercise in Test 1 and Test 2. Error bars show the standard deviation of the mean.

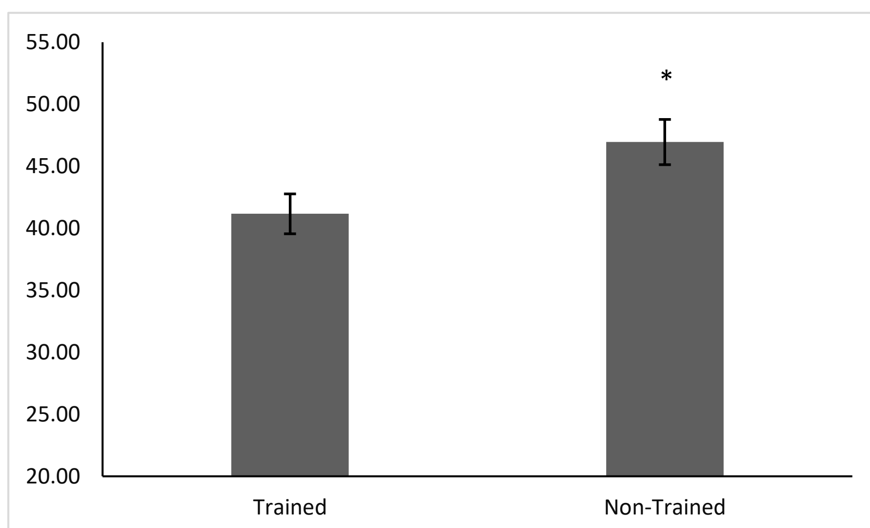


Figure 10. shows the mean activity of all activity samples, significance (*) indicates a significant difference between the two groups.

Table 4. Shows the mean percentage change between test one and test two in each group. No significant differences were found within or between the two groups.

% Change Test 1 to Test 2	**Trained**		**Non-Trained**		
	Mean	**SD (+/-)**	**Mean**	**SD (+/-)**	**P Value**
Pre	113.6	12.3	111.4	30.0	.862
Mid	105.5	29.8	94.2	13.4	.379
Post	119.1	60.2	112.0	46.5	.809

XANTHINE OXIDASE EXPRESSION

Western blot images were obtained as described using Licor imaging software. Images depicting the raw western blot data are shown in image 1. Using a negative control, we identified a band that appeared on the sample blots that was not on the negative control (image 2). We used the top band of the sample blots as a loading control to normalise that data when quantifying the blots (images 1 and 2) as this band appeared in all blots consistently.

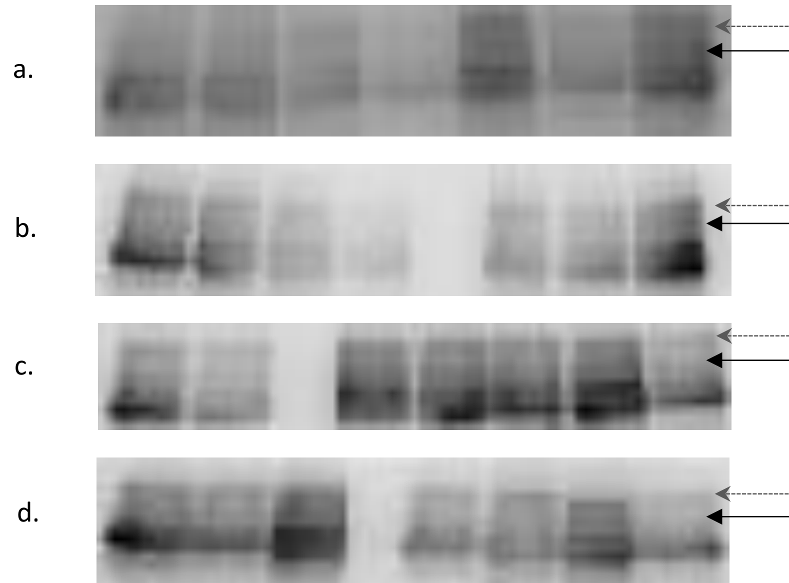


Image 1. Depicts the western blot images for XO protein. (a.) is for the trained group of swimmers pre-intervention and (b.) is the same group post-intervention. (c.) is for the group of non-trained swimmers pre-intervention and (d.) is the same group post intervention. The solid arrow indicates the band for XO and the dashed arrow indicates the band used to normalise the quantification for analysis.

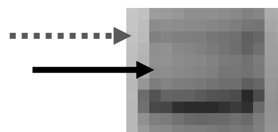


Image 2. The negative control western blot sample. The solid arrow indicates where the XO band sits on the sample blots that is missing from this blot image. The dashed arrow indicates the band that appeared on all samples that was used to normalise the data (respective bands for each sample).

The quantified data obtained from the images is represented in the graph in figure 11. From these data, we observe a statistically significant increase in the amount of XO protein content

in trained group of swimmers in the pre- to post-intervention samples (M= 0.763, SD= 0.043 (pre) M= 0.857, SD= 0.077 (post), p= .017). The non-trained group showed no significant change in XO protein content in the pre- to post-intervention samples (M= 0.820, SD= 0.049 (pre) M= 0.832, SD=0.076 (post), p= .738).

There was a significant difference between the two groups in the pre-intervention samples (M= 0.763 (trained) M= 0.820 (non-trained), SD= .024, P= .036), showing the trained swimmers had less XO protein content pre-intervention than the non-trained group of participants. There was no significant difference between the two groups in the post-intervention samples (M= 0.857 (trained) M= 0.832 (non-trained), SD= .041, p= .559). The mean XO content of the two samples measured was not significant between the two groups (M= 0.810, SD= 0.066 (trained) M= 0.826, SD= 0.008 (non-trained), p= .503).

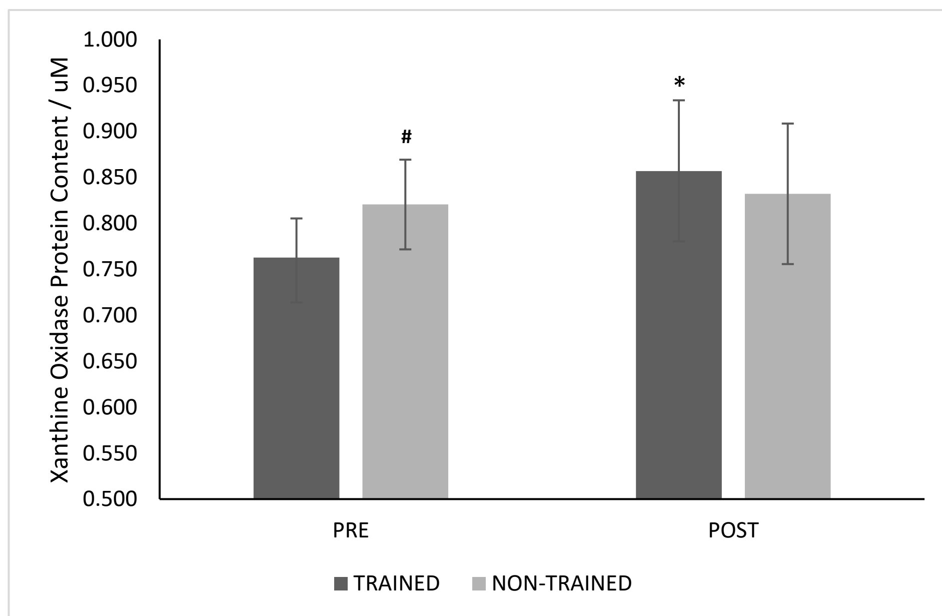


Figure 11. Shows the quantified data from the western blot images for trained and non-trained swimmers pre and post intervention. Significant increase in protein content in the trained group of swimmers from pre- to post-intervention is indicated with an (*). The

significantly higher protein content in the non-trained group for the pre-intervention sample is indicated on the graph by (#). Error bars show the standard deviation of the mean.

TOTAL ANTIOXIDANT CAPACITY (FRAP ASSAY)

The results of the FRAP assay for total antioxidant capacity are displayed in figure 12. The data shows an increase in total antioxidant capacity in the group of trained swimmers, whereas the TAC for the non-trained group shows little change from pre- to post-intervention. Statistical analysis revealed no significant difference between the two groups for mean FRAP of analysed samples (M= 102.67, SD= 6.94 (trained) M= 102.21, SD= 0.64 (non-trained), p= .914), no significant difference between the two groups in both pre-intervention (M= 97.76, SD= 6.48 (trained, pre) M= 102.67,M SD= 11.23 (non-trained, pre), p= .335) and post-intervention samples (M= 107.59, SD= 13.20 (trained, post) M= 101.77, SD= 9.33 (non-trained, post), p= .360), and no significant difference within the groups pre- to post-intervention (trained, pre/post p= .088; non-trained, pre/post p= .867).

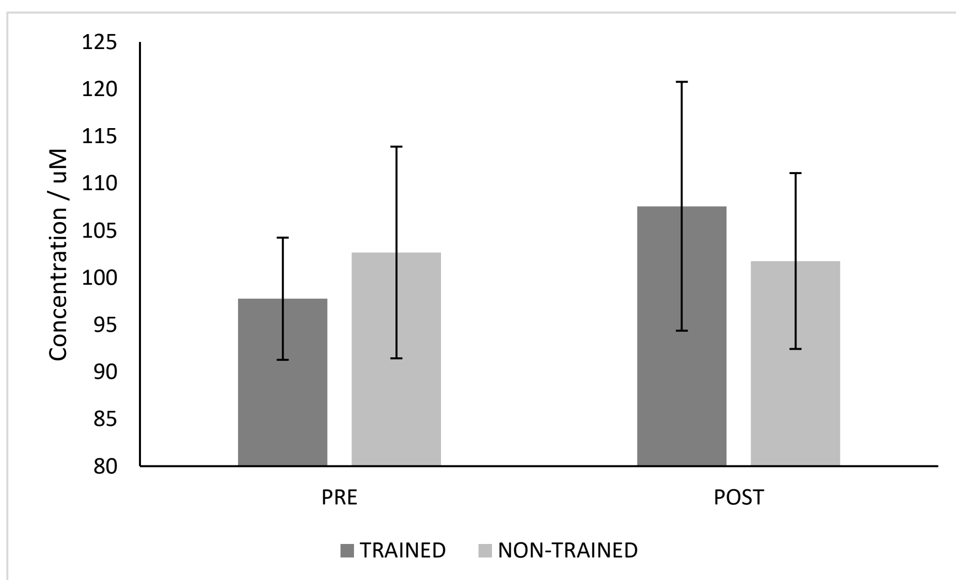


Figure 12. Displays the data for FRAP in the pre and post intervention blood samples. The graph demonstrates an increase in FRAP in the trained swimmer group samples, however analysis reveals no significant differences between or within the two groups. Error bars show the standard deviation of the mean.

DISCUSSION

The aim of this study was to investigate the activity and potential adaptative capacity of the enzyme XO in response to swimming exercise, comparing participants trained in competitive swimming to participants who are not trained, but purposefully active.

Results of the participant exercise data confirmed that the desired group conditions were successfully created. Baseline measures showed no significant differences in participant BMI and no differences in their $VO_{2\ max}$ (Table 3). These data show that both groups of participants were similarly fit and active and the difference between the groups is focused on their swimming ability and the response of XO to this mode of exercise specifically. Trained swimmers showed significantly faster swimming times for 50-metres in comparison to the non-trained swimmers with a mean difference of 15.23 seconds between the two groups, which was also stable throughout the experimental protocol (figure 3). The swimming exercise was required to be completed at high intensity to create the temporary condition of hypoxia required to activate XO. RPE (figure 4) reported by participants during the swimming exercise also showed no significant differences between the two groups, with each group reporting a mean RPE of 16 – defined on the scale as between 'Hard (Heavy)' and 'Very Hard'. This also highlights the effect of training in the trained group of swimmers, as they swam significantly faster for the same amount of perceived effort. As RPE is a subjective measure of effort (exposed to demand characteristics), HR data were also recorded as a more objective way to monitor the intensity participants were working at during swimming exercise. These data were also non-significant between the two groups and within the participant groups (figure 5), confirming RPE results suggesting that all participants were working at a similar intensity. Observation of the HR data calculated as a percentage of

VO_{2max} HR revealed a pattern to seemingly suggests that trained participants were working at a slightly lower percentage of their $VO_{2\ max}$ HR compared to the non-trained participants (Figure 6), however the difference between the two groups was not significant. This could suggest that the trained group were able to swim in a more efficient way compared to the non-trained group, due to their experience in swimming. In a review of literature on competitive swimming, Aspenes and Karlsen, (2012) found competitively trained swimmers have an increased respiratory capacity compared to their non-athletic counterparts and those of similar conditioning from other sports, due to the restriction of breathing during training. In addition, EE was not significantly different between the two groups at any point where measurements were taken throughout the intervention (figures 7 and 8). We chose to estimate the values of EE to further consolidate the data confirming that swimming training status was the sole difference between the two participant groups, despite there being a number of limitations and an estimation error to accept. We did so based on research that suggests HR is a good predictor of EE (Keytel *et al.*, 2005), and research showing the use of HR to predict EE at varying levels of exercise intensity (Hiilloskorpi *et al.*, 2003). The values are an estimate of the EE both at $VO_{2\ max}$ and especially during the swimming exercise, as gas analysis measurements were not taken during the swimming exercise. The measurement of VO_2 is the most influential in calculation of EE, as errors in this measurement have larger implications for errors in EE (Singer, 2016). Research on EE in swimming exercise was also consulted, showing VO_2 predicted in swimming is highly correlated to measured VO_2 (Costill *et al.*, 1985) and therefore a predictor of EE. The lack of difference between the participant groups is important to confirm suitable conditions to activate XO had been created and that swimming experience and technique were the only differences between the two groups.

It was hypothesised that XO activity would be greater in those who were not trained to swimming exercise and that an adaptive response would occur in both participant groups, with the greatest changes occurring amongst the non-trained participant group. Therefore, key findings to highlight from the blood sample measures include the results of XO activity, XO protein content, and total antioxidant capacity (FRAP) results.

Swimming exercise was able to induce some activation of XO as small increases in XO activity were observed from the pre-exercise sample in the mid- and post- exercise samples, compared to rest samples in both exercise tests, however these differences were not statistically significant. A significant difference was found between the two groups for the mean XO activity, of all samples analysed, with the non-trained group exhibiting a higher mean level of XO activity as a group than the trained group (figure 10). The non-trained group exhibited higher XO activity than the trained group across all samples taken during the exercise tests, however the difference between the two groups at each stage was not significant. Lower activity in trained participants compared to non-trained participants at rest, could be related to the differences in swimming frequency between the two groups, as those in the trained group were recruited because they swam eight or more hours per week, compared to the non-swimming trained condition who swam less than three hours per week. Therefore, there are differences in exposure to intense, hypoxic type swimming exercise between the two groups which may explain the differences at rest. Upon further analysis of the XO activity data, small increases in activity between the two exercise tests can be observed. This could suggest that with increased frequency of exposure to anaerobic, hypoxia inducing exercise training a change could be elicited in the enzyme’s activity. Statistical analysis revealed there were no significant differences found within the groups, showing no

differences between the first exercise test (test 1) and the second exercise test following the training sessions (test 2), evident from table 4 and figure 9.

The identification of the presence of XO protein in the samples, is corroborated by the image of blot data produced by Cappola *et al.*, (2001) (figure 3, page 2410). This blot shows the band identified as XO to be in the same position of the band identified in the present study, confirming the correct identification of XO on the blots. In the present study, significant differences were found in the XO protein content between trained and non -trained groups pre-intervention, with the trained group samples containing significantly less XO protein than the non-trained group samples. This result was expected and could be attributed to the greater exposure to hypoxia inducing exercise from competitive swimming training.

Contrary to our hypothesis, the trained group also exhibited a significant increase in the amount of XO protein from pre- to post-intervention, increasing to a similar level to the non-trained group as no significant differences were found between the two groups post-intervention. Observation of the graph would even suggest that the trained groups protein content exceeded the amount in the non-trained samples. The results of the XO protein content data were therefore largely inconclusive as the trained swimmers started the experiment with a significantly lower amount of XO present in their blood samples compared to the non-trained swimmers – which was expected in our hypothesis. The post intervention increase in XO protein was unexpected, whereas there was no significant change in the non-trained group. A possible explanation for this change could be related to the amount of training the trained groups of swimmers were taking part in outside of the protocol. The study utilised participants from the University swimming club therefore all participants were students. The point in the academic year that the study took place coincided with students gaining an increase in workload in preparation for exams, therefore, the amount of training

taken part in by the participants in the trained group may have decreased to allow for the increase in academic commitments. This would mean their exposure to swimming exercise and conditions suitable to activate XO will have decreased, similar to the amount swimming of the non-swimming trained group. This could explain why the results of the XO activity increased, as it is possible that they encountered effects that occur when training reduces or ceases (Ready, Eynon and Cunningham, 1981; Mujika and Padilla, 2000).

Interestingly, total antioxidant capacity (FRAP analysis) demonstrated an identical pattern to the XO protein content results (figure 12). Although no significant differences were found between or within the groups for the total antioxidant capacity results, unlike the significant changes found in the trained group XO protein, the results suggest changes in XO protein content prompt simultaneous with antioxidant capacity.

Regarding the aim of investigating a potential adaptive response of XO, the results obtained from the current experiment are inconclusive, possibly due to certain limitations surrounding swimming training volume as previously mentioned. The pre-intervention sample XO expression/protein content would suggest that swimming training causes a reduction in the level of XO protein present, as the concentrations were significantly lower than the non-trained group's samples at the same time-point. Over time, the non-trained group showed no change whereas the trained group showed a significant increase in XO protein. This contrasts the pre-intervention results as the concentration increased to a similar level to the non-trained group – a result that would lead to suggest that XO protein content increases with swimming training.

However, the identical pattern of the FRAP analysis is useful in confirming that XO has a contribution to antioxidant capacity. The results show that XO protein content is mimicked by

antioxidant capacity, which suggest that XO could play a potential role in the contribution of antioxidant defence. UA is a major contributor to antioxidant defences (Fabbrini *et al.*, 2014) and is included in the FRAP analysis (Benzie and Strain, 1996). In exercise, Green and Fraser, (1988) show serum UA significantly increases with supra-maximal exercise from pre- to mid-, to post-exercise, and also increases over consecutive days. As UA is the product of XO reaction with hypoxanthine (Enroth *et al.*, 2000; Ardan, Kovačeva and Čejková, 2004), and is antioxidant in nature (Waring *et al.*, 2003), this could provide support for the increase in FRAP observed with the increase in XO protein observed in the results of the present study. This would also be consistent with the lack of change in FRAP and XO protein in the non-trained participant group.

The results of the FRAP analysis could be suggestive of the potential contribution XO has to adaptive processes. Endogenous antioxidant enzymes have been shown to adapt with exercise training, often through the ROS stimulated transcription and consequential protein synthesis (Ji, Stratman and Lardy, 1988; Ji *et al.*, 2004). As the pattern of the FRAP analysis is so close to the pattern on the XO blot data, this could suggest that the ROS produced from XO is involved in the adaptive processes of the up-regulation of endogenous antioxidant enzymes such as superoxide dismutase and glutathione through ROS activation of NF-κB (Gomez-Cabrera *et al.*, 2006; Ji *et al.*, 2004). This would therefore be a beneficial property of XO action.

Higher XO activity level in the non-trained group is consistent with the concentrations of XO protein present in the blood. The XO activity of the trained swimmers is suggestive of an adaptive quality being evident. A key observation is the increase in XO protein content in the trained group from pre- to post-intervention was not reflected in the XO activity, as activity remained almost constant from the first to second test and was not significantly different from

those in the non-trained group. Further to this, activity did not significantly change from test one to test two, despite their post-intervention protein content increasing to a similar level as the non-trained group, activity remained the same and was still less than the non-trained group. These results would suggest that adaptation lies within the activity of XO, rather than in the content of the protein.

The limited change in XO activity found in the results could be due to the age of the participants recruited. The participant cohort consisted of young, healthy individuals aged between eighteen and twenty-five. Research by Lambertucci *et al.*, (2007) measured XO activity in young and aged rats, finding no change in XO activity amongst the young rats with exercise training. The results did show a significant decrease in XO activity resulting from exercise training in the aged rats. In addition, training caused an increase, however non-significant, in antioxidant glutathione peroxidase in the young rats, and showed no change in the aged rats, therefore the lack of change in XO activity could be due to an increase in antioxidant defence in the young rats. The results from the present study are also consistent with these findings. The participants were young individuals, showed an increase in total antioxidant capacity and no change in XO activity with training.

Dupont *et al.*, (1992) investigated XDH and XO activity and gene expression in cultured rat endothelial cells. Their research found new protein is need for XDH and XO activity to increase, deriving from an increase in XDH/XO mRNA, concluding that new protein is required to elicit an increase in XDH and XO activity. The results obtained from the non-trained group are consistent with this as their protein content did not change and neither did their XO activity.

A study by Poss *et al.*, (1996) investigated *in vitro* hypoxia stimulated cultured bovine aortic endothelial cells, measuring for XDH and XO activity, gene and protein expression. In their results, they found a significant two-fold increase in the amount of activity of both XDH and XO in response to hypoxia, however no significant differences in both gene and protein expression, suggesting that hypoxia induces post-translational modifications that increase activity of the enzymes. Research by Terada *et al.*, (1997) also supports this notion of post-translational modification of XO by hypoxia, finding significant increases in XO activities under hypoxic conditions in cultured cells derived from a handful of different species. This could explain the results found in the present study, as the results show the activity of XO in the trained participants to be seemingly independent of the amount of XO protein content. This would suggest that swimming exercise created conditions of hypoxia in both groups of participants. The low protein content of the trained swimmers is supportive of this research, as the amount of activity did not change even with more protein present.

Further to this, research previously outlined by Hellsten *et al.*, (1997) demonstrated increases in XO attributed to an increase in expression of the enzyme protein. An eightfold increase in XO was reported by the group, which was observed four days post eccentric type exercise. The results in the present study also show an increase in protein expression in the trained group of participants, however the timing of our sampling could have had an impact on the results. We took a blood samples 24 hours post intervention, which although previous research (Hellsten *et al.*, 1997) and our results show significant increases in XO protein at this time point, the peak in expression in Hellsten et al.'s research is observed later at 96 hours post exercise. A limitation therefore is that sampling was limited and perhaps with more sample time points, more XO expression would be observed.

IMPLICATIONS

Trained participants show lower XO activity than non-trained participants (pre-intervention), throughout the four-week intervention. This would suggest that longitudinal swimming training, and repeated induction of hypoxic conditions associated with competitive swimming training (Aspenes and Karlsen, 2012), leads to a more controlled activation of the enzyme's activities, therefore reducing the amount of radical oxygen species produced from this extracellular source. This appears to be independent of the protein content of XO as this changed significantly in the trained group, yet activity did not change. The addition of swimming exercise in the not swimming trained group did not have any significant effects on activity or protein content, possibly due to the acute nature of the study.

Broadly, XO is most influential in the vasculature and is highly associated with ischaemic reperfusion injury (Friedl *et al.*, 1990) due to the paradoxical nature of the enzyme and the ischaemic conditions created in the pathology of some vascular diseases. Decreasing XO activity through exercise training therefore could have beneficial implications for some disease pathophysiology, particularly diseases with associated with endothelial dysfunction. Evidence of XO having a role in disease pathophysiology has been researched in Chronic Heart Failure (CHF) (Doehner *et al.*, 2002), metabolic syndrome (Feoli *et al.*, 2014), and diabetic complications (Liu *et al.*, 2015) to name a few.

Research has suggested XO activity is increased in such conditions, and that inhibition of XO with allopurinol can be used in the treatment of disease pathology (Doehner *et al.*, 2002; Butler *et al.*, 2000), however if exercise could reduce the action of XO, or even maintain the levels of activity as shown in the results of the current study, then this could provide a method of treatment for some mild cases of endothelial dysfunction disease states before drug

treatments become necessary and could also provide a longer-term preventative therapy. The utilisation of exercise to reduce or maintain XO activity as an alternative to oral allopurinol administration would also be beneficial on other beneficial adaptation to exercise such as increases in antioxidant enzyme superoxide dismutase and increases in $NO^{\bullet}$ synthases eNOS and iNOS (Gomez-Cabrera *et al.*, 2005), as research shows that allopurinol is detrimental to such signalling (Wadley *et al.*, 2013) and adaptive processes (Gomez-Cabrera *et al.*, 2005).

As the activity of XO can produce large amounts of superoxide, which can then interact with $NO^{\bullet}$ synthesis – should both ROS and $NO^{\bullet}$ production occur in close proximity - leading to the rapid formation of the powerful oxidant, peroxynitrite (Pacher, Beckman and Liaudet, 2007). This formation has an impact on the endothelial dysfunction (Berry and Hare, 2004) as it can impair the endothelium-derived relaxant factor of $NO^{\bullet}$, leading to an increase in blood flow (Beckman and Crow, 1993). It has been shown that allopurinol inhibition of XO improves endothelial dysfunction via $NO^{\bullet}$-stimulated blood flow in smokers (Guthikonda *et al.*, 2003) and in diabetic populations with hypertensive traits, returning blood flow to normal levels (Butler *et al.*, 2000). Therefore, the results of the current study showing a lower level of XO activity in swimming-trained participants, implies that reduction of XO activity with this type of exercise training, could have a positive effect on the regulation of blood flow via $NO^{\bullet}$ mediated responses, *via* decreased release of ROS. Because activity did not change with the changes in XO protein content demonstrated in the results, this could suggest that the adaptation of swimming exercise on XO activity is independent of protein content.

As previously mentioned, UA is a powerful scavenger of peroxynitrite. Administration of UA has shown to have a greater antioxidant impact than the administration of antioxidant compound vitamin C (W. Waring, J. Webb and R. J. Maxwell, 2001), and high UA concentrations are associated with increased total antioxidant capacity and decreases in

oxidative stress following acute exercise (Waring *et al.*, 2003). The results demonstrate an increase in total antioxidant capacity, following the same pattern to the increase in XO in both groups of participants. This suggests that XO contributes to TAC and would therefore be a beneficial action of the enzyme on health to increase clearance of peroxynitrite. Although this apparent relationship between XO and antioxidant capacity needs further exploration, it provides a platform for further research to investigate whether XO is influential on antioxidant enzymes and their regulatory processes. This could therefore have a positive impact on health to combat oxidative stress in populations concerning some of the outlined disease states.

FUTURE DIRECTION

Given the results and the limitations of this study, future investigation should look at the response of XO to more frequent swimming exercise bouts. It would be helpful to measure adaptive markers, such as signalling markers like NF-κB, which has been cited multiple times to have a role in the up-regulation of antioxidants (Gomez-Cabrera *et al.*, 2005; Gomez-Cabrera *et al.*, 2006; Ji *et al.*, 2004), to determine activation of specific pathways that could involve XO in an adaptive role.

Testing trained and untrained participants in older age, and in disease pathology may also prove to be more explicit and provide insight to a potential use of exercise as a preventative measure of disease in older age or as a treatment in disease states. As suggested in research in rodents (Lambertucci *et al.*, 2007), XO activity did not change in young healthy rats. Therefore, repetition of the current study in a population of older aged participants, or in a population of participants with mild endothelial dysfunction could prove to show a more definitive change in the activity.

As discussed, the influence of XO ROS production on the up-regulation of antioxidant enzymes following the results of the XO blot data and FRAP analysis, provides a platform for future research investigating whether ROS produced from XO leads to regulation of these antioxidant proteins through ROS signalling pathways. Research should also investigate how much UA catalysed by XO contributes to TAC, and whether this is beneficial to health.

CONCLUSION

The present study assessed the response of XO activity and protein content in trained and non-trained human participants, fulfilling the aims of the study. The novelty of this study is that previous research using swimming, and research investigating XO, is mostly undertaken using animal models of rats or mice, and it is rare that swimming exercise is utilised as a modality involving human participants, despite swimming being a large participation sport.

The findings of this study suggest XO activity in young healthy individuals does not change, consistent with previous research in rodent studies. It was also found that XO protein content/expression can significantly change, without significantly inducing an alteration in the activity of the enzyme.

It is clear the multitasking action of XO does play a role in exercise and could have benefit to our health. XO is source of ROS, however with the correct level of activity such as in healthy individuals, has the capacity to lead to the activation of cellular signalling processes to regulate antioxidant proteins amongst others. The results suggest prolonged exposure to swimming exercise, such as in competitively trained individuals could have an effect on the oxidative activity of XO. In addition, XO catalyses the reaction that produces a highly powerful antioxidant molecule, that evidently increases our antioxidant capacity. This study

also demonstrated changes in antioxidant capacity with changes in XO protein content, which should be considered for further investigation to determine the relationship between XO's oxidant and antioxidant capacity and how this could be exploited to benefit health.

Further research is required to fully understand the role of XO and to determine whether swimming exercise can stimulate any beneficial adaptive effects on the activity of XO or the protein concentrations. This study looked at young, healthy individuals, however it would also be interesting to investigate the response of XO to exercise in other populations – such as an older, disease state population and in populations where swimming may be more practical - to investigate whether XO has an impact on endogenous antioxidant defence through ROS signalling to have a positive impact on our health.

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