EXERCISE, OXIDATIVE STRESS, AND INFLAMMATION

THE EFFECT OF ACUTE EXERCISE ON THE PRODUCTION OF REACTIVE OXYGEN SPECIES AND INFLAMMATORY MARKERS IN HEALTHY PRE-PUBERTAL AND ADULT MALES

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TITLE: The effect of acute exercise on the production of reactive oxygen species and inflammatory markers in healthy pre-pubertal and adult males

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ABSTRACT

An acute bout of exercise causes short-term changes in the immune system in both children and adults. It has been well-established that exercise induces an inflammatory response. Especially in children, cytokines play an important role in balancing anabolic and catabolic processes of growth. Existing evidence suggests cross-talk between inflammation and oxidative stress. Reactive oxygen species are also found to transiently increase in response to exercise, affecting muscle adaptation post-exercise. Characterizing the exercise-induced inflammatory and oxidative stress responses in children compared to adults will start clarifying the transition from the child phenotype to that of an adult. Ten children aged 8-10 and 12 adults aged 19-21 performed 2×30min bouts of continuous cycling, separated by a 6min rest period, at a target work rate of 60% of their maximum aerobic capacity. Blood samples were collected pre-exercise and immediately post-exercise, and analyzed for neutrophil count, systemic oxidative and inflammatory markers (tumor necrosis factor alpha, interleukin 6, protein carbonyls, malondialdehyde, elastase), intracellular neutrophil-derived reactive oxygen species (using 3 fluorescent markers detected by flow cytometry), and *in vitro* production of neutrophil-derived myeloperoxidase and interleukin 8. Compared to the post-exercise increase in absolute neutrophils in men, boys showed no change. However, intracellular neutrophil reactive oxygen species production increased for boys and not for men. Boys also demonstrated higher overall protein carbonyl levels, whereas men showed higher overall malondialdehyde. Both boys and men showed a positive correlation between tumor necrosis factor alpha and elastase, with a steeper slope seen in boys. Although

there were other correlations observed in boys and men, no others existed in both. The differences observed in the exercise-induced inflammatory and oxidative stress response may indicate growth-mediated adaptive responses to exercise during childhood development.

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DEDICATION

This thesis is dedicated to my family who has walked with me every step of the way.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
DCFH	2',7'-dichlorfluorescein-diacetate
DHR	Dihydrorhodamine 123
ELISA	Enzyme-linked immunosorbent assay
H_2O_2	Hydrogen peroxide
HE	Hydroethidine (or dihydroethidine)
IL-6	Interleukin 6
IL-8	Interleukin 8
$O_2^{\cdot -}$	Superoxide anion
PMA	Phorbol 12-myristate 13-acetate
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor alpha

CHAPTER 1: INTRODUCTION

Davies et al. (1982) were the first group to quantify and show an increase in free-radical production after acute exercise (Davies, Quintanilha, Brooks, & Packer, 1982; Finaud, Lac, & Filaire, 2006). This fundamental work, which started out in an animal model, has quickly become a very popular area of exercise research in human models (Finaud et al., 2006). Much of the literature has shown that short-term acute exercise does indeed increase free-radical production, specifically reactive oxygen species (ROS) production in humans (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). Compared to the resting state, the body requires more oxygen during exercise and this leads to a transient increase in mitochondrial function and ROS generation (Finaud et al., 2006; Gougoura et al., 2007; Nikolaidis et al., 2007; Timmons & Raha, 2008). Although high levels of ROS can possess detrimental effects to adult health, emerging research has suggested a positive role in children's growth and development (Timmons, 2006; Timmons, Tarnopolsky, Snider, & Bar-Or, 2006).

1.1 ROS generation

Free radicals possess one or more unpaired electrons, thus rendering them very unstable and susceptible to react with other molecules (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). ROS are a sub-population of free-radical molecules that originate from oxygen. There are many types of ROS but the important ones for this thesis are the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2).

ROS production originates from both exogenous and endogenous sources. Pollution, radiation, hyperoxia, xenobiotic consumption, and chemical reactions in the ozone are responsible for most exogenous sources of ROS (Finaud et al., 2006; Klaunig et al., 1998). However, the bulk of scientific research has focused on both unprogrammed and programmed ROS production in the body.

During heightened metabolic processes such as exercise, there is an increased flux of ROS that are formed as a by-product (Fisher-Wellman & Bloomer, 2009). The majority of the unprogrammed ROS pool is generated by the mitochondria, specifically through leakage from complexes 1 and 3 as electrons pass through the electron transport chain (Finaud et al., 2006). Non-mitochondrial sources also contribute to the unprogrammed production of ROS through the xanthine oxidase enzyme during purine catabolism (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009; Timmons & Raha, 2008).

One of the main foci of this thesis is the programmed endogenous production of ROS in response to exercise. Neutrophils are phagocytic cells involved in the first line of defence against foreign molecules (Pyne, Baker, Smith, Telford, & Weidemann, 1996; Walrand et al., 2003). During the immune response, they are recruited to the site of inflammation and proceed with a microbicidal reaction termed neutrophil oxidative burst (Pyne et al., 1996; Smith & Weidemann, 1993; Walrand et al., 2003). These immune reactions induce neutrophil production of $O_2^{\cdot-}$, and H_2O_2, used to produce the antimicrobial agent, hypochlorous acid (Finaud et al., 2006; Smith & Weidemann, 1993; Walrand et al., 2003). The above-mentioned intermediate and end products from the

oxidative burst contribute to the endogenous ROS pool when neutrophils are recruited in response to inflammation (van Eeden, Klut, Walker, & Hogg, 1999).

1.2 Oxidative stress: positive and negative effects

Because of their highly reactive nature, the body has developed antioxidants to counteract the damaging effects of ROS (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). Various enzymatic and non-enzymatic antioxidants are involved in either stopping or slowing down ROS activity, essentially preventing the accumulation of ROS to harmful levels (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009; Klaunig et al., 1998; Thomas, 2000). When ROS activity surpasses antioxidant capacity, a state of oxidative stress is reached (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009; Sen, 2001). Thus ROS levels are used as an indication of the severity of oxidative stress in the system, and measuring ROS levels provides a quantifiable assessment of the body's oxidative stress (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009).

Chronically-high levels of oxidative stress can induce negative downstream complications, causing disruptions in cell function and regulation (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009; Quindry, Stone, King, & Broeder, 2003). Downstream effects of ROS reactions can result in, among others, loss of membrane function, inflammation, dysregulation of protein homeostasis, and DNA mutations (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009; Klaunig et al., 1998; Nasca, Zhang, Super, Hazen, & Hall, 2010). Oxidative stress has therefore been linked to

various diseases such as arthritis, chronic obstructive pulmonary disease, and cancer (Fisher-Wellman & Bloomer, 2009; van Eeden et al., 1999; Woo et al., 2010).

Despite these adverse effects, novel research has given insight into the positive aspects of oxidative stress (Sen, 2001). ROS affect many important metabolic processes through both direct interaction and secondary messengers (Castro Fernandes, Bonatto, & Laurindo, 2010). They are involved in enzyme activation and drug detoxification (Reid, 2001). In addition to their potent role in neutrophil antibacterial defense, ROS have also been widely shown to regulate functions such as muscle development, blood flow, and contractile processes (Reid, 2001; Sen, 2001). Studies looking at oxidative stress in skeletal muscle indicate an optimal ROS level for maximal isometric force production (Reid, 2001). In light of these multi-functional effects of ROS, it seems that there exists a constant regulation of ROS to keep levels at homeostasis in order to maintain health. Investigating the changes in ROS in both children and adults, especially after exercise stimulation, will contribute to understanding how this regulation evolves during growth.

1.3 Potential link between oxidative stress and inflammation

New research has begun to show a link between oxidative stress and regulation of various gene transcription processes, especially downstream cytokine gene transcription (Castro Fernandes et al., 2010; Timmons & Raha, 2008). In addition to muscle contraction or muscle injury, acute exercise also induces a transient rise in inflammatory cytokines, including tumor necrosis factor alpha (TNF-α) and interleukin 8 (IL-8) (Quindry et al., 2003; Reid & Li, 2001; Timmons & Raha, 2008). Parallel to this, there is

also an observed increase in free-radical generation post-exercise, including ROS (Reid & Li, 2001; Timmons & Raha, 2008). Although the direct connection between exercise, ROS generation, and cytokine production has not been well established to date, there have been proposed models suggesting possible cellular links.

TNF-α is a polypeptide cytokine involved in the body's antitumour response and immune cell recruitment (Timmons & Raha, 2008). Reid and colleagues postulated that through oxidative activation of nuclear factor-κB (transcription factor), TNF-α induces muscle protein loss (Reid & Li, 2001). Further studies in this lab showed that through individual administration of TNF-α and antioxidants (indicating ROS involvement), skeletal muscle force contraction decreased and increased respectively (Reid & Li, 2001). Ryan and colleagues, using bacterial lipopolysaccharide interaction with macrophage receptors, found that nuclear factor-κB demonstrated downstream gene transcription regulation of the proinflammatory cytokine IL-8 (Ryan, Smith, Sanders, & Ernst, 2004). IL-8 is a potent neutrophil chemoattractant, which may cause further ROS production through the oxidative burst pathway, as discussed previously (Quindry et al., 2003; Timmons & Raha, 2008). Taken together, these data suggest a link between cytokine production and oxidative signalling in controlling muscle function.

1.4 Inflammation and oxidative stress after exercise in children

An acute bout of exercise causes short-term changes in the immune system in both children and adults (Timmons & Raha, 2008). The inflammatory response due to exercise has been an increasing area of interest in research due to its numerous

downstream effects in the body's physiology, especially when implicated in disease. Although much research has been done on the effects of exercise on inflammation in adults, the literature on children's exercise is scant (Cooper, Nemet, & Galassetti, 2004). This is surprising given the possible role that inflammatory mediators may play in children's growth (Cooper et al., 2004; Timmons & Raha, 2008).

Cytokines, primarily involved in the inflammatory response, play a large role in balancing the anabolic and catabolic processes of growth (De Benedetti et al., 1997; Timmons, 2006; Timmons & Raha, 2008). Rate of growth is at its highest in children, and thus regulation of these cytokines is especially important for healthy development (De Benedetti et al., 1997; Timmons, 2006; Timmons & Raha, 2008). This is apparent in children presenting with chronic inflammatory diseases and in transgenic mice expressing high levels of interleukin 6 (IL-6), where both groups are characterized by stunted growth (De Benedetti et al., 1997). However, presence of IL-6 during growth is also crucial as it is involved in regulating vascular endothelial growth factor expression (important in angiogenesis) (Cohen, Nahari, Cerem, Neufeld, & Levi, 1996; Timmons & Raha, 2008). Thus for healthy development in children, it is evident that cytokines must be tightly regulated in order to achieve the desired anabolic and catabolic balance.

Relatively few studies have been done in the area of immunological change due to exercise in children; even fewer studies have looked into oxidative stress due to exercise in children. Although oxidative stress is most often interpreted in the context of being detrimental to adult health, it has been speculated that oxidative stress generated through exercise, may play a role in children's muscle development (Timmons, 2006; Timmons et

al., 2006). Nikolaidis and colleagues tested 22 pre-pubertal swimmers (11 males and 11 females) for ROS production after a sub-maximal intensity exercise protocol (Nikolaidis et al., 2007). Post-exercise data, using a combination of ROS and antioxidant markers, indicated an increased oxidative stress profile (Nikolaidis et al., 2007). Two other studies, testing prepubescent and pubescent male participants with an acute bout of exercise or participation in a track and field training camp, also resulted in an increase oxidative profile (Benitez-Sillero et al., 2009; Nasca et al., 2010). In studying the oxidative stress and inflammatory response due to acute exercise comparing boys to men, not only will this thesis contribute to understanding adaptive exercise effects in the pediatric population, it will also start elucidating physiological differences in the transition from child to adulthood. This will provide unprecedented insight into the possible mechanisms relating exercise and healthy childhood development.

1.5 Purpose

Despite the depth in adult literature in terms of exercise and the inflammatory and oxidative stress response, similar responses in children still require extensive examination. This thesis focused on oxidative stress generation after acute bouts of cycling exercise in healthy pre-pubertal boys and adult men. Although there is apparent cross-talk between cytokine and ROS production, more research is needed to understand the cellular mechanisms involved in the adaptive response to exercise. There is a significant gap in the literature between adults and children in terms of exercise and oxidative stress generation. This thesis attempted to start bridging that gap and clarifying

the transition from child to adult, and the extent to which exercise-induced ROS generation may be linked to the normal growth process.

1.6 Objectives

1. The primary objective was to compare the production of exercise-induced ROS as measured by 1) intracellular levels, 2) systemic levels, and 3) neutrophilic (cellular) production between boys and men.

2. The secondary objective was to assess the strength of association between systemic inflammatory cytokines and oxidative stress markers.

1.7 Hypothesis

1) ROS production will increase as a result of exercise across both groups; however, like the blunted exercise-induced cytokine response that is characteristically observed in children, boys will show a blunted increase in ROS production compared to adult participants.

2) Inflammatory markers will be closely correlated with ROS markers, showing similar changes as a result of exercise.

CHAPTER 2: LITERATURE REVIEW

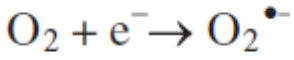
2.1 Exercise and ROS production

2.1.1 Unprogrammed ROS generation

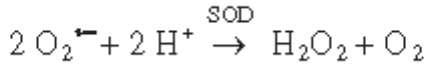
Intense physical activity of any nature where adenosine triphosphate (ATP) is generated through muscle recruitment can lead to a high production of free radicals during exercise (Finaud et al., 2006; Sen, Packer, & Hänninen, 2000). Free radicals are molecules that are capable of existing with one of more unpaired electrons rendering them very unstable, often causing addition of electrons to other molecules which also makes them highly reactive (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). ROS, though they can be non-radical species, are a subgroup of free radicals stemming from oxygen (Fisher-Wellman & Bloomer, 2009). Exercise results in physiological increases in both ROS and reactive nitrogen species. $O_2^{\cdot -}$ and H_2O_2 make up the largest portions of ROS produced as a result of exercise (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009).

Most unprogrammed ROS are by-products from oxygen metabolism through the electron transport chain, a process that is accelerated during exercise (Finaud et al., 2006; Sen et al., 2000). Although 95-99% of oxygen consumed is turned into water in the electron transport chain, 1-5% forms $O_2^{\cdot -}$ (Sen et al., 2000). $O_2^{\cdot -}$ is derived from an addition of one electron to dioxygen through oxygen metabolism, making it highly reactive and very short-lived (Finaud et al., 2006). This is seen via leakage from the nicotinamide adenine dinucleotide dehydrogenase enzyme at complex 1 and oxidation of

$CoQH_2$ at complex 3 from the electron transport chain (Finaud et al., 2006). This process results in production of lone electrons that react with O_2 to produce $O_2^{\cdot-}$ (Finaud et al., 2006):



H_2O_2 is produced as a result of $O_2^{\cdot-}$ breakdown by the enzymatic antioxidant superoxide dismutase in an acidic environment (Finaud et al., 2006). This intermediate reaction occurs during ROS and antioxidant homeostatic balance:



H_2O_2 is not considered a free radical since it lacks unpaired electrons, however it is still considered a ROS because of its toxicity and capacity to cause ROS formation. While this form of ROS is produced in most cells, H_2O_2 can be further broken down by the leukocyte enzyme myeloperoxidase into hypochlorous acid, a strong antimicrobial agent involved in immune reactions (Finaud et al., 2006).

H_2O_2 is also an intermediate product in Fenton's reaction, which breaks down H_2O_2 and produces other ROS in the breakdown mechanism (Finaud et al., 2006). This process plays a major role in changing the oxidative states of iron (Finaud et al., 2006). The end product of Fenton's reaction is the hydroxyl radical. In the presence of ferrous ions (Fe^{2+}), H_2O_2 is broken down resulting in the very reactive and toxic hydroxyl radical, which causes lipid and protein oxidation (Finaud et al., 2006).

The second major source of ROS formation occurs during ischaemia reperfusion, a phenomenon that occurs when certain tissues receive a burst of blood flow (oxygen supply) after being in a hypoxic environment, such as during anaerobic exercise, surgical intervention, or after shocks (Finaud et al., 2006; Groussard et al., 2003). Xanthine oxidase is activated during the production of uric acid from ATP degradation and catalyzes the formation of $O_2^{\cdot -}$ (Finaud et al., 2006). Some studies have shown that ischaemia reperfusion also increases mitochondrial turnover and thus free-radical production through the electron transport chain (Powers & Jackson, 2008).

Exercise-induced muscle damage further contributes to downstream unprogrammed ROS production, through disruption of calcium homeostasis (Sen et al., 2000). An increase in intramuscular calcium activates proteolytic enzymes, which can lead to disruption of membrane integrity. This disruption, especially in the mitochondria, will lead to failure of ATP production through the electron transport chain, giving rise to increased O_2^- by-products (Sen et al., 2000). Also contributing to the pool of unprogrammed ROS due to exercise are processes such as oxidation of haemoglobin and myoglobin, increased body temperature, and increased production of catecholamines and lactic acid (Finaud et al., 2006; Sen et al., 2000).

2.1.2 Programmed ROS

Neutrophils are the first line of defense in the immune response (Finaud et al., 2006; Lekstrom-Himes & Gallin, 2000; Nathan, 2006). Especially during processes that cause muscle injury, such as unaccustomed exercise, neutrophils are recruited from the

blood and interstitium to the area of trauma (Sen et al., 2000). Their function is to destroy foreign antigens through processes such as phagocytosis, degranulation, and a process called oxidative burst. During the exercise-induced inflammatory response, neutrophils are mobilized to the area of inflammation, initiating ROS formation, however it is yet unclear the physiological role of this exercise-induced ROS formation (Papayannopoulos, Metzler, Hakkim, & Zychlinsky, 2010; Smith & Weidemann, 1993).

Neutrophil oxidative burst is actively initiated by the combination of two O_2 molecules with the nicotinamide-adenine dinucleotide phosphate oxidase enzyme to yield two $O_2^{\cdot -}$ molecules (Finaud et al., 2006; Smith & Weidemann, 1993). Subsequently, $O_2^{\cdot -}$ is converted to H_2O_2 by superoxide dismutase through Fenton's reaction (Smith & Weidemann, 1993). The release of myeloperoxidase from azurophilic granules catalyzes the final step in the production of hypochlorous acid, a potent microbicidal reagent responsible for antigen degradation (Finaud et al., 2006; Smith & Weidemann, 1993).

In addition to its direct role in bactericidal activity, it has recently been found that ROS are also crucial to the generation of neutrophil extracellular traps (Fuchs et al., 2007; Papayannopoulos et al., 2010; Yan et al., 2012). Neutrophil extracellular traps are formed via a type of cell death, thought to trap and kill bacteria, fungi, and parasites through release of a dense network of cell-free DNA and antimicrobial enzymes such as neutrophil elastase and myeloperoxidase (Papayannopoulos et al., 2010; Sen et al., 2000; Yan et al., 2012). Recent exercise studies have demonstrated a rise in cell-free DNA which is thought to be attributed in part to these neutrophil extracellular traps (Breitbach, Tug, & Simon, 2012). This newly discovered source may also contribute to the pool of

ROS produced due to exercise, though it is yet unknown how significant a role it plays in the adaptive mechanisms after exercise. Much of the literature has shown that exercise increases ROS production; however the degree of ROS generation depends on the type, intensity, and duration of exercise, in addition to oxygen consumption and mechanical stress on muscle tissue due to exercise (Sen et al., 2000).

2.2 Antioxidants

Antioxidants are substances that counteract the effects of ROS activity by either forming less active radicals or by quenching the reactions completely (Finaud et al., 2006). Although this thesis did not measure antioxidants to quantify oxidative stress, it should be noted that it is used not only in adult exercise testing, but frequently in pediatric studies as well. Efficiency of antioxidant capacity is dependent on various factors such as fitness level, nutrition, and age (Finaud et al., 2006). There are two categories of antioxidants; enzymatic and non-enzymatic. Enzymatic antioxidants are made endogenously whereas non-enzymatic antioxidants are mainly procured through food sources (Finaud et al., 2006).

Some of the main endogenous antioxidants involved in quenching ROS are superoxide dismutase, catalase, and glutathione peroxidase (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). Superoxide dismutase is the first line of defense against $O_2^{\cdot -}$ radicals and is most active in the mitochondria where the majority of $O_2^{\cdot -}$ is reduced through mitochondrial superoxide dismutase (Finaud et al., 2006; Powers & Jackson, 2008). This reaction produces another ROS, H_2O_2, which is broken down by both

catalase and glutathione peroxidase to yield water (Fisher-Wellman & Bloomer, 2009). Exercise plays a very important role in increasing superoxide dismutase, catalase, and glutathione peroxidase activity.

Non-enzymatic exogenous antioxidants, mostly vitamins, flavonoids, thiols, and various micronutrients, come mainly from ingestion of fruits and vegetables in the diet (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). The main antioxidant vitamins are vitamin A, C, and E. Vitamin A and E are fat-soluble, known to deactivate ROS and decrease lipid peroxidation (Finaud et al., 2006; Powers & Lennon, 1999). Vitamin C is water-soluble and is present in the cytosol, but more abundant in extracellular fluid (Palmer et al., 2003). More importantly, tissues with high oxidative stress also demonstrate higher vitamin C levels, indicating an adaptive antioxidant response (Palmer et al., 2003).

Most studies show that although antioxidant supplementation does not enhance sport performance in non-deficient athletes, it does help protect against detrimental effects of ROS as a result of exercise (Evans, 2000; Singh, Moses, & Deuster, 1992). Supplementation has been shown to protect against exercise-induced harmful effects on muscle tissue and maintain optimal health, however they have also shown pro-oxidant effects when doses over physiological concentrations are taken (Clarkson & Thompson, 2000). It seems that for antioxidants to be effective in athletes' health and performance, supplementation should be carefully controlled for composition, duration, and dose.

2.3 Approaches to measuring ROS during exercise

There are 3 main methods for ROS detection: direct detection of free radicals, measurement of ROS damage, and antioxidant enzyme activity or concentration.

2.3.1 Direct detection of free radicals

It is very difficult to measure ROS directly since they are very short-lived and quite reactive (Finaud et al., 2006). The electron spin resonance technique uses spectroscopic methods to directly measure ROS paramagnetic properties (Ashton et al., 1999). Although these levels can be precisely measured *in vitro, ex vivo*, and *in vitro* in animal models, these measures can not be used in humans due to the high toxicity of markers used to tag ROS in the human body (Clarkson & Thompson, 2000). Because of their high reactivity and volatility, ROS stabilizers have been used in whole blood during centrifugation, enabling direct quantification of ROS in serum samples through spectroscopy (Ashton et al., 1999). However, difficulties in this method lie in the interpretation of ROS since they are highly reactive, have short half-lives, and are weak in concentration (Ashton et al., 1999).

2.3.2 Detection of oxidative by-products

One of the more commonly used methods for ROS detection is measurement of oxidative damage to lipids, proteins, and DNA. Lipid peroxidation is measured by quantifying by-products from lipid breakdown (Finaud et al., 2006). Measurement of primary oxidation products, such as conjugated dienes and lipid hydroperoxides, are

advantageous due to their quantification of initial lipid peroxidation (Ashton et al., 1999; Clarkson & Thompson, 2000). Other less specific methods measure secondary oxidation by-products such as malondialdehyde, F2-isoprostanes, and expired pentane, ethane, and hexane. Malondialdehyde, although secondary, is a commonly used marker for lipid peroxidation, produced through fatty acid auto-oxidation (Clarkson & Thompson, 2000; Groussard et al., 2003). Measuring hydrocarbon end-products such as pentane, hexane, and ethane in expired air is non-invasive, however these gases can be formed by other mechanisms other than oxidative injury and thus may lead to inaccurate estimations of ROS (Rimbach et al., 1999). In this thesis, lipid peroxidation was measured using malondialdehyde as a marker of oxidative stress in plasma samples.

Oxidative damage to proteins leads to protein modifications, most commonly forming carbonyl groups on amino acid side chains (Finaud et al., 2006). Protein carbonyls are the most common form of ROS measurement for protein damage due to their long half-life and high stability (Levine, 2002). In this regard, protein carbonyl can be used to measure ROS accumulation for long-term exercise studies in athletes (Finaud et al., 2006). Oxidized amino acids can also be used to measure ROS interaction with proteins; the method is non-invasive, products are stable, and they exist in high concentrations (Finaud et al., 2006). However, there is not much known about the amino acid kinetics, which limits the interpretation of results (Levine, 2002). This thesis measured protein oxidation by quantifying protein carbonyl levels in plasma.

2.3.3 Detection of antioxidants

An alternate method of quantifying oxidative stress activity is by measuring antioxidant activity, with the assumption that the activity is a direct reflection of oxidative stress injury. Such characteristics can be determined using enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, or using vitamins (A, C, and E) (Marzatico, Pansarasa, Bertorelli, Somenzini, & Della Valle, 1997; Miyazaki et al., 2001; Rimbach et al., 1999). Evaluating enzyme activity can be very useful not only in determining baseline conditions, but also adaptations in activity due to oxidative stress specifically after exercise (Marzatico et al., 1997; Miyazaki et al., 2001). Although concentration of circulating vitamins are also a common indirect measure of oxidative stress, interpretation of these levels must be done with caution since many vitamins are very short-lived and may redistribute between tissue and plasma (Rimbach et al., 1999). Since there tends to be difficulty in measuring one single antioxidant marker, total antioxidant capacity has also been quantified. This method uses pro-oxidants to quantify oxygen radical absorbance capacity of the tested sample (Prior & Cao, 1999). Limitations to this kind of analysis lie in the dependence on individual diet and short-term adaptations to oxidative stress (Prior & Cao, 1999).

It is clear that there is no single test that is sufficiently accurate to quantify the global effects of oxidative stress. With each type of analysis there exist limitations, and interpretation of a marker can be a source of error in itself. Thus, for the most accurate measure of ROS activity, it has been recommended that a variety of tests should be used in combination (Finaud et al., 2006).

2.4 Negative effects of oxidative stress

Much of popular media exposure has focused on the negative effects of oxidative stress. An example of this is the reaction of ROS with lipids, which induce lipid peroxidation (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). This reaction is more common with poly-unsaturated fatty acids than mono-unsaturated fatty acids due to the characteristic double-bond (Powers & Jackson, 2008). Such a reaction can cause structural damage, resulting in altered fluidity and rigidity in the membrane (Finaud et al., 2006; Klaunig et al., 1998; Nasca et al., 2010; Powers & Jackson, 2008). These structural damages can affect membrane function leaving proteins more susceptible to attack and resulting in loss of enzyme activity and transporter function, of particular importance is the function of ATP and ADP coupling (Klaunig et al., 1998; Nasca et al., 2010; Powers & Jackson, 2008).

Another negative effect of ROS is protein oxidation which results in the formation of altered protein carbonyl groups (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009; Klaunig et al., 1998). These protein carbonyls are not as easily degraded as normal proteins, which slows down protein turnover, thus disturbing protein homeostasis (Finaud et al., 2006). The more severe effects are seen in DNA oxidation where ROS interaction with DNA can cause strand breaks and damage to base repair systems, which may lead to DNA mutation, development of cancer, and increased cell aging (Cooke, Evans, Dizdaroglu, & Lunec, 2003; Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009; Klaunig et al., 1998). Many diseases such as arthritis and cancer, demonstrate a

characteristic elevated oxidative stress profile (Fisher-Wellman & Bloomer, 2009; van Eeden et al., 1999; Woo et al., 2010).

2.5 Positive effects of oxidative stress

The positive effects of oxidative stress have been a relatively new topic of research (Sen, 2001). As mentioned previously, ROS production in neutrophils is the primary reaction involved in the antimicrobial oxidative burst response. ROS are involved in enzyme activation and drug detoxification, and directly and indirectly affect many important cellular signaling pathways such as the mitogen-activated protein kinase and nuclear factor-κB (Castro Fernandes et al., 2010; Powers & Jackson, 2008; Reid & Li, 2001). These signaling pathways modulate ion transport, transcription, neuromodulation, apoptosis and affect release of hormones, cytokines, and growth factors (Powers & Jackson, 2008). This finding was supported by the work done by Gomez et al who showed that with antioxidant administration in rat muscle (decrease in ROS), the exercise-induced adaptive changes in gene expression through mitogen-activated protein kinase and nuclear factor-κB were lost (Gomez-Cabrera et al., 2005).

ROS have also been widely shown to regulate functions such as muscle development, blood flow, and contractile processes (Reid, 2001; Sen, 2001). Studies have shown that ROS are required in muscle for normal force production (Supinski & Callahan, 2007). It has been found that maximal muscle force production is mediated by an optimal level of ROS. Lower and higher levels result in decreased force production, and this force production is time- and dose-dependent (Reid, Khawli, & Moody, 1993).

Although certain levels of ROS and their respective by-products may be detrimental to various molecules in the body, they are also essential for physiological processes such as cell signalling and muscle function. Most likely, ROS levels are regulated to balance the positive and negative effects of oxidative stress. This process may be especially important in children since many hormones and mediators are tightly regulated to ensure a healthy growth environment. Exercise may be an effective contributor in inducing and maintaining this oxidative balance.

2.6 Inflammation and inflammatory cytokines

Inflammation is a protective mechanism in the body, orchestrated by the immune system usually in response to trauma or tissue injury (Cone, 2001). Such a response typically induces redness, swelling, and local pain due to recruitment of leukocytes, stimulation of nerve endings, and vasodilation (Pickup, 2004). Although inflammation was initially characterized in the innate immune defense response, it also plays a role in the exercise-induced acute phase response, eliciting a systemic rise in signaling proteins called cytokines (Pedersen & Hoffman-Goetz, 2000; Pickup, 2004). Cytokines, usually proteins or glycoproteins, are the main mediators of the inflammatory response (Reid & Li, 2001). They are involved in intercellular signaling, mediating cell function, proliferation, and various adaptive responses (Reid & Li, 2001). The major cytokines in the exercise response, which will be one of the foci of this thesis are IL-6, TNF-α, and IL-8.

2.6.1 Interleukin 6

There is much debate over IL-6 as a pro-inflammatory or anti-inflammatory cytokine, since it displays both properties (Bishop, Gleeson, Nicholas, & Ali, 2002; Nielsen & Pedersen, 2007). On the one hand, it is secreted by leukocytes including monocytes, macrophages, neutrophils, B and T lymphocytes (Biffl, Moore, Moore, & Peterson, 1996), by adipocytes as a defense mechanism in response to injury (Balagopal et al., 2005), and by skeletal muscles during exercise (Nielsen & Pedersen, 2007). IL-6 then stimulates the liver to upregulate the systemic inflammatory response (Pickup, 2004). On the other hand, secretion of IL-6 also induces release of anti-inflammatory mediators such as interleukin 1ra and interleukin 10 for regulation of homeostasis (Nielsen & Pedersen, 2007), and is also seen to counterbalance TNF-α, a pro-inflammatory cytokine (Timmons & Raha, 2008). These effects suggest that IL-6 may also possess anti-inflammatory properties (Nielsen & Pedersen, 2007).

IL-6 also plays a prominent role in catabolic and anabolic processes (Timmons & Raha, 2008). Mice genetically modified to express high levels of IL-6 demonstrate stunted growth, an effect reversed by the administration of IL-6 antibodies (De Benedetti et al., 1997). Children and adolescents with systemic juvenile rheumatoid arthritis who have symptoms of stunted growth also show higher than normal levels of circulating IL-6 (De Benedetti et al., 1997). Its angiogenic properties are seen in its role in upregulating the expression of vascular endothelial growth factor (Timmons & Raha, 2008). Aside from its function in the inflammatory and growth response, IL-6 also helps mediate

glucose production in muscles, and increase lipolysis and fat oxidation during exercise (Nielsen & Pedersen, 2007).

2.6.2 Tumor necrosis factor alpha

TNF-α is a pro-inflammatory polypeptide involved in the antitumour and immune response (Reid & Li, 2001). It is mostly secreted by macrophages, but also produced from skeletal muscles (Reid & Li, 2001). Its catabolic properties lie primarily in muscle pathology where chronic exposure to TNF-α in animal studies resulted in a loss of muscle mass (Buck & Chojkier, 1996). In humans, TNF-α inhibits muscle protein synthesis and subsequent contractile dysfunction, commonly seen in inflammatory diseases involving sepsis (Reid & Li, 2001; Timmons & Raha, 2008). ROS and reactive nitrogen species production in various cell types have been linked to upstream TNF-α signaling, which are thought to contribute to the catabolic symptoms from TNF-α seen in muscle cells (Reid & Li, 2001).

Studies have shown a vital link between oxidant signalling and TNF-α, which is thought to compromise muscle function either by protein loss or disruption of contractile processes (Li & Reid, 2000). Evidence shows that TNF-α-induced redox signaling of the transcription factor nuclear factor-κB, regulates protein loss in skeletal muscle (Sen & Packer, 1996). Further support for the link between TNF-α and ROS was elucidated when over expression of TNF-α in animals studies resulted in a depression of force production, which was reversed through both antioxidant administration and pre-incubation of the muscle with antioxidants (Li & Reid, 2000; M. B. Reid & Li, 2001).

2.6.3 Interleukin 8

IL-8 is a potent chemo-attractant and induces inflammation through leukocyte recruitment, especially neutrophils in the immune response (Nielsen & Pedersen, 2007; Paulsen et al., 2005). IL-8, although less well studied compared to other cytokines such as IL-6 and TNF-α, is associated with angiogenesis, acting through receptors on endothelial cells (Nielsen & Pedersen, 2007). Skeletal muscles produce IL-8 locally in small transient increases during exercise bouts (Nielsen & Pedersen, 2007). Frydelund-Larsen and colleagues detected IL-8 receptor mRNA and protein expression in endothelial cells present in muscle fibers, suggesting a mechanism of action for IL-8 in angiogenesis (Frydelund-Larsen et al., 2007).

2.7 Exercise and the oxidative stress response: children vs adults

The adult literature on oxidative stress is relatively abundant, with most studies geared towards high performance athletes, investigating the effects of antioxidant supplementation, the effects of ROS in overtraining, or the beneficial adaptive effects of chronic exercise for antioxidant profile and attenuation of oxidative damage (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). Compared to the inflammatory data, effects of exercise on oxidative stress in adult populations have been relatively inconclusive, depending on the participant's training and nutrition history, and even the method of detection itself (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). Even less comparable is the exercise research in pediatric populations.

Pediatric exercise and oxidative stress is a novel area of scientific research. There exists only a handful of studies that have looked at the effect of any type of exercise on the production of ROS in pediatric populations. A large contributing factor to the absence of research in this area is due to the fact that various markers can only be interpreted through systemic sources (e.g. blood), due to ethical considerations with collection of tissue (e.g. muscle) (Timmons & Raha, 2008). Broadening our knowledge on changes in physiological components and mechanisms linking these chemicals during growth may help overcome this barrier. Studies such as the one conducted by Vassilakopoulos and colleagues showed blunted cytokine responses to exercise after patients ingested antioxidants, compared to the antioxidant-absent response (Vassilakopoulos et al., 2003), suggesting a connection between oxidative markers and inflammatory cytokines during exercise. Because exercise-induced changes in certain cytokines are different between children and adults, it has been proposed that these intercellular messengers may play an important role in healthy growth and development (Timmons, Tarnopolsky, & Bar-Or, 2004; Timmons & Raha, 2008). Thus, oxidative stress, which is intricately linked to inflammation and cytokine production (Reid & Li, 2001), may be important in elucidating the adaptive changes due to exercise in children.

To our knowledge, no studies have simultaneously looked at the effect of acute exercise on a variety of oxidative stress and inflammatory markers comparing children to adults under the same exercise conditions. This thesis specifically investigated the effect of an acute bout of exercise on markers of oxidative stress. Due to the varying results

from different types of exercise, the following literature review of exercise in children compared to adults will be solely concerned with acute aerobic exercise.

2.7.1 Effect of acute exercise on systemic oxidative stress

Overall lipid peroxidation is commonly measured by using by-products such as thiobarbituric acid-reactive substances, malondialdehyde, and F_2-isoprostanes (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). The less invasive methods can also test by-products in expired air, such as pentanes or ethanes (Dillard, Litov, Savin, Dumelin, & Tappel, 1978; Leaf, Kleinman, Hamilton, & Barstow, 1997). For adults, lipid peroxidation increases due to exercise are only observed at near-maximal intensity levels (Sen et al., 2000). Various studies have reported substantial increases in thiobarbituric acid-reactive substances after aerobic activities such as maximal exercise performed on a treadmill or cycle ergometer (VO_{2max}) (Miyazaki et al., 2001; Vider et al., 2001). There is more controversy in the effects of exercise on malondialdehyde concentrations. Most studies do not observe an increase in malondialdehyde after maximal or sub-maximal exercise, however the few significant increases that were reported used maximal or near maximal (>75% VO_{2max}) exercise protocols (Fisher-Wellman & Bloomer, 2009). A more specific marker of lipid peroxidation is F_2-isoprostane, shown to increase as a function of increasing cycling intensity in healthy young men (Fisher-Wellman & Bloomer, 2009; Goto et al., 2007). Of the few pediatric exercise studies, two tested healthy adolescents for lipid peroxidation after exercise. Contrary to adults, they found no changes in F_2-isoprostanes or lipid hydroperoxides after acute bouts of intermittent and continuous cycling exercise (Rosa et al., 2011; Youssef et al., 2009).

Protein oxidation is most commonly detected by quantifying the accumulation of protein carbonyl by-products (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). Several studies have shown an increase in protein carbonyls due to exercise in a duration-dependant manner (Fisher-Wellman & Bloomer, 2009). Especially in healthy untrained adults, significant increases in protein carbonyls were seen after acute bouts of submaximal and maximal running exercise (Goldfarb, Patrick, Bryer, & You, 2005; Michailidis et al., 2007). In addition, protein carbonyl levels remained elevated for 8 hours into recovery (Michailidis et al., 2007). Some studies show null findings in the adult literature; these contrary results may have been attributed to short exercise protocols or not enough samples taken into recovery (Fisher-Wellman & Bloomer, 2009). The few pediatric exercise studies are more controversial, resulting in both increases after acute swimming exercise in adolescent boys and girls (Nikolaidis et al., 2007), and no change after one hour of 75% VO_{2max} cycling exercise in boys (Timmons & Raha, 2008).

Other pediatric exercise and oxidative stress studies have used antioxidants, most commonly glutathione through minimally invasive samples such as blood or saliva. Adolescents did show significant decreases in glutathione after an acute bout of swimming and an incremental 20-meter shuttle test (Benitez-Sillero et al., 2009; Nikolaidis et al., 2007). Pediatric exercise studies demonstrate an increase in oxidative stress due to exercise, however this response is not as conclusive or robust as that observed in adults under similar exercise conditions. Especially in pediatric populations, ethical considerations limit quantification of ROS to systemic measures. However, the systemic oxidative stress response has not been clearly characterized in this population; in

order to conclusively determine this response, more testing at varying intensities and durations are required. In doing so, this may enable effective comparisons of the adaptive changes in oxidative stress due to exercise between children and adults.

2.7.2 Effect of acute exercise on intracellular ROS production

Detection of ROS pools is certainly not limited to the systemic circulation. It is thought that neutrophilic ROS production is also a contributing factor during exercise (Quindry et al., 2003; Syu, Chen, & Jen, 2011). High-intensity exercise induces neutrophil mobilization to the active muscle, resulting in ROS expulsion from the neutrophil oxidative burst (Ji, 1999; Walrand et al., 2003). These oxygen free-radicals may be important in post-exercise adaptive mechanisms, which is especially important to characterize in growing children (Quindry et al., 2003; Timmons & Raha, 2008).

The adult literature for exercise effects on neutrophilic ROS production is much less abundant compared to studies using systemic markers. This is likely due to the complexity of detection methods and analysis since it usually involves isolating neutrophils and detecting ROS through flow cytometry or chemiluminescence. Studies show significant increases in $O_2^{\cdot -}$ and fluorescent ROS stains, only with maximal or near maximal acute exercise protocols (Quindry et al., 2003; Syu et al., 2011). It is interesting to note that significantly higher levels of $O_2^{\cdot -}$ were found 2 hours into recovery, even when adjusted for neutrophil increases (Quindry et al., 2003). These findings suggest an adaptive role in response to exercise. On the contrary, other studies have observed no increases or have even seen decreases in neutrophil ROS production after cycling or

running exercise (Peake et al., 2004; Pyne, Smith, Baker, Telford, & Weidemann, 2000b). The difference in results may have been due to participant training status since the latter two studies tested highly trained cyclists whereas the others tested healthy, but untrained individuals. Thus far, there have been no studies in children looking at the changes in neutrophil intracellular ROS production after exercise, therefore, this thesis will use three different stains to quantify the respective exercise-induced response.

2.7.3 Effect of acute exercise on in vitro ROS production

It is well established that neutrophils produce ROS when stimulated by exercise; this thesis will further explore the chemicals produced by neutrophils mobilized by exercise. Most studies investigating neutrophils *in vitro* have detected myeloperoxidase and elastase to determine neutrophil activity. Specifically in regards to neutrophils, elastase and myeloperoxidase are essential for degranulation and the oxidative burst, respectively, contributing to the overall ROS pool (Bishop et al., 2002; Pyne et al., 1996; Smith & Weidemann, 1993). Most exercise studies have not found changes in these neutrophilic enzymes (Bishop et al., 2002; Pyne et al., 1996; Pyne, Smith, Baker, Telford, & Weidemann, 2000a; Syu et al., 2011); non-exercise studies have found increases in these enzymes during the production of neutrophil extracellular traps, however it is yet unclear if this process plays a substantial role in exercise (Papayannopoulos et al., 2010; Yost et al., 2009). Mitochondrial ROS produced in neutrophils is characterized by a change in membrane potential and apoptosis; an exercise study tested healthy young males on a cycling protocol, and found increased apoptosis and immediate change in membrane potential after maximal cycling exercise (Syu et al., 2011). To fill a

significant gap in the field of pediatric exercise, this thesis studied the neutrophil oxidative response *in vitro* in order to characterize exercise-primed neutrophils in children compared to adults.

2.8 Exercise and the inflammatory response: children vs adults

Exercise stimulates various changes in the inflammatory response in both children and adults (Timmons, 2006). During exercise, cytokines are produced in skeletal muscle, facilitating the recruitment of leukocytes, which is then accompanied by a systemic acute phase response (Pedersen & Hoffman-Goetz, 2000). Although exercise-induced immunological changes in adults have been extensively studied (Pedersen & Hoffman-Goetz, 2000), such changes in pediatric populations have been a relatively new area of research. Marked differences are seen in cytokine levels after exercise in children compared to adults, and this has been hypothesized to play an important role in adaptive mechanisms during healthy growth and development (Timmons & Raha, 2008).

Although adult studies investigating the exercise-induced cytokine response have used different types, intensities, and durations, all have shown a marked increase in IL-6 levels (Pedersen & Hoffman-Goetz, 2000). Exhaustive acute exercise results in an exponential increase in IL-6, which then decreases during recovery, likely due to the secretion of anti-inflammatory cytokines such as IL-1ra and IL-10 (Febbraio & Pedersen, 2002; Nielsen & Pedersen, 2007). Although an increase in IL-6 levels is seen in the pediatric population, the response is not as robust as the adult response (Nemet, Oh, Kim, Hill, & Cooper, 2002; Nemet, Rose-Gottron, Mills, & Cooper, 2003; Nemet et al., 2003).

There seems to be an age-dependant increase in exercise-induced cytokine changes, showing increasing IL-6 levels with increasing age (Timmons & Raha, 2008). Upon closer examination of pediatric exercise studies, it has been observed that the rise in IL-6 for children is about 50% lower compared to adults (Timmons, 2006). Since IL-6 demonstrates both anabolic and catabolic effects, it is possible that such blunted increases in IL-6 may demonstrate optimal levels for growth-related adaptations (Timmons & Raha, 2008).

The TNF-α cytokine response to exercise has also been extensively studied in the adult population. These results have not always been consistent, some showing no changes whereas others report increased levels (Pedersen & Hoffman-Goetz, 2000). This could be a result of the methods used for testing. In general, more strenuous exercise does induce an increase in TNF-α, however not as dramatic as IL-6 (Pedersen & Hoffman-Goetz, 2000). In pediatric populations, exercise either does not change or induces a decrease in TNF-α concentrations (Nemet et al., 2002; Nemet et al., 2003; Timmons et al., 2004). Due to its catabolic properties and potentially negative effects in high quantities, it has been proposed that this tight regulation of TNF-α levels serves as a protective function in developing children (Timmons & Raha, 2008).

IL-8 has primarily been detected locally in muscle biopsies after exercise in adults (Nielsen & Pedersen, 2007). Studies showed an increase in IL-8 mRNA and protein in skeletal muscle cells throughout exercise, and into recovery (Nieman et al., 2003). Although this localized effect has been observed in other exercise studies (Chan, Carey, Watt, & Febbraio, 2004), systemic rises in IL-8 levels are only seen in response to

exhaustive eccentric exercise (Akerstrom et al., 2005; Nielsen & Pedersen, 2007; Nieman et al., 2003). There are very little data for IL-8 levels during exercise in children. Children did not show variability during exercise, however, IL-8 increased after one hour of recovery (Timmons, Tarnopolsky, Snider, & Bar-Or, 2006). Since most of the adaptive processes to exercise occur during the recovery period, it is hypothesized that IL-8 may play a role through recruitment of immune cells to the muscle for tissue adaptation (Mahoney, Parise, Melov, Safdar, & Tarnopolsky, 2005).

2.9 Summary

The biochemistry of oxidative stress demonstrates both negative and positive downstream effects on the body's physiology. These effects are dictated by fluctuating levels of systemic and intracellular ROS. It is of the belief that factors such as antioxidants and especially exercise, play a role in maintaining homeostasis in ROS levels. Research has shown a potential link between inflammation and oxidative stress, and both demonstrate exercise-mediated changes. The exercise-induced inflammatory response is different in boys compared to men, with boys showing a blunted response, which is potentially attributed to growth processes. If signalling mechanisms exist between cytokines and ROS, then it is hypothesized that exercise would also induce differences in ROS production between boys and men. There is limited research in the pediatric field in terms of oxidative stress and exercise. More importantly, there have been no studies to date testing the same exercise conditions and oxidative markers in conjunction with inflammatory markers between children and adults. Thus, this thesis will begin to address this gap.

CHAPTER 3: METHODS

3.1 Participants

This cross-sectional study tested ten healthy pre-pubertal boys ages 8-10 and twelve healthy adult men ages 19-21. Participants were recruited from around McMaster University and from the lab study participant database. Healthy participants were determined first by a screening phone call for average weekly physical activity, and then by a maximal aerobic fitness test (VO_{2max}). Participants had to score a minimum VO_{2max} of 35mL/kg/min to be eligible for the study. Participants were excluded if they were currently taking any supplements, multivitamins, or medication. In the case that participants were taking supplements, they were required to abstain for a 1-month washout period before their first visit. Exclusion criteria also outlined no allergies or family history of inflammatory disease. Participant characteristics are outlined in Table 1.

Table 1. Participant characteristics

	Boys (n=10)	Men (n=12)	P-value
Chronological age (years)	9.5 ± 1.2	20.9 ± 1.0	p<0.001
Height (cm)	140.1 ± 9.4	178.2 ± 9.8	p<0.001
Weight (kg)	34.7 ± 6.9	78.3 ± 18.8	p<0.001
% Body fat	14.7 ± 7.4	12.8 ± 4.0	0.723
VO2max (mL/kg/min)	51.4 ± 8.1	49.5 ± 13.3	0.564
Tanner	1.2 ± 0.4		

Values are expressed as mean ± SD. Data were analyzed through independent t-tests with significance set at $p<0.05$. %body fat: Percent body fat was calculated using bioelectrical impedance analysis technology. Tanner stage (pubertal maturity) was self-assessed using five diagrams depicting stages of pubic hair development for boys according to (Taylor et al., 2001).

3.2 Experimental design

Participants came to the Children's Exercise and Nutrition Centre for two visits, with a span of at least 3 days between visits to allow for completion of the 3-day food record.

Visit 1: Upon arrival, the study coordinator explained the progression of the study to the participants, including the risks and discomforts and compensation. If the participant decided to join in the study, children were asked to sign an assent form, and parents and adult participants were asked to sign an informed consent form. They were then asked to fill out a contact form, a medical and activity questionnaire, and given a 3-day food record to complete over 2 weekdays and one weekend. Anthropometric measurements were then taken, followed by a maximal aerobic fitness test, assessed on a cycle ergometer.

Visit 2: Participants were asked to refrain from doing heavy exercise 24 hours prior, and from eating and drinking (other than water) 3 hours prior to the session. Upon arrival, trained personnel inserted a venous catheter in the antecubital area of the arm, and resting blood samples were taken. This was followed by the continuous exercise protocol on a cycle ergometer. Immediately after the exercise session, blood samples were taken in the same manner and amounts as the pre-exercise blood sample.

3.3 Measurements

3.3.1 Sexual maturity – Tanner stage

To determine sexual maturity, the boys were asked to evaluate their pubic hair development according to the method of Tanner through self-assessment. They were given a sheet with five diagrams depicting stages of pubic hair development for boys (Taylor et al., 2001). Verbal instructions were given to the boys and their parent, and they were asked to write down the number that corresponded to the picture that was most similar to them. They were given privacy with their parent to complete the assessment.

3.3.2 Height, weight, percent body fat

Standing height was measured to the nearest millimeter using a Harpenden Stadiometer. The participant was asked to stand with their back against the stadiometer and heels against the plate on the ground without shoes or socks. They were instructed to stand with their back straight, shoulders back, looking forward with their head level. When the head plate was lowered onto their head, participants were asked to take a deep breath in, and the measurement was recorded.

Weight and percent body fat were measured using an Inbody 520 bioelectrical impedance analyzer (model MW160, BioSpace Co. Ltd., Korea). Weight was recorded to the nearest 0.01kg, with participants lightly dressed, usually in a T-shirt and shorts, in bare feet. They were asked to stand very still on the machine, feet on the metal plates and hands holding the handles.

3.3.3 Three-day food record

Due to the importance of accounting for antioxidant intake, and its effect on measured ROS, participants were asked to record their diet over 3 days prior to visit 2. They were instructed to record the type and quantity of all foods consumed for 2 regular week days and one weekend day. Three-day food records were analyzed by a Registered Dietitian using The Food Processor SQL Version 10.10.0 (Esha Research, Salem, Oregon, 2012) to provide an average daily intake (Table 2). Based on age, sex, and estimated physical activity levels, values were reported as a percent of recommended daily intake (Table 2); most participants for this study were in the moderate to very active categories.

Table 2. Daily intake of macronutrients per kilogram body weight and major antioxidants from 3-day food record.

Nutrient	Total or %Rcmd	Boys (n=8)	Men (n=11)	P-value
Energy (kcal)	Total	2041.7 ± 152.9	2805 ± 1143	0.052
	%Rcmd	78.4 ± 13.3	84.1 ± 33.2	0.614
Energy (kcal/kg)	Total	58.8 ± 12.1	36.5 ± 14.9	**0.003***
Protein (g/kg)	Total	2.6 ± 0.6	1.9 ± 0.8	0.071
	%Rcmd	270.7 ± 65.3	241.8 ± 96.3	0.474
Carbohydrates (g/kg)	Total	7.2 ± 2.1	4.4 ± 1.9	**0.008***
	%Rcmd	69.8 ± 18.9	73.8 ± 32.8	0.766
Fat (g/kg)	Total	2.3 ± 0.7	1.3 ± 0.6	**0.006***
	%Rcmd	97.1 ± 27.8	95.6 ± 45.1	0.936
Vitamin A (RAE/kg)	Total	22.2 ± 9.5	10.8 ± 8.4	**0.013***
	%Rcmd	147.7 ± 75.2	90.9 ± 71.6	0.113
Vitamin C (mg/kg)	Total	3.7 ± 2.2	1.7 ± 1.5	**0.030***
	%Rcmd	315.6 ± 141.6	141.4 ± 119.8	**0.010***
Vitamin E (mg/kg)	Total	0.082 ± 0.022	0.081 ± 0.072	0.982
	%Rcmd	29.2 ± 7.1	41.3 ± 35.6	0.298

Values are expressed as means ± SD. *significant difference, $p<0.05$, NS: no significance.
%Rcmd: Percent recommended based on age, sex, and physical activity levels (most participants were in the moderate to very active categories), RAE: retinol activity equivalents for vitamin A.

3.4 Exercise testing

3.4.1 Maximal aerobic fitness test

Maximal aerobic fitness was measured via a VO_{2max} test using one of two cycle ergometers (Fleish-Metabo, Geneva, Switzerland; Lode, Groningen, Netherlands), using the *McMaster All-Out Progressive Continuous Cycling Test*. The initial workload (i.e. resistance) was determined based on the participant's height and estimated level of physical activity, increasing the same workload every 2 minutes. Participants cycled at

60 revolutions per minute, with verbal encouragement from the test administrator until they were unable to maintain the pedaling rate. Breath-by-breath VO_2 and VCO_2 were collected during the entire test using a metabolic cart (Vmax29, Sensormedics), with appropriate mouthpieces (most participants used the pediatric mouthpieces). VO_{2max} was determined to be the highest VO_2 attained during a 30 second interval period. Rating of perceived exertion was taken 30sec before the end of each workload, and heart rate at the end of each workload. Participants were presented with the Borg 6-20 scale (Appendix 2) and asked "How hard do you feel you're working?". Heart rate was recorded using a Polar heart rate monitor (Polar Electro Oy, Kempele, Finland).

3.4.2 Exercise protocol

Participants performed the exercise protocol on the same cycle ergometer as was used for their VO_{2max} test. The exercise protocol consisted of 2x30 min bouts of cycling exercise, with a rest of 6 min between bouts, the workload was set at 60% of VO_{2max} (Figure 1). Participants warmed up for 3 min at either very low or no resistance. Initial resistance was set at 50% of maximum workload from the VO_{2max} test, and adjusted in the first 6 min of exercise to elicit 60% VO_{2max}. This resistance remained unchanged throughout the rest of the exercise session. Breath by breath VO_2 and VCO_2 were taken in the same manner as the aerobic fitness test during the first 6 min, and last 6 min of each exercise bout to ensure steady state and approximate work intensity (slight drift towards increased oxygen uptake was observed in most participants). Heart rate and rating of perceived exertion were recorded every 5 min and 10 min, respectively. Participants were allowed to drink water ad libitum.

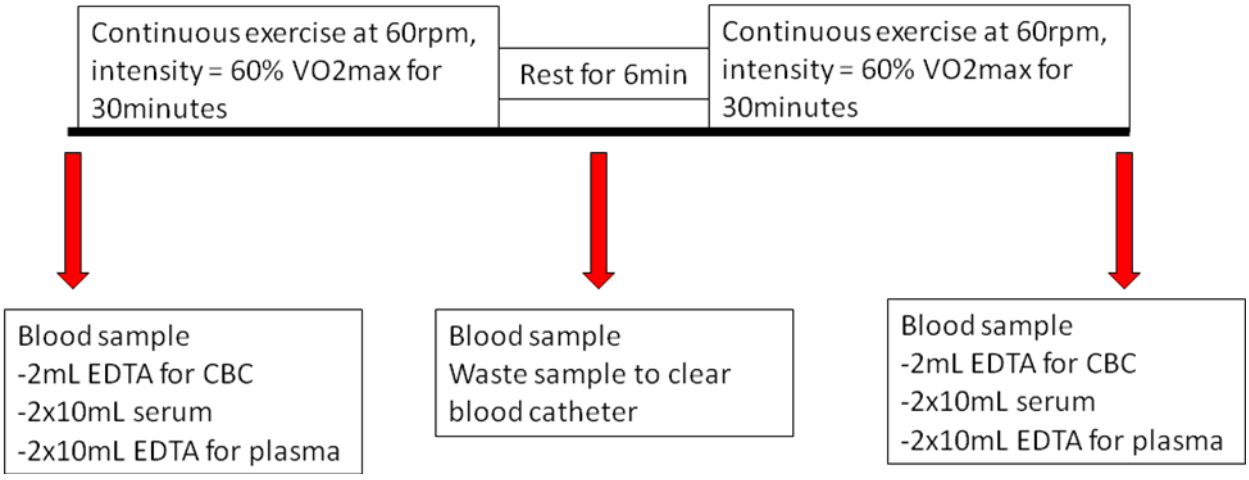


Figure 1. Continuous exercise protocol – total exercise time was 60min, with 6min rest between 30min cycling bouts. Blood samples were taken from a blood catheter before and after exercise with a small sample during rest to clear the blood catheter.

3.5 Blood collection

Blood samples were taken at two time-points, before and immediately after exercise (Figure 1). Prior to the first blood collection, participants were asked to rest in a supine position on a bed for 10min, to ensure resting blood samples were obtained. An in-dwelling venous catheter was then inserted in a forearm vein by a trained investigator. The pre-exercise sample collection consisted of one 5mL EDTA tube for complete blood count sent to the core lab, 2x10mL serum tubes, and 2x10mL EDTA tubes for plasma (Figure 1). Sterile saline was used to flush the catheter after blood collection. All samples were then put on ice.

A waste blood sample was taken, and the catheter was flushed with sterile saline, during the rest period between exercise bouts to ensure clearance of the catheter. Immediately after exercise, the participants were asked to dismount the cycle ergometer and immediately sit on a chair for blood collection. Blood samples were taken in

identical method and amounts as the pre-exercise sample. Figure 2 outlines all tests done with each blood sample.

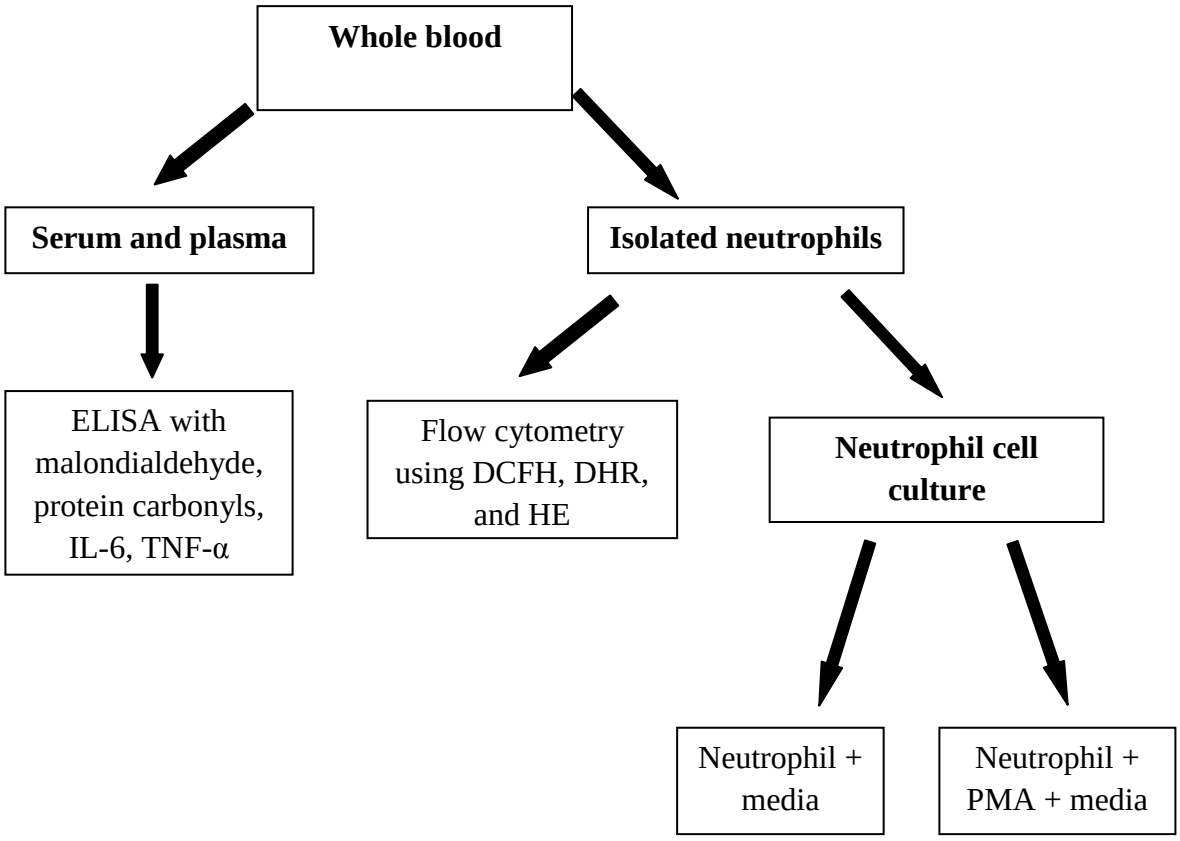


Figure 2. Blood sample processing protocol.

3.5.1 Complete blood count

The two 5mL EDTA tubes (pre- and post-exercise) were delivered to the Hamilton Regional Lab Medicine Program Clinical Laboratories – Core Laboratories at McMaster Hospital for the analysis of complete blood count using an automated Coulter counter. Data used from the complete blood count report included cell counts for

leukocytes, hemoglobin, hematocrit, relative neutrophils, absolute neutrophils. Changes in blood and plasma volume were accounted for according to Dill & Costill (Dill & Costill, 1974) using hemoglobin and hematocrit.

3.5.2 Serum and plasma

Serum tubes were centrifuged at 2000g for 20min at room temperature, serum was aliquotted into 1.5mL portions, and frozen at -80^{o}C for later analysis of cytokine and enzyme content. All serum samples were analyzed for cytokines IL-6 and TNF-α, and elastase using enzyme-linked immunosorbent assay (ELISA) kits. IL-6 and TNF-α ELISA kits were purchased from R&D systems (Minneapolis, MN), with sensitivities reported at 0.016-0.110pg/mL (IL-6) and 0.038-0.191pg/mL (TNF-α). Intra-assay coefficients ranged from 2.50%-3.59% (IL-6) and 3.97%-6.64% (TNF-α). The elastase ELISA kit was purchased from Merck Millipore Canada (Mississauga, ON), with a sensitivity reported at 3.0ng/mL. The intra-assay coefficient for the elastase kit was 2.73%.

Plasma was obtained from the neutrophil isolation process as explained below, was aliquotted into 1.5mL portions, and frozen at -80^{o}C for later analysis of oxidative by-products malondialdehyde and protein carbonyl. Both the malondialdehyde assay kit and protein carbonyl ELISA kit were ordered from Northwest Life Science Specialties (Vancouver, WA), with sensitivities reported at 0.03μM (malondialdehyde), and 0.05ng/mL (protein carbonyls). The malondialdehyde assay was read using a Cary 300 bio spectrophotometer (Varian – now Agilent Technologies, Ontario, CA) and analyzed

using Cary WinUV software, version 3.0. Absorbance was measured using the 400nm-700nm method (explained in detail in the kit insert), transformed using third-derivative analysis, and the lowest absorbance data point around 532nm was recorded for concentration. The protein carbonyl assay outlined 3 different sample preparation protocols; the highest protein concentration (35-80mg/mL) protocol was used for this experiment. To determine which protocol to use, protein content for each sample was determined using the Coomassie Bradford Protein Assay Kit, ordered from Thermo Scientific (Rockford, IL). All samples fell within 35-80mg/mL of protein. Intra-assay coefficients were 8.05% (malondialdehyde) and 3.65%-4.50% for protein carbonyls. All samples were re-run if the coefficient of variance was higher than 15%. ELISA plates were read on a Thermo Scientific Multiskan Spectrum reader (Vantaa, Finland).

3.5.3 Neutrophil isolation

Neutrophils were isolated using both Histopaque 1077 and Histopaque 1119 according to the manufacturer's instructions (Sigma Aldrich, Oakville, ON). Briefly, 3mL of Histopaque 1119 was overlayed with 3mL of Histopaque 1077, which was then overlayed with 6mL of whole blood. Tubes were then centrifuged at 700g for 30min. The third layer, containing granulocytes, was pooled into a separate tube, and washed with 10mL of RPMI 1640, and centrifuged at 200g for 10min. The supernatant was discarded, the pellet resuspended in 2.5mL of BD Pharm Lyse lysing buffer (BD Biosciences, Ontario, CA), incubated for 5min, and centrifuged again at 200g for 5min. The cells were washed twice more, and resuspended in 4mL of RPMI 1640. RPMI 1640

was supplemented with 2.0g sodium bicarbonate ($NaHCO_3$)/L for the RPMI 1640 medium, both ordered from Fisher Scientific (Ottawa, ON).

Cell viability and count for the suspended neutrophils were assessed using a Countess cell counter. Based on previous methods (H. Nguyen, O'Barr, & Anderson, 2007; J. A. Smith & Weidemann, 1993; van Eeden et al., 1999; Walrand et al., 2003), a final cell concentration of $1x10^6$ cells/mL was used. Thus, the 4mL suspended cell solution was adjusted to a final concentration of $1x10^6$ cells/mL using the equation $C_1V_1=C_2V_2$ (C = concentration, V = volume, 1&2 = initial and final samples). The final cell solution for each time-point was aliquotted into 7 samples of 500µL portions into polystyrene tubes for flow cytometry analysis, and 2 samples of 1mL portions into polypropylene tubes for cell culture.

3.5.4 Neutrophil cell culture

Cells were incubated with either 10µM phorbol 12-myristate 13-acetate (PMA) or without for 1hour at 37^{o}C. For the first 10 participants, cell viability was recorded after culture to ensure survival of cells, which ranged from 80%-95%. An optimal culture period of 1hr was determined from previous pilot testing, recording cell count and viability every hour up to 24hrs. After incubation, cells were centrifuged at 1200rpm for 10min, and the supernatant was collected and frozen at -80^{o}C for later analysis. Cell culture supernatant was tested for IL-8 cytokine and myeloperoxidase enzyme content, both analyzed using ELISA kits ordered from R&D systems (Minneapolis, MN), with sensitivities reported from 1.5-7.5pg/mL for IL-8 and 0.003-0.062ng/mL for

myeloperoxidase. Intra-assay coefficients of variation ranged from 6.1%-8.5% for IL-8 and from 4.0%-4.9% for myeloperoxidase.

3.5.5 Flow cytometry

Isolated neutrophils were analyzed for intracellular ROS content using specific fluorescent probes detected by flow cytometry. Three probes were used to detect for different ROS: 2',7'-dichlorofluorescein diacetate (DCFH) as a general ROS marker, dihydrorhodamine 123 (DHR) for H_2O_2, and hydroethidine or dihydroethidium (HE) for O_2^-, with the neutrophil stimulant PMA. Relatively small in size and capable of diffusing across cell membranes, the probes are trapped in the cell and emit a highly fluorescent signal upon reaction with ROS (Walrand et al., 2003). The PMA stimulant activates the nicotinamide adenine dinucleotide phosphate oxidase enzyme present in neutrophils by increasing protein kinase C activity, resulting in an upregulation of ROS production (Walrand et al., 2003). HE, PMA, and dimethyl sulfoxide (used to make working solution of DCFH) were all ordered from Fisher Scientific (Ottawa, ON). DCFH and DHR were both ordered from Sigma Aldrich (Oakville, ON).

Neutrophils were aliquotted into 7 polystyrene tubes; one for control, and two for each fluorescent probe (one with PMA and one without). Cells were incubated for 20 min at 37^{o}C with each probe; 0.625μM DCFH, 1μM DHR, 10μM HE, and 10μM PMA for the respective tubes. Samples were stored on ice immediately after incubation to stop the reactions until flow cytometry analysis.

Flow cytometry analysis was performed using a three-laser (488nm, 633, 350nm UV), 15-color capable LSRII instrument, using the 488nm excitation. FACsDiva software from BD Biosciences was used for data acquisition. Forward and side-scatter plots were used to locate the neutrophil scatter (Figure 3A). Once the neutrophils were located on the scatter plot, a gate was placed around the group of cells (Figure 3A). The threshold was increased until a majority of debris cells were excluded. A total of 10^4 events were collected within the neutrophil gate.

A. **B.**

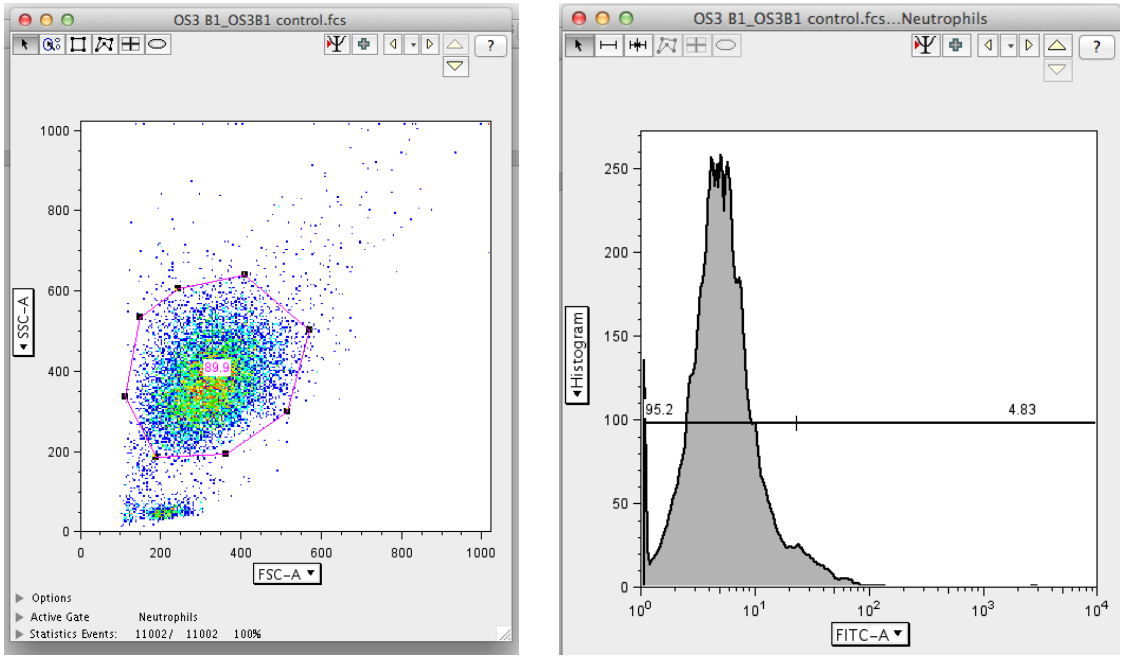


Figure 3. Isolated neutrophils samples analyzed by flow cytometry from a representative participant. A. Forward and side-scatter plot, depicting the neutrophil population, B. histogram showing the fluorescence intensity of the gated neutrophils through the FITC filter.

DCFH and DHR were measured in the FL1 channel for green fluorescence using FITC emission, and HE was measured in the FL2 channel for red fluorescence. Analysis was

completed using the program FlowJo version 8.7 for Macintosh (Tree Star Inc 2008), gating around the neutrophil scatter and using 95% of the control cells to determine negative fluorescence (Figure 3B). All cells above this gate were determined to be positive, and ROS production was quantified by the median fluorescence intensity recorded.

3.6 Statistics

3.6.1 Differences between groups for anthropometric measures

All values were presented as means ± SD. Independent t-tests (SPSS 18.0) were used to determine differences between boys and men for chronological age, height, weight, percent body fat, and VO_{2max} values. Significance was set at $p<0.05$.

3.6.2 Group-dependant and time-dependant differences for analysis of blood samples

A two-way repeated measures ANOVA (Statistica version 5.0) for group x time interaction (boys vs men; pre- vs post-exercise, respectively) was used to analyze relative neutrophils, absolute neutrophils, IL-6, TNF-α, protein carbonyl, elastase, malondialdehyde, and fold change in stimulated IL-8, myeloperoxidase, DCFH, DHR, HE. Significance was set at $p<0.05$. A significant group x time interaction was analyzed with Tukey's post-hoc test (Statistica version 5.0).

Independent t-tests (SPSS 18.0) were used to analyze percent change in unstimulated cells for DCFH, DHR, and HE. Dependent t-tests (SPSS 18.0) were used to determine differences between pre-exercise and post-exercise values in each group (boys and men) for relative neutrophils, absolute neutrophils, IL-6, TNF-α, protein carbonyl,

elastase, malondialdehyde, and fold change in stimulated IL-8, myeloperoxidase, DCFH, DHR, HE. Significance was set at $p<0.05$.

3.6.3 Effect size

Cohen's *d* equation was used to calculate effect size, based on the difference in means between groups and the average standard deviation (equation below) (Field, 2009). This was calculated for time and between group effects.

$$d = \frac{\bar{x}_1 - \bar{x}_2}{s}$$

where x_1 *and* x_2 = means for each group and s = average standard deviation for each group.

3.6.4 Correlations

To determine if there was a correlation between changes in cytokines and ROS markers after exercise, Pearson's *r*-correlation coefficient (SPSS 18.0) with $p<0.05$, was used. These correlations were performed comparing fold change from baseline to post-exercise in IL-6 and TNF-α to protein carbonyls, malondialdehyde, myeloperoxidase, elastase, and percent changes in DCFH, DHR, HE fluorescence. To determine if the cytokine response changes neutrophil activation after exercise, Pearson's *r*-correlation (SPSS 18.0) with $p<0.05$, was used. These correlations were performed comparing fold change from baseline to post-exercise in IL-6 and TNF-α to fold change from stimulation in post-exercise DCFH, DHR, and HE fluorescence intensity.

All significant correlations were plotted with boys and men's data together on Statistica (Statistica version 5.0) and analysis of slopes was performed to determine statistical difference between the two slopes.

3.6.5 Sample size calculation

Sample size was determined using the change in protein carbonyls in post-exercise values compared to resting conditions reported previously (Timmons & Raha, 2008), because this was the only previous investigation directly comparing boys and men using a marker of oxidative stress. In boys, resting protein carbonyl samples, average around 0.22 ± 0.02nmol/mg (mean±SD), increased to approximately 0.24 ± 0.02nmol/mg (mean±SD) post-exercise. An alpha value set at 0.05 (Zalpha=1.96) and beta value set at 0.10 (power of 90%, Zbeta=-1.28) was used in the equation below. Based on the equation by Downson-Saunders (1994) (Downson-Saunders & Trapp, 1994), a sample size of 15 was required for each experimental group. Because experiments from this thesis have not been tested on both boys and adults, this sample size calculation was used as a reference value for recruitment.

$$n = 2\left[\frac{\left(\left(Z_{alpha} - Z_{beta}\right) \times \left(SD_{CF} - SD_{Controls}\right)\right)}{Mean_{CF} - Mean_{Controls}}\right]^2$$

CHAPTER 4: RESULTS

4 .1 Immune cell response

A group effect was seen for relative neutrophils with boys showing lower percentages compared to men *(p=0.03)* with an effect size *(d)* of 0.88 (Figure 4A). A time effect was not seen for relative neutrophils (*p=0.88)*. A group × time effect was not seen for relative neutrophils *(p=0.40)*. The paired samples t-test did not show significantly different values for pre- compared to post-exercise relative neutrophils in boys or men *(p=0.27; p=0.59*, respectively*)*.

A group effect was not seen for absolute neutrophils *(p=0.52*). A time effect was seen for absolute neutrophils with approximately two-fold higher levels post-exercise *(p=0.007)* with a *(d)* of 0.90 (Figure 4B). A group × time effect was seen for absolute neutrophils, and from Tukey's post-hoc test, it was determined that post-exercise levels were significantly higher in men (*p=0.008)* (Figure 4B). The paired samples t-test did not show significantly different values for pre- compared to post-exercise absolute neutrophils in boys *(p=0.15)*, however significance was observed for men *(p=0.02)*.

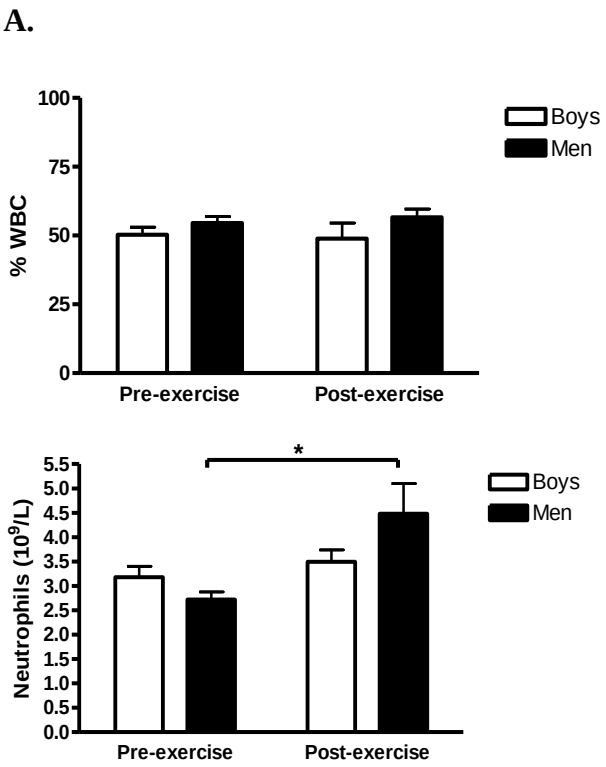


Figure 4. Change in neutrophils from baseline after an acute bout of exercise in boys (n=9) and in men (n=10). A. Relative neutrophils, as percentage of white blood cells, B. Absolute neutrophils ($\times 10^9$cells/L). Values are expressed as means ± SD. Open bars represent boys and solid black bars represent men. * post-exercise values are significantly higher at $p<0.05$ compared to pre-exercise.

4.2 Systemic oxidative stress and inflammatory markers

4.2.1 Oxidative stress markers

A group effect was seen for protein carbonyl concentrations with boys showing higher concentrations than men *($p<0.001$)* with a *(d)* of 2.39 (Figure 5A). A time effect was not seen for protein carbonyls *($p=0.15$)*. A group × time effect was not seen for protein carbonyls *($p=0.35$)*. The paired sample t-test did not show significantly different values for pre- compared to post-exercise protein carbonyls in boys (*$p=0.76$)* but showed a decrease trending toward significance in men (*$p=0.064$)* (Figure 5A).

A group effect was seen for malondialdehyde concentrations with men showing higher concentrations compared to boys *($p<0.001$)* with a *(d)* of 1.88 (Figure 5B). A time effect was not seen for malondialdehyde *($p=0.19$)*. A group × time effect was not seen for malondialdehyde *($p=0.82$)*. The paired samples t-test did not show significantly different values for pre- compared to post-exercise malondialdehyde in boys or men *($p=0.22$; $p=0.36$*, respectively).

A group effect was not seen for elastase concentrations *($p=0.54$)*. A time effect was not seen for elastase *($p=0.09$)*. A group × time effect was not seen for elastase *($p=0.77$)*. The paired samples t-test did not show significantly different values for pre- compared to post-exercise elastase in boys or men *($p=0.22$; $p=0.25$*, respectively) (Figure 5C).

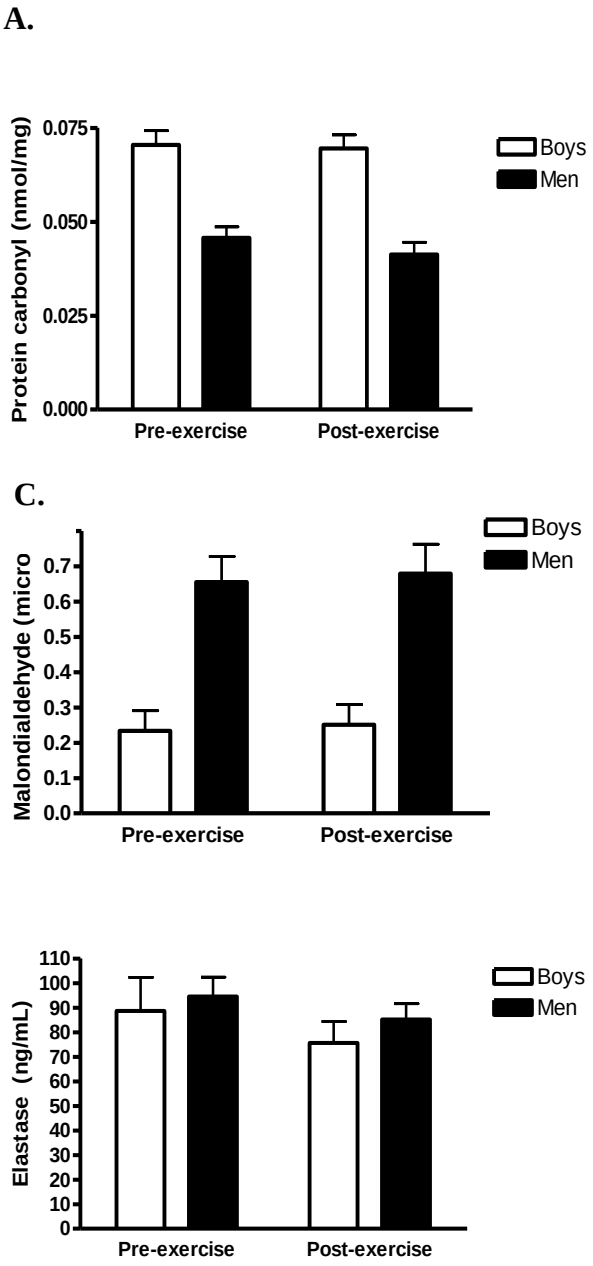


Figure 5. Change in protein carbonyls, malondialdehyde, and elastase from baseline after an acute bout of exercise in boys (n=10) and in men (n=12, n=9 for elastase). A. protein carbonyls (nmol/mg protein), B. malondialdehyde (μM), C. Elastase (ng/mL). Values are expressed as means ± SD. Open bars represent boys and solid black bars represent men.

4.2.2 Systemic inflammatory markers

A group effect was seen for TNF-α concentrations with boys showing higher concentrations than men *($p<0.001$)* with a *(d)* of 1.59 (Figure 6A). A time effect was not seen for TNF-α *($p=0.08$)*. A group × time effect was not seen for TNF-α *($p=0.07$).* The paired sample t-test did not show significantly different values for pre- compared to post-exercise TNF-α concentrations for boys *($p=0.99$)* but verified that TNF-α was significantly higher post-exercise in men *($p=0.008$)* (Figure 6A).

A group effect was not seen for IL-6 concentrations *($p=0.11$)*. A time effect showed approximately 2.5-fold higher post-exercise IL-6 compared to pre-exercise *($p<0.001$)* with a *(d)* of 1.84. A group × time effect was seen for IL-6 *($p=0.005$)*, and from Tukey's post-hoc test, it was determined that post-exercise IL-6 concentrations were significantly higher than baseline in both boys and men, and that boys showed significantly lower post-exercise IL-6 levels compared to men (exercise effect: boys $p<0.001$, men $p<0.001$; post-exercise boys vs post-exercise men $p=0.01$ with a *(d)* of 1.07 (Figure 6B). The paired sample t-test verified that IL-6 was significantly higher post-exercise in both boys *($p=0.001$)* and in men ($p<0.001$) (Figure 6B).

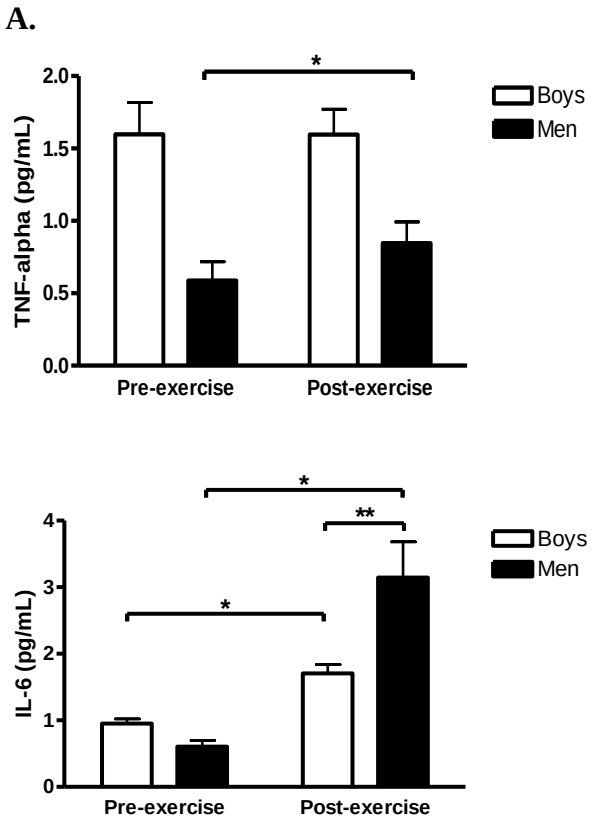


Figure 6. Change in TNF-α and IL-6 from baseline after an acute bout of exercise in boys (n=10) and men (n=12). A. TNF-α: pg/mL, B. Interleukin-6 (IL-6: pg/mL). Values are expressed as means ± SD. Open bars represent boys and solid black bars represent men. * post-exercise values are significantly higher at $p<0.05$ compared to pre-exercise, **post-exercise values are significantly different at $p<0.05$ between boys and men.

4.3 Neutrophil cell culture

4.3.1 IL-8

A condition effect (unstimulated vs stimulated) was not seen for IL-8 concentrations within boys *(p=0.43)*. A time effect was not seen for IL-8 within boys *(p=0.70*). A condition × time effect was not seen for IL-8 within boys *(p=0.72)*. The paired sample t-test did not show significantly different values for pre- compared to post-exercise unstimulated and stimulated IL-8 concentrations for boys *(p=0.43; p=0.60*, respectively) (Figure 7A). A condition effect was not seen for IL-8 within men *(p=0.18)*. A time effect was not seen for IL-8 within men *(p=0.19)*. A condition × time effect was not seen for IL-8 within men *(p=0.46)*. The paired sample t-test did not show significantly different values for pre- compared to post-exercise unstimulated and stimulated IL-8 concentrations for men *(p=0.30; p=0.11*, respectively) (Figure 7B).

When comparing IL-8 concentrations between boys and men, from pre- to post-exercise, and between unstimulated and stimulated conditions; a group effect was not seen *(p=0.50)*, a time effect was not seen *(p=0.16)*, and a condition effect was not seen *(p=0.58)*. Neither was a group × time × condition effect seen for IL-8 *(p=0.40)*.

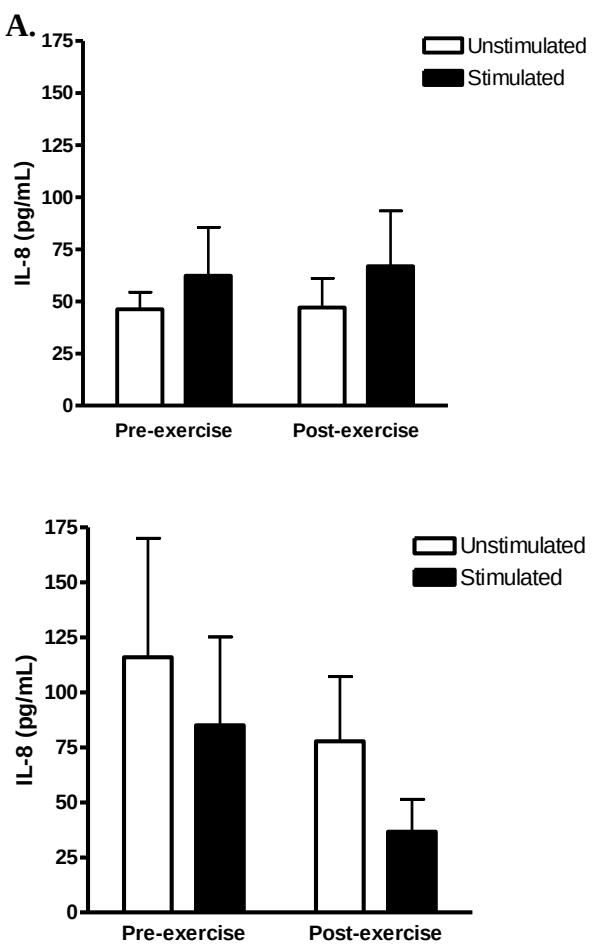


Figure 7. Change in IL-8 supernatant concentrations in unstimulated and PMA-stimulated cultured neutrophils from baseline after an acute bout of exercise in boys (n=10) and in men (n=8). Unstimulated and stimulated neutrophil supernatant IL-8 (pg/mL) in A. Boys, and B. Men. Values are expressed as means ± SD. Open bars represent unstimulated and solid black bars represent stimulated. **PMA = phorbol 12-myristate 13-acetate**

4.3.2 Myeloperoxidase

A condition effect was seen for myeloperoxidase concentrations within boys, with higher levels in unstimulated cells compared to PMA-stimulated cells *(p=0.009)* with a *(d)* of 0.40 (Figure 8A). A time effect was not seen for myeloperoxidase within boys *(p=0.60)*. A condition × time effect was not seen for myeloperoxidase within boys *(p=0.49)*. The paired sample t-test did not show significantly different values for pre- compared to post-exercise unstimulated and stimulated myeloperoxidase concentrations for boys *(p=0.93; p=0.35*, respectively) (Figure 8A). Since these experiments were mainly exploratory, and no significant differences were found in unstimulated samples for boys and men nor for stimulated samples in boys, stimulated samples in men were not measured for myeloperoxidase levels.

When comparing unstimulated myeloperoxidase concentrations between boys and men, from pre- to post-exercise; a group effect was not seen *(p=0.20)*, and a time effect was not seen *(p=0.89)*. A group × time effect was not seen for myeloperoxidase *(p=0.78)*.

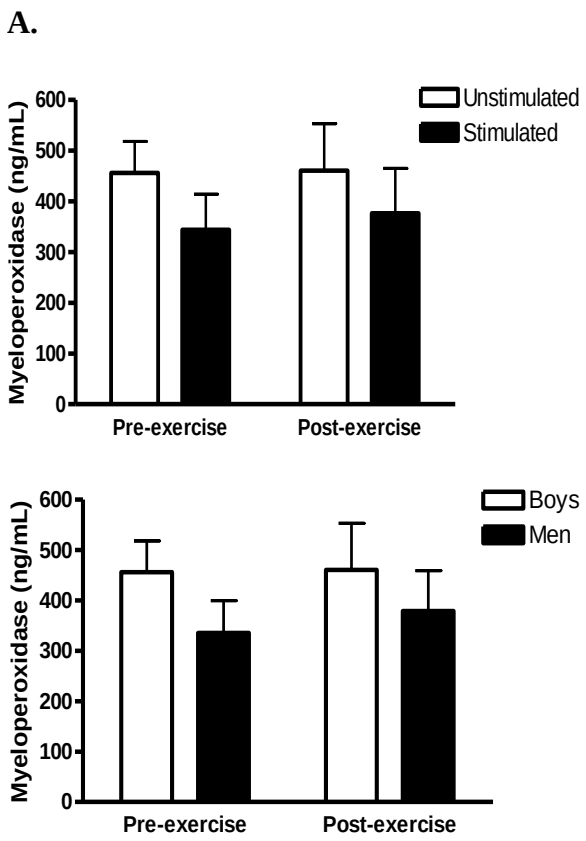


Figure 8. Change in myeloperoxidase supernatant concentrations in unstimulated and PMA-stimulated cultured neutrophils from baseline after an acute bout of exercise in boys (n=10) and in men (n=8). Unstimulated and stimulated neutrophil supernatant myeloperoxidase (pg/mL) in A. Boys, and B. unstimulated neutrophil supernatant myeloperoxidase (pg/mL) in boys compared to men. Values are expressed as means ± SD. Open bars represent unstimulated and solid black bars represent stimulated (A), or boys and men, respectively (B). **PMA = phorbol 12-myristate 13-acetate**

4.4 Intracellular ROS production

4.4.1 DCFH

There was no observed difference in percent change after exercise in DCFH-stained unstimulated cells comparing boys to men *(p=0.98)* (Figure 9).

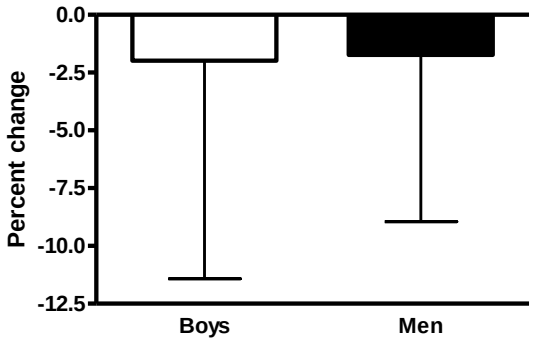


Figure 9. Percent change in intracellular ROS production in unstimulated neutrophils, measured by DCFH fluorescence intensity, from baseline after an acute bout of exercise in boys (n=10) and men (n=12). Values are expressed as means ± SD. Open bars represent boys and solid black bars for men.

A group effect was not seen for stimulated cells stained with DCFH *(p=0.34*). A time effect was seen for DCFH, with a higher post-exercise fold change compared to pre-exercise *(p=0.04*) with a *(d)* of 1.81 (Figure 10). A group × time effect was not seen for DCFH *(p=0.11).* The paired sample t-test showed a significant increase in DCFH fold-change post-exercise in boys *(p=0.018)* but not in men (*p=0.71*) (Figure 10).

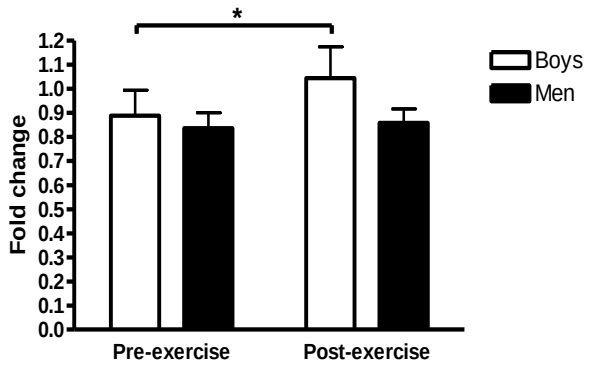


Figure 10. Fold change in intracellular ROS production in stimulated conditions compared to unstimulated counterparts, measured by DCFH fluorescence intensity, from baseline after an acute bout of exercise in boys (n=10) and men (n=12). Values are expressed as means ± SD. Open bars represent boys and solid black bars for men. * post-exercise values are significantly higher at *p<0.05* compared to pre-exercise.

4.4.2 DHR

There was no observed difference in percent change after exercise in DHR-stained unstimulated cells comparing boys to men *(p=0.40)* (Figure 11).

Figure 11. Percent change in intracellular ROS production in unstimulated neutrophils, measured by DHR fluorescence intensity, from baseline after an acute bout of exercise in boys (n=10) and men (n=12). Values are expressed as means ± SD. Open bars represent boys and solid black bars for men.

A group effect was not seen for stimulated cells stained with DHR *(p=0.58)*. A time effect was seen for DHR, with a higher post-exercise fold change compared to pre-exercise (*p=0.02*) with a *(d)* of 0.48 (Figure 12). A group × time effect was not seen for DHR *(p=0.39)*. The paired sample t-test showed an increase trending toward significance for DHR fold-change post-exercise in boys (*p=0.097)* but not in men *(p=0.13)* (Figure 12).

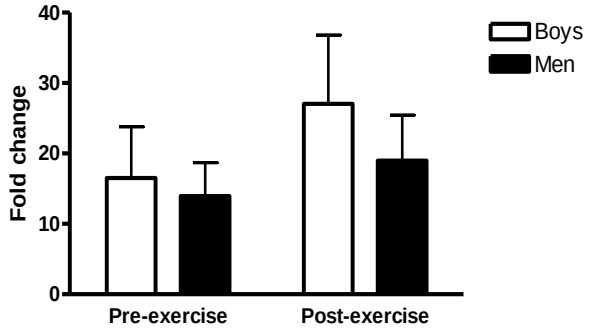


Figure 12. Fold change in intracellular ROS production from pre- to post-exercise in stimulated conditions compared to unstimulated counterparts, measured by DHR fluorescence intensity after an acute bout of exercise in boys (n=10) and men (n=12). Values are expressed as means ± SD. Open bars represent boys and solid black bars for men.

4.4.3 HE

Although Figure 13 shows an apparent difference, there was no statistically significant difference in percent change after exercise in HE-stained unstimulated cells comparing boys to men (*p=0.096)* (Figure 13).

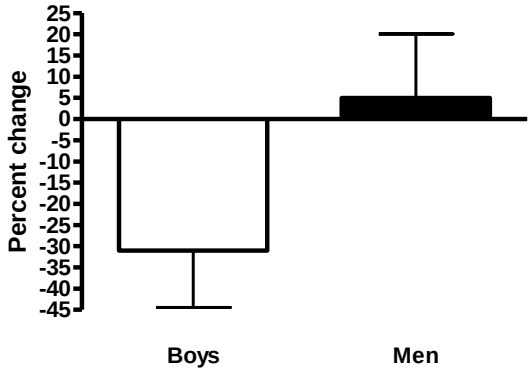


Figure 13. Percent change in intracellular ROS production in unstimulated neutrophils, measured by HE fluorescence intensity, from baseline after an acute bout of exercise in boys (n=10) and men (n=12). Values are expressed as means ± SD. Open bars represent boys and solid black bars for men.

A group effect was not seen for stimulated cells stained with HE *(p=0.718)*. A time effect was seen for HE, with a higher post-exercise fold change compared to pre-exercise *(p=0.05*) with a *(d)* of 0.59 (Figure 14). A group × time effect was not seen for HE *(p=0.38)*. The paired sample t-test did not show a significant difference post-exercise in boys or in men *(p=0.14; p=0.26*, respectively).

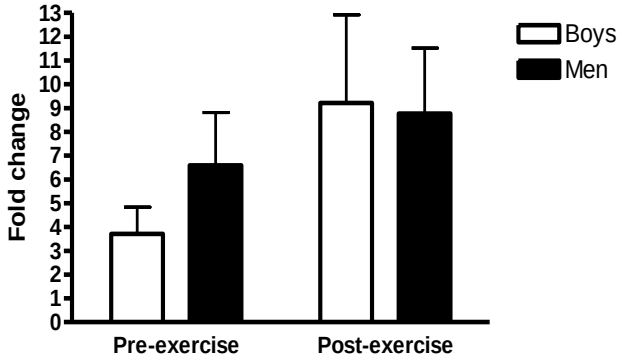


Figure 14. Fold change in intracellular ROS production from pre- to post-exercise in stimulated conditions compared to unstimulated counterparts, measured by HE fluorescence intensity after an acute bout of exercise in boys (n=10) and men (n=12). Values are expressed as means ± SD. Open bars represent boys and solid black bars for men.

4.5 Correlations between inflammatory cytokines and ROS markers

When comparing exercise-induced changes in inflammatory cytokines to changes in ROS markers, only one significantly positive correlation was observed consistently in both boys and men. Baseline to post-exercise fold-change in TNF-α and elastase concentrations showed a positive correlation in boys *r=0.788 (p=0.007)* and men *r=0.791 (p=0.011)* (Figure 15). Slopes were significantly different *(p=0.0049).*

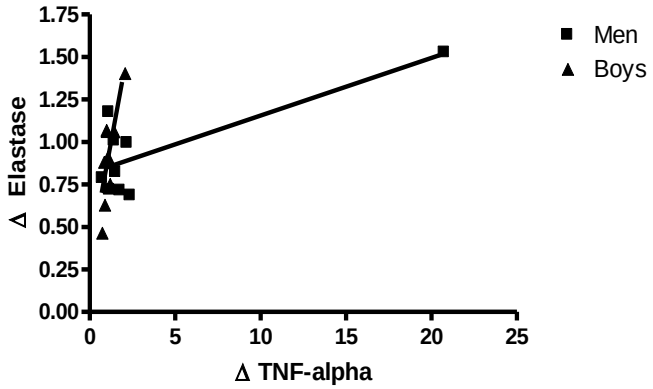


Figure 15. Correlation between baseline to post-exercise fold change in systemic TNF-α and elastase concentrations in boys (closed triangles; n=10) and in men (closed squares; n=9). Values are expressed as means. Boys *r=0.788 (p=0.007),* men *r=0.791 (p=0.011).*

Baseline to post-exercise fold-change in IL-8 and myeloperoxidase concentrations showed a positive correlation in boys *r=0.681 (p=0.03)* but not in men *r=0.039 (p=0.91)* (Figure 16). Slopes were not significantly different *(p=0.25)*, therefore the combined slope was 0.033.

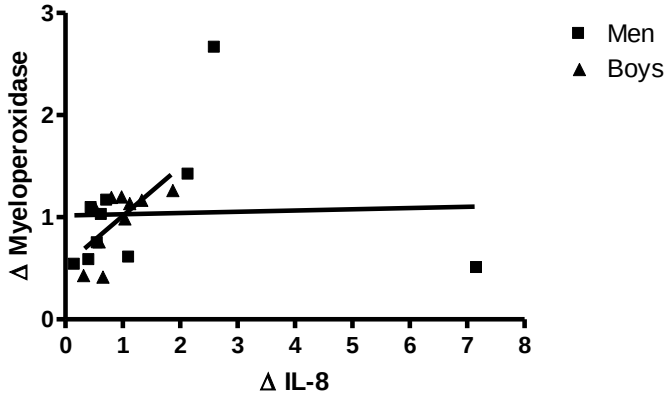


Figure 16. Correlation between baseline to post-exercise fold change in systemic IL-8 and myeloperoxidase concentrations in boys (closed triangles; n=10) and in men (closed squares; n=10). Values are expressed as means. Boys *r=0.681 (p=0.0.03)*, men *r=0.039 (p=0.914).*

Baseline to post-exercise fold-change in myeloperoxidase concentrations and percent change in unstimulated DCFH intensity showed a negative correlation in boys *r=-0.735 (p=0.016)* but not in men *r=0.237 (p=0.46)* (Figure 17). Slopes were significantly different *(p=0.025)*.

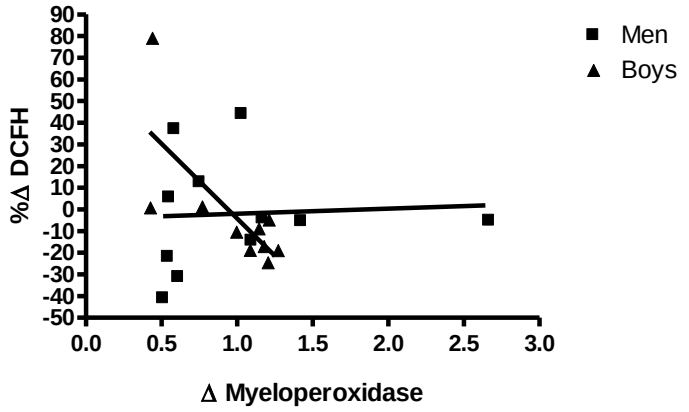


Figure 17. Correlation between baseline to post-exercise fold change in myeloperoxidase concentration and percent change in unstimulated DCFH intensity in boys (closed triangles; n=10) and in men (closed squares; n=11). Values are expressed as means. Boys *r=-0.735 (p=0.016)*, men *r=0.057 (p=0.868)*.

Baseline to post-exercise fold-change in protein carbonyl concentrations and percent change in unstimulated DHR intensity showed a negative correlation in men *r=-0.658 (p=0.02)* but not in boys *r=0.144 (p=0.69)* (Figure 18). Slopes were significantly different *(p=0.034)*.

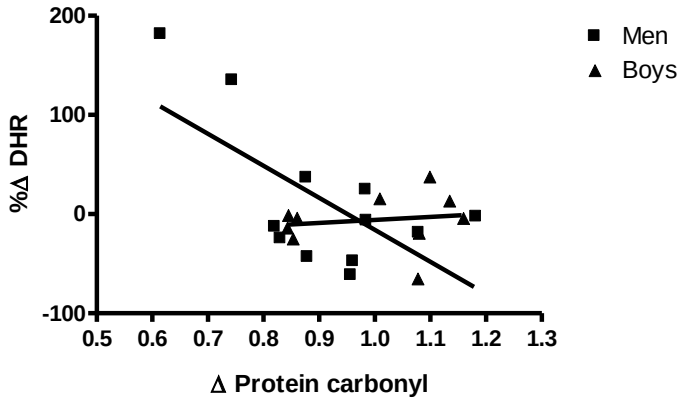


Figure 18. Correlation between baseline to post-exercise fold change in protein carbonyl concentration and percent change in unstimulated DHR intensity in boys (closed triangles; n=10) and in men (closed squares; n=12). Values are expressed as means. Boys *r=0.144 (p=0.692),* men *r=-0.658 (p=0.02).*

There were no significant correlations when comparing fold-change in inflammatory cytokines from baseline to post-exercise to fold-changes after stimulation post-exercise, in any of the fluorescent markers, in either boys or men.

CHAPTER 5: DISCUSSION

Despite the large body of evidence describing physiological adaptations gained from exercise in adults, such literature is scarce within the pediatric population. On the one hand, this is surprising given the role that physical activity and exercise may play at this crucial time of development; on the other hand, limitations in biological samples preclude an in depth understanding of biological processes. To address gaps in our understanding, the main objective of this thesis was to identify the age-related transition in the exercise-induced production of ROS, comparing boys to men. It was found that although men showed larger increases in circulating immune cells, boys showed higher stimulated intracellular ROS production in neutrophils mobilized with exercise. Boys also showed overall higher circulating protein carbonyl concentrations compared to men, whereas men had higher circulating malondialdehyde than boys. The secondary objective of this thesis was to assess the strength of association between systemic inflammatory cytokines and oxidative stress markers. Previous researchers have hypothesized a link between inflammatory mediators and oxidative stress (Reid & Li, 2001; Timmons & Raha, 2008; Vassilakopoulos et al., 2003), although the nature of such relationships are yet unknown (Reid & Li, 2001; Timmons & Raha, 2008; Vassilakopoulos et al., 2003). In this thesis, only one consistent link was found in both boys and men, showing a positive correlation between TNF-α and elastase fold-change.

The first part of this discussion will be divided into two sections. The first section will focus on the results related to the main objective of this study, including exercise-induced effects on immune cells, on neutrophil-derived intracellular ROS, and neutrophil

related cytokines and enzymes. The second section will focus on inherent differences in oxidative markers observed in boys compared to men. The latter part of the discussion will discuss results pertinent to the secondary objective correlating inflammatory cytokines and oxidative stress markers, and any additional interactions observed from the analysis.

5.1 Exercise effects in boys compared to men

5.1.1 Neutrophils

The changes in neutrophils before to after exercise showed an approximately 1.5-fold increase in men, but no change in boys (Figure 3B). In contrast, the proportion of neutrophils remained constant with neither group showing an exercise-induced change (Figure 3A). This indicates that acute exercise induces a more rigorous mobilization of neutrophils into the circulation in men than that observed in boys. These results are consistent with the limited existing research directly comparing children to adults, where acute exercise generally elicits a smaller neutrophil response in children (Shore & Shephard, 1998; Timmons et al., 2004; Timmons, 2006).

5.1.2 Intracellular ROS production

The oxidative stress response due to exercise stems mainly from the endogenous production of ROS from various sources (Finaud et al., 2006). Aside from mitochondrial ROS leakage, it is thought that the neutrophil oxidative burst response also contributes substantially to the ROS pool (Pyne, Smith, Baker, Telford, & Weidemann, 2000b; Smith

& Weidemann, 1993; Walrand et al., 2003). The main finding of this thesis showed increased neutrophil production of intracellular ROS, as measured by flow cytometry (Figure 10). Three fluorescent markers were used to gauge different types of ROS; DCFH is a general ROS marker, DHR is specific to H_2O_2, and HE is specific to O_2^- (Walrand et al., 2003). Cells were stimulated with PMA, which up regulates ROS production in neutrophils through protein kinase C, as both a positive control for ROS formation and as a determinant of exercise effects on the ability of neutrophils to produce ROS. Although there was no exercise effect on the production of ROS by unstimulated neutrophils between boys and men (Figures 9), the fold increase in stimulated ROS production by neutrophils (above that measured in unstimulated neutrophils) was higher after exercise (Figures 10). Interestingly, this effect was only observed for DCFH and for boys (Figure 10). The lack of significance for changes in other markers could have been due to the short half-life of H_2O_2 and O_2^-. Since there was a delay from blood sampling after completion of the exercise protocol and staining of neutrophils for flow cytometry due to the process of neutrophil isolation, some of the ROS may have disintegrated resulting in a decreased signal. To the author's knowledge, this is the first study comparing exercise-induced neutrophil ROS production between boys and men.

The higher stimulated neutrophil ROS production in boys, taken together with no change in the neutrophil response to exercise may be explained, in part, if the boys demonstrated a more sensitized oxidative response to exercise. This possibility is supported by the fact that other ROS markers responded in a similar fashion, but did not reach statistical significance. The notion of sensitized oxidative response to exercise may

stem from neutrophils primed for increased ROS formation after exercise, or this sensitization may originate from the mobilization of functionally different neutrophil populations. The following discussion further examines the literature supporting the two possibilities.

Despite the wealth of research on the exercise-induced rise in neutrophils and the resulting increase in ROS, not much is known as to what systemic factors activate this ROS cascade (Peake et al., 2004). Smith et al attributed neutrophil priming and activation to growth hormone produced after a bout of exercise (Smith et al., 1996). After a 1hr cycling bout (heartrate around 140 beats/min), 8 untrained males showed a three-fold increase in intracellular H_2O_2 production in PMA-stimulated neutrophils, along with a ten-fold increase in growth hormone (Smith et al., 1996). To determine if growth hormone and neutrophil-derived ROS were mechanistically linked, the participants also consumed glucose during exercise, which substantially attenuated the rise in growth hormone post-exercise and also induced a blunted response in H_2O_2 levels (Smith et al., 1996). In further support of the link between growth hormone and neutrophil priming and stimulation, in vitro studies have provided direct evidence for growth hormone binding to neutrophil receptors, resulting in a rise in superoxide anions (Fu, Arkins, Wang, & Kelley, 1991; Fu et al., 1992). Exercise studies involving children have shown increases in growth hormone after an acute bout of exercise in healthy participants (Nemet & Eliakim, 2010; Nguyen et al., 2011; Tirakitsoontorn, Nussbaum, Moser, Hill, & Cooper, 2001). Although the exercise-induced growth hormone response in adults is higher in absolute concentrations, children typically show a relatively larger percent

change from baseline with exercise, which is most likely important for the anabolic processes during their development (Timmons, 2006).

Another possible mechanism to explain the sensitization effect of exercise on neutrophils focuses on the type of neutrophils mobilized with exercise. Exercise can induce recruitment of neutrophils from circulation in the blood, or induce mobilization from the bone marrow (Peake et al., 2004) or other marginated pools. To the author's knowledge, there are no exercise studies that have distinguished the types of neutrophils mobilized in response to exercise in children, although there have been such studies involving adults. An earlier study identified two subpopulations of neutrophils by flow cytometry, characterized as high and low activity in terms of stimulated ROS production (Smith et al., 1996). ROS was measured in neutrophils using flow cytometry; results revealed varying levels of intracellular H_2O_2 produced per neutrophil after PMA-stimulation, and this was defined as neutrophil activity which was used to distinguish two subpopulations of neutrophils (Smith et al., 1996). The authors attributed the separate neutrophil populations to circulating versus demarginated neutrophils from capillary beds and blood vessel walls, the latter having intrinsically higher activity than those in circulation (Smith et al., 1996).

Two more recent studies tested adult male participants on treadmill protocols of various intensities (Peake et al., 2004; Quindry et al., 2003). Peake and colleagues determined that the decrease in the specific neutrophil CD16 receptor after an acute bout of high intensity exercise, could be explained by a shift toward the release of newly recruited neutrophils from bone marrow (Peake et al., 2004). This is supported by the

fact that previous studies have found an increase in immature neutrophils after exercise (Bishop, Walsh, & Scanlon, 2003; Suzuki et al., 1999; Suzuki et al., 2003), and that neutrophils shed their CD16 receptors when they are mobilized from the bone marrow (Huizinga et al., 1994). A similar explanation was provided for exercise recovery samples in adult males after maximal aerobic treadmill exercise (Quindry et al., 2003). It was found that both post-exercise and 2hr recovery samples had higher levels of O_2^- production, however when these levels were normalized for neutrophil numbers, only the 2hr sample's O_2^- levels remained significantly higher than baseline (Quindry et al., 2003). The neutrophils tested for the 2hr sample were likely mobilized from the bone marrow (Quindry et al., 2003; Suzuki et al., 1996). Because boys show a higher fold-change in ROS after exercise than men, which cannot be attributed to increase in absolute neutrophils, it is possible that exercise-induced neutrophil recruitment in boys originate from the bone marrow opposed to circulating pools in the blood. Since children tend to possess lower baseline inflammatory profiles compared to adults (Timmons, 2006), circulating immune cells would be relatively low, resulting in a higher ratio of mobilized neutrophils from the bone marrow compared to those from the blood.

5.1.3 Inflammatory markers

Acute exercise did not affect systemic TNF-α concentrations in boys, but induced a small increase in men (Figure 6). A group effect was seen with higher levels in boys overall compared to men (Figure 6). These results are consistent with existing literature especially for children, where TNF-α either does not change or has been seen to decreases due to exercise (Nemet et al., 2002; Scheett et al., 2002; Timmons et al., 2004). Adults

do not generally demonstrate an increase in TNF-α levels after acute exercise; increases are mostly seen as a result of long duration exercise protocols (Moldoveanu, Shephard, & Shek, 2001; Timmons, 2006).

Acute exercise significantly increased systemic IL-6 levels from baseline in both boys (2-fold increase) and men (4-fold increase), and post-exercise IL-6 concentrations were lower in boys compared to men (Figure 6). These results are all consistent with the literature. Although children generally do show an increase in IL-6 after acute exercise, it is of much smaller magnitude compared to adults (Nemet et al., 2002; Scheett et al., 2002; Timmons, 2006; Timmons et al., 2004). A review of 67 exercise trials with various modes of testing showed acute exercise cycling stimulates an increase in IL-6 by 5-fold from baseline in adult participants (Fischer, 2006).

Because IL-6 and TNF-α both possess anabolic and catabolic roles in growth (Cohen et al., 1996; De Benedetti et al., 1997), tight regulation of these, and most likely other cytokines, may be essential during growth during childhood (Timmons & Raha, 2008). However, these adaptive pathways are yet unknown. Due to ethical limitations specific to pediatric testing, invasive measures to determine the exact pathways and mediators involved in the exercise adaptive response cannot be implemented. If the inflammatory mediators are in fact associated with oxidative stress, then examining the exercise effects on ROS can potentially give more insight into the pathways involved in regulating proper growth.

5.1.4 Neutrophilic production of IL-8 and myeloperoxidase in culture

Neutrophils isolated from pre- and post-exercise samples were cultured for 1hr with and without PMA-stimulation, and supernatants were analyzed for IL-8 and myeloperoxidase content. There were no exercise-induced changes in IL-8 between boys and men in either stimulated or unstimulated samples (Figure 7). Although there were no exercise-induced changes in myeloperoxidase between boys and men, overall unstimulated neutrophils produced higher myeloperoxidase levels than stimulated in boys (Figure 8). It is possible that with stimulation of neutrophils, the original myeloperoxidase half-life of 4-5hours (Srivastava, Rado, Bauerle, & Broxmeyer, 1991) was substantially decreased due to its increased activity. To the author's knowledge, no exercise study has used a similar protocol to compare neutrophil characteristics between boys and men. This exploratory test revealed that acute exercise induces similar responses in neutrophilic production of oxidative stress-related mediators (IL-8 and myeloperoxidase) in boys as in men. The analogous outcomes may indicate a minimal role of neutrophil-derived IL-8 and myeloperoxidase in the child to adult transition, or it may be that the exercise intensity or duration was not strong or long enough to elicit a vigorous response. This result may also be due to methodological circumstances, such as sub-optimal culture conditions or culture supplementation.

5.1.5 Summary of exercise effects in boys compared to men

One of the main findings of this thesis revealed different neutrophil and oxidative stress responses to exercise between boys and men. Circulating neutrophils increased

after exercise in men, but not in boys. However, stimulated intracellular ROS production in neutrophils increased in post-exercise samples for boys, which was not seen in men. Taken together, these data indicate that in boys, exercise may induce a sensitizing effect on neutrophils.

Priming of neutrophils to produce ROS may play a role in the exercise adaptive response for children since increased neutrophilic superoxide production is stimulated by growth hormone (Fu et al., 1992), and growth hormone is tightly regulated for optimal development during childhood (Nemet & Eliakim, 2010). Alternatively, exercise may primarily induce mobilization of highly active neutrophils from bone marrow more so in children than in adults. A likely explanation is that these mechanisms are not mutually exclusive, and that exercise induces to some extent, a combination of both these mechanisms in children.

5.2 Secondary findings: oxidative markers in boys versus men

5.2.1 Protein carbonyls

An acute bout of cycling exercise did not induce significant increases in protein carbonyls post-exercise in either boys or men (Figure 5). To the author's knowledge, only two pediatric studies have tested the effect of acute exercise on circulating protein carbonyl levels. Trained male and female pre-pubertal swimmers performed an intermittent exercise protocol with 12x50m bouts at 70%-75% of the participant's VO_{2max} (Nikolaidis et al., 2007). Among other oxidative stress markers, protein carbonyls were found to be approximately two-fold higher in both boys and girls after exercise

(Nikolaidis et al., 2007). However, such an effect in boys, was not seen in another study testing boys on a 1hr exercise protocol set at an intensity of 70%VO_{2max} (Timmons & Raha, 2008). The results of this thesis obtained for boys do align with those of Timmons and colleagues (Timmons & Raha, 2008), which is logical since the exercise conditions and participant histories were very similar.

Adult literature on protein carbonyl levels after a bout of acute exercise is conflicting, despite the number of studies done to date (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). The majority of studies show evidence for increases in protein carbonyls in an intensity dependent fashion, with a sustained elevated level several hours after exercise (Fisher-Wellman & Bloomer, 2009; Michailidis et al., 2007). This thesis showed no change in plasma protein carbonyl levels as a result of an acute bout of cycling exercise (Figure 5), which is consistent with results obtained from exercise studies using similar protocols such as graded exercise tests or short testing periods (within 1hr) (Bloomer, Goldfarb, Wideman, McKenzie, & Consitt, 2005; Fisher-Wellman & Bloomer, 2009; Miyazaki et al., 2001). It has been proposed that the lack of change observed in protein carbonyl levels is due to short sampling time such that blood samples taken before and immediately after the exercise period, the time points used for this thesis, do not capture the full extent of exercise effects on protein carbonyls (Fisher-Wellman & Bloomer, 2009). Post-exercise recovery samples after a 30min bout of cycling exercise have revealed a significant rise in protein carbonyl levels at 6hrs and 24hrs after exercise, compared to baseline (Bloomer et al., 2005). Since protein carbonyls possess relatively long half-lives (Levine, 2002), it is possible these increases

after recovery reflect the accumulation of protein carbonyls as a more chronic result of exercise in adults and the sampling time used in this study did not yet reflect such an effect.

To date, there is only one known study to have tested both boys and men for oxidative stress markers under identical exercise conditions; Timmons et al tested pre-pubertal boys and adult men using a 1hr cycling protocol set at an intensity of 70%VO_{2max}, and measured protein carbonyls before and immediately after exercise (Timmons & Raha, 2008). They found an overall exercise-induced increase in protein carbonyl levels; although boys showed a smaller although non-significant increase, there were no significant differences between boys and men (Timmons & Raha, 2008). When comparing protein carbonyl levels after exercise in boys and men in the current study, there was no such time effect, but a group effect was observed with boys presenting 1.5-fold higher levels compared to men (Figure 5). Because this was a group effect and not a time-effect, this indicates intrinsic physiological differences in circulating protein carbonyl levels. There are a few possible explanations that may contribute to this effect; increased availability of circulating substrate for oxidation in boys, the novel role of protein carbonyls in promoting cell growth, higher protein diet intake, and possibly an immature antioxidant defense system.

During growth and development, whole body protein turnover in children is twice that of adults (Welle, 1999). Protein carbonyls disrupt the physiological protein balance because they are more difficult to break down than normal proteins (Finaud et al., 2006). As such, the increased new protein substrate generated, combined with disruptions in the

protein cycle, it is possible that the higher protein carbonyl levels seen in boys may be due to a buildup of protein carbonyls, a result of the higher protein turnover. It has been observed that increased lipid peroxidation in overweight and obese children is attributed to fundamentally higher circulating levels of lipids in the blood (Youssef et al., 2009). In addition, it has been shown that a main target of protein oxidation is albumin (Stadtman & Levine, 2006). Children also tend to have higher circulating albumin levels compared to adults, further emphasizing the differences between children and adults and substrate availability for oxidation (Weeke & Krasilnikoff, 1972).

It is possible that the higher protein carbonyl levels may act as a signaling mechanism instead of being detrimental by-products from protein oxidation. Wong and colleagues showed protein carbonyl formation through receptor-ligand binding of endothelin-1, which promotes vasoconstriction and vascular remodeling, to annexin-1, responsible for apoptosis and inhibiting cell proliferation (Wong, Cheema, Zhang, & Suzuki, 2008; Wong, Marcocci, Liu, & Suzuki, 2010). Binding of endothelin-1 to annexin-1 induced production of ROS and subsequent carbonylation of annexin-1, which essentially deactivated its function (Wong et al., 2008; Wong et al., 2010). This was proposed to be a mechanism to promote cell growth and survival (Wong et al., 2008), which is evidently essential for children's growth. Lastly, confounding contributors may have originated from higher protein intake in boys compared to men (although there were no group differences in protein intake relative to body mass), or due to the fact that children may have an underdeveloped antioxidant defense system compared to adults (Benitez-Sillero et al., 2011; Nikolaidis et al., 2007).

5.2.2 Malondialdehyde

Similar to protein carbonyls, a bout of acute exercise did not elicit significant changes in malondialdehyde concentrations in either boys or men (Figure 5). There have not been any studies done on pediatric populations looking at the effects of acute exercise on malondialdehyde production. However, only three primary research studies have been found testing the effect of acute exercise on markers of lipid peroxidation (Nikolaidis et al., 2007; Rosa et al., 2011; Youssef et al., 2009). Only one of the three studies showed a significant increase in thiobarbituric acid-reactive substances, testing trained pre-pubescent swimmers with an intermittent high-intensity swimming protocol (Nikolaidis et al., 2007). Control subjects from the other two studies did not show any significant changes in F_2-isoprostanes (common marker for lipid peroxidation) after an acute intermittent bout of 30min cycling exercise, or an incremental cycling exercise to exhaustion (Rosa et al., 2011; Youssef et al., 2009). Results from this thesis parallel the latter two studies, which is not unexpected due to the similarity in the nature of exercise protocols and participant history (healthy untrained individuals).

The use of malondialdehyde as an oxidative stress marker, is much more commonly seen in adult exercise studies (Finaud et al., 2006). Contrary to most lipid oxidative stress markers such as thiobarbituric acid-reactive substances and F_2-isoprostanes, malondialdehyde has shown to be generally unchanged after an acute bout of exercise, increasing only at maximal or near maximal intensities of exercise (Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). This was directly shown in the study by Lovlin et al where increases in cycling intensity, from 40%VO_{2max}, to 70%VO_{2max}, and

100%VO_{2max} resulted in a decrease, no change, and increase in malondialdehyde levels, respectively, in untrained adult participants (Lovlin, Cottle, Pyke, Kavanagh, & Belcastro, 1987). Thus, the resulting unchanged malondialdehyde levels after exercise for adults in the current thesis were expected, given the general consensus in literature.

Although exercise did not seem to stimulate increased lipid peroxidation, a group effect was observed between boys and men. Contrary to the trend in protein carbonyls, boys had lower overall malondialdehyde levels compared to men (Figure 5). It is important to note that because there is so little existing literature on using this marker in children, with the already conflicting adult literature, in addition to the current controversy in using malondialdehyde as an accurate marker of lipid peroxidation, the author will not be putting much emphasis on these results. Regardless, from the malondialdehyde data gathered from this study, a possible explanation for intrinsically lower malondialdehyde levels in children may be attributed to their increased resistance to physiological insult (Timmons, 2006; Timmons & Raha, 2008). This resistance is evident in immune cells showing smaller percent increases and faster recovery, and smaller changes in cytokines under similar exercise conditions comparing children to adults (Timmons, 2006). Additionally, higher levels of malondialdehyde may indicate upstream catabolic processes such as cell injury, or downstream inflammation and cell death (Begriche, Igoudjil, Pessayre, & Fromenty, 2006; Bloomer et al., 2005). Taking into account the negative side-effects of malondialdehyde which are seen primarily in adults, a higher circulating concentration in malondialdehyde observed from this study

may suggest that either adults are more prone to physiological stress and injury, or children are more protected against such effects.

5.2.3 Summary of secondary findings for oxidative stress markers in boys compared to men

Secondary findings from this study show a difference in intrinsic levels of protein carbonyls and malondialdehyde between boys and men, but no effect of acute exercise. Protein carbonyl levels were significantly higher in boys whereas malondialdehyde levels were higher in men. Due to their higher protein turnover that is required for growth, disturbances in protein homeostasis may result in a buildup of protein carbonyls in boys. At the same time, protein carbonyl molecules may play an integral part in promoting anabolic processes in children. Higher circulating malondialdehyde levels in men could have been due to either men being more prone to physiological insult, or to boys being more resistant. Not unexpectedly, intrinsic differences in protein carbonyl and malondialdehyde levels were most likely attributed to the physiological differences in growth between boys and men.

5.3 Secondary objective: link between inflammation and oxidative stress

The secondary objective for this thesis was to assess the strength of association between systemic inflammatory cytokines to oxidative stress markers. Although exercise studies have shown changes in both inflammatory and oxidative stress markers, there have been very few to examine changes in both under the same exercise conditions, and none to the author's knowledge, that have directly compared these changes between

children and adults. In vitro studies have attempted to elucidate the mechanistic link between ROS production and cytokine response. For example, a combination of in vitro studies have demonstrated that through activation of TNF-α, the transcription factor nuclear factor-κB can be stimulated to up-regulate downstream gene transcription of various pro-inflammatory cytokines, such as the potent chemo-attractant, IL-8 (Reid & Li, 2001; Ryan et al., 2004). In this thesis, correlations were performed between the exercise-induced change in inflammatory markers, IL-6 and TNF-α, and the exercise-induced change in oxidative indicators protein carbonyls, malondialdehyde, myeloperoxidase, elastase, IL-8, DCFH, DHR, and HE in boys and in men (Appendix 4: A, B, C, D). Although the initial goal was to compare changes in cytokines to changes in oxidative stress markers, other correlations were also found; boys showed a positive correlation between cultured supernatant IL-8 and myeloperoxidase fold-change (Figure 16) and a negative correlation between myeloperoxidase fold-change and DCFH percent change (Figure 17), men showed a negative correlation between protein carbonyl fold change and DHR percent change (Figure 18). Because these correlations were exploratory in nature, they will not be discussed further. When all data were taken into consideration (including outliers), among the primary correlations observed within boys and men, there was only one that was consistently significant for both groups: a positive correlation between TNF-α and elastase fold-change (Figure 15).

TNF-α is produced primarily by monocytes and macrophages as an inflammatory mediator with catabolic properties, which can induce injury in muscle tissue (Buck & Chojkier, 1996; Reid & Li, 2001; Timmons & Raha, 2008). It is possible a rise in

elastase, an indirect indicator of neutrophil degranulation with release of ROS, induces local tissue damage and inflammation (Bishop et al., 2002; Pyne et al., 1996; Smith & Weidemann, 1993). Subsequently, the rise in TNF-α originates from the inflammatory response, migrating to the site of damage (Reid & Li, 2001; Timmons & Raha, 2008). Recruitment of inflammatory markers may induce the production of IL-8, a potent chemo-attractant for neutrophils, inducing further neutrophil migration to the location of damage (Reid & Li, 2001; Scuderi, Nez, Duerr, Wong, & Valdez, 1991). Regulation of these signaling processes is yet unclear, and warrants further investigation.

Despite TNF-α and elastase being significantly correlated in both boys and men, the slope for boys was much steeper than that for men, indicating a larger change in elastase corresponding to a small change in TNF-α in the boys group (Figure 15). Scuderi and colleagues overlaid isolated human neutrophils with human leukocyte elastase, and tested neutrophil culture supernatants for TNF-α content (Scuderi et al., 1991). Using ELISAs and western blots, they found both a decrease in TNF-α concentrations in the supernatant, and evidence for degradation of the cytokine with elastase incubation (Scuderi et al., 1991). Because children demonstrate an overall blunted immune response compared to adults (Timmons, 2006; Timmons & Raha, 2008), it follows that children may possess a more efficient elastase response in order to maintain lower circulating levels of TNF-α. Further support for this notion comes from work done by Ding et al who found that neutrophil elastase also decreases TNF-α binding receptors on phagocytes (Ding & Porteu, 1992). This down-regulating response would likely negatively feedback further cytokine recruitment, and also decrease the cytokine-

induced response to maintain the balance in inflammatory mediators important for healthy development during childhood.

5.3.1 Summary of secondary objective: link between inflammation and oxidative stress

It was hypothesized that significant associations would be observed for exercise-induced changes in inflammatory and ROS markers, due to the proposed links between inflammation and oxidative stress. The associated rise in TNF-α and elastase in both boys and men may have been attributed to local and systemic inflammation, inducing neutrophil recruitment and ROS release catalyzed by elastase. Due to the differences in correlation slopes, it is possible that elastase was produced as a regulatory molecule to degrade circulating levels of TNF-α. The steeper rise in elastase may decrease and down-regulate the catabolic effects of TNF-α, contributing to the blunted exercise-induced cytokine response typical in growing children, a response that tightly regulates fluctuations in anabolic and catabolic mediators to optimize growth conditions.

CHAPTER 6: CONCLUSION

The aim of this thesis was to investigate the changes in oxidative stress and inflammatory mediators after an acute bout of cycling exercise in boys compared to men. Results from the primary objective, in comparing production of exercise-induced ROS between boys and men, demonstrated that although acute exercise did not induce an increase in absolute neutrophils in boys, an increase in neutrophil-stimulated ROS production was observed. Men, however, responded differently to exercise than boys, with an increase in neutrophils post-exercise and no change in neutrophil-stimulated ROS production. These novel findings point towards exercise as a potential stimulant for a sensitized neutrophil response in boys, which has not been previously characterized.

Intrinsic differences were also found in systemic protein carbonyls and malondialdehyde in boys compared to men. Although there were no exercise-induced effects, boys demonstrated higher protein carbonyl levels and men showed higher malondialdehyde levels. These results reveal that although acute exercise may not have altered systemic oxidative stress levels, there may be pre-existing differences in oxidation-derived mediators that change in the transition from boys to men.

The secondary objective was to explore the strength of association between systemic inflammatory cytokines and oxidative stress markers. A significant correlation was found for both boys and men, between TNF-α and elastase. This association reveals that there is a potential link between oxidative stress and inflammation which warrants further research to determine the exact cellular mechanisms.

CHAPTER 7: FUTURE DIRECTION

In terms of pediatric exercise medicine, there are endless possibilities for future directions because it is a relatively novel area limited in research. From the results and existing literature pertaining to the main objective of this thesis, it is evident that in order to understand the exercise-induced adaptive response that may be involved in children's growth and development, recovery samples should be acquired and analyzed. From the adult literature, recovery samples of up to 24hrs reveal changes in oxidative stress markers, including changes from post-exercise to 24hrs after recovery (Michailidis et al., 2007). In addition, children's immune cells' recovery to baseline seems to be faster compared to that of adults (Timmons, 2006). Changes observed in adults may be involved in the adaptive immune or fitness response to exercise. Consequently, in children, fluctuations in oxidative stress and inflammatory mediators may provide better insight into adaptive growth mediators.

In further pursuit of elucidating the transition from the child to the adult phenotype, additional markers of growth and oxidative defense should be examined. Growth hormone plays a significant role in mediating healthy growth in children, and pediatric exercise studies have shown an increase in growth hormone immediately after exercise (Nemet & Eliakim, 2010). Since growth hormone has been shown to mediate ROS release, studying the exercise-induced fluctuations in both will give more insight into the effects of exercise during childhood. Also directly linked to oxidative stress are antioxidants, and studies have suggested that children may possess an underdeveloped antioxidant defense system (Nikolaidis et al., 2007). Athletes who are chronically trained

have higher antioxidant activity (Finaud et al., 2006), thus it may be beneficial to examine the effects of exercise training on antioxidant activity in children in the efforts of decreasing potentially detrimental effects of oxidative stress due to exercise.

Further examining the exercise-induced adaptive mechanisms in children compared to adults will lend a better understanding of healthy growth and development in children. Only by increasing the breadth of testing such as duration of sample acquisition or variety of hormones, inflammatory mediators, and oxidative stress markers, can there be a complete understanding of pediatric exercise medicine in healthy children. This will hopefully facilitate the transition from scientific research to clinical application of exercise in early intervention and treatment of diseases or conditions acquired during childhood.

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APPENDIX 1: Daily intake of macronutrients per kilogram body weight and major antioxidants from 3-day food record.

Nutrient	Total or %Rcmd	Boys (n=8)	Men (n=11)	P-value
Energy (kcal)	Total	2041.7 ± 152.9	2805 ± 1143	0.052
	%Rcmd	78.4 ± 13.3	84.1 ± 33.2	0.614
Energy (kcal/kg)	Total	58.8 ± 12.1	36.5 ± 14.9	**0.003***
Protein (g/kg)	Total	2.6 ± 0.6	1.9 ± 0.8	0.071
	%Rcmd	270.7 ± 65.3	241.8 ± 96.3	0.474
Carbohydrates (g/kg)	Total	7.2 ± 2.1	4.4 ± 1.9	**0.008***
	%Rcmd	69.8 ± 18.9	73.8 ± 32.8	0.766
Fat (g/kg)	Total	2.3 ± 0.7	1.3 ± 0.6	**0.006***
	%Rcmd	97.1 ± 27.8	95.6 ± 45.1	0.936
Saturated fat (g/kg)	Total	1.0 ± 0.3	0.4 ± 0.2	**<0.001**
	%Rcmd	132.9 ± 42.4	87.0 ± 37.4	**0.023***
Mono fat (g/kg)	Total	0.5 ± 0.2	0.4 ± 0.2	0.113
	%Rcmd	64.7 ± 28.5	75.3 ± 46.8	0.576
Poly fat (g/kg)	Total	0.2 ± 0.1	0.2 ± 0.2	0.701
	%Rcmd	27.0 ± 13.8	54.6 ± 47.5	0.093
Vitamin A (RAE/kg)	Total	22.2 ± 9.5	10.8 ± 8.4	**0.013***
	%Rcmd	147.7 ± 75.2	90.9 ± 71.6	0.113
Vitamin C (mg/kg)	Total	3.7 ± 2.2	1.7 ± 1.5	**0.030***
	%Rcmd	315.6 ± 141.6	141.4 ± 119.8	**0.010***
Vitamin E (mg/kg)	Total	0.082 ± 0.022	0.081 ± 0.072	0.982
	%Rcmd	29.2 ± 7.1	41.3 ± 35.6	0.298

Values are expressed as means ± SD. * significant difference, *p<0.05*. %Rcmd: Percent recommended based on age, sex, and physical activity levels (most participants were in the moderate to very active categories), mono fat: mono-unsaturated fat, poly fat: poly-unsaturated fat, RAE: retinol activity equivalents for vitamin A.

APPENDIX 2: BORG scale for rating of perceived exertion

6	
7	VERY VERY LIGHT
8	
9	VERY LIGHT
10	
11	FAIRLY
12	
13	SOMEWHAT HARD
14	
15	HARD
16	
17	VERY HARD
18	
19	VERY VERY HARD
20	

APPENDIX 3: Results from complete blood count test

	Boys		Men	
	Pre	**Post**	**Pre**	**Post**
Leukocytes ($\times 10^9$/L)	6.32 ± 1.29	7.15 ± 1.35	5.01 ± 0.74	**7.66 ± 2.48***
Relative neutrophils (%WBC)	50.33 ± 2.74	48.89 ± 5.67	54.50 ± 7.60	56.60 ± 9.60
Absolute neutrophils ($\times 10^9$/L)	3.18 ± 0.67	3.49 ± 0.73	2.72 ± 0.49	**4.48 ± 1.97***

Values are expressed as means ± SD. *significant difference from pre-exercise, $p<0.05$.

APPENDIX 4A: Results from Pearson Correlation between fold-change in inflammatory markers and fold-change in oxidative markers in men.

		IL6	TNF-α	IL8	Protein carbonyls	Malon-dialdehyde	Myelo-peroxidase	Elastase	DCFH	DHR	HE
IL6	Pearson Correlation	1	.015	.243	-.139	.077	-.011	.466	-.043	-.258	-.017
	Sig. (2-tailed)		.964	.499	.666	.812	.975	.206	.894	.418	.959
	N	12	12	10	12	12	11	9	12	12	12
TNF-α	Pearson Correlation	.015	1	-.103	-.112	-.078	.050	.791*	-.067	.177	-.091
	Sig. (2-tailed)	.964		.778	.729	.810	.884	.011	.837	.583	.779
	N	12	12	10	12	12	11	9	12	12	12
IL8	Pearson Correlation	.243	-.103	1	.155	.047	.039	-.256	-.516	-.284	.148
	Sig. (2-tailed)	.499	.778		.668	.897	.914	.507	.127	.426	.684
	N	10	10	10	10	10	10	9	10	10	10
Protein carbonyls	Pearson Correlation	-.139	-.112	.155	1	-.141	.357	-.343	.077	-.658*	-.494
	Sig. (2-tailed)	.666	.729	.668		.663	.281	.366	.812	.020	.103
	N	12	12	10	12	12	11	9	12	12	12
Malon-dialdehyde	Pearson Correlation	.077	-.078	.047	-.141	1	.139	.270	.237	-.081	.092
	Sig. (2-tailed)	.812	.810	.897	.663		.683	.483	.458	.801	.776
	N	12	12	10	12	12	11	9	12	12	12
Myelo-peroxidase	Pearson Correlation	-.011	.050	.039	.357	.139	1	.377	.057	-.074	-.082
	Sig. (2-tailed)	.975	.884	.914	.281	.683		.317	.868	.829	.810
	N	11	11	10	11	11	11	9	11	11	11
Elastase	Pearson Correlation	.466	.791*	-.256	-.343	.270	.377	1	-.091	.260	-.038
	Sig. (2-tailed)	.206	.011	.507	.366	.483	.317		.816	.499	.923
	N	9	9	9	9	9	9	9	9	9	9
DCFH	Pearson Correlation	-.043	-.067	-.516	.077	.237	.057	-.091	1	.260	-.250
	Sig. (2-tailed)	.894	.837	.127	.812	.458	.868	.816		.414	.433
	N	12	12	10	12	12	11	9	12	12	12
DHR	Pearson Correlation	-.258	.177	-.284	-.658*	-.081	-.074	.260	.260	1	.479
	Sig. (2-tailed)	.418	.583	.426	.020	.801	.829	.499	.414		.115
	N	12	12	10	12	12	11	9	12	12	12
HE	Pearson Correlation	-.017	-.091	.148	-.494	.092	-.082	-.038	-.250	.479	1
	Sig. (2-tailed)	.959	.779	.684	.103	.776	.810	.923	.433	.115	
	N	12	12	10	12	12	11	9	12	12	12

*. Correlation is significant at the 0.05 level (2-tailed).

APPENDIX 4B: Results from Pearson Correlation between fold-change in inflammatory markers and fold-change in oxidative markers in boys.

		IL6	TNF-α	IL8	Protein carbonyls	Malon-dialdehyde	Myelo-peroxidase	Elastase	DCFH	DHR	HE
IL6	Pearson Correlation	1	.106	.375	-.181	-.241	.398	-.360	-.169	-.052	-.280
	Sig. (2-tailed)		.770	.285	.617	.502	.255	.307	.641	.888	.433
	N	10	10	10	10	10	10	10	10	10	10
TNF-α	Pearson Correlation	.106	1	-.138	.469	-.115	.178	.788**	-.321	.532	.067
	Sig. (2-tailed)	.770		.705	.171	.751	.622	.007	.366	.113	.854
	N	10	10	10	10	10	10	10	10	10	10
IL8	Pearson Correlation	.375	-.138	1	.185	-.266	.681*	-.534	-.546	-.557	-.127
	Sig. (2-tailed)	.285	.705		.609	.457	.030	.112	.103	.095	.727
	N	10	10	10	10	10	10	10	10	10	10
Protein carbonyls	Pearson Correlation	-.181	.469	.185	1	-.534	.250	.591	-.471	.144	-.091
	Sig. (2-tailed)	.617	.171	.609		.112	.486	.072	.169	.692	.803
	N	10	10	10	10	10	10	10	10	10	10
Malon-dialdehyde	Pearson Correlation	-.241	-.115	-.266	-.534	1	-.251	-.110	.112	.063	.170
	Sig. (2-tailed)	.502	.751	.457	.112		.484	.762	.757	.862	.638
	N	10	10	10	10	10	10	10	10	10	10
Myelo-peroxidase	Pearson Correlation	.398	.178	.681*	.250	-.251	1	-.245	-.735*	.098	.267
	Sig. (2-tailed)	.255	.622	.030	.486	.484		.494	.016	.788	.456
	N	10	10	10	10	10	10	10	10	10	10
Elastase	Pearson Correlation	-.360	.788**	-.534	.591	-.110	-.245	1	-.066	.590	.124
	Sig. (2-tailed)	.307	.007	.112	.072	.762	.494		.857	.072	.732
	N	10	10	10	10	10	10	10	10	10	10
DCFH	Pearson Correlation	-.169	-.321	-.546	-.471	.112	-.735*	-.066	1	-.243	-.502
	Sig. (2-tailed)	.641	.366	.103	.169	.757	.016	.857		.499	.139
	N	10	10	10	10	10	10	10	10	10	10
DHR	Pearson Correlation	-.052	.532	-.557	.144	.063	.098	.590	-.243	1	.523
	Sig. (2-tailed)	.888	.113	.095	.692	.862	.788	.072	.499		.121
	N	10	10	10	10	10	10	10	10	10	10
HE	Pearson Correlation	-.280	.067	-.127	-.091	.170	.267	.124	-.502	.523	1
	Sig. (2-tailed)	.433	.854	.727	.803	.638	.456	.732	.139	.121	
	N	10	10	10	10	10	10	10	10	10	10

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

APPENDIX 4C: Results from Pearson Correlation between fold-change in inflammatory markers and fold-change in neutrophil-stimulated fluorescent markers in men.

		IL6	TNFalpha	IL8	DCFH	DHR	HE
IL6	Pearson Correlation	1	.015	.243	-.139	-.003	.078
	Sig. (2-tailed)		.964	.499	.666	.992	.810
	N	12	12	10	12	12	12
TNFalpha	Pearson Correlation	.015	1	-.103	-.152	-.271	-.275
	Sig. (2-tailed)	.964		.778	.638	.394	.386
	N	12	12	10	12	12	12
IL8	Pearson Correlation	.243	-.103	1	.166	.178	.330
	Sig. (2-tailed)	.499	.778		.647	.624	.351
	N	10	10	10	10	10	10
DCFH	Pearson Correlation	-.139	-.152	.166	1	-.436	-.156
	Sig. (2-tailed)	.666	.638	.647		.156	.628
	N	12	12	10	12	12	12
DHR	Pearson Correlation	-.003	-.271	.178	-.436	1	.885**
	Sig. (2-tailed)	.992	.394	.624	.156		.000
	N	12	12	10	12	12	12
HE	Pearson Correlation	.078	-.275	.330	-.156	.885**	1
	Sig. (2-tailed)	.810	.386	.351	.628	.000	
	N	12	12	10	12	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

APPENDIX 4D: Results from Pearson Correlation between fold-change in inflammatory markers and fold-change in neutrophil-stimulated fluorescent markers in boys.

		IL6	TNFalpha	IL8	DCFH	DHR	HE
IL6	Pearson Correlation	1	.106	.375	.233	.164	.011
	Sig. (2-tailed)		.770	.285	.517	.650	.976
	N	10	10	10	10	10	10
TNFalpha	Pearson Correlation	.106	1	-.138	.037	-.452	-.403
	Sig. (2-tailed)	.770		.705	.920	.190	.248
	N	10	10	10	10	10	10
IL8	Pearson Correlation	.375	-.138	1	.301	-.041	-.307
	Sig. (2-tailed)	.285	.705		.398	.910	.389
	N	10	10	10	10	10	10
DCFH	Pearson Correlation	.233	.037	.301	1	-.769**	-.745*
	Sig. (2-tailed)	.517	.920	.398		.009	.013
	N	10	10	10	10	10	10
DHR	Pearson Correlation	.164	-.452	-.041	-.769**	1	.829**
	Sig. (2-tailed)	.650	.190	.910	.009		.003
	N	10	10	10	10	10	10
HE	Pearson Correlation	.011	-.403	-.307	-.745*	.829**	1
	Sig. (2-tailed)	.976	.248	.389	.013	.003	
	N	10	10	10	10	10	10

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).