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# Muscle Specific Alterations in Mitochondrial Function in Adult and Aged Rats Selectively Bred for High versus Low Running Capacity

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Muscle Specific Alterations in Mitochondrial Function in Adult and Aged Rats Selectively Bred for High versus Low Running Capacity" submitted by Constance L. Tweedie in partial fulfillment of the requirements for the degree of Masters of Science.

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**Abstract**

Selective breeding for high (HCR) and low (LCR) running capacity has concurrently resulted in significant differences in propensity for obesity (80; 91; 104), insulin resistance (80; 104) and cardiovascular disease (20; 52; 104). Differences in mitochondrial function between HCR and LCR rats have been speculated, although little functional data has been obtained. To investigate this possibility, we examined mitochondrial function (respirometry, reactive oxygen species [ROS] production) in small bundles of the soleus and gastrocnemius muscle in adult and aged HCR and LCR animals. Measurements were also taken of citrate synthase activity, protein carbonylation, and oxidative DNA damage to provide an overall view of mitochondrial function and the consequences for oxidative stress. The results of this study showed dramatically different adaptations between the two muscle types. Specifically, the soleus muscle bundles of HCR animals had greater respiratory capacity and ROS production in adult animals, but not in aged animals. On the other hand, gastrocnemius muscle bundles of HCR animals had similar respiratory capacity at both ages, and greater ROS production in both adult and aged animals. There were no significant differences in protein carbonylation between selection groups at either age; however, HCR animals had lower oxidative DNA damage than LCR animals in both muscles in both age groups. In conclusion, it appears that selecting for high innate running capacity has yielded muscle mitochondrial adaptations that maximize muscle respiratory capacity while minimizing damage due to ROS production. Several mechanisms may be at play to result in the above findings, with the most prominent hypotheses being hormetic changes to antioxidant and scavenging abilities in HCR animals.

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## Dedication

I would like to dedicate this thesis to those who have been there for me during my ups and downs throughout this project, giving me unconditional love and support. Namely, my fiancée Daniel Kane, my mother Norma Milne, my stepfather Alex Milne, my father Murray Tweedie, and my siblings David Tweedie, Jennifer Tweedie, Jonathan Tweedie, and Catherine MacDow. Without the support and guidance I would not be where I am today.

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## List of Symbols and Abbreviations

Δp- Proton motive force

8-OHdG- 8-hydroxy-2'-deoxyguanosine

ADP-Adenosine diphosphate

AMP- Adenosine monophosphate

AMPK-AMP activated protein kinase

ANOVA- Analysis of variance

ANT-Adenosine nucleotide transporter

Asc- Ascorbate

ATP-Adenosine triphosphate

β-HAD-β-hydroxyacyl CoA dehydrogenase

Complex I- NADH:ubiquinone oxidoreductase

Complex II- Succinate-coenzyme Q reductase

Complex III- Cytochrome c oxidoreductase

Complex IV- Cytochrome c oxidase

Complex V- ATP synthase

COX- Cytochrome c oxidase

CS- Citrate synthase

CVD- Cardiovascular disease

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

DNP- Dinitrophenol

ECT- Electron transport chain

F344BN- Fisher 344 X Brown Norway

FAD- flavin adenine dinucleotide

FFA- Free fatty acids

HCR- High running capacity

HRP- horseradish peroxidase

i.p.- Intraperitoneal

LCR- Low running capacity

MnSOD- Manganese superoxide dismutase

NADH- Nicotinamide adenine dinucleotide

NRF-1- Nuclear respiratory factor 1

OAA- Oxalacetic Acid

PPAR γ- peroxisome proliferator-activated receptor gamma

PGC1-α- Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha

RCR- respiratory control ratio

ROS- Reactive oxygen species

OXPHOS- Oxidative phosphorlyation system

SDH- Succinate dehydrogenase

TFAM- Transcription factor A, mitochondrial

UCP- Uncoupling protein

UQCR 2- ubiquinol-cytochrome c reductase

$VO_2$-maximal oxygen uptake

# Chapter 1. General Introduction

In today's society, high calorie diets and sedentary lifestyle have become increasingly common, and associated with these lifestyle choices is an increase in type II diabetes and obesity, as well as other serious diseases. Investigation into preventative/ therapeutic approaches to these lifestyle associated diseases have found that increasing aerobic capacity, primarily via endurance exercise training, has beneficial effects in reducing or even eliminating said diseases (23; 67; 83). Aerobic capacity and changes in aerobic capacity are very individual traits and are controlled by the interaction of both genes for innate capacity and a second set of genes controlling one's trainability (15; 60). Due to the complexity of the control of aerobic capacity, selective breeding for both innate high and low running capacities has been undertaken to more carefully investigate the role of high aerobic capacity on health and disease (60). Investigations of animals selectively bred for high running capacity have found decreased susceptibility for insulin resistance, obesity, hypertension and CVD versus their low running capacity counterparts (20; 39; 51; 80; 91; 104). Although few analyses on the mitochondrial function of these animals has been undertaken, several studies have inferred a diminished mitochondrial capacity in LCR animals as an important contributor to their poorer health outcomes (39; 46; 104).

Similar to endurance exercise capacity, mitochondrial function appears to play an important role in health and disease, with indices of decreased oxidative capacity found in conjunction with type II diabetes (14; 58), obesity (48), and aging (65; 100). As mitochondria are the primary sites of energy production, their proper function is integral to health and life itself. When mitochondria become dysfunctional, aged, or damaged

they are also the primary sites for production of reactive oxygen species and thus oxidative damage (53; 69; 70; 77; 96; 100). Due to the interplay of mitochondrial function with endurance exercise capacity, a next logical step would be to determine the differences in mitochondrial function in these high and low running capacity animals, and how this may be associated with health and longevity.

## Chapter 2. Review of Literature

*Mitochondrial function*

The mitochondrion is a very complex and important cellular organelle, vital to the energy production of nearly all eukaryotic. The central aspect of energy transduction in a mitochondrion is its ability to generate an ionic gradient via the pumping of protons ($H^+$) across the inner membrane. This pumping is accomplished by coupling the $H^+$ - translocation from the mitochondrial matrix with concurrent electron flow down an "electrochemical staircase" (i.e., the electron transport chain, or *ETC*). This pumping action of the mitochondrial proteins results in a proton motive force ($\Delta p$) that is the driving force for ATP production at $F_0F_1$ATP synthase, located at the end of the ETC. Mitochondria utilize glutamate, malate and pyruvrate to generate NADH, the reducing equivalent for NADH:ubiquinone oxidoreductase (complex I); and succinate to generate $FADH_2$, the reducing equivalent for succinate-coenzyme Q reductase (complex II). Both complex I and II shuttle electrons to the quinone pool, which then passes them off to cytochrome c oxidoreductase (complex III), and finally to cytochrome c oxidase (complex IV) via cytochrome c. At complex IV, the electrons are finally used to reduce diatomic oxygen to water. Electron transfer at complexes I, III and IV results in the pumping of $H^+$ into the intermembrane space. $H^+$ flow back through the $F_0$ aspect of $F_0F_1$ATP synthase (complex V), which provides the energy for conversion of ADP and inorganic phosphate ($P_i$) to ATP at the $F_1$ aspect of complex V. For production of one molecule of ATP it has been estimated that 3-4 $H^+$ must pass though complex V (79). The rate of ATP production, besides being linked to the $\Delta p$, is also dependent on the concentration of substrate (ADP + $P_i$) available. When ADP is limiting, complex V will

function sub-maximally, and the Δp will not be quickly dissipated. Thus, the pumping of $H^+$ across the inner membrane is linked (or coupled) to the production of ATP (and thus to the availability of ADP). However, this complex circuit is not perfect, and not all the Δp goes to the production of ATP. There is always some leak of $H^+$ across the inner mitochondrial membrane, via specific channels (ionophores) or passive diffusion across damaged areas of the membrane (77; 79). This inefficient leak is referred to as 'uncoupling' and occurs during both basal states and during active states, albeit less so in the latter condition (17; 77; 79). The $H^+$ -pumping that is used for ATP synthesis relative to the non-phosphorylating proton leak represents the 'coupling efficiency' of the mitochondrion, which during high rates of ATP production reaches 90% (17; 43).

Cellular energy production is dependent on the proper function of the ETC and complex V (79). Since this relationship is both complex and interdependent, any dysfunction in ATP production may therefore affect proper cell function. For example, a decrease in complex I, but not complexes II, III or IV is found to be present in Parkinson's Disease (81) highlighting the importance of determining the ETC function in disease states. Many different diseases are related to mitochondrial dysfunction including Huntington's Disease (103), atherosclerosis (38; 69), and Alzheimer's Disease (103). Also associated with mitochondrial dysfunction is the aging process (41). Some hypothesize that the reason that we age (and most importantly why we become weak, unhealthy and diseased in old age) is primarily due to a dysfunction in mitochondrial proteins and/or the mitochondrial membrane, resulting in decreased energy production, increased oxidative stress and eventually cell death (apoptosis) (25; 71; 77). This hypothesis is commonly known as the *mitochondrial theory of aging* (25; 71; 77; 96).

*Effects of aging on muscle mitochondrial function.*

The mitochondrial theory of aging states that accumulation of mitochondrial damage, produced from oxidative stress, results in mitochondrial DNA mutations/deletions, protein degradation, and ultimately the induction of apoptosis, all of which are held to be key factors in the onset of sarcopenia (age-related muscle loss) and aging (25; 29; 71; 77; 96). Aged muscles also have fewer, yet larger mitochondria, which are frequently seen with defects such as abnormal cristae and vacuoles (96). There are several processes working concurrently that contribute to oxidative damage as a cell ages: 1) There is a decrease in biosynthesis of new mitochondria. This is a result of decreased PGC-1α (mitochondrial synthesis protein) mRNA (7) and protein (25). There is evidence that the decrease in PGC-1α with aging may be due to an inefficiency in the activator, adenosine monophosphate kinase (AMPK) (25). AMPK detects increased energy requirement via increases in AMP, and signals the nucleus to produce PGC-1-α. PGC-1-α then begins the biosynthesis of new mitochondria to fulfill the energy requirement (25). In aged muscle it has been shown that AMPK is not being activated in high AMP levels, thus the cascade of events leading to increased/new mitochondria is arrested (25); 2) There is a decrease in mitochondrial degradation, via insufficient lysosomal activity, thus resulting in a decreased mitochondrial turnover in the aged cell (25; 100); and 3) Due to the decreased turnover of mitochondria, old/"dysfunctional" complexes within the mitochondria are around the cell longer (95). These old mitochondria are less effective at energy transduction, and therefore less oxygen is being reduced, and less ATP is being produced (77; 100).

Older mitochondria have a diminished capacity to utilize the incoming oxygen, which impairs the electron transfer upstream of complex IV. This slowing of the electron flow through the respiratory chain results in an elevation of membrane potential and thus the protonmotive force. When the ETC is in this slowed state of electron flow it results in the reduction of the ETC complexes, increasing the potential for ROS formation. Specifically, unreduced oxygen may bind to complex I and/or III and 'steal' an electron, thus forming $O_2^{\cdot}$, a form of ROS that can damage the cell (17). These increases in ROS cause defects in both mitochondrial and nuclear DNA, as well as damaging mitochondrial proteins and lipids directly (17; 86; 100). As the mitochondrial proteins are being damaged by ROS, they become even more inefficient, and thus increased ROS production occurs. This vicious cycle, along with the decreased mitochondrial turnover, is the basis for the mitochondrial theory of aging.

Along with oxidative damage to the mitochondria, gene expression for oxidative phosphorlyation (OXPHOS) is decreased. In aged rats it has been found that 39 genes for OXPHOS (6 for the krebs cycle and 14 for substrate metabolism) were down-regulated (85). A large portion of these genes coded for complex I and V. Although gene expression for complex IV (cytochrome c oxidase) does not appear to be strongly affected by aging (94), there is evidence of reduced activity of this enzyme (7; 94), and increased mitochondrial cytochrome c release (94). Diminished cytochrome c oxidase activity limits the electron transfer, and thus also the respiratory capacity of the aged mitochondria (94). Cytochrome c release triggers one of the apoptotic pathways, leading to cell death, and is hypothesized to contribute to sarcopenia (64; 72; 94).

One proposed theory of defense against ROS damage to the mitochondria is uncoupling of the inner mitochondrial membrane (1; 17; 38; 43; 92). The basis of this theory is that by reducing the coupling of respiration to ATP-synthase (effectively decreasing the control by ATPsynthase on electron transfer ) the cell can maintain relatively high oxidation at complex IV, and decreased reduction of the electron transport chain complexes, specifically complex I , and thus prevent unwanted oxidation at complex I and III, decreasing ROS emissions. Interestingly, the expression of uncoupling proteins (UCP's) and their incorporation into the mitochondrial inner membrane is signaled by ROS emissions, thus providing a means for the cell to adapt to conditions of elevated ROS production under normal circumstances (6; 43). Research on coupling levels has found the tibialis anterior muscle to have greater oxygen uptake but no difference in ATP flux in comparison to the first dorsal interosseus muscle in adult humans, indicative of mild uncoupling seen with UCP3 (1). In aged humans there was no change in the coupling level or the ATP concentration in the tibialis anterior muscle, however the first dorsal interosseus muscle had decreased coupling associated with a loss of ATP concentration compared to adult muscle (1). This decreased coupling with concurrent ATP drop is indicative of uncoupling due to damage, and associated mitochondrial dysfunction. The mild uncoupling of the tibialis anterior muscle, therefore, appears to have protective benefits, preventing mitochondrial damage into old age (1). This concept may be especially important in preventing loss of muscle mass in old age, as dysfunctional/damaged mitochondria are more likely to produce ROS, which would in turn increase damage to the mitochondria and cell, potentially triggering cell death. Along with lowering ROS production by uncoupling, it has been found in animal models

that uncoupling (via induction of uncoupling protein 1, UCP1) can decrease the pathological effects of aging, albeit not aging per say (38). Models have also shown delayed/decreased atherosclerosis, cancer, and lymphoma in mice over-expressing UCP1 (38). The activity of AMPK is increased following induction of UCP1, thus counteracting the decreased PGC1-α effect associated with aging (38). It was shown that induction of UPC1 also helped to reverse the effects of obesity, decreased glucose metabolism and hypertension (38). UCP's positive effect of increasing basal metabolic rate, which thus aids in the decrease of obesity, has resulted in the pharmacological industry investigating the potential benefits of uncoupling as an anti-obesity tool. Progress in that area, however, has yet to produce effective and safe treatments (43).

Two methods that have been found to aid in the decrease of oxidative damage and its associated role on aging are calorie restriction (7; 47) and endurance training (8; 13; 25; 57; 78). Both calorie restriction and endurance training result in increased mitochondrial biosynthesis, decreased ROS and decreased sarcopenia (8; 13; 25; 49; 57; 66; 78). Surprisingly, calorie restriction has also been found by some to be accompanied by a decrease in proton leak (increased coupling to complex V)(13), however others have found increases in proton leak (66). Although there is strong evidence for the health/age related benefits of calorie restriction (97), it does require caloric consumption to be *reduced* (while still maintaining adequate vitamin and mineral consumption). As much of the population is now overweight/obese and fast food is increasingly common, it is difficult for health professionals to convince the population to eat only the recommended caloric intake, let alone less than that. Endurance training may be a preferred alternative for some, as well endurance training has many other health related benefits including

improved heart and pulmonary function, increased stamina and increased bone health (83) .

*Effects of endurance exercise on mitochondrial function*

Endurance training has many effects on mitochondria in both the young and aged. Chronic training has been shown to increase levels of PGC1-α in skeletal muscle, resulting in increased mitochondrial density (61; 78). Mitochondrial density is also increased following an exercise protocol in cardiac tissue, as is evidenced by increased succinate dehydrogenase (SDH) (8). Along with increases in mitochondrial density, mitochondrial enzyme activities (NADH-cytochrome c reductase, COX) increase following endurance training (78), resulting in elevated routine and state 3 ADP stimulated respiration (78).

Oxidative damage due to ROS generation was found to be decreased following chronic training in mice, as the age-related decrease in endogenous antioxidants, superoxide dismutases and catalaze seen in the control group, were prevented (78). Endurance exercise training results in increased oxygen sent to the tissue, which might be thought to increase the chance for ROS production. However, the increased mitochondrial content, along with increased endogenous antioxidants, result in increased oxygen utilization by complex IV and free radical scavenging, respectively (78). These effects of endurance training not only help to decrease the short-term effects of high oxygen flux into active tissues, they continue to attenuate ROS generation and oxidative damage between bouts of exercise.

Following chronic endurance exercise, uncoupling protein 3 (UCP3, found within skeletal muscle) (3) has been shown to acutely rise (3). The function of UCP3 has yet to

be concretely determined; however, it is believed to increase during lipid metabolism and is also stimulated by superoxide (31). UCP3 is hypothesized by some to allow leakage of $H^+$ across the inner mitochondrial membrane, decreasing $\Delta p$, allowing a faster rate of oxygen reduction at complex IV (increasing $VO_2$), and thereby reducing the probability for damage due to oxidative stress. Others have hypothesized UCP3 is in fact not a proton transporting protein like UCP1, but solely aids in transporting lipids across the inner mitochondrial membrane (42). In either case, UCP3 is known to be associated with decreased ROS generation, although the mechanism may not be fully clear.

Whereas it is clear that endurance training facilitates a multitude of positive adaptations within the muscle and the mitochondria, it is unknown if these effects occur in those who are genetically pre-disposed for high aerobic capacity but who do not engage in a training stimulus. To investigate this possibility, two lines of rats have been selectively bred for high and low aerobic capacity, respectively (60). With these strains of rat, it is possible to tease apart the effects of innate aerobic capacity versus the effects of exercise training specifically.

*Effects of artificial selection for high versus low aerobic capacity*

Selective breeding by (60) has resulted in two strains of rats that are pre-disposed for either high (HCR) or low (LCR) running capacity, with running capacity differences of 347% at generation 11 (104). As one might expect, these differences in running capacity are accompanied by significant differences in the muscle aerobic machinery, with reports of higher capillary density (due to smaller fiber number) (50) and higher mitochondrial enzyme activities (39; 80; 104) in the skeletal muscle of HCR rats versus

their LCR counterparts. Despite the evidence of augmented mitochondrial enzyme activities in the muscles of HCR rats (40), specific investigation of the scope of mitochondrial adaptations in HCR rats has been very limited to date.

Consistent with the expected consequences of lower oxidative capacity, LCR rats have increased levels of triglycerides, adipose tissue, FFA, and have a higher body mass in comparison to HCR rats (80; 91). LCR rats also have decreased PGC1-α, PPARγ, COX1 UQCR2, UCP2, and UCP3 in their skeletal muscles (80; 104). These results indicate that LCR rats likely have a decreased mitochondrial content versus HCR rats. LCR rats also showed signs of insulin resistance, higher risks of cardiovascular dysfunction (68; 104), and high blood pressure (104). Wisloff et al (104) investigated mitochondrial and metabolic differences in five week old pups and found that LCR rats showed increased plasma triglycerides and glucose than their HCR counterparts; however, at this age their lean/visceral masses were equal. As such, these observations indicate that the metabolic differences observed between LCR and HCR rats are present at birth, and are not due to secondary factors such as a greater accumulation of adipose tissue during postnatal life.

When investigating the differences in metabolic utilization, it was found that phosphofructokinase levels were greater in the LCR rats, whereas HCR rats showed greater levels of citrate synthase activity in skeletal muscle (50). These differences are indicative of a greater reliance on glycolytic energy supply by the LCR group. Correspondingly, Bye et al (20)investigated cardiac gene expression pre and post exercise training in both HCR and LCR rats. They found that pre-training HCR rats had a greater expression of genes for lipid metabolism, whereas LCR rats had a greater expression of

genes for glucose metabolism. Following exercise training all but one gene responded similarly between the strains; however, the function of this one gene is unknown. The preference for LCR rats to utilize glucose rather than fatty acids in the untrained state is similar to what is seen in diseased hearts of humans, indicating possible mitochondrial dysfunction in the LCR animals (20).

When sustained on a high fat diet, LCR rats gained excess body adipose tissue, and their already compromised insulin sensitivity was exacerbated. HCR rats, however, were resistant to diet-induced obesity, and showed significantly higher levels of UCP3 and higher FFA oxidation in the gastrocnemius muscle than LCR rats. These results imply that the HCR rats were able to adapt their substrate utilization to fatty acid oxidation when fed a high fat diet, thereby avoiding storing excess intramuscular fat (80). The increase in UCP3 following high fat feeding in the HCR rats may attenuate the increased ROS generation that is normally seen during fatty acid oxidation. Consistent with this idea, one study done by Anderson et al. (2007) showed that during recovery from exercise in control mice UCP3 was highly expressed and ROS emission was reduced to near control levels, whereas $UCP3^{-/-}$ animals had no up-regulation of UCP3 during exercise recovery and subsequently showed significantly higher rates of ROS emission. Thus, it can be proposed that expression of UCP3 may occur to counteract ROS formation during metabolic states in which ROS formation is promoted (fatty acid utilization, post exercise, etc.). The HCR rat, therefore, may be better able to prevent excess ROS formation during both high fat feeding and chow feeding via their greater expression of UCP3, and this in turn may help minimize oxidative damage.

The differences between HCR and LCR rats are consistent with the latter being in a pre-diabetic stage, having higher adipose tissue, insulin insensitivity, hypertension (104) and metabolic inflexibility (80). Research done with Type 2 diabetes patients has shown that oxidative metabolism in skeletal muscle is decreased when utilizing carbohydrate fuel sources (44; 58), and that this results in increased muscle lipid content (44), and a decrease in PGC1-α (75). As was previously stated, mitochondria that become less efficient during old age are associated with declines in muscle function and disease states (1).

As the greater maximal aerobic capacity during whole body exercise ($VO_2max$) seen in HCR rats has been hypothesized to be due in part to greater oxidative capacity in skeletal muscles of HCR rats, it seems pertinent to investigate the mitochondrial capacity of these two strains of rats. Due to the aforementioned differences between HCR and LCR animals, several hypotheses have been formed in respect to the mitochondrial function of these animals. Firstly, we hypothesize that HCR rats will have a higher oxidative capacity (to account for the higher $VO_2max$), lower coupling and lower ROS generation which will result in less oxidative damage to the skeletal muscle. Secondly, we hypothesize that these beneficial alterations in the HCR rats will be carried over into old age, thus contributing to their greater life span and lower disease risk.

# Chapter 3. Muscle Type-specific Alterations in Mitochondrial Function in Rats Selectively Bred for High versus Low Running Capacity

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## Abstract

Oxidative capacity and associated mitochondrial function has been known to play a role in an array of diseases such as diabetes and obesity. Selection for high (HCR) and low (LCR) running capacity in rats has shown HCR animals to not only have greater $VO_{2max,}$ but they are also protected from such diseases as obesity, insulin resistance, and cardiovascular disease. Investigation into the mitochondrial function of adult HCR and LCR animals was therefore undertaken to decipher if mitochondrial differences could be attributed to the disease protection that occurs in the HCR animals. Soleus and the red region of gastrocnemius muscles were investigated, with results indicating alterations in mitochondrial function that were distinct between the two muscles examined. The soleus muscle of HCR animals had: no detectable difference in citrate synthase (CS) activity, greater respiration both per mg tissue and per CS activity, greater $H_2O_2$ emission both per mg tissue and per CS activity, and no detectable difference in protein carbonylation but lower total 8-OHdG content versus LCR animals. The red region of gastrocnemius muscle of HCR animals had: greater CS activity, no greater respiration per mg tissue and lower respiration per CS activity, a lower respiratory control ratio, greater $H_2O_2$ emission per mg tissue but lower $H_2O_2$ emission per CS activity, and no detectable difference in protein carbonylation but a lower total 8-OHdG content than LCR animals. These differences in mitochondrial function between the two muscle types appear to maximize respiratory capacity while limiting oxidative damage within each muscle in HCR rats. Effects of stress-induced hormesis, including increased antioxidant capacity, may play a role in explaining the oxidative damage protection observed in HCR animals.

## Introduction

Mitochondria play many physiological roles in the cell, including ATP production, heat production, regulation of intracellular $Ca^{2+}$, and regulating key pathways of programmed cell death (77). For this reason, it is logical to hypothesize that mitochondrial dysfunction may be involved in pathologies of the body. The contribution of mitochondrial dysfunction to disease is not clear in the literature. This is due to several factors, one being that the idea of mitochondria playing a role in disease states is relatively new. More importantly, the term, "mitochondrial dysfunction" is used rather loosely in the literature with very few studies being clear in what is meant by this term. While some studies refer to a decrease in mitochondrial density as "dysfunction," others use decreased oxidative capacity per mitochondrion as their definition of dysfunction. As mitochondrial content is extremely malleable in response to normal physiological stimuli (e.g., increased muscle use with exercise training causes an increase, whereas reduced muscle use due to bed rest causes a decrease), a reduced mitochondrial density per se is insufficient to warrant the label "dysfunction". While the latter definition of reduced oxidative capacity per mitochondrion is more specific and consistent with the notion of "dysfunction", it is but one of many changes in mitochondrial function that could influence disease susceptibility. For this reason, it is argued here that use of the term "mitochondrial dysfunction" should, to a large extent, be replaced by more specific statements of changes in mitochondrial function. This approach will provide a better appreciation of the scope of mitochondrial functional plasticity and its potential role in disease risk and healthy aging.

Alterations in mitochondrial function have been indicated in a vast array of diseases, including type II diabetes (16; 75; 82), obesity (6; 14; 28; 45; 59; 90), and aging (25; 29; 30; 41; 55). The impact of impaired mitochondrial function on the body can be poignantly observed in cases of mitochondrial myopathies. Research on the extent of exercise intolerance in a large cohort of patients with mitochondrial myopathies of various causes revealed an inverse correlation between the extent of mitochondrial DNA (mtDNA) mutation and oxidative phosphorlyation capacity (93). As the mtDNA mutation load increased, the ability to extract oxygen from the blood stream during exercise decreased (93), resulting in decreased ability for oxygen utilization during exercise. This data demonstrates 1) that impaired mitochondrial function can greatly affect one's health and well being, and 2) the importance of the electron transport system in aerobic capacity during exercise.

Recently, selective breeding has permitted the development of rats exhibiting high running capacity (HCR) and low running capacity (LCR) (60). Unlike training studies, this selection experiment allows one to investigate the impact of genetic predisposition for high versus low endurance capacity rather than the effect of physical activity per se. Past investigations have shown LCR animals to be susceptible to obesity (80; 104), insulin resistance (80; 104), abnormal lipid handling (91) and to have greater risk for diabetes (80; 104) and cardiovascular disease (20; 104), whereas HCR animals show protection from said disease and dysfunction. These differences have been suggested to be due, in part, to differences in mitochondrial function (80; 91; 104). Limb skeletal muscles from HCR animals have increased PGC1-α (80; 91; 104), complex IV content (80; 91; 104) and citrate synthase activity (50; 80) versus LCR rats. Similarly, muscle

from HCR rats has higher mitochondrial uncoupling proteins (80), which could decrease mitochondrial ROS generation.

Studies comparing LCR and HCR animals have implicated increased mitochondrial content in HCR rats, on the basis of the greater citrate synthase activity (51; 80) and greater expression of mitochondrial proteins (COX subunits (80; 91; 104) and ATP synthase in HCR versus LCR rats (104)). Studies have also indicated possible differences in the capacity for oxidative phosphorlyation, due to the greater mitochondrial proteins (80; 91; 104), greater expression of genes for fat oxidation (20), and increased uncoupling proteins (80; 104) in HCR rats. However the above studies have inferred these differences due to changes in gene or protein expression, and not mitochondrial respiratory chain activity per se. Walsh et al. (101) investigated respiratory activity using a permeabilized muscle fiber bundle technique in soleus muscle, and found no detectable difference in respiratory capacity (101), although HCR rats did show a greater sensitivity to creatine than LCR rats. Walsh et al (101) thus provided the first insight into the functional differences evident in mitochondria from HCR and LCR animals. Although this first study found no detectable difference in muscle respiratory capacity between HCR and LCR rats, two limiting factors may have reduced their ability to detect higher mitochondrial respiration in HCR animals. First, the tests were performed on only one muscle: the soleus, which is composed of 87% type I fibers (4). The significance of this is that fiber type has a significant impact on respiratory capacity (33), as well as ROS production (2; 84), and further to this, much of the protein content data suggesting increased respiration was performed on muscles comprised of largely type II fibers (51; 80; 91), meaning that there may be differences in muscle respiratory capacity that were

missed by Walsh et al. Second, respiration was only studied using complex I-linked substrates (101). Not only do mitochondria respire on multiple substrates in vivo, but it has been shown *in situ* that respiration supported by the complex II-linked substrate succinate is greater than that supported by complex I-linked substrates (2). Investigation into the muscle respiratory capacity of HCR and LCR animals during different substrate conditions may reveal differences in mitochondrial function not seen previously. Investigating mitochondrial respiration during various substrate protocols, as well as the mitochondrial ROS emitting potential, will increase our understanding of the role that mitochondria may play in the protection from disease that occurs in HCR animals. Specifically, we hypothesize that HCR animals will have greater mitochondrial respiration and lower mitochondrial coupling. Following from lower mitochondrial coupling, we hypothesize that HCR animals will emit lower levels of ROS, and thus have less oxidative damage. To test these hypotheses, we measured respiratory function and $H_2O_2$ emission (normalized to both tissue weight and CS activity), in small permeabilized muscle fiber bundles, and examined two indices of muscle oxidative damage, protein carbonylation and 8OHdG content (marker of DNA damage).

## Methods

*Animals.*

The selection of animals for HCR and LCR has been described in detail previously (60). In brief, animals were selectively bred for treadmill running capacity from a starter population of 96 male and 96 female heterogeneous rats from N:NIH stock. Intrinsic running capacity was tested when the rats were 11 weeks old, via treadmill test time to exhaustion, at 15° beginning at 10 m x $min^{-1}$ and increasing 1 m x $min^{-1}$ every 2

min. The 13 highest time-to-exhaustion animals were bred and the 13 lowest time-to-exhaustion animals were bred to produce two strains of rats that at generation six differed in running distance by 171% (60). All testing of running capacity and breeding was done at the University of Michigan in Ann Arbor, MI, by our collaborators Dr. Steve Britton and Dr. Lauren Koch. Following maximal treadmill testing animals were housed without access to a running wheel.

Nineteen male (10 HCR and 9 LCR) rats obtained from the 21$^{st}$ generation of selection were used for this study. Animals were sacrificed between 13-15 months of age (adulthood). A second cohort of 18 animals (10 HCR and 8 LCR) obtained from the 19$^{th}$ generation were also used in this study for determination of protein carbonyls, and these animals were sacrificed between 8-9 month of age (adulthood). Upon arrival at the University of Calgary animals were housed individually at the biological sciences vivarium (12:12-h light-dark cycle, ambient temperature = 22°C) without access to a running wheel to prevent exercise induced adaptations. Animals were given standard chow and water *ad libitum*.

*Muscle harvesting*

Animals were anesthetized with 50-60 mg x kg$^{-1}$ sodium-pentobarbital ip. Upon deep surgical anesthesia the left soleus and gastrocnemius were harvested, weighed and frozen in liquid nitrogen and stored at -80°C. The right soleus and gastrocnemius were harvested, weighed and placed in ice cold buffer X containing 7.23 mM $K_2EGTA$, 2.77 mM $CaK_2EGTA$, 20 mM Imidazole, 0.5 mM Taurine, 5.7 mM ATP, 14.3 mM PCr, 6.56 mM $MgCl_2$-$6H_20$, and 50 mM MES (pH 7.1). Animals were euthanized by cardiac removal following muscle harvest.

The Soleus and $Gas_{red}$ muscles were chosen for this study for several reasons. Firstly, the soleus muscle has primarily (87%) type I fibers while the $Gas_{red}$ is composed of a mixture of type I (30%) and type IIa (62%) fibers (4), thus allowing us to investigate the differences in mitochondrial function between fiber types. Secondly, it has previously been shown that the gastrocnemius muscle has a greater ROS emitting potential than the soleus muscle (2), and this has been found to be accompanied by lower endogenous antioxidants to quench this production of ROS (2). Thus it appears fiber type greatly affects ROS generation and possibly oxidative damage to the muscle. Lastly, as a very practical approach the soleus and the gastrocnemius muscles were chosen as much of the available data on HCR/LCR animals has been studied in these two muscles, thus allowing us to make comparisons to previous studies.

*Preparation of permeabilized of muscle fiber bundles*

Small fiber bundles (~5 mg) of soleus muscle and red region of gastrocnemius muscle ($Gas_{red}$) were manually teased apart with sharp ended needles to maximize surface area, and then either incubated in buffer X with 100 uL saponin for 30 min on ice with mild stirring, or cryopreserved (see below). The bundles were then washed three times in ice cold buffer Z containing 105 mM K-MES, 30 mM KCL, 10 mM $KH_2PO_4$, 5 mM $MgCl_2$-$6H_2O$, 1mM EGTA, and 5 mg x $ml^{-1}$ BSA (pH 7.4) to remove saponin and metabolites. Fiber bundles were stored (no more than 60 min) on ice in buffer Z until respirometry measures.

*Cryopreservation of muscle fiber bundles*

Cryopreservation has been shown to have minimal negative effects on the mitochondrial structure, mitochondrial function (63) or ROS production (see Appendix A) in teased muscle bundles. Following manual teasing, two muscle bundles were cryopreserved from each muscle as was described in detail previously (63). In short, muscle bundles were placed in eppendorffs containing 100 µl buffer X supplemented with 30% dimethyl sulfoxide (DMSO) and 10 mg x $ml^{-1}$ bovine serum albumin (BSA). Muscle bundles were exposed to the solution for five seconds, then frozen in liquid $N_2$ and stored at -80°C for analysis of $H_2O_2$ emission.

*Mitochondrial respiration measurements*

Respiration was measured in 2 mL of buffer Z at 37°C with a polarographic oxygen sensor, designed for high-resolution respirmetry (Oxygraph-2k, Oroboros, Innsbruck, Austria). All measures were done following required oxygraph calibration. Two substrate protocols were performed in parallel, with each substrate addition being allowed to reach a steady state before the subsequent addition was made (approximately 3-5 minutes between additions). The protocols were as follows: 1) 5 mM glutamate + 2.5 mM malate (gm), 2 mM adenosine diphosphate (ADP), 10 mM succinate (succ), 8 µM cytochrome c (cyt C), 8µg x $ml^{-1}$ oligomycin, and 2.5 µM Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP); 2) 5 mM glutamate + 2.5 mM malate (gm), 2mM adenosine diphosphate (ADP), 10 mM succinate (succ), 10 µM antimycin A (aa), 0.6 mM N,N,N',N'-tetramethyl-p-phenylendiamine (TMPD) + 2.4 mM ascorbate (Tmpd

& Asc), and 2 mM potassium cyanide (kcn). Respiratory Control Ratio (RCR) was calculated as the quotient of ADP-stimulated respiration (gm substrates) and gm-stimulated respiration. Following respirometry measures bundles were weighed, then frozen in liquid $N_2$ and stored at -80 °C until used for analysis of citrate synthase enzyme activity (see below).

*Mitochondrial $H_2O_2$ production*

Measurements were performed with a Hitachi F-2500 fluorescence spectrophotometer, to detect resorufin, a byproduct of the 1:1 reaction of Amplex red and $H_2O_2$. Resorufin is detectable at an excitation/emission wavelength of 563 nm/587 nm, and is catalyzed by the addition of horseradish peroxidase. $H_2O_2$ measurements were done on previously cryopreserved muscle bundles. For analysis, bundles were thawed in a water bath at 37 °C. Once cryopreservation solution was fully thawed (around 10 sec) bundles were washed three times in buffer X supplemented with 2mg x $ml^{-1}$ BSA to remove any trace of DMSO. Measurements were done in 1mL of buffer Z supplemented with 5 μM Amplex red and 0.17 U x $mL^{-1}$ HRP at 37°C. After baseline measures, substrate additions were introduced as follows: 5 mM glutamate + 2 mM malate; 3 mM succinate; 10 μM ADP; 100 μM ADP; 1 mM ADP. Each substrate addition was allowed to reach steady state (approximately 3 minutes) before the subsequent addition.

*Measurement of Citrate Synthase Enzyme Activity*

Stored muscle bundles from the respirometry measurements were used for analysis of citrate synthase (CS) activity. Bundles were weighed and homogenized on ice in 1:200 ratio of buffer containing 100 mM $KPO_4$ + 5 mM EDTA + 5 mM EGTA (pH 7.4) with a hand homogenizer for approximately 40 seconds. Homogenate was then vortexed and subjected to three freeze-thaw cycles in liquid $N_2$. After the third thaw samples underwent a second dilution of 1:2 for a final dilution of 1:400. Measurement of citrate synthase (CS) enzyme activity was determined spectrophotometrically via mercaptide ion production, at 412 nm on a microplate reader. This involved aliquoting 20 µL homogenate 170 µL reaction buffer containing 0.3 mM acetyl Co A, 0.1 mM 5.5'-Dithiobis(2-nitrobenzoic acid), and 100 mM TRIS buffer in triplicate into a 96-well plate and reading the resulting absorbance change for endogenous levels of thiol or deacetylase activity. Following initial reading, 10 µl of 10 mM OAA was added to each well to start the enzyme reaction that resulted in mercaptide ion production. CS activity calculated by subtracting the endogenous activity from the final reading, and expressed as µmol x $g^{-1}$ x $min^{-1}$. Both respirometry and $H_2O_2$ measures were normalized to CS activity as a proxy measure of respiration and ROS emission per mitochondrion, respectively.

*Measurement of Protein Carbonyls*

Protein Carbonyls were measured in soleus and $Gas_{red}$ muscles of the 19$^{th}$ generation of HCR/LCR animals via a protein carbonyl enzyme immuno-assay kit

(BioCell Corp. Ltd., Auckland, New Zealand). Whole muscle was weighed (20-50 mg) and homogenized on ice in 1:40 dilution of buffer containing 20 mM $KH_2PO_4$ and 20 mM $Na_2HPO_4$. An aliquot of homogenate was taken to determine protein content via Bradford assay. The homogenate was then vortexed and incubated in dinitrophenylhydrazine (DNP) for 45 minutes. Sample was then diluted in enzyme immunoassay (EIA) buffer (potassium di-hydrogen phosphate and sodium chloride), aliquoted into a 96 well plate, sealed and incubated overnight. Following overnight incubation the plate was washed five times in EIA buffer and incubated in blocking solution for 30 minutes. The plate was then washed as above and incubated in anti-DNP-biotin-antibody for 60 minutes at 37°C. The plate was again washed and streptavidin-HRP was added for a final incubation for 60 minutes. Following the final incubation the plate was washed and chromatin reagent was added and set for seven minutes. Stopping reagent was then added and the plate was read at 450 nm. Carbonyl content was measured in nmol x $mg^{-1}$ protein.

*Measurement of 8-OHdG*

Total genomic DNA (nuclear and mitochondrial) was extracted from skeletal muscle (*gastrocnemius* and *soleus muscles*) using QIAmp DNA Mini kit (Qiagen, Mississauga, ON) as per manufacturer instructions. The concentration of the isolated DNA was determined using NanoDrop ND-1000 UV-Vis spectrophotometer (ThermoScientific, Ottawa, ON). The DNA concentrations ranged from 400 – 800 ng x $\mu L^{-1}$ and the average absorbance ratio of $A_{260}/A_{280}$ was between 1.7 – 1.9. One microgram of total DNA from each sample was dot blotted on a nitrocellulose membrane

(Amersham, Piscataway, NJ). 8-OHdG immunoblotting was carried out using mouse monoclonal 8-OHdG (N45.1) antibody (Japan Institute for the Control of Aging, Fukuroi, Japan). Membranes were then incubated with anti-mouse horseradish peroxidase-linked secondary antibody (Bio-Rad Laboratories, Burlington, ON) and were visualized by enhanced chemiluminescence detection reagent (Amersham, Piscataway, NJ). Relative intensities of the circular dots were digitally quantified by using NIH ImageJ analysis software (version 1.37, Scion Image, NIH).

*Statistics*

Respiratory and $H_2O_2$ measures were analyzed with a two-way ANOVA, with substrate condition and selection group (HCR/LCR) as fixed factors, the Bonferonni post hoc test was used when interactions were found. RCR, CS content and protein carbonyls were analyzed with an independent t-test. Values are expressed as means ± SE .

## Results

*Running Capacity and Body weight*

LCR rats were significantly heavier than HCR rats ($p<0.001$). All body mass, hind limb mass and running capacity are expressed in Table 1. Overall LCR animals were heavier and had larger gastrocnemius muscles than HCR animals. There was no difference in the weight of the soleus muscle between HCR and LCR animals. HCR animals ran significantly longer and for greater distances than LCR animals.

*Citrate synthase activity*

HCR rats had significantly greater CS activity than LCR rats in $Gas_{red}$ muscle bundles (36.4 ± 6.0 μmol x min $^{-1}$ x g$^{-1}$, 26.4 ± 4.9 μmol x min $^{-1}$ x g$^{-1}$, p=0.001) (*Figure 1. 1a*); however, there was no detectable difference in the soleus muscle bundles between selection groups (25.5 μmol x min $^{-1}$ x g$^{-1}$ ± 3.5 μmol x min $^{-1}$ x g$^{-1}$, 22.5 μmol x min $^{-1}$ x g$^{-1}$ ± 3.6 μmol x min $^{-1}$ x g$^{-1}$, p=0.169) (*Figure 1. 1b*).

*Respirometry*

Two-way ANOVA revealed significant interactions between selection and substrate condition for $Gas_{red}$ muscle (p=0.02), with post hoc tests revealing significantly higher flux in HCR animals during TMPD + Asc stimulated respiration (*Figure 1. 2a*). Soleus muscle did not show any interaction of substrate condition and selection group (p=0.14), but did have a significant main effect for selection group (p=0.025), with HCR having higher $O_2$ flux than LCR (*Figure 1. 2b*). Normalization of respirometry measures to CS activity resulted in no interactions; however, main effects indicated significant differences, with higher respiratory capacity in soleus muscle of HCR (p<0.001), and lower respiratory capacity in $Gas_{red}$ muscle of HCR rats (p<0.001) (*Figure 1. 2c&d*). This difference in respiration per unit of CS activity was found in conjunction with significantly lower RCR in $Gas_{red}$ muscle (p=0.01), whereas soleus muscle RCR was not different between HCR and LCR (p=0.12).

*$H_2O_2$ production*

No significant interactions between selection group and substrate condition were found in either $Gas_{red}$ or soleus muscles. Significant main effects were found, with higher $H_2O_2$ production in both $Gas_{red}$ ($p=0.001$) and soleus ($p=0.004$) muscles in HCR than in LCR rats (*Figure 1. 3a&b*). Due to greater CS activity in $Gas_{red}$ muscle in HCR rats, normalization resulted in decreased $H_2O_2$ production per unit of CS activity in $Gas_{red}$ muscle of HCR vs. LCR rats ($p=0.017$) (*Figure 1. 3c*). Normalization to CS activity of soleus muscle did not change the greater $H_2O_2$ production in HCR than LCR ($p=0.001$) (*Figure 1. 3d*).

*Oxidative stress*

Protein carbonyls, an indicator of cellular damage often caused by oxidative stress, were not different between HCR and LCR rats in either the $Gas_{red}$ or soleus muscles ($p=0.64$ and $p=0.38$, respectively) (*Figure 1. 4a&b*). 8-OHdG content, an indicator of oxidative DNA damage, was significantly lower in HCR animals than LCR animals in both the gastrocnemius muscle ($p=0.04$) and the soleus muscle ($p=0.00$) (*Figure 1. 4c&d*).

## Discussion

The purpose of this study was to examine mitochondrial function through high resolution respirometry and fluorometric measurement of $H_2O_2$ production, and to

examine two measures of oxidative damage (protein carbonylation, oxidative DNA damage), to gain an understanding of the role that differences in mitochondrial function may play in health and disease of HCR and LCR animals. In the present study selective breeding for high and low running capacity resulted in differences in mitochondrial function that were dependent upon the muscle examined. In the soleus muscle, HCR animals showed no detectable difference in CS activity, suggesting no difference in mitochondrial content; however, soleus muscle bundles in HCR rats did have significantly higher maximal ADP-stimulated respiration whether expressed per milligram tissue or per unit of CS activity. The $Gas_{red}$ of HCR animals had higher CS activity, suggesting higher mitochondrial content, yet this did not translate into higher maximal ADP-stimulated respiration per milligram of tissue. On the other hand, TMPD+Asc stimulated respiration was higher in $Gas_{red}$ of HCR rats, indicating a higher complex IV activity (*Figure 1. 3)*. Interestingly, normalization per unit of CS activity resulted in a lower maximal ADP-stimulated respiration in $Gas_{red}$ of HCR rats. RCR, a proxy measure of the coupling efficiency of the mitochondria, was lower in $Gas_{red}$ of HCR rats but not different in soleus muscle of HCR rats. Free radical emission, as tested by $H_2O_2$ emission, was also found to have muscle type-specific differences between HCR and LCR animals. Contrary to our hypothesis, $H_2O_2$ emission was greater in HCR than LCR animals in bundles of both the soleus and $Gas_{red}$ muscles. However, upon normalization to CS activity, HCR animals had lower $H_2O_2$ emission than LCR in the $Gas_{red}$ muscle, whereas normalization to CS activity in the soleus muscle indicated a greater $H_2O_2$ emission relative to LCR animals in this muscle. In other words, our results suggest that ROS production per mitochondrion is higher in the predominantly slow

twitch soleus muscle but lower in the faster twitch $Gas_{red}$ muscle of HCR rats, but because of greater mitochondrial content in $Gas_{red}$ and no detectable difference in soleus muscle versus LCR rats, there was a greater ROS burden at the whole muscle level in both muscles of HCR rats. The greater $H_2O_2$ emission at the whole muscle level in HCR animals could be expected to lead to greater oxidative damage; however, when investigating for protein carbonyls, we found no detectable differences between HCR and LCR animals, and further, DNA damage was lower in HCR animals despite the greater ROS emission. Therefore, although there was decreased oxidative damage to the muscles of HCR animals this appears not to be due to decreased ROS emission. We were, however, correct in hypothesizing a greater oxidative capacity in HCR animals than LCR animals, although the muscle type-specificity of these changes result in more complex characterization than we had previously anticipated.

*Mitochondrial Content*

The muscle type-specific differences in mitochondrial function due to selection for high running capacity seen in the current study are distinct from those found by Burelle et al. (19) in trained versus untrained rats. Burelle et al. found that four weeks of endurance training resulted in increases in ADP-stimulated respiration in soleus muscle, yet not in $Gas_{red}$ muscle, which is similar to the findings in the current study. However the adaptations of maximal respiratory rate in response to exercise training paralleled changes in mitochondrial content as the soleus muscle showed increases in CS activity, and the $Gas_{red}$ did not and these muscle type-specific differences likely reflect the degree to which exercise training increased the recruitment of these muscles (19). In the present

study, selective breeding for high running capacity, unlike exercise training, resulted in greater CS activity in $Gas_{red}$ but not the soleus muscle of HCR animals, and in contrast to what was seen with exercise training, these differences in CS activity did not always parallel differences in maximal ADP-stimulated respiration, indicating functional differences in mitochondria rather than simply differences in mitochondrial content between HCR and LCR rats. The findings of elevated CS activity in the $Gas_{red}$ of HCR rats seen here are consistent with previous investigations on the HCR/LCR strain. Both Noland et al. (80) and Howlett et al. (50) found greater CS activity in mixed gastrocnemius muscle. Although no prior studies have investigated CS activity in soleus muscle in HCR/LCR animals, other markers of mitochondrial content such as the protein expression of subunits of complex IV and F1-ATPase synthase are elevated in soleus muscle of HCR rats (80; 104), although these protein levels may not necessarily translate into increased mitochondrial content (e.g., if the subunits are not assembled into functional complexes) or an increased respiratory capacity.

*Respiratory function and ROS generation in the gastrocnemius muscle*

Greater CS activity in $Gas_{red}$ muscle in HCR rats did not translate to greater maximal mitochondrial respiration; however, there was increased TMPD + Asc, and TMPD-KCN stimulated respiration (*Figure 1. 2*). Greater respiration with artificial complex IV substrates indicates greater complex IV activity in HCR animals, consistent with other investigations which found increased subunits for complex IV in the gastrocnemius muscle (80; 91) of HCR animals. The increased TMPD+Asc stimulated respiration, along with increased complex IV protein levels, indicate an excess of

complex IV in comparison to the other complexes of the electron transport chain in mitochondria of HCR rats. As complex IV is the terminal electron acceptor, reducing oxygen into water, an increase of this complex allows oxygen to be converted at a lower $PO_2$, thus reducing the possibility of oxygen interacting at other sites and producing reactive oxygen species (ROS).

Intuitively, one would suspect an increase in citrate synthase activity to coincide with increased respiratory capacity; however, as was mentioned above, this was not the case in the $Gas_{red}$ of HCR animals. Since a higher CS activity is suggestive of an elevated mitochondrial content in $Gas_{red}$ of HCR rats, each individual mitochondrion would be taxed to a lesser extent to produce the amount of ATP required under any given level of tissue oxygen uptake, whereas the mitochondria of LCR animals must work at a higher respiration at any given rate of tissue oxygen uptake to make up for a lower mitochondrial content. The lower ATP demand from each mitochondrion within the $Gas_{red}$ of HCR rats would allow for a decrease in substrate flux throughout the entire electron transport chain. As decreased substrate flux through the system results in less reduction of mitochondrial complexes (importantly complex I and III) this would also allow for decreased occurrence of oxygen binding to the reducing equivalents to produce ROS (9), as was observed in this study with lower $H_2O_2$ production per unit of CS activity in the $Gas_{red}$ of HCR animals. One other factor that is know to aid in reduction of ROS production is a low coupling status of ATP synthesis with substrate oxidation (3; 17; 32). In this study HCR animals showed decreased RCR, an indicator of respiratory coupling, in $Gas_{red}$ in comparison to their LCR counterparts, due predominately to a decreased state 3 respiration per mitochondrion. Uncoupling of the mitochondrial inner

membrane can be accomplished in a number of ways, including induction of uncoupling proteins (predominately UCP3 in skeletal muscle) (3; 5; 17; 31; 32; 92), damage to the inner membrane due to oxidative stress (1), and increases in ANT (18). In this case, it is unclear what mechanism is responsible for the increased uncoupling found in HCR animals. Past investigations of HCR/LCR animals have, however, shown increased (although not statistically detectable) levels of UCP3 in mixed gastrocnemius (80), indicating the increased uncoupling found in $Gas_{red}$ of HCR animals may, in fact, be due to increased UCP3. This possibility, amongst others, should be examined in future investigations.

*Respiratory function and ROS generation in the soleus muscle*

The $Gas_{red}$ muscle in HCR animals appears to have developed mechanisms to aid in lower ROS generation, by compromising efficiency (ATP production) per mitochondrion, allowing the greater mitochondrial content to fulfill the energy requirement. The soleus muscle of HCR animals, however, has developed strikingly different adaptations due to selective breeding for running capacity. Unlike the $Gas_{red}$ muscle, the soleus muscle has increased mitochondrial respiration per mg tissue (*Figure 1. 2*) in HCR animals, and due to equal CS activity in HCR and LCR within the soleus muscle, HCR rats also exhibited greater mitochondrial respiration per CS activity, suggesting greater respiratory capacity per mitochondrion. Unlike what was observed in the $Gas_{red}$ muscle, the mitochondria were more efficient in the soleus muscle of the HCR than in LCR animals, as the mitochondrial respiration in HCR animals was 28% higher than LCR animals. The enhanced mitochondrial respiration observed in this study is

consistent with increased protein levels of ATP-synthase, as was previously found in the soleus muscle of HCR animals (104). Similar to our findings, Walsh *et al.* (2006) had found HCR animals to have 13% higher maximal ADP-stimulated respiratory capacity in the soleus muscle than LCR animals when respiring on complex I-linked substrates, although they did not find statistical significance (101). They did, however, find HCR animals to have a significantly higher sensitivity to creatine, which would allow the muscle to have an increased sensitivity to ADP during exercise. This finding, along with our data may indicate that *in vivo*, where phospocreatine is present, HCR animals may be even more sensitive to ADP and thus have an even greater mitochondrial respiratory capacity than observed here, further aiding the muscle's ability to oxidize substrates and aid in their increased running capacity.

Increased oxidative capacity of the soleus muscle mitochondria in HCR animals was found in conjunction with significantly greater ROS production versus the LCR animals. This appears to be due to the greater amount of oxygen being reduced in the HCR animals than the LCR animals. When looking at the ratio of $H_2O_2$ produced per $O_2$ consumed (*Figure 1. 5*), we can see that there is in fact no detectable difference between the selection groups within each substrate condition. Therefore, we can see that the greater $O_2$ flowing through the electron transport system provided increased possibilities for ROS formation. Thus the soleus muscle of HCR animals has developed adaptations for increased mitochondrial respiration per mitochondrion, and with that comes a parallel increase in $H_2O_2$ emission. Why the soleus muscle adapted in such a way as to allow increased ROS formation may be due to the fiber type-specific ROS scavenging abilities of the soleus muscle. Anderson *et al.* (2006) have found the soleus muscle to have greater

ability to convert superoxide into hydrogen peroxide than either the red or white sections of the gastrocnemius muscle (2). Due to this apparent greater antioxidant ability of the soleus muscle, increases in ROS generation may be more easily handled in the soleus than in the gastrocnemius muscle. If this is in fact true, then the soleus muscle can increase its respiration per mitochondrion with little consequence for the associated increases in ROS generation, as was observed in this study. Unfortunately antioxidant capacity of HCR versus LCR animals has not been investigated in these animals.

*Oxidative damage to the soleus and gastrocnemius*

Protein carbonylation and 8-OHdG content are indicators of oxidative damage to the cell. With both muscle types emitting greater levels of $H_2O_2$ per mg tissue in HCR animals, it might be expected that there would be increased oxidative damage to the muscle tissue of HCR rats. This was not the case, as neither muscle type showed any detectable difference in protein carbonyl concentration between HCR and LCR animals, and 8-OHdG content was actually lower in HCR rats than LCR rats. For this to occur, several mechanisms of ROS quenching must be at play to prevent ROS accumulation and the resultant damage. The cell has many mechanisms of preventing oxidative damage due to ROS, including cytosolic (CuZn) SOD and mitochondrial (Mn) SOD to convert the highly reactive superoxide radical to hydrogen peroxide, catalaze and glutathione to convert hydrogen peroxide to water, as well as Myoglobin's ability to react with both hydrogen peroxide and superoxide to form water (36). The up-regulation of any of the above mentioned antioxidant mechanisms would help attenuate oxidative damage, and

may help explain the decreased oxidative damage in the HCR animals compared to the LCR animals.

One tempting hypothesis for the unusual adaptation in both the soleus muscle and gastrocnemius muscle of HCR animals may be involve "stress response hormesis" (73). Hormesis, the bi-phasic response to a chemical or environmental factor known to be detrimental at high doses (21), has become an increasingly investigated phenomenon, especially in regards to antioxidant expression and mitochondrial biogenesis (73; 88). Previous studies have shown that moderate levels of ROS can trigger the induction of NRF-1 (88), resulting in a downstream increase in mitochondrial formation (88). Likewise, another study has found that moderate ROS generation can actually induce increases in antioxidants, specifically MnSOD (56), thus giving the cell protection during periods of greater ROS emission where it would be otherwise susceptible to oxidative damage. The concept of hormesis, therefore appears to be very applicable to our findings, as the soleus muscle and red region of gastrocnemius muscle of HCR animals produce greater ROS emission than LCR animals, yet these muscles have less oxidative damage (*Figure 1.4*). Anderson *et al* (2006) has previously shown increased ROS scavenging ability in the soleus muscle compared to the red and white sections of the gastrocnemius (2). With the soleus muscle's inherent high ROS scavenging capacity, and the probable increase in antioxidants due to ROS-induced up regulation (hormesis), it becomes clear why there was not increased oxidative damage in this muscle in HCR, regardless of the significantly higher ROS emission, even per CS activity.

As previously stated, increased (although not excessive) levels of ROS emission has been shown to result in increased NRF-1, and thus increased PGC1-α (88). In

agreement with this concept, both gastrocnemius (50; 80; 91) and soleus (104) muscles have been found to have higher protein levels of PGC-1α in HCR animals than in LCR animals, while our data shows both soleus and $Gas_{red}$ muscles to have greater ROS emission per mg tissue. These findings, taken together, may indicate that the greater ROS emission by HCR animals may be inducing a positive effect on mitochondrial production. As neither the HCR nor the LCR animals are exposed to conditions which would actively increase mitochondrial content (such as endurance training), we can assume that a potentially high level of mitochondrial biogenesis (as indicated by greater PGC-1α) seen in HCR animals would be complemented with an equal rate of mitochondrial degradation, so as to maintain a steady mitochondrial content throughout the adult life of the animals. This increased PGC-1α may therefore be an indicator of increased mitochondrial turnover, especially as there is not increased mitochondrial content in the soleus muscle, according to our findings. As oxidative damage accumulates in the mitochondria, the damaged mitochondrial proteins (especially those of the electron transport chain) increase ROS emission and thus further increase oxidative damage during one's lifetime. The increase in mitochondrial turnover would aid in reducing the oxidative damage, by maintaining relatively stable ROS emissions.

*In summary*, HCR rats have developed muscle type-specific adaptations to the selection for high running capacity. The soleus muscle has developed greater oxidative capacity per mitochondrion, while the gastrocnemius muscle has greater mitochondrial content, which has allowed the mitochondria to be less coupled and thus reducing $H_2O_2$ emission per mitochondrion. We speculate that these adaptations permit an optimization of maximal oxidative capacity within the muscle, while limiting the ROS emission,

thereby limiting oxidative damage to the cell. These changes to the muscle may aid in prevention/slowing of a multitude of disease states, including diabetes, aging, and obesity, by maintaining optimal functioning of the mitochondria and preventing cellular damage that can ultimately result in cell death and muscle wasting.

# Chapter 4. Changes in Mitochondrial Function of Aged Rats Bred for High versus Low Running Capacity

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## Abstract

Selection for high (HCR) and low (LCR) running capacity in rats has resulted in protection from a vast array of health problems including obesity, insulin resistance, and cardiovascular disease in HCR animals. Recent investigations into potential mitochondrial differences between HCR and LCR revealed muscle-specific adaptations that maximized oxidative capacity while preventing oxidative damage to the muscle. Due to the greater lifespan of HCR animals, we investigated whether the mitochondrial adaptations found in adult HCR animals were carried into old age. Soleus and the red region of the gastrocnemius muscle were studied for respiratory function, $H_2O_2$ emission, and oxidative damage. The soleus muscle of HCR animals had: no detectable difference in citrate synthase (CS) activity, no detectable difference in respiration both per mg tissue and per CS activity, no detectable difference in $H_2O_2$ emission both per mg tissue and per CS activity, and no detectable difference in protein carbonylation but decreased total 8-OHdG content versus LCR animals. The red region of gastrocnemius muscle of HCR animals had: greater CS activity, no detectable difference in respiration per mg tissue but lower respiration per CS activity, increased $H_2O_2$ emission per mg tissue but decreased $H_2O_2$ emission per CS activity, and no detectable difference in protein carbonylation but lower total 8-OHdG content versus LCR animals. It appears that the HCR rats have maintained a mechanism to prevent accumulation of oxidative damage, whereas LCR animals have not. Possible mechanisms for the increased protection from oxidative damage found in HCR animals include decreased ROS emission, hormesis, and possibly augmented antioxidant response versus LCR animals. These changes observed during

aging of HCR animals may help to explain the increased life span, and health of HCR animals verses LCR animals.

## Introduction

Aging is associated with a host of physiological declines, including loss of muscle mass and function (sarcopenia) (87), decreased aerobic capacity (24), and decreased mitochondrial function (34). At the cellular level aging is associated with increased oxidative damage (100), decreases in the ROS buffering capacity of the cell (54), and decreased mitochondrial turnover (27; 62), resulting in the progression of oxidative damage. One of the prominent theories of why this occurs is the *mitochondrial theory of aging* proposed by Harman in 1971, which states that as oxidative stress builds up in the mitochondria, damage to proteins and DNA occurs, leading to the loss of function with age. It follows from this theory that if mitochondrial integrity can be maintained with aging, decreased oxidative damage would occur and organism function would be maintained longer.

Investigation into ways to slow the cycle of mitochondrial damage and oxidative stress has found that endurance exercise training can have a positive impact on mitochondrial function. Endurance exercise training has been shown to increase mitochondrial density (8), increase oxidative capacity of the muscle (10; 78), prevent decreases in oxidative capacity with aging (67), increase lifespan, decrease oxidative stress and increase antioxidant content (78). Past investigations into the effects of endurance capacity on aging have used an exercise training stimulus (8; 12; 24; 78; 89); however, this gives little insight into the impact of an innate genetic ability for high endurance exercise capacity versus the exercise stimulus itself. To investigate the role of innate capacity for exercise on health, artificial selection for running capacity was used to generate two strains of rats that differ markedly in intrinsic running ability (60).

High running capacity (HCR) rats had a 347% higher running capacity (104) and a 50% higher $VO_{2max}$ (40) versus low running capacity rats (LCR) at Generation 11. Along with a greater $VO_{2max}$, HCR animals appear to be protected from obesity (80; 104), insulin resistance (80; 104), abnormal lipid handling (91) and are at decreased risk for diabetes (80; 104) compared to LCR rats. Recently our lab investigated the mitochondrial function (oxidative capacity and ROS emission per mitochondria) and oxidative stress levels of adult HCR and LCR animals and we found muscle type-specific adaptations at the mitochondrial level that may help to explain the decreased susceptibility to disease in HCR animals. The soleus muscle of HCR animals, while not having greater CS activity (suggesting no difference in mitochondrial content), had greater oxidative capacity and indications of a greater mitochondrial turnover versus LCR animals (99). Unlike the soleus muscle, the red gastrocnemius ($Gas_{red}$) muscle had greater CS activity (suggesting greater mitochondrial content), but a reduced respiratory capacity per unit of CS activity, an adaptation we speculate was adopted to facilitate a decrease in $H_2O_2$ emission per mitochondrion. Both adaptations appear to be functioning so as to maximize oxidative capacity while preventing oxidative damage to the cell. Our working hypothesis is that these adaptations have come about due to the effects of stress induced hormesis.

It is thus clear that HCR animals have some unique differences in mitochondrial function that allow them not only to have an increased $VO_{2max}$, but also improved health, and in some respects these mitochondrial adaptations are different from those associated with exercise training. The question that now arises is whether these positive adaptations are maintained into old age and have a favorable impact on the health and lifespan of HCR animals. We hypothesize that HCR animals will have better maintained oxidative

capacity, less oxidative damage and a smaller age-related increase in $H_2O_2$ generation versus LCR animals. We therefore assessed mitochondrial respiratory function, $H_2O_2$ emission, and protein and DNA oxidation in aged HCR and LCR animals to see if the differences in mitochondrial function observed between adult HCR/LCR animals are preserved in old age.

## Methods

*Animals.*

The selection of animals for HCR and LCR has been described in detail previously (60). In brief, animals were selectively bred for treadmill running capacity from a starter population of 96 male and 96 female heterogeneous rats from N:NIH stock. Intrinsic running capacity was tested when the rats were 11 weeks old, via treadmill test time to exhaustion, at 15° beginning at 10 m x $min^{-1}$ and increasing 1 m x $min^{-1}$ every 2 minutes. The 13 highest time to exhaustion animals were bred and the 13 lowest time to exhaustions animals were bred to produce a stain of rat that at generation six differed in running distance by 171% (60). All testing of running capacity and breeding was done at the University of Michigan in Ann Arbor, MI, by our collaborators, Dr.'s Lauren Koch and Steven Britton. Following maximal treadmill testing animals were housed without access to a running wheel (60).

Nine male (6 HCR and 3 LCR) rats obtained from the $20^{th}$ generation of selection were used for this study. Animals were sacrificed between 22-24 months of age (approximately the 50% survival age for LCR rats; Koch and Britton, unpublished

results). A second cohort of 11 animals (6 HCR and 5 LCR) obtained from the 18$^{th}$ generation was also used in this study for determination of protein carbonyls, and these animals were sacrificed between 25 and 28 month of age. Upon arrival at the University of Calgary, animals were housed individually at the biological sciences vivarium (12:12-h light-dark cycle, ambient temperature = 22°C) without access to a running wheel to prevent exercise-induced adaptations. Animals were given standard chow and water *ad libitum*.

*Muscle harvesting*

Animals were anesthetized with 50-60 mg x kg$^{-1}$ sodium-pentobarbital *i.p.* Upon deep surgical anesthesia the left soleus and gastrocnemius muscle were harvested, weighed and frozen in liquid nitrogen and stored at -80°C. For Generation 21 animals the right soleus and gastrocnemius muscle were harvested, weighed and placed in ice cold buffer X containing 7.23 mM $K_2$EGTA, 2.77 mM Ca$K_2$EGTA, 20 mM Imidazole, 0.5 mM Taurine, 5.7 mM ATP, 14.3 mM PCr, 6.56mM $MgCl_2$-6$H_20$, and 50 mM MES, pH 7.1. Animals were euthanized by cardiac removal following muscle harvest.

The Soleus and $Gas_{red}$ muscles were chosen for this study for several reasons. Firstly, the soleus has primarily (87%) type I fibers while the $Gas_{red}$ is composed of a mixture of type I (30%) and type IIa (62%) fibers (4), thus allowing us to investigate the differences in mitochondrial function between fiber types. Secondly, it has previously been shown that the gastrocnemius muscle has a greater ROS emitting potential than the soleus muscle (2), and this has been found to be accompanied by lower endogenous antioxidants to quench this production of ROS in gastrocnemius muscle (2). Thus it

appears fiber type greatly affects ROS generation, and antioxidant enzyme levels and this likely translates to differences in oxidative damage to the muscle. Thirdly, fiber type has been greatly implicated in aging in the literature. Specifically it has been conjectured that type II fibers are selectively targeted for atrophy and apoptosis during aging. Lastly, as a very practical approach the soleus and the gastrocnemius muscles were chosen as much of the available data on HCR/LCR animals has been studied in these two muscles, thus allowing us to make comparisons to previous studies.

*Preparation of permeabilized of muscle fiber bundles*

Small fiber bundles (~5mg) of soleus and red gastroc ($Gas_{red}$) muscle were manually teased apart with sharp ended needles to maximize surface area, and then either incubated in buffer X with 100 uL saponin for 30 minutes on ice with mild stirring or cryopreserved (see below). The bundles were then washed three times for 10 minutes in ice cold buffer Z containing 105 mM K-MES, 30 mM KCL, 10 mM $KH_2PO_4$, 5 mM $MgCl_2$-$6H_2O$, 1 mM EGTA, and 5 mg x $ml^{-1}$ BSA, pH 7.4 to remove saponin and metabolites. Fiber bundles were stored (no more than 60 min) on ice in buffer Z until respirometry measures.

*Cryopreservation of muscle fiber bundles*

Cryopreservation has been shown to have minimal negative effects on the mitochondrial structure, mitochondrial function (63) or ROS production (see Appendix A) in teased muscle bundles. Following manual teasing, two muscle bundles were

cryopreserved from each muscle as was described in detail previously (63). In short, muscle bundles were placed in eppendorffs containing 100 µl buffer X supplemented with 30% dimethyl sulfoxide (DMSO) and 10 mg x $ml^{-1}$ bovine serum albumin (BSA). Muscle bundles were exposed to the solution for five seconds, then frozen in liquid $N_2$ and stored at -80°C for analysis of $H_2O_2$ emission.

*Mitochondrial respiration measurements*

Respiration was measured in 2 mL of buffer Z at 37°C with a polarographic oxygen sensor, designed for high resolution respirmetry (Oxygraph-2k, Oroboros, Innsbruck, Austria). All measures were done following required oxygraph calibration. Two substrate protocols were performed in parallel, with each substrate addition being allowed to reach a steady state before the subsequent addition was made, requiring approximately 3-5 minutes between additions. The protocols were as follows: 1) 5 mMglutamate + 2.5 mM malate, 2 mM adenosine diphosphate, 10 mM succinate, 8 µM cytochrome c, 8µg x $ml^{-1}$ oligomycin, and 2.5 µM FCCP; 2) 5 mMglutamate + 2.5 mM malate (gm), 2 mM adenosine diphosphate (ADP), 10 mM succinate (succ), 10 µM antimycin A (aa), 0.6 mM N,N,N',N'-tetramethyl-p-phenylendiamine + 2.4 mM ascorbate (Tmpd&Asc), and 2 mM potassium cyanide (kcn). Respiratory Control Ratio (RCR) was calculated as the quotient of ADP-stimulated respiration (gm as substrates) and gm-stimulated respiration. Following respirometry measures bundles were weighed, and then frozen in liquid $N_2$ and stored at -80 °C until later analysis for CS activity (see below).

*Mitochondrial $H_2O_2$ production*

Measurements were performed with an Hitachi F-2500 fluorescence spectrophotometer, to detect resorufin, a byproduct of the 1:1 reaction of Amplex red and $H_2O_2$. Resorufin is detectable at an excitation/emission wavelength of 563 mn/ 587 nm, and is catalyzed by the addition in HRP. For analysis, bundles were thawed in a water bath at 37 °C. Once cryopreservation solution was fully thawed bundles were washed three times for 10 minutes in buffer X supplemented with 2 mg x $ml^{-1}$ BSA to remove any trace of DMSO. Measurements were done in 1 mL of buffer Z supplemented with 5 μM Amplex red and 0.17 U/ mL HRP at 37°C. After baseline measures, substrate additions were injected as follows: 5 mM glutamate + 2 mM malate; 3 mM succinate; 10 uM ADP, 100 uM ADP, 1 mM ADP. Each substrate addition was allowed to reach steady state (approximately 3 minutes) before the subsequent addition

*Measurement of Citrate Synthase content*

Stored muscle bundles from the respirometry measurements were used for analysis of citrate synthase (CS) activity. Bundles were weighed (3-7 mg) and homogenized on ice in 1:200 ratio of buffer containing 100 mM $KPO_4$ + 5 mM EDTA + 5 mM EGTA, pH 7.4 with a hand homogenizer for approximately 40 seconds. Homogenate was then vortexed and subjected to three freeze-thaw cycles in liquid $N_2$. After the third thaw samples underwent a second dilution if 1:2 for a final dilution of 1:400. Measurement of citrate synthase (CS) enzyme activity was determined

spectrophotometrically via mercaptide ion production, at 412 nm on a microplate reader. This involved aliquoting 20 μL homogenate, and 170 μL reaction buffer containing 0.3 mM acetyl Co A, 0.1 mM 5.5'-Dithiobis(2-nitrobenzoic acid), and 100 mM TRIS buffer were aliquoted in triplicate into a 96 well plate and reading absorbance changes for endogenous levels of thiol or deacetylase activity. Following initial reading, 10 μl of 10 mM OAA was added to each well to start the enzyme reaction that results in mercaptide ion production. CS activity calculated by subtracting the endogenous activity from the final reading, and expressed as μmol x $g^{-1}$ x $min^{-1}$. Both respirometry and $H_2O_2$ measures were normalized to CS activity as a proxy measure of respiration and ROS emission per mitochondrion.

*Measurement of Protein Carbonyls*

Protein Carbonyls were measured in soleus and $Gas_{red}$ muscles of the $18^{th}$ generation of HCR/LCR animals. Protein carbonyl concentration was determined via a protein carbonyl enzyme immuno-assay kit (BioCell Corp. Ltd., Auckland, New Zealand). Whole muscle was weighed (20-50 mg) and homogenized on ice in 1:40 dilution of buffer containing 20 mM $KH_2PO_4$ and 20 mM $Na_2HPO_4$. An aliquot of homogenate was taken to determine protein content via Bradford assay. The homogenate was then vortexed and incubated in dinitrophenylhydrazine (DNP) for 45 minutes. Sample was then diluted in enzyme immunoassay (EIA) buffer (potassium di-hydrogen phosphate and sodium chloride), aliquoted into a 96 well plate, sealed and incubated overnight. Following overnight incubation the plate was washed five times in EIA buffer and

incubated in blocking solution for 30 minutes. The plate was then washed as above and incubated in anti-DNP-biotin-antibody for 60 minutes at 37°C. The plate was again washed and streptavidin-HRP was added for a final incubation for 60 minutes. Following the final incubation the plate was washed and chromatin reagent was added and set for seven minutes. Stopping reagent was then added and the plate was read at 450 nm. Carbonyl content was measured in nmol x $mg^{-1}$ protein.

*Measurement of 8-OHdG*

Total genomic DNA (nuclear and mitochondrial) was extracted from skeletal muscle (*gastrocnemius* and *soleus*) using QIAmp DNA Mini kit (Qiagen, Mississauga, ON) as per manufacturer instructions. The concentration of the isolated DNA was determined using NanoDrop ND-1000 UV-Vis spectrophotometer (ThermoScientific, Ottawa, ON). The DNA concentrations ranged from 400 – 800 ng x $\mu L^{-1}$ and the average absorbance ratio of $A_{260}/A_{280}$ was between 1.7 – 1.9. One microgram of total DNA from each sample was dot blotted on the nitrocellulose membrane (Amersham, Piscataway, NJ). 8-OHdG immunoblotting was carried out using mouse monoclonal 8-OHdG (N45.1) antibody (Japan Institute for the Control of Aging, Fukuroi, Japan). Membranes were then incubated with anti-mouse horseradish peroxidase-linked secondary antibody (Bio-Rad Laboratories, Burlington, ON) and were visualized by enhanced chemiluminescence detection reagent (Amersham, Piscataway, NJ). Relative intensities of the circular dots were digitally quantified by using NIH ImageJ, version 1.37, analysis software (Scion Image, NIH).

*Statistics*

Respiratory and $H_2O_2$ measures were analyzed with a two-way ANOVA, with substrate condition and selection group (HCR/LCR) as fixed factors, Bonferroni post hoc was used when interaction were found in the data. RCR, CS content and protein carbonyls were analyzed with an independent t-test. Values are expressed as means ± SE.

## Results

*Body weight and Running Capacity*

Body HCR rats ran longer, and a farther distance than LCR rats, and weighed less. Weight and running capacity are summarized in table 2.

*Citrate synthase activity*

HCR animals had significantly higher CS activity than LCR rats in $Gas_{red}$ (30.9 +/- 1.6 μmol x $min^{-1}$ x $g^{-1}$ versus 22.0 +/- 4 μmol x $min^{-1}$ x $g^{-1}$); p=0.048 (*Figure 2.1a*), but no detectable difference in the soleus (23.4 +/- 1.8 μmol x $min^{-1}$ x $g^{-1}$ versus 18.8 +/- 0.9 μmol x $min^{-1}$ x $g^{-1}$; p=0.10) (*Figure 2. 1b*).

*Respirometry*

Two-way ANOVA revealed no significant interaction between selection group and substrate condition in either $Gas_{red}$ ($p=0.85$) (*Figure 2. 2a*) or soleus ($p=0.42$) (*Figure 2. 2b*) muscle. Similarly, there were no significant main effects for selection group in either muscle. Normalization to CS, used as a marker for mitochondrial content, resulted in significant main effects for selection group in $Gas_{red}$ muscle ($p=0.000$) and soleus muscle ($p=0.01$), with LCR having significantly higher respiratory capacity than HCR (*Figure 2. 2c&d*). Respiratory control ratio (RCR) did not differ by selection group in either soleus ($p=0.11$) or $Gas_{red}$ (0.98) muscle.

*$H_2O_2$ production*

Two-way ANOVA revealed no significant interaction between selection group and substrate condition. There were significant main effects for selection group in $Gas_{red}$ muscle ($p=0.02$), with HCR animals having a higher flux of $H_2O_2$ than LCR animals (*Figure 2. 3a*). There were no significant main effects for selection group in the soleus muscle (*Figure 2. 3b*). Normalization to CS activity resulted in significant interaction between selection group and substrate condition in the succinate condition in $Gas_{red}$ muscle only (*Figure 2. 3c*), with higher $H_2O_2$ production in LCR than in HCR. Normalization to CS activity had no impact on the lack of differences in $H_2O_2$ between selection groups seen in soleus muscle (*Figure 2. 3d*).

*Oxidative Stress*

There was no detectable difference between HCR and LCR in protein carbonyl concentration in either $Gas_{red}$ (p=0.79) (*Figure 2. 4a*) or soleus (p=0.71) muscle (*Figure 2. 4b*). There was however, significantly lower DNA 8-OHdG content in the gastrocnemius muscle (p=0.02) and soleus muscle (p=0.02) of HCR animals than of LCR animals (*Figure 2. 4c&d*).

## Discussion

Previously we reported muscle type-specific mitochondrial adaptations due to selective breeding for high running capacity, including increased CS activity and decreased RCR in $Gas_{red}$ muscle, and increased oxidative capacity and $H_2O_2$ per mitochondrion in the soleus muscle of adult HCR animals (99). These adaptations may aid in the protection of HCR animals from diseases such as obesity, insulin resistance, and CVD, all of which are seen in the progression of aging. The purpose of the present study was to investigate whether these adaptations are carried over in old age and translate to lower indices of oxidative damage with aging. The main findings of this study were that, with the exception of RCR, the adaptations found in the red region of gastrocnemius muscle of adult HCR animals were carried into old age; however, the adaptations in the soleus muscle were not. On the other hand, whereas protein carbonyls were not different between selection groups in the aged animals (similar to observations in adult animals), 8OHdG content increased dramatically with aging in the LCR rats and this was attenuated in the HCR rats, suggesting continued protection from oxidative

damage with aging in the HCR rats. These differences may play an important role in explaining the superior health and longevity observed in aged HCR rats.

*Mitochondrial Content and Respiration*

The greater CS activity found in $Gas_{red}$ of aged HCR animals, like that of the adult animals, resulted in decreased respiration per CS activity, permitting each mitochondrion to have a lower electron flux during any required ATP demand, thus decreasing the demand on any individual mitochondrion. The greater CS activity seen in old HCR animals is similar to that seen in aerobic exercise training of both old animals and humans. Endurance exercise training performed on late middle aged F344BN (Fisher 344 X Brown Norway) rats resulted in increased CS activity (12), as did endurance exercise training in human subjects ranging from 20 to greater than 70 years old (11; 22; 37). In one epidemiological study of young and old subjects who had been moderately active over the past four years, trained subjects showed greater CS activity over their sedentary counterparts, with no significant difference in CS activity between old and young trained individuals observed (67). Associated with increased CS activity due to endurance training in older populations, other markers of increased mitochondrial content, mitochondrial biosynthesis and improved glucose uptake were found in previous papers, including: increases in cytochrome oxidase (COX) activity (12; 89), Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1-α) content (67; 89), Nuclear respiratory factor 1 (NRF-1) (67; 89), mitochondrial Transcription factor A (TFAM) (67; 89), Glucose transporter 4 (GLUT4) (89) and silent mating type information regulation 2 homolog 3 (SIRT3) (67). One common theme in all of the

endurance exercise training studies was an increase in $VO_{2max}$ (12; 67; 89). Contrary to the impacts of exercise training, however, we did not observe greater muscle respiratory capacity in the old HCR animals versus their aged LCR counterparts. Our findings show no significant difference in maximal ADP-stimulated respiration per mg tissue between older HCR and LCR in either $Gas_{red}$ or Soleus muscle, in any substrate condition (*Figure 2. 2)*. These findings are in stark contrast to that in adult HCR/LCR animals, where HCR rats were found to have increased complex IV substrate respiration in $Gas_{red}$ (99) and higher respiration in all substrate conditions in the soleus muscle (99). Likewise, past investigation on young adult HCR/LCR animals had found a significantly higher whole body $VO_{2max}$ during treadmill exercise and associated increased enzymes for oxidative capacity in skeletal muscle: CS (50; 80), and βHAD (50), as well as increased protein concentration for subunits for complex IV (80; 91; 104). The current findings suggest that the possibly protective augmentation of respiration in HCR animals may be lost with aging, increasing their susceptibility to age-related diseases such as sarcopenia, diabetes and obesity. With this in mind, it appears that although HCR animals do present with increased CS activity in the $Gas_{red}$ into old age, other indices of muscle respiratory capacity, such as is observed with endurance exercise training, are not maintained. Thus, they may not have the health benefits (decreased disease risk) associated with maintaining physical activity. Conversely, changes associated with this decrease in respiration, namely decreased $H_2O_2$ emission per mg tissue, may play a more important role in preserving the health of HCR animals.

*ROS generation*

Overall $H_2O_2$ emission in $Gas_{red}$ of aged HCR animals was significantly higher than that of aged LCR rats, as was previously seen in adult animals. However, upon closer inspection, aged HCR rats appeared to have lower $H_2O_2$ emission in the succinate + gm condition (although not statically significantly, p=0.0542) (*Figure 2.3*), whereas this was not previously seen in adult animals. Although this difference did not reach statistical significance (no interaction), this was likely confounded by the low sample size in LCR rats and may have reached statistical significance with a greater number of animals. This limitation acknowledged, a lower H2O2 emission in the succinate + gm condition (where ROS generation is usually at its greatest level) may still have pronounced effects on the cellular redox state. The redox potential of muscle is known to decrease with age, primarily due to a decrease in total glutathione (54), and thus there is less capacity for any increase in $H_2O_2$ to be efficiently buffered by the cell (54). As cellular *in vivo* conditions typically have both complex I and II substrates supplying energy demand, this state may represent a more physiologically relevant level of $H_2O_2$ emission seen in the $Gas_{red}$, and may explain in the differences in accumulation of ROS and associated oxidative damage in the muscle of HCR versus LCR animals. Although there remains some controversy whether the observed maximal $H_2O_2$ emission during gm + succinate supported respiration occurs under physiologically normal but lower succinate concentrations (26), recent findings have contradicted this point (76) and it now appears that the combined effect of succinate and glutamate or succinate and palmatiol carnitine results in higher $H_2O_2$ emission than succinate alone, even at low succinate

concentrations (less than 1mM) (76). With this finding, it appears that reverse electron flow is in fact an *in vivo* occurrence, and thus should be carefully taken into consideration, as any increase in ROS production would continue to shift the redox state of the cell into a more oxidized state, further decreasing the redox potential in aged muscle. The near significant decreased $H_2O_2$ emission during respiration per CS activity with succinate in HCR animals is found despite no significant differences between HCR and LCR in any other substrate condition. As metabolism and electron transfer occur with both NAD and FAD linked substrates concurrently, our observations suggest that aged HCR animals emit less $H_2O_2$ per mitochondrion than their aged LCR counterparts.

*Oxidative Damage to the Soleus and Gastrocnemius Muscle*

As it is believed that mitochondria are a primary target for ROS-induced damage (55; 100), differences in oxidative damage accumulation to the mitochondria in HCR versus LCR rats with aging could be important to the greater health and longevity of the HCR rats. To investigate the extent of oxidative damage to the muscle, protein carbonylation in whole muscle homogenate and measures of 8-OHdG in DNA were assessed as measures of protein and DNA damage, respectively. There were no detectable differences found in either muscle in the concentration of protein carbonyls between aged HCR and LCR animals. There were, however, differences in total 8-OHdG in the gastrocnemius and soleus muscles, with aged HCR rats having significantly lower 8-OHdG content than aged LCR rats, as was observed in adult HCR/LCR animals (*Figure 2. 4*) (99). Note, however, that the absolute 8-OHdG content with aging in the HCR and LCR rats was much greater than in Adult HCR and LCR animals, respectively. Thus,

although HCR animals had lower total 8-OHdg content than LCR animals in old age, both HCR and LCR animals increased their total 8-OHdG content from adulthood into aging.

The protection from oxidative damage found in both the gastrocnemius and soleus muscle of aged HCR animals is similar to that seen in adult HCR animals (99). Our primary hypothesis to explain how HCR animals could have lower oxidative damage despite a greater muscle ROS production in adult animals was a hormetic stress response to $H_2O_2$ emission via an increase in antioxidants such as CuZnSOD, MnSOD, catalase, and glutathione peroxidase. The concept of stress response hormesis is that relatively low levels of a potential toxin will elicit positive adaptations (in this instance increased antioxidant mechanisms), which may ultimately aid in prevention of damage when exposed to higher levels of toxin, and this may confer overall health benefits as a result (21) (74). As adult HCR animals were emitting greater levels of $H_2O_2$ in both soleus and $Gas_{red}$ muscle, the hormetic connection was evident. The current cohort of aged HCR animals, however, are not emitting greater levels of $H_2O_2$ in the soleus, and thus we do not know if stress-induced hormesis is still at play in aged animals.

Relative to adult animals, aged animals have increased oxidative damage (35) and decreased mechanisms of protection such as antioxidant defense (56). These changes may affect the amount of ROS production that can be sufficiently buffered and thus reduce the optimal amount required for beneficial effects such as increased antioxidants and repair mechanisms (56) and increased mitochondrial formation (88). Due to these age-related changes, the optimal amount of ROS required for hormetic associated benefits may be lower in aged animals than in adult animals. If this is the case, the question arises as to

why both HCR and LCR animals are emitting the same amount of $H_2O_2$ with aging, yet only the HCR rats appear to be having decreased oxidative stress in the form of less 8-OHdG. If stress response hormesis was in fact occurring in adult animals, then HCR animals would have increased antioxidants (56) and an increased rate of mitochondrial turnover (88). As the animals aged, the HCR rats would have a greater innate scavenging ability, and thus would have maintained their ROS scavenging ability into old age far better than LCR animals. Thus, the level of $H_2O_2$ emission seen in the aged HCR and LCR animals may not have been too high to cause harm for the HCR animals. However, as the aged LCR animals seem likely to have less antioxidants to scavenge ROS, the $H_2O_2$ emission may have been beyond the beneficial dose to elicit a positive hormetic effect.

In summary, it appears that the muscle type-specific adaptations to mitochondrial function found in adult HCR/LCR animals were predominately carried over with aging in the $Gas_{red}$ muscle but not in the soleus muscle. Aged HCR animals maintained a greater level of CS activity (and thus we assume increased mitochondrial content) over their aged LCR counterparts in $Gas_{red}$ muscle; however, the coupling of ATP production was no longer decreased. Along with these findings, aged HCR rats appeared to emit slightly less $H_2O_2$ per mg tissue in the succinate condition than aged LCR rats, an event not apparent in adult animals. The soleus muscle had no detectable difference between HCR and LCR rats in either oxidative capacity or $H_2O_2$ emission in old age, unlike their adult counterparts where HCR animals had higher oxidative capacity and $H_2O_2$ emission. The loss of oxidative capacity in the soleus muscle with aging in HCR rats may be less of a negative change, as the associated decrease in $H_2O_2$ emission may play a more important

role in keeping the oxidative damage of the muscle to low levels. As in adult animals, hormesis is still an attractive hypothesis to explain the protection from oxidative damage seen in HCR animals. Overall, the adaptations found in soleus muscle of adult HCR animals were not found in old HCR animals; however, alternative changes seem to have occurred to maintain the health of the mitochondria, and the surrounding muscle. Based on the current findings, life span measures, as well as other measures of health and fitness, should be investigated in old HCR/LCR animals, as it appears HCR animals may be protected from diseases common to old age.

## Chapter 5. General Discussion

The studies reported in the preceding chapters were originally undertaken to investigate the differences in mitochondrial function between HCR and LCR animals and to investigate changes in said function during aging (98; 99). Unfortunately, four of the original seven aged LCR animals and one of the original seven aged HCR animals died before testing could be undertaken, leaving only three aged LCR and six aged HCR animals for study. Thus, possible conclusions about changes during aging cannot be fully verified with such low numbers.

Along with low numbers in the aged cohort, another limitation of this study may be found in the protein carbonyl assay. Although the ELISA has been determined to have an intra-assay CV between 1-4%, we found much greater intra-assay CV (average 10%). Although we found a relatively high CV, the protein carbonylation of our samples (after sufficient dilution) was found to be above the detectable range of our standard curve, thus changes in protein carbonylation may not have been able to be sufficiently detected. It is our belief that the large intra-assay differences that may have resulted in null findings between young and aged animals, and thus the apparent conflict with the 8-OHdG results. Also we found our protein content of our samples was relatively low, which also may have affected our outcome.

Of the available data, we can see that the mitochondrial function between HCR and LCR animals is different both between the muscles investigated (soleus and gastrocnmeius muscles) as well as between adult and aged animals. In adulthood, the soleus muscle of HCR animals had: similar CS activity, higher respiratory capacity (both per muscle weight as well as normalized to CS activity), higher $H_2O_2$ generation (both

per muscle weight as well as normalized), and lower oxidative damage than LCR animals. The gastrocnemius muscle of HCR animals during adulthood had: higher CS activity, higher complex IV stimulated respiration per muscle weight but lower respiratory capacity when normalized, higher $H_2O_2$ emission per muscle weight but lower $H_2O_2$ emission when normalized to CS activity, and lower oxidative damage than LCR animals. In aged animals the soleus muscle of HCR animals had: similar CS activity, similar respiratory capacity per muscle weight but lower respiratory capacity when normalized to CS activity, similar $H_2O_2$ emission (both per muscle weight as well as normalized to CS activity), and lower oxidative damage than LCR animals. The gastrocnemius muscle of aged HCR animals had: higher CS activity, similar respiratory capacity per muscle weight but lower respiratory capacity when normalized to CS activity, higher $H_2O_2$ emission per muscle weight but similar $H_2O_2$ emission when normalized to CS activity, and lower oxidative damage than LCR animals. In brief, the differences between HCR and LCR animals were quite different in the soleus muscle between adulthood and aging, as the higher respiratory capacity and $H_2O_2$ emission seen in adulthood were not carried into old age. However almost all of the differences between HCR and LCR rats in adult animals in the gastrocnemius were carried into old age, with the exception of lower RCR and lower $H_2O_2$ emission when normalized.

***Changes within HCR and LCR from adulthood to aged***

The majority of this paper has investigated the differences *between* HCR and LCR animals, once in the adult stage, and again in the aged stage. We have not however, emphasized the changes within one strain (HCR *or* LCR) from adulthood *into* aging. This has not been greatly emphasized as we believe the above mentioned limitations may

result in null findings where there may in fact be differences had we greater numbers of old animals. I will now however, outline the observed changes during aging in both the HCR and the LCR animals. When investigating the changes in mitochondrial function and oxidative stress during aging (adult HCR compared to aged HCR), we can see that the soleus muscle of HCR animals changed from adult to aged more than the gastrocnemius muscle (an overview of changes from adulthood to aged in the HCR and LCR can be found in Table 3, statistics not shown). Why there was such a disparity between soleus and gastrocnemius muscles during aging may be due to a protective mechanism to decrease the level of $H_2O_2$ emission with aging. As we had seen, the soleus muscle of adult HCR animals had a higher $H_2O_2$ emission than LCR animals, which was correlated to the higher respiratory capacity (See Figure 1.5). One possible explanation for this would be that during aging it may have become more important to decrease ROS emission than to maintain a high respiratory capacity. The reasoning behind this idea, as well as an explanation for the differences found between HCR and LCR both in adulthood and aging could be stress-induced hormesis, as is indicated in the above chapters.

Based on these findings, it appears that the phenomenon of stress-induced-hormesis may be acting to preserve the muscles in both the adult and aged HCR animals. Although it appears evident that hormesis is occurring in the HCR animals due to the decreased oxidative damage despite the greater total $H_2O_2$ emission (in the adult animals), the actual mechanism explaining how this is occurring is yet to be answered. Several different enzymes may be unregulated with hormesis, the most common being mitochondrial (Mn) SOD, although cytosolic (CuZn) SOD, catalase, and glutathione

content may also be increased. As all of these enzymes have the potential to convert either superoxide to hydrogen peroxide (SOD) or hydrogen peroxide to water (catalase and glutathione peroxidase), and any increase in their content would considerably decrease oxidative damage. The up-regulation of any of the above mentioned antioxidant mechanisms would help attenuate oxidative damage, and may help explain the decreased oxidative damage in the HCR animals compared to the LCR animals.

Furthermore, an important issue to resolve is how the ROS-induced stress in HCR rats is coupled to positive adaptations which protect the muscle from oxidative damage, despite ROS emission being equal between HCR and LCR animals in the aged condition. A possible hypothesis to explain the decreased oxidative damage found in HCR animals is that the hormetic curve of LCR animals may be shifted to the left (and potentially downwards) relative to HCR animals. A left-shifting of the curve in LCR rats would result in maladaptations in LCR animals but positive adaptations in HCR animals for the same amount of ROS emission (see Figure 3.1 for pictorial explanations). Possible mechanisms which would result in this shifting include: greater antioxidant content in HCR animals, and/or increased ROS-buffering capacity via a more reduced redox environment. Research into stress-induced-hormesis has found that a moderate level of ROS generation can result in significant increases in mitochondrial superoxide dismutase (56) (thus possibly other antioxidant and buffering enzymes) as well as increases in mitochondrial biogenesis (88). If hormesis is to be considered as the primary explanation for the unusual observances of decreased oxidative damage with concurrent increased $H_2O_2$ emission, then measurements of antioxidant enzymes, and mechanisms of DNA repair must be undertaken to find the underlying reason for how this is actually occurring.

One final question arises concerning why these differences in mitochondrial function are found in HCR animals versus LCR animals. It is clear from the previous two chapters in both adult and aged HCR and LCR animals that there are differences in both oxidative capacity of the mitochondria as well as the ROS emission and scavenging capacity. However, are these differences due to a genetic change from selective breeding for high running capacity, or are there other factors contributing, such as an increase in non-exercise activity thermogenesis? A previous study has found that given access to a running wheel, HCR animals are more likely to run or run longer, for more bouts and at a faster rate than LCR animals (102). Even without access to a running wheel it is possible that HCR animals are expending more energy from daily movement, which may be contributing to their differences in muscle function. To determine the extent that daily activity is contributing to changes in mitochondrial function, measures of daily movement should be recorded, as this has the potential to have great effects on muscle function as it has been found that chronic (at least 4 years) of training can significantly reduce age-related decline in oxidative capacity and mitochondrial proteins in humans (67).

Regardless of why these differences have occurred, it is likely that mitochondrial function plays a role in the health and disease of HCR and LCR animals, both as adult animals and into old age. To fully understand how these differences in mitochondrial function are occurring future studies on these animals must be undertaken. Specifically, investigation into the antioxidant capacity and redox buffering capacity for HCR and LCR animals needs to be carried out in both the soleus and the gastrocnemius muscles to increase our understanding of why there are such drastic differences in both oxidative

damage and $H_2O_2$ emission between these two different muscles. Finally, future studies into the life and health span of HCR/LCR animals will help us to more fully connect the changes in mitochondrial function to improved lifespan and health of HCR animals.

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## Tables

Table 1. Running Capacity, Body and Hind Limb Masses of Adult HCR and LCR rats. Animals body and muscle weights were taken at time of sacrifice (adulthood), maximum time and distance ran were collected when animals were 10 weeks old. Measurement of distance/time ran was performed at the University of Michigan, Ann Arbour MI (Personal Communication, Britton & Koch). Generation 19 animals were used for measurement of protein carbonyls, and generation 21 animals were used for all other tests. Values are shown as mean ± SD, missing values were indicated as an X.

| | HCR | LCR | HCR | LCR |
|---|---|---|---|---|
| Generation | 19 | 19 | 21 | 21 |
| Maximum distance ran | 1428 m (± 16 m) | 370 m (± 8 m) | 1776 m (±143 m) | 186 m (± 60 m) |
| Maximum time ran | 58.9 min (± 0.4 min) | 23.9 min (± 0.4 min) | 67.4 min (± 3.2 min) | 14.1 min (±3.6 min) |
| Body mass | 339 g (±21 g) | 503 g (±20 g) | 451 g (± 14 g) | 616 g (± 28 g) |
| Right Gastrocnemius mass | 1.65 g (±0.010 g) | 2.34 g (±0.08 g) | 2.14 g (± 0.05 g) | 2.45 g (±0.07 g) |
| Left Gastrocnemius mass | X | X | 2.04 g (±0.08 g) | 2.27 g (±0.08 g) |
| Right Soleus mass | 0.139 g (±0.01 g) | 0.169 g (±0.05 g) | 0.23 g (±0.01 g) | 0.22 g (±0.01 g) |
| Left Soleus mass | X | X | 0.21 g (± 0.01 g) | 0.22 g (± 0.01 g) |

Table 2. Running Capacity, Body and Hind Limb Masses of Aged HCR and LCR rats. Animals body and muscle weights were taken at time of sacrifice (aged), maximum time and distance ran were collected when animals were 10 weeks old. Measurement of distance/time ran was performed at the University of Michigan, Ann Arbour MI (Personal Communication, Britton & Koch). Generation 18 animals were used for measurement of protein carbonyls, and generation 20 animals were used for all other tests. Values are shown as mean ± SD, missing values were indicated as an X.

| | HCR | LCR | HCR | LCR |
|---|---|---|---|---|
| Generation | 18 | 18 | 20 | 20 |
| Maximum distance ran | X | X | 1666.41 m (± 62.5m) | 232.08 m (± 3.7m) |
| Maximum time ran | X | X | 64.77 min (± 1.5 min) | 16.91 min (± 0.2 min) |
| Body mass | 398.1 g (±17.85g) | 559.7 g (±21.76g) | 470.3 g (±29.89g) | 624.0 g (±11.44g) |
| Right Gastrocnemius mass | 1.28 g (±0.06g) | 1.37 g (±0.09g) | 2.08 g (±0.07g) | 1.89 g (±0.18g) |
| Left Gastrocnemius mass | X | X | 1.84 g (±0.05g) | 2.06 g(±0.10g) |
| Right Soleus mass | 0.12 g (±0.01g) | 0.14 g (±0.01g) | 0.23 g (±0.02g) | 0.17g (±0.01g) |
| Left Soleus mass | X | X | 0.18 g(±0.013g) | 0.18 g(±0.01g) |

Table 3. Changes in Mitochondrial Function and oxidative damage during aging in HCR and LCR rats in the soleus and gastrocnemius muscles. Indication of direction of change from adulthood to aging of each animal strain (HCR or LCR). The majority of change during aging was seen in the soleus muscle of HCR animals, with apparent decrease in both respiration and ROS emission.

| **Soleus** | **Aged HCR vs. Adult HCR** | **Aged LCR vs. Adult LCR** |
|---|---|---|
| CS activity | no change | Decreased |
| Oxidative respiration | Decreased | no change |
| Respiration per CS activity | Decreased | Increased |
| Respiratiory control ratio | no change | no change |
| ROS emission | Decreased | no change |
| ROS emission per CS activity | Decreased | no change |
| Protien Carbonylation | no change | no change |
| 8-OHdG content | increased | increased |
| **Gastrocnemius** | | |
| CS activity | Decreased | no change |
| Oxidative respiration | no change | no change |
| Respiration per CS activity | no change | no change |
| Respiratiory control ratio | no change | no change |
| ROS emission | no change | Decreased |
| ROS emission per CS activity | decreased | no change |
| Protien Carbonylation | no change | no change |
| 8-OHdG content | increased | increased |

# Figures

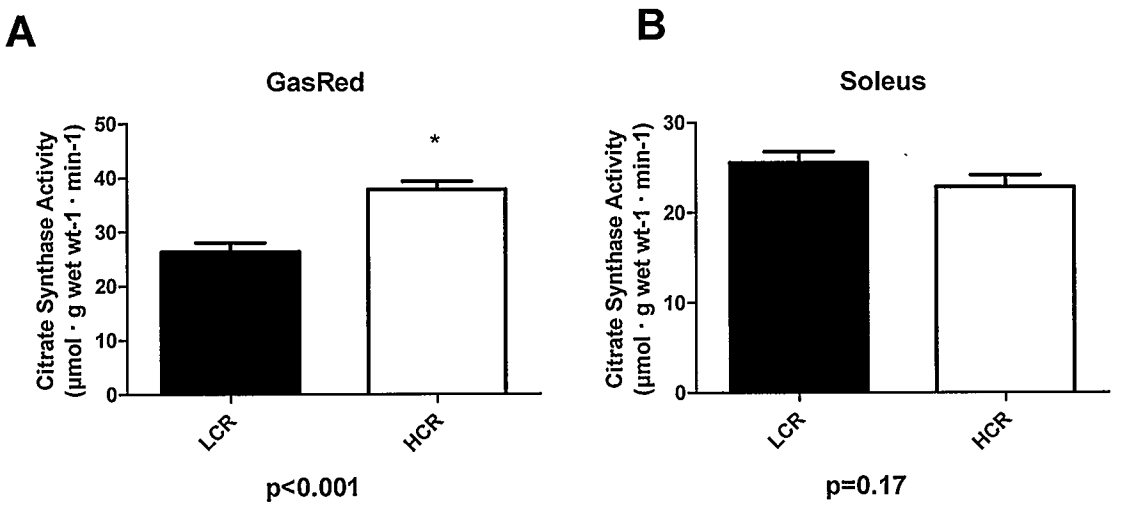



Figure 1.1. Citrate synthase activity in A) red gastrocnemius muscle and B) Soleus muscle in LCR= low running capacity animals and HCR= high running capacity animals. Data are presented as mean +/- SE.

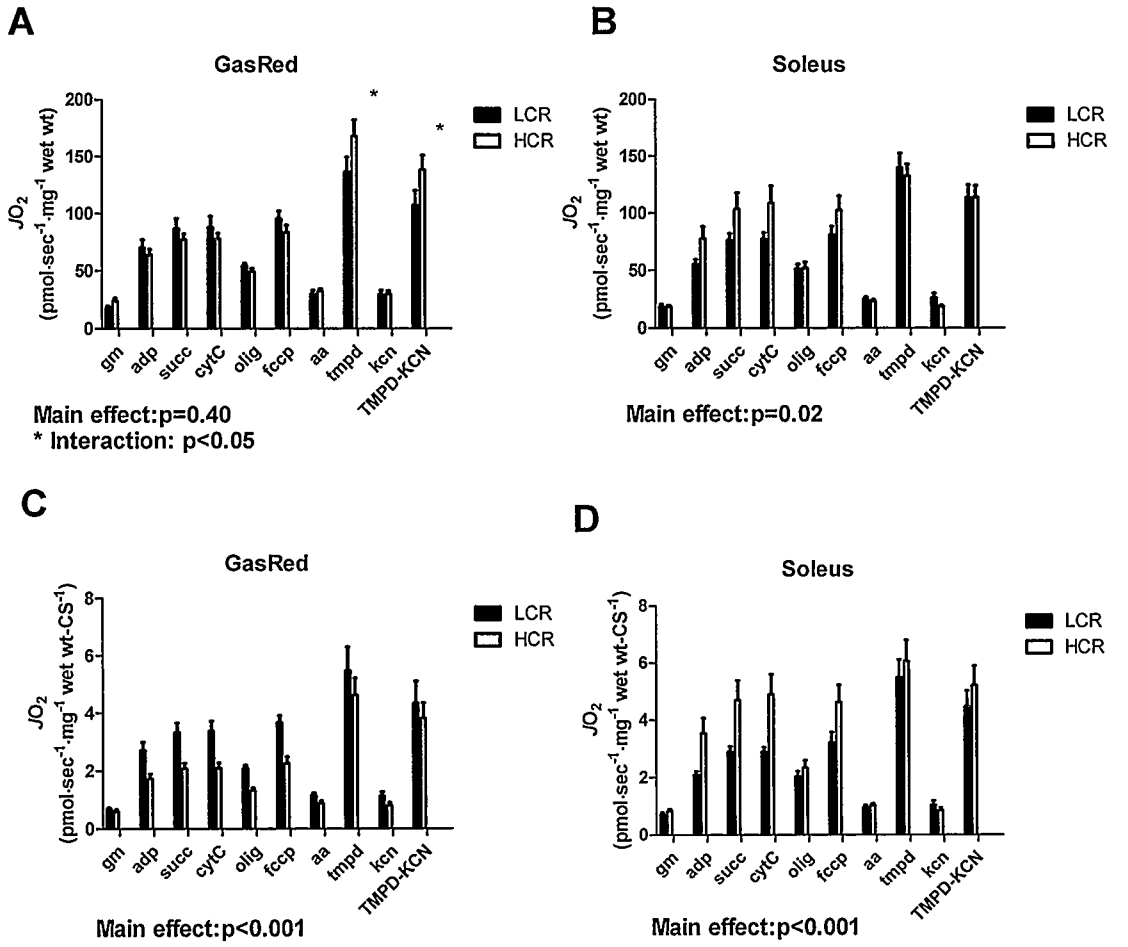



Figure 1.2. Respirometry measurements during different substrate conditions per tissue weight in A) red gastrocnemius and B) soleus muscle and per citrate synthase activity in C) red gastrocnemius and D) soleus muscle in HCR=high running capacity and LCR= low running capacity animals. *p<0.05 Data are presented as mean +/- SE

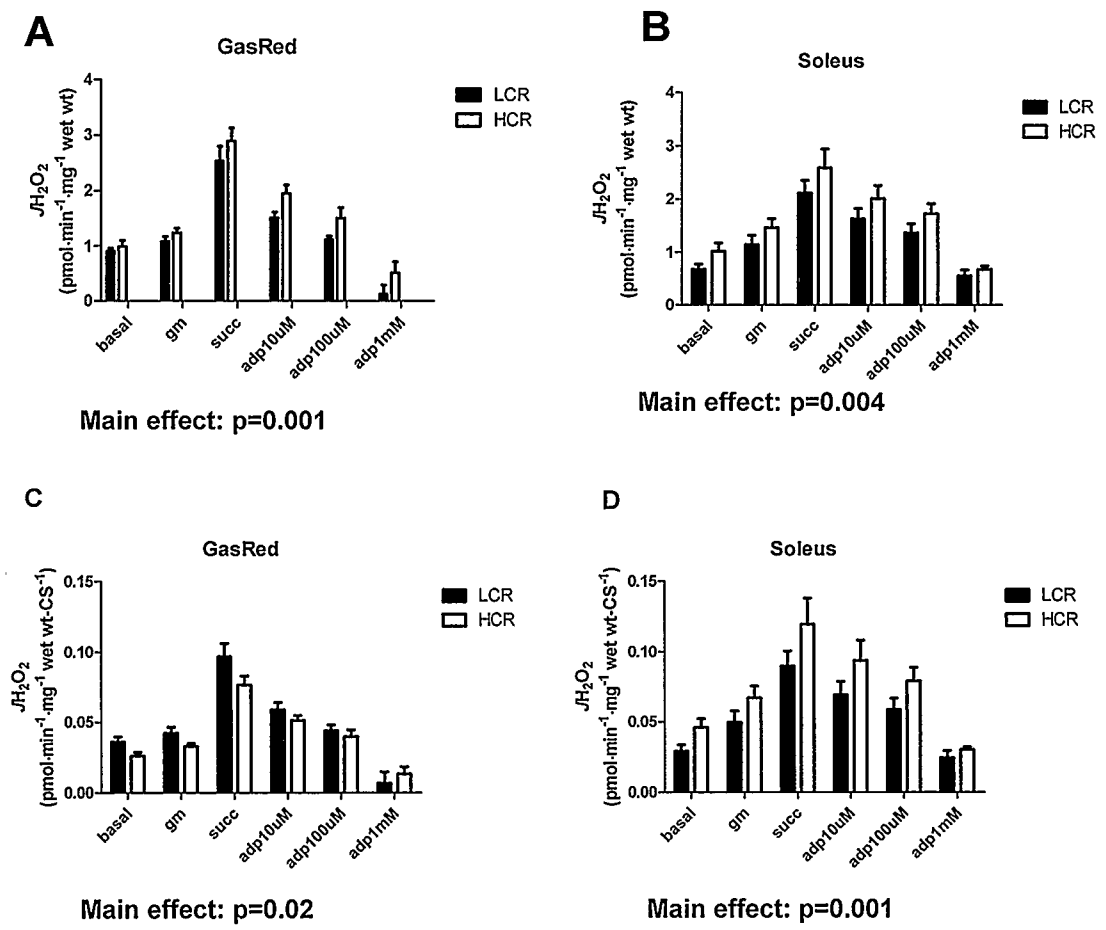



Figure 1.3. $H_2O_2$ emission during different substrate conditions per tissue weight in A) red gastrocnemius and B) soleus muscle; and per citrate synthase activity in C) red gastrocnemius and D) soleus muscle in HCR=high running capacity and LCR= low running capacity animals. Data are presented as mean +/- SE.

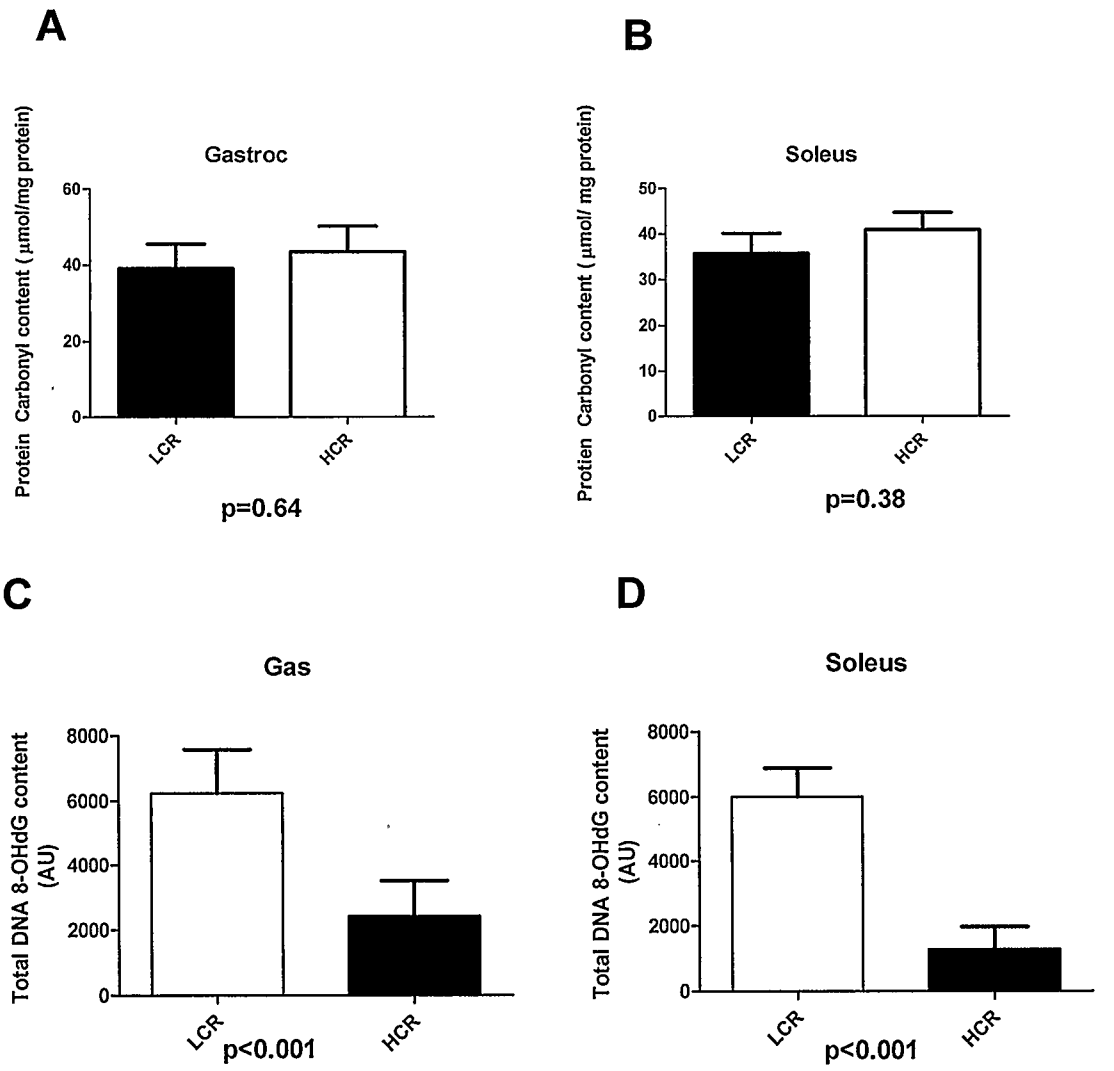



Figure 1.4. Measures of oxidative damage as measured by protein carbonylation content in red gastrocnemius (A) and soleus (B) muscle; and DNA 8-OHdG content in gastrocnemius (C) and (D) soleus muscle in HCR=high running capacity and LCR= low running capacity animals. Data are presented as mean +/- SE

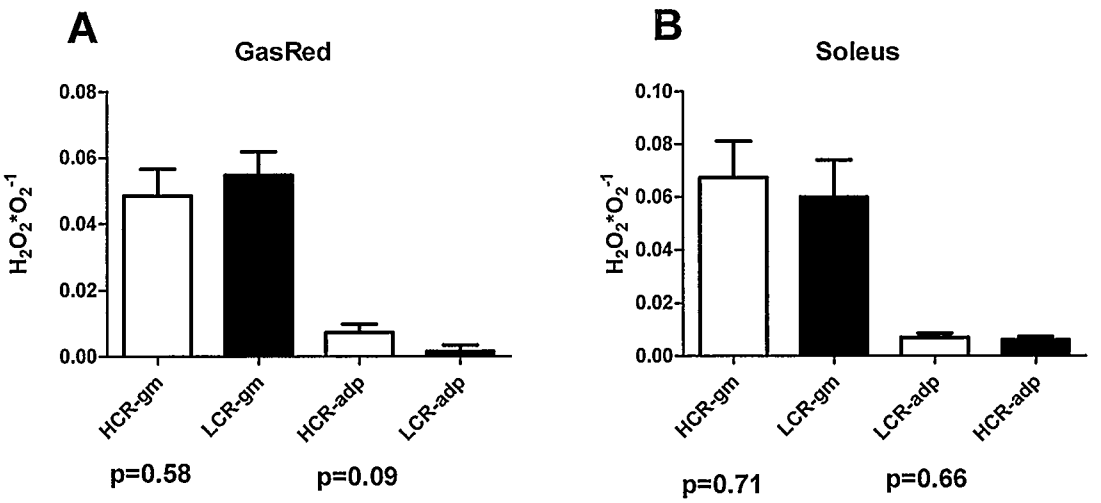



Figure 1.5. Free radical leak, as percentage of $H_2O_2$ formed per $O_2$ consumed in (A) red gastrocnemius and (B) soleus muscle during state 2 (gm) and state 3 (adp) conditions in HCR=high running capacity and LCR= low running capacity animals. Data are presented as mean +/- SE.

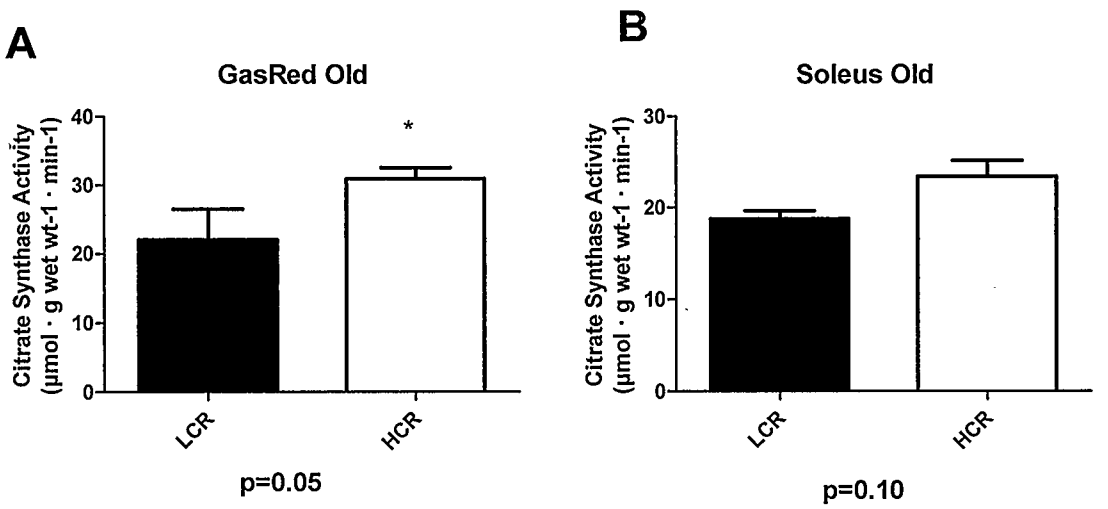



Figure 2.1. Citrate synthase activity in A) red gastrocnemius muscle and B) Soleus muscle in LCR= low running capacity animals and HCR= high running capacity animals. *$p<0.05$ different than LCR group. Data are presented as mean +/- SE

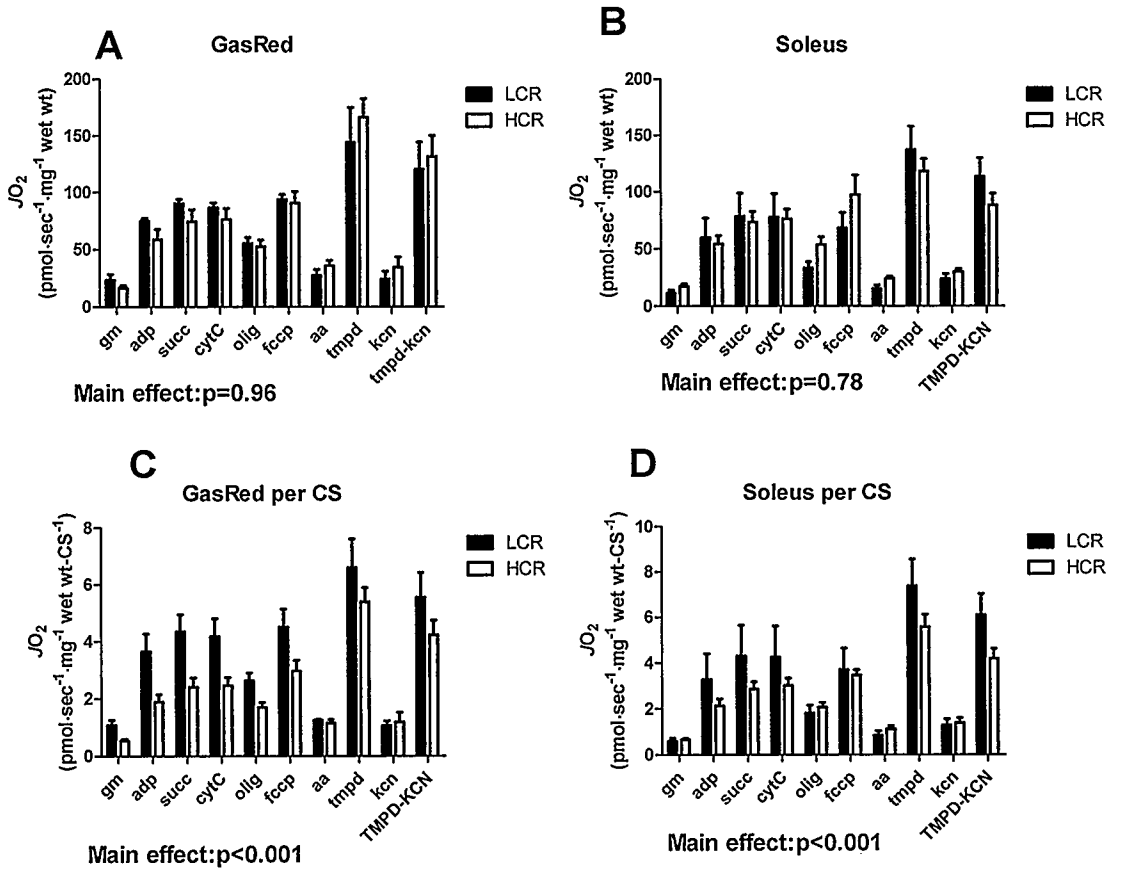



Figure 2.2. Respirometry measurements during different substrate conditions per tissue weight in A) red gastrocnemius and B) soleus muscle and per citrate synthase activity in C) red gastrocnemius and D) soleus muscle in HCR=high running capacity and LCR= low running capacity animals. Data are presented as mean +/- SE

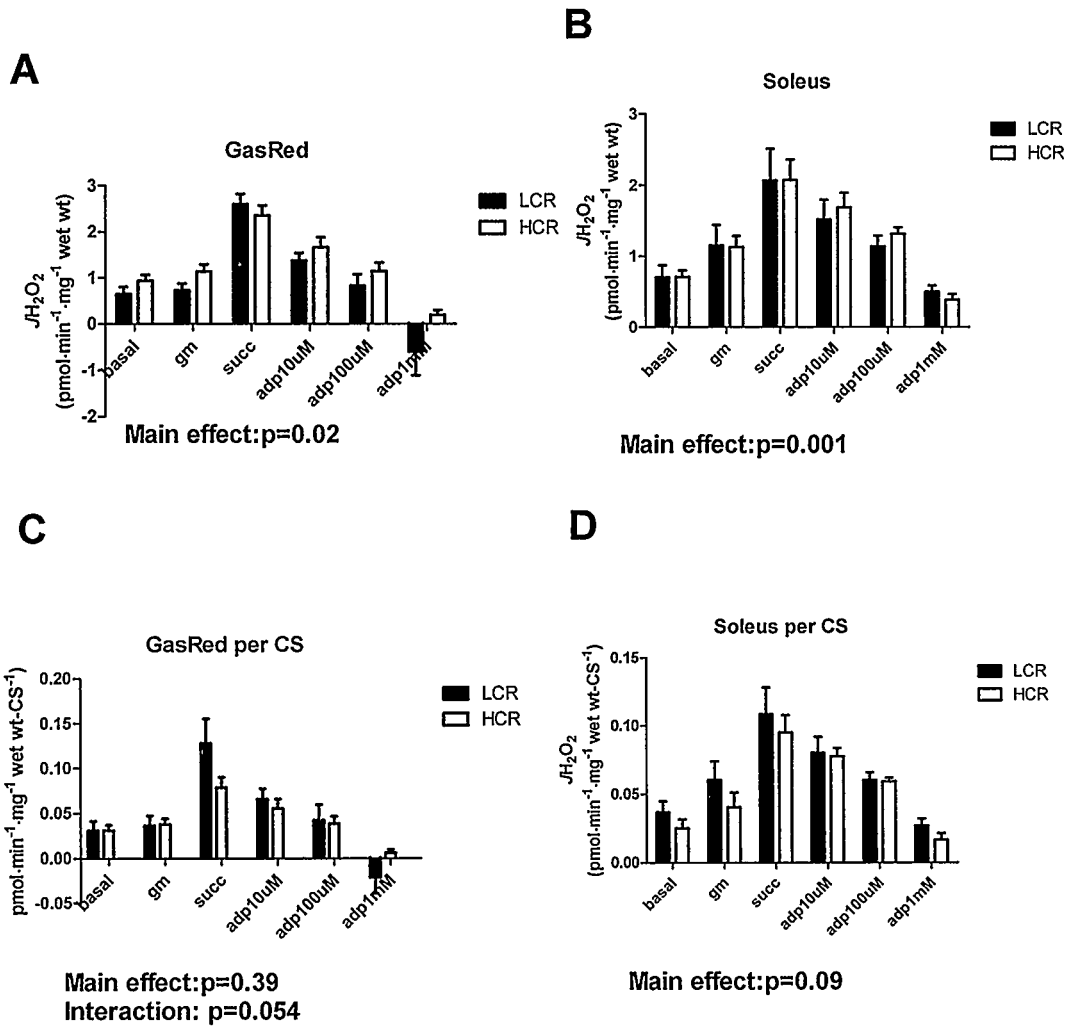



Figure 2.3. $H_2O_2$ emission during different substrate conditions per tissue weight in A) red gastrocnemius and B) soleus muscle; and per citrate synthase activity in C) red gastrocnemius and D) soleus muscle in HCR=high running capacity and LCR= low running capacity animals. Data are presented as mean +/- SE.

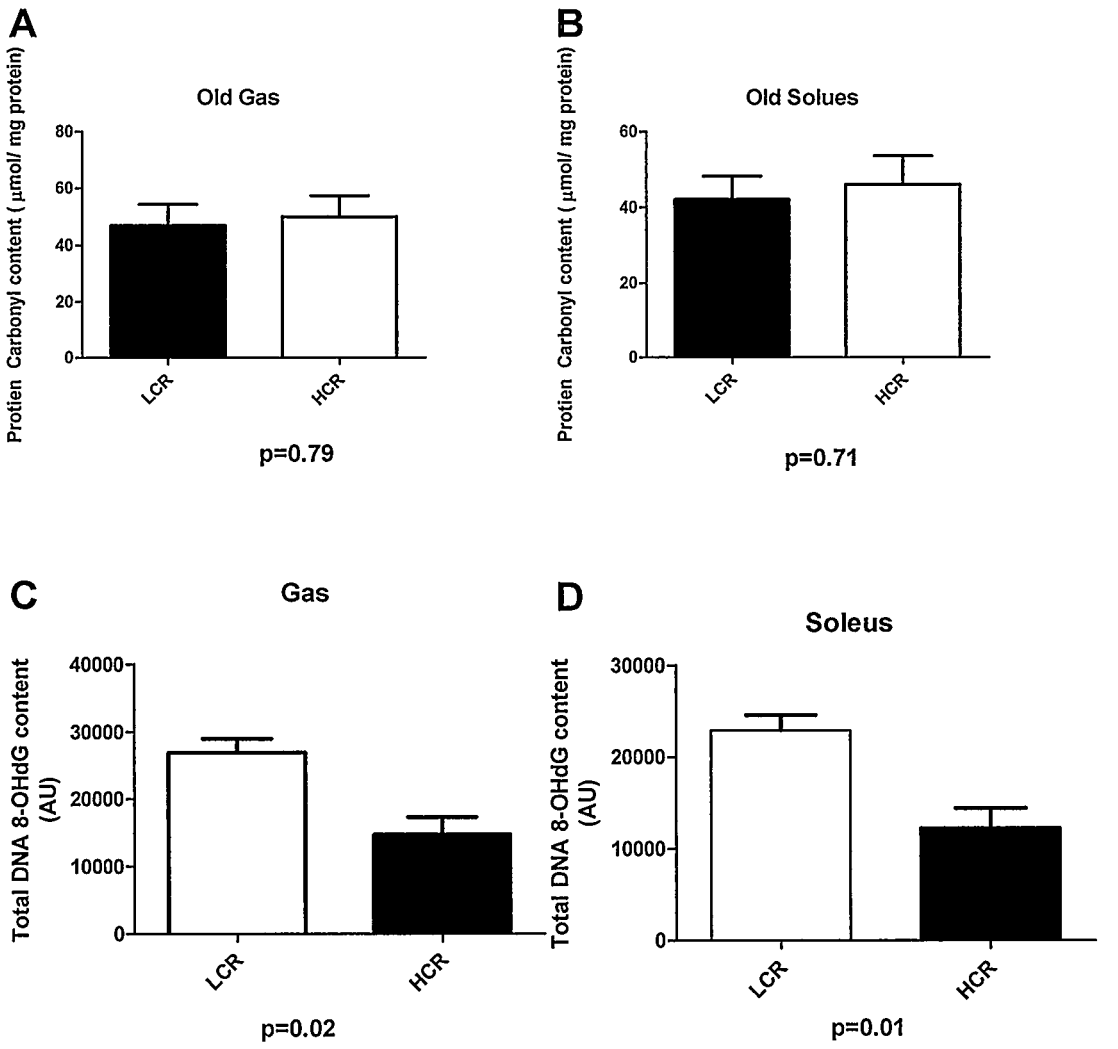



Figure 2.4. Measures of oxidative damage as measured by protein carbonylation content in red gastrocnemius (A) and soleus (B) muscle; and DNA 8-OHdG content in gastrocnemius (C) and (D) soleus muscle in HCR=high running capacity and LCR= low running capacity animals. Data are presented as mean +/- SE.

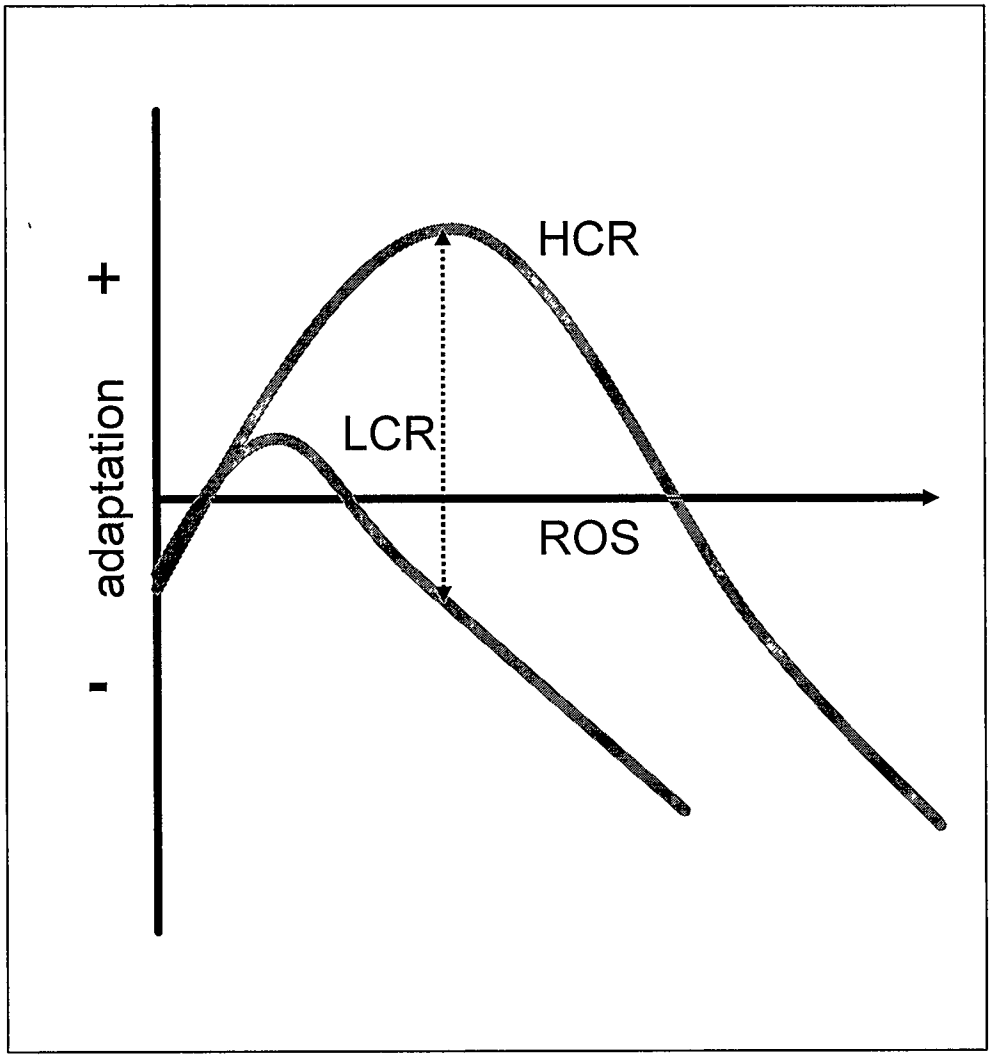
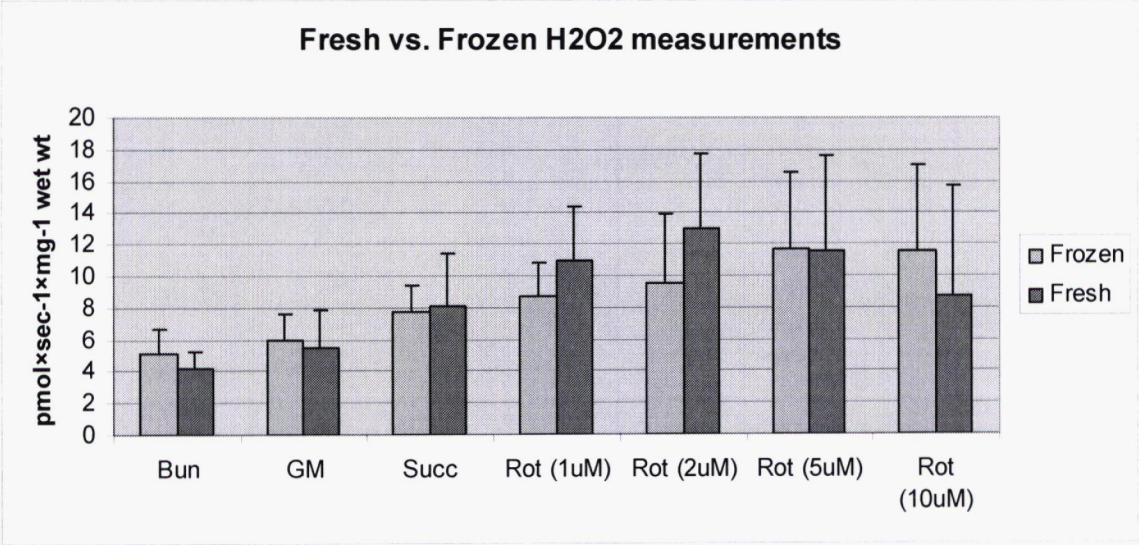



Figure 3. Hormetic curve indicating adaptation with increasing levels of a stressor (ROS generation). HCR animals are indicated in the red line, LCR animals are indicated in the blue line. The x-axis is increasing from right to left. See text for explanation of hormesis.

# Appendix A




Measurement were done so as to verify the validity of using cyropreserved muscle bundles to measure mitochondrial $H_2O_2$ emission. The methods of this study were the same as is found in Chapter 3 and Chapter 4, found above. Following manual teasing, muscle bundles were either used imediatally, or cryopreserved for later study. Cryopreservation was performed by placing teased muscle bundles in eppendorffs containing 100 µl buffer X supplemented with 30% dimethyl sulfoxide (DMSO) and 10 mg x $ml^{-1}$ bovine serum albumin (BSA). Muscle bundles were exposed to the solution for five seconds, then frozen in liquid $N_2$ and stored at -80°C for analysis of $H_2O_2$ emission. In this study 5 bundles were tested immediately following exctaction from the animals, and eight were testing following the above outlined cyropreservation.

UNIVERSITY OF CALGARY

Protocol BI 2009-11

# Certification of Animal Protocol Approval

Applicant: Russell T. Hepple

Faculty/Department: Kinesiology

Project Title: Determinants of skeletal muscle mass and function in health and aging

Sponsoring Agency(s): CIHR, NSERC

Effective: April 1, 2009 Expires: March 31, 2010

The Animal Care Committee,
having examined the animal care and treatment protocol,
approves the experimental procedures proposed and certifies
with the applicant that the care and treatment of animals
used will be in accordance with the principles
outlined in the most recent policies and
"Guide to the Care and Use of Experimental Animals"
By The Canadian Council on Animal Care.

Applicant — Date: May 29, 2009

Chair of Animal Care Committee or University Veterinarian — Date: May 29, 2009

Original - ACC File