The effects of physiological acidosis on skeletal muscle mitochondrial function, ROS balance, and intracellular signalling.

Submitted by

Christopher P. Hedges

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Supervisor: Professor David J. Bishop

Co-supervisors: Associate Professor Anthony J.R. Hickey

Professor Glenn K. McConell

Institute of Sport, Exercise and Active Living

College of Sport and Exercise Science

Victoria University, Melbourne, Australia

Abstract

Mitochondrial adaptation in skeletal muscle is promoted by a diverse array of stimuli, and changes in mitochondrial plasticity have been noted as a result of a many exercise modalities. High-intensity interval training is one such modality that promotes mitochondrial adaptation in response to repeated short-duration bouts of intense effort. Another result of intense muscular effort is a decrease in muscle pH, resulting in intracellular acidosis. The effect of this acidosis on oxygen consumption in muscle has received attention previously, with mixed findings. An aspect of skeletal muscle mitochondrial function that has received limited attention is the production of reactive oxygen species. To date a small number of studies have also provided evidence that attenuating the development of intracellular acidosis may have beneficial effects for mitochondrial adaptation.

This thesis aimed to further investigate the effect of acidosis on mitochondrial function, and on intracellular signalling for mitochondrial biogenesis. The first study looked at whether acute acidosis of a physiological range affects mitochondrial function, and/or activities of enzymes involved in reactive oxygen species balance, in rat soleus homogenate (*ex vivo*). Decreasing pH from 7.1 to 6.5 increased apparent ADP affinity and decreased maximal oxygen consumption rates, but no effect was seen at a pH of 6.9 or 6.7. Additionally, the net effect of decreasing pH on the enzymes tested appears to favour reactive oxygen species disposal through the glutathione pathway. This suggests mitochondrial function can be influenced by pH changes in the intracellular space (cytosol *in vivo*), and that acidosis may increase reactive oxygen species disposal.

The second study looked at whether a period of voluntary exercise altered the response of skeletal muscle mitochondria to acute acidosis. Assessment of mitochondrial function *in situ* in permeabilised soleus muscle fibres showed that rats that had performed voluntary wheel running for 49-53 days had significantly higher oxygen consumption during oxidative phosphorylation compared to sedentary caged rats. Additionally, soleus muscle homogenates from exercise trained rats had significantly greater muscle buffering capacity, and significantly greater enzyme activities of citrate

synthase, lactate dehydrogenase and mitochondrial electron transport complex enzymes in comparison to sedentary rats. In contrast to the first study, pH did not affect oxygen consumption when mitochondria were maximally phosphorylating, across a greater pH range (7.1-6.2). Lowering pH significantly decreased ROS emission from permeabilised fibres in all non-phosphorylating states, except in the case of leak respiration with both complex I and II substrate. In this state, ROS emission significantly declined as pH decreased in sedentary rats, but was not significantly changed by lowering pH in rats that were exercised. Rats that were exercised also showed significantly greater ROS emission in all activity states measured. However, when ROS emission was normalised to oxygen consumption, pH had no significant effect on the proportion of oxygen converted to ROS, although the proportion of ROS emission through complex I is significantly higher from exercised rats. Decreasing pH significantly depressed activity of complex I, II and III, and significantly increased the activity of complex IV in both exercised and sedentary groups. However, activity of complex II and III was less sensitive to pH in exercised rats. Additionally, enzyme activities of all ETS complexes were significantly higher in exercised rats. This shows that acidosis depresses ROS release from mitochondria, either through depressed ROS emission or increased ROS consumption. However, exercise prevented this decrease in a complex I and II supported leak state, possibly mediated by a smaller effect of pH on complex III activity.

The third study investigated whether acidosis could play a direct role in modifying the intracellular signalling response to muscle contraction. Rat hind limbs were isolated and perfused with modified Krebs-Henseleit buffer at pH 7.5 and pH 7.0, with subgroups of perfusion only, and perfusion with electrically-stimulated muscle contraction, at each pH. Force production, muscle pH, and perfusate lactate concentration were determined, as well as protein expression of total and phosphorylated forms of: AMPK, p38 MAPK, ERK1, ERK2, ATF2, ACC, and phosphorylated HDAC 4, 5 and 7. Decreasing perfusion pH resulted in significantly greater ratio of phosphorylation of AMPK and ERK1, but significantly lower phosphorylation of ATF2. Stimulated contraction resulted in significantly greater phosphorylation of AMPK, ERK1, ERK2, HDAC4 and HDAC5/7. There was no significant effect of either pH or stimulated contraction on phosphorylation of p38 MAPK or ACC. There was also no

significant effect of perfusion pH on contraction force or perfusate lactate concentration, while decreasing perfusion pH resulted in significantly decreased muscle pH. Stimulating contraction significantly increased perfusate lactate concentration, and significantly decreased muscle pH. These results show that a lower perfusion pH resulted in greater AMPK and ERK1 phosphorylation, and that this result is not explained by a difference in contraction force or lactate production. Furthermore, despite differences in phosphorylation of upstream signals (AMPK, ERK1, ERK2), downstream targets (ACC, ATF2 and HDAC 4, 5 and 7) did not show results consistent with the expected effects of upstream signals. This suggests that pH may affect early signalling response but also has some yet-to-be-determined effect on the propagation of this response.

Collectively, the results in this thesis provide evidence that decreasing pH does not inhibit mitochondrial respiration during oxidative phosphorylation, but increases mitochondrial affinity for ADP. Decreasing pH also lowers ROS emission from mitochondria and xanthine oxidase, while also potentially aiding ROS disposal through increased glutathione resynthesis. The depression of ROS emission at lower pH is not seen in rats that have been voluntarily running, and this is possibly mediated by a smaller effect of pH on complex III activity. Finally, decreasing pH up-regulates initial signalling in some of the pathways associated with contraction-induced mitochondrial biogenesis in skeletal muscle, however, this effect is not transferred to targets downstream of these initial signal transducers, suggesting other level(s) of regulation in controlling the effect of pH on mitochondrial biogenesis.

Declaration

I, Christopher Paul Hedges, declare that the PhD thesis entitled *the effects of physiological acidosis on skeletal muscle mitochondrial function, ROS balance and intracellular signalling* is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature:  Date: 21/03/2017

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Contents

Abbreviations

[ ]	Concentration
µg	Microgram
µL	Microlitre
µM	Micromole
31P-NMRS	Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy
95% CI	95% confidence interval
ACC	Acetyl coenzyme-A carboxylase
ADP	Adenosine Diphosphate
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate-activated Protein Kinase
ANOVA	Analysis of variance
ANT	Adenine nucleotide transporter
AO	Antioxidant
ATF2	Activating transcription factor 2
ATP	Adenosine Triphosphate
ATPase	Adenosine-5'-triphosphatase
CaMK (II/IV)	Ca^{2+} calmodulin-dependent protein kinase (II/IV)
CaMKK	Ca^{2+} calmodulin-dependent protein kinase kinase
cAMP	Adenosine 3´,5´-cyclic monophosphate
CaN	Calcineurin
CAT	Catalase
CCCP	Carbonyl cyanide-m-chlorophenyl hydrazone
CK	Creatine kinase
CO_2	Carbon dioxide
CoA	Coenzyme-A
COX IV	Cytochrome c oxidase/ complex IV
CREB	Cyclic AMP response element binding protein

CV	Coefficient of variation
d.w.	Dry weight
DHAP	Dihydroxyacetone phosphate
DHPR	dihydropyridine receptor
DNA	Deoxyribose nucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol tetra-acetic acid
ERK	Extracellular signal-regulated kinase
ETFDH	Electron transfer flavoprotein dehydrogenase
ETS	Electron transport system
FAD	Flavin Adenine Dinucleotide
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone
Fe-S	Iron-sulfur heme protein
FMN	Flavin Mononucleotide
G3P	Glycerol-3-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GCN5	General control of amino acid synthesis protein 5
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GR	Glutathione reductase
GSSG	Glutathione disulfide
GTPase	Guanosine-5'-triphosphatase
H^+	Hydrogen ion
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
ICDH	Isocitrate dehydrogenase
IMP	Inosine monophosphate
IMS	Intermembrane space
$K_{Mapp\text{-}ADP}$	Apparent Michaelis-Menten constant for ADP

KO	Knockout (gene modification)
La	Lactate
LDH	Lactate dehydrogenase
LKB1	Liver kinase b1
MDA	Malondialdehyde
MEF2	Myocyte enhancing factor 2
MEK	Mitogen/extracellular signal kinase
mGPDH	Mitochondrial glycerol-3-phosphate dehydrogenase
mM	Millimole
MOPS	3-(N-Morpholino) propanesulfonic acid
mRNA	Messenger ribose nucleic acid
Mt	Mitochondrial
mtEMP	Mitochondrially-encoded mitochondrial protein
Na^+	Sodium ion
NAC	N-acetyl cysteine
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NFAT	Nuclear factor of activated T-cells
NNT	Nicotinamide Nucleotide Transhydrogenase
NOS	Nitric Oxide Synthase
NOX	NADPH oxidase
NRF	Nuclear respiratory factor
NuEMP	Nuclear-encoded mitochondrial protein
NUGEMP	Nuclear genes encoding mitochondrial proteins
Oxphos	Oxidative phosphorylation
p38 MAPK	p38 mitogen activated protein kinase
PAGE	Polyacrylamide gel electrophoresis
PCr	Phosphocreatine
PDH	Pyruvate dehydrogenase
PGC-1α	Peroxisome proliferator gamma coactivator -1 alpha

PLA2	Phospholipase-a2
POLRMT	Mitochondrial RNA polymerase
PPAR	Peroxisome proliferator-activated receptor
PVDF	polyvinylidene fluoride
RET	Reverse electron transfer
RIPA	Radioimmunoprecipitation assay
RM	Repeated measures
RNS	Reactive Nitrogen Species
ROS	Reactive oxygen species
SD	Standard deviation
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SERCA	Sarcoplasmic/endoplasmic reticulum calcium-ATPase
SIRT	Sirtuin
SOD	Superoxide Dismutase
SR	Sarcoplasmic reticulum
TBARS	Thiobarbituric acid reactive substances
TBST	Tris-buffered saline with Tween-20
Tfam	Mitochondrial transcription factor a
Tfb1m	Mitochondrial transcription factor b1
Tfb2m	Mitochondrial transcription factor b2
TTF	Time to fatigue
TTFA	Thenoyl trifluoroacetone
U	Standard enzyme activity unit (rate of 1 μM substrate per minute)
UCP	Uncoupling protein
v/v	Volume per volume
Vit	Vitamin
V_{max}	Maximum enzyme system catalytic rate
VO_2	Rate of oxygen consumption

w.w.	Wet weight
w/v	Weight per volume
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase
A	Alpha
β_m	Muscle buffering capacity
Γ	Gamma
Δ	Change in
Σ	Sum
Ψ	Mitochondrial membrane potential

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Publications and presentations.

Publications arising during candidature:

Wyckelsma, V.L., Levinger, I., Murphy, R.M., Petersen, A.C., Perry, B.D., Hedges, C.P., Anderson, M.J., McKenna, M.J. *Intense interval training in healthy older adults increases skeletal muscle [^{3}H]ouabain binding site content elevates and Na^+,K^+-ATPase α_2 isoform abundance in Type II fibers*. Physiological Reports, (Accepted for publication).

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Chapter 1: Introduction

Mitochondria are essential to production of ATP within muscle cells. This thesis further investigated the role that acidosis, such as that which occurs during high-intensity exercise, may play in regulating mitochondrial function and adaptation.

Chapter two of this thesis comprises a literature review, divided into three sections. The first section outlines skeletal muscle energy metabolism, with a particular focus on the role of mitochondria in energy provision at rest and during exercise. Furthermore, the change in pH that can occur following high-intensity exercise (exercise-induced acidosis) is established. This provides context on how mitochondria work, and what parameters are of use in assessing mitochondrial function, and how these may change with alterations in cellular pH. The second section of chapter two focuses on reactive oxygen species, unstable oxygen-centred molecules that are naturally formed within cells. This section focuses on skeletal muscle ROS, as ROS metabolism is also altered in exercising muscle. Different sites of ROS production within skeletal muscle are discussed, and also different mechanisms of ROS disposal. The balance between ROS production and ROS disposal is central to the role ROS may have in inducing beneficial or deleterious effects on cells. This section also discusses how ROS production and disposal may change in the context of acidosis. The third section of chapter two examines some of the established signalling pathways that result in mitochondrial adaptation in muscle. Though there is interaction between these pathways, this section is broadly divided into categories, based around initial signalling molecules. This provides context on how mitochondria adapt as a result of exercise-induced metabolic stress.

Chapter three addresses one of the inconsistencies in studies investigating the effect of acidosis on some skeletal muscle mitochondrial function, ADP concentration. Specifically, soleus mitochondrial oxygen consumption was measured while ADP was titrated to determine ADP sensitivity and maximal ADP-stimulated mitochondrial respiration. Additionally, activities of various enzymes involved in ROS production and disposal in muscle were measured. Both oxygen consumption and enzyme activity

measurements were made across a physiological pH range, to determine the effect of pH on mitochondrial function and ROS balance.

Chapter four addresses whether exercise can alter the effects of pH on muscle mitochondrial function. Rats were given access to running wheels for a period of 7-8 weeks, and compared to sedentary rats. Soleus mitochondrial oxygen consumption, ROS emission, citrate synthase activity, lactate dehydrogenase activity, and muscle buffering capacity were measured. Following initial findings, activities of individual electron transport system complexes were determined. These measures were made across a slightly greater pH range than was used in chapter three, to determine whether exercise could alter the effect of changing pH on mitochondrial function.

Chapter five addresses whether acidosis may have a direct role in altering intracellular signalling pathways for mitochondrial adaptation. Phosphorylation of various signal transducing kinases and transcription regulators was determined by western blot, following hindlimb perfusion, or perfusion with electrical stimulation. This was measured in addition to muscle pH, force production, and lactate production. Perfusion media pH was altered, in order to determine the role pH may have in regulating signalling pathways.

The studies in chapters three, four, and five all provide novel data on the role pH has in regulating mitochondrial function and adaptation. However, they also raise further questions, and have limitations. The collective implications of the findings in this thesis, as well as potential extensions to these experiments, are presented in chapter six.

Chapter 2: Literature Review.

2.1 Skeletal muscle energy metabolism at rest and during exercise

2.1.1. Overview

Skeletal muscle contraction requires energy in the form of ATP, proportional to the intensity at which the exercise is performed. There are varying accounts as to the amount of ATP stored in resting muscle cells, though consensus appears to be between 20-25 $mM.kg^{-1}$ dry weight (d.w.) [222, 272, 418, 483]. During maximal stimulated activity in skinned muscle fibres, actomyosin ATPase activity consumes ATP at a rate of 0.1-0.4 $mM.L^{-1}.s^{-1}$ [492], depending on muscle fibre type. These values have been used to estimate an ATP consumption rate of 6.5-26.6 $mM.s^{-1}.kg^{-1}$ d.w. during maximal exercise [449]. At this rate, muscle would deplete ATP stores entirely in under 5 s, depending on muscle fibre type composition. However, following 90 s of dynamic knee extension exercise Krustrup et al. [272] found no significant decrease in muscle ATP content. Similarly, Hultman and Sjoholm [222] found only a slight (c.a. 5 $mM.kg^{-1}$ d.w.) decrease in muscle ATP following 50 s of electrically-stimulated muscle contraction. Finally, following 25 s of "all-out" maximal intensity cycling, average muscle ATP content only decreased by ~50% (from an average of 22.6 to 10.5 $mM.kg^{-1}$ d.w.) [253]. Collectively, these results indicate that muscle ATP levels are highly conserved during exercise, despite the high capacity of muscle ATP turnover. In order to achieve this, muscle has a variety of mechanisms for maintaining cellular ATP stores; namely the phosphagen system, glycolytic metabolism, and oxidative phosphorylation [410]. The capacity and rate of these systems to regenerate ATP are varied, and as such they are used to different extents depending on the intensity and duration of muscle contraction. In this section the key concepts underpinning these energy systems will be described, as well as how they relate to exercise-induced acidosis.

2.1.2. The phosphagen energy system

The most rapid system for regeneration of ATP is phosphocreatine (PCr), via a system known as the PCr shuttle. During contraction, myosin creatine kinase (myoCK) breaks down PCr and the phosphate group binds to ADP to form ATP and creatine. As this reaction consists of a single enzyme catalysing a single reaction, it is extremely quick to regenerate ATP. Muscle stores of PCr are larger

than those of ATP, around 75 $mM.kg^{-1}$ d.w. [222, 272, 418, 483]. Following intense, short-duration exercise, depletion of muscle PCr is much greater than that of ATP [24, 273, 449]. This indicates the importance of creatine phosphate derived energy for short-term bursts of activity.

An additional aspect of the phosphagen energy system is the adenylate kinase reaction. Hydrolysis of ATP results in the formation of ADP, and adenylate kinase can cleave a further phosphate from one ADP and add it to a second ADP molecule, to form one ATP and one AMP molecule [595]. AMP deaminase further removes an amine group from AMP to form inosine monophosphate (IMP).

2.1.3 Glycolysis

As PCr concentration is progressively depleted following multiple sprint efforts, ATP production is compensated by the increased utilisation of glycolysis, resulting in the two systems combining to maintain ATP resynthesis [158]. Glycolysis is a 10-step process, with each step regulated by a specific enzyme, and results in the production of either 2 or 3 ATP molecules, two pyruvate molecules, and two hydrogen ions (H^+) [243]. The process can be separated into an "investment" phase and a "payout" phase. In the investment phase, ATP must be consumed to break down the initial substrate, while in the payoff phase ATP is produced by substrate level phosphorylation. In strict terms, glycolysis is the breakdown of glucose and glycogenolysis is the breakdown of glycogen; however, they are commonly both referred to as glycolysis. Glycolysis from glucose requires one more ATP during the investment phase; as such glycolysis results in the net production of 2 ATP, and glycogenolysis in the production of 3 ATP.

Glycolysis directly produces a limited amount of ATP. However, it also results in the production of pyruvate and reduced nicotinamide adenine dinucleotide (NADH), both substrates for oxidative phosphorylation in mitochondria. Paradoxically, muscles rich in glycolytic enzymes have decreased mitochondrial densities [604] meaning less pyruvate is able to be oxidised than in a more oxidative muscle. As glycolysis is an important source of rapid ATP generation during high-intensity exercise [427, 530], the enzyme lactate dehydrogenase (LDH) functions to prevent these glycolytic end products building up and inhibiting the process itself. The role of LDH is to convert pyruvate to lactate,

with concomitant oxidation of NADH + H^+ to NAD+, in times when pyruvate flux exceeds pyruvate oxidation. This explains the high muscle lactate concentrations (~13-fold increase from rest) that are associated with repeat sprint exercise [104]. LDH also catalyses the reverse reaction, meaning that lactate can be transported to muscle where more abundant oxygen supply allows the reconversion of pyruvate and oxidation through the Krebs' cycle. Systemically-produced lactate may also be an important cerebral energy source [529], or can be transported to the liver for gluconeogenesis via the Cori cycle, though the exact role of lactate in whole-body metabolism is still a subject of debate [157].

2.1.4 Mitochondrial metabolism – the electron transport system

Ultimately, cellular homeostasis is dependent on ATP derived from oxidative phosphorylation in mitochondria. Production of ATP by mitochondria is dependent on the electron transport system (ETS) and oxidative phosphorylation (oxphos) complexes. The traditional ETS consists of a series of proteins bound to the inner mitochondrial membrane [472]. Nominally labelled complex I, II, III and IV, these proteins catalyse the transfer of electrons between several intermediaries, a process that will be further discussed in the following sections. In brief, complexes I and II, mitochondrial glycerophosphate dehydrogenase, and electron transferring flavoprotein dehydrogenase all reduce the electron shuttle ubiquinone to ubiquinol. Ubiquinol is then oxidised by complex III, which reduces cytochrome c, which in turn transfers electrons to complex IV. Complex IV utilises electrons to bind and reduce oxygen and protons (H^+) to form molecular water (H_2O). During this process, each time an electron is transferred it transitions down energy states, and subsequently releases energy. This energy released is used by complexes I, III and IV to actively transport H^+ into the intermembrane space against the H^+ concentration gradient [472]. The net result of this is a greater H^+ concentration in the intermembrane space relative to the mitochondrial matrix.

2.1.4.1 Complex I

Mammalian complex I, also known as NADH-ubiquinone oxidoreductase [79], is arranged in an 'L' shape, comprising a hydrophobic arm bound to the inner mitochondrial membrane, and a hydrophilic arm protruding perpendicularly into the mitochondrial matrix [603]. Complex I was thought to consist of 45 individual subunits [70, 71]; however, recent research indicates a subunit originally

attributed to complex I may in fact be part of complex IV [21], and complex I may instead be made of 44 subunits [26, 542]. At complex I electrons are transferred from the reduced NADH to a flavin mononucleotide (FMN) and subsequently passed through 7 iron-sulfur (Fe-S) clusters to ubiquinone [603]. Concurrently, complex I pumps H^+ from the mitochondrial matrix into the mitochondrial intermembrane space. With the two electron reduction of one ubiquinone molecule to ubiquinol (QH_2) from one NADH, an estimated four H^+ are concomitantly translocated across the inner mitochondrial membrane [79, 420].

Complex I can be in part regulated by supply of NADH, which is formed by several dehydrogenases in the citric acid cycle [160, 269]. NADH can also be generated by the nicotinamide nucleotide transhydrogenase (NNT) enzyme, which reversibly transfers electrons between NADP+ and NAD+, depending on the ratio of each [210]. NNT can also modulate the membrane potential, as H^+ is translocated from the matrix to the intermembrane space when NADPH is used to reduce NAD+, forming NADH (and NADP+) [210]. Complex I can be inhibited by a range of organic compounds (such as rotenone, piericidin A, and capsaicinoids), which act by blocking the ubiquinone binding site, and thus inhibiting the reduction of ubiquinone to ubiquinol [99, 373]. This has implications for mitochondrial ROS production, which is further discussed in section 2.3 of this review.

2.1.4.2 Complex II

Complex II, also known as succinate dehydrogenase (SDH), has a dual role as an enzyme in the Krebs' cycle [269], and a catalyst for the transfer of electrons. It consists of four subunits, two of which (SdhC and SdhD) are hydrophobic and form a dimer which anchors SDH to the inner mitochondrial membrane, with the remaining two hydrophilic subunits (SdhA and SdhB) in the mitochondrial matrix [72]. Succinate is oxidised to fumarate by SdhA, and electrons are transferred to a flavin adenine nucleotide (FAD) cofactor, reducing it to $FADH_2$. SdhB then transfers electrons from $FADH_2$ through 3 Fe-S proteins to the SdhC/SdhD dimer, where ubiquinone is bound, reduced to ubiquinol, and released [72, 312]. SDH does not cross the inner mitochondrial membrane, and as such does not directly contribute to the membrane H^+ gradient. Other than the structural limitation of not crossing the phospholipid bilayer, it is possible that the reason complex II does not also act as an H^+

pump is that the energy released from electron transfer in complex II is not sufficient to translocate H^+ [472]. However, complex II indirectly contributes to H^+ flux through subsequent complexes by reducing ubiquinone. SDH can be inhibited by compounds structurally similar to succinate at the succinate binding site, such as malonate, oxaloacetate and beta-nitropropionic acid [109, 220, 400, 573]. In the case of malonate and oxaloacetate, inhibition appears to be competitive and can be reversed by an increase in succinate concentration [266, 573].

2.1.4.3 Complex III

Complex III (CoQ-cytochrome c oxidoreductase) consists of 11 subunits [230] and catalyses the transfer of electrons from ubiquinol molecules to cytochrome c [419]. This occurs via the Q-cycle, in which the two electrons from a single ubiquinol follow different paths. Ubiquinol first binds to a quinol binding site on the intermembrane space (IMS) side of the inner membrane, the Qo-site. One electron reduces an Fe-S protein, and then is used to reduce cytochrome-c (cyt-c). The other electron bound to the quinol at the Qo-site is passed down a cytochrome-b chain, reducing low-potential cytochrome-b (cyt-b_L), then high-potential cytochrome-b (cyt-b_H), before reducing a second quinol bound at the Qi-site, on the matrix side of the inner membrane. As this results in an incomplete reduction (in the case of ubiquinone bound to the Qi-site), a partially-reduced ubisemiquinone is formed, which is bound until it is further reduced to ubiquinol by a second turn of the Q-cycle. In this reaction another four H^+ are released into the IMS, and two H^+ are taken up from the mitochondrial matrix [94]. Thus the entire reaction sequence can be summarised as follows:

$$2QH_2 + 2\text{cyt-c}_{(ox)} + 2H^+_M \longrightarrow QH_2 + Q + 2\text{cyt-c}_{(red)} + 4H^+_{IMS}$$

This reaction mechanism appears at first to be inefficient due to the recycling of a ubiquinol, but the end effect allows complex III to have a greater contribution to the H^+ gradient than if both electrons from a single ubiquinol were used to reduce cyt-c. There are many known inhibitors of complex III, which appear to target either the Qi-site or Qo-site [45, 482, 520]. Many are quinone analogues, but in experimental comparisons the most effective inhibitor appears to be the Qi-site inhibitor antimycin, closely followed by the Qo-site inhibitor myxothiazol [520].

2.1.4.4 Complex IV

The terminal step in the ETS is complex IV, cytochrome c oxidase. Complex IV has 13 identified subunits [335], with debate over a possible 14th subunit originally categorised as a subunit of complex I [21, 249]. Through a series of copper-centred intermediates and cytochrome a, complex IV ultimately transfers four electrons from four molecules of cytochrome c, and binds one oxygen molecule (O_2) at cytochrome a [592]. The net yield of the reaction produces two water molecules for each O_2 consumed, with an average of one H^+ pumped per electron transferred [58], totalling four H^+ per oxygen molecule consumed. Cyanide (CN^-) and azide (N_3^-) bind to heme groups of cytochrome a, preventing binding of oxygen and thereby inhibiting respiration. *In vivo* regulation of complex IV can occur by nitrosylation of heme groups by nitric oxide (NO), or allosteric inhibition by ATP [15, 16].

2.1.4.5 Other electron donors and interactions of the electron transfer system

In addition to electron supply by complexes I and II, ubiquinone can also be reduced from other sources. Electron transfer flavoproteins (ETF) reduce ubiquinone via a family of electron transfer flavoprotein dehydrogenases (ETFDH) [142, 596]. This forms part of electron supply through beta oxidation of lipids [435]. Additionally, ubiquinone can be reduced by mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH), which transfers electrons from glycerol-3-phosphate (G3P), using FAD/$FADH_2$ as a cofactor [347]. G3P can be formed from the glycerol backbone of a triglyceride molecule, again part of lipid metabolism, and GPDH has been called the 'crossroad' between lipid and carbohydrate metabolism as the product of the reaction, dihydroxyacetone phosphate (DHAP), is also a glycolytic intermediate [347]. In mammalian tissues, brown adipose tissue possesses the highest measured expression and activity of mGPDH; however, skeletal muscle has higher expression and activity than other tissues [267, 414].

In this way, the total amount of ubiquinol available to complex III is increased by multiple sources acting in concert with each other to augment the quinone pool. It should also be noted that the traditional view of the respiratory complexes as a random assortment of membrane bound proteins, a model which relies on a random collision model of activity, has been challenged by evidence of respiratory supercomplexes (reviewed in [463-465]). While this is largely beyond the scope of this

review, the key point is that this theory proposes that individual complexes aggregate to form functional units (respirasomes) that could comprise a more efficient system than a random collision model.

2.1.5 Mitochondrial metabolism – oxidative phosphorylation

The ETS generates an electrochemical H^+ gradient across the inner mitochondrial membrane, with a higher H^+ concentration in the intermembrane space than in the mitochondrial matrix. These H^+ are then able to flow through ATP synthase back to the mitochondrial matrix, helping to drive the formation of ATP [137]. In addition to passing through ATP synthase, H^+ can also 'leak' back into the matrix via other protein channels and/or carriers, which does not result in ATP synthesis. The ATP synthase unit has two components; F_0 is a membrane-spanning ion channel that anchors the unit to the mitochondrial membrane, and allows the movement of H^+ back into the mitochondrial matrix, and F_1 is a multi-unit structure that catalyses the formation of ATP from ADP and inorganic phosphate (P_i). The structure of F_1F_0-ATP synthase, and the process by which ATP formation happens, has been previously elucidated [137, 485]. In brief, the translocation of H^+ through F_0 provides the energy required to bind ADP and P_i in F_1. In this manner, oxygen is consumed by the ETS and the resulting energy from the H^+ gradient used to phosphorylate ADP, thus the term oxidative phosphorylation. When generation of a membrane potential is impaired in hypoxia, the F_1F_0-ATP synthase can reverse, consuming ATP and translocating H^+ into the mitochondrial matrix to maintain membrane potential [84].

Once ATP has been produced it must be transported from the mitochondrial matrix to the cytosol where it can then eventually be used to provide energy to other cellular processes. The movement of ATP across the membrane occurs via the adenine nucleotide transporter (ANT) [28, 369]. This protein complex exports one molecule of ATP to the intermembrane space in exchange for one molecule of ADP into the matrix [128]. However, the rate of this transport may vary between tissue types, which have different isoforms of ANT [85]. From the intermembrane space, ATP is used to resynthesise phosphocreatine by mitochondrial creatine kinase (mtCK), which is coupled to ANT [292, 454]. The result of this is a tight and constant cycling of ADP and ATP through ANT in myocytes. In

addition to supplying ADP for ATP synthase activity, ANT may itself be a H^+ channel in mitochondria [54].

2.1.6 Proton leak and uncoupling

ATP synthase is not the only channel for H^+ to pass back from the intermembrane space into the mitochondrial matrix. As mentioned previously, the nictotinamide nucleotide transhydrogenase (NNT) can either contribute to, or dissipate, the mitochondrial membrane potential, while simultaneously reducing either NAD^+ or $NADP^+$ (respectively) [114]. While this reaction appears to be reversible *in vitro*, when a membrane potential is generated (as would be the case *in vivo*), NNT strongly shifts towards dissipation of membrane potential and production of NADPH [438].

Another potential avenue for management of membrane potential are the uncoupling proteins (UCP). UCP1, also known as thermogenin, was first identified in mitochondria from brown adipose tissue [359]. Brown adipose tissue contributes to heat production through adaptive thermogenesis [360], mediated by UCP1 dissipation of mitochondrial membrane potential [354], meaning that the potential energy of the membrane potential is converted to heat. Homologues of UCP1, UCP2 and UCP3, have been found in both human and animal tissue [49, 139, 155, 537]. UCP2 shows varied tissue expression, seeming to localise to lung and skeletal muscle tissue in humans, but being more widely expressed in mouse [139]. UCP3 expression is more limited, being expressed in skeletal muscle in human tissue, and skeletal muscle and brown adipose tissue in animals [49, 537].

While it is clear that UCP1 can influence membrane potential, the role of UCP2 and 3 is less clear. In response to a 24-hour fast, skeletal muscle UCP2 and UCP3 mRNA content, and UCP3 protein expression, all increase in rats; but without any change in mitochondrial H^+ leak [62]. However, when UCP3 is overexpressed in transgenic mice H^+ leak is significantly increased in comparison to wild-type controls, but no difference in mitochondrial oxygen consumption is seen in the presence of ETS substrate alone [63]. To further cloud the role of UCP3, no differences are seen in either oxygen consumption or H^+ leak when UCP3-knockout mice are compared to wild-type littermate controls [63]. Indeed Cadenas et al. [62] summarise many studies that display unexpected results if UCP2 and UCP3

do indeed contribute to H^+ leak, and elegantly conclude "Clever secondary hypotheses may explain the apparent anomaly, but the simplest explanation is that UCP2 and UCP3 do not catalyse the proton leak" (p.259 [62]).

Despite inconsistent evidence of their role in regulating mitochondrial membrane potential, UCP2 and UCP3 expression is clearly influenced by exercise. Endurance-trained humans show a decrease in mRNA expression of two isoforms of UCP3, and a trend towards decreased UCP2 mRNA expression, when compared to untrained controls [471]. In humans, UCP3 protein expression is also fibre-type specific, with the highest expression in fast-glycolytic (type IIx/IIb) fibres, followed by fast-oxidative (type IIa) fibres, and the lowest expression in slow oxidative (type I) fibres [201, 434]. This finding is consistent in both trained and untrained individuals, though trained individuals express less UCP3 in each fibre type compared to untrained [434]. This is a curious finding given that type IIx fibres have the lowest mitochondrial density, and indicates that UCP3 expression is inversely related to mitochondrial density. If UCP3 does not have a role in regulating membrane potential, but UCP3 expression can be altered by training, this suggests it has some other role in regulating mitochondrial function. It has been proposed that this role may be related to substrate metabolism, as UCP3 appears to act as a channel for export of fatty acids from mitochondria, and may prevent deleterious effects of substrate accumulation [47, 302, 469, 470]

2.1.7 States of mitochondrial function

Mitochondrial function is transiently regulated by substrate supply. Chance & Williams [75] proposed five different states of mitochondrial function based on the observation of changes in oxygen consumption with different limiting factors, as detailed below:

Table 2.1: States of mitochondrial function – from Chance & Williams [75].

	State 1	**State 2**	**State 3**	**State 4**	**State 5**
Characteristics	Aerobic	Aerobic	Aerobic	Aerobic	Anaerobic
ADP level	Low	High	High	Low	High
Substrate level	Low-endogenous	Low-approaching 0	High	High	High
Respiration rate	Slow	Slow	Fast	Slow	None
Rate-limiting component	Phosphate acceptor (ADP)	Substrate	Respiratory chain	Phosphate acceptor (ADP)	Oxygen

By these definitions, mitochondria are transiently active between state 3 and state 2/4 respiration *in vivo*. Entry of pyruvate into mitochondria is regulated by the pyruvate dehydrogenase (PDH) enzyme complex (for review see [487]), which is inhibited by ATP, NADH, or acetyl-CoA, which are high in inactive muscle, and stimulated by calcium. During muscle contraction, calcium levels increase, and ATP, NADH and acetyl-CoA are consumed, so PDH activity is increased, in an intensity-dependent manner [218, 409]. Substrate usage is also intensity-dependent, with greater carbohydrate oxidation seen during high-intensity, short-term exercise, while low-intensity, longer-duration exercise results in greater lipid oxidation [427, 522], and lipid entry into mitochondria is increased by exercise [214]. Thus it is generally accepted that during exercise substrate and ADP supply is high (due to ATP turnover), and therefore state 3 best describes mitochondrial function. Resting mitochondrial function *in vivo* is harder to define by these terms, as it is not truly state 2 or state 4 as both substrate and ADP concentrations could be considered 'low' in relative terms. For the purpose of this thesis, respiration in the presence of substrates without ADP, or in the presence of substrate, ADP,

and an inhibitor of ATP synthase activity is termed ‘leak’ respiration. Respiration during phosphorylation in the presence of substrate and ADP is termed ‘oxphos’.

2.1.8 Measurement of mitochondrial function

In vivo mitochondrial function can be assessed with the use of phosphorus-31 nuclear magnetic resonance spectroscopy (31P-NMRS) to evaluate concentrations of phosphate groups (such as ATP, ADP, PCr) in skeletal muscle [105, 245, 279]. 31P-NMRS correlates well with measurement of oxygen consumption (Pearson’s r = 0.664 – 0.717 for correlation of phosphorylating respiration measured in isolated mitochondria with ATP synthesis rate measured by 31P-NMRS, [279]). However, *in vivo* measurement represents a double-edged sword – *in vivo* measurement reflects the actual physiological effect of an intervention, but at the expense of being able to make precise, well-controlled manipulations to the extracellular environment. To achieve greater control on an intervention, many studies have used either the aforementioned *in vitro* isolated mitochondria model to investigate mitochondrial function (for example [202, 247, 262, 413]) or the *in situ* method of permeabilised muscle fibres (for example [388, 389, 516, 552]). For extensive review and comparison of *in vivo*, *in situ* and *in vitro* methods see Kuznetsov et al. [275] and Lanza & Nair [280]. In brief, mitochondrial isolation involves homogenising the tissue and then differential centrifugation to firstly remove cellular debris and then to pellet out mitochondrial protein. Permeabilising muscle fibres involves careful separation of tissue and then incubation in a detergent (commonly saponin), which selectively permeabilises the cell membrane due to an affinity for cholesterol content [455]. Both the isolated mitochondria and permeabilised fibre approach allow manipulations of specific enzyme substrates and inhibitors to assess mitochondrial function. However, both models still possess distinct advantages and disadvantages, which have been well-reviewed and summarised by and Picard et al. [392]. Of primary concern with isolated mitochondria is the disruption of the cytoskeletal architecture, which may have important effects on the regulation of respiratory activity as many studies have noted [255, 256, 452, 453, 532]. Additionally Picard et al. [393] found that isolation procedures lead to compromised oxygen consumption and increased reactive oxygen species production in skeletal muscle mitochondria.

2.1.9 Exercise-induced acidosis

Intense exercise can result in decreases in both blood and muscle pH in humans [197, 221, 442, 446], though both the cause and role of acidosis in skeletal muscle remain very contentious issues [423, 424, 541]. At rest, muscle intracellular pH is close to 7.1, and can drop to 6.5-6.7 following fatiguing exercise [64, 440, 441, 443, 446]. Resting arterial and venous blood pH are both close to 7.4, though arterial pH is consistently higher than venous [441], and both arterial and venous blood pH can drop to around 7.0 following fatiguing exercise [442, 443]. As discussed in section 2.1.4 and 2.1.5, mitochondrial synthesis of ATP is dependent on a H^+ concentration gradient that is used to drive ATP synthase. This means that changes in pH have the potential to affect mitochondrial function. However, how changes in cytosolic pH affect mitochondrial pH is not clearly understood.

2.1.10 Acidosis and mitochondrial function

Different cellular compartments show varied pH under resting conditions, and mitochondrial matrix pH in cell lines has been estimated to be 7.7-8.0 with various fluorescent dyes [1, 23, 301]. Collapsing of the membrane potential with either FCCP or CCCP decreases matrix pH by 0.3 to 1.0 units to a minimum value of around 7.0 [1, 23, 301]. Similarly matrix pH in beef heart mitochondria is around 8.0 under resting conditions, and decreases an unspecified amount with CCCP, again when estimated using fluorescent dye [248]. Under exercising conditions, specific subcellular pH changes are as yet undetermined. However, there is evidence that acidosis may be detrimental to mitochondrial function.

The outer mitochondrial membrane has a relatively high permeability to molecules smaller than 3 kDa, due to the presence of mitochondrial porin [35]. While this should permit diffusion of H^+ between the cytosolic space and intermembrane space, targeted fluorescent proteins reveal a pH gradient between the cytosol, (pH ~7.6) and intermembrane space (pH ~6.9), and also between the intermembrane space and mitochondrial matrix (pH ~7.8) in cultured EVC-304 cells [399]. A higher cytosolic pH indicates significant partitioning of H^+ between cytosolic and intermembrane compartments, i.e. that the outer mitochondrial membrane can impede H^+ flux despite access for much larger molecules from the cytosol. The classic chemiosmotic coupling hypothesis proposed that a pH

gradient (ΔpH) of approximately 3.5 pH units was necessary for ATP synthesis in the absence of an ion driven membrane potential (Δψ) [339]. However, it was proposed that any combination of Δψ and ΔpH drives ATP synthesis, provided it met a necessary protonmotive force, estimated to be 210 mV. Later studies showed that a combination of ΔpH and Δψ is necessary to maximise ATP production in bacterial F-ATPase, but that once a ΔpH of 3.5 is reached no further increase in ATP synthesis rate occurs with increasing the pH gradient (Figure 2.1, originally figure 3 in Fischer & Graber [138]).

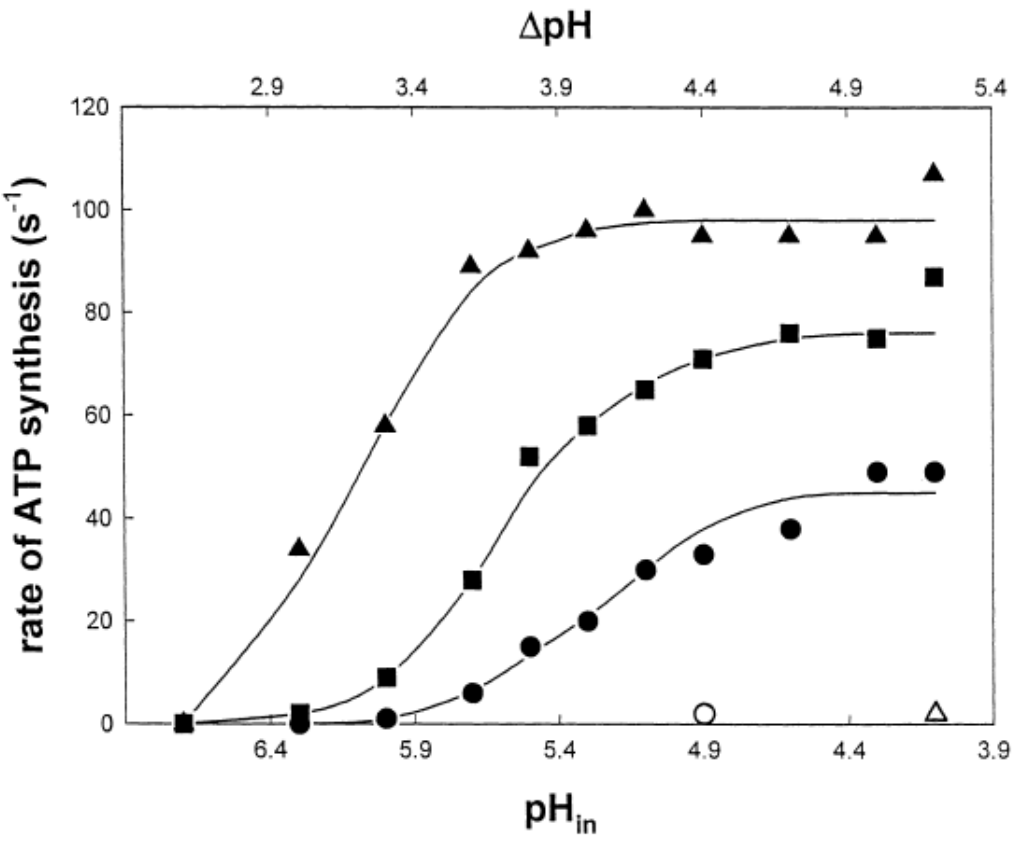


Figure 2.1: Rate of ATP synthesis as a function of ΔpH in *E. coli* ATPases.

Traces are K^+ Nernst potential of 80 mV (filled circles), 108 mV (filled squares) or 152 mV (filled triangles). Absence of Nernst potential due to equilibrated K+ minimises ATP production rate (open symbols). Reproduced from [138].

An aspect of the data in figure 2.1 that is not well discussed in the original work is that regardless of Nernst potential, the ATP synthesis rate is minimal when ΔpH is < ~2.0, indicating that some pH gradient is critical for ATP generation. The ΔpH required is also much larger than the measured ΔpH across the inner mitochondrial membrane. Strauss et al. [494] and Davies et al. [103]

provide structural insights of the organisation of the inner membrane, where the negatively charged phosphate heads of the lipid bilayer and cristae fold/ridge structures may concentrate and preserve a constant H^+ gradient in micro-domains. This means that a substantial gradient of pH occurs within the intermembrane space at the apex of the cristae, while an average pH of 6.9 [399] and may gradually rise to be similar to cytosolic pH in the space immediately adjacent to the outer membrane (figure 2.2). ATP synthase dimers appear to be arranged along these ridges [103, 494], meaning that the site of phosphorylation may be located in a region where pH gradient is enhanced, and may also be isolated from changes in cytosolic pH dynamics. This further raises the possibility that there may be some excess capacity of ATP synthase to use an even larger pH gradient.

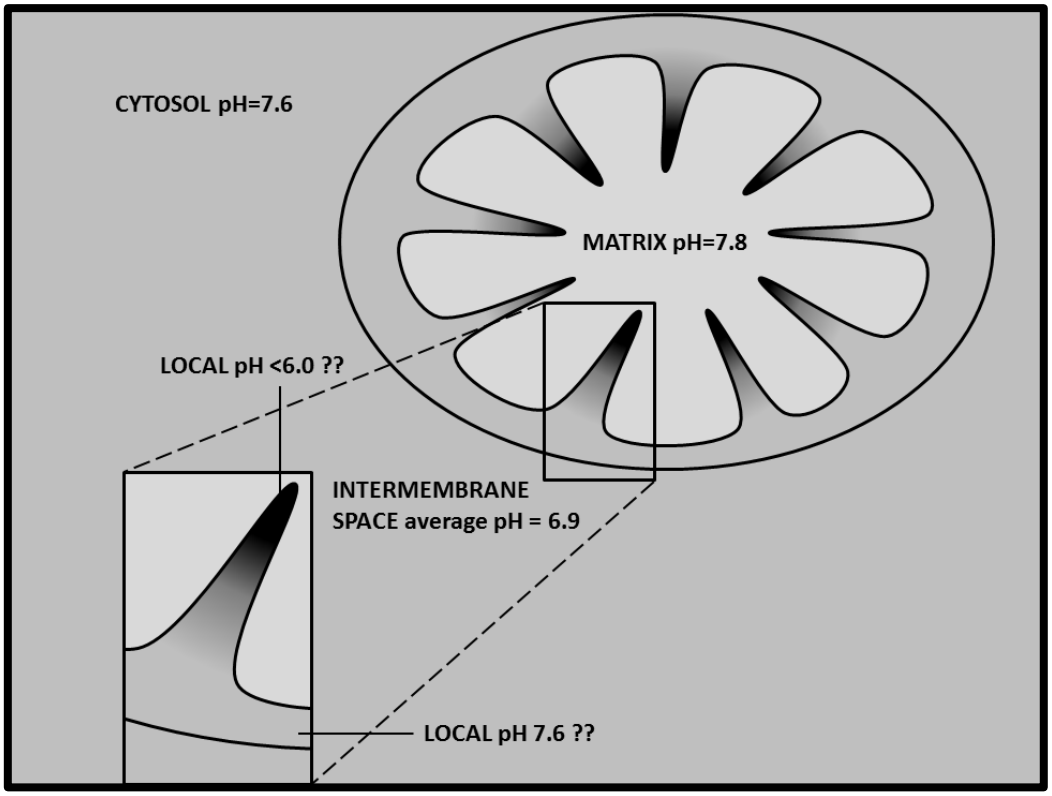


Figure 2.2: Theoretical graduation of proton concentration in mitochondrial inner membrane.

Local pH (darker shade = lower pH) in the intermembrane space may be concentrated to enhance pH gradient across the apices of cristae, the site of ATP synthase dimers.

Rat hearts show a depression in oxygen consumption when perfused with pH 6.8 buffer, an effect that is not seen when perfused with pH 7.4 buffer [496]. In this study, a variety of substrates (glucose, pyruvate and succinate) were tested to determine that this depression was not simply due to impairment of glycolysis by the pH-sensitive enzyme phosphofructokinase [315, 518], however, the finding of depressed oxygen consumption was consistent across all 3 substrates. Studies on skeletal muscle *in vivo* using 31P-NMRS have identified that resynthesis of PCr (a process dependent on mitochondria) is depressed when muscle becomes acidotic [187, 245]. Additionally, cultured cortical neurons undergo a pH-induced change in morphology in pH 6.5 media, indicating pH may somehow regulate mitochondrial structure, potentially leading to changes in mitochondrial function [259]. How pH may affect individual respiratory complexes remains unknown.

Measures of mitochondrial function *in situ* provide mixed results. When acidosis is induced while mitochondria are in a phosphorylating state, oxygen consumption rates are not depressed [517]. Given that mitochondria will certainly be phosphorylating in an active muscle, this would seem to contrast the idea that acidosis inhibits mitochondrial function *in vivo*. However, *in situ* measures are often made at supraphysiological substrate concentrations [447], and in a later study from the same group it was determined that at a more physiological concentration of ADP (100μM), acidosis did depress mitochondrial oxygen consumption [551]. Thus it appears that concentration of substrate may influence the effect of acidosis.

2.1.11 Summary

Mitochondria are essential for maintaining and/or recovering muscle ATP supply at rest and during/following exercise. Mitochondrial ATP synthesis requires a gradient of H^+ between the IMS and matrix. This suggests that modifying this H^+ gradient may affect mitochondrial function. Exercise-induced is one example of an alteration in muscle pH which may affect mitochondrial function, and evidence from skeletal muscle *in vivo* or isolated hearts *in vitro* suggest that acidosis depresses mitochondrial function. However, studies on mitochondria *in situ* do not consistently show and effect of acidosis on mitochondrial function, possibly due to varied concentrations of ADP. Thus it is necessary to investigate how kinetic parameters such as ADP affinity may be affected by acidosis.

2.2 Skeletal muscle reactive oxygen species

2.2.1 Overview

Cellular respiration is not a perfect system. Under normal conditions, a very small amount of the oxygen consumed by cells is reduced to superoxide ($O_2^{\bullet-}$). This occurs when an additional unpaired electron is bound to molecular oxygen. Superoxide is a strong oxidant and very unstable, with a half-life around 10^{-6} s [156, 406]. Due to the reactivity of superoxide, it is the "parent radical" for a number of other molecules such as hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\bullet-}$) and perhydroxyl (HO_2) radicals; collectively these are termed reactive oxygen species (ROS). Like their superoxide parent, ROS have strong oxidant properties and are extremely reactive; H_2O_2 has the longest half-life (thus is most stable) of around 10^{-5} s [156, 406].

In addition to ROS, there are reactive nitrogen species (RNS), a group of nitrogen-centered radicals, formed from the parent radical nitric oxide (NO). Superoxide and nitric oxide can react with each other to form peroxynitrite ($ONOO^-$) [263]. Peroxynitrite has been implicated as a mediator of skeletal muscle contractile dysfunction [116, 515], and does not appear to have a direct role in intracellular signalling. NO is formed by nitric oxide synthase (NOS) and serves several biological functions, most relevant to exercise being smooth muscle relaxation for blood vessel dilation; however, there is also evidence for involvement of NO in intracellular signalling networks for mitochondrial biogenesis [365, 366]. While RNS appear to be involved in at least basal mitochondrial biogenesis, it does not appear there is a role of RNS in exercise-induced mitochondrial biogenesis as both inhibition [547] and knockout [546] of NOS does not impair acute up-regulation of signalling pathways in skeletal muscle. This thesis focuses on the role of ROS and as such RNS will not be reviewed here. For reviews on the role of RNS in skeletal muscle see [363, 364, 405]. The following section of the review will focus on the production, regulation and effects of reactive oxygen species in the context of exercise.

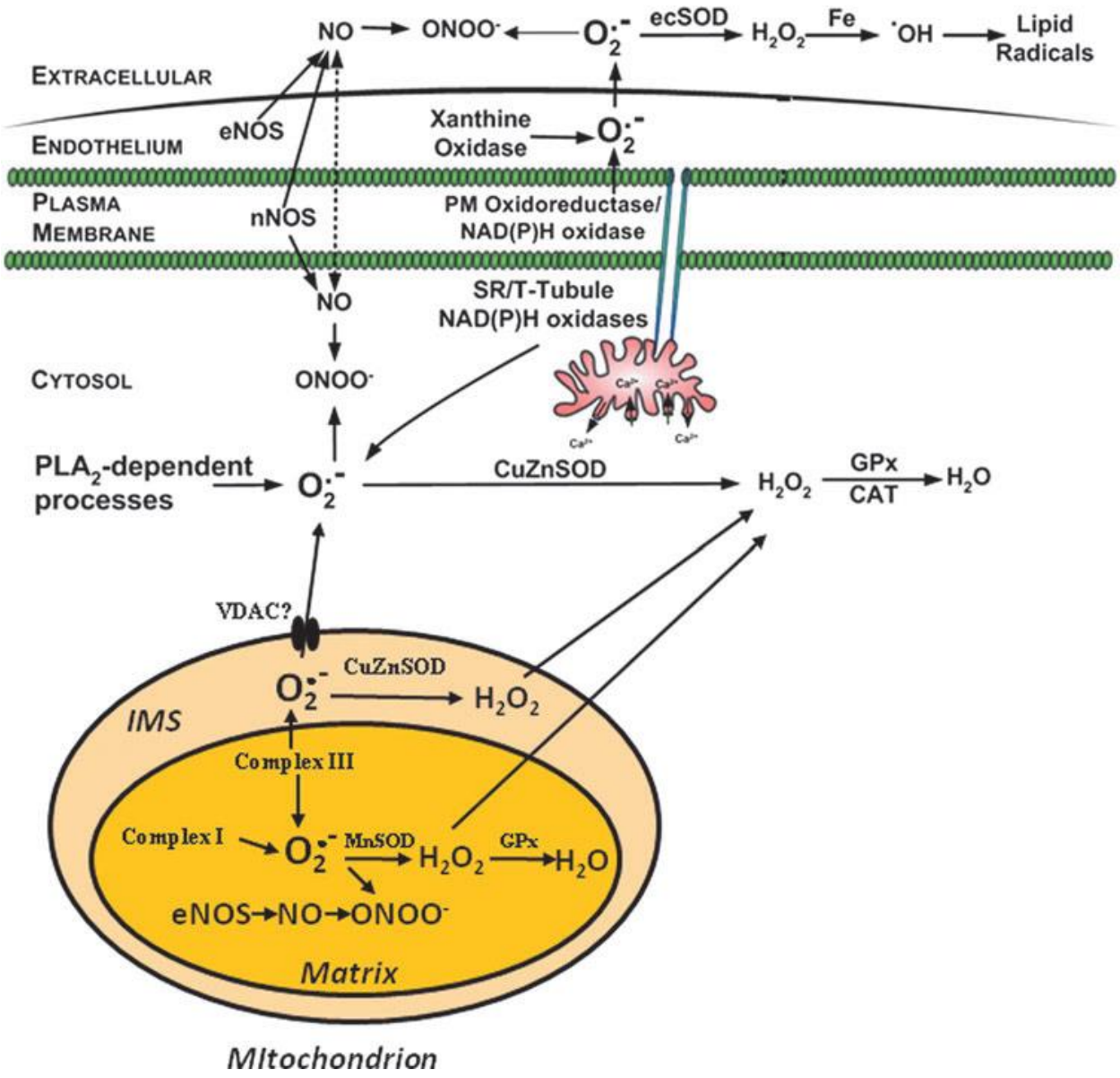


Figure 2.3: Sites of endogenous reactive species production in skeletal muscle.

Originally figure 1 in Jackson, M. J. (2011) [231].

Abbreviations: NO - Nitric Oxide; NOS - Nitric Oxide Synthase (e - Endothelial, n - Neuronal); $ONOO^{\bullet}$ - Peroxynitrite; $O_2^{\bullet-}$ - Superoxide; H_2O_2 - Hydrogen Peroxide; $^{\bullet}OH$ - Hydroxyl Radical; H_2O - Water; SOD - Superoxide Dismutase (Mn - Manganese, CuZn - Copper Zinc, ec - Extracellular); GPx - Glutathione Peroxidase; CAT - Catalase; PLA_2 - Phospholipase A_2; Fe - Iron; PM - Plasma Membrane; SR - Sarcoplasmic Reticulum; IMS - Intermembrane Space; VDAC - Voltage Dependent Anion Channel.

2.2.2 Sites of skeletal muscle ROS production

Production of ROS occurs from a number of oxidase enzymes within the cell, shown in figure 2.3. The primary sites of ROS production in skeletal muscle are thought to be mitochondrial electron transport complexes I and III, and cytosolic enzymes NADPH oxidase and xanthine oxidase [403, 405]. Boveris, Oshino and Chance [51] estimated that 5% of cellular oxygen uptake resulted in H_2O_2 formation; however, the majority of H_2O_2 production was attributed to enzymes external to mitochondria. Additionally, they estimated that approximately 1-2% of oxygen consumed during resting respiration (in the absence of ADP) was converted to H_2O_2 – an effect abolished during ADP-stimulated phosphorylating respiration.

2.2.2.1 Mitochondria

Like many other biological systems, the ETS operates on a dynamic equilibrium, and appears to obey Le Chatelier's principle [277, 474, 545]. When membrane potential is high, as is the case in state 4 respiration, the protonmotive force opposes the movement of H^+ from the matrix into the inter-membrane space. This in turn opposes the normal transitions of electrons through the ETS complexes. This can lead to a situation where oxygen interacts with complexes earlier in the system than complex IV, the usual site of oxygen reduction to water. This can result in the single electron reduction of oxygen, forming $O_2^{\bullet-}$. As such, increases in ROS production are noted when mitochondrial membrane potential is high [265]. However, there is extensive discussion in the literature as to the exact sites of mitochondrial ROS generation [14, 78, 296, 351, 356, 474, 490, 523].

Complex I is one known site of mitochondrial ROS production. Isolated complex I sub-particles generate increased ROS in the presence of excess NADH [503, 525]. Inhibition of complex I with rotenone causes an increase in ROS production [199, 207, 503, 525], which implicates a mechanism within complex I itself, as opposed to somewhere else in the ETS that is simply supplied electrons through complex I.

Complex III has also been implicated as a site of ROS production, as incubation of complex III sub-particles with succinate and succinate dehydrogenase leads to an increase in ROS production. This

is further increased with the addition of complex III inhibitor antimycin, and is abolished when the membrane potential is collapsed with an uncoupler (carbonyl cyanide *m*-chlorophenyl hydrazine, CCCP) [597]. Addition of either rotenone or complex II inhibitor thenoyltrifluoroacetone (TTFA) to rat heart mitochondria respiring with succinate alone decreases ROS production [199]. This indicates two things; firstly that succinate in some way causes an increase in complex I ROS production in the absence of complex I substrate, and secondly that complex II itself does not produce ROS, but supplies electrons to other sites of ROS production.

There are other sources of superoxide production in mitochondria, though their role in exercise-induced ROS production is unclear, and is not a focus of this thesis. Mitochondrial sources of superoxide in skeletal muscle not expounded upon in this thesis include dihydroorotate dehydrogenase (DHODH), and oxoacid dehydrogenase (OADH). Furthermore, there are other sources of superoxide in other tissues (such as monoamine oxidases, involved in neural tissues). DHODH is involved in pyrimidine synthesis, which is either depressed or unchanged during exercise [521], therefore unlikely to be contributing to exercise-induced ROS production. OADH is involved in metabolism of amino acids, which is typically low during exercise when sufficient carbohydrate or lipid substrate is present [417], thus again unlikely to be contributing to exercise-induced ROS production. Some enzymes of the Krebs' cycle can produce ROS (such as pyruvate dehydrogenase or alphaketoglutarate dehydrogenase) but evidence of them having a role in exercise-induced ROS production is not apparent. Investigation of mitochondrial ROS production in states mimicking different intensities of physical activity show that when mild aerobic exercise is simulated, over 75% of ROS produced are from complex I and III of the mitochondria, and when intense exercise is simulated, mitochondrial ROS production is almost exclusively from complex I [574].

It is clear that different substrates influence levels of ROS production from different sites in mitochondria. In addition to complex I and II linked substrates, lipids also induce oxidative stress [278, 317, 488]. Paradoxically however, in sufficient quantity lipids can induce uncoupling of mitochondria and decrease ROS production [264]. Evidence supports the idea that this lipid-induced uncoupling may be the role of UCP3, as UCP3 expression can be changed by diet [48, 242], inhibitors of fat metabolism

increase UCP3 expression [468], and UCP3 can translocate oxidised lipids [302]. Collectively, these results suggest that lipids can induce oxidative stress, and UCP3 expression may be a protective response to an accumulation of lipid in mitochondria in order to decrease lipid-induced ROS production [469, 470]. There are multiple potential sites for lipid-induced ROS production, as beta oxidation provides NADH for complex I, and both the electron transfer flavoprotein dehydrogenase [473, 488] and glycerol-3-phosphate dehydrogenase [341, 348, 349] are potential sources of ROS. While much of the mechanistic evidence on the topology of ROS production has been from organs other than skeletal muscle, it has been demonstrated that skeletal muscle exhibits the same characteristics of ROS production with complex I and II substrates [350] and lipid substrate [278, 488].

A proposed mechanism for mitochondrial ROS production is a phenomenon known as reverse-electron-transfer (RET) [336, 375]. The premise of RET is that when membrane potential is high, intermediates such as the Fe-S clusters and cytochromes are maintained in a reduced state and electrons are forced to flow in the opposite direction through the ETS. This movement of electrons is thermodynamically challenging, as they are in theory transitioning to more negative redox potential centres. As mentioned previously, when mitochondria are given complex I substrates (such as malate, glutamate or pyruvate) and rotenone, ROS production increases [27, 199, 488]. However, when rotenone is added to preparations respiring on succinate, ROS production is depressed [66, 199, 277, 296, 477]. If succinate enters the ETS at complex II, and yet ROS production can be suppressed by rotenone blocking the quinone binding site of complex I, this suggests that some of the ROS produced during oxidation of succinate occurs as a result of electrons moving backwards through the quinone pool into complex I.

2.2.2.2 NADPH Oxidase (NOX)

NADPH oxidase (NOX) is an enzyme that catalyses the oxidation of NADPH to $NADP^+$ and the reduction of molecular oxygen to superoxide. NOX has been extensively studied in neutrophils and vascular endothelial tissue (for review see [285]), but is also located in skeletal muscle transverse tubules, the sarcolemma, and the sarcoplasmic reticulum [204, 235, 586]. NOX-derived superoxide appears to have a role in calcium release during excitation-contraction coupling, through modulation of

ryanodine receptor function [130, 204, 498]. There is evidence that NOX may oxidise NADH in addition to NADPH. Javesghani et al. [235] noted that there was significant oxidation of NADH by muscle membrane fractions, even in the presence of inhibitors of traditional NADH consumers (cyclooxygenases, mitochondrial NADH dehydrogenase and nitric oxide synthase). However, this NADH consumption was diminished by NOX inhibitor apocynin, indicating that NOX may be responsible for the observed oxidation of NADH. A possibility not explored by these researchers is the involvement of the nicotinamide nucleotide transhydrogenase (NNT) enzyme. NNT can exchange electrons between the nicotinamide nucleotides NAD and NADP [210], so the large increase in the free pool of NADH in the above study may have resulted in NNT-catalysed production of NADPH. The role of NNT in ROS metabolism is further discussed in section 2.2.4.2.

NOX has been well characterised in neutrophils as an enzyme consisting of 6 subunits; a regulatory GTPase and 5 catalytic phagocytic oxidase subunits [285, 538]. These subunits appear mostly conserved, with rat skeletal muscle expressing 4 of the 5 catalytic subunits, while the 5th subunit was located only in blood vessels [235]. Homologues for some of these subunits have also been found in human cell cultures [504].

2.2.2.3 Xanthine Oxidoreductase (XOR)

Xanthine oxidoreductase (XOR) is an enzyme that catalyses the oxidation of hypoxanthine to xanthine, and the reduction of oxygen to superoxide as a part of purine metabolism. The enzyme exists as both xanthine oxidase (XO) and xanthine dehydrogenase (XDH), and conversion to the XO state is regulated reversibly by increased thiol-group oxidation, or irreversibly by proteolytic conversion of XDH to XO by calcium-calmodulin-dependent proteases [223, 322, 362]. Both forms of XOR catalyse the sequential 2-electron oxidation of hypoxanthine to xanthine and then xanthine to uric acid – the distinction between the two is in the substrate they reduce. XDH preferentially performs the 2-electron reduction of NAD^+ to NADH, whereas XO is unable to reduce NAD^+ and instead reduces molecular oxygen to superoxide [206, 493]. It has been reported that human XOR *ex vivo* is mostly present in XDH form, with limited XO activity detectable [550]. Furthermore, within skeletal muscle, XO activity is detectable only in the endothelial cells of capillaries [194] – it is important to note that the data from

both Wajner and Harkness [550] and Hellsten-Westing [194] were obtained from cadaveric tissue samples, and the properties of XOR strongly suggest conversion to the superoxide producing XO form during exercise.

2.2.2.4 Phospholipase/Lipoxygenase

Phospholipase-A2 (PLA2) enzymes have been implicated in indirect ROS production through production of arachidonic acid. PLA2 cleaves glycerol to form lysophospholipids and arachidonic acid, and inhibition of PLA2 enzymes has reduced ROS production in rat fibroblast cells [575, 576] and in mouse diaphragm muscle [356, 357]. Subsequent investigations of downstream enzymes that use arachidonic acid as a substrate (lipoxygenases, cyclooxygenases and cytochrome p450) revealed inhibition of lipoxygenases, but not cyclooxygenases or cytochrome p450, could decrease ROS production [606]. PLA2 enzyme isoforms expressed in muscle can be either constitutively active, calcium-independent (iPLA2) or a calcium-dependent inducible form located in the cytosol (cPLA2) – these being just two of the multiple enzymes in the PLA2 superfamily [480]. However, it appears that both the iPLA2 and cPLA2 isoforms present in human skeletal muscle act independently of calcium, suggesting their role may be more in maintaining a basal level of ROS as opposed to increasing ROS production during exercise [22, 313, 394, 505, 527, 570]. So while PLA2 enzymes clearly have a role in cellular redox signalling, it is unlikely they have a role in exercise-induced ROS production as opposed to basal levels.

2.2.3 Exercise-induced ROS production

Exercise is a known stimulus for oxidative stress in muscle. In 1982, Davies et al. [102] demonstrated that exercise to exhaustion in rats resulted in an increase in free radical production measured by electron paramagnetic spectroscopy (EPRS) and lipid peroxidation measured by thiobarbituric acid reactive substances (TBARS) assay. Since this work, many studies have corroborated the finding that exhaustive physical exercise induces oxidative stress, through a variety of measures [30, 135, 168, 250, 460, 536]. However, markers of ROS production have been seen to increase in non-exhaustive endurance exercise modalities in both rats and humans [252, 598]. This lead to the opinion that cellular oxygen flux ($\dot{V}O_2$) dictated ROS production, and, consistent with this theory,

it has been shown that ROS production increases during endurance-type exercise, both with exercise duration [448] and intensity [10, 20]. While it would seem intuitive that oxygen demand would be a key driver of oxygen radical production, markers of oxidative stress still increase during exercise without high oxygen flux rates, such as isometric exercise [11] and resistance exercise [320]. In additional conflict with the cellular $\dot{V}O_2$ theory, ROS production is increased during hypoxia [87, 180], which decreases cellular oxygen flux. These findings have now lead to the theory that it is the decrease in partial pressure of oxygen in the cell (pO_2) that may be the important determinant of ROS production, as opposed to total oxygen flux [19]. To further cloud this area, hyperbaric oxygen levels have also been seen to increase ROS production from mitochondria [50, 74]. This highlights the poorly understood nature of molecular-level regulation of ROS production in skeletal muscle, and conflict within current theories. What is apparent from these findings is that elevated ROS production seems to occur as a result of any shift away from cellular oxygen tension homeostasis. This may explain the role of ROS as a signalling molecule for skeletal muscle adaptation.

A central challenge to understanding exercise-induced ROS production is that the identification of ROS sources *in vitro* does not necessarily translate to their activity *in vivo* in response to exercise. As there are a multitude of subcellular sites where ROS can be produced, deciphering which are active and inactive during exercise is challenging, and much of the evidence for sources of ROS during exercise is either from inhibition of certain target sources, or extrapolation of *in vitro* data.

As initial opinion favoured the cellular $\dot{V}O_2$ theory, mitochondria were suspected to be a major source of muscle ROS production during exercise, as mitochondrial oxygen consumption increases manifold with the onset of exercise. However, mitochondrial ROS production is potentiated when membrane potential is high, such as when they are in a resting leak state [340]. When mitochondria are supplied with ADP, such as during exercise, ROS production is decreased – despite increased oxygen consumption [306]. Additionally, type II muscle fibres produce greater amounts of ROS than type I muscle fibres [12], despite having lower mitochondrial density. As previously mentioned, mitochondria produce more ROS when oxidising lipid substrates [278, 488], and during exercise at higher intensities

preferential oxidation of glucose occurs. This suggests that during exercise mitochondria may not be the main source of skeletal muscle ROS.

There is reasonable evidence that allopurinol, an inhibitor of xanthine oxidase [558], can prevent oxidative damage during, or resulting from, exercise [165, 168, 436, 536, 539, 549]. Purine degradation occurs during high-intensity exercise as AMP formed through the adenylate kinase reaction is broken down to inosine monophosphate (IMP), a precursor for hypoxanthine production by purine nucleotide phosphorylase. Following strenuous exercise, total adenine nucleotide pools (ΣAMP + ADP + ATP) are smaller [499], indicating some degradation through this pathway, and blood markers show increases in xanthine [444] and uric acid [444, 499], markers of xanthine metabolism during exercise. This in conjunction with the effects of xanthine oxidase inhibition decreasing exercise-induced oxidative stress implicate xanthine oxidase as a source of skeletal muscle ROS production during exercise. ROS appear tied to regulating basal SR calcium release, with NOX4 being present in the transverse tubule and an increase in NOX activity resulting in increased calcium release from the SR [204, 498].

It is important to note that ROS have beneficial roles within cells, and that an increase in ROS production does not in and of itself indicate a pathological situation. For example, addition of pro- or antioxidants to diaphragm muscle can increase or decrease (respectively) contractile force produced by diaphragm muscle *in vitro* [416]. Similarly, incubation of cardiac muscle SR with combinations of hypoxanthine xanthine + xanthine oxidase, superoxide dismutase, ryanodine and calmodulin demonstrated a superoxide-dependent increase in cytosolic calcium concentration [254]. This indicates that superoxide may be necessary for regulation of calcium release in both skeletal and cardiac muscle. Furthermore, a study by Tweedie and colleagues [526] demonstrated that rats selectively bred to have a high capacity for running (HCR) produced more ROS than rats bred to have a low capacity for running (LCR); however, the HCR strain show less oxidative damage than LCR, despite producing more ROS. Thus the production of ROS is not an inherently negative event – indeed it is necessary for normal cellular function – though chronic dysregulation of ROS balance may have deleterious effects.

2.2.4 Cellular antioxidant defences

Antioxidants represent the ROS removal side of the ROS balance equation. Due to their instability and reactivity, when ROS levels are not managed, they have the potential to cause widespread cellular damage, such as mutations of mitochondrial and cellular DNA, and oxidation of lipid membranes. As a defence against the damaging properties of ROS, cells contain antioxidant compounds and enzyme systems that convert ROS into more stable compounds. Antioxidants can broadly be classified as either enzymatic or non-enzymatic, and skeletal muscle possesses a combination of both. An increase in ROS production that exceeds cellular antioxidant capacity is known as oxidative stress. Oxidative stress is a characteristic of numerous disease states in multiple organs, highlighting the critical role of cellular antioxidant capacity [174, 178, 236, 289, 514, 531].

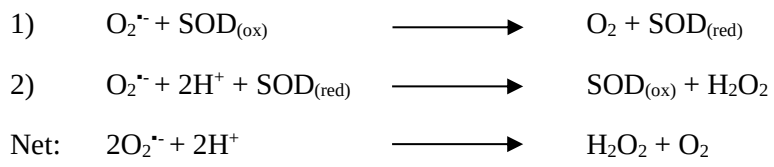
2.2.4.1 Superoxide Dismutase

One of the enzymatic antioxidants is superoxide dismutase (SOD), which catalyses the simultaneous oxidation and reduction (dismutation) of superoxide into oxygen and hydrogen peroxide. As previous mentioned, superoxide is the parent radical for many of the reactive species, and hydrogen peroxide is a more stable form of ROS (though still reactive in and of itself).

SOD exists as two isoenzymes within skeletal muscle, which differ in their subcellular distribution, and also the metal co-factor bound to the enzyme's active site. These isoforms are Copper/Zinc SOD (CuZn-SOD), encoded by the SOD1 gene and located primarily in the cytosol; and Manganese SOD (Mn-SOD), encoded by the SOD2 gene and primarily confined to the mitochondrial matrix [402, 404]. There is a third extracellular variant encoded by SOD3, which also has a copper/zinc centre, but it has extremely limited expression in skeletal muscle [374]; however, this can be increased following acute exercise [208]. The different SOD isoenzymes have different activity constants, and Mn-SOD displays a decrease in rate constant with elevation in pH, while Cu/Zn-SOD is largely unaffected [140]. However, two important things should be noted. Firstly, the concentration of SOD enzymes is vastly greater than the concentration of superoxide [145], meaning the rate of superoxide dismutation is still extremely rapid even when the enzyme is not functioning at optimal pH; and secondly that the pHs studied in [140] (being 6.0, 7.8, 8.5 and 10.2) are all likely to be outside of

physiological pH range. Nevertheless it is important to consider the trend shown, especially when considering the subcellular location of these enzymes.

Dismutation of superoxide involves a transient change in the oxidation state of the metal centre of SOD, temporarily "storing" an electron and oxidising a superoxide molecule. This stored electron is then combined with another superoxide anion and two H^+ to form hydrogen peroxide. These two half reactions and the resulting total reaction are shown below.



2.2.4.2 Glutathione Peroxidase/Reductase

The H_2O_2 generated from SOD is still a reactive species, even though it is more stable than superoxide. Thus there is still a need to metabolise H_2O_2 before it reacts with other cellular structures. Glutathione peroxidase (GPX) is one enzyme that serves this purpose. In skeletal muscle, GPX exists in different isoforms that have different preferred substrates. GPX1 has high affinity for H_2O_2 whereas GPX4 possesses a higher affinity for lipid hydroperoxides formed when ROS react with lipid membranes [178]. The GPX reaction utilises reduced glutathione (GSH) as an electron donor and produces the oxidised glutathione disulphide (GSSG), along with either water (in the case of reaction with H_2O_2) or a lipid-bound alcohol (in the case of reaction with lipid hydroperoxide). GPX4 appears to be critical to life, as mice with complete genetic ablation of GPX4 die during gestation, while mice with a single copy knockout of GPX4 are more sensitive to induction of oxidative stress [590]. This single copy knockout of mitochondrial GPX4 is not embryonic lethal, but resulting male offspring are infertile [467].

Glutathione reductase (GR) completes these cycles by reducing GSSG back to two GSH molecules via the electron donor NADPH. Essentially GPX and GR act synergistically to oxidise one NADPH and reduce a radical species to a non-radical, utilising GSH/GSSG as intermediate cofactors. Only one ubiquitous GR form is known, and it is conserved between yeast, animals and humans.

The reliance of the glutathione cycle on NADPH presents a crossroad of mitochondria, ROS production, and ROS disposal. As mentioned previously the NNT can utilise the mitochondrial membrane potential to produce NADPH and NAD^+ from NADH and $NADP^+$ [114, 210]. In doing so, NNT may help decrease oxidative stress via a range of mechanisms. By lowering the membrane potential and consuming NADH, ROS production resulting from reverse electron transfer through ETS complexes may be decreased. Further, provision of NADPH may assist in the recycling of GSH from GSSG, though it could in theory also increase ROS production through NOX.

2.2.4.3 Mitochondria

In addition to mitochondrial isoforms of antioxidant enzymes, mitochondria may act as a sink for ROS through oxidative phosphorylation. In a study by Mailer [310], cardiac mitochondria incubated with a superoxide generating system of xanthine and xanthine oxidase were able to phosphorylate ADP to form ATP. ATP production was prevented by the addition of excess SOD to the reaction mix, and was increased with increasing amounts of xanthine oxidase present (with fixed xanthine concentration and mitochondrial protein content). Reduction of cytochrome-c was also increased by generation of superoxide; the collective implication of which is that an electron from superoxide can reduce cytochrome-c, which can then participate in the electron transport system through complex IV as it normally would. This indicates that to some degree the mitochondrial electron transport system can act as an antioxidant, and that superoxide could even be considered a substrate for oxidative phosphorylation.

2.2.5 Measurement of skeletal muscle ROS production

As superoxide has an extremely short half-life, indices of ROS production are reliant on measuring its downstream products rather than the parent molecule itself. While it is possible to fluorometrically measure superoxide “flashes” [308, 555, 556, 560], it is often secondary markers that are used to quantify ROS production. These mainly rely on either fluorescent or spectrophotometric detection methods of secondary radical species [129, 216, 303], electron paramagnetic resonance (EPR) detection [89, 183], measurement of antioxidant balance such as GSSG/GSH levels [240, 316, 460, 479], or measurement of products of specific oxidation events in fluids and tissue (such as TBARS,

malondialdehyde (MDA), protein carbonyls [173, 461, 510] or protein oxidation via the likes of an OxyBlot™ [535]). Each approach has respective advantages and limitations. A general advantage of a fluorescence or EPR approach is the ability to quantify rates of production of radicals, which measuring an endpoint such as TBARS or GSSG does not (though inferences can be made during time course sampling). However, a limitation of fluorescent/EPR approaches is that radical species are conjugated to or modified by other compounds, which mean that endogenous antioxidants are no longer capable of removing the radical, meaning this method does not account for the capacity of the tissue to eliminate ROS. Thus a measure of net oxidation (such as Oxyblot™, TBARS, or GSSG) may more accurately reflect the *in vivo* effects of a particular intervention on a target tissue. Further complicating measurement of tissue ROS production is that it is difficult to quantify the role of endogenous antioxidants that may mask true levels of ROS production. As such, data presented in this thesis are referred to as "ROS emission", as this better describes the detection of ROS which have not already been disposed by any native antioxidants.

ROS production may be muscle fibre type specific, as H_2O_2 production relative to oxygen consumption is higher in type II than type I muscle fibres [12]. This study also measured H_2O_2 consumption, and found that when normalised to citrate synthase (CS) activity (a common marker of mitochondrial content [282]), H_2O_2 consumption was greater in type I fibres than type II. Additionally, when rat soleus (primarily type I muscle), red vastus (primarily type IIa muscle) and white vastus (primarily type IIb muscle) are compared after exercise, a similar continuum of MDA, accumulation is noted (smallest increase in MDA content in soleus, followed by red vastus, with white vastus showing largest increase in MDA content) [10]. This suggests a property of more oxidative muscle fibres that both diminishes their capacity to produce ROS, and possibly increases their ability to consume ROS. In human skeletal muscle, slow-twitch (type I) muscle fibres are accepted to have the greatest oxidative capacity, followed by fast-twitch oxidative (type IIa) muscle, with fast-twitch glycolytic (type IIx) fibres possessing the lowest oxidative capacity [604]. Contractile twitch speed and force show the opposite relationship, with fastest twitch and highest force in humans seen in type IIx fibres, followed by type IIa fibres, with type I fibres having the slowest twitch speed and lowest force [604]. In rodent

skeletal muscle, muscle citrate synthase activity was found to correlate best with proportion of type IIa fibres, rather than type I [107], indicating that rodent type IIa fibres may be more oxidative than type I fibres. However, rodent muscle fibre contractile properties (twitch speed and force) have a similar relationship to humans [412]. This is of interest, as rodent models are commonly used in research, and as such careful attention should be paid to the fibre type of any muscle used. Due to the difference between rats and humans in properties of type IIa muscle fibres, research on muscles high in type IIa fibre content may have limited translatability between rodents and humans.

2.2.6 Acidosis and oxidative stress

Studies on Wistar rats have shown that either addition of lactic acid, or exposure to hypercapnia increased the appearance of TBARS in brain homogenate [415]. Similarly, addition of lactic or phosphoric acid to cortical brain slices also increased appearance of TBARS [53]. These findings indicate that acidosis, induced in a number of different ways, can increase lipid peroxidation in brain. The influence of acidosis on mitochondria appears to be tissue specific, as demonstrated by Bento et al. [34] who showed that inducing acidosis with ammonium chloride (NH_4Cl) decreased membrane potential and increased the rate of calcium uptake in rat kidney mitochondria, but not liver mitochondria. This has implications for ROS production, as both increased mitochondrial membrane potential and increased calcium concentration have been implicated as factors in potentiating mitochondrial ROS generation [14, 55]. It is therefore important to investigate what effect exercise-induced acidosis may have on skeletal muscle ROS production.

To date there is only one study on the effects of pH on skeletal muscle ROS metabolism. Banh & Treberg [25] measured respiration, H_2O_2 production, H_2O_2 consumption and estimated membrane potential in isolated mitochondria from rat skeletal muscle, in media of pH 6.8, 7.2 and 7.6 at 37°C. They found that membrane potential was unaffected by pH of media, but respiration was lower at pH 6.8 than either 7.2 or 7.6 (which were not significantly different). H_2O_2 production rate was lowest at pH 6.8, increased approximately 2.5 fold from pH 6.8 to pH 7.2, and increased again (but much smaller change) from pH 7.2 to 7.6. H_2O_2 consumption followed the opposite trend, with H_2O_2 consumption lowest at pH 7.6, and approximately 1.5 fold higher at pH 6.8. Finally, the rates of H_2O_2 consumption

were far greater than rates of H_2O_2 production. While this is the first paper to describe the potential role of acidosis on oxidative stress in skeletal muscle, there are a number of reasons to take caution when considering their results. Firstly, the authors identify themselves that the conditions used to measure H_2O_2 consumption (addition of 1-3μM H_2O_2 to assay media) are likely to be supraphysiological; thus the difference in H_2O_2 consumption rate may simply be an artefact of the assay which would not be observed *in vivo*. Secondly, as previously mentioned, the isolated mitochondria model has been shown to alter characteristics of mitochondrial ROS metabolism [393], and therefore may affect the relationships observed in this study. Thirdly, the substrates used in this investigation were malate and glutamate, meaning that only complex I function was observed, though succinate may be lost from the TCA cycle in assay preparations, and therefore maybe necessary for maximum flux to be achieved (summarised in [159]). Lastly, the authors mention correction of H_2O_2 consumption rates when drift was observed from their sensor after H_2O_2 was completely consumed, but no mention is made of a control of the effect of the change in pH on H_2O_2 stability. In the presence of iron salts, H_2O_2 decomposition is decreased at lower pH ranges (i.e. H_2O_2 stability is increased) [561], and at highly alkaline pH (>10) even small variations in pH have marked effects on the stability of H_2O_2. It is reasonable to infer from the results of Nicoll & Smith [361] that at 37°C and a relatively narrow pH range that the authors either did not see a difference, or accounted for any difference if one was there, however, it remains a possibility.

2.2.7 Summary

ROS can be produced from a number of sites within skeletal muscle. Production of ROS increases during exercise, and specific sites responsible for exercise-induced ROS production are located in both the mitochondria and in the cytosol of muscle. One recent study describes that mitochondria may produce less ROS, and can consume more ROS, in an acidotic environment. However, mechanisms resulting in this observation are yet to be determined. It is therefore important to investigate what properties of muscle and mitochondria may change that would enable lower ROS production and/or greater ROS consumption in response to acidosis.

2.3 Exercise-induced mitochondrial biogenesis

2.3.1 Overview

As ATP production is ultimately dependent on mitochondrial oxidative phosphorylation, mitochondria adapt to changes in cellular energy demand (such as exercise) [288]. An intricate network of transcriptional activators, co-activators and repressors all function in an integrated fashion to bring about changes in mitochondrial protein expression, termed mitochondrial biogenesis. [293, 294, 300, 580, 601] Mitochondrial biogenesis requires co-ordination of transcription between two genomes, nuclear genes encoding mitochondrial proteins (NUGEMPs) and mitochondrial DNA (mtDNA), which encode different functional units of the organelle as a whole. This process is regulated by upstream signalling cascades.

A key protein involved in the regulation of mitochondrial biogenesis is peroxisome proliferator-activated receptor gamma (PPARγ) co-activator 1-alpha (PGC-1α), the so-called 'master-regulator' of mitochondrial biogenesis. Identification of PGC-1α first emerged in studies on metabolic regulation and non-shivering thermogenesis in brown adipose tissue cells [408]. PGC-1α is a transcriptional co-activator, and in its inactive form is located in the cytosol. Phosphorylation of PGC-1α protein causes it to accumulate within the cell nucleus, and deacetylation enables it to activate the transcription of genes relating to aerobic metabolism from nuclear DNA. Among these are the nuclear respiratory factor (NRF) family, which are transcription factors for both nuclear DNA and mtDNA [215], and allow the co-expression of both genomes that is necessary for mitochondrial biogenesis to occur.

It is well established that cell signalling pathways mediating mitochondrial biogenesis can be modulated through both exercise [152, 293, 294, 300, 386], and inactivity or disease [3, 281, 439]. PGC-1α integrates a variety of signals to stimulate exercise-induced mitochondrial biogenesis. There are a multitude of upstream signals that can alter the expression or activity of PGC-1α, such as changes in 1) cytosolic calcium concentration, 2) cellular energy status (in the form of adenosine phosphate groups and also redox state), and 3) cellular stress. This section of the review will focus on pathways leading to the up-regulation of mitochondrial biogenesis in response to exercise.

2.3.2 Calcium-mediated signalling

Muscle contraction begins with an action potential, which eventually results in release of calcium from the sarcoplasmic reticulum (SR), and an increase in cytosolic calcium ion concentration [276, 377, 501, 502]. Calcium transients are activity-dependent, meaning they are determined by the frequency and the duration of neural stimulation, which is dependent on the contraction force required [562, 564]. In addition to enabling muscle contraction, calcium can also activate transcription factors involved in muscle adaptation. In cultured rat (L6) myocytes, treatment with a calcium ionophore results in increased PGC-1α protein content, mitochondrial transcription factor A (Tfam), and NRF1 and 2 gene expression [371], as well as increased mitochondrial enzyme activities [372]. Both the calcium-calmodulin dependent protein kinase (CaMK), and the phosphatase calcineurin have been implicated in promoting mitochondrial biogenesis in response to exercise [82, 129, 225, 303, 580, 582]. The calcium-calmodulin complex can also autoregulate the release of calcium into cells [507], as one of the CaMK isoforms may regulate the ryanodine receptor [6, 112, 559].

While several CaMK isoforms have been discovered [217], it appears likely that the isoform responsible for skeletal muscle adaptation to exercise is CaMK II. Protein expression of CaMK IV in human skeletal muscle is extremely limited [430] and occasionally undetectable [429], and while CaMK I protein expression can be detected in human skeletal muscle, activity of both CaMK I and CaMK IV as a calmodulin-dependent kinase is not seen in skeletal muscle [430]. Additionally, mRNA expression of CaMK IV is not detectable in rat skeletal muscle [82]. Transgenic mouse models reveal that overexpression of CaMK IV in skeletal muscle leads to increased oxidative fibre type I and IIa content, and increases in PGC-1α and mitochondrial protein content relative to wild-type controls [582]. However, it has also been shown that CaMK IV knock-out mice show the same adaptation to training as wild-type controls, though they voluntarily run less and, somewhat paradoxically, also show increased expression of type I muscle fibres in soleus muscle [8]. Taken together these findings suggest that CaMK IV is sufficient, but not necessary, to induce mitochondrial biogenesis, and may not have an important role in contraction-induced mitochondrial biogenesis.

One of the ways CaMK II acts to enable the transcription of PGC-1α is through nuclear exclusion of the transcriptional repressor histone deacetylase (HDAC) [328]. DNA is organized within the nucleus as chromatin, which comprises DNA strands wound around a histone octamer. DNA transcription is regulated by histone acetyl transferase (HAT) and HDAC enzymes, which function (respectively) to either add or remove an acetyl group from histones [330, 331]. Addition of an acetyl group relaxes chromatin and allows transcriptional machinery to access DNA strands, and subsequently promote transcription of target genes. In the case of skeletal muscle mitochondrial biogenesis, HDACs regulate the function of myocyte enhancer factor 2 (MEF2) [73, 98, 307, 401]. Class II HDAC's are expressed primarily in skeletal muscle and the brain [330], and when two serine residues are phosphorylated by CaMK, a chaperone protein 14-3-3 enables HDAC's translocation out of the nucleus [328, 329, 353]. With HDAC no longer active in the nucleus, HAT's can acetylate histones, chromatin is relaxed, and transcription is enabled. As such, low-frequency (10 Hz) electrical stimulation of skeletal muscle results in efflux of HDAC from the nucleus of isolated muscle fibres, but this effect is not observed with higher frequency (100 Hz) stimulation [297], potentially representing one way that fibre-type-specific mitochondrial content may be determined. It has also been noted that MEF2 binding to DNA in humans is increased following 60 minutes of cycling [325].

CaMK II and IV also phosphorylates the transcription factor cyclic AMP response element binding (CREB) protein [182, 582]. CREB binds to a cyclic AMP response element (CRE) sequence of DNA in the PGC-1α gene which activates PGC-1α transcription [584]. As such, incubation of mouse (C2C12) myocytes with the cyclic AMP stimulator forskolin results in increased expression of PGC-1α, as does induced over-expression of CaMK IV, but these effects are prevented in cells with a non-functional CREB mutation [182]. Using recombinant protein it has be shown that CREB can be phosphorylated at Serine 133 by both CaMK II and IV, but that CaMK II can also phosphorylate Serine 142 [497]. Phosphorylation at serine-142 in fact acts to repress transcription, whereas phosphorylation of Ser133 enhances transcription [497]. However, two studies from the same group showed that CREB phosphorylation is either not significantly changed [567] or depressed [566] following cycling exercise

in humans, though in both cases the authors note an extremely variable response of CREB. So while PGC-1α is regulated *in vitro* by CREB, evidence for a role of CREB in exercise adaptation is unclear.

The calcium-calmodulin-dependent protein phosphatase calcineurin is also responsive to increased cytosolic calcium ion concentration, and may be a key determinant of muscle fibre type [83]. Calcineurin de-phosphorylates the transcription co-factor nuclear factor of activated T-cells (NFAT), which in turn allows it to translocate into the cell nucleus [83, 149]. Once in the nucleus, NFAT can associate with MEF2 to upregulate transcription of PGC-1α [583]. In mice engineered to constitutively express calcineurin, PGC-1α protein expression and mitochondrial respiration are significantly increased relative to control mice [241], and in another study overexpression of calcineurin lead to an increase in cytochrome b and PGC-1α mRNA, as well as an increase in cytochrome c protein content [582]. Conversely, treatment with calcium chelators (EDTA and/or EGTA), SR calcium release inhibitor dantrolene, calcineurin antagonist cyclosporine A, or CaMK inhibitor KN93, all blunt increases in mitochondrial gene and protein expression, and increases in mitochondrial enzyme activities [83, 149, 371, 372]. This demonstrates an important role of calcium-mediated pathways in mitochondrial adaptation, though some of these pathways have limited evidence of an *in vivo* role in adaptation to exercise.

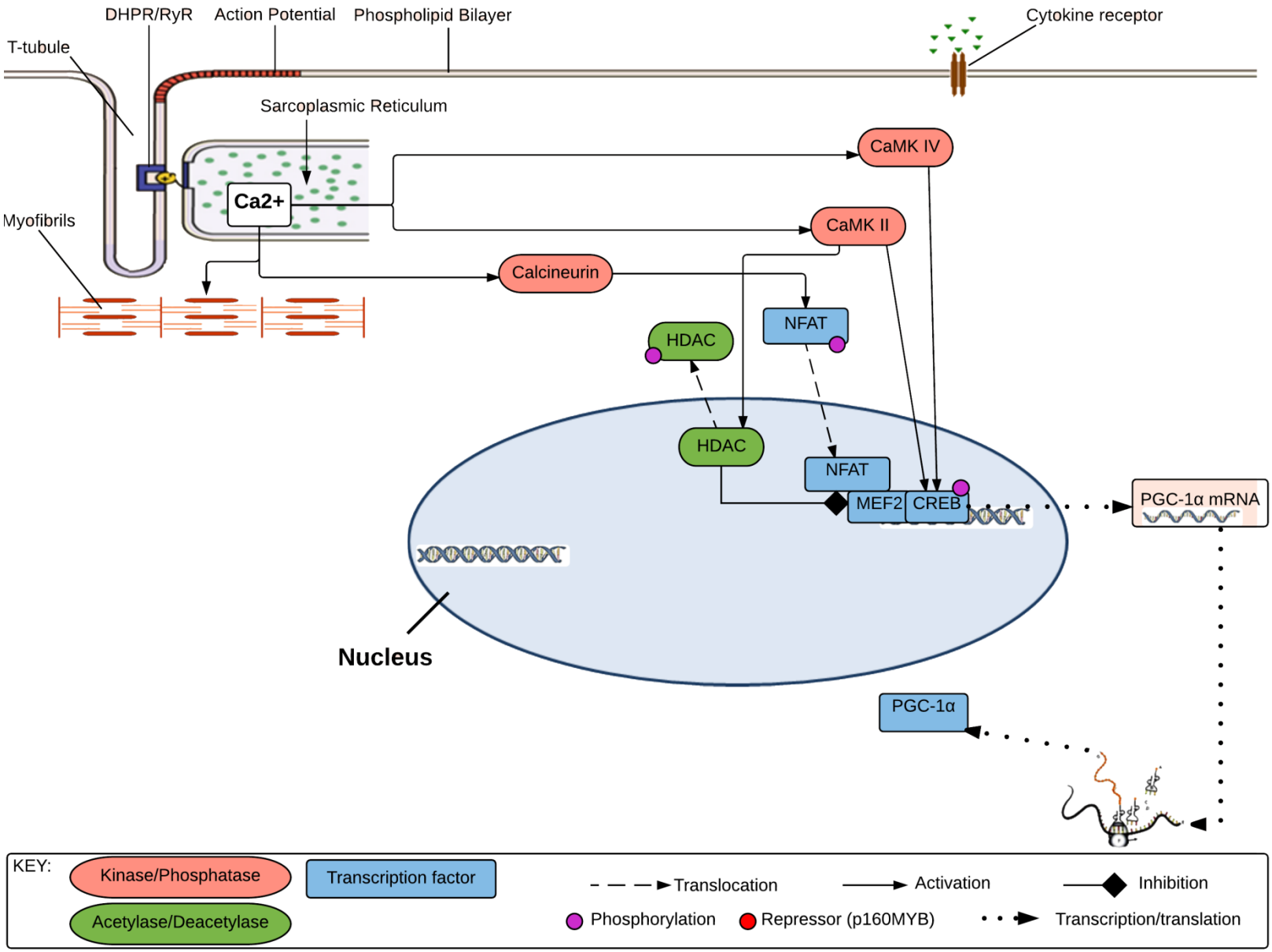


Figure 2.4: Calcium-mediated signalling for mitochondrial biogenesis.
See in text for abbreviations.

2.3.3 Energy-dependent signalling

Sustained contractile activity requires hydrolysis of ATP for many events. For example, cross-bridge cycling of myofibrils during contraction, SR calcium reuptake via sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pumps, and membrane repolarisation via the sodium-potassium antiporter (Na^+-K^+-ATPase), are all ATP dependent. The energetic demands of exercise and the systems that supply this demand have been reviewed previously in section 2.1. The important points to recap are that during high-intensity exercise, muscle ATP content may halve [253], and concentrations of AMP increase as a by-product of ADP degradation to support ATP regeneration [190, 445]. Increased cellular AMP concentration ultimately results in the activation of adenosine monophosphate-dependent protein kinase (AMPK), and can increase mitochondrial biogenesis [36, 370, 398, 569].

AMPK is allosterically regulated by the binding of AMP or ATP to the gamma subunit [4]. Binding of ATP inhibits AMPK activity, while binding of AMP then enables AMPK to be phosphorylated [343, 587]. As such, AMPK phosphorylation is lower in adenylate kinase-deficient mice, which have a diminished capacity to produce AMP from ADP [181]. In addition to activation as AMP concentration increases, there is evidence that AMPK can be phosphorylated by a pathway responsive to the glycolytic intermediate 3-phosphoglycerate [125]. Also, creatine /phosphocreatine can activate/inhibit AMPK activity respectively [398]. Because AMPK is responsive to metabolites that indicate the level of energy demand a cell is under, it has been described as an cellular energy sensor [185, 186] or fuel gauge [184].

Activation of AMPK occurs by phosphorylation at the threonine-172 residue in the activation loop of the alpha subunit [193]. This indicates that one or more upstream AMPK kinases (AMPKKs) are required to activate AMPK. One upstream kinase that appears responsible for increasing AMPK activity is the serine/theronine kinase 11, also known as liver kinase B1 (LKB1) [192, 299, 578]. Mice with a muscle specific LKB1 deficiency (mLKB1-KO) display a decrease in expression of PGC-1α protein and markers of mitochondrial complexes I-V [512]. Phosphorylation of target AMPK protein acetyl coenzyme A carboxylase (ACC) is also depressed in mLKB1-KO mice, along with AMPK activity [513]. In both the previous studies mLKB1-KO mice show decreased running capacity and

citrate synthase activity [512, 513]. Exercise training can increase the expression of both LKB1 and MO25 in rat skeletal muscle [508, 509]; however, this increase alone does not induce an increase in AMPK kinase activity [508]. As mentioned in the preceding section, CaMKK may be another upstream kinase that phosphorylates AMPK. Stress-induced increases in AMPK activity can be depressed by treatment with STO-609, a CaMKK inhibitor [577], and additional treatments with calcium ionophores, CaMKK inhibitors and CaMKK small interfering RNA molecules also demonstrate a role for CaMKK in phosphorylating AMPK independently of LKB1 [224, 577]. This has been seen in contracting muscle, where hindlimb perfusion with STO-609 depresses CaMKK activity and also AMPK-α2 phosphorylation in response to electrically-stimulated muscle contraction relative to vehicle treatment [2]. AMPK-α1 phosphorylation was not different in STO-609 perfusions, another potential explanation for a difference in regulation of the different alpha isoforms.

It appears that AMPK has an array of activities in promoting mitochondrial biogenesis. Treatment of C2C12 cells with an AMPK agonist, the AMP analogue 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), results in increased PGC-1α mRNA expression [227]. In this study, further experiments with a cloned section of PGC-1α promoter DNA lead the authors to postulate that the increase in PGC-1α mRNA may be mediated by removal of MEF2 repression by HDAC, consistent with previous evidence [98]. AMPK also interacts directly with PGC-1α protein, inducing phosphorylation and may have a role in regulating post-translational acetylation of PGC-1α. Structurally, AMPK is a heterotrimer consisting of three subunits, an alpha (α), beta (β) and gamma (γ) subunit [489]. The alpha unit is the catalytic unit, and exists as either α1 or α2; however, stability and activity of AMPK is still influenced by the other subunits [117, 148]. Knock-out of the AMPK-α2 (-α2 KO) subunit in mice results in decreased basal expression of markers of mitochondrial density (citrate synthase, cytochrome oxidase (COX) subunit I and β-hydroxyacyl-CoA dehydrogenase), but no significant difference in training-induced increases in these markers [244]. The data from this study also showed that the -α2 KO mice had increased activity of AMPK-α1, indicating a degree of redundancy and compensation between the isoforms. A different model of -α2 KO used by Rockl et al. [425] also demonstrates lowered basal levels of selected markers (citrate synthase activity, PGC-1α

protein and fast oxidative muscle fibre content), but similar adaptive response to training in comparison to wild type controls.

Regulation of AMPK by exercise in humans demonstrates intensity-dependent responses. It appears that moderate-intensity exercise may preferentially increase AMPK-α2 catalytic domain (AMPK-α2) activity [146, 244, 491]. Furthermore, it seems there is a threshold for activation, as Fuji. et al. [146] found no significant change in either AMPK-α1 or -α2 following 20 minutes of cycling at 50% of VO_2max, but significant increases in -α2 activity following 20 minutes of cycling at 70% VO_2max. McGee et al. [324] observed an increase in -α2 translocation into the nucleus following 60 minutes of cycling at around 70% VO_2max, an effect which was independent of -α2 mRNA or protein content. In a study by Egan et al. [122] volunteers were asked to cycle at either 40% or 80% of VO_2max for a duration whereby energy expenditure was matched between groups. In both groups, PGC-1α mRNA expression was up-regulated post-exercise, but to a greater degree in the group exercising at 80% VO_2max (3.8 fold increase in the 40% group compared to 10.2 fold increase in the 80% group). Both groups showed phosphorylation of p38 MAPK and CREB, but only the 80% group showed increased phosphorylation of CaMK, AMPK and ATF2. This could explain the difference in PGC-1α expression. Following a protocol of four 30-second all-out cycling efforts, separated by four minutes of rest, Gibala et al. found increases in both -α1 and -α2 activity in humans, but a larger increase in -α2 [154]. This is similar to results seen by Chen et al. [80] after a single 30-second all-out bout, where both -α1 and -α2 AMPK isoforms showed approximately two-fold increase in activation. Thus it seems that maybe exercise at near-maximal intensities is necessary for activation of the -α1 isoform, and even at sub-maximal intensities there is a threshold for activation of the -α2 isoform. Differences in AMP-dependence may explain differential activation of -α1 and -α2 isoforms of AMPK, as -α2 isoforms from rat liver shows a greater increase in activity as AMP concentration increases [458]. This could mean that *in vivo* AMPK-α2 responds to smaller increases in AMP, explaining the preferential activation of -α2 at lower intensities than -α1.

While the α-subunit of AMPK appears to regulate the response to exercise, it has been demonstrated that a gain-of-function mutation of the γ3 subunit can increase mitochondrial biogenesis

in glycolytic mouse muscle (white gastrocnemius) [150]. This was an activity-dependent effect, as both over-expression and knock-out of the γ3 subunit did not alter mitochondrial biogenesis. The importance of the γ3 subunit in regulating AMPK signaling has also been demonstrated *in vivo* in humans. Following a bout of either 30 seconds of all-out cycling, 120 seconds of cycling at 110% peak power, or 20 minutes of cycling at around 75% VO_2max, activity of the α2/β2/γ3 isoform was significantly increased, whereas activity of the α2/β2/γ1 isoform significantly decreased [39]. This effect was consistent across all trials, except for the 20-minute cycle bout where there was no significant change in α2/β2/γ1 activity. In both the 120-second and 30-second bouts of higher-intensity exercise, there was a significant decrease in α1/β2/γ1 activity, the only AMPK-α1 isoform in skeletal muscle. This confounds the previous assertion that AMPK-α1 may be induced by higher-intensity exercise; thus the role of AMPK-α1 in response to exercise is unclear.

Another indicator of cellular energy state is the ratio of NAD^+ to NADH, also considered an index of cellular redox state. When substrate supply is limited, less NADH can be produced, resulting in an increase in NAD^+ concentration relative to NADH. Increases in cellular NAD^+ concentration can activate the silent information regulator 2 (sirtuins), a family of proteins with NAD^+-dependent deacetylase enzyme activity [367], that can regulate PGC-1α activity [355].

Sirtuin 1 (SIRT1) was first associated with regulating cell survival, due to the capacity of SIRT1 to deacetylate the cell cycle regulatory protein p53 [533]. It has been noted that caloric restriction induces increases in SIRT1 expression in rat brain, kidney, liver and adipose tissue [90]; however, another study found that caloric restriction decreased SIRT1 expression in liver, but increased SIRT1 expression in muscle and adipose tissue [76]. Caloric restriction also increases mitochondrial biogenesis [86], and co-immunoprecipitation in cells reveals there is an interaction between SIRT1 and PGC-1α [355]. It is thought that SIRT1 may regulate mitochondrial biogenesis by translocation to the cell nucleus, where it may deacetylate PGC-1α [355, 506] and MEF2 [600].

SIRT1 protein expression can be increased by exercise in rat skeletal muscle [261], but protein content does not seem to correlate to changes in mitochondrial function in both rats and humans. Rather,

SIRT1 activity provides a better association [177], and SIRT1 activity can be increased by high-intensity training [176]. However, mice with whole-body SIRT1 deficiency do not show any decrease in muscle oxidative capacity, and both cells and mice overexpressing SIRT 1 do show a decrease in oxygen consumption capacity relative to controls [44, 355]. Additionally, mice with a muscle-specific SIRT1 knockout (mSIRT1-KO) show no difference in the upregulation of mitochondrial biogenesis markers in response to training, voluntary running capacity, or time to fatigue [391]. The acetyltransferase general control of amino acid synthesis protein 5 GCN5 has been proposed as another potential mediator of PGC-1α acetylation. GCN5 can repress PGC-1α through lysine acetylation [113, 287]. In mSIRT1-KO mice GCN5 is still active, however, following exercise there is less GCN5 present in the cell nucleus [391]. So while acetylation of PGC-1α may cause transcriptional repression, a decline in PGC-1α acetylation following exercise may occur as a result of nuclear exclusion of the acetyltransferase GCN5, rather than an increase in activity of the deacetylase SIRT1.

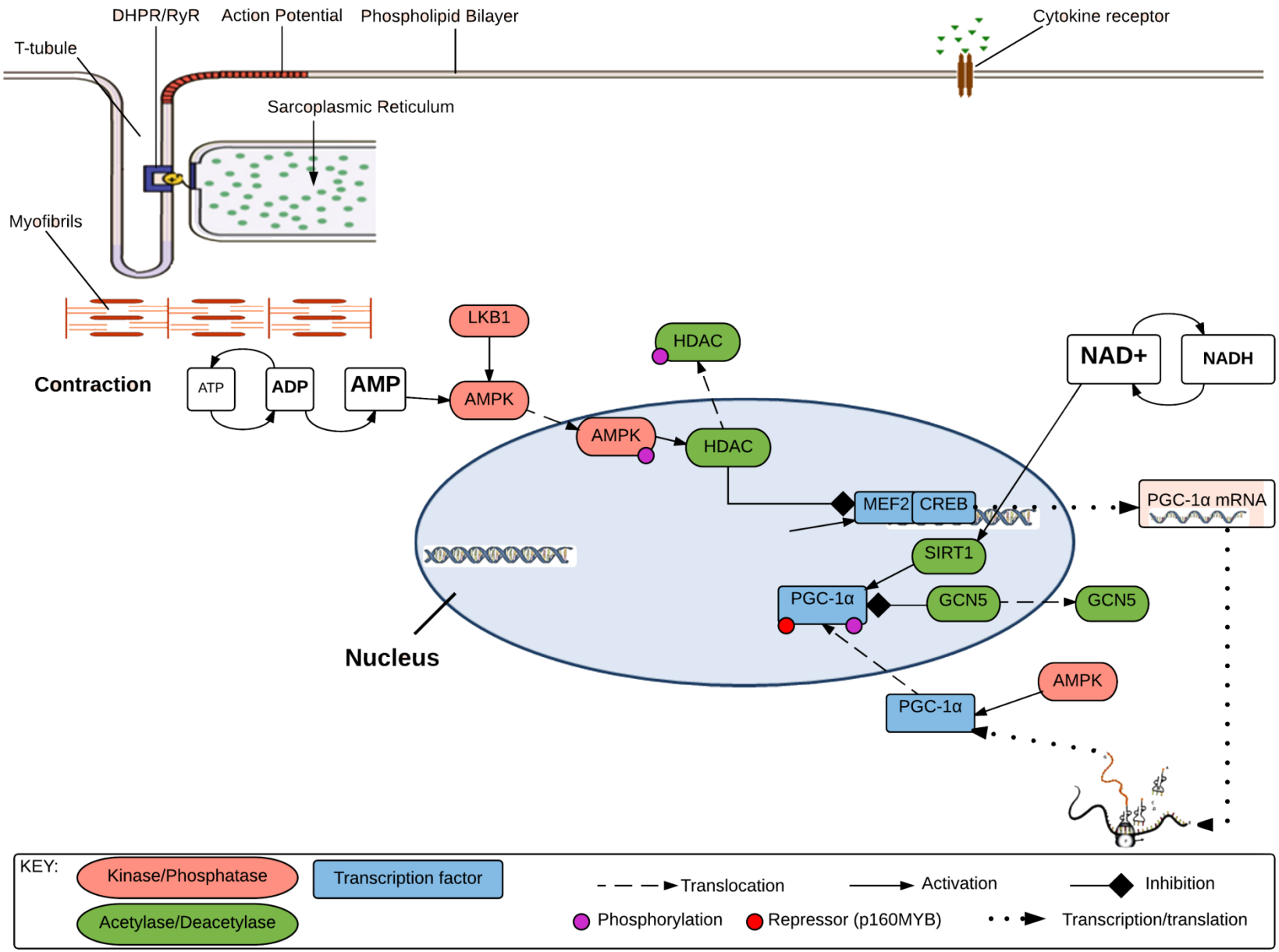


Figure 2.5: Energy-dependent signalling for mitochondrial biogenesis.
See in text for abbreviations.

2.3.4 Cellular-stress signalling

Cellular response to a wide range of perturbations is mediated by a family of stress-activated protein kinases (SAPKs). In the case of exercise, SAPKs are responsive to mechanical stress from contraction, release of growth factors, and also to the effects of muscle damage [304]. Skeletal muscle damage is elicited in a manner dependent on the type and intensity of the exercise performed [88]. Damage is greater following high-intensity exercise, and is also pronounced with eccentric-type muscle contraction [118, 368]. In response to muscle damage, muscle and plasma cytokine levels are elevated [65, 385], and increases in cytokine concentration stimulate p38 mitogen-activated protein kinase (p38 MAPK) [407].

There are 4 isoforms of p38 MAPK expressed in skeletal muscle (alpha (α), beta (β), gamma (γ), and delta (δ)), with the γ isoform being most abundant [96, 161]. As such, several studies have demonstrated the importance of p38γ in various skeletal muscle processes and adaptive responses, including glucose uptake [209], myogenesis [141, 258] and mitochondrial biogenesis [397]. Activation of any of the isoforms is due to dual phosphorylation of a conserved threonine-glycine-tyrosine residues by a cascade of upstream MAPK kinase (MKK) enzymes [95]. There is also evidence of a role for p38 MAPK in post-translational control of PGC-1α, by phosphorylation of PGC-1α at threonine 262, serine 265, and threonine 298, which are in the binding site of a repressor protein ($p160^{MBP}$) [134, 260]. Thus phosphorylation of PGC-1α by p38 MAPK may also enhance transcriptional activity by inhibiting PGC-1α repression. Cell culture studies have shown that incubation of C2C12 cells with various pro-inflammatory cytokines induces expression of PGC-1α, as does activation of endogenous p38 MAPK pathways. This can be abolished by mutation of the activation loop, meaning it is unable to be phosphorylated [407]. Calcium may also play a role in p38 MAPK activation, as Wright et al. [581] demonstrated that raising calcium concentration induced phosphorylation of p38 MAPK and that this could be blocked by a CaMK inhibitor. Upregulation of PGC-1α expression via a p38 MAPK pathway may occur through the transcription factor MEF2, which is phosphorylated by p38 MAPK [599]. Another target is ATF2 [7, 175], as the p38 MAPK-mediated increase in PGC-1α mRNA expression is blunted by inactivation of ATF2 [7].

Exercise studies have also demonstrated an important role for p38 MAPK in exercise-induced mitochondrial biogenesis. Following four weeks of voluntary wheel running in mice there was a 2-fold increase in type IIa muscle fibre content of the plantaris muscle, at the expense of IIb fibres [7]. Furthermore, after a single night of voluntary wheel running, there was an increase in phosphorylation of MKK, and increased phosphorylation of p38 MAPK. Additionally, transgenic mice overexpressing MKK in skeletal muscle have increased PGC-1α and COXIV protein content in white gastrocnemius [7]. In humans, both sprint interval training and submaximal continuous exercise upregulate p38 MAPK phosphorylation in a manner that appears related to total work performed [122, 154]. However, Egan et al. [122] found that while p38 MAPK phosphorylation was not different between work-matched high- and low-intensity exercise, increased ATF2 phosphorylation only occurs following high-intensity exercise.

Another stress-inducible pathway in skeletal muscle involves the extracellular signal-regulated kinases 1 and 2 (ERK1/2). While the precise upstream signal for ERK1/2 activation is unclear, ERK1/2 phosphorylation is increased in response to stimulated contraction in rats, and ERK1/2 phosphorylation in humans increases with both cycling [17, 566, 594] and marathon running [593]. ERK1 can be phosphorylated at threonine 202 and tyrosine 204, and ERK 2 at threonine 185 and tyrosine 187, by mitogen/extracellular signal kinase 1/2 (MEK1/2), and phosphorylation increases ERK1/2 kinase function [431, 459]. Human exercise-induced ERK phosphorylation is intensity dependent, and rapidly declines upon cessation of exercise with ERK phosphorylation returned to baseline levels within 1 hour of recovery [566, 567].

There is an element of redundancy present between different members of the SAPK family, as ERK1/2 are also proposed activators of ATF2 [376]. Another potential downstream target of both p38MAPK and ERK1/2 are mitogen/stress activated kinase 1 and 2 (MSK1/2), which may mediate CREB binding with DNA [91, 106]. MSK1/2 activity is increased in response to exercise in humans along with phosphorylation of both ERK1/2 and p38MAPK [593, 594].

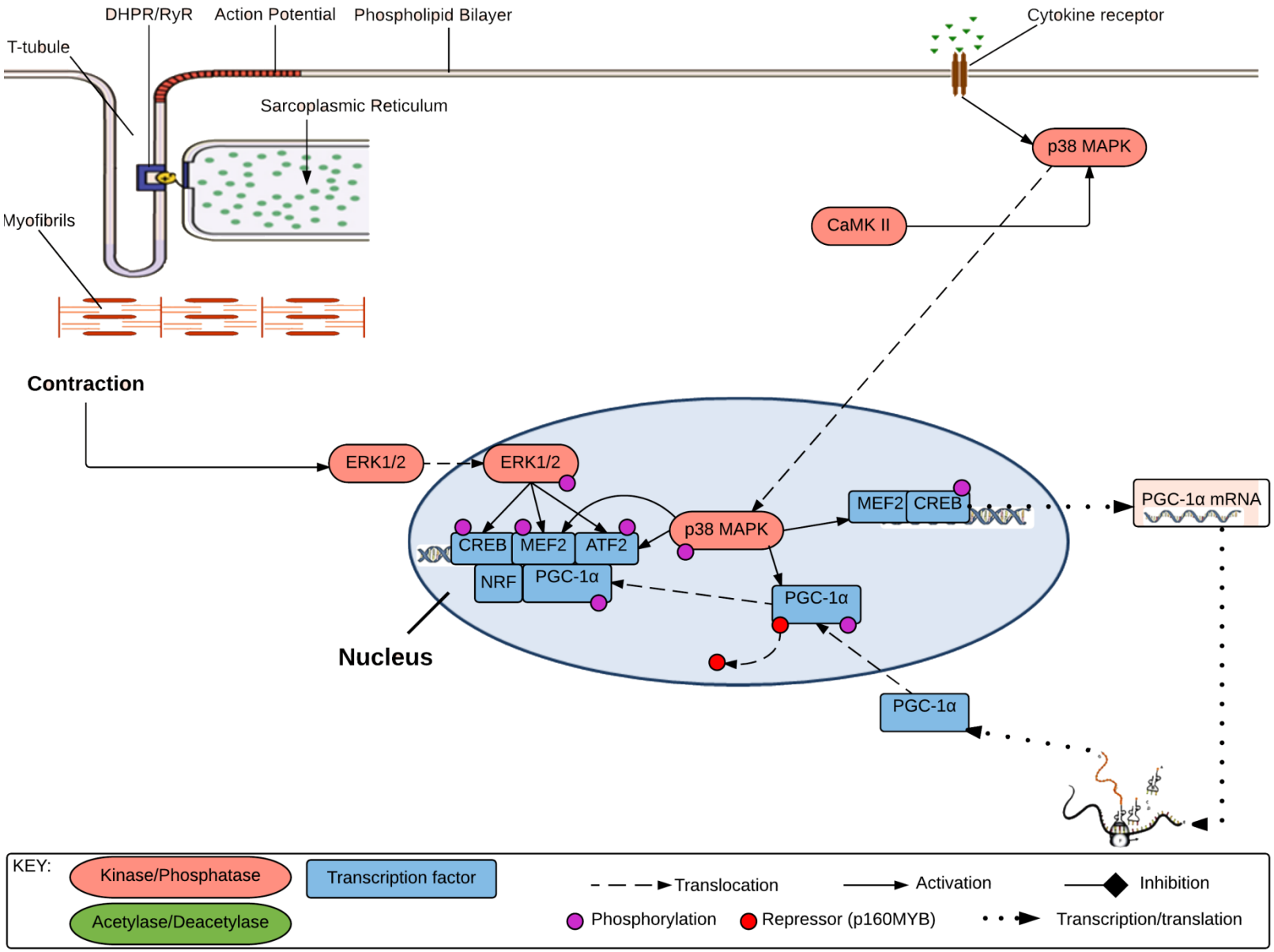


Figure 2.6: Cellular-stress signalling for mitochondrial biogenesis
See in text for abbreviations.

2.3.5 From PGC-1α to transcription of mitochondrial proteins.

The role of PGC-1α in mitochondrial biogenesis is ultimately as a transcription co-activator, and as such it co-operates with these transcription factors to increase transcription of mitochondrial proteins. Many nuclear encoded mitochondrial proteins contain a binding site for nuclear respiratory factor (NRF) 1 or 2 [131, 132, 462], and NRF-1 has been shown to physically interact with PGC-1α [585] and MEF2 [411]. Additionally, supressing NRF-1 in C2C12 cells that also overexpress PGC-1αdepresses mitochondrial biogenesis [585]. However, mice that overexpress NRF-1 do not show increased muscle oxygen consumption capacity, though some specific protein and enzyme content is increased [18]. Thus, it appears that a coactivation between NRF-1 and PGC-1α is necessary for transcription of mtDNA, but NRF-2 is still necessary for transcription of other nuclear genes necessary for mitochondrial biogeneisis. While this enables the transcription of the nuclear encoded mitochondrial proteins (NuEMPs), there remain a small number of proteins encoded in mtDNA that encode subunits of ETS complexes I, II, IV and V, and are thus important for formation of functional respiratory complexes [211].

Human mtDNA contains 37 genes; 13 subunits of the ETS/OXPHOS complexes, 2 ribosomal RNAs, and 22 transfer RNAs, necessary for mitochondrial protein synthesis [13, 46, 500]. As with nuclear DNA, transcription of mtDNA and control of mitochondrial-encoded mitochondrial protein (mtEMP) synthesis is regulated. In the case of mtDNA, transcription is regulated by the mitochondrial transcription factors A, B1 and B2 (Tfam/Tfb1m/Tfb2m) [151, 268]. Tfam is involved in binding and replicating mtDNA from both promoters, and mtDNA copy number is therefore well correlated with Tfam content [284, 568]. The role of Tfb1m and Tfb2m in the regulation of mitochondrial adaptation is not as clear; both Tfb1m and Tfb2m are capable of combining with Tfam to enable transcription of human mtDNA, though transcription is higher with Tfb2m than it is with Tfb1m [133].

In summary, as a result of the interaction of multiple intracellular signalling pathways, mitochondria-encoded mitochondrial proteins combine with nuclear-encoded mitohcondrial proteins to assemble new functional mitochondrial proteins (summarised in figure 2.7).

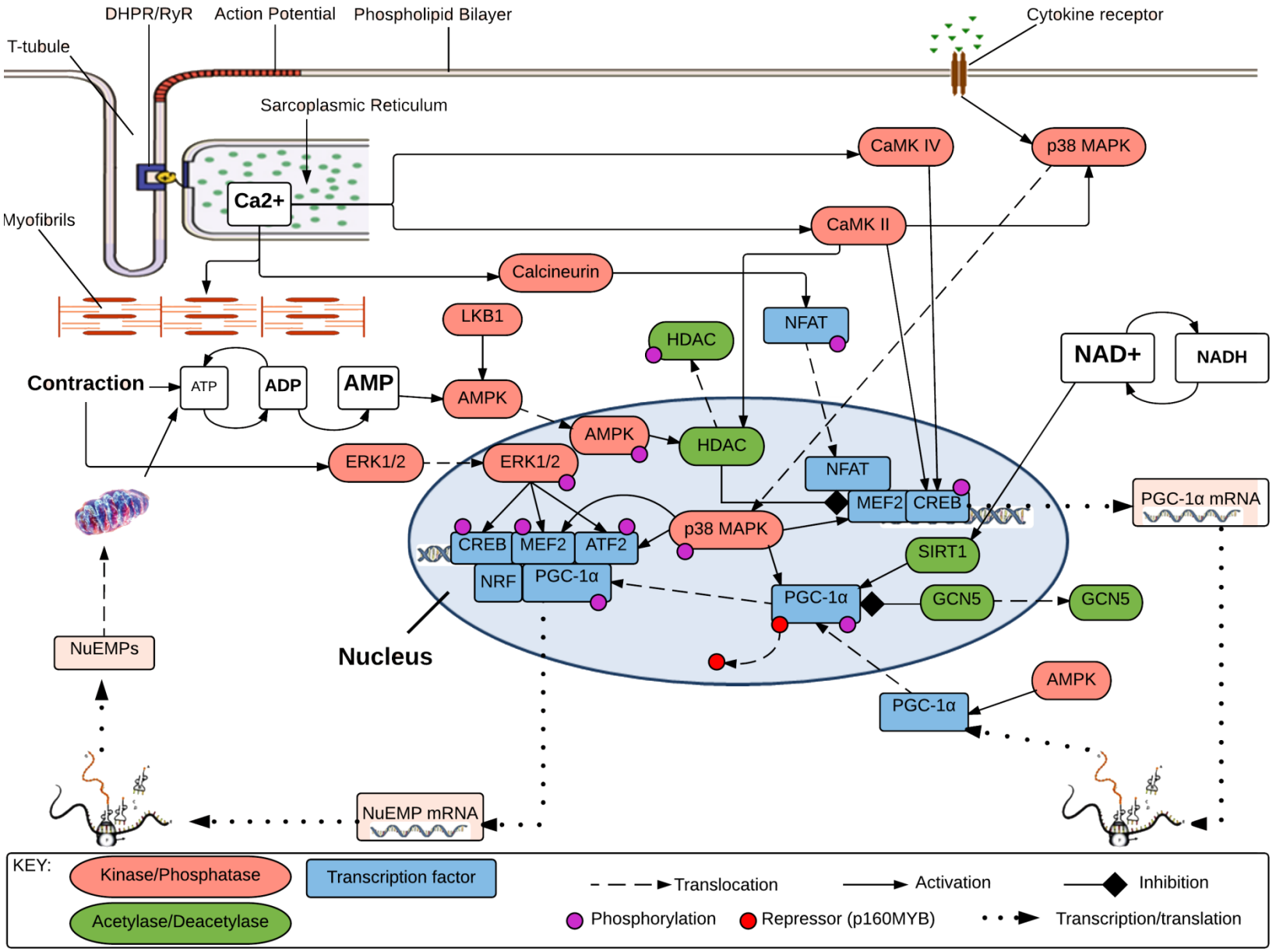


Figure 2.7: Summary of intracellular signalling for mitochondrial biogenesis.
See in text for abbreviations.

2.3.7 ROS-mediated signalling and mitochondrial biogenesis

Recent evidence suggests that within specific levels, ROS have a beneficial, or hormetic effect on mitochondria [421]. One proposed pathway for ROS-mediated mitochondrial biogenesis is through the energy sensor AMPK. Incubation of skeletal muscle cells (C2C12) with 300 μM H_2O_2 for 24 hours increases phosphorylation of AMPK, and this effect is diminished by incubation with the combination of 300 μM H_2O_2 and the antioxidant precursor N-acetyl-cysteine (NAC) [226]. As noted previously, it has been independently noted that ROS production increases with increasing exercise intensity [20]; and that phosphorylation of both the AMPK–α1 and AMPK–α2 isoforms is dependent on exercise intensity [80, 154]. Though no causal link has been established between these observations, it raises the possibility that ROS may contribute to the intensity-dependent response of AMPK to exercise. Further to this, ingestion of antioxidants (vitamin C, vitamin E, and alpha-lipoic acid) appears to blunt AMPK phosphorylation following sprint exercise in humans *in vivo* [345].

ROS-mediated signalling for mitochondrial biogenesis may also occur through cell stress pathways. Following hypoxia the p38 MAPK pathway is phosphorylated in cardiomyocytes [274]. These authors tested the role of mitochondrial ROS, and found that p38 MAPK phosphorylation was decreased when mitochondrial ROS production was attenuated by rotenone, thenoyltrifluoroacetone or myxothiazol (inhibitors of complex I, II and III respectively). Furthermore, treatment with antimycin-a or sodium azide (inhibitors of complex III and IV respectively) increased ROS production and p38 MAPK phosphorylation. Finally, when cardiomyocytes were incubated in H_2O_2 for 15 min at normoxia, there were similar increases in p38 MAPK phosphorylation as with hypoxia, which could be attenuated by the non-specific antioxidant NAC [274]. This finding was corroborated by Guyton et al. [179], who found that H_2O_2 treatment increased p38 MAPK phosphorylation in a range of cell types. Furthermore, H_2O_2 administration also increased ERK2 phosphorylation, an affect that could be prevented by NAC administration. They also demonstrated that increasing concentrations of H_2O_2 had a detrimental effect on cell survival, but this effect could be lessened by overexpression of MEK (the upstream ERK kinase) [179]. As p38 MAPK and ERK both play a role in skeletal muscle mitochondrial biogenesis, ROS-

induced phosphorylation of these kinase pathways may increase downstream phosphorylation of PGC-1α and other transcription regulators, and act to upregulate mitochondrial biogenesis.

There is also evidence to suggest ROS may have a role in calcium signalling. Several studies in cultured hepatocytes reveal that hydroperoxide incubation leads to an increase in cytosolic calcium concentrations [31, 32, 450], and this effect appears to be dependent on NOX [31, 32]. As mentioned previously, NOX has also been shown to regulated calcium release from the sarcoplasmic reticulum in skeletal muscle [204, 498], and cardiac muscle calcium concentration is dependent on superoxide [254]. Thus if ROS are capable of increasing calcium concentration, possibly through a NOX-dependent mechanism, then they may potentiate calcium-mediated signalling pathways for mitochondrial biogenesis in skeletal muscle.

In support of a role for ROS in mitochondrial adaptation in skeletal muscle, markers of mitochondrial biogenesis are decreased following antioxidant supplementation in rats, though their adaptation to training was maintained [495]. In contrast, antioxidant supplementation diminishes adaptation to training in both previously sedentary and habitually active humans [422]. A potential explanation for this difference (other than the obvious distinction of rats compared with humans) is that Strobel et al. [495] did not see an effect of their antioxidant supplement on skeletal muscle malondialdehyde (MDA) content, a marker of lipid peroxidation, where Ristow et al. [422] noted that exercise induced a 2-fold increase in muscle thiobarbituric acid reactive substances (TBARS), a different measure of lipid peroxidation. As these studies show different effects of the antioxidant supplement used on markers of exercise-induced oxidative stress, the difference seen in markers of adaptation may be due to differences in the effectiveness of the supplement used.

Similarly, Gomez-Cabrera et al. [166] found that increases in markers of mitochondrial adaptation to training are completely prevented, and time-to-fatigue (TTF) improves by only 27 minutes in rats supplemented with vitamin C. In rats not given vitamin C, signalling pathways are up-regulated by exercise, and TTF improves by 187 minutes [166]. However, Wadley & McConell [548] found no effect of antioxidants on the acute up-regulation of markers of mitochondrial biogenesis in response to

a single exercise bout in rats, when using the same vitamin C supplementation protocol as in Gomez-Cabrera et al. [166]. In addition, Higashida et al. [205] showed that an even higher dose of Vitamin C, in combination with Vitamin E, had no effect on increases in mitochondrial electron transport proteins and antioxidant content in rats, compared to a placebo supplement.

While in most cases antioxidants decrease markers of exercise-induced oxidative stress, there are some cases where touted antioxidants have had no effect [495], or even increased markers of oxidative damage post-exercise [81, 548]. To further cloud the role of ROS in the response to exercise, it has been suggested that both vitamin C and vitamin E may both have pro-oxidant roles. In one study, blood was drawn from humans supplemented with a placebo or 500 mg of vitamin C per day for 3 weeks in a crossover fashion. Two markers of oxidative DNA damage was were measured at several time points, and it was found that vitamin C supplementation decreased one marker (8-oxoguanine) but increased the other (8-oxoadenine) when compared to baseline, placebo supplementation, and after a washout period [396]. While inconclusive as to the net effect, this raises the possibility that vitamin C may induce some oxidative DNA damage and therefore have a pro-oxidant role at levels touted as having antioxidant effects. This study should be treated with caution, as the methods were criticised by Carr & Frei [69], who also concluded that the balance of evidence strongly favours an antioxidant role for vitamin C *in vivo*. Similarly, in some situations Vitamin E can potentiate lipid peroxidation and reviews conclude that beneficial effects of vitamin E are more likely to occur when given in concert with another antioxidant (like vitamin C) to augment vitamin E activity [52, 528].Thus there is both an unexplained inconsistency between the acute and long-term effects of antioxidants on mitochondrial response to training, and even a lack of agreement on the effects of antioxidants on training-induced oxidative stress. Two double-blinded, randomised control trials in humans have shown no effect of antioxidant supplementation on exercise-induced increases in VO_2max/peak, however, some markers of cellular adaptations (COX IV, SOD and Tfam protein content) were attenuated by antioxidants [346, 384]. Thus evidence of the role of antioxidants in mediating both cellular and performance changes remains unclear. A summary table of studies on antioxidants, oxidative stress and adaptation is presented in table 2.2.

Table 2.2: The effects of antioxidants on exercise-induced oxidative stress and markers of mitochondrial adaptation.

Model	Intervention duration	Exercise duration (intensity)	Exercise mode	Antioxidant supplement*	Effects on oxidative stress markers	Effects on adaptation and signaling	Ref.
Wistar rat	14 wk, 4 d/wk	90 min (70% VO_2max)	Treadmill running	VitE 1000 IU/kg + ALA 1.6 g/kg	Ex. and ex. + AO ↑ MDA similarly.	AO ↓ PGC-1α mRNA, ↓ PGC-1α + COX IV protein, ↓ CS + Mn-SOD activity. All markers ↑ similarly with ex. and ex.+AO.	[495]
Wistar rat	6wk, 5d/wk	25-85 min (75% VO_2max)	Treadmill running	VitC 500 mg/kg	N.R.	Ex. ↑ PGC-1α, NRF-1 and Tfam protein, TTF ↑185±107min. Ex.+ AO ↔ PGC-1α, NRF-1 and Tfam protein, TTF ↑ 27±47min.	[166]
Wistar rat	3 wk, 5 d/wk	25-85 min (75% VO_2max)	Treadmill running	VitC 500 mg/kg	N.R.	Ex. ↑ NRF-1, Tfam, Mn-SOD and GPX1 mRNA. Ex. + AO ↔ NRF-1, Tfam, Mn-SOD and GPX1 mRNA.	[166]

Wistar rat	3 wk, 6 d/wk	2 x 3 hours with 45 min break	Swimming	VitC 750 mg/kg + VitE 150 mg/kg†	N.R.	Ex. and ex. + AO ↑ Mn-SOD, CS protein and ↑ subunits of CI, CII, CIV and CV similarly. Ex. and ex. + AO ↔ CuZn-SOD. (PGC-1α N.R.)	[205]
Wistar rat	3 d	2 x 3 hours with 45 min break	Swimming	VitC 750 mg/kg + VitE 150 mg/kg#	Ex. ↑ plasma TBARS Ex. + AO ↔ plasma TBARS	Ex. and ex. + AO ↑ PGC-1α, CuZn-SOD, and Mn-SOD protein, and ↑ expression of subunits of CI, CII and CIV similarly (CS and CV N.R.)	[205]
Sprague-Dawley rat	Single bout	60 min	Treadmill running	VitC 500 mg/kg (7d)	AO ↑ muscle GSSG (sed. and ex.) Ex. ↑ muscle GSSG Ex., AO ↔ protein carbonylation	Ex. and ex. + AO ↑ phosphorylation of p38 MAPK, AMPK and ATF2, ↑ mRNA of PGC-1a, NRF1, NRF2, Tfam, GPX1, Mn-SOD or ecSOD. AO ↑ NRF-1 and NRF-2 mRNA.	[548]
Human (sed.)	4 wk, 3 d/wk	10 x 4 min (90% VO_2 peak), 2 min rest	Cycle	VitC 2 x 500 mg + VitE 400IU	Ex. and Ex. + AO ↔ skeletal muscle GSH, GSSG, 8-isoprostane.	Ex. ↑ Tfam and Mn-SOD protein, SOD activity Ex. + AO ↔ Tfam and Mn-SOD protein, SOD activity	[346]
Human (sed.)	11 wk, 3-4 d/wk	4-6 x 4-6 min (> 90% HR_{max}) + 30-60 min (70-90% HR_{max})	Running	VitC 1000 mg + VitE 235 mg		Ex. and ex. + AO ↑ VO_2 max, shuttle run test Ex. ↑ COX IV and PGC-1α protein Ex. + AO ↔ COX IV and PGC-1α protein	[384]

Human (sed.)	8 wk, 3 d/wk	40 min (65-90% VO_2max)	Cycle	VitC 1000 mg/d	N.R.	Ex. ↑ VO_2max 8.2±2.9 mL/kg/min Ex. + AO ↑ VO_2max 4.4±4.2 mL/kg/min (no significant difference)	[166]
Human (activity N.R.)	Single bout	30 min (75% VO_2max)	Treadmill running	VitC 500 mg/d or 1000 mg/d (2 wks)	Ex. ↑ whole blood GSSG/TGSH ratio, no effect of either AO dose. Ex. ↔ plasma TBARS, no effect of either AO dose. Ex. ↑plasma protein carbonylation, smaller ↑ with 500 mg VitC and smaller again with 1000 mg VitC.	N.R.	[162]
Human (sed. and trained)	4 wk, 5d/wk	20 min + 45 min	Run/cycle + circuit training	VitC 1000 mg/d + VitE 400 IU/d	Ex. ↑ muscle TBARS, ex. + AO ↔ muscle TBARS	Sed: Ex. ↑ mRNA of PGC-1α and PGC-1β, PPARγ, CuZn-SOD, Mn-SOD and GPX1. Ex. + AO ↔ mRNA of PGC-1β, CuZn-SOD, Mn-SOD and GPX1. ↑ mRNA of PGC-1α and PPARγ (much smaller ↑ than ex. alone) Trained: Ex. ↑ mRNA of PGC-1α and PGC-1β, PPARγ, CuZn-SOD, Mn-SOD and GPX1 Ex. + AO ↔ mRNA of PGC-1α, PGC-1β, PPARγ, Mn-SOD and GPX1. ↑ mRNA of CuZn-SOD (much smaller ↑ than ex. alone)	[422]

Human (activity N.R.)	Single bout	30min (80% VO_2max)	Treadmill running	VitC 1000 mg/d (2 wks)	Ex. ↑ plasma TBARS, ex. + AO ↔ plasma TBARS	N.R.	[9]
Human (activity N.R.)	Single bout	30+5min (60+90% VO_2max)	Treadmill running	VitC 1000 mg/d + VitE 592 mg/d + β-carotene 30mg/d (6 wks)	AO ↓ serum MDA content and exhaled pentane at rest and both ex. intensities, but did not affect rate of increase from placebo.	N.R.	[251]
Human (sed.)	Single bout	3 x 10 rep. (80% 1RM)	Eccentric bicep curl	VitC 12.5 mg/kg + NAC 10 mg/kg (AO given post-ex for 7d)	Ex. + AO ↑ lipid hydroperoxides more than ex. alone.	N.R.	[81]
Human (sed.)	Single bout	70 rep.	Eccentric bicep curl	VitC 3000 mg/d (2 wks)	Ex. ↑ whole blood GSSG/TGSH ratio 4 h and 24 h post-ex., ex. + AO GSSG/TGSH ratio not significantly changed post-ex.	N.R.	[57]

Human (activity N.R.)	Single bout	30min (80% VO_2max)	Treadmill running	VitC 1000 mg/d (2 wks)	Ex. ↑ plasma TBARS, ex. + AO ↔ plasma TBARS	N.R.	[9]
Human (activity N.R.)	Single bout	30+5min (60+90% VO_2max)	Treadmill running	VitC 1000 mg/d + VitE 592 mg/d + β-carotene 30mg/d (6 wks)	AO ↓ serum MDA content and exhaled pentane at rest and both ex. intensities, but did not affect rate of increase from placebo.	N.R.	[251]
Human (sed.)	Single bout	3 x 10 rep. (80% 1RM)	Eccentric bicep curl	VitC 12.5 mg/kg + NAC 10 mg/kg (AO given post-ex for 7d)	Ex. + AO ↑ lipid hydroperoxides more than ex. alone.	N.R.	[81]
Human (sed.)	Single bout	70 rep.	Eccentric bicep curl	VitC 3000 mg/d (2 wks)	Ex. ↑ whole blood GSSG/TGSH ratio 4 h and 24 h post-ex., ex. + AO GSSG/TGSH ratio not significantly changed post-ex.	N.R.	[57]

Abbreviations: d – days; wk – week; d/wk – days per week; /d - per day; rep. – repetitions; h –hour; HR_{max} –maximum heart rate; Vit(C/E) – vitamin (C/E); ALA – alpha-lipoic acid; AO – antioxidant; ex. – exercise; TGSH – total glutathione; N.R. – not reported; sed. – sedentary; ↑ - increase; ↔ - no change; ↓ - decrease.

***** For all acute studies (single bout of exercise) the duration of the supplement period is provided in brackets after the dose.

† Supplementation carried out for 5 weeks prior to 3 week training period (8 weeks total).

Supplementation was carried out for 6 days prior to first day of exercise (9 days total).

2.3.8 Summary of intracellular signalling for mitochondrial biogenesis

Mitochondrial biogenesis occurs in response to a number of stimuli, which can be influenced by exercise. Multiple pathways ensure there is a level of redundancy in the system, though there are some critical signal transduction mechanisms without which mitochondrial biogenesis cannot occur. Fluctuations in cellular calcium, AMP, NAD^+, and ROS concentrations activate a number of kinase, phosphatase, and deacetylase enzymes, which converge on the 'master regulator' protein PGC-1α. Deacetylation of PGC-1α and histones of DNA enable transcriptional activation, which results in transcription of nuclear DNA which encodes mitochondrial proteins. Among many nuclear-encoded mitochondrial proteins are the mitochondrial transcription factors (Tfam, Tfb1m and Tfb2m), which are necessary for transcription and translation of mitochondrial DNA. Mitochondrial transcription factors are then imported into mitochondria (along with other nuclear encoded proteins). Once inside mitochondria, they induce the transcription of mitochondrial DNA, which encodes a small number of subunits of some of the electron transport complexes. These mtEMPs are then integrated with NuEMPs to expand the mitochondrial reticulum (figure 2.7). As mitochondrial biogenesis is a result of coordination of multiple different stimuli and pathways, mitochondrial adaptation to different training paradigms is an active area of research. High-intensity training has emerged as one time-efficient method for promoting mitochondrial adaptation.

2.3.9 High-intensity training for promoting mitochondrial adaptation

Endurance-style training, typified by relatively long-duration, moderate-intensity exercise, has long been known as an effective way to promote mitochondrial adaptation (for review, see [213]). This intuitively makes sense, as anaerobic metabolism only provides the majority of ATP during muscular contraction for around 3 minutes of maximal exercise, after which aerobic metabolism (and thus mitochondria) are the dominant provider of ATP supply [332-334]. Alternatively, there is a growing body of evidence suggesting that short-term, high-intensity training is also capable of provoking mitochondrial adaptation. Table 2.3 summarises a number of studies that have investigated various markers of aerobic adaptation to high intensity exercise, and shows that only the studies which used

15+ -second sprint durations showed increases in citrate synthase activity or meaningful increases in VO_2peak – markers of mitochondrial density and whole-body aerobic capacity respectively.

Subsequent studies have compared markers of aerobic adaptations to endurance and sprint training. Gibala et al. [153] showed that repeated 30-second maximal efforts (sprint interval training, SIT) showed improvements in time-trial performance, cytochrome c oxidase activity, and protein content of two cytochrome c oxidase subunits comparable to increases seen in participants doing a more traditional endurance training protocol. SIT has also been shown to induce similar increases in protein content of PGC-1α in comparison to endurance training in 6 weeks of training [60]. Other high-intensity interval training (HIIT) studies have shown increases citrate synthase and cytochrome c oxidase activity and protein content, increases in nuclear localisation of PGC-1α, and increases in SIRT1 and Tfam protein content [295] as well as increases in mitochondrial respiration [100]. .

This shows that mitochondrial adaptation can be achieved at intensities above what are considered to be ‘aerobic’, but do not require maximal efforts.

Table 2.3: Studies on sprint interval training and markers of mitochondrial adaptation.

Sprint duration	Sprint intensity	Rest duration	Work:rest ratio	Repetitions	Training frequency	Training duration	Marker(s) of aerobic adaptation	Ref.
5 s	80% F_{max}	55s	1:11	2 x 8 (15 min between blocks)	4/wk	7wk	↔CS activity	[290]
5 s	*	55s	1:11	2 x 15 (15 min between blocks)	4/wk	7wk	↔CS activity, ↑~3% rel. VO_2peak†	[291]
10 s	Maximal effort	50s	1:5	15	3/wk	6wk	↔CS activity	[195]
15 + 30 s	Maximal effort	45s + 15min	1:3 + 1:30	2-6	2-3/wk	6wk	↑~10% CS activity	[233]
30 s	100% VO_2peak	30s	1:1	15	3/wk	8wk	↔CS activity, ↑~11% abs.VO_2peak	[170]
30 s	Maximal effort	4min (wks 1-4), ↓30s/wk thereafter	1:8 – 1:5	4-10	3/wk	7wk	↑~35% CS activity, ↑~7% abs. VO_2peak	[309]
30 s	Maximal effort	4min	1:8	4-7	3/wk	2wk	↑~38% CS activity	[61]
30 s	Maximal effort	4min	1:8	4-7	3/wk	2wk	↑~11% CS activity	[59]

Abbreviations: s – seconds; d – days; wk – weeks; /wk – per week; /d - per day; ↑ - increase; ↔ - no change; ↓ - decrease; CS – citrate synthase, abs. – absolute, rel. -relative.

*Intensity was altered between the two blocks of 15 sprints, the first block was the resistance that allowed a maximal pedalling velocity of 150 rpm during pretesting and was maintained throughout. The second block was adjusted weekly to a resistance that allowed a maximal velocity of 150 rpm during training.

† Though the increase seen was determined to be statistically significant, in absolute terms it was an increase of 1.5 mL/kg/min and likely to be within the measurement error of the test.

The results in table 2.3 showing changes in markers of aerobic adaptation following high-intensity training suggest that there must be some exercise-induced signals for mitochondrial biogenesis that are activated by high-intensity exercise. As mentioned in sections 2.3.3 and 2.3.4, some of the pathways for mitochondrial biogenesis show intensity-dependent responses to exercise, and this finding of increase in PGC-1α protein content is consistent with previous observations that high-intensity exercise acutely up-regulates both p38 MAPK [154] and AMPK [80, 154] signalling. One key difference between high-intensity exercise and sustained endurance exercise is the decrease in muscle and blood pH following intense exercise (section 2.1.9). Acidosis may influence the activity of some intracellular signalling pathways, and potentially plays a role in the adaptation of mitochondria to high-intensity exercise.

2.3.10 Acidosis and mitochondrial biogenesis

Very little work has been done investigating whether acidosis influences any mediators of mitochondrial biogenesis. In one study, Bishop et al. demonstrated that supplementing rats with 0.05 g.kg body mass^{-1} sodium bicarbonate, which induces alkalaemia, promoted increases in soleus mitochondrial respiration in response to training in comparison to a placebo supplement [43]. Some caution should be taken with this finding, as when normalised to citrate synthase activity - an estimate of mitochondrial mass, there was no significant difference between untrained control and trained rats supplemented with placebo, no significant difference between trained rats supplemented with placebo and trained rats supplemented with sodium bicarbonate, but there was a significant difference between the untrained controls and the trained rats supplemented with sodium bicarbonate. The lack of difference between the two supplemented groups could be interpreted as a lack of effect of sodium bicarbonate in comparison to placebo; however, this may also simply be an artefact of the number of rats used in the study – seven rats were included in each group and as such the study may not have had adequate statistical power to detect a difference between placebo and sodium bicarbonate supplemented rats.

Percival et al. [387] looked to investigate whether a sodium bicarbonate supplement (0.4 g.kg body mass^{-1}) influenced signalling for mitochondrial adaptation in humans. Participants completed a counterbalanced design where each participant was given a placebo or sodium bicarbonate, before a

HIIT cycling protocol. It was found that there were no differences between placebo or sodium bicarbonate supplementation in the acute response of CaMK II, AMPK, p38 MAPK or nuclear localisation of PGC-1α protein. However, in comparison to placebo the sodium bicarbonate supplement promoted a greater increase in PGC-1α mRNA expression 3 hours post exercise.

Edge et al. [121] used a different approach to assessing human skeletal muscle mRNA increase post-exercise in response to acidosis. Participants ingested either NH_4Cl (to lower blood pH) or a placebo, with mRNA of a number of mitochondrial and metabolic markers analysed from muscle biopsies taken pre-exercise, immediately post-exercise, and two hours post-exercise. At rest, NH_4Cl supplementation lead to greater expression of PGC-1α, CS and cytochrome-c mRNA, however, NH_4Cl supplementation prevented the exercise-induced increases in PGC-1α, CS, cytochrome-c, hexokinase II, GLUT4, and FOXO1 that were seen in the placebo group. This suggests that at rest, provoking acidosis enhances basal mRNA levels for mitochondrial proteins, but attenuates the exercise-induced increase in the same mRNA.

The results of Percival et al. [387] and Edge et al. [121] together show that increasing blood pH (with $NaHCO_3$) or decreasing blood pH (with NH_4Cl) causes a corresponding increase or decrease (respectively) in post-exercise PGC-1α mRNA expression. This may be caused by a difference in the regulation of signalling pathways that increase expression of PGC-1α mRNA. While blood pH seems to correlate to post-exercise PGC-1α mRNA expression, there are other effects associate with a change in blood pH, so it is unclear whether the observed effects seen are a direct effect of pH *per se*. It is therefore worth investigating what effect altering pH has on different signalling pathways upstream of PGC-1α.

2.4 Summary, Aims and Hypotheses

2.4.1 Summary

Mitochondrial biogenesis in skeletal muscle occurs in response to exercise, and is mediated by a complex network of transcription and translational regulators. These regulators are responsive to various stimuli, which represent challenges to homeostasis and place demand on mitochondria. Thus,

in accordance with the theory of supercompensation, mitochondria adapt to enable a greater response to future challenges, and to minimise future perturbations. Relatively recent research has found that high-intensity exercise is effective in promoting mitochondrial adaptation, and requires much shorter time commitment than traditional endurance training regimes. One of the many unique elements of high-intensity exercise is the development of an intramuscular acidotic state, and the effects that this acidotic state may have on mitochondrial adaptation are largely unknown.

Mitochondrial membrane potential is partly driven by a pH gradient, and thus pH is an important part of mitochondrial function. Mitochondria consume oxygen to maintain membrane potential and to facilitate ATP synthesis; however, studies on mitochondrial function are inconclusive as to whether acidosis has a detrimental effect on mitochondria, with a lack of agreement between *in vivo* and *in vitro* data. *In vitro* evidence with high ADP concentrations suggests mitochondrial function is not impaired by acidosis, but evidence from both *in vivo* experiments and *in vitro* experiments with physiological ADP concentrations suggests some impairment exists. Given the differences noted between different experimental concentrations of ADP, mitochondrial affinity for ADP is an important parameter that may explain why the effect of acidosis is not consistent between studies. Furthermore, while there is evidence of pH-induced changes in mitochondrial function, to date there has been no investigation of whether the different respiratory complexes of mitochondria are equally or disparately affected by acidosis. Thus determining the response of individual respiratory enzymes to changing pH would provide data on the response of the individual enzymes that comprise the ETS.

Previous studies demonstrate that induction of an acidotic state (such as that which occurs in high-intensity exercise) increases lipid peroxidation in rat brain, but decreases ROS production from skeletal muscle mitochondria, though there are limitations with the approaches used. However, it has also been shown that skeletal muscle ROS production increases during high-intensity exercise, suggesting a role for other sources of ROS from skeletal muscle. While skeletal muscle mitochondria have been investigated, there has been little attention given to how acidosis may affect other intracellular ROS sources that arguably play a greater role in exercise-induced ROS production. Further to this, activity of various pro- and antioxidant enzymes has been investigated, but little attention has

been given to the activity of these enzymes at a physiological pH range as opposed to an optimal pH. Investigating how these enzymes function within a physiological range of pH may help in determining why mitochondria produce less ROS and consume more ROS in an acidotic state.

In addition to promoting the adaptation of mitochondria, one of the many benefits of exercise is improvement in muscle pH buffering capacity. This means there may be differences in the effect of changing pH on muscle from trained and untrained individuals. Increasing muscle buffer capacity may confer a greater resistance to acidosis, and thus pH may have less effect on trained muscle than untrained muscle. To date this has not been investigated.

Mitochondrial adaptation is promoted by a number of stimuli associated with changes in cellular and mitochondrial homeostasis (such as AMP, intracellular calcium concentration, mechanical stress, cytokines, and ROS). If acidosis alters mitochondrial function, then some of these signalling molecules (and their associated intracellular signalling pathways) may be affected. Therefore, it seems reasonable to suggest that acidosis may also influence intracellular signalling. To date, one study has suggested that inducing alkalaemia prior to training in rats, while two other studies suggest that inducing acidosis or alkalaemia may impair/enhance the acute post-exercise increase in PGC-1α mRNA, a mediator of mitochondrial adaptation. However, the signalling pathways upstream that regulate expression of PGC-1α, and may be affected by acidosis, warrant further attention.

2.4.2 Aims

The purpose of this thesis was to address three of these questions regarding the effects of a physiologically relevant range of pH manipulation on skeletal muscle mitochondrial function, ROS emission, and intracellular signalling networks:

2.4.2.1 Study One

Does a physiological range of acidosis affect endogenous skeletal muscle mitochondrial function and ROS balance?

2.4.2.2 Study Two

Does exercise training affect the response of skeletal muscle mitochondria to an induced acidotic state?

2.4.2.3 Study Three

Does induction of an acidotic state affect the response of intracellular signalling networks that promote mitochondrial adaptation in response to contractile activity?

2.4.3 Hypotheses

2.4.3.1 Study One

It was hypothesised that at a lower pH, mitochondrial affinity for ADP may be increased as membrane potential may be artificially enhanced by the surrounding milieu. Furthermore, it was hypothesised that acidosis would impair antioxidant enzyme activity and either impair to a lesser extent or possibly even promote pro-oxidant enzyme activity, consistent with observations that skeletal muscle ROS production increases during high-intensity exercise.

2.4.3.2 Study Two

It was hypothesised that exercise would increase ROS emission capacity, but also increase muscle pH buffering capacity, leading to a diminished effect of pH on ROS emission and mitochondrial function from trained muscle.

2.4.3.3 Study Three

It was hypothesised that acidosis would impair the contraction-induced up-regulation of intracellular signalling networks, with a particular focus on those purportedly responsive to ROS (AMPK, ERK1/2 and p38 MAPK).

Chapter 3: The influence of acute acidosis on mitochondrial respiration, ADP sensitivity, and ROS balance enzymes in rat skeletal muscle.

3.1 Introduction

The mitochondrial reticulum is an integral and multifunctional facet of nearly all eukaryotic cells. In contracting skeletal muscle, one of the primary functions of mitochondria is the formation of adenosine triphosphate (ATP). Mitochondrial ATP synthesis occurs via oxidative phosphorylation, and is driven by a membrane potential that is governed in part by the maintenance of a proton gradient across the inner mitochondrial membrane [137, 485]. This proton gradient is formed by complexes I, III and IV of the electron transport system (ETS) pumping protons to the intermembrane space [472]. The permeability of the inner mitochondrial membrane to protons is limited in order to maintain this proton gradient; however, the outer mitochondrial membrane is permeable to protons. Therefore, mitochondrial function may be influenced by an decrease in intracellular pH, such as that which may occur during high-intensity exercise [197], as protons are effectively a substrate for ATP synthesis.

Acidosis may have a role in regulating mitochondrial function. A decrease in pH of only 0.1 to 0.2 pH units inhibits oxidative phosphorylation *in vivo* in contracting human skeletal muscle [245], and a decrease of ~0.5 pH units has a similar effect *in vitro* in isolated contracting feline soleus muscle [187]. Such studies rely on measurements of metabolites to estimate mitochondrial function, and are unable to investigate the precise mechanism(s) of impairment. In contrast, measurement of oxygen consumption from isolated mitochondria or permeabilised muscle fibres assesses mitochondrial function in isolation of glycolytic regulation, as mitochondria can be supplied with Krebs' cycle intermediates. However, the use of maximal oxygen flux *in vitro* or *in situ* may not provide a valid estimate of *in vivo* mitochondrial function, as small amounts or working muscle seem to be limited by mitochondrial capacity, whereas whole-body exercise is more limited by oxygen supply [432]. Measurement of oxygen flux *in situ* suggests that maximal ADP- and creatine-stimulated respiration are inhibited by acidosis, but submaximal ADP-stimulated respiration is unaffected [517, 551]. This indicates experimental ADP concentration is an important determinant of the effect of acidosis. As such, parameters such as ADP affinity also need to be considered – particularly when the import of some

substrates is membrane potential dependent (i.e., electrogenic) [378], and could be affected by increased proton concentration.

Another mitochondrial process dependent on membrane potential is the generation of reactive oxygen species (ROS). Superoxide (O_2^-) can be produced from a number of sites within muscle during rest and contraction (for review see [231, 232, 318, 319, 382]), and ROS production from skeletal muscle is known to increase during exercise [11, 20, 102, 540]. Hydrogen peroxide (H_2O_2) is formed via disproportionation of the very unstable superoxide by the enzyme superoxide dismutase (SOD) [144, 305]. Once thought of as detrimental, H_2O_2 appears to play a signalling role in muscle adaptation [166, 239]. Studies on brain tissue reveal that acidosis may have a role in enhancing lipid peroxidation [53, 415], and that mitochondria isolated from skeletal muscle produce less ROS, and/or consume more ROS at a lower pH [25]. However, there is no research on whether extra-mitochondrial sites of superoxide production and disposal in skeletal muscle may be affected by a change in pH. The main system used to consume ROS in rat skeletal muscle is the glutathione cycle, with oxidative muscle having relatively high concentrations of glutathione and activity of glutathione cycle enzymes [240]. This system uses a series of enzymes to convert superoxide into H_2O_2 through superoxide dismutase (SOD), and then to water by oxidising glutathione (GSH) to glutathione disulfide (GSSG) through glutathione peroxidase (GPX). GSSG can then be reduced back into GSH by glutathione reductase (GR), which uses NADPH as an electron donor. In skeletal muscle NADPH is largely supplied by isocitrate dehydrogenase (ICDH) [286] or glucose-6-phosphate dehydrogenase (G6PDH) [311]. The many enzymes involved in ROS production and disposal in skeletal muscle may also be sensitive to physiological pH shifts, and understanding how these systems respond to acidosis should provide insights into how physiological changes in pH may affect oxidative stress in skeletal muscle.

The method used to produce acidosis is an important methodological consideration. Studies investigating the effects of acidosis on tissues have used a variety of approaches to alter pH, such as the addition of lactic acid [53, 415, 517], or hypercapnia [187, 415]. In human cells (lung, fibroblast and adenocarcinoma lines) exposed to high CO_2 levels (60-120 mmHg), cell cycle progression and oxygen consumption are depressed in comparison to normal CO_2 levels (40 mmHg). However, this effect is

seen without change in media pH, and manipulating media pH to match hypercapnia-induced acidotic pH (but still maintaining 40 mmHg CO_2) does not show these effects [543]. Similarly, lactic acid dissociates and forms a proton in addition to a lactate anion, the latter of which may have a role as a mitochondrial substrate [126, 234]. Though the precise subcellular location of this process is debated, it is generally accepted that oxidation of lactate to pyruvate can support further mitochondrial pyruvate oxidation [126, 234, 591]. Therefore, while hypercapnia and lactic acidosis are physiological in nature, they can also confound interpretations as both lactate and CO_2 will have metabolic effects independent of the acidosis they induce.

The degree of acidosis in tissue is another important methodological consideration, as average intramuscular pH drops from a resting pH of ~7.1 to as low as 6.5 following maximal intensity exertion [64, 440, 441, 443, 446]. Though some studies have investigated the pH sensitivity of different aspects of mitochondrial function and/or skeletal muscle ROS balance, many of these have been conducted outside of physiologically relevant ranges. For example, Patel & Katyare (2006) determined that there were tissue-specific differences in the pH sensitivity of SOD measured from different rat tissues (not including skeletal muscle); however, enzyme function was determined at non-physiological levels of pH 8.0 and pH 9.2 [381]. It is therefore important that the pH range assessed is physiologically relevant to the tissue being investigated.

The aim of this study was to clarify the specific role of acidosis on skeletal muscle mitochondrial function and enzymes involved in modulating cellular oxidative stress. Specifically, this study investigated the effects of a physiological decrease in pH on skeletal muscle mitochondrial ADP affinity, respiration, and ROS emission, and on the activity of enzymes involved in ROS emission and disposal. It was hypothesised decreasing pH may inhibit maximal oxygen consumption, but increase affinity for ADP. Further it was hypothesised that decreasing pH may decrease oxidative stress, by way of decreasing mitochondrial ROS emission. Additionally, it was hypothesized that decreasing pH would influence enzyme activities such that net cellular ROS emission would be decreased, either by way of increased antioxidant activity or decreased oxidant production, or a combination of both.

3.2 Methods

3.2.1 Study animals

Male Sprague-Dawley rats aged 22.5±0.9 weeks old and weighing 680±22g (mean ±SD) were used in this study. These animals were used as no *in vivo* manipulations took place for this study, therefore it was considered ethical that muscle be taken from adult males being culled as part of colony maintenance. Though these rats may be considered older than other typically used, a review of rat aging based on life stages concluded an age of 6 months in a rat is roughly equivalent 18 years in humans [475]. Furthermore, studies on muscle function and aging using rat models have used rats aged 3-9 months old as 'young' groups, while 'old' groups are typically >20 months old [38, 67, 68, 219]. Animals in this study were housed in pairs in standard cages, with access to standard rat chow (Teklad Global 18% protein rodent diet, Harlan Laboratories) and tap water ad libitum. Environmental conditions were maintained at a temperature of 19-21°C and humidity 55-70%, with a 12-hour light-dark cycle. Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich Pty, and chemical concentrations are presented in mM.

3.2.2 Sample collection

Animals were anaesthetised with 3.5% vol/vol isoflurane mixed with medical grade oxygen (BOC gases), introduced into a closed chamber at a rate of 3 L per minute. Anaesthesia was continued until animals were unresponsive to both tail and toe pinch, at which point animals were killed by cervical dislocation, according to standard operating procedure as approved by the Animal Ethics Committee of the University of Auckland (SOP 517/2). The soleus muscle from both limbs was rapidly excised, a portion (~50 mg) of which was immediately placed in ice-cold Histidine-Tryptophan-α-Ketoglutarate (Custodiol® HTK, Alsbech Hähnlein, Germany) transplant buffer (180 histidine, 30 mannitol, 18 histidine.HCl, 15 NaCl, 9 KCl, 4 $MgCl_2$, 2 tryptophan, 1 potassium hydrogen 2-ketoglutarate, 0.015 $CaCl_2$.). This portion was always taken from the right hindlimb, which was arbitrarily but consistently the first muscle excised. The remaining portion of the right soleus and the entire left soleus were then snap frozen in liquid nitrogen and stored at -80°C until future analysis. HTK

buffer was selected after pilot work determined it to be a superior preservative in comparison to a different preserving solution (pilot work further detailed in appendix 2).

3.2.3 High-resolution respirometry

Muscles from HTK were dissected free of visible fat and connective tissue under microscope while in ice-cold assay medium (120 KCl, 10 KH_2PO_4, 10 HEPES, 10 MES, 3 $MgCl_2$, 0.5 EGTA, with 250 $mg.L^{-1}$ BSA, pH 7.1 at 37°C (modified from Komary et al., [262]). Muscle portions were then blotted dry on lint-free filter paper and the wet weight measured. Muscle tissue was then diluted 20-fold (weight/volume) in fresh ice-cold assay media and finely minced with scissors, following which they were homogenised on ice with an Omni TH rotor-stator homogeniser fitted with a hard tissue homogenising probe (Omni International, Georgia USA). Muscle homogenate was elected as a model which balanced the need for sensitive detection of changes in oxygen consumption to determine ADP affinity, with the intent to maintain a completely representative population of mitochondria (which can be lost during mitochondrial isolation).

Oxygen consumption measurement and ROS emission measurements were performed using high-resolution respirometry (Oxygraph-O2K, Oroboros Instruments, Innsbruck, Austria) with in-house made fluorescence LED modules (Tony Hickey, University of Auckland, as used in [203]) on 40 µL of crude muscle homogenate (corresponding to 2 mg wet tissue weight) in 2 mL of assay medium containing 10U horseradish peroxidase (HRP), 25U superoxide dismutase (SOD) and 50 µM Amplex UltraRed™. Each muscle was assayed simultaneously at four different pH levels (pH 6.5, 6.7, 6.9, and 7.1, at 37°C) to represent a physiological range of muscle pH. This was achieved by titration of the assay buffer with concentrated (10 M) potassium hydroxide (KOH) from native pH (pH ~5.0) prior to experiments. Immediately prior to assay, 20 mM of creatine hydrate was added to the assay medium to facilitate mitochondrial ADP transport through creatine kinase [454, 455, 553]. A multiple substrate-uncoupler-inhibitor-titration (SUIT) protocol was used to assess respiratory sensitivity to ADP at each pH. Malate (2), glutamate (10) and pyruvate (10) were added to support respiration through electron transport system (ETS) complex I (CI), and succinate (10) to support respiration through complex II (CII). Following substrate addition, ADP was titrated in steps from 5-2500 µM to measure ADP

sensitivity. Leak respiration was then induced by addition of carboxyatractyloside (5 μM) to inhibit mitochondrial adenine nucleotide transporter (ANT), then uncoupled electron transport system respiration was measured by addition of ionophore carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (FCCP, 1-1.5 μM). Residual oxygen consumption was determined by adding the inhibitor antimycin-a (4 μM). Respiratory measurements were conducted in duplicate at 37°C, with continuous stirring at 750 rpm. Mass-specific respiration rate ($pmol.O_2.s^{-1}.mg^{-1}$ wet weight) was determined for each concentration of ADP using Oroboros Datlab 5.1 software. ROS emission was not quantified (see section 3.3.2).

Apparent K_M for ADP ($K_{Mapp\text{-}ADP}$) and maximal oxygen consumption (V_{max}) during oxidative phosphorylation was determined by curve fitting experimental data using the Michaelis-Menten enzyme kinetics model in Prism 6 (GraphPad Software Inc., La Jolla, California). Briefly, residual error between measured flux rate and flux rate estimated by the Michaelis-Menten equation $F_{[ADP]} = (V_{max} \times [ADP])/(K_{Mapp\text{-}ADP} + [ADP])$ was minimised, where [ADP] is the final concentration of ADP in the chamber; $F_{[ADP]}$ is the flux rate for a given [ADP]; V_{max} is the maximal flux rate; and $K_{Mapp\text{-}ADP}$ is the apparent Michaelis-Menten constant, the [ADP] require to reach 50% of V_{max}.

3.2.4 Enzyme analyses

Approximately 10-20 mg of muscle was removed from -80°C storage and diluted 20 fold in ice-cold homogenising buffer (in mM: 50 KCl, 25 Tris-HCl, 2 $MgCl_2$, 1 EDTA with 0.5% vol/vol Triton X-100, pH 7.8 at 0°C) in 2 mL microcentrifuge tubes. A sterile stainless steel bead was added to each tube and samples were mechanically homogenised for 6 minutes at an oscillation frequency of 30 Hz in a Tissue Lyser II (Qiagen, Dusseldorf, Germany). Following homogenising, beads were removed from tubes and samples were centrifuged at 14,000 rcf for 10 min at 4°C. Analyses were conducted on the resulting supernatant fraction.

Enzyme activity of isocitrate dehydrogenase (ICDH), glucose-6-phosphate dehydrogenase (G6PDH), and glutathione peroxidase (GPX) were measured by following the production (ICDH and G6PDH) or consumption (GPX) of NADPH by measuring absorbance at 340 nm. Glutathione reductase

(GR) was measured by following the formation of 2-nitro-5-thiobenzoate anions (TNB^{2-}) by measuring absorbance at 412 nm. Xanthine oxidase (XO) and NADPH oxidase (NOX) activity were measured by following the fluorescence of Amplex Ultra Red (A36006, Life Technologies New Zealand Limited, Auckland, NZ) using an excitation filter of 535 nm and an emission filter of 595 nm. Superoxide dismutase (SOD) was measured using a colourimetric assay kit (ab65354, Abcam®, Victoria, Australia), measuring the formation of the formazan salt of water soluble tetrazolium-1, (WST-1) monitored by measuring absorbance at 450 nm. ICDH, G6PDH, GPX, GR and SOD activities were measured in clear 96-well plates in a Spectramax 340PC plate reader (Molecular Devices, California, USA). NOX and XO activities were measured in opaque black 96-well plates in an EnVision plate reader (Perkin-Elmer, Massachusetts, USA).

All enzymes except SOD, XO and NOX were measured in assay media containing(in mM): KCl (120), KH_2PO_4 (20), HEPES (10), $MgCl_2$ (5), DTT (1), EGTA (1). All enzymes were tested at three different pH values; 6.5, 7.0, 7.5, and all samples were measured in quadruplicate at 37°C. This range of pH values was decided after analysis of respirometry data suggested that a slightly wider range of pH may provide greater clarity. The use of 3 experimental pH values (rather than 4) allows quadruplicate measurement to be completed within a single 96 well microplate, decreasing technical variation that may be experienced conducting assays over multiple plates.

ICDH and G6PDH assays contained $NADP^+$ (0.5) and 1 mM substrate (isocitrate or glucose-6-phosphate respectively). GPX assay contained apocynin (0.4), NADPH (0.5), reduced glutathione (GSH, 0.5), and H_2O_2 (0.1), with excess GR as a linking enzyme. GR assay contained apocynin (0.4), 5-5’ dithiobis nitrobenzoic acid (DTNB, 1), NADPH (0.5) and oxidised glutathione (GSSG, 0.5).

NOX and XO assays were measured in assay media containing: Tris-HCl (140), 10 µM amplex ultra red, 1 U/mL SOD and 1 U/mL horseradish peroxidase. NOX assay contained NADPH (0.5), and 1 µM rotenone. Xanthine oxidase assay contained xanthine (0.5).

SOD activity was measured with minor modification to manufacturer instructions. The assay buffer provided was pH 8.0, close to the pH optimum of the enzyme 7.8 [257]. This was titrated down

to pH 6.5, 7.0 and 7.5 for assays using hydrochloric acid, and the volume of acid used was recorded. The buffer adjusted to pH 7.5 and 7.0 then had appropriate amounts of sample dilution buffer added so that the dilution of all 3 buffers from stock was equivalent.

Absorbance slope and pathlength for ICDH, G6PDH, GPX, GR and SOD reactions were recorded using Softmax Pro version 5 software (Molecular Devices) and used to calculate enzyme activities. ICDH, G6PDH, and GPX activities were calculated using a millimolar extinction coefficient of 6.22 $mM^{-1}.cm^{-1}$ for NADPH at 340 nm. GR activity was calculated using a millimolar extinction coefficient of 13.7 $mM^{-1}.cm^{-1}$ for TNB^{2-} at 412 nm [337]. SOD activity was calculated using a millimolar extinction coefficient of 37.0 $mM^{-1}.cm^{-1}$ for WST1-formazan at 450 nm. The activity of both NOX and XO were determined by interpolating the concentration of H_2O_2 from a standard curve at each pH. For all enzyme analyses, the coefficient of variation (CV) was determined from sample replicates. Assays were repeated if CV of replicate measures was >10%, after removal of a maximum of 1 outlier value (defined as ± 2 standard deviations from the mean). Enzyme data is expressed as enzyme units per milligram tissue ($U.mg^{-1}$), where 1 U is the amount of enzyme that catalyses 1 $\mu M.min^{-1}$ of substrate turnover. All mean enzyme activities and standard deviations are reported in Appendix 3.

3.2.5 Statistical analyses

Statistical analyses were conducted in Prism 6 (GraphPad Software, La Jolla California, USA) with statistical significance determined as $P < 0.05$. Data are presented as mean values ± standard deviation. For mitochondrial experiments, Km and Vmax values determined from Michaelis-Menten kinetic models were compared, and for enzyme analyses, mean activity rate of enzymes was compared. A Shapiro-Wilk test found that all variables were distributed normally; therefore data were analysed by 1-way repeated-measures ANOVA for effect of pH. Post-hoc tests with Sidak correction were used to compare means between all groups. Differences between means are reported as 95% confidence intervals of the magnitude of the difference (95% CI).

3.3 Results

3.3.1 V_{max} and $K_{Mapp\text{-}ADP}$

A main effect of pH was seen for V_{max} ($P = 0.023$). Post-hoc testing showed that respiration rate at pH 6.5 was significantly different to pH 7.1 ($P = 0.044$, 95%CI: 0.2 to 29.7 $pmol.O_2.s^{-1}.mg^{-1}$ wet weight). Similarly, a main effect of pH was seen for $K_{Mapp\text{-}ADP}$ ($P = 0.002$), and post-hoc testing showed $K_{Mapp\text{-}ADP}$ was lower at pH 6.5 compared with pH 7.1 ($P = 0.001$, 95%CI: 3.5 to 16.5 μM). No other pairwise comparisons reached statistical significance for either V_{max} or $K_{Mapp\text{-}ADP}$.

Table 3.1: Apparent $K_{Mapp\text{-}ADP}$ and V_{max} values measured in different pH conditions.
Data presented as mean ± standard deviation, n = 11. * = $p < 0.05$ compared to pH 7.1.

pH	$K_{Mapp\text{-}ADP}$ (μM)	V_{max} ($pmol.O_2.s^{-1}.mg^{-1}$ wet weight)
7.1	25.9 ± 12.2	102.2 ± 20.6
6.9	20.1 ± 7.5	101.8 ± 14.0
6.7	21.5 ± 10.9	99.4 ± 21.1
6.5	15.9 ± 5.5*	87.2 ± 13.4*

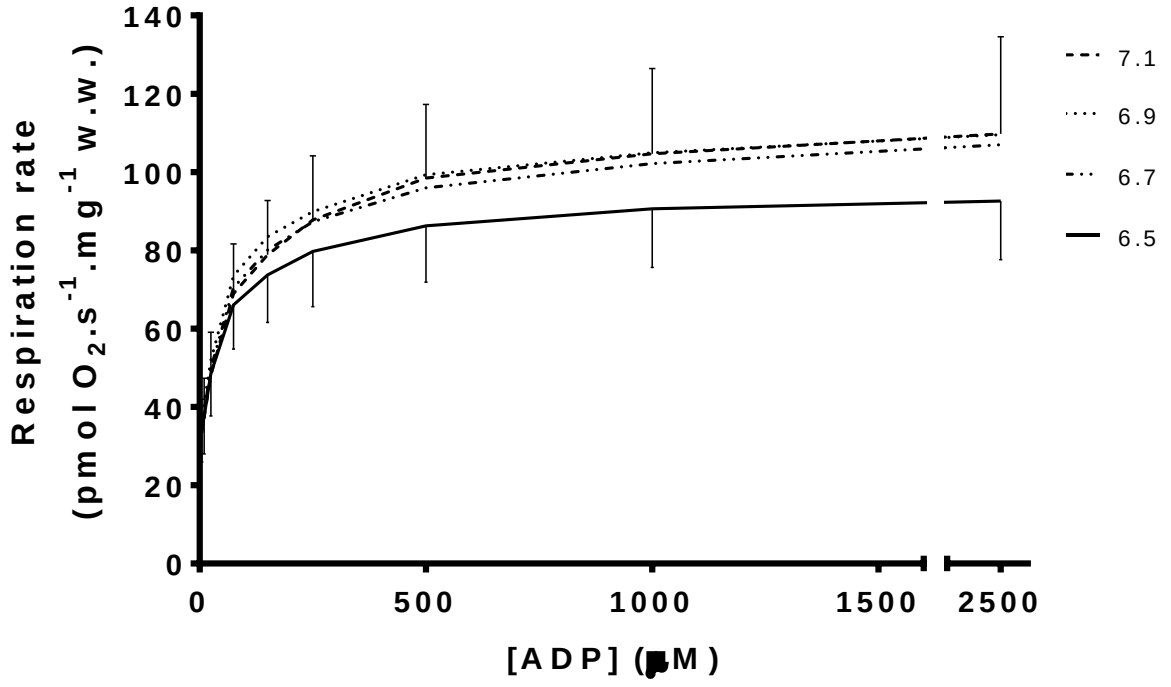


Figure 3.1: Respiration rate as a function of ADP concentration in different pH conditions.
Data are presented as mean values, n = 11 for each pH, and the error bars indicate the standard deviation (positive error for pH 7.1, negative for pH 6.5, error for pH 6.7 and 6.9 not shown).

Enzyme catalytic efficiency, estimated by dividing catalytic turnover rate (K_{CAT}) by substrate affinity constant (K_M), is sometimes used to compare an enzyme's affinity for different substrates [124]. An analogous comparison can be made between V_{max} and $K_{Mapp\text{-}ADP}$ (figure 3.2) to see whether the catalytic efficiency of ATP synthase may be affected by pH, essentially providing the rate of turnover for a given amount of substrate at each pH. There was no statistically significant difference for the estimated catalytic efficiency of ATP synthase between different pH conditions (P = 0.152).

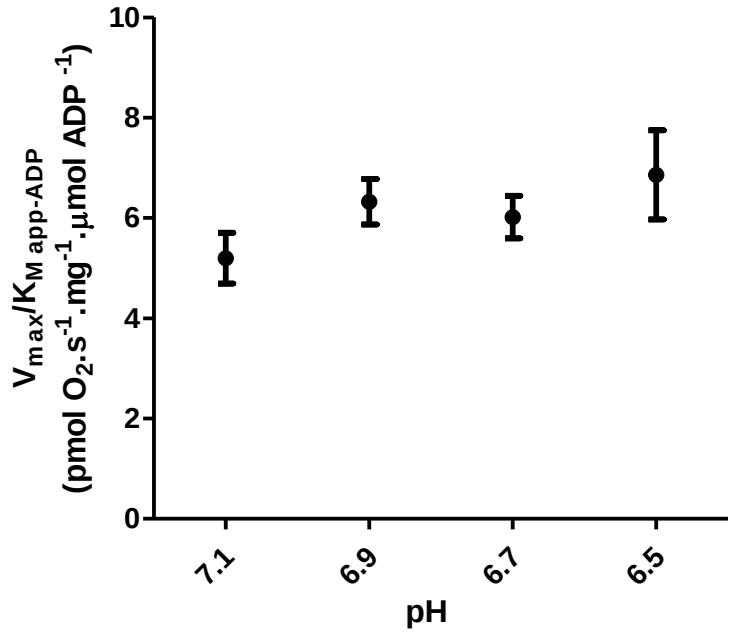


Figure 3.2: Catalytic efficiency of ATP synthesis ($V_{max}/K_{Mapp\text{-}ADP}$) in different pH conditions.
Data are presented as the mean ± standard deviation, n = 11.

3.3.2 ROS emission

ROS emission was not detectable from skeletal muscle samples. The assay was able to detect hydrogen peroxide during calibration, and proof-of-concept tests showed increases in signal with the addition of menadione (figure 3.3), which can reduce oxygen to superoxide [321, 338]. Additionally, ROS emission was detected when the assay was conducted on heart tissue (figure 3.4), indicating that it was possible to measure ROS released from tissue, but not the skeletal muscle samples.

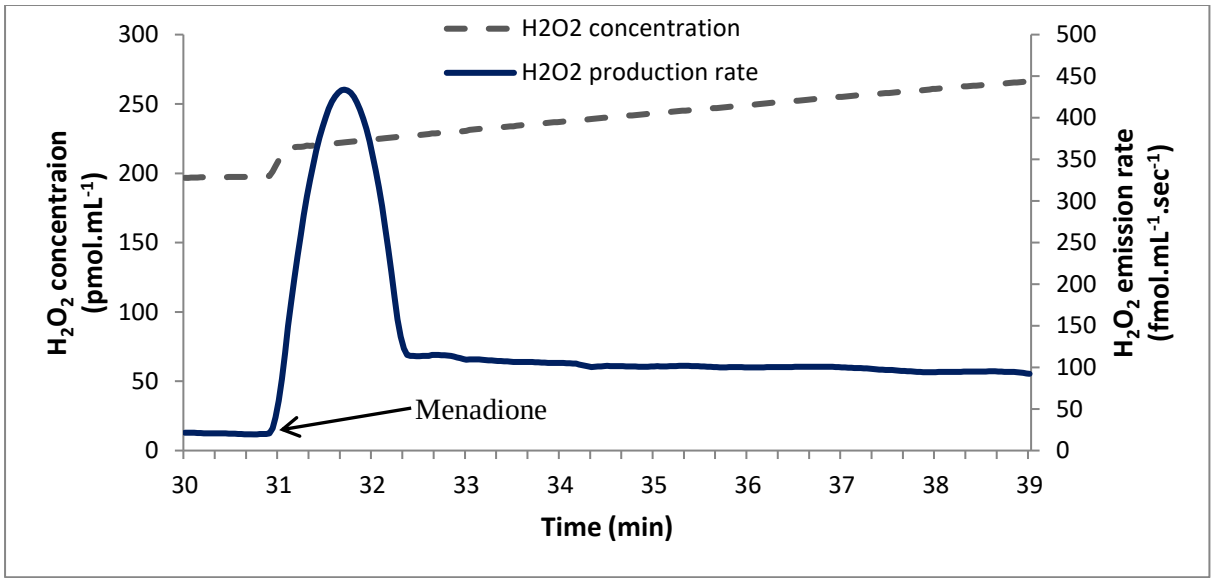


Figure 3.3: Addition of menadione generates a detectable increase in the rate of H_2O_2 production.

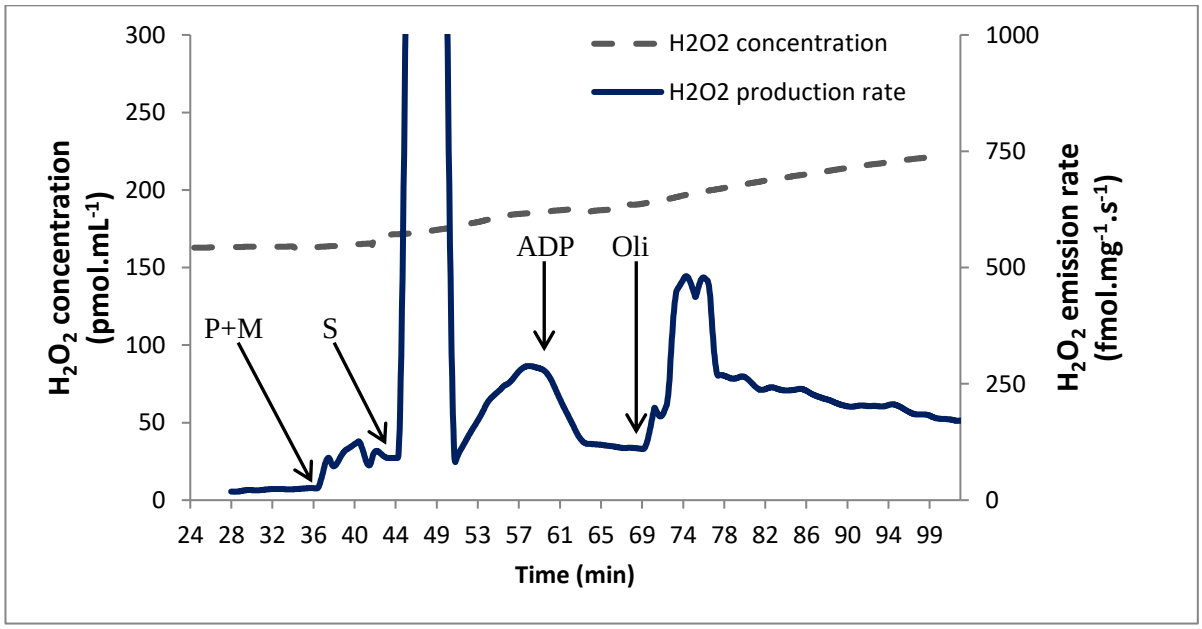


Figure 3.4: **Measurement of ROS emission from heart homogenate.**
Abbreviations: P+M – Pyruvate + Malate; S – Succinate; ADP – Adenosine diphosphate; Oli – Oligomycin.

3.3.3 Enzyme activities

3.3.3.1 Muscle NADPH Supply

Activity of ICDH decreased as pH decreased ($P < 0.001$, figure 3.5). Post-hoc tests indicate mean activity at pH 6.5 was lower at both pH 7.0 ($P < 0.001$, 95% CI: 10.16 to 14.41 $U.mg^{-1}$) and pH 7.5 ($P < 0.001$, 95% CI: 13.49 to 17.73 $U.mg^{-1}$). Mean activity at pH 7.0 was also lower than at pH 7.5 ($P = 0.004$, 95% CI: 1.20 to 5.45 $U.mg^{-1}$).

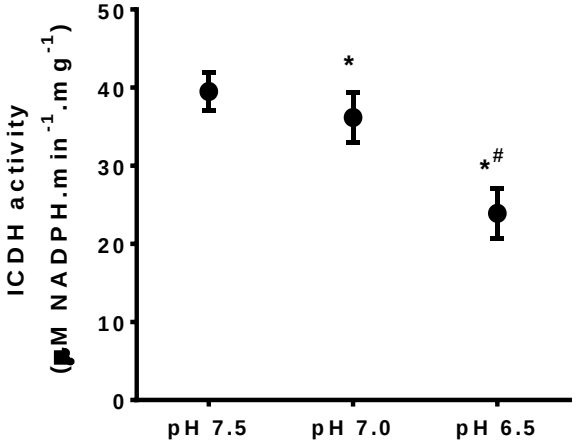


Figure 3.5: Soleus isocitrate dehydrogenase activity decreases as pH decreases.
Data presented as the mean ± standard deviation, n = 7. * = $p < 0.05$ vs pH 7.5, # = $p < 0.05$ vs pH 7.0.

Activity of G6PDH decreased as pH decreased ($P < 0.001$, figure 3.6). Post-hoc tests indicate mean activity was lower at pH 6.5 than at pH 7.5 ($P < 0.001$, 95% CI: 1.93 to 3.25 $U.mg^{-1}$), and lower at pH 7.0 than pH 7.5 ($P < 0.001$, 95% CI: 2.32 to 3.64 $U.mg^{-1}$). Mean activity was not significantly different between pH 7.0 and pH 6.5 ($P = 0.358$).

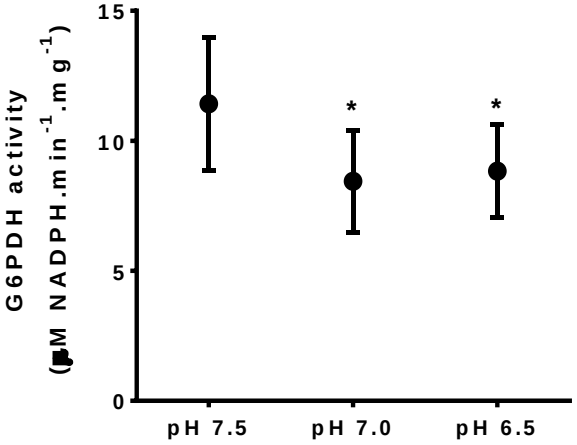


Figure 3.6: Soleus glucose-6-phosphate dehydrogenase activity decreases as pH decreases.
Data presented as the mean ± standard deviation, n = 11. * = $p < 0.05$ vs pH 7.5.

3.3.3.2 The glutathione cycle

Activity of GR increased as pH decreased ($P < 0.001$, figure 3.7). Post-hoc tests indicate mean activity was higher at pH 6.5 than at both pH 7.0 ($P < 0.001$, 95% CI: 0.87 to 2.80 $U.mg^{-1}$) and pH 7.5 ($P < 0.001$, 95% CI: 1.84 to 3.77 $U.mg^{-1}$). There was no significant difference in activity at pH 7.0 and pH 7.5 ($P = 0.058$, 95% CI: -0.03 to 1.97 $U.mg^{-1}$).

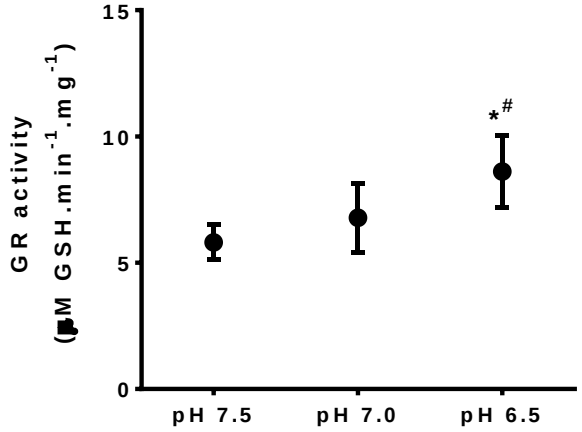


Figure 3.7: Soleus glutathione reductase activity increases as pH decreases.
Data presented as the mean ± standard deviation, n = 11. * = $P < 0.05$ vs pH 7.5, # = $P < 0.05$ vs pH 7.0.

There was no effect of pH on the activity of GPX when reducing H_2O_2 ($P = 0.836$, figure 3.8).

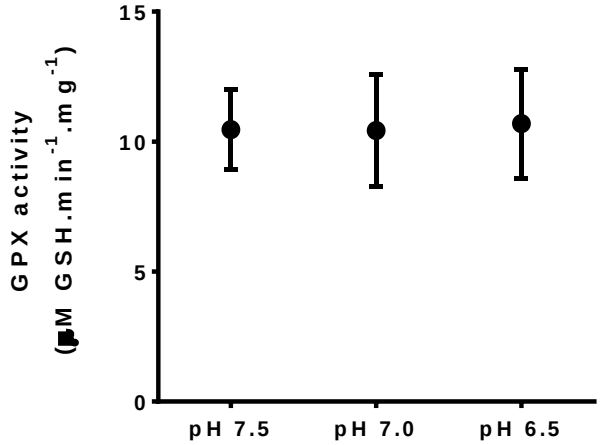


Figure 3.8: Soleus glutathione peroxidase activity is unaffected by lowering pH.
Data presented as the mean ± standard deviation, n = 11.

3.3.3.3 H_2O_2 Production

Activity of xanthine oxidase decreased as pH decreased ($P < 0.001$, figure 3.9). Post-hoc tests indicate mean activity was lower at pH 6.5 than at both pH 7.0 ($P < 0.001$, 95% CI: 0.77 to 1.67 $U.mg^{-1}$) and pH 7.5 ($P < 0.001$, 95% CI: 1.09 to 1.99 $U.mg^{-1}$). There was no significant difference between pH 7.0 and 7.5 ($P = 0.234$).

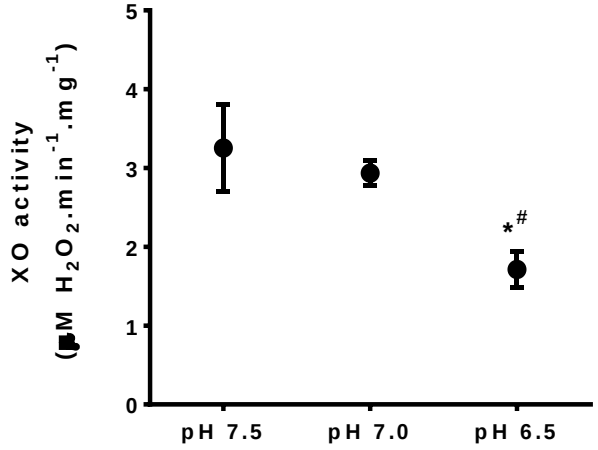


Figure 3.9: Soleus XO activity decreases as pH decreases.
Data presented as the mean ± standard deviation, n = 11. * = $P < 0.05$ vs pH 7.5, # = $P < 0.05$ vs pH 7.0.

Activity of SOD decreased as pH decreased ($P < 0.001$, figure 3.11). Post-hoc tests indicate mean activity was lower at pH 6.5 than at both pH 7.0 ($P = 0.002$, 95% CI: 1.13 to 3.17 $U.mg^{-1}$) and pH 7.5 ($P < 0.001$, 95% CI: 1.88 to 3.92 $U.mg^{-1}$). Mean activity was lower at pH 7.0 than at pH 7.5 ($P = 0.025$, 95% CI: 0.14 to 2.19 $U.mg^{-1}$).

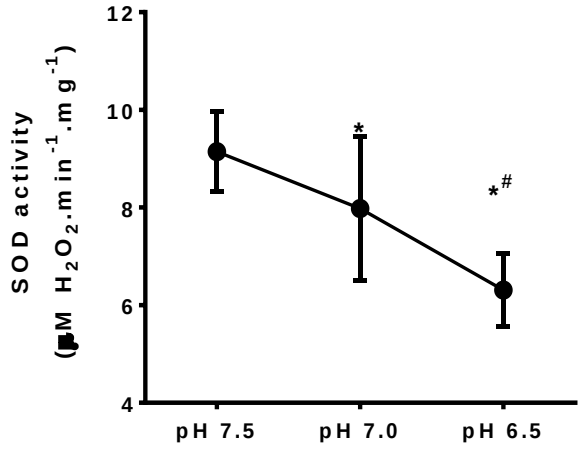


Figure 3.10: Soleus SOD activity decreases as pH decreases.
Data presented as the mean ± standard deviation, n = 11. * = $P < 0.05$ vs pH 7.5, # = $P < 0.05$ vs pH 7.0

3.4 Summary of findings

These results show a decrease in $K_{Mapp\text{-}ADP}$ and a trend for a decrease in maximal respiration rate at pH 6.5, but these parameters were similar between other pH values tested. Activity rates of ICDH, G6PDH, SOD, and XO were decreased by lowering pH, while the activity of GR was increased. The activity of GPX was unaffected by pH.

3.5 Discussion

3.5.1 Influence of pH on mitochondrial respiration

The present results show that decreasing pH depressed V_{max} during oxidative phosphorylation. It is possible that this depression of respiration may be a threshold event, as these data show respiration rate at pH 7.1 is very similar to pH 6.9 and pH 6.7, and then is depressed at pH 6.5. This contrasts with the findings of Jubrias et al [245] found that only a small decline (~0.2 pH units) was sufficient to depress oxidative phosphorylation in humans *in vivo*. Tonkonogi & Sahlin [517] showed a significant decline in respiration from pH 7.4 to pH 6.4, but this only occurs in a leak state, without ADP. This suggests that maximal oxygen consumption capacity of mitochondria *in vivo* may be inhibited by lowering pH; however, this may not be seen as consistently in *in situ* experimental models, as it may in part be offset by an increase in affinity for ADP (lower $K_{Mapp\text{-}ADP}$) in combination with supraphysiological concentrations of ADP.

As pH is decreased, $K_{Mapp\text{-}ADP}$ also decreases, indicating that changing the pH of the extra-mitochondrial space (the cytosol *in vivo*) may improve the rate of oxidative phosphorylation at lower ADP concentrations (i.e., in states approaching the leak state). This finding is consistent with similar work by Tonkonogi and Sahlin, who determined mitochondrial $K_{Mapp\text{-}ADP}$ of 20.0 ± 1.2 at pH 7.4, 10.3 ± 2.0 at pH 6.4 [517]. Though the present study ultimately finds similar results, this study was designed to measure $K_{Mapp\text{-}ADP}$ at a physiological temperature of 37 °C across a physiological pH range, rather than testing only two different pH levels at 25 °C as in [517]. Within maximally working muscles, the amount of ADP should rise before pH declines, so that when pH drops ADP should not be rate-limiting to mitochondrial oxidative phosphorylation. However, $K_{Mapp\text{-}ADP}$ in permeabilised muscle fibres has

previously been reported as ~100-200 μM [389], an order of magnitude higher than the results found in homogenate in the present study, and this may reflect a diffusion limitation of ADP in fibres. As such, even small changes in mitochondrial $K_{Mapp\text{-}ADP}$ may become important as pH decreases. An explanation for the decline in $K_{Mapp\text{-}ADP}$ with decreased pH could result from the increase in $[H^+]$, as ATP synthesis is dependent on ADP, P_i, and H^+. This could suggest that H^+ may be limiting, and that that oxidative phosphorylation at pH 6.5 can proceed more efficiently with a low [ADP], while changes in V_{max}, or approximately ½ V_{max} which may more closely reflect *in vivo* flux, differ little at pH 6.5. By analogy to the k_{cat}/K_M measure of enzymatic efficiency, the $V_{max}/K_{Mapp\text{-}ADP}$ indicates that the greatest efficiency of OXPHOS appears to be at a lower pH, as there is minimal change in the V_{max} as the $K_{Mapp\text{-}ADP}$ drops. However, it must be considered that estimation of $K_{Mapp\text{-}ADP}$ is not independent of V_{max} respiration, and in this instance it is possible that the decrease in $K_{Mapp\text{-}ADP}$ seen is simply due to lower V_{max} respiration. Measurement of ATP synthesis rates and/or membrane potential would help clarify whether these observed differences in V_{max} respiration and $K_{Mapp\text{-}ADP}$ are in fact impairments of mitochondrial function, or simply physiological responses to changes in proton supply/demand.

Both the present and previous studies have used oxygen consumption as a proxy of mitochondrial function in the context of ATP synthesis, where electron flow and delivery to oxygen maintains membrane potential to power ATP synthesis. However, oxygen consumption is not equivalent to ATP production, and the relationship between oxygen consumption and ATP production can vary [457]. Also, while it is generally assumed that a depression of oxygen flux reflects some form of mitochondrial dysfunction, in some circumstances it may indicate a normal physiological event. The arrangement of ATP synthase dimers along cristae apices should isolate dynamic changes in cytosolic pH through a localised enhancement of the pH gradient at cristae apices within mitochondria. However, if changes in cytosolic pH do impact mitochondrial pH gradients, then a depression in oxygen consumption could simply reflect a different origin of the mitochondrial membrane ΔpH. In this instance, if H^+ from the cytosol can pass through the outer mitochondrial membrane and maintain a constant ΔpH across the inner mitochondrial membrane, the ETS would need to pump fewer H^+ into the intermembrane space. Although speculative, this may explain why a depression of oxygen

consumption seems to occur only during low pH states – not because there is any mitochondrial dysfunction, but simply that the ETS does not have to pump as many electrons (and thus consume as much oxygen) to provide the ΔpH necessary for ATP synthesis when there is H^+ diffusion from the cytosol.

Further information could be gained by looking at ATP synthesis rate measurements, and ratios of ATP production to oxygen consumption (P:O ratio) under different pH conditions, to see if ATP synthesis is maintained in the event of an apparent depression in mitochondrial oxygen consumption with decreasing pH. Measurement of membrane potential could also answer whether membrane potential is indeed greater under more acidotic conditions. Membrane potential is also linked to ROS production, and one of the aims of this study was to measure mitochondrial ROS emission in different pH conditions. In this instance, ROS emission was unable to be detected from skeletal muscle mitochondria, even though proof-of-concept work shows increases in H_2O_2 with addition of menadione (figure 3.3), and detection of ROS from cardiac muscle (figure 3.4). It is possible that the sensor system used was not sensitive enough to detect ROS emission rates from skeletal muscle. The effect of pH on mitochondrial ROS emission remains worthy of investigation. However, mitochondria are not the only source of ROS in skeletal muscle, and other sources of ROS emission and disposal may also be affected by changes in pH.

3.5.2 Influence of pH on myocyte ROS dynamics

These present results show that low pH may enhance the ability of muscle to dispose of ROS through the glutathione pathway. This is shown by an increase in GR activity as pH decreases, while GPX activity is unaffected with changing pH. Thus, under lower pH conditions GR may be able to supply more GSH to meet the demands of GPX than at higher pH. GR also requires NADPH to resynthesise GSH, and though NADPH supply from ICDH and G6PDH does decline as pH decreases, the rate of NADPH supply remains in excess of the maximal rate of NADPH consumption by GR (figures 3.5 to 3.7). Therefore, NADPH supply is unlikely to limit glutathione resynthesis in a physiological pH range. Furthermore, these results also suggest that G6PDH is less sensitive to a decrease in pH than ICDH. This has implications for specific subcellular compartments, as inhibition

of mitochondrial G6PDH leads to the depletion of matrix NADPH pools and suggests G6PDH is a main contributor of NADPH within mitochondria [311]. In the cytosol, ICDH is the main contributor of NADPH [286], and also in mitochondria with limited glucose as a substrate [311]. Thus if ICDH activity is more sensitive to alterations in pH, cytosolic NADPH supply may be more prone to fluctuation with changes in cell pH, while mitochondrial NADPH supply may remain more consistent over the same fluctuation. The results of the present study suggest that the ability of isolated mitochondria to consume more exogenous H_2O_2 at a low pH observed by Banh & Treberg [25] could be explained by a greater capacity of the mitochondrial glutathione system to consume H_2O_2 at lower pH.

From the perspective of whole-cell ROS emission in skeletal muscle, production of H_2O_2 (and by extension superoxide) from XO is depressed at lower pH. Though at rest mitochondria convert a small fraction of consumed oxygen to superoxide [51], evidence suggests that during exercise mitochondrial ROS production declines during activity [110, 198], and thus does not explain the increase in ROS production with contractile activity in muscle. XO and NOX have now been suggested to be the main producers of ROS during contractile activity (reviewed in [405]). Considering pH alone, the present results would suggest that ROS production from XO should decrease during strenuous activity, however, these rates were determined with maximal substrate concentrations, which are not reflective of *in vivo* conditions. For example, *in vivo* substrate for xanthine oxidase (hypoxanthine) would be very low at rest as it is a product of AMP breakdown. However, AMP breakdown occurs to a greater extent during high-intensity exercise [499], so despite the maximal activity of XO to produce ROS is lower during acidotic conditions, the available substrate concentration would be higher during exercise strenuous enough to induce acidosis. The net result *in vivo* is likely to be an increase in ROS production from XO due to greater substrate availability at lower pH, despite the lower maximal enzyme activity.

This raises a limitation of this study that warrants further attention, the substrate affinity of these ROS balance enzymes. Some of the enzymes measured share common substrates (for example G6PDH and specific isoforms of ICDH both consume $NADP^+$, and NOX and GR both consume NADPH). Much like mitochondria, *in vivo* these enzymes will not have saturating presence of

substrates, so while this represents the maximal capacity of these enzymes under different pH conditions, it may not necessarily reflect how these systems cooperate *in vivo*. Hypothetically, if GR has a much greater affinity for NADPH than NOX does, a majority of NADPH could be used in an antioxidant role rather than the pro-oxidant role of NOX, despite maximal capacity of NOX being greater.

Spatial relationships between sites of ROS production and disposal have also not been considered in this experiment. The location of xanthine oxidase in skeletal muscle appears limited to the capillary endothelium [194], while NADPH oxidase is present around both the sarcolemma and blood vessels [235]. In skeletal muscle, GPX1 is expressed in the cytosol and mitochondria, while GPX4 is limited to the cytosol [111]. Two different isoforms of SOD are expressed, one cytosolic (CuZn-SOD) and one mitochondrial (Mn-SOD) [144]. Potentially, each isoform differs in pH sensitivity, and therefore specific subcellular compartments may be more affected by a change in pH than others. Moreover, as sites of ROS production and disposal are not immediately juxtaposed (that is, sites of ROS production are largely membrane-bound, whereas ROS disposal enzymes are not membrane bound and distributed more ubiquitously), there may be localised areas where ROS production exceeds disposal, despite an apparently greater average ROS disposal capacity.

3.6 Conclusion

Physiological acidosis appears to depress maximal ADP-stimulated mitochondrial respiration, and increase mitochondrial affinity for ADP, which may enhance oxidative phosphorylation at physiological concentrations of ADP. Measurement of additional parameters of mitochondrial function would help to clarify the physiological relevance of these observations. Skeletal muscle ROS disposal may be increased under acidotic conditions through increased activity of glutathione reductase. Measurement of XO activity suggests acidosis would depress XO-mediated ROS production, however, substrate supply was not considered, and there remains scope for investigation of other non-mitochondrial sources of ROS.

Chapter 4: Does voluntary exercise alter the effects of acute acidosis on skeletal muscle mitochondrial respiration and ROS emission?

Summary of preceding chapter.

In chapter 3 it was reported that acidosis increases mitochondrial ADP affinity and depresses maximal respiration, without effect on mitochondrial catalytic efficiency, in skeletal muscle. Results suggest that depression of respiration may only be seen if pH were to be lowered beyond an undetermined threshold point. Acidosis may also enhance ROS disposal through the glutathione cycle, while simultaneously depressing non-mitochondrial ROS emission, resulting in a net enhancement of ROS consumption capacity. Further work to address some of the questions raised by these findings is detailed in chapter 6.

This chapter sought to build on the work completed in chapter 3, by investigating whether exercise alters the effect of acidosis on skeletal muscle mitochondrial function. Respiration and ROS emission in permeabilised muscle fibres was determined from sedentary and rats that were exercised-trained by voluntary wheel running, across a broader pH range. Measurements of mitochondrial ROS emission are also presented, providing a measure of mitochondrial ROS generation that was not achieved in chapter 3.

4.1 Introduction

Skeletal muscle is a plastic organ that responds dynamically to various stimuli, so as to better resist future challenges to homeostasis. One example of this is mitochondrial adaptations to muscle contraction [5]. Early studies noted that trained individuals had higher oxidative enzymes activities in their muscle, and that activity of oxidative enzymes increased in response to exercise training interventions [163, 164]. While specific training methodologies remain under constant research and refinement, high-intensity interval training (HIIT) appears to mediate mitochondrial adaptations similar to longer-duration, steady-state, endurance exercise training [60, 93, 101, 554]. In rodents, typical running behaviour consists of short bursts of fast-paced activity, lasting on average 40 to 90 s at speeds around 40 $m.min^{-1}$ [426]. As such, voluntary running behaviour in rats could be called interval training in nature, though of a more moderate intensity. Skeletal muscle is responsive to voluntary wheel running in rats, showing increases in oxidative capacity, fibre area and capillarisation [228, 229, 605]. Additionally, mitochondrial ROS production is decreased in cardiac muscle following voluntary wheel running [246]. In rodent skeletal muscle, citrate synthase activity, a proxy for mitochondrial density [282], is more closely correlated with muscle type IIa content than type I content [107]. This suggests rodent type IIa muscle fibres may be more oxidative than type I fibres, and rodents may be phenotypically suited to aerobic high-intensity efforts.

An associated effect of high- or maximal-intensity exercise is the lowering of intramuscular pH, or exercise-induced acidosis [197, 442]. Acidosis has been proposed to inhibit oxidative phosphorylation [187, 245, 517, 551], though conclusive evidence of a direct effect of acidosis on mitochondrial function remains equivocal. Another function of mitochondria that may be influenced by acidosis is the production of reactive oxygen species (ROS). Mitochondrial ROS production generally occurs when high membrane potential opposes electron flow through the electron transport system (ETS) [265, 340]. Current data suggests that a lower pH decreases production of ROS by mitochondria isolated from skeletal muscle, and increases their capacity to consume ROS [25]. Data presented in chapter 3 of this thesis indicates that glutathione reductase activity is also increased at a lower pH, which may help explain the greater antioxidant capacity at lower pH. There are numerous sites of ROS

production within mitochondria, and complexes I and III in particular have large capacities for ROS production [78, 172, 524]. The pH difference across the mitochondrial inner membrane forms part of membrane potential, and superoxide production from these complexes is dependent on the membrane pH gradient [115, 277]. Decreased ROS production with decreasing pH could therefore be explained by an impact of changing pH on mitochondrial electron transport complexes, particularly complexes I and III. However, there has been no research to date on how changes in pH might affect individual complexes of the electron transport system.

Modulating changes in pH during exercise can also affect the response of mitochondria to exercise training. Skeletal muscle mitochondrial respiration increases post-training in rats supplemented with sodium bicarbonate ($NaHCO_3$) prior to each training session, relative to those given a placebo intervention [43]. This suggests a role of pH in regulating mitochondrial function and adaptations to exercise training. However, for pH to impact the ETS the pH must change within mitochondria, and there are endogenous mechanisms that buffer intracellular pH within muscle. Muscle buffering capacity (βm) represents the resistance of muscle to changes in pH, and comprises various transport proteins, the dipeptide carnosine, and bicarbonate. βm can be assessed *in vitro* by looking at expected vs observed changes in muscle pH following titration with acid [314], and $\beta m_{in\ vitro}$ often elevates in response to training status and correlates with repeat-sprint performance [120], but optimal training stimulus for improving βm is unknown. While some studies have shown $\beta m_{in\ vitro}$ increases following high-intensity exercise [478, 519, 565], others have demonstrated a decrease in $\beta m_{in\ vitro}$ and components of muscle buffering following high-intensity exercise [40, 42, 188, 358]. Ingestion of $NaHCO_3$ does not diminish the response of $\beta m_{in\ vitro}$ to training in comparison to a placebo [43], though this is not necessarily unexpected given that NaHCO3 primarily affects extracellular pH, and adaptation of buffering capacity is likely to be mediated by intracellular mechanisms. However, the effect that a voluntary exercise protocol may have on buffering capacity, and the subsequent response of mitochondria to acidosis is as yet unknown.

The aim of the present study was to investigate the effects of 8 weeks of voluntary wheel running on skeletal muscle $\beta m_{in\ vitro}$, and mitochondrial oxygen consumption and ROS emission, and ETS complex enzyme activities of rat soleus when exposed to a physiological range of pH challenges. It was firstly hypothesised that mitochondrial respiration and ETS activities would be higher, and ROS emission lower, in exercise-trained rats compared to sedentary rats. It was further hypothesised that decreasing pH would depress respiration, ROS emission, and enzyme activities of ETS complexes of muscle from sedentary rats, but exercise-trained rats would have greater muscle buffering capacity and therefore the effects of pH seen in sedentary rats would be less pronounced in exercise-trained rats.

4.2 Methods

4.2.1 Study Animals

Twenty age-matched (10-week old) male Sprague-Dawley rats were used in this study. One cohort (Sedentary, n = 10) was housed in pairs in standard cages, a second cohort (Exercise-trained, n = 10) was housed in pairs in activity cages (cage model 80859, Lafayette Instruments, Indiana, USA). Both cohorts had access to standard rat chow (Teklad Global 18% protein rodent diet, diet 2018, Harlan Laboratories, Wisconsin, USA) and tap water *ad libitum*. Environmental conditions were maintained at a temperature of 19-21°C, and humidity of 55-70%, with a 12 hour light-dark cycle. All procedures were approved by the Animal Ethics Committee of the University of Auckland (R001432).

4.2.2 Exercise and body composition measurement

For a period of 49-53 days, pairs of animals in the exercise-trained cohort had access to a running wheel, and paired voluntary activity was monitored by Activity Wheel Monitoring (AWM) software (Lafayette instruments). Briefly, an infrared (IR) LED and photodiode were positioned adjacent to the top of the wheel, which had 6 evenly-spaced tabs around the circumference of the wheel. The breaking of the IR beam was used to determine the number of revolutions (or part thereof) of the wheel. This was converted to a distance measurement based on a conversion factor of 1 complete revolution being equal to 1.1 metres distance.

Throughout the duration of the study, body mass and paired food intakes were recorded from both the sedentary and exercise-trainedd groups. One week prior to the commencement of experimental culls, rats were subjected to a dual-energy x-ray absorptiometry (DEXA) scan (Lunar Prodigy, GE Medical Systems, Wisconsin, USA). For this rats were anaesthetised using 3.5% v/v isoflurane in medical oxygen introduced into a closed chamber, and anaesthesia maintained using 2% v/v isoflurane delivered via nosecone while the DEXA scan was conducted.

During the study, the activity sensor on one cage appeared to be faulty as unexpectedly low daily distance measurements were recorded from day 23-36 of the study (daily distance was < 7% of that recorded in the other cages). When this sensor was replaced, measures went from an average daily

distance of 401 meters per day in the week prior to sensor replacement to 14418 meters per day in the week following sensor replacement. Based on assessment of body composition data, these rats were not determined to be outliers from the cohort and thus their muscle data are included for all parameters, but their data excluded for description of rat activity for the total duration of the study.

4.2.3 Muscle sampling

At the end of the exercise training period, exercise-trained animals were removed from wheel cages, and all animals were housed in standard cages and fasted overnight prior to being culled. Cage mates were culled in pairs by decapitation following anaesthesia with intraperitoneal injection of sodium pentobarbitone (60 $mg.kg^{-1}$). The right hindlimb was rapidly skinned, disarticulated and removed, following which the soleus was rapidly excised and a portion placed into ice-cold HTK buffer. The remainder was frozen in liquid nitrogen and stored at -80°C for future analysis of enzyme activities and buffering capacity. Soleus muscle was selected due to it having a high type I muscle fibre content, and properties of rat type I muscle fibres being more similar to humans than type IIa fibres [107, 412].

4.2.4 High-resolution respirometry

Muscle from HTK was dissected free of visible fat and connective tissue under dissection microscope while in ice-cold high energy relaxing solution (BIOPS, in $mM.L^{-1}$: 50 K-MES, 30 sucrose, 20 taurine, 20 imidazole, 15 PCr, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 6.56 $MgCl_2$, 5.7 ATP, and 0.5 dithiothreitol, pH 7.1 at 0°C). Fine tip forceps were then used to gently tease apart muscle fibre bundles, which were then permeabilized by placing them into fresh ice-cold BIOPS with 50 $\mu g\ mL^{-1}$ saponin. Bundles were gently agitated in this solution for 30 minutes on ice, before being placed into mitochondrial respiration medium (MiR05, in $mM.L^{-1}$: 110 sucrose, 60 K-lactobionate, 20 HEPES, 20 taurine, 10 KH_2PO_4, 3 $MgCl_2$, 0.5 EGTA and 1 mg/ml fraction V BSA, pH 7.1 at 37°C) for three 10 minute washes to remove substrates. Following the final wash, fibres were blotted dry on lint-free filter paper and bundles weighed.

Respiration and ROS emission were measured simultaneously on ~1.5 mg of permeabilised soleus muscle fibres in 2 mL of MiR05 using high-resolution respirometry with fluorescence LED

modules (Oxygraph-O2F, Oroboros Instruments, Innsbruck, Austria). Due to the apparent threshold of lowering pH seen in chapter 3, the pH range was lowered further to pH 6.2 for this study. Each muscle was assayed simultaneously at four different pH levels (pH 6.2, 6.5, 6.8, and 7.1, at 37°C). A multiple substrate-uncoupler-inhibitor-titration (SUIT) protocol was used to assess respiration and ROS emission at each pH. Prior to assay exogenous horseradish peroxidase (HRP, 10U), superoxide dismutase (SOD, 25U) and 50μM Amplex Ultra Red™ (Life Technologies A36006, Thermofisher Scientific) were added to media to facilitate fluorescent measurement of H_2O_2, and fluorescent sensors were calibrated with a known concentration of H_2O_2 added. After sensor calibration, fibres were added, followed by malate (2 mM) and pyruvate (10 mM) to support leak respiration through complex I (CI Leak). Following CI substrate addition, 5 mM ADP added to measure maximal oxidative phosphorylation respiration supported by complex I (CI Oxphos). Succinate (10 mM) was then added to support maximal oxidative phosphorylation respiration supported by complex I and II (CI&II Oxphos). Leak respiration was then induced by addition of carboxyatractyloside to inhibit mitochondrial ANT, and then addition of oligomycin to inhibit ATP synthase (CI&II Leak). Uncoupled electron transport system respiration was measured by addition of ionophore carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (FCCP). Residual oxygen consumption was determined by adding the inhibitor antimycin-a. Respiratory measurements were conducted at 37°C, with continuous stirring at 750 rpm, with media oxygen concentration maintained between 300-500 nmol $O_2.mL^{-1}$. Mass-specific respiration rate (pmol $O_2.s^{-1}.mg^{-1}$ wet weight) and total ROS emission rate (fmol $H_2O_2.s^{-1}$) were determined using Oroboros Datlab 5.1 software. ROS emission rates were corrected to tissue mass (fmol $H_2O_2.s^{-1}.mg^{-1}$ wet weight) during analysis in Microsoft Excel.

4.2.5 Enzyme analyses

Approximately 10-20 mg of muscle (wet weight) was diluted 20-fold in ice-cold homogenising buffer (in mM: 50 KCl, 25 Tris-HCl, 2 $MgCl_2$, 1 EDTA with 0.5% vol/vol Triton X-100, pH 7.8 at 0°C) in 2 mL microcentrifuge tubes. A sterile stainless steel bead was added to each tube and samples were mechanically homogenised for 6 minutes at an oscillation frequency of 30 Hz in a Tissue Lyser II (Qiagen, Dusseldorf, Germany). Following homogenising, beads were removed from tubes and samples

were centrifuged at 10,000 rcf for 5 min at 4°C. Analyses were conducted on the resulting supernatant fraction.

Citrate synthase (CS) activity was determined by measuring the production of 2-nitro-5-thiobenzoate anions (NTB^{2-}) at 412 nm. NTB^{2-} is formed from the reaction between dithiobisnitrobenzoic acid (DTNB) and sulfhydryl coenzyme A (CoA-SH), the latter formed from the CS catalysed production of citrate from oxaloacetate and acetyl-coenzyme A. CS was measured in buffer containing (in mM): 50 Tris-HCl, 0.2 DTNB, 0.1 acetyl-coenzyme A, 0.5 oxaloacetate, pH 8.0. Lactate dehydrogenase activity (LDH) was determined by measuring the oxidation of NADH at 340 nm from the LDH catalysed reaction between pyruvate and NADH to form lactate and NAD^+. LDH was measured in buffer containing (in mM): 50 mM Tris-HCl, 1 EDTA, 2 $MgCl_2$, 1 DTT, 0.15 NADH, 0.2 pyruvate, pH 7.0. CS and LDH activity of each sample were corrected to protein concentration determined by biuret method using BSA as a standard.

Sensitivity of ETS complex I, II, III and IV enzyme activities to pH were determined with minor modification to a published protocol [486]. Complex I activity was measured in buffer containing (in mM): 50 potassium phosphate buffer, 0.3 potassium cyanide, 0.1 NADH, 0.06 coenzyme Q_1, with 3 mg/mL BSA. Activity rate was determined by measuring the oxidation of NADH at 340 nm from the complex I catalysed reduction of coenzyme Q. Complex II activity was measured in buffer containing (in mM): 75 potassium phosphate buffer, 0.3 potassium cyanide, 20 succinate, 0.075 DCPIP, 0.075 decylubiquinone, with 1 mg/mL BSA. Activity rate was determined by measuring the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm. DCPIP is reduced by decylubiquinol formed from the oxidation of succinate by complex II. Complex III activity was measured in buffer containing (in mM): 75 potassium phosphate buffer, 0.5 potassium cyanide, 0.1 EDTA, 0.1 decylubiquinol, 0.075 cytochrome-c, and 0.025% v/v Tween-20. Activity rate was determined by measuring the reduction of cytochrome-c at 550 nm from the complex III catalysed oxidation of decylubiquinol. Complex IV activity was measured in buffer containing (in mM): 75 potassium phosphate buffer, and 0.06 reduced cytochrome-c. Activity rate was determined by measuring the oxidation of cytochrome-c at 550 nm

catalysed by complex IV to produce water. All ETS complex enzyme activities were measured at pH 6.2, 6.5, 6.8 and 7.1 on a pooled sample homogenate from each group (exercise-trained vs sedentary).

For all enzymes, samples were analysed in quadruplicate at 37°C in clear 96-well plates using a Spectramax 340PC plate reader (Molecular Devices, California, USA). Absorbance slope and pathlength for all reactions were recorded using Softmax Pro version 5 software (Molecular Devices), and used to calculate enzyme activities using extinction coefficients of 6.22 $mM^{-1}.cm^{-1}$ for LDH and complex I, 13.7 $mM^{-1}.cm^{-1}$ for CS, 19.1 $mM^{-1}.cm^{-1}$ for complex II, and 18.5 $mM^{-1}.cm^{-1}$ for complex III and IV. For all enzyme analyses, the coefficient of variation (CV) was determined from sample replicates. Analysis was repeated if CV of replicate measures was >10%, after removal of a maximum of 1 outlier value (defined as ± 2 standard deviations from the mean). Statistical analyses were performed on the mean activity determined from at least three replicates for each animal.

4.2.6 Muscle buffering capacity

$\beta m_{in\ vitro}$ was determined using an adaptation of the technique described by Mannion et al., 1993 [314]. Briefly, muscle stored at -80°C was freeze-dried and subsequently dissected free of connective tissue and blood. Dry muscle (~5-10 mg) was diluted 500-fold (dw/vol.) in 10 mM sodium fluoride and homogenised using a rotor-stator homogeniser (Omni TH, Omni International, Georgia, USA). Baseline homogenate pH was determined, and if necessary adjusted to > 7.1 with 10 mM NaOH. pH was titrated from baseline to < 6.5 using 10mM HCl, and the resulting titration curve fitted with a linear trendline. Buffer capacity was interpolated from this curve as the amount of HCl necessary to shift the pH from 7.1 to 6.5, and is presented as mM $H^{+}.kg^{-1}.pH^{-1}$.

4.2.7 Statistical analyses

Data are presented as arithmetic means ± standard deviation, unless otherwise specified. Statistical analyses were conducted in SPSS version 22 (IBM, New York, USA), with statistical significance determined as $p < 0.05$. Where statistically significant differences were determined, 95% confidence intervals of the difference (95%CI) are presented (with the exception of mitochondrial respiratory complex enzyme activities due to the use of a pooled sample).

Mitochondrial respiration and ROS emission were analysed by mixed linear modelling for each respiration state, with fixed effects of pH and exercise training, and a random effect attributed to each individual rat. Comparisons between respiration states were not made, as this would require a 3-way repeated measures ANOVA model, greatly diminishing the statistical power of the test. Moreover, differences between states are expected and not the comparison the study is investigating. Where significant main effects were found, post-hoc pairwise comparisons were made with Sidak correction. Where a significant interaction effect was determined, pairwise comparisons were made by custom hypothesis testing using Helmert contrast coding (custom test syntax presented in appendix 3). Custom test P values were then assessed using false discovery rate comparison [97].

Complex I, II, III and IV enzyme activities were analysed by repeated measures 2-way ANOVA for main effects of pH and exercise training, and an interaction effect between exercise training and pH. Where a significant main effect of pH was determined, post-hoc pairwise comparisons were made with Sidak correction. Body composition parameters were assessed by 1-way ANOVA for a difference between sedentary and exercise-trained animals. $\beta m_{in\ vitro}$, CS activity, and LDH activity were analysed by independent sample t-tests for differences between sedentary and exercise-trained animals. For muscle buffering capacity a significant effect was seen in Levene's test for equality of variances, thus equal variance was not assumed when interpreting the t-test result.

4.3 Results

4.3.1 Voluntary wheel exercise

Average daily running distance in the exercise-trained cohort was 6949±1853 metres per day per pair of rats (n=8 rats) for the total duration of the study. In the week prior to the DEXA scan, average daily distance was 9095±2960 meters per day per pair of rats (n=10 rats).

4.3.2 Body composition

Exercise-trained rats had a lower total body mass, less fat mass, and lower bone mineral content than sedentary rats, with no difference in lean mass (table 4.1 and figure 4.1).

Table 4.1: Exercise-trained rats have lower body mass and less fat-mass than sedentary rats.
Values are presented as mean (standard deviation), n = 10 (each group). * = P < 0.001 (Sedentary vs Exercised)

	Sedentary	Exercise-trained
Lean mass (g)	396.1 (34.8)	395.2 (27.4)
Bone mineral content (g)	13.4 (1.6)	10.5 (0.8)*
Fat mass (g)	163.6 (45.5)	51.4 (19.7)*
Total Body mass (g)	573.0 (55.5)	457.3 (39.9)*
Body fat (%)	28.9 (6.3)	11.4 (3.4)*

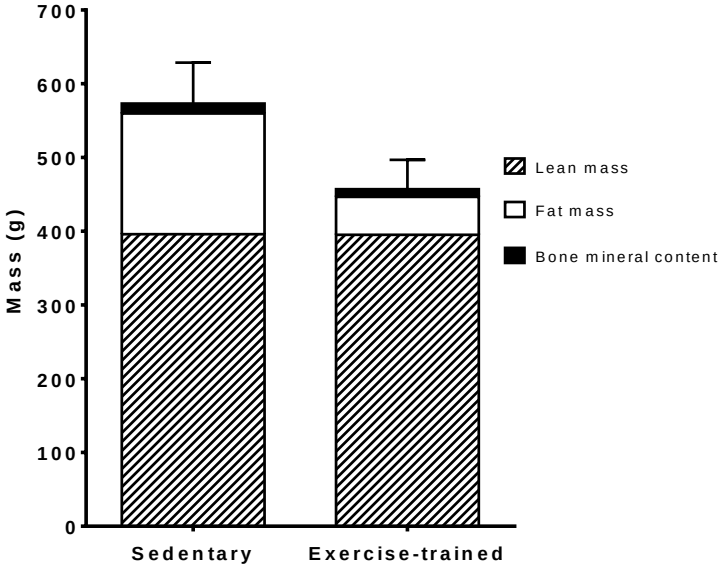


Figure 4.1: Exercise-trained rats have lower body mass due to less fat mass.
Bars represent mean data, error bar is standard deviation of total body mass. N = 10 (each group)

4.3.3 Muscle buffering capacity

Mean buffering capacity ($\beta m_{\ in\ vitro}$) of soleus was significantly greater in exercise-trained rats (152.6±5.4 mM $H^+.kg^{-1}.pH^{-1}$) compared to sedentary rats (138.3±11.4 mM $H^+.kg^{-1}.pH^{-1}$, P = 0.004, 95% CI: 5.6 – 22.9 mM $H^+.kg^{-1}.pH^{-1}$, figure 4.2).

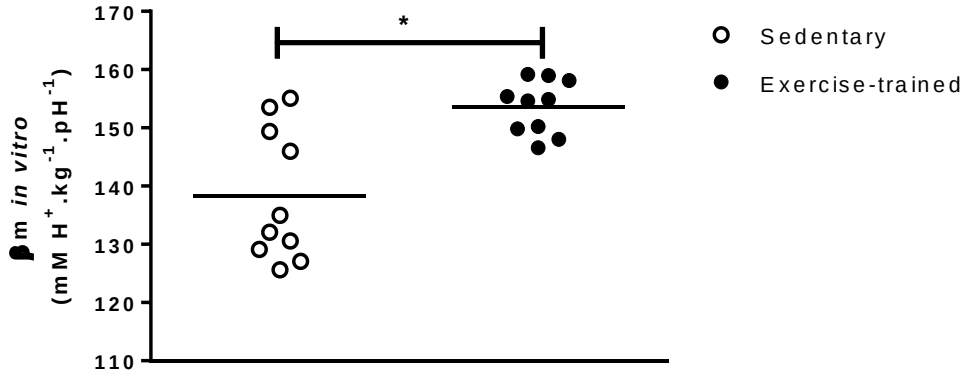


Figure 4.2: **Exercise elevates soleus βm *in vitro* in exercise-trained rats (n = 10) relative to sedentary rats (n=10).**
Data presented as individual samples (filled/unfilled circles) with group mean (horizontal bar).
* = P < 0.05 (Sedentary vs Exercise-trained).

4.3.4 Enzyme activities

Mean CS activity was 42% higher in soleus from exercise-trained rats (1625 ± 387 nM citrate.min^{-1}.mg $protein^{-1}$) than sedentary rats (1139 ± 387 nM citrate.min^{-1}.mg $protein^{-1}$, P = 0.011, 95% CI: 125 – 874 nM citrate.min^{-1}.mg $protein^{-1}$, figure 4.3).

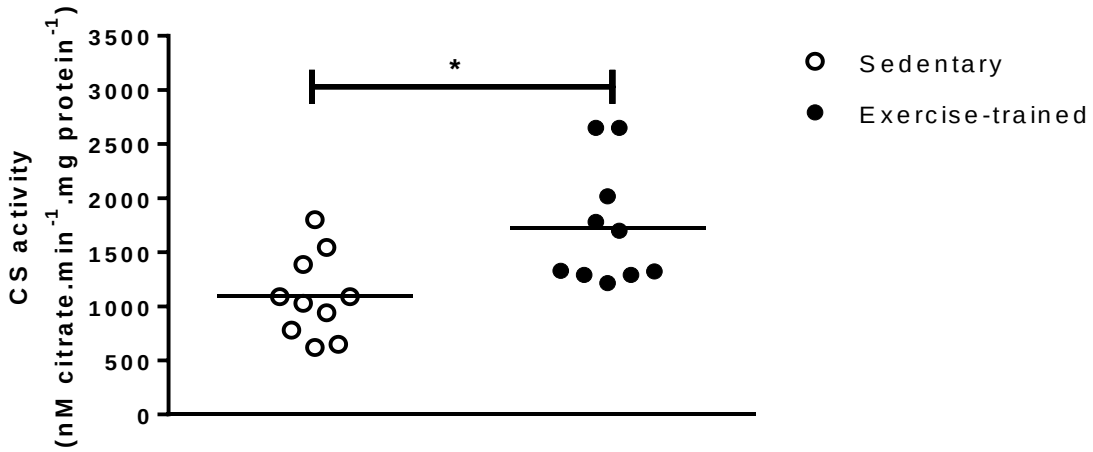


Figure 4.3: Soleus citrate synthase (CS) activity is greater in exercise-trained rats (n=10) in comparison to sedentary rats (n=10).
Data presented as individual samples (filled/unfilled circles) with group mean (horizontal bar).
* = p < 0.05 (Sedentary vs Exercise-trained).

Mean LDH activity was 40% higher in soleus from exercised rats (2373 ± 475 nM NADH.min^{-1}.mg protein^{-1}) than sedentary rats (1694 ± 361 nM NADH.min^{-1}.mg protein^{-1}, P = 0.005, 95% CI: 282 – 1075 nM NADH.min^{-1}.mg protein^{-1}, figure 4.4).

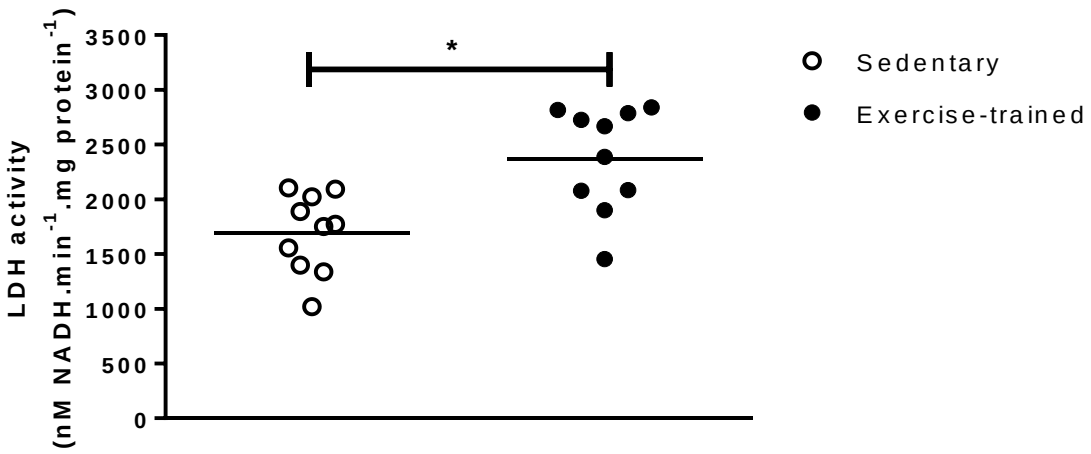


Figure 4.4: Soleus lactate dehydrogenase (LDH) activity is greater in exercise-trained rats (n=10) in comparison to sedentary rats (n=10).
Data presented as individual samples (filled/unfilled circles) with group mean (horizontal bar).
* = p < 0.05 (Sedentary vs Exercise-trained).

Buffer capacity showed a significant positive correlation with CS activity (Pearson r = 0.452, p = 0.045, Fig. 4.5).

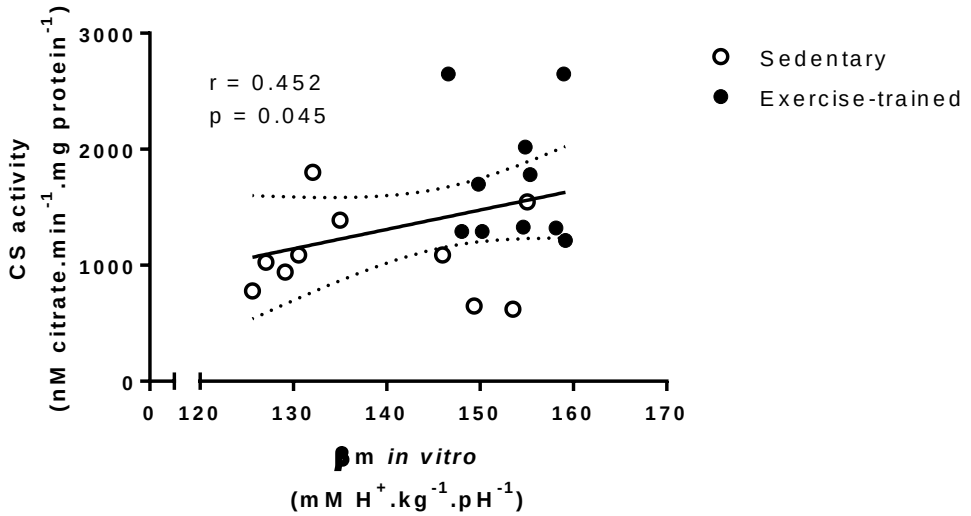


Figure 4.5: βm *in vitro* weakly correlates with CS activity in sedentary (n=10) and exercise-traine d (n=10) rats.
Data presented as individual samples (filled/unfilled circles), with trendline ± 95% CI.

Buffer capacity also showed a significant positive correlation with LDH activity, which was stronger than the correlation with CS activity (Pearson r = 0.647, p = 0.002, Fig. 4.6).

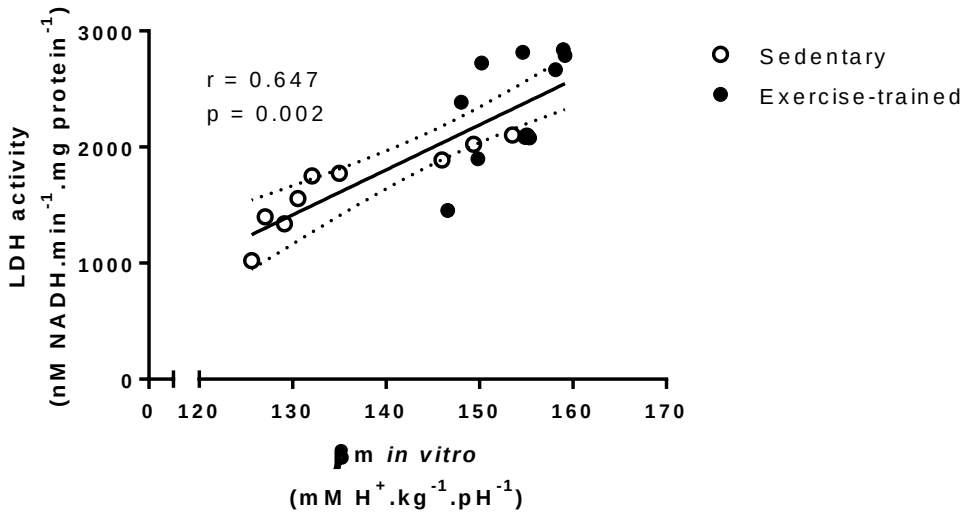


Figure 4.6: βm *in vitro* correlates with LDH activity in sedentary (n=10) and exercise-trained (n=10) rats.
Data presented as individual samples (filled/unfilled circles), with trendline ± 95% CI.

Complex I activity was ~15% higher in exercise-trained animals (main effect of exercise training, $P < 0.001$, figure 4.7A), and in both cohorts activity was depressed by ~14% per 1.0 unit decrease in pH (main effect of pH, $P < 0.001$, figure 4.7A). There was no significant interaction effect between exercise training and pH on complex I enzyme activity ($P = 0.846$). Complex II activity was ~80-90% higher in exercise-trained animals (main effect of exercise training, $P < 0.001$, figure 4.7B), and depressed with decreasing pH in both cohorts (main effect of pH, $P < 0.001$, figure 4.7B). There was a significant interaction effect between exercise training and pH ($P < 0.001$, figure 4.7B), however, the magnitude of this difference was small (complex II activity was depressed by 20% per 1.0 unit decrease in pH in the sedentary cohort, in comparison to 18% per 1.0 unit decrease in pH in the exercise-trained cohort). Complex III activity was ~10-50% higher in exercise-trained animals (main effect of exercise training, $P < 0.001$, figure 4.7C), and in both cohorts decreasing pH depressed enzyme activity (main effect of pH, $P < 0.001$, figure 4.7C). There was a significant interaction effect between exercise training and pH on complex III activity ($P = 0.003$, figure 4.7B), with depression of complex III activity less in exercise-trained animals (23% decrease in activity per 1.0 unit pH decrease) than in sedentary

animals (47% decrease in activity per 1.0 unit pH decrease). Complex IV activity was ~110-140% higher in exercise-trained animals (main effect of exercise training, $P < 0.001$, figure 4.7D), and in both cohorts complex IV activity increased as pH decreased (main effect of pH, $P < 0.001$, figure 4.7D). There interaction effect between exercise training and pH on complex IV activity approached significance ($P = 0.06$), with a 32% increase in activity per 1.0 unit pH decrease in exercise-trained animals and a 39% increase in activity per 1.0 unit pH decrease in sedentary animals. Activity rates of mitochondrial respiratory complexes ($nM.min^{-1}.mg\ protein^{-1}$) from both cohorts at each pH are presented in figure 4.7, and mean values can be found in appendix 3.

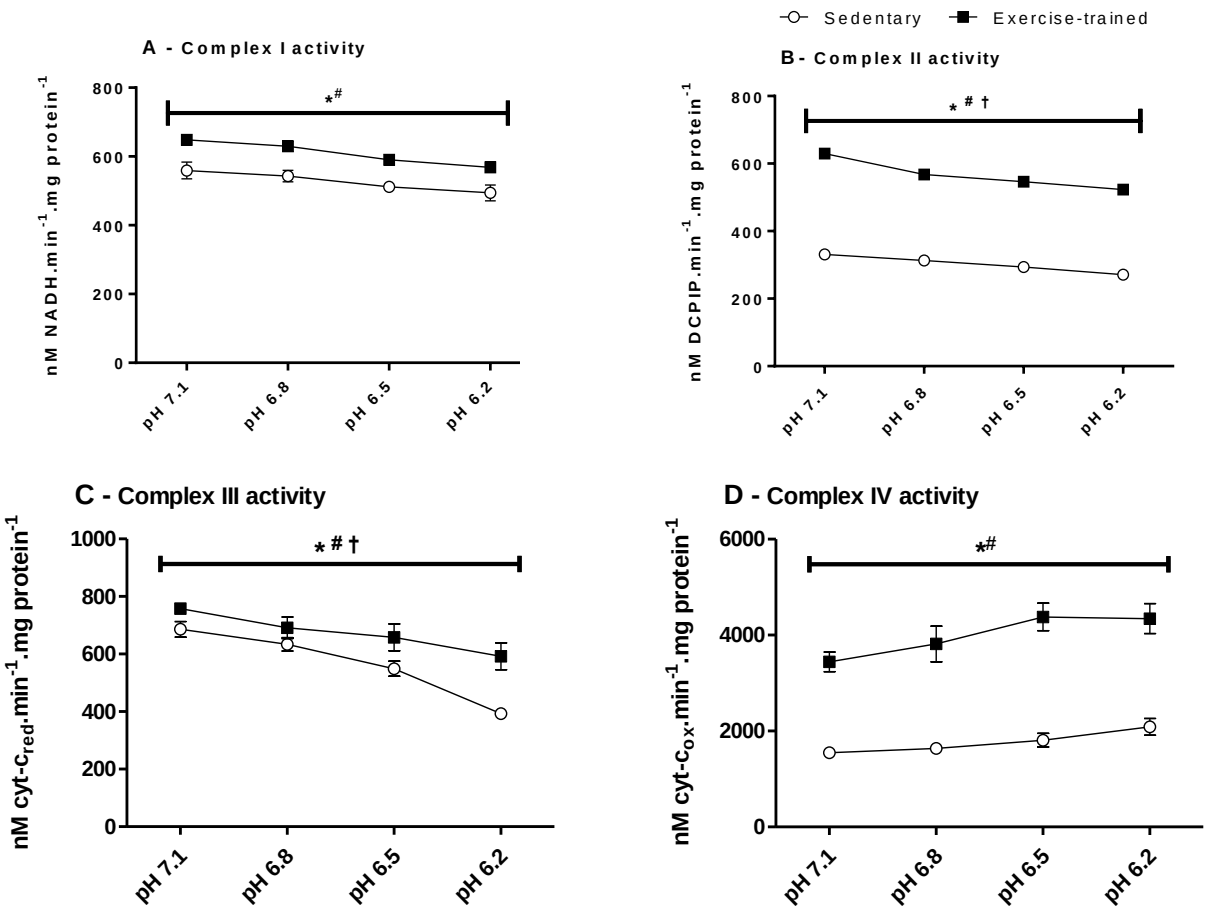


Figure 4.7: Effects of pH and exercise on mitochondrial respiratory complex enzyme activities in pooled rat soleus samples from sedentary (n=10) and exercise-trained (n=10) rats.
Data are presented as the mean ± standard deviation. Symbols indicate $p < 0.05$ for main effect of pH (*); main effect of exercise training (#); or interaction effect between pH and exercise training (†).
Abbreviations: NADH – Reduced nicotinamide adenine dinucleotide; DCPIP – 2,6-dichlorophenolindolphenol; cyt-c_{red} – reduced cytochrome-c; cyt-c_{ox} – oxidised cytochrome-c.

4.3.5 Mitochondrial respiration

Lowering pH decreased mitochondrial respiration during Leak respiration with both complex I and II substrate (main effect of pH, P = 0.001 figure 4.8B). Pairwise comparison showed lower respiration at pH 6.2 compared to pH 7.1 (P = 0.001, 95% CI: 1.8 to 9.0 pmol.mg^{-1}.s^{-1}), and pH 6.5 compared to pH 7.1 (P = 0.004, 95% CI: 1.2 to 8.4 pmol.mg^{-1}.s^{-1}). All other states were not significantly affected by altering pH.

Exercise training increased mitochondrial respiration during complex I and II oxidative phosphorylation (main effect of exercise training, P = 0.04, 95% CI: 1.0 to 36.7 pmol.mg^{-1}.s^{-1}, figure 4.2 D). Uncoupled respiration showed a trend of being higher in exercise-trained animals, but this effect did not reach statistical significance (P = 0.09).

There were no significant interaction effects in any states, though complex I oxidative phosphorylation approached significance (P = 0.076). P values for all main effects and interaction effects for all states are presented in table 4.2.

Table 4.2: P values for main and interaction effects of pH and exercise training on mitochondrial respiration.

* = P < 0.05.

State	Main effect: pH	Main effect: exercise training	Interaction effect: pH*exercise training
Complex I leak	0.944	0.222	0.846
Complex I & II leak	0.001*	0.142	0.456
Complex I oxphos	0.864	0.828	0.076
Complex I & II oxphos	0.585	0.040*	0.655
Uncoupled	0.201	0.090	0.790

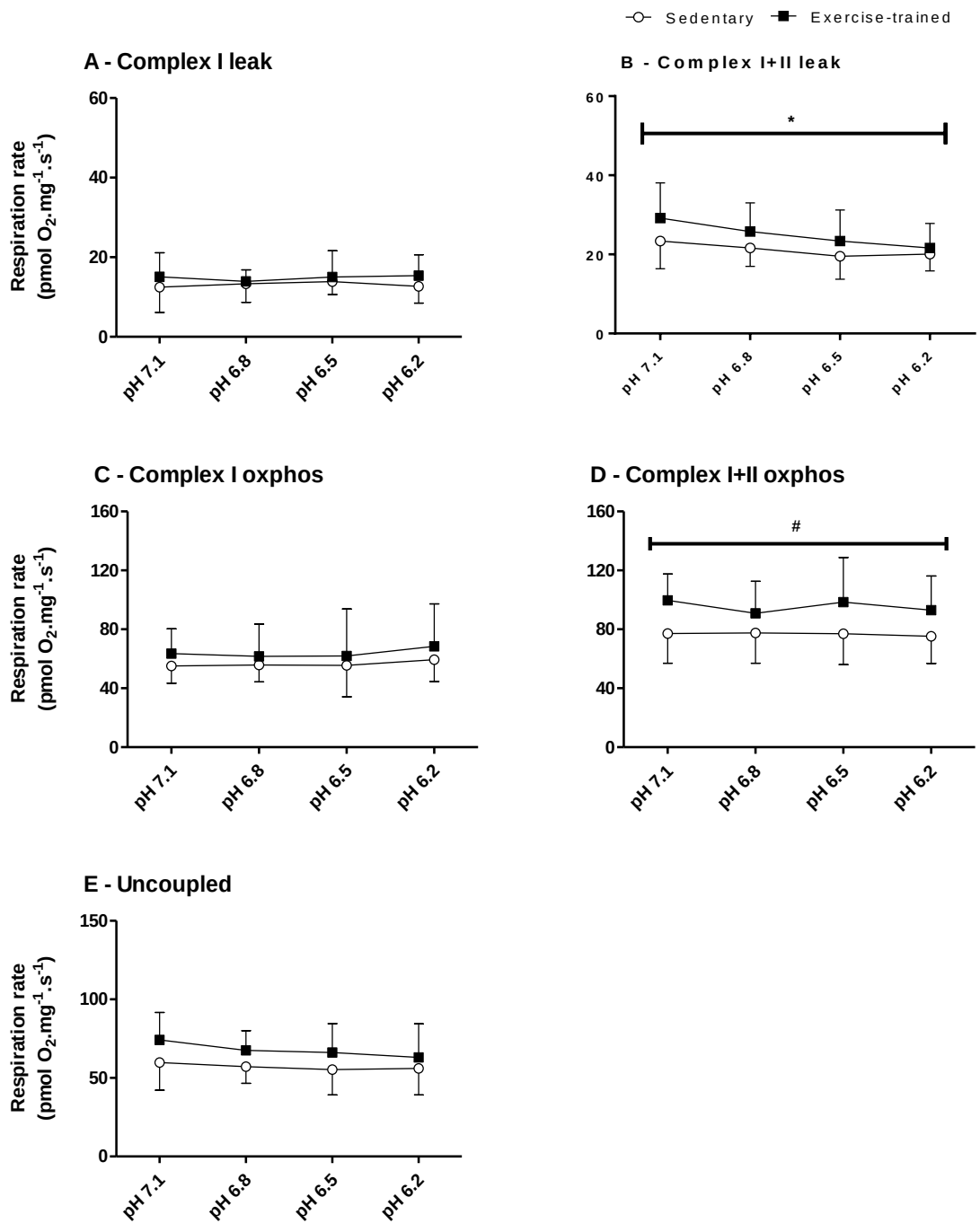


Figure 4.8: Effect of pH on mass-specific mitochondrial respiration from sedentary (n=10) and exercise-trained (n=10) rats.

Data are presented as the mean ± standard deviation.
States are complex I leak (A), complex I and II leak (B), complex I oxidative phosphorylation (C), complex I and II oxidative phosphorylation (D) and uncoupled respiration (E).
Symbols indicate $P < 0.05$ for: main effect of pH (*); main effect of exercise training (#).

4.3.6 ROS emission

ROS emission showed main effects of pH in all non –phosphorylating states (complex I leak, complex I & II leak, uncoupled, and antimycin-A induced), and a main effect of exercise training in all states. An interaction effect between pH and exercise training was seen in complex I & II Leak ROS emission only. P values for all main effects and interaction effects for all states are presented in table 4.3.

Table 4.3: P values for main and interaction effects of pH and exercise training on mass-specific ROS emission.

* = P < 0.05.

State	Main effect: pH	Main effect: exercise training	Interaction effect: pH*exercise training
Complex I leak	0.030*	0.004*	0.284
Complex I & II leak	<0.001*	0.034*	0.007*
Complex I oxphos	0.065	0.001*	0.715
Complex I & II oxphos	0.130	0.005*	0.202
Uncoupled	0.005*	0.001*	0.542
Antimycin-A induced	< 0.001*	0.008*	0.260

ROS emission in complex I leak state decreased as pH decreases (main effect of pH, P = 0.030, figure 4.9 A). Pairwise comparisons showed ROS emission at pH 6.5 was significantly lower when compared to pH 7.1 (P = 0.003, 95% CI: 6.6 to 31.1 fmol.mg^{-1}.s^{-1}), and there was a trend for ROS emission to be lower at pH 6.2 when compared to pH 7.1 (P = 0.062, 95% CI: -0.6 to 24.0). ROS emission was also higher in exercise-trained animals (main effect of exercise training, P = 0.004, 95% CI: 12.7 to 57.8 fmol.mg^{-1}.s^{-1}, figure 4.9A). No interaction effect was seen (P = 0.284).

ROS emission in complex I & II leak state also decreased with decreasing pH (main effect of pH, P < 0.001, figure 4.9 B). Pairwise comparison showed ROS emission being lower at pH 6.2 when compared to pH 7.1 (P < 0.001, 95% CI: 35.9 to 130.2 fmol.mg^{-1}.s^{-1}); lower at pH 6.5 when compared

to pH 7.1 (P = 0.001, 95% CI: 23.8 to 118.1 fmol.mg^{-1}.s^{-1}); and lower at pH 6.8 when compared to pH 7.1 (P = 0.029, 95% CI: 3.4 to 96.8 fmol.mg^{-1}.s^{-1}). ROS emission was also higher in exercise-trained animals (main effect of exercise training, P = 0.034, 95% CI 3.6 to 81.6 fmol.mg^{-1}.s^{-1}, figure 4.9B). An interaction effect between pH and exercise training was seen (pH*exercise training interaction effect, P = 0.007, figure 4.9B), and subsequent pairwise analyses determined that pH did not affect ROS emission in muscle from exercise-trained animals, but ROS emission at pH 6.2, 6.5 and 6.8 were significantly lower than pH 7.1 in sedentary animals.

ROS emission during complex I-supported oxidative phosphorylation showed a trend for decrease in ROS emission as pH decreased (main effect of pH, P = 0.065), and that exercise-trained rats had higher ROS emission (main effect of exercise training, P = 0.001, 95% CI: 18.6 to 59.2 fmol.mg^{-1}.s^{-1}, figure 4.9C). No interaction effect was seen (P = 0.715).

ROS emission during complex I & II-supported oxidative phosphorylation was not affected by changing pH (main effect of pH, P = 0.130), but ROS emission was higher in exercise-trained animals (main effect of exercise training, P = 0.005, 95% CI: 11.7 to 56.0 fmol.mg^{-1}.s^{-1}, figure 4.9D). No interaction effect was seen (P = 0.202).

ROS emission during uncoupled respiration decreased as pH decreased (main effect of pH, P = 0.005, figure 4.9E), and subsequent pairwise comparisons showed ROS emission at pH 6.2 was lower when compared to pH 7.1 (P = 0.011, 95% CI 5.0 to 53.5 fmol.mg^{-1}.s^{-1}), and lower at pH 6.5 when compared to pH 7.1 (P = 0.011, 95% CI 4.8 to 53.3 fmol.mg^{-1}.s^{-1}). ROS emission was higher in exercise-trained animals compared with sedentary animals (main effect of exercise training, P = 0.001, 95% CI: 22.8 to 78.1 fmol.mg^{-1}.s^{-1}, figure 4.9E). No interaction effect was seen (P = 0.542).

ROS emission induced by complex III inhibition with antimycin-A decreased as pH decreased (main effect of pH, P < 0.001, figure 4.9F), and subsequent pairwise analysis showed ROS emission was lower at pH 6.2 when compared to pH 6.8 (P < 0.001, 95% CI: 55.4 to 154.4 fmol.mg^{-1}.s^{-1}); lower at pH 6.2 when compared to pH 7.1 (P < 0.001, 95% CI: 208.3 to 309.6 fmol.mg^{-1}.s^{-1}); lower at pH 6.5 when compared to pH 6.8 (P < 0.001, 95% CI: 36.3 to 135.3 fmol.mg^{-1}.s^{-1}); lower at pH 6.5 when

compared to pH 7.1 ($P < 0.001$, 95% CI: 189.2 to 290.6 fmol.mg^{-1}.s^{-1}); and lower at pH 6.8 when compared to pH 7.1 ($P < 0.001$, 95% CI 103.7 to 204.4 fmol.mg^{-1}.s^{-1}). ROS emission was higher in exercise-trained animals (main effect of exercise training, $P = 0.008$, 95% CI: 26.6 to 151.4 fmol.mg^{-1}.s^{-1}, figure 4.9F). No interaction effect was seen ($P = 0.260$).

When ROS emission is normalised to oxygen consumption, no effect of pH is seen in any state (table 4.4). Exercise-trained animals had higher ROS emission per oxygen consumed in complex I leak state (main effect of exercise training, $P = 0.011$, 95% CI: 0.6 to 3.8 fmol H_2O_2.pmol O_2^{-1}, figure 4.10 A), and during complex I oxidative phosphorylation (main effect of exercise training, $P = 0.002$, 95% CI: 0.3 to 1.2 fmol H_2O_2.pmol O_2^{-1}, figure 4.10 C). No states showed statistically significant interaction effects between pH and exercise training (table 4.4).

Table 4.4: P values for main effects and interation effects of pH and exercise training on mitochondrial ROS emission per oxygen consumed.
* = $P < 0.05$.

State	Main effect: pH	Main effect: exercise training	Interaction effect: pH*exercise training
Complex I leak	0.442	0.011*	0.055
Complex I & II leak	0.322	0.194	0.089
Complex I oxphos	0.110	0.002*	0.274
Complex I & II oxphos	0.577	0.159	0.779
Uncoupled	0.761	0.068	0.690

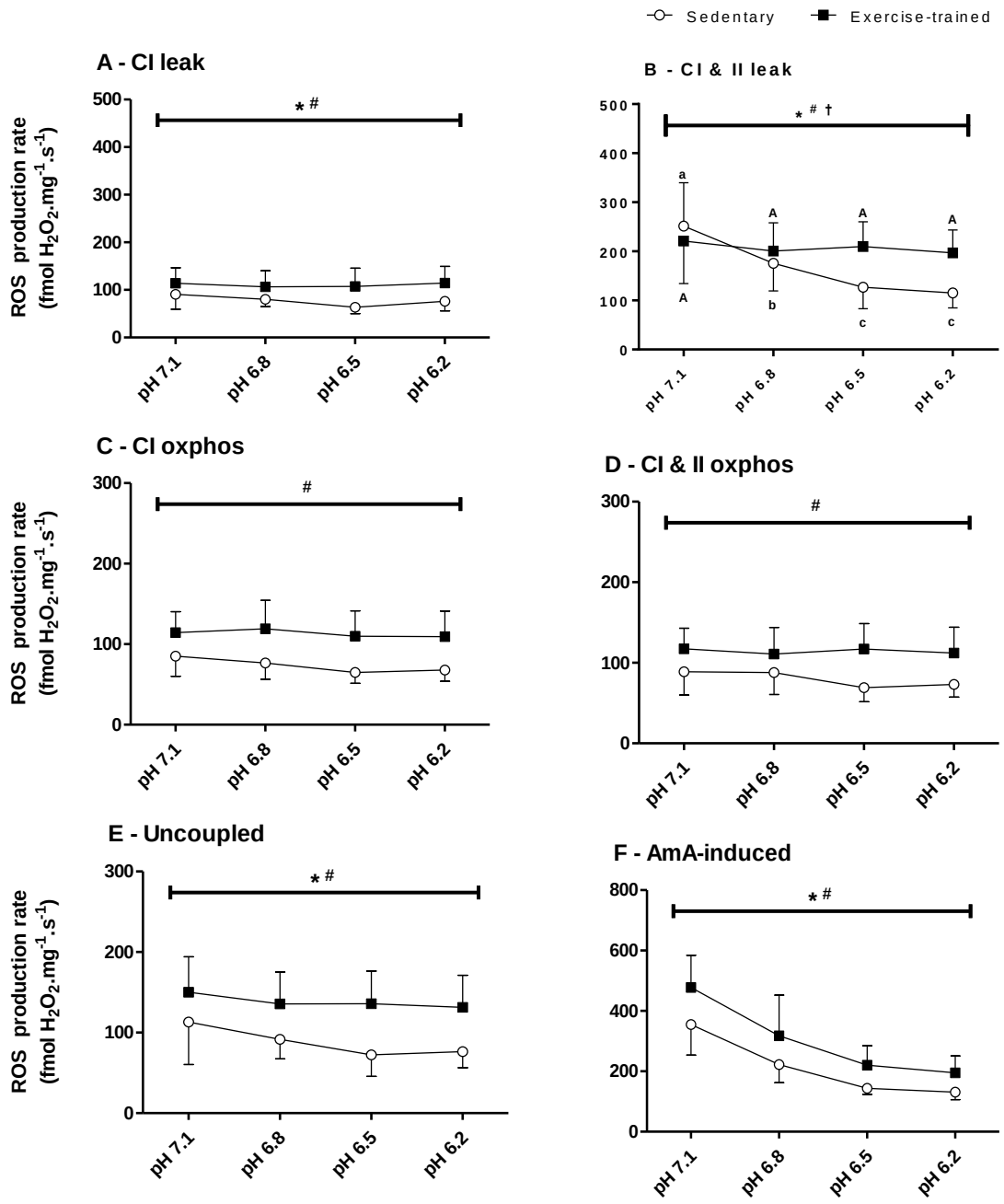


Figure 4.9: Effect of pH on mass-specific mitochondrial ROS from sedentary (n=9) and exercise-trained (n=9) rats.

Data are presented as the mean ± standard deviation.
States are complex I leak (A), complex I and II leak (B), complex I oxidative phosphorylation (C), complex I and II oxidative phosphorylation (D), uncoupled respiration (E), and antimycin-a-induced ROS (F).
Symbols indicate $p < 0.05$ for: main effect of pH (*); main effect of exercise training (#); or an interaction effect between pH and exercise training (†). Where an interaction effect was seen, Tukey's groupings are used to indicate differences in exercise-trained (capital letters) and sedentary (lower case letters) animals. Different letters indicate significant differences within cohort.

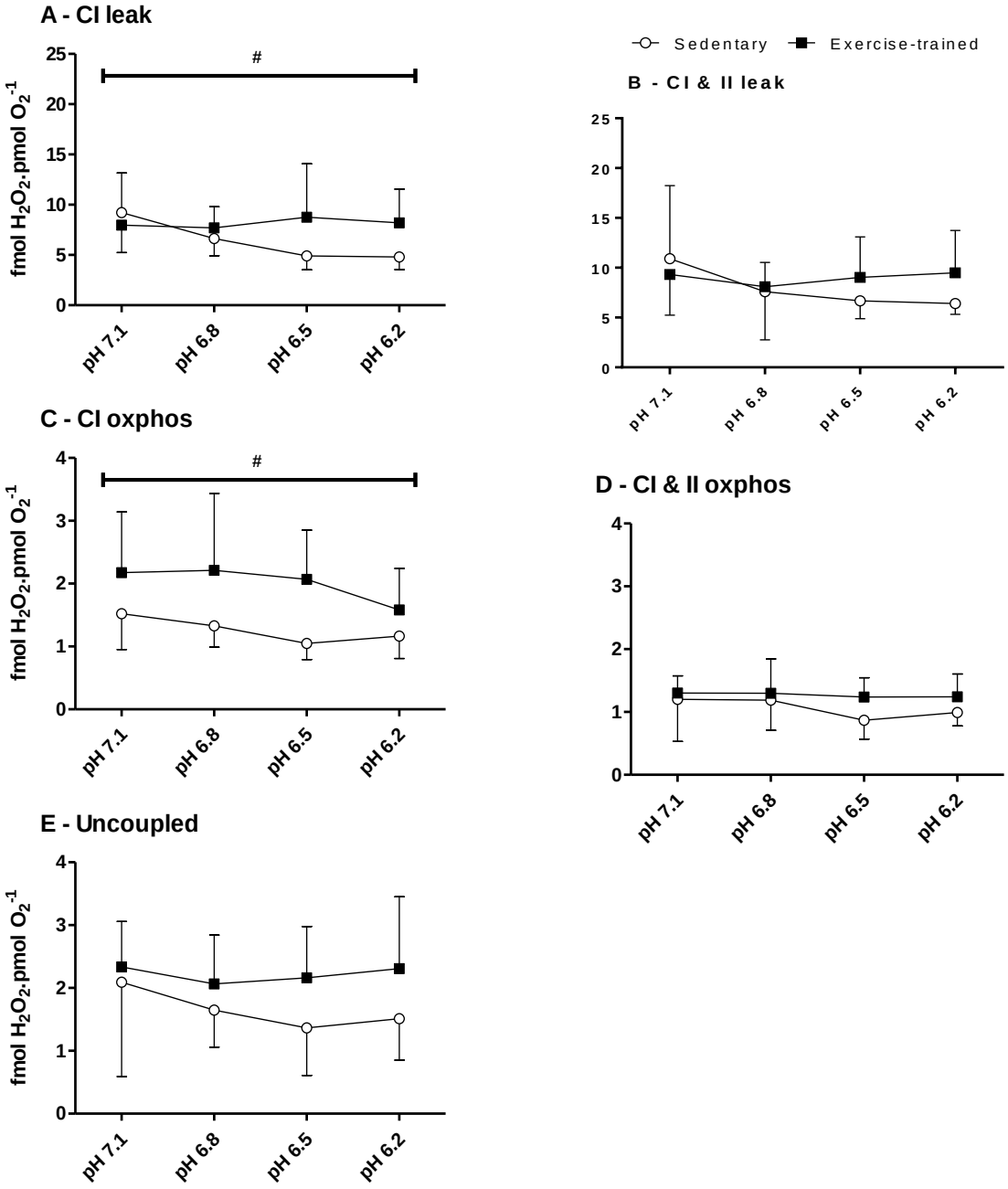


Figure 4.10: Effect of pH on mitochondrial ROS per oxygen consumed from sedentary (n=9) and exercise-trained (n=9) rats.

Data are presented as the mean ± standard deviation.

States are complex I leak (A), complex I and II leak (B), complex I oxidative phosphorylation (C), complex I and II oxidative phosphorylation (D) and uncoupled respiration (E).

Symbols indicate $p < 0.05$ for: main effect of pH (*) and main effect of exercise training (#).

4.4 Summary of findings

In sedentary animals, decreasing pH depressed complex I and II leak respiration, depressed ROS emission in all non-phosphorylating states, and depressed enzyme activity of mitochondrial complexes I, II, and III. Complex IV activity was increased by increasing pH. Exercise-trained rats had a lower total body mass, lower fat mass, and slightly lower bone mass. Muscle from exercise-trained rats had greater respiration during complex I & II-supported oxidative phosphorylation, greater ROS emission in all states, greater ETS complex enzyme activities, and a greater *in vitro* buffering capacity. The response of mitochondrial respiration to a pH challenge was not significantly altered by 8 weeks of voluntary wheel running in any state. ROS emission in exercise-trained rats showed a similar depression with decreasing pH as sedentary rats, except during maximal leak respiration, where ROS emission was unaffected by decreasing pH. Though all ETS complex activities were higher in exercise-trained rats, complex II and complex IV showed larger differences compared to complex I or complex III; this indicates a disparity in the effect of exercise training on individual ETS complexes. Additionally, complex II and III activity in exercise-trained rat muscle was less pH sensitive than sedentary animals.

4.5 Discussion

4.5.1 Effects of pH on mitochondrial function

In contrast to chapter three, this study corroborates the findings of previous research that decreasing pH supresses leak respiration, yet oxidative phosphorylation is unaffected by changes in pH [517]. This contrast between chapters is addressed further in chapter six. It is possible that substrate type, electron flow rate, and/or competition for ubiquinone may play roles in the effect pH has, as leak respiration through complex I alone with malate and pyruvate appears unaffected by pH, but on succinate addition an effect of pH is noted. Activity assays indicate there is a small depression of complex I activity with decreasing pH, but this depression is only ~12% from pH 7.1 to pH 6.2. This magnitude of difference is possibly below the technical sensitivity to detect in the oxygraph for complex I leak respiration rate, as a 12% difference in complex I leak respiration is between 1-2 pmol O_2.mg^{-1}.s^{-}

[1]. Additionally, when functioning as part of an intact electron transport system, complex I may be subject to more regulation or constraints than when measured as an isolated enzyme. Considering also the biological variation of samples, such a small magnitude of difference in respiration would be difficult to detect. The effect of decreasing pH on complex II activity is larger (17-18% depression from pH 7.1 to pH 6.2), and, as such, when the effect of pH on complex I is combined with the effect of pH on complex II, a difference is detectable. The effect of pH on combined complex I and II oxygen flux is larger (~15% depression in activity from pH 7.1 to pH 6.2), and the combination of a slightly larger effect with a larger signal from combined substrate input means the effect of pH on respiration is more readily detected (and meets the significance threshold) with complex I and II.

Mitochondrial ROS emission was depressed with decreased pH in non-phosphorylating states. When ADP was added and mitochondria were phosphorylating, there was no effect of pH on ROS emission. Depression of ROS emission by decreasing pH agrees with previous research on isolated skeletal muscle mitochondria [25]. However, when corrected for changes in oxygen consumption, the relative amount of oxygen converted to ROS does not change with pH in all states, suggesting that pH does not potentiate or depress ROS production *per se*, rather this happens as a result of pH-induced changes in oxygen flux.

In both groups, lowering pH increases complex IV activity. This means that the capacity of complex IV to consume oxygen is in fact enhanced, despite no change in respiration with decreasing pH (aside from complex I & II leak state, where respiration was depressed). The disparity in effects of pH on respiration and mean complex IV activity can be explained by depression of complexes I, II and III. As the complexes function as a system, even though complex IV capacity is increased, maximal electron supply to complex IV is regardless still regulated through these pathways. So the functional significance of an increase in complex IV capacity with decreasing pH is unclear.

4.5.2 Effects of exercise training on mitochondrial function

Voluntary exercise training provided a sufficient stimulus to induce a small but significant increase in maximal oxidative phosphorylation respiration in these rats, although only a trend was evident in all other respiratory states. Previous literature on the effect of voluntary wheel running on mitochondrial respiration is highly variable. For example, Servais et al. [477] found that 5 months of voluntary wheel running did not increase mitochondrial oxygen consumption in either leak or phosphorylating states in the gastrocnemius muscle of male Lou/C rats, while Zoll et al. [605] reported that 8 weeks of voluntary wheel running resulted in a 57% increase in maximal phosphorylating respiration, and a 72% increase in CS activity, in plantaris muscles from female Sprague-Dawley rats. The data presented in this thesis fall in the middle of these observations, with a 25% increase in maximal phosphorylating respiration (averaged across all pH conditions, 29% at pH 7.1) and a 42% increase in CS activity. There are a number of potential explanations for the variability seen with studies investigating the effects of voluntary wheel running on mitochondrial function, with different rat strains, sexes, and muscles tested, different experimental conditions used, and different lengths of interventions.

As with the effect of pH, the effect of exercise training in the present study was more pronounced with a combination of complex I and II substrate than with complex I alone. This could be explained by 85% higher mean complex II enzyme activity, compared to 15% higher mean complex I activity, in exercise-trained rats. Exercise training also resulted in greater activity of ETS complexes III and IV, although this effect was more pronounced in complex IV (125% greater mean activity in exercise-trained rats) than in complex III (20% greater mean activity in exercise-trained rats). This shows that exercise training seems to increase activity of complex II and IV to a greater extent that complex I and III. Why the difference in response of individual ETS complex activities occurs remains unclear. As complex I and complex II both provide electrons to complex III, then on to complex IV, this implies that exercise training results in proportionally greater increase in capacity of complex II to provide electrons to complex III than complex I, and an increase in capacity of complex IV to use electrons, but without concomitant increase of complex III activity to facilitate this transfer. A similar pattern of disproportional remodelling is seen in the male offspring of maternal rats fed a high fat diet

[395]. These male offspring show decreased gene and protein expression of complexes I and III, and lower enzyme activity when complex I and III are measured together, but no effect of maternal diet is seen on complex II or IV expression, or enzyme activity [395]. So it appears that there is specific remodelling of different complexes in response to different types of metabolic stress. Complex II ultimately contributes less to membrane potential development than complex I, as complex II itself does not pump protons into the intermembrane space [472]. Also, for each pyruvate molecule that enters the citric acid cycle, there is a theoretical maximal yield of four NADH for oxidation by complex I and one succinate for oxidation by complex II through the TCA cycle. So electron entry through complex I not only provides more H^+ per reducing equivalent, but also has greater supply of ETS substrate. Based on current estimates of proton pumping stoichiometry of the complexes (summarised in [472]), total proton pumping yield for a single complete revolution of the citric acid cycle should be 46 H^+ pumped into the intermembrane space, of which only 6 are from succinate entry at complex II. Thus it is intriguing that the difference in complex II activity between groups is so much greater than the difference in complex I activity between groups.

One explanation could be that NADH consumption is not limited to complex I. In particular, nicotinamide nucleotide transhydrogenase (NNT) can oxidise NADH to produced NADPH, which may be necessary for synthesis of antioxidant intermediates or other biosynthesis pathways. Therefore the NADH flux through complex I may be less than the maximum NADH yield from the citric acid cycle. A second possibility is that there is some adaptive mechanism for regulating membrane proton motive force. This proton motive force (Δpmf) consists of an ionic component ($\Delta\psi_m$) and a pH component (ΔpH_m), such that $\Delta pmf = \Delta\psi_m + \Delta pH_m$ [138]. Mitochondria have a variety of ion transporters and can buffer levels of calcium, sodium and potassium in addition to hydrogen ions (reviewed in [37]). Fluctuations in these cations may contribute to $\Delta\psi_m$, and the Δpmf needs to be regulated in order to maintain ATP synthesis. However, if there is hyperpolarisation of the inner membrane, mitochondrial ROS production could be elevated [277, 336]. In turn, this could down-regulate reverse electron flow through mitochondrial complex I so that when $\Delta\psi_m$ is increased, electron flux occurs preferentially through complex II. This would decrease ΔpH_m while maintaining Δpmf. Another explanation is that

succinate dehydrogenase/complex II is formed by 4 nuclear-encoded subunits, whereas complex I is formed from a total of 44 subunits, 37 of which are nuclear-encoded, and the remaining 7 mitochondria-encoded. Complex II may be more readily assembled than complex I.

Greater complex IV capacity following exercise training seems counterintuitive, given that mean activity of complex IV is greater than the other ETS complexes measured. However, *in vivo*, complex IV is regulated by interactions with other compounds, including adenylates [29], myoglobin [588, 589] and nitric oxide [56]. It is possible that *in vivo* regulation of complex IV in fact means it is limiting to oxidative phosphorylation, and as such expression and/or activity of complex IV increase with exercise training. Indeed, complex IV activity correlates with oxidative phosphorylation capacity in human skeletal muscle [282], although training induced changes in complex I and complex IV activity are similar in human muscle [544].

Voluntary activity was also associated with greater rates of tissue-specific ROS emission in all respiratory states. However, when ROS emission is normalised to oxygen flux, higher ROS emission in exercise-trained rats is only noted from complex I states (complex I leak and complex I oxidative phosphorylation). This suggests there may be a mechanism by which exercise training may preferentially increase ROS production relative to oxygen consumption from complex I. An increase in ROS emission following voluntary wheel running contrasts with previous research showing that 10 weeks of swimming training results in lower ROS production from Wistar rats [534], and that after 5 months of voluntary wheel running, no difference is seen in either mass-specific ROS production or proportion of oxygen converted to ROS between sedentary and exercise-trained Lou/C rats [477]. It is possible that different sub populations of mitochondria respond differently, as the data of Servais et al. [477] suggest that there may have been an increase in ROS production in subsarcolemmal mitochondria, but not intermyofibrillar mitochondria, something that was not focussed on in their paper. Subsarcollemal mitochondria are more responsive to training [270], and as the training stimulus of voluntary activity is difficult to quantify, differences in training-induced ROS emission between the results presented here and Servais et al. [477] may be explained by differences in training load.

4.5.3 Effects of exercise training on the pH sensitivity of mitochondrial function

Exercise training appears to alter the effect of pH on ROS emission during complex I and II leak respiration. In this state, ROS emission was depressed as pH decreased in muscle from sedentary rats, but remained constant as pH decreased in exercise-trained rats. Additionally, ROS emission at pH 7.1 was not different between exercise-trained and sedentary rats. This suggests that ROS emission during maximal leak respiration is affected by pH in the sedentary cohort but not in the exercise-trained cohort. This may be mediated by complex III activity, as the decline in complex III activity with pH is approximately twice as large in the sedentary cohort than the exercise-trained cohort. Complex II showed a statistically significant difference in the rate of decline with decreasing pH between exercise-trained and sedentary rats; however, the magnitude of this effect (2% per pH unit) suggests it is unlikely to be responsible for the difference in complex I & II leak ROS emission.

A difference between sedentary and exercise-trained animals in the effect of pH on complex II activity, complex III activity, and ROS emission in complex I & II leak state is potentially explained by a 10% greater *in vitro* muscle buffering capacity in exercise-trained rats. However, as *in vitro* buffering capacity is a ubiquitous property, it seems unusual it would specifically affect some measures and not others. For example, the difference in the effect of pH on complex III activity between the groups suggests that hydrogen ions may regulate complex III function, through some mechanism other than inhibition of the proton pumping function of the enzyme due to high membrane ΔpH, perhaps allosteric modification. Alternately, there may be no direct effect of H^+, but that a difference in capacity to buffer H^+ may affect another variable.

Current literature on training methodologies to improve muscle buffering capacity do not show consensus on the optimal intensity and duration to improve muscle buffering. High-intensity interval training typically produces increases in βm that are not seen with continuous training in recreational team sport players [119], but an acute maximal-intensity exercise bout [42] or interval training at near-maximal intensities [41] decreases muscle buffering capacity. Given that buffering capacity is correlated with training status and repeat-sprint ability [120], and that intramuscular acidosis is most pronounced after high-intensity exercise [197, 446], it stands to reason that high-intensity exercise

would provide a greater stimulus for adaptation than lower-intensity paradigms. Nonetheless, the higher $\beta m_{in\ vitro}$ of exercise-trained rats in this study indicates that at least some change is possible with voluntary exercise, although the magnitude of this difference is lower in comparison to a 41% higher buffering capacity in Wistar rats following five weeks of high-intensity interval training [511]. The increase in buffering capacity seen here may also be mediated by the use of soleus in this study, as previous research shows more glycolytic muscle (extensor digitorum longus) has a greater baseline buffering capacity, and increases less following training [511].

The greater muscle buffering capacity seen in exercise-trained rats is also coincident with greater LDH activity. LDH is a commonly-used enzyme marker for anaerobic capacity, thus it seems reasonable to expect that the difference in buffering capacity might mirror the difference in LDH activity. In this case, they are both higher in exercise-trained rats, and across both groups there is a significant correlation between LDH activity and muscle buffering capacity. It is also important to note that the range of values is such that there are some animals in the sedentary group with buffering capacities and LDH activities similar to those at the lower end of the exercise-trained group (figure 4.2).

Whether an increase in *in vitro* buffering capacity reflects an actual *in vivo* physiological benefit is unclear. The muscle buffering capacity assay by titration method [314] involves homogenising a sample and reacting it with acid to determine the amount of H^+ necessary to alter the pH of the homogenate. In this instance, buffering capacity of the homogenate is determined by both a protein component (histidine side-chains of proteins) and non-protein component (generally bicarbonate buffering). *In vivo*, buffering capacity is dependent on these buffering systems but also on transport proteins which compartmentalise H^+ accumulation. The actions of these proteins are not tested by this titration method; moreover protein buffering may be overestimated as the H^+ can react with any histidine present in the milieu, even that which would not be available to buffer *in vivo*. Mechanistically, it is possible that the increase in buffering capacity seen is due to an increase in protein content, as protein concentration of homogenate from exercise-trained rats was ~25% greater than protein concentration of muscle from sedentary rats (data not shown), while buffering capacity is ~10% greater in exercise-trained rats compared to sedentary rats.

One unexpected finding in this study was that exercise trained rats had significantly lower bone mineral content compared to sedentary rats (table 4.1). It seems as though this is most likely due to the difference in total body mass between sedentary and exercise-trained rats, as sedentary rats had ~25% greater total body mass, and ~27% greater bone mineral content.

4.6 Conclusion

Decreasing pH depresses non-phosphorylating respiration and ROS emission in skeletal muscle, though different effects of pH are seen between sedentary and exercise-trained rats. Exercise training may ameliorate some of the effects of decreased pH through an increase in buffering capacity, though this is not seen in all states. Exercise training also seems to favour an increase complex II and IV activity more than an increase in complex I and III activity, indicating that they may be more rate-limiting to *in vivo* performance, or that preferentially increasing them may confer some additional functional effect not tested here.

Chapter 5: The influence of extracellular pH on rat skeletal muscle force production, metabolism, and acute signalling responses for mitochondrial biogenesis in response to electrical stimulation.

Summary of preceding chapters

Chapters 3 & 4 both examined aspects of mitochondrial function in the context of physiological changes in pH, both at rest and following a period of voluntary activity. Collectively, chapters 3 & 4 indicate that acute changes in pH within a physiological range affect some aspects of mitochondrial function. Specifically, there is lower net ROS emission at a lower pH, depressed leak respiration rates, but the balance of evidence suggests mitochondrial respiration in the presence of ADP can be maintained. If mitochondrial function is changed in response to acute exposure to a reduced pH, then concentrations of the molecules which are produced and/or influenced by mitochondria (AMP, NADH, Ca^{2+}, ROS) may also change. As these molecules play a role in intracellular signalling, this may translate to differences in the activation of intracellular signalling pathways associated with mitochondrial biogenesis. This may have implications for mitochondrial biogenesis, both at rest and in response exercise training. However, whether there is a direct role of pH in modulating either resting or contraction-induced intracellular signalling in skeletal muscle is unknown. This chapter sought to investigate whether several canonical signalling pathways showed different activation in response to altering muscle pH, with and without stimulating muscle contraction.

5.1 Introduction

Muscle contraction results in multiple perturbations to cellular homeostasis, such as increases in cytosolic calcium concentration, consumption of ATP, oxidative stress (increased ROS production), changes in pH, and other delayed responses such as cytokine production and inflammation. These perturbations trigger a number of signalling cascades to result in adaptation, including those associated with mitochondrial biogenesis. Initial transduction occurs via activation of kinases sensitive to these perturbations, such as calcium-calmodulin kinase II (CaMKII) [143], AMP-activated protein kinase (AMPK) [451], p38 mitogen activated protein kinase (p38 MAPK), and extracellular-regulated protein kinases 1 and 2 (ERK1/2) [17, 304]. Extensive reviews of contraction-induced signalling for mitochondrial biogenesis can be found elsewhere [123, 215].

There is a growing body of evidence to suggest that changes in pH may modulate signalling for mitochondrial adaptation. In rats, increases in mitochondrial respiration post-training are greater when rats are supplemented with sodium bicarbonate ($NaHCO_3$) prior to each training session [43]. This effect is seen in soleus, an oxidative, mitochondria-rich muscle type, but not extensor digitorum longus (EDL), a more glycolytic muscle type, indicating that fibre-type composition may determine the response of mitochondria to $NaHCO_3$. Additionally, increases in PGC-1α mRNA content following high-intensity interval training in humans are greater when supplemented with $NaHCO_3$ [387], and blunted when supplemented with ammonium chloride (NH_4Cl) [121]. Both $NaHCO_3$ and NH_4Cl alter blood pH (to raise or lower blood pH respectively), and this suggests that raising blood pH may augment signalling responses in favour of mitochondrial biogenesis and/or function, while lowering blood pH has the opposite effect. Changes in blood pH naturally occur following high intensity exercise, where blood pH may decrease to pH ~7.1 from resting levels of pH ~7.5 [441-443].

One confounding effect of changing blood pH is that an increase or decrease in blood pH causes a corresponding increase or decrease in the affinity of haemoglobin for oxygen. This promotes the respective binding or dissociation of oxygen from haemoglobin, through a process known as the Bohr effect. The Bohr effect acts to enhances oxygen loading at the more alkaline respiratory surfaces, and unloading in regions of local acidification such as within working tissues [557]. Changes in blood pH

may therefore affect tissue oxygenation. Hypoxia can affect mitochondrial function [379], and also post-exercise intracellular signalling [344], offering a potential indirect effect of changing blood pH on subsequent adaptation. Thus to investigate the effects of pH on regulating adaptation, it is important to control for potential confounding variables, such as haemoglobin oxygen saturation, that are also affected by changing pH.

Another important consideration is that altering blood pH also has consequences for energy metabolism. Post-exercise accumulation of blood lactate is greater following $NaHCO_3$ ingestion [387], and lowered following NH_4Cl ingestion [121]. This indicates that blood lactate concentration correlates with alterations in pH. Muscle lactate accumulation post-exercise is also greater following NaHCO3 ingestion [212]. This presents another potential confounding influence, as lactate has been hypothesised to be a signalling molecule in skeletal muscle [191], and thus the apparent effects of altering blood pH on PGC-1α mRNA content could also be mediated by lactate metabolism, rather than a direct effect of pH *per se*.

The aim of the present study was to clarify whether altering extracellular pH may have a direct role in altering contraction-induced signalling that modulates mitochondrial biogenesis in skeletal muscle. To investigate this, an erythrocyte-free hindlimb perfusion model was employed, using buffers of pH 7.5 (reflecting circulating pH in a resting state), and pH 7.0 (reflecting circulating pH post-high-intensity exercise), in order to alter perfusion pH without the potential confounding effect of pH on blood oxygen saturation. Signalling response was investigated for signal transducers (CaMK II, AMPK, p38 MAPK, ERK1/2) and transcriptional regulators (ATF2, HDAC4/5/7), as well as the metabolic regulator ACC, a target of AMPK. It was hypothesized that signalling response would be diminished under lower pH (7.0) conditions relative to higher pH (7.5) conditions.

5.2 Methods

5.2.1 Surgical preparation

Adult male Wistar rats (3 month old, n=24) were used to study the effects of altering extracellular pH on soleus muscle contractile function, pH, lactate production, and intracellular signalling for mitochondrial biogenesis. All procedures were approved by the Animal Ethics Committee of the University of Auckland (R001127). Rats were allocated to four groups: pH 7.5 perfusion with electrically-stimulated contraction (7.5 STIM, n=8), pH 7.0 perfusion with electrically-stimulated contraction (7.0 STIM n = 8), pH 7.5 perfusion only control (7.5 REST, n = 4) and pH 7.0 perfusion only (7.0 REST, n = 4). Surgical techniques used were an adaptation of Ruderman et al. [433]. Firstly, rats were anaesthetised using with isoflurane (3.5% vol/vol in medical oxygen), and anaesthesia was maintained throughout surgery with 2.5-4% isoflurane delivered through a nosecone. Following induction of anaesthesia, the rat was placed on a heated pad. Reflex response was assessed by pinching the tail and checking for foot withdrawal. Once rats were non-responsive to a tail pinch or foot movement, the femoral vessels of the right hindlimb were exposed by incision through the fur and skin on the ventral side of the animal, along the line where the leg meets the body. This cut was extended around the circumference of the leg and the leg was carefully de-sheathed to expose the underlying leg from the ankle to the body.

5.2.2 Isolated hindlimb perfusion

The femoral artery, vein, and nerve were all carefully separated from one another and a section of the artery and vein approximately 20 mm in length was freed from any underlying tissue using forceps. A 4-0 silk tie was passed under both the artery and vein individually, at both the proximal and distal end of the exposed section of the vessels. A haemostat clip was placed on the distal end of the artery and the proximal tie on the artery tied with a triple knot to occlude blood flow. A small cut was made in the upper wall of the artery using Vannas microdissection scissors, through which a 22-gauge cannula was inserted at least 1 cm past the incision point and tied in place with the distal silk suture. A second suture was then placed and tied around the cannulated section of the vessel. Isotonic heparinised saline was injected into the vessel to flush the cannula clear and prevent clotting of blood in the leg.

Following cannulation of the artery, the proximal suture on the vein was tied, the vessel expanded by injecting a small amount of saline through the arterial line, and then cut similarly to the artery. An 18-gauge cannula was inserted into the vein and double tied in place as with the arterial line. Once both lines were secured in places, 10-15 mL of isotonic heparinised saline was injected through the arterial line to confirm flow and to flush blood from the leg. The total time for this part of the procedure was always less than 10 minutes, and loss of oxygen supply to the leg was minimised as much as possible (routinely less than 5 minutes). Measurement of hindlimb oxygen consumption was attempted through use of in-line oxygen sensors to determine dissolved oxygen concentration in the perfusion media before and after passing through the hindlimb. Pilot experiments using this apparatus required slower flow rates due to media viscosity, and also resulted in significant tissue oedema due to the greater flow resistance the transducers applied to the system. This made them unsuitable for use for the experiment. However, dissolved oxygen concentration of the two media used for perfusion into the limbs was not different (mean oxygen partial pressure 675.4 ± 5.7 mmHg).

Following cannulation of both the artery and vein, the arterial line was connected to the perfusion apparatus and infusion commenced at a rate of 5 mL/min. The perfusion media was a modification of Krebs-Henseleit buffer containing, in mM; 122 NaCl, 1 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 10 glucose, 50 MOPS and 5% w/v Dextran 40. The pH of the buffer was adjusted with sodium hydroxide to be either 7.0 or 7.5 at 37°C when equilibrated with 95% O_2/5% CO_2. Perfusion media was then pumped through an in-house designed oxygenator consisting of Silastic™ tubing coiled around a copper pipe inside a perspex outer cylinder. The outer perspex chamber was continuously gassed with 95% O_2/5% CO_2, while a waterbath was used to circulate water through the inner copper pipe at 37°C. This served to maintain pH and oxygenation, and pre-warm the perfusion media.

Isolation of the hindlimb was achieved using the rat inguinal ligament to support a suture as a tourniquet. Forceps were used to make a hole under the cranial end of the ligament and a suture tied around the ligament. Another hole was made near the caudal end of the ligament, and the suture was passed under the leg (dorsal side of the rat), through the hole at the caudal end of the ligament, and passed back under the leg. The suture was pulled tight, causing the ligament to be pulled down on the

leg and compress underlying tissue. Finally the suture was tied once again to the cranial end of the ligament to secure the suture in place. Following isolation in this manner, perfusion was continued until perfusate showed no trace of remaining blood. Once isolated leg perfusion was achieved, rats were killed by ventricular destruction.

5.2.3 Electrically stimulated muscle contraction and force measurement

Electrically stimulated contraction force was measured in n=16 animals, n=8 perfused with pH 7.00 buffer and n=8 perfused with pH 7.50 buffer. Forceps were used to blunt dissect an opening between the Achilles tendon and tibia close to the ankle. A non-compliant silk tie was tied securely around the tendon ensuring no muscle tissue was compressed by the knot. The Achilles tendon below the tie was then severed. The opposite end of the tie was attached to a Grass model FT-03 force transducer connected via a Maclab™ signal conditioner (ADInstruments Pty Ltd, Dunedin, New Zealand) to a Powerlab™ (ADInstruments). To provide counter-tension and minimise movement of the leg, a hook was inserted beneath the patellar tendon and attached to a fixed anchor point. The position of the force transducer was then adjusted to ensure the correct line of pull to the force transducer.

Hindlimb plantarflexors were stimulated to contract by electrical stimulation of the sciatic nerve. An opening was blunt dissected in the posterior aspect of the hamstring muscles with care taken to avoid damage to the proximal tendons of the plantarflexors and the sciatic nerve. Once exposed, a perspex-encased platinum stimulating electrode was placed around the sciatic nerve. The wires for the electrode were secured to the tail of the rat via cable tie to ensure the electrode did not twist or stretch the nerve. In both the STIM and REST groups, the length of the muscle was then adjusted to provide maximal twitch tension in response to a single stimulus pulse, before being fixed at this optimal length. The leg was then rested for a 10-minute period before baseline measures were taken. To simulate exercise in the STIM group, the nerve was stimulated with 200 ms duration pulses delivered every 2 seconds (0.5 Hz). This was alternated for 2 minutes of stimulation followed by 2 minutes of rest for 5 cycles, then a 6th final stimulation period not followed by a rest period. The total duration from the beginning of the first stimulation period to completion of the final stimulation period was 22 minutes. The REST group were instrumented in the same manner as the STIM group, but did not undergo any

electrical stimulation after initial length adjustment, but remained perfused for the same time period as the STIM group.

5.2.4 Perfusate and muscle sampling

In all groups, perfusate was collected during the last 10 seconds of each two-minute period of rest or stimulation cycles. The perfusate was then frozen at -20°C and stored for later analysis of lactate concentration. At the end of the experiment (immediately following perfusate sampling of the final STIM/REST period) ~5 mL of isotonic saline was flushed through the cannulae, and the soleus was then rapidly removed, weighed, and snap-frozen in liquid nitrogen. Frozen muscle was stored at -80°C until further analysis. Figure 5.1 shows the experimental timeline.

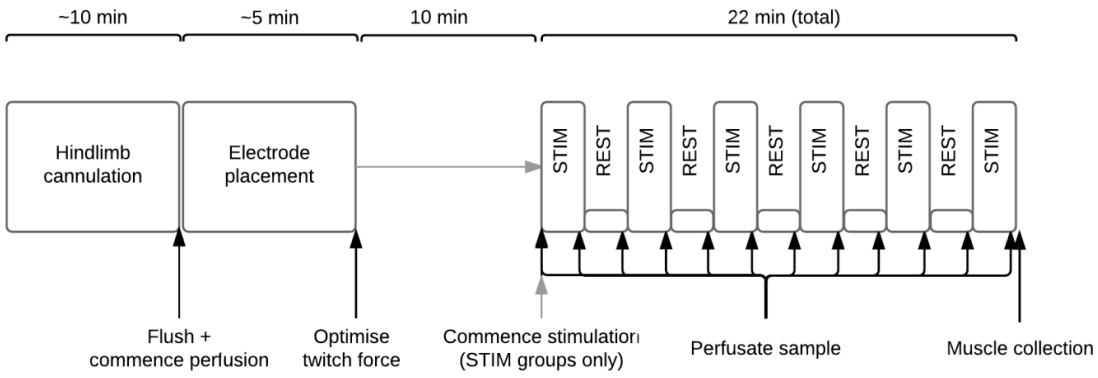


Figure 5.1: Experimental timeline for STIM group trial.
REST group trials follow the same timeline, but without the STIM periods.

5.2.5 Force measurement

Muscle force production was determined from the average peak height during each two-minute contraction cycle using LabChart™ software (ADInstruments).

5.2.6 Perfusate lactate measurement

Perfusate lactate concentrations were measured using an automated lactate oxidase enzyme system (YSI 2300 STAT, Yellow Springs Instruments, Ohio, USA).

5.2.7 Muscle pH measurement

A small portion of frozen muscle was freeze-dried, dissected free of connective tissue, and powdered. Approximately 3 to 5 mg of powdered muscle was then diluted 100 fold (dry weight/volume) in 10 mM sodium fluoride (NaF). Diluted muscle was then homogenised on ice with an Omni TH power homogeniser fitted with a hard tissue homogenising probe (Omni International, Georgia, USA). Muscle pH was then measured using an ion-sensitive field-effect transistor probe (model PHW77-SS, Hach, Colorado, USA) connected to a pH meter (IQ Scientific Instruments model IQ240, Cole-Parmer Instruments, Illinois, USA).

5.2.8 Immunoblotting

Frozen muscle was ground with a mortar and pestle on dry ice and small portions weighed into bead mill homogenising tubes containing 7 ceramic beads to shear tissue (Omni International). Muscle was then diluted 15 fold (weight/volume) with ice-cold RIPA lysis buffer (Merck Millipore #20-188, Dusseldorf, Germany) supplemented with 0.1% sodium dodecyl sulphate (SDS) and protease inhibitor cocktail (Halt™ Protease and Phosphatase Inhibitor Cocktail, Thermo Fisher Scientific, Massachusetts, USA). Samples were then homogenised for 2 cycles of 1 minute at 5 m/s in a bead mill homogeniser (Omni Ruptor, Omni International). After homogenisation samples were mixed for 1 hour at 4 °C on a mechanical turntable, before being centrifuged at 14,000 g for 10 minutes at 4 °C. Protein concentration of each sample was determined using a commercially available bicinchoninic acid assay (Pierce™ BCA Protein assay kit, Thermo Fisher Scientific), with samples measured in triplicate against a BSA standard curve. A fixed volume (100 µL) of 4x Laemmli's buffer was added to each sample, and water to dilute each sample to a final protein concentration of 1.5 mg/mL, after which samples were boiled at 95 °C for 5 minutes. Samples were then mixed by vortexing, and a small amount (25 µL) taken from each sample to make a pooled homogenate, which was included on every gel to standardise expression between different gels.

Up-regulation of proteins involved in acute signalling for mitochondrial biogenesis was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for the following proteins: total and phosphorylated forms of (phosphorylation site(s) in brackets) AMPK

(threonine 172); CaMKII (threonine 286); ERK1/2 (threonine 202, tyrosine 204); p38 MAPK (threonine 180, tyrosine 182); ACC (serine 79) and ATF2 (threonine 71). In addition phosphorylation only of HDAC4 (serine 246); HDAC5 (serine 259); and HDAC7 (serine 155) was determined. Further details of antibodies and dilutions used can be found in table 5.1.

Table 5.1: Antibodies used in immunoblotting of intracellular signalling pathways.
Abbreviations used: CST = Cell Signalling Technology; SCBT = Santa Cruz Biotechnology; p- = phosphorylated; t- = total.

Primary antibodies					
Protein	Expected molecular weight	Host	Dilution	Catalogue	Supplier
p-AMPK	62	Rabbit IgG	1:1000	#2535	CST
t-AMPK	62	Rabbit	1:2000	#2532	CST
p-CaMKII	50	Rabbit	1:2000	#3361	CST
t-CaMKII	50	Rabbit	1:2000	sc-13082	SCBT
p-ERK1/2	42/44	Rabbit IgG	1:2000	#4370	CST
t-ERK1/2	42/44	Mouse IgG	1:2000	#4696	CST
p-p38MAPK	43	Rabbit IgG	1:2000	#4511	CST
t-p38MAPK	43	Rabbit	1:2000	#9211	CST
p-ACC	280	Rabbit	1:2000	#3661	CST
t-ACC	265	Rabbit IgG	1:2000	04-322	Millipore
p-ATF2	70	Rabbit IgG	1:2000	#5112	CST
t-ATF2	70	Rabbit IgG	1:2000	#9226	CST
p-HDAC4/5/7	140/124/120	Rabbit IgG	1:2000	#3443	CST

Secondary antibodies				
Primary host	Antibody reactivity	Dilution	Catalogue	Supplier
Rabbit	Donkey anti-Rabbit IgG	1:20000	715-035-151	Jackson Immuno Research
Mouse	Goat anti-mouse IgG	1:20000	115-035-146	Jackson Immuno Research

Denatured protein samples (30 µg total protein) were resolved in 15-well 4-15% Mini-PROTEAN® TGX Stain-Free™ gels (Bio-Rad Laboratories, Sydney, Australia). All gels included a pre-stained protein standard (Precision Plus Protein™ Kaleidoscope™, Bio-Rad Laboratories) and the pooled sample. Following protein resolution, images of gels were obtained using Stain-Free activation using a Chemi-Doc MP imaging system (Bio-Rad Laboratories). Protein was then transferred to polyvinylidene fluoride (PVDF) membranes using Trans-Blot® Turbo™ transfer system (Bio-Rad Laboratories) according to manufacturer specifications. Following transfer, images of both gels and membranes were obtained using Stain-Free activation as above in order to verify transfer.

Following post-transfer imaging, all membranes were blocked for 2 hours with 5% BSA in Tris-buffered saline with 0.1% Tween-20 (TBST) at room temperature. After transfer, membranes were cut using the stained ladder as a guide for expected molecular weight of target proteins. Primary antibodies were diluted in 5% BSA in TBST, and membranes were incubated overnight at 4 °C with primary antibody. Following primary antibody incubation, membranes were washed with TBST, and then incubated for 1 hour at room temperature with secondary antibody diluted in 5% BSA in TBST. Following secondary antibody incubation, membranes were again washed in TBST before imaging. Protein bands were detected following a 3-minute incubation with Amersham enhanced chemiluminescent substrate (GE healthcare RPN2235, Thermofisher Scientific) using a ChemiDoc MP (Bio-Rad Laboratories). Images were taken and densitometry performed using ImageLab version 5.1 (Bio-Rad Laboratories). All protein expression was normalised first to protein loaded, which was assessed by quantifying optical density of lanes from Stain-Free images. Expression was then normalised to optical density of the pooled sample for comparison between different gels.

5.2.9 Statistical analyses

Statistical analyses were conducted in Prism 5, with statistical significance determined as $p < 0.05$. Data distribution was first assessed by Shapiro-Wilk test, and no variables were found to violate the assumption of normality. Animal mass, soleus mass, and soleus to body mass ratio were analysed by 1-way ANOVA for differences between groups. Muscle pH and signalling protein expression was analysed by 2-way ANOVA for effects of pH and stimulated contraction.

Where differences were found by ANOVA, post-hoc tests were used to determine 95% confidence intervals (95%CI) of the magnitude of the difference, and are presented with the exception of protein expression. As protein expression units are arbitrary, magnitude inferences cannot be made and thus significant differences are reported without 95%CI. Muscle force production and perfusate lactate were analysed by linear regression to determine the rate of change of force and lactate over time for each experiment. These slopes were compared between groups for effects of pH and stimulated contraction.

5.3 Results

5.3.1 Animal characteristics

There were no baseline differences in body mass (P = 0.840), soleus mass (P = 0.910), or soleus to body mass index (P = 0.358) between groups.

Table 5.2: Rat anthropometric data.
Values presented as mean (standard deviation).

	Body mass (g)	Soleus mass (mg)	Soleus/body mass index (mg/g)
7.0 STIM (n = 8)	437.8 (37.5)	200.2 (8.7)	0.46 (0.02)
7.5 STIM (n = 8)	453.9 (43.4)	200.0 (9.3)	0.44 (0.02)
7.0 REST (n = 4)	439.3 (15.1)	197.6 (4.9)	0.45 (0.01)
7.5 REST (n = 4)	444.2 (39.1)	202.7 (15.7)	0.46 (0.01)

5.3.2 Force production

Force production decreased significantly over time in both groups, but there was no significant effect of pH.

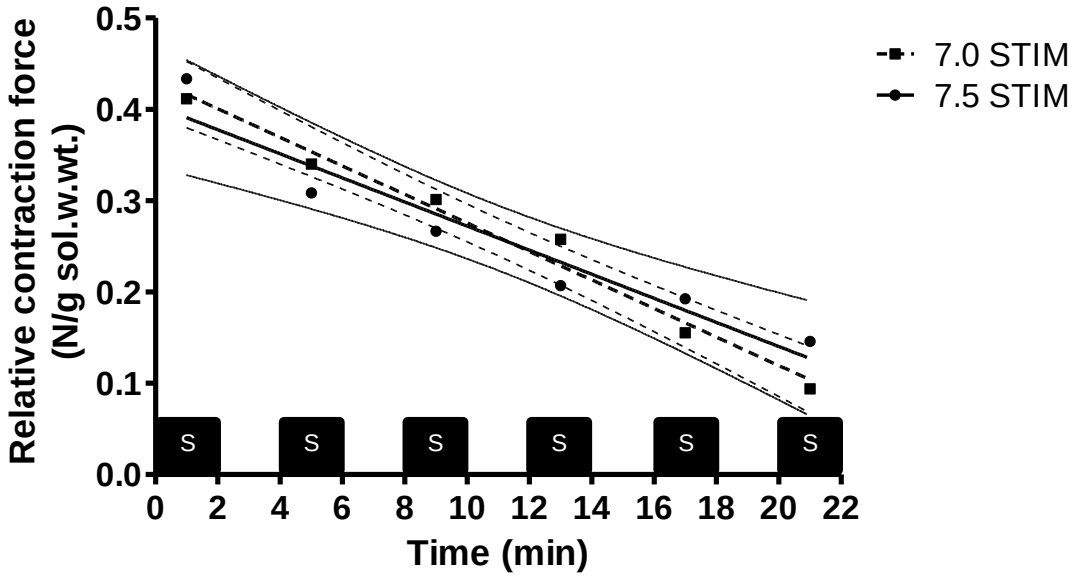


Figure 5.2: Mass-specific contraction force decreases over time in both pH groups.
Data presented as mean values, with linear regression line ±95%CI for both pH 7.0 (squares, dashed lines, n = 8) and pH 7.5 (circles, solid lines, n = 8) perfusion. "S" boxes represent stimulation periods.

5.3.3 Muscle pH and perfusate lactate

There was a main effect of stimulation on muscle pH ($P < 0.001$, table 5.3), with lower pH seen in STIM groups compared to REST groups (95%CI pH 7.5 STIM vs pH 7.5 REST 0.04 to 0.25 pH units, 95%CI pH 7.0 STIM vs pH 7.0 REST 0.09 to 0.30 pH units). There was also a main effect of perfusion pH ($P = 0.001$, table 5.3), however, from confidence intervals this is only apparent following muscle stimulation (95%CI 7.5 STIM vs 7.0 STIM 0.04 to 0.20 pH units, 7.5 REST vs 7.0 REST 0.18 to -0.05). Despite this, there was no interaction effect between perfusion pH and stimulation ($P = 0.285$).

Table 5.3: Muscle pH of perfused soleus.
Data presented as mean (standard deviation).
* = significant effect of contraction (STIM vs REST, $P < 0.05$); # = significant effect of perfusion pH (7.0 vs 7.5, $P < 0.05$).

Perfusion pH	STIM	REST
7.0	6.83 (0.07)*#, n = 8	7.03 (0.02), n = 4
7.5	6.95 (0.05)*, n = 8	7.09 (0.05), n = 4

The rate of increase in perfusate lactate concentration was higher in 7.5 STIM (186.1 ± 9.6 $uM.g^{-1}.min^{-1}$) and 7.0 STIM (177.9 ± 8.7 $uM.g^{-1}.min^{-1}$) in comparison to 7.5 REST (46.7 ± 13.7 $uM.g^{-1}.min^{-1}$) and 7.0 REST (54.9 ± 3.9 $uM.g^{-1}.min^{-1}$), but there was no effect of pH seen in either exercise or control conditions.

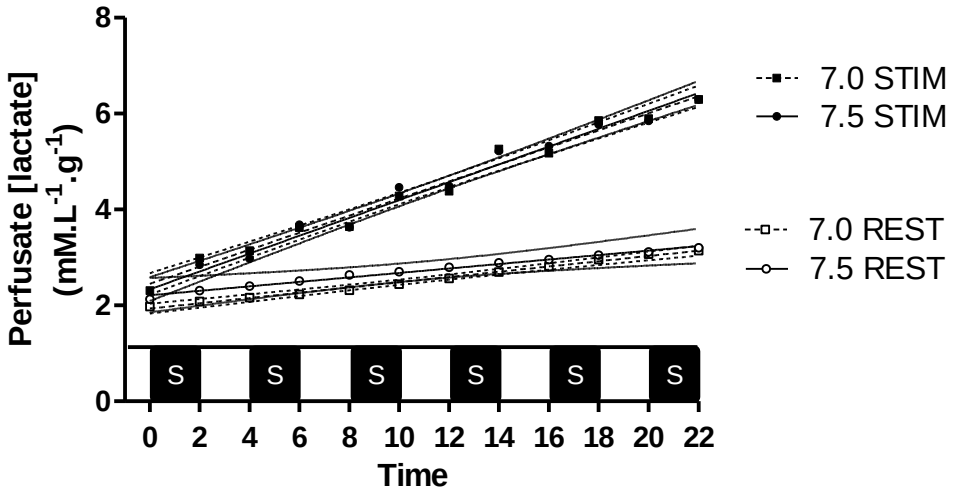


Figure 5.3: Rate of increase in perfusate lactate concentration in contracted (filled symbols, n = 8 each pH) and rested control (open symbols, n = 4 each pH) legs.
Data presented as mean with linear regression line for pH 7.0 (squares, dashed lines) and pH 7.5 (circles, solid lines) perfusion. "S" boxes represent stimulation periods in the STIM groups.

5.3.4 Phosphorylation of signalling proteins

When normalised to pooled sample, no difference was seen between any of the groups for total amounts of any of the proteins investigated, though a trend was evident for greater total p38 MAPK expression in pH 7.0 perfused muscle compared to pH 7.5 perfused muscle (main effect of pH, P = 0.16). No difference in total protein expression is expected, due to the time course of exercise-induced protein synthesis [390]. Accordingly, the ratio of phosphorylated to total protein expression is presented for all proteins, except HDAC 4 and HDAC5/7, which are presented as phosphorylated protein normalised to protein loaded. HDAC5 and HDAC7 were quantified together due to there not being clear separation between their respective bands. All protein expression data are presented as mean ± standard deviation.

5.3.4.1 Calcium signalling

The antibody used to detect phosphorylation of CaMK II resulted in a large amount of non-specific binding (fig 5.4), and has since been discontinued from production by the company. When compared to the antibody against total CaMK II, there was not in fact a band present in the same site. As such, a result for CaMK II was not able to be assessed.

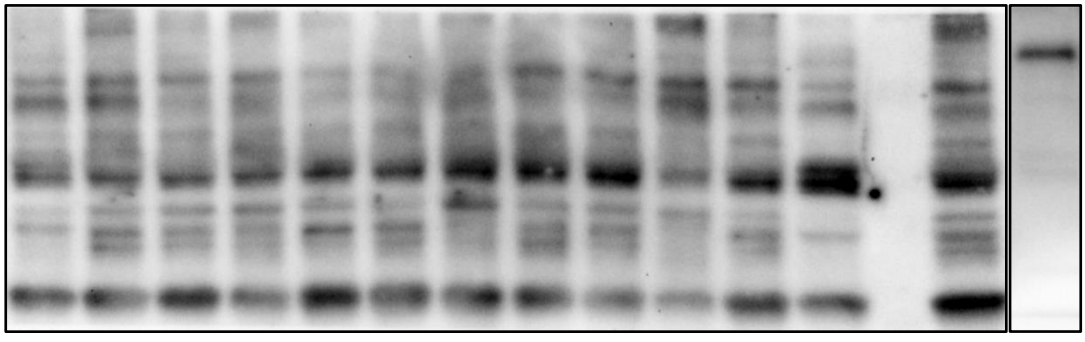


Figure 5.4: Western blot images of threonine-286 phosphorylated CaMK II (left) and total CaMK II from pooled sample (right).

5.3.4.2 Energy-dependent signalling

There was a main effect of pH, with greater AMPK phosphorylation in muscles perfused at pH 7.0 compared to pH 7.5 ($P = 0.008$), and main effect of muscle stimulation, with greater AMPK phosphorylation in STIM groups compared to REST ($P = 0.004$). There was no interaction effect between pH and muscle stimulation ($P = 0.267$).

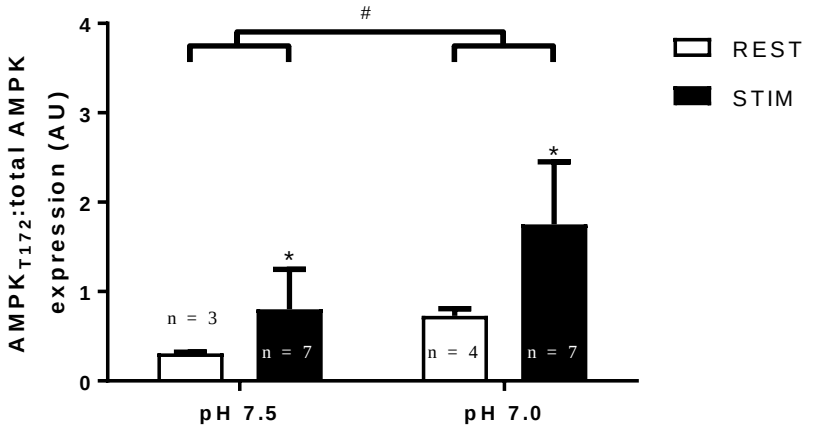


Figure 5.5: AMPK phosphorylation at Thr172 is higher following muscle stimulation and low pH perfusion.
Data are presented as mean ± standard deviation * = $P < 0.05$ (STIM vs REST); # = $P < 0.05$ (pH 7.5 vs pH 7.0).

ACC phosphorylation was not significantly different between perfusion pHs ($P = 0.161$) or muscle stimulation ($P = 0.580$), and there was no interaction effect between pH and muscle stimulation ($P = 0.377$).

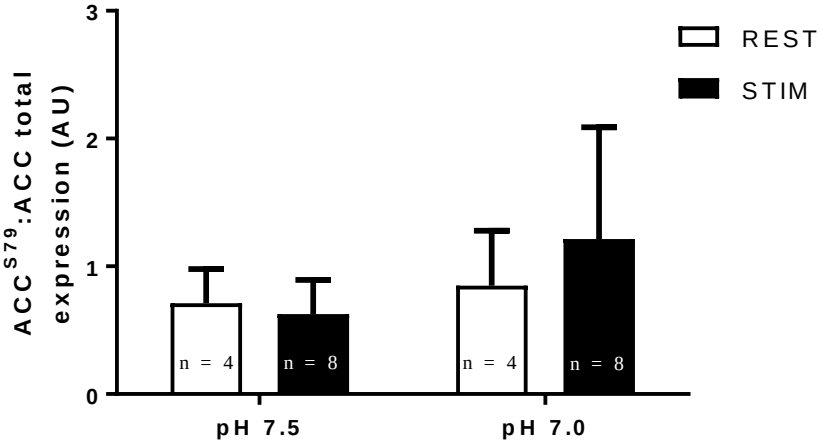


Figure 5.6: ACC phosphorylation at Ser79 is unaffected by muscle stimulation or perfusion pH.
Data are presented as mean ± standard deviation

5.3.4.3 Cellular stress signalling

Phosphorylation of p38 MAPK was not significantly different between perfusion pHs (P = 0.151) or muscle stimulation (P = 0.844), and there was no interaction effect between pH and muscle stimulation (P = 0.293).

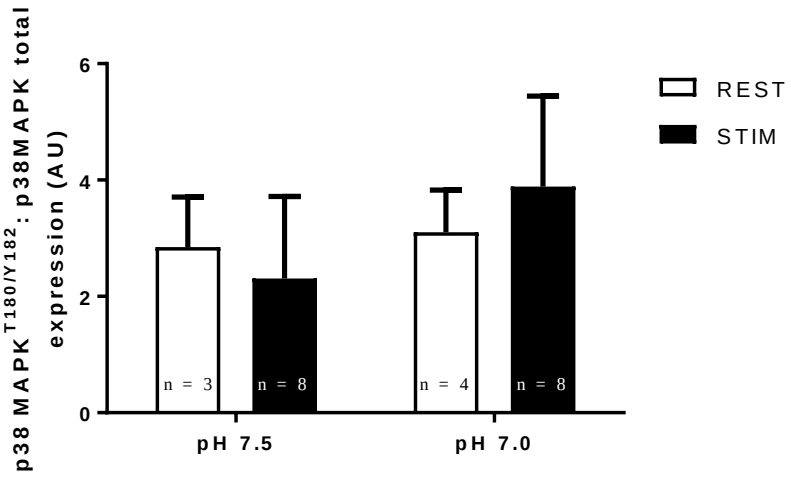


Figure 5.7: p38 MAPK phosphorylation at Thr180 and Tyr182 is unaffected by muscle stimulation or perfusion pH.
Data are presented as mean ± standard deviation.

There was a main effect of pH, with greater ERK1 phosphorylation in muscles perfused at pH 7.0 compared to pH 7.5 (P = 0.020), and main effect of muscle stimulation, with greater ERK1 phosphorylation in STIM groups compared to REST (P = 0.009). There was no interaction effect between pH and muscle stimulation (P = 0.131).

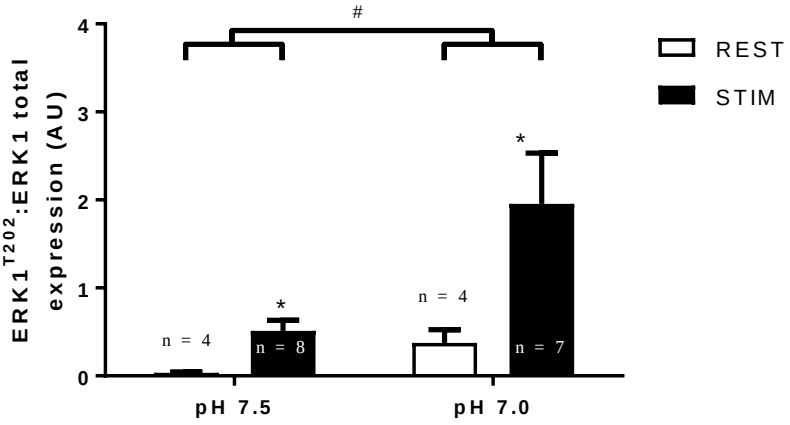


Figure 5.8: ERK1 phosphorylation at Thr202 is higher following muscle stimulation, and higher when perfused at lower pH.
Data are presented as mean ± standard deviation. * = P < 0.05 (STIM vs REST); # = P < 0.05 (pH 7.5 vs pH 7.0).

There was a main effect of muscle stimulation, with greater ERK2 phosphorylation in STIM groups compared to REST groups (P = 0.002), but there was no significant difference between perfusion pHs (P = 0.130), or an interaction effect between pH and muscle stimulation (P = 0.575).

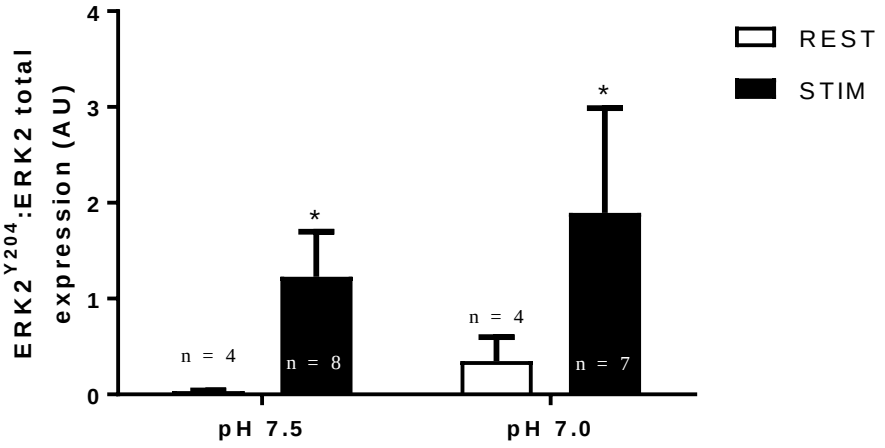


Figure 5.9: ERK2 phosphorylation at Tyr204 is higher following muscle stimulation but unaffected by perfusion pH.
Data are presented as mean ± standard deviation. * = P < 0.05 (STIM vs REST).

5.3.4.4 Transcriptional regulation

There was a main effect of pH, with lower ATF2 phosphorylation in muscles perfused at pH 7.0 compared to pH 7.5 (P = 0.002), but there was no significant effect of muscle stimulation (P = 0.457), or an interaction effect between pH and muscle stimulation (P = 0.223).

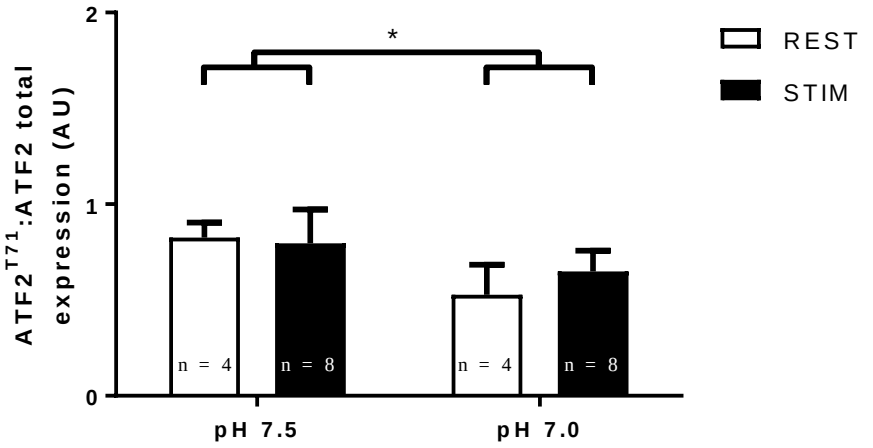


Figure 5.10: ATF2 phosphorylation at Thr71 is lower following low pH perfusion but unaffected by muscle stimulation.
Data are presented as mean ± standard deviation. # = P < 0.05 (pH 7.5 vs pH 7.0).

Phosphorylation of HDAC4 was greater following muscle stimulation in both pH conditions (P = 0.011), but there was no significant difference between perfusion pHs (P = 0.906), or an interaction effect between pH and muscle stimulation (P = 0.747).

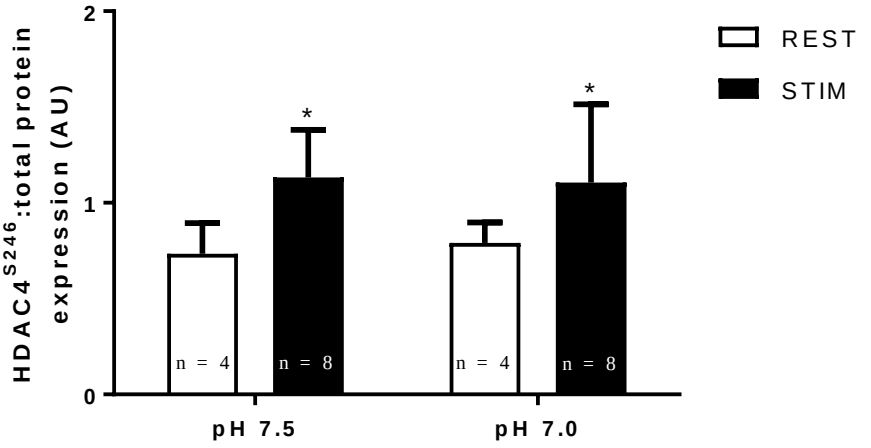


Figure 5.11: HDAC4 phosphorylation at Ser246 is higher following muscle stimulation but unaffected by perfusion pH.
Data are presented as mean ± standard deviation. * = P < 0.05 (STIM vs REST).

Similarly, phosphorylation of HDAC5 and HDAC7 was also greater following muscle stimulation in both pH conditions (P = 0.004), but there was no significant difference between perfusion pHs (P = 0.202), or an interaction effect between pH and muscle stimulation (P = 0.700).

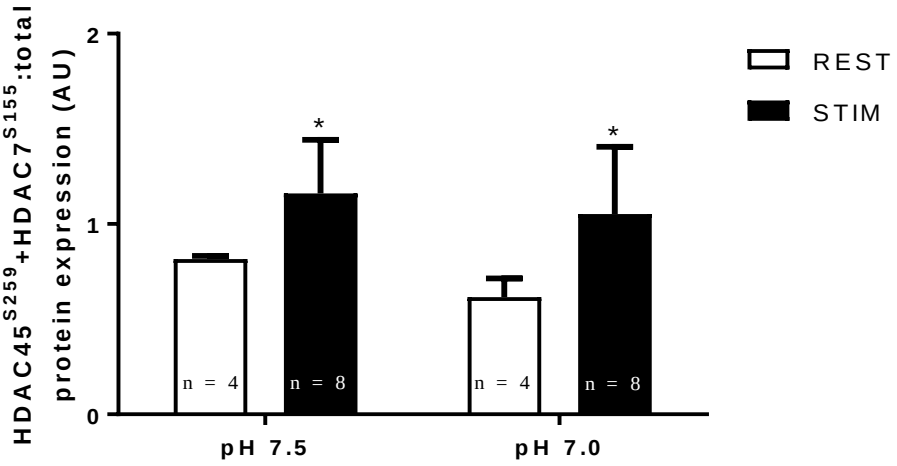


Figure 5.12: HDAC5 phosphorylation at Ser259 and HDAC7 phosphorylation at Ser155 is higher following muscle stimulation, but unaffected by perfusion pH.
Data are presented as mean ± standard deviation. * = P < 0.05 (STIM vs REST).

5.4 Summary of findings

Electrical stimulation was sufficient to decrease muscle pH following perfusion, which further decreased with lowering perfusion pH. However, lowering perfusion pH did not have a significant effect on the rate of muscle fatigue. Lactate efflux from muscle increased over time in all groups, and was increased with stimulation, but unaffected by pH in either the rest or stimulation groups.

Phosphorylation of AMPK and ERK1 was higher with pH 7.0 perfusion in comparison to pH 7.5 perfusion, and phosphorylation of AMPK, ERK1, and ERK2 was higher in stimulation groups compared to rest. However, downstream targets of these kinases did not show similar responses. Phosphorylation of ACC, a target of AMPK, was unaffected by perfusion pH or stimulating contraction. Phosphorylation of ATF2, a target of ERK1/2, was lower following pH 7.0 perfusion, and unaffected by muscle stimulation. Lastly, phosphorylation of HDAC4, 5 and 7, targets of AMPK and CaMK, were higher following stimulation, but unaffected by pH. Thus, there are inconsistent effects of both contraction and pH on short-term signalling responses in skeletal muscle.

5.5 Discussion

5.5.1 Effects of perfusion pH on muscle metabolism and force production

Decreasing the pH of the perfusion media significantly decreased muscle pH in stimulation groups, but did not have any effect on the rate of fatigue development, as both pH perfusions fatigued at a similar rate. This supports the contention that altering muscle pH does not have an effect on muscle force production *per se*. Consistent with this result, previous research on isolated muscle fibres has reported that acidosis has minimal or no effect on muscle contractile function, at physiological temperatures (as used in the present study) [380, 563]. The present study also found that decreasing perfusion pH did not affect the rate of lactate appearance in perfusate in either REST or STIM groups. This suggests that there was no effect of altering extracellular pH on the rate of lactate efflux from muscle, despite different effects on muscle pH in STIM and REST groups. Previous research suggests that glycolytic enzyme phosphofructokinase is extremely pH sensitive (complete inhibition of enzyme activity with a decrease of ~0.2 to 0.4 pH units) [518]. As such, the rate of glycolysis may be expected

to slow with a decrease in pH; however, the pH sensitivity of phosphofructokinase can be offset by increasing substrate and/or AMP concentration [315, 518], both of which happen as a result of intense muscle contraction or exercise [169, 189, 196]. Collectively, this means that differences seen in activation of different signalling pathways at different pH levels are not explained by differences in contraction force or lactate production.

5.5.2 Effects of perfusion pH on intracellular signalling pathways

Phosphorylation of signal transducing kinases AMPK and ERK1 was higher following low pH perfusion, both at rest and following muscle stimulation. There was no significant effect of pH on ERK2 phosphorylation ($P = 0.100$); however, the lack of a main effect for pH may be due to the variability in this measure for the perfusion + stimulation groups. There was also no effect of pH on p38 MAPK phosphorylation. This suggests a low pH can upregulate signalling through at least the AMPK and ERK1 pathways, but these results do not support a role of pH in modulating ERK2 or p38 MAPK signalling. Metabolic acidosis induced by administration of ammonium chloride (blood pH 7.16 compared with controls 7.38 following 10 days supplementation) has previously been shown to increase phosphorylation of both ERK1 and ERK2 in rat kidney, with accompanying renal hypertrophy [33]. Additionally, lactic acidosis (25 mM lactic acid, final media pH 6.7) increases AMPK phosphorylation in breast cancer cell culture models [77], however, this effect again cannot be separated from lactate. The results of the present study are the first to suggest a direct role of acidosis in modulating the phosphorylation of the AMPK and ERK1 pathways in skeletal muscle, pathways that are both associated with mitochondrial biogenesis.

While some signal transduction responses were affected by pH, most of the downstream targets of these early signalling kinases were not affected by pH. Despite higher AMPK phosphorylation with a lower pH, phosphorylation of HDAC4, HDAC5/7, and ACC, were not affected by pH. Phosphorylation of ACC by AMPK is well-established in skeletal muscle [136, 428], and AMPK is a known regulator of HDACs in skeletal muscle [327] although it is not essential for HDAC phosphorylation [326]. Nonetheless, the hypothesis that changes in ACC and HDAC phosphorylation would parallel changes in AMPK phosphorylation was not observed. Similarly, ERK1 phosphorylation

was higher at a low pH, but phosphorylation of ATF2 was lower in low pH conditions. ATF2 is a target of both p38 MAPK and ERK1/2, and phosphorylation of ATF2 is increased via ERK1/2 in response to stress induced by UV light exposure in skin cells [602]. Thus, ATF2 phosphorylation might be expected to follow a similar pattern of activity to ERK; however, in this case, an inverse relationship is seen. Collectively, these indicate that while changes in pH may alter early signalling responses, this may not translate to an effect of pH on transcriptional regulation. It is possible that this is simply due to muscle samples being taken at only a single time point, and that further changes may have occurred over a longer time scale. However, this seems unlikely in the case of AMPK-ACC interactions, which mirror each other over very short (< 5 minute) time frames [428], even though it is noted that different phosphorylation sites were investigated in this study (serine 79) and the previous study (serine 221) [428].

Previous studies have demonstrated that alkalising blood may be beneficial for adaptation and gene transcription [43, 121, 387]. Collectively, the present results suggest that a lower pH may increase activity of early signal transduction pathways, but this regulation is then not evident at the transcriptional regulation level. This is somewhat discordant with the idea that buffering changes in pH may be beneficial to adaptation and/or gene transcription. There are several potential explanations for this. Firstly, the proteins measured in this study are not an exhaustive array of all possible pathways regulating mitochondrial biogenesis. Secondly, events such as post-translational stability of mRNA transcripts, ribosome biogenesis and post-translation protein folding/chaperoning have not been measured, all of which could affect training-induced protein adaptations. Thirdly, as mentioned previously, the time for signal transduction was not accounted for in this study, and it remains possible that delaying muscle sampling would allow time for other pathways to be modified. Thus, despite acidosis seemingly enhancing initial signal transduction, acidosis may cause more inhibitory effects downstream which negate and/or counteract the initial signalling enhancement.

5.5.3 Effects of muscle stimulation on intracellular signalling pathways

Phosphorylation of AMPK, and both ERK1 and ERK2, was higher following muscle stimulation. However, no effect of muscle stimulation was seen on phosphorylation of p38 MAPK.

Previous research on rat EDL muscle showed that concentric contraction induces phosphorylation of ERK1/2, but not p38 MAPK, while eccentric contraction results in greater phosphorylation of p38 MAPK [579]. However, other papers have seen increases in p38 MAPK phosphorylation in rat EDL muscle following 20 minutes of electrical stimulation [484], and mouse EDL muscle following 15 minutes of stimulation [108]. Rat epitrochlearis shows time-dependent effects of stimulation on both p38 MAPK and ERK1/2 phosphorylation [437]. Whole-body exercise studies show that an acute bout of intermittent cycling exercise (one minute cycling, one minute rest) is sufficient to increase phosphorylation of AMPK and p38 MAPK in humans [92]. Additionally, in rats, p38 MAPK phosphorylation is increased in gastrocnemius after one hour of treadmill running [549]. Thus the increases in AMPK and ERK1/2 phosphorylation seen in the present work are consistent with previous findings on electrically-stimulated muscle contraction, while the lack of phosphorylation of p38 MAPK is consistent with the previously reported pattern following electrically-stimulated concentric muscle contraction [579]. However, it is possible that this model does not accurately reflect the response of these proteins to whole-body exercise.

As with the observed effects of pH, there appear to be some inconsistencies between early signalling responses and downstream target activation. HDAC 4 and HDAC 5/7 phosphorylation increased following muscle stimulation, consistent with increased AMPK phosphorylation, and also consistent with observations of increased HDAC phosphorylation post-exercise in humans [323]. However, both ACC and ATF2 phosphorylation were not affected by muscle stimulation, despite increases in AMPK, ERK1 and ERK2 phosphorylation. Once again, it is possible that the timing of muscle sampling may have prevented the effects of stimulation being detected, although previous research has shown intensity-dependent phosphorylation of both ACC and ATF2 immediately post-exercise [122, 476]. This suggests that perhaps increasing the intensity of the stimulated contraction protocol used (either through increased stimulus voltage or increased stimulation frequency), may be necessary to induce phosphorylation of ACC or ATF2, despite the increase in phosphorylation of kinases upstream of ACC and ATF2.

5.5.4 Interactions between pH and muscle stimulation

There were no interactions detected between pH and muscle stimulation. Thus the results of the present study are unable to account for differences seen in previous research, such as Edge et al. [121], which observed that acidosis enhanced PGC-1α expression at rest, but attenuated the increase in PGC-1α expression following exercise. This suggests that acidosis plays a different role during a resting state than following exercise, possibly through stress-activated pathways.

5.5.5 Limitations

There are limitations to the model used in this study. Cell-free (erythrocyte-free) perfusion is rarely used in contraction studies due to the limitations of diffusion-mediated oxygen delivery, where inadequate oxygen supply may limit oxidative metabolism. Previous studies that have used cell-free perfusion and muscle contraction models have typically done so to avoid the interference of blood cell metabolism on a variable of interest [2, 237, 571, 572]. In the present study, the use of cell-free media was used to avoid the effect of pH on oxygen dissociation from haemoglobin, as this would alter oxygen delivery between the two conditions. The rate of fatigue observed was not different between contraction groups, and was lower than that reported in a previous cell-free contraction model [2], and contraction force at 15 minutes was comparable with results seen at the same time point in another study [200]. Additionally, perfusate lactate concentration did not increase during rest periods in the contraction groups, suggesting muscle oxygenation was sufficient to prevent increases in lactate. Collectively, the force production and perfusate lactate concentration data suggest that an oxygen limitation that may have occurred did not affect one pH group more than another, and does not explain the differences seen in signalling pathway phosphorylation. However, while this is a methodological limitation in terms of descriptive data, transient anaerobic energy demands coupled with progressive fatigue is a characteristic of repeat-sprint type training [147], and thus this model may in fact represent signalling response to this training paradigm well. Nonetheless, the limitation remains that the aerobic and anaerobic demand of the contraction protocol could not be determined with the equipment available.

Another potential limitation is that during the cannulation of the hindlimb vessels, there was a small period of ischaemia between the vessels being cannulated, and the start of the perfusion protocol.

Stress-signalling kinases measured in this study (ERK 1 and 2, and p38 MAPK) are responsive to hypoxia/ischaemia [127, 274, 383], and this period may have induced some signalling effect that is unaccounted for. In the case of ERK 1 and 2, it does not seem that any effect of ischaemia detracts from the findings on stimulation and pH responses. However, no effect of either pH or stimulation for p38 MAPK phosphorylation. It is possible that there was a phosphorylation effect seen from this brief ischaemia which may have masked any effects of pH or contraction. This does seem unlikely however, as perfusate lactate remained low in both rest groups – if there had been a significant ischaemic insult, lactate levels would be expected to rise substantially [298], which was not seen here. This suggests any ischaemia which may have occurred was minor, and unlikely to affect the results seen.

Another potential issue is the more 'traditional' approach taken to western blotting. There is growing support for more rigorous methodologies that allow for fully-quantitative assessment of proteins using western blotting [342, 352], as traditional immunoblotting techniques are usually considered 'semi-quantitative' due to the assumptions surrounding proportionality of antibody binding and imaging. The premise of quantitative western blotting is that a protein standard curve can be included in each gel, to allow for calibration of antibody binding proportionality and signal intensity. While this is certainly a methodological improvement in many respects, this approach was not used in this study for the reason that it does not further aid the interpretation of the results seen. There is no known quantifiable amount of phosphorylation that is required for the proteins of interest in this study to be active. Thus quantification of the absolute change in protein phosphorylation does not aid in the interpretation of the protein data, and true quantification requires the use of recombinant peptides, which considerably increases the cost of the research. Use of recombinant peptides also relies on the assumption that both the recombinant and protein from the sample of interest exhibit the same binding characteristics with the antibody used. Accordingly, this approach was deliberated but ultimately considered not to be a meaningful improvement from the perspective of the outcome objectives.

5.6 Conclusion

The key finding of the present study is that lower pH perfusion increases phosphorylation of some of the kinases known to have a role in signalling mitochondrial biogenesis. Importantly, these changes are not explained by a difference in contraction force or lactate production. However, despite differences in early signalling response, measured markers of transcriptional regulation are unaffected by changing pH either basally or acutely post-contraction. Collectively, this suggests that changes in pH may be able to modulate some early signalling events in the mitochondrial biogenesis cascade, but that there may be further regulatory events that prevent this translation of this early response to downstream targets.

Chapter 6: Future directions and conclusion

This thesis has focussed on the role acidosis may have in regulating mitochondrial function, skeletal muscle ROS balance, and contraction-induced signalling associated with mitochondrial biogenesis. The experiments conducted have added to the evidence on how pH changes may affect mitochondrial function and adaptation; however, they also raise further questions. Speculation on further experiments to help explain some of the data presented in this thesis follows.

6.1 Mitochondrial function

In chapter three, it was shown that acidosis decreases the apparent Km of mitochondria for ADP, with a concomitant decrease in maximal respiration, however, the decrease in maximal respiration was challenged by the results in chapter four, where no decrease in maximal respiration was seen over a larger pH range. As identified in chapter three, a central premise of these results is that consumption of oxygen provides an index of mitochondrial function, with the assumption that accelerated oxygen consumption rates are indicative of increased function. However, consumption of oxygen occurs via the electron transport system, which serves to pump protons to support ATP synthesis. Thus if 1) ATP synthesis can be supported by an influx of protons from the cytosol to the intermembrane space; and 2) ATP synthase activity rate is not rate limiting to ATP synthesis, then the following results are explained; firstly, affinity for ADP is increased at pH $\leq$ 6.5 (table 3.1, figure 3.1; [517]), and leak respiration, but not maximal ADP-stimulated respiration, is depressed at pH 6.4 [517]. However, this would not explain the frequent observation that uncoupling the electron transport system from oxidative phosphorylation can result in higher rates of oxygen consumption. Measurement of ATP synthesis rates at different pH remains something worth further investigation. This would effectively test the premise that an acidotic intracellular space could contribute to a mitochondrial inner membrane potential solely through proton diffusion through the outer mitochondrial membrane.

It is also possible that intracellular acidosis may contribute indirectly to ionic membrane potential through the multitude of mitochondrial ion transport proteins (reviewed in [37]), some of which are proton symporters/antiporters. This means that accumulation of hydrogen ions may

potentially affect other ionic gradients, either through acting as a substrate for sodium/potassium - hydrogen exchangers, or simply altering the enzyme activity of any other ion pumps. This in turn would affect the ionic component of membrane potential, independent of any effect on hydrogen ion distribution. There are numerous fluorescent dyes available that could be used to infer the effect of acidosis on membrane potential (for example JC-1/safranin to measure net membrane potential [271]). Further mechanistic information could be determined using ion-selective fluorophores to investigate changes in specific transmembrane ion gradients (for example calcium-green™). Collectively, this would provide information on whether an acidotic intracellular space may alter a) ATP synthesis, b) membrane potential, and in the event membrane potential is affected, c) which ion(s) contribute to any difference in membrane potential seen.

In chapter four, it was shown that voluntary exercise activity resulted in increased mitochondrial respiration and ROS emission, but in most cases did not affect the response of mitochondria to acute changes in pH. One notable interaction was that ROS emission from mitochondria during maximal leak respiration was maintained as pH dropped in muscle from exercised animals, but dropped in muscle from sedentary animals. This effect may be mediated by complex III, as activity assays indicate a pH-induced decline in complex III activity in both sedentary and exercised groups, but the rate of decline was half as much in the exercised cohort compared to sedentary. This suggests that the voluntary exercise protocol in some way made complex III activity less sensitive to changes in pH, and the mechanism(s) mediating this effect merit further investigation. One important thing to note is that permeabilised muscle has no intact circulation, and should have lost intracellular material – so any exercise-induced increase in carnosine or bicarbonate buffering capacity from the exercise training in chapter 4 should be washed out during the tissue preparation. This means that the decreased sensitivity to pH seen in the exercise-trained rats may be an innate property of the mitochondria themselves, rather than due to an increase in muscle buffering capacity.

One finding in chapter four which contrasts with previous findings was that mass-specific mitochondrial ROS emission was higher in exercised rats (figure 4.8), where previous studies have either shown no change, or a decrease in ROS emission [246]. It is difficult to compare results given

the voluntary nature of the intervention, and as such an obvious further addition to this study would be the inclusion of an exercise training group, to compare the magnitude of effect between voluntary activity and a more fixed training stimulus. This could further be divided into a prescribed endurance training protocol compared to high-intensity interval type training.

Chapters three and four do not show agreement on whether acidosis does indeed depress mitochondrial respiration. In chapter three, maximal ADP-stimulated respiration was depressed at pH 6.5 compared to pH 7.1, but was not different at pH 6.9 or pH 6.7. As this suggested there may be a threshold for an effect of pH, this range was extended in chapter four. However, no depression of respiration was evident during phosphorylating states in a pH range from 7.1 to 6.2. In looking collectively at the data, the conclusion could be drawn that the difference seen in chapter 3 between pH 7.1 and pH 6.5 could just be a statistical anomaly – the lower limit of the 95% confidence interval of the difference is 0.2 pmol $O_2.mg^{-1}.s^{-1}$, which indicates that though statistically significant, the confidence limits of the difference mean it may actually not be meaningful. Coupled with no difference seen at pH 6.2 in chapter four, this suggests lower pH does not affect respiration during oxidative phosphorylation. However, one key difference between the two chapters was that in chapter three, maximal respiration was determined by curve-fitting following titration of ADP up to 2.5 mM, while in chapter four maximal respiration was determined following addition of a single 5 mM bolus of ADP. So the final concentration of ADP in each experiment is different, and there is a different time course over which ADP was provided.

Voluntary exercise also resulted in higher mean activities of all mitochondrial enzyme complex activities; however, complex II and complex IV showed greater differences than complex I or III. This suggests there is preferential increase in complex II and IV activity with exercise, and the mechanisms responsible warrant further attention. It should also be noted that no effort was made to look at supercomplex assembly, and this could be an interesting addition – particularly in comparison with a training intervention. Mitochondrial function is ultimately a synergy between different enzyme complexes, and the formation of ‘supercomplexes’, which are formed when respiratory complexes associate together [466], represents another level of control of mitochondrial function. Exercise

increases supercomplex formation [171], however how supercomplex assembly and/or composition is affected by acidosis is unknown.

6.2 Non-mitochondrial ROS balance

In chapter three, various pro- and antioxidant enzyme activities were investigated, with the collective implication that glutathione resynthesis may be promoted during acidosis. However, to fully characterise the effect of acidosis on skeletal muscle antioxidant capacity, substrate affinity and spatial arrangement of sites of ROS production and disposal should be further investigated. Though maximal enzyme activities suggest there may be greater glutathione resynthesis at a lower pH, this may not occur if there is competition for NADPH, or if GR affinity for NADPH is decreased at a lower pH. This could be achieved in a manner similar to the ADP titration used for determining mitochondrial affinity for ADP. Measuring the activity of enzymes with titrations of different substrate concentration would allow the estimation of K_M as pH is changed, and this data could be used to generate a model of the net effect of pH on ROS disposal capacity. This could be further tested by incubating muscle homogenates with a superoxide-generating system such as xanthine with xanthine oxidase, and measuring ROS disposal across the pH range tested.

One key ROS producing enzyme in skeletal muscle which was not presented is NADPH oxidase. Attempts were made to measure NADPH oxidase activity using both the Oroboros-O2F system and the Enspire plate reader system, and initially seemed successful. However, further testing revealed that there seemed to be autofluoresence of amplex ultra red in the presence of NADPH, or that NADPH was somehow able to directly oxidise amplex ultra red (which seems unlikely given that NADPH is a reducing agent). Experiments in the oxygraph were unable to distinguish a difference between different amounts of sample, or indeed between sample and no sample. However, differences were seen with different amounts of NADPH added. Measurement of NADPH oxidase activity would be a strong addition to the data presented in chapter three, both from the perspective of NADPH oxidase being a ROS producer in skeletal muscle, and also being a competitor for NADPH.

In chapter four, antioxidant capacity was not compared between exercised and sedentary rats, and it is possible that the permeabilised fibre preparation will lose antioxidant capacity due to loss of cytosolic contents. This could in part explain why ROS emission was higher in exercised animals, as previous findings of lower ROS emission following exercise training may be a result of greater skeletal muscle antioxidant capacity, rather than lower ROS production [167, 238].

6.3 Mitochondrial adaptation

Data presented in chapter five examined how pH affected various signalling pathways associated with mitochondrial biogenesis in skeletal muscle. The central challenge to testing this theory was to develop a system where muscle pH could be manipulated, without manipulating other variables known or implicated in altering signalling (in this case, force production, lactate production, and oxygen availability). The hindlimb perfusion model used achieved this; however, there are improvements that could be made.

Firstly, only two different pH buffers were examined, and the addition of more would be useful. Pilot experiments originally included a pH 6.5 perfusion, but perfusion + contraction could not be sustained at pH 6.5, with loss of excitability occurring after ~10 minutes. This meant that there was not an equal contraction stimulus for pH 6.5 perfusion, and thus any signalling results seen could not be attributed to a change in pH. Thus, perfusion at pH 6.5 was not continued, and only pH 7.0 and pH 7.5 were used. Testing a range of other pH buffers to give greater resolution within physiological range (for example pH 7.25) would be a useful addition to see if the effect of pH may be a linear or threshold event.

Secondly, the design of the experiment was to look at intracellular signalling pathways, and as such muscle was collected immediately post-contraction. The results seen imply that some early signalling response are affected by changing pH, but this response is somehow ‘lost’ at the level of transcriptional regulation. In hindsight, it would have been good to have another group at each pH which had the perfusion and contraction intervention, and then remained perfused for 2-4 hours before collection of muscle, to then look at mRNA response. This would determine what the actual effect of

altering pH was on mRNA expression. Some muscle samples remain for determining mRNA expression immediately post-contraction, though no change is expected from the immediate time point.

Thirdly, this study focussed on the canonical, well-known signalling pathways (CaMK, AMPK, p38 MAPK and ERK1/2). However, the list of proteins investigated here are not exhaustive, with targets such as calcineurin, MEF2, CREB, and SIRT1, as well as CaMK which could not be determined. There are also other candidate pathways in regulating mitochondrial biogenesis which are growing in support in the literature (for example the tumour suppressor protein p53 [456]).

6.4 Conclusions

The experiments conducted in this thesis have determined that, in sedentary muscle, a physiological range of acidosis increases mitochondrial affinity for ADP, depresses leak respiration rates, but has no conclusive effect on respiration rates during oxidative phosphorylation. Activities of electron transport system complexes I, II and III are depressed with lowering pH, but activity of complex IV is increased. Mitochondrial ROS emission is depressed by lowering pH in non-phosphorylation states, though this is possibly mediated by enhanced ROS disposal. Glutathione reductase activity is enhanced at lower pH, though supply of NADPH is decreased, and superoxide dismutase and xanthine oxidase activities are both depressed as pH is decreased. The net effect of these changes is unknown.

Voluntary exercise increases muscle buffering capacity, but does not seem to alter the effects of pH on mitochondrial function. The exception to this is that voluntary exercise eliminates the effect of pH on ROS emission in a leak state, meaning that as pH lowers, ROS emission is maintained in exercised muscle, but in sedentary muscle is depressed. This is possibly mediated by an intrinsic effect of exercise on complex III activity, which is depressed approximately 2-fold more in sedentary muscle than exercise muscle as pH decreases. Voluntary exercise does increase respiration during oxidative phosphorylation, and increases mitochondrial ROS emission. Voluntary exercise also increases the activity of all mitochondrial electron transport system complexes, though complexes II and IV show greater increase than complex I or III.

Decreasing pH up-regulates AMPK and ERK1 phosphorylation, but down-regulates ATF2 phosphorylation, both at rest and post-contraction. Contraction induces increases in AMPK, ERK1, ERK2, HDAC4 and HDAC5 + HDAC7 phosphorylation. Neither pH nor contraction had an effect on p38 MAPK or ACC phosphorylation, and pH did not influence the contraction-induced increase in phosphorylation of any proteins investigated. Inconsistencies between the effects of pH on AMPK and ERK1 and their respective targets ACC and HDAC are unexplained, but suggest further regulation. Importantly, the effects of pH on AMPK and ERK1 are not explained by differences in muscle oxygen supply, lactate production, or force production.

Appendices

Appendix 1: References

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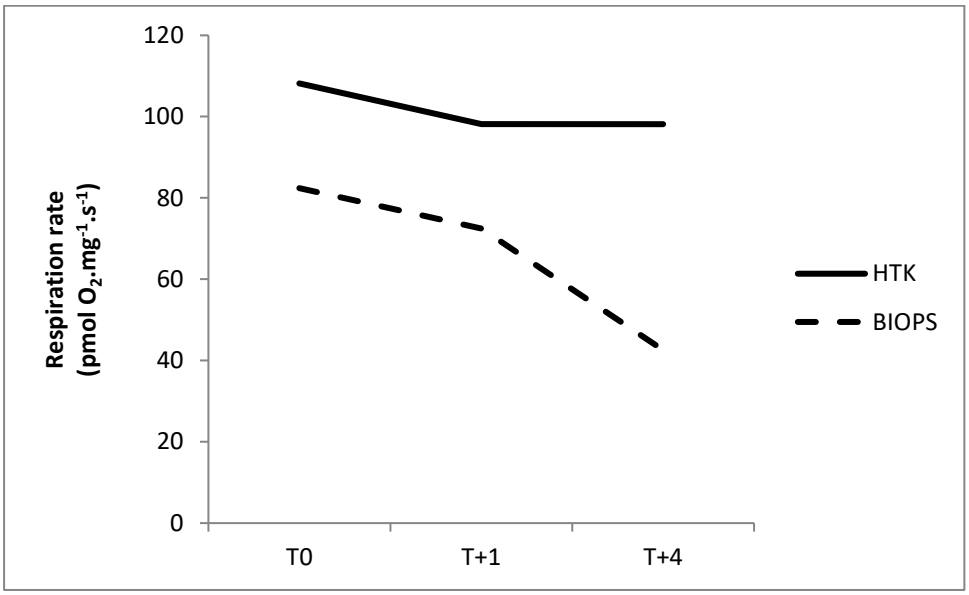
Appendix 2: Preservation solution for respiration analyses

For respiration assays, tissue is often preserved in a high-energy solution (BIOPS), described by Oroboros (the manufacturers of the Oxygraph-O2k system). This solution contains (in mM) 10 Ca-EGTA buffer (2.77 CaK_2EGTA, 7.23 K_2EGTA, allowing 0.1mM free calcium) 5.7 Na_2ATP, 6.56 $MgCl_2(H_2O)_6$, 20 Taurine, 15 Na_2PCr, 20 Imidazole, 0.5 Dithiothreitol, 50 K-MES, and 30 Sucrose, pH 7.1 at 0°C. The purpose of this solution is to allow pH changes to be buffered and provide ample phosphocreatine and ATP to maintain membrane potential (through consumption of ATP by ATP synthase). In our hands this solution allows viable preservation of skeletal muscle samples for ~3 hours with minimal loss of mitochondrial function, though others have claimed up to 29 hour stability of samples [481]. A potential alternative available was Custodiol® Histidine-Tryptophan-α-Ketoglutarate (HTK) organ transplant buffer. The principle behind HTK is almost the opposite of BIOPS, in that it induces membrane hyperpolarisation through a low extracellular sodium composition, thus inactivating tissue (rather than attempting to support activity as BIOPS does).

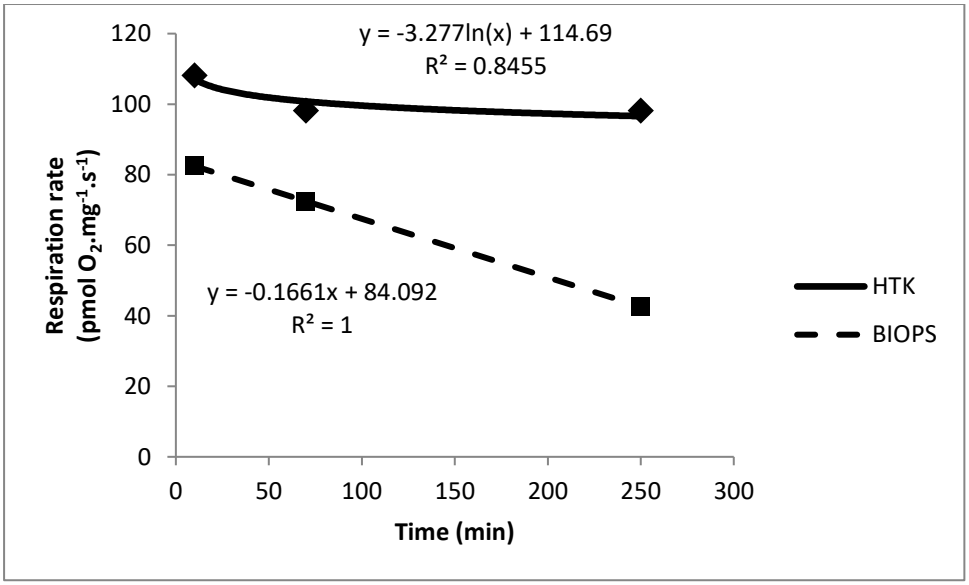
To test the efficacy of these solutions in preserving skeletal muscle mitochondrial function, soleus muscle from n=2 rats was excised as per study 1 (removed following anaesthesia induced by isoflurane and death by cervical dislocation), divided into two portions lengthways along the muscle, with half placed in BIOPS, the other half placed in HTK. This muscle was kept on wet ice and stored in a 4°C room. Muscle fibers were prepared and permeabilised immediately (T_0) after collection (time in BIOPS/HTK < 10 minutes), 1 hour post-collection (T_{+1}), 4 hours post-collection (T_{+4}) and 24 hours-post collection (T_{+24}). Permeabilisation was performed in BIOPS regardless of preservation media as described by Kuznetsov et al. [275].

At T_0, maximal OXPHOS respiration measured from HTK muscle was ~1.3 fold higher than BIOPS muscle, a similar fold difference was again observed at T_{+1}, and at T_{+4} HTK muscle was 2.3 fold higher than BIOPS muscle. This was due to a drop in respiration from BIOPS muscle. In terms of absolute respiration values over time, both solutions dropped slightly (~10%) from T_0 to T_{+1}, but HTK muscle did not drop further from T_{+1} to T_{+4}. Neither sample was still viable at T_{+24}, with very little

complex I linked respiration detected (though complex II function appeared to still be intact from HTK muscle).



Plotting the average respiration recorded against time in minutes reveals that maximal OXPHOS recorded from BIOPS muscle declines linearly with time, whereas HTK muscle does not (HTK muscle is best modelled by a logarithmic function, but the key point is that function is maintained at a higher rate for a longer time).



From these results it was concluded that HTK is the superior preservation solution for measurement of mitochondrial function from permeabilised skeletal muscle fibres, with minimal loss of function up to 4 hours post excision from animal.

This finding is incongruous with original reports on high-energy preservation solution (HEPS), a precedent to BIOPS, in which human skeletal muscle fibres showed minimal loss of function over 24 hours [481]. HEPS contained contains (in mM) 10 Ca-EGTA buffer (allowing 0.1μM free calcium) 9.5 $MgCl_2$, 20 Taurine, 5 ATP, 15 PCr, 3 KH_2PO_4, 29 Imidazole, 49 K-MES, pH 7.1. These concentrations are similar to BIOPS, though BIOPS contains the addition of sucrose and DTT. However, in this report, maximal ADP-stimulated mitochondrial oxygen consumption rate at baseline is not reported, and results provided suggest that mitochondrial function is not compromised when comparing 6.5 hours of cold storage to 29 hours of cold storage. However, with no baseline comparison it is unknown how function was compromised from 0 hours to 6.5 hours of cold storage, and the rates shown appear low (~10 pmol $O_2.mg^{-1}.s^{-1}$). This could potential be a reflection of the age of the participants (40-82 years) and/or the substrate choice (complex I respiration supported by malate and glutamate). However, these results are still approximately 3-fold lower than those of Larsen et al., who report maximal ADP-stimulated respiration with malate and glutamate in lean males aged 53±3 years as ~30 pmol $O_2.mg^{-1}.s^{-1}$ [283].

Appendix 3: Supplementary enzyme data

Enzyme activities (mean ± SD, U.mg tissue^{-1}) for ROS balance enzymes presented in chapter 3 are as follows:

Enzyme	**pH 6.5**	**pH 7.0**	**pH 7.5**
ICDH	23.89 ± 3.18	36.18 ± 3.19	39.50 ± 2.49
G6PDH	8.84 ± 1.79	8.44 ± 1.95	11.42 ± 2.56
GR	8.61 ± 1.44	6.78 ± 1.36	5.81 ± 0.69
GPX	5.35 ± 1.05	5.21 ± 1.09	5.23 ± 0.77
NOX	4.80 ± 0.64	8.22 ± 0.45	8.39 ± 0.89
XO	1.71 ± 0.23	2.94 ± 0.16	3.25 ± 0.55
SOD	6.23 ± 0.50	8.38±1.06	9.13±1.01

Enzyme activities (mean ± SD, nM.min^{-1}.mg protein^{-1}) of mitochondrial respiratory complexes presented in chapter 4 are as follows:

pH	**Cohort**	**Complex I**	**Complex II**	**Complex III**	**Complex IV**
6.2	Sedentary	494 ± 23	271 ± 8	393 ± 9	2087 ± 176
	Exercised	569 ± 8	523 ± 9	592 ± 47	4345 ± 311
6.5	Sedentary	512 ± 16	293 ± 8	549 ± 26	1809 ± 141
	Exercised	590 ± 12	547 ± 5	658 ± 47	4378 ± 287
6.8	Sedentary	543 ± 17	313 ± 5	634 ± 23	1639 ± 114
	Exercised	630 ± 10	567 ± 15	691 ± 37	3816 ± 371
7.1	Sedentary	559 ± 24	331 ± 7	686 ± 27	1546 ± 82
	Exercised	648 ± 10	629 ± 5	758 ± 13	3443 ± 207

Appendix 4: Custom statistical analyses

Custom SPSS pairwise analyses used in study 2 were developed with the guidance of Dr. Kathy Ruggiero, Department of Statistics and School of Biological Science, University of Auckland.

As SPSS does not offer pairwise comparisons as a default post-hoc analysis for an interaction effect in a mixed linear model, custom hypothesis tests were generated to compare between groups. This involved contrast coding pH and activity levels to generate a comparison matrix. Contrasts were not made involving comparing different pH measures and different activity levels (for example sedentary muscle, pH 7.1 was not compared with exercised muscle, pH 6.8). Comparisons were made between sedentary and exercised muscle at each pH, and within sedentary and exercised muscle each pH were compared.

For a comparison between sedentary and exercised muscle at a given pH, sedentary muscle was coded as 1, and exercised muscle coded as -1. The pH to be tested was coded as 1, and the remaining pH values coded as 0, resulting a matrix. For example, to test between sedentary and exercised muscle at a pH of 6.2, the matrix appears as follows:

		6.2	6.5	6.8	7.1
		1	0	0	0
Sedentary	1	1	0	0	0
Exercised	-1	-1	0	0	0

This coding system could then be entered into SPSS using the syntax editor, which interprets a numerical string to weight particular group(s) for analysis based on user specification. Thus for 4 pH levels (a-d) and 2 activity levels (1-2), a pH*Activity interaction results in 8 groups, entered in order as a1, a2, b1, b2, c1, c2, d1, d2. For example, the above comparison requires that the user specify that Activity is coded as 1 and -1, the interaction being tested, and then the resulting code of each group as a string of 8 numerical codes to represent the contrast, ie:

/test = 'pH[6.2]sed vs pH[6.2]ex' Activity 1 -1 pH*Activity 1 -1 0 0 0 0 0 0

To test a contrast of the difference between sedentary and exercised animals at a pH of 6.5, the second pH level, the matrix now appears as:

		6.2	6.5	6.8	7.1
		0	1	0	0
Sedentary	1	0	1	0	0
Exercised	-1	0	-1	0	0

And the resulting custom test is coded as follows:

/test = 'pH[6.5]sed vs pH[6.5]ex' Activity 1 -1 pH*Activity 0 0 1 -1 0 0 0 0

For a test of different pH groups within a single activity group a similar principle is applied but instead the specified contrast is within the 4 pH groups, so a matrix for a test between pH 6.2 and 6.5 in sedentary animals would appear as follows:

		6.2	6.5	6.8	7.1
		1	-1	0	0
Sedentary	1	1	-1	0	0
Exercised	0	0	0	0	0

Importantly, the order of groups in a pH*Activity contrast is not changed, so the syntax is slightly changed as follows:

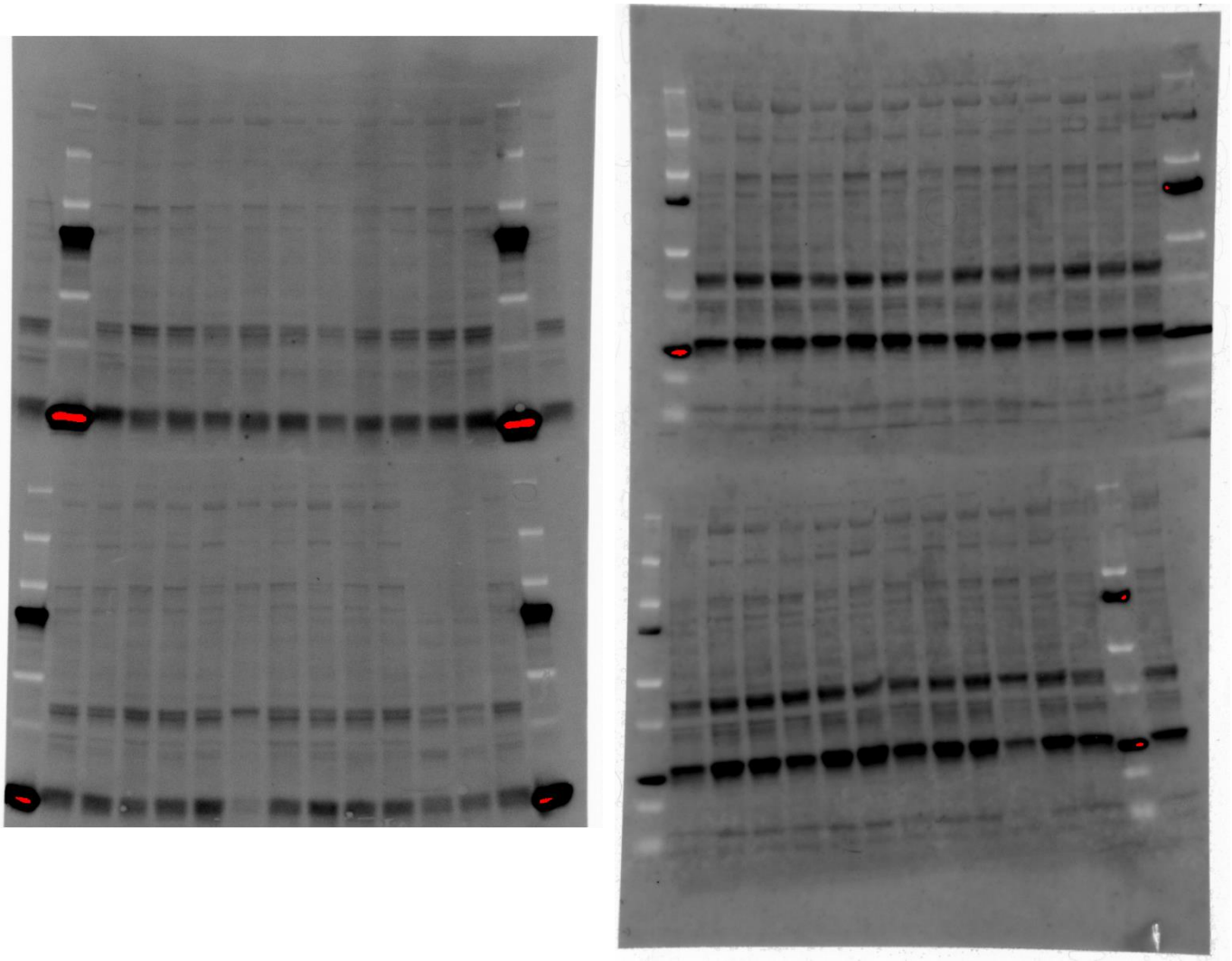
/test = 'pH[6.2]sed vs pH[6.5]sed' pH 1 -1 0 0 pH*Activity 1 0 -1 0 0 0 0 0

These tests were then replicated to give pairwise comparisons between activity groups at each pH (totalling 4 tests), and pairwise comparisons between all possible pH pairs within both activity

groups (totalling 12 tests). This resulted in a total of 16 custom hypothesis tests performed. It is important to note that the p-values generated by these tests do not adjust for multiple comparisons, thus significance was assessed using the false discovery rate according to Curran-Everett, D (2000) [97].

Appendix 5: Immunoblot images

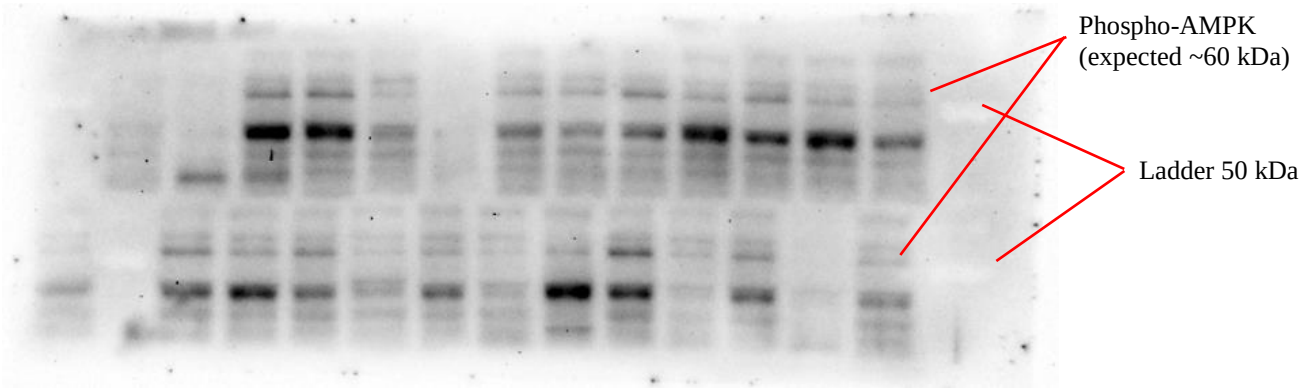
Stain free gel activation, used for total protein loading control



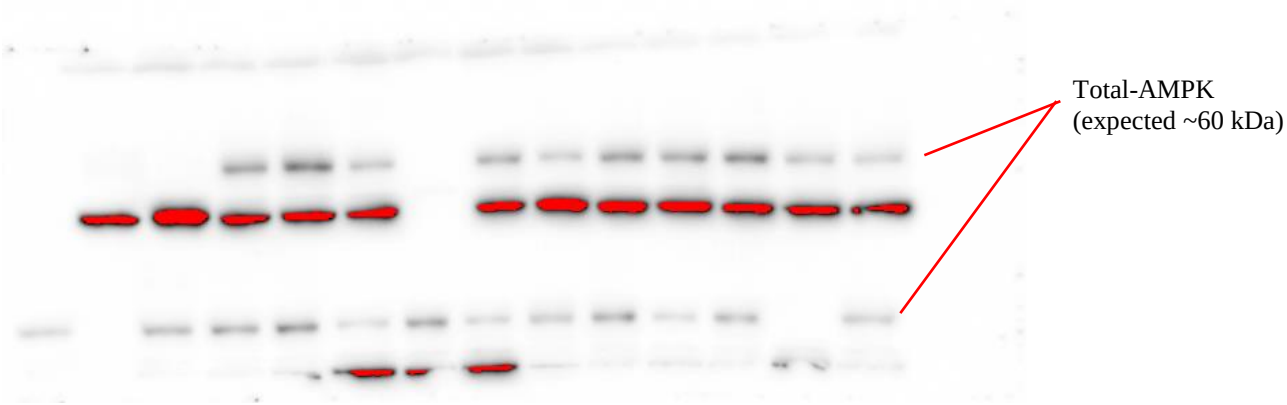
Immunoblot images:

All blots are presented as two membrane sections, the top being pH 7.0 perfused samples, and bottom being pH 7.5 perfused samples. Each gel contains two distinct lane which are protein ladders, and a pooled sample, in addition to experimental samples. By convention, experimental samples were loaded in an order of: rest, stim, stim (x4). Some blots had to be overexposed to detect certain proteins of interest, these images show saturated pixels as red (and these bands were not part of the image quantification).

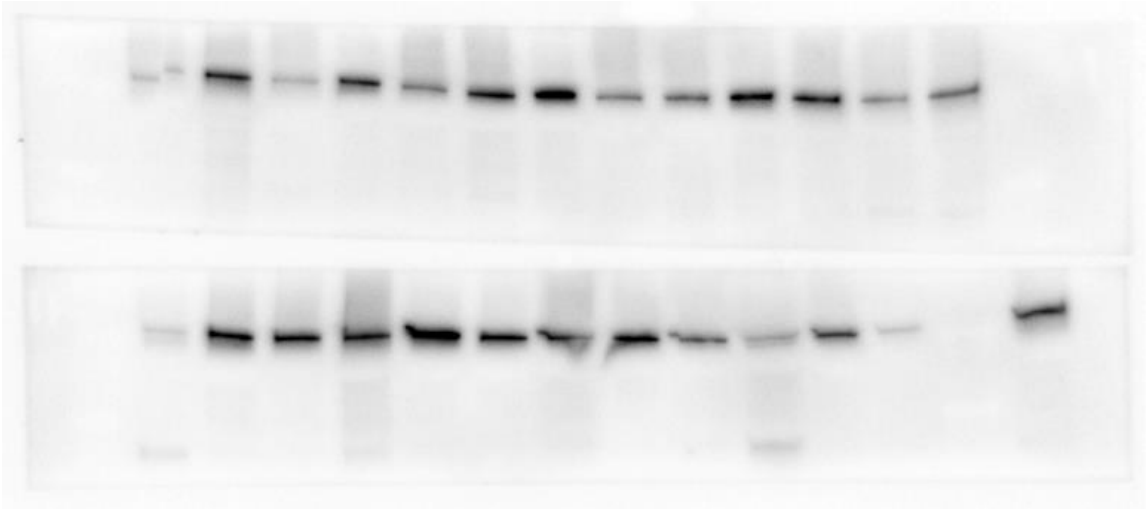
Phospho AMPK



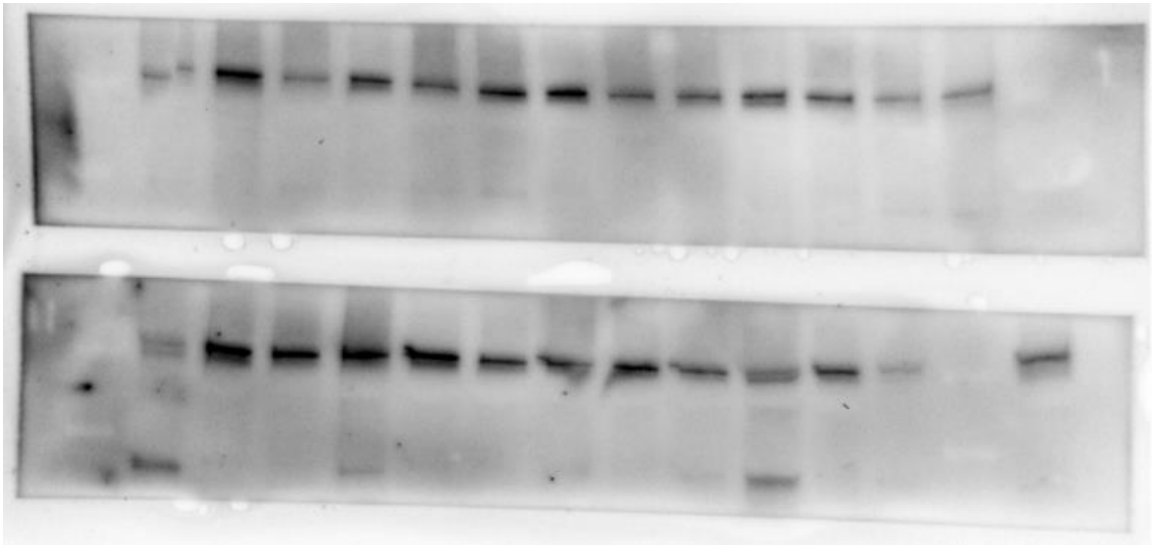
Total AMPK



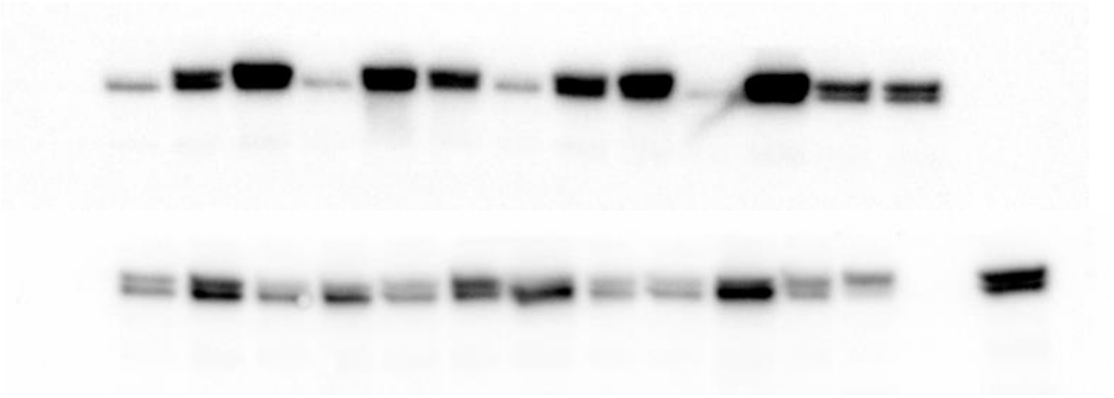
Phospho ACC



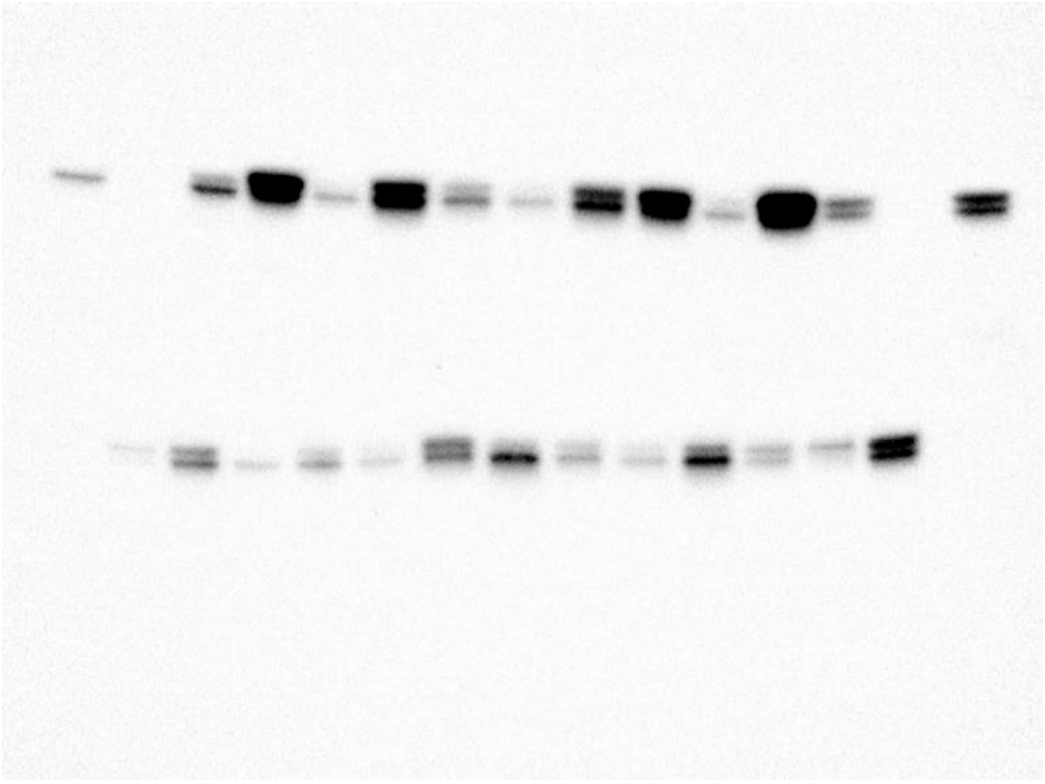
Total ACC



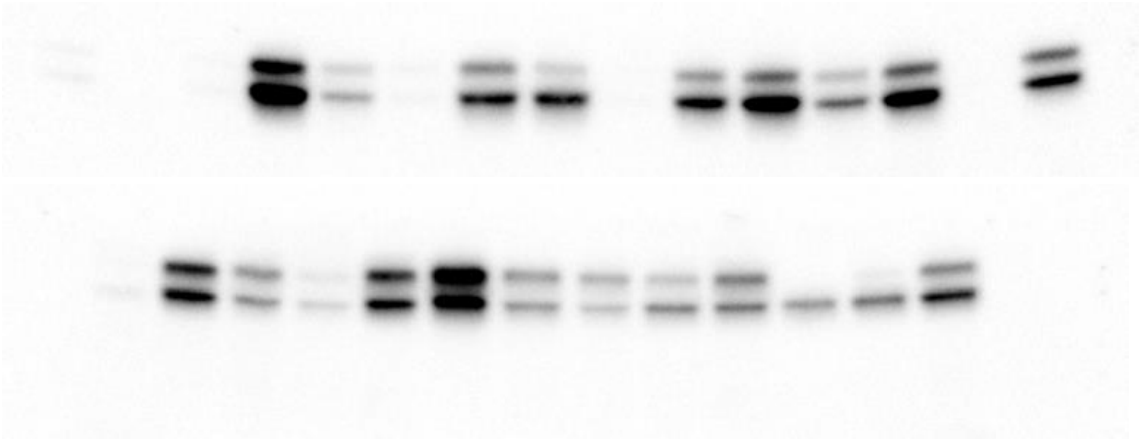
Phosphorylated p38 MAPK



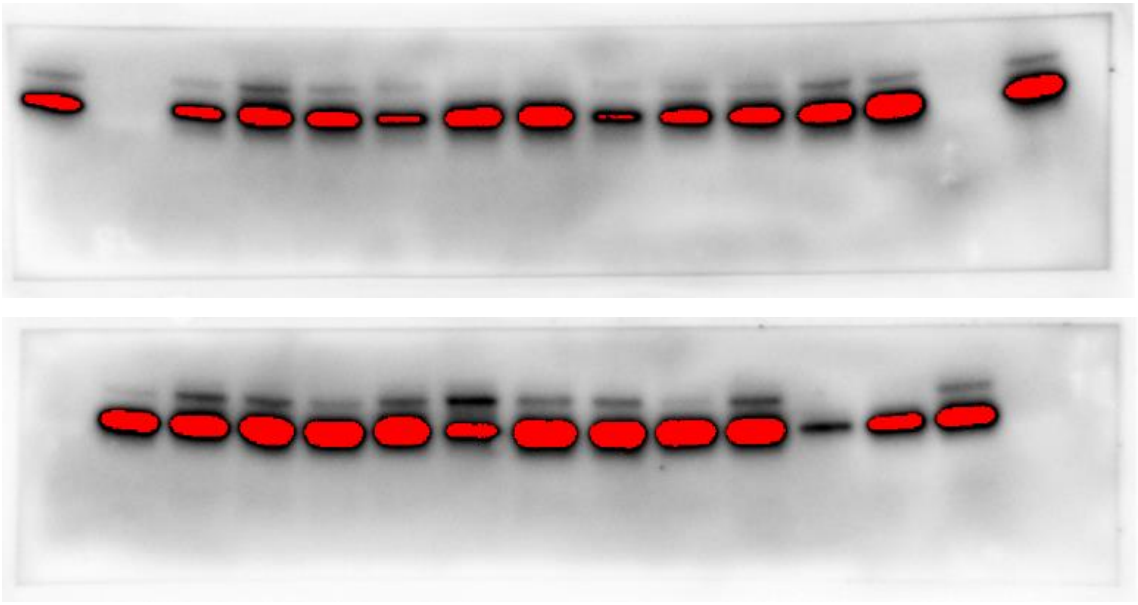
Total p38 MAPK



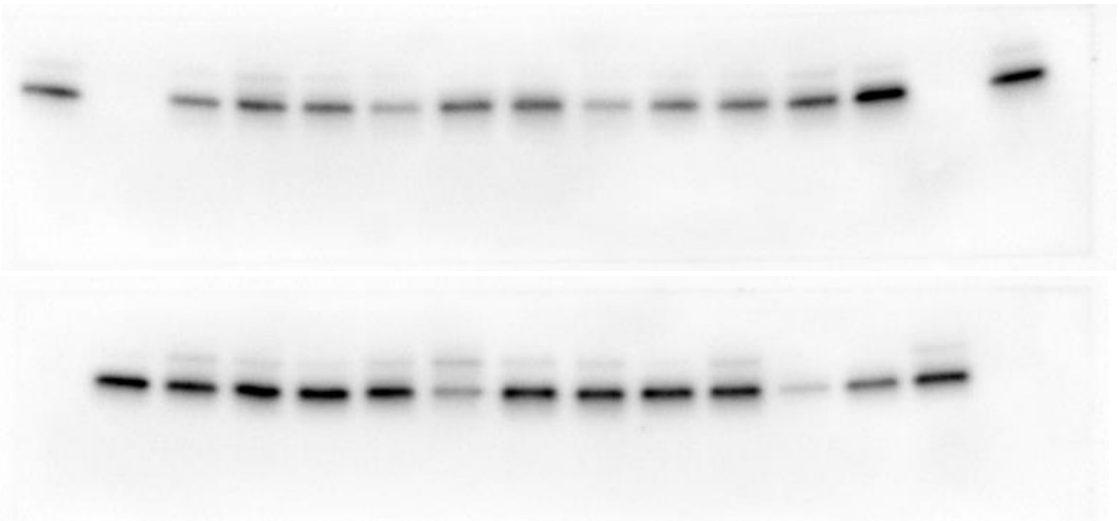
Phospho ERK 1/2



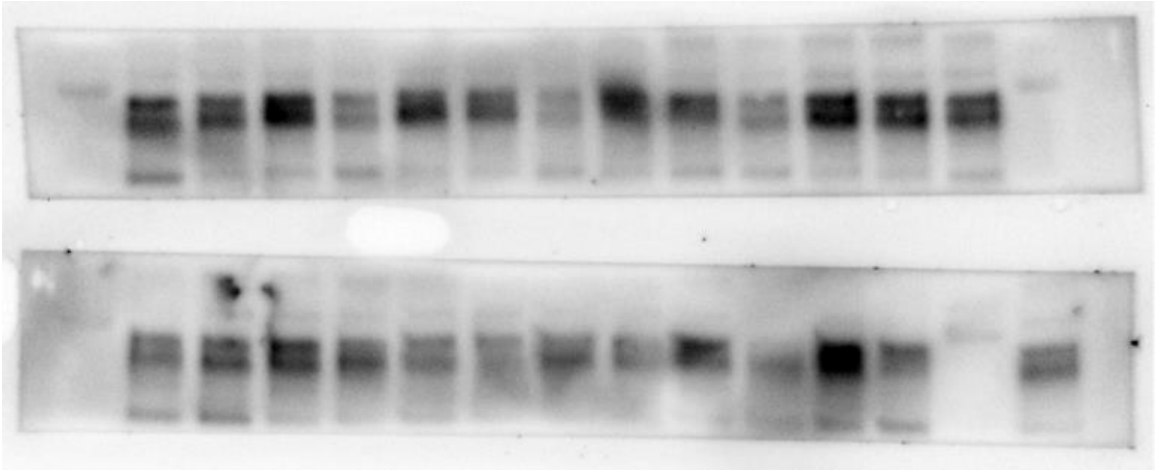
Total ERK 1



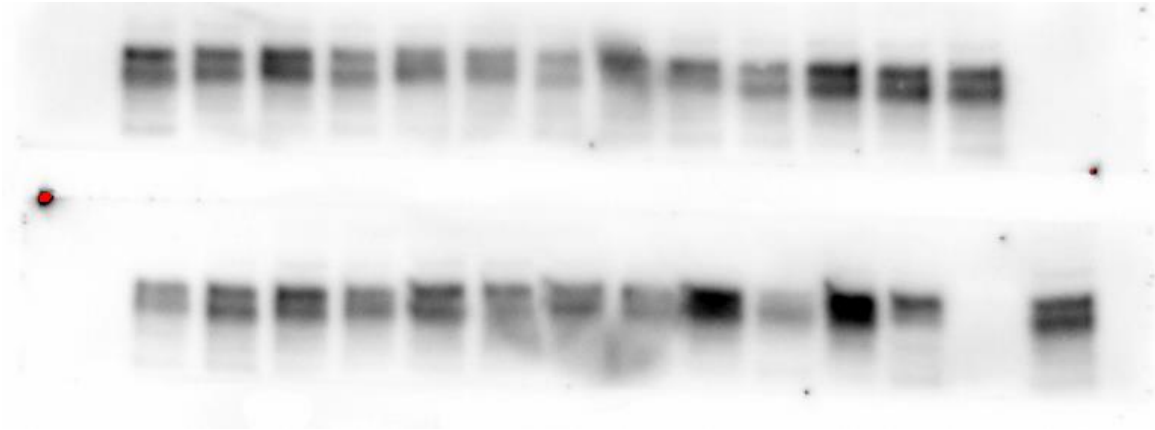
Total ERK 2



Phosphorylated ATF2



Total ATF2



Phosphorylated HDAC 4/5/7