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The Mitochondrial Basis of Ageing and Neurodegeneration

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Abstract

One of the greatest achievements of the past 150 years is the extraordinary extension of average human lifespan. However, increasing average lifespan is coupled to an insidious rise in age-related cognitive decline and muscle loss. Indeed, progressive age is the number one risk factor for non-communicable diseases. Understanding the underlying biological mechanisms which drive ageing is a mammoth task. Mitochondrial dysfunction has emerged as a critical feature of ageing. This Thesis explored the biochemical changes to mitochondria with age and diseases of age.

Firstly, in mouse brain and muscle mitochondria, the expression of potential biological markers of ageing, carbonic anhydrase-II and -III were shown across adulthood. A window of increasing carbonic anhydrase expression between 12-18 months of age was identified. Targeted inhibition of *C. elegans CA* orthologue, *cah-2*, at middle age significantly increased lifespan. Treatment of *C. elegans* with the carbonic anhydrase-II inhibitor, dorzolamide hydrochloride, at middle age showed a trend toward increased lifespan, but this did not reach significance.

Secondly, the long-lived bat (*P. pipistrellus)* was shown to have high levels of fatty acid binding protein 3 (FABP3) in muscle mitochondria, compared to short-lived mice. Increasing FABP3 levels was connected to high free fatty acid levels. Knockdown of *FABP3 C. elegans* orthologues altered mitochondrial morphology, oxygen consumption and significantly reduced lifespan.

Thirdly, the healthspan promoting lifestyle intervention, exercise, was assessed. *D. melanogaster* were exercised on the bespoke Ingram Counterbalanced Exercise machine. The proteomic profiles of mitochondria isolated from exercised and non-exercised wild-type and *Pink1*$^{-/-}$ *D. melanogaster* were examined. The mitochondrial proteome displayed exercise-related changes. Interestingly, exercise caused sweeping mitochondrial protein expression reductions to *Pink1*$^{-/-}$ *D. melanogaster* with exercise.

Fourthly, the cerebellar mitochondrial inflammatory marker profiles of females and males with and without Parkinson's disease were surveyed. This study found that inflammatory marker changes manifest differently with disease progression between sexes. Interestingly, the control female group showed a distinctive inflammatory profile, characterised by highly variant inflammatory marker levels.

This Thesis describes biochemical alterations to mitochondria with ageing and diseases of age. These findings highlight the diverse changes that mitochondria undergo with ageing, potential markers of longevity and interventions that may improve healthspan.

List of Publications

Ingram, T. L., & Chakrabarti, L. (2016). Proteomic profiling of mitochondria: what does it tell us about the ageing brain? Aging, 8(12), 3161–3179. https://doi.org/10.18632/aging.101131

Pollard, A. K., Ingram, T. L., Ortori, C. A., Shephard, F., Brown, M., Liddell, S., … Chakrabarti, L. (2019). A comparison of the mitochondrial proteome and lipidome in the mouse and long-lived Pipistrelle bats. Aging, 11(6), 1664–1685. https://doi.org/10.18632/aging.101861

Wang, Y., Ingram, T.L., Marshall, S., Shephard, F., & Chakrabarti, L. (2020) E-liquids reduce brood size in Caenorhabditis elegans. Frontiers in Physiology (*in peer-review*).

Ingram, T.L., Shephard, F., Sarmad, S., Ortori, C., Barrett, D., Chakrabarti, L. (2020). Sex specific inflammatory profiles are characteristic of mitochondria from Parkinson's disease cerebellum. (*Pre-print – MitoEagle*).

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In the words of Stephen Jay Gould, *"Universities operate one of the few surviving old apprenticeship programmes, and at no other time other than nursery are you more dependent on your mentor"*.

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Declaration

I hereby declare that this thesis has been composed by myself and has not been, or will be, submitted for any other degree elsewhere. The work presented herein is my own and all work of other authors and material from other sources is duly recognised. Collaboration with other persons during this work has been fully acknowledged.

Thomas Ingram

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Abbreviations

AA	Arachidonic acid
AD	Alzheimer's disease
AMPK	AMP-activated protein kinase
AP	Antagonistic pleiotropy
ATP	Adenosine triphosphate
CA-II /-III	Carbonic anhydrase-II /-III
CNS	Central nervous system
CR	Caloric restriction
CSF	Cerebrospinal fluid
DH	Dorzolamide hydrochloride
DLB	Dementia with Lewy bodies
DS	Disposable soma
dsRNA	Double-stranded RNA
EDL	Extensor digitorum longus
FABP3	Fatty acid binding protein 3
FFA	Free fatty acids
FOXO	Forkhead box protein
GH	Growth hormone
H_2O_2	Hydrogen peroxide
ICE	Ingram counterbalanced exerciser
IGF-1	Insulin-like growth factor-1
IMM	Inner mitochondrial membrane
LA	Linoleic acid
LB	Lewy body
LCFA	Long chain fatty acid
L-DOPA	levodopa (L-3,4-dihydroxyphenylalanine)
MA	Mutation accumulation
MAM	Mitochondria associated membrane
MCI	Mild cognitive impairment
MFRTA	Mitochondrial free radical theory of ageing
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	Mitochondrial DNA
NAD	Nicotinamide adenine dinucleotide
nDNA	Nuclear DNA
NGM	Nematode growth medium
NSAID	Non-steroidal anti-inflammatory drug
$O^{\cdot}$	Superoxide
OMM	Outer mitochondrial membrane

OXPHOS	Oxidative phosphorylation
PD	Parkinson's disease
PG	Prostaglandin
PGC1α	Peroxisome proliferator-activated receptor γ coactivator 1-α
PINK1	PTEN-induced kinase 1
PT	Power Tower
PTGS2	Prostaglandin-endoperoxide synthase 2
PUFA	Polyunsaturated fatty acid
REQS	Rotational exercise quantification system
RNAi	RNA interference
ROS	Reactive oxygen species
SNpc	*Substantia nigra pars compacta*
SOD	Superoxide dismutase
SOL	Soleus
TCA	Tricarboxylic acid
TRF	Time-restricted feeding
WT	Wild type
α-syn	α-synuclein

1 Introduction

1.1 Ageing

1.1.1 What is ageing?

Evolutionary theory states that ageing is a non-adaptive process and is explained by the weakening force of natural selection and reduced genetic effects with age (McCay CM, Crowell MF, 1989). Phenotypically the most apparent signs of ageing in humans are greying hair, reduced eyesight, immobility and skin wrinkling. On the cellular level, ageing manifests as progressive loss of function resulting in the demise of the cell - homeostasis is less readily maintained and response to cellular insults are dealt with ineffectually (Toescu, 2005). Many theories explaining ageing have been put forward, however, none fully encapsulate the phenomenon, and the underlying mechanisms remain to be elucidated.

1.1.2 An ageing population

Since 1850 human life expectancy has increased dramatically, from 36 to 80 years of age today, in Europe (Riley, 2005b). Infection and malnutrition affect increasingly fewer individuals due to improving care and understanding of health. People are living longer and the proportion of elderly is increasing - recent projections indicate that 22% of the global population will be represented by older adults (>60 years) by 2050 (Riley, 2005b). This means that diseases rarely seen 150 years ago are increasing in prevalence. Indeed, ageing

is a primary risk factor for cancer, neurodegenerative and cardiovascular disease, and current data suggest these diseases account for 25% of the global disease burden (Prince *et al.*, 2015). Improvements in our molecular understanding of ageing is imperative in order to face these modern challenges.

1.1.3 What is healthy ageing?

A dichotomy between healthy ageing and age-related disease is difficult to distinguish as age-related pathologies such as osteoporosis, insulin resistance and sarcopenia are normal with age. Indeed, based on autopsy records, the prevalence of prostate tumours is almost universal in old men (Haas *et al.*, 2008). Ageing as a disease is a view which has long been debated, however, the argument has recently been revitalised (Faragher, 2015). Defining an elderly person as healthy is problematic, however, if we position a definition of healthy in the context of elderly people only it is clearly possible to designate a 'healthier ageing' group by phenotypic differences or disease prevalence. A definition would account for the handicap of ageing – for example, an elderly person who lives independently and is in relatively good health, may be defined as ageing healthily. Currently, little is known about the molecular differences between healthy ageing and non-healthy ageing. Distinguishing ageing changes may enable targeting of them to improve our healthspan.

1.2 Theories of ageing

1.2.1 Learning is the price for immortality

Cellular ageing results in a build-up of cellular "garbage", including aberrant proteins, defective mitochondria and lipofuscin (Terman, 2001). Post-mitotic cells (neurones, cardiomyocytes) show the most dramatic accumulation of dysfunctional and damaged cellular material with age (Strehler, 1965). As the proportion of dysfunctional material builds, cells are incapable of maintaining cellular function, ultimately resulting in cell death. On the other hand, age-related accumulation of this waste is almost undetectable in proliferative cell populations (bone marrow, intestinal epithelium) (Strehler, 1965; Sheldrake, 1974). The failure of long-lived cells has been reported as the principle cause of death in animals (Terman, 2001) and evidence supporting this comes from *Hydra vulgaris*, a simple multi-cellular organism with no long-lived post-mitotic cells. Laboratory observation of the organism over 4 years showed extremely low mortality which did not increase with age (Martínez, 1998). Further, the closely related sea anemones have been reported to be immortal. In the 1860s a species of sea anemone (*Sagartia troglodytes*) were collected in jars containing sea-water. They exhibited no obvious signs of ageing for more than 80 years, eventually dying of "neglect" (Ashworth and Annandale, 1906; Comfort, 1964). To my knowledge, no contemporary work has continued to study the apparent immortality of these intriguing animals.

Non-ageing animals are a small minority of multicellular life, and according to the fossil record, they are among the earliest multicellular animals (Holland,

2003). The cost of a limited lifespan must have been worth the trade. A tantalising hypothesis is that ageing is an epiphenomenon of the appearance of long-lived neurones and memory (Terman and Brunk, 2005). It is unknown whether the continuous replacement of neurones would permit memory formation. *Hydra vulgaris* neurones and synaptic connections are constantly replaced and unless information is able to be transferred to newly differentiated neurones, which has never been demonstrated, information likely cannot be stored long-term (Smalheiser, Manev and Costa, 2001). Complex behaviour, which is requisite of learning and memory, may have only become possible with the appearance of long-lived post-mitotic neurones (Terman and Brunk, 2005).

1.2.2 Evolutionary theory of ageing

The evolutionary theory of ageing suggests that natural selection (a measure of the effect of selection upon survival or reproduction as a function of age) declines with age (Rose *et al.*, 2007). In 1942, Haldane presented evidence in support of this and suggested that the declining force of natural selection with age may explain the prevalence of Huntington's disease - since symptoms appear after 30 years of age, it would not have been eliminated by selection in ancestral populations because most would have died before experiencing the disease (Haldane, 1942). Therefore, selection ignores favourable or deleterious effects when these are expressed after reproduction has ceased, and therefore it will not affect reproductive success nor be efficiently selected for or against.

In the 1940 and 50s, Medawar took this a step further in his mutation accumulation (MA) hypothesis. He outlined the steps that would enable deleterious mutations that are restricted in their effects to late life, to be passed on. Over evolutionary time these mutations, which natural selection works only weakly upon, would accumulate in a population by genetic drift. Only once extrinsic mortality decreases and individuals live to an age where they may express symptoms of ageing, would such a deleterious mutation manifest (Medawar, 1946).

The second cornerstone of ageing was originally outlined by George C. Williams in 1957. He argued that mutations or alleles may exist which have opposing affects at early and old age – beneficial effects which promote fitness in early life, but deleterious effects in later life when selection is weak. This is known as the antagonistic pleiotropy (AP) hypothesis (Williams, 1957). Ageing would therefore be described as a by-product of selection for increased fitness in early age. AP suggests that genotypes which promote high early-life reproductive fitness should be shorter lived than those with low reproduction (Hughes and Reynolds, 2005). An extension of this model is Kirkwood's 1977 disposable soma (DS) hypothesis. This states that the level of investment into somatic repair and maintenance will always be below that required for indefinite survival. A high investment in somatic repair and maintenance is unlikely to evolve due to the high likelihood of never realising a return due to extrinsic mortality. Conversely, investment into high early-life reproductive fitness may shift resources away from somatic repair and

maintenance (Kirkwood, 1977). In agreement, fruit flies (*Drosophila melanogaster; D. melanogaster*) that are selected for late-life reproductive success are long-lived at the expense of early-life fertility (Rose and Charlesworth, 1980; Rose, 1984).

MA and AP stand as the current evolutionary theories of ageing and although both theories differ slightly, in actuality it is likely both models occur in real populations, however, their relative frequencies are unknown.

1.2.3 Why do animals have different lifespans?

Lifespan varies between animals and reproductive lifespan adapts in response to selection for increased success. In general, a longer lifespan implies greater reproductive success – low adult mortality and high juvenile mortality both tend to increase reproductive lifespan (Searns, 1992). Selection favours increased reproductive success, which carries with it a longer lifespan, however intrinsic trade-offs between reproduction and survival (AP theory) may limit lifespan (Williams, 1957). Lifespan may therefore be described as a balance between reproductive success and the intrinsic age-dependent causes of mortality. In support of extrinsic factors influencing lifespan, *D. melanogaster* exposed to either high or low levels of extrinsic adult mortality showed that exposure to low levels significantly increased lifespan, whereas *D. melanogaster* exposed to high extrinsic adult mortality aged more rapidly (Stearns *et al.*, 2000).

1.2.3.1 Biological theories of lifespan

In 1928, Pearl published his rate-of-living theory, which stated that lifespan and metabolic rate are inversely correlated. It predicted that, in general, long-lived species have lower basal metabolic rates in comparison to shorter-lived species (Pearl, 1928). To explain this mechanistically, in 1956, Denham Harman put forward the free radical theory of ageing. He proposed that free radicals, produced as by-products during metabolic processes, cause damage to biomolecules. Accumulation of damaged molecules would ultimately lead to decreased cellular functioning and eventual cell and organismal death. The theory predicted that animals that produce more free radicals should have a shorter lifespan (Harman, 1956). Initially, there was little support for Harman's theory, however, the discovery of the anti-oxidant enzyme, superoxide dismutase (SOD) in 1969 changed this - if the cell had detoxification mechanisms then free radicals must be produced *in vivo* (McCord and Fridovich, 1969). Four years later, the production of hydrogen peroxide by mitochondria were described and, in addition, the group showed that oxygen is reduced incompletely, giving rise to reactive oxygen species (ROS), even during normal mitochondrial respiration (Boveris and Chance, 1973). In 1972, Harman published an addendum to the free radical theory of ageing, singling out the mitochondrion as the primary producer of ROS and the primary target of ROS damage – the mitochondrial free radical theory of ageing (MFRTA) was born (Harman, 1972).

For decades, the prevailing theory to explain the biological basis of lifespan has been the MFRTA (Harman, 1972). In support of the MFRTA, short lived species generally produce ROS at a greater rate than long lived species (Ku and Sohal, 1993; G. Barja *et al.*, 1994; Lambert *et al.*, 2007a); caloric restriction, which increases lifespan, significantly reduces the rate of ROS production (GREDILLA *et al.*, 2001; Qiu *et al.*, 2010); mutagenesis of polymerase gamma, required for replication and repair of mtDNA, increases accumulation of mutations, increases rate of ROS production and accelerates ageing (Kujoth *et al.*, 2005). However, the MFRTA has not fully withstood experimental testing. Antioxidant therapy consistently fails to produce increases in maximal lifespan (Lopez-Torres *et al.*, 1993; G Barja *et al.*, 1994; Pérez-Campo *et al.*, 1994). The long lived bat (*M. lucifigus*) has a maximal lifespan four times longer than the white footed mouse (*P. leucopus*) and yet electron-leak from the respiratory chain is greater in the bat (Brunet-Rossinni, 2004a). The naked mole rat (*H. glaber*) is another exception to the MFRTA – it has a maximal lifespan of 28 years and yet produces a similar ROS at a similar rate to the mouse (maximal lifespan four years) (Munro *et al.*, 2019). Oxidative damage to biomolecules also does not correlate clearly with lifespan. Oxidative damage to proteins is negatively correlated with lifespan in mammals (Barja and Herrero, 2000), and yet, when mitochondrial carbonyl modifications are investigated in the naked mole rat, this correlation becomes positive (Andziak, Timothy P. O'Connor, Qi, Dewaal, *et al.*, 2006). Further, the longer-lived small fruit flies (*Drosophila spp*) contain higher levels of protein carbonyls than the shorter-lived blow flies (*Calliphora vicina*) (Sohal, Sohal and Orr, 1995). Even

more convincing, low-doses of the mitochondrial respiratory chain toxins, rotenone or paraquat, which increase the rate of ROS production, extend lifespan of *C. elegans* (Yang and Hekimi, 2010a).

These data suggest the MFRTA is insufficient to explain the molecular basis of ageing. It is true that data support a correlation between mitochondrially derived ROS and lifespan in animals, however, this does not mean cause. Maximal lifespan is also correlated to degree of membrane saturation (Hulbert, 2005), methionine content of proteins (Obata and Miura, 2015; Lee, Kaya and Gladyshev, 2016), and guanine and cytosine content of mtDNA (McLain and Faulk, 2018). Long lifespan appears to be correlated with an improved capacity of biomolecules to resist oxidative stress, thereby maintaining cellular function for longer (Austad and Fischer, 1991; Barreto-Estrada *et al.*, 2004; Austad, 2010; Pride *et al.*, 2015). Regardless of whether mitochondrial ROS are the key biological drivers of ageing, mitochondria and their dysfunction are implicit in the process (Bratic *et al.*, 2013a; López-Otín *et al.*, 2013).

More recent biological theories suggest cell-wide changes drive the ageing process. Nine hallmarks of cellular ageing have been described: genomic instability, telomere attrition, loss of proteostasis, epigenetic alterations, deregulated nutrient-sending, cellular senescence, altered intercellular communication and mitochondrial dysfunction (López-Otín *et al.*, 2013). These hallmarks are not separate – extensive interconnections exist and mitochondria appear central. Research is now investigating mitochondrial

dysfunction with age beyond simply the MFRTA – burgeoning evidence suggest wide-scale age-related alterations to oxidative phosphorylation, protein and lipidomic alterations, mtDNA mutations and altered mitochondrial morphology (Bratic *et al.*, 2013a; Finkel, 2015; Ingram and Chakrabarti, 2016).

1.3 Age-related changes to mitochondria

Mitochondria are intracellular organelles present in almost all eukaryotic cells. They are dynamic, displaying heterogeneous morphology, number and constituent molecules dependent on cell type, cellular localisation, and stress (Westermann, 2012). Since Denham Harman's seminal paper developing the free radical theory of ageing mitochondria have maintained their position as critical to ageing, despite the theory not wholly standing up to experimental testing (Harman, 1956). Mitochondrial dysfunction has been shown to take many forms – altered protein and lipid composition, morphological changes, altered membrane potential, increased production of reactive oxygen species and downstream damage to biomolecules (Navarro and Boveris, 2007; Staunton *et al.*, 2011; A. M. Joseph *et al.*, 2012; Stauch, Purnell and Fox, 2014a; Grimm and Eckert, 2017). The primary focus of this thesis are the ageing brain and skeletal muscles as these organs are composed of long-lived, post-mitotic cells - any alterations which occur may be expected to exert profound ageing effects (Terman *et al.*, 2010). Indeed, age-related alterations to these tissues manifest phenotypically as cognitive decline and sarcopenia (Yankner, Lu and Loerch, 2008; Walston, 2012). The following sections shall be focussed on the literature surrounding the known biochemical changes to mitochondria of the brain and skeletal muscles.

1.3.1 The mitochondrial proteome

The mitochondrial proteome consists of around 1000-1500 different proteins, which vary in expression by tissue (Nunnari and Suomalainen, 2012; Friedman and Nunnari, 2014). The brain has the highest per gram demand for ATP than any other organ, accounting for 2% of body weight yet consuming around 20% of glucose-derived energy (Mergenthaler *et al.*, 2013). Glucose oxidation is accountable for the vast majority of ATP generated in the brain. In contrast, muscle is exceptionally versatile and capable of oxidising both fat and glucose and switching to anaerobic respiration for short periods (Piec *et al.*, 2013; Wolfe, 1998). The different roles and requirements of the brain and skeletal muscle manifest as differences in mitochondrial constituents – mitochondrial alterations with age should therefore be considered in brain and skeletal muscle separately.

1.3.1.1 Oxidative phosphorylation

Mitochondria are best known for their capacity to generate ATP through oxidative phosphorylation (OXPHOS), which provides more than 90% of the cellular energy requirements (Mounsey and Teismann, 2011). Electrons are primarily generated from glycolysis, the TCA cycle and β-oxidation. Electrons are shuttled by redox couples (NADH and $FADH_2$) to the electron transport complexes where a series of oxidation and reduction reactions shuttle electrons through complexes I (NADH:ubiquinone oxidoreductase), II (succinate:ubiquinone oxidoreductase, III (ubiquinol:cytochrome *c* oxidoreductase) and IV (cytochrome *c* oxidase) situated on the inner

mitochondrial membrane (IMM). A proton gradient is created by the transfer of protons from the matrix to the intermembrane space, which drives the F_0F_1 ATP synthase activity, transferring four electrons to oxygen and synthesising ATP. This process is not wholly efficient and small losses of electrons may occur resulting in the creation of singlet oxygen, generating superoxide anions (O^-) (Brookes, 2005). O^- is reduced by antioxidant enzyme, superoxide dismutase (SOD), to hydrogen peroxide (H_2O_2) and both O^- and H_2O_2 are both capable of causing damage to biomolecules (Brookes, 2005).

Many tissues show changes to oxidative phosphorylation with ageing – the rate of electron transfer and capacity have been shown to decrease in the ageing brain (Link *et al.*, 1999) with the most prominent changes seen in complexes I, III, IV and V (Gevaert *et al.*, 2008; Pitt, 2009; Xie *et al.*, 2011).

1.3.1.2 Mitochondrial dynamics are altered

Mitochondria are dynamic, continuously fusing (fusion) and dividing (fission) in response to stress, hormones, exercise and nutrient status (Bereiter-Hahn and Vöth, 1994). Overexpression of fusion-related proteins, MFN1 (mitofusin), MFN2 and OPA1 (optic atrophy protein 1) cause the formation of large mitochondria or long mitochondrial tubules (Chan, 2012). Fusion processes are necessary for the maintenance of mitochondrial function by the redistribution of mitochondrial components of normal and defective mitochondria, thereby diluting dysfunctional components (Gomes, Di Benedetto and Scorrano, 2011). The overexpression of fission-related proteins, DRP1 (dynamin-related protein 1), FIS1 (mitochondrial fission protein 1),

MIRO (mitochondrial rho), endophilin B1, MFF (mitochondrial fission factor), MTP18 (mitochondrial protein 18) and GDAP (ganglioside-induced differentiation associated protein) results in the formation of small, fragmented, mitochondria (Pich *et al.*, 2005). Fission processes segregate irreversibly damaged and depolarised mitochondria for subsequent elimination by autophagy (Priault *et al.*, 2005).

Mitochondrial number is tightly controlled in a process known as mitochondrial biogenesis. At its crux is peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α), which coordinates the activity of transcription factors (NRF1, PPARs, mtTFA) to promote additional coactivators for downstream mitochondrial production (Puigserver *et al.*, 1998; López-Lluch *et al.*, 2008). Gene-expression analyses suggest mitochondrial biogenesis involves close to 1000 genes and affects 20% of cellular proteins (Annex *et al.*, 1991; Freyssenet, Berthon and Denis, 1996; Lee *et al.*, 2002; Lee and Wei, 2005). Tight control of mitochondrial morphology by these competing processes, and number by mitochondrial biogenesis, ensures mitochondria respond to the cellular environment appropriately. If this balance is lost, mitochondrial and cellular functions are altered (Lee *et al.*, 2002; López-Lluch *et al.*, 2008).

In the ageing brain evidence points to a shift toward increased fusion events (or decreased fission), resulting in enlarged and elongated mitochondria (Stauch, Purnell and Fox, 2014b; Stauch *et al.*, 2015). Indeed, 'giant' mitochondria are a commonality of senescent cells (Terman, Gustafsson and

Brunk, 2006). Proteomic studies of mouse brain have shown a reduction in fission (decreased expression of DRP1 and MFF) from 12-24 months of age (Stauch *et al.*, 2015). Synaptic populations of mitochondria from mouse brain revealed an increased expression of MFN1, MFN2 and OPA1 from 12-24 months of age (Stauch, Purnell and Fox, 2014a). As mentioned, a propensity toward increased fusion is seen in the ageing brain and evidence suggest elongated mitochondria result in greater intracellular ROS production and lowered rate of mitochondrial respiration (Yoon *et al.*, 2006; Lee *et al.*, 2007). It may be that these associated costs are outweighed by the stress ageing exerts upon a cell – fusion maintains ATP production, increases the threshold for mitochondrial membrane depolarisation, inhibits cytochrome *c* release and apoptosis (Terman *et al.*, 2010). In this setting, mitochondrial fusion may be important to maintain cell function with age.

Similar to the brain, ageing muscle appears to show an imbalance of fusion and fission processes, however, the directionality is unclear. Studies in rodent skeletal muscle suggest either an increase in fission (Iqbal *et al.*, 2013; Sebastián *et al.*, 2016), an increase in fusion (Joseph, Peter J. Adhihetty, *et al.*, 2013; Leduc-Gaudet *et al.*, 2015), or no change (Distefano *et al.*, 2017). The lack of consensus is likely due to differences in muscle type analysed, mitochondrial fraction (subsarcolemma or intermyofibrillar), age comparisons or species.

The ageing brain (Sas, Szabó and Vécsei, 2018) and skeletal muscle (Konopka *et al.*, 2014) both show decreased mitochondrial biogenesis and PGC-1α has been shown to decline in expression in the skeletal muscle of rodents

(Konopka *et al.*, 2014). Another key factor regulating this reduction appears to be AMPK, which senses AMP/ATP ratio and regulates nutrient deprivation pathways. A decrease in AMPK activity with age is apparent and its chronic inactivation is linked to decreased activation of PGC-1α and subsequent mitochondrial biogenesis with ageing (Reznick *et al.*, 2007; Ljubicic and Hood, 2009).

1.3.1.3 Mitophagy

The brain and skeletal muscle are primarily composed of post-senescent long lived cells (Campisi and d'Adda di Fagagna, 2007) – many of these cells are maintained for the entire lifespan (La Rosa, Ghibaudi and Bonfanti, 2019). Normal ageing is associated with damage to organelles and cellular biomolecules and maintenance of cellular homeostasis requires the selective degradation of damaged molecules and organelles by autophagy. Excessive damage or failure of the autophagic systems results in cellular demise (Cuervo *et al.*, 2005; Twig, Hyde and Shirihai, 2008a). Organelle specific forms of autophagy, with characteristic protein and pathway involvement, have been described (Parzych and Klionsky, 2014).

Mitochondrial-specific autophagy, termed mitophagy, is concerned with mitochondrial quality control and ensures a pool of high-quality mitochondria are maintained (Sun, Youle and Finkel, 2016). Mitophagy involves numerous proteins and best characterised are PINK1 (PTEN-induced kinase 1), MFN2 and PARKIN. Upon membrane depolarisation or irreparable mitochondrial

damage, PINK1 is stabilised on the OMM. PINK1 subsequently phosphorylates MFN2, which serves as a docking site for PARKIN, an E3 ubiquitin ligase. Parkin ubiquitinates multiple outer mitochondrial membrane (OMM) target proteins, signalling LC3-positive phagophore formation and mitochondrial engulfment by a double-membrane autophagosome. Autophagosomes fuse with a lysosome, forming an autolysosome. The acidic autolysosomal pH and enzyme-mediated hydrolysis degrade the contents (Heo *et al.*, 2015; Sun, Youle and Finkel, 2016; McWilliams and Muqit, 2017).

Ageing is associated with an accumulation of damaged organelles and protein aggregates (Terman, 2001; Terman, Gustafsson and Brunk, 2006; Terman *et al.*, 2010). Due to technical limitations of measuring a transient process, such as mitophagy, direct evidence for *in vivo* activation of PINK1/PARKIN-mediated mitophagy is lacking (Um *et al.*, 2018). Traditional direct methods employed electron or fluorescent microscopy (Dolman *et al.*, 2013), these methods are aimed at recognising mitochondrial remnants within autophagosomes, however, sampling is only possible on a small fraction of the cell population making quantification difficult (Sun *et al.*, 2015a). Indirect measurements may be used to measure mitochondrial turnover rates (Kim *et al.*, 2012). Recent work employing mitochondria-Keima (mt-Keima), a pH sensitive fluorescent reporter that co-localises to ATP synthase subunit-β (ATP5β), has gone some way to rectifying the limited quantifiable autophagic-turnover data (Kim *et al.*, 2019). Fluorescence imaging revealed increased mitophagy levels with age in the muscle and dopaminergic neurones of WT *D. melanogaster*, and that PINK1

and PARKIN were necessary for mitophagy (Kim *et al.*, 2019). Conversely, vertebrate data has shown that mitophagy may be maintained independently of PINK1 (McWilliams *et al.*, 2018), however, this work utilised a different fluorescent reporter, *mito*-QC, which is targeted to the OMM protein FIS1 (McWilliams *et al.*, 2016). Many OMM proteins are ubiquitinated during mitophagy, including FIS1 (Sarraf *et al.*, 2013), therefore, FIS1 may be ubiquitinated and delivered to lysosomes reducing the sensitivity of *mito*-QC as a reporter.

Fission as a process is intimately linked to mitophagy as fission segregates damaged mitochondria, targeting them for degradation by mitophagy (López-Lluch *et al.*, 2008). Current data show a propensity toward larger mitochondria in the brain with age (López-Lluch *et al.*, 2008), which suggests an age-related reduction in mitophagy. Indeed, utilising mt-Keima fluorescent reporter, in the dentate gyrus of 21 month old mice revealed that mitophagy was markedly reduced compared to 3 month old mice (Sun *et al.*, 2015b). Within aged muscle, changes in basal mitophagy rate are still subject to debate. Both mice and humans have impaired mitophagy in muscle satellite cells with ageing (Brunk and Terman, 2002; Terman, Gustafsson and Brunk, 2006), however, three studies in aged mouse muscle demonstrated increased mitophagic protein levels (O'Leary *et al.*, 2013; Sakuma *et al.*, 2016), and increased levels of mitophagic receptors (Carter *et al.*, 2018). These studies are limited in their quantifiability due to usage of more traditional methodologies and differences in species, age, selected proxies for autophagy, and cell type

all add to the nuance and complexity of evaluating these data. With the advent of fluorescent reporters of mitophagy, it is necessary to revisit the question of autophagy with ageing.

1.4 Diseases of ageing

Age is the principle risk factor of the non-communicable diseases that trouble modern life, including cancer, cardiovascular disease, diabetes, osteoporosis, sarcopenia, dementia and Parkinson's disease (Prince *et al.*, 2015). Life expectancy and the proportion of elderly in populations are both increasing (Bloom *et al.*, 2015) and, in turn, it is expected that the burden of these diseases will increase. To combat this, it is necessary to understand the aetiology and biological mechanisms which drive these diseases.

1.4.1 Sarcopenia

First proposed as a term in 1989, sarcopenia comes from the Greek meaning "flesh-loss" (Rosenberg, 1997). Sarcopenia is the age-related loss of muscle and strength. It is clinically defined as muscle mass two standard deviations below the young adult mean (Cruz-Jentoft *et al.*, 2010). Sarcopenia effects 14% of those aged 65-70 years of age and by 80 years of age it is estimated to impact 53% of individuals (Santilli *et al.*, 2014).

In the human body, muscle accounts for 60% of the body's stores of protein. Decreased muscle mass is directly implicated in strength loss, disability, increased likelihood of falls, reduced autonomy, and all-cause mortality (Janssen, Heymsfield and Ross, 2002). In the ageing population, sarcopenia is likely highly multi-factorial with a long onset duration. It has been suggested that reduced energy intake, reduced protein intake, lack of vitamin D, lack of

exercise, and a shift toward higher pro-inflammatory internal milieu are all contributors toward sarcopenia (Jensen, 2008; Zamboni *et al.*, 2008). As the proportion of elderly individuals in society continues to rise, sarcopenia will become more of a burden. It is essential to better understand the biology of sarcopenia and lifestyle factors which reduce muscle and protein loss with age.

1.4.1.1 Sarcopenia and mitochondria

Studies into the biological mechanisms of sarcopenia have shown an association with motor neurone death (Tomlinson and Irving, 1977; Mittal and Logmani, 1987), impaired innervation and a 25-30% reduced motor unit pool (Hepple and Rice, 2016; Pannérec *et al.*, 2016). Loss of muscle cell innervation has been shown to lead to muscle atrophy (Siu and Alway, 2005; Pannérec *et al.*, 2016). Both motor neurones and muscle cells share a common theme of mitochondrial dysfunction (Joseph, Peter J Adhihetty, *et al.*, 2013; Rygiel, Grady and Turnbull, 2014).

At present, few studies have directly investigated mitochondria in the context of sarcopenia in humans. Comparisons of muscle mitochondria from sarcopenic or pre-frail individuals (criteria: slow walking speed, low strength, low muscle mass, rapid exercise exhaustion (Fried *et al.*, 2001)) with physically active age-matched individuals have been studied. A study of Dutch pre-frail individuals showed that skeletal muscle mitochondrial volume density and mtDNA/nDNA ratio were lower; respiratory complexes I, IV and V protein levels were reduced; complexes I, II and IV showed reduced activity; and

expression of mitochondrial genes were markedly lowered (Andreux *et al.*, 2018). In a study of 25 elderly individuals admitted for hip fractures, 10 of which were clinically diagnosed with sarcopenia, mitochondrial quality control processes were altered - sarcopenic individuals had significantly lower MFN2 and LC3B, and a trend toward increased FIS1 and lower OPA1 was also apparent. LC3B-II/LC3B-I ratio, a proxy for autophagic flux, was observed to be decreased, suggesting sarcopenic individuals had lowered autophagy processes (Marzetti *et al.*, 2016). Mitochondrial fusion upregulation appears to be characteristic of normally ageing muscle (Twig, Hyde and Shirihai, 2008b) and older frail women have decreased *MFN2* mRNA expression than non-frail, age-matched, women (Drummond *et al.*, 2014). Further, upregulated fission without concomitant upregulation of fusion processes was shown to cause muscle atrophy in rodents (Romanello *et al.*, 2010). Sarcopenic muscle may distinguish itself from normally ageing muscle by imbalanced fusion/fission processes, resulting in disease-characteristic muscle loss. Recently, proteomics of skeletal muscle from ageing individuals revealed wide-ranging changes to mitochondrial proteins. Of the 173 mitochondrial proteins identified as changed, 70% declined with age. The majority of altered proteins from complex I, III, IV and V declined with age, while complex II proteins increased (Ubaida-Mohien *et al.*, 2019). These findings describe a sarcopenic mitochondrial protein profile which engenders dysfunctional OXPHOS.

The few studies describing the molecular alterations in sarcopenic individuals compared with age-matched healthy individuals suggest mitochondrial dysfunction is implicit in the disease. Wide-spread mitochondrial alterations have been described, however, it is not clear whether these alterations are causal or consequential – much more work is required to delineate sarcopenic and healthy muscle.

1.4.2 Parkinson's disease

Parkinson's disease (PD) was defined by James Parkinson who first medically described the disease in 1817, in his essay "An Essay on the Shaking Palsy" (Parkinson, 2002). Interestingly, this was not the first source – traditional Indian texts from approximately 1000BCE provide descriptions (Manyam, 1990). As lifespan has increased during the past century and a half so too have ageing-related neurodegenerative disease. Today PD is the second most prevalent neurodegenerative disease and affects 1% of the population at age 65 and at age 85 incidence rises to 4-5% (de Rijk *et al.*, 1995). Classical motor symptoms include: resting tremor, dyskinesia, postural instability and rigidity (Miller and Cronin-Golomb, 2010). More recently, non-motor symptoms of PD have been acknowledged: depression, anxiety, anosmia, insomnia, addictive behaviour (Ziemssen and Reichmann, 2007; Smith and Dahodwala, 2014a). Motor symptoms coincide with the loss of dopaminergic neuronal projections from the *substantia nigra pars compacta* (*SNpc*) to the striatum (Reeve, Simcox and Turnbull, 2014). PD pathology is further identified by the hallmark

presence of cytoplasmic Lewy Bodies (LB). These inclusions are described as 2-5μm in diameter with a dense core and clear halo (Wakabayashi *et al.*, 2007). Although most commonly situated in neuronal bodies, they have also been located in neurites (Volpicelli-Daley *et al.*, 2014). α-synuclein (α-syn) is a major component of LBs and the presence of α-syn containing inclusions at sites of major cell loss has justified much research into their role (Maries *et al.*, 2003; Wakabayashi *et al.*, 2007; Banerjee *et al.*, 2010).

Despite classification of the disease since the 1800s contemporary PD therapy only targets symptoms (Goetz, 2011). Levodopa (L-3,4-dihydroxyphenylalanine; L-DOPA) therapy was first introduced for the treatment of PD in 1967 (Cotzias, Van Woert and Schiffer, 1967). In principle, the same high-dose oral L-DOPA regimen is still used today. Efficacy varies with symptoms – bradykinesia and rigidity generally respond well, however, tremor responsiveness is poor (Ovallath and Sulthana, 2017). Additionally, chronic L-DOPA treatment is associated with dyskinesia – an estimated 30-50% of individuals on L-DOPA therapy for more than five years experience dyskinesia (Schrag and Quinn, 2000). Clearly it is necessary to develop interventions which target the underlying mechanisms, rather than symptoms.

1.4.3 Mitochondrial dysfunction is a feature of Parkinson's disease

In order to treat a disease, an understanding of the causal mechanisms is necessary - currently, this understanding is lacking in PD. The discovery of pathological hallmarks, including LBs (Rodrigues e Silva *et al.*, 2010) and α-syn (Spillantini *et al.*, 1997), initially garnered much effort toward their targeting. Mitochondrial dysfunction is increasingly understood to feature in PD; complex I is consistently shown to be affected (Moon and Paek, 2015; Flønes *et al.*, 2018), and evidence suggest mitophagic processes are inhibited or less active (Lazarou *et al.*, 2012a; Ryan *et al.*, 2015; Anzell *et al.*, 2018). Further, α-syn has been shown to bind mitochondria and alter their function (Devi *et al.*, 2008; Nakamura, Venu M. Nemani, *et al.*, 2011; Ludtmann *et al.*, 2016). Indeed, many heritable forms of the disease either directly impact or indirectly exacerbate mitochondrial dysfunction (Polymeropoulos *et al.*, 1997; Maries *et al.*, 2003; Clark *et al.*, 2006a; Li, Tan and Yu, 2014; Chen *et al.*, 2019).

1.4.3.1 α-synuclein

α-syn is a complex protein and knowledge of its physiological role is still incomplete. α-syn may exist in a variety of states – physiologically it presents as an intrinsically disordered monomer, however, it may form a folded helical tetramer or undergo α-helical folding (Li *et al.*, 2018). α-syn is highly enriched at presynaptic terminals and evidence suggest a role in the regulation of synaptic vesicles (Maroteaux, Campanelli and Scheller, 1988). α-syn appears to be an essential component of soluble *N*-ethylmaleimide-sensitive factor

attachment receptor (SNARE) complex assembly, which is involved in docking and fusion of synaptic vesicles with the presynaptic membrane of neurones (Burre *et al.*, 2010). A lipid binding function was first postulated in 1995, on work in the Zebrafinch, and was shown to share high sequence homology with apolipoproteins (George *et al.*, 1995), including fatty acid binding protein (Ronit Sharon *et al.*, 2001). However, the mechanism of binding, lipid class preference and downstream pathway activation remain unknown. Data suggest certain lipids, such as arachidonic acid (AA), are capable of influencing physiological and pathogenic features of α-syn (and vice versa), however, knowledge of how this occurs is lacking (Alza *et al.*, 2019).

α-syn has long been associated with PD pathogenesis (Galvin *et al.*, 1999). Indeed, point mutations, duplication and triplication of the α-syn gene *SNCA* cause early-onset familial PD (Harada *et al.*, 2009). Mutant α-syn overexpression in animal models causes protein aggregates to form and clinical PD symptomology to manifest (Yavich *et al.*, 2005; Unger *et al.*, 2006). Of interest is the capacity for misfolded α-syn to propagate its pattern of misfolding to nearby proteins – inoculation of misfolded α-syn caused endogenous protein misfolding in non-transgenic mice (Bernis *et al.*, 2015). Recent data point toward α-syn lipid-binding as accelerating misfolding and pathogenicity (Shioda *et al.*, 2014b). α-syn is capable of binding polyunsaturated fatty acids (PUFAs): α-linolenic acid, docosahexaenoic acid and eicosapentaenoic acid (R. Sharon *et al.*, 2001). Elevated levels of PUFAs

have been shown to be present in soluble brain fractions in PD and dementia with Lewy Bodies (DLB) (Sharon *et al.*, 2003). Indeed, in the presence of PUFAs recombinant α-syn has been observed to exacerbate aggregation and oligomer formation (Shioda *et al.*, 2014b).

1.4.3.1.1 α-synuclein and mitochondria

α-syn proteins are enriched at presynapses, particularly of the central catecholaminergic system, which includes those relevant to PD pathogenesis (such as: olfactory bulb, DMN, *SNpc*) (Li, Henning Jensen and Dahlström, 2002). α-syn aggregates correlate with reduced dendritic spines in post-mortem DLB brains, suggesting they contribute to synaptic loss (Taguchi *et al.*, 2019). One mechanism by which α-synuclein may cause synaptic loss and neurodegeneration is its influence upon mitochondrial function (Faustini *et al.*, 2017). Efficient synaptic transmission is highly reliant on Ca^{2+} regulation and ATP production by a properly functioning pool of mitochondria. Indeed, synapses contain a dense population of mitochondria (Lee *et al.*, 2018). As mitochondrial dysfunction is a major feature of PD (Mounsey and Teismann, 2011) it may also be that α-syn-mitochondrial interactions promote neurodegeneration by reducing the number of functional mitochondria at the synapse.

Although no evidence for a mitochondrial targeting sequence exists, α-syn preferentially binds cardiolipin, which is almost exclusively localised to the inner mitochondrial membrane (Nakamura, Venu M. Nemani, *et al.*, 2011). Recently, an alternate mitochondrial localisation site has been uncovered - α-

syn aggregates in neurones derived from an *SNCA* triplicate patient were localised to the mitochondrial ATP synthase complex (Ludtmann *et al.*, 2018). Interestingly, evidence suggest a differential pattern of α-syn localisation and action due to its folding (Faustini *et al.*, 2017). It has been shown that monomeric α-syn can bind complex I (Devi *et al.*, 2008) and mitochondria-associated endoplasmic reticulum membranes (MAMs) (Guardia-Laguarta *et al.*, 2014). Oligomers of α-syn have also been shown to interact with mitochondrial membranes, acting upon the translocase of the outer membrane 20 (TOM20) (Di Maio *et al.*, 2016). α-syn may also elicit functional alterations to mitochondria - transgenic mice overexpressing wild-type α-syn show reduced ATP production and elevated rate of ROS production, with concomitant mitochondrial oxidative damage (Reeve *et al.*, 2015). Additionally, α-syn bound mitochondria are functionally altered: Ca^{2+} signalling (Luth *et al.*, 2014), morphology (Chinta *et al.*, 2010), protein import (Di Maio *et al.*, 2016), and complex I activity, are all affected (Reeve *et al.*, 2015). It is plausible that mitochondrial dysfunction in PD is, at least in part, due to the accumulation of α-syn aggregates on (or in) mitochondria, interfering with mitochondrial proteins and lipids.

1.4.3.2 Complex I activity decreases in PD

Complex I inhibition was initially identified in the pathogenesis of PD during the early 1980s (Langston *et al.*, 1983). The opioid 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) was produced by clandestine laboratories in California during the 1980s as a "synthetic heroin". However, unfavourable

reaction conditions can result in the synthesis of the toxic by-product 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (LEE *et al.*, 1947). In 1982, four patients were admitted to hospital with Parkinsonism, due to intravenous administration of MPTP (Langston *et al.*, 1983). Further investigation revealed that the oxidation product of MPTP, 1-methyl-4-phenylpyridine (MPP^+) inhibits complex I (A. H. V. Schapira *et al.*, 1990). Identification of MPTP causing Parkinsonism provided the tools necessary for development of PD animal models and mechanistic progress in the field to be made. Complex I deficiency in PD individuals was later observed - individuals with idiopathic PD showed reduced complex I activity and protein levels in the *SNpc* (A. H. V. Schapira *et al.*, 1990; A. H.V. Schapira *et al.*, 1990), platelets (Haas *et al.*, 1995) and skeletal muscle (Bindoff *et al.*, 1991). At present, data regarding complex I changes in other brain regions are less consistent: the frontal cortex of PD individuals both decreased complex I activity (Keeney *et al.*, 2006), or no changes have been observed (A. H.V. Schapira *et al.*, 1990; Janetzky *et al.*, 1994); the prefrontal cortex and striatum have some evidence suggesting a reduction in complex I activity in PD individuals (Mizuno *et al.*, 1989; Fløthings *et al.*, 2018);. within the cerebellum, both reduced complex I activity and expression or no difference have been observed (Fløthings *et al.*, 2018).

α-syn has also been implicated in interfering with complex I function (Chinta *et al.*, 2010; Luth *et al.*, 2014; Faustini *et al.*, 2017). Interestingly, this effect was amplified in the presence of PD-associated A53T *SNCA* mutation (Chinta *et al.*, 2010; Choubey *et al.*, 2011).

Although data are inconsistent, evidence points to complex I deficiency, in a subset of PD individuals, arising from mtDNA mutations. Sequencing of the seven mitochondrial genes encoding subunits of complex I in the frontal cortex of twenty-eight idiopathic PD individuals and age-matched controls revealed a common mutation in a narrow region of mitochondrial complex I gene, *MT-ND5* (Smigrodzki, Parks and Parker, 2004; Parker and Parks, 2005; Smigrodzki *et al.*, 2005; Parker, Parks and Swerdlow, 2008). Transcription of mitochondrial genes are downregulated in PD individuals and nuclear signalling of mitochondrial gene transcription is impacted in the *SNpc* of PD individuals – both transcription factor A, mitochondrial (TFAM) and TFB2M were shown to be down-regulated, and neurones contained fewer mtDNA copies and less 7S DNA, which may explain the complex I deficiency (Grünewald *et al.*, 2016). Whether or not complex I deficiencies are a primary cause, a compensation to disease-related oxidative stress, or a consequence of an underlying dysfunction remains an open question.

1.4.3.3 Mitochondrial quality control defects drive PD?

Idiopathic PD accounts for 90-95% of all cases (Kalinderi, Bostantjopoulou and Fidani, 2016). Many familial forms have now been identified: α-syn (*SNCA*) (Polymeropoulos *et al.*, 1997), PARKIN (*PRKN*) (McWilliams and Muqit, 2017), PTEN-induced putative kinase 1 [(PINK1) (*PARK6*)] (Puschmann *et al.*, 2017), protein deglycase-1 [(DJ-1) (*PARK7*)] (Hong *et al.*, 2010), Leucine-rich repeat kinase 2 (*LRRK2*) (Li, Tan and Yu, 2014) and more (Liao *et al.*, 2014). Interestingly, many of these proteins are involved in mitochondrial quality

control and lysosomal function, and these patients display mitochondrial defects (Kalinderi, Bostantjopoulou and Fidani, 2016). Evidence suggests dysfunctional mitophagy to be implicit in PD pathogenesis (McWilliams and Muqit, 2017).

The best characterised mitophagic pathway requires PINK1-dependent activation of PARKIN: PINK1 is translocated across the mitochondrial inner membrane, where it is cleaved by presenilin-associated rhomboid-like protein (PARL) (Jin *et al.*, 2010). Import of proteins into mitochondria, including PINK1, requires an active proton gradient. Damage to mitochondria and subsequent membrane depolarisation, results in full-length PINK1 accumulation on the outer mitochondrial membrane (OMM) and its auto-phosphorylation (Lazarou *et al.*, 2012b). PINK1 accumulation on the OMM phosphorylates ubiquitin (Ub) and PARKIN on damaged mitochondria (Narendra *et al.*, 2008). PARKIN phosphorylation acts as a signal amplifier, leading to the ubiquitinylation of mitochondrial membrane proteins and production of poly-Ub chains. Subsequently, the autophagy receptors: optineurin (OPTN), nuclear dot protein 52kDa (NDP52), ubiquitin-binding protein 62/sequestosome-1 (p62/SQSTM1), neighbour of BRCA1 gene 1 (NBR1) and Tax1-binding protein 1 (TAX1BP1), are recruited to the poly-Ub chains, followed by formation of light chain-3 (LC3)-positive phagophores and the ensuing lysosomal degradation of mitochondria (Geisler *et al.*, 2010; Heo *et al.*, 2015; McWilliams and Muqit, 2017).

Mitophagic deficits are observed in both toxin-induced and genetically altered PD models. Homo- or heterozygous mutations of *PINK1* are the most common causes of recessive early-onset PD (usually below the age of 40) (Sarraf *et al.*, 2013). These patients display the cardinal symptoms and pathophysiology of idiopathic PD, including Lewy bodies, alongside more frequent psychiatric symptoms (Samaranch *et al.*, 2010). Alterations to PINK1 have consistently been shown to regulate mitochondrial function. *Pink1* knockout *D. melanogaster* display mitochondrial morphological abnormalities, locomotor deficits, muscle degeneration, male sterility and dopaminergic neuronal cell loss (Clark *et al.*, 2006b; Park *et al.*, 2006a; Yang *et al.*, 2006; Guo, 2012).

1.4.3.4 How is inflammation implicated in PD?

1.4.3.4.1 Microglia

The central nervous system (CNS) was once considered an immunologically privileged tissue due to the absence of dendritic cells, an immunosuppressed microenvironment under physiological conditions, and the presence of the blood-brain-barrier separating the brain parenchyma and peripheral nervous system (Medawar, 1948). However, recent discoveries of the presence of immune cells in the meninges and lymphatic vessels of the CNS, which drain and interface with the peripheral nervous system, have led to a re-evaluation of CNS status as immunologically privileged (Louveau, Harris and Kipnis, 2015). The CNS evokes an immune response against potential pathogens and cellular stress. Within the brain, microglia initiate the immune response (Saijo

et al., 2009). Appropriate conclusion of the immune response, once the insult is dealt with, is required in order to avoid chronic inflammatory activation. Inflammation in PD was first proposed in 1988, when PD patients were shown to have upregulated major histocompatibility complexes (McGeer *et al.*, 1988). Further evidence has since emerged – reactive microglia production and increased pro-inflammatory factor upregulation, such as tumour necrosis factor α (TNF-α), TGF- α, TGF-β, IL-1β, IL-2 and IL-6, are found in the brains of PD individuals (Gerhard *et al.*, 2006; Bartels *et al.*, 2010). Indeed, animal models of PD have added credence to this, revealing that dopaminergic neurone loss is accompanied by chronic neuroinflammation (Ferrari *et al.*, 2006a; Melissa K McCoy *et al.*, 2006). The rate-limiting eicosanoid pathway enzyme, cyclooxygenase-2/prostaglandin-endoperoxide synthase-2 (COX-2/PTGS2) has also been implicated in PD-associated chronic inflammation (Teismann *et al.*, 2006).

The primary glial cells responsible for initiation of the innate immune response in the CNS are microglia (Saijo *et al.*, 2009). CNS macrophages include, meningeal macrophages, epipexus cells, choroid plexus macrophages and perivascular macrophages. Microglia constitute 5-10% of total brain cells (Li and Barres, 2018). In the absence of activator signals microglia are quiescent - this is maintained by the expression of immunoregulatory molecules binding to microglia, including: CX3CL1, CD200, CD22, CD47, CD95, and neuronal cell adhesion molecule (NCAM) (Mott *et al.*, 2004; Sheridan and Murphy, 2013). Microglia are localised to specific regions of the

brain: the medulla oblongata, pons, basal ganglia, and the *SNpc* (Savchenko *et al.*, 1997; Kofler and Wiley, 2011). Microglial activation may be caused by exogenous insults (bacteria or virus), disease-related proteins, misfolded proteins (amyloid-β and α-syn) or neuronally-released molecules (ATP, mtDNA, matrix metalloproteinase-3, neuromelanin) (Li and Barres, 2018). Microglial activation results in the formation of reactive microglia. Two classical reactive microglia phenotypes have been described: M1 (pro-inflammatory) and M2 (anti-inflammatory), however, more recent evidence suggest extensive overlap between the two phenotypes exists (Ransohoff, 2016). Reactive microglia upregulate major histocompatibility complexes and cell-surface markers for cytokine and chemokine receptors (Li and Barres, 2018). Inflammasomes, protein complexes mainly formed of adaptor protein apoptosis-associated speck-like protein containing CARD (ASC), caspase-1 and an inflammasome component (NLRP3, NLRP1, NLRC4, AIM2), are present within microglia, neurones and astrocytes. Their major role is to enhance and sustain inflammation, via cleavage of pro-IL-1β to IL-1β, or production of IL-18 (Kofler and Wiley, 2011); these microglial inflammasomes have been implicated in PD (Codolo *et al.*, 2013).

1.4.3.4.2 Neuroinflammation may be directly activated by mitochondria

The pesticides rotenone and tebufenpyrad inhibit complex I function and increase the rate of ROS production. The neurotoxicity of tebufenpyrad has not been well studied (Charli *et al.*, 2016), however, the application of rotenone

generates a Parkinsonism phenotype in model species (Greenamyre *et al.*, 2010). Rotenone and tebufenpyrad have been shown to induce NLRP3 inflammasome activation in primary microglial cells. Further, mitochondrial ROS production drives NLRP3 inflammasome activation and was attenuated by decreasing superoxide generation with the mitochondrial-targeted antioxidant, mito-apocynin (Sarkar *et al.*, 2017). In both microglia and astrocytes the NLRP3 complex inflammasome has been reported to translocate to damaged mitochondria (Zhou *et al.*, 2011; Sarkar *et al.*, 2017). Inhibition of mitophagy and release of mtDNA have both been shown to induce activation of the NLRP3 inflammasome (Zhou *et al.*, 2011; Shimada *et al.*, 2012). Indeed, mitophagic disturbances and lysosomal dysfunction are both implicated in inflammasome activation in PD (Lynch-Day *et al.*, 2012).

1.4.3.4.3 α-synuclein activation of microglia

Growing evidence describes activation of microglia by aggregated α-syn, inducing a pro-inflammatory response, which includes increased IL-1β, IL-6, TNF-α, iNOS, PTGS-1 and -2, and ROS (Su *et al.*, 2008; Lee *et al.*, 2009; Su, Federoff and Maguire-Zeiss, 2009; Roodveldt *et al.*, 2010; Couch *et al.*, 2011). Comparative analysis of fibrillary, oligomeric and monomeric α-syn showed specific inflammatory responses – fibrillary α-syn caused the greatest increase in TNF-α and IL-1β (Hoffmann *et al.*, 2016). Oligomeric α-syn has been observed to be phagocytosed by microglia, leading to downstream production of ROS and increased prostaglandin E2 (PGE2) (Zhang *et al.*, 2005a). PGE2 is directly implicated in PD pathogenesis - prostaglandin E receptor subtype 2

deletion improved microglia degradation of α-syn (Jin *et al.*, 2007; Liu *et al.*, 2007).

1.4.3.4.4 Prostaglandin-endoperoxide synthase-2 and the eicosanoids

Prostaglandin (PG) synthesis is driven in large part by the Prostaglandin-endoperoxide synthase (PTGS) enzymes. Three PTGS isoforms exist: PTGS1, -2 and -3. Current evidence suggest that both PTGS1 and PTGS3 are constitutively expressed (Chandrasekharan *et al.*, 2002), and that PTGS1 is expressed in nearly all cell types and maintains a constant low-level generation of PGs for physiological functions (Pannunzio and Coluccia, 2018). Whereas, PTGS2 expression is primarily induced in response to stimuli and is encoded by an immediate-early response gene and inducible by cytokines, mitogens, ROS, hormones and other pro-inflammatory mediators (Yamagata *et al.*, 1993). In addition, within the CNS PTGS2 may be expressed constitutively at low levels (Beiche *et al.*, 1998). Since the discovery of PTGS3 in 2002, little progress has been made toward understanding its functional role. Indeed, PTGS3 may entirely lack PTGS activity in rodents and humans (Kis, Snipes and Busija, 2005). PTGS1 and -2 enzymes catalyse the formation of PGs from the lipid, AA (AA). They catalyse a two-step reaction: the first PTGS reaction involves AA and two O_2 molecules to form PGG2; the second peroxidase reaction reduces PGG2 by two electrons to form PGH2 (Smith, DeWitt and Garavito, 2000). PTGS1 and PTGS2 enzymatic differences are primarily in their peroxidase activity - PGH2 generation by PTGS2 is more

rapid. Secondly, while PTGS1 carries out solely two-electron reduction, PTGS2 carries out two-electron reduction 60% of the time; the remaining 40% being one-electron reduction (Landino *et al.*, 1997). Classically, PTGS2 generation of ROS by one-electron reduction was thought to occur (Nelson *et al.*, 1992; Chan, 1996), however, emerging evidence suggest the peroxidation reaction of PTGS2 only generates carbon-centred radicals which may indirectly cause oxidative stress (Jiang *et al.*, 2004).

Study of mouse models have further implicated PTGS2 and PGs in PD. The MPTP-treated PD mouse model is neuro-protected by non-steroidal anti-inflammatory drugs (NSAID) acetylsalicylic acid, salicylate, or meloxicam (Teismann and Ferger, 2001; Aubin *et al.*, 2002). However, treatment with the NSAID diclofenac showed no neuroprotective effect, although this may be due to its inability to cross the blood-brain-barrier (Teismann and Ferger, 2001). Further, the *Ptgs2* knockout mouse model exhibited a significant reduction in MPTP-induced neurodegeneration (Feng *et al.*, 2002; Teismann, Tieu, D. K. Choi, *et al.*, 2003). More support for the role of PTGS2 in PD comes from its direct involvement in the oxidation of dopamine to highly reactive dopamine-quinone (Hastings, 2002). Dopamine-quinone may react with cysteinyl residues in proteins, altering their structure and, potentially, function (Grünewald *et al.*, 2016).

PTGS2 may also localise to mitochondria and influence mitochondrial function as shown in nasopharylgeal cancer - mitochondrial PTGS2 co-localised with p53 and regulated the expression of p53-responsive genes. In

doing so, mitochondrial fission protein DRP1 was phosphorylated, inducing mitochondrial fission (Zhou *et al.*, 2017).

Metabolites of AA are altered in Parkinson's disease. Of the eicosanoids, PGE2 has the largest body of evidence implicating it. PGE2 may drive pathogenesis through increased ROS production and activation of astrocytes and microglia (Bezzi *et al.*, 1998; NH, 1999). In addition, pro-inflammatory IL-6 is regulated by PGE2 in numerous cell-types - inhibition of PGE2 resulted in reduced IL-6 and a reduction in inflammation, in a carrageen-induced paw-inflammation model (Hinson, Williams and Shacter, 1996; Williams and Shacter, 1997). The parallel rise in PGE2 and IL-6 is also observed in PD (Rojo *et al.*, 2010; Rojanathammanee, Murphy and Combs, 2011), although it remains to be seen whether PTGS2 and IL-6 rise due to a shared pathway or are independent of one another.

Chronic inflammation is a hot topic in disease research and reviews describe pro-inflammatory processes as chronically activated in PD (Bower *et al.*, 2006; Su *et al.*, 2008; Tufekci *et al.*, 2012). The role of inflammation in disease onset and/or progression is not clear - in part, this lack of understanding is simply due to the complexity surrounding inflammation, including the plethora of initiators, signalling cascades, and vast molecular alterations that are induced making itx difficult to conceptualise. What is clear is that inflammatory markers are differentially expressed in PD and further work into understanding these alterations will enable a fuller understanding of the disease and its underlying mechanisms.

1.5 How do we study ageing?

The advent of public health initiatives has caused the average lifespan to dramatically increase. However, longer life has brought with it an increased incidence of age-related diseases. Making advanced age healthy is the next challenge and in order to do this ageing research laboratories utilise various methodologies and model species. Organisms age at different rates: the laboratory mouse has a maximal lifespan of 4 years, whereas the naked-mole rat has a maximal lifespan of 30 years (Austad, 2010). Even within the same animal, cells may senesce at varying rates. Comparative biology and easily manipulatable organisms, with well-understood biology, are tools commonly employed for the interrogation of biological ageing.

1.5.1 *C. elegans* in ageing research

The nematode has provided many insights into the biology of ageing, including the identification of the insulin/IGF-1 pathway as a master regulator of longevity (Kenyon *et al.*, 1993; Kenyon, 2005). The success of *C. elegans* is due to its simple biology, coupled with its straight-forward husbandry and relatively short lifespan (Kenyon, 2010; Mack, Heimbucher and Murphy, 2018). Furthermore, *C. elegans* display phenotypic signs of ageing – pharyngeal pumping rate declines (Chow *et al.*, 2006), movement speed decreases and coordination is lost (Bolanowski, Russell and Jacobson, 1981), decreased responsiveness to noxious stimuli (Glenn *et al.*, 2004) and decreased reproductive rate (Hughes *et al.*, 2007).

A further advantage is that *C. elegans* have distinct tissues, including a fully mapped nervous system of 302 neurones (White *et al.*, 1986; Yan *et al.*, 2017). In 1998, *C. elegans* became the first multicellular organism to have its whole genome sequenced (*C. elegans* Sequencing Consortium, 1998). Although they contain 1/30th the number of base-pairs as the human genome, remarkably the number of protein coding genes is similar at ~20,000. Further, about 38% of *C. elegans* genes have predicted human orthologues (Shaye and Greenwald, 2011; Kim *et al.*, 2018). The *C. elegans* genome is easily manipulatable – gene knockdown by feeding RNAi-producing bacteria is a widely used technique for reverse genetic studies (Kamath *et al.*, 2003a). Further, RNAi can be fed transiently, thereby separating developmental and ageing phenotypes (Dillin, Crawford and Kenyon, 2002). The introduction of transgenes by microinjection (Stinchcomb *et al.*, 1985) or microparticle bombardment (Praitis *et al.*, 2001) is also readily used, and tissue-specific or transient expression can be achieved with promoters (Bacaj and Shaham, 2007). CRISPR/Cas-9 genome editing has also been adapted for *C. elegans* (Dickinson *et al.*, 2013).

The nine hallmarks of ageing that have been described are all able to be investigated in *C. elegans*. Specifically, alteration of key molecular pathways within these pathways has demonstrated lifespan changes. Autophagy (Meléndez *et al.*, 2003), mitophagy (Palikaras, Lionaki and Tavernarakis, 2015), nutrient signalling pathways (Friedman and Johnson, 1988; Kenyon *et al.*, 1993; Vellai, Kriztina Takacs-Vellai, *et al.*, 2003; Apfeld *et al.*, 2004), unfolded protein response regulators (Hsu, Murphy and Kenyon, 2003; Henis-Korenblit

et al., 2010; Taylor and Dillin, 2013), histone and chromatin remodelling (Samuelson, Carr and Ruvkun, 2007; Curran *et al.*, 2009) have all been demonstrated to regulate longevity in *C. elegans.* Given our understanding of the highly conserved molecular mechanisms underpinning ageing, numerous findings from *C. elegans* have produced insights into human ageing and age-related diseases - for example, the discovery of the role of the insulin/IGF-1 signalling pathway in longevity) (Klass, 1983; Kenyon *et al.*, 1993; Kenyon, 2010).

Work has progressed to screening age-delaying drug interventions in *C. elegans* (Petrascheck, Ye and Buck, 2009). The *Caenorhabditis* Intervention Test Program (CITP) is in place to test and identify drugs that have the potential to improve human health- and lifespan (CITP, 2019).

1.5.2 Comparative biology in ageing research

Comparative biology as a tool requires the simultaneous appreciation of species similarities and differences. Ageing in mammals is a phenomenon which presents with a typical phenotype – old mammals may be easily distinguished from young mammals due to shared distinguishing features - for example, hair loss, loss of skin elasticity, visual impairment and frailty. However, the rate of ageing is very different across mammalian species – the maximal lifespan of the house mouse is 4 years, whereas bowhead whales live for over 200 years (George *et al.*, 1999). Outwardly these mammals look dissimilar, however, the underlying biology is surprisingly alike. Comparative

biological investigation of evolutionarily similar species, with dissimilar maximal lifespans, should reveal biochemical differences relevant to longevity.

The rate of living theory suggests that ageing and longevity are an inverse function of metabolic rate (Pearl, 1928). Larger animals do tend to have a slower basal metabolic rate, and this suggests that metabolic rate and ageing are closely related. Indeed, the MFRTA states that an accumulation of oxidative damage, driven by mitochondrial free radical production, is the driving force behind ageing (Harman, 1992). Therefore, an increased rate of metabolism equates to more free radicals produced, subsequent cellular damage is increased, and overall lifespan should be shorter. However, accumulating evidence suggests the MFRTA is not an adequate explanation of biological ageing and ROS production may not be the primary driver of the ageing rate (Andziak, O'Connor and Buffenstein, 2005; Andziak, Timothy P O'Connor, *et al.*, 2006; Csiszar *et al.*, 2008; Copeland *et al.*, 2009; Van Raamsdonk and Hekimi, 2009; Yang and Hekimi, 2010b).

Assessment of mammalian longevity across species has generally been carried out by examining the log-log plot of longevity vs body mass (Speakman, 2005). Plotting longevity information of over 600 mammalian species reveals a linear relationship between body size and longevity for mammals above 1kg. However, this relationship disappears below 1kg (Austad and Fischer, 1991) – the longevity of bats and marsupials are independent of body mass. Removal of these species, termed the "non-flying eutherian regression line", restores

the linear relationship in mammals (Austad and Fischer, 1991). The longevity quotient (LQ), the ratio of actual observed longevity to that predicted by the non-flying eutherian regression line, highlights species with exceptional longevity. By definition mean LQ is 1. Since the beginning of the 19th century and the development of public health initiatives and modern medicine, humans have emerged as an exceptionally long-lived species (Riley, 2005a; Roser, 2015). Taking 100 years of age as a representative maximum lifespan (1 in 10,000 people live to 100 years of age (Perls *et al.*, 2002a)) human LQ is about 4.5. Nineteen mammalian species are known to exceed this LQ. One is the naked mole rat, with a LQ of 5.3 (Allard *et al.*, 1999). Interestingly, if the human record for maximum longevity is used to generate a LQ score, this translates to the same LQ as the naked mole rat, 5.3 (Allard *et al.*, 1999). The remaining 18 species are bats. For bats that we currently have longevity data (65+ species) the mean LQ is 3.52, which means that on average a bat lives 3.5-times longer than would be expected by its body mass (Austad and Fischer, 1991). Intriguingly, at present the majority of lifespan estimate data are primarily from wild-caught bats, whereas records for the majority of species come from captive species (Austad, 2010). Due to this, it is possible that the lifespan of many bats exceeds that of current maxima.

1.5.2.1 Bats

Any mammal that does not fit the non-flying eutherian regression line trend is of interest to investigate. The longevity of bats has been known for many decades, however, it was originally dismissed as a consequence of hibernation

and torpor intermittently reducing metabolism (Bourliere, 1958). It was thought that accounting for these reduced metabolic rates would bring bat LQ in line with other mammals (Jürgens and Prothero, 1987). Multiple lines of evidence now question these early ideas. Firstly, tropic bat species do not hibernate yet are exceptionally long lived (Herreid, 1964; Austad and Fischer, 1991; Turbill, Bieber and Ruf, 2011; Wilkinson and Adams, 2019); secondly, while comparisons of hibernating and non-hibernating bats suggest hibernation accounts for up to six additional years of life, bats are still exceptionally long lived compared to similarly sized mammals (Wilkinson and South, 2002).

Although research is limited, due in part to the difficulty in obtaining bats for research, laboratories all over the world are increasingly taking an interest in this exceptional mammal. Maintenance of mitochondrial function may explain the lifespan of bats - bats are the only mammal capable of flight and flight requires extreme energy utilisation and a very high metabolic rate. On average, bat metabolic rate in flight is three times higher than that of similar sized mammals during exercise (Austad and Fischer, 1991). During flight, the average oxygen consumption rate of bats reaches 20-30x its basal level (Thomas and Suthers, 1972; Maina and King, 1984). The MFRTA suggests increased oxygen consumption should result in increased ROS production and, consequently, an increased rate of ageing (Harman, 1972, 1983), however, bats contradict this theory as data from *M. lucifugus* have shown bats produce less ROS per unit of oxygen consumed compared to similar sized mammals

(Brunet-Rossinni and Austad, 2004). Bat heart mitochondria H_2O_2 production was ~50% less than short-lived rodents (Lambert *et al.*, 2007b). Recently, mtDNA heteroplasmy with age was investigated in *M. myotis*. The researchers found that although oxidative stress affected mtDNA of individual bats, at subsequent time points (one-year intervals), removal of the majority of mtDNA mutations was clear, which suggests an efficient mtDNA repair system. Additionally, mutation sites predicted to be functionally deleterious were very low in *M. myotis* (Jebb *et al.*, 2018). Repeat regions (8-10bp) in mtDNA are prone to mutagenesis and bats have been shown to have a lower number of mtDNA repeat regions than other similar mammals (Khaidakov, Siegel and Shmookler Reis, 2006).

The long-term maintenance of a healthy population of mitochondria is largely due to effective proteostasis. Proteostasis mechanisms are known to become less efficient with age (Joseph, Peter J. Adhihetty, *et al.*, 2013), however, evidence suggests bats have a highly capable proteostasis system - analysis of the long-lived little brown bat (maximal lifespan about 34 years), compared with a close phylogenetic relative, the evening bat (maximal lifespan about 6 years), revealed enhanced autophagy in the little brown bats fibroblasts. Interestingly, the same study showed that a secondary proteostasis system in the long-lived bat was unchanged between the two bat species – heat shock protein response and proteasome (20S) activity were similar between bats (Pride *et al.*, 2015). In contrast, a comparative study between the mouse and two bat species [Myotis velifer (maximal lifespan ~12 years) and Tadarida

brasiliensis (maximal lifespan ~12 years)] revealed enhanced resistance to urea-induced protein unfolding in bats compared with mice (Salmon *et al.*, 2009). It may be that the improved chaperone response is a feature of both short and long-lived bats, compared with mice, and that additional mechanisms, beyond simply improved proteostasis, underlie the lifespan of the longest-lived bats.

Sparse evidence exists for the role of antioxidants in bat longevity. No difference in cardiac superoxide dismutase activity was observed between bats and rodents (Brunet-Rossinni, 2004b), however, the contents of α-tocopherol and β-carotene in multiple organs were shown to be 1-2 orders of magnitude greater in bats than rodents (Yin *et al.*, 2016).

According to contemporary biological theories of longevity, bat mitochondria are adapted for long-life (Hamon, Bulteau and Friguet, 2015). These initial findings are tantalising, however, data remain scant and more research is necessary to determine how bats achieve their exceptional longevity.

1.6 Strategies to fix ageing

As our understanding of the biological basis of ageing has grown the identification of potential behavioural interventions and molecular targets for improving health- and lifespan are becoming apparent. The health and lifespan of model species are consistently improved by environmental interventions, such as, caloric or dietary restriction (Qiu *et al.*, 2010; Mattison *et al.*, 2012; López-Domínguez *et al.*, 2015; Lee, Kaya and Gladyshev, 2016; Tinsley and Horne, 2018) and exercise (Lanza *et al.*, 2008; Garcia-Valles *et al.*, 2013; Barnes, 2015). These behavioural lifestyle modifications elicit biochemical changes. Interestingly, many of the known behavioural and molecular alterations, which improve lifespan, also regulate nutrient-sensing pathways (Kapahi *et al.*, 2004; Kenyon, 2010; López-Otín *et al.*, 2013; Rebelo-Marques *et al.*, 2018).

More recently, bioinformatic approaches have aided the discovery of repurposable drugs for the field of ageing. The Connectivity Map, a database of drug-induced gene expression changes, has been used to identify drug mimetics that cause expression changes similar to dietary restriction in rodents and monkeys (Calvert *et al.*, 2016). In the future, identification of molecular markers of ageing coupled with the application of bioinformatics should enable rapid and specific targeting of the biological ageing process.

1.6.1 Molecular markers of ageing

Molecular markers of ageing would allow for the differentiation of individuals based upon their rates of ageing, the identification and quantification of healthy ageing markers, and prediction of lifespan. In addition, identification of molecular markers could pinpoint new mechanisms underlying the ageing process and their eventual targeting.

This thesis focusses upon three proteins that can be evaluated as markers for health and longevity: carbonic anhydrase II and III (CAII and CAIII), and fatty acid binding protein 3 (FABP3). The following subsections are focussed on these proteins and evidence for their role in the ageing process.

1.6.1.1 Carbonic anhydrases II and III

Carbonic anhydrases (CA) are zinc metalloenzymes that catalyse the reversible reaction between carbon dioxide hydration and bicarbonate dehydration:

$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$.

They are essential for many processes, including the maintenance of tissue acid-base balance, CO_2 transport and bodily fluid formation (Henry, 1996; A. Pollard *et al.*, 2016). The CA family are exceptionally well conserved, the numerous CA isoforms found in the animal kingdom are termed α-type, while phylogenetically unrelated, but functionally similar, β and γ families exist in plants and prokaryotes (C. Supuran, 2008).

In humans, the CAII isoform is the most widely expressed CA, expressed in almost all tissues (Lindskog, 1997). CAII has diverse physiological functions - neurologically these include the production of aqueous humour and cerebrospinal fluid by the ciliary body of the eye and the choroid plexus, respectively (Sly and Hu, 1995a). While CAII is a cytosolic isoform, it has been linked to mitochondrial function as areas of high CAII expression and activity have been identified in mitochondria (Schroeder *et al.*, 2013; A. Pollard *et al.*, 2016). CAII may act as an acid-base buffer of the mitochondrial matrix – it regulates the activity of the Na^+/H^+ exchanger, a ubiquitous membrane glycoprotein which exchanges one intracellular proton for one extracellular sodium ion, altering intracellular pH (Li *et al.*, 2002).

In muscle, CAIII is the dominant isoform, found in the sarcoplasm of type I (Sly and Hu, 1995b) and type IIa (Feng and Jin, 2016a) muscle fibres. Classically, CAIII has been thought to support the transport of CO_2 out of muscles by catalysing its conversion to the more soluble bicarbonate (Côté, Ambrosio and Perreault, 1999a). However, no clear correlation exists between CAIII activity and CO_2 production in muscle (Côté, Ambrosio and Perreault, 1999a). CAIII has a lower catalytic function than any other member of the α-CA family (Feng and Jin, 2016b) - this may indicate that the enzyme has other or additional functions. Indeed, CAIII is thought to have roles in lipid and carbohydrate metabolism and to act as a protein phosphatase (Côté, Ambrosio and Perreault, 1999b), although contrary evidence exists (Kim, Selengut and Levine, 2000). Evidence supports its role as a ROS sink, due to the presence of

high numbers of reactive cysteines present on its protein surface (Di Fiore *et al.*, 2018).

1.6.1.1.1 Carbonic anhydrases II and III in ageing and diseases of age

CAII is most highly expressed in brain tissue and is widely distributed across many brain regions (Rogers and Hunt, 1987). Pathologically, increased CAII expression in the eye is associated with glaucoma (Scozzafava and Supuran, 2014). The most common form of glaucoma, primary open-angle glaucoma, presents with high intraocular pressure (IOP), causing trauma and death of retinal ganglion cells. High IOP is caused by excessive aqueous humour (AH) fluid levels, produced by the ciliary body (Scozzafava and Supuran, 2014) - CAII is abundant in epithelial cells of the ciliary body. Although the exact causal mechanisms behind excessive AH are unknown, topical CAII inhibitors dorzolamide and brinzolamide, which are commonly used to treat glaucoma, improve ocular blood flow, reduce AH volume and thereby decrease IOP (Scozzafava and Supuran, 2014). CAII has also been shown to be differentially expressed or modified in other neurodegenerative conditions. In the familial PD mouse model, A30P α-syn, higher CAII carbonylation levels, corresponding to a decrease in activity, were observed (Fai Poon *et al.*, 2005). Similarly, in AD CAII activity was lower in AD individuals and in those diagnosed with mild cognitive impairment (MCI), which suggests CAII may

be an early marker of cognitive impairment (Meier-Ruge, Iwangoff and Reichlmeier, 1984; Sultana *et al.*, 2010). Further, proteomic analysis of the hippocampus of AD individuals found increased CAII levels (Sultana *et al.*, 2007). Taken together, in AD an increase in protein levels may act to compensate for the lowered activity.

CAIII has been proposed as a diagnostic marker of muscular diseases (Brancaccio, Lippi and Maffulli, 2010). A mouse model of muscular dystrophy, *mdx* mouse, showed an increase in CAIII protein levels with disease progression (Carberry *et al.*, 2012).

Recently, CA has been implicated in ageing - increased CAII and CAIII were observed in brain and muscle of 18 month old mice compared to 2 month old mice. Further, the group showed that *C. elegans* fed recombinant CA had a 50% reduction in lifespan, suggesting high levels of CA are life-limiting (A. Pollard *et al.*, 2016). Additional evidence comes from elderly humans who exhibited increased activity and expression of CAIII in skeletal muscles (Staunton *et al.*, 2012a; Gueugneau *et al.*, 2014). However, some discrepancy in the literature exists as the gastrocnemius muscle of 30 month old Wistar rats had reduced CAIII protein levels, compared with middle aged rats (Doran *et al.*, 2008). An explanation may be the disparate ages used in these studies – 30-month-old rats are equivalent to 75 year old humans – the rats used in this study were older than the mouse or human studies and may have had age-related muscle wasting. Increased CAIII levels with age have been proposed to be due to a shift in fibre type with age (Staunton *et al.*, 2012b) as evidence

consistently points to slow twitch fibres containing higher CAIII protein levels, and ageing is associated with a fast-to-slow twitch fibre transition (Sly and Hu, 1995a; Feng and Jin, 2016b). CAIII expression changes may simply be due to muscle or fibre-type changes with age, or may be due to a functional demand.

1.6.1.2 Fatty acid binding protein 3

Fatty acid binding proteins (FABP) are a family of 10 abundant intracellular proteins, which are expressed in almost all tissues (Kitanaka *et al.*, 2003, 2006; Yun *et al.*, 2004; Nourani *et al.*, 2006). They are small (15kDa) proteins which show moderate sequence homology (~20-70%), yet are highly similar in tertiary structure (Smathers and Petersen, 2011). All isoforms show strong binding affinity for long chain saturated or unsaturated fatty acids. All isoforms have capacity to bind a single long chain fatty acid (LCFA), except liver-type FABP (FABP1) which may bind two LCFAs (He *et al.*, 2007). Some evidence suggest the tissue-specificity of FABP isoforms reflects their binding affinity for the fatty acids present within the tissue (Storch and McDermott, 2009; Smathers and Petersen, 2011). In brain and muscle, the predominant isoform is known as heart-type FABP, or FABP3 (Smathers and Petersen, 2011).

FABP3 is strongly associated with the maintenance of an adequate energy supply. Firstly, FABP3-null mice show decreased palmitate oxidation by mitochondrial β-oxidation, and a shift in cardiac muscle fuel selection from LCFA to glucose (Furuhashi and Hotamisligil, 2008). Secondly, during skeletal

muscle cell differentiation, FABP3 is upregulated alongside a concomitant increase in utilisation of LCFA via β-oxidation and esterification (Zhu *et al.*, 2011). Some data also suggest FABP3 may interact with the PPAR family of proteins, further supporting its role in β-oxidation (Tsukahara *et al.*, 2014a).

FABP3 modulation has been connected with age and diseases of age. Recent work showed increased protein levels of FABP3 in response to longevity promoting activities, fasting (Sánchez-Gurmaches *et al.*, 2011) and exercise (Mulya, Jacob M Haus, *et al.*, 2017). In AD and Down-syndrome, significantly lower protein levels of FABP3 are observed (Cheon *et al.*, 2003) and growing interest surrounds the role of FABP3 in PD, where it has been observed to be increased in the CSF of PD patients (Mollenhauer *et al.*, 2007). FABP3 is highly expressed in dopaminergic neurones of the *SNpc* and it binds to dopamine D2 receptors and regulates D2 receptor function in the mouse brain (Wang *et al.*, 2000). FABP3 shows favourable binding to n-6 fatty acids, including arachidonic acid (AA) (Hanhoff, Lücke and Spener, 2002). FABP3 and AA are shown to accumulate in post-mortem PD brains (Julien *et al.*, 2006). Lastly, α-syn has been shown to be induced to fold by FABP3 in the presence of AA (Cheng *et al.*, 2019).

Less is known concerning the role of FABP3 in the ageing process. Its responsiveness to longevity promoting lifestyle interventions is intriguing though and it may be a potential molecular marker of ageing, whose restoration may promote longevity or delay diseases of ageing.

1.6.2 Lifestyle interventions that delay the ageing process

Ultimately, the goal is to extend the number of healthy years (healthspan) an individual has. Reducing the disease burden of a population improves healthspan and simultaneously increases average lifespan. It is well known that lifestyle interventions, such as dietary or caloric restriction (Luhtala *et al.*, 1994; Goldberg *et al.*, 2009; Mattison *et al.*, 2012), and exercise (O'Callaghan, Griffin and Kelly, 2009; Harrison *et al.*, 2013), are sufficient to improve healthspan, therefore, an active and growing area of research is aimed at investigating the molecular changes these interventions promote.

1.6.2.1 Longevity pathways

The first longevity pathway to be described was the insulin/IGF-1/FOXO pathway. In 1993, Cynthia Kenyon's lab discovered that inhibition of *C. elegans daf-2* (orthologue of insulin/IGF-1 receptor) doubled lifespan, and that *daf-16* (a forkhead-family (FOXO) transcription factor) was required for this lifespan enhancement (Kenyon *et al.*, 1993). Members of the pathway are implicated in regulating lifespan in both *D. melanogaster* and mice (Longo and Fabrizio, 2002; Kenyon, 2005). FIRKO mice, which have disrupted insulin receptor activity in adipose tissue, are long-lived (Bluher, Kahn and Kahn, 2003). Small dogs have a greater maximal lifespan than large dogs – small dogs carry a single *IGF-1* single-nucleotide polymorphism haplotype, which is nearly absent from large dogs (Sutter *et al.*, 2007). In humans, IGF-1 receptor deficiency is linked to longer life in Ashkenazi Jews (Suh *et al.*, 2008), and FOXO allelic variants have been observed in other long lived populations

(Flachsbart *et al.*, 2009). Both progeroid diseases and normal ageing consistently correlate with insulin resistance and type II diabetes incidence, further associating this pathway with longevity (Rosenbloom *et al.*, 1983; Mordarska and Godziejewska-Zawada, 2017).

More recently, the evolutionarily conserved serine-threonine kinase, mechanistic (also referred to as mammalian) target of rapamycin (mTOR) has emerged as another central molecular regulator of lifespan (Fabrizio *et al.*, 2001; López-Otín *et al.*, 2016). mTOR is a member of the phosphatidylinositol-3 kinase (PI3K)-related kinase family and is a catalytic subunit of the mTOR protein complexes. Two complexes, mTORC1 and mTORC2 exist in mammals. mTOR is named for its inhibitor, identified in the 1970s from the soil bacteria on Easter Island (Rapa Nui) (Vézina and Kudelski, 1975). Rapamycin acts upon FK506-binding protein 12 (FKBP12), which interacts with the mTOR1 complex, decreasing mTOR activity (Chung *et al.*, 1992). mTORC2 has been shown to be insensitive to rapamycin treatment (Saxton and Sabatini, 2017). Rapamycin was originally approved as an anti-fungal agent, then later as an immunosuppressant, due to its potent anti-proliferative effect on T-lymphocytes (Vézina and Kudelski, 1975; Martel, Klicius and Galet, 1977). Physiological activation of mTOR is controlled by diverse pro-growth signals: plentiful amino acids, glucose, lipids, high ATP/AMP ratio and oxygen are capable of activating mTOR (Saxton and Sabatini, 2017).

The early 2000s presented the first hint of mTOR as a regulator of lifespan: *C. elegans*, *D. melanogaster* and *S. cerevisiae* all exhibit lifespan extension with TOR

pathway inhibition (Vellai, Krisztina Takacs-Vellai, *et al.*, 2003; Kapahi *et al.*, 2004; Kaeberlein *et al.*, 2005). Later, work in rodents confirmed its lifespan extension and conserved role in mammals (Harrison *et al.*, 2009). In genetically heterozygous mice, rapamycin treatment at 600 days of age (equivalent to 60 human years) was shown to increase lifespan 10-18%, with females showing greater benefit (Harrison *et al.*, 2009). Rapamycin treatment of younger mice increased lifespan in a dose-dependent manner, however, the improvement in lifespan was similar to mice treated from 600 days (Miller *et al.*, 2014; Johnson *et al.*, 2015). Deletion of mTORC1 substrate S6K1, a nutrient response transducer, increased median and maximal lifespan of female mice. Interestingly, male mice showed no change to either median or maximum lifespan increase (Selman *et al.*, 2009).

1.6.2.2 Caloric restriction

Insulin/IGF-1/FOXO and mTOR pathways sense nutrient status and respond accordingly. In 1935, the seminal work of McCay and colleagues, revealed a link between caloric restriction (CR), without malnutrition, and lifespan (McCay CM, Crowell MF, 1989). Since then, CR has been demonstrated to extend lifespan in all organisms tested (Madeo *et al.*, 2015).

Clearly CR will result in weight reduction - reducing white adipose depots decreases adipose derived pro-inflammatory and diabetogenic activity (Bianchi, 2018). CR is a potent inducer of autophagy, thereby maintaining a healthy and efficient pool of cellular organelles (Finkel, 2015). Mechanistically, both the insulin and mTOR pathways are inhibited by CR, whilst SIRT1,

AMPK and FOXO1 are activated (Galluzzi *et al.*, 2014). In *C. elegans* and mice the longevity benefit of CR appears to be mediated in part by SIRT1 activation – the *C. elegans* SIRT1 orthologue is required for CR-mediated activation of autophagy (Madeo *et al.*, 2015). Ketogenesis and increased β-oxidation have also been shown to be upregulated with CR - calorie restricted hepatocytes release fibroblast growth factor 21 (FGF21), which enhances ketogenesis and β-oxidation, and inhibits GH/IGF-1. Interestingly, mice overexpressing FGF21 are longer-lived (Zhang *et al.*, 2012). CR also promotes increased expression of insulin-like growth factor binding protein 1 in non-obese adults, which inhibits free IGF-1 levels, thereby directly reducing IGF-1 signalling (Fontana *et al.*, 2016). The anti-inflammatory properties of CR are also well documente: increased cortisol, increased β-hydroxybutyrate and FOXO3A act to promote an anti-inflammatory cellular environment, in part by inhibiting the NLRP3 inflammasome (Shimazu *et al.*, 2013; Youm *et al.*, 2015; Yang *et al.*, 2016).

1.6.2.3 Time-restricted feeding

Evidence suggest time-restricted feeding (TRF) as another intervention which promotes health and longevity. No strict TRF definition currently exists - studies conducted with a TRF paradigm generally define TRF as eating within an 8-hour period and fasting for 16-hours. CR together with TRF is a growing lifestyle trend, however, TRF independent of CR has been shown to elicit health benefits in both mice (Hatori *et al.*, 2012; Sherman *et al.*, 2012; Zarrinpar *et al.*, 2014; Olsen *et al.*, 2017; Delahaye *et al.*, 2018) and humans (Antoni *et al.*, 2017; Sutton *et al.*, 2018; Tinsley and Horne, 2018; Ravussin *et al.*, 2019).

Restricting access to food to a few hours per day in mice, compared to an ad libitum access group, protected time-restricted mice from obesity, hepatic steatosis, hyperinsulinemia, inflammation, and metabolic disease, despite consuming equal calories (Sherman *et al.*, 2012; Chaix *et al.*, 2014; Sundaram and Yan, 2016; Olsen *et al.*, 2017; Delahaye *et al.*, 2018; Sun *et al.*, 2018; Sutton *et al.*, 2018).

1.6.2.4 Dietary restriction

Dietary restriction, rather than caloric restriction, is another intervention shown to extend lifespan. Protein restriction of humans aged 50-65 resulted in decreased IGF-1 levels and was associated with reduced all-cause mortality and cancer incidence (Levine *et al.*, 2014). Individual amino acid restriction has also shown efficacy in extending lifespan. From yeast to rodents, methionine restriction promotes longevity (Madeo *et al.*, 2015). Methionine levels are in equilibrium with S-adenosyl methionine (SAM), an essential donor of methyl groups for DNA methyltransferases and therefore vital for epigenetic regulation. SAM catabolism has been linked to lifespan extension in *D. melanogaster* (Obata and Miura, 2015) and, furthermore, methionine restriction also acts to promote the synthesis of hydrogen sulphide (H_2S), which promotes autophagy and has been shown to be necessary for lifespan extension by CR in multiple model species (Hine *et al.*, 2015).

1.6.2.5 Exercise

Sedentary living accelerates the ageing process. It is estimated to underlie 5.3 million deaths per year globally (9% of all-cause mortality) (I.-M. Lee *et al.*,

2012). Regular exercise has consistently been observed to promote long-term health of humans. Indeed, estimates from the US Office of Disease Prevention and Health suggest exercise reduces all-cause mortality risk by 30-40% (Office of Disease Prevention and Health Promotion, 2008). Interestingly, evidence supporting lifespan extension due to exercise are mixed, although this may be a finding unique to mice (Garcia-Valles *et al.*, 2013; Kujala, 2018; Nilsson *et al.*, 2019). Conversely, healthspan and median lifespan are reliably improved in humans and other model species (O'Callaghan, Griffin and Kelly, 2009; Ahlskog, 2011; Gomez-Cabrera *et al.*, 2013; Harrison *et al.*, 2013; Di Loreto *et al.*, 2014; Svensson, Lexell and Deierborg, 2014; Rebelo-Marques *et al.*, 2018).

Exercise elicits a holistic response and alleviates symptoms of ageing more effectively than any currently available drug. In the nervous system, exercise promotes neurogenesis and attenuates cognitive loss with age; cardiovascular and respiratory functions are improved; enhanced metabolic function, including muscle protein synthesis and fat oxidation; improvements to bone and muscle size and strength, increased lean/fat ratio, and reduced visceral fat adiposity (Nelson *et al.*, 2007; Garatachea *et al.*, 2015b).

1.6.2.6 How is age-related inflammation influenced by exercise?

Exercise is a transient stressor, which means it causes a change in cellular stress signalling (ROS, pH, hypoxia, Ca2+), metabolic intermediates (AMP/ATP, NAD+/NADH) and circulating hormones and factors. Repeated bouts of exercise result in physiological adaptations to a more stress resistant homeostatic level (Barnes, 2015). A chronic pro-inflammatory state underpins

ageing and age-related disease (Ferrucci and Fabbri, 2018). Lifelong exercised rodents show a dampened pro-inflammatory response with wheel-running exercise, and these rodents were protected against "inflammageing" and cancer. Further, the same group showed that key pro-inflammatory cytokines were reduced (TNFα, IL-6 and IL-1β) and anti-inflammatory (IL-10, CXCL1) cytokines were enhanced. Although biological ageing markers were reversed, the exercised mice had only a modest increase in median lifespan (Nilsson *et al.*, 2019).

Exercise-induced muscle contraction causes an array of myokines to be released. Interestingly, the muscle-released IL-6, induced by contracting muscles during and after exercise, has been observed to act in an anti-inflammatory manner, inducing the promotion of anti-inflammatory cytokines (IL-1ra, IL-10, soluble TNF receptors) (Pedersen, 2012; Schett, 2018). In support, several groups have reported lower inflammatory levels associated with lifelong aerobic exercise training, particularly C-reactive protein, IL-6 and TNFα (Beavers, Brinkley and Nicklas, 2010; Mikkelsen *et al.*, 2013). Another mechanism by which exercise promotes an anti-inflammatory milieu may be regulation of methylation. Six months of high-intensity interval walking exercise increased methylation levels of the pro-inflammatory apoptosis-associated speck-like protein caspase gene, which regulates pro-inflammatory cytokines IL-18 and IL-1β, in leukocytes of older adults (Nakajima *et al.*, 2010). Epigenetic alterations due to exercise are not well

characterised, however, existing data hint that epigenetic mechanisms are likely an important regulator of the effects exercise mediates.

1.6.2.7 How does exercise impact ageing mitochondria?

Exercise requires cellular adaptations in order to cope with the increased demand for energy. Mitochondrial adaptive responses are pivotal to exercise-induced changes – their size, number and content all alter (A. J. Trewin, Berry and Wojtovich, 2018). Metabolic changes concerned with fat transport, oxidative enzymes, and respiratory components are regulated to match requirements. Endurance trained humans display higher mitochondrial protein expression, mtDNA number and TFAM (Norrbom *et al.*, 2010). The central mitochondrial biogenesis controller, PGC-1α, is upregulated with exercise (Patki and Lau, 2011; Ruas *et al.*, 2012; Mulya, Jacob M. Haus, *et al.*, 2017a) and exercise promotes lipolysis and enhances fuel utilisation of fatty acids by upregulation fatty acid metabolism proteins (long-chain acyl-CoA dehydrogenase) fatty acid transport proteins (FAT/CD36 and FABP) and oxidation proteins (CPT1B) (Wolfe, 1998; Mulya, Jacob M. Haus, *et al.*, 2017a). Ageing is associated with mitochondrial dysfunction and aged muscle mitochondria are generally characterised by fragmented, round-shape networks or unusually large mitochondrial fragments (Yoon *et al.*, 2006; Leduc-Gaudet *et al.*, 2015). Mitochondrial adaptations to exercise have been observed in both young and aged muscle - endurance trained elderly humans consistently show widespread improvements in mitochondrial markers of ageing, including proteasomal and autophagic activity, antioxidant defence

and mitochondrial biogenesis (Fiuza-Luces *et al.*, 2013; Garatachea *et al.*, 2015a). Endurance trained elderly individuals showed no age-related decline in mitochondrial oxidative capacity, had increased levels of mitochondrial transcription factors and mtDNA, and high SIRT3 protein levels, compared with age-matched sedentary individuals (Lanza *et al.*, 2008).

Resistance exercise is an effective measure to reduce ageing-related muscle atrophy and weakness. At present, the literature describing resistance exercise mediated mitochondrial alterations is sparse. An acute period of resistance exercise training in elderly men increased mRNA levels of PGC-1α and TFAM (Ogborn *et al.*, 2015). Conversely, 12 weeks of resistance exercise in >60 year olds did not improve mitochondrial markers in skeletal muscle (Flack *et al.*, 2016).

Collectively, studies show that exercise strongly influences mitochondrial turnover. Acute exercise training induces a transient swelling and/or fragmentation of mitochondria and training is associated with an increase in mitochondrial volume and connectivity (A. Trewin, Berry and Wojtovich, 2018). Fusion and fission processes are altered in the skeletal muscle of exercised young mice (López-Lluch *et al.*, 2008) and a similar change is also apparent in aged rat skeletal muscle – 6 weeks of treadmill exercise upregulated FIS1 and MFN1 protein levels in old rats (Koltai *et al.*, 2012). In humans, the data are less conclusive - lifelong active, older women, had elevated levels of *MFN2* and *DRP1* mRNA in skeletal muscle compared to age-matched inactive women (Drummond *et al.*, 2014). A single bout of

running training reversed the age-related increase in *MFN1* mRNA (Bori *et al.*, 2012), however, other groups have found no change to *MFN1* or *MFN2* with exercise (A. M. Joseph *et al.*, 2012; Fealy *et al.*, 2014). Similarly, the literature on exercise-regulation of fission proteins DRP1 and FIS1 in the elderly is contradictory (A.-M. Joseph *et al.*, 2012; Bori *et al.*, 2012; Koltai *et al.*, 2012; Drummond *et al.*, 2014; Fealy *et al.*, 2014; Konopka *et al.*, 2014). Many factors may account for this, including study duration, interval before muscle-biopsy, age and exercise protocol. Although a consensus describing individual protein regulation by exercise cannot be determined at present, it remains that changes to mitochondrial dynamics are detected due to exercise.

Exercise has also been observed to influence autophagic processes. A single bout of treadmill exercise increased skeletal muscle levels of LC3-II and p62 immediately after exercise and this was sustained during recovery. Interestingly, data from the same group suggest PGC-1α is required for this response to exercise – in the absence of PGC-1α autophagic protein levels did not rise (Vainshtein, Tryon, *et al.*, 2015). In agreement, *Pgc-1α* knockout mice showed reduced denervation-induced mitophagic flux in skeletal muscle, whilst denervation of wild-type mice promoted mitophagic flux (Vainshtein, Desjardins, *et al.*, 2015). Endurance exercise trained old mice showed an increase in muscle expression of autophagic markers ATG7 and Beclin-1, which are key to autophagosome formation, and increased LC3-II to LC3-I ratio [LC3-I is ostensibly non-active, and is lipidated (binds to phophatidylethanolamine) to form LC3-II, which is associated with completed

phagosomes] (Kim *et al.*, 2013; Garvey *et al.*, 2015; White *et al.*, 2016). In humans, physically active elderly individuals showed increased ATG7, Beclin-1, p62, BNIP3 and Parkin (Drummond *et al.*, 2014; Zampieri *et al.*, 2015). Resistance exercise appears to accelerate autophagic flux, although a dearth of research currently exists. Six weeks of ladder climbing exercise downregulated LC3-II to LC3-I and p62 protein abundance in old rat muscle, whilst upregulating Beclin-1, ATG7 and cathepsin L (Luo *et al.*, 2013). Similarly, in exercised elderly humans, the muscle protein LC3-II to LC3-I ratio was decreased, suggesting resistance exercise reversed age-related declines in autophagy (Fry *et al.*, 2013).

1.6.3 Drug Repurposing

Realising new applications for existing drugs widens the potential market and thereby increases profits (Dudley, Deshpande and Butte, 2011). Repurposing skips costly development, which regularly cost billions of dollars, clinical trials for safety and efficacy are already passed for the original application, all of which substantially reduce the overall development time (Adams and Van Brantner, 2006). Approaches to drug repurposing include drug-centred or disease-centred: drug-centred focusses on matching chemical similarity or biological effects of a drug, or the target, with another. Commonly shared side-effects are another factor used to determine drug similarity. Bioinformatic approaches have improved speed and scale of analysis and computational modelling of drug-protein interactions is increasingly being utilised; disease-

centred assesses similarities between diseases in order to determine potentially useful drugs. If the underlying disease mechanisms are similar between diseases, it is likely therapies used to treat one may reliably treat another (Dudley, Deshpande and Butte, 2011).

Commonalities exist in the underlying mechanisms that drive the ageing process (López-Otín *et al.*, 2013). Further, comparative biology and healthy lifestyle behaviours clearly show that differences in lifespan are mediated in a non-random manner and that shared features characterising the rate of ageing exist (Salmon *et al.*, 2009; Austad, 2010; Dammann, 2017). Elucidation of these mechanisms and features will enable their targeting, indeed, the immunosuppressant rapamycin and Type-II diabetes treatment metformin have received tremendous attention in the anti-ageing field for their success at increasing health and lifespan of multiple model species (Kapahi *et al.*, 2004; Miller *et al.*, 2014; Faragher, 2015; Johnson *et al.*, 2015; Aliper *et al.*, 2017). In middle-aged companion dogs, rapamycin trialling to modulate healthy ageing has been ongoing since 2014 - early results have shown that ten weeks of rapamycin treatment improves heart function (Urfer *et al.*, 2017). If researchers are able to show that health- or lifespan can be improved in an animal that shares our environment, it is likely to reduce funder and investor doubts toward supporting anti-ageing trials in humans. Human trialling is in the pipeline for metformin and after a four year delay, the Targeting Ageing using MEtformin (TAME) trial is reported to begin at the end of this year (2019). The study aims to prospectively measure the rate and onset of multiple ageing-

related diseases in non-diabetic, healthy, older adults taking metformin daily (American Federation for Aging Research, 2019).

A growing number of molecules are being connected to ageing and diseases of age. Excitingly, many of these have drugs that have been used for decades, for example, the CAII inhibitor dorzolamide hydrochloride is regularly used to treat of open-angle glaucoma (Balfour and Wilde, 1997; Scozzafava and Supuran, 2014). As our understanding of ageing and its cellular manifestation improves, repurposing of approved drugs presents an appealing means of rapidly answering ageing research questions, as well as the prospect of improving human healthspan, without the need for protracted and costly drug development and testing.

1.7 *Drosophila melanogaster* as a tool for exercise experimentation

Professor William Ernest Castle, at Harvard University in 1901, was the first recorded scientist to use fruit flies (*D. melanogaster*) for research purposes. His research was targeted at understanding the genetics of coat-colour in mice and guinea pigs. He saw *D. melanogaster* as a simple and cheap model to aid his work (Carlson, 2013). This practical benefit of *D. melanogaster*, together with a short generation time and lifespan, has ensured they remain a highly attractive model to this day.

D. melanogaster have a complex nervous system which is comprised of ~100,000 neurones (Whitworth, Wes and Pallanck, 2006). They exhibit behaviours such as learning and memory, circadian rhythm, aggression and sleep (Chan and Bonini, 2000). Fundamental cell biological functions, such as gene expression regulation, cell death and membrane trafficking are conserved in *D. melanogaster* (Lu and Vogel, 2009). *D. melanogaster* have been important in research on the mutability of ionising radiation, circadian rhythm, neurogenesis and nervous system development (Stephenson and Metcalfe, 2013).

In the last three decades, *D. melanogaster* research has extended to investigating human diseases as close to 75% of human disease-related loci have a *D. melanogaster* orthologue (Cauchi and van den Heuvel, 2006). A long tradition of open access and sharing of fly research tools, reagents, informational resources and community engagement has produced one of the

most supportive research ecosystems in science. Due to this, engineering the *D. melanogaster* genome is a relatively simple task - existence of comprehensive RNAi collections, cDNA libraries, insertional mutagenesis collections and CRISPR/Cas9 allow for multiple ways to tailor genetic modification (Venken *et al.*, 2016). As an example, insight into the mechanisms and development of genetic forms of PD have come primarily from *D. melanogaster* (Whitworth, Wes and Pallanck, 2006).

1.7.1 Utility of fruit flies in Parkinson's Disease research

Genetic forms of PD account for 5-15% of incidence (Satake *et al.*, 2009). Genes encoding, α-syn, leucine-rich repeat kinase 2 (LRRK2), Parkin, phosphatase and tensin homologue deleted on chromosome 10-induced putative kinase 1 (PINK1), DJ-1, vacuolar protein sorting 35 (VPS35) and glucocerebrosidase (GBA), and others have been identified as genetic forms of PD closely resembling idiopathic PD (Guo, 2012; Li, Tan and Yu, 2014; Liao *et al.*, 2014; Kalinderi, Bostantjopoulou and Fidani, 2016; Chen *et al.*, 2019). Polymorphic variants of genes associated with familial PD also contribute to idiopathic PD (Kalinderi, Bostantjopoulou and Fidani, 2016). Familial and idiopathic forms share similar disease presentation, therefore an improved understanding of PD-associated genes should aid our understanding of the mechanisms underpinning idiopathic PD. Due to difficulties in obtaining human brain samples for analysis, the vast majority of which are post-mortem, there is a limit to the usefulness of human samples in understanding disease onset and

progression, therefore, PD model species, including *D. melanogaster*, are invaluable in determining the mechanisms underlying PD. Within its 100,000 neurones, *D. melanogaster* have ~200 dopaminergic neurones and display behaviours mimicking dopamine-dependent human behaviours (Julienne *et al.*, 2017). In comparison to rodent PD models, *D. melanogaster* recapitulate disease symptomology more closely, including motor (loss of coordination and slow movement speed) and non-motor (sleep disturbances and smell sense loss) symptoms (Guo, 2012).

1.7.1.1 PINK1 and D. melanogaster

PINK1 protein is a serine-threonine kinase with a mitochondrial targeting sequence (Clark *et al.*, 2006a). Loss of PINK1 causes a recessive form of PD and, together with Parkin, implicated mitochondrial dysfunction as a major cause of PD pathogenesis (Clark *et al.*, 2006a). Studies in *D. melanogaster* have allowed construction of the PINK1/Parkin pathway and enabled major advances in understanding the process of mitophagy (Park *et al.*, 2006b). *D. melanogaster* studies shown that the PINK1/Parkin pathway enhances fission and/or inhibits fusion, and that it is involved in mitochondrial motility, PINK1 binds the microtubule protein Miro, leading to its degradation and loss of mitochondrial motility (Wang *et al.*, 2011). These studies were the first evidence that alterations in mitochondrial dynamics, caused by PINK1 inhibition, are sufficient to result in whole organismal changes (motor symptoms, muscle degeneration, dopamine loss, circadian rhythm alterations) (Wang *et al.*, 2011).

1.7.2 *D. melanogaster* in exercise and ageing research

Exercise is a lifestyle intervention which eleviates multiple symptoms of ageing and disease. It is necessary to 1) understand the usefulness and biological effect of different exercise regimes and durations; 2) to develop this knowledge into personalised exercise regimes to target ageing and disease. Human exercise research is fraught with potential confounding factors – biological age, diet, genetics and lifestyle factors that cannot be readily controlled. To overcome this, exercise research has long employed rodents (Patten *et al.*, 2015; Strickland and Smith, 2016; Pitcher, 2018; Eschke *et al.*, 2019). Although rodents are exceptionally useful, with a recent study describing a high level of similarity in the molecular changes elicited in humans and rodents undergoing similar exercise (Goutianos *et al.*, 2015), they are expensive and do not provide a high-throughput method for timely answers.

1.7.2.1 *D. melanogaster* exercise machines

Over the past decade, *D. melanogaster* have emerged as a tool for exercise research. In order to exercise *D. melanogaster,* novel machines have been developed by a small number of research groups (Piazza *et al.*, 2009; M J Tinkerhess *et al.*, 2012; Mendez *et al.*, 2016; Watanabe and Riddle, 2017; Lowman *et al.*, 2018). The first paper detailing a *D. melanogaster* exercise "Power Tower" (PT) machine was published in 2009 (Piazza *et al.*, 2009). Since then, iterations on the original design have been developed, however, they all

share a similar mode of exercise activation - *D. melanogaster* have an innate negative geotaxis response that the machines are designed to prime (Gargano *et al.*, 2005).

The original PT used a motorised lever system to lift vials of *D. melanogaster* followed by a sudden drop (Piazza *et al.*, 2009). The drop would knock the *D. melanogaster* to the bottom of the vial thus priming their innate climbing response. Repeated bouts of dropping and climbing equates to exercise. A limitation of the PT is the impact of dropping upon *D. melanogaster*, which is relatively harsh and may cause physical trauma and/or death (Piazza *et al.*, 2009). Indeed, control *D. melanogaster* that were never placed in the PT showed increased climbing ability than those on the machine (Piazza *et al.*, 2009). More gentle variants, including partial and full rotation systems have since been developed. These act by altering the relative "up" orientation. Partial rotational systems (Swing Boat) have been used in Alzheimer's Disease (AD) model *D. melanogaster* and showed that exercise improved lifespan (Berlandi *et al.*, 2017). Full rotational systems [Treadwheel and Rotating Exercise Quantification System (REQS)] are similarly being used with success (Mendez *et al.*, 2016; Watanabe and Riddle, 2017). Like the PT, partial and full rotational systems are not without limitations - *D. melanogaster* rapidly habituate to the rotational motion and stop responding to the gravitational cue (Watanabe and Riddle, 2017). Until recently, another distinct limitation of the early work in *D. melanogaster* exercise research was a lack of quantitative methods to ensure animal activity. *D. melanogaster* are highly heterogeneous in their locomotor

activity and it has been shown that activity may be regulated by group behavioural dynamics (Ramdya, Schneider and Joel D Levine, 2017). Recently, locomotor detection by infrared beam has been incorporated to quantify movement in the Swing Boat and REQS (Berlandi *et al.*, 2017; Watanabe and Riddle, 2017). Infrared beam detection has the added benefit of removing the need for additional handling of *D. melanogaster* to manually test locomotion changes with exercise (for example by classical climbing assays).

Exercise in *D. melanogaster* has been shown to cause alterations to physical fitness, cardiac function, age-related mobility and disease progression (Piazza *et al.*, 2009; Martin J. Tinkerhess, Ginzberg, *et al.*, 2012; Berlandi *et al.*, 2017; Lowman *et al.*, 2018). Early studies using the PT device showed that exercised wild-type *D. melanogaster* have improved cardiac stress resistance, improved mobility with age and increased mitochondrial activity (Piazza *et al.*, 2009; Martin J. Tinkerhess, Ginzberg, *et al.*, 2012). REQS exercised wild-type *D. melanogaster* showed decreased body weight, triglyceride and glycogen storage while increasing protein content and mobility (Watanabe and Riddle, 2017).

Both REQS and PT exercise have revealed a sex specific response to exercise. Male *D. melanogaster* consistently show improvements in climbing ability with exercise training and females do not. Mendez *et al* showed improvements in climbing ability in four genotypes of female *D. melanogaster* (Mendez *et al.*, 2016), whereas other groups have shown no improvements (Piazza *et al.*, 2009; Tinkerhess *et al.*, 2012; Watanabe and Riddle, 2017). Sex and genotype are

clearly important factors to account for in analyses of data generated from exercised *D. melanogaster*.

Data from humans and vertebrate models have shown clear benefit of exercise in alleviating symptoms of neurodegenerative diseases (Lanza *et al.*, 2008; Fiuza-Luces *et al.*, 2013; Asano *et al.*, 2014; Barnes, 2015; Cass, 2017; Nystoriak and Bhatnagar, 2018). The Swing Boat device has recently been utilised to exercise an AD model *D. melanogaster*. Exercised male AD model *D. melanogaster* had alleviated disease symptoms, improved circadian rhythm and lifespan (Berlandi *et al.*, 2017).

With the large variety of methods and libraries available for probing and monitoring the *D. melanogaster* genome, and with the publication of successful exercise training modalities, the scope for future exercise research is huge. At present, several hurdles must be overcome to entice researchers to utilise *D. melanogaster* as an exercise model. Measurement of respiratory rate during exercise and recovery parallels workload variations across exercise sessions - this is readily measurable during mammalian exercise (Petrosino *et al.*, 2016; Nicolò, Massaroni and Passfield, 2017). Although *D. melanogaster* respiration is measurable, at present no method to measure respiratory rate during exercise has been developed. Further, exercise modalities investigate only endurance exercise. No methodologies exist to measure strength or balance training in *D. melanogaster.* Indeed, *D. melanogaster parkin* mutants are observed to have improved motor symptoms, including balance, with exercise (Bajracharya and Ballard, 2018). Video-recording to qualitatively observe changes is possible,

however, an ability to quantify this would enable systematic replication and improve data reliability.

1.8 Research aims and objectives

As discussed in the introduction to this thesis, ageing and age-related diseases are multifaceted biochemical process that result in cellular dysfunction and eventual disease or death. Although broad biochemical hallmark alterations are known, including mitochondrial dysfunction, the precise nature of these alterations are not. The studies in this thesis aimed to elucidate the changes to the mitochondrion during healthy ageing and in PD. Identifying mitochondrial changes due to ageing or healthy ageing interventions may present novel treatment strategies toward ageing and degenerative diseases.

The identification of mitochondrial biomarkers of ageing would allow for the determination of a biological stage of ageing, which would be useful to quantify an individual's biological age. In addition, their targeting may slow the ageing process. Determination of biomarkers of ageing and diseases of age featured heavily across all four studies in this thesis.

Study one investigated the potential of carbonic anhydrases as a mitochondrial biomarker of ageing. CA levels and activity were determined across the lifespan of mouse brain and muscle by Western blotting and spectrophotometry. Early and mid-life RNAi-knockdown of CA orthologues, or inhibition by dorzolamide hydrochloride, in *C. elegans* were used to determine whether controlling CA levels modulated lifespan.

Study two used comparative biology to compare the mitochondrial proteome differences between the long-lived bat and short-lived mouse. Bats show exceptional longevity and previous comparative mitochondrial proteome

analysis revealed FABP3 as highly expressed in muscle. FABP3 and free fatty acid levels were compared between mouse and bat by Western blotting and spectrophotometric analysis. Similar to study one, knockdown of FABP3 in *C. elegans* was carried out to better understand its role in the ageing process. Before this study, FABP3 had not been investigated for its role in the ageing process.

For the first time, mitochondrial changes due to exercise were investigated in *D. melanogaster*. *D. melanogaster* present a tractable organism for biomedical research – in recent years their use has extended to exercise research using novel "fly exercise machines". Study three subjected both wild-type and *Pink1*$^{-/-}$ *D. melanogaster* to exercise, followed by proteomic analysis using 2D-gel electrophoresis. Identifying mitochondrial protein changes due to exercise may present novel biomarkers for promoting healthy ageing.

A comparison of mitochondrial inflammatory mediators from cerebellar tissue of PD individuals was conducted in study four. Neuroinflammation is strongly implicated in PD onset and progression, however, the cerebellum has not been investigated in this context before. Panels of inflammatory proteins and lipids were analysed using an inflammatory antibody array and ultra-high-performance liquid chromatography coupled with high resolution mass spectrometry, respectively. These techniques give relative quantification of the proteins and lipids from the samples. Determining biochemical changes to the cerebellum during PD is likely to support a role for the cerebellum in PD pathology and highlight the necessity to include this region in future research.

Overall, the studies in this thesis have increased the body of literature supporting wide-spread changes to the mitochondria of ageing tissues. The biomarkers investigated and discovered from this work may have potential translational implications for improving age-related mitochondrial dysfunction.

2 Materials & Methods

2.1 Human samples

Human brain sections and frozen brain samples were obtained from, Human Tissue Authority approved, Nottingham Health Science Biobank (Nottingham University Hospitals NHS Trust) and Parkinson's UK Brain Bank (Imperial College London). The tissue banks granted us use of the tissue as end users. The Parkinson's tissue bank has approval from the Research Ethics Committee for Wales ref. 08/MRE09/31 + 5. The tissue collection and procedures at the Nottingham University Hospitals Biobank have been ethically approved by the Greater Manchester National Research Ethics Service. This study was granted specific ethics approval by both ethics' committees serving the Biobanks and also by the local ethics committee at the School of Veterinary Medicine and Science at the University of Nottingham (ref. 2035 170519). Tissues from human cerebellum were used for mitochondrial isolations and were frozen at the time of post-mortem (PM). Diagnoses of Parkinson's disease were confirmed at PM. Age at death varied from 69 to 85 years for Parkinson's disease brains, non-degenerative human control brains were age matched to within five years of Parkinson's disease samples (Table 2.1). PD Braak staging was performed according to published classification (Alafuzoff *et al.*, 2009).

ID	Diagnosis	Age (yr)	Sex	Disease duration (yr)	α-syn	Cause of death	Post mortem delay (hr)
75/02	Control	61	F	N/A	N/A	Not reported	N/A
CO48	Control	68	M	N/A	N/A	Metastatic colon cancer	10
CO22	Control	69	F	N/A	N/A	Lung cancer	33
PDC008	Control	71	F	N/A	N/A	Myocardial infarction	17
PDC030	Control	77	M	N/A	N/A	Conductive cardiac failure	17
CO45	Control	77	M	N/A	N/A	Multiple (old age)	22
CO26	Control	78	F	N/A	N/A	Myeloid leukaemia	33
61/07	Control	81	M	N/A	N/A	Not reported	N/A
PDC029	Control	82	M	N/A	N/A	Metastatic liver/lung cancer	48
PDC005	Control	58	M	N/A	N/A	Not reported	9
PDC022	Control	65	M	N/A	N/A	Lung carcinoma	12
PDC034	Control	90	M	N/A	N/A	Respiratory failure	12
PDC016	Control	93	F	N/A	N/A	Bronchial pneumonia/old	22
PDC026	Control	80	F	N/A	N/A	Breast carcinoma	23
PDC023	Control	78	F	N/A	N/A	Not reported	23
PDC008	Control	71	F	N/A	N/A	Myocardial infarction	17
PDC028	Control	84	F	N/A	N/A	Pancreatic cancer	11
PDC027	Control	79	M	N/A	N/A	Cardiac arrest &pneumonia	21
PDC092	Control	79	M	N/A	N/A	Brainstemstroke, bronchopneumonia	25
PDC014	Control	64	M	N/A	N/A	Cardiac failure	18
PDC078	Control	91	M	N/A	N/A	Not reported	18
PDC105	Control	95	M	N/A	N/A	Pneumonia, cellulitis of leg	11
PDC053	Control	89	F	N/A	N/A	Not reported	22
PDC040	Control	61	F	N/A	N/A	Ovarian cancer	15
002/08	Late PD	69	M	1	Not available	Not reported	N/A
PD131	Late PD	76	F	11	6	Not reported	22
044/07	Late PD	78	M	6	Not available	Not reported	N/A
PD014	Late PD	79	M	12	3	Parkinson's	21
PD063	Late PD	80	F	13	4	Old age and PD	10
PD028	Late PD	82	M	18	6	Not reported	14
PD050	Late PD	82	F	14	6	Chest infection &CVA	18
PD099	Late PD	82	M	11	6	Pneumonia	10
PD016	Late PD	85	F	18	6	Bronchopneumonia/PD	14
PD045	Late PD	80	M	19	6	Not reported	16
PD028	Late PD	82	M	18	6	Not reported	14
PD079	Late PD	78	F	19	6	Chest infection &CVA	22
PD016	Late PD	85	F	18	6	Bronchopneumonia/PD	14
PD180	Late PD	85	F	15	6	Chest infection &PD	15
PD093	Late PD	81	F	14	6	Not reported	22
PD203	Late PD	84	F	18	6	PD	19
PD413	Late PD	72	F	21	6	Pneumonia	14
PD081	Early PD	73	M	9	6	Not reported	19
PD036	Early PD	76	M	10	3	Not reported	10
PD041	Early PD	77	M	10	6	Not reported	6
PD007	Early PD	78	M	10	3	Pneumonia	22
PD067	Early PD	83	M	9	6	Not reported	10
PD051	Early PD	80	M	5	5	Not reported	7
PD067	Early PD	83	M	9	Not available	Not reported	10
PD081	Early PD	73	M	9	Not available	Not reported	19
PD084	Early PD	78	M	9	5	Ischaemic bowel and atrial fibrillation	3
PD074	Early PD	85	M	5	5	bronchopneumonia	7
PD153	Early PD	76	F	7	6	PD	12
PD125	Young PD	74	M	25	6	Bronchopneumonia/PD	20
PD117	Young PD	77	F	31	5	Not reported	6
PD021	Young PD	76	M	27	6	Not reported	17
PD020	Young PD	75	M	34	6	Not reported	2
PD117	Young PD	77	F	31	5	Not reported	6
PD017	Young PD	75	M	18	6	Not reported	22

Table 2.1 Clinical diagnosis and description of the human samples used for all experimentation in the proceeding chapters.

2.2 Non-human samples

2.2.1 Mice

Male C57BL/6J mice were obtained from Charles River and were aged 3, 5, 6, 9, 12 and 18 months old. Brain and skeletal muscle were dissected and stored at -80°C until use. Purkinje cell degeneration mice, pcd^{5J}, aged 9-17 weeks old were bred and kept under licence at the University of Nottingham.

2.2.1.1 Ethical Approval

Tissues were obtained after animals were humanely killed, by a trained individual, using a Home Office approved Schedule 1 method of killing. Animals were bred and housed in accordance with strict Home Office stipulated conditions. The overall programme of work (in respect to the original UK Home Office Project Licence application) is reviewed by the Animal Welfare and Ethical Review Body at the University of Nottingham and then scrutinised by the UK Home Office Inspectorate before approval by the Secretary of State. Individual study protocols link to the overarching Home Office Project Licence and are made available to the Named Animal Care and Welfare Officer, the Named Veterinary Surgeon (both are members of the AWERB), the animal care staff and the research group. The Project Licence Number for the breeding and maintenance of this genetically altered line of mice is PPL 40/3576. The mice are typically group housed and maintained within solid floor cages containing bedding and nesting material with additional environmental enrichment including chew blocks and hiding tubes. Cages are Individually Ventilated Cage Units within a barrier SPF unit

to maintain biosecurity. Animals are checked daily by a competent and trained animal technician. Any animal giving cause for concern such as subdued behaviour, staring coat, loss of weight or loss of condition will be humanely killed using a Home Office approved Schedule 1 method of killing.

2.2.2 Common Pipistrelle bats

Bats were obtained from the West Yorkshire Bat Hospital with permission from *Natural England*. Only those animals that died from natural causes were collected. Adult bats were selected for older age by an experienced bat handler and based on observations that older bats have a rougher coat and general signs of ageing, including tattered wings and ears. All bats used were common Pipistrelle bats (*Pipistrellus pipistrellus*). The bats were transported to the laboratory on dry ice and were then stored at -80 °C until use. The bats were thawed on ice in a category II fume hood and the brain and skeletal muscle tissues were dissected. Mitochondrial preparations were carried out immediately after the tissue was dissected to reduce the number of freeze/thaw cycles.

2.2.3 Drosophila melanogaster (D. melanogaster)

Collaboration with the laboratory of Dr. Nicoleta Moisoi (De Montfort University, Leicester) made available age-synchronised wild-type, W^{1118}, and $Pink1^{B9}$ (*Pink1*$^{-/-}$) *D. melanogaster* strains from their stocks (Table 2.2).

Strain	Genotype/ID	Mutation	Description
W^{1118}	Dmel\w^{1118} (FBal0018186)	Partial deletion of w.	Loss of function of white (w). *w* encodes a member of the ABCG2 class of transporters
PINK1^{B9}	Dmel\Pink1^{B9} (FBal0193144)	Imprecise excision of the P element of PINK1^{Ge271}	Loss of function of PINK1

Table 2.2 Identification and description of *D. melanogaster* strains used in the proceeding chapters.

2.2.4 Caenorhabditis elegans (C. elegans)

C. elegans strains (Table 2.3) were originally obtained from the laboratories of Prof. Nathaniel Szewczyk [University of Nottingham (CB5600 and CC46)] and Dr. Timothy Etheridge [University of Bristol (RB1643 and RB2268)]. *C. elegans* stocks were subsequently maintained at 20°C in a temperature-controlled incubator.

Strain	Symbol/ID	Mutation	Description
CB5600	ccIs4251 I; him-8(e1489) IV	N/A	Superficially wild-type. Expresses GFP in nuclei and mitochondria of body wall
CC46	*ccIs4251* I; *pink-1* (*tm1779*) II; *him-8* (*e1489*) IV	*pink-1* -/-	*pink-1* knockout. Expresses GFP in nuclei and mitochondria of body wall and muscles
RB1643	cah-1(ok2032) III	*cah-1* -/-	*cah-1* knockout
RB2268	cah-5(ok3070) X	*cah-5* -/-	*cah-5* knockout

Table 2.3 Identification and description of *C. elegans* strains used in the proceeding chapters.

2.3 Tissue and fraction preparations

2.3.1 Vertebrates

Brain and skeletal muscle were dissected from the animals. Human cerebellar sections were supplied from Parkinson's UK and Nottingham BioBanks. Mitochondrial extraction buffer (50mM Tris-Cl pH 7.4, 100mM KCL, 1.5mM $MgCl_2$, 1mM EGTA, 50mM HEPES and 100mM sucrose) was added to the samples (1.5ml per $1cm^3$) and the samples were homogenised in a C-tube using a GentleMACS Dissociator (Miltenyl Biotec). The C-tube was spun at 5000g for 30 seconds and the homogenised sample transferred to two Eppendorfs. The homogenate was spun at 850g for 10 minutes at 4°C. The supernatant was kept and spun at 1,000g for 10 minutes at 4°C. The supernatant was saved and spun at 10,000g for 10 minutes at 4°C. The supernatant contained the cytoplasmic fraction which was saved and stored at -80°C. The pellet contained the mitochondrial fraction, and this was stored at -80°C. The whole procedure was carried out on ice.

2.3.2 D. melanogaster

2.3.2.1 Whole cell homogenisation

Whole *D. melanogaster* underwent 5 minutes of manual homogenisation using a 1.2-2ml Eppendorf micro-pestle (Sigma-Aldrich) in mitochondrial extraction buffer (50mM Tris-Cl pH 7.4, 100mM KCL, 1.5mM MgCl2, 1mM EGTA, 50mM HEPES and 100mM Sucrose). 100µl of buffer was added for every ten *D.*

melanogaster. Homogenates were centrifuged at 800g for 15 minutes at 4°C. Supernatants were transferred to fresh Eppendorfs and stored at -80°C. The whole procedure was carried out on ice. Individual whole *D. melanogaster* were homogenised directly in 140µl 1X rehydration buffer (see 2.5.1), on ice. Homogenates were spun at 800g for 15 minutes at 4°C. Supernatants were pipetted directly into cassette lanes.

2.3.2.2 Mitochondrial preparation

Ten *D. melanogaster* were homogenised in 250µl mitochondrial isolation buffer and subjected to 5 minutes of manual homogenisation using a 1.2-2ml Eppendorf micropestle (Sigma-Aldrich). Homogenates were centrifuged at 800g to remove the pelleted insoluble protein fraction. Supernatants were centrifuged at 1,000g. Supernatants from this spin were further centrifuged at 13,200g. The pellet contained the mitochondrial fraction and was stored at -80°C. The supernatant contained the cytoplasmic fraction which was saved and stored at -80°C. The whole procedure was carried out on ice.

2.4 Determination of protein concentration

A Bradford assay was carried out to determine the protein concentrations of the samples. The standard 3.1ml assay protocol as described in the technical bulletin provided by Sigma-Aldrich was followed to perform the Bradford assay (Sigma-Aldrich, 2013). The bovine serum albumin protein standards (0 to 2mg/ml) were diluted using Tris-Buffer (pH 7.6) before addition to the Bradford reagent. The mitochondrial samples (1µl) were diluted in 100µl 0.1M Tris-Buffer (pH 7.6) before addition to the Bradford reagent. The cytosolic samples (5-10µl) were diluted in 100µl 0.1M Tris-Buffer (pH 7.6). The *D. melanogaster* and *C. elegans* samples (20µl) were diluted in 100µl 0.1M Tris-Buffer (pH 7.6). The protein concentrations µg/µl were determined using a linear regression of the protein standards.

2.5 2D-gel electrophoresis

2.5.1 Isoelectric focussing (IEF)

2D gel electrophoresis of single or mitochondrial preparations of pools of 20 *D. melanogaster* were prepared. An individual fly was homogenised directly in 140µl rehydration solution. Mitochondrial 2D gel electrophoresis contained 135µl rehydration solution and 5µl mitochondrial sample. Rehydration solution contained 8M urea, 2% CHAPS, 2% IPG Buffer, 0.1% bromophenol blue. 20mM DTT was added to an aliquot of rehydration solution directly before use. The standard protocol was followed according to manufacturer instructions (Life Technologies, 2012b). Briefly, 2D non-linear Zoom IPG Strips pH 3-10 (Life Technologies) were rehydrated for an hour at room temperature. The strips were then focussed for one hour and 30 minutes with an increasing voltage of 175-2000V.

Strips were incubated in 2ml equilibration solution (9ml 1X NuPAGE LDS sample buffer and 1ml of 100mM DTT) for 15 minutes at room temperature, followed by 2ml alkylation solution (232mg iodoacetamide and 10ml 1X NuPAGE LDS sample buffer) for 15 minutes at room temperature.

2.5.2 Size separation

NuPAGE 4-12% Bis-Tris gels and NuPAGE MES SDS running buffer (1X) were used in the Mini-Cell Chamber (Fisher Scientific). Strips were placed in the gel well with the negative end closest to the marker lane. 5µl of NEB Broad

Range pre-stained protein marker (New England BioLabs Inc) was added to the marker lane. Gels were subsequently washed according to manufacturer instructions (Life Technologies, 2012a). Briefly, gels were dismantled from their cassettes and washed three times in 100ml ddH_2O, followed by staining in 25ml SimplyBlue™ SafeStain (Life Technologies) at room temperature for one hour. The stain was removed from the gels and the gels washed twice in 100ml ddH_2O for an hour. Gels were imaged using an ImageQuant 300 (GE Healthcare Life Sciences).

2.5.3 SameSpots analysis and protein identification

Gel images were uploaded to SameSpots software (Totallab) and comparisons between protein spots of each group were carried out (Table 2.4). Protein spots identified as significantly altered between conditions were excised from the gel using a sterile scalpel.

Group Comparison		**Female (WT)**		**Male (WT)**		***Pink1*** $^{-/-}$	
		E	**N.e**	**E**	**N.e**	**E**	**N.e**
Female (WT)	**E**		x				
	N.e				x		
Male Group (WT)	**E**				x		
	N.e		x	x		x	x
Pink1 $^{-/-}$	**E**				x		x
	N.e				x	x	

Table 2.4 Group comparisons made using SameSpots (Totallab). E = exercised; N.e = non-exercised.

Samples were analysed by the Centre of Excellence in Mass Spectrometry at University of York (Centre for Excellence in Mass Spectrometry, 2019). Briefly, proteins were reduced and alkylated, followed by digestion in-gel with trypsin. Matrix Assisted Laser Desorption Ionization Tandem Time-of-Flight mass spectrometry (MALDI-TOF/MS) was used to analyse the samples. The generated tandem MS data was compared against the NCBI database using the MASCOT search programme to identify the proteins. De novo sequence interpretation for individual peptides were inferred from peptide matches.

2.6 Western Blotting

2.6.1 Gel electrophoresis and transfer

30µg/µl of mitochondrial sample was prepared using 3µl 4X LDS sample buffer, 5µl PBS, 3µl 4mM DTT. The sample was mixed gentle pipetting and boiled for 10 minutes at 90°C. The sample was centrifuged at 7,000 rpm for 30 seconds. The samples were run on a 4-12% Bis-Tris gel (Invitrogen) in 1X MES SDS running buffer for 35 minutes at 200V. Each lane had 10µl of sample added to it. The marker lane contained 5µl of pre-stained protein broad range marker (New England BioLabs Inc). After running the gels were transferred to a nitrocellulose membrane (0.45µm) (Bio-Rad). The transfer was carried out in cooled 1X NuPAGE transfer buffer. The transfer was run at 30V for 60 minutes.

2.6.2 Immunoblotting

Membranes were blocked for 60 minutes at room temperature in 5% Bovine Serum Albumin (BSA) or 5% Milk Powder. The membranes were incubated in the specific concentration of primary antibody overnight at 4°C.

2.6.2.1 Human samples

PTGS2 ab62331 (Abcam) 1:2000 dilution in 5% (w/v) BSA in 1xTBS-T; β-actin ab8227 (Abcam) in 5% (w/v) BSA in 1xTBS-T.

2.6.2.2 Mouse and bat samples

CAII ab6621 (Abcam) 1:5000 dilution in 3% (w/v) BSA in 1xTBS-T; β-actin ab8227 (Abcam) 1:5000 dilution in 3% (w/v) BSA in 1xTBS-T; CAIII AP7633a (ABGENT) 1:2500 dilution in 3% (w/v) BSA in 1xTBS-T; GAPDH ab9458 (Abcam) 1:5000 dilution in 3% (w/v) BSA in 1xTBS-T; FABP3 PA5-13461 (ThermoFisher) 1:1000 in 3% (w/v) BSA in 1xTBS-T; β-tubulin ab6046 (Abcam) 1:2000 in 3% (w/v) BSA in 1xTBS-T; HSP-60 ab45134 (Abcam) 1:2000 in 5% (w/v) BSA in 1xTBS-T.

2.6.2.3 D. melanogaster samples

FABP3 PA5-13461 (ThermoFisher) 1:1000 in 5% (w/v) milk powder in 1xTBS-T; Troponin-C ab51106 (Abcam) 1:1000 in 5% (w/v) milk powder in 1xTBS-T; Tropomyosin-1α ab50567 (Abcam) 1:1000 in 5% (w/v) milk powder in 1xTBS-T; α-actinin ab50599 (Abcam) 1:1000 in 5% (w/v) milk powder in 1xTBS-T; β-tubulin ab6046 (Abcam) 1:2000 in 5% (w/v) milk powder in 1xTBS-T; HSP-60 ab45134 (Abcam) 1:2000 in 5% (w/v) milk powder in 1xTBS-T; ATP5β sc-16690 (Santa Cruz) 1:1500 in 5% (w/v) milk powder in 1xTBS-T; VDAC ab15895 (Abcam) 1:1000 in 5% (w/v) milk powder in 1xTBS-T.

2.6.2.4 Secondary antibodies

Goat anti-Rabbit IgG (HRP) A16096 (Invitrogen) 1:1000 – 1:5000 in primary blocking solution; Goat anti-Rat IgG (HRP) ab205720 (Abcam) 1:1000 – 1:5000 in primary blocking solution.

2.6.3 Imaging and densitometry

Membranes were incubated in Pierce ECL Plus (ThermoFisher) for 5 minutes and imaged using ChemiDoc (Bio-Rad). The software ImageJ was used to measure intensity for the band of interest and the loading control, normalised to the background. Protein of interest measurements were compared to the loading control (protein of interest/loading control). The results were plotted on graphs along with the SEM of the values.

2.7 Carbonic anhydrase enzymatic assay

The esterase activity of carbonic anhydrase was measured by monitoring the hydrolysis of p-nitrophenyl acetate to 4-nitrophenol at A348nm using a Varioskan spectrophotometer and standard methods (Verpoorte et al, 1967). The test well of a 96-well plate contained 50µl 15mM Tris Sulfate Buffer, pH 7.6 at 0°C (Sigma-Aldrich) and 25µl of 3mM p-nitrophenyl-acetate (Sigma-Aldrich). 30µg/µl of mitochondrial sample was added to the buffer and substrate solution. The assay was measured every 30 seconds for 30 minutes. Three mitochondrial preparations were used for each condition [3-months-old (mo), 6mo, 9mo, 12mo, 18mo mice] and the average absorbance calculated for each mitochondrial preparation. The rate of change in absorbance of the assay was plotted (n-3). two-tailed, unpaired *t*-tests were carried out to compare the rate of change between young and old mitochondria at each time point. Statistical analyses were carried out using GraphPad Prism.

2.8 Inflammation antibody array

Abcam human inflammation antibody array (ab197451) kit was used for the detection of forty cytokines: BLC, eoxtaxin-1, eotaxin-2, G-CSF, GM-CSF, I-309, ICAM-1, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-6sR, IL-7, IL-8, IL-10, IL-11, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17. MCP-1, MCSF, MIG, MIP-1α, MIP-1β, MIP-1δ, PDGF-BB, RANTES, TIMP-1, TIMP-2, TNFα, TNFβ, sTNF RI, sTNF RII. Glass slides were supplied with 16 identical antibody arrays, with a 16-well gasket for addition of separate samples to each array. Each array spotted cytokines in quadruplicate. Human cerebellar mitochondria samples were prepared to 500μg/ml in 1X TBS-T. Cytokine standards (manufacturer supplied) were prepared immediately before use. Standards were supplied lyophilised. Serial dilution of standards in 1X Sample Diluent (manufacturer supplied) was carried out. The assay was carried out according to manufacturer instructions (Abcam, 2018). Briefly, glass slides were blocked followed by addition of cytokine standards or samples to each well for 1 hour at room temperature. Slides were washed in 1X Wash Buffer 1 (manufacturer supplied) followed by 1X Wash Buffer 2 (manufacturer supplied). 1X Detection Antibody (manufacturer supplied) was added to each well and incubated overnight at 4°C. Following overnight incubation, slides were washed in 1X Wash Buffer (manufacturer supplied). Cy3 dye-conjugated streptavidin (manufacturer supplied) was added. Slides were covered in foil to protect from light and incubated for 1 hour at room

temperature with gentle rocking. Slides were centrifuged at 1500rpm until completely dry and stored at room temperature.

Slides were scanned at 532nm and data analysed using GenePix Pro Software (Axon GenePix). The median local background was subtracted from the median fluorescence of each spot and the corrected fluorescence was used to calculate the average fluorescence signal and standard deviation. Two positive control spots were present in each array – POS1 and POS2. POS1 signal is several times that of POS2. A reference array was selected and a ratio of POS1 to POS2 was calculated. The ratio was used to calculate an adjusted average of POS1 and POS2 in each array. Array data were normalised to the reference array using the adjusted average and the POS1 value for the reference array. Linear regression of standards was used to generate a standard curve for extrapolation of sample data. Results were plotted on graphs along with the SEM of the values.

2.9 Oxylipin quantification

LC-ESI-MS/MS was used for analysis of twenty-nine oxylipins in mitochondrial extracts of human cerebellar tissue: AA, LA, 12-HETE, TXB-2, PGE2, 5-HETE, 5-HPETE, 12-HPETE, 9-HETE, 20-HETE, LTB-4, 9-oxoODE, 9-HODE, 5,6-EET, 5,6-DHET, 8,9-DHET, 11,12-DHET, 14,15-DHET, 8-HETE, 11-HETE, 15-HETE, 16-HETE, 8,9-EET, 11,12-EET, 14,15-EET, 17-HDoHE, 13-oxoODE, 13-HODE, 8,15-DiHETE. Oxylipin analyses were carried out by the Centre for Analytical Bioscience, School of Pharmacy, University of Nottingham. Briefly, HPLC (Shimadzu SCL 10Avp) was run for 20 minutes at 4°C. Injection volume was 20µl. The MS system used was a triple quadrupole ion-trap 4000 QTRAP (ABI, UK), equipped with Turbo Spray ionisation interface. Standards were purchased from Cambridge Biosciences, Cambridge, UK. One batch of QC human plasma standard samples was used to confirm the intra-day accuracy of the method.

2.10 *N*-acylethanolamine quantification

LC-ESI-MS/MS was used for quantification of four *N*-acylethanolamines: arachidonyl ethanolamide (anandamide, AEA), 2-arachidonyl glycerol (2-AG), palmitoyl ethanolamide (PEA), oleoyl ethanolamide (OEA). *N*-acylethanolamine quantification were carried out by the Centre for Analytical Bioscience, School of Pharmacy, University of Nottingham. Briefly, the uHPLC system used was modular Exion series LC (Sciex, UK). The injection volume was 5µl. The MS system used was a Qtrap 6500+ equipped with an electrospray ionisation (ESI0 interface (Sciex, UK). Standards were purchased from Cambridge Biosciences, Cambridge, UK. One of QC human plasma standard samples were prepared to confirm intra-day accuracy.

2.11 Free fatty acid quantification

Total free fatty acid concentrations were measured using the Free Fatty Acid Quantification Kit (ab65341, Abcam), according to manufacturer instructions (Abcam, 2019). Fatty acids are converted to their coenzyme A derivatives, which are oxidised generating measurable colour (A570nm). All materials and solutions were equilibrated to room temperature before use. 0-1 nmol/well palmitic acid standards were generated by dilution in Assay Buffer (manufacturer supplied).

2.11.1 Cerebellar samples

30µg/µl cerebellar mitochondrial samples were suspended in 200µl chloroform (1% Triton X-100) and homogenised by 30 strokes in a glass Dounce homogeniser. The samples were placed on ice for 30 minutes, followed by centrifugation at 16,000g for 10 minutes. The lower (organic) phase of the samples was collected and vacuum dried to remove trace chloroform. The dried lipids were subsequently dissolved in 200µl fatty acid buffer (manufacturer supplied). 50µl of samples or standards were added to a 96-well plate. All samples and standards were measured in duplicate. 2µl acyl-CoA synthetase was added to each well. Plates were incubated at 37°C for 30 minutes. A light-sensitive Reaction Mix (manufacturer supplied) was added to each well. The plate was wrapped in foil before a second 30 minute 37°C incubation was carried out. Colourimetry was measured on a Varioskan LUX multimode microplate reader at A570nm.

2.11.2 Mouse and bat samples

30µg/µl mouse (20 week old mouse brain, 73 week old mouse brain, 20 week old mouse muscle, 73 week old mouse muscle) and bat (adult bat brain, adult bat muscle) were suspended in 200µl chloroform (1% Triton X-100). Samples underwent manual homogenisation with a 1.2-2ml Eppendorf micro-pestle (Sigma-Aldrich) for 5 minutes. Samples were prepared as described (see 2.11.1). Each group were measured in duplicate.

2.11.3 C. elegans

Four plates per condition (control, *lbp-4*, *-5*, *-6* knockdown) of age-synchronised *C. elegans* were cleaned of any contamination by sucrose flotation. *C. elegans* were collected in M9 buffer and centrifuged at 200g for 2 minutes at 4°C. The supernatant was discarded and fresh M9 buffer used to resuspend the pellet. *C. elegans* were centrifuged at 3,000g for 4 minutes at 4°C. The supernatant was discarded, and the pellet resuspended in 60% sucrose solution, before centrifuging at 3,000g for 3 minutes at 4°C. The top layer, containing the live *C. elegans*, was moved to a new tube, M9 buffer added and cooled for 3 minutes on ice. A final centrifugation at 3,000g for 3 minutes at 4°C and removal of supernatant was carried out.

Cleaned, L4 stage *C. elegans* were suspended in chloroform/Triton X-100 (1% Triton X-100) and homogenised by 5 minutes of manual homogenisation with

a 1.2-2ml Eppendorf micropestle (Sigma-Aldrich). Samples were subsequently prepared as described (see 2.11.1).

2.11.4 Data analyses

Duplicated measurements were averaged, and linear regression carried out to generate a standard curve. Free fatty acid concentrations (nmol/μl) were plotted. Statistical analyses (two-tailed, unpaired *t*-tests) were carried out using GraphPad Prism.

2.12 *C. elegans* lifespan assay

2.12.1 NGM agar plate preparation with *E. coli*

C. elegans were maintained on solid NGM agar plates seeded with *Escherichia coli* (*E. coli*) strain OP50. NGM agar plates were prepared using standard methods (Stiernagle, 2006). Briefly, the NGM agar was prepared with 3g NaCl, 17g Bacto-agar (Oxoid LP0011) and 2.5g Bacto-peptone (Oxoid LP0037) along with 975ml $_{dd}H_2O$. The NGM agar was autoclaved, followed by addition of 1ml cholesterol whilst still very hot. Once the NGM solution cooled to approximately 60°C, 1ml of calcium chloride solution, 1ml of magnesium sulphate solution, 25ml of potassium phosphate buffer and 50µg/ml ampicillin was added to the NGM agar solution. Approximately 25ml of NGM agar solution was poured into medium sized petri dishes (90mm). The petri dishes were left to dry overnight before being wrapped in Parafilm and stored at room temperature until use. The procedure described above was carried out in a lamina flow hood to maintain sterile conditions.

The nutrient agar solution contained 28g of nutrient agar made to 1 litre of $_{dd}H_20$ and autoclaved. The nutrient agar solution was poured onto medium sized petri dish (90mm) in a lamina flow hood. *E. coli* strain OP50 or dsRNA expressing *E. coli* (see 2.11.1.1) was streaked on to a nutrient agar plate and incubated at 37°C overnight. 5g of TB growth media [high nutrient growth media for growing *E. coli* (MoBio catalog #12105-05)] was made to 100ml $_{dd}H_20$ and autoclaved. One colony of *E. coli* was removed from the nutrient agar plate using a pipette tip. The pipette tip was then added to 25ml of the TB

growth media solution and put in an incubator overnight at 37°C with gentle agitation. The *E. coli* solution was removed from the incubator after 12-16 hours and kept at room temperature. The NGM agar plates were seeded with *E. coli*: 500µl of *E. coli* solution was added to the petri dish and left to dry.

2.12.1.1 RNAi knockdown

Gene knockdown of *C. elegans* was achieved by delivering dsRNA by feeding. The dsRNA is transcribed in *E. coli*, which *C. elegans* subsequently ingest. Briefly, a specific cDNA corresponding to a gene of interest is cloned into a bacterial expression vector between phage T7 polymerase promoter sites. This is transformed into *E. coli* strain HT115, which carries the DE3 lysogen and thereby provides IPTG-inducible expression of phage T7 RNA polymerase. RNAi food was prepared using the methods outlined in 2.11.1, with the addition of IPTG to the NGM plates. Briefly, 1mM IPTG was added to 60°C NGM solution before pouring into petri dishes. Refer to table 2.5 for RNAi food used for the subsequent chapters. RNAi were originally received from the laboratory of Prof. Nathaniel Szewczyk.

RNAi target	ORF ID	Vector	Host
lbp-4	ZK742.5	pL4440-DEST	HT115(DE3) (Fire lab)
lbp-5	W02D3.7	pL4440-DEST	HT115(DE3) (Fire lab)
lbp-6	W02D3.5	pL4440-DEST	HT115(DE3) (Fire lab)
cah-2	D1022.8	pL4440-DEST	HT115(DE3) (Fire lab)
cah-3	K05G3.3	pL4440-DEST	HT115(DE3) (Fire lab)
cah-4	R01E6.3	pL4440-DEST	HT115(DE3) (Fire lab)

Table 2.5 RNAi used in these works. RNAi were obtained from the laboratory of Prof. Nathaniel Szewczyk.

2.12.2 Maintaining stock populations

C. elegans strains (refer to table 2.3) were thawed from -80 °C and poured on to seeded NGM agar plates. *C. elegans* were kept in an incubator (ThermoScientific MaxQ 4000) at 20°C for 12-18 hours before transferring the living C. *elegans* to new plates. *C. elegans* were kept in the incubator at 20 °C for a week before a chunk was cut out from the plate using a sterile needle and transferred to a newly seeded plate. Each week *C. elegans* would be transferred to a new plate to maintain the colony. The old plates containing the *C. elegans* were kept in the incubator.

2.12.3 Age synchronisation

A well populated plate containing L1 larvae was needed to start the age-synchronisation. 3ml of M9 buffer (3g KH_2PO_4, 6g Na_3HPO_4, 1ml 1M $MgSO_4$ made to 1 litre using $_{dd}H_2O$) was pipetted on to the surface of the plate to wash off the *C. elegans*. The M9 buffer on the plate containing the suspended *C. elegans* was pipetted back up and transferred to a 15ml tube. The *C. elegans* were left to settle in the tube for 2.5 minutes. The large *C. elegans* settle quickest to the bottom of the tube leaving the young L1 larvae at the top of the buffer. The top 500µl of M9 buffer (containing the L1 larvae) was pipetted and added to either one or two seeded plates with OP50 depending on how well populated the stock plate was for the age synchronisation. The plates

containing the L1 larvae were then incubated at 20°C for 48 hours until adults (L4 stage).

2.12.4 Survival assay

C. elegans were maintained on solid NGM agar seeded with either *E. coli* strain OP50 or RNAi food, using standard methods (Stiernagle, 2006). *C. elegans* were aged-synchronised (see 2.11.3). *C. elegans* were transferred daily to new plates using standard techniques and were scored every day. *C. elegans* that were not moving and did not respond to stimulation with a needle were recorded as dead. *C. elegans* were raised at 20°C throughout the experiment. Each plate contained 500µl *E. coli* (OP50 or RNAi food) and 10 newly adult (L4 stage) *C. elegans*. Statistical analysis was performed using GraphPad Prism.

For survival assays involving treatment of *C. elegans* (see table 2.6), before the *C. elegans* were transferred on to the NGM agar plates, 20µl of treatment was spotted on to the surface. For these experiments, control plates had 20µl of $_{dd}H_20$ spotted on to the surface. Each plate contained 500µl *E. coli* strain OP50, 20µl treatment (if applicable) and ten newly adult (L4 stage) *C. elegans*. Statistical analysis was performed using GraphPad Prism.

Treatment	Solvent	Source
Recombinant carbonic anhydrase II - 150 units	DMSO	Sigma-Aldrich (C2522)
Dorzolamide Hydrochloride - 25mM	$_{dd}H_2O$	Sigma-Aldrich (BP1069)
Acetazolamide - 25mM	DMSO	Sigma-Aldrich (A6011)

Table 2.6 *C. elegans* treatments used in these works. 20µl of treatments were spotted onto plates containing CB5600 strain *C. elegans* only.

2.13 Sequence similarity and phylogeny

Multiple sequence alignment containing the sequences of human and *C. elegans* FABP3 orthologues LBP-1 to -9 was created from UCSC genome browser database, accession numbers for which are given in Table 2.7. Similarly, multiple sequence alignment was created for human and *C. elegans* carbonic anhydrase II and III orthologues CAH-1 to -6.

Species	Protein	UCSC Accession Number
H. sapiens	CA-II	ENST00000285379.10
	CA-III	ENST00000285381.3
	FABP3	ENST00000373713.7
C. elegans	CAH-1	NP_001309597
	CAH-2	NP_495567
	CAH-3	NP_510674
	CAH-4	NP_510264
	CAH-5	NP_509186
	CAH-6	NP_491189
	LBP-1	NP_508557.1
	LBP-2	NP_508558
	LBP-3	NP_001041249.1
	LBP-4	NP_505016.1
	LBP-5	NP_491928.1
	LBP-6	NP_491926.1
	LBP-7	NP_506440.1
	LBP-8	NP_506444.2
	LBP-9	NP_001033511.1

Table 2.7 Predicted amino acid sequences of *H. sapiens* CA-II, -III and FABP3, and *C. elegans* orthologues. Predicted amino acid sequences were generated using the UCSC genome database.

Amino acid residues are coloured shades of blue signifying their sequence similarity; lightest is 30%, darkest is 100%. Sequence similarity data were

generated using multiple sequence comparison by log-expectation (MUSCLE) and are shown in the relevant Chapters (Tables 3.1 and 4.1) (European Molecular Biology Laboratory, 2018). Sequence alignments were carried out using Jalview and phylogeny produced using PAM 250 (Waterhouse *et al.*, 2009).

2.14 Fluorescence imaging GFP-tagged muscle mitochondria and nuclei

CB5600 (*ccls4251 (Pmyo-3::Ngfp-lacZ; Pmyo-1::Mtgfp)* I; *him-8 (e1489)* IV) with GFP fusion proteins localised to mitochondria and nuclei (Gaffney *et al.*, 2014). Age synchronised L1 *C. elegans* were grown for 48-60 hours at 20°C until they reached L4 stage. Twenty *C. elegans* were picked into 20µl M9 buffer on a microscope slide. A coverslip was applied, and *C. elegans* were imaged using the Leica CTR5000 under a GFP filter (460-500nm, blue light excitation) at 100X magnification.

2.15 Oxygen consumption

2.15.1 Microplate reader set up

Oxygen consumption measurements were carried out using a glass 24-well plate (Loligo Systems) containing sensor spots in contact with a sensor dish reader (SDR) (Precision Sensing). A weighted, parafilm lined, silicone pad was placed on top of the microplate to seal the wells (Figure 2.1). The SDR USB-PC adaptor facilitates oxygen consumption data output to the SDR software (Loligo Systems). Software parameters were setup: ambient temperature, measurement time interval, and measurement parameter (oxygen saturation %). A one-point calibration was used to artificially adjust oxygen saturation of all wells to 100% prior to beginning.

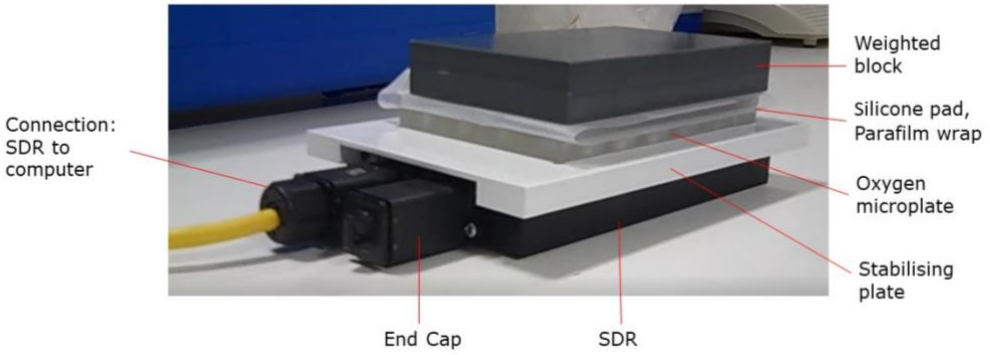


Figure 2.1 Oxygen microplate set up.

2.15.2 D. melanogaster

Oxygen consumption of *D. melanogaster* were measured. Wells were equilibrated to 100µl mitochondrial isolation buffer for 30 minutes before addition of *D. melanogaster.* A single, freshly homogenised, fly was added to each well. At least three biological replicates were included for each condition.

Oxygen consumption was measured at 15 second intervals continuously for two hours. Negative control wells of mitochondrial isolation buffer only were used to correct for background oxygen loss.

2.15.3 C. elegans

Oxygen consumption of knocked-down *C. elegans* were measured across their lifespan. Wells were equilibrated to 100µl M9 buffer for 30 minutes before adding *C. elegans*. Oxygen consumption trials were carried out using five *C. elegans* per well with four replicate wells for each knockdown and control. The first oxygen consumption measurements were conducted on newly adult *C. elegans* (L4 stage), 48-72 hours after age-synchronisation (termed adult age day zero). Oxygen consumption was measured at 15 second intervals continuously for two hours. Additional trials were conducted at adult age days four, eight and twelve. Negative control wells of M9 buffer only were used to correct for background oxygen loss.

2.15.4 Data analyses

Oxygen consumption was measured for 60-120 minutes. Oxygen consumption over the lifespan was calculated using data generated from the first 15 minutes of each trial. All data were corrected for volume of buffer and total protein per well, expressed as µl/min/mg protein.

2.16 ATP determination assay

ATP concentration was measured using luciferin-luciferase (ATP Determination Kit; Molecular Probes). Briefly, freshly homogenised *D. melanogaster* were collected and frozen at -80°C for 5 minutes, followed by boiling at 90°C for 15 minutes, and placed on ice for 5 minutes. Samples were centrifuged at 13,400g for 10 minutes at 4°C. Supernatants were transferred to fresh tubes and immediately used for ATP determination. To determine ATP concentration, an aliquot of 10µl *D. melanogaster* supernatant was added to 100µl of reaction solution (0.5mM D-luciferin, 1.25µg/mL firefly luciferase, 25mM tricine buffer, pH 7.8, 5mM $MgSO_4$, 100µM EDTA and 1mM DTT). Light emission was quantified using a Varioskan LUX (Thermofisher Scientific). ATP standard curves were generated for each individual experimental run. ATP concentrations were calculated from spectrophotometric absorbance at 560nm. Data were normalised for protein content by Bradford assay. Data were plotted on graphs and two-tailed, unpaired *t*-tests conducted (GraphPad Prism).

2.17 D. melanogaster exercise

2.17.1 D. melanogaster food

20g of pre-made 'Bloomington food' mix (Genesee Scientific, NutriFly™ MF) was added to 90ml $_{dd}H_2O$ and mixed thoroughly with a spatula before microwaving on the highest setting for one minute. Food was kept at 75°C and mixed on a heated stirring plate. 0.297g of propionic acid (Acros Organics) and 130.5µl of 10% nipagin (in 100% ethanol) was added while mixing. 5ml of food was added to autoclaved glass vials. Food set overnight before adding *D. melanogaster*.

2.17.2 Maintaining stock populations

D. melanogaster stocks were originally purchased from Blades Biological or were from the laboratory of Dr. Nicoletta Moisoi. *D. melanogaster* were kept in 9.5cm tall glass vials in an incubator at 25°C. Stock populations of *D. melanogaster* were monitored for over-crowding and food quality. Stock populations of adult *D. melanogaster* were moved to autoclaved glass vials with fresh food every two weeks. The old vials contained pre-adult stage *D. melanogaster*. These were kept in the incubator until eclosion. Newly eclosed adult *D. melanogaster* would subsequently be transferred to fresh food.

2.17.3 *D. melanogaster* sex determination

Newly eclosed adult *D. melanogaster* were separated into sex-specific vials. *D. melanogaster* were exposed to 33% triethylamine solution (FlyNap) for 2 minutes. Immobilised *D. melanogaster* were poured onto white paper and visualised under a benchtop microscope. Sex was determined by identifying at least two sex-specific *D. melanogaster* features (sex combs, size, abdominal colouration) (Lab, 2015). Separated sexes were immediately moved into autoclaved glass vials with fresh food and allowed to recover overnight. Vials were stoppered with cotton wool.

2.17.4 Climbing assay

D. melanogaster were transferred into autoclaved, empty, glass vials. Vials were placed within our bespoke "Waring Rack" (Figure 2.2). The Waring Rack was designed to minimise visual obstruction of the tubes. It has a white background for ease of identification and a quadrant scale on each side for climbing ability scoring. A light source was directed onto the rack to make *D. melanogaster* identification easier.



Figure 2.2 Climbing assay rack including tubes and quadrant scale.

D. melanogaster were acclimatised to the new vials for 15 minutes. A video camera was placed on a level surface 30cm from the Waring Rack and recording initiated. Once per minute for 3-5 minutes, *D. melanogaster* were encouraged to climb by sharply tapping the rack against the benchtop 3 times, knocking them to the vial floor. After completion of the climbing assay, recording was stopped. *D. melanogaster* were transferred to vials with fresh food and allowed to recover for at least one hour before any further experimentation.

2.17.5 Climbing index

An adjusted version of the quadrant method used by Tinkerhess et al., 2012 was employed. The scale on the Waring Rack divided the tubes into four equal height sections (see figure 2.2). The base of the tube was labelled quadrant zero. From the base to the top of the tube the quadrants were labelled in ascending order from 1 to 4. Video recordings were uploaded and viewed on VLC software (VideoLAN). 4 seconds post-tap the video was paused and still frame analysed. *D. melanogaster* were assigned a score corresponding to the quadrant they were in. If the *D. melanogaster* did not move from the bottom it was assigned a score of 0. The Index score was then calculated by adding the values of all the *D. melanogaster* together and dividing by the number of *D. melanogaster* within each. Climbing index scores were plotted on a graph with SEM and two-tailed, unpaired *t*-tests carried out (GraphPad Prism).

2.17.6 Ingram counter-balanced exerciser

Our bespoke Ingram Counter-balanced Exercise (ICE) machine was adapted from the PT design of Piazza *et al.,* by Mr. John Ingram (my father) (Piazza *et al.*, 2009). The exerciser fits into an incubator with internal dimensions of 48 x 48 x 35cm and provides a vertically moving tray measuring 30 x 34cm. A solenoid-powered counterbalanced lever causes the tray to be lifted 3cm. The tray is lifted and immediately dropped every 15 seconds.

Counterbalancing the lever are a series of springs, which can be adjusted to allow lifts of up to 3.0kg. Springs efficiently store and release energy enabling a more rapid drop than would be the case if weights were used. They also reduce the size and overall weight of the device.

Lift is provided by a solenoid wound onto a nylon core and fixed to the body of the exerciser. The steel armature passing through the solenoid is attached to the counterbalanced lever. Activation of the solenoid causes the armature to rise which lifts the lever.

Solenoid activation is controlled by an astable timer. This triggers a relay to pulse the applied AC voltage. Two capacitors acting as a loss-less resistor allow the voltage to be reduced without producing excess heat, before it is rectified, smoothed and finally applied to the solenoid. Powering with DC current causes less vibration and heat generation in the solenoid but, as capacitors and solenoids both work more efficiently at lower temperatures,

any excess heat is subsequently dissipated by proximate fans. Using a pulse of current as described rather than the discharge from a large capacitor to activate the solenoid saves space, it is also safer as there is far less stored

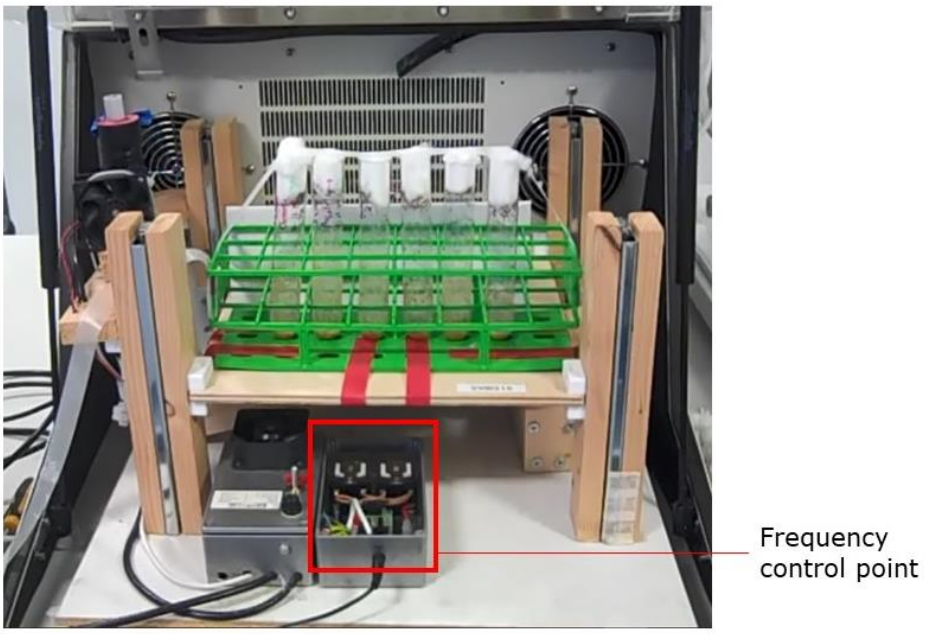


Figure 2.3 The exercise machine, including vial rack and a set of vials. Vials are secured with sticky tape to maintain upright position during taps.

energy.

2.17.7 Exercise regimes

Vials of approximately 20 newly eclosed adult WT or Pink1^{B9} *D. melanogaster* were separated into exercised or non-exercised groups. Exercised group vials were stoppered with cotton wool 6cm from the food; non-exercised group vials were stopped with cotton wool 1cm from the food, creating a physical barrier to activity. Both exercised and non-exercised groups were subjected to the ICE machine for either 30 minutes or 10 minutes per day for 7 days [refer to table 2.8 (female exercised, female non-exercised, male exercised, male non-

exercised; Pink1^{B9} exercised, Pink1^{B9} non-exercised)]. Similarly, vials of approximately 20 Pink1^{B9} *D. melanogaster* were exercised for either 30 minutes or 10 minutes per day for 7 days [refer to table 2.8 (Pink1^{B9} exercised, Pink1^{B9} non-exercised)]. All exercise was carried out in the morning. *D. melanogaster*

Exercise duration	Strains	Groups
30 minutes	W^{1118} (WT) Pink1^{B9}	WT male non-exercised WT male exercised WT female non-exercised WT female exercised Pink1^{B9} non-exercised Pink1^{B9} exercised
10 minutes	W^{1118} (WT) Pink1^{B9}	WT male non-exercised WT male exercised WT female non-exercised WT female exercised Pink1^{B9} non-exercised Pink1^{B9} exercised

were sacrificed by freezing at -80°C one hour after the final exercise bout.

Table 2.8 Strains and groups involved in each exercise regime.

3 Carbonic anhydrase modulation from middle life improves lifespan

3.1 Introduction

By 2026, it is estimated that over 20% of residents of the United Kingdom will be over 65 years of age; by 2046 this is estimated to climb to 25% (*Overview of the UK population - Office for National Statistics*, 2017). Increasing life expectancy is certainly a commendable achievement, however, ageing is strongly implicated with reduced quality of life. In particular, it is associated with declines in cognition (Geda, 2012) and muscular weakening (Walston, 2012). Growing evidence suggests mitochondrial function to be a critical regulator of ageing and underpin age-related disease risk (Sun, Youle and Finkel, 2016). Understanding the changes that occur to mitochondria with age, as well as their relevancy to the ageing process, is likely to elucidate pathways of interest as novel targets for anti-ageing.

Carbonic anhydrases (CAs) are ubiquitous zinc metalloenzymes present in all cells (Aggarwal *et al.*, 2013). Physiologically they catalyse the reversible hydration of carbon dioxide to bicarbonate and proton. They are a highly conserved group of proteins, which carry out functions vital to life in a plethora of disparate and divergent species (Banerjee and Deshpande, 2016) – acid-base balance, respiration, ureagenesis, dioxide and ion transport, bone resorption, gluconeogenesis, and CSF formation (Chegwidden, Dodgson and Spencer, 2000).

Despite its abundance and ubiquity across tissues, a dearth of research into the physiological role of CAs exist. Beyond healthy individuals, while rare, CA-II deficiency, an autosomal recessive disorder, has been shown to result in renal tubular acidosis, osteopetrosis, renal stones, short stature, growth and mental retardation (Alsharidi, Al-Hamed and Alsuwaida, 2016). Clinically, CA-II inhibition is a target of glaucoma therapy (Masini *et al.*, 2013). More recently, CA-IX has been strongly implicated in the progression of various cancers (Ward *et al.*, 2018).

Previous work by Pollard *et al.,* elucidated alterations in levels of mitochondrial CA-II and CA-III with age in wild-type mice (C57BL/6J), and in the Purkinje Cell Degeneration (*pcd*^{5J}) mouse model of neurodegeneration (A. Pollard *et al.*, 2016). It was shown that CA-II and CA-III are increased in mitochondria of aged brains and skeletal muscle, respectively, compared with wild-type. Cerebellar mitochondrial fractions from *pcd*^{5J} mice were also characterised by increased protein levels of CA-II, compared with age-matched controls. Further, using the model species *C. elegans*, it was shown that treatment with recombinant CA-II from early-adulthood resulted in a 58% reduction in lifespan, compared with control (A. Pollard *et al.*, 2016).

The subsequent work is a continuation, characterising CA as a determinant of the ageing process. Here, CA protein levels and activity are characterised across the adulthood of mice to isolate a precise time-point at which CA changes occur. *C. elegans* are then used to determine how knockdown of CA at different stages of life impacts worm lifespan and mitochondrial function. The

aim of the work in this Chapter is to add to the data supporting CA as a potential biomarker of ageing whose targeting may improve age-related outcomes. It is hypothesised that targeting CA at a critical time-point of life will improve lifespan by remediating the biomarker to healthy levels.

3.2 Methods

3.2.1 Ethics

Animals were bred and housed in accordance with strict Home Office stipulated conditions. The programme of work was reviewed by the Animal Welfare and Ethical Review Body at the University of Nottingham and then scrutinised by the UK Home Office Inspectorate before approval by the Secretary of State. The Project Licence Number for the breeding and maintenance of this genetically altered line of mice is PPL 40/3576.

3.2.2 Mitochondrial preparations

Brain and skeletal muscle were dissected from 3, 6, 9, 12 and 18-month-old C57BL/6J mice (Charles River). Mitochondria were isolated as described previously (A. K. Pollard *et al.*, 2016).

3.2.3 Western Blotting

Western blotting was carried out as described previously (Shephard *et al.*, 2014). Antibody dilutions: CAII ab6621 (Abcam) 1:5000 dilution in 3% (w/v) BSA in 1xTBS-T; CAIII AP7633a (ABGENT) 1:2500 dilution in 3% (w/v) BSA in 1xTBS-T; β-tubulin ab6046 (Abcam) 1:2000 in 3% (w/v) BSA in 1xTBS-T.; HSP-60 ab45134 (Abcam) 1:2000 in 5% (w/v) BSA in 1xTBS-T. Mouse muscle and brain mitochondrial samples were normalised to either β-tubulin or HSP-60 levels. The average of three samples for each condition were plotted showing the mean +/- SEM. Statistical analyses were carried out using GraphPad Prism (two-tailed, unpaired *t*-tests).

3.2.4 Carbonic anhydrase enzymatic assay

The esterase activity of carbonic anhydrase was measured by monitoring the hydrolysis of p-nitrophenyl acetate to 4-nitrophenol at A348nm in a Varioskan LUX Multimode Microplate Reader using standard methods (Verpoorte, Mehta and Edsall, 1967). 30µg/µl of mitochondrial sample was added to the buffer and substrate solution. Absorbance measurements were taken every 30 seconds for 30 minutes. Esterase activity assessment for each condition (3-, 6-, 9-, and 12-month-old mice) was carried out in triplicate and the average absorbance of each time-point calculated. The rate of change of absorbance was plotted. Two-tailed, unpaired *t*-tests were carried out at each time point using GraphPad Prism.

3.2.5 Lifespan assay

C. elegans strain CB5600 (ccIs4251) [(pSAK2) myo-3p::GFP::LacZ::NLS + (pSAK4) myo-3p::mitochondrial GFP + dpy-20(+)] were used for all control and knockdown lifespan assessments (Gaffney *et al.*, 2014). *cah-1* [(ok2032)(F54D8.4)] and *cah-5* [(ok3070)(R173.1)] gene knockout *C. elegans* were also used for lifespan experiments. *C. elegans* were maintained on NGM agar plates and age synchronised using standard methods (A. Pollard *et al.*, 2016). RNAi *E. coli* [HT115 (DE3)] against *cah-2* (D1022.8), *-3* (K05G3.3) and *-4* (R01E6.3a) were used. Control plates were seeded with *E. coli* strain OP50 or RNAi food as appropriate. Twenty age synchronised L4 stage *C. elegans* were added to each plate. *C. elegans* were scored each day for activity and those not moving, and that did not respond to gentle needle stimulation, were scored as

dead. For middle age *cah-2* knockdown experimentation, *C. elegans* strain CB5600 were maintained on OP50 strain *E. coli* until adult age day 8, 10 or 13. at which point they were maintained on *cah-2* RNAi food for the remainder of their lifespan. Kaplain-Meier survival curves and log-rank statistical analyses were carried out using GraphPad Prism.

For lifespan assays using recombinant CA-II (C2522 SIGMA) *C. elegans* were treated with 150 units, dissolved in DMSO, as used previously (A. Pollard *et al.*, 2016). The CA-II inhibitors dorzolamide hydrochloride (DH) (Sigma-Aldrich) and acetazolamide (Sigma-Aldrich) were spotted onto plates at a volume of 20µl and concentration of 0.25mM. To test the effect of CA drug inhibition from middle age, 20µl of DH was applied to separate groups of CB5600 strain *C. elegans* each day following adult age day 8, 10 or 12.

3.2.6 Sequence similarity and phylogenetic tree

Multiple sequence alignment of the amino acid sequences of human CA2 and CA3 and *C. elegans* orthologous family (CAH-1 to -6) were created from UCSC genome browser database, accession numbers for which are given in Table 2.7 (James Kent *et al.*, 2002). Multiple sequence alignment was carried out using EMBL-EBIs MUSCLE (MUltiple Sequence Comparison by Log-Expectation) tool (R. C. Edgar, 2004; Robert C Edgar, 2004). Sequence similarity data are shown in Table 3.1. Amino acid residues are coloured shades of blue signifying extent of sequence similarity; lightest is 30%, darkest is 100%. Sequence alignment was visualised using Jalview2 and phylogeny produced using PAM 250 (Waterhouse *et al.*, 2009).

3.2.7 Fluorescence imaging GFP-tagged muscle mitochondria and nuclei

CB5600 were used for all control and knockdown fluorescence imaging assessments (Gaffney *et al.*, 2014). *C. elegans* were age-synchronised using standard methods and grown for 48-60 hours at 20°C until they reached L4 stage. Twenty *C. elegans* were transferred into 20µl M9 buffer on a microscope slide. A coverslip was applied and *C. elegans* were imaged using the Leica CTR5000 under a GFP filter (460-500nm, blue light excitation) at 100X magnification.

3.2.8 Oxygen consumption

Oxygen consumption trials were carried out using five *C. elegans* per well with four replicate wells for each group. CB5600 strain *C. elegans* were age-synchronised onto OP50 *E. coli* or RNAi food. 48-72 hours following age synchronisation, newly adult day 0 (L4 stage). After 24 hours oxygen consumption was measured. Oxygen consumption was measured at 15 second intervals continuously for 30-120 minutes. Additional trials were conducted at adult days 4, 8 and 12. Oxygen consumption over time was calculated using the first 15 minutes of each trial. All data were corrected for volume of buffer and total protein per well, expressed as µl/min/mg protein.

3.2.9 ATP Concentration

One hundred newly adult (L4 stage) age-synchronised *C. elegans*, fed OP50 or RNAi food were collected, frozen, boiled and centrifuged. Supernatants were

transferred to fresh tubes and immediately used for ATP determination. An aliquot of 10µl freshly homogenised *C. elegans* was added to 100µl of reaction solution (0.5mM D-luciferin, 1.25µg/mL firefly luciferase, 25mM tricine buffer, pH 7.8, 5mM $MgSO_4$, 100 µM EDTA and 1mM DTT). Light emission was quantified in a Varioskan LUX (Thermofisher Scientific). ATP standard curves were generated for each individual experimental run. ATP concentrations were calculated from spectrophotometric absorbance at 560nm.

3.3 Results

3.3.1 Mouse muscle mitochondria display altered carbonic anhydrase activity and protein expression across the lifespan.

Pollard *et al.* showed an age-related increase in CA esterase activity and protein levels, in muscle, brain and retina of wild-type (WT) mice (A. Pollard *et al.*, 2016). Addition of recombinant CA to *C. elegans* significantly reduced lifespan. If CA is relevant to ageing, pinpointing the onset of its age-related increase may present a window of opportunity to target ageing. This work examined WT mouse muscle mitochondria of four age groups for CA activity [3-, 6-, 9- and 12-month-old (n=3)] and five age groups for protein levels of the primary muscle isoform, CA-III [3-, 6-, 9-, 12- (n=3) and 18-month-old (n=2)].

No significant change in carbonic anhydrase enzymatic activity was seen between 3 and 12 months of age (Figure 3.1A). However, there is a trend toward increased enzymatic activity in 3-month-old muscle mitochondria. CA-III protein levels have clearer age-related changes. Differences in the expression of CA-III across adulthood are apparent (Figure 3.1B). 3-month-old mouse muscle mitochondria show comparatively high levels of CA-III, which is subsequently significantly decreased in 9-month-old muscle mitochondria. 18-month-old mouse muscle mitochondria is characterised by significantly increased CA-III protein levels, compared to 3-, 9- and 12-month-old; p=0.0254, p=0.0135 and p=0.0467, respectively.

3.3.2 Increasing age is accompanied by CA-II activity and protein expression changes in mitochondria from brains of 3- to 12-month-old mice

3, 6, 9 and 12-month-old WT mouse brain mitochondria CA activity was also assessed (Figure 3.1C). A significant drop in activity from 3-month to 6- and 9-months were shown. The reduction in activity at 6- and 9-months was followed by an increase at 12-months-old, however, this did not reach the significance threshold. There was no significant difference in enzymatic activity between 3- and 12-month-old mouse brain mitochondria.

Mitochondrial CA-II protein levels are high at 3-months-old, followed by a drop, and recovers at 12-months-old (Figure 3.1D). Brain CA-II mitochondrial protein levels are significantly higher at 3 months compared with 6- or 9-months-old; p=0.01 and p=0.0254, respectively. From 6- or 9-month-old to 12-month-old a trend toward increased brain mitochondrial CA-II protein levels was apparent, however, this did not reach statistical significance. No statistically significant difference in CA-II protein levels between 3- and 12-month-old mouse brain mitochondria was seen.

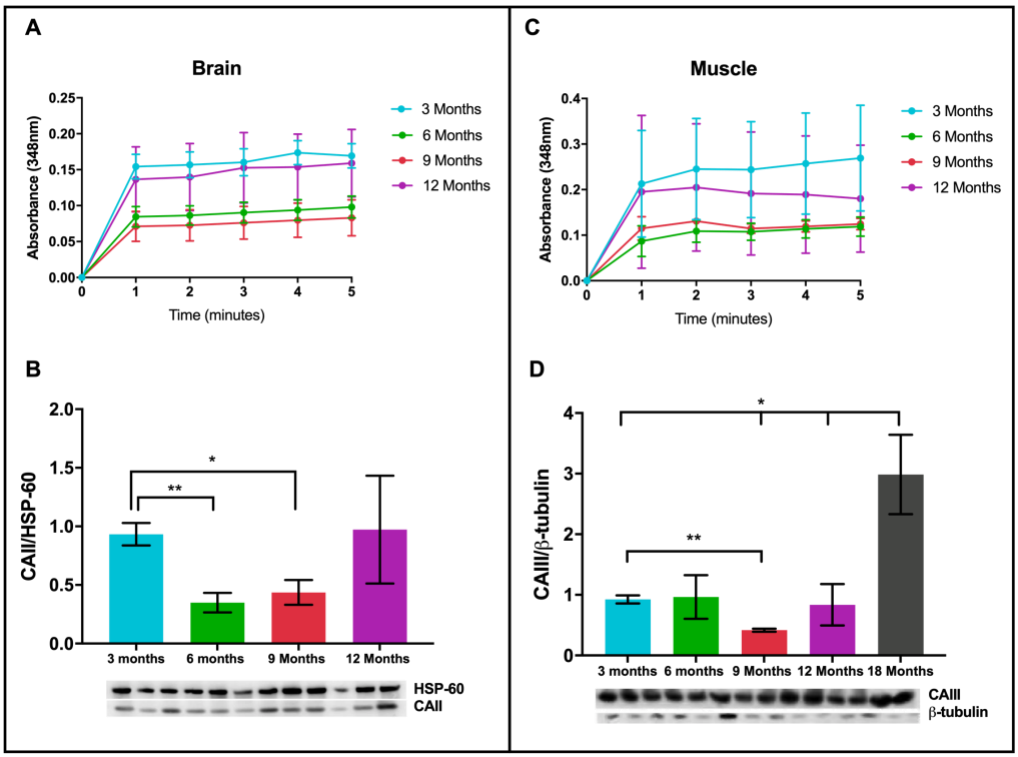


Figure 3.1 (A) CA esterase activity is increased in young mouse brain. CA activity is higher in the brain mitochondria of 3-month-old mice, compared with 6- and 9-month-old mice. No significant difference in the enzymatic activity of 3- and 12-month-old mice is seen. **(B)** CA-II protein levels are reduced in 6- and 9-month-old brain mitochondria. Protein levels of CA-II were measured in 3- to 12-month-old mouse brain mitochondria. CA-II protein levels are significantly reduced in 6- and 9-month-old brain mitochondria compared to 3-month-old; p =0.01 and p =0.0254; two-tailed, unpaired t-test, respectively. **(C)** CA esterase activity of mouse muscle mitochondria is unchanged between 3- and 12-months-old. No statistically significant change in the enzymatic activity of CA is seen in mouse muscle mitochondrial samples of 3- to 12-month-old mice. **(D)** CA-III protein levels display differential expression with age in muscle mitochondria. CA-III significantly decreases in 9-month-old muscle mitochondria compared to 3-month-old; p =0.0022; two-tailed, unpaired t-test. 18-month-old mouse muscle mitochondria has significantly higher protein levels than 3-, 9- and 12-month-old mice; p =0.0254, p =0.0135 and, p=0.0467; two-tailed, unpaired t-test, respectively. Three mitochondrial samples from different mice were used for each age group, except 18-month-old mouse muscle mitochondria where two different mice were used. Each mitochondrial sample contained 30µg/µl protein. Columns display mean activity ±SEM. *= p <0.05. **= p <0.01 two-tailed, unpaired *t*-test.

3.3.3 Early-life knockdown of *cah* significantly reduces the lifespan of *C. elegans*

Pollard *et al.* previously showed that newly adult worms treated with recombinant CA-II have significantly shortened lifespan (A. Pollard *et al.*, 2016). *C. elegans* contain six proteins orthologous to human CA-II. Amino acid sequence alignment analysis revealed high sequence similarity between all orthologues and *H. sapiens* CA-II (Table 3.2 and Figure 3.2). Further, *H. sapiens* CA-II residues 198-199, which are concerned with substrate binding, are highly conserved in *C. elegans* CAH proteins (Figure 3.2A) (Briganti *et al.*, 1999). These key sequence similarities support the use of *C. elegans* as a model for investigating the role of CA in ageing research. In mice higher protein levels of CA-II are found in early adulthood (Figure 3.1B). CA orthologues in newly adult worms are required for normal lifespan (Figure 3.3). Knockdown of CA-II orthologues, *cah-2*, *-3* or *-4*, in newly adult worms significantly reduces lifespan (p=0.0122, p=0.0007 and p=0.0009, respectively). Similarly, knockout of CA orthologues *cah-1* (RB1643) or *cah-5* (RB2268) significantly reduced lifespan (p=0.0004 and p=0.0024, respectively).

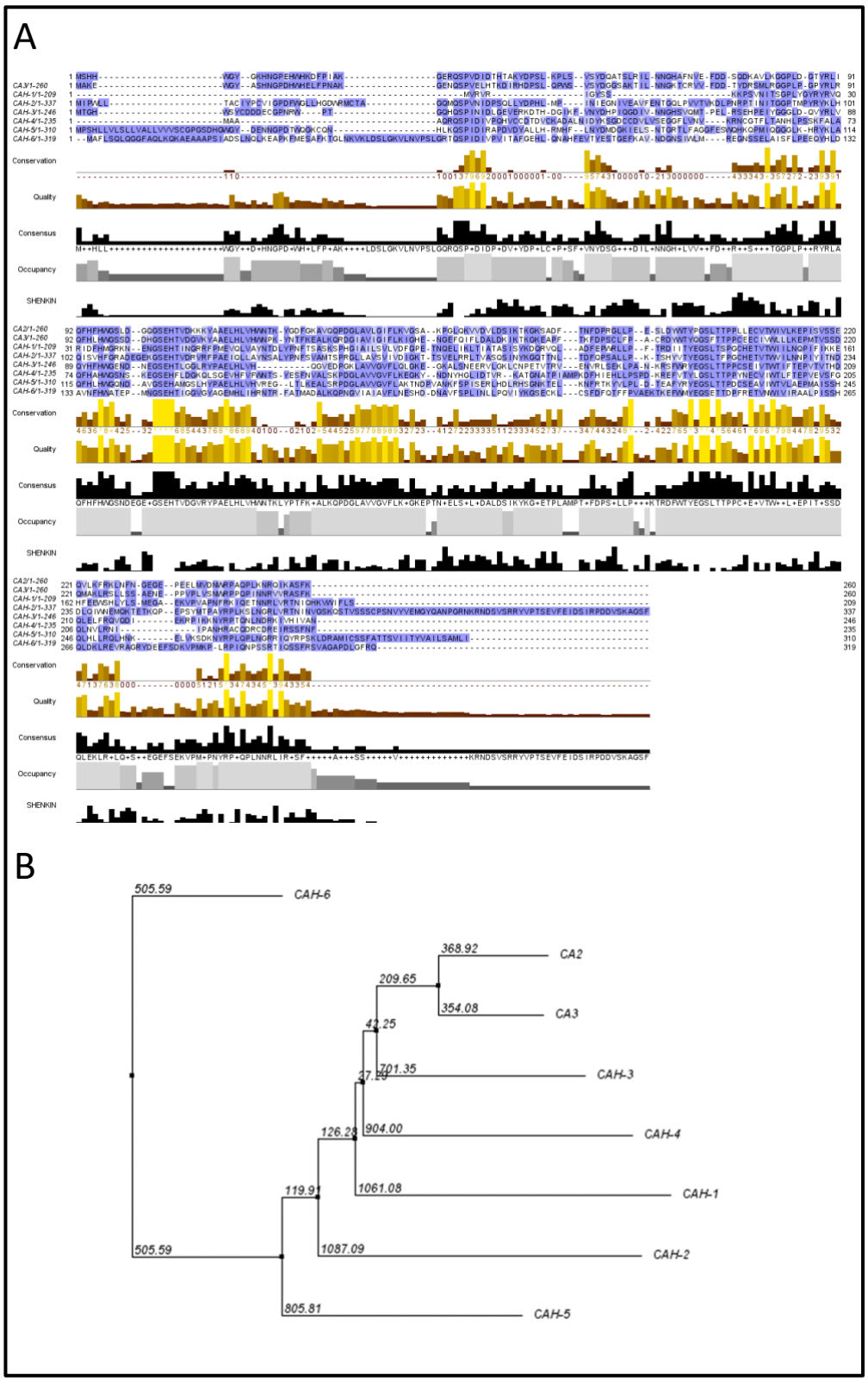


Figure 3.2 (A) Multiple sequence alignment of C. elegans CA orthologues CAH-1 to -6, compared to *H. sapiens* CA-II and CA-III. Amino acid residues are shaded blue signifying their sequence similarity; lightest is 30%, darkest is 100%. Sequence similarity to *H. sapiens* CA-II is 34%, 31%, 32%, 31%, 35% and 31% for CAH-1, -2, -3, -4, -5 and -6, respectively. Sequence similarity to *H. sapiens* CA-III is 34%, 31%, 35%, 33%, 36% and 28% for CAH-1, -2, -3, -4, -5 and -6, respectively. Sequence alignments were carried out using Jalview. **(B)** A phylogenetic tree of CA was produced using PAM 250. Accession data can be found in Table 2.7.

Alignment reference	Orthologue (*C. elegans*)	Sequence Homology
CA-II (*H. sapiens*)	CAH-1	34%
	CAH-2	31%
	CAH-3	35%
	CAH-4	33%
	CAH-5	36%
	CAH-6	28%
CA-III (*H. sapiens*)	CAH-1	34%
	CAH-2	31%
	CAH-3	32%
	CAH-4	31%
	CAH-5	35%
	CAH-6	31%

Table 3.1 CA-II and -III *C. elegans* protein orthologues CAH-1 to -6 sequence data. Sequence data were generated using UCSC genome browser database accession data (Table 2.7). Sequence similarity was determined using EMBL-EBIs MUSCLE (MUltiple Sequence Comparison by Log-Expectation) tool.

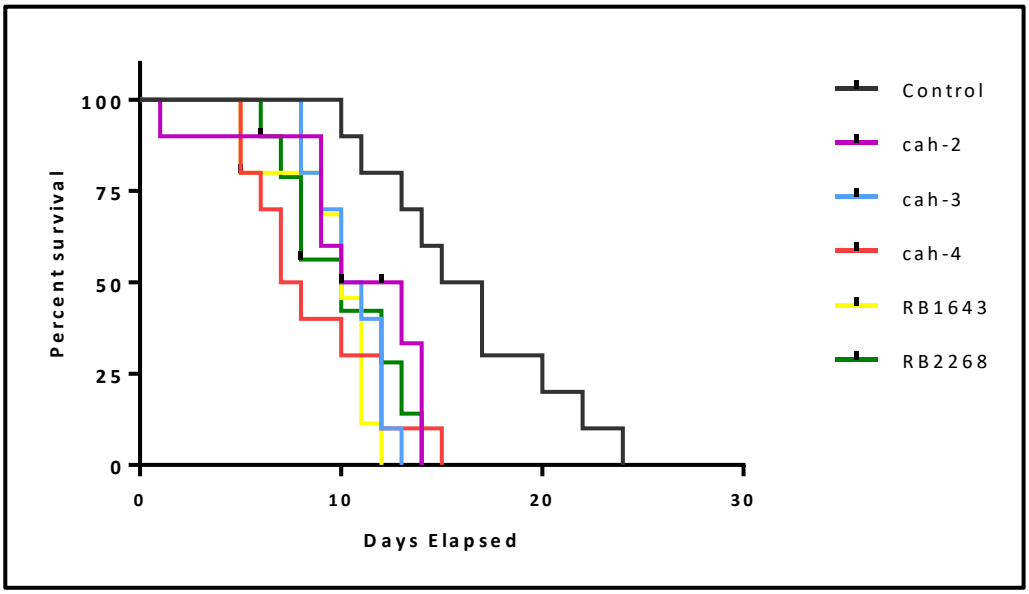


Figure 3.3 Knockdown and knockout of *C. elegans CA* orthologues significantly reduces lifespan. *C. elegans* treated with either *cah-2, -3 or -4* RNAi, and *cah-1* and *cah-5* knockout *C. elegans* (RB1643 and RB2268, respectively) have a significant reduction in lifespan; p=0.0010 (Log-rank test). Median lifespans were 16, 11.5, 10.5 and 7.5, 10 and 10 days for control (OP50 fed), *cah-4* RNAi, *-5* RNAi, *-6* RNAi, RB1643 and RB2268, respectively. Twenty *C. elegans* were used for each group.

3.3.4 Knockdown of CA orthologues in newly adult *C. elegans* alters mitochondrial function

The mechanism behind the lifespan alteration elicited by recombinant CA-II addition or orthologue knockdown is unknown. This work suggests functional and morphological alterations are apparent in the mitochondria of *cah-2* knockdown *C. elegans*.

Knockdown of *cah-2* or *cah-3* in newly adult *C. elegans* resulted in a significant increase in oxygen consumption, compared with age-matched control *C. elegans* (p=0.0221 and p<0.0001, respectively) (Figure 3.4A). Knockout of *cah-1* or *cah-5* also significantly increased oxygen consumption (p<0.0001 and p<0.0001, respectively). Conversely, knockdown of *cah-4* showed a decrease in oxygen consumption (p=0.0001).

Knockdown of *cah* revealed a marked reduction in maximal lifespan (Figure 3.3). Daily feeding of L4 stage *C. elegans* either OP50 or *cah-2*, *-3* or *-4* RNAi was carried out for 12 days and the rate of oxygen consumption (μl/min/mg protein) was monitored at days 0, 4, 8 and 12 (Figure 3.4B). Of note, OP50 fed *C. elegans* had a remarkably stable rate of oxygen consumption between 0 and 12 days of age.

At day 4 a significant increase in rate of oxygen consumption was seen in *cah-4* knockdown *C. elegans* compared to control (p=0.0479). A trend toward a higher rate of oxygen consumption is apparent in *cah-2* and *-3* knockdown *C. elegans*, however, this did not reach the significance threshold (p=0.0636 and p=0.0787,

respectively). *cah-4* knockdown *C. elegans* retained a significantly higher rate of oxygen consumption at days 8 and 12, compared with OP50 fed (p=0.0162 and p=0.0113, respectively). Further, *cah-4* knockdown *C. elegans* show a significant increase in rate of oxygen consumption between 0-4 and 4-8 days of age (p=0.0191 and p=0.0256, respectively). *cah-3* knockdown *C. elegans* show a significant increase in rate of oxygen consumption at day 8 only (p=0.0006), compared with OP50 fed. The increased rate is due to a significant increase in rate of oxygen consumption between days 4-8. At day 12, *cah-2* knockdown *C. elegans* show a significantly greater rate of oxygen consumption than OP50 fed *C. elegans* (p=0.0432).

The majority of oxygen utilisation in a cell is for ATP generation, with minor uses by NADPH oxidases and the production of reactive oxygen species (Abdel-Rahman *et al.*, 2016). These data show that the increase in oxygen consumption with *cah* knockdown is coupled to an increase in ATP generation (Figure 3.4C). *C. elegans* fed *cah-2* RNAi show significantly increased ATP concentration compared to age-matched control *C. elegans* (p=0.0368). Mitochondria are dynamic organelles capable of sensing and responding to the environment. By doing so they adapt to cellular needs accordingly even in times of stress. CB5600 strain *C. elegans* express a transgene for GFP-bound muscle wall nuclei and mitochondria. Age synchronised *C. elegans* were fed either OP50 or *cah-2* RNAi for 48-72 hours. OP50 fed *C. elegans* displayed elongated and orderly mitochondria (Figure 3.4D). In comparison, *cah-2*

knockdown *C. elegans* show considerably altered mitochondrial dynamics – smaller, more fragmented and disorderly mitochondria phenotype

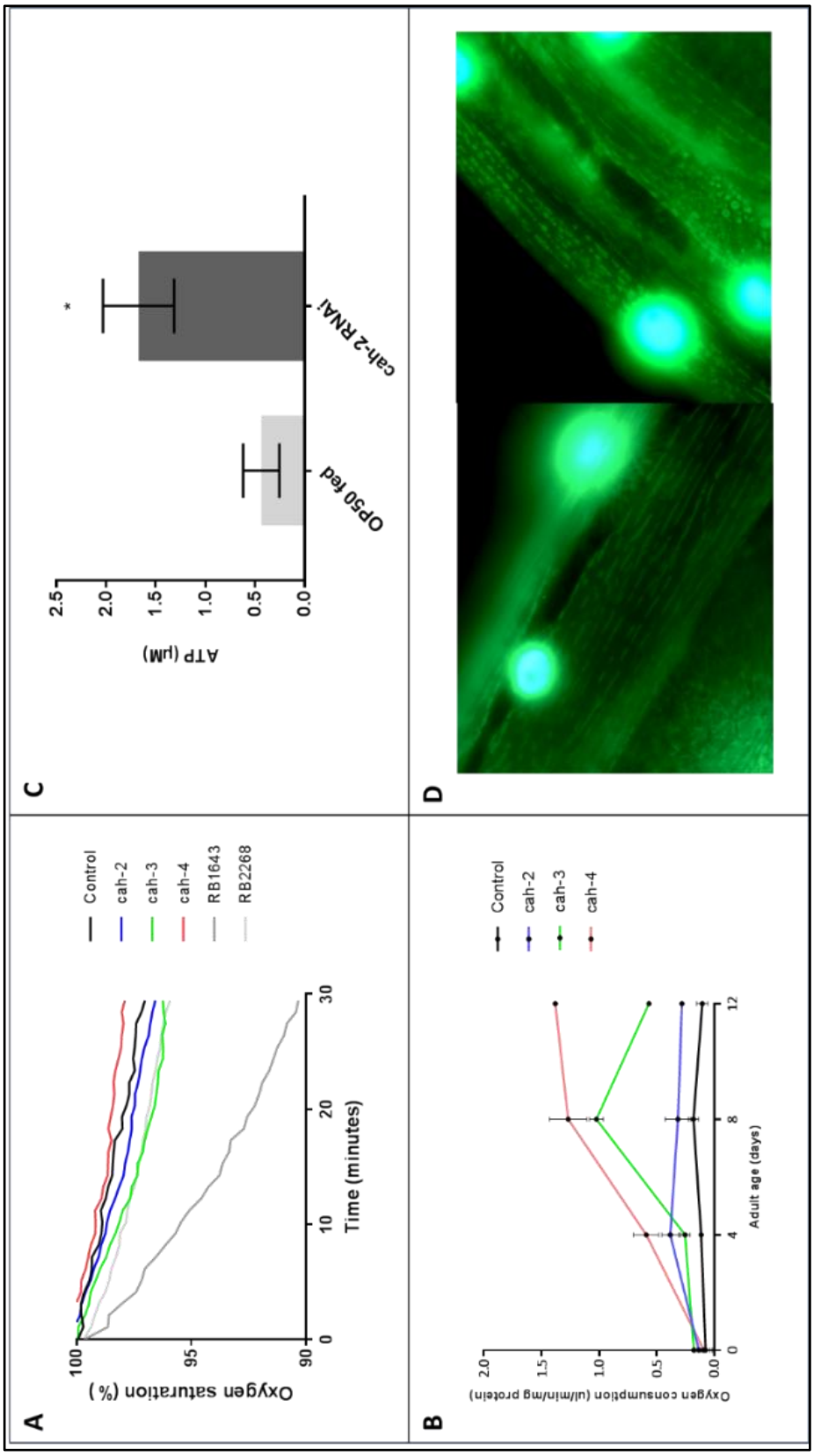


Figure 3.4 (A) Knockout or knockdown of *cah* in newly adult *C. elegans* significantly increases oxygen consumption compared to control. Knockdown of *cah-2* (p=0.0221), *cah-3* (p<0.0001), and knockout of *cah-1* (RB1643) (p<0.0001) and *cah-5* (RB2268) (p<0.0001) significantly increased oxygen consumption. Conversely, knock-out of cah-4 significantly decreased oxygen consumption

(p<0.0001). One-way ANOVA was used for all statistical analysis. **(B)** Knockdown of *CA* orthologues from newly adult *C. elegans* increases rate of oxygen consumption across the lifespan. Knockdown of *cah*-2, -3, or -*4* continuously in newly adult *C. elegans* increases the rate of oxygen consumption with age, compared with control. Oxygen consumption of 15 Day 0 adult CB5600 strain *C. elegans* were measured every 15 seconds for 30 minutes, at 25°C (Loligo Systems). Data points are the mean value of three different measurements. **(C)** ATP concentration is increased in *cah-2* knockdown worms. Knockdown of *cah-2* from newly adult *C. elegans* increases ATP generation compared with control *C. elegans*; p=0.0368 (two-tailed, unpaired t-test). *C. elegans* homogenates were equalised for protein content by Bradford assay. **(D)** *cah-2* knockdown alters mitochondrial dynamics. CB5600 strain *C. elegans* grown on OP50 **(left)** exhibit an elongated mitochondrial shape but show dispersed and disordered mitochondria when grown on *cah-2* RNAi **(right)**. *C. elegans* in this figure are newly adult and express the transgene ccIs4251 with GFP fusion proteins localised to the body muscle mitochondria and nuclei.

3.3.5 Knockdown of *cah-2* at middle age significantly increases *C. elegans* lifespan

Cumulatively this work suggests an early life requirement for CA (Figure 3.3) and that mitochondria are altered by its removal. Additionally, previously published data reveal a picture of ageing and neurodegeneration that is characterised by increased levels of CA proteins localised to the mitochondria of mouse brain, retina, and muscle (A. Pollard *et al.*, 2016). Targeted inhibition of CA during middle age may increase longevity.

This work shows that knockdown of *C. elegans* CA orthologue, *cah-2*, from day 13 significantly extends lifespan, resulting in a median lifespan of 20 days, compared to 14 days for control *C. elegans* (p=0.0034) (Figure 3.5). Knockdown of *cah-2* from day 8 or 11 also exhibits a trend toward increased median

lifespan (15 and 18 days, respectively), however, these did not reach the significance threshold (p=0.0945, p=0.1012, respectively). As previously shown (Figure 3.3), knockdown of *cah-2* from day 0 of adulthood significantly reduced lifespan compared to control *C. elegans* (p=0.0293).

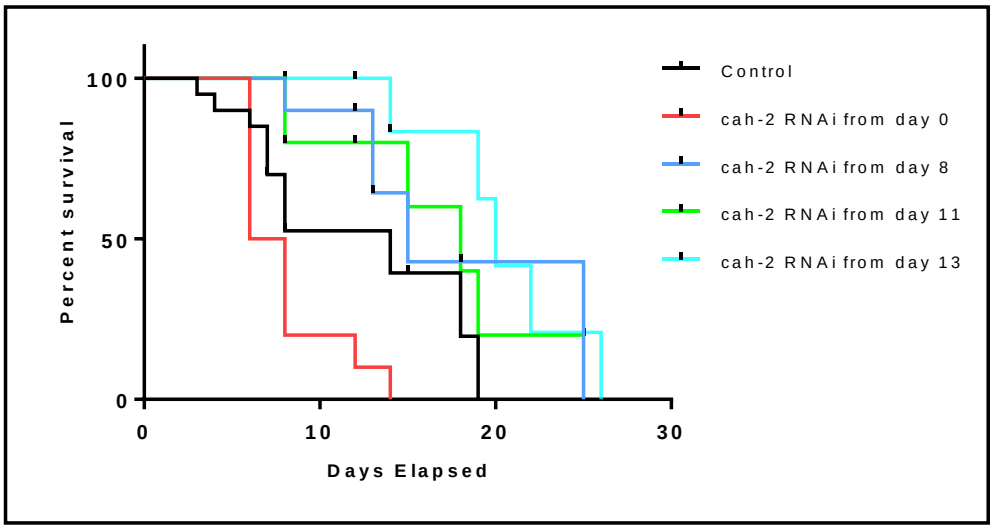


Figure 3.5 Knockdown of *C. elegans CA* orthologue, *cah-2*, from day 13 significantly increases lifespan**.** *C. elegans* treated with *cah-2* RNAi from day 13 have a significantly increased lifespan (p=0.0034; Log-rank test), compared with OP50 fed control *C. elegans*. Knockdown of *cah-2* at days 8 and 11 increased median and maximal lifespan, however, this did not reach the significance threshold (p=0.0945, p=0.1012, respectively). Median lifespans were 14, 7, 15, 18 and 20 days for OP50 fed, *cah-2* RNAi from day 0, day 8, day 11 and day 13, respectively. Twenty *C. elegans* were used for each knockdown group; twenty *C. elegans* were used for the control group.

3.3.6 Dorzolamide hydrochloride rescues the lifespan of *C. elegans* fed recombinant CA-II

The work of Pollard *et al.* showed a significant reduction in lifespan with the addition of recombinant CA-II to newly adult (L4 stage) *C. elegans* (A. Pollard *et al.*, 2016). The present data is consistent with their finding - addition of

recombinant CA-II significantly reduced the lifespan of *C. elegans* (Figure 3.6A).

Dorzolamide hydrochloride (DH) is a specific inhibitor of CA-II, and acetazolamide (AZ) is a pan-CA inhibitor; both are used primarily in the treatment of glaucoma (Scozzafava and Supuran, 2014). Here, simultaneous exposure to recombinant CA-II and DH significantly increased *C. elegans* lifespan compared to CA-II treatment alone (p=0.0282), restoring lifespan to that of control.

Unlike knockdown of *cah-2* from early adulthood, continuous DH treatment in newly adult *C. elegans* did not alter lifespan (Figure 3.6A). In opposition to the *cah-2* knockdown results, CA orthologue inhibition by DH in newly adult *C. elegans* did not significantly alter oxygen consumption compared to controls (p=0.1125) (Figure 3.6B). However, the pan-carbonic anhydrase inhibitor, AZ, significantly increased oxygen consumption compared with control (p<0.0001).

3.3.7 Middle age inhibition of *C. elegans* CA orthologues by dorzolamide hydrochloride does not extend lifespan

This work has shown that middle age knockdown of *cah-2* improves median and maximal lifespan of WT *C. elegans* (Figure 5). Preliminary work with the specific CA-II inhibitor, dorzolamide hydrochloride, suggests it does not cause negative functional mitochondrial alterations (Figure 3.6B), and it wholly rescues the lifespan reduction of *C. elegans* caused by recombinant CA-II

treatment. Mimicking the survival assay methodology of middle age *cah-2* RNAi knockdown, middle age DH treatment of *C. elegans* (from day 8, 10 or 12) did not alter median or maximal lifespan, compared with control (Figure 3.6C). Interestingly, as figure 3.6A also shows, daily DH treatment in day 0 adult *C. elegans* does not alter lifespan.

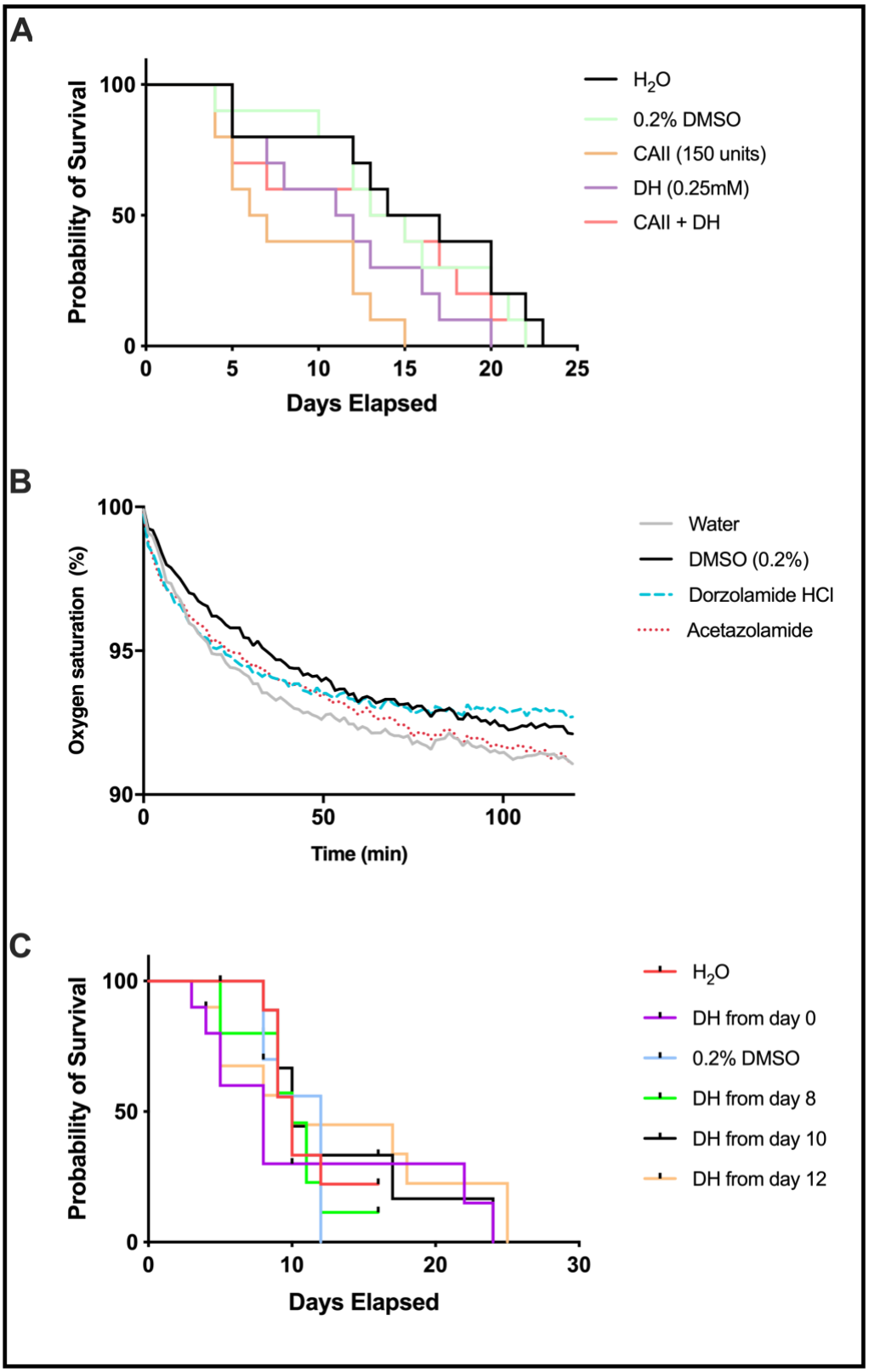

A
Probability of Survival
100
50
0
0
5
10
15
20
25
Days Elapsed
H2O
0.2% DMSO
CAII (150 units)
DH (0.25mM)
CAII + DH
B
Oxygen saturation (%)
100
95
90
0
50
100
Time (min)
Water
DMSO (0.2%)
Dorzolamide HCl
Acetazolamide
C
Probability of Survival
100
50
0
0
10
20
30
Days Elapsed
H2O
DH from day 0
0.2% DMSO
DH from day 8
DH from day 10
DH from day 12

Figure 3.6 (A) CA-II inhibitor dorzolamide hydrochloride (DH) restores normal lifespan to *C. elegans* fed recombinant CA-II. *C. elegans* fed CA-II from adult day 0 show a significant decrease in lifespan; p=0.0061 (Log-rank test). However, this decrease is attenuated upon addition of the CA-II inhibitor DH in parallel with CA-II and restores lifespan to that of control; p=0.0282 (Log-rank test). Newly adult *C. elegans* treated continuously with DH alone do not have altered lifespan; p=0.1727 (Log-rank test). The median lifespans were 15.5, 14, 6.5, 11.5 and 14 days, for control (H_2O), vehicle control (DMSO 0.2%), CAII (150 units), DH (0.25mM), and CAII + DH, respectively. Twenty *C. elegans* were used for each group. **(B)** Oxygen consumption of newly adult *C. elegans* treated with dorzolamide hydrochloride is unchanged. Treatment with DH shows no significant changes in oxygen consumption over a 120-minute-period, compared with vehicle (DMSO 0.2%) or H_2O treatment. However, *C. elegans* treated with the pan-CA inhibitor, acetazolamide have a significant increase in oxygen consumption, compared with vehicle control; p<0.0001 (one-way ANOVA). Oxygen consumption of 15 newly adult CB5600 strain *C. elegans* was assessed every 15 seconds for 120 minutes, at 25°C (Loligo systems). Data points are the mean value of three different measurements. **(C)** Middle age targeted inhibition of *C. elegans* CA-II orthologues by DH does not increase lifespan. Daily DH treatment from either day 0, 8, 10 or 12 did not significantly alter lifespan, compared with OP50 fed *C. elegans*. Median lifespan of each group was not significantly different: 10, 12, 8, 10, 10, 10 days for control (H_2O), vehicle control (DMSO 0.2%), or daily DH treatment from day 0, 8, 10 and 12, respectively. Twenty *C. elegans* were used for each condition.

3.4 Discussion

This work is a continuation of previous findings from Pollard *et al.*, which highlighted an ageing-related and age-related disease increase in mitochondrial CA-II and CA-III in the brain and muscle, respectively, of mice (A. Pollard *et al.*, 2016). This study describes CA activity and expression changes across mouse adulthood, it more precisely pinpoints where the age-related increase begins, and shows that abnormal CA levels affect mitochondria.

Across adulthood, differences in mitochondrial CA protein expression and enzymatic activity, in mouse brain and muscle, are clear. Adulthood is characterised by higher brain CA-II protein levels and CA activity in early (3-month-old) and middle-adulthood (12-month-old). Mouse muscle mitochondrial CA-III protein levels and CA activity display a similar pattern to brain, although a larger standard error around the mean at 6-months-old did not fully recapitulate the pattern of changes in the brain. As shown in previous work (A. Pollard *et al.*, 2016), muscle CA-III protein levels were significantly increased at 18 months of age. Due to a lack of 18-month-old brain tissue, these data were not duplicated in this work, however, Pollard *et al.* found an age-related increase in CA-II protein expression between 12 and 18 months of age (A. Pollard *et al.*, 2016).

3.4.1 The role of carbonic anhydrase across ageing - hypotheses

This is the first-time CA expression and activity across mouse adulthood has been described. Changes to CA levels with age have been described by others, however, none have looked specifically in brain tissue (Diedrich *et al.*, 2007; Laimer *et al.*, 2010; Feng *et al.*, 2018). CA-II is most prevalent in the brain and its activity rivals that of the most active enzymes known (Supuran, 2007). In agreement with Pollard *et al.* and this work (A. Pollard *et al.*, 2016), CA-II protein expression was shown to be increased in older female human heart, aged mouse heart (Diedrich *et al.*, 2007) and older human foreskin (Laimer *et al.*, 2010). The impact increasing CA-II levels with ageing have is unknown. Ageing is associated with an increase in intracellular ROS production, resulting in an accumulation of damage to intracellular biomolecules (Bratic *et al.*, 2013b). Evidence exists for CA proteins acting as a sink for ROS due to a high proportion of cysteines (Monti *et al.*, 2017). Further, respiration is accompanied by the generation of ATP, H_2O, CO_2, and ROS. Excess CO_2 is converted into bicarbonate and proton by CA proteins (Supuran, 2007). Indeed, this may be an additional mechanism whereby increasing CA becomes detrimental. CAIX and CAXII, which are primarily cell membrane isoforms, are upregulated in various solid tumours and maintain intracellular alkalinity while promoting extracellular acidification (Mboge *et al.*, 2018). Disturbances to intracellular pH, or increased variation in intracellular/extracellular pH ratio is known to disturb biological processes

such as mitochondrial membrane potential and ATP synthesis (Parks, Chiche and Pouyssegur, 2011). It may be that CA levels beyond a certain point promote alterations to pH, causing mitochondrial dysfunction. To further interrogate this question measurements of cellular and mitochondrial pH should be conducted with altered CA-II and -III levels.

Within the muscle, CA-III is the most prevalent isoform (Kim *et al.*, 2004). CA-III is fibre-type specific, preferentially expressed in type I muscle fibres (Feng and Jin, 2016b). Interestingly, unlike other isoforms, CA-III has exceptionally poor enzymatic activity (Feng and Jin, 2016b). It may be pleiotropic in its roles. Evidence for a role in fatty acid metabolism exists (Feng and Jin, 2016b). Interestingly, CA-III has been shown to be key in muscle fatigue resistance. CA-III positive fast twitch transverse abdominis (TA) is more resistant to fatigue than CA-III negative fast twitch extensor digitorum longus (EDL). Further, mouse homologue *Car-3* knockout TA muscle is less tolerant to fatigue than wild-type TA muscle (Feng and Jin, 2016b). Fundamentally, muscle fatigue is an accumulation of intracellular inorganic phosphate and hydrogen ions, impairing Ca^{2+} handling and contractile proteins. The subsequent decreased pH plays a causative role in muscle fatigue onset (Feng and Jin, 2016b). Low CA-III may not be able to adjust the intracellular pH to counter acidosis. CA-III has been shown to contain many more cysteine residues in its amino acid sequence than other CA isoforms (Monti *et al.*, 2017). These residues are highly reactive and the high levels suggest CA-III may scavenge ROS. Ageing is associated with type II muscle fibre conversion to

type I (Feng and Jin, 2016b). As mentioned, type I fibres express higher levels of CA-III (Feng and Jin, 2016b). The age-related CA-III increase in mouse muscle mitochondria may reflect this conversion. In agreement, muscle from aged rat gastrocnemius muscle (Capitanio *et al.*, 2009), middle aged vs old age subjects (sex not specified) (Staunton *et al.*, 2012b), and post-menopausal women (Gueugneau *et al.*, 2014) showed increased CA-III. In contrast, rat gastrocnemius muscle showed an age-related decrease in CA-III (Doran *et al.*, 2008). As shown in this work, CA-III expression is clearly altered across adulthood in the mouse. These differences suggest the discrepancy between the rat gastrocnemius muscle studies may be due to differences in the ages of the groups analysed (8 month old vs 22 month old Sprague Dawley rats (Capitanio *et al.*, 2009); 3 month old vs 30 month old Wister rats (Doran *et al.*, 2008)).

3.4.2 Mitochondria and carbonic anhydrase

Pollard *et al.* found that addition of recombinant CA-II to newly adult *C. elegans* significantly reduced lifespan, suggesting excess levels to be life-limiting (A. Pollard *et al.*, 2016). Similarly, this work has shown that selective knockdown of newly adult *C. elegans* CA orthologues mirrored the lifespan reduction caused by an excess. This suggests that the high CA-II and CA-III levels in 3-month-old mice are necessary. Mitochondrial function is reduced with age (A. M. Joseph *et al.*, 2012). Here, it is shown that mitochondrial function and morphology is altered in *cah* knockdown *C. elegans*, and phenotypically they mimic stressed or dysfunctional organelles. Indeed,

knockdown of *cah-2* caused increased oxygen consumption and a disordered, fragmented, mitochondrial phenotype. Mitochondria are the primary sources of ROS and it is likely that the increase in oxygen consumption is not simply a reflection of increased ATP production (Abdel-Rahman *et al.*, 2016). Greater ROS levels have been shown to be associated with increased fission and mitochondrial fragmentation – the anti-cancer agent cisplatin, which damages both nDNA and mtDNA was shown to cause mitochondrial fission with concomitant increased ROS generation (Choi *et al.*, 2015). mtDNA are considered especially vulnerable to ROS insult, due to their close proximity to sites of oxidative metabolism (Shokolenko *et al.*, 2009). Increased ROS may increase the propensity for mtDNA mutagenesis (Shokolenko *et al.*, 2009). Core subunits of mitochondrial oxidative phosphorylation are coded for by mtDNA – mutation of these may result in further mitochondrial dysfunction, thus perpetuating a vicious cycle until, ultimately, cell death (Gustafsson, Falkenberg and Larsson, 2016). With CAs putative role as a ROS sink, at least some of the mitochondrial functional and morphological changes that occur may be due to a loss of ROS buffering capacity (Monti *et al.*, 2017).

It is relevant to note that mitochondrial isoforms of CA exist, however, the isoforms of interest in this work, CA-II and -III, are classically cytosolic forms (Supuran *et al.*, 2016). A necessary next step would be a more precise interrogation of CA-II and -III upon mitochondrial function, including how increased expression affect mitochondrial parameters (respiration, membrane potential, ROS production) and its binding or localisation

(immunohistochemistry, differential centrifugation of mitochondria). CA-II and -III orthologue cah-2 is an extracellular isoform in *C. elegans* (UniProt, 2019). It may be that inhibition of CA-II or -III do not alter mitochondria and the effect is specific to *C. elegans* and the *cah-2* isoform, or extracellular isoforms. However, recent evidence points to inhibition of transmembrane isoforms, CA-IX and -XII, reducing mitochondrial biogenesis (Bernardino *et al.*, 2019). Regardless, it will be necessary to determine whether, and if so how, CA-II and -III modulate mitochondrial function.

3.4.3 The lifespan extension disparity

CA expression and activity changes across mouse adulthood have been described here. Excess CA-II given to newly adult *C. elegans* (A. Pollard *et al.*, 2016), or newly adult *C. elegans* knockdown of CA orthologues resulted in significantly reduced lifespan. In mice, between 12 and 18 months of age, a significant increase in CA protein levels are observed. If high CA protein levels later in life is life-limiting, then inhibiting it when it begins to increase may be an effective treatment to improve health- or lifespan. Here, middle age knockdown of *cah-2* in *C. elegans* significantly increased lifespan. CA is an exciting potential marker of ageing as inhibitors of it have been utilised in the clinic for many decades (Scozzafava and Supuran, 2014). Dorzolamide hydrochloride (DH) is a sulphonamide class drug which specifically inhibits CA-II. It is used as an adjunctive therapy to β-blockers and more commonly in the treatment of glaucoma (Scozzafava and Supuran, 2014). Newly adult *C. elegans* fed DH showed reduced oxygen consumption. Caloric restriction, the

only intervention known to consistently improve lifespan, has also been shown to reduce oxygen consumption (Ferguson *et al.*, 2007) and, interestingly, promote slight intracellular acidity (Dolz-Edo *et al.*, 2019). Interestingly, *C. elegans* fed DH from early adulthood showed no significant difference in lifespan compared with control. This disparity to the significant lifespan reduction by *cah* knockdown will require investigation, however, it may be that DH is only partially inhibiting CA, as it has been shown that <1% CA-II enzymatic activity is required to maintain physiological functions (Balfour and Wilde, 1997). In order to determine this, it would be necessary to purify *cah-2* and measure its activity with and without DH present. Alternatively, an indirect method would entail the measurement of CA activity of *cah-2* knockdown *C. elegans* and compare this to DH-treated *C. elegans*. DH has been shown to display antioxidant-type effects and that its presence exerts protective activity toward oxidative stress in the presence of intact mitochondria (Saccà, 2011). *C. elegans* were treated with DH using the same protocol as middle age knockdown of *cah-2*. A trend toward increased lifespan was apparent, however, this did not reach significance. Alternatively, as mentioned, DH has been shown to have antioxidant activity (Saccà, 2011). Antioxidant therapy does not consistently positively impact longevity (Ristow and Schmeisser, 2011) – *C. elegans* exposed to an inhibitor of glycolysis have increased respiration and concomitant increase in ROS formation, which leads to increased lifespan. Interestingly, antioxidant treatment abolished the lifespan extension (Schulz *et al.*, 2007). Further, antioxidant therapies have been shown to inhibit the beneficial effects of exercise (Gomez-Cabrera *et al.*,

2013). It is plausible that *cah-2* knockdown or inhibition by DH increases ROS production, however, the antioxidant activity of DH negates any ROS-stimulated lifespan benefits. It may be that no lifespan extension by DH is a true result, however, healthspan may be improved. Indeed, exercise is an intervention known to be beneficial and significantly improve healthspan, however, data for lifespan extension are conflicting (Kujala, 2018; Nilsson and Tarnopolsky, 2019). Further work should include measures of healthspan to interrogate this question. Finally, this survival study suffered from an unusually high level of *C. elegans* death, across all groups, in the early portion of the experiment. It would be necessary to repeat these experiments with a greater sample of worms (~100 per group).

In this work no confirmation of *CA* orthologue knockdown was carried out. Induction of a phenotype is often a reliable indicator of a successful RNAi result (in this case the reduced lifespan exhibited), as the level of false positives in *C. elegans* has been shown to be extremely low (Kamath *et al.*, 2003b), however, validating mRNA knockdown by RT-PCR would be prudent. In addition, some protein products are stable over a number of days and may continue to exert normal physiological activity even with a successful RNAi event (Tencer *et al.*, 1994) – Western blotting would confirm whether residual protein was present.

3.5 Conclusion

Pollard *et al.* identified potential markers of ageing, CA-II and CA-III (A. Pollard *et al.*, 2016). The present work found that knockdown of *C. elegans* orthologue, *cah-2*, during middle age increases lifespan. CAs are inhibited clinically by sulphonamides, including DH. Inhibition of CA-II for the purpose of "anti-ageing" presents an exciting opportunity to rapidly test the potential of biological markers of ageing by utilising available drugs, such as DH. It will be necessary to extend the work with DH, to assess its potential for healthspan improvements, which early evidence suggests may be imparted by its influence on mitochondria.

4 Lower fatty acid binding protein 3 levels characterise short lived animals and neurodegenerative disease models

4.1 Introduction

Comparative biology requires perceiving species as simultaneously similar, yet different. The phenotype of ageing is shared among all species – it is easy to recognise an old dog from a young one – coat quality, strength, gait, cognitive ability – these are all measurable and reliably alter with age across species (M J Tinkerhess *et al.*, 2012). However, the rate of this alteration, the rate of ageing, is vastly different between species. Mammalian maximal lifespan (MLS) varies greatly, from 3 years in mice to over 200 years in bowhead whales (Keane *et al.*, 2015). Even between closely related species, divergence in MLS appears quickly – humans and macaques diverged around 30 million years ago, however their MLS has diverged three-fold over this period (Colman *et al.*, 2014). In order to explain this disparity, numerous theories have been postulated.

Some common observations have been used to generate theories of ageing. The rate-of-living theory proposes to explain the disparity in mammalian lifespans and suggests that longevity is an inverse function of metabolic rate (Pearl, 1928). Mechanistically, the mitochondrial free radical theory of ageing states that animals with high basal metabolic rates will generate higher levels

of ROS, have an increased rate of cellular damage and have shorter lifespans. In general, body size, metabolic rate and longevity do correlate well, however, of interest are species which are exceptions – those that live far longer than their body size and metabolic rate should allow (Austad, 2010) – one such group are the bats. Microbats are exceptionally long-lived considering their small body size and high metabolic rates. The maximum lifespan of the bat *Myotis lucifugus,* weighing 8g, is 34 years, whereas the maximum lifespan of a mouse, weighing 30g, is 4 years (Brunet-Rossinni and Austad, 2004). In addition, bats expend double the amount of energy and yet live on average 3 years longer than non-flying mammals (Jürgens and Prothero, 1987). Elucidating how bats maintain such high metabolic rates and extreme longevity, without accumulating cellular damage, is of particular interest.

Recently published data show the adult *P. pipistrellus* bat has a mitochondrial lipid profile which more closely resembles young mice than old (Pollard *et al.*, 2019). This "youthful" profile is characterised by increased levels of brain and muscle docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), and AA (AA), which have been linked to neuroprotection and inflammation mediation (Zárate *et al.*, 2017). Proteomic analysis of *P. pipistrellus* mitochondria revealed a potential player in the promotion of the bat lipid profile: heart-type fatty acid binding protein (FABP3) – which was found to be significantly increased in adult bats compared to both young and old mice (Pollard *et al.*, 2019).

In the brain and skeletal muscle, the predominant FABP is heart-type fatty acid binding protein (FABP3). Several lines of evidence implicate FABP3 in the

maintenance of an adequate energy supply. Firstly, FABP3-null mice show decreased palmitate oxidation by β-oxidation, and a shift in cardiac muscle fuel selection from long-chain fatty acids (LCFA) to glucose (Furuhashi and Hotamisligil, 2008). Secondly, during skeletal muscle cell differentiation, FABP3 is upregulated alongside a concomitant increase in utilisation of LCFA via β-oxidation and esterification (Zhu *et al.*, 2011). Some data also suggest FABP3 may interact with the PPAR family of proteins, further supporting its role in β-oxidation (Tsukahara *et al.*, 2014a).

FABP3 modulation is connected with age and diseases of age. Expression of FABP3 is increased in response to longevity promoting activities: fasting (Sánchez-Gurmaches *et al.*, 2011) and exercise (Mulya, Jacob M Haus, *et al.*, 2017). In Alzheimer's disease and Down syndrome (a disease of premature ageing), individuals have significantly lower levels of FABP3 (Cheon *et al.*, 2003). There is growing interest surrounding the role of FABP3 in Parkinson's disease (PD), where it has been shown to be increased in the CSF of PD patients (Mollenhauer *et al.*, 2007).

This Chapter aims to characterise a role for FABP3 in lifespan, its influence upon mitochondrial function and its implication in age-related disease. Western blotting was used to assess levels of FABP3 in long-lived bat and short-lived mouse muscle. To determine a role in longevity, FABP3 orthologues were knocked-down in *C. elegans* and mitochondrial function analysed to determine mechanistic underpinnings. In addition, *D. melanogaster* and the Ingram Counterbalanced Exercise (ICE) machine were used to

determine exercise-dependent effects on this understudied protein. This study is predicted to inform upon the utility of FABP3 as a potential targetable biomarker for ageing and diseases of age.

4.2 Methods

4.2.1 Ethics

This study was approved by the School of Veterinary Medicine and Science Ethics and Clinical Review Panel, University of Nottingham (ERN# 1922 170103). Mouse tissues were obtained from Charles River Laboratories. All mice were humanely killed by a trained individual using approved schedule 1 methods, in full accordance with UK Home Office guidelines.

4.2.2 Tissue and fraction preparation

Brain and skeletal muscle were dissected from adult (20-week-old) and old (73-week-old) C57BL/6J mice (Charles River) and adult *P. pipistrellus* bats (donation from the West Yorkshire Bat Trust). Purkinje cell degeneration mice, *pcd*^{5J}, aged 9-17 weeks old were bred and kept under licence at the University of Nottingham. Bat and mouse mitochondria were isolated as described previously (A. K. Pollard *et al.*, 2016). Whole *D. melanogaster* underwent 5 minutes of manual homogenisation using a 1.2-2ml Eppendorf micro pestle (Sigma-Aldrich) in mitochondrial extraction buffer (50mM Tris-Cl pH 7.4, 100mM KCL, 1.5mM MgCl2, 1mM EGTA, 50mM HEPES and 100mM Sucrose). 100µl of buffer was added for every 10 *D. melanogaster*. Homogenates were centrifuged at 800g for 15 minutes at 4°C. Supernatants were transferred to fresh Eppendorf's and stored at -80°C.

4.2.3 Western Blotting

Western blotting was carried out as described previously (Shephard *et al.*, 2014). Antibody dilutions: FABP3 PA5-13461 (ThermoFisher) 1:1000 in 3% (w/v) BSA in 1xTBS-T (mouse and bat); FABP3 PA5-13461 (ThermoFisher) 1:1000 in 5% (w/v) milk powder in 1xTBS-T (*D. melanogaster*); GAPDH ab9458 (Abcam) 1:5000 dilution in 3% (w/v) BSA in 1xTBS-T; β-tubulin ab6046 (Abcam) 1:2000 in 3% (w/v) BSA in 1xTBS-T (bat and mouse); β-tubulin ab6046 (Abcam) 1:2000 in 5% (w/v) milk powder in 1xTBS-T (*D. melanogaster*). Bat and mouse muscle mitochondrial samples were normalised to GAPDH levels. *Pcd*^{5J} mouse mitochondrial samples were normalised to β-tubulin. *D. melanogaster* samples were normalised to β-tubulin levels. The average of three samples for each condition (bat, mouse and fruit fly) were plotted showing the mean +/- SEM. Statistical analyses were carried out in GraphPad Prism.

4.2.4 Sequence similarity and Phylogenetic tree

Multiple sequence alignment containing the sequences of *H. sapiens* FABP3 and *C. elegans* FABP3 orthologues LBP-1 to -9 was created from UCSC genome browser database, accession numbers for which are given in Table 2.6. Amino acid residues are coloured shades of blue signifying their sequence similarity; lightest is 30%, darkest is 100%. Sequence similarity data are shown in Table 2.7. Sequence alignment graphic was produced using Jalview and phylogeny determined by percentage accepted mutation (PAM) 250 matrix (Waterhouse *et al.*, 2009).

4.2.5 Lifespan assay

C. elegans strain CB5600 (ccIs4251) [(pSAK2) myo-3p::GFP::LacZ::NLS + (pSAK4) myo-3p::mitochondrial GFP + dpy-20(+)] were used for all control and knockdown lifespan assessments (Gaffney *et al.*, 2014). *C. elegans* were maintained on NGM agar plates and age synchronised using standard methods (A. Pollard *et al.*, 2016). RNAi *E. coli* [HT115 (DE3)] against *lbp-4* (ZK742.5), *-5* (W02P3.7) and *-6* (W0203.5) were used. Each RNAi agar plate was grown overnight in nutrient broth. Control plates were seeded with *E. coli* strain OP50 or RNAi food as appropriate. Twenty age synchronised L4 *C. elegans* were added to each plate. Animals were scored each day for activity and those not moving and that did not respond to gentle needle stimulation were scored as dead. The experiment was maintained at 20°C. Kaplain-Meier curve and log-rank statistical analysis were carried out using GraphPad Prism.

4.2.6 Fluorescence imaging GFP-tagged muscle mitochondria and nuclei

C. elegans strain CB5600 (ccIs4251) [(pSAK2) myo-3p::GFP::LacZ::NLS + (pSAK4) myo-3p::mitochondrial GFP + dpy-20(+)] with GFP fusion proteins localised to mitochondria and nuclei were used to assess mitochondrial morphology (Gaffney *et al.*, 2014). *C. elegans* were age-synchronised using standard methods and grown for 48-60 hours at 20°C until they reached L4 stage. Twenty *C. elegans* were picked into 20µl M9 buffer on a microscope slide. A coverslip was applied and *C. elegans* were imaged using the Leica

CTR5000 under a fluorescence filter (460-500nm, blue light excitation) at 100X magnification.

4.2.7 Oxygen consumption

Oxygen consumption trials were carried out using 5 *C. elegans* per well with four replicate wells for each knockdown and control. *C. elegans* were age synchronised as described previously (A. Pollard *et al.*, 2016). Oxygen consumption measurements were conducted on newly adult *C. elegans*, 48-72 hours after age synchronisation (termed: adult day 0). Oxygen consumption was measured at 15 second intervals continuously for 1 hour. Additional trials were conducted at adult days 4, 8 and 12. Oxygen consumption over time was calculated using the first 15 minutes of each trial. A one-point calibration was used to artificially adjust oxygen saturation of all wells to 100% prior to beginning. All data were corrected for volume of buffer and total protein per well, expressed as μl/min/mg protein.

4.2.8 Free fatty acid quantification

Total fatty acid concentration was measured using a Free Fatty Acid Quantification Kit (AB65341, Abcam), according to manufacturer's instructions. Briefly, bat and mouse mitochondrial samples, or 4 plates of cleaned adult day 0 *C. elegans* were suspended in chloroform/Triton X-100 (1% Triton X-100) and homogenised by 30 strokes of a 1.5ml mini-pestle, vortexed and centrifuged at 16,000g for 10 minutes. Lower phases of the samples were collected and vacuum dried for 30 minutes. Fatty acid concentrations were

quantified by fluorometric analysis (Varioskan LUX Multimode Microplate Reader). Bat and mouse measurements were carried out in duplicate.

4.2.9 D. melanogaster exercise

4.2.9.1 Maintaining stock populations

D. melanogaster stocks ($Pink1^{B9}$ and W^{1118}) were a kind gift from the laboratory of Dr. Nicoletta Moisoi. *D. melanogaster* were kept in 9.5cm tall glass vials in an incubator at 25°C. Stock populations of *D. melanogaster* were monitored for over-crowding and food quality. Stock populations of adult *D. melanogaster* were moved to autoclaved glass vials with fresh food every two weeks. Newly eclosed adult *D. melanogaster* were separated from stock populations and used for the following exercise regime.

4.2.9.2 Exercise regimes

D. melanogaster were exercised using the Ingram Counter-balanced Exercise (ICE) machine, which is designed to prime the innate negative geotaxis response of *D. melanogaster* (Figure 2.3).

Vials of approximately twenty newly eclosed wild-type adult male (W^{1118}) or $Pink1^{B9}$ *D. melanogaster* were exercised for 30 minutes per day for 7 days. Exercised group vials were stoppered with cotton wool 6cm from the food; non-exercised group vials were stopped with cotton wool 1cm from the food, creating a physical barrier to activity. Both exercised and non-exercised groups were subjected to the ICE machine. *D. melanogaster* were exercised in

the morning each day. *D. melanogaster* were sacrificed by freezing at -80°C one hour after the final exercise bout.

4.3 Results

4.3.1 Greater FABP3 and free fatty acid levels characterise bat muscle mitochondria

Significant differences have been seen in the brain and muscle mitochondrial lipidome of the long-lived *P. pipistrellus* bat compared with young and old mice. In addition, proteomic studies identified differences in the protein expression of the intracellular lipid binding protein FABP3, with bats showing increased levels compared to mice (Pollard *et al.*, 2019). The present work was motivated by continued interest in FABP3 as potentially important for exceptional longevity.

2D-gel electrophoresis coupled with LC/MS-MS identified FABP3 as increased in bat muscle mitochondria (Pollard *et al.*, 2019). This observation was verified by Western blot - bat muscle mitochondrial FABP3 protein expression is significantly higher than adult mouse (20-week-old) muscle mitochondria (p=0.0367) (Figure 4.1A). Further, comparison between adult (20-week-old) and old (73-week-old) mouse muscle mitochondria showed increased FABP3 protein levels with age (Figure 4.1B) (p=0.0413).

FABP3 plays a significant role in intracellular lipid transport (Tsukahara *et al.*, 2014b). Analysis of bat, adult and old mouse free fatty acid (FFA) levels was carried out (Figure 4.1C). Bat muscle mitochondrial FFA levels paralleled the high protein levels of FABP3 – bats have significantly greater FFA compared to adult and old mice (p=0.0049, and p=0.0024, respectively). Interestingly,

although a significant increase in FABP3 protein levels is clear in old compared to adult mouse muscle mitochondria, this was not paralleled by

FFA levels – old mouse muscle mitochondrial FFA levels are not significantly different to adult mouse.

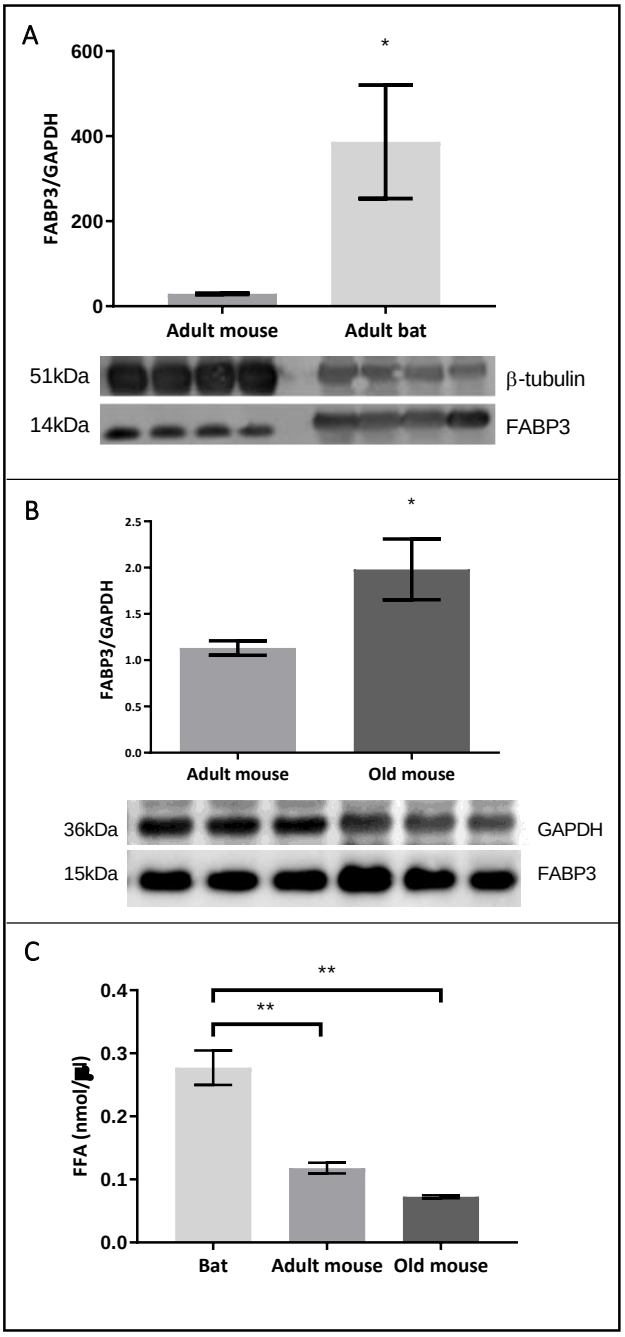


Figure 4.1 (A) Fatty acid binding protein 3 (FABP3) protein levels are significantly greater in bat muscle mitochondria compared to adult mouse muscle mitochondria. FABP3, normalised to β-tubulin, is increased in the muscle mitochondria of *Pipistrelle pipistrelle* bats compared with adult (20-week-old) C57BL/6J mice; p=0.0367 (Unpaired t-test). Columns display mean protein expression ± SEM (Adult bat muscle mitochondria n=4; 20-week-old

mouse muscle mitochondria n=4). **(B)** Fatty acid binding protein 3 protein levels significantly increase with age in mouse muscle mitochondria. FABP3, normalised to GAPDH, is increased in the brains of old (73-week-old) C57BL/6J mice, compared to adult (20-week-old) mice; p=0.0413 (Unpaired T-test). Columns display mean protein expression ± SEM (20-week-old mouse muscle mitochondria n=3; 73-week-old mouse muscle mitochondria n=3). **(C)** Free fatty acid (FFA) concentration is greater in bat muscle mitochondria compared to mouse. FFA of adult bat, adult (20-week-old) and old (73-week-old) C57BL/6J mouse muscle mitochondrial isolates were compared (n=2 each condition). FFA concentration was significantly increased in mitochondria of adult bat muscle compared with adult or old mouse muscle (p=0.0049, and p=0.0024; one-way ANOVA, respectively). Data shown are mean ± SD; statistical analysis was carried out using GraphPad Prism 7.03.

4.3.2 Knockdown of *C. elegans FABP3* orthologues significantly reduces lifespan and alters physiological parameters

There is a lack of information regarding the role of FABP3 in mitochondrial function, ageing and diseases of ageing. The FABP family have an exceptionally well-conserved role (Storch and McDermott, 2009). *C. elegans* contain a family of nine lipid binding proteins (LBP). Sequence alignment of LBP family proteins (Figure 4.2) show reasonable sequence similarity (Table 4.1) of many LBP isoforms to *H. sapiens* FABP3. Indeed, key residues required for formation of the beta-barrel structure which houses lipid ligands are conserved, including multiple N-terminal residues and the arginine binding site residue 127 (*H. sapiens*) (Offner *et al.*, 1988; Spillantini *et al.*, 1997) (Figure 4.2).

RNA interference (RNAi) is a powerful technique to temporally knockdown genes of interest. An advantage of *C. elegans* is the ease of RNAi utilisation and the broad library of available *E. coli* expressing specific dsRNA. In the present work, accessibility to *lbp-4, -5, -6* RNAi drove the selection to knockdown these sequences.

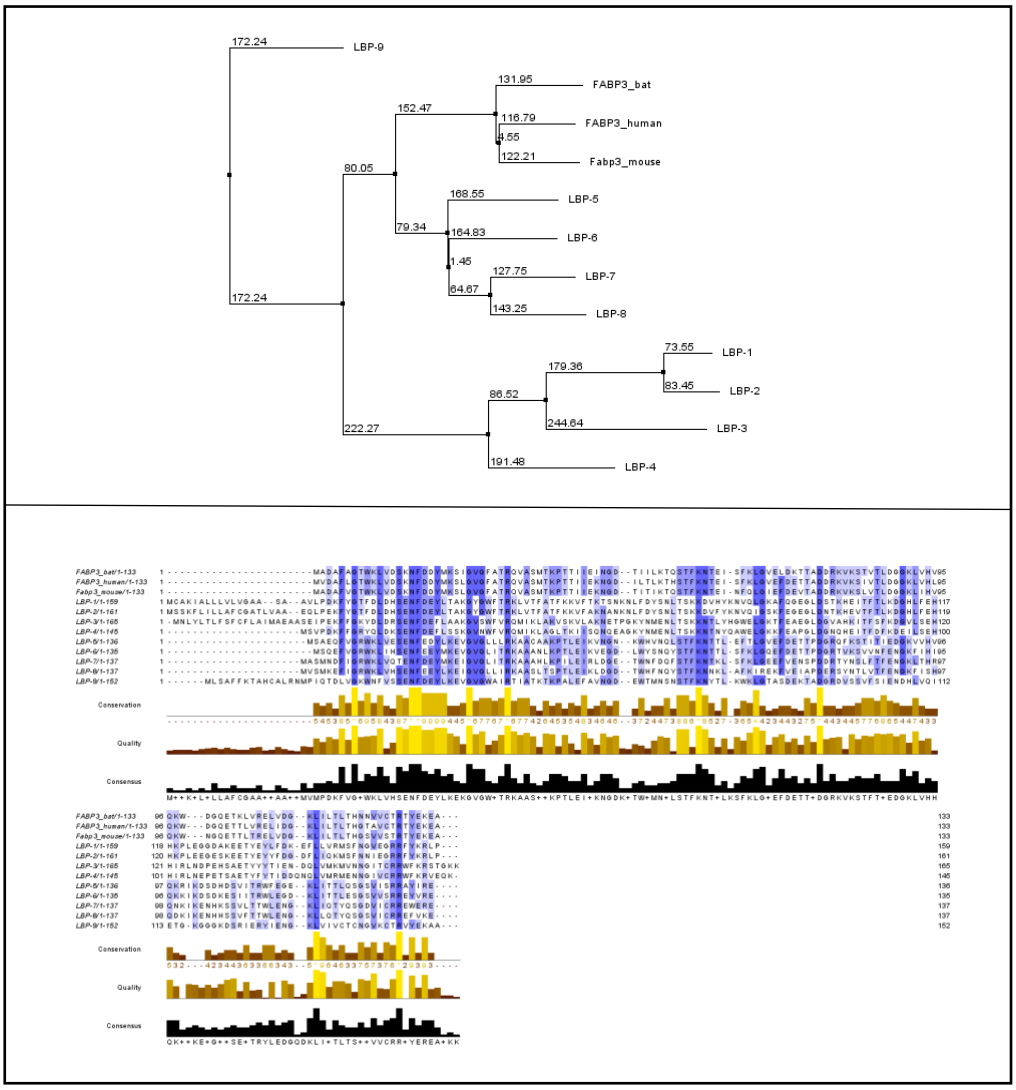
Day 0 adult (L4 stage) WT *C. elegans* were fed *E. coli* strain OP50 or *lbp-4, -5 or -6* RNAi food. *lbp-4* and *lbp-6* knockdown significantly reduced lifespan compared to control worms (p=0.0452 and p=0.0004, respectively) (Figure 4.3A). Although a trend toward decreased lifespan was observed with *lbp-5* knockdown this did not reach the significance threshold (p=0.1588).

Next, *C. elegans* were assessed for their oxygen consumption across the lifespan (Figure 4.3B). All knockdown and control conditions showed a trend toward increasing oxygen consumption with age. *lbp-5 and lbp-6* knockdown *C. elegans* showed significantly increased oxygen consumption over their lifespan, compared to OP50 fed (p= 0.0029 and p=0.0035, respectively). A trend toward increased oxygen consumption was apparent in *lbp-4* knockdown *C. elegans*, however, this did not reach the significance threshold. The rate of oxygen consumption is greatest in *lbp-5* knockdown *C. elegans*; significance is reached at day 4, compared with control (p=0.0266). *lbp-6* knockdown *C. elegans* display a trend toward significance at day 4 (p=0.0933). Similarly, *lbp-5* and *-6* knockdown *C. elegans* oxygen consumption displays a trend toward increased oxygen consumption at day 8, compared with control (p=0.0644 and

p=0.0783, respectively). At day 12, no significant differences in oxygen consumption between knockdown groups and control is apparent.

Due to the role of LBP in intracellular lipid binding and transport, FFA concentration was assessed (Figure 4.3C). Knockdown of *lbp-4* significantly reduced FFA concentration (*p*=0.0177). No significant reduction in FFA concentration is observed in *lbp-5* or *-6* knockdown *C. elegans*, compared to OP50 fed.

C. elegans strain CB5600 are superficially WT and contain GFP-labelled muscle wall nuclei and mitochondria, enabling assessment of mitochondrial morphology by microscopy. OP50 fed *C. elegans* display elongated and orderly mitochondria. In comparison, *lbp-4, -5 and -6* knockdown *C. elegans* have considerably altered mitochondrial dynamics – a dispersed and disordered mitochondria phenotype (Figure 4.3D).


172.24
LBP-9
131.95
FABP3_bat
152.47
116.79
FABP3_human
4.55
122.21
Fabp3_mouse
80.05
168.55
LBP-5
79.34
164.83
LBP-6
1.45
127.75
LBP-7
64.67
143.25
LBP-8
172.24
73.55
LBP-1
179.36
83.45
LBP-2
86.52
244.64
LBP-3
222.27
191.48
LBP-4

Alignment reference	Orthologue (*C. elegans*)	Sequence Homology
FABP3 (*H. sapiens*)	LBP-1	27.46%
	LBP-2	28.85%
	LBP-3	21.32%
	LBP-4	23.74%
	LBP-5	45.52%
	LBP-6	48.15%
	LBP-7	44.03%
	LBP-8	38.17%
	LBP-9	41.22%

Table 4.1 FABP3 *C. elegans* protein orthologues LBP-1 to -9 percent sequence homology data. Sequence data were generated using UCSC genome browser database accession data (Table 2.7). Sequence homology was determined using EMBL-EBIs MUSCLE (MUltiple Sequence Comparison by Log-Expectation) tool.

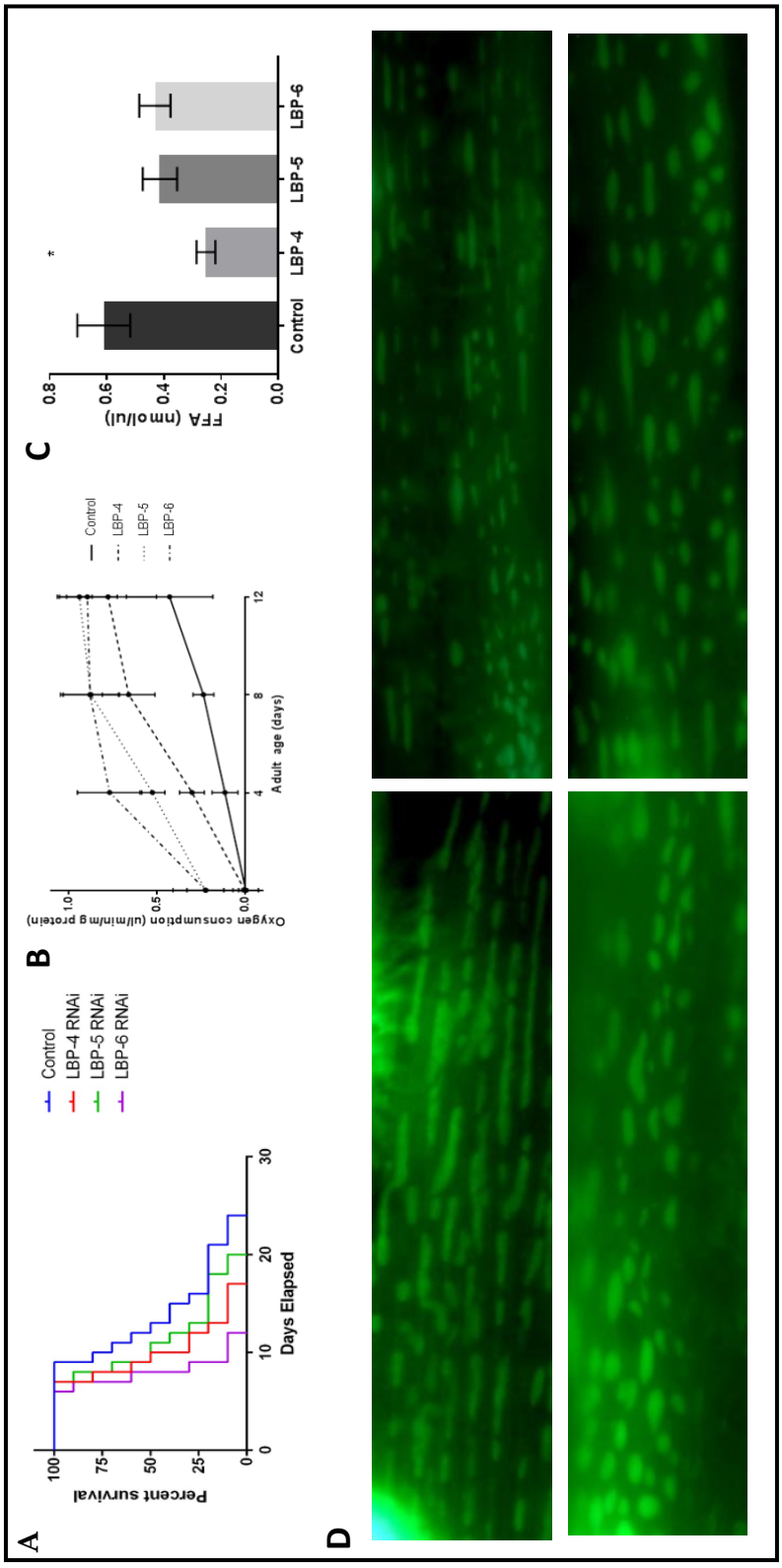


Figure 4.3 (A) Knockdown of *C. elegans* FABP3 orthologues *lbp-4* and *-6* significantly decreased lifespan. *C. elegans* fed *lbp-4* or *-6* RNAi from adult day 0 showed a significant decrease in lifespan (p=0.0452 and p= 0.0004; Log-rank test, respectively), compared with control. Knockdown of *lbp-5* from adult day 0 did not significantly alter lifespan (p=0.1588; Log-rank test). Median lifespans were 12.5, 9.5, 10 and 8 days respectively, for control, *lbp-4*, *-5* and *-6*

RNAi, respectively. **(B)** Oxygen consumption rate is significantly greater across the lifespan in *lbp-5* and *-6* knockdown than control. Oxygen consumption increased with age in all worms. *lbp-5* and *-6* knockdown worms showed significantly increased oxygen consumption across their lifespan; p= 0.0029 and p=0.0035; two-way ANOVA, respectively. *lbp-4* oxygen consumption was not significantly greater than control. Oxygen consumption rates were measured over 15 minutes at 25°C. Each data point is the mean value of four different measurements, where error bars represent the standard error mean. N=20 for each group; 5 *C. elegans* per well. **(C)** Free fatty acid concentration is decreased in LBP-4 knock-down worms. FFA analysis of CB5600 strain *C. elegans* and *lbp-4*, *-5* and *-6* knock-down worms. A significant reduction in free fatty acid concentration was observed in *lbp-4* knockdown worms, compared with control; p=0.0177; one-way ANOVA. FFA concentration was normalised to protein content of worm homogenates. Data are presented as mean ± SEM. **(D)** *C. elegans* mitochondrial dynamics are altered upon knockdown of *lbp-4*, *-5* or *-6*. CB5600 strain *C. elegans* grown on OP50 strain *E. coli* (top left) exhibit an elongated mitochondrial shape but show dispersed and disordered mitochondria when grown on *lbp-4* (top right), *-5* (bottom left) or *-6* (bottom right) RNAi food. All *C. elegans* in this figure were adult and carry the transgene ccIs4251 with GFP fusion proteins localised to the body muscle mitochondria and nuclei.

4.3.3 FABP3 protein levels are reduced in animal models of neurodegeneration

Alterations in lipid content have been shown in the brains of individuals with neurodegenerative disease (Janssen and Kiliaan, 2014; Zárate *et al.*, 2017). Indeed, FABP3 has recently been implicated in the pathogenesis of PD (Shioda *et al.*, 2014a; Cheng *et al.*, 2019). In this work, two animal models of neurodegeneration were used to assess FABP3 changes: *Pink1*$^{-/-}$ *D. melanogaster*, and *pcd*^{5J} mouse. *Pink1*$^{-/-}$ whole *D. melanogaster* homogenate and *pcd*^{5J} mouse brain have significantly reduced FABP3 protein levels compared

with age-matched wild-type (p=0.0034 and p=0.0202, respectively) (Figure 4.4B).

4.3.4 Exercise restores FABP3 protein levels in *Pink1*$^{-/-}$ *D. melanogaster*

Exercise alleviates symptoms of ageing and delays the onset of age-related disease (Short *et al.*, 2004; Mikkelsen *et al.*, 2013; Viña *et al.*, 2016) and it is therefore prudent to investigate the molecular underpinnings of this benefit. To do this, *Pink1*$^{-/-}$ and WT male *D. melanogaster* were exercised using the ICE machine. *D. melanogaster* were subjected to 30 minutes of exercise per day, for 7 days. Exercise significantly increased FABP3 protein levels in *Pink1*$^{-/-}$ *D. melanogaster* compared to non-exercised (p=0.0299) (Figure 4.4A). Further, the new protein level was not significantly different from WT; exercise restored *Pink1*$^{-/-}$ FABP3 protein levels to that of WT. Exercise did not significantly alter the protein levels of FABP3 in male WT *D. melanogaster*.

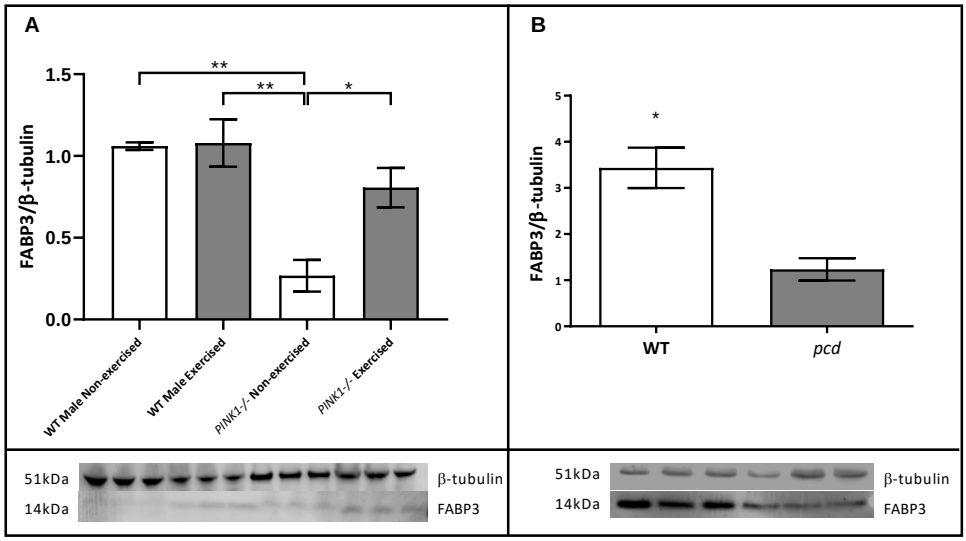


Figure 4.4 (A) FABP3 protein levels are decreased in the *Pink1*$^{-/-}$ *D. melanogaster*. FABP3, normalised to β-tubulin, is significantly decreased in non-exercised *Pink1*$^{-/-}$ whole cell homogenate compared to non-exercised wild type *D. melanogaster*; p=0.0034; two-tailed, unpaired t-test. Exercise restored FABP3 protein levels in *Pink1*$^{-/-}$ to WT levels; p=0.0299; two-tailed, unpaired t-test (wild type exercised n=3; wild-type non-exercised n=3; *Pink1*$^{-/-}$ exercised n=3; *Pink1*$^{-/-}$ non-exercised n=3). Homogenates were obtained from twenty pooled *D. melanogaster*; replicates were obtained from separately pooled *D. melanogaster*. All *D. melanogaster* were sacrificed one hour after the final exercise bout. Columns display mean protein expression ± SEM. * = $p<0.05$, **=$p<0.005$ **(B)** FABP3 levels are decreased in the Purkinje Cell Degeneration (*pcd*^{5J}) mouse model. FABP3 protein levels, normalised to β-tubulin, are significantly decreased in *pcd*^{5J} mouse brain mitochondria compared to age-matched WT; p=0.0202; two-tailed, unpaired t-test. *pcd*^{5J} mice n=3; WT mice n=3. Columns display mean protein expression ± SEM.

4.4 Discussion

Mitochondrial lipid and protein differences have been shown between the mouse and long-lived *P. pipistrellus* bat (Pollard *et al.*, 2019). The analysed tissue types in this study were brain and muscle, as these contain mainly post-mitotic cells, which show declines with age. Further, mitochondria from brain and muscle are known to exhibit distinct age-related changes due to the metabolic requirements of these tissues. Identifying biological differences with age is likely to improve our understanding of how ageing manifests.

Lipidomic analysis, carried out by Pollard *et al*, identified numerous lipid species to be more abundant in bat mitochondria than mouse – ceramide (32:1), *N*-acylethanolamines (18:2 and 22:6) and a docosanoid species. In addition, metabolic precursors of inflammatory lipids, *N*-acylethanolamines, DHA, DPA and AA, were found to be more highly enriched (Pollard *et al.*, 2019). Evidence supports the benefits of high dietary PUFA levels, and in particular DHA, on ageing tissues and membranes (Dyall, 2015; Mulya, Jacob M. Haus, *et al.*, 2017b) – the enrichment in bat tissues is likely to be protective. Proteomic analysis identified FABP3 to be significantly upregulated in bat muscle mitochondria (Pollard *et al.*, 2019). PUFAs are transported intracellularly by FABP3 suggesting that high mitochondrial levels of FABP3 may be due to the higher quantities of PUFAs, or vice versa. Muscle FABP3 changes with age have not been characterised, however, the lipidomic and proteomic data suggest that increased fatty acids and their intracellular transport are relevant to bats exceptional longevity (Pollard *et al.*, 2019).

4.4.1 Increased FABP3 in bat muscle is connected to increased FFA concentration

This Chapter investigated the potential role FABP3 has in promoting longevity. Western blotting verified an increased abundance of FABP3 protein in bat muscle mitochondria. In parallel, an increased concentration of FFA levels was apparent in bat muscle mitochondria. Further, isolated mitochondria from adult and old mouse muscle revealed an age-dependent increase in FABP3 levels. Interestingly, this increase with age was not mirrored by an increase in FFA – adult and old mouse mitochondrial FFA concentration is not significantly different. The FABP3 protein data are in agreement with another study showing increased FABP4 levels in the muscle of old rats (Tucker and Turcotte, 2002). The same study also showed that ageing was associated with a marked increase in muscle triglyceride (TG) levels (Tucker and Turcotte, 2002). TGs are not FFA and would therefore not be measured with the FFA quantification kit used in this study. FABP3 in old mice may increase the content of esterified fatty acids, as ageing is associated with lipid droplet accumulation within skeletal muscle cells (Crane *et al.*, 2010; Copes *et al.*, 2015; Nielsen *et al.*, 2017). In agreement, Pollard *et al.* showed an increase in abundance of triglycerides in old compared with young skeletal muscle (Pollard *et al.*, 2019). Increasing intracellular TGs has been shown to be associated with ageing (Crane *et al.*, 2010; Copes *et al.*, 2015) and diseases of age, including diabetes and cardiovascular disease (Nielsen *et al.*, 2017) – it would be interesting to elucidate when age-related TG accumulation begins in

normal ageing, as this may be another important target for healthy ageing. 18-month-old mice were chosen in this work in order to interrogate the earliest stages of ageing – it is possible that FFA levels show marked differences with more advanced age than investigated here. Indeed, Tucker and Turcotte used rats and compared 5-, 15- and 24-month-old muscle (Tucker and Turcotte, 2002).

4.4.2 FABP3 expression is linked to mitochondrial function

To investigate the influence of FABP3 on mitochondria, *FABP3 C. elegans* orthologues, *lbp-4, -5* and *-6* were knocked down. RNAi knockdown reduced FFA levels and lifespan. In agreement, others have shown that knockdown of *lbp-8* regulates of longevity in *C. elegans* (Folick *et al.*, 2015). In this study, *lbp-4* knockdown caused mitochondrial functional changes and altered morphology. *lbp-5* mutation has previously been shown to increase glycolysis, possibly to compensate for dysfunctional mitochondria (Xu, Choi and Paik, 2014). Like study one, no confirmation of *FABP3* orthologue knockdown was carried out. Validation of a successful knockdown by RT-PCR and the presence of residual protein by Western blotting would be wise.

Much of the current literature surrounding FABP3 is connected to its role in cardiac health. In humans, patients with post-operative atrial fibrillation show reduced *FABP3* gene expression, suggesting a link between atrial fatty acid transport and increased atrial fibrillation onset after cardiac surgery (Shingu *et al.*, 2018). FABP3 has been suggested as a marker of myocardial damage – FABP3 protein levels are decreased in LPS administered heart muscle (Memon

et al., 1999). In addition, it is suggested to be an important factor in mitochondrial β-oxidation. *Fabp3*$^{-/-}$ mice show decreased palmitate oxidation (Binas *et al.*, 1999; Das, 2016), and a shift in cardiac fuel selection from LCFA to glucose, with concomitant reduction in exercise tolerance and cardiac hypertrophy in older animals (Binas *et al.*, 1999). Ageing is associated with a decrease in fatty acid oxidation at rest, following a meal, and during exercise (Toth and Tchernof, 2000). Conversely, exercise and the long-lived *C. elegans* strain, *eat-2*, show an upregulation in genes associated with β-oxidation (Chamoli *et al.*, 2014; Bustos and Partridge, 2017). Collectively, current data point to an intimate connection between FABP3, β-oxidation and fatty acid fuel utilisation. It would be interesting to study the β-oxidation pathway in bats and mice, as evidence suggest changes with age, a connection to longevity, and the involvement of FABP3 in its regulation.

4.4.3 FABP3 is altered in neurodegenerative models and diseases of age

Neurodegenerative disorders and their symptoms are associated with alterations in lipid profiles across intracellular compartments. Decreases in plasma and brain levels of DHA have been observed in individuals with Alzheimer's disease (AD) (Schaap *et al.*, 1999), and diets high in *ω*-3 PUFA are correlated with a decrease in risk of depression and/or dementia (Schaap *et al.*, 1999). FABP3 is decreased in both Down syndrome and AD (Cheon *et al.*, 2003), suggesting the altered lipid content and decreased FABP3 in AD may be

relevant to the disease. Indeed, in this study FABP3 levels are decreased in muscle mitochondria of the *pcd*^{5J} mouse and whole *Pink1*$^{-/-}$ *D. melanogaster*.

Exercise prevents and alleviates symptoms of ageing and age-related disease (Mikkelsen *et al.*, 2013; Viña *et al.*, 2016). Here, exercise training reversed the reduced FABP3 levels in *Pink1*$^{-/-}$ *D. melanogaster*. Increased FABP3 with exercise has previously been shown and suggested to be due to enhanced muscle utilisation of fatty acids for β-oxidation (Samieri *et al.*, 2011). Together, these data suggest higher FABP3 levels are protective and that mechanisms which raise its levels warrant further study.

4.5 Conclusion

This Chapter has shown that decreased levels of FABP3 orthologues in *C. elegans* are associated with mitochondrial dysfunction and reduced longevity. In agreement with data from progeroid syndromes and age-related neurodegenerative diseases (Cheon *et al.*, 2003), the present study showed decreased FABP3 protein levels in *Pink1*$^{-/-}$ and *pcd* animal models. Further, exercise partially reversed this decline in *Pink1*$^{-/-}$ *D.* melanogaster.

Increasing levels of FABP3 or specific fatty acids within mitochondria are likely supportive of mitochondrial health – mechanisms which assist this, including exercise, warrant further investigation.

5 Mitochondrial proteomic analysis of exercised *Pink1*$^{-/-}$ *D. melanogaster* reveals wide-spread reductions in protein expression

5.1 Introduction

The Greek physician, Hippocrates of Kos, prescribed exercise as central to what he termed 'regimen' - "eating alone will not keep a man well; he must also take exercise" (Sallis, 2009). Galen of Pergamum, physician to the Roman emperor Marcus Aurelius built upon the philosophy of Hippocrates and included exercise as one of his six factors that described his "hygiene" (named after the goddess of health, Hygieia) theory of health. Galen proposed that exercise should be used in moderation, as too much or little would put the body in imbalance and lead to disease or illness (Sallis, 2009). Only relatively recently, since the 20th Century, has medicine reduced its focus on Galenic laws and cast its gaze on disease, drugs and surgery.

Strong evidence showing chronic diseases are influenced by lifestyle has caused a resurgence of interest in the Hippocratic regimen and Galenic laws. With the advent of the modern scientific method, exercise has been shown effective at combating numerous chronic diseases, including: cardiovascular diseases (Nystoriak and Bhatnagar, 2018), diabetes (Asano *et al.*, 2014), cancers (Cormie *et al.*, 2017) and neurodegenerative diseases (Cass, 2017; Oliveira de Carvalho *et al.*, 2018). Further, all-cause mortality is reduced with regular exercise and it extends healthspan (Garcia-Valles *et al.*, 2013).

Parkinson's disease (PD) is characterised by a progressive loss of the dopaminergic neurones of the *substantia nigra pars compacta* (*SNpc*). The cardinal motor symptoms are tremor, rigidity, dyskinesia, and postural instability. It affects 1-2% of the population and is the second most common neurodegenerative disease (Schapira and Jenner, 2011). The majority of PD presents as idiopathic, however, autosomal dominant and recessive familial forms have been identified, all of which present with similar symptomology (Geisler *et al.*, 2010; Hong *et al.*, 2010; Heo *et al.*, 2015). Interestingly, the identified mutations influence autophagic processes at various points, strongly implicating autophagic dysfunction in PD aetiology.

Although end-point outcome measures in human studies are not standardised, there is broad agreement that aerobic exercise training in PD individuals is beneficial and improves motor symptoms significantly (Oliveira de Carvalho *et al.*, 2018). Evidence supports resistance training for improving muscle strength, volume, and mobility, in PD individuals (Ramazzina, Bernazzoli and Costantino, 2017). Non-motor symptoms, such as executive function, depression and sleep disturbances, are positively affected by exercise (Wu, Lee and Huang, 2017).

Understanding the molecular mechanisms underlying the exercise-related benefits for ageing and diseases of ageing is a major goal. In vertebrates, the scientific community has made headway toward characterising the broad molecular changes that exercise evokes. These changes include: increased mitochondrial efficiency via upregulated PGC1α (Anderson *et al.*, 2008),

altered energy metabolism (Short *et al.*, 2004), AMPK downstream regulatory changes (Musi *et al.*, 2001), neurotrophic responses (Sleiman *et al.*, 2016), ROS-scavenging enzyme alterations (Hollander *et al.*, 1999), stress-response changes (Bejma, Ramires and Ji, 2000), lipid metabolism alterations (Tunstall *et al.*, 2002), and increased autophagic flux (Garvey *et al.*, 2015). Furthermore, sex-specific variation in the response to exercise may exist. Women undergoing exhaustive exercise show menstrual cycle phase specific differences in anti- and pro-inflammatory gene expression (Timmons *et al.*, 2005). In addition, the expression of genes encoding metabolic responses, cell cycle, apoptosis, brain function and transcriptional regulation were differentially modulated between sexes (Abbasi *et al.*, 2016). The cellular changes that occur due to exercise are clearly holistic. Picking apart the key mediators and precise alterations in a timely fashion, to meet the demands of modern medicine, will require a more rapid approach.

D. melanogaster offer a well characterised and understood model system for exercise studies. Rodent models are costly and ethically fraught - a shift away from vertebrate experimentation is a target of numerous governments and organisations (NC3Rs, 2019). *D. melanogaster* are easy to maintain in large isogenic populations with fully controlled environmental backgrounds (Jennings, 2011). Further, *D. melanogaster* boast a well-established genetics model, with a fully sequenced and annotated genome and large choice of mutants and populations (Mackay *et al.*, 2012). Additionally, over 75% of

human disease genes also exist in *D. melanogaster* (Ugur, Chen and Bellen, 2016).

A number of research groups have developed bespoke automated machinery to exercise *D. melanogaster* (Piazza *et al.*, 2009; Watanabe and Riddle, 2017; Damschroder *et al.*, 2018; Lowman *et al.*, 2018). These machines prime their innate negative geotaxis response repeatedly over a fixed time period. These groups have shown that exercise modifies *D. melanogaster* behaviour and causes molecular changes reminiscent of improved healthspan in vertebrates – reduced age-related decline in mobility, improved cardiac performance and improved mitochondrial efficiency (Piazza *et al.*, 2009; Martin J. Tinkerhess, Healy, *et al.*, 2012; Watanabe and Riddle, 2017).

The present work aims to extend the body of evidence supporting *D. melanogaster* as a model for exercise research and its translatability to currently known work in humans and other vertebrate species. With the growing interest in sex-specific biological differences, and the value that exercise has for promoting healthspan and improving outcomes in PD individuals, this work utilises both male and female wild-type (WT) and the PD model, $Pink1^{B9}$ ($Pink1^{-/-}$), to interrogate the effect of exercise on behavioural and functional outcomes. It is hypothesised that males and females will elicit disparate behavioural and biochemical changes due to exercise, that *Pink1-/-* will have a favourable response, and that exercise will cause widespread cellular changes not limited to any one cellular compartment.

5.2 Methods

5.2.1 Maintaining stock populations

5.2.1.1 D. melanogaster

D. melanogaster stocks were obtained from the laboratory of Dr. Nicoletta Moisoi (W^{1118} and $Pink1^{B9}$). *D. melanogaster* were kept in 9.5cm tall glass vials in an incubator at 25°C. Stock populations were monitored for over-crowding and food quality. Stock populations of adult *D. melanogaster* were moved to autoclaved glass vials with fresh food each week or when food quality appeared poor.

5.2.1.2 C. elegans

The superficially WT *C. elegans* strain CB5600 [ccIs4251 I; him-8(e1489) IV] and *Pink1*$^{-/-}$ strain CC46 [ccIs4251 I; *pink-1*(*tm1779*) II; *him-8*(*e1489*) IV] were used in this work (Gaffney *et al.*, 2014). *C. elegans* were maintained on NGM agar plates, seeded with *E. coli* strain OP50, and age synchronised using standard methods (A. Pollard *et al.*, 2016). Each week a chunk from *C. elegans* stock plates were cut out using a sterile needle and transferred to a newly OP50-seeded plate. Each week *C. elegans* would be transferred to a new plate to maintain the colony.

5.2.2 D. melanogaster exercise

Approximately twenty WT or *Pink1*$^{-/-}$ *D. melanogaster*, 1-4 days post-eclosion, were separated into glass vials filled with 5ml food. Exercised group vials were stoppered with cotton wool 6cm from the food; non-exercised group

vials were stopped with cotton wool 1cm from the food, creating a physical barrier to activity. Both exercised and non-exercised groups were subjected to the ICE machine for 30 minutes or 10 minutes per day for 7 days. *D. melanogaster* that underwent climbing and ATP concentration assays had 10 minutes of exercise per day; *D. melanogaster* that underwent proteomic analysis had 30 minutes of exercise per day. *D. melanogaster* were exercised in the morning each day. *D. melanogaster* were sacrificed by freezing at -80°C one hour after the final exercise bout in order to determine biochemical changes immediately after the exercise regime.

5.2.3 Climbing assay

Climbing assays were carried out on day 0 and day 7, before the morning exercise. *D. melanogaster* were transferred into empty, flat-bottomed, glass vials. Vials were placed in the Waring rack (Figure 2.2), which has no viewing obstruction of the tubes, a white background for ease of identification and a quadrant scale for scoring. *D. melanogaster* were acclimatised to the new vials for 15 minutes before the video and timer were started. Once every minute for 5 minutes, the rack was tapped sharply against the desk three times to ensure all *D. melanogaster* fell to the bottom. Subsequently, *D. melanogaster* were left for a minute to move freely before the process was repeated.

5.2.4 Climbing index

A modified version of the protocol by Tinkerhess *et al.*, 2012 was used in this work (Tinkerhess *et al.*, 2012). Briefly, each vial was separated into four quadrants. The base of the tube was labelled quadrant zero. From the base to

the top of the tube the quadrants were scored 1 to 4. Climbing score was determined by taking still frames four seconds after the last tap and scoring *D. melanogaster* climbing by the value of the quadrant they reached. If *D. melanogaster* did not move from the bottom it was assigned a 0. An index score was calculated by adding all scores together and dividing by the number of *D. melanogaster* within each vial. Data were analysed using GraphPad Prism (two-way ANOVA).

5.2.5 Fraction preparation

Groups of 10-20 WT or *Pink1*$^{-/-}$ *D. melanogaster* were homogenised in 100-200µl mitochondrial extraction buffer (50mM Tris-Cl pH 7.4, 100mM KCL, 1.5mM $MgCl_2$, 1mM EGTA, 50mM HEPES and 100mM sucrose) by 5 minutes of manual homogenisation using a 1.2-2ml Eppendorf micro-pestle (Sigma-Aldrich). Subsequently, *D. melanogaster* were centrifuged at 800g for 10 minutes, at 4°C, to remove the insoluble fraction. For downstream work involving whole homogenates, the supernatant of this centrifugation step was used. For preparation of mitochondrial samples further centrifugation steps were carried out. Supernatants from the first centrifugation were centrifuged at 1,000g for 10 minutes at 4°C to pellet the nuclear fraction. Supernatants from this centrifugation were centrifuged at 13,200g for 30 minutes at 4°C to pellet the mitochondrial fraction.

5.2.6 Western Blotting

Western blotting was carried out as described previously (Shephard *et al.*, 2014). Antibody dilutions: Troponin-C ab51106 (Abcam) 1:1000 in 5% (w/v)

milk powder in 1xTBS-T; Tropomyosin-1α ab50567 (Abcam) 1:1000 in 5% (w/v) milk powder in 1xTBS-T; α-actinin ab50599 (Abcam) 1:1000 in 5% (w/v) milk powder in 1xTBS-T; ATP5β sc-16690 (Santa Cruz) 1:1500 in 5% (w/v) milk powder in 1xTBS-T; β-tubulin ab6046 (Abcam) 1:2000 in 5% (w/v) milk powder in 1xTBS-T; VDAC ab15895 (Abcam) 1:1000 in 5% (w/v) milk powder in 1xTBS-T. *D. melanogaster* samples were normalised to either β-tubulin or VDAC levels. Where possible, the average of three samples for each condition were plotted showing the mean +/- SEM. Statistical analyses were carried out using GraphPad Prism (two-tailed, unpaired *t*-tests).

5.2.7 2D-gel electrophoresis

Due to practical limitations relating to sample size, preparation of whole *D. melanogaster* homogenates was altered for all 2D-gel electrophoresis work. Briefly, individual whole *D. melanogaster* were subjected to 5 minutes of manual homogenisation using a 1.2-2ml Eppendorf micro-pestle (Sigma-Aldrich) in 140µl 1x Rehydration buffer [8M urea, 2% CHAPS, 2% IPG Buffer, 0.1% bromophenol blue, 20mM DTT (added directly before use)]. Homogenates were spun at 800g for 5 min at 4°C. Supernatants were pipetted directly into cassette lanes.

D. melanogaster mitochondrial fraction or whole homogenates were then subjected to iso-electric focussing using the ZOOM IPG (Life Technologies) system and pH 3-10 (non-linear) ZOOM IPG strips, following the manufacturers protocol (Life Technologies, 2012b). Gels were stained (SimplyBlue™ SafeStain, Life Technologies) and imaged (ImageQuant 300, GE

Healthcare Life Sciences). Analyses were performed using SameSpots software (Totallab) (one-way ANOVA).

Samples were analysed out-of-house by the Centre of Excellence in Mass Spectrometry at University of York (Centre for Excellence in Mass Spectrometry, 2019). Briefly, proteins were reduced and alkylated, followed by digestion in-gel with trypsin. Matrix Assisted Laser Desorption Ionization Tandem Time-of-Flight mass spectrometry (MALDI-TOF/MS) was used to analyse the samples. The generated tandem MS data was compared against the NCBI database using the MASCOT search programme to identify the proteins. De novo sequence interpretation for individual peptides were inferred from peptide matches.

5.2.8 Oxygen consumption

A 24-channel optical fluorescence, oxygen sensing system (Loligo Systems, Copenhagen, Denmark) was used for the analysis of oxygen consumption of *D. melanogaster* and *C. elegans*. Oxygen consumption of freshly homogenised whole *D. melanogaster* was measured using a single *D. melanogaster* per well; at least three replicate *D. melanogaster* were included per trial. Fifteen adult *C. elegans* were added directly to wells containing M9 buffer and oxygen consumption was measured at 15 second intervals.

Briefly, for *D.* melanogaster-containing wells, 100µl mitochondrial extraction buffer was added to individual wells. For *C. elegans*-containing wells, 100ul M9 buffer was added to individual wells. Freshly homogenised *D. melanogaster* or age-synchronised day 0 adult *C. elegans* were moved directly

into the buffer. Control wells of mitochondrial extraction buffer or M9 buffer were used to correct for background levels of oxygen consumption. A one-point calibration was used to artificially adjust oxygen saturation of all wells to 100% prior to beginning. All data were corrected for volume of buffer and total protein per well, expressed as µl/min/mg protein. Data were analysed using GraphPad Prism (two-tailed, unpaired *t*-test).

5.2.9 ATP Concentration

Total cellular ATP was measured using luciferin-luciferase (ATP Determination Kit; Molecular Probes). Briefly, freshly homogenised whole *D. melanogaster* were collected and frozen at -80°C for 5 minutes, followed by boiling at 90°C for 15 minutes, and then placed on ice for 5 minutes. Samples were centrifuged at 13,400g for 10 minutes at 4°C. Supernatants were transferred to fresh tubes and immediately used for ATP determination. To determine ATP concentration, an aliquot of 10µl whole *D. melanogaster* supernatant was added to 100µl of reaction solution (0.5mM D-luciferin, 1.25µg/mL firefly luciferase, 25mM tricine buffer, pH 7.8, 5mM $MgSO_4$, 100µM EDTA and 1mM DTT). Light emission was quantified in a Varioskan LUX (Thermofisher Scientific). ATP standard curves were generated for each individual experimental run. ATP concentrations were calculated from spectrophotometric absorbance at 560nm. Data were normalised for protein content by Bradford assay. Data were analysed using GraphPad Prism (two-tailed, unpaired *t*-test).

5.3 Results

5.3.1 *Pink1*$^{-/-}$ model species have an irregular mitochondrial phenotype and increased oxygen consumption

Mitochondrial changes are inherent to PD (Moon and Paek, 2015). In order to better characterise these changes and elucidate targets for therapy, *Pink1*$^{-/-}$ *D. melanogaster* and *C. elegans* have been generated. *Pink1*$^{-/-}$ *C. elegans* strain CC46 contain the transgene ccls4251 with GFP-tagged muscle wall nuclei and mitochondria, making this model well placed to interrogate mitochondrial changes.

Day 0 adult (L4 stage) CC46 strain *C. elegans* have altered mitochondrial morphology - they display disorganised, irregular mitochondria (Figure 5.1B) compared with age-matched WT *C. elegans* (Figure 5.1A). Further, oxygen consumption of *Pink1*$^{-/-}$ *C. elegans* and *D. melanogaster* were significantly increased over the period measured, compared to WT controls (p<0.0001).

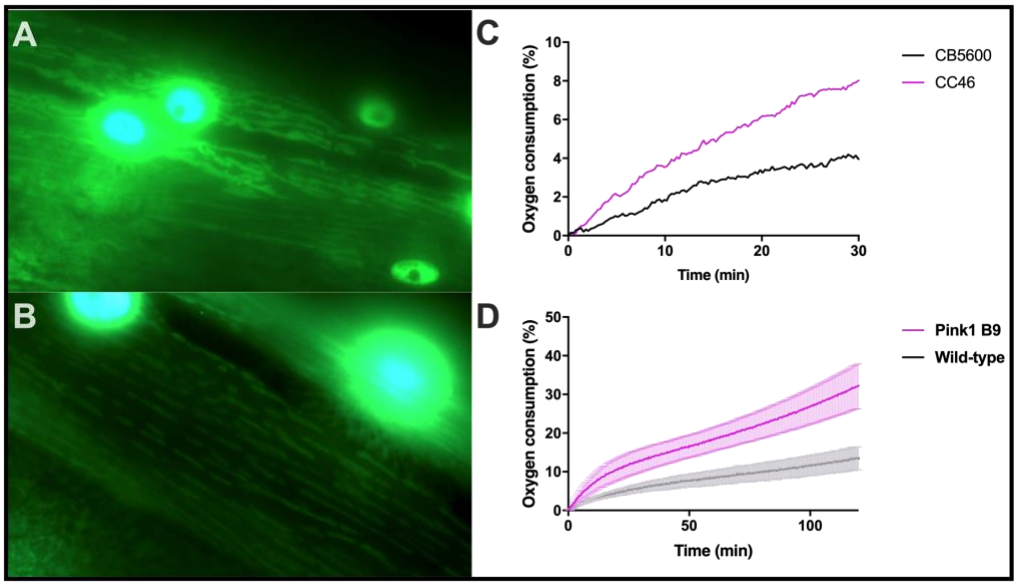


Figure 5.1 *Pink1*$^{-/-}$ **(A)** show tubular, long and irregular mitochondria; WT *C. elegans* **(B)** exhibit an elongated mitochondrial morphology. All *C. elegans* in this figure were day 0 adults and carry the transgene ccIs4251 with GFP fusion proteins localised to body muscle mitochondria and nuclei. **(C)** Oxygen consumption of day 0 adult *Pink1*$^{-/-}$ *C. elegans* is significantly increased. A significant increase in oxygen consumption of the *Pink1*$^{-/-}$ compared with WT is clear (p<0.0001; two-tailed, unpaired t-test). Using the Loligo Microplate System oxygen consumption of fifteen day 0 adult *Pink1*$^{-/-}$ or WT *C. elegans* were measured every 15 seconds for 30 minutes, at 25°C. **(D)** *Pink1*$^{-/-}$ *D. melanogaster* oxygen consumption is significantly increased. *Pink1*$^{-/-}$ *D. melanogaster* consume oxygen at a significantly greater rate than WT (p<0.0001; two-tailed, unpaired t-test). Using the Loligo Microplate System, oxygen consumption of individual freshly homogenised newly eclosed *D. melanogaster* was carried out. Data were collected every 15 seconds for 120 minutes, at 25°C. Each data point is the mean value of three biological replicates and graphs show mean±SEM.

5.3.2 Ten minutes of exercise per day increases ATP generation

WT *D. melanogaster* have previously shown improvements in climbing ability after one hour and two hours exercise per day, for one to three week regimes (Piazza *et al.*, 2009; M J Tinkerhess *et al.*, 2012; Mendez *et al.*, 2016; Watanabe and Riddle, 2017). In this study shortened regimes of 30 minutes and 10 minutes per day for one week were employed. *D. melanogaster* subjected to the

10-minute regime for seven days did not show any significant changes in climbing score, in either the male or female WT *D. melanogaster* investigated (Figure 5.2A). Female *D. melanogaster* had a significantly lower climbing score than males on both day one and day seven ($p<0.0001$).

Although climbing ability was not significantly altered over the seven day exercise period, ATP levels were significantly increased with exercise in both male and female exercised *D. melanogaster* (Figure 5.2B) ($p=0.0012$ and $p=0.0008$). Paralleling the lower climbing ability in females, non-exercised and exercised female *D. melanogaster* have significantly lower ATP levels than the respective male groups ($p<0.0001$).

5.3.3 Exercise halts the age-related decrease in climbing ability of *Pink1*$^{-/-}$ D. melanogaster

Pink1$^{-/-}$ *D. melanogaster* show phenotypic similarities to the motor disability of PD (Clark *et al.*, 2006a). *Pink1*$^{-/-}$ *D. melanogaster* climbing ability was significantly worse than WT males at day 0 and day 7 ($p=0.0020$ and $p<0.0001$, respectively) (Figure 5.2A). In addition, a significant age-related decline in climbing score was apparent in non-exercised *Pink1*$^{-/-}$ *D. melanogaster* ($p=0.0064$). However, this decline is ameliorated in exercised *Pink1*$^{-/-}$ *D. melanogaster* – no significant differences in climbing ability are apparent with 10 minutes exercise per day for seven days.

5.3.4 Exercise normalises ATP concentration of Pink1$^{-/-}$ D. melanogaster

Non-exercised *Pink1*$^{-/-}$ *D. melanogaster* have significantly greater ATP concentration than non-exercised WT males and females (p<0.0001) (Figure 5.2B). However, these levels are significantly reduced with exercise (p=0.0057). The new ATP concentration is not significantly different to WT males non-exercised or WT females exercised, suggesting that exercise normalises ATP concentration.

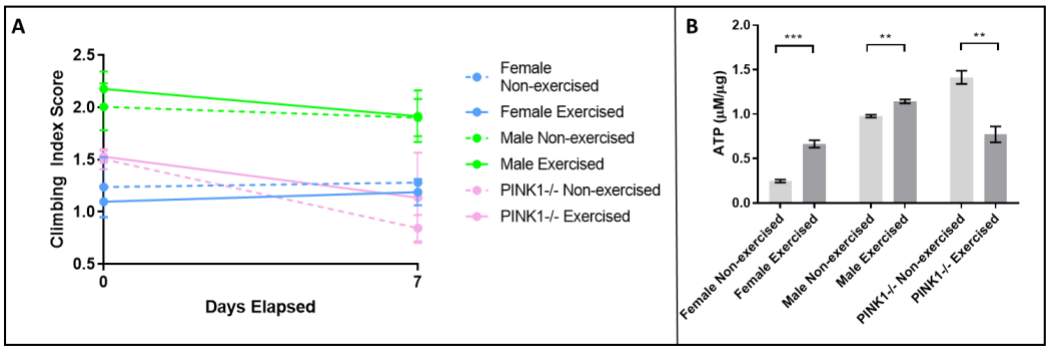


Figure 5.2 (A) 10 minutes of exercise for 7 days does not significantly alter climbing ability of WT or *Pink1*$^{-/-}$ *D. melanogaster*. Neither WT nor *Pink1*$^{-/-}$ *D. melanogaster* showed improvements in climbing ability after 10 minutes of exercise per day for 7 days. Climbing ability was significantly reduced over 7 days in *Pink1*$^{-/-}$ non-exercised *D. melanogaster* (p=0.0064; one-way ANOVA), whereas, exercised *Pink1*$^{-/-}$ show no significant change in climbing index score after 7 days. Climbing assays were carried out prior to the days exercise. Data are the mean of three vials of *D. melanogaster* for males and *Pink1*$^{-/-}$; two vials for females; ± SEM. **(B)** ATP concentration is significantly decreased with exercise in *Pink1*$^{-/-}$ *D. melanogaster*. *Pink1*$^{-/-}$ have significantly increased ATP concentration compared to WT male and female *D. melanogaster* (p<0.0001; one-way ANOVA). 10 minutes of exercise for 7 days significantly lowered *Pink1*$^{-/-}$ ATP concentration (p=0.0057; unpaired t-test with Welch's correction), and not significantly different to WT male non-exercised. Fly homogenates were equalised for protein content by Bradford assay. Data are the mean of three biological replicates ± SEM.

5.3.5 2D-gel electrophoresis of wild-type exercised *D. melanogaster* reveals novel protein alterations

Exercise elicits broad protein expression changes. To capture this, 2D-gel electrophoresis (2DGE) was employed on whole *D. melanogaster* homogenates from WT males and females that had undergone 30 minutes exercise per day for seven days. Mass spectrometry revealed the identity of proteins altered with exercise and between sex (Table 5.1).

5.3.5.1 Glycolytic proteins

2DGE with subsequent LC/MS-MS identified differentially expressed proteins between exercised and non-exercised, and male and female, WT *D. melanogaster*. The glycolytic protein glycerol-3-phosphate dehydrogenase [NAD+] is decreased 1.7-fold in female exercised vs female non-exercised (p=0.032). A second glycolysis protein, fructose-bisphosphate aldolase shows sex-dependent expression differences - a 2.4-fold increase in expression in female non-exercised vs male non-exercised (p=0.0009).

5.3.5.2 Mitochondrial proteins

Three proteins identified as differentially expressed are involved in mitochondrial function: aconitate hydratase increased 1.5-fold in female exercised vs female non-exercised (p=0.047). Dihydrolipoyl dehydrogenase decreased 1.7-fold in male exercised vs male non-exercised (p=0.0005). A sex-dependent difference was also shown - pyruvate dehydrogenase E1

component subunit beta increased 2.2-fold in male non-exercised vs female non-exercised (p=0.001).

5.3.5.3 Vitellogenin-3

Vitellogenin is a major yolk protein of eggs, used primarily as a food source during embryogenesis, and thus expressed only in females. It is synthesised in the fat body and ovarian follicle cells (Bownes *et al.*, 1993). 2DGE revealed increased vitellogenin-3 protein levels in female exercised *D. melanogaster* compared with female non-exercised.

5.3.6 Profiling of whole *Pink1*$^{-/-}$ *D. melanogaster* and WT males by 2D-gel electrophoresis reveals exercise-related protein expression differences

5.3.6.1 Exercise decreases cytoskeletal protein levels in *Pink1*$^{-/-}$ *D. melanogaster*

Two cytoskeletal proteins were identified as reduced in *Pink1*$^{-/-}$ *D. melanogaster* compared with WT (Table 5.1). Myosin heavy chain (MYH) and actin are both reduced, 5-fold (p=0.0002) and 2.1-fold (p=0.002) respectively, in *Pink1*$^{-/-}$ non-exercised compared with WT non-exercised.

MYH was also identified in *Pink1*$^{-/-}$ exercised compared to WT non-exercised – here it is similarly reduced in expression in *Pink1*$^{-/-}$ *D. melanogaster.*

Interestingly, the magnitude of this reduction is less with exercise; 3.3-3.9-fold

decrease with exercise (p=0.0006 and p=0.0009, respectively), compared with a 5-fold decrease without exercise (p=0.0002). The data suggest exercise partially reverses the reduced levels of MYH in *Pink1*$^{-/-}$ *D. melanogaster.*

5.3.6.2 Homeobox protein slou is reduced in *Pink1*$^{-/-}$ *D. melanogaster*

Proteomic analysis revealed a 2.6-fold reduction in expression of homeobox protein slou (slouch) in *Pink1*$^{-/-}$ compared with WT male *D. melanogaster* (p=0.0003) (Table 5.1). Intriguingly, this protein was also identified and shown to increase in *Pink1*$^{-/-}$ exercised versus non-exercised (p=0.045; one-way ANOVA). Like MYH, the data suggest exercise partially reverses *Pink1*$^{-/-}$ *D. melanogaster* protein slouch deficits.

5.3.6.3 Arginine kinase is increased in *Pink1*$^{-/-}$ *D. melanogaster*

The invertebrate equivalent of vertebrate creatine kinase (CK) is arginine kinase (AK). Like CK, AK belongs to the phosphotransferase family. In this work, 2DGE identified AK to be increased 1.9-fold in *Pink1*$^{-/-}$ non-exercised compared with WT non-exercised (p=0.007) (Table 5.1).

5.3.6.4 Exercise normalises *Pink1*$^{-/-}$ *D. melanogaster* ATP5β protein levels

2DGE showed ATP synthase subunit beta (ATP5β) increased 2.9-fold in *Pink1*$^{-/-}$ non-exercised, compared to WT non-exercised *D. melanogaster* (p=0.001) (Table 5.1). ATP5β is a crucial component of complex V of the oxidative phosphorylation machinery and its alteration in *Pink1*$^{-/-}$ *D. melanogaster* or PD has not previously been shown. Western blotting corroborated the proteomics,

confirming a similar fold change increase in non-exercised *Pink1*$^{-/-}$ compared to WT non-exercised *D. melanogaster* (Figure 5.3).

A dramatic reduction in ATP5β protein expression in *Pink1*$^{-/-}$ *D. melanogaster* was found after the seven-day exercise regime. Exercise normalised *Pink1*$^{-/-}$ ATP5β to WT levels. WT *D. melanogaster* showed no change in ATP5β levels with exercise.

Whole *D. melanogaster* : wild-type males vs females					
Group Comparison	**Spot no.**	**Protein identity**	**Anova (*p*)**	**Fold Change**	**Increased in**
Female exercised vs female non-exercised	351	Glycerol-3-phosphate dehydrogenase [NAD(+)]	0.032	1.7	Female ne
	363	Aconitate hydratase	0.047	1.5	Female e
	375	Vitellogenin-3	0.039	1.6	Female e
Male non-exercised vs female non-exercised	472	Pyruvate dehydrogenase E1 component subunit beta	0.001	2.2	Male ne
	486	Fructose-bisphosphate aldolase	0.0009479	2.4	Female ne
Male exercised vs male non-exercised	487	Dihydrolipoyl dehydrogenase	0.0005776	1.7	Male ne

Whole *D. melanogaster* : wild-type males vs *PINK1-/-*					
Group Comparison	**Spot no.**	**Protein identity**	**Anova (*p*)**	**Fold Change**	**Increased in**
WTne vs *PINK1-/-* ne	631	ATP synthase subunit beta	0.001	2.9	*PINK1-/-* ne
	306	Myosin heavy chain	0.000191	5	WT ne
	330	Actin	0.002	2.1	WT ne
	358	Homeobox protein slou	0.0002802	2.6	WT ne
	359	Arginine kinase	0.007	1.9	*PINK1-/-* ne
Wtne vs *PINK1-/-* e	309	Myosin heavy chain	0.000697	3.9	WT ne
	311	Myosin heavy chain	0.0009122	3.3	WT ne
PINK1-/- e vs *PINK1-/-* ne	702	Homeobox protein slou	0.045	1.2	*PINK1-/-* e

Table 5.1 Protein level changes with exercise or sex in *D. melanogaster*. Excised protein spots were identified by MALDI-TOF/MS. One-way ANOVA revealed statistically significant protein expression differences between WT and *Pink1*$^{-/-}$ exercised, non-exercised, male and female *D. melanogaster* (SameSpots, Totallab). Data are the average of three biological replicates. See **Appendix** Figure 5.1 for representative 2D-gels.

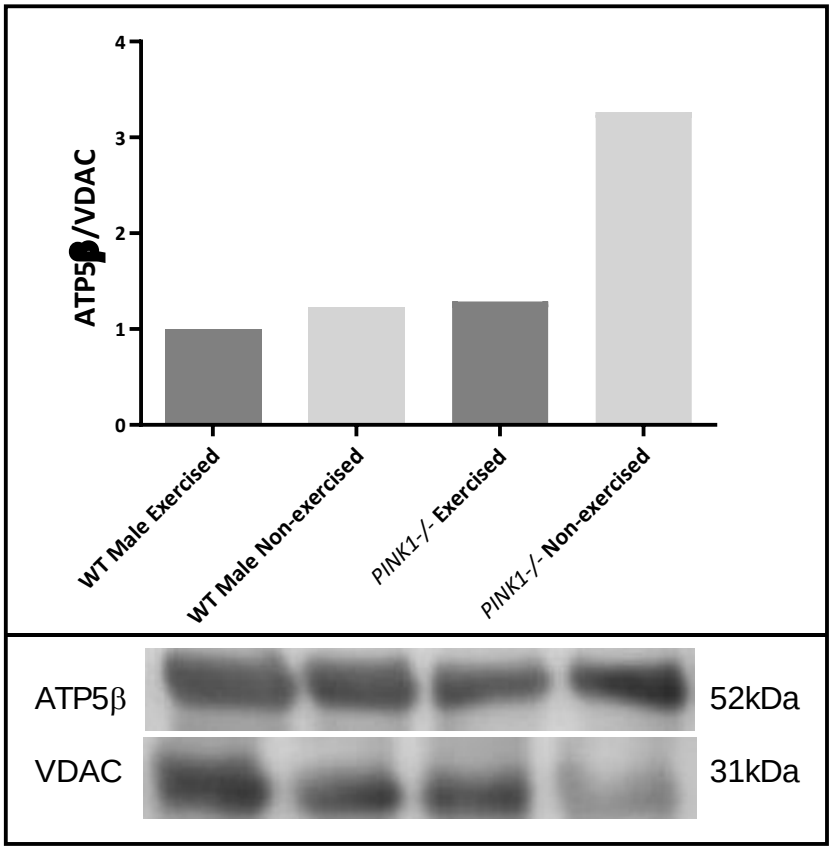


Figure 5.3 *Pink1*$^{-/-}$ ATP5β protein levels are reduced with exercise. ATP5β, normalised to VDAC, is decreased in whole *D. melanogaster* homogenates of exercised *Pink1*$^{-/-}$ compared to non-exercised. The new ATP5β protein levels of exercised *Pink1*$^{-/-}$ are more similar to WT. Columns display protein expression of homogenates of twenty WT male or *Pink1*$^{-/-}$ *D. melanogaster* (n=1 biological replicate).

5.3.7 Mitochondrial 2D-gel electrophoresis identified exercise-induced protein alterations in male WT *D. melanogaster*

Proteomic analysis of mitochondrial fractions was carried out. WT male *D. melanogaster* underwent 30 minutes of exercise per day for 7 days and mitochondria were compared to WT non-exercised males. Seven proteins were identified as affected by exercise (Table 5.2).

5.3.7.1 Electron transport chain proteins

Male WT *D. melanogaster* showed electron transport complex subunit alterations. The complex one subunit NADH-ubiquinone oxidoreductase 75kDa subunit (NDUFS1) was increased 1.5-fold in exercised compared with non-exercised *D. melanogaster* (p=0.004). Exercise reduced complex V subunit ATP synthase subunit alpha 1.8-fold (p=0.037).

5.3.7.2 Other proteins

Proteins with a range of biological functions were identified as decreased in expression in the mitochondrial fractions. The cytoskeletal protein actin decreased 2.7-fold with exercise (p=0.024) and arginine kinase (AK) decreased 2-fold (p=0.003). Two protein spots identified as altered in expression were TCA cycle intermediates - malate dehydrogenase and succinate-CoA ligase subunit alpha both decreased in expression 1.8-fold (p=0.037). Alcohol dehydrogenase, which catalyses the oxidation and reduction of alcohols and aldehydes, was similarly reduced with exercise 1.7-fold (p=0.012).

5.3.8 Proteomic analysis of mitochondria from *Pink1*$^{-/-}$ *D. melanogaster* reveals wide-spread reduction in protein expression with exercise

All proteins identified as significantly changed, after 30 minutes of exercise per day for seven days, were reduced in expression (Table 5.2). Eight proteins were identified as reduced with exercise.

5.3.8.1 Mitochondrial proteins

Exercised *Pink1*$^{-/-}$ *D. melanogaster* show decreased expression of proteins involved in mitochondrial bioenergetics. The pyruvate dehydrogenase complex protein pyruvate dehydrogenase E1 component subunit beta was identified as altered; a 1.3 and 1.5-fold decrease (p=0.048 and p=0.015, respectively). Further, two TCA cycle proteins were identified as reduced in expression with exercise; CG5028 (probable isocitrate dehydrogenase (NAD^+) activity) (p=0.008), and probable isocitrate dehydrogenase [NAD^+] subunit alpha (p=0.015) (1.4- and 1.5-fold decreased, respectively). Mitochondrial protein acyl-coenzyme A dehydrogenase catalyses the initial step of fatty acid β-oxidation. Here, it was shown to decrease 1.4-fold with exercise (p=0.015). The pleiotropic enzyme glycerol-3-phosphate was identified as reduced with exercise. Alcohol dehydrogenase, which was identified as reduced in exercised WT, also shows a 1.3-fold decrease in exercised *Pink1*$^{-/-}$ *D. melanogaster* (p=0.024).

5.3.8.2 Exercise reduces protein levels of tropomyosin-1 and -2 in *Pink1*$^{-/-}$ *D. melanogaster*

Additionally, proteomic analysis identified the actin binding proteins, tropomyosin-1, isoforms 33/34, and tropomyosin-2 as reduced 1.4-fold with exercise in *Pink1*$^{-/-}$ *D. melanogaster* (p=0.022).

Mitochondrial fraction: wild-type males				
Spot no.	**Protein identity**	**Anova (*p*)**	**Fold Change**	**Exercise-related change**
519	Arginine kinase OS=Drosophila melanogaster	0.003	2	Reduced
784	NADH-ubiquinone oxidoreductase 75 kDa subunit	0.004	1.5	Increased
773	Alcohol dehydrogenase	0.012	1.7	Reduced
791	Actin, larval muscle	0.024	2.7	Reduced
788	IP09655p (malate dehydrogenase) Succinate-CoA ligase [ADP/GDP-forming] subunit alpha ATP synthase subunit alpha	0.037	1.8	Reduced

Mitochondrial fraction: *PINK1-/-*				
Spot no.	**Protein identity**	**Anova (*p*)**	**Fold Change**	**Exercise related change**
716	Tropomyosin-1, isoforms 33/34 Tropomyosin-2	0.022	1.4	Reduced
695	CG3902 (acyl-coenzyme A dehydrogenase)	0.015	1.4	Reduced
709	CG5028	0.008	1.4	Reduced
687	Enolase	0.022	1.3	Reduced
684	Probable isocitrate dehydrogenase [NAD] subunit alpha Glycerol-3-phosphate dehydrogenase [NAD(+)] Pyruvate dehydrogenase E1 component subunit beta	0.015	1.5	Reduced
719	Pyruvate dehydrogenase E1 component subunit beta CG6084, isoform C	0.048	1.3	Reduced
707	Alchohol dehydrogenase CG9992, isoform A	0.024	1.3	Reduced

Table 5.2 Proteins identified as altered with exercise in wild-type male or *Pink1*$^{-/-}$ *D. melanogaster*. One-way ANOVA revealed statistically significant protein expression differences between WT male or *Pink1*$^{-/-}$ exercised and non-exercised *D. melanogaster* (SameSpots, Totallab). Excised protein spots were identified by MALDI-TOF/MS. Data are the average of three biological replicates. See Appendix Figure 5.2 for representative 2D-gels.

5.3.9 Exercise reduces expression of muscle contractile proteins in *Pink1*$^{-/-}$ *D. melanogaster*

Mitochondrial proteomic analysis of *Pink1*$^{-/-}$ *D. melanogaster* identified reduced levels of tropomyosin-1α with exercise (Table 5.2). Western blotting confirmed this change, although the magnitude of reduction was considerably greater; a 4-fold reduction, compared to 1.4-fold shown by 2D-gel electrophoresis (Figure 5.4A). This finding spurred further investigation into other proteins involved in the muscle contractile complex – troponin and α-actinin. Western blotting of troponin and α-actinin showed no significant reduction in

exercised *Pink1*$^{-/-}$ *D. melanogaster*, however, a trend toward reduction in protein level was apparent (Figure 5.4A).

5.3.9.1 Wild-type *D. melanogaster* display wide-spread muscle contractile protein changes with exercise

In contrast to *Pink1*$^{-/-}$ *D. melanogaster*, WT showed a significant increase in tropomyosin-1α with exercise. Troponin and α-actinin are also significantly altered in WT, showing decreased levels with exercise (Figure 5.4B).

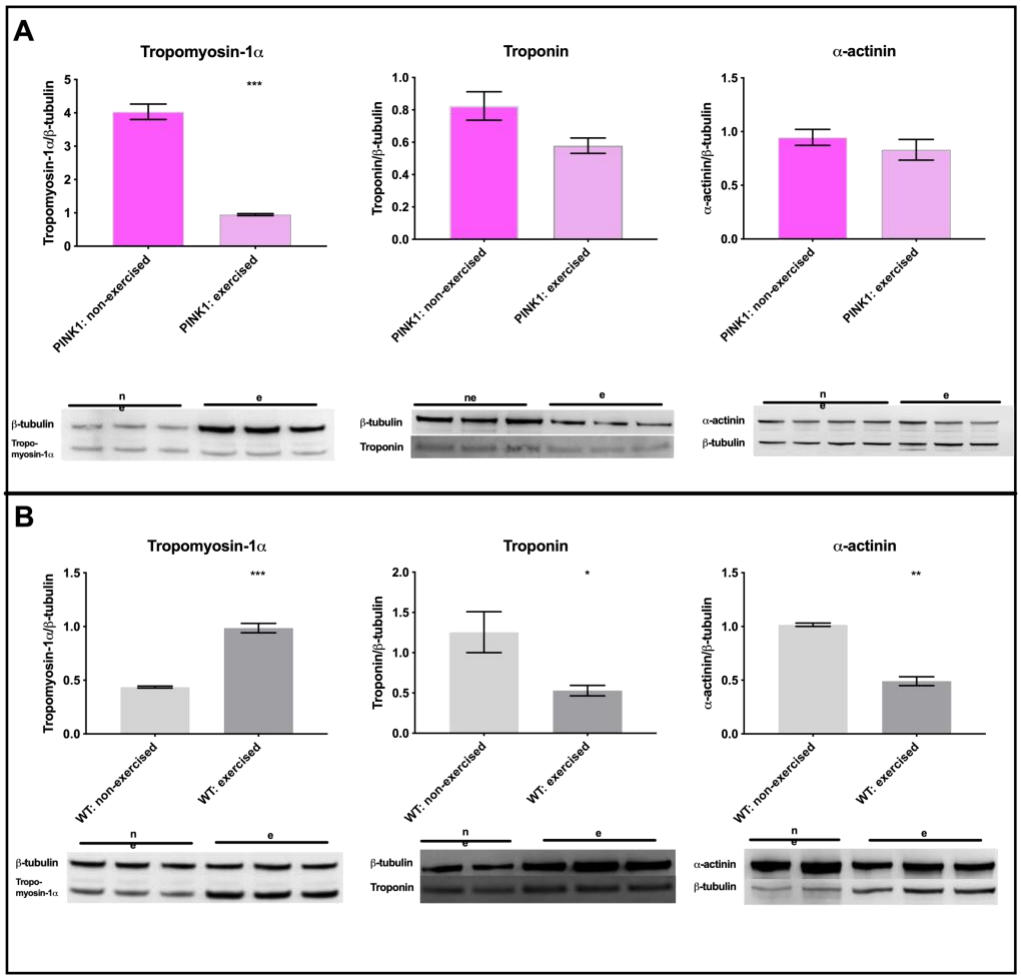


Figure 5.4 Muscle contractile complex proteins are altered with exercise in *Pink1*$^{-/-}$ and WT *D. melanogaster*. Tropomyosin-1α, troponin and α-actinin protein levels were measured in exercised and non-exercised *Pink1*$^{-/-}$ and male WT *D. melanogaster*. **(A)** *Pink1*$^{-/-}$ tropomyosin-1α protein levels are significantly reduced with exercise. **(B)** All three interrogated proteins were altered with exercise in WT *D. melanogaster*. Lanes display biological replicates of three sets of 10 pooled whole *D. melanogaster* homogenates. Columns display mean protein expression normalised to β-tubulin loading control. Error bars represent ±SEM. Statistical analyses were carried out using GraphPad Prism (unpaired, two-tailed, *t*-tests).

5.4 Discussion

Exercise is an intervention known to promote health, wellbeing and delay the onset and reduce symptom severity of age-related diseases (Mikkelsen *et al.*, 2013; Viña *et al.*, 2016). The importance of measuring and reporting biology with respect to sex differences is relevant to developing therapeutics. Evidence suggest males and females present different molecular profiles in response to exercise, and females exercised at different stages of their menstrual cycle show regulatory differences (Timmons *et al.*, 2005). The present work utilised male and female WT and PD model *D. melanogaster* ($Pink1^{B9}$; $Pink1^{-/-}$) and subjected them to daily exercise to assess biological alterations.

In humans, loss of function of PINK1 can lead to autosomal recessive PD (Pickrell and Youle, 2015; Puschmann *et al.*, 2017) and $Pink1^{-/-}$ *D. melanogaster* display motor symptoms reminiscent of PD individuals (Guo, 2012). Mitochondrial dysfunction is implicated in PD (Bose and Beal, 2016) and this study supports this - $Pink1^{-/-}$ have altered mitochondrial morphology in *C. elegans* and increased oxygen consumption in both *C. elegans* and *D. melanogaster*. $Pink1^{-/-}$ *C. elegans* displayed disorganised and fragmented mitochondria. Mitochondrial morphological alterations consistent with those seen here have been shown in the *SNpc* of $Pink1^{-/-}$ mice, which was also associated with increased ROS generation and depolarised mitochondria (Heeman *et al.*, 2011). The increased oxygen consumption of $Pink1^{-/-}$ *C. elegans* and *D. melanogaster* likely reflects, in part, an increased generation of ROS

through one-electron reduction to form superoxide. The oxygen consumption assay in the present work cannot distinguish the precise localisation of oxygen consumption. Ideally, more precise analysis of individual mitochondrial respiratory complex activity (O_2K, Oroboros, Innsbruck) and ROS production assays (O_2K and aconitase assay) would be performed to more accurately characterise the influence of *Pink1*$^{-/-}$ on increasing oxygen consumption.

D. melanogaster were subjected to 10 or 30 minutes of exercise per day for seven days, the shortest that has been reported in *D. melanogaster*. Justification for the shortened regime was three-fold: 1) Previous interventions utilising "Power Tower" type devices have proven to be stressful to *D. melanogaster*, leading to dropping-induced deaths. Further, exercised *D. melanogaster* climbing ability has been shown to be less than control, likely due to physical impairments to limbs due to dropping (Piazza *et al.*, 2009; Tinkerhess *et al.*, 2012). 2) Preliminary work (data not shown) suggested that *Pink1*$^{-/-}$ *D. melanogaster* are less able to cope with dropping stress, causing many to die. 3) A 10-minute duration would be more comparable to the "gentler" exercise regimes that PD individuals might be capable of carrying out.

5.4.1 10 minutes of exercise for seven days

10 minutes of exercise per day for seven days was sufficient to elicit molecular and behavioural changes to *D. melanogaster*. Non-exercised *Pink1*$^{-/-}$ *D. melanogaster* showed a decline in motor function with age. Exercised *Pink1*$^{-/-}$ *D. melanogaster* did not show a decline and had a significantly improved climbing ability than non-exercised, suggesting that exercise slows *Pink1*$^{-/-}$ age-related

mobility declines. This finding matches human data, which show motor symptom improvements in PD individuals taking exercise (Oliveira de Carvalho *et al.*, 2018). *Pink1*$^{-/-}$ non-exercised *D. melanogaster* had significantly higher ATP concentration than WT, which reflects the higher oxygen consumption in *Pink1*$^{-/-}$. Exercise normalised ATP to WT concentration. In disagreement with these data, work in *Pink1*$^{-/-}$ mice showed impairments to mitochondrial respiration and a concomitant decrease in oxidative phosphorylation (Heeman *et al.*, 2011). Neither male nor female WT *D. melanogaster* climbing ability was significantly different over the exercise regime. Groups have previously shown improvements in WT male climbing ability with exercise - 10 minutes may be too short for improvements to manifest. In agreement with others (Piazza *et al.*, 2009; Tinkerhess *et al.*, 2012; Mendez *et al.*, 2016), this work showed significantly higher climbing index scores in WT males compared with females. Although no improvements in WT *D. melanogaster* climbing ability were apparent, molecular alterations were. Exercise increases ADP/ATP ratio and demand for ATP (Fentz *et al.*, 2015). ATP concentration was increased in WT male and female *D. melanogaster* that underwent 10 minutes of exercise per day for 7 days.

5.4.2 30 minutes of exercise for seven days – whole cell proteomics

WT and *Pink1*$^{-/-}$ *D. melanogaster* underwent 30 minutes of exercise per day for 7 days. Proteomic analysis of whole *D. melanogaster* homogenates revealed alterations to functionally diverse proteins, including those involved in energy

metabolism, cytoskeletal maintenance and contractile activity. Eight proteins were selected that changed in level between *Pink1*$^{-/-}$ and WT whole *D. melanogaster* homogenates. *Pink1*$^{-/-}$ *D. melanogaster* had a 5-fold reduction in the level of myosin heavy chain (MYH) compared to WT non-exercised *D. melanogaster.* Interestingly, MYH was also identified as altered between *Pink1*$^{-/-}$ exercised and WT non-exercised. With exercise, *Pink1*$^{-/-}$ showed a smaller difference in MYH compared to WT non-exercised (5-fold vs 3.3-3.9 fold, respectively). *Pink1*$^{-/-}$ *D. melanogaster* phenotypically show apoptotic muscle degeneration (Clark *et al.*, 2006b). The exercise-related increase in MYH present in *Pink1*$^{-/-}$ may reflect partial recovery from this phenotype. Indeed, MYH protein level recovery may be associated with the improvement in *Pink1*$^{-/-}$ climbing ability after seven days of exercise.

Protein slouch was another protein that was shown to be lower in *Pink1*$^{-/-}$ non-exercised compared with WT non-exercised. Like MYH, slouch was increased in expression with exercise. In *D. melanogaster*, larval muscle development is configured by a single mesodermal cell, known as a muscle founder. The pattern of gene expression tailors the muscle founder to a specific fate. Slouch plays a role in specifying the identity of various somatic muscles (Rushton *et al.*, 1995). No direct evidence for a post-development role of slouch currently exists, however, it is plausible it may be involved in adult muscle development and differentiation. The lower levels in *Pink1*$^{-/-}$ *D. melanogaster* may mirror the phenotypical muscle atrophy. Exercise appears to remediate this to some degree. This is a novel finding and one which should be

investigated further to determine the post-developmental role of slouch and its relevance to ageing pathology.

Arginine kinase (AK) is a highly abundant ATP phosphotransferase found in invertebrates. It buffers, cellular ATP and ADP concentrations by catalysing the reversible exchange of phosphate bonds between phosphoarginine and ADP (Wu *et al.*, 2007). It plays an analogous role to creatine kinase (CK) in vertebrates. In mammals, serum CK is raised for an extended period following exercise (Evans *et al.*, 1986). Additionally, chronic high levels of serum CK levels are associated with damaged muscle tissue and diseases, such as, rhabdomyolysis, myocardial infarction, brain damage and mitochondrial myopathies (Brancaccio, Lippi and Maffulli, 2010). Here, AK was shown to be higher in non-exercised *Pink1*$^{-/-}$ compared with non-exercised WT *D. melanogaster.* In agreement, the serum of PD individuals is characterised by increased CK levels (Takubo, Shimoda-Matsubayashi and Mizuno, 2003). This increase may be due to the phenotypical muscular atrophy of *Pink1*$^{-/-}$ *D. melanogaster.* In addition, the increase may be related to mitochondrial dysfunction – in PD, CK may act as a buffer to compensate for inefficiencies of ATP synthesis due to mitochondrial dysfunction.

ATP synthase subunit beta (ATP5β) was significantly increased in non-exercised *Pink1*$^{-/-}$ compared to non-exercised WT, and this was verified by Western blotting. Limited literature describing ATP5β alterations within PD currently exists. DJ-1 (Chen *et al.*, 2019) and α-syn (Ludtmann *et al.*, 2016), mutations of which are implicated in PD, directly interact with ATP synthase

catalytic subunits. In this work, *Pink1*$^{-/-}$ *D. melanogaster* had increased ATP5β protein levels. This increase may simply reflect a build-up of dysfunctional mitochondria, due to PINK1 loss of function, resulting in inefficiencies of mitophagy. However, the mitochondria of *Pink1*$^{-/-}$ *D. melanogaster* are likely still functionally capable of oxidative phosphorylation - the increase in ATP5β, together with the increased AK levels, may explain the high ATP concentration compared to WT.

5.4.3 30 minutes of exercise for seven days – mitochondrial proteomics

Proteomic analysis of mitochondria from WT *D. melanogaster* exercised for 30 minutes per day for seven days identified seven altered proteins. Surprisingly, AK was found to be reduced with exercise. In vertebrates, exercise is known to produce an increase in serum CK for days following activity. However, peak CK levels are apparent 1-5 days post-exercise (Evans *et al.*, 1986) – here *D. melanogaster* were homogenised one hour following exercise, which may account for the acute reduction. Further, Power Tower type exercise devices are known to be relatively harsh on *D. melanogaster* (Piazza *et al.*, 2009; Tinkerhess *et al.*, 2012). In vertebrates, muscular damage releases CK into the serum (Brancaccio, Lippi and Maffulli, 2010) – a similar phenomenon may occur with AK and invertebrate muscular damage. Even with the reduced dropping height, it is reasonable to suggest the ICE machine could have caused muscular damage to non-exercised *D. melanogaster*, increasing AK protein levels. Alcohol dehydrogenase (ADH), actin, malate dehydrogenase

(MDH), ATP synthase subunit alpha (ATP5α) and succinate-CoA ligase subunit alpha (SCSα) were also reduced with exercise. ADH is involved in the metabolism of alcohol to acetylaldehyde. In agreement with our finding, *adh* mRNA expression has been shown to be decreased in the intestines of rats following exercise (Choi and So, 2018). TCA cycle proteins, MDH and SCSα, and ATP5α, were reduced in exercised WT *D. melanogaster*. These findings were unexpected as vertebrate exercise literature suggests increased mitochondrial density and increased aerobic respiratory proteins levels with exercise (Norrbom *et al.*, 2010; Tadaishi *et al.*, 2011; Ruas *et al.*, 2012; Garcia-Valles *et al.*, 2013; Vainshtein, Tryon, *et al.*, 2015). However, it is relevant to note that this is the first-time proteomics has been carried out to interrogate mitochondrial changes to *D. melanogaster* with exercise, and that the invertebrate response may be quite different. Indeed, utilising the TreadWheel and exercising *D. melanogaster* for two hours per day for five days, Mendez et al, showed results opposing vertebrate exercise data, noting a decrease in mRNA of *CYTC*, *ETFB*, and *PPARGC1A* with exercise (Mendez *et al.*, 2016). Another possible explanation for these findings is that culling of *D. melanogaster* one-hour-post the final exercise session may be capturing a transient turnover of proteins or catabolic state, as it is known that exercise promotes an autophagic response (Carter *et al.*, 2018; Nilsson and Tarnopolsky, 2019). A single mitochondrial protein was shown to be increased in exercised WT *D. melanogaster*: NADH-ubiquinone oxidoreductase 75kDa subunit (NDUFS1). NDUFS1 protein is the largest subunit of mitochondrial complex I (Zhu, Vinothkumar and Hirst, 2016). High intensity interval

training in rodents has previously been shown to increase protein expression of NDUFS1 (Yamaguchi *et al.*, 2010) and protein expression of complex I subunit NDUFB8 (Henríquez-Olguín *et al.*, 2019). Further, five days of treadmill and resistance exercise in WT mice increased activity of complex I (Fiuza-Luces *et al.*, 2019). In addition, PD is associated with complex I dysfunction, and exercise is known to delay onset and reduce symptoms (Parker *et al.*, 2008). The exercise-related benefits for PD may, in part, be due to maintenance and regulation of complex I subunits. The effect of exercise on NDUFS1 in *Pink1*$^{-/-}$ *D. melanogaster* was not investigated in the present work, but would be useful, as others have shown deficits to *Pink1*$^{-/-}$ *D. melanogaster* complex I function (Morais *et al.*, 2009). Intriguingly, overexpression of complex I subunit 10 (*ND42*) in *Pink1*$^{-/-}$ *D. melanogaster* significantly restored climbing and flight ability, and partially restored mitochondrial disruption (Pogson *et al.*, 2014).

Interestingly, all significantly altered proteins in *Pink1*$^{-/-}$ *D. melanogaster* with exercise were reduced in expression. PINK1 protein is recognised as having a central role in mitophagy, ensuring a healthy pool of mitochondria are maintained (Clark *et al.*, 2006a; Park *et al.*, 2006b). Dysfunctional mitophagy results in an accumulation of damaged and dysfunctional mitochondria and their contents (Anzell *et al.*, 2018). It is enticing to imagine that alternate mitophagic pathways compensate for its knockout, are upregulated during exercise, and that this accounts for the sweeping reductions in protein levels observed here. Indeed, alternative proteins have been identified which

promote PINK1/PARKIN-independent mitophagy: AMBRA1, HUWE1 and IKKα (Di Rita *et al.*, 2018). Analysis of these alternative proteins, together with autophagosome markers, would be a next step. In addition, electron microscopy of the flight muscles from non-exercised and exercised *D. melanogaster*, would allow visualisation of qualitative changes to contractile proteins, which could be coupled to the accumulated data of this Chapter.

5.4.4 Muscle contractile protein changes

Tropomyosin-1, isoforms 33/34, and tropomyosin-2 were identified as reduced in *Pink1*$^{-/-}$ exercised *D. melanogaster*. Tropomyosin, together with the troponin complex, is central to the calcium-dependent regulation of muscle contraction. To date, investigation of contractile proteins with exercise in *D. melanogaster* has not been described. This work investigated protein levels of three muscle contraction complex proteins in WT and *Pink1*$^{-/-}$: tropomyosin-1α, troponin and α-actinin. WT *D. melanogaster* showed tropomyosin-1α to significantly increase, and troponin and α-actinin to significantly decrease with exercise. In agreement, exercise trained rats showed increased muscle protein levels of tropomyosin-1α (Chaĭkovskiĭ *et al.*, 1987). High cardiac troponin is used as a diagnostic marker for elevated cardiovascular risk (Brancaccio, Lippi and Maffulli, 2010; deFilippi *et al.*, 2012). Moderate exercise in older humans has been shown to lower cardiac troponin levels, and the incidence of heart failure was found to be inversely associated with the amount of exercise (deFilippi *et al.*, 2012). This study shows exercised WT *D. melanogaster* have reduced troponin levels, paralleling the findings in humans. In humans, α-actinin has

muscle (α-actinin-2, and -3) and non-muscle isoforms (α-actinin-1). In muscle, α-actinin creates actin-actin cross-links in the Z-lines of sarcomeres (Ogura *et al.*, 2009). Structurally, it aids transmission of contractile force and maintenance of a regular myofibril formation. α-actinin shows fluctuating expression with time post-exercise – eccentric exercise in humans showed α-actinin loss immediately post-exercise, which recovered seven to eight days later (Yu, Fürst and Thornell, 2003). Male Wistar rats treadmill exercised for nine weeks showed α-actinin isoform expression differences – α-actinin-2 expression was increased in all muscles, α-actinin-3 was unchanged between exercised and non-exercised rats (Ogura *et al.*, 2009). *D. melanogaster* also contain both muscle and non-muscle specific isoforms, and are orthologous to human α-actinin-1 and -2 (Fyrberg, 1990). In this work, exercise decreased α-actinin protein expression in WT *D. melanogaster.* Culling one-hour post-final exercise session may have captured a catabolic muscle protein state. Further, multiple isoforms, both muscle and non-muscle, are present in *D. melanogaster* (Fyrberg, 1990) - homogenates were isolated from whole *D. melanogaster* and antibodies were not specific, so the data may not be reflective of alterations to individual isoforms with exercise.

Unlike WT *D. melanogaster*, *Pink1*$^{-/-}$ showed decreased tropomyosin protein levels with exercise. Individuals with PD have been shown to have increased levels of five isoforms of non-muscle tropomyosin (Lippolis *et al.*, 2015). Normal cytoskeletal function is required in order to regulate mitochondrial mobility to meet local intracellular requirements (Fletcher and Mullins, 2010).

Non-muscle tropomyosin isoforms are intimately linked to the actin-filament cytoskeletal system, which functions to enable cell motility, intracellular transport, cell morphology and size (Gunning *et al.*, 2005). Limited data exist describing over-expression of tropomyosin and its subsequent effect. It is plausible that high levels are detrimental and interfere with normal cytoskeletal system function. The exercise-related decline in tropomyosin-1α may be a revision to healthy levels by an increase in exercise-stimulated autophagic turnover (Fry *et al.*, 2013; Luo *et al.*, 2013; Garvey *et al.*, 2015; White *et al.*, 2016). Further work would be necessary to test autophagic turnover with exercise in *D. melanogaster*, as well as the broader question regarding the impact of tropomyosin overexpression. Alternatively, multiple tropomyosin isoforms exist in *D. melanogaster* and the antibody used in this work was not specific to an individual isoform – determining isoform expression differences may reveal a clearer picture.

Exercise is becoming appreciated as medicine. Like other medicines, dose is exceptionally important. The present study could rapidly determine whether exercise dose is apparent. Indeed, others have shown exercise dose effects are apparent in markers of metabolic and cardiovascular health in humans (Wasfy and Baggish, 2016). Significantly altered proteins highlighted by proteomic analysis of *D. melanogaster* exercised for the 30-minute regime could undergo Western blotting to compare protein expression changes between exercise durations to elucidate whether an exercise dose effect is apparent.

5.4.5 Chapter limitations

As mentioned throughout this discussion, a limitation of this Chapter is its data transferability to the wider literature. *D. melanogaster* were culled one hour after the final exercise session. This time point was chosen in order to capture protein changes immediately post-exercise. Exercise elicits numerous changes, however, many of these transient and fluctuate with time. Exercised rodents are generally culled two days after exercise (Bednarczyk *et al.*, 2009; Tine Kartinah *et al.*, 2018); and *D. melanogaster* are regularly culled one-three days after exercise (Sujkowski *et al.*, 2017; Watanabe and Riddle, 2017). The data collected from this study present an opportunity to compare changes in protein levels at the earliest stage post-exercise, to studies which collect one-three days post-exercise. How protein expression changes emerge over a longer duration would be useful to characterise the relevance of this work to the wider literature.

D. melanogaster are not a homogenous group, individuals are more or less active than others - complex group dynamics exist (Ramdya, Schneider and Joel D. Levine, 2017). Quantitative network analysis together with genetic tools have provided compelling evidence for the complexity of *D. melanogaster* social networks and collective behaviour (Ramdya, Schneider and Joel D Levine, 2017). Researchers monitored *D. melanogaster* for time spent in an unpleasant odour and showed that individual odour avoidance was increased when they were placed in group densities similar to those found on a food source (Ramdya *et al.*, 2015). Transgenic *D. melanogaster* unable to smell an

unpleasant odour were still herded out of the odour zone by interactions with WT, despite remaining in the odour when alone (Ramdya *et al.*, 2015). Individual activity differences exist between *D. melanogaster* and a vial containing an exceptionally active individual may dispose the rest to follow suit, or the opposite. Until recently, a limitation of *D. melanogaster* exercise research was the inability to monitor the amount of exercise each was receiving. The advent of the Locomotor Activity Monitor (Trikinetics), to the TreadWheel exercise device has rectified this. The device records the number of laser beam crossings through the centre of each vial and is capable of tracking multiple *D. melanogaster* at once (Watanabe and Riddle, 2017). Future work would benefit from the addition of this technology. Another limitation is that the present work used whole *D. melanogaster* homogenates. It is known that exercise in vertebrates elicits tissue and cell specific alterations (Ross *et al.*, 2019). This work did distinguish protein expression changes of specific tissues. An area of burgeoning interest is the impact of exercise on the brain (Liu *et al.*, 2019). Specific tissue analysis would enable more precise delineation of the effect of exercise.

D. melanogaster are a fantastic model species that research groups can cheaply and rapidly incorporate into their laboratories. The growth of interest into exercise and its longevity promoting affects necessitates the development of new tools to accurately and affectively interrogate the underlying biological mechanisms. Exercising *D. melanogaster* is a concept that at first blush is greeted with scepticism, however, validation of their usefulness has already

been confirmed (Piazza *et al.*, 2009; M J Tinkerhess *et al.*, 2012; Tinkerhess *et al.*, 2012; Sujkowski *et al.*, 2015, 2017; Berlandi *et al.*, 2017; Watanabe and Riddle, 2017). I can imagine the paradigm of exercise research using *D. melanogaster* will be as ordinary and accepted as rodents by the scientific community in the next decade.

5.5 Conclusion

Regular exercise is a lifestyle intervention that is well evidenced to improve healthspan (Garcia-Valles *et al.*, 2013). *D. melanogaster* are different to us in many ways, and yet they continue to offer insights into human health and disease – it is plausible they will yield translatable findings in exercise research. The present work adds support for the use of *D. melanogaster* as a model for exercise research. Here, 10 minutes of exercise was sufficient to elicit behavioural and biological changes, and proteomic analysis of *Pink1*$^{-/-}$ and WT *D. melanogaster* revealed novel protein expression changes, including sweeping alterations to key muscle contractile proteins. It is increasingly clear that *D. melanogaster* are a valid model for exercise research.

6 Levels of mitochondrial inflammatory markers reveal sex differences in cerebellum which are attenuated in Parkinson's disease

6.1 Introduction

Ageing is associated with changes to the immune system, which include chronic innate immune system activation (Salminen *et al.*, 2008). Although of fundamental physiological importance as a defence against pathogens, inappropriate activation of immune responses in later life are detrimental to health (Ferrucci and Fabbri, 2018). It is not clear whether immunity and inflammation are causal drivers, or a consequence of other factors of ageing. However, changes in immunity with age correlate with general poor health and premature death (Rodríguez-Hernández *et al.*, 2013). Recent data have revealed a relationship between the level of immune system disruption and severity of Parkinson's disease (PD) (Tufekci *et al.*, 2012).

PD is a neurodegenerative disorder most frequently associated with advancing age. Pathophysiologically PD is characterised by the progressive loss of dopaminergic neurones of the *Substantia Nigra pars compacta* (*SNpc*), and intracellular accumulation of Lewy bodies (Tufekci *et al.*, 2012). The aetiology of PD is unknown although excessive reactive oxygen species (Jiang, Sun and Chen, 2016), mitochondrial dysfunction (Federico *et al.*, 2012; Bhat *et al.*, 2015; Pickrell and Youle, 2015), autophagy dysfunction (Hong *et al.*, 2010; McLelland *et al.*, 2014; Pickrell and Youle, 2015; Ryan *et al.*, 2015), disruption of

calcium homeostasis (Surmeier *et al.*, 2017), loss of trophic support (Bose and Beal, 2016; Raefsky and Mattson, 2017), and neuroinflammation (Reeve, Simcox and Turnbull, 2014; Bose and Beal, 2016) have all been implicated.

Clinical symptoms of PD appear once 50-60% of dopaminergic neurones of the *SNpc* are lost and when striatal dopamine concentration falls below 70% (Blesa *et al.*, 2017). Compensatory mechanisms must be operating in this pre-symptomatic phase, however, they are not understood (Blesa *et al.*, 2017). A potential site of compensation may be the cerebellum (Palmer *et al.*, 2010; Sen *et al.*, 2010; Wu *et al.*, 2011). The cerebellum is hyperactivated in PD individuals and has been correlated with motor symptom severity (Payoux *et al.*, 2010). fMRI of mild-moderate PD individuals showed enhanced putamen-cerebellum functional connectivity, which was associated with better performance. The enhanced connectivity was reduced upon levodopa administration, pointing to a compensatory feedback loop in action (Simioni, Dagher and Fellows, 2017). Substantial connectivity between cerebello-thalamo-cortical pathways has also been shown during motor task performance in PD individuals (Palmer *et al.*, 2010). Further, those indiviudals with greater activation of this pathway fare better in motor symptom scores, further supporting this pathway as compensatory (Simioni, Dagher and Fellows, 2017). Intriguingly, many symptoms of PD can be linked to a cerebellar role. The cerebellum is concerned with maintaining postural stability, motor coordination, emotional stress and anxiety, sleep and bladder function, all of which are affected in PD (Griffiths *et al.*, 2005; Grabli *et al.*, 2012;

Imamizu, Kawato and Imamizu, 2012; DelRosso and Hoque, 2014; Lange *et al.*, 2015; Rajan *et al.*, 2017). Deep brain stimulation has seen success at treating PD tremor – the circuits necessary for this improvement have been shown to utilise a striatal-cerebellar interaction (Hanssen *et al.*, 2019). Targeting compensatory mechanisms in PD is a strategy to delay or halt symptom onset and could be used therapeutically to manage symptoms.

When considering the treatment of PD, it is apparent that disease onset and progression are heterogeneous. An obvious yet largely overlooked variation is sex disparity in disease risk, time of onset, and severity. PD has earlier onset in males and an age-standardised incidence male to female ratio of 2:1. Females are more likely to present with tremor as the primary symptom, have a higher mortality rate and more rapid disease progression (Glenda E. Gillies *et al.*, 2014; Kumagai *et al.*, 2014; Smith and Dahodwala, 2014a; Picillo *et al.*, 2017). Sex disparity may have both behavioural and genetic determinants. Behaviourally, role expectations and social attitudes may weight lifestyle risk factors including exercise, smoking, caffeine and diet (Bellou *et al.*, 2016). Molecular evidence points to sexual dimorphism in the basal ganglia. Females have a greater number of dopaminergic neurones in the *SNpc* (Smith and Dahodwala, 2014b). Research into oestrogen and PD risk has received some attention (Glenda E. Gillies *et al.*, 2014; Smith and Dahodwala, 2014a; Litim, Morissette and Di Paolo, 2016). Epidemiological evidence for oestrogen-replacement therapy (ORT) in post-menopausal women and PD risk is mixed – data suggest ORT reduces risk (Currie *et al.*, 2004) or does not change it

(Popat *et al.*, 2005; Simon *et al.*, 2009). Further, post-menopausal women show increased bioavailability of L-DOPA than males (Kumagai *et al.*, 2014). Mechanistically, oestrogen directly affects dopaminergic functioning – dopaminergic neurones contain oestrogen receptors (Paolo, 1994; Quesada, Romeo and Micevych, 2007). Administration of oestrogen ameliorates dopamine depletion in MPTP (Bains, Cousins and Roberts, 2007) and 6-OHDA (McArthur *et al.*, 2007) mouse models. Evidence points toward oestrogen modulating inflammation, resulting in a favourable neuroinflammatory pattern (Straub, 2007; Au *et al.*, 2016). Indeed, sex differences in inflammatory regulation are known, but not well understood. Broadly, females are significantly less prone to infectious diseases and considerably more at risk of auto-immune disorders (Whitacre, 2001; Marriott and Huet-Hudson, 2006) – evidence describes differences in toll-like receptor densities, a more robust pro-inflammatory and anti-inflammatory resolution, and differences in active immunity regulation (Ashdown *et al.*, 2007; Xia *et al.*, 2009; Kawai and Akira, 2010; Scotland *et al.*, 2011).

Neuroinflammation has emerged as a common feature of PD (Thakur and Nehru, 2015). The pathological hallmark of PD, Lewy bodies, are largely composed of aggregated α-synuclein (α-syn), a potent immune response activator. Under conditions of stress α-syn release by neurones into the extracellular space is high (Jang *et al.*, 2010). α-syn is phagocytosed by astrocytes, which secrete pro-inflammatory factors in response (Lee, Kim and Lee, 2010). Under stress conditions astrocytes become chronically activated,

secreting pro-inflammatory cytokines, which activate microglial cells (Sekiyama *et al.*, 2012). Dying neurones also release aggregated α-synuclein, hastening microglial cell recruitment and pro-inflammatory factor production (Zhang *et al.*, 2005b). In addition, α-synuclein directly associates with mitochondria causing a loss of mitochondrial membrane potential (Banerjee *et al.*, 2010), inhibiting mitophagy (Winslow *et al.*, 2010), increasing mitochondrial fragmentation (Nakamura, Venu M Nemani, *et al.*, 2011), and disrupting mitochondrial transport along axons by tubulin depolymerisation (Chen *et al.*, 2007). Indeed, inherent to PD is mitochondrial dysfunction (Federico *et al.*, 2012; Bhat *et al.*, 2015; Pickrell and Youle, 2015). Mitochondria themselves propagate a pro-inflammatory phenotype - treatment of human chondrocytes with respiratory chain inhibitors antimycin A or oligomycin, generating excessive oxidative stress, triggered production of IL-1β, IL-8, TNFα, PTGS2 and PGE2 (Vaamonde-García *et al.*, 2012). Further, ROS, mitochondrial associated membranes and mitochondrial damage-associated molecular patterns stimulate inflammatory responses (Codolo *et al.*, 2013; Reeve *et al.*, 2015; Rieusset, 2018; Sliter *et al.*, 2018; Hazeldine *et al.*, 2019). Aggregation of α-syn sets off a feedback loop of events, linking mitochondrial dysfunction, neuroinflammation, and neurodegeneration.

Oxylipins and inflammatory cytokines are critical to inflammatory action. Oxylipins are important anti- and pro-inflammatory mediators, derived from polyunsaturated fatty acids (PUFAs) by prostaglandin-endoperoxide synthase (PTGS), lipoxygenase (LOX) and cytochrome P450 pathways (Massey and

Nicolaou, 2013). These enzymes form a wide array of short lived, tightly regulated, oxylipins, which are synthesised *de novo* and utilised in a para- or autocrine manner (Tourdot, Ahmed and Holinstat, 2014). The type of oxylipin produced is regulated by dietary PUFA consumption, enzyme specificity for particular PUFAs and the presence of particular oxygenases for PUFA metabolism (Gabbs *et al.*, 2015). PTGS enzymes convert AA (AA) into prostaglandins (PGs), thromboxanes (TXBs) and some hydroxy- metabolites (11-HETE and 9-HODE); LOX enzymes metabolise AA into mid-chain HETEs (5-, 8-, 9-, 11-, 12- and 15-); CYP enzymes have either epoxygenase or hydroxylase activity and metabolise AA into long-chain HETEs (16-, 17-, 18-, 19- and 20-) or metabolise AA into EETs, which may be further metabolised to dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH). Linoleic acid (LA) may similarly be metabolised by PTGS, LOX or CYP450. LA forms EpOMEs by CYP epoxygenases, which may be further hydrated by sEH, to form DiHOMEs; LOX enzymes convert LA into HODEs (Nayeem, 2018). Oxylipins and their enzymatic mediators have been implicated in PD. Long term non-steroidal anti-inflammatory drugs, which inhibit the rate-limiting AA metabolising enzyme PTGS2, is associated with reduced risk of PD (Rees *et al.*, 2011). Increased levels of PTGS2 have been shown in the *SNpc* of PD individuals (Knott, Stern and Wilkin, 2000; Teismann, Tieu, D.-K. Choi, *et al.*, 2003). In the MPTP-treated mouse model, increased prostaglandin E2 levels have been shown, and neurodegeneration was mitigated in PTGS2 knockout mice treated with MPTP (Teismann, Tieu, D.-K. Choi, *et al.*, 2003). In addition, EETs and sEH have been recently implicated in PD. EETs are potent

anti-inflammatory mediators, however, metabolism of EETs by sEH to DHETs reduces the anti-inflammatory effect, and inhibition of sEH has been shown to prolong EETs anti-inflammatory action (Morisseau and Hammock, 2005). Administration of an sEH inhibitor, or sEH gene deletion, to MPTP-treated mice attenuated the neurotoxicity, while sEH overexpression enhanced it. Extending the work, the group showed decreased levels of 8-EET, PGF2a and 9-DiHOME in MPTP treated mice, and significantly increased sEH protein levels in the striatum of individuals with DLB, and sEH mRNA in PARK2 iPSC-derived dopaminergic neurones (Ren, Ma, Yang, Nonaka, Yamaguchi, K. I. Ishikawa, *et al.*, 2018).

Inflammatory cytokines are central to immune processes, regulating acute and chronic inflammatory states by complex networks of interactions. The most well characterised pro-inflammatory cytokines are interleukin-1 (IL-1), IL-6 and tumour necrosis factor (TNFα) (Turner *et al.*, 2014). They are tasked with initiation and propagation of inflammation by multiple mechanisms (Turner *et al.*, 2014). A family of cytokines, known as chemokines, are primarily involved in recruiting leukocytes to sites of injury or infection; however, evidence points to a pleiotropic role: initiation of immune responses, immune surveillance and haematopoiesis (Moser *et al.*, 2004). Inflammatory cytokines have been implicated in PD. Post-mortem analyses have shown elevated levels of pro-inflammatory cytokines and activated microglia in the *SNpc* of PD individuals (Imamura *et al.*, 2003; Flood *et al.*, 2011). In mice, IL-1β levels increase rapidly after LPS administration into the *SNpc*. Further, the

neurodegeneration this caused was abolished with concomitant IL-1β inhibitor treatment (Arai *et al.*, 2004). In a rat model of PD, treatment with a TNFα inhibitor was neuroprotective and prevented DA neuronal degeneration (Melissa K. McCoy *et al.*, 2006).

Inflammation can also involve the *N*-acylethanolamine group of lipids. The best studied are the endocannabinoids, 2-arachidonoylglycerol (2-AG) and anandamide (AEA), and the non-endocannabinoids, palmitoylethanolamide (PEA) and oleoyl-ethanolamide (OEA). *N*-acylethanolamines bind cannabinoid or PPARα receptors with varying affinity, eliciting a variety of biological activities, including anti-inflammation (Tsuboi *et al.*, 2018). Some evidence suggest that PEA and 2-AG are neuroprotective in MPTP-mouse models (Mounsey *et al.*, 2015; Brotini, Schievano and Guidi, 2017). AEA has been shown to increase in a 6-OHDA neurotoxin mouse model of PD (Gorman *et al.*, 2010). *N*-acylethanolamines and their cannabinoid receptors are present in the cerebellum (Skaper *et al.*, 1996; Buczynski and Parsons, 2010; Rodríguez-Cueto *et al.*, 2014).

This study aims to present a survey of oxylipins, *N*-acylethanolamines, and inflammatory cytokines of mitochondrial fractions from post-mortem cerebellar tissue of PD and age-matched control cerebellum using antibody arrays and HPLC. This is the first study to investigate the biochemical changes to the cerebellum within the setting of PD. Due to burgeoning evidence from imaging studies that the cerebellum is impacted in PD, it is hypothesised that

the cerebellum will contain biochemical differences in PD brains and that these differences will manifest differently due to sex.

6.2 Methods

6.2.1 Human tissues

Human brain sections and frozen brain samples were obtained from, Human Tissue Authority approved, Nottingham Health Science Biobank (Nottingham University Hospitals NHS Trust) and Parkinson's UK Brain Bank (Imperial College London). The tissue banks granted the use of the tissue as end users. The Parkinson's tissue bank has approval from the Research Ethics Committee for Wales ref. 08/MRE09/31 + 5. The tissue collection and procedures at the Nottingham University Hospitals Biobank have been ethically approved by the Greater Manchester National Research Ethics Service. This study was granted specific ethics approval by both ethics committees serving the Biobanks and also by the local ethics committee at the School of Veterinary Medicine and Science at the University of Nottingham (ref. 2035 170519). Tissues from human cerebellum were used for mitochondrial isolations and were frozen at the time of post-mortem (PM). Diagnoses of PD were confirmed at PM. Age at death varied from 69 to 85 years for PD brains, non-degenerative human control brains were age matched to within two years of PD samples (Table 2.1). PD Braak staging was performed according to published classification (Alafuzoff *et al.*, 2009).

6.2.2 Mitochondrial fraction preparation

Cerebellar samples were homogenised in mitochondrial extraction buffer (50mM Tris-HCl pH 7.4, 100mM KCl, 1.5mM $MgCl_2$, 1mM EGTA, 50mM HEPES and 100mM sucrose) using a GentleMACS Dissociator (Miltenyi

Biotec). Homogenates were centrifuged at 850g for 10 minutes. The supernatant obtained was subsequently centrifuged at 1,000g for 10 minutes. Finally, this supernatant was centrifuged at 13,200g for 30 minutes to produce the mitochondrial pellet; the remaining supernatant contained the cytosolic fraction. Samples were kept at 4˚C throughout. All fractions were stored at -80˚C.

6.2.3 Western Blotting

Western blotting was carried out as described previously (Shephard *et al.*, 2014). Antibody dilutions: PTGS2 ab62331 (Abcam) 1:2000 dilution in 5% (w/v) BSA in 1xTBS-T; β-actin ab8227 (Abcam) in 5% (w/v) BSA in 1xTBS-T; Goat anti-Rabbit IgG (HRP) A16096 (Invitrogen) 1:1000 in 5% (w/v) BSA in 1xTBS-T. Mitochondrial samples were normalised to β-tubulin level. Blots were visualised using ChemiDoc MP Imaging System (Bio-Rad) and ECL Plus reagent (Pierce). Densitometry were carried out using ImageJ (Schneider, Rasband and Eliceiri, 2012).

6.2.4 Free fatty acid quantification

Total fatty acid concentration was measured using a Free Fatty Acid Quantification Kit (AB65341, Abcam), according to manufacturer's instructions. Briefly, 30µg/µl cerebellar mitochondrial samples [PD Braak male stage 3-4 (n=5), PD Braak male stage 5-6 (n=5), age-matched control male (n=10)] were suspended in 200µl chloroform (1% Triton X-100) and homogenised by 30 strokes of a 1.5ml mini-pestle, vortexed and centrifuged at 16,000g for 10 minutes. Lower phases of the samples were collected and

vacuum dried for 30 min. Fatty acid concentrations were quantified by fluorometric analysis (Varioskan LUX Multimode Microplate Reader).

6.2.5 Absolute quantitation of *N*-acylethanolamine

LC-ESI-MS/MS was used for absolute quantification of four *N*-acylethanolamines: arachidonyl ethanolamide (anandamide, AEA), 2-arachidonyl glycerol (2-AG), palmitoyl ethanolamide (PEA), oleoyl ethanolamide (OEA). *N*-acylethanolamine quantification were carried out by the Centre for Analytical Bioscience, School of Pharmacy, University of Nottingham. Briefly, the uHPLC system used was modular Exion series LC (Sciex, UK). The injection volume was 5µl. The MS system used was a Qtrap 6500+ equipped with an electrospray ionisation (ESI0 interface (Sciex, UK). Standards were purchased from Cambridge Biosciences, Cambridge, UK. One of QC human plasma standard samples were prepared to confirm intra-day accuracy.

6.2.6 Absolute quantitation of oxylipins

LC-ESI-MS/MS was used for analysis of twenty-nine oxylipins in mitochondrial extracts of human cerebellar tissue: AA, LA, 12-HETE, TXB-2, PGE2, 5-HETE, 5-HPETE, 12-HPETE, 9-HETE, 20-HETE, LTB-4, 9-oxoODE, 9-HODE, 5,6-EET, 5,6-DHET, 8,9-DHET, 11,12-DHET, 14,15-DHET, 8-HETE, 11-HETE, 15-HETE, 16-HETE, 8,9-EET, 11,12-EET, 14,15-EET, 17-HDoHE, 13-oxoODE, 13-HODE, 8,15-DiHETE. Oxylipin quantitation was carried out by the Centre for Analytical Bioscience, School of Pharmacy, University of Nottingham. Briefly, HPLC (Shimadzu SCL 10Avp) was run for 20 minutes at

4°C. Injection volume was 20µl. The MS system used was a triple quadrupole ion-trap 4000 QTRAP (ABI, UK), equipped with Turbo Spray ionisation interface. Standards were purchased from Cambridge Biosciences, Cambridge, UK. One batch of QC human plasma standard samples (from in-house stock) was used to confirm the intra-day accuracy of the method.

6.2.7 Human Inflammation Antibody Array

Abcam human inflammation antibody array (ab197451) kit was used for the detection of forty cytokines: BLC, eoxtaxin-1, eotaxin-2, G-CSF, GM-CSF, I-309, ICAM-1, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-6sR, IL-7, IL-8, IL-10, IL-11, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17. MCP-1, MCSF, MIG, MIP-1α, MIP-1β, MIP-1δ, PDGF-BB, RANTES, TIMP-1, TIMP-2, TNFα, TNFβ, sTNF RI, sTNF RII. Glass slides were supplied with sixteen identical antibody arrays, with a 16-well gasket for addition of separate samples to each array. Antibodies were spotted in quadruplicate on each array. The manufacturer's protocol was followed (Abcam, 2018). Briefly, gasket wells were blocked for 30 minutes in Sample Diluent (manufacturer supplied), followed by addition of a standard cytokine cocktail or 100µg of sample. The cocktail was diluted three-fold across eight dilutions and 100µl was added to each well for one hour. Slides were washed and incubated in antibody (manufacturer supplied) overnight at 4°C. Subsequently, Cy3 equivalent dye-conjugated streptavidin was added and incubated at room temperature for one hour. Slides were dried by centrifugation. Slides were scanned at 532nm and data analysed using GenePix Pro Software (Axon GenePix). The median local background was

subtracted from the median fluorescence of each spot and the corrected fluorescence was used to calculate the average fluorescence signal as well as the standard deviation.

6.2.8 Statistical analyses

Data for all experiments were presented as mean ±SEM. Mann-Whitney U-tests were performed on Western blot data. Kruskall-Wallis test was performed on FFA data. Mann-Whitney U and Kruskell-Wallis tests were performed on *N*-acylethanolamine, oxylipin and inflammatory array data using GraphPad Prism (7.03).

6.3 Results

6.3.1 Cerebellar mitochondrial FFA levels increase and PTGS2 levels decrease in PD Braak 5-6 males

This study showed a significant (~14%) increase in free fatty acid (FFA) levels in PD Braak 5-6 male cerebellar mitochondria compared to age-matched control males (p=0.0047) (Figure 6.1A). Increased FFA are consistent with increased inflammatory lipid mediators, however, the increase was not necessarily due to this. Therefore, oxylipin pathways were interrogated directly.

This work found a significant decrease (~40%) in cerebellar mitochondrial PTGS2 levels in PD Braak 5-6 males compared to both age matched PD Braak 3-4 and control males (p=0.0397 and p=0.0070, respectively) (Figure 6.1B). No difference in the levels of PTGS2 were apparent in mitochondrial fractions of control and PD Braak 3-4 cerebellar tissue.

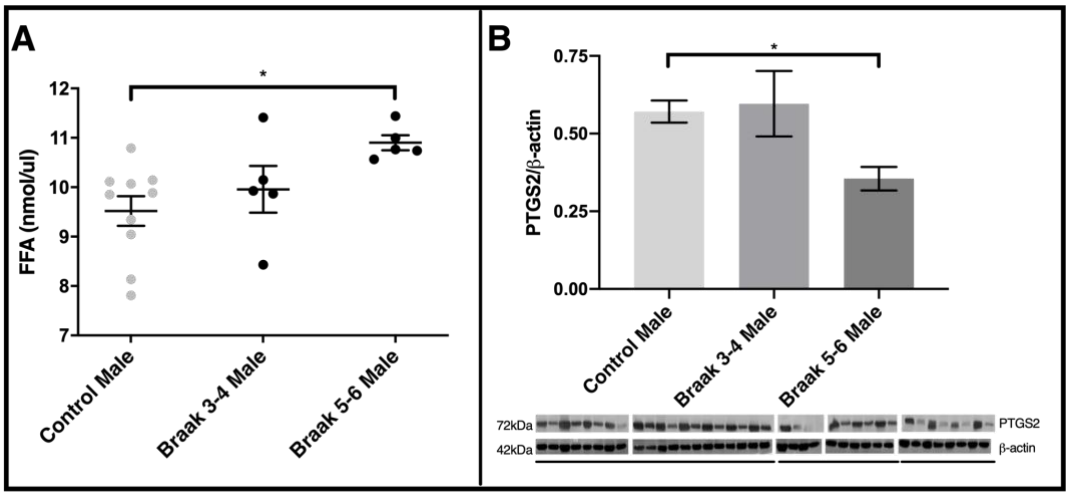


Figure 6.1 (A) FFA concentration increases in males with PD Braak 5-6. A significant increase in FFA levels is apparent in PD Braak 5-6 males compared

with control males (p=0.0047). A significant increase in FFA levels was shown in cerebellar mitochondria of PD Braak 5-6 males compared to age-matched controls (p=0.0164). No significant differences were apparent between PD Braak 3-4 and control. PD Braak 3-4 (n=5) or stage 5-6 (n=5), or age-matched control males (n=10). Plots display mean levels ± SEM. Statistical analyses were carried out using GraphPad Prism (Kruskall-Wallis test). **(B)** PD Braak 5-6 males cerebellar mitochondria contains significantly lower PTGS2 protein compared to either PD Braak 3-4 or control males. PTGS2 localised to mitochondria was found to be significantly lower in the cerebellum of PD Braak 5-6 males compared to PD Braak 3-4 and control males (p=0.0397 and p=0.0070, respectively). No significant difference in PTGS2 levels were seen between PD Braak 3-4 and age-matched control. Mitochondrial PTGS2 levels, normalised to β-actin, were determined by Western blotting using mitochondrial and cytosolic fractions extracted from cerebellar tissue from age-matched control male (n=10) and PD Braak 3-4 (n=5) and 5-6 (n=4). Columns show the mean ±SEM. Statistical analyses were carried out using GraphPad Prism (Mann-Whitney U-test).

6.3.2 Cerebellar mitochondrial oxylipins show distinct PD stage expression changes in males

A panel of twenty-nine oxylipins derived from PTGS, LOX and CYP pathways were interrogated. PD Braak 3-4, 5-6 and control cerebellar mitochondrial samples from males were available for oxylipin analyses. Interestingly, a distinctive pattern was observed across all oxylipins: increasing oxylipins in PD Braak 3-4 compared to controls, followed by a return to control male levels in PD Braak 5-6 (Figure 6.5). 4 oxylipins were significantly increased in PD Braak 3-4 compared with control (LTB-4, 12-HETE, 8-HETE, 8,9-EET). 5 oxylipins were significantly decreased in PD Braak 5-6 compared with PD Braak 3-4 (TXB-2, LTB-4, 9-HODE, 11,12-EET, 13-HODE). No significant differences were apparent between PD Braak 5-6 and control (Figure 6.2A).

Analysis of intergroup variance showed that AA levels are highly variant in disease compared to control males (Figure 6.2B). Control male cerebellar mitochondria showed highly homogenous AA levels, which was subsequently lost in PD.

6.3.3 PD Braak 5-6 males have increased inflammatory cytokine levels

PD Braak 5-6 and control male samples were available for inflammatory protein analysis. PD Braak 5-6 males had significantly greater levels of inflammatory cytokines compared to control males (Figure 6.3). Eight inflammatory proteins were differentially expressed, all of which were increased in PD Braak 5-6 cerebellar mitochondria. Seven were pro-inflammatory (Eotaxin-1, I-309, IL-1β, IL-12p40, IL-12p70, MIG, TNF-RII) and one was anti-inflammatory (IL-1ra) (Refer to Table 6.1 for *p* values).

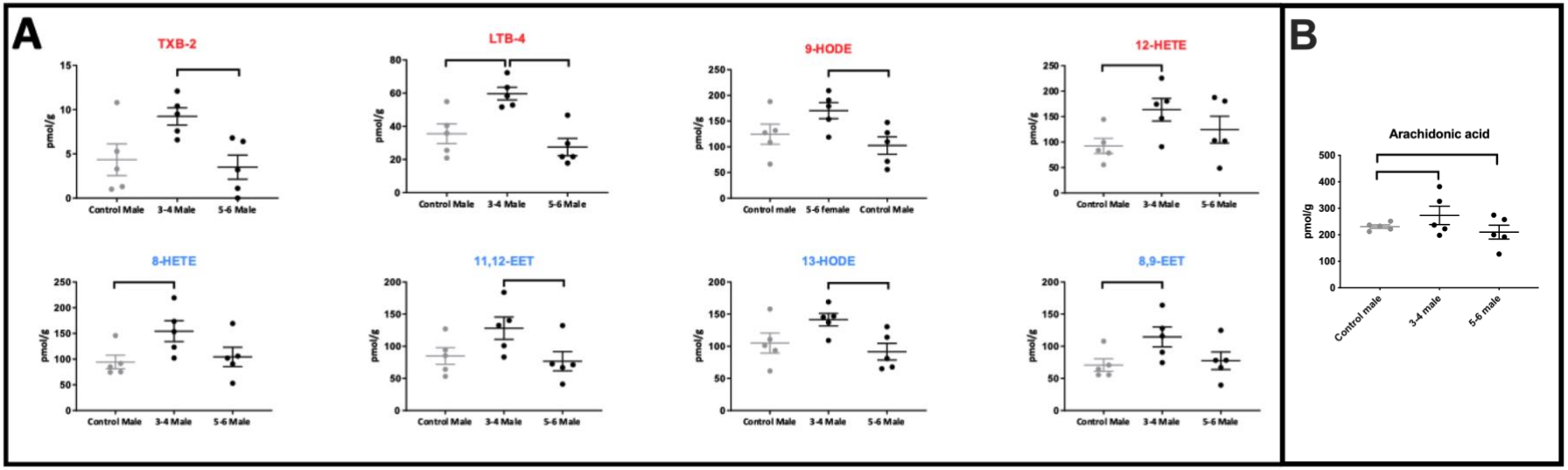

A
TXB-2
pmol/g
Control Male
3-4 Male
5-6 Male
LTB-4
9-HODE
Control male
5-6 female
12-HETE
8-HETE
11,12-EET
13-HODE
8,9-EET
B
Arachidonic acid
Control male
3-4 male
5-6 male

Figure 6.2 (A) A distinctive disease-specific pattern of oxylipins was found in cerebellar mitochondria of males. Four oxylipins were significantly increased in cerebellar mitochondria of males with PD Braak 3-4, compared with control males. Five eicosanoids are significantly decreased in PD Braak 5-6 compared with PD Braak 3-4 males. No significant changes were shown between control males and PD Braak 5-6 males. Red or blue titles represent pro- or anti-inflammatory, respectively. Plots display mean levels ± SEM. Refer to Table 6.1 for *p* values (Mann-Whitney U-test). **(B)** Levels of AA are heterogenous in PD. Cerebellar mitochondria AA levels are homogenous amongst control individuals, however, significant variation is clear in PD Braak 3-4 and 5-6 males. AA Box plots display interleaved high and low. The horizontal line represents the mean. Refer to Table 6.2 for *F* values. All samples were age matched. PD Braak 3-4 male n=5; PD Braak 5-6 male n=5; control male n=5.

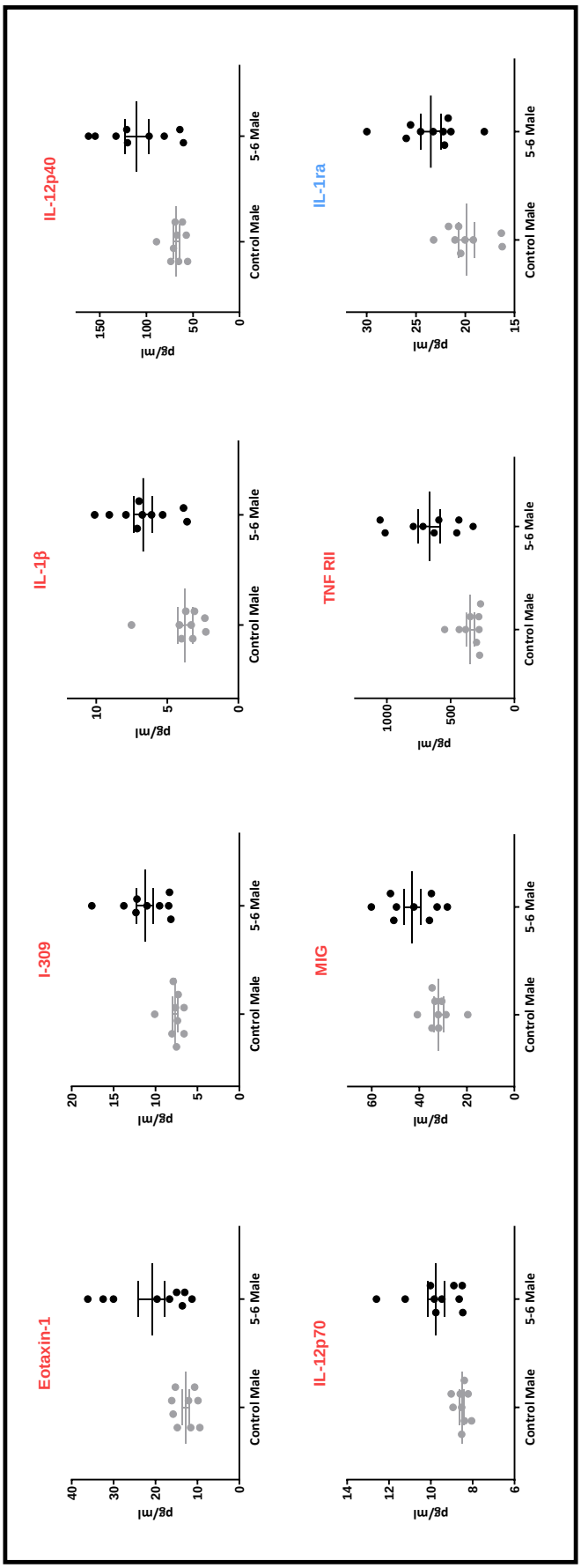

IL-12p40
pg/ml
150
100
50
0
Control Male
5-6 Male
IL-1ra
pg/ml
30
25
20
15
Control Male
5-6 Male
IL-1β
pg/ml
10
5
0
Control Male
5-6 Male
TNF RII
pg/ml
1000
500
0
Control Male
5-6 Male
I-309
pg/ml
20
15
10
5
0
Control Male
5-6 Male
MIG
pg/ml
60
40
20
0
Control Male
5-6 Male
Eotaxin-1
pg/ml
40
30
20
10
0
Control Male
5-6 Male
IL-12p70
pg/ml
14
12
10
8
6
Control Male
5-6 Male

Figure 6.3 Levels of inflammatory cytokines are increased in cerebellar mitochondria of males with PD Braak 5-6. Eight inflammatory cytokines were increased in cerebellar mitochondria of Braak 5-6 males compared with control males. PD Braak 5-6 male n=10; control male n=9. Red /blue titles represent pro- /anti-inflammatory, respectively. Plots display mean levels ± SEM (Mann-Whitney test). Refer to Table 6.1 for *p* values.

6.3.4 A distinct pattern of inflammatory marker expression change in PD Braak 5-6 female cerebellar mitochondria

Cerebellar mitochondria from PD Braak 5-6 females and control females were analysed for oxylipin and inflammatory cytokine content. Unlike males, PD Braak 5-6 females have significantly decreased levels of two inflammatory cytokines, compared to control females (ICAM-1 and TIMP-2) (Figure 6.4B). Mean quantities of ICAM-1 were 891.9 and 535.4 pmol/g, for control female and Braak 5-6 female, respectively. Mean quantities of TIMP-2 were 604.5 and 298.5 pmol/g, for control female and Braak 5-6 female, respectively. Oxylipins similarly have a different pattern of change compared to males (Figure 6.4A). Twelve oxylipins and AA were significantly reduced in PD Braak 5-6 females compared to control females (Table 6.1). Of the significantly altered oxylipins, seven were pro-inflammatory (12-HETE, TXB-2, 5-HETE, 5-HPETE, 12-HPETE, 9-oxoODE, 9-HODE) and five were anti-inflammatory (5,6-EET, 16-HETE, 17-HDoHE, 13-oxoODE, 13-HODE).

6.3.5 *N*-acylethanolamines levels do not significantly change in cerebellar mitochondria of PD Braak 5-6 females or males

No significant changes in cerebellar mitochondrial *N*-acylethanolamines (2-AG, AEA, OEA, PEA) were apparent between females and males with or without PD Braak 5-6 PD (Figure 6.6B). The measured quantities are in line with previous studies measuring *N*-acylethanolamines in human CSF and serum (Nicholson *et al.*, 2015; Kantae *et al.*, 2017).

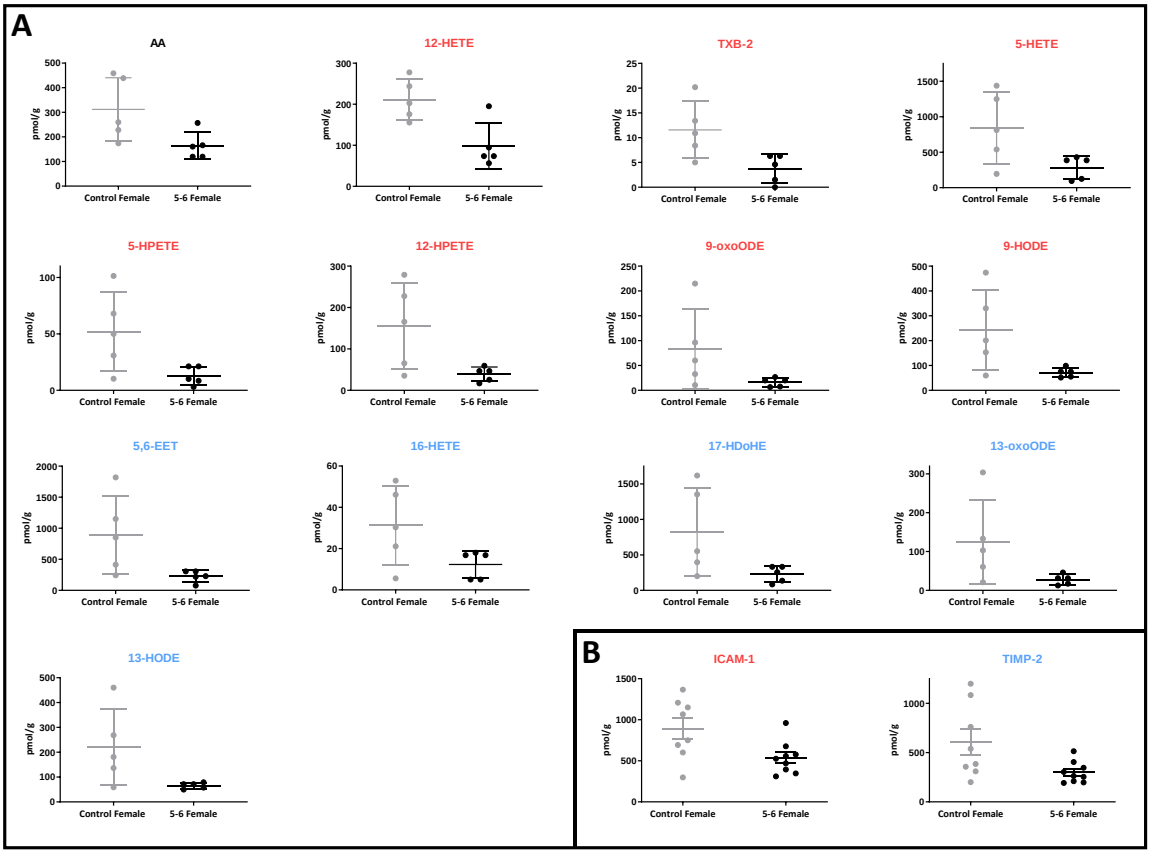


Figure 6.4 (A) Oxylipin levels are lower in cerebellar mitochondria of females with PD Braak 5-6 PD. Twelve oxylipins and AA are significantly reduced in cerebellar mitochondria of females with PD Braak 5-6, compared with controls. All samples were age matched. PD Braak 5-6 female n=5; control female n=5. **(B)** Two inflammatory cytokines are significantly lower in cerebellar mitochondria of PD Braak 5-6 females. ICAM-1 and TIMP-2 were significantly reduced in cerebellar mitochondria of PD Braak 5-6 females compared with control females. PD Braak 5-6 female n=9; control female n=8. Plots display mean levels ± SEM. Red or blue titles represent pro- or anti-

inflammatory, respectively. Refer to Table 6.1 for *p* values (Mann-Whitney U-test).

6.3.6 Variation in inflammatory marker expression characterises the control female group.

It was clear that females and males both had a unique pattern of oxylipin expression (Figure 6.5). The control female group showed considerably more variation than any other group, and this heterogeneity appeared to be lost in PD Braak 5-6 females. In contrast, control males showed much greater oxylipin expression homogeneity. This observation drove a reanalysis of oxylipin, inflammatory protein and *N*-acylethanolamine data to investigate group expression variation.

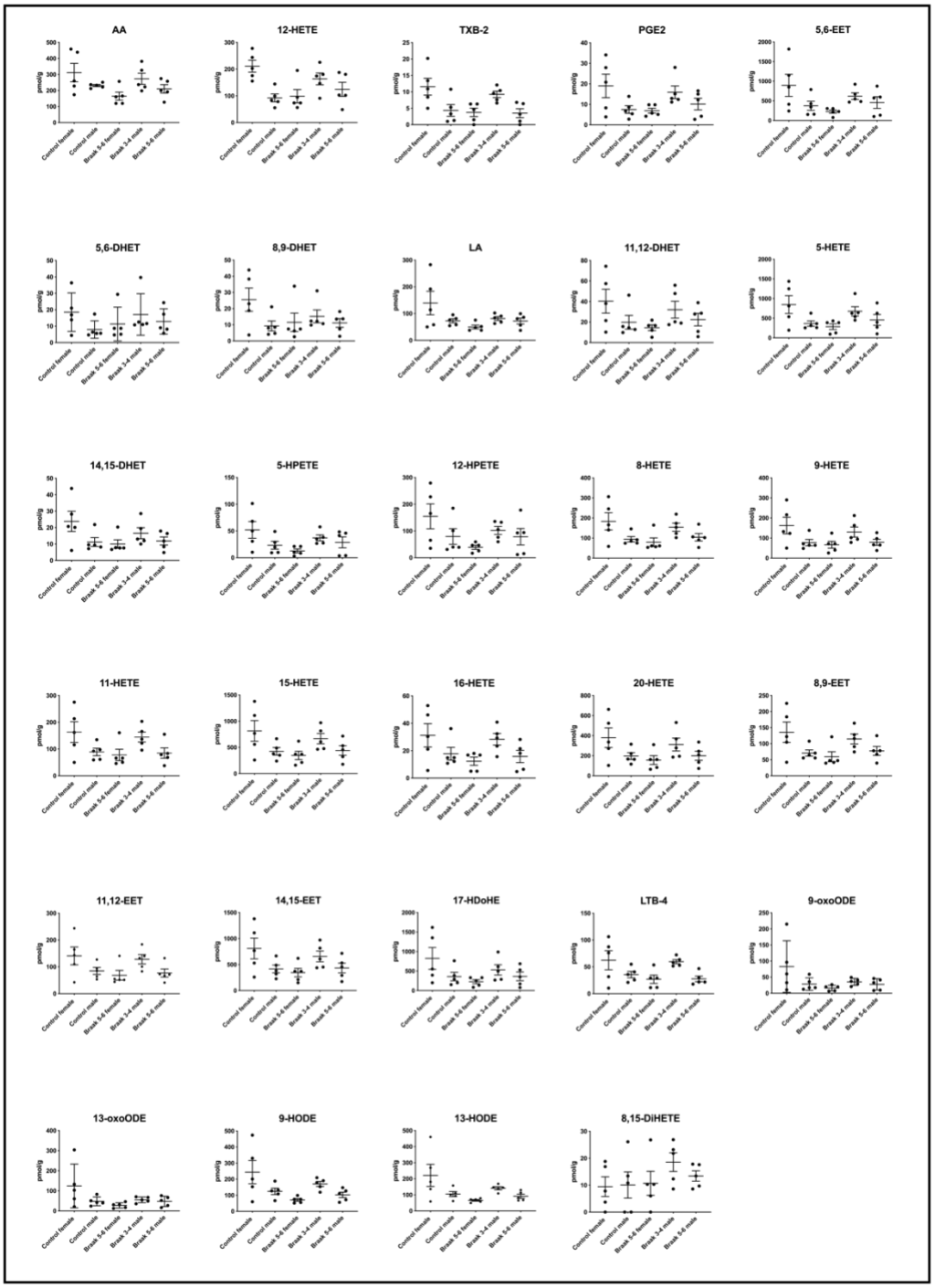


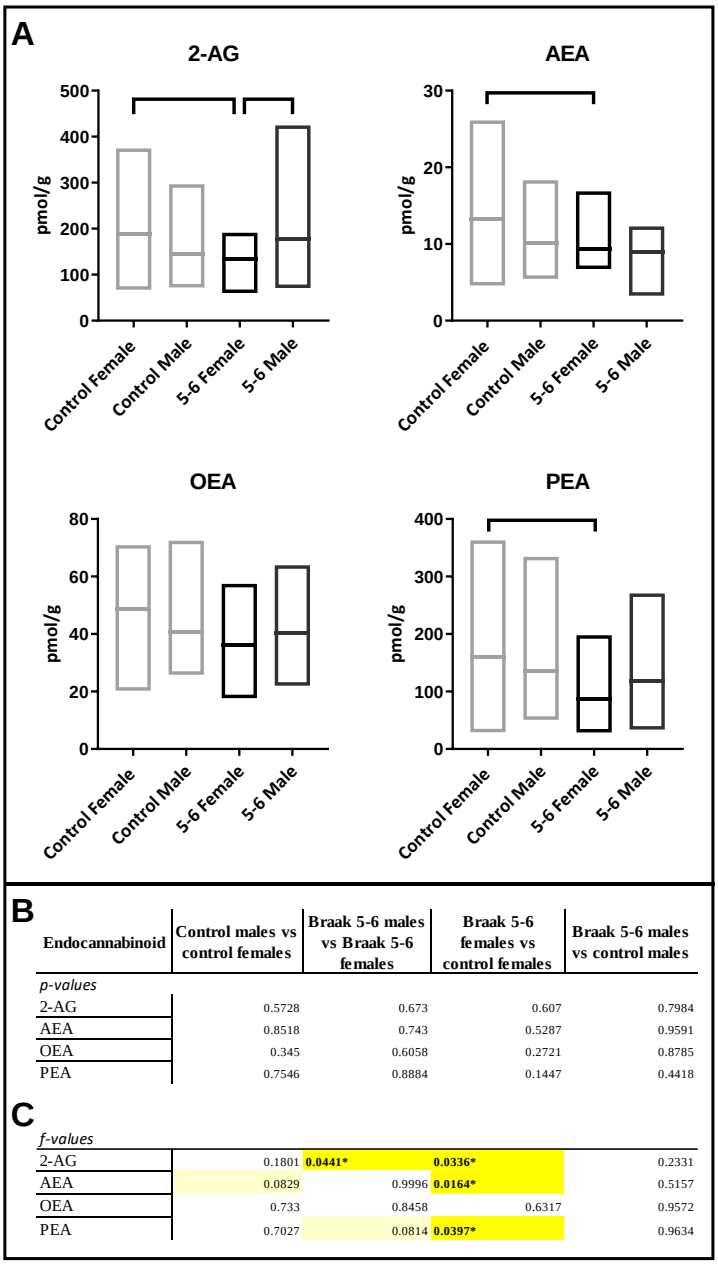
Figure 6.5 Control female group show variance in expression of oxylipins. Comparisons of all measured oxylipins in cerebellar mitochondria from males (PD Braak 3-4 and 5-6) and females (PD Braak 5-6) with PD and age-matched controls. PD Braak 3-4 male n=5; PD Braak 5-6 male n=5; PD Braak 5-6 female n=5; control male n=5; control female n=5. Red or blue titles represent pro- or anti-inflammatory, respectively. Plots display mean levels ± SEM. Statistical analyses were carried out using GraphPad Prism (Mann Whitney U-test). For all *p* or *F* values refer to Supplementary Tables 6.1 or 6.2, respectively.

Group comparison		Inflammatory marker	*p* value
Braak 3-4 Males vs Control Males	Oxylipins	LTB-4	0.0317*
		12-HETE	0.0317*
		8-HETE	0.0317*
		8,9-EET	0.0317*
Braak 5-6 Males vs Control Males	Inflammatory Cytokines	Eotaxin-1	0.04*
		I-309	0.0005***
		IL-1β	0.0057**
		IL-12p40	0.0188*
		MIG	0.0188*
		TNF-RII	0.0012**
		IL-1ra	0.0057**
		IL-12p70	0.0101*
Braak 3-4 Males vs Braak 5-6 Males	Oxylipins	TXB-2	0.0159*
		LTB-4	0.0079**
		9-HODE	0.0317*
		11,12-EET	0.0317*
		13-HODE	0.0317*
Braak 5-6 Females vs Control Females	Oxylipins	AA	0.0317*
		12-HETE	0.0317*
		TXB-2	0.0317*
		5-HETE	0.0476*
		5-HPETE	0.0476*
		12-HPETE	0.0476*
		9-oxoODE	0.0476*
		9-HODE	0.0476*
		5,6-EET	0.0317*
		16-HETE	0.0476*
		17-HDoHE	0.0476*
		13-oxoODE	0.0476*
		13-HODE	0.0476*
	Inflammatory Cytokines	ICAM-1	0.0274*
		TIMP-2	0.036*
Control Males vs Control Females	Oxylipins	12-HETE	0.0079**
	Inflammatory Cytokines	GM-CSF	0.0206*
		RANTES	0.007**

Table 6.1 Statistical comparisons of significantly altered inflammatory marker levels in cerebellar mitochondria of males and females with PD Braak 3-4, 5-6 or age-matched controls. Red /blue font represent pro- /anti-inflammatory markers, respectively. Statistical analyses were carried out using GraphPad Prism (Mann-Whitney U-test). Refer to Supplementary Tables 6.1 and 6.3 for *p* values of all twenty-nine analysed oxylipins and forty inflammatory cytokines.

6.3.7 Control female group *N*-acylethanolamine variance is reduced with PD

As discussed (refer to 6.3.5), no significant differences in the mean levels of *N*-acylethanolamines were seen in this study. However, variance analysis revealed significant differences between control females and PD Braak 5-6 females (Figure 6.6A). 2-AG, AEA and PEA levels were highly heterogenous in control females and this heterogeneity was subsequently lost in PD Braak 5-6 individuals (Figure 6.6C). In addition, 2-AG levels were significantly variant between PD Braak 5-6 females and males.



B

Endocannabinoid	Control males vs control females	Braak 5-6 males vs Braak 5-6 females	Braak 5-6 females vs control females	Braak 5-6 males vs control males
p-values				
2-AG	0.5728	0.673	0.607	0.7984
AEA	0.8518	0.743	0.5287	0.9591
OEA	0.345	0.6058	0.2721	0.8785
PEA	0.7546	0.8884	0.1447	0.4418

C

f-values				
2-AG	0.1801	**0.0441***	**0.0336***	0.2331
AEA	0.0829	0.9996	**0.0164***	0.5157
OEA	0.733	0.8458	0.6317	0.9572
PEA	0.7027	0.0814	**0.0397***	0.9634

Figure 6.6 (A) Control female groups *N*-acylethanolamine variance is diminished in the PD Braak 5-6 female group. Control female group show heterogenous expression of 2-AG, AEA and PEA compared with PD Braak 5-6 female group. Male groups do not show significant variation in *N*-acylethanolamine levels. **(B)** Statistical analyses were carried out using GraphPad Prism (Mann-Whitney U-test). No significant differences in mean *N*-acylethanolamine levels were seen between any groups. **(C)** Statistical analyses were carried out using GraphPad Prism (*F*-test). Bright yellow shaded numbers refer to significance; pale yellow shaded numbers refer to near significance ($p<0.1$). Braak 5-6 male n=8; Braak 5-6 female n=9; control male n=8; control female n=6. All samples were age matched (±2 years). Box plots display interleaved high and low. The horizontal line represents the mean.

6.3.8 PD Braak 5-6 females have less anti-inflammatory marker variance than controls

Group variance comparison of cerebellar mitochondria from control females and PD Braak 5-6 females revealed eleven oxylipins with significant variance (Figure 6.7A). LA and ten oxylipins had significantly more variation in the control female group than PD Braak 5-6 female: LA, six pro-inflammatory (PGE2, 5-HETE, 5-HPETE, 12-HPETE, 9-oxoODE, 9-HODE) and four anti-inflammatory (5,6-EET, 17-HDoHE, 13-oxoODE, 13-HODE) (Table 6.2). This pattern was repeated across twenty-eight of the twenty-nine analysed oxylipins (Figure 6.5 and Supplementary Table 6.2). The data suggest a loss of cerebellar mitochondrial oxylipin expression heterogeneity is a characteristic of the PD Braak 5-6 female group.

Control female group showed alterations in inflammatory cytokine variance compared with PD Braak 5-6 female group (Figure 6.7B). Six pro-inflammatory cytokines had significant variance. Four of the seven pro-inflammatory cytokines had greater variance in PD Braak 5-6 females (IL-1β, MIP-1δ, RANTES and TNF-RII). Three of the Four anti-inflammatory cytokines were shown to have greater variance in control female group than PD Braak 5-6 female group (IL-10, IL-11, TIMP-2), suggesting a less dynamic or robust anti-inflammatory response in PD Braak 5-6 females.

6.3.9 Male inflammatory marker variance increases in PD

Seventeen inflammatory proteins showed significant variance between PD Braak 5-6 male and control male groups (Figure 6.7C). All seventeen had greater variance in PD Braak 5-6 male group; Fourteen were pro-inflammatory and three were anti-inflammatory (Table 6.2). Extending the threshold of significance (p<0.1) revealed another four inflammatory proteins with greater variance in PD Braak 5-6 male than control male groups (Supplementary Table 6.4).

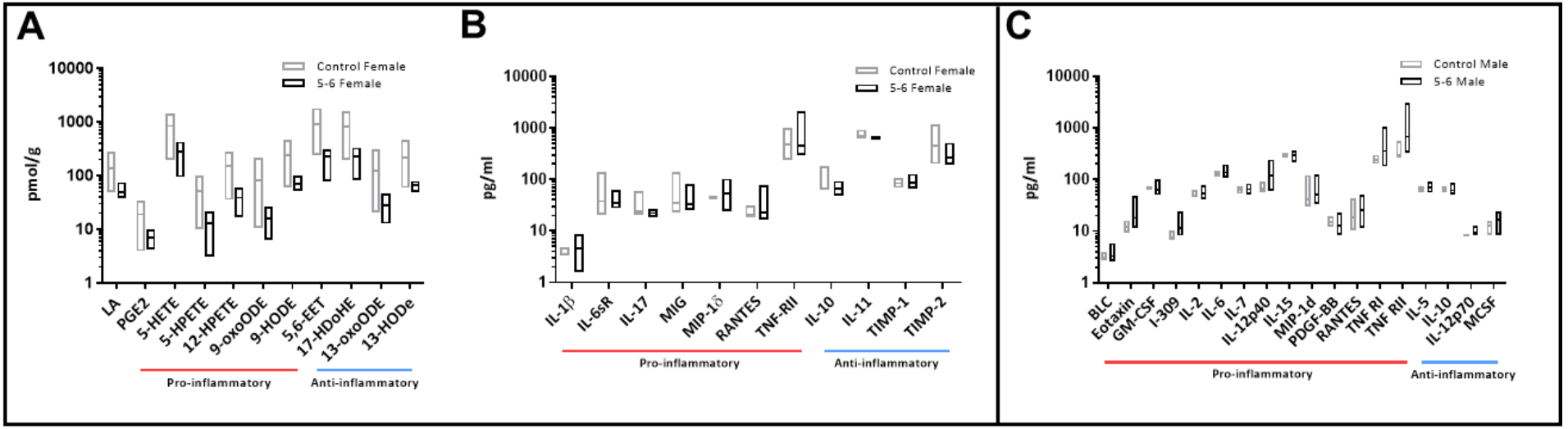

A
pmol/g
10000
1000
100
10
1
Control Female
5-6 Female
LA
PGE2
5-HETE
5-HPETE
12-HPETE
9-oxoODE
9-HODE
5,6-EET
17-HDoHE
13-oxoODE
13-HODe
Pro-inflammatory
Anti-inflammatory
B
pg/ml
10000
1000
100
10
1
Control Female
5-6 Female
IL-1β
IL-6sR
IL-17
MIG
MIP-1δ
RANTES
TNF-RII
IL-10
IL-11
TIMP-1
TIMP-2
Pro-inflammatory
Anti-inflammatory
C
pg/ml
10000
1000
100
10
1
Control Male
5-6 Male
BLC
Eotaxin
GM-CSF
I-309
IL-2
IL-6
IL-7
IL-12p40
IL-15
MIP-1d
PDGF-BB
RANTES
TNF RI
TNF RII
IL-5
IL-10
IL-12p70
MCSF
Pro-inflammatory
Anti-inflammatory

Figure 6.7 (A) Control female group have greater variation in cerebellar mitochondria oxylipin expression than PD Braak 5-6 female group. Control female group show significantly greater variance in eleven oxylipins than the PD Braak 5-6 female group. PD Braak 5-6 female n=5; control female n=5. **(B)** Cerebellar mitochondria from PD Braak 5-6 female group have greater pro-inflammatory cytokine variation and less anti-inflammatory variation than the control female group. Seven pro-inflammatory and four anti-inflammatory cytokines have significant variation between PD Braak 5-6 female and control female group. The PD Braak 5-6 female groups showed increased and decreased variance in pro- and anti-inflammatory cytokines, respectively, compared with the control female group. **(C)** PD Braak 5-6 male group show considerably greater variance in cerebellar mitochondrial inflammatory cytokine levels than the control male group. All seventeen inflammatory cytokines are significantly more variant in the PD Braak 5-6 male group than the control male group. PD Braak 5-6 male n=10; PD Braak 5-6 female n=9; control male n=9; control female n=8. Box plots display interleaved high and low. The horizontal line represents the mean. Data shown are oxylipins (A) and inflammatory cytokines (B+C) with significant variances (*F*-test). Refer to Table 6.2 for *F* values.

6.3.10 Inflammatory markers have high levels of variance in females when compared to the male control group

Females and males were directly compared. Inflammatory profiles of cerebellar mitochondria from the control female group are highly varied, whereas, the control male group shows homogenous levels. This pattern is reflected in twenty-eight of the twenty-nine oxylipins analysed (Figure 6.5 and Supplementary Table 6.2). Female group were significantly more variant in the expression of seven oxylipins, AA and LA (Figure 6.8B). PUFAs AA and LA showed significantly greater variance in control female group than male. Three oxylipins with significant variance were pro-inflammatory (5-HETE, 9-oxoODE, 9-HODE) and four were anti-inflammatory (8-HETE, 8,9-EET, 13-oxoODE, 13-HODE).

Substantial variation in cerebellar mitochondrial inflammatory cytokine variance was also apparent when comparing control female and male groups (Figure 6.8A). Twenty-one pro- and anti-inflammatory cytokines showed significant variance between control female and male groups (Table 6.2). Twenty inflammatory cytokines were significantly more heterogenous in control females. Pro-inflammatory IL-1β was the only cytokine with significantly greater variance in the control male group than female group.

Two cytokines were shown to be differentially expressed between female and control males (Figure 6.8E). RANTES and GM-CSF were the only inflammatory proteins with sex-specific mean expression changes – both were significantly increased in control female compared with male. RANTES mean levels were 22.91 and 17.39 pg/ml, for control female and control male, respectively. GM-CSF mean levels were 75.62 and 68.62 pg/ml, for control female and control male, respectively.

6.3.11 Comparison between PD Braak 5-6 female and male groups show they are similar

Unlike the significant variance between female and control males, PD Braak 5-6 female and male groups show little variation in inflammatory marker levels. Indeed, there was significantly less inflammatory cytokine variation than between control groups (Fig 6.8C). Seven inflammatory cytokines had significant differences in variation (Table 6.2). PD Braak 5-6 female group had

increased variance in expression of pro-inflammatory cytokines IL-12p70, MIP1α, MIP-1β and TNFα. Anti-inflammatory IL-4, and MCSF had greater variance in PD Braak 5-6 female group, whereas the PD Braak 5-6 male group showed greater variance in expression of TIMP-1.

Pro-inflammatory 12-HPETE and anti-inflammatory 5,6-EET were the only significantly variant oxylipins between PD Braak 5-6 female and male groups (Figure 6.8D).

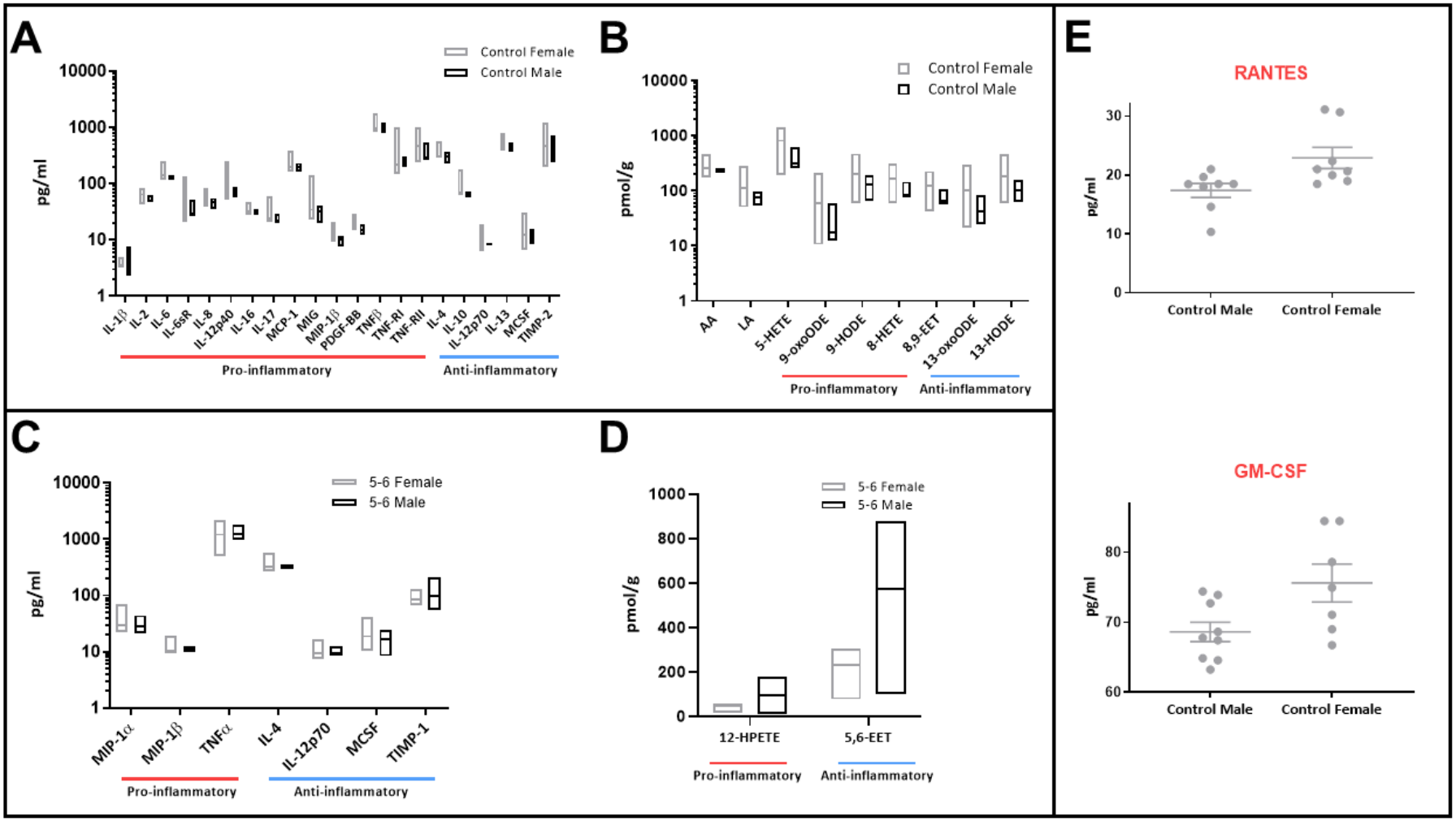

A
Control Female
Control Male
pg/ml
10000
1000
100
10
1
IL-1β
IL-2
IL-6
IL-6sR
IL-8
IL-12p40
IL-16
IL-17
MCP-1
MIG
MIP-1β
PDGF-BB
TNFβ
TNF-RI
TNF-RII
IL-4
IL-10
IL-12p70
IL-13
MCSF
TIMP-2
Pro-inflammatory
Anti-inflammatory
B
Control Female
Control Male
pmol/g
10000
1000
100
10
1
AA
LA
5-HETE
9-oxoODE
9-HODE
8-HETE
8,9-EET
13-oxoODE
13-HODE
Pro-inflammatory
Anti-inflammatory
C
5-6 Female
5-6 Male
pg/ml
10000
1000
100
10
1
MIP-1α
MIP-1β
TNFα
IL-4
IL-12p70
MCSF
TIMP-1
Pro-inflammatory
Anti-inflammatory
D
5-6 Female
5-6 Male
pmol/g
1000
800
600
400
200
0
12-HPETE
5,6-EET
Pro-inflammatory
Anti-inflammatory
E
RANTES
pg/ml
30
20
10
0
Control Male
Control Female
GM-CSF
pg/ml
80
70
60
Control Male
Control Female

Figure 6.8 (A) Cerebellar mitochondria of the control female group have highly variant inflammatory protein expression compared with control male group. Twenty-one inflammatory cytokines had significant variation between control female and male groups. Twenty of these were significantly more variant in female control group. Control male n=9; control female n=8. **(B)** Oxylipin variance is significantly greater in cerebellar mitochondria of control female group than control male group. Control female group have significantly greater variance in nine oxylipins. Control male group cerebellar mitochondrial oxylipin levels are more homogenous. Control male n=5; control female n=5. **(C)** Cerebellar mitochondria from PD Braak 5-6 female group show greater variance in inflammatory cytokine expression than PD Braak 5-6 male group. Six of the Seven significantly different inflammatory cytokines showed higher variance in PD Braak 5-6 female group than male. PD Braak 5-6 male n=10; PD Braak 5-6 female n=9. **(D)** PD Braak 5-6 female and male groups show little variance in cerebellar mitochondrial oxylipin levels. Two oxylipins are significantly more variant in PD Braak 5-6 males than females. PD Braak 5-6 male n=5; PD Braak 5-6 female n=5. **(E)** The mean levels of two cytokines are significantly increased in control females compared to control males. RANTES and GM-CSF were significantly increased in control females compared to control males. All samples were age matched ±2 years. A-D present inflammatory cytokines and oxylipins with significantly different variances (*F*-test). Refer to Table 6.2 for *F* values. Box plots display interleaved high and low. The horizontal line represents the mean. E shows cytokines with significantly different means. Red font titles represent pro-inflammatory cytokines. Displayed are mean levels ± SEM. (Mann-Whitney U-test). Refer to Table 6.1 for *p* values.

Group comparison		Inflammatory marker	*F* value
Braak 3-4 Males vs Control Males	Oxylipins	AA	0.0072**
Braak 5-6 Males vs Control Males	Oxylipins	AA	0.0209*
	Inflammatory Cytokines	BLC	0.0024**
		Eotaxin-1	0.0017**
		GM-CSF	0.0004***
		I-309	0.0054**
		IL-2	0.0202*
		IL-6	0.0065**
		IL-7	0.0419*
		IL-12p40	0.0013**
		IL-15	0.0263*
		PDGF-BB	0.0396*
		RANTES	0.0046**
		TNF-RI	<0.0001****
		TNF-RII	0.0123*
		IL-5	0.0301*
		IL-10	0.0095**
		IL-12p70	0.0004***
		MCSF	0.0367*
Braak 5-6 Females vs Control Females	Oxylipins	LA	0.0036**
		PGE2	0.0104*
		5-HETE	0.0468*
		5-HPETE	0.0149*
		12-HPETE	0.0043**
		9-oxoODE	0.0008***
		9-HODE	0.0011**
		5,6-EET	0.0027**
		17-HDoHE	0.006**
		13-oxoODE	0.0012**
		13-HODE	0.0002***
	Inflammatory Cytokines	IL-6sR	0.0145*
		IL-17	0.0138*
		MIG	0.0155*
		MIP-1δ	<0.0001****
		RANTES	0.0026**
		TNF-RII	0.0479*
		IL-10	0.0097**
		IL-11	0.024*
		TIMP-1	0.0087**
		TIMP-2	0.0022**
Braak 5-6 Males vs Braak 5-6 females	Inflammatory Cytokines	IL-1β	0.0008***
		MIP-1α	0.0419*
		MIP-1β	0.0023**
		TNFα	0.0413*
		IL-4	0.0005***
		IL-12p70	0.0408*
		MCSF	0.0439*
		TIMP-1	0.0265*
Control Males vs Control Females	Oxylipins	AA	0.001**
		LA	0.0048**
		5-HETE	0.0402*
		9-oxoODE	0.0173*
		9-HODE	0.0272*
		8-HETE	0.0431*
		8,9-EET	0.0408*
		13-oxoODE	0.0085**
		13-HODE	0.0138*
	Inflammatory Cytokines	IL-1β	<0.0001****
		IL-2	0.0155*
		IL-6	0.0001***
		IL-6sR	0.0002***
		IL-8	0.0117*
		IL-12p40	<0.0001****
		IL-16	0.0166*
		IL-17	0.0279*
		MCP-1	0.0028**
		MIG	<0.0001****
		MIP-1β	0.0044**
		MIP-1δ	0.0002***
		PDGF-BB	0.0203*
		TNFβ	0.0155*
		TNF-RI	<0.0001****
		TNF-RII	0.0079**
		IL-4	0.0349*
		IL-10	<0.0001****
		IL-12p70	<0.0001****
		IL-13	0.0072**
		MCSF	0.0052**
		TIMP-2	0.0139*

Table 6.2 Significantly changed group variances of analysed inflammatory cytokines and oxylipins from cerebellar mitochondria of PD females and males or age-matched controls. Red /blue font represent pro- /anti-inflammatory cytokines or oxylipins, respectively. Statistical analyses were carried out using GraphPad Prism (*F*-test). Refer to Supplementary Tables 6.2 and 6.4 for *F* values of all 29 analysed oxylipins and 40 inflammatory cytokines.

6.4 Discussion

The cerebellum is a brain region that has received interest in the context of PD in recent years. However, its role in PD has almost solely been determined by imaging techniques – molecular changes to this region are not well characterised. In this study, molecular analysis has shown inflammatory alterations to be present within the cerebellum of individuals with PD, thereby positioning the cerebellum as having an active role in the disease process.

6.4.1 PTGS2 levels may reflect the level of cerebellar compensation

Many critical mediators of inflammation are lipid derived and previous work have shown inflammatory lipids (oxylipins, *N*-acylethanolamines) to be altered in chronic inflammatory diseases, including PD (Tourdot, Ahmed and Holinstat, 2014; Ren, Ma, Yang, Nonaka, Yamaguchi, K. Ishikawa, *et al.*, 2018; Diab *et al.*, 2019). This work showed that FFA concentration was increased in PD Braak 5-6 males. To assess whether the increased FFA concentration was reflected in oxylipin and *N*-acylethanolamine levels, the inflammatory lipid enzymatic pathway with the most robust evidence supporting its involvement in PD, PTGS2, and an array of twenty-nine oxylipins and four *N*-acylethanolamines, were investigated. With its central, rate-limiting role, in the enzymatic conversion of AA to downstream inflammatory eicosanoids, PTGS2 has been implicated in PD (Teismann, Tieu, D. K. Choi, *et al.*, 2003). Two groups (Teismann, Tieu, D. K. Choi, *et al.*, 2003; Przybyłkowski *et al.*, 2004) have shown that PTGS2 levels are upregulated in the initial stage of

MPTP administration to mice, corresponding to the degeneration phase of injury, and that they fall by day 14 post-MPTP treatment. Thus, the maximum PTGS2 expression appears to coincide with maximal neuronal insult. This, together with evidence pointing to a peak pro-inflammatory period early in disease (Stojkovska, Wagner and Morrison, 2015), are in agreement with the present study, showing reduced PTGS2 levels in PD Braak 5-6 males compared with PD Braak 3-4 and control males.

Another interesting hypothesis is that cerebellar PTGS2 levels parallel the level of compensation. At present, the fMRI literature describing greater cerebellar activity in PD individuals has been conducted in either early-middle stage (Benninger *et al.*, 2009) or early stage PD individuals (Liu *et al.*, 2013; Festini *et al.*, 2015). To my knowledge, no fMRI data exist describing cerebellar activity in later stage PD. If we take the cerebellum to act in a compensatory fashion in PD, it may be that greater cerebellar activity occurs in earlier disease stages, but this is not maintained into later stages. Support for this comes from evidence suggesting PTGS2 expression is not simply induced by stress, but by excitatory glutamatergic synaptic activity (Kaufmann *et al.*, 1997). N-methyl-D-aspartate (NMDA)-type glutamate receptor activation, which are prevalent in the cerebellum (Llansola *et al.*, 2005), increases PTGS2 levels, and levels are modulated in an NMDA-dependent manner (Kaufmann *et al.*, 1997). Further work should be directed at assessing cerebellar activation at different PD stages.

6.4.2 Inflammation peaks early in Parkinson's disease

Oxylipin levels also showed a temporal pattern. Evidence suggest that symptom severity is not worsened linearly with dopaminergic neurone loss and that in mild-moderate PD enhanced cerebellar circuit functional connectivity is apparent and is associated with improved motor function (Sen *et al.*, 2010; Wu *et al.*, 2011; Simioni, Dagher and Fellows, 2017). The increased oxylipin levels in PD Braak 3-4 males adds evidence for cerebellar involvement and may reflect hyperactivation in earlier disease. PD Braak 5-6 males oxylipin levels revert to control male levels. Interestingly, mice overexpressing wild-type α-syn support a temporal distribution of inflammatory markers – they showed increased TNFα levels until 6 months of age despite maintaining microglial activation until 14 months of age (Watson *et al.*, 2012). Cumulatively, growing evidence suggests inflammation is an early process in PD, beginning and peaking before the development of motor impairments (Stojkovska, Wagner and Morrison, 2015) - the present data are in agreement: increased oxylipin levels in PD Braak 3-4 male individuals, which subsequently fall in PD Braak 5-6, reflected by decreasing oxylipin levels and PTGS2 expression. There is a dearth of research regarding *N*-acylethanolamines in PD. Evidence points to PEA and 2-AG neuroprotection in the MPTP-treated mouse (Mounsey *et al.*, 2015; Brotini, Schievano and Guidi, 2017), and AEA was increased in the 6-OHDA neurotoxin mouse (Gorman *et al.*, 2010). *N*-acylethanolamines and their cannabinoid receptors are present in the cerebellum (Skaper *et al.*, 1996; Buczynski and Parsons, 2010;

Rodríguez-Cueto *et al.*, 2014), however, their alterations in PD have not been conducted. This study found no change in the levels of *N*-acylethanolamines in cerebellar mitochondria of PD Braak 5-6 males. This work did not investigate its receptors nor enzymes involved in *N*-acylethanolamine metabolism. Fatty acid amide hydrolase (FAAH), the main *N*-acylethanolamine metabolising enzyme, has been shown to be decreased in activity in 6-OHDA treated mice, and that its further inhibition was sufficient to restore normal cortico-striatal function (Gubellini *et al.*, 2002). An investigation of other *N*-acylethanolamine pathway molecules, including FAAH, its transporters and receptors would be a prudent next step. Further, as the data from oxylipins showed PD Braak 3-4 modulation, quantitation of *N*-acylethanolamines using PD Braak 3-4 and 5-6 would be interesting.

It is not apparent where the PD Braak 5-6 males FFA increase originates. Over one hundred oxylipins have so far been characterised, derived from multiple PUFAs (Gabbs *et al.*, 2015) - this work interrogated primarily AA-derived. Whether there is an attempted, yet dysfunctional, shift toward inflammatory resolution in later stage PD is an open question. CSF and plasma DHA derived resolvin D1 (RvD1) are reduced in patients with early PD (motor symptom duration 13±5 months) (Krashia *et al.*, 2019), suggesting that inflammation resolution processes are dysfunctional. It should be noted that Krashia *et al.*, did not specify the sex of their participants – it is plausible that sex differences in inflammation resolution markers exist (Krashia *et al.*, 2019). A more comprehensive panel of oxylipins would be necessary to characterise

inflammation resolution markers in PD and how they are altered across disease stages and sexes. The current body of research suggests anti-inflammatory oxylipins do not exhibit high levels in later PD, therefore, the high FFA levels in this work are likely not accounted for by these lipids. The pathological hallmark of PD, α-syn, is a lipid binding protein, which shares high sequence homogeneity to fatty acid binding proteins and has been shown to influence lipid homeostasis (R. Sharon *et al.*, 2001; Shioda *et al.*, 2014b). α-syn overexpression in WT mice and a human embryonic stem cell line carrying the PD causing E46K mutation showed increased monounsaturated fatty acid species, particularly oleic acid (OA) binding, and this exacerbated α-syn toxicity (Fanning *et al.*, 2019). Cerebellar tissue from PD individuals have been shown to harbour α-syn aggregates (Seidel *et al.*, 2017). Finally, lipid transporter alterations have been implicated in PD. FABP3, as described in chapter 4, has been shown to be increased in the CSF and serum of PD individuals (Mollenhauer *et al.*, 2007; Wada-Isoe *et al.*, 2008). Further, it has been shown to accelerate α-syn oligomerisation and its subsequent neurotoxicity (Shioda *et al.*, 2014a; Cheng *et al.*, 2019). It is plausible that an accelerated accumulation of oligomeric α-syn by FABP3, with progressive disease, increases monounsaturated FA levels in cerebellar mitochondria, and this is the most significant cause of the PD Braak 5-6 males FFA concentration increase found in this work – lipidomic analysis, together with quantification of FABP3 protein levels, would be necessary to confirm this.

6.4.3 Parkinson's disease males - Changes to the mean levels of inflammatory markers

A chronic pro-inflammatory state characterises PD brain (Tufekci *et al.*, 2012) and inflammatory cytokines are known to be intimately linked to the regulation of oxylipin expression and vice versa (Nayeem, 2018; Ren, Ma, Yang, Nonaka, Yamaguchi, K. Ishikawa, *et al.*, 2018). Further, mitochondrial dysfunction drives and is driven by inflammatory protein production (Vaamonde-García *et al.*, 2012; Ferrucci and Fabbri, 2018; Sliter *et al.*, 2018). In the present work, IL-1β was shown to be increased in cerebellar mitochondria of PD Braak 5-6 males. IL-1β is a primary pro-inflammatory driver and has consistently been shown to be increased in the serum and brains of PD individuals (Mogi, Harada, Kondo, *et al.*, 1994; Mogi, Harada, Riederer, *et al.*, 1994; Monteiro-Junior *et al.*, 2015; Sliter *et al.*, 2018) and in mouse models (Gao *et al.*, 2011; Depboylu *et al.*, 2012; Sliter *et al.*, 2018). IL-12p40 and IL-12p70 were both shown to be increased in PD Braak 5-6 males. The IL-12p40 subunit contributes to the pro-inflammatory IL-12p70 or IL-23 heterodimers (Gee *et al.*, 2009). IL-12p70, but not IL-23, was included in the inflammatory protein panel. This work showed IL-12p70 to be increased in PD Braak 5-6 individuals. In agreement, IL-12p70 was also increased in the serum of PD individuals (Rentzos *et al.*, 2009) and *Prkn*$^{-/-}$ and *Pink1*$^{-/-}$ mice (Sliter *et al.*, 2018). Of the eight significantly increased inflammatory proteins in PD Braak 5-6 males only one was anti-inflammatory: IL-1ra, which has been corroborated by another group (Walker *et al.*, 2016). Others have similarly shown an

imbalance of pro- to anti-inflammatory proteins in brain regions and serum of PD individuals (Mogi, Harada, Riederer, *et al.*, 1994; Blum-Degena *et al.*, 1995; Mogi *et al.*, 1996; Brodacki *et al.*, 2008). Inflammatory cytokines eotaxin-1, MIG and I-309 were also increased in PD Braak 5-6 male cerebellar mitochondria. Eotaxin-1 has previously been shown to be increased in the *SNpc* of post-mortem PD individuals and its inhibition in MPTP-treated mice suppressed pro-inflammatory marker expression and activation of glial cells (Chandra *et al.*, 2016). Similarly, MIG has been shown to be increased in PD *SNpc* compared with control (Walker *et al.*, 2016). I-309, a potent chemoattractant, has not previously been shown to be upregulated in PD. Interestingly, the antibiotic minocycline has been shown to inhibit I-309 (Saito *et al.*, 2017), and treatment of the MPTP mouse model of PD with minocycline inhibited microglial function and was neuroprotective (Wu *et al.*, 2002). The increased levels of chemokines may enhance the recruitment of immune modulatory glial cells in PD Braak 5-6 males, which may be crucial to maintaining a pro-inflammatory environment. Additionally, evidence suggest chemokines trigger activation of the NLRP3 inflammasome (Schroder, Zhou and Tschopp, 2010) and subsequent release of IL-1β (Maedler *et al.*, 2002), maintaining a persistent pro-inflammatory environment.

PD Braak 3-4 males were not included in the inflammatory cytokine analysis due to lacking these samples. Although the levels of eight inflammatory cytokines were significantly increased in cerebellar mitochondria of PD Braak 5-6 males it is plausible that a similar pattern to oxylipin expression may be

present in Braak 3-4 males cytokines. Further work should look to characterise the temporal expression of inflammatory cytokines across disease stage in PD.

6.4.4 Parkinson's disease females - Changes to the mean levels of inflammatory markers

The female group showed a distinctive profile of inflammatory markers with and without PD. In contrast to PD Braak 5-6 males, oxylipins were significantly reduced in PD Braak 5-6 females compared to control females. This work lacked PD Braak 3-4 female cerebellar samples, however, the total absence of significant changes in oxylipin levels between PD Braak 5-6 males and control males in comparison with the respective female groups is glaring. Once again, conversely to PD Braak 5-6 males, PD Braak 5-6 females showed limited significant changes and an opposite expression pattern – two cytokines were significantly reduced in expression compared with control females; ICAM-1 and TIMP-2. Pro-inflammatory ICAM-1 has previously been shown to be increased in the *SNpc* of a mixed analysis of males and females with PD and MPTP-treated rhesus monkeys. ICAM-1 has not been described in the PD cerebellum. It is interesting to speculate that reduced ICAM-1 in females may act to blunt the pro-inflammatory cascade. TIMPs are inhibitors of matrix metalloproteinases (MMPs), which have been shown to cause neuronal damage and have been implicated in PD (Backstrom, Miller and Tökés, 1992; Miyazaki *et al.*, 1993; Asahina, Yoshiyama and Hattori, 2001; Lorenzl *et al.*, 2002). Previous work in the *SNpc* of a mixed analysis of females and males with PD found an increase in TIMP-1, but no change to TIMP-2

expression (Lorenzl *et al.*, 2002). It is possible that the reduced TIMP-2 in PD Braak 5-6 female cerebellum reflects a limited need. An analysis of MMP levels in females and males would be interesting, as evidence has implicated MMP-3 to interact with α-syn and be critically important to dopaminergic neurone degeneration in MPTP-treated mice, and that its inhibition was neuroprotective (Choi *et al.*, 2011).

These data suggest the pro-inflammatory state in cerebellar fractions of PD Braak 5-6 males is more severe than females. In agreement with the sex differences in inflammatory profiles shown here, evidence from females and males with chronic depression show that males have a characteristic high pro-inflammatory response, whereas females show very little change in inflammatory markers (Majd *et al.*, 2018). Further, the inflammatory marker changes females do show are reductions, perhaps suggestive of a less aggressive pro-inflammatory state, or alternatively, simply starting at a higher set-point.

6.4.5 Group variation in levels of inflammatory markers

Quantitation of oxylipin levels revealed a temporal expression pattern across disease in males and a reduction in PD Braak 5-6 females compared with control. Intriguingly, this pattern was shared amongst almost the entire panel of oxylipins. The control female group showed high heterogeneity in levels of oxylipins, inflammatory cytokines and *N*-acylethanolamine. Further, the highly heterogenous inflammatory marker levels of the control female group was attenuated in the PD Braak 5-6 female group. Oxylipin levels showed a

global reduction in variance in the PD Braak 5-6 female group. Inflammatory cytokines in the PD Braak 5-6 female group showed a pattern of increased variation in pro-inflammatory cytokines, and reduced variance in anti-inflammatory cytokines. Central pro-inflammatory cytokine IL-1β was highly homogenous in expression in the control female group, whereas the PD Braak 5-6 female group showed high expression heterogeneity. Five anti-inflammatory cytokines were identified as having significant variance between PD Braak 5-6 female and control female groups – of these, four were more variant in the control female group. Together, the data suggest PD Braak 5-6 females lose anti-inflammatory expression variance, which may engender a reduced capacity to resolve a pro-inflammatory scenario (Krashia *et al.*, 2019). *N*-acylethanolamines 2-AG, AEA and PEA were significantly more variant in the control female group compared with the PD Braak 5-6 female group. The present data suggest the control female group have an innate high heterogeneity of inflammatory marker expression and that they lose this heterogeneity in PD. Some evidence exists which shows females mount more acute and robust inflammatory states (Marriott and Huet-Hudson, 2006; Scotland *et al.*, 2011). This may be reflected in the heterogenous inflammatory levels shown here – females may be more capable of mounting an affective immune response and resolution, which may manifest as reduced prevalence of PD. In contrast, males show an opposite pattern. The control male group show inflammatory marker level homogeneity, whereas the PD Braak 5-6 male group are increasingly variant. Collectively, the present data suggest PD Braak 5-6 males show increased pro-inflammatory marker levels and that the

increase is highly heterogenous across individuals. Further, a substantial pro-inflammatory bias exists – IL-1ra was the only significantly increased anti-inflammatory marker, while anti-inflammatory IL-10, IL-5 and MCSF were shown to be significantly more variant in the PD Braak 5-6 male group compared to the control male group.

A growing body of evidence describes a phenomenon of reduced severity, reduced incidence and improved outcomes from innate immune conditions, such as post-surgery infections, in females (Marriott and Huet-Hudson, 2006). In adult mice, LPS administration has been shown to induce a heightened febrile response, prolonged hypothermic response and more pronounced sickness in males than females (Scotland *et al.*, 2011). Leukocytes, including macrophages, provide tissue immunosurveillance in order to initiate an immune response upon insult. Insults are detected by antigen binding to leukocyte membrane receptors, including toll-like receptors (TLRs), which release cytokines (Kawai and Akira, 2010). Interestingly, female rodents have been shown to have more leukocytes and a greater density of TLRs than males (Scotland *et al.*, 2011). Increased levels of leukocytes have also been shown in female humans (Amadori *et al.*, 1995) and other primates (Xia *et al.*, 2009). Recruitment of leukocytes are controlled by chemokine activity. The greater levels of leukocytes in female rodents suggest increased chemokine levels or activity. Indeed, in this work leukocyte attractants GM-CSF and RANTES had significantly greater mean levels in control females than males. In agreement,

Scotland *et al.*, previously showed increased RANTES in female mice exposed to a noxious stimuli compared to males (Scotland *et al.*, 2011).

6.4.6 Inflammatory level flexibility may characterise control females

Inflammatory marker expression variance comparisons between the female and male groups revealed a clear picture – the control male group have homogenous levels of inflammatory cytokines and lipids, whereas the control female group have heterogeneous levels. The levels of seven oxylipins, AA and LA were heterogenous in the control female group. 8,9-EET showed significantly greater variance in the control female group than control male group, and recent evidence has implicated reduced levels of this oxylipin in DLB and PD (Ren, Ma, Yang, Nonaka, Yamaguchi, K. I. Ishikawa, *et al.*, 2018). Twenty of the twenty-one significantly variant inflammatory cytokines were in the control female group. Many key pro-inflammatory cytokines, that have evidence for expression increases in PD, were highly variant in the control female group, including IL-2, IL-6, and TNF-RI (Bessler *et al.*, 1999; Reale *et al.*, 2009; Scalzo *et al.*, 2010; Alrafiah *et al.*, 2019). Equally, critical anti-inflammatory proteins were also highly variant, including IL-4, IL-10 and MCSF. Interestingly, centenarians show a persistent immune system activation, characterised by elevated natural killer cell activity, and this correlated with preservation of endocrine function and muscle mass (Mariani *et al.*, 1999). Interestingly, pro-inflammatory IL-6 receptor, IL-6sR, was shown to be reduced in centenarians despite IL-6 levels being up to 10-times that of

young subjects. It was speculated that the low IL-6sR levels may act to mediate the higher IL-6 levels (Giuliani *et al.*, 2001). The present work did not show a reduction in IL-6sR, in fact, it was shown to be significantly more variant in the control female group. The control female group pro-inflammatory marker variance may reflect a propensity to have greater activation of innate immune system. Similarly, the control female group variation in anti-inflammatory markers suggest a robust inflammation resolution response.

The single more varied inflammatory protein in the control male group was pro-inflammatory IL-1β. In this work mean expression of IL-1β was significantly increased in PD Braak 5-6 males. PD Braak 5-6 females did not show a significant increase in IL-1β expression, compared with control females, however, the PD Braak 5-6 female group IL-1β variance was reminiscent of males. It is plausible that highly variant IL-1β expression in the control male group reflects a lesser capacity to appropriately resolve pro-inflammatory states and may support the hypothesis that males are more prone to pro-inflammatory states or have a higher baseline pro-inflammatory environment, without an appropriate anti-inflammatory activation. In support of the critical position of IL-1β in neurodegeneration, the IL-1ra-/- mouse, a model of overactive IL-1β, exhibits an age-related decline in motor function, a reduction in dopaminergic neurones and increased microglial activation (Stojakovic *et al.*, 2017). In agreement, central injection of IL-1β to the hippocampus or *SNpc* of rats causes neurotoxicity (Carvey *et al.*, 2005; Depino

et al., 2005; Ferrari *et al.*, 2006b). Tight regulation of IL-1β, as seen in the control female group, may be important to the prevalence of PD.

6.4.7 Females with PD have a more 'masculinised' inflammatory marker profile

Inflammatory marker expression and variance were remarkably similar between PD Braak 5-6 females and males. The mean levels of inflammatory markers were not significantly different between PD Braak 5-6 females and males, and group variance was substantially reduced compared to control groups.

Collectively, this work illustrates distinct inflammatory marker patterns between stages and sexes. Females may have an increased flexibility of response to injury or infection - greater variance in control female anti-inflammatory markers may safeguard against highly variant pro-inflammatory markers. In agreement, the literature points to females mounting a more acute and efficient immune response, and subsequent resolution, more effectively than males (Giuliani *et al.*, 2001; Marriott and Huet-Hudson, 2006; Scotland *et al.*, 2011). Indeed, the heterogeneity shown here may play a role in the sex-differences in disease manifestation. It is enticing to imagine that an improved buffer to inflammation in the cerebellum, acting to compensate for dopaminergic neuronal loss, enables females to maintain cerebellar compensation for longer. Further, PD Braak 5-6 females show a similar inflammatory profile to PD Braak 5-6 males, suggesting that females that succumb to PD are more "male" in their

inflammatory profile. Autism spectrum disorder (ASD) is a male dominated neurological disorder. Early life neuroinflammation has been hypothesised to drive ASD onset. However, why neuroinflammation causes this in males and rarely in females is an open question, but differences in inflammatory states and the role of inflammatory markers in the brain have been hypothesised (McCarthy and Wright, 2017). Interestingly, females with ASD show a "masculinised" brain profile (Lai *et al.*, 2013). Translating this to PD, it may be that the male inflammatory profile pre-disposes them to chronic inflammatory diseases. The remarkably variant control female profile in comparison to the more homogenous inflammatory profile of PD males and females may represent female brain masculinisation. Sub-group analysis of female inflammatory profiles and their predispositions to diseases with a chronic inflammatory component may highlight female populations with higher or lower risk. Alternatively, but not dichotomous, it may be that high heterogeneity in expression of inflammatory markers is characteristic of any population of females and males until a certain threshold "biological age", at which point inflammatory marker heterogeneity begins to constrict. Males may reach this threshold earlier than females, thus explaining the finding that women are diagnosed with PD on average two years later than men (Glenda E Gillies *et al.*, 2014; Smith and Dahodwala, 2014a), and may even contribute to the higher average life expectancy of women (Zarulli *et al.*, 2018). A thorough search of the literature did not reveal any research that had previously quantified an array of inflammatory markers in healthy individuals, therefore comparing the expression heterogeneity of the control female group observed

in this work to the wider literature has not been possible. This appears to be an area ripe for interrogation. An investigation of healthy female and male inflammatory marker expression at various ages would make headway toward revealing whether this heterogeneity is specific to females or whether males share this yet lose heterogeneity earlier than females. Further, what increased heterogeneity of expression means and whether its causal to sex differences in PD prevalence is an open question.

Assuming the pattern of inflammatory marker expression is relevant to the innate PD-protection afforded to females, the highly heterogeneous inflammatory marker levels of the control female group may be important. Interestingly, this heterogeneity is not isolated to anti-inflammatory markers and neither is there a trend toward decreased expression in control females. Hormesis describes how stress-inducing stimuli, such as exercise, elicit mild stress and result in favourable adaptations to deal with future stresses (Calabrese *et al.*, 2010; Ristow and Schmeisser, 2011). Exercise is known to increase pro-inflammatory marker expression including IL-1β, -6 and TNFα. Concomitantly, a number of compensatory mechanisms are upregulated, including the production of antioxidants and anti-inflammatory markers (Cotman, Berchtold and Christie, 2007; Beavers, Brinkley and Nicklas, 2010; Nilsson and Tarnopolsky, 2019). The menstrual cycle has not previously been described as hormetic, however, it fits the description. It is a regularly occurring stress that is maintained over decades and characterised by cycles of pro- and anti-inflammatory marker expression (Clancy, Baerwald and Pierson,

2013). It is plausible that this regular cycle may result in favourable adaptations that improve outcomes against future stresses. Indeed, female mice that undergo ovariectomy do not have the usual innate protection from infection and show similar outcomes to males (Bird, Karavitis and Kovacs, 2008; Baeza *et al.*, 2011). The underlying mechanisms that engender this are likely to be varied, but data regarding the inflammatory profiles of these mice, alongside ovariectomy mice given hormone replacement therapy and wild type, would go some way to determining whether the menstrual cycle is hormetic. If it is, this may translate to an increased capacity and flexibility of the female immune response to stress, including the underlying neuroinflammatory stress that contributes to PD.

Earlier research has not distinguished between male and female PD individuals when analysing the PD brain (Lorenzl *et al.*, 2002; Brodacki *et al.*, 2008; Kwak *et al.*, 2010; Liu *et al.*, 2013; Festini *et al.*, 2015); in fact, neglecting to account for sex within PD research is still on-going (Krashia *et al.*, 2019). The literature regarding sex differences in immune response is clear and sex is a significant determinant of risk and severity of infection and disease (Marriott and Huet-Hudson, 2006; Scotland *et al.*, 2011; McCarthy and Wright, 2017). Indeed, in agreement with the data presented here, fundamental immunological differences exist between the sexes. Leukocyte number, toll-like receptor densities and adaptive immune system responses are different (Amadori *et al.*, 1995; Xia *et al.*, 2009; Scotland *et al.*, 2011). Oestrogen has been shown to be neuroprotective in multiple animal models (Bird, Karavitis and

Kovacs, 2008; Au *et al.*, 2016), however, direct evidence for a neuroprotective role in PD has not yet been shown. Indeed, elderly females show increased bioavailability of levodopa despite lowered oestrogen due to menopause, hinting at an explanation other than oestrogen (Kumagai *et al.*, 2014). Disregarding sex in PD, and more broadly, is an error. The data generated from the present study bolsters support toward future work taking sex into account.

6.4.8 Sample size and the limitations of *p* values

A limitation of the present study was low sample size for some datasets. Whether the distinctive control female group inflammatory profile is reflective of an average control female population or is simply an abnormal subset will remain to be determined until the work is repeated with more samples. However, the samples used for each quantitation (*N*-acylethanolamines, inflammatory cytokines and oxylipins) were different, which strengthens support for these patterns being representative of general populations. It is relevant to note that throughout this Chapter reliance on *p*-values and significance was appreciably less than Chapters 3-5, and multiple references were made to "patterns" and "trends" described by the data. Increased appreciation for the limitations and inappropriate use of *p*-values has permeated outside the realm of statistics (Halsey, 2019). For example, *p*-values are a function of sample size, hence, *p*-values are likely to decrease when a sample size increases. Equally, smaller *p*-values do not of necessity imply greater significance (Faber and Fonseca, 2014; Kennedy-Shaffer, 2019).

Therefore, an awareness of limited sample sizes and inherent limitations of *p*-values were considered when describing the overall patterns of inflammatory markers between groups.

6.5 Conclusion

The findings of the present work depict sex-dependent inflammatory marker expression differences in the cerebellar mitochondria of PD females and males. This is the first-time molecular changes consistent with inflammatory process dysregulation in post-mortem human cerebellar fractions have been quantified – these data establish the cerebellum as affected in PD.

Inflammatory marker changes were apparent between sexes and with disease progression. The female control group stands out as having a distinctive inflammatory profile – highly heterogeneous inflammatory marker levels. The underlying mechanisms of these changes are unknown, however, it is enticing to imagine the distinctive inflammatory profile of the control female group may, in part, manifest as reduced PD prevalence, delayed onset and symptomatic differences.

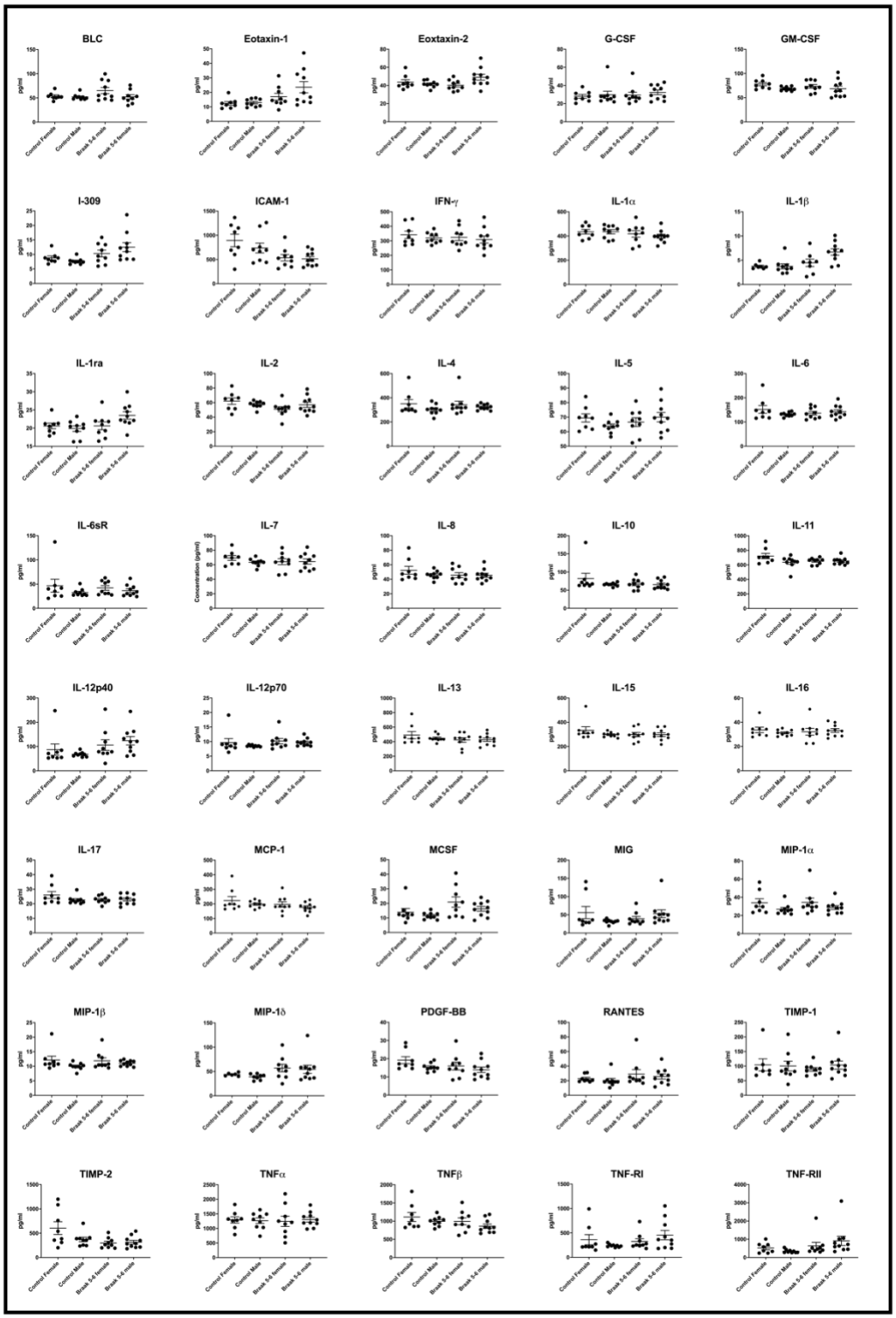
6.6 Supplementary data

Oxylipin	Control males vs control females	Braak 5-6 males vs Braak 5-6 females	Braak 3-4 males vs control males	Braak 3-4 males vs Braak 5-6 males	Braak 3-4 males vs control females	Braak 5-6 females vs control females	Braak 5-6 males vs control males
AA	0.4206	0.1429	0.5476	0.3095	0.6905	0.0317*	0.6905
LA	0.4206	0.1984	0.4206	0.5476	0.6905	0.0556	>0.9999
12-HETE	0.0079**	0.5159	0.0317*	0.6905	0.2222	0.0317*	0.3095
TXB-2	0.0556	0.8571	0.0952	0.0159*	0.5476	0.0317*	0.8413
PGE2	0.2222	0.6032	0.0556	0.4206	>0.9999	0.2778	0.6905
5-HETE	0.2222	0.3968	0.0556	0.3095	0.6905	0.0476*	0.6905
5-HPETE	0.0952	0.381	0.2222	>0.9999	0.5476	0.0317*	>0.9999
12-HPETE	0.3095	0.6508	0.3095	0.5476	0.5476	0.0476*	0.6905
9-HETE	0.2222	0.8016	0.0556	0.1508	0.5476	0.0873	0.8413
20-HETE	0.2222	0.5238	0.1508	0.3095	0.8413	0.0873	>0.9999
LTB-4	0.4206	0.7857	0.0317*	0.0079**	0.6905	0.2063	0.3889
9-oxoODE	0.3095	0.1905	0.3095	0.6905	0.4206	0.0476*	0.8413
9-HODE	0.2222	0.1905	0.1508	0.0317*	0.6905	0.0476*	0.5952
5,6-EET	0.1508	0.381	0.0952	0.6905	0.8413	0.0317*	>0.9999
5,6-DHET	0.3095	0.5238	0.0952	0.4206	0.5476	0.381	0.1667
8,9-DHET	0.2222	0.5397	0.0952	>0.9999	0.3095	0.2063	0.8413
11,12-DHET	0.2222	0.3968	0.0556	0.5476	0.5476	0.1349	>0.9999
14,15-DHET	0.3095	0.5159	0.0952	0.3095	0.4206	0.1984	0.8413
8-HETE	0.2222	0.381	0.0317*	0.0952	0.6905	0.0873	0.5476
11-HETE	0.2222	0.5	0.0556	0.0556	0.8413	0.1349	0.8413
15-HETE	0.1508	0.5238	0.1508	0.1508	0.6905	0.0873	>0.9999
16-HETE	0.3095	0.3968	0.0952	0.0952	0.8413	0.0476*	>0.9999
8,9-EET	0.2222	0.2778	0.0317*	0.1508	0.6905	0.0873	0.5476
11,12-EET	0.2222	0.5159	0.0952	0.0317*	0.6905	0.1984	0.8413
14,15-EET	0.1508	0.5238	0.1508	0.3095	0.6905	0.0873	>0.9999
17-HDoHE	0.2222	0.381	0.4206	0.5476	0.6905	0.0476*	>0.9999
13-oxoODE	0.2222	0.2857	0.5476	0.6905	0.3095	0.0476*	>0.9999
13-HODE	0.2222	0.1905	0.1508	0.0317*	0.5476	0.0476*	0.8413
8,15-DiHETE	>0.9999	0.5238	0.2857	0.3333	0.0952	0.8968	0.5476

Supplementary Table 6.1 Comparisons of all twenty-nine quantified oxylipins from cerebellar mitochondria. PD Braak 3-4 male n=5; PD Braak 5-6 male n=5; PD Braak 5-6 female n=5; control male n=5; control female n=5. Statistical analyses were carried out using GraphPad Prism (Mann-Whitney U-test). Red /blue font oxylipins represent pro- /anti-inflammatory, respectively. Bright yellow shaded numbers refer to significance; pale yellow shaded numbers refer to near significance ($p<0.1$).

Oxylipin	Control males vs control females	Braak 5-6 males vs Braak 5-6 females	Braak 3-4 males vs control males	Braak 3-4 males vs Braak 5-6 males	Braak 3-4 males vs control females	Braak 5-6 females vs control females	Braak 5-6 males vs control males
AA	0.001**	0.9319	0.0072**	0.5974	0.3514	0.1354	0.0209*
LA	0.0048**	0.3855	0.9381	0.4176	0.0041**	0.0036**	0.4618
12-HETE	0.4458	0.9151	0.453	0.7467	0.9901	0.8379	0.2903
TXB-2	0.5039	0.903	0.2706	0.5359	0.0895	0.2078	0.6142
PGE2	0.0517	0.125	0.349	0.8676	0.2616	0.0104*	0.4369
5-HETE	0.0402*	0.2352	0.3246	0.7657	0.2306	0.0468*	0.2075
5-HPETE	0.1729	0.0686	0.6102	0.2696	0.0715	0.0149*	0.538
12-HPETE	0.3939	0.0208*	0.2237	0.1959	0.0503	0.0043**	0.9326
9-HETE	0.0878	0.8281	0.3884	0.3553	0.3558	0.1145	0.948
20-HETE	0.0641	0.9358	0.2454	0.5188	0.4326	0.1447	0.5881
LTB-4	0.0567	0.454	0.3996	0.5632	0.0108*	0.1338	0.7851
9-oxoODE	0.0173*	0.2025	0.2913	0.3717	0.0019**	0.0008***	0.8634
9-HODE	0.0272*	0.2072	0.6756	0.8803	0.0119*	0.0011**	0.7879
5,6-EET	0.1154	0.0308*	0.5482	0.2996	0.0385*	0.0027**	0.6494
5,6-DHET	0.1545	0.5922	0.1216	0.3628	0.8868	0.8066	0.4865
8,9-DHET	0.1304	0.1762	0.6324	0.4639	0.28	0.6597	0.7949
11,12-DHET	0.3047	0.158	0.7037	0.5873	0.5078	0.0169	0.8692
14,15-DHET	0.137	0.9187	0.669	0.5498	0.2709	0.1214	0.8623
8-HETE	0.0431*	0.8093	0.4253	0.8786	0.1791	0.2063	0.516
11-HETE	0.0707	0.8643	0.5844	0.9402	0.1819	0.2665	0.535
15-HETE	0.0759	0.8024	0.5574	0.8881	0.2066	0.108	0.6538
16-HETE	0.268	0.4558	0.8615	0.9181	0.2051	0.0674	0.9429
8,9-EET	0.0408*	0.8163	0.3884	0.8263	0.1907	0.1947	0.5151
11,12-EET	0.0946	0.7192	0.5743	0.7844	0.2406	0.274	0.7711
14,15-EET	0.0859	0.7794	0.5518	0.8582	0.2331	0.1098	0.675
17-HDoHE	0.0951	0.1531	0.7221	0.764	0.174	0.006**	0.9555
13-oxoODE	0.0085**	0.2321	0.4311	0.2845	0.0016**	0.0012**	0.7652
13-HODE	0.0138*	0.1104	0.3799	0.5811	0.0022**	0.0002***	0.7363
8,15-DiHETE	0.5978	0.1323	0.5292	0.2795	0.9173	0.7143	0.0999

Supplementary Table 6.2 Group variance analyses of all twenty-nine quantified oxylipins from cerebellar mitochondria. PD Braak 3-4 male n=5; PD Braak 5-6 male n=5; PD Braak 5-6 female n=5; control male n=5; control female n=5. Statistical analyses were carried out using GraphPad Prism (F-test). Red /blue font oxylipins represent pro- /anti-inflammatory, respectively. Bright yellow shaded numbers refer to significance; pale yellow shaded numbers refer to near significance ($p<0.1$).



Supplementary Figure 6.1 Graphical comparisons of all quantified inflammatory cytokines from cerebellar mitochondria of males and females with PD Braak 5-6 and age-matched controls. PD Braak 5-6 male n=10; PD Braak 5-6 female n=9; control male n=9; control female n=8. Red /blue titles represent pro- /anti-inflammatory, respectively. Statistical analyses were carried out using GraphPad Prism (Mann Whitney U-test). Plots display mean

levels ± SEM. For all *p* or *F* values refer to Supplementary Tables 6.3 or 6.4, respectively.

Inflammatory Cytokine	Control males vs control females	Braak 5-6 males vs Braak 5-6 females	Braak 5-6 males vs control males	Braak 5-6 females vs control females
BLC	0.4807	0.1564	0.1823	0.5414
Eotaxin	0.6058	0.4363	**0.04***	0.0745
Eotaxin-2	0.8884	0.077	0.0503	0.3704
G-CSF	0.8884	0.3562	0.3154	>0.9999
GM-CSF	**0.0418***	0.4002	0.6607	>0.9999
I-309	0.0745	0.5457	**0.0005*****	0.4234
ICAM-1	0.4234	>0.9999	0.0789	**0.0274***
IFNγ	0.8148	0.447	0.3562	0.6058
IL-1α	0.9626	0.447	0.2428	0.743
IL-1β	0.536	0.0545	**0.0057****	0.3357
IL-2	0.3213	0.447	0.7197	0.1388
IL-6	0.4807	0.6607	0.549	0.5414
IL-6sR	0.743	0.3562	0.6038	0.5414
IL-7	0.3213	0.9048	0.9682	0.6058
IL-8	0.743	0.9048	0.549	0.3213
IL-12p40	0.8148	0.4894	**0.0188***	0.1672
IL-15	0.2766	0.9048	0.8421	0.3213
IL-16	0.5414	0.8421	0.4967	0.6058
IL-17	0.2359	0.8421	0.9048	0.6058
MCP-1	0.8148	0.4002	0.1333	0.4807
MIG	0.4807	0.2581	**0.0188***	0.8148
MIP-1α	0.1388	0.3154	0.3154	>0.9999
MIP-1β	0.0927	>0.9999	0.0653	0.8148
MIP-1δ	0.1812	0.4967	0.1728	0.2721
PDGF-BB	0.1139	0.6038	0.3154	0.2359
RANTES	**0.007****	0.9682	0.1011	0.6058
TNFα	0.9626	0.6607	0.9048	0.5414
TNFβ	0.9626	0.3154	0.0789	0.6058
TNF RI	0.9626	0.4967	0.1564	0.3704
TNF RII	0.3213	0.2224	**0.0012****	0.9626
IL-1ra	0.6058	0.0535	**0.0057****	0.8884
IL-4	0.5414	0.9682	0.1823	0.8148
IL-5	0.2359	0.4967	0.1823	0.673
IL-10	0.4234	0.7802	0.6607	0.6058
IL-11	0.1672	0.9048	0.7802	0.1996
IL-12p70	0.743	>0.9999	**0.0101***	0.3213
IL-13	0.673	0.9682	0.6607	0.8148
MCSF	0.743	0.4967	0.0653	0.1996
TIMP-1	0.9182	0.4967	0.6607	0.9182
TIMP-2	0.3704	0.7802	0.2428	**0.036***

Supplementary Table 6.3 Comparisons of all forty quantified inflammatory cytokines from cerebellar mitochondria. PD Braak 5-6 male n=10; PD Braak 5-6 female n=9; control male n=9; control female n=8. Statistical analyses were carried out using GraphPad Prism (Mann-Whitney U-test). Red /blue font represent pro- /anti-inflammatory, respectively. Bright yellow shaded numbers refer to significance; pale yellow shaded numbers refer to near significance ($p<0.1$).

Inflammatory Cytokine	Control males vs control females	Braak 5-6 males vs Braak 5-6 females	Braak 5-6 males vs control males	Braak 5-6 females vs control females
BLC	0.489	0.2784	**0.0024****	0.1595
Eotaxin	0.4921	0.4269	**0.0017****	0.0723
Eotaxin-2	0.0547	0.4614	0.0586	0.429
G-CSF	0.0785	0.5697	0.269	0.1925
GM-CSF	0.1593	0.2312	**0.0004*****	0.2614
I-309	0.114	0.7636	**0.0054****	0.1258
ICAM-1	0.6282	0.4518	0.0546	0.1108
IFNγ	0.0864	0.7262	0.057	0.8382
IL-1α	0.9418	0.1935	0.8813	0.321
IL-1β	**0.0221***	0.8982	0.4273	**0.0042****
IL-2	**0.0155***	0.7287	**0.0202***	0.5979
IL-6	**0.0001*****	0.7721	**0.0065****	0.0755
IL-6sR	**0.0002*****	0.5675	0.2664	**0.0145***
IL-7	0.1184	0.8129	**0.0419***	0.5199
IL-8	**0.0117***	0.6105	0.2609	0.2745
IL-12p40	**<0.0001******	0.129	**0.0013****	0.9745
IL-15	**0.0007*****	0.728	**0.0263***	0.2327
IL-16	**0.0166***	0.1326	0.0653	0.4724
IL-17	**0.0279***	0.3974	0.5947	**0.0138***
MCP-1	**0.0028****	0.1306	0.3733	0.3504
MIG	**<0.0001******	0.1617	0.0902	**0.0155***
MIP-1α	0.0575	**0.0419***	0.7264	0.7378
MIP-1β	**0.0044****	**0.0023****	0.5406	0.5712
MIP-1δ	0.2335	0.732	**0.0006*****	**0.0005*****
PDGF-BB	**0.0203***	0.4083	**0.0396***	0.6927
RANTES	0.3007	0.1473	**0.0046****	**0.0026****
TNFα	0.8404	**0.0413***	0.7458	0.1598
TNFβ	**0.0155***	0.2411	0.3658	0.569
TNF RI	**<0.0001******	0.1133	**<0.0001******	0.1464
TNF RII	**0.0079****	0.0696	**0.0123***	**0.0479***
IL-1ra	0.9013	0.998	0.362	0.3329
IL-4	**0.0349***	**0.0005*****	0.0917	0.8174
IL-5	0.1335	0.7278	**0.0301***	0.755
IL-10	**<0.0001******	0.6593	**0.0095****	**0.0097****
IL-11	0.5622	0.7733	0.1148	**0.024***
IL-12p70	**<0.0001******	**0.0408***	**0.0004*****	0.337
IL-13	**0.0072****	0.5229	0.1929	0.3199
MCSF	**0.0052****	**0.0439***	**0.0367***	0.3129
TIMP-1	0.793	**0.0265***	0.7116	**0.0087****
TIMP-2	**0.0139***	0.7664	0.6342	**0.0022****

Supplementary Table 6.4 Group variance analyses of all forty quantified inflammatory proteins from cerebellar mitochondria. PD Braak 5-6 male n=10; PD Braak 5-6 female n=9; control male n=9; control female n=8. Statistical analyses were carried out using GraphPad Prism (F-test). Red /blue font represent pro- /anti-inflammatory, respectively. Bright yellow shaded numbers refer to significance; pale yellow shaded numbers refer to near significance ($p<0.1$).

7 Thesis discussion

Thirty-six years ago, the first long lived strains of *C. elegans* were identified (Klass, 1983). In the early 1990's, Cynthia Kenyon and colleagues showed that the lifespan of *C. elegans* may be extended with the knockout of a single gene (Kenyon *et al.*, 1993). Since then, research groups across the globe have extended this work, showing common conserved pathways which are implicated in ageing and whose modulation extends it (Dillin, Crawford and Kenyon, 2002; Hsu, Murphy and Kenyon, 2003; Vellai, Kriztina Takacs-Vellai, *et al.*, 2003; Finkel, 2015). Interestingly, lifespan extension by modulation of these pathways also improves the health of the organism, reducing disease burden and prevalence (Hsu, Murphy and Kenyon, 2003; Harrison *et al.*, 2009; Fontana *et al.*, 2016; Saxton and Sabatini, 2017). This suggests that pathways which promote longevity are also implicated in healthy ageing. Ageing is now a hot topic of research, and health- and lifespan extension in humans are increasingly appreciated as realistic ambitions.

This Thesis aimed to expand our understanding of the role of mitochondria in ageing and neurodegeneration. The Chapters were as follows: the identification of biological markers of ageing that could be modulated to enhance health- and /or lifespan (Chapter 3); comparative biology of differently ageing species (Chapter 4); exercise as a lifestyle intervention for healthy ageing (Chapter 5); mitochondrial cerebellar inflammation in Parkinson's disease (Chapter 6). The following sub-sections will attempt to tie together the converging themes of this Thesis.

7.1 The use of model (and non-model) organisms

Model organisms have been used since the fields of ageing's inception. Even before the use of *C. elegans*, the seminal work by McCay *et al*., in 1935, showed caloric restriction of rats, without malnutrition, could significantly improve lifespan (McCay, Crowell and Maynard, 1935). A movement away from the use of rodents in scientific research has been promoted in recent years, which has led to a greater reliance on alternative tools, including invertebrates such as *C. elegans* and *D. melanogaster* (NC3Rs, 2019). Indeed, this Thesis utilised both invertebrates to reduce reliance on mice, while taking advantage of the inherent benefits that the organisms each have.

Mice were used to distinguish how the expression of potential biological markers of ageing are changed across the lifespan (CA-II, -III and FABP3), and for comparative biology alongside the bat (*P. pipistrellus*). Although themes exist to explain the disparity in mammalian lifespan differences [for example, the reasonable mammalian correlation between body size, metabolic rate and lifespan (Pearl, 1928; Keane *et al.*, 2015)], exceptions are apparent. Amongst these are the bats (Herreid, 1964). Comparative biology of long- and short-lived species have helped identify biological mechanisms relevant to ageing. The naked mole rat, which has a maximal lifespan of ~30 years, has been shown to have improved proteostasis, increased membrane lipid saturation, and other cellular alterations relevant to long-life (Andziak, O'Connor and Buffenstein, 2005; Andziak, Timothy P. O'Connor, Qi, Dewaal, *et al.*, 2006; Mitchell, Buffenstein and Hulbert, 2007; Pride *et al.*, 2015). Working with bats,

Chapter 4 followed a precedent of using exceptional species to highlight differences that may be relevant to lifespan.

The field of ageing has become widely accepted as a standalone research field in no small part due to the nematode. Since discovery of long-lived IGF-1 mutants, *C. elegans* have been at the forefront (Kenyon *et al.*, 1993). In 1977, Klass published a methodology for measuring the lifespan of *C. elegans* (Klass, 1977), which inspired widespread use in labs across the World. This, coupled with the practical suitability of *C. elegans* [cheap, easy to maintain, short generation time, large progeny, fully sequenced genome, easily manipulatable genome (Mack, Heimbucher and Murphy, 2018)], have cemented their place in the ageing field. This Thesis heavily utilised *C. elegans* to determine whether proteins implicated in ageing from mammalian experimentation were relevant to lifespan. As mentioned, the *C. elegans* genome is easily edited. *C. elegans* fed *E. coli* expressing a dsRNA of interest is a powerful tool to easily (and transiently if preferred) knockdown a gene of interest (Kamath *et al.*, 2003a; Samuelson, Carr and Ruvkun, 2007). Indeed, CA and FABP3, highlighted as potential biological markers of ageing in Chapters 3 and 4, respectively, were firmly connected to ageing in this manner. Another benefit of *C. elegans* is that they are transparent. The overarching theme of this Thesis is the mitochondrial changes due to the ageing process – *C. elegans* mutants containing GFP-tagged mitochondria are well placed to quickly determine morphological alterations in living animals. Indeed, this was a technique used repeatedly to determine that both CA (Chapter 3) and FABP3 (Chapter 4) are

connected to mitochondrial function, and that *Pink1*$^{-/-}$ (Chapter 5) alters mitochondrial morphology.

Disadvantages to *C. elegans* use are apparent – as a model for human ageing they are not ideal. They lack many defined organs and tissues, have a simple body plan and are evolutionarily distant from humans. Biochemical analyses are difficult in *C. elegans* – experimentation is carried out on whole worm extracts, thereby limiting an understanding of tissue-specific signaling or cell compartment specific analyses (such as mitochondria). *D. melanogaster* rectify some of these limitations, plus their genome is fully characterised and they have a long history of use in scientific research (He and Jasper, 2014). Their use in ageing research is not as illustrious as *C. elegans*, however, they are regularly used and have been shown to share common lifespan regulatory pathways (IGF-1 /TOR) and that modulation of these extends *D. melanogaster* lifespan (Partridge *et al.*, 2011). This Thesis employed *D. melanogaster* for use in exercise research – a paradigm which is increasing in acceptance and popularity (Piazza *et al.*, 2009; M J Tinkerhess *et al.*, 2012; Watanabe and Riddle, 2017). Exercise is a lifestyle intervention that promotes improvements in healthspan and mean lifespan (Lanza *et al.*, 2008; Garcia-Valles *et al.*, 2013; Luo *et al.*, 2013; Nilsson *et al.*, 2019). It appears to do this by promoting the regulation of global compensatory pathways that result in superior stress-resistance to future insults. Chapter 5 utilised *D. melanogaster* heavily to determine the molecular changes caused by exercise. Further, Chapter 4 also showed that exercise altered the expression of FABP3 in *D. melanogaster*.

Ideally, this would have been extended to investigate the effect of exercise on CA in *D. melanogaster*.

7.2 Carbonic anhydrases, pH and cellular dysregulation

Chapter 3 delved into the role of CAs in ageing. Some hints in the literature existed describing increasing expression across the lifespan (Diedrich *et al.*, 2007; Laimer *et al.*, 2010; A. Pollard *et al.*, 2016; Feng *et al.*, 2018), however, this study has helped firmly place CAs as having a central role in lifespan determination. Chapter 3 showed that knocking down a *CA* orthologue at middle age extended *C. elegans* lifespan and that a potential mechanism is CAs influence upon mitochondria. What is unclear, is a molecular and biochemical explanation for the lifespan alteration.

CAs are zinc metalloenzymes, which catalyse the interconversion of CO_2 to bicarbonate and proton. In this way they maintain and influence pH, fluid balance, bone resorption, urea production and many other functions (C. T. Supuran, 2008). Cellular pH was not discussed during this thesis, however, it is relevant to touch upon as the mechanistic reasons for CAs' impact upon longevity may theoretically be due to this critical function. pH maintenance is critically important to correct physiological function and mitochondrial oxidative phosphorylation is a major cause of pH alteration, due to the production of protons (Llopis *et al.*, 1998). Membrane lipids are prone to alterations by changes in pH, due to their acid and basic properties (Mannella, 2006). Further, mitochondrial cristae are altered by the influence of pH on cardiolipin (Angelova *et al.*, 2018). Cardiolipin is a lipid found almost entirely in the IMM (Horvath and Daum, 2013). Abnormalities in its synthesis and

structure lead to mitochondrial functional changes, including loss of ATP synthesis and cristae morphology (Mannella, 2006).

Chapter 3 showed increasing CA-II and CA-III with ageing and that targeted inhibition of *C. elegans* orthologue *cah-2* by RNAi at middle age improved lifespan. It is unclear whether increasing CA acts to increase or decrease pH with age, however, aged rat hippocampal slices have lower pH (Roberts and Sick, 1996), and prevention of media acidification in yeast promotes a longer life (Murakami *et al.*, 2011). The decreased pH has been associated with decreased resistance to periods of anoxia (Hochachka and Mommsen, 1983), decreased activation of glycolysis during periods of high energy demand (Hochachka and Mommsen, 1983), and increased formation of ROS (Bralet *et al.*, 1991). Coupling this with Chapter 3, which showed that knockdown of *cah-2* improved lifespan, it is likely increasing CA is decreasing intracellular pH, and inhibition remediates this acidification. Normalising intracellular pH likely restores structural stability to membrane lipids, including cardiolipin, and shifts cellular function closer to physiological.

Interestingly, pH dysregulation has been linked to processes inherent to the pathogenesis of PD. An acidic pH has also been shown to promote the formation of insoluble α-syn oligomers (Pham *et al.*, 2009) and that an acidic pH increases autooxidation of dopamine and the formation of the reactive 6-hydroxy-dopamine (García-Moreno *et al.*, 1991), which may promote PD-relevant neurodegeneration. Few studies have been conducted regarding pH alterations in neurodegeneration or ageing. This seems like an

underappreciated topic, and pH dysregulation appears to be a commonality of ageing and neurodegenerative pathogenesis. Carbonic anhydrase may be a viable target for reducing the prevalence of PD by modulating pH, and perhaps for improving healthspan generally.

7.3 FABP3 and free fatty acids

FABP3 is an intracellular fatty acid transporter expressed primarily in the skeletal and cardiac muscle (Furuhashi and Hotamisligil, 2008), however, evidence also exists for its presence in brain, and a wide variety of other tissues to a lesser degree (Yamamoto *et al.*, 2009; Uhlén *et al.*, 2015). Chapter 4 described FABP3 and its known role in detail, but to recap – FABP3 shuttles FFA intracellularly, and is relevant to lipid droplet formation (Liang *et al.*, 2014), β-oxidation (Furuhashi and Hotamisligil, 2008) and inflammation (Ozawa *et al.*, 2015).

Chapter 4 determined that long-lived bats (*P. pipistrellus*) have increased levels of FABP3 compared with short-lived mice, and that this was coupled to increased levels of FFA. Pollard *et al.,* using the same bat species, carried out lipidomic profiling and determined that the bat muscle and brain mitochondrial lipid profile is highly enriched in *N*-acylethanolamines, DHA, EPA and AA (Pollard *et al.*, 2019). Chapter 4 also showed that knockdown of *FABP3* orthologues in *C. elegans* significantly reduced lifespan. Healthspan extending lifestyle interventions, exercise (Mulya, Jacob M Haus, *et al.*, 2017) and fasting (Sánchez-Gurmaches *et al.*, 2011), have also been shown to raise FABP3 levels. In Chapter 4, it was shown that PD model *D. melanogaster Pink1-/-* have low levels of FABP3 compared to WT. In agreement, AD and Down syndrome individuals have significantly lower levels of FABP3 (Cheon *et al.*, 2003). Conversely, FABP3 was shown to be raised in a clinical trial of overweight adolescents with low-grade inflammation (Klein-Platat *et al.*, 2005)

and the mouse model of diabetic nephropathy, eNOSKO (Ozawa *et al.*, 2015). Clearly, increasing FABP3 to high levels is not of necessity 'healthful'. More so, it is likely that FABP3 simply shuttles FFA that are present – if an intracellular inflammatory scenario is signaled, and the only FFA available are AA and its oxylipin derivatives, these are what are shuttled. Increasing FABP3 may improve efficiency of transport and FFA utilisation, however, the constituent FFA are likely important.

As an example, the diet of *P. pipistrellus* is highly insectivorous, containing a high proportion of ω-3 and ω-6 FAs (Wilkinson and South, 2002). High levels of these FFA coupled with high FABP3 may predispose the bat to effectively and efficiently mount immune defense mechanisms. Indeed, it is known that bats are exceptionally resilient to infection – a postulated mechanism for this is the bats propensity to avoid over-induction of a pro-inflammatory response (Subudhi, Rapin and Misra, 2019), it may be that efficient resolution capacity, via, beside others, increased DHA content and resolvin oxylipins aid this.

This fits neatly with Chapter 6 and the studies analyses of inflammatory response in females and males with and without PD. FFA were markedly increased in Braak 5-6 males and oxylipin levels altered with disease stage. The oxylipins quantified were primarily AA-derived and so a full picture of known oxylipins derived from DHA was not possible. Interestingly, evidence exists to suggest DHA supplementation in PD individuals shows efficacy – one study showed ω-3 supplementation improved depression symptoms. Animal studies have shown that ω-3 supplementation is neuroprotective,

reducing apoptosis of dopaminergic neurones (Ozsoy *et al.*, 2011), increasing endogenous antioxidant enzyme expression (Wang and Saito, 2003) and displacing ω-6 PUFAs in the brains of MPTP-treated mice (Bousquet *et al.*, 2008). The place of FABP3 in PD is an open question, however, as stated before, I think it plausible the constituent FFA are critically important, and that increasing FABP3 levels may act to improve utilisation of these constituents. In this case, supplementation with DHA, alongside an appropriate level of FABP3, may act to improve inflammatory resolution capacity. Direct evidence for involvement of FABP3 in the pathogenesis of PD has come to light - it has been shown to be increased in the CSF of PD patients (Mollenhauer *et al.*, 2007) and is highly expressed in dopaminergic neurones of the *SNpc*. FABP3 shows favourable binding to ω-6 fatty acids, including AA (Hanhoff, Lücke and Spener, 2002), and both FABP3 and AA have been shown to accumulate in post-mortem PD brains (Julien *et al.*, 2006). Lastly, a hallmark aggregate of PD, α-syn, has been shown to be influenced and induced by FABP3, in the presence of AA (Cheng *et al.*, 2019). Cumulatively, these data suggest the interplay of high FABP3 expression, coupled with high levels of AA, act to increase the rapidity of transport and utilisation of AA-derived oxylipins, thereby acting to propagate α-syn aggregation, a more pro-inflammatory intracellular milieu and neurodegeneration. Finally, Chapter 6 also showed that the control female group had a distinctly different inflammatory profile to the control male group – whether differences in FABP3 and class of FFA present played a role in this was not tested, but is worth considering as a possibility.

Altering what FABP3 is shuttling may directly impact disease prevalence, whilst altering the levels of FABP3 will likely improve health or exacerbate disease depending on the intracellular FFA present.

7.4 Can these be modulated?

Chapters 3 and 4 both identified potential biological markers relevant to longevity. In the case of CA-II and -III, knockdown of *cah-2* at middle age improved *C. elegans* lifespan. Chapter 3 also tested the clinically available drug dorzolamide hydrochloride (DH) in middle age *C. elegans* and showed a non-significant trend toward increased lifespan, and improvements in markers of health (reduced oxygen consumption). The treatment of *C. elegans* with DH lifespan experiment suffered from using few worms and would also benefit from extended testing of markers of healthspan (fertility, gut motility, motility). The ultimate goal would be to translate this work to the clinic as a treatment for healthy ageing. Two drugs have received most attention in the past decade for their repurposing to the field of healthy ageing. Rapamycin and metformin both show great promise for translation to humans, as model species consistently show age-related improvements with treatment (Kapahi *et al.*, 2004; Aliper *et al.*, 2017; An *et al.*, 2017; Sills *et al.*, 2019). However, metformin has been shown to blunt the stress-response to exercise, thereby inhibiting functional improvements over time (Malin and Braun, 2016). It is likely rapamycin may similarly blunt this effect to a degree, as mTORC1 is activated following exercise (Song *et al.*, 2017). Further, chronic systemic treatment with mTORC1 has been shown to retard recovery from skeletal and cardiac muscle injury (Dickinson *et al.*, 2011; Aoyagi *et al.*, 2012), and negatively affect memory consolidation (Stoica *et al.*, 2011). This means that these inhibitors may be most suited to transient or situational use and /or

specifically to certain tissues. Translating this to CA inhibition, it may be that global inhibition will not be as effective at improving lifespan as was shown in *C. elegans*, which lack many defined organs. Indeed, the wider literature suggest that the use of activators of CA may have a clinical setting. Administration of CA activators have been shown to enhance synaptic efficiency, memory and spatial learning (Sun and Alkon, 2001, 2002), and improve bone mineralization processes (Wang *et al.*, 2014). Ageing is associated with a loss of bone density (Khosla, 2013) and cognitive impairment (Geda, 2012) – it may be that global CA inhibition will be detrimental in certain settings. Indeed, exercise has been shown to modulate CA activity – during six weeks of altitude exercise training athletes showed a 50% increase in erythrocyte CA activity (Tas *et al.*, 2019). Like metformin, inhibition of CA during exercise may not be wise. Extending this work to *D. melanogaster* is a sensible next step, to test whether the lifespan extension shown in *C. elegans* extends to the fly, and how CA inhibition acts tissue specifically.

As discussed in Chapter 7.3, it would be prudent to consider the modulation of FABP3 together with the modulation of the profile of FFA. FABP3 has a key role in β-oxidation - FABP3-null mice show decreased palmitate oxidation by β-oxidation, and a shift in cardiac muscle fuel selection from long-chain fatty acids (LCFA) to glucose (Furuhashi and Hotamisligil, 2008) Further, FABP3 was shown to be upregulated in elderly individuals undergoing a twelve week aerobic exercise intervention (Mulya, Jacob M Haus, *et al.*, 2017), and this

has also been corroborated in exercised adults (Tunstall *et al.*, 2002; Talanian *et al.*, 2010). Indeed, exercise training consistently improves lipid utilisation by improving entry of fat into the mitochondria (Tunstall *et al.*, 2002; Mulya, Jacob M. Haus, *et al.*, 2017b). Exercise improves utilisation of FA and this appears to be in part by increasing the expression of FABP3. In an exercised scenario, increasing FABP3 will primarily function to shuttle LCFAs for β-oxidation – decreased adiposity itself has numerous health benefits (Rodríguez-Hernández *et al.*, 2013). However, an epiphenomenon of exercise increasing FABP3 could plausibly be realised if the FFA profile is healthful. Indeed, dietary modulation of FA intake has been shown to impact cellular membrane profiles and intracellular lipid profiles – in healthy adults, twelve months of EPA and DHA supplementation displaced ω-6 FA, reducing the intracellular ω-6: ω-3 PUFA ratio (Walker *et al.*, 2015). The dual hit of increasing FABP3, via exercise, and a healthy profile of intracellular lipids, via diet, may be critical to improving healthspan.

7.5 Endogenous modulation of ageing processes

Although all humans age, the rate and how it manifests is quite different between individuals. Surviving to extreme old age is much more likely if your sibling or a relative does too (Perls *et al.*, 2002b). This suggests that the endogenous processes underlying ageing are different between individuals – innate protection from ageing may be afforded to some. Centenarians have been shown to have a lipidome suited to adapting and responding to oxidative and chronic inflammatory states. A cohort of Italian centenarians were shown to have higher circulating levels of anti-inflammatory oxylipins, 15-HETE, 8,9-EET and decreased levels of 9-HODE, which is a marker of lipid peroxidation (Collino *et al.*, 2013). Indeed another group also showed that centenarians have reduced markers of lipid peroxidation (Pradas *et al.*, 2019). The authors postulated that the group of Italian centenarians have a specific mechanism of AA metabolism that may result in an effective activation of antioxidant response (Collino *et al.*, 2013). Interestingly, it appears the high anti-inflammatory oxylipin levels in centenarians are matched with a persistent immune system activation, characterised by elevated natural killer cell activity (Mariani *et al.*, 1999). It is plausible centenarians have an immune response that is more vigilant, capable of responding and resolving efficiently, due to robust levels of both pro- and anti-inflammatory mediators. Chapter 6 presented a survey of inflammatory markers of females and males with and without PD. This study revealed control females and males to have distinctive inflammatory profiles. The control female group showed inflammatory

marker level variation, whereas males were homogenous as a group. The control female groups innate variance may underpin the reduced prevalence of females to diseases with chronic inflammatory components (Marriott and Huet-Hudson, 2006), and their longer average lifespan (Ginter and Simko, 2013). Indeed, it is known that centenarians are similarly less prone to inflammatory diseases across their lifespan (Bürkle *et al.*, 2007), and the data described above suggest this to be due to robust pro- and anti-inflammatory marker regulation (Mariani *et al.*, 1999; Collino *et al.*, 2013). Females appear to have an immune regulatory system that affords them superior protection compared with men.

No direct evidence exists to describe a population with endogenous modulation of either CA-II, -III or FABP3. However, there are clear hints that health-promoting pathways, which these proteins are features of, do improve longevity, and it is feasible that these are differently modulated in long-lived populations. Overexpressing FA β-oxidation genes in *D. melanogaster* has been shown to extend lifespan (S. H. Lee *et al.*, 2012). Further, healthspan promoting lifestyle interventions, exercise (Mulya, Jacob M Haus, *et al.*, 2017) and caloric restriction (Sánchez-Gurmaches *et al.*, 2011), also increase FA utilisation by β-oxidation. FABP3 levels have also been shown to directly determine the rate of FA oxidation - brown adipose tissue, through both gain- and loss-of-function approaches, dose dependently effected FA oxidation efficiency (Vergnes *et al.*, 2011). As mentioned, whether some individuals have endogenous high expression of FABP3 is unclear, however, the circulating

lipid transporter lipoprotein(a) has been long known to be significantly increased in centenarians (Pepe *et al.*, 1998; Thillet *et al.*, 1998) – evidence strongly supports lipids and their efficient transport as critical to longevity. It is enticing to imagine that high endogenous expression of FABP3, in some individuals, may predispose to longevity by promoting FA oxidation and playing a role in ensuring a robust pro- and anti-inflammatory response.

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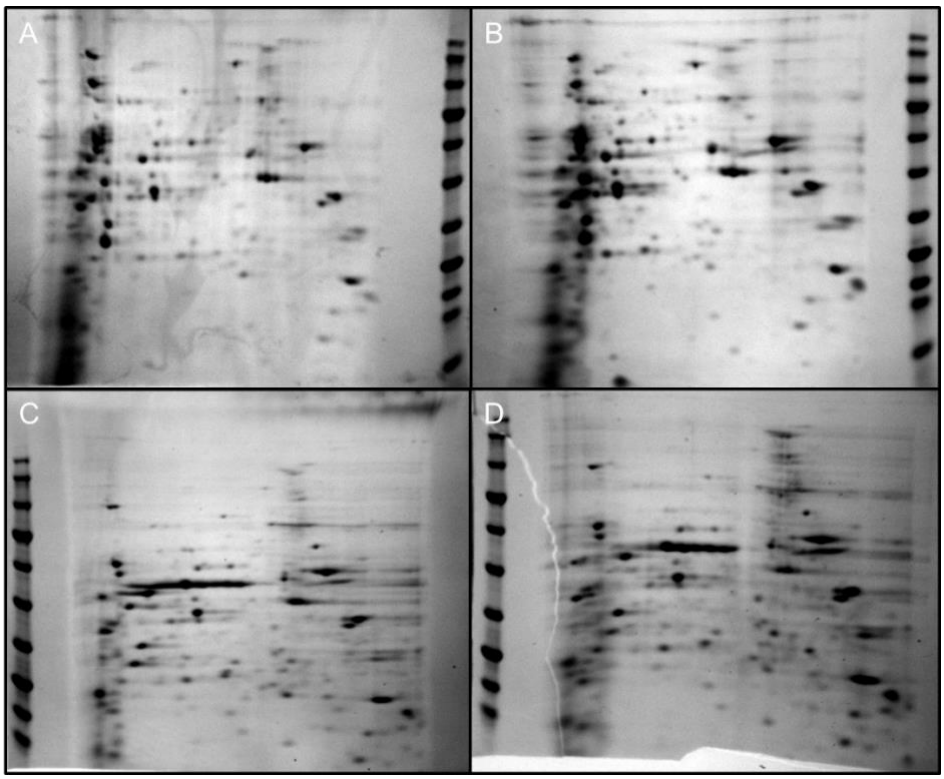
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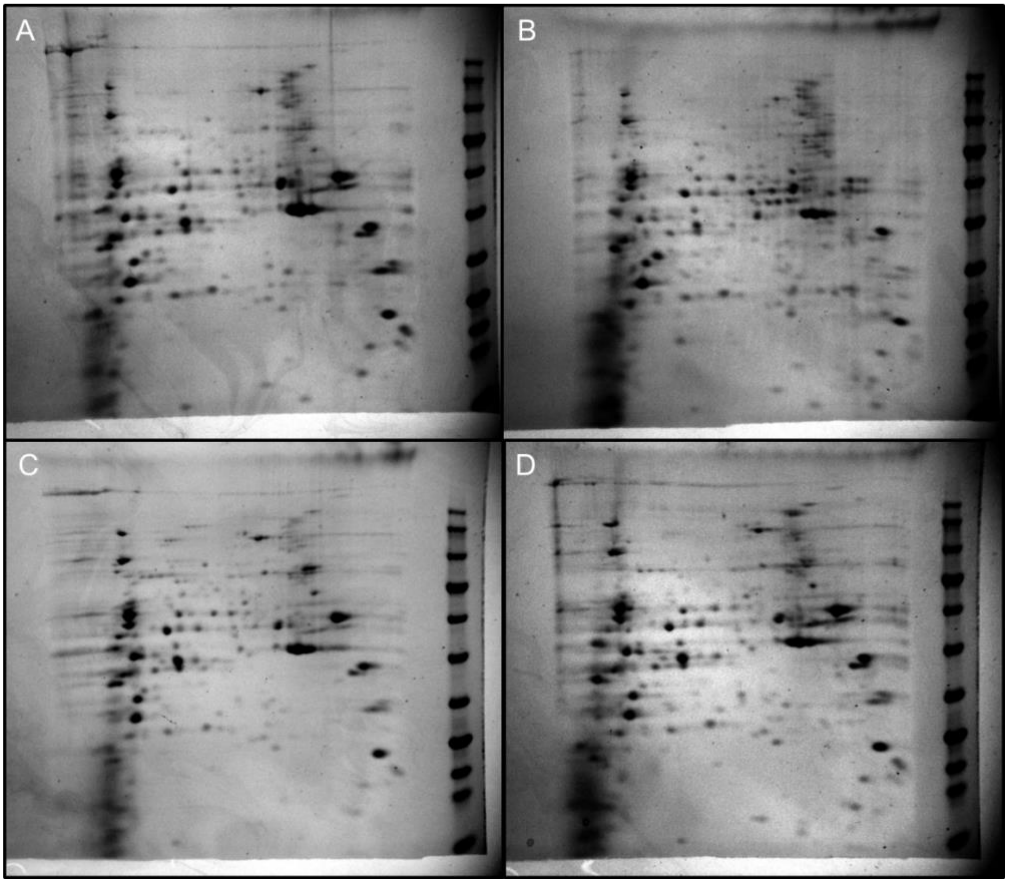
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Appendix 1



Appendix Figure 7.1 Representative 2D-gels for WT exercised and non-exercised, female and male, whole-cell *D. melanogaster* homogenates. Shown are representative gels of male exercised (A), male non-exercised (B), female exercised (C), and female non-exercised (D). Three biological replicates were carried out for each group. Significantly altered protein spots (one-way ANOVA) were determined by SameSpots (Totallab) and identified by MALDI-TOF/MS (Centre of Excellence in Mass Spectrometry at University of York).



Appendix Figure 7.2 Representative 2D-gels of WT and *Pink1*$^{-/-}$ exercised and non-exercised *D. melanogaster* mitochondrial fractions. Shown are representative gels of WT exercised (A), WT non-exercised (B), Pink1-/- exercised (C), and Pink1-/- non-exercised (D). Three biological replicates were carried out for each group. Significantly altered protein spots (one-way ANOVA) were determined by SameSpots (Totallab) and identified by MALDI-TOF/MS (Centre of Excellence in Mass Spectrometry at University of York).

Appendix 2

Note to examiners

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory three month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

PIP Reflective Statement

For my three-month placement I worked with the Education & Learning (E&L) team at the charity, Wellcome Trust. The E&L team is split into three major areas: 1) outreach to support schools to deliver engaging science lessons; 2) fund research to inform and improve science education; 3) advocate for policy change. I dipped my toes into each of these areas to some degree, but primarily, I was tasked with aiding the science education research branch.

In brief, my day-to-day role involved three parts – 1) evaluating education research funding applications alongside my team; 2) promoting E&L funded projects to teachers; 3) managing the logistics and setup of a Wellcome funded and hosted educational neuroscience conference [European Association for Research on Learning and Instruction (EARLI) biennial Neuroscience and Education conference].

The Wellcome Trust E&L team funds RCTs to be carried out in schools across the UK, to systematically test educational interventions with robust supporting evidence that they may positively impact students. I took an active role in meetings with funders and fundees. Knowing how to write effective

applications (and what a funder likes to see!), is a skill that all academics need to hone. Application writing is a regular part of an academic's life. This experience will serve me well should I continue to post-doctoral studies.

The Wellcome Trust E&L team have been funding numerous education-related projects for a number of years. A number of these are online projects that aim to improve teachers' access to the "science of learning". For example, their "Science of Learning Zone" aims to connect teachers to science-based teaching methods, while dispelling science teaching myths. In my second week working for the E&L team, I gave a talk at the BrainCanDo conference in Caversham, promoting Wellcome Trust funded projects. The audience were primarily primary and secondary school teachers. This opportunity instilled a lot of confidence and I undertook many other speaking opportunities during my placement.

The culmination of my 3-month placement was an educational neuroscience conference. This brought in close to 150 academics from all over the World to discuss pedagogical research and related topics. My role in this conference was to manage ticket sells, liaise with speakers and other organisers/attendees, advertise at events, write promotional materials and day-to-day practicalities and logistics. This tested my planning, attention-to-detail and communication skills. Further, it required a precise schedule to ensure multiple deadlines were adhered to.

The Wellcome Trust was a fantastic opportunity, and one which will stick with me. Due to the resources at their disposal, The Wellcome Trust hosts

tours for their staff at all Wellcome Trust funded partner institutes. I took advantage of this and visited Harwell Syncotron, Crick Institute, and a staff tour around the Houses of Parliament.

In the later part of my time working with the E&L team, I was entrusted as a member of an interview panel for a new E&L team member – being given the chance to see how this process worked was a fantastic opportunity and great experience to see a large sample of interviews and decipher what makes a successful applicant.

Working in a professional, office based, environment was an opportunity I had not previously had. A certain feeling of self-assuredness comes with wearing a suit each day, and I must say I missed that upon my return to academia. The E&L team graciously awarded me a "bonus" at the end of my placement for going "above and beyond", however, this is likely something they do to all leavers to maintain their superb reputation.