Testing Sulforaphane for chemoprevention against ageing and functional decline in male *Drosophila* models

---

A Research Master's Thesis

Esme Bellew-Dunn BSc (Hons)

Lancaster University

March 2020

I, Esme Bellew-Dunn, confirm that the work presented in this thesis is my own and has not been submitted in substantially the same form for the award of a higher degree elsewhere. Where information has been derived from other sources, I confirm this has been indicated in the thesis.

Signed……..

Submitted in part fulfilment of the requirements for the degree of Research Masters.

ABSTRACT

Esme Bellew-Dunn BSc (Hons)

Testing Sulforaphane for chemoprevention against ageing and functional decline in male *Drosophila* models

A Research Master's Thesis

March 2020

Sulforaphane (SFN) has been a phytochemical of interest in targeting several diseases. Many of these diseases are age related. Further, we can find evidence in the literature that SFN affects nearly every understood hallmark of ageing. Despite this, very little work has been carried out with SFN in the context of ageing, lifespan and functional health with age. Previous unpublished work has supported the possibility that SFN acts as a hormetic drug to improve lifespan at sub-toxic doses.

In this study, SFN was tested in the *Drosophila melanogaster* model organism, and its effects in lifespan, performance and biochemical hallmarks of ageing were monitored. These were assessed through three full lifespan/survival studies, a negative geotaxis assay, and a quantitative Real-Time Polymerase Chain Reaction experiment to investigate the impact of the drug on antioxidant gene expression. A High-Performance Liquid Chromatography experiment was also carried out to investigate our early findings, and test SFN absorption into fly food.

A significant improvement in lifespan was associated with SFN in only very limited conditions. White Dahomey flies reared on sugar-yeast food given 100µg/ml SFN between days 9 and 45 of their lives showed an improvement of 5.74%. Notably, Lancaster flies under identical conditions showed a significant decline (6.34%). No other significant findings were made.

These findings may indicate SFN has limited potential as an anti-ageing intervention and its effect is dramatically altered by food conditions, genetic background, and timing of dosage. However, we cannot rule out that these findings were caused by chance, and so recommend further work to verify our findings. We also hypothesise that another compound found in broccoli extract, glucoraphanin, may represent a better target for these studies, as previous work has shown low-SFN content broccoli extract to have a stronger effect than pure SFN.

Contents

1. Acknowledgements

I would like to thank the following people for their help during my studies;

My supervisor, Dr. David Clancy, for his knowledge, guidance, patience and understanding throughout this long and difficult process. Without his input and constant support, I feel I would never have been able to see this work through.

My loving partner Russell, for his love and support as well as helping me to finally come to terms with and begin to address many of the deep-seated issues and obstacles that have made this time so difficult for me. Because of him, I have finally been able to recover to the point of being able to complete my degree.

Lisa Butler, who carried out the practical work of the third lifespan experiment, "*Can Sulforaphane administration at different time-points in a fly's life confer an effect on longevity?*", during my intercalation period. This provided valuable data that allowed me to draw a well-rounded conclusion on this study.

My family, for their support particularly during my intercalation when I struggled with financial support. In particular, my great aunt Kay for allowing me to stay in her house while I finished this work.

Dr. Daniel Palmer, for guiding and training me during the first few months of my work, introducing me to the basic methods I would use throughout my studies.

Dawn McCracken, Dr. Ed Parkin, and the BLS postgraduate office for their kindness, understanding and help during my time here. Especially for their support up to, during, and after my intercalation period.

Dr. Tim Gregson, for his operation of the High-Performance Liquid Chromatogram and assistance throughout the related experiment and analysis.

Dimitra Papatziamou, Shiqi Li, and Emma Houchen, for their extensive work on the protein oxidation experiment and the insight they brought into it.

Finally, to all my other friends and associates who have provided support to me in this difficult time.

2. Literature Review

2.1. Ageing Research

Ageing is the process by which organisms lose function over the course of time. It occurs at every level of an organism; cellular, tissue, organ and organ systems, and ultimately culminates in the death of the organism (Lees, 2016), usually due to an inability to maintain the integrity of organs and physiological systems.

The cause of ageing, and how the process can be altered through medical interventions, has been of interest for most if not all human history. However, much of the understanding of the biological nature of ageing has only been developed over the course of the last few decades, with the development of multiple theories of ageing.

The goals of research into ageing are first to understand the natural ageing process, its cause and how it develops over time at a biochemical level, and then to attenuate the ageing process in meaningful ways with interventions that can ameliorate the progressive decline of health with age. This may or may not include extension of lifespan itself, but certainly would involve the extension of 'healthspan', the longevity of an organism's functional health.

Ageing is also associated with several disease states, distinct from but connected to normal life progression. These include most cancers, cardiovascular disorders, diabetes and neurodegenerative diseases (Jaul and Barron, 2017). Understanding normal ageing can also help to understand and begin to treat these debilitating conditions.

Ageing interventions in model organisms are commonplace experiments in ageing studies. This study considers one candidate drug, sulforaphane (SFN), and assesses the literature supporting its potential as an anti-ageing intervention. We will explain our choice of model organism (*Drosophila melanogaster*) in the study and assess the most relevant theory of ageing (the mitochondrial free radical theory) for SFN's potential action. We will then investigate the background of SFN in terms of its chemical properties and origin, and current studies.

SFN's most well-understood mechanism of action, the Nrf2 pathway (Kubo et al., 2017), has been a focus of ageing research in the past and represents to most obvious potential vector for SFN to act as an anti-ageing drug under the free radical theory.

Due to the lack of data on the effects of SFN in our chosen model, *Drosophila melanogaster*, and the very scant research of SFN into ageing specifically, the bulk of evidence for SFN as an anti-ageing drug must be inferred indirectly. To this end, we assess SFN in terms of studies that demonstrate its impact on the hallmarks and indicators of ageing. Finally, we discuss the previous, unpublished findings in our lab that form the foundation of this study.

2.1.1 Model Organisms in Ageing Studies

Model organisms are used in ageing studies due to the impracticality and ethical constraints of using human subjects and the limited value of focusing only on cell or tissue cultures when ageing is a process that consists of many interlinking factors across organ systems. The most commonly used models are the nematode worm, *Caenorhabditis elegans*, fruit fly *Drosophila melanogaster* and common mouse *Mus musculus*.

The nematode, though useful for its short lifespan and simple nutritional needs, has been criticised as a model for human ageing due to its distinct metabolic profile – most notably the presence of the dauer pathway, which enables it to enter a low-ageing state of zero mobility and reproduction (Clancy and Birdsall, 2013).

The fruit fly is a good compromise between genetic and physiological similarity with humans and the practical requirements of keeping good population sizes whose lifespan can be easily measured. It is important to note that there are areas where the fly may be lacking as a model – several pathways that have been connected to ageing are not present or significantly different in the fly. These include some target genes of the Nrf2 pathway such as NQO1 (Vasiliou et al., 2006).

The mouse model has clear advantages given its nature as a mammal with strong evolutionary proximity to humans compared to invertebrate models. However, it has a longer lifespan of a 2-3 years on average and requires more care and expense when rearing. Further, there are greater ethical concerns with using mammalian models and these add to the burden of experimental design. In some cases, aged mouse tissues are now shared between researchers to reduce time, cost and ethical concerns (Duran et al., 2013), which may help to increase the use of this model.

For the purposes of this study, the fly was selected. Had the study a longer duration, following any confirmed effects on age-related factors in the fly, mice would also have been studied for changes in lifespan, functional, neurological and cellular health.

2.1.2 Oxidative Damage and the Free Radical Theory of Ageing

The Mitochondrial Free Radical Theory of Ageing (mFRTA) was first proposed by Harman (1956, updated 1992), based on the concept that ageing, at its core, is the result of accumulated cellular damage over time. As a by-product of oxidative phosphorylation in the mitochondrial electron transport chain, oxygen free radicals are produced. These radicals are highly reactive and disrupt multiple cellular structures and processes by inflicting ‘Oxidative Damage’.

The mFRTA has had a turbulent history; studies carried out between the first proposals by Harman and the late-2000s and the found support for the theory with observations of correlations between high oxidative damage, carbonylated proteins and the presence of free radicals being higher with advanced age (Beckman and Ames, 1998).

However, in the late 2000s, papers emerged finding evidence contrary to the proposals of the theory. For instance, multiple criticisms of the role of superoxide dismutase, an enzyme that converts superoxide into either oxygen or hydrogen peroxide, were produced in this time. Reducing SOD expression in multiple genetic backgrounds of *C. elegans* and in mice could increase oxidative damage, but did not *reduce* lifespan

(Yang et al., 2007, Doonan et al., 2008, Jang et al., 2009). Other antioxidant targets such as glutathione peroxidase were also found to have little impact on lifespan (Zhang et al., 2009). Van Raamsdonk and Hekimi (2012) found that complete loss of SOD in *C. elegans* did not affect lifespan but did lead to sensitivity to acute stress in the worm.

Despite these findings, later work suggested the theory still held merit. Clancy and Birdsall (2013) criticised the use of the *C. elegans* model organism because of its specific metabolic profile and the dauer pathway. They also suggested that inducible transgenic studies would be preferable to embryonic interventions as the reduction of early-life mortality can mask whether an intervention meaningfully enhances lifespan. They concluded that oxidative damage, while not the sole factor in ageing, is important to late-life mortality.

In a review on the then-current relevancy of the mFRTA, Speakman et al., (2015) considered the issues surrounding obtaining good results in studies of oxidative damage and ageing. Damage is often tissue specific, with different tissues showing different levels of damage due to re-direction.

Further, Speakman et al. criticised the assessment of damage in studies as too simplistic, rather than considering the functional relevance of the damaged areas within the context of the organism. The lack of alternative hypotheses to the mFRTA was also noted by the paper.

Recent works have also taken to seeing the damage caused by oxidative stress as a component of a more complex understanding of chronic damage in organisms over time (Pomatto and Davies, 2018, Golubev et al., 2018). Here, a more complex role is given to homeostatic pathways that handle an organism's response to chronic damage over time. This includes the pathway most associated with SFN, the Nrf2 pathway.

2.2. Sulforaphane

Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)butane) is an isothiocyanate – a class of compounds formed from the enzymatic reactions of glucosinolates in cruciferous vegetables.

Sulforaphane exists in plants as glucoraphanin, which can be converted to the active SFN compound in a two-step reaction involving first the myrosinase-driven hydrolysis into unstable aglycone thiohydroximate-O-sulfonate, which then undergoes a non-enzymatic, intramolecular (Lossen) rearrangement into sulforaphane (**Figure 1**; Gu et al., 2012).

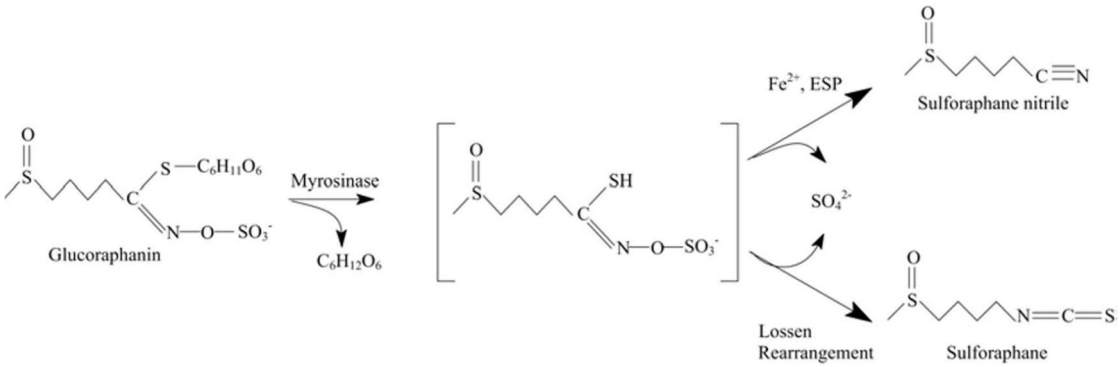


Figure 1: Reaction of Glucoraphanin by Myrosinase to produce Sulforaphane. Sulforaphane nitrile, also shown, is produced in the presence of epithiospecifier protein (ESP) and Fe^{2+} ions. Yield of SFN can be increased by avoiding this alternative reaction through Fe^{2+} ion manipulation (Gu et al., 2012).

SFN is unstable in the plant and glucoraphanin and myrosinase are held separately, in the vacuole and myrosin grains respectively, and react only when the plant is damaged (Gu et al., 2012). Sulforaphane's primary role in the plant is a deterrent to insect herbivores and other consumers, as it shows antifeedant, cytotoxic and selective antibiotic properties (Fahey et al., 2015).

Pure sulforaphane is an unstable compound which is highly sensitive to pH, light and temperature. It is most stable at a pH of around 5.0-6.0, low temperatures, in the dark and in organic solvents (Franklin et al., 2014). Sulforadex, which combines sulforaphane with a stabilising alpha-cyclodextrin, shows much higher stability (www.evgen.com/technology/, 2019), as does Prostaphane, a patented capsule-based commercial sulforaphane drug, with no apparent loss of potency or bioavailability (Fahey et al., 2017). These technologies are therefore necessary if the compound is to be employed as a commercial drug.

Sulforaphane has multiple advantages over other isothiocyanates due to its good bioavailability, multiple antioxidant and anti-cancer mechanisms, and that normal cells are resistant to SFN-induced cell death (Ullah, 2015). It can cross the blood-brain barrier, suggesting its potential use in the treatment of neurological disorders and ageing phenotypes (Tarozzi et al., 2013).

Other than cancer, SFN has been investigated as a potential treatment for many diseases, including diabetes mellitus (Zhang et al., 2014), obesity (Choi et al., 2014) and neurodegenerative disorders (Angeloni et al., 2015, Tarozzi et al., 2013).

Currently, evidence for Sulforaphane's efficacy in humans is lacking, though some clinical trials in certain human cancers have begun in recent years (Cornblatt et al., 2007, Lozanovski et al., 2014, Alumkal et al., 2015).

We became interested in SFN's potential as an anti-ageing drug candidate due to the many age-related diseases it has been used to treat, and the age-related intracellular processes it is known to interact with. A full summary of the effects of SFN can be found at the beginning of the section "Sulforaphane and the Hallmarks of Ageing", in **Figure 3**. However, the most well-understood mechanism by which SFN could potentially affect the ageing process is through its up-regulation of the anti-oxidative Nrf2 pathway.

2.2.1. Sulforaphane and Oxidative Damage: The Nrf2 Pathway

Sulforaphane's best characterised role is as an antioxidant in cancer research (Zhang and Tang, 2007). A well-understood mechanism by which Sulforaphane exerts its role is as an up-regulator of antioxidant genes through the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) Pathway (Kubo et al., 2017; Lee and Lee, 2011).

The Nrf2 pathway is a well-characterised metabolic pathway that regulates multiple genes containing Antioxidant Response Element (ARE) sites. These include Phase II detoxification genes, elements of the glutathione response, and NADPH generating enzymes such as NQO1 (Lee and Lee, 2011). The Nrf2 gene and its pathway are well-conserved evolutionarily, being found in all mammals (Ma, 2013; Sykiotis and Bohmann, 2008) and many invertebrate models including *D. melanogaster*, where it was first identified as the Cap n' Collar, or *cnc* gene (Sykiotis and Bohmann, 2008).

There are multiple models of activation of the Nrf2 pathway. In the classic model, Nrf2 is constitutively expressed in the cytoplasm, but is sequestered by Kelch-like ECH-associated protein 1 (Keap1), which binds the Neh2 terminal of Nrf2, preventing it from translocating to the nucleus and inducing gene expression (Itoh et al., 1999, Zhang et al., 2013). Keap1 also works with Cullin-3 to promote the ubiquitination and proteasomal degradation of Nrf2, suppressing expression levels (Zhang et al., 2013).

Activation of the pathway occurs when an activator (classically an electrophilic attack against cysteine residues) targets Keap1 and induces a conformational change that unbinds it from Nrf2. Nrf2 is then free to heterodimerize with sMAf and translocate to the nucleus to bind ARE sites (Zhang et al., 2013). This is summarised in **Figure 2** below.

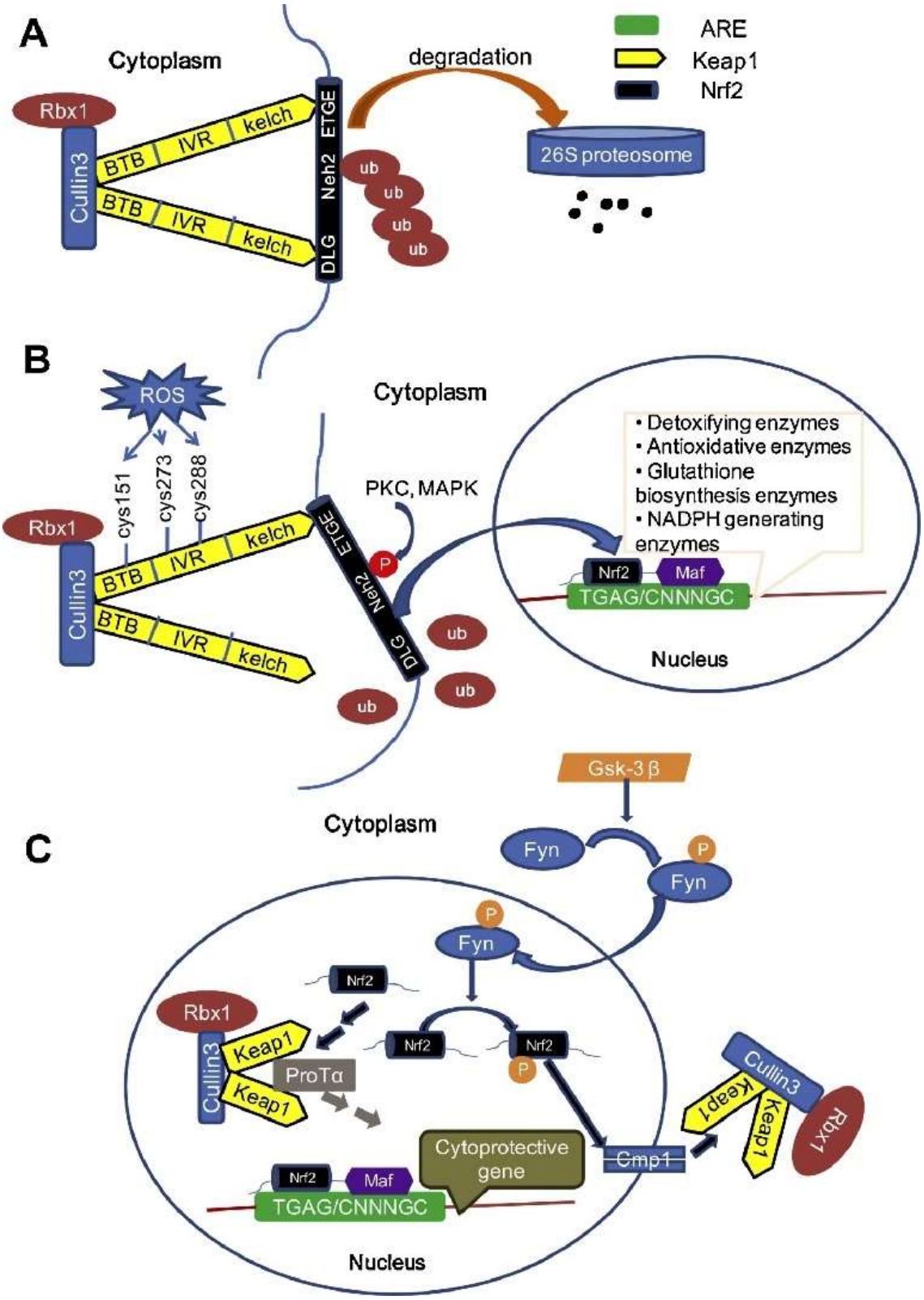


Figure 2: The conventional mechanism of Nrf2 activation. A: In basal state, Nrf2 is sequestered in the cytoplasm by the Keap1 complex (Keap1, Rbx1, Cullin3) and is signalled for degradation by the 26S Proteosome by ubiquitination. B: An electrophilic attack by ROS on key cysteine residues induces a conformational change in Keap1 and releases Nrf2 to translocate to the nucleus and promote expression of target genes via the ARE site. C: Nuclear Nrf2 is returned to the cytoplasm by Fyn phosphorylation and the Cmp1 system, or alternatively through Cul3-

Keap1 complexes imported into the nucleus by ProTα (Image: Zhang et al., 2013, Permission to reproduce this image has been granted by Elsevier).

Alternative methods of activating Nrf2 include phosphorylation of Nrf2 to disturb the Keap1-Nrf2 complex by various protein kinases. PKC, JNK1 and ERK2 promote the Nrf2 pathway, MAPKs interact in various ways depending on the specific MAPK, and may be carried out by an indirect mechanism, while GSK-3 is an inhibitor of Nrf2 that promotes its degradation (Huang et al., 2015. Sun, Huang and Zhang, 2009).

P62 is a competitive inhibitor of Keap1 that can promote Nrf2 signalling (Komatsu et al., 2010). It forms a feedback loop with Nrf2, as its expression is promoted by activation of the pathway (Jain et al., 2010). Persistent activation of Nrf2 by p62 is associated with cancer (Komatsu et al., 2010), but Nrf2's relationship to cancer is somewhat complicated by the fact that transcription is repressed in some prostate tumours by hypermethylation of certain CpGs in its promoter region (Yu. et al, 2010).

MicroRNAs miR-144, miR-153, miR-27a, and miR-142-5 reduce Nrf2 mRNA and protein levels, while miR-34a reduces Keap1 mRNA (Huang et al., 2015).

Nrf2 can also be upregulated by targeting specific UPS enzymes that regulate its degradation, avoiding the need for antioxidants (Plafker, 2010).

Nrf2 in Drosophila melanogaster:

The fly Nrf2 equivalent is Cap n' Collar (cnc) with the gene product CncC being the closest homologue to mammalian Nrf2 (McGinnis et al., 1998). The N-terminus of CncC shows close homology with the Nrf2 group Neh (Sykiotis and Bohmann, 2008). CncC is mostly expressed in the fly alimentary canal (analogous to mammalian gut). Maf and dKeap1, also homologues to sMaf and Keap1, conserve their biochemical roles in the fly in heterodimerization with, and repressing Nrf2, respectively.

The cnc gene lends its name to the family of proteins Nrf2 belongs to, being first identified in the early 1990s (Mohler et al., 1991). Despite this, it's role in the fruit fly Keap1/Nrf2 signalling system equivalent went under-studied compared to the mammalian Nrf2 (Sykiotis and Bohmann, 2008). It has been found to behave similarly to the mammalian protein, with a conserved pathway and similar target genes, with the ARE sequence also found in CncC targets. These targets have included the GstD family of Glutathione S-Transferases (Sykiotis and Bohmann, 2008), and several xenobiotic defence genes including p450 gene, Jheh1 and Gnmt (Misra et al., 2011).

D. melanogaster male dKeap1 heterozygotes have displayed enhanced lifespan, though this was not conserved in female heterozygotes (Sykiotis and Bohmann, 2008). Though this study suffers from the issues of using genetic intervention and a low sample size, a second study corroborated it by finding a slight increase in lifespan associated with Keap1 knock-down in both male and female flies (Pomatto et al., 2017). The CncC pathway also appears to be critical to xenobiotic defence in the fly, accounting for about 70% of gene induction when exposed to phenobarbital (Misra et al., 2011). Any compound that promotes CncC activation should then lead to measurable changes in the expression and lifespan in male *D. melanogaster* flies.

One difficulty the fly model presents is that some of the best-characterised ARE-sequence genes that respond to Nrf2 in mammals, such as NAD(P)H:Quinone

Oxidoreductase 1 (NQO1) are absent, in any form, in the insect model (Vasiliou et al., 2006).

Sulforaphane activates the Keap1-Nrf2 pathway primarily via thiocarbamylation (forming thionoacyl adducts) of Cys151 in Keap1. Like the classical pathway of Nrf2 activation, this induces a conformational change that destabilizes the Keap1-Nrf2 complex. Sulforaphane also regulates Nrf2 at the level of protein synthesis by promoting ribosomomal internalization of Nrf2 mRNA (Li et al., 2010).

SFN is known to reduce the phosphorylation of MAPK signalling pathways ERK1/2, JNK and p38 (Angeloni et al., 2015). This may reduce the efficacy of Nrf2 induction, however SFN's relationship with the MAPK Kinases is complex, as it can act to increase the activation of the same signalling pathways in some cancer cells (Lin et al., 2017). This is likely mediated by its dual-role effect in mitochondrial dynamics (Negrette-Guzmán et al., 2013).

There is evidence that Sulforaphane can regulate Nrf2 by epigenetic pathways to allow easier induction. It has also been demonstrated to demethylate the first 15 CpGs of the Nrf2 promoter, which leads to it being more easily induced under stress conditions (Su et al., 2013). In mouse models of Alzheimer's Disease SFN was able to promote Nrf2 nuclear translocation by decreasing DNA methylation levels of the Nrf2 promoter (Zhao et al., 2018).

2.2.1. Sulforaphane and *Drosophila melanogaster*

Sulforaphane has not been studied in *Drosophila melanogaster* models in the context of ageing outside of our lab as far as we know. Very little literature on the effects of Sulforaphane in *D melanogaster* is available. What is known is that SFN has been used to ameliorate neuronal death in *Drosophila* models of Parkinson's (Parkin mutants) (Trinh et al, 2008). More recently, SFN administration is linked to a change in expression of Cyp6g1 and Cyp6a2 (Vázquez-Gómez et al., 2010), both genes associated with the *Drosophila* Nrf2 homologue, CncC (Misra et al., 2011), and a reduction in the genotoxic damage induced by methyl methanesulfonate (Dueñas-García et al., 2012). In both studies Sulforaphane exhibited a different effect in a high bioactivation strain of the flies than in the standard, which may indicate the importance of fly background in any study.

These studies link to ageing in that the conditions they replicated are considered age-related, but since neither of them directly studied ageing, the rationale for our study must instead be based on the indirect wealth of evidence that Sulforaphane should impact ageing in *Drosophila*.

Although only scant research has centred *Drosophila melanogaster*, SFN has been studied in a wide variety of alternative models for its impact in several different diseases. Most of this work has been focused on understanding its effect on the Nrf2 pathway and oxidative damage, but more recent studies have found several unrelated secondary mechanisms of the compound. Many of these findings touch biological systems that are tied to the physiological hallmarks of ageing.

2.3. Sulforaphane and the Hallmarks of Ageing

In 2013, López-Otín et al. produced a list of nine physiological 'hallmarks' of ageing, to define the biological markers are associated with the ageing phenotype. These were genomic instability, telomere attrition, regulation of cell senescence, mitochondrial dysfunction, epigenetic regulation, regulation of proteostasis, stem cell exhaustion and altered cell communication.

Below, we look at the evidence for an impact of Sulforaphane in each of the hallmark areas, and a few other areas that have been studied in recent work, such as Advanced Glycation End-Product Formation (summarised in **Figure 3**).

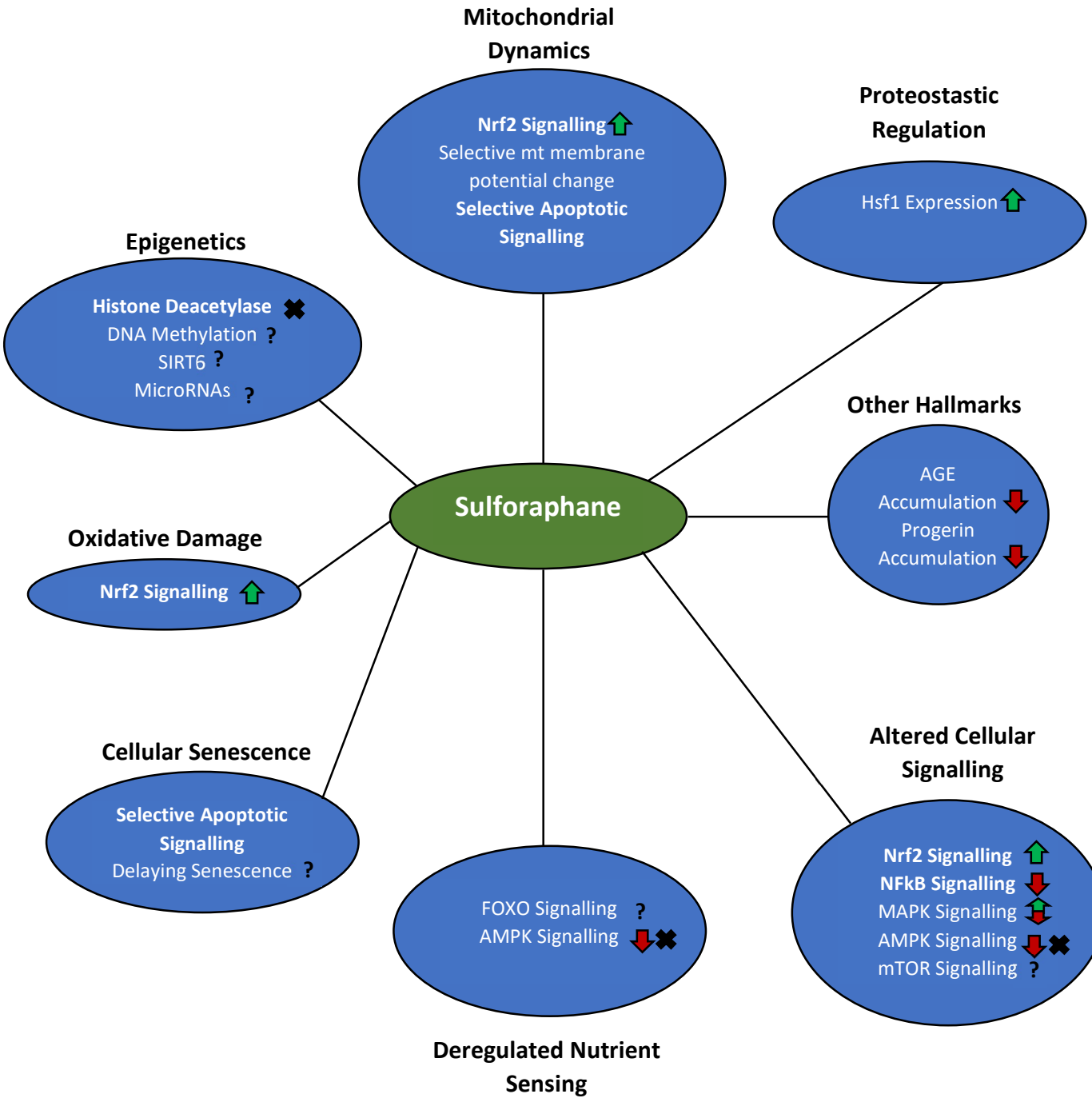


Figure 3: Summary of sulforaphane's known impact on various components of the hallmarks of ageing. Bold; a well-understood effect with known mechanisms, Non-Bold; Effect with some evidence but not well-documented. Symbols; Arrows indicate direction of regulation, cross indicates inhibition, question mark indicates an unclear effect, or that it is unclear whether an anti-ageing effect can be found.

2.3.1. Major Hallmarks of Impact with Sulforaphane

2.3.1.1. Cellular Senescence and Apoptosis

Senescence is the stable arrest of the cell cycle and accompanying phenotypic changes, often preceding apoptosis, the stable death of a cell.

Cellular senescence acts as a protective system in healthy organisms, preventing the propagation of damaged cells. However, it may contribute to age-related decline when the clearance system becomes inadequate to handle the burden of senescent cells (López-Otín et al., 2013).

Apoptosis follows cell senescence and is corolled by multiple mechanisms. The two major apoptotic pathways are the extrinsic 'death-receptor' pathway, and the intrinsic mitochondrial pathway (Tower, 2015; Negrette-Guzmán et al., 2013). The relationship between apoptosis and ageing is complicated, with the rate of apoptosis increasing in some cell types with age and decreasing in others (Van Deursen, 2014; Negrette-Guzmán et al., 2013; López-Otín et al., 2013). However, overall a decline in apoptotic markers is observed in advanced age, due to increased senescence and reduced cell turnover (Tower, 2015).

Drugs that can selectively kill senescent cells, senolytics, were developed relatively recently, and show promise in mouse models (Kirkland and Tchkonia, 2015; Zhu et al., 2015). These drugs target pro-survival network components such as EFNB1 or 3, PI3Kδ, p21, BCL-xL, and plasminogen-activated inhibitor-2, yet do not seem to harm non-senescent cells (Zhu et al., 2015).

Sulforaphane may be able to delay senescence in human fibroblasts via its Nrf2-mediated action, due to changes to the glucose and glycolytic response (Hariton et al., 2018). Nrf2 silencing has also been demonstrated to accelerate cellular senescence (Kapeta et al., 2010).

Numerous studies have confirmed that Sulforaphane can induce apoptosis through the mitochondria-dependent pathway, in both p53-dependent and -independent manners (Negrette-Guzmán et al., 2013). The mechanism is achieved through pro-apoptotic signalling pathways such as ERK1/2, JNK and PI3K/Akt. Interestingly, Sulforaphane is reported to activate these pathways to promote apoptosis in many studies, but a low state of activation for PI3K/Akt and ERK1/2 is associated with SFN-mediated inhibition of some tumours (Shankar et al., 2008). SFN has also been reported to induce apoptosis in the extrinsic pathway, with caspase-8 participation, in numerous studies (Negrette-Guzmán et al., 2013).

Since these mechanisms are specific to cancerous or damaged cells, it is possible for SFN to prevent the accumulation of damaged cells by encouraging more efficient early removal. In this way, Sulforaphane is akin to a senolytic, as it shows selectivity with regards to the mitochondrial state of target cells.

2.3.1.2. Mitochondrial Dysfunction and Sulforaphane's 'Dual-Role'

Mitochondrial Dynamics:

In addition to the impact of the respiratory chain's efficiency diminishing with age, as covered by the mitochondrial Free Radical Theory of Ageing, dysfunctional mitochondrial contribute to ageing via apoptotic signalling and can trigger inflammatory reactions. They may also directly impact cellular signalling and inter-organellar cross talk via mitochondrion-associated membranes that constitute an interface between the outer mitochondrial membrane and endoplasmic reticulum (Raffaello and Rizzuto, 2011).

Mitochondrial dynamics can be thought of as the balance between the biogenesis of mitochondria, and their degradation through mitophagy. A reduction in the biogenesis of mitochondria is associated with ageing, and is possibly linked to telomere attrition, as it can be partly reversed by the activation of telomerases (Bernades de Jesus et al., 2012).

Mitophagy is the process by which mitochondria are selectively degraded by autophagy. It acts as a quality control system to eliminate damaged mitochondria. A decline in mitophagy is associated with advanced age (Picca et al., 2019; López-Otín et al., 2013). Mitophagy is known to be regulated by the gene Atg32 and is possibly induced by increased ROS levels (Wang and Klionsky, 2011; Kanki et al., 2009). Dysregulation of mitophagy leads to an increased number of damaged mitochondria (Picca et al., 2019; Tower, 2015) which can be cytotoxic and reduce other cell recycling systems such as proteostasis.

Mild mitochondrial poisons that induce a low energy state characterized by increased AMP and activation of AMP-Kinase can increase lifespan in model organisms (Ulgherait et al., 2014; López-Otín et al., 2013). This is due to them triggering repair mechanisms that surpass the cost of repairing the damage they inflict and improve overall mitochondrial fitness, in a concept known as mitohormesis (Bárcena et al., 2018; López-Otín et al., 2013).

SFN may be a mitohormetic compound. When introduced to healthy cultured cells, Sulforaphane promotes the fusion of mitochondria and the peroxisome and thereby protect cells against apoptotic threats in a Nrf2-independent mechanism (O'Mealey et al., 2016). It is speculated that SFN may target the GTPase domain of Dynamin-related protein 1 (Drp1) for acylation, thereby inhibiting it. As Drp1 is the main catalyst for mitochondrial fission, this inhibition would promote mitochondrial fusion (O'Mealey et al., 2016).

SFN could induce mitochondrial biogenesis through the Nrf2 pathway. NRF-1 is required for mitochondrial biogenesis and contains an ARE site (Piantadosi et al., 2008), therefore SFN may up-regulate biogenesis indirectly through the Nrf2 pathway. SFN dosing also correlates with an increase in mitochondrial mass in multiple types of human fibroblasts (Brose et al., 2012), supporting this idea.

Dual Role of Sulforaphane:

The body of literature on Sulforaphane shows considerable evidence of contradictory effects when used as an intervention in cellular cultures – it has been found repeatedly to be cytotoxic when administered to cancer cells (Negrette-Guzman et al., 2013; Yu et al., 1998), but there is also evidence of cytoprotective effects in cases when applied to healthy cells and whole organisms (Guerrero-Beltrán et al. 2012).

This may be governed by its effect on the membrane potential of mitochondria – for example, in healthy cells, such as porcine renal proximal tubular LLC-PK1 cells, and in animal models, SFN can prevent the disruption of the mitochondrial membrane potential and the induction of apoptosis following exposure to nephrotoxic drugs such as cisplatin (Guerrero-Beltrán, 2010). In cancerous cells SFN instead disrupts the mitochondrial membrane potential and induces apoptosis (Negrette-Guzmán et al., 2013a).

Negrette-Guzmán et al. (2013) proposed an integrative hypothesis. In non-cancerous cells SFN will induce Nrf2 action, activate phase II enzymes and induce mitochondrial biogenesis. This will enable mitochondrial fission to separate out damaged and non-damaged sister mitochondria. The damaged mitochondria will be targeted for autophagy, and higher levels of biogenesis will then replenish the healthy mitochondria. This allows SFN to act as a cytoprotective.

In cancerous cells, most mitochondria are already damaged, so SFN will primarily induce Bax oligomerization, leading to outer membrane permeabilization and disruption of mitochondrial membrane potential. This promotes pro-apoptotic factors, which are then multiplied by increased mitochondrial biogenesis, leading to cell death.

Which way a cell responds is thought to be due to a certain threshold level of Reactive Oxygen Species damage to mitochondria present prior to the intervention of SFN. In this way, the same dose of sulforaphane can exhibit different effects across different cells and tissues.

2.3.1.3. Epigenetic Regulation

Epigenetics are a particularly important target in ageing studies because they, unlike direct DNA damage, can be relatively easily reverted, giving opportunities for anti-ageing treatment (López-Otín et al., 2013). There is considerable evidence that shows Sulforaphane targets many key epigenetic regulatory systems, including histone methylation and acetylation, and the regulation of sirtuins and microRNAs.

Histone Methylation:

It has long been established that Histone Methylation is a hallmark of ageing in invertebrates and that the deletion of components of histone methylation complexes can extend longevity in these models (López-Otín et al., 2013). Global DNA methylation has a complex relationship with ageing, as global hypomethylation is correlated with age, but some genes receive hypermethylation instead (Maegawa et al., 2010). No direct experimental evidence has been found to indicate lifespan can be extended by altering DNA Methylation patterns.

SFN has been linked to global DNA hypomethylation, decreased levels of DNA methyltransferases DNMT1 and DNMT3B, and decreased m6A RNA methylation in breast cancer cells (Lewinska et al., 2017). Also, in some gastric carcinoma cells, SFN has been linked to the downregulation of the histone methyltransferase SMYD3 (Dong et al., 2018). However, as with many cases of Sulforaphane triggering potentially anti-survival responses, these appear to be cancer specific.

Histone Acetylation:

Histone Deacetylase Inhibitors show promise as anti-ageing drugs. Early research demonstrated that histone acetylation increases in some sites of aged organisms with increased histone acetylation represents a beneficial state age, while decreasing in others (López-Otín et al., 2013). It is now believed that increased histone acetylation represents a beneficial state that helps to combat loss of transcription in genes that are involved in maintaining homeostasis, metabolism and healthspan (Pasyukova and Vaiserman, 2017). In *D. melanogaster,* DNA Methylation is almost entirely absent in adult flies, and acetylation is thought to be the primary mechanism of epigenetic regulation (Deshmukh et al., 2018).

Sulforaphane is known to exert an inhibitory effect on Histone Deacetylases (HDACs). The current proposed mechanism by which SFN acts against HDACs is through SFN-Cys, a metabolite of SFN that can be produced in multiple ways. The most common way that SFN-Cys forms is through deacetylation of SFN-NAC, which can be a consequence of HDAC action on SFN (Ho, Clarke and Dashwood, 2009). Inhibition is then caused by the occupation of the HDAC active site by SFN-Cys acting as a bidentate Zn ligand (Ho, Clarke and Dashwood, 2009). This proposed mechanism is based on computer modelling of the metabolite's structure (Myzak et al., 2004).

SFN treatment has been found to correspond to increased histone acetylation, however not all studies agree on this point, with one finding no increase in the acetylation of histones H3 and H4 by SFN treatment (Abbaoui et al., 2017). Notably, SFN has been successful in selectively inducing apoptosis via a HDACi-mediated mechanism in cancer cells. This did not affect healthy cells (Ho, Clarke and Dashwood, 2009).

Sirtuin Regulation:

Sirtuins are known to be involved in the ageing process, however, this role is complex. In mammals, SIRT6 has shown some promise as a target for lifespan extension. It regulates NF-kB signalling and glucose homeostasis through histone deacetylation, both of which are key targets of ageing. Overexpression can lead to the extension of male mouse longevity (Kanfi et al., 2012) while deficiency leads to accelerated ageing (Mostoslavsky et al., 2006).

SIRT6 reduction has been reported in Sulforaphane treatment (Rajendran et al., 2013), in the context of cancer cells. No evidence exists for an effect of SFN on SIRT6 levels in healthy cells.

MicroRNAs:

MicroRNAs are implicated in most metabolic pathways, including those associated with ageing (Harries 2014) and are known to modulate longevity in invertebrate models (Liu et al., 2012).

Sulforaphane intervention changes the miRNA profile of breast cancer cells (Lewinska et al., 2017). Although the effect Sulforaphane exerts on miRNA in healthy cells is not known, this supports the potential of SFN to interact with them and warrants further investigation.

In summary, Sulforaphane represents a potential non-genetic intervention against HDACs, as well as interventions in a vast array of other epigenetic components, which may contribute to an improvement in lifespan and amelioration of the aged phenotype.

2.3.1.4. Altered Cellular Communication

Ageing is associated with several changes to the neuronal, endocrine and neuroendocrine systems, all of which regulate cell signalling pathways. Sulforaphane impacts several of these pathways, including Nrf2 (discussed previously), Nuclear Factor-kappa B (NF-kB) and multiple MAPK Kinase pathways.

NF-kB Pathway:

NF-kB is a pleiotropic transcription factor involved in cell function and inflammation. In resting cell state NF-kB is inactive, sequestered in the cytoplasm by Inhibitory kB (IkB). In response to stimuli such as cytokines, inhibitory kB Kinases (IKKs) phosphorylate IkB and target it for proteosomal degradation, thereby releasing NF-kB, which can then translocate to the nucleus and activate certain genes associated with an inflammatory response (Sivandzade et al., 2019; Lawrence, 2009). In this way, it can be thought of as inverse to the Nrf2 pathway. It can also alternatively be triggered by CD40 and lymphotoxin activation of IKKa through NF-kB inducing kinase (NIK) (Lawrence, 2009). NFkB over-activation promotes cellular senescence and is a transcriptional signature of ageing (Balistreri et al., 2013; Rovillain et al., 2010).

NF-kB also drives the ‘Bystander Effect’, where senescent cells can induce senescence in neighbours via gap-junction mediated contacts or ROS (Nelson et al., 2012). Senescent cells produce a ‘Senescence-Associated Secretory Phenotype (SASP)’ and produce pro-inflammatory cytokines such as IL-1, 6 and 8. This is driven by the NF-kB pathway, which itself is activated by ROS production (Nelson et al., 2018).

SFN exhibits an inhibitory effect on the NF-kB pathway. In cultured human prostate cancer cells, SFN treatment at 20 and 30uM decreased transcriptional activity of the NF-kB pathway and the expression of NF-kB regulated genes (Xu et al., 2005). SFN can attenuate microglia-mediated neuronal necroptosis, an age-associated pathology, in BV-2 microglia via a process partially mediated by NF-kB down-regulation. This inhibition of NF-kB may be downstream of SFN’s inhibition of MAPK Kinases ERK1/2, JNK and p38 (Qin et al., 2018).

Since Nrf2 regulates NF-kB by reducing ROS levels and preventing the degradation of IkB-α (Sivandzade et al., 2018), SFN may also partially inhibit NF-kB through its up-regulation of Nrf2.

Regulation of MAPK Kinases:

The relationship of SFN with MAPK Kinases is complex. In many studies SFN is reported as an activator of MAPK Kinases such as ERK1/2, JNK and p38 (Negrette-Guzmán et al., 2013), however in other studies SFN has an inhibitory effect on these same pathways, most importantly the JNK-1 pathway, which regulates pro-apoptotic signalling (Subedi et al., 2019). This is likely to be tied to the dual-role Sulforaphane expresses as a result of differing mitochondrial dynamics in different cell types. Notably, SFN is found to activate these pathways when it acts as a pro-apoptotic in cancer cells and inhibits these pathways when acting as an anti-apoptotic or pro-survival treatment in healthy cells (Negrette-Guzmán et al., 2013).

2.3.1.5. Deregulated Nutrient Sensing

Insulin and IGF-1 Signalling (IIS) and FOXO Pathways:

The IIS pathway is the most conserved ageing-controlling pathway in evolution. It targets the FOXO family of transcription factors and the mTOR complexes, which are also evolutionarily conserved ageing-involved factors (Lopez-Ortin et al., 2013). It mediates the effect of Caloric ('Dietary') Restriction in increasing lifespan, an effect that has been seen in all investigated eukaryotic species (Lopez-Ortin et al., 2013; Fontana et al., 2010). IGF1 levels decline during both normal ageing and premature ageing conditions (Schumacher, 2008), however, early constitutive decreased IIS extends longevity (Lopez-Otin et al., 2013). Organisms with decreased IIS live longer due to lower rates of cell growth and metabolism, which means they accrue damage at a slower overall rate (Garinis et al., 2008).

The FOXO family of transcription factors are required to mediate many of the effects of the IIS pathway. Mammals possess 4 FOXO family members. They are affected differently by Caloric Restriction – under CR, young mice showed a decrease in FOXO1 and 4 while older mice experienced upregulation of FOXO1, 3 and 4 (Sheng et al., 2017).

There is no evidence of Sulforaphane directly affecting IGF-1, with one study finding a negative outcome from broccoli extract (Riso et al., 2009), though this study also failed to find an impact on HDAC and lacked specificity to Sulforaphane. SFN may affect insulin signalling in *diabetes mellitus* (Xu et al., 2018) but no specific mechanism has been elucidated and the relevance this would have to ageing is unclear.

FOXO signalling is important in Sulforaphane's control of cell cycle arrest and apoptosis (Yu et al., 2015, Roy et al., 2010). Activity of FOXO pathways is a requirement for Sulforaphane action in multiple studies (Son et al., 2017, Davis et al., 2009). In flour beetles (*Tribolium castaneum*), knock-down of Foxo-1 and Jnk-1 prevented lyophilized broccoli-mediated lifespan benefit, though in this study the presence of SFN was not absolutely or exclusively confirmed (Grünwald et al., 2013).

mTOR Signalling Pathways:

The mTOR kinase, found in multicomplexes mTORC1 and mTORC2, regulates anabolic metabolism. Down-regulation of mTORC1 in invertebrates extends longevity, and this extension attenuates any further benefits from DR (Johnson et al., 2013). AMPK acts in opposition to mTOR, and there is evidence AMPK up-regulation can extend lifespan through drug interventions such as metformin (Martin-Montalvo et al., 2013; Onken and Driscoll, 2010).

Inhibition of TOR activity has undesirable side effects, including impaired wound healing, insulin resistance, cataract development and testicular degeneration (Wilkinson et al., 2012). Notably, all these side effects are common phenotypical changes seen in normal ageing. These side effects can be mitigated by carefully assessing dosage, and/or the co-application of drugs that counteract these side-effects without disrupting the desired function of rapamycin (Walters and Cox, 2018).

SFN can up-regulate mTOR signalling. This leads to an inhibition of inflammation and prevention of damage in mice exposed to lipopolysaccharide, a compound that induces neuroinflammation (Gao et al., 2018).

Sulforaphane has been shown to prevent and reverse the inhibition of AMPK induced by diabetes in mouse models. This appears to be related to the effect of up-regulating the Nrf2 pathway, as downstream products of Nrf2 (NQO1 and HO-1) were correlated with up-regulation of LKB1 and the enhancement of LKB1/AMPK signalling (Zhang et al., 2014). This mechanism is also implicated in SFN's ability to inhibit adipogenesis in obesity studies (Choi et al., 2014).

2.3.1.6. Proteostatic Regulation

Proteostasis refers to the mechanisms that stabilize correctly folded proteins while signalling the degradation of mis-folded or excess proteins, is found to become deregulated with age (Dahlmann et al., 2007). This is particularly important to the understanding of key age-related diseases such as Alzheimer's and Parkinson's, which are caused by mis-folded proteins (Ross and Piorier, 2004).

The decline of molecular chaperones is implicated in ageing. This decline is due to both dysregulation of transcription and the sequestration of chaperones such as the heat-shock family proteins (HSPs) by protein aggregates (Labbadia and Morimoto, 2015). Interventions such as overexpressing chaperones in *Drosophila melanogaster* can increase their lifespan (Morrow et al., 2004), while mice deficient in Hsp70-interacting protein exhibit accelerated ageing phenotypes (Min et al., 2008).

Sulforaphane has been found to induce proteasome activation in a variety of cancer cell lines, as well as protect normal cells from the cytotoxic effect of hydrogen peroxide. This protection was eliminated by disrupting proteasome functionality, and overexpression of the proteasomal catalytic subunit PSMB5 prevented further benefit from SFN treatment (Kwak et al. 2007).

Though this was originally believed to be linked to the role of the Nrf2 pathway (Kwak et al., 2007), it is now understood that Sulforaphane induces proteasomal activation through activation of heat shock transcription factor 1 (hsf1) via dissociation with Hsp90 and Hsp70 in an Nrf2-independent manner, and silencing Hsp27 blocks proteasomal activation with SFN (Gan et al., 2010). Hsf1 regulates the expression of heat-shock family chaperones.

Recently, it has been proposed that SFN may mediate proteasomal activity by ERK1/2 activation. Using an ERK1/2 inhibitor can prevent 26S proteasome activation by SFN (Geng et al., 2017).

The Proteasomal degradation activated by SFN is linked to the induction of apoptosis (Geng et al., 2017), loss of Bmi-1 and Ezh2, leading to a reduction in polycomb group proteins and a suppressive effect on skin cancer cells (Balasubramanian, Chew and Eckert, 2011 and Balasubramanian et al., 2012).

2.3.2. Other Hallmarks of Ageing

2.3.2.1. Advanced Glycation End-Product (AGE) formation

AGEs are a heterogenous group of compounds formed when a carbonyl group of reducing sugars couples covalently, in a non-enzymatic reaction (glycation), to multiple compounds including proteins, lipids and nucleic acids.

AGEs first form as precursor molecules, often during the glycolytic process, such as Glyoxal or Methylglyoxal reducing sugars. These molecules undergo the Maillard reaction, a reaction where these sugars react with amino acids (mostly lysine) to form unstable Schiff bases. These then stabilize as Adamori products (Fishman et al., 2018). Finally, the adamori products undergo further reactions to form dicarbonyl intermediates that react with arginine and lysine residues to form irreversible crosslinked AGEs (Thornalley, 2005). **Figure 4** summarizes these reactions.

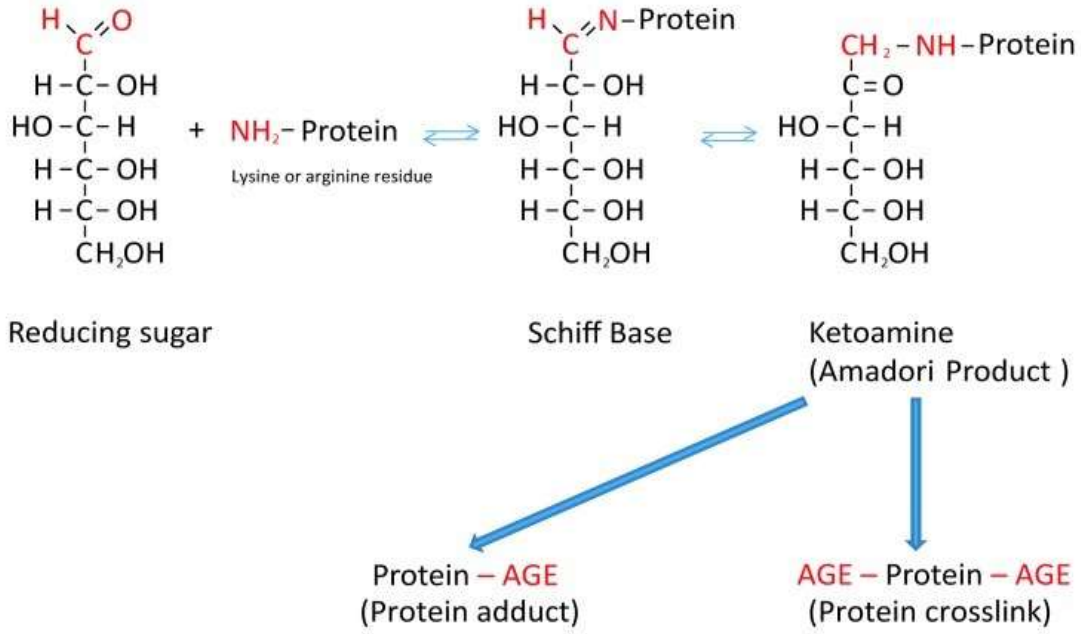


Figure 4: Schema of the Maillard Reaction. Reducing sugars, formed as by-products of glycolysis, react with the free amino groups of amino acids (often lysine or other basic amino acids). These form Schiff Bases, which can freely rearrange into Adamori products and back. Adamori products form AGEs when they form crosslinks to other proteins, stabilizing them in the adamori form and preventing rearrangement into Schiff Bases (Gkogkolou and Bohm, 2012).

A high accumulation of AGEs is correlated to advanced are, as well as multiple age-related diseases (Fishman et al., 2018). In addition, AGEs are known to interfere with cell signalling and the proteasome, leading to pro-inflammatory cell profiles and age-related tissue decline (Fishman et al., 2018; Xie et al., 2013).

Only scant research has been carried out into the impact of Sulforaphane on AGEs. All evidence points to it acting to inhibit the formation of AGEs, possibly through reducing the expression of RAGE, the receptor for AGEs (Matsui et al., 2016). More recent work has found that Sulforaphane up-regulates Glyoxalase1 (Glo1), which defends against the accumulation of Methylglyoxal, thus preventing AGE formation (Alfarano et al.,

2018). Given this preliminary evidence, SFN's impact on AGE accumulation is worth studying further.

2.3.2.2. Genomic Instability

Genetic damage is accumulated throughout life, and many premature ageing conditions are caused by increased DNA damage accumulation (Burtner and Kennedy, 2010). However, the role that DNA damage plays in ageing is questionable (Clancy and Birdsall, 2013), though it may be indicative of an aged phenotype.

It is not currently believed that sulforaphane plays a significant role in ameliorating the accumulation of direct DNA damage, though through its actions to up-regulate antioxidant genes it may improve some preventative mechanisms.

SFN may reduce progerin accumulation in Hutchinson-Gilford Progeria Syndrome (HGPS) cells by activation of the proteasome and enhancement of autophagy (Gabriel et al., 2015). Progerin is an aberrant isoform of prelamin A (a nuclear scaffold protein) found overexpressed in HGPS (Jiang et al., 2018) and correlated with advanced age in humans (Ragnauth et al., 2010).

2.3.2.3. Telomere Attrition

Telomeres are a special region of DNA found at the end of chromosomes. They have been thought important in ageing due to the inability for most mammalian cells to replicate them properly due to the absence of the specific DNA Polymerase telomerase. They are also particularly susceptible to damage due to the presence of Shelterin, a nucleoprotein complex that prevents normal DNA repair (Fumagalli et al., 2012). Studies have linked telomerase expression to a delay, but not complete prevention, of physiological ageing (Bernardes de Jesus et al., 2012; de Jesus et al., 2011)

Enforced expression of telomerase is associated with increased risk in some cancers, such as epithelial (González-Suárez et al., 2001) or breast cancer (Artandi et al., 2002), though this is only a slight effect. Recent research has shown some possibility of expressing telomerase without this risk (Muñoz-Lorente et al., 2018).

Sulforaphane is capable of inhibiting hTERT, the human reverse transcriptase and catalytic subunit of telomerase, through apparent alteration to chromatin structures, in cancer cells, but has no effect in healthy cells (Abbas et al., 2016; Martin et al., 2018).

There is no evidence that SFN can induce telomerase expression in healthy mammalian cells, and therefore it does not appear to be a component of the phytochemical's potential anti-ageing effects.

2.3.2.4. Stem Cell Exhaustion

Stem cell availability declines with age, preventing tissue regeneration. It is linked to telomere shortening (Flores and Blasco, 2010) and increased cellular senescence (Janzen et al.,2006).

Exhaustion occurs when the need for cell proliferation exceeds the capacity of stem cells. Excessive stem cell proliferation leads to premature ageing in flies (Rera et al., 2011) and mice (Kippin et al., 2005).

Sulforaphane is not known to directly impact Stem Cell Exhaustion. SFN may be able to influence this hallmark indirectly through its effects on cellular senescence and apoptosis.

2.3.3. Summary of Sulforaphane and the Hallmarks of Ageing

Sulforaphane directly acts on cellular signalling and epigenetics, with resulting impacts in nearly every aspect of physiological ageing. Primarily, Sulforaphane acts by modulation of cysteine residues in a variety of important protein structures. Through this it can up-regulate Nrf2, inhibit Histone Deacetylase, and modulate apoptotic signalling, among other mechanisms of action.

It is also important to note is its dual role. Sulforaphane can act as either a pro-survival or pro-apoptotic signal (Negrette-Guzman et al, 2013), depending on the mitochondrial dynamics and physiological state of the target cell. Further, this is dose-dependent, whereby extremely high doses tend towards cell death even in healthy cells. However, at ideal doses, SFN can simultaneously promote the survival and preserve the health of normal cells, while improving the clean-up of damaged/cancerous cells. Both aspects can improve the overall health of an organism as they age.

It is clear, then, that Sulforaphane is a strong candidate for survival studies, with a focus on both lifespan and physiological health with age.

2.4. Introduction

2.4.1. Previous Research in Sulforaphane and the lifespan of D. melanogaster.

As noted, there is little direct research on sulforaphane as a potential drug intervention on lifespan. Villatoro-Pulidoa et al. (2012) investigated the effect of *Eruca* extracts and SFN in *D. melanogaster*. Although they found reasonably positive results, this study was weakened by its reliance on impure extract, non-wild type genetic backgrounds, a slow cycling of food medium, and a very low number of flies (10 per treatment).

In a study of red flour beetles (*Tribolium castaneum*), a broccoli extract (1% and 5% w/w lyophilized broccoli powder added to food medium) and pure SFN (0.01% w/w, added to food medium via ethanol solvent) were able to improve beetle lifespan moderately under normal conditions, and dramatically under heat stress. This effect was abolished by knocking down Nrf2, Jnk-1 and foxo1 (Grünwald, 2013). However, this study used a relatively low number of beetles and did not test multiple concentrations of SFN.

In unpublished work at Lancaster University, Grimes (2016) observed an increase in lifespan of approximately +9.8% in *Drosophila melanogaster* fed between 0 and 2μM broccoli extract (made from capsules stated to contain 400ug SFN by their supplier, and dissolved in ethanol to appropriate concentrations). This effect was only found when flies were reared on a sugar-yeast food type. In addition, an improvement in functional health as assessed by negative geotaxis, and a decline in AGE accumulation were also found between these concentrations, though oddly the improvement in negative geotaxis results was only recorded on a different, cornmeal-based food type.

However, a qPCR experiment did not find a significant change in the expression of genes CG1707 (Glyoxalase 1) and CG4365 (Glyoxalase 2), both important genes in the antioxidant response of flies.

The broccoli extract was found to contain very little Sulforaphane compared to a sample of the pure SFN in HPLC analysis. It was important, therefore, to distinguish that SFN, and not some other compound, was responsible for the improvements seen. Further studies have also suggested that the content of broccoli extracts may include compounds that prevent proper glucoraphanin hydrolysis, limiting SFN availability (Han, 2011).

Mifsud (2017) followed-up Grimes work with a study using both the extract and pure SFN. Pure SFN of 20μg/ml produced a lifespan extension of up to 3.4%. The broccoli extract produced an improvement of 11.5% in flies raised on Oatmeal-Dextrose food at a dosage of 0.8μg/ml. 20μg/ml extract also gave an improvement to performance in negative geotaxis, with a 16.9% improvement recorded after 39 days. Pure SFN showed an improvement at both 29 and 39 days at 20μg/ml for SY flies. An AGE assay was unable to detect a significant change in AGE levels between control and dosed flies, though there was an insignificant decline overall.

2.4.2. Project Aims and Research Design

This study follows the work of the previous Lancaster students to investigate the effect of sulforaphane on the lifespan, functional health and biomarkers of ageing in *Drosophila melanogaster*. As the previous studies were of limited scope due to the time-pressure of an undergraduate research project, this represented an opportunity to run complete lifespan assays and investigate the ideal dose and effect of conditions such as diet, dose-timing, and genetic background on the effect of sulforaphane.

The study ultimately aimed to assess the potential of SFN as an anti-ageing drug intervention. To achieve this, we needed to determine its effect on lifespan in *Drosophila* models, to determine the ideal dose for maximising lifespan extension, and to determine the impact of SFN dose on the functional health of *Drosophila* with age. Further, we also looked into possible mechanisms of action by which SFN might exert these effects, concentrating on the Nrf2/CncC pathway.

2.4.3. Research Questions and Experiments

A lifespan experiment was conducted to confirm the effect of Mifsud (2017), as well as further investigating the ideal dose range. Based on those previous results we hypothesised that SFN would have a positive effect on *D. melanogaster* lifespan at a dose range close to 20µg/ml, though we could not be certain of the exact ideal dosage. We also expected to see a detrimental effect at a very high dose, such as 200µg/ml.

This was followed further lifespan experiments to investigate the impact of different genotypes, food types and the timing of the dose on the impact of SFN and investigate the lack of outcome in the first experiment.

Other experiments were run alongside these lifespan experiments to assess secondary effects of SFN dose. These included a negative geotaxis experiment to compare the decline in fly functional fitness over time with SFN dose, we expected that increasing SFN dose should ameliorate the decline of fly fitness with age, based on previous results in our lab (Mifsud, 2017; Grimes, 2016).

A reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) experiment to investigate the effect of SFN dose on the regulation of GstD1, a *Drosophila* CncC (Nrf2) target, based on the principle that if SFN was upregulating the fly CncC pathway we should see a corresponding increase in GstD1 expression in the fly tissue (Sykiotis and Bohmann, 2008).

An investigation into the absorption and retention of SFN in fly food using HPLC analysis of dosed food was carried out as part of our investigation into the lack of results in our early lifespan experiments. This was intended as speculative, and could be expanded upon further in later experiments.

Finally, an assay of protein oxidation was trialled for use in this study, to compare levels of protein carbonylation between different SFN doses in flies at different ages, but no reliable protocol was produced, and this assay was cut from the experiment.

3. Materials and Methods

3.1. Lifespan Research

3.1.1. Determining the idea dose of sulforaphane for lifespan Improvement in *D. melanogaster*.

Fly Husbandry:

Lancaster strain *Drosophila* were reared for use in the first lifespan experiment. These flies were collected by aspirator from Williamson's Park Butterfly House and a domestic glasshouse in Lancaster, UK, October 2013. Around 20 individuals were collected at each location and created a mass-bred strain. Flies from this genotype were also used in later experiments.

Flies were kept in mixed-sex bottles and allowed to mate and feed on live yeast for 1-2 days before being introduced to egg-laying cages. Cage food was prepared with 2.5% agar content sugar-yeast food (otherwise standard, see Food Preparation) and kept in the dark under humid conditions for 1 day to allow flies to lay. Eggs were then transferred to fresh bottles via egg-squirting method and raised to adulthood in standard conditions (Clancy and Kennington, 2001). This process was performed three times to produce flies reared from optimal conditions for at least three generations.

Only male flies were selected for the experiment as female flies are known to respond differently to male flies in lifespan experiments because of the effect of egg production on metabolism (Spencer et al., 2003). This was decided upon with the intent that if an effect can be found in males, females can be tested in a follow-up experiment. Using only one sex also reduced the number of variables to be controlled in each experiment, saving time and labour costs.

Flies were gassed with CO_2 for less than three minutes on average during sorting. Flies were sorted into ten groups, by food type and by drug dosage concentration. Eight vials of twenty male flies were set up for each group.

Food Preparation:

Two food types were prepared - a simple sugar-yeast (SY) recipe (standard or 'low' food) and a more complex oatmeal-molasses recipe ('high' food).

SY was prepared as 80g sugar (sucrose, granulated), 80g brewer's yeast and 16g agar per litre of water. Oatmeal-Molasses was prepared as 50g cornmeal, 50g oatmeal, 10g agar, 120g molasses and 50g brewer's yeast per litre of water. To both foods, 30ml of nipagin (Methylparaben) (10% w/v in ethanol) and 5ml of propionic acid per litre were added as a preservative.

Foods were prepared in vials with approximately 1cm depth of food. Food was prepared at least one day in advance of being used. Food was stored in the cold room at 15°C when not in use.

Dosing with SFN:

Sulforaphane (25mg/ml) (>97%, LKT Laboratories, Inc., St. Paul, MN, USA) was diluted in 100% ethanol to produce necessary concentrations. Large volumes of the correct concentrations were produced prior to the experiment and stored in the freezer at -18°C. The stability of SFN is sensitive to the solvent. SFN is more stable in organic solvents than water (Franklin et al., 2014), and though there are better solvents available, ethanol is cost effective and when stored at our low temperature, SFN should be stable for at least 2 years (caymanchem.com/pdfs/14797.pdf, 2020).

Vials were dosed with appropriate concentrations of sulforaphane in ethanol. 70µl of SFN per dose was added by pipette and given 48 hours to diffuse into the food. This was performed alongside fly vial changeover. Flies were sorted into ten groups, by food type and by drug concentration. For the first experiment these were 0, 5, 10, 20 and 200µg/ml. This gave us a broad range of doses up to that found most effective in Mifsud (2017), and one comparable to the toxic dose reported by Grimes (2016). Eight vials of twenty male flies were set up for each treatment. Flies were changed onto new food vials every 2 days.

Flies were briefly gassed with CO_2 before being tipped to new vials with fresh food. Care was taken that flies did not become stuck and dead flies were recorded before and after tipping to ensure an accurate measurement of death over time was obtained. Flies that escaped or were killed by extrinsic factors (e.g. mishandling) were recorded as censors.

3.1.2. Determining the ideal dose of sulforaphane for lifespan extension in Multiple Genotypes.

This experiment was performed with overall similar methodology to the previous lifespan experiment. Two genotypes were used, White Dahomey (wDah) (Grandison et al., 2009), a wild-caught mass bred strain made white-eyed, and Zim53 (Sengwa Wildlife Preserve, Zimbabwe, 1990 (Ballard, 2000)), a wild-caught mass bred strain. All populations were reared in the same manner as the first experiment.

In place of the oatmeal-molasses food, a modified sugar-yeast food using 120g yeast and 80g sugar per litre water made up the alternative “higher” content food type. The preparation was otherwise identical to the standard SY food type.

Flies were given only three drug dosages – 50, 20 and 0µg/ml per each food and genotype. These dosages were prepared in the same manner as in the previous experiment. A fresh vial of sulforaphane from the same source was used to prepare the doses.

3.1.3. Can sulforaphane administration at different time-points in a fly’s life confer an effect on longevity?

This experiment was carried out by Lisa Butler (see Acknowledgements) and analysed by me. Deaths were scored and vials changed Monday, Wednesday, Friday. Lancaster strain flies and White Dahomey flies were selected as genotypes.

Flies were mated for 4 days at 17°C before being separated by sex 2 days before the first count. 5 vials of 20 male flies each were set per treatment.

A cornmeal-based food type was used as the higher-content food. This was prepared as 132g sugar, 33g yeast, 57g cornmeal, and 10g agar, with 30ml nipagin and 5ml propionic acid added per litre food.

The dosing procedure for this experiment differed from our standard model. All fly groups were divided into two 'time' groups. Early groups were given sulforaphane between day 9 and day 42, late groups from day 42 until the conclusion of the experiment. Concentrations of sulforaphane were given at 0, 100 and 400µg/ml. While flies were not receiving sulforaphane, a pure ethanol dose, as in the 0µg/ml controls, was applied to their food.

3.2. Negative Geotaxis Assay

The negative geotaxis assay to determine the impact of Sulforaphane on *Drosophila* physical performance was performed using distance climbed in 15 seconds in 40cm x 1cm perspex tubes using photographs. The assay was performed concurrently with the second lifespan experiment, starting on days 27, 31, 35 and 39 (after eclosion). Four vials from each group were used consistently across negative geotaxis days, giving ~80 flies per experimental group (exact numbers vary slightly due to deaths and censors from the lifespan experiment).

Each day flies were taken from the same vials from each of the experimental groups in the corresponding lifespan experiment, and one vial was loaded into each of the 12 geotaxis tubes at a time. Flies were allowed two minutes to adjust to the change, then knocked down with manual force. Photos were taken 15 seconds after force was applied to capture the height reached. This was repeated three times with each set of flies to obtain triplicate photographs, and flies were then returned to their original vials, before the next set of vials were loaded.

Analysis of the photographs was performed using ImageJ with a custom script. The distances flies travelled were measured in pixels from a defined base of each tube, and then these values were averaged across all flies from each treatment group within each day. Zim53 flies were excluded due to their poor lifespans resulting in low numbers early into the experiment.

3.3. High-Performance Liquid Chromatography

Preparation:

Samples of food prepared for HPLC analysis were treated exactly as food vials had been during the second lifespan experiment. Only standard (80g sugar, 80g yeast) SY food was used in this experiment. Tested doses were 100, 200, 500 and 5000µg/ml. After 48hrs scrapings were taken from the surface of the food using a spatula and added to pre-weighed Eppendorf tubes. These scrapings were ~2mm deep. The Eppendorf tubes were then re-weighed to calculate the weight of the scrapings.

500µl ethanol was added to each tube and then left overnight for the ethanol to leach sulforaphane from the solid food. On the following day, tubes were spun at 13,000rpm for 5 minutes and 200µl of supernatant was extracted for use in the HPLC. These samples of supernatant were stored at -70°C before HPLC analysis.

Standard controls were produced by taking 500ml samples of Sulforaphane diluted in ethanol.

HPLC Procedure:

10µl of the supernatant per sample was injected into a Phenomenex Gemini 5u C18 110A column, 250 (length) x 4.6 mm (diameter). The mobile phase was 30% Acetonitrile, isocratic at 0.6ml/min. Flow rate was 0.6ml/min. The run time was 30 mins with peak of interest ~10 mins. The column temperature was maintained at 36C. The absorbance of the peaks at 202nm was monitored using a Dionex ICS-3000 with Ultimate UV detector. 202nm was selected based on the findings of Campas-Baypoli et al. (2010) in their validation of HPLC to detect SFN in broccoli by-products.

Due to a limited supply of SFN standards and the speculative nature of the work, we did not carry out a full validation of this experiment, though a standard curve demonstrating linearity over four concentrations (short of the five which is the minimum standard for typical validation) was produced.

Graphical data was produced using MS Excel (2010, Microsoft) to produce the standard curve and plot estimates of Sulforaphane concentration in food samples based on average peak area.

Using the calculated concentrations produced by the analysis, the approximate dose ultimately available to the flies was calculated. It is difficult to determine the amount a fly consumes in a day, as it varies with sex (Wong et al., 2009, Lushchak et al., 2011) and nutrient content of food (Wong et al., 2009), however we estimated that a male fly consumes ~36µg/day (extrapolated from Wong et al., 2009) of our recipe and weighs ~800µg (Ong et al., 2015). After converting concentration to the consumed amount of food per fly, the dose in µg/mg body mass can be calculated.

Due to the qualitative nature of the study and the small number of samples, statistical analysis was not performed.

3.4. Quantitative Reverse-Transcription Polymerase Chain Reaction

Fly Preparation:

White Dahomey flies were raised separately to the populations used in the lifespan experiments. Fly populations were raised and maintained on standard sugar-yeast mix food. Flies received SFN doses after ~20 days, twice over two weeks, at doses of 0, 100, 400 and 800µg/ml. Three days after the second dosage flies were transferred to non-dosed food.

Single flies (n = 4/treatment) were collected two days later and flash-frozen with liquid nitrogen. Collected flies were stored at -80°C until use.

RNA Extraction:

RNA Extraction was performed using Norgen Biotek Total RNA Purification Kits. Single flies were isolated and placed into 1.7mm zirconium bead tubes (OPS Diagnostics) and homogenised in 600µL Lysis buffer (Norgen Biotek, Ontario) at 6m/s for 7 seconds. Tubes were set on ice for 5mins, then centrifuged at 12000rpm for 2 min and 450µl lysate supernatant was extracted.

Equal volume 70% EtOH was added to extracted lysate and briefly vortexed. 450µl of this solution was added to the column of collection tubes (from kit) and centrifuged 1min, 10,000rpm. Flowthrough was discarded and the processes repeated once. 400µl of wash solution (Norgen Biotek, Ontario) was applied and the column centrifuged 1 min at 10,000rpm and flow-through discarded. Wash was repeated twice before column was centrifuged for 1 min/10,000rpm to dry the resin prior to elution. 10µl of elution solution was applied to the centre of the resin and the column placed into an RNA-free 1.7ml elution tube. The tube was incubated at room temperature for 15mins before being centrifuged for 2 mins at 2000rpm and 1 min at 14000rpm. The columns were discarded, and the final elute was collected and stored at -18°C.

1µl of extracted RNA was quantified using a nandrop 2000c spectrophotometer (Thermo Fisher Scientific).

Reverse Transcription:

A master mix containing 2µl of 5x Prime Script buffer, 0.5µl Prime Script RT Enzyme mix, 0.5µl 50uM oligo-dT primers and 0.5µl 100µM random hexamers per sample was prepared on ice. 3.5µl Master Mix was added to 6.5µl RNA and incubated at 37°C for 15 min, 85°C for 5 mins and held at 4°C to produce cDNA transcripts.

Quantitative Polymerase Chain Reaction

Two master mixes were created for each gene (household EF1g and test gene GstD1). These were composed (per sample) of 5µl Ssoadvanced*TM SYBR Green Supermix (Biorad, US), 3.4µl water and 0.3µl each of the 10µM Left and Right primers for their respective gene.

The samples were added in triplicate into a 96-well plate using 9µL of each master mix and 1µL of RNA sample and then the plate was centrifuged. The qPCR was run on a CFX96 Thermal Cycler (Bio-Rad, Hertfordshire, UK) using the program: 95°C for 30 seconds, 39 cycles of 95°C for 15seconds and 15 seconds at 58°C.

3.5. Statistical Analysis

Lifespan Analysis:

Statistical analysis of Lifespan data was carried out using SPSS (Version 24.0, IBM Corp.). Survival estimates were produced using Kaplan-Meier survival curves with post-hoc Tarone Ware pairwise comparison of strata, cases weighted by deaths.

A model fitting with Cox proportional hazards regression was employed to analyse interaction effects between covariates (food, genotype, and dose timing where applicable).

Negative Geotaxis Analysis:

Negative geotaxis analysis was carried out in SPSS using a Repeated Measures Mixed Models (ANOVA) analysis comparing factors day, food type and dosage, with triplicates nested within biological replicate nested within day X food X dose. Means for display in results are estimated marginal means.

QPCR:

QPCR results were analysed using REST2009 software (Pfaffl et al., 2001). This software performs complex hypothesis testing using a random reallocation technique (Pfaffl et al., 2002), which can take into account reaction efficiency and normalization of reference genes, which create a challenge for normal statistical tests (Pfaffl et al., 2002, gene-quantification.de/REST_2009_Software_User_Guide.pdf, 2020).

3.5. Protein Oxidation Assay

This experiment was intended to work as an investigation into the effect of Sulforaphane on protein oxidation by measuring carbonyl groups on fly proteins.

The assay was performed based on the methodology of Wehr et al. (2012). The principle of this method is that fly protein can be solubilized and bound to a PVDF membrane using a DMSO-based solution, the carbonyl groups derivatized by dinitrophenolhydrazine, then anti-DNPH antibodies are hybridized followed by a fluorescence-labelled 2^{o}. Typically, 4 flies were tested at a time.

Individual flies were homogenised in 30µl of a solution of 92.5% Dimethyl Sulfoxide (DMSO) and 7.5% dH_2O, acidified with 0.5% Trifluoroacetic acid (TFA), then centrifuged at 13000G for 5 minutes and 2µl of the supernatant was extracted for the experiment while 18µl was set aside for an accompanying BCA Protein Assay.

Oxidised BSA standards were produced by reacting 40mg/ml BSA solution (in PBS) with 50mM Hypochloride solution for 24hrs at 37°C. 50µl of derivatizing solution (DS), a second solution of homogenising solution and Dinitrophenol (DNPH) balanced to give a final molar concentration of 20mM DNPH, was added to each standard sample. All samples were then wrapped in foil and incubated for 15mins at 200rpm (rocker) to allow complete derivatization of carbonyl groups. An Immoblin FL PVDF membrane was spotted with 1µl of each standard sample in quadruplicate.

2µl fly homogenate was mixed with 28µl derivatizing solution and then diluted further in DS to a 1 in 8 dilution. Fly samples were then incubated in foil for 15mins at 200rpm. Fly samples were then applied to the same membrane as the standards, 1µl in quadruplicate.

The membrane was left to dry for 30 minutes, then washed twice with acetic acid (2 mins per wash) and placed in water to equilibrate for 5 minutes. Membrane was then immersed in a solution of 5% w/v milk-TBST (skimmed milk powder, 1x TBS, 0.1% Tween 80) buffer for 1 hour. The buffer solution was then replaced with another 5% w/v milk-TBST buffer containing 1:10,000 dilution of 1mg/ml goat anti-DNPH primary antibody (A150-117A, Bethyl Laboratories, Montgomery, USA) for 2 hours. The membrane was then rinsed three times with PBST (1x PBS, 0.1% Tween 80).

Membrane was then immersed in a solution of Milk-TBST containing the secondary 1:2,500 of 0.5 mg/ml dilution donkey anti-Goat antibody conjugated to Cy3 (ab6949, Abcam, Cambridge, UK), while wrapped in foil, for 1 hour. The membrane was then rinsed three times with PBST and equilibrated in water for 5 minutes.

Analysis was performed by scanning on a Typhoon FLA 9500 (GE Healthcare Life Sciences), typically at 600V and 25µm to produce fluorescence values for each sample. Standard samples were used to construct a standard curve.

Total protein measurement was obtained by testing small (1µl) samples of protein from the saved 18µl homogenate with the nanodrop 2000c spectrophotometer (Thermo Fisher Scientific).

Graphical data was produced using MS Excel. No statistical analysis was carried out as the method never advanced to the point of producing a viable protocol that could be used to test fly samples with confidence in obtained results.

This experiment was cut from the project due to a lack of consistent results in trial runs. No appropriate standard curve was ever produced, and large degrees of triplicate variation made results unreliable. Multiple attempts were made with numerous changes to the procedure, but ultimately Sulforaphane-treated flies were never tested.

4. Results

To assess the effect of sulforaphane on ageing, a study was undertaken consisting of several lifespan experiments using increasing doses of sulforaphane in *Drosophila melanogaster* food, as well as tandem investigations into the functional health of flies and biological markers of ageing.

4.1. Determining the ideal dose of sulforaphane for lifespan improvement in *D. melanogaster*.

Preliminary evidence that sulforaphane might convey a lifespan-extending effect in flies had been found in the work of Grimes and Mifsud (2016 and 2017, unpublished). From this work it seemed an ideal dosage to produce lifespan extension might be found around 20µg/ml, and a detrimental dose above 200µg/ml.

This experiment sought to verify that finding with a complete lifespan experiment testing the effect of multiple dosages of sulforaphane on fly longevity. Male Lancaster flies were treated with a range of sulforaphane concentrations (0, 10, 20, 50 and 200ug/ml) applied to their food throughout the whole of their natural lifespan beginning 4 days after eclosion. Two food types were used, a simple sugar-yeast mix and a complex oatmeal-molasses recipe. The findings of this experiment are summarised in **Figure 5**.

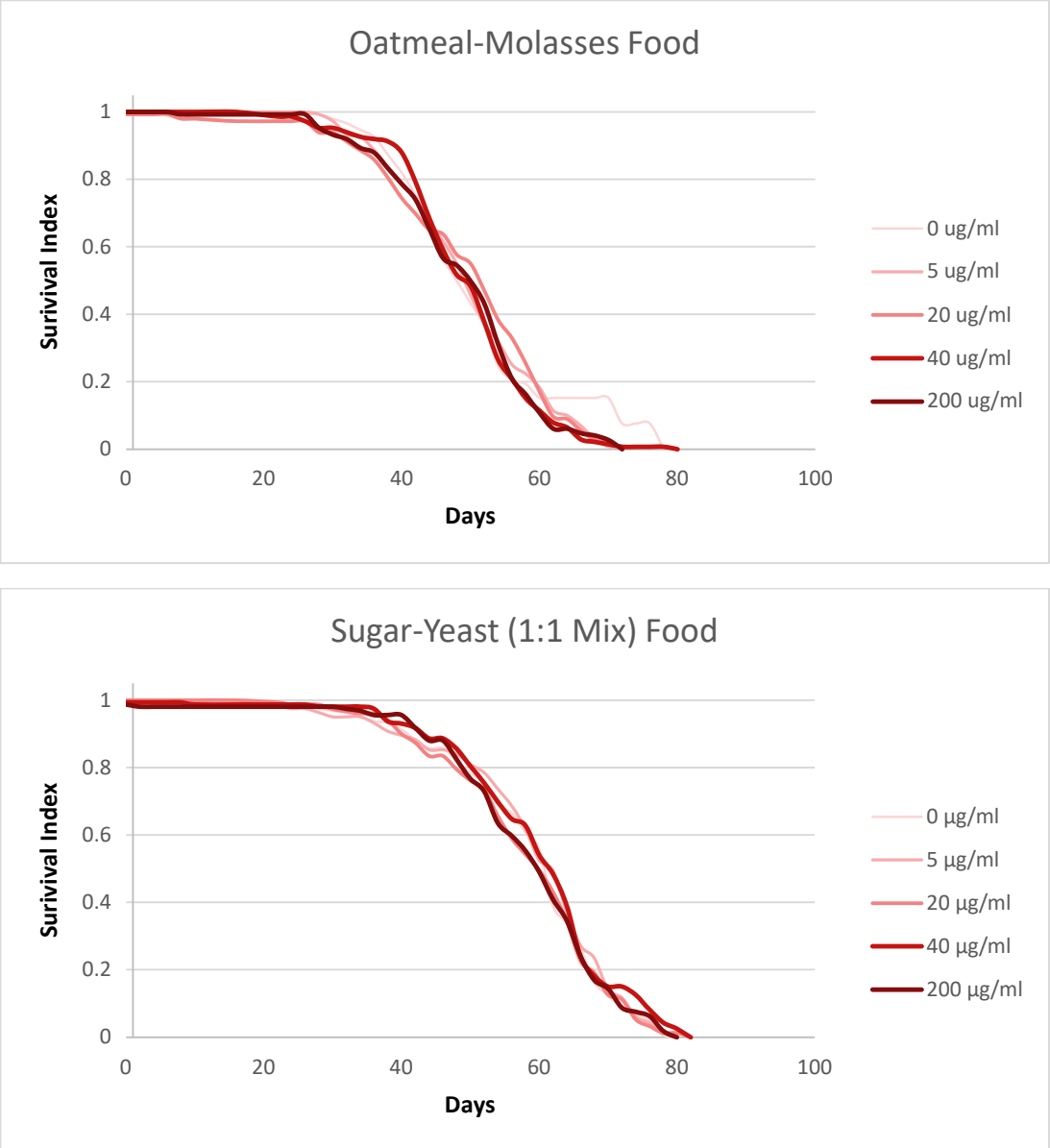


Figure 5: Effect of SFN dosage on Lancaster-strain male fly lifespan (n=160 per treatment) in each food-type. Concentrations indicate SFN dose added to food. No significant differences between dosages were detected (all P > 0.05) by Kaplan-Meier lifespan analysis with tarone-ware pairwise analysis.

No expected consistent improvement can be seen in any dose in the lifespan curves, and besides the expected effect of food type, no significant change in lifespan was found between any treatment groups. Cox-Regression analysis confirmed that there was no significant effect of dosage (P = 0.724) or interaction between dosage and food type (P = 0.650) on risk of death.

The curve for oat 0 (oatmeal control) appears erratic towards the end due to an incident involving a poorly-made batch of food that compromised this result around Day 60 of the study, leading to a large censorsing of flies in this group. Adjusting the data to replace the 0µg/ml with the 5µg/ml as control dosage for both food types did

not produce qualitatively different results, so it is unlikely that this could explain the lack of findings.

4.2. Determining the ideal dose of sulforaphane for lifespan extension in multiple genotypes.

In a revision of the first lifespan experiment, White Dahomey and Zim53 flies were used instead of just the Lancaster strain. Both are well-established laboratory strains, so the possibility of already-active stress response mechanisms is lowered. Oatmeal-Molasses Food was excluded due to the incident involving the poorly-made batch in the previous experiment. An altered Sugar-Yeast food type that contained 1.5x the yeast content of the standard recipe was substituted in its place. The SFN dosage range was also narrowed to between 0 and 50µg/ml, since we didn't see a toxic dose at 200µg/ml. Flies were kept on non-dosed food until 17 days after eclosion. The findings of the experiment are summarised in **Figure 6**.

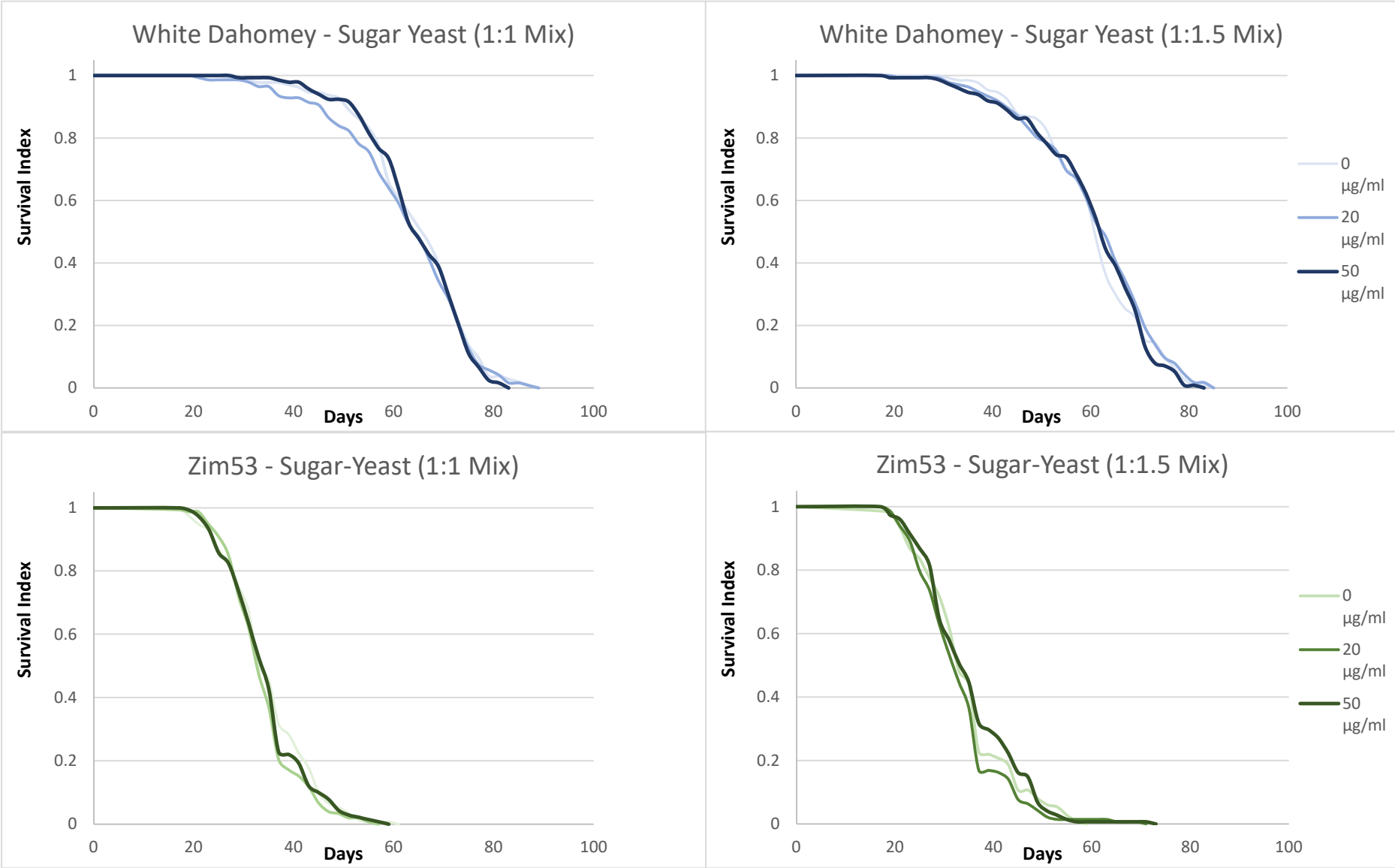

White Dahomey - Sugar Yeast (1:1 Mix)
Survival Index
Days
White Dahomey - Sugar Yeast (1:1.5 Mix)
Survival Index
Days
0 µg/ml
20 µg/ml
50 µg/ml
Zim53 - Sugar-Yeast (1:1 Mix)
Survival Index
Days
Zim53 - Sugar-Yeast (1:1.5 Mix)
Survival Index
Days
0 µg/ml
20 µg/ml
50 µg/ml

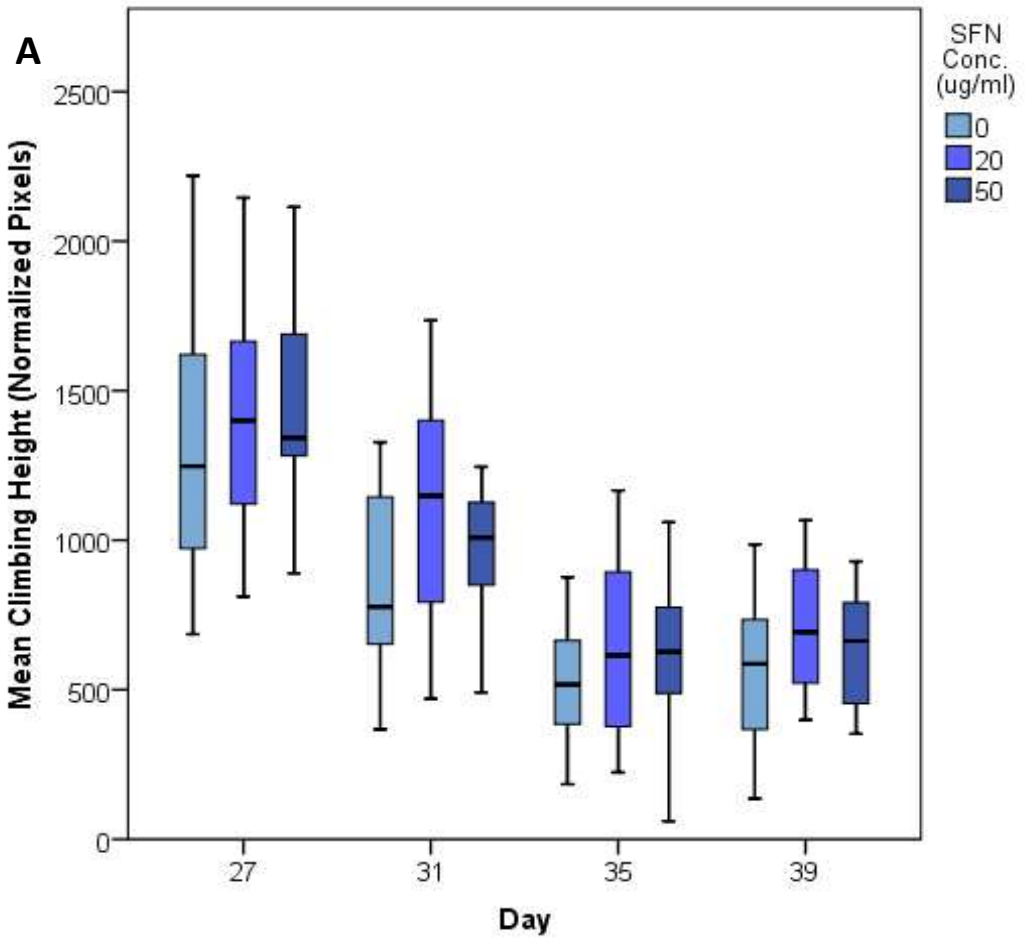
Figure 6: Effect of SFN dosage on White Dahomey and Zim53 fly lifespan (n = 160 per treatment). Concentrations indicate SFN dose added to food. No significant differences between dosages were detected (all P > 0.05) by Kaplan-Meier lifespan analysis with Tarone-Ware pairwise analysis.

Expected differences were observed for genotype and food type. Zim53 survived less well than White Dahomey. No significant difference was observed between Sulforaphane dosages within the tested groups. No significant interactions were found between SFN dosage and any other parameter by Cox-Regression.

4.3. Investigating the effect of sulforaphane on the performance of *D. melanogaster* with age.

Negative geotaxis is a well-established measure of *D. melanogaster* performance that has been demonstrated to decline with age (Feany and Bender, 2000, LeBourg and Lints, 1992). This study employed a method of assessing negative geotaxis using a measure of climbing height achieved in 15 seconds.

Flies from the same vials as the survival experiment described previously were removed and tested for negative geotaxis to ascertain whether sulforaphane administration affected their performance across lifespan (**Figure 5**).



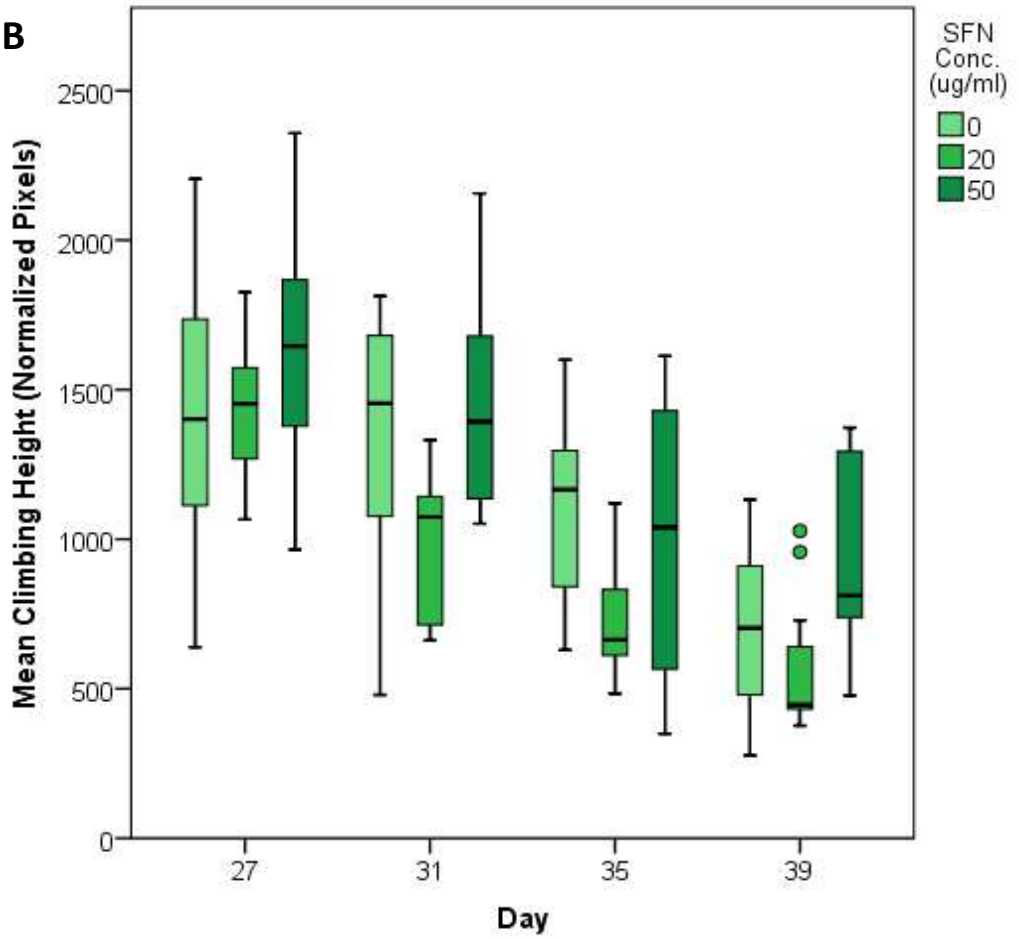


Figure 7: The effect of Sulforaphane dosage on the performance of male WDah flies by food type over time. Based on estimated least squares marginal means. (n=~80 per treatment, subject to losses in lifespan experiment) A; Low/Standard Sugar-Yeast mix (1:1 sugar to yeast ratio), B; High Sugar-Yeast mix (1:1.5 sugar to yeast ratio). Pixel distances are normalized by position of tube base. See **Table 1** for detailed analysis.

Low yeast results show high degrees of overlapping range between doses, with a general decline over time but no clear difference by dose. High yeast results are more varied though still show significant overlap and skewed medians.

Repeated Measures Mixed-Model ANOVA found no significant effect of dosage on fly performance. See **Table 1** for full interaction effects.

Table 1: Repeated Measures Mixed Model ANOVA Tests of Fixed Effects. Food-type and dose are fixed effects. Random effects were triplicates nested within biological replicates, and biological replicates within food*dosage. (n=~80 per treatment, subject to losses in lifespan experiment).

Source	Numerator df	Denominator df	F	P Value
Intercept	1	29.676	678.773	<0.001
Day	3	72.736	33.75	<0.001
food	1	76.019	13.312	<0.001
dosage	2	76.019	2.135	0.125
Day * food	3	72.736	1.199	0.316
Day * dosage	6	72.736	0.25	0.958
food * dosage	2	76.019	4.562	0.013

The expected decline in climbing height with time was observed across all treatments. No significant effect of SFN dosage was found across the study or at any individual time point. No change in the decline over time was observed with dosage (no significant interaction between day and dosage).

High-yeast food showed an overall significant improvement of ~19% mean height. However, this was not seen in the first and final time points (Day 27 and Day 39), where the effect of food was non-significant (P = 0.193 and P = 0.325). A weak interaction between food and dosage was observed, with 20µg/ml showing a small decline on high-yeast food, followed by a small improvement at 50µg/ml. These changes were still overall non-significant at P <0.05.

4.4. Using High Performance Liquid Chromatography to measure the content of sulforaphane present in dosed food.

Due to the lack of findings in both lifespan experiments and negative geotaxis, an investigation took place into what could have caused the lack of effect despite previous findings in Grimes (2016) and Mifsud (2017). As SFN is known to degrade over time, especially when stored at room temperature and in well-lit conditions (Franklin et al., 2014), and we did not know how well it would be absorbed onto the food.

In a speculative study using High Performance Liquid Chromatography, we measured the concentration of Sulforaphane present in food samples 24 hours after dosing. Scrapings were taken from food samples and SFN was leached from them by ethanol immersion at 4^{o}C over an additional 24 hours. The low temperature, though it may reduce the rate of leaching, was used to reduce the degradation of SFN within the samples. The size of scraping, concentration of SFN used and number of days between administration and extraction of food were varied during the experiment. To measure these results, we also generated a standard curve from samples of pure SFN diluted to a linear range of concentrations in ethanol (**Figure 8**).

Absorbance of SFN was measured at 202nm, this value is based on the work of Campas-Baypoli et al. (2008), which identified this as the ideal wavelength for detecting SFN in *Brassica* by-products.

A consistent peak for Sulforaphane was identified in the standard samples at around 10.15 minutes retention time. This peak was also seen in all SFN-dosed food samples, though consistently at lower heights (mAU), indicating a smaller presence, than at equivalent standard samples.

Calculations were performed based on the obtained concentration of the samples from HPLC to estimate the concentration of Sulforaphane that flies were receiving, factoring in the mass of individual flies and estimated consumption of food per day. Estimations of dosage were then given in mg/kg (**Table 2**).

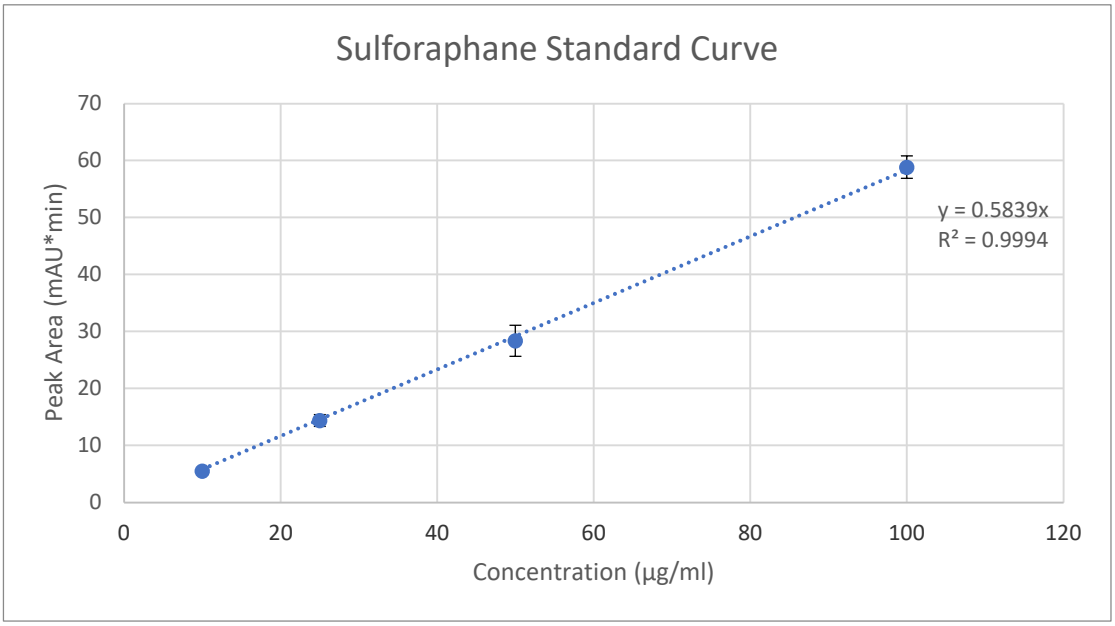


Figure 8: Standard curve produced from HPLC assay of samples of SFN in ethanol at known concentrations (10, 25, 50 and 100µg/ml).

Table 2: Concentration of Sulforaphane administered into food, calculated concentration of sulforaphane detected in food samples, and estimated dose received by the flies. Size of scraping indicates whether a scraping was taken from the centre to the edge (radius) or from edge to edge (diameter) of the food. One tested concentration duplicate (200µg/ml after 2 days, radius scraping) was lost during the work. Estimation of final dose is based on the formula "(((([concentration]/2)/[weight of food sample])/1000)*36)*0.8 = Dose in ug/mg body mass, then converted to mg/kg body mass" see methods for justifications.

Sample Initial Concentration (µg/ml SFN)	**Days Given for SFN to Soak In**	**Size of Scraping**	**Obtained Concentration (µg/ml SFN)**	**Weight of Food (mg)**	***Estimated Dosage (mg/kg)***
100	1	Radius	0.156	54.6	0.041
100	1	Radius	0.168	63.4	0.038
200	1	Radius	0.212	74.5	0.041
200	1	Radius	0.237	66.8	0.051
500	1	Radius	0.587	72.4	0.117
500	1	Radius	1.287	109.8	0.169

100	2	Radius	0.153	79.7	0.028
100	2	Radius	0.167	89.8	0.027
200	2	Radius	0.217	103.1	0.030
500	2	Radius	0.642	129.6	0.071
500	2	Radius	0.609	92.1	0.095
100	1	Diameter	0.295	244.0	0.017
100	1	Diameter	0.406	270.1	0.027
200	1	Diameter	0.441	241.5	0.026
200	1	Diameter	0.380	248.7	0.022
500	1	Diameter	1.013	352.8	0.041
500	1	Diameter	0.994	375.8	0.038
5000	2	Radius	4.069	97.78	0.599
5000	2	Diameter	8.519	258.0	0.476

SFN appears near undetectable at the lowest tested concentrations. The bioactive dosage determined in rat models is 0.1 – 0.5 mg/kg (Hu et al., 2004), and of our tested concentrations only doses of 500µg/ml (1-day, diameter scraping) administered to the food or more give a final dose that meets the minimum dose of this range. Concentrations generally appear to be lower after 2 days and in larger scrapings.

One data point (200µg/ml, 2 days, radius scraping) was lost due to mishandling.

4.5. Can sulforaphane administration at different time-points in a fly's life extend lifespan?

The HPLC results suggested sulforaphane may require a higher dosage to be bioactive in flies. Furthermore, given Grimes and Mifsud both carried out incomplete lifespan experiments, Sulforaphane may confer a different effect on lifespan depending on whether it is administered early or late in life.

A final lifespan experiment was conducted to test both hypotheses – much higher doses of Sulforaphane were administered, and the treatments were divided between two treatment times. This work was conducted by Lisa Butler (Acknowledgements) and analysed by me. 'High' food type was again changed to a cornmeal-based recipe that is closer to (though not identical) that used by Grimes and Mifsud. Results are summarised in **Figure 9**.

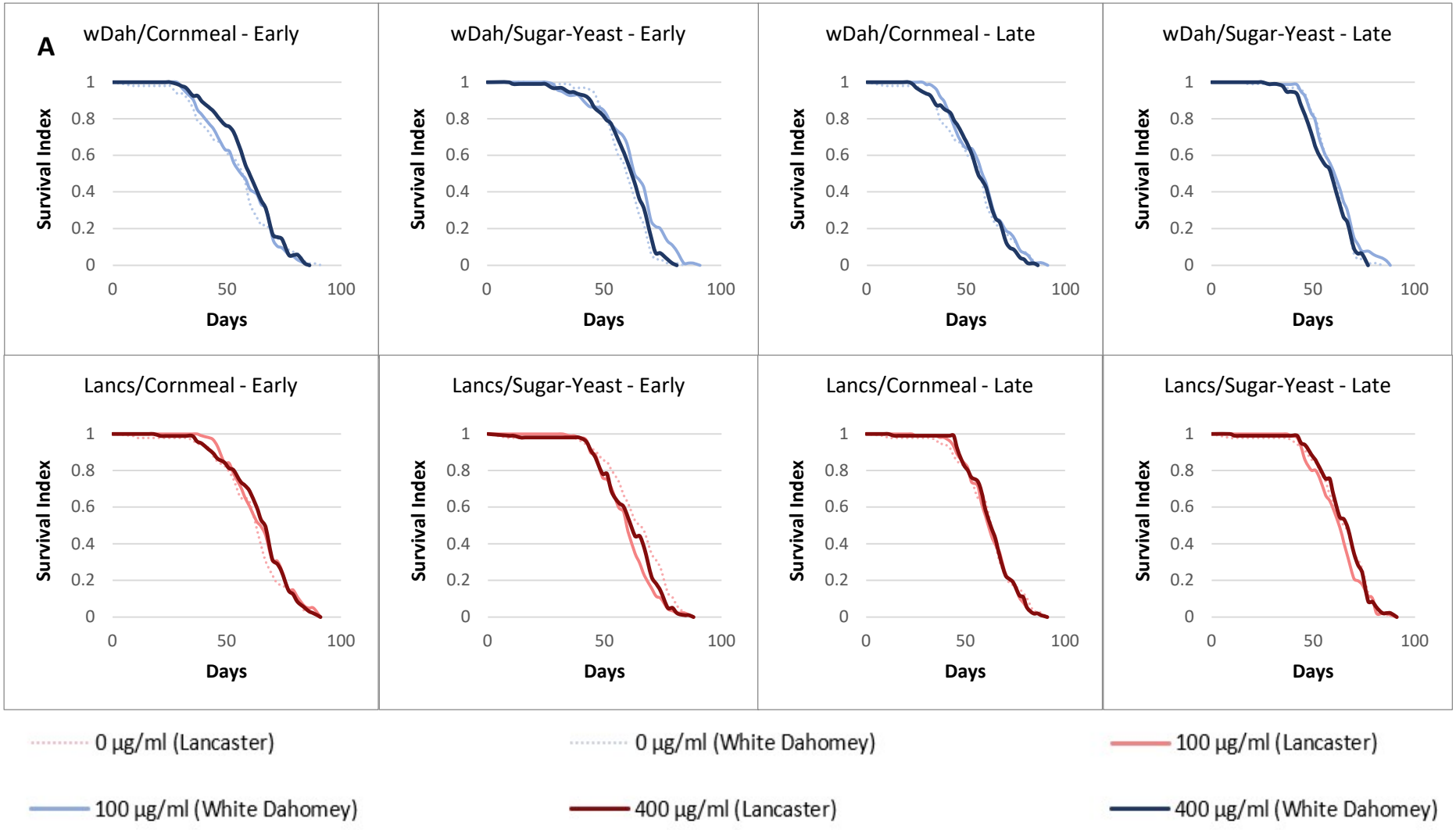

A
wDah/Cornmeal - Early
wDah/Sugar-Yeast - Early
wDah/Cornmeal - Late
wDah/Sugar-Yeast - Late
Lancs/Cornmeal - Early
Lancs/Sugar-Yeast - Early
Lancs/Cornmeal - Late
Lancs/Sugar-Yeast - Late
Survival Index
Days
0
0.2
0.4
0.6
0.8
1
0
50
100
0 µg/ml (Lancaster)
0 µg/ml (White Dahomey)
100 µg/ml (Lancaster)
100 µg/ml (White Dahomey)
400 µg/ml (Lancaster)
400 µg/ml (White Dahomey)

Figure 9: Effect of Sulforaphane on *Drosophila* lifespan when administered at different time points. Lifespan charts split according to factors genotype, food-type and time administered. Concentration indicates Sulforaphane dose added to food. Genotypes: Lancaster and White Dahomey. Food Types: Sugar-Yeast (SY) and Cornmeal. Time Administered; Early; Dosage given between 10 and 45 days old, Late; Dosage given after 45 days old. (n = 100) for all treatments.

By Kaplan-Meier survival analysis with Tarone-Ware pairwise comparison, a significant effect was found between 0 and 100µg/ml SFN in both Lancaster and WDah flies on sugar-yeast food in the early cohort. WDah shows an improvement of 5.74% (P= 0.003), while Lancaster shows a decline of 6.34% (P = 0.003). Significance is lost between both 0 and 400µg/ml, and 0 and 100µg/ml comparisons.

Late flies of both genotypes showed closest significance improvement on sugar-yeast food for 0 vs 100µg/ml (Lancs P = 0.09, wDah P = 0.096).

No significant change in lifespan is recorded for either genotype under any dosage on cornmeal food, however wDah shows an improvement of ~9% with P = 0.067, which is close to significance.

Table 3: Proportional Hazards analysis of data with Cox-Regression significance of main and interaction effects. (n = 100) for all treatments.

Hazard Source	P Value
food	0.6152
genotype	<.0001*
time	0.9830
dosage	0.7649
food*genotype	0.4782
food*time	0.9559
food*dosage	0.6670
genotype*time	0.9671
genotype*dosage	0.0145*
time*dosage	0.7024
food*genotype*time	0.0214*
genotype*time*dosage	0.4537
food*genotype*dosage	0.0127*
food*time*dosage	0.5120

Proportional Hazards analysis with cox regression (**Table 3**) showed that Lancaster has a significantly longer average lifespan of approximately 5 days across all other factors and carrying a significantly smaller hazard of death (P < .0001) than White Dahomey. There were modestly significant interactions between genotype and dosage (P = 0.0145), genotype, food and time of dosing (P = 0.0214), and food, genotype and dosage (P = 0.0127). This is consistent with the visible trend of Lancaster genotype showing less change with treatment in all lifespan curves, however with so many interactions proportional hazards analysis can be less accurate, and there is overall no significant effect of any main factor aside from genotype.

Excluding the possibly toxic 400µg/ml dose and all ‘late’ results from proportional hazards analysis results in similar significant interactions in genotype, genotype*dosage and food*genotype*dosage. Notably, the latter becomes much more significant (P = 0.0015).

The lack of impact of food type is unexpected, given previous lifespan results. There was also no significant change to hazard with dosage. The timing of dosage had almost no effect at all (P = 0.9830).

4.6. Effect of sulforaphane on the regulation of GstD1, a known target of *Drosophila* Nrf2.

Sulforaphane is known to up-regulate the Nrf2 detoxification pathway in humans, mice and other species. The Nrf2 pathway regulates a variety of genes associated with Phase II detoxification, the glutathione response and NADPH generating enzymes. In *Drosophila*, the Nrf2 homologue isoform CncC has been demonstrated to regulate many of the same genes, including GstD1 (Sykiotis and Bohmann, 2009).

To help determine if Sulforaphane can regulate this pathway in the same way it regulates Nrf2 in mammals, a Reverse-Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) experiment was designed to test the expression of GstD1 relative to housekeeping gene eF1γ, in flies with increasing SFN dose. **Table 4** shows the results of this experiment.

Table 4: Change in fold expression of GstD1 with SFN dose with eF1γ reference gene, determined by RT-qPCR (n = 4). Fold expression is relative to control (0µg/ml) expression of 1. Statistical test performed by REST2009 software, via randomization test.

Comparison of SFN Concentration (µg/ml)	GstD1 Relative Expression	95% C.I.	P-Value
0 vs 100	0.413	0.033 - 3.035	0.462
0 vs 400	0.629	0.040 - 5.200	0.652
0 vs 800	0.32	0.048 - 1.196	0.305

One control sample showed anomalous expression of the household gene, eF1γ, for unknown reasons (>5 cycles higher than other controls). **Table 5** shows the effect of excluding this anomaly from the results.

Table 5: Change in fold expression of GstD1 with SFN dose with eF1γ reference gene, determined by RT-qPCR (n = 4). Control outlier has been excluded. Fold expression is relative to control (0µg/ml) expression of 1. Statistical test performed by REST2009 software, via randomization test.

Comparison of SFN Concentration (µg/ml)	GstD1 Relative Expression	95% C.I.	P-Value
0 vs 100	1.043	0.365 - 3.095	0.865
0 vs 400	1.587	0.433 - 5.251	0.553
0 vs 800	0.808	0.539 - 1.198	0.594

No significant change in the relative fold expression of GstD1 compared to control (0µg/ml) dose SFN was detected at any SFN concentration. Eliminating the anomalous control sample did not produce significant changes to the results. Melt curves were singular at 79.5°C for GstD1 and 80°C for eF1γ.

This study was attempted twice with no significant result either time.

4.7. Investigation into the effect of Sulforaphane on Protein Oxidation with age in *D. melanogaster*.

Measuring protein carbonyl levels in whole fly samples:

In order to measure protein carbonyl levels in small volumes of material, such as that contained in a single fly, a protocol was developed based on the work of Wehr et al. (2012). This method, based on the reaction of Dinitrophenol (DNPH) on carbonyl groups to derivatize protein and allow immunoassay, is supposed to provide a detection limit of 0.19 ± 0.04 pmol of carbonyl and be sensitive enough to detect carbonylation with a minimum of 60ng protein.

Figure 10 shows the results of a trial run according to our final standard methodology. **Figure 11** shows the results of an alternative method.

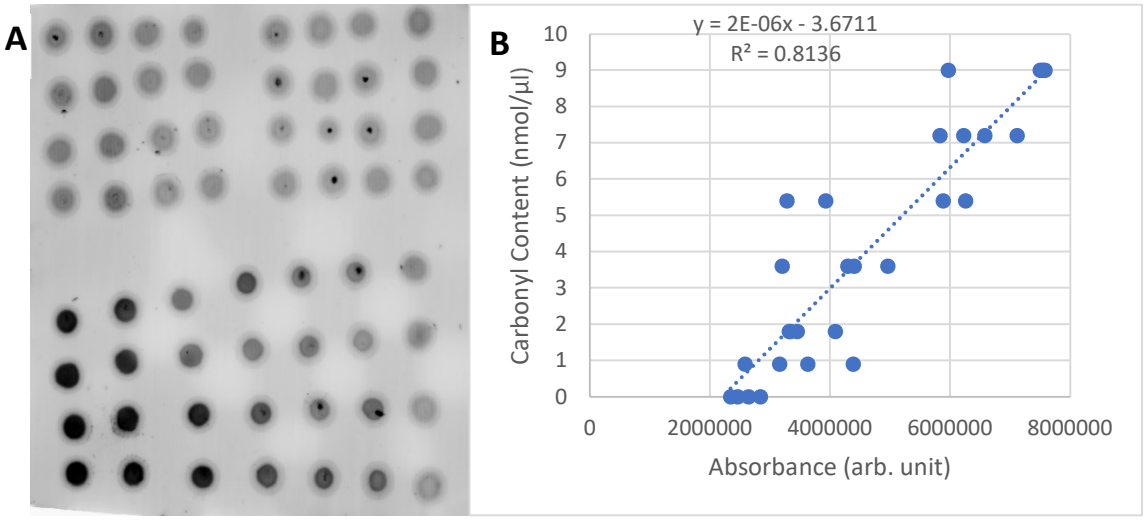


Figure 10: A; PVDF Membrane showing protein carbonyl assay results (8 December 2017). Top Left; Four columns show fly samples at a 1in8 dilution, Top Right; Fly samples repeated at a 1in16 dilution, Bottom; 6 Standard Oxidised BSA samples ranging from to 9nmol/ul to 0.9nmol/ul, with a seventh negative control column. B; Standard curve produced from analysis of A.

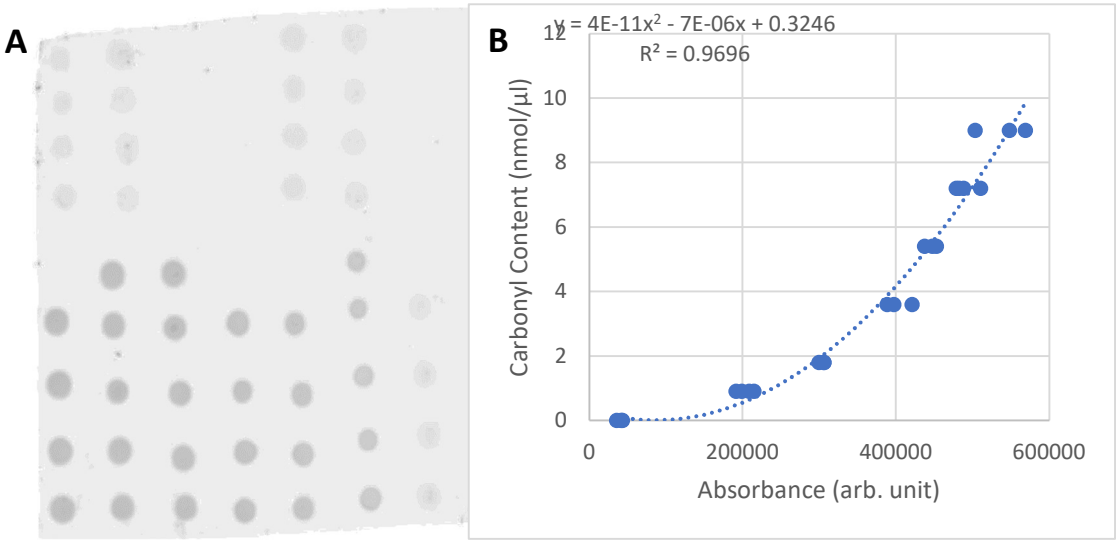


Figure 11: A; PVDF Membrane showing protein carbonyl assay results (27 September 2017). Top Left; Week-old fly samples at 1 in 8 and 1 in 16 dilutions. Top Right; 45-day old fly at 1 in 8 and 1 in 16 dilutions Bottom; 6 Standard Oxidised BSA samples ranging from to 9nmol/µl to 0.9nmol/µl, with a seventh negative control column. B; Standard curve produced from analysis of A.

In the alternative method, standard samples and fly samples were derivatized together at the same time, and fly samples were diluted after derivatization took place, rather than before. Both 1 in 8 and 1 in 16 dilutions were tested for fly samples. Finally, significantly larger amounts of antibody were employed (Primary 7µl in 13ml buffer, Secondary 27µL in 13ml buffer).

While in the 27th September experiment, the standard curve is acceptable, this was not obtained in repeat attempts. The fly samples in this experiment were too light to be placed on the curve and no useful analysis could take place. Further attempts using the same protocol, but different fly samples resulted in a significantly different curve ($r^2 = 0.9064$).

In the 8th December experiment the fly samples fit into the curve, but the curve is not accurate enough to give a reliable estimate of the carbonylation level. Again, repeat attempts failed to produce similar curves without changing method. It should be noted that in this second experiment several spots appear to have dark marks in the centre, which may indicate places where the pipette tip touched the membrane, something that is difficult to control in this procedure.

Over the course of the study, multiple attempts were made to produce a working version of this protocol. Every result produced showed too much variation in either standard oxidised BSA, fly samples or both. Sulforaphane-dosed flies were never tested, and the experiment was abandoned.

5. Discussion

This study aimed to investigate the isothiocyanate sulforaphane, a compound studied in numerous contexts related to ageing, and determine if it could have a positive impact on the lifespan, functional health and/or biochemical markers of ageing in the *Drosophila melanogaster* model. We had hypothesized based on the previous research of students in our lab, that an ideal SFN dose around 20µg/ml should increase lifespan and ameliorate physiological decline as tested by negative geotaxis, in the fly model (Mifsud, 2017). In addition, we expected that SFN may up-regulate the CncC pathway and increase expression of target ARE genes such as GstD1.

This work only identified an extension of lifespan under very limited conditions, and no effect was found in our assay of functional health or our investigation into the effect of SFN on the regulation of GstD1, a known CncC target gene. Significant challenges were encountered in attempting further experiments, such as an assay of protein oxidation, and so this study did not cover its full intended scope.

5.1. Assessment of the results of this study

5.1.1. No change in lifespan associated with sulforaphane dose in *Drosophila melanogaster.*

This first experiment attempted to establish the effect on lifespan and ideal dose of sulforaphane in *D. melanogaster,* building on the unpublished work of Grimes (2016) and Mifsud (2017). Mifsud found an effective dose for SFN around 20µg/ml, while Grimes found a mildly toxic dose at 100µM broccoli extract. This extract was made from capsules stated by the manufacturer to contain 400µg SFN dissolved in ethanol. Due to SFN's high solubility in ethanol, Grimes and Mifsud assumed that all of it should have dissolved. Given the reduction of lifespan by Grimes' 100µM extract dosage, we expected that a high dose of pure SFN, such as 200µg/ml, should have reduced lifespan as well.

The findings did not support this expectation. No significant effect on lifespan was found for any SFN dose. Several possibilities for this were considered, such as that the dose was insufficient, that the Lancaster genotype displayed unusual stress resistance, or the vial of sulforaphane was in some way at fault. Other possible causes included the storage conditions of the drug and the flies, or failures on the part of the operator.

To eliminate some of these possibilities the experiment was repeated with a fresh vial of Sulforaphane from the same source, a different food type in place of the high food, and two different genotypes of flies. The fresh vial would likely eliminate the possibility of a faulty vial, the high food was changed because Mifsud (2017) suggests SFN has a stronger effect on the high food, but this recipe ran the risk of extrinsic harm to flies when a batch failed, so we replaced it with a recipe that would be less likely to fail. Finally, Lancaster flies were exchanged to two different established laboratory strains to control for variance in stress resistance.

Dose was kept consistent as it was supported by Mifsud (2017). Conditions and operation were considered unlikely causes at this time as no major issues were obvious in either.

5.1.2. No change in lifespan associated with SFN dose in multiple food and genotypes.

An interesting development in this experiment was that Zim53 did not survive well at all, showing an overall lifespan ~25% less than White Dahomey. The reason for this may be that their progenitors were subject to an event where the temperature control on their incubator failed and they were exposed to unusually high temperature stress, inadvertently selecting for flies which were very heat stress resistant but with poor longevity. Alternatively, this might be due to an unrecognised issue during the generation of the tested flies, or an even earlier problem.

This did provide some opportunity to see if sulforaphane would 'rescue' this damaged phenotype. SFN is known to be capable of rescuing impaired phenotypes in cultured cells (Kubo et al., 2017). However, the value of this would be compromised by the uncertain nature of the genotype's deficiency.

The experiment failed to find any effect of sulforaphane dose on lifespan in either genotype or food type. This suggests that SFN was not able to rescue the short-lived phenotype of Zim53. It also suggests that food type, drug vial and food were not the causes of failure in the previous experiment, as all of these factors were changed.

5.1.3. No change to performance or performance decline over time with sulforaphane dose in *Drosophila melanogaster.*

Based on the findings of Mifsud (2017) we could expect to see an improvement in the performance of flies treated with SFN. Mifsud (2017) found an improvement of 16.9% at 20µg/ml SFN for 39-day old flies. We could also expect to see that, over time, the decline in performance with age might be slowed in appropriately dosed flies.

We failed to see either outcome, with no significant change to fly performance with dose at any given time point, nor any change in performance decline over time. There is some evidence of a trend in the spread of the data as in the low-yeast cohort there is a tendency towards better performance for 20µg/ml, while this group shows some decline in the high-yeast cohort. However, no significant effect was detected by mixed-model ANOVA analysis. This may indicate a very slight change that could not be detected by this study, possibly due to diminishing power as a result of deaths over time between readings, though negative geotaxis was stopped once large numbers of deaths were recorded in the concurrent lifespan experiment.

It is also plausible that whatever caused the lack of results in the concurrent lifespan experiment also caused the negative result here. A limitation of this experiment was that it was not repeated alongside the next lifespan experiment, due to my absence at that time.

Food effect was important, as high-yeast food showed a significant improvement ($P < 0.001$) to climbing height across time. The lack of effect in the earliest and last time points may indicate that it affects only the decline over time, and not the overall performance of the flies. Although weak, the interaction between food and dosage supports the similar interaction effect found in the last lifespan experiment.

5.1.4. Sulforaphane levels detectable by HPLC reduce considerably when in food.

After the issues with the early lifespan experiments, we decided to investigate the sulforaphane content of food at the time flies would consume it (48 hours after dosing). We expected to find measurable levels of SFN that would indicate it was sufficiently available for a bioactive effect in flies.

This work acted as a speculative study, rather than a full quantitative analysis of SFN content in *Drosophila* food, to identify a potential cause of the problems we encountered during the lifespan experiments. We also had limited time and supply of SFN standard to spare. As such, a full validation procedure was not employed, which would have consumed considerably more time and resources. Had the project extended to include a full analysis of the consumption of SFN by *Drosophila*, this experiment would have been repeated and a full validation procedure employed. This would need to include a wider range of standards and repeated testing, to establish a reliable limit of detection, limit of quantification, and linearity. This is particularly important going forward, as our results indicate the concentration of SFN detected declines considerably, so we need to know if the method can give an accurate reading of these relatively small concentrations.

We detected at least some presence of sulforaphane in every tested sample. In some cases, however, particularly with samples dosed with <200µg/ml SFN, the amount of sulforaphane detected was very low, ~0.15µg/ml SFN.

Assessing whether the concentrations reported could provide a bioactive dose to flies is also difficult. There is no known estimate of the bioactive dose in flies – in the few published studies testing SFN in *D. melanogaster* that exist, only the dose added to food is provided. There is a figure available for rats, where bioactivity is found between 0.1-0.5mg/kg/day (Hu et al., 2004). By calculating the received dose in flies considering the amount a fly would consume in a day and the mass of an average fly, we can determine if any of our doses fit the range suggested for rats. Only those doses of 500µg/ml were estimated to be close to the minimum 0.1mg/kg. Doses of 5000µg/ml exceed this dose range. Our work also suggests SFN dose is lower in samples tested after 2 days, which may indicate degradation over time, but a more focused study testing samples over a longer period would be needed to confirm this.

A problem with this estimation is that it is difficult to find a good estimation for the amount a single fly eats. Ultimately, we used an estimation of 36µg/day based on extrapolation from a single study (Wong et al. 2009). The best method to determine the average food consumption of a fly per day is contentious (Shell et al., 2018; Deshpande et al. 2014; Lushchak et al., 2011; Wong et al. 2009), and so we cannot be completely confident in our results. It would be beneficial in future work to carry out our own assay of this question.

Another problem with the work is that our method of leaching SFN from the food samples by EtOH may not be completely effective, since we carried out the work at a low temperature of 4°C, which would have reduced the rate of leaching. This should have been compensated for by our allowing of >24 hours for leaching to take place, though this has not been proven. The use of a lower temperature can be justified by

the need to prevent degradation of SFN at room temperature, something our findings do support, as obtained concentrations of SFN were lower in samples given 48hrs instead of 24.

If this experiment is to be validated and repeated, temperature should be considered as a variable, with both 4°C and room temperature at least included in the study. In Grimes (2016) and Mifsud (2017), active agitation was also applied when dissolving the 'Sulforaphane' capsules in ethanol, something which was not used here, and may be worth testing.

We took the results of this work as advisory, and decided to include higher doses in the final lifespan experiment and in the RT-qPCR assay of GstD1. The findings of the final lifespan experiment, assuming they can be confirmed, seem to indicate that the bioactive range may be lower than this estimation, as positive findings were found around 100µg/ml.

5.1.5. Sulforaphane can influence lifespan in limited conditions when administered early in *D. melanogaster* lifespan.

It was hypothesised that the effect of SFN dose might be dependent on the timing of the drug's administration. If SFN can prevent but not repair damage, then an early-life intervention may yield better results than administration in already aged flies. Further, it is possible that SFN's impact becomes negative later in life as more fly cells begin to show damaged profiles, leading to SFN acting as a cytotoxin rather than cytoprotective. SFN is made in response to plant wounding and may very well be harmful to insects at sufficient doses (Fahey et al., 2015). It is important to note here that *D. melanogaster* adults display very low rates of cellular regeneration (Shwartz and Rhiner, 2018; Repiso, 2011) and therefore lack the ability to replace cells that are destroyed, at least outside of their imaginal discs, and so the destruction of cells by SFN would be likely to have a net negative impact.

Alternatively, SFN's effects may have more of an impact later in life, due to correcting existing damage and rescuing the aged phenotype, like its ability to rescue impaired phenotypes in cultured cells.

We found a single positive result for lifespan extension by SFN. White Dahomey flies on sugar-yeast food displayed an increase of 5.74% (P= 0.003) on 100µg/ml SFN when administered early in life. This effect was not however seen between 0 and 400µg/ml or 100 and 400µg/ml. Furthermore, no significant increase was recorded on cornmeal food, or the late wDah cohort.

Lancaster flies under the same conditions also displayed a significant effect, however this was a decline of about 6.34%. Again, no effect on cornmeal food, at 400µg/ml or the late cohort.

However, the comparison of 100 to 400µg/ml does come close to significance (P= 0.058), and the estimated means show an expected trend – increasing from ~60 days to ~63 between 0 and 100µg/ml, then falling back to ~60 at 400µg/ml. This curve would be anticipated from a hormetic drug.

Comparison to previous lifespan experiments:

The findings here contradict the earlier lifespan experiments of this study. In the first lifespan experiment with Lancaster strain flies, flies were treated almost equally to the Lancaster flies in this study. No significant increase was observed between 0 and 5, 20 or 40µg/ml SFN. It is possible, though unlikely based on our previous work, that the ideal concentration is between 40 and 100µg/ml.

In the second experiment, White Dahomey flies on the normal SY food should be comparable to those used in this experiment. However, the largest dose in that experiment, 50µg/ml, was smaller than the working 100µg/ml in the last lifespan experiment. Also, the first dose was received 8 days later than in this experiment's early cohort, and dosing continued throughout. Again, no significant increase was found between any dosage. This may indicate the importance of treating flies as early as possible to obtain the increase in lifespan.

It may also be that the difference between this experiment and the two previous is due to the cessation of treatment after 45 days. This may infer that the lifespan extension effect can be abolished by continuing SFN treatment beyond a certain point in age. If so, this may seriously limit its potential as an effective anti-ageing intervention.

Practical issues with the experiment:

The study suffers from some lack of range in the tested doses. This was necessary for practical reasons due to the large number of treatments already involved. However, no dose between 0 and 100µg/ml was tested. This may account for the findings in the Lancaster strain. If we overshot the target window, then we may only see a detrimental effect of the drug. Further, there might be a stronger increase in lifespan in White Dahomey at a lower, or higher (but not as high as 400µg/ml) dose than 100µg/ml.

Clearly, the large number of treatments also mean significance may be found by chance, and these findings need to be confirmed in further work.

5.1.6. No effect of SFN found in expression of GstD1 in *Drosophila melanogaster.*

The most well-studied mechanism for sulforaphane cytoprotection in cultured cells and model organisms is its effect on the Nrf2 pathway. The Nrf2 pathway is well conserved through invertebrate and mammal models and we would expect that SFN could target it in flies as well. Based on the findings of Sykiotis and Bohmann (2008) we reasoned that if *Drosophila* CncC was activated by SFN, we could expect to see an increase in the expression of GstD1.

We did not see this – there was no significant increase in GstD1 at any tested dose of SFN, compared to control. Notably, the melt curves for the EF1g housekeeping gene were unusual (varying between 80 and 82.5°C) and the resultant Cqs were considerably higher than we typically expect from our lab. Previous work in *Drosophila* of the Lancaster strain with this gene has yielded Cqs in the low 20s, rather than the high 20s-to low 30s we saw.

The experiment was repeated, as there were clear problems with the qPCR in the first experiment, with several poor triplicates and high variation between individual fly samples, as well as abnormal melt curves. This first experiment's results are not reported here but showed consistency with our repeat. We felt confident enough from the second attempt to conclude a lack of effect in the results.

There are several possible reasons for this – it may be that one of the genes we selected, either the housekeeping gene or target gene was a poor choice for this study. The EF1g housekeeping gene is a well-established gene of reference in RT-qPCR experiments and previous work in our lab has yielded good results using it in *D. melanogaster* studies.

GstD1 was chosen as the target gene due to both the previous work indicating its expression increases when CncC is activated (via manipulation of *Drosophila* Keap1) (Sykiotis and Bohmann, et al., 2008) and its increase in expression with paraquat (flybase.org/reports/FBgn0001149, 2019). Alternative genes that show similar characteristics would be GstD2, a related gene, or Cyp6g1. It is unclear if the latter gene is expressed in adult male flies, as Misra et al. (2011) implies it does, while flybase indicates it does not. Notably, Dueñas-García et al. (2012) showed that expression of this gene in larval flies is increased by sulforaphane dosing.

Since an increase in GstD1 expression following CncC activation is supported by the previous literature, it is possible that a failure of SFN to elicit the same response indicates that the compound is unable to activate CncC. This could be due to a low dose, but this is unlikely as our maximum dose was the extremely high 800µg/ml.

Alternatively, it could be due to a structural difference between *Drosophila* dKeap1 and mammalian Keap1. SFN acts on Cys151 of mammalian Keap1 to induce the conformational change necessary to release Nrf2. dKeap1 may not share this or an orthologous structure with the mammalian counterpart. dKeap1 shows 47% identity and 64% alignment with human Keap1 (flybase.org/reports/FBgn0038475, 2019), and Cys151 does not align perfectly in the primary structure. However, there is a cysteine residue close in sequence. It is likely that 3D modelling would be needed to further determine if the difference in structure could account for the lack of effect seen in this work.

A single reference gene was used in this experiment, eEF1γ. The use of only one reference gene has been common practice in ageing studies for some time (Ponton et al., 2011), however, it is not best practice. Studies have demonstrated that very few, if any, 'housekeeping genes' are stable under all treatment conditions (Ponton et al., 2011; Van Hiel et al., 2009), and so must be carefully selected for based on the nature of the treatments being tested.

eEF1γ has been demonstrated to be a very stable reference gene in arthropods including *Drosophila melanogaster* when treatments involve dietary manipulation (Ponton et al., 2011; Van Hiel et al., 2009). eEF1γ shows stable expression across *Drosophila* lifespan, and with the administration of paraquat, an oxidising agent (flybase.org/reports/FBgn0029176, 2020). eEF1γ has also been used previously for SFN-treatment in *Drosophila* in our lab (Grimes, 2016).

It is nonetheless preferred to use multiple reference genes. If this work were to be repeated or expanded upon, future qPCR work should ideally select a second reference gene as well. Suitable candidates include α-tubulin or Rpl32, both of which display similar stability to eEF1γ (Ponton et al., 2011; flybase.org/reports/FBgn0003884, 2020; flybase.org/reports/FBgn0002626, 2020).

Finally, it should be noted that in this experiment the flies were only exposed to two consecutive doses of SFN. This may not be enough to cause a measurable elevation in GstD1 levels, and future work should consider using flies treated for a longer duration.

5.1.7. Failure to produce working assay for protein oxidation in *Drosophila*

The protein carbonylation assay was intended to act as a measure of protein oxidation in flies, so that oxidation could be measured against increased doses of SFN. Unfortunately, a working protocol for this assay that could deliver reliable results was never produced.

The most likely reason for the failure is the combination of small volumes of material and the need for a derivatization process with DNPH, that leads to unstable protein products. Given the multitude of attempts by several different researchers and many revisions to the protocol it seems sensible that the experiment was abandoned.

There are alternative methods of measuring protein carbonyl, such as western blot assay (Wehr et al., 2012) or similar DNPH assays based on larger volumes (used internally by Lancaster University), but these would necessitate multiple flies to be used, which would be taken from our lifespan experimental groups, reducing the available sample in those experiments.

5.2. Limitation of sulforaphane as an effective treatment in *Drosophila melanogaster.*

Our experiments suggest that SFN can have a positive effect on *D. melanogaster* lifespan under limited conditions, but this is not conclusive. Our only positive finding was in a single experiment, and while the significance of the improvement is not weak, the large number of interaction effects and the lack of support in our other, similar experiments means a repeat will be necessary to determine if the result stands.

Even if these results are confirmed however, it leaves many implications about the efficacy of SFN in improving *D. melanogaster* lifespan.

First, food type is clearly important. A dose*food type interaction was significant by cox regression, and cornmeal food showed no significant improvement in White Dahomey flies. However, a near-significant improvement of 9% was recorded in early White Dahomey flies on cornmeal food at 400µg/ml SFN. This might mean that on cornmeal food a higher dose is required to provoke an effect on lifespan. This suggests that the effect of sulforaphane can be altered by food type, possibly altering the sensitivity of the flies to the drug.

The opposite effect in Lancaster flies raises interesting questions. We can see some, albeit non-significant, evidence from the experiment that high doses of SFN (400µg/ml) in White Dahomey flies led to loss of lifespan improvement. This might have, with even greater doses, caused a reversal of effect, as was seen in Grimes (2016) with broccoli extract. Therefore, it is possible Lancaster flies are more sensitive to SFN, and have a lower target dose than White Dahomey, which we overshot. Differences in sensitivity with genotype are not uncommon in humans and other animals.

Lancaster flies showed no change to lifespan on cornmeal food, which may be due to their already increased sensitivity to the drug and the high concentrations we tested.

The most striking result is that the improvement was only seen in flies that were given sulforaphane only in the early part of their life. In flies given SFN later, or in comparably treated flies given SFN throughout their lives, no significant extension is found. If there are no technical issues to explain these results, and SFN genuinely does enhance the lifespan of the fly, then this may indicate that at some point SFN ceases to act as an anti-ageing drug and can even abolish the early benefit of taking it. This would greatly limit its potential as a therapeutic intervention.

Given the number of compounding issues surrounding these findings we cannot state with certainty that the lifespan enhancement is due to the SFN dosage and this experiment must be reproduced before any further conclusion can be drawn.

5.3. Further Implications and Future Experiments

5.3.1. Reflections on this study

Several problems with the design and execution of many experiments in this study have been discussed here. It is important to reflect on this and consider what changes could have be made to the study design to produce better work. This can then better inform repeat experiments and future work on this topic.

First, lifespan experiments were performed linearly, from start to end, before the next began. This reduced the amount of time available to conduct multiple experiments, as each experiment takes approximately 12 to 14 weeks in total to complete. Although the workload of each experiment is high during the peak time of death (typically between day 40 and 60), it is possible and preferable to begin breeding a second experiment's flies before the first concludes. Improved time management would allow for more experiments and may improve the performance of researchers involved.

The lack of time as a result of this failing created further issues. For example, the negative geotaxis experiment was not repeated alongside further lifespan experiments, due to researcher unavailability. If this study were to be repeated, it would be beneficial to run a negative geotaxis experiment alongside each lifespan study.

Our work with HPLC analysis of the retention of SFN on food was useful, but given more time, could have been expanded upon with a fully validated method and follow-up procedure to verify those findings.

The qPCR experiment was also only repeated once due to limited time, and only used one reference gene. Though this is common practice (Ponton et al., 2011), it would

have been ideal to use a second reference gene, and to repeat the experiment at least once more, to provide three repeats of the data.

Even with improved time management, lifespan studies still take considerable time to complete. This creates the possibility for simple mistakes to harm the usefulness of several months' worth of data, which will take even more time to repeat. It is important, then, to reduce as much as possible, the potential for mistakes to be made. This is highlighted by the food melting issue that occurred during the first lifespan experiment. In a repeat of this study, the benefits of food types that require such care must be considered before they are used in an experiment.

Female flies were not used in this experiment. This is common practice in our field (He and Jasper, 2014; Spencer et al., 2003), since female flies respond differently to male flies in lifespan intervention. There are complex reasons for this, including differences in metabolism from egg production (Spencer et al., 2003), as well as endocrine and genetic differences (He and Jasper, 2014). In particular, varying levels of reproductivity has been used to justify choosing males over females in single-sex studies before (Koudounas et al., 2012).

In our case, the decision to only use one sex of flies was to reduce the number of covariates in our experiments, which was important given limited time and labour. Males were selected over females to eliminate the effect of varying reproductivity on lifespan. Any further work on this topic should test female flies, either in a separate experiment or in comparison to male flies, as ignoring the effect of sex, and the complex factors that underpin these differences, may cause an oversight of variables that could be important to consider when attempting to apply our findings to other organisms.

The use of CO2 to stun the flies during routine changing of their food vials does create a possible problem. Given SFN was believed, based on its impact on Nrf2 (Kubo et al., 2017), to act to improve stress resistance in flies in some way, the gassing may have been producing a stress on the flies that prevented SFN from functioning correctly. Control flies were given the same treatment, but it may be useful to trial in at least one experiment not using this method.

Finally, the protein oxidation assay consumed more time and effort than it was reasonably worth. The protocol should have been replaced much sooner, with an alternative study of a biochemical impact. Alternatives to study protein oxidation, such as western blotting (Wehr et al., 2012) do exist. We could also have replaced this aspect of the study with a different investigation altogether, such as the study of sleep cycles (Chiu et al., 2010) to determine if there is an effect of SFN on *Drosophila* behaviour.

5.3.2. Confirming and investigating our findings further.

After our original negative findings in the first pilot lifespan experiment, this study has tried to determine why SFN did not have the effect we expected, eliminate any extrinsic cause, and further investigate the biological mechanisms underpinning out findings.

We were able to find a positive result but only in limited circumstances. Therefore, a repeat experiment to both confirm our findings and investigate them further is required. Furthermore, our results suggest that a simple difference in genotype or food type can change the effect of SFN dramatically. The differences in genotype and food type might indicate their importance in determining the sensitivity of flies to the drug. Work is also then required to determine what exactly is the cause of these varying effects, even if we do confirm the result of our previous experiment.

Further lifespan investigations:

The most useful test going forward would be to compare flies given SFN between days 9 and 45 only, and those given it throughout their entire adult lives. These flies should otherwise be the same as the group in which the positive result was found – White Dahomey, on normal SY food, with 100µg/ml SFN. We could also investigate the effect of different food types, such as a 1:1.5 Sugar-Yeast ratio, again.

To test if Lancaster flies are more sensitive to SFN, and we overshot our target dose with them, we could run a lifespan experiment that uses a range of doses, but only treats them until day 45 as in our last lifespan experiment. If we determine that this is not the case, and Lancaster flies show no lifespan improvement at any dosage, it would be likely that a difference between these genotypes is the cause – we know that SFN can show different effects in cell cultures based on their mitochondrial profile (Negrette-Guzman et al., 2013) and this, or another factor, may be the case in whole organisms as well.

Either or both experiments should also be accompanied by a repeat of our negative geotaxis assay, which currently suffers from a lack or repetition. It may also be that the lack of results in our single negative geotaxis assay were due to the same reasons as the lack of results in its accompanying lifespan experiment, and if this method can produce a working result in lifespan, it may produce a more interesting finding in fly performance as well.

Further investigation into genetic and biochemical pathways of SFN mechanisms:

Based on our findings on the change in expression of GstD1 in flies with SFN dosage, we have found that possibly, SFN does not up-regulate CncC (fly Nrf2). If this is the case, then our positive lifespan result would not be due to the activation of Phase II detoxification and the cytoprotective effect of Nrf2 up-regulation. However, our qPCR results are questionable and of limited scope. This finding should be confirmed, possibly by testing different genes and flies that had been consuming SFN in food for longer.

Bioavailability of SFN in Drosophila melanogaster:

Our work to determine the retention of SFN in fly food underpins the need to be certain that flies treated with SFN absorb the compound into their tissues. To be certain of this, the uptake of SFN by flies should be tested.

Our HPLC work relied upon an estimation of the average food intake of our test flies. A more effective method of accounting for this factor would be to produce an assay testing the uptake of our specific solid food types in our fly treatment groups. There are a number of assays developed for testing food uptake in Drosophila, though none are without issues (Wong et al., 2009). The use of labelled or coloured dye is perhaps best for our purposes, as it allows the use of solid food media (Shell et al., 2018). This is preferable to other techniques, like capillary feeder (CAFE) (Diegelmann et al., 2017; Wong et al., 2009) which use liquid food media.

SFN presence in fly tissues may be possible to test via HPLC. A challenge might be posed by the small amounts of SFN that might be found in single flies. Liquid Chromatography-Mass Spectrometry techniques have been used in other research to successfully measure levels of neurotransmitters in fly heads (Lakkappa et al, 2018; Makos et al., 2009), so a similar technique that is sensitive enough to detect SFN in the whole fly should be possible to design. This would allow us to detect if SFN is present in our treated flies' tissues, and if this presence scales with dosage.

5.3.3. Other areas of interest and potential experimental investigations.

There are a many other processes and hallmarks of ageing that SFN is known to effect that were not tested in this study, usually due to time constraints and practical priorities. Investigating these areas may yield interesting results.

Histone Deacetylase inhibition:

Sulforaphane is well-understood as an inhibitor of histone deacetylases. This is thought to play a role in its regulation of autophagy and anti-cancer properties (Yang et al., 2018, Ho, Clarke and Dashwood, 2009). There is no study of whether it can exert this effect in fruit flies, and this mechanism is thought to be unrelated to its activation of Nrf2. It is therefore still possible that an effect could be found. Histone deacetylase inhibition can be tested by HDAC Activity assay (Yuan et al., 2009), which could be used on whole fly tissue samples of dosed flies.

Regulation of autophagy:

Since SFN can regulate autophagy (Yang et al., 2018; Wang et al., 2018; Zhou et al., 2016) assays for autophagy could be run to examine if this effect can be found at appropriate doses in *Drosophila*. An assay of *Drosophila* autophagy regulatory protein Atg8a by Western Blot is a good method of measuring autophagy using whole fly tissue extracts (Lőrincz et al., 2017). It is recommended that any western blot-based autophagy assay be accompanied by an assay of autophagic flux such as a Ref(2)P/p62 antibody blot (Lőrincz et al., 2017; Pircs et al., 2012), as The level of

Ref(2)P/p62 is usually inversely proportional to autophagic degradation (Lőrincz et al., 2017).

Regulation of cell signalling pathways:

SFN is known to inhibit some MAPK kinases, such as JNK and ERK1/2 (Subedi et al., 2019). Assays of MAPK activity can be performed using western blot analysis (Caelles et al., 2004; Qin et al., 2018). This can be tested by commercially available kits on tissue samples extracted from dosed flies.

Downstream of these is the NFkB pathway, which SFN exhibits and inhibitory effect on (Qin et al., 2018). The inhibition of NFkB can be tested by RT-qPCR assay of one of its target genes, similar to our work on Nrf2.

Other potential targets:

Our RT-qPCR does not definitively prove no role of CncC in lifespan extension. This could be tested in a lifespan experiment using a cohort of flies that have an anti-Nrf2 intervention of some kind, for example CncC can be conditionally knocked down by RNAi in flies (Sykiotis and Bohmann, 2008).

There are a few other known effects of SFN that are thought to be Nrf2-independent. These include its up-regulation of heat-shock factor 1 (Gan et al., 2010), its promotion of mitochondrial fusion and possibly at least part of its regulation of apoptotic signalling (O'Mealey et al., 2016). Investigating any of these might be useful in establishing the mechanistic basis of its lifespan extension, especially if we can confirm the non-involvement of the Nrf2/CncC pathway.

Although the protein oxidation assay failed, a similar assay has been developed in our lab to test the level of AGEs in single fly samples. Sulforaphane administration has been found to correlate with a decrease in AGEs in a few studies (Alfarano et al., 2018, Matsui et al., 2016). Whether it up-regulates glyoxalase1, as found in Alfarano et al. (2018) in flies (Glo1 in *D. melanoagaster* (flybase.org/reports/FBgn0283450, 2019)) could also be interesting.

5.4. Could broccoli extract be a better option than sulforaphane in improving *D. melanogaster* lifespan?

Grimes (2016) showed clear improvement to the lifespan of Lancaster strain flies using an extract of broccoli. Later work using HPLC found that chromatograms of the extract did not contain any peaks matching pure SFN samples, and that further investigation with mass spectrometry found only trace amounts of the chemical in the extract (Mifsud, 2017). Mifsud further showed that, while pure SFN did improve lifespan under limited circumstances, a stronger effect was found using the broccoli extract.

Given that the extract contained no detectable SFN, another compound may be responsible for this effect. A possible candidate is glucoraphanin, the precursor of SFN. Previous studies have found that glucoraphanin administration can achieve some of

the effects of SFN in rats, such as up-regulating NQO1, an ARE-containing gene, in colon, liver and lung tissue samples (Lai et al., 2008).

To test if glucoraphanin is the main active ingredient in the extract, we could use HPLC to compare the peak to that of a sample of pure glucoraphanin. If it is found to be a likely match, we could then test glucoraphanin on lifespan in *D. melanogaster*.

It is not clear why glucoraphanin could have a stronger effect than SFN, though it may be due to SFN being released during digestion having a longer-lasting effect due to less time to degrade, or possibly some kind of interference one stage of fly digestion has on pure SFN that does not affect glucoraphanin. Such questions would need to be investigated should glucoraphanin be found to be the more effective compound.

In addition to glucoraphanin, *Brassica* species contain several other compounds which have been suggested to hold potential in researching age-related disorders such as cancer and cardiovascular disease (Pérez-Balibrea et al., 2011, Moreno et al., 2006). It is possible that one or more of these other compounds, which may have been present in the extract, could be responsible for the more pronounced effect of it over the pure SFN. This could be through a direct mechanism, or by acting in some way to enhance the effect of glucoraphanin/SFN.

Since Mifsud (2017) demonstrated that the information given by the supplier of the extract cannot be relied upon to inform us of its contents, we do not know which of these compounds might be found within the extract. As the supplier aimed to produce sulforaphane, or at least glucoraphanin, glucosinolates or their hydrolysed products would be the best compounds to focus on in future work with this extract, though consideration should be given to other compounds with the potential to ameliorate ageing, or attenuate age-related disease, such as flavonoids (Samieri et al., 2014, Prasain et al., 2010) or polyphenolics (Queen and Tollefsbol, 2010).

Table 6 gives a breakdown of the most relevant compounds found in green broccoli sprouts, the plant that the extract used in Grimes (2016) was isolated from (Swanson Health Products, Fargo, ND, USA).

Table 6: Glucosinolate and phenolic composition of green broccoli sprouts (*Brassica oleracea L. var. italica*). Adapted from Pérez-Balibrea et al. (2011). Content values are for control subjects from source article 5 days after sowing. *Indicates that source did not provide discrete values for individual compounds.

Type	Sub-Type	Compounds	µmol g−1 d.w.
Glucosinolates	Aliphatic Glucosinolates	Glucoraphanin	15.3 ± 0.8
		Glucoiberin	6.5 ± 0.4
		Glucoerucin	3.9 ± 0.5
	Indole Glucosinolates	Glucobrassicin	1.8 ± 0.3
		4-hydroxy-glucobrassicin	6.6 ± 0.4
		Neoglucobrassicin	2.2 ± 0.2
		4-Methoxyglucobrassicin	1.7 ± 0.6
Phenolics	Flavonoids*		38.7 ± 1.5
	Caffeoyl-quinic acid derivatives*		1.7 ± 0.2
	Sinapic and ferulic acid derivatives*		12.9 ± 0.9

Brassica species are also rich in β-Carotene, Ascorbate (Vitamin C) and α-Tocopherol (Moreno et al., 2006), though glucosinolates and phenolics are more likely candidates. Testing these other compounds might be useful if studies fail to isolate a lifespan-enhancing effect in the glucosinolates or phenolics.

5.5. Conclusion

With these findings we cannot conclude that SFN is a strong candidate anti-ageing drug intervention. For the most part, we failed to find sufficient evidence that SFN can, at an appropriate dosage, increase *Drosophila* lifespan. We also found no evidence of improvement in mobility or increase of the expression of GstD1 with increased SFN dose.

We did find a single enhancement of *D. melanogaster* lifespan in one (White Dahomey) genotype, fed with one food type, and at high dose of 100ug/ml. Without being able to reproduce this result however, it cannot be relied upon as proof of SFN's potential. Further, even if it was such, the narrow range of conditions seemingly required for this effect greatly limit sulforaphane's usefulness as a drug intervention.

As such, this work requires further investigation to both attempt to reproduce these findings and, if upheld, the reason that lifespan extension is only found in these narrow circumstances.

A good investigation to carry out next would be to test SFN's effect in flies dosed only in the 'early' part of their lives (before day 45) against flies dosed continuously throughout life. This may also be an opportunity to try different food types and genotypes, and a further negative geotaxis experiments could be run alongside this work.

Other important investigations should be carried out to find the reason Lancaster strain flies showed a different effect, and the possible biological mechanisms underlying the lifespan extension, given our preliminary work suggesting Nrf2/CncC may not be the important pathway in *Drosophila*.

Consideration should also be given to the possibility that glucoraphanin or another compound found in extracts of broccoli may be more effective than SFN, either in *D. melanogaster* or more broadly across species.

Another important possibility is that *Drosophila melanogaster* is not an appropriate model organism to test the effect of SFN, as the compound acts as part of plant defence against insect pests, via anti-feedant and cytotoxic properties (Fahey et al., 2015). *Brassica* plants are not a component of typical *Drosophila* diets (Brankatschk et al., 2018), but they could still be susceptible to these defensive properties. Further, the lack of NQO1 (Vasiliou et al., 2006) likely alters their response to Nrf2-enhancing compounds such as SFN.

Depending on future work, SFN may still hold some value in ageing research. The evidence from this study, however, is insufficient to make that claim. It should be noted however, that even if we fail to confirm an effect on lifespan, this negative result will be a valuable addition to the body of literature in sulforaphane and health.

6. References

Abbaoui, B. et al. (2017). The impact of cruciferous vegetable isothiocyanates on histone acetylation and histone phosphorylation in bladder cancer. *Journal of proteomics,* 156, pp. 94-103.

Abbas, A. et al. (2016). Sulforaphane modulates telomerase activity via epigenetic regulation in prostate cancer cell lines. *Biochemistry and Cell Biology*, 94 (1), pp. 71-81.

Alfarano, M. et al. (2018). The Effect of Sulforaphane on Glyoxalase I Expression and Activity in Peripheral Blood Mononuclear Cells. *Nutrients,* 10 (11).

Alumkal, J. J. et al. (2015). A phase II study of sulforaphane-rich broccoli sprout extracts in men with recurrent prostate cancer. *Investigational new drugs*, 33 (2), pp. 480-489.

Angeloni, C. et al. (2015). Neuroprotective Effect of Sulforaphane against Methylglyoxal Cytotoxicity. *Chemical Research in Toxicology,* 28 (6), pp. 1234-1245.

Artandi, S. E. et al. (2002). Constitutive telomerase expression promotes mammary carcinomas in aging mice. *Proceedings of the National Academy of Sciences of the United States of America*, 99 (12), p. 8191.

Balasubramanian, S., Chew, Y. C. & Eckert, R. L. (2011). Sulforaphane Suppresses Polycomb Group Protein Level via a Proteasome-Dependent Mechanism in Skin Cancer Cells. *Molecular Pharmacology*, 80 (5), pp. 870-878.

Balasubramanian, S., Kanade, S., Han, B. & Eckert, R. L. (2012). A proteasome inhibitor-stimulated Nrf1 protein-dependent compensatory increase in proteasome subunit gene expression reduces polycomb group protein level. *The Journal of biological chemistry,* 287 (43), pp. 36179-36189.

Balistreri, C. R. et al. (2013). NF-κB pathway activators as potential ageing biomarkers: targets for new therapeutic strategies. *Immunity & ageing*, 10 (1), p. 24.

Ballard, J. W. O. (2000). When One Is Not Enough: Introgression of Mitochondrial DNA in Drosophila. *Molecular Biology and Evolution,* 17 (7), pp. 1126-1130.

Bárcena, C., Mayoral, P. & Quirós, P. M. (2018). Mitohormesis, an Antiaging Paradigm. In: *International review of cell and molecular biology*, 340, pp. 35-77.

Beckman, K. B. & Ames, B. N. (1998). The Free Radical Theory of Aging Matures. *Physiological Reviews,* 78 (2), pp. 547-581.

Bernardes de Jesus, B. et al. (2011). The telomerase activator TA-65 elongates short telomeres and increases health span of adult/old mice without increasing cancer incidence.. *Aging cell,* 10 (4), pp. 604-621.

Bernardes de Jesus, B. et al. (2012). Telomerase gene therapy in adult and old mice delays aging and increases longevity without increasing cancer. *EMBO molecular medicine,* 4 (8), pp. 691-704.

Brankatschk, M. and Gutmann, T. (2018). A Temperature-Dependent Switch in Feeding Preference Improves Drosophila Development and Survival in the Cold. *Developmental Cell*, 46, pp: 781-793.

Brose, R. D. et al. (2012). Activation of the stress proteome as a mechanism for small molecule therapeutics. *Human molecular genetics,* 21 (19), pp. 4237-4252.

Burtner, C. R. & Kennedy, B. K. (2010). Progeria syndromes and ageing: what is the connection? *Nature Reviews Molecular Cell Biology,* 11 (8), pp. 567-578.

Caelles C., Morales M. (2004) Assays to Measure Stress-Activated MAPK Activity. In: Brady H.J.M. (1st edn) Apoptosis Methods and Protocols. Methods in Molecular Biology, vol 282. Humana Press.

Campas-Baypoli, O. N., et al. (2009). HPLC method validation for measurement of sulforaphane level in broccoli by-products. *Biomedical Chromatography*, 24, pp: 387-392.

Chiu, J. C., et al. (2010). Assaying Locomotor Activity to Study Circadian Rhythms and Sleep Parameters in *Drosophila*. *J. Vis. Exp.,* 2010 (43), pp: 2157.

Choi, K.-M.et al. (2014). Sulforaphane attenuates obesity by inhibiting adipogenesis and activating the AMPK pathway in obese mice. *The Journal of Nutritional Biochemistry,* 25 (2), pp. 201-207.

Clancy, D. & Birdsall, J. (2013). Flies, worms and the Free Radical Theory of ageing. *Ageing Research Reviews*, 12 (1), pp. 404-412.

Clancy, D. J. & Kennington, W. J. (2001). A simple method to achieve consistent larval density in bottle cultures. *Dros. Inf. Serv*, 84, pp: 168-169.

Cornblatt, B. S. et al. (2007). Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast. *Carcinogenesis*, 28 (7), pp. 1485-1490.

Dahlmann, B. (2007). Role of proteasomes in disease. *BMC biochemistry*, 8 (Suppl 1), p. S3.

Deshmukh, S. et al. (2018). Levels of DNA cytosine methylation in the *Drosophila* genome. *PeerJ,* 6, p. e5119.

Deshpande, S. A. et al. (2014). Quantifying *Drosophila* food intake: comparative analysis of current methodology. *Nature methods,* 11(5), pp. 535-540.

Diegelmann, S. et al. (2017). The Capillary feeder assay measures food intake in *Drosophila melanogaster*. *J. Vis. Exp.,* 2017 (121).

Dong, Q.-Q.et al. (2018). SMYD3-associated pathway is involved in the anti-tumor effects of sulforaphane on gastric carcinoma cells. *Food Science and Biotechnology,* 27 (4), pp. 1165-1173.

Doonan, R. et al. (2008). Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in *Caenorhabditis elegans*. *Genes & development,* 22 (23), pp. 3236-3241.

Dueñas-García, I. et al. (2012). Interactions of sulforaphane and dimethyl sulfoxide with methyl methanesulfonate, urethane, 4-nitroquinoline-1-oxide and hydrogen

peroxide in the *Drosophila melanogaster* wing spot test. *Food and Chemical Toxicology,* 50 (12), pp. 4479-4486.

Duran, A. et al. (2013). Shared Ageing Research Models (ShARM): a new facility to support ageing research. *Biogerontology*, 14 (6), pp: 789-794.

Fahey, J. W. et al. (2015). Sulforaphane Bioavailability from Glucoraphanin-Rich Broccoli: Control by Active Endogenous Myrosinase. *PLOS ONE,* 10 (11), p. e0140963.

Feany, M. B. & Bender, W. W. (2000). A *Drosophila* model of Parkinson's disease. *Nature,* 404 (6776), pp. 394-398.

Fishman, S. L. et al. (2018). The role of advanced glycation end-products in the development of coronary artery disease in patients with and without diabetes mellitus: a review. *Molecular Medicine,* 24 (1), p. 59.

Flores, I. & Blasco, M. A. (2010). The role of telomeres and telomerase in stem cell aging. *FEBS Letters,* 10 9, 584(17), pp. 3826-3830.

Fontana, L., Partridge, L. and Longo, V. (2010). Extending healthy life span-from yeast to humans. *Science*, 328 (5976), pp: 321-326.

Franklin, S. J. et al. (2014). Stability of sulforaphane for topical formulation. *Drug Development and Industrial Pharmacy,* 40 (4), pp. 494-502.

Fumagalli, M. et al. (2012). Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. *Nature cell biology,* 14 (4), pp. 355-365.

Gabriel, D., Roedl, D., Gordon, L. B. & Djabali, K. (2015). Sulforaphane enhances progerin clearance in Hutchinson-Gilford progeria fibroblasts. *Aging cell,* 14 (1), pp. 78-91.

Gan, N. et al. (2010). Sulforaphane activates heat shock response and enhances proteasome activity through up-regulation of Hsp27. *The Journal of biological chemistry,* 285 (46), pp. 35528-35536.

Gao, J. et al. (2018). Sulforaphane Alleviates Lipopolysaccharide-induced Spatial Learning and Memory Dysfunction in Mice: The Role of BDNF-mTOR Signaling Pathway. *Neuroscience,* 388, pp. 357-366.

Garinis, G. A., van der Horst, G. T. J., Vijg, J. & Hoeijmakers, J. H. J. (2008). DNA damage and ageing: new-age ideas for an age-old problem. *Nature cell biology,* 10 (11), pp. 1241-1247.

Geng, Y. et al. (2017). Sulforaphane Induced Apoptosis via Promotion of Mitochondrial Fusion and ERK1/2-Mediated 26S Proteasome Degradation of Novel Pro-survival Bim and Upregulation of Bax in Human Non-Small Cell Lung Cancer Cells. *Journal of Cancer,* 8 (13), pp. 2456-2470.

Gkogkolou, P. and Bohm, M. (2012). Advanced glycation end products: Key players in skin aging? *Dermatoendocrinol*, 4 (3), pp: 259-270.

Golubev, A., Hanson, A. D. & Gladyshev, V. N. (2018). A Tale of Two Concepts: Harmonizing the Free Radical and Antagonistic Pleiotropy Theories of Aging. *Antioxidants & Redox Signaling,* 29 (10), pp. 1003-1017.

González-Suárez, E. et al. (2001). Increased epidermal tumors and increased skin wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes. *The EMBO journal,* 20 (11), pp. 2619-2630.

Grandison, R. C. et al. (2009). Effect of a Standardised Dietary Restriction Protocol on Multiple Laboratory Strains of *Drosophila melanogaster*. *PLoS ONE,* 4 (1), p. e4067.

Grünwald, S. et al. (2013). Longevity in the red flour beetle *Tribolium castaneum* is enhanced by broccoli and depends on nrf-2, jnk-1 and foxo-1 homologous genes. *Genes & nutrition,* 8 (5), pp. 439-448.

Gu, Z. X., Guo, Q. H. & Gu, Y. J. (2012). Factors Influencing Glucoraphanin and Sulforaphane Formation in *Brassica* Plants: A Review. *Journal of Integrative Agriculture*, 11 (11), pp. 1804-1816.

Guerrero-Beltrán, C., et al. (2012). Protective effect of sulforaphane against oxidative stress: Recent advances. *Experimental and Toxicologic Pathology*, 64 (5), pp: 503-508.

Hariton, F. et al. (2018). Sulforaphane Delays Fibroblast Senescence by Curbing Cellular Glucose Uptake, Increased Glycolysis, and Oxidative Damage. *Oxidative medicine and cellular longevity,* 2018, p. 5642148.

Harries, L. W. (2014). MicroRNAs as Mediators of the Ageing Process. *Genes,* 5 (3), pp. 656-670.

He, Y. and Jasper, H. (2014). Studying aging in *Drosophila*. *Methods*, 68 (1), pp: 129-133.

Ho, E., Clarke, J. D. & Dashwood, R. H. (2009). Dietary sulforaphane, a histone deacetylase inhibitor for cancer prevention. *The Journal of nutrition,* 139 (12), pp. 2393-2396.

Hu, R. et al. (2004). In Vivo Pharmacokinetics and Regulation of Gene Expression Profiles by Isothiocyanate Sulforaphane in the Rat. *The Journal of Pharmacology and Experimental Therapeutics*. 310 (1), pp: 263-271.

Huang, Y., Li, W., Su, Z.-y. & Kong, A.-N. T. (2015). The complexity of the Nrf2 pathway: beyond the antioxidant response. *The Journal of nutritional biochemistry,* 26 (12), pp. 1401-1413.

Itoh, K. et al. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes & development,* 13 (1), pp. 76-86.

Jain, A. et al. (2010). p62/SQSTM1 Is a Target Gene for Transcription Factor NRF2 and Creates a Positive Feedback Loop by Inducing Antioxidant Response Element-driven Gene Transcription. *Journal of Biological Chemistry,* 285 (29), pp. 22576-22591.

Jang, Y. C. et al. (2009). Overexpression of Mn superoxide dismutase does not increase life span in mice. *The journals of gerontology. Series A, Biological sciences and medical sciences,* 64 (11), pp. 1114-1125.

Janzen, V. et al. (2006). Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature,* 443 (7110), pp. 421-426.

Jaul, E. & Barron, J. (2017). Age-Related Diseases and Clinical and Public Health Implications for the 85 Years Old and Over Population. *Frontiers in public health,* 5, p. 335.

Jiang, Y. & Ji, J. Y. (2018). Understanding lamin proteins and their roles in aging and cardiovascular diseases. *Life Sciences,* 212, pp. 20-29.

Johnson, S. C., Rabinovitch, P. S. & Kaeberlein, M. (2013). mTOR is a key modulator of ageing and age-related disease. *Nature,* 493 (7432), pp. 338-345.

Kanfi, Y. et al. (2012). The sirtuin SIRT6 regulates lifespan in male mice. *Nature,* 483 (7388), pp. 218-221.

Kanki, T. et al. (2009). Atg32 is a mitochondrial protein that confers selectivity during mitophagy. *Developmental cell,* 17 (1), pp. 98-109.

Kapeta, S., Chondrogianni, N. & Gonos, E. S. (2010). Nuclear erythroid factor 2-mediated proteasome activation delays senescence in human fibroblasts. *The Journal of biological chemistry,* 285 (11), pp. 8171-8184.

Kippin, T. E., Martens, D. J. & van der Kooy, D. (2005). p21 loss compromises the relative quiescence of forebrain stem cell proliferation leading to exhaustion of their proliferation capacity. *Genes & development,* 19 (6), pp. 756-767.

Kirkland, J. and Tchkonia, T. (2015), Clinical strategies and animal models for developing senolytic agents. *Experimental Gerontology*, 68, pp. 19-25.

Komatsu, M. et al. (2010). The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nature Cell Biology,* 12 (3), pp. 213-223.

Koudounas, S., Green, E. and Clancy, D. (2012). Reliability and variability of sleep and activity as biomarkers of ageing in *Drosophila*. *Biogreontology*, 13 (5), pp: 489-499.

Kubo, E. et al. (2017). Sulforaphane reactivates cellular antioxidant defense by inducing Nrf2/ARE/Prdx6 activity during aging and oxidative stress. *Scientific Reports,* 7 (1), p. 14130.

Kwak, M-K. et al. (2007). Role of increased expression of the proteasome in the protective effects of sulforaphane against hydrogen peroxide-mediated cytotoxicity in murine neuroblastoma cells. *Free Radical Biology and Medicine,* 43 (5), pp. 809-817.

Labbadia, J. & Morimoto, R. I. (2015). The biology of proteostasis in aging and disease.. *Annual review of biochemistry,* 84, pp. 435-464.

Lai, R. H. et al. (2008). Evaluation of the safety and bioactivity of purified and semi-purified glucoraphanin. *Food and Chemical Toxicology*, 46 (1), pp. 195-202.

Lakkappa, N., et al. (2018). Evaluation of antiparkinson activity of PTUPB by measuring dopamine and its metabolites in *Drosophila melanogaster*: LC–MS/MS method development. *J. Pharm. Biomed. Anal.,* 149, pp: 457-464.

Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harbor perspectives in biology*, 1 (6).

Le Bourg, E. & Lints, F. A. (1992). Hypergravity and Aging in *Drosophila melanogaster* Climbing Activity. *Gerontology,* 38 (1-2), pp. 59-64.

Lees, H., Walters, H. & Cox, L. S. (2016). Animal and human models to understand ageing. *Maturitas,* 93, pp. 18-27.

Lee, Y.-J. & Lee, S.-H. (2011). Sulforaphane induces antioxidative and antiproliferative responses by generating reactive oxygen species in human bronchial epithelial BEAS-2B cells. *Journal of Korean medical science,* 26 (11), pp. 1474-1482.

Lewinska, A., Adamczyk-Grochala, J., Deregowska, A. & Wnuk, M. (2017). Sulforaphane-Induced Cell Cycle Arrest and Senescence are accompanied by DNA Hypomethylation and Changes in microRNA Profile in Breast Cancer Cells. *Theranostics,* 7(14), pp. 3461-3477.

Liu, N. et al. (2012). The microRNA miR-34 modulates ageing and neurodegeneration in *Drosophila*. *Nature*, 482 (7386), pp. 519-523.

Li, W. et al. (2010). An internal ribosomal entry site mediates redox-sensitive translation of Nrf2. *Nucleic acids research*, 38 (3), pp. 778-788.

López-Otín, C. et al. (2013). The hallmarks of aging. *Cell*, 153 (6), pp. 1194-1217.

Lőrincz, P., Mauvezin, C. & Juhász, G. (2017). Exploring Autophagy in *Drosophila*. *Cells,* 6 (3).

Lozanovski, V. J. et al. (2014). Pilot study evaluating broccoli sprouts in advanced pancreatic cancer (POUDER trial) - study protocol for a randomized controlled trial. *Trials,* 15, p. 204.

Lushchak, O. V. et al. (2012). Balance Between Macronutrients Affects Life Span and Functional Senescence in Fruit Fly *Drosophila melanogaster*. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 67A (2), pp. 118-125.

Ma, Q. (2013). Role of Nrf2 in Oxidative Stress and Toxicity. *Annual Review of Pharmacology and Toxicology*, 53 (1), pp. 401-426.

Maegawa, S. et al. (2010). Widespread and tissue specific age-related DNA methylation changes in mice. *Genome research*, 20 (3), pp. 332-340.

Makos, M., et al. (2009). Chemical measurements in *Drosophila*. *Trends in Analytical Chemistry*, 28 (11), pp: 1223-1234.

Martin-Montalvo, A. et al. (2013). Metformin improves healthspan and lifespan in mice. *Nature communications,* 4, p. 2192.

Martin, S. L., Kala, R. & Tollefsbol, T. O. (2018). Mechanisms for the Inhibition of Colon Cancer Cells by Sulforaphane through Epigenetic Modulation of MicroRNA-21 and Human Telomerase Reverse Transcriptase (hTERT) Down-regulation. *Current cancer drug targets,* 18 (1), pp. 97-106.

Matsui, T. et al. (2016). Sulforaphane reduces advanced glycation end products (AGEs)-induced inflammation in endothelial cells and rat aorta. *Nutrition, Metabolism and Cardiovascular Diseases*, 26(9), pp. 797-807.

McGinnis, N., Ragnhildstveit, E., Veraksa, A. & McGinnis, W. (1998). A cap 'n' collar protein isoform contains a selective Hox repressor function. *Development (Cambridge, England)*, 125(22), pp. 4553-4564.

Min, J.-N.et al. (2008). CHIP deficiency decreases longevity, with accelerated aging phenotypes accompanied by altered protein quality control. *Molecular and cellular biology*, 28 (12), pp. 4018-4025.

Misra, J. R., Horner, M. A., Lam, G. & Thummel, C. S. (2011). Transcriptional regulation of xenobiotic detoxification in *Drosophila*.. *Genes & development*, 25 (17), pp. 1796-1806.

Moreno, D., et al. (2006). Chemical and biological characterisation of nutraceutical compounds of broccoli. *J. Pharm. Biomed. Anal.*, 41 (5), pp: 1508-1522.

Morrow, G., Samson, M., Michaud, S. & Tanguay, R. M. (2004). Overexpression of the small mitochondrial Hsp22 extends *Drosophila* life span and increases resistance to oxidative stress. *The FASEB Journal*, 18 (3), pp. 598-599.

Mostoslavsky, R. et al. (2006). Genomic Instability and Aging-like Phenotype in the Absence of Mammalian SIRT6. *Cell*, 124 (2), pp. 315-329.

Muñoz-Lorente, M. A. et al. (2018). AAV9-mediated telomerase activation does not accelerate tumorigenesis in the context of oncogenic K-Ras-induced lung cancer. *PLOS Genetics*, 14 (8), p. e1007562.

Myzak, M. C., Karplus, P. A., Chung, F.-L. & Dashwood, R. H. (2004). A Novel Mechanism of Chemoprotection by Sulforaphane. *Cancer Research*, 64 (16), pp. 5767-5774.

Negrette-Guzmán, M., Huerta-Yepez, S., Tapia, E. & Pedraza-Chaverri, J. (2013). Modulation of mitochondrial functions by the indirect antioxidant sulforaphane: A seemingly contradictory dual role and an integrative hypothesis. *Free Radical Biology and Medicine*, 65, pp. 1078-1089.

Nelson, G., Kucheryavenko, O., Wordsworth, J. & von Zglinicki, T. (2018). The senescent bystander effect is caused by ROS-activated NF-κB signalling. *Mechanisms of ageing and development,* 170, pp. 30-36.

Nelson, G. et al. (2012). A senescent cell bystander effect: senescence-induced senescence. *Aging cell*, 11 (2), pp. 345-9.

O'Mealey, G. B., Berry, W. L. & Plafker, S. M. (2017). Sulforaphane is a Nrf2-independent inhibitor of mitochondrial fission. *Redox Biology*, 11, pp. 103-110.

Ong, C. et al. (2015). *Drosophila melanogaster* as a model organism to study nanotoxicity. *Nanotoxicology*, 9 (3), pp. 396-403.

Onken, B. & Driscoll, M. (2010). Metformin induces a dietary restriction-like state and the oxidative stress response to extend *C. elegans* Healthspan via AMPK, LKB1, and SKN-1.. *PloS one,* 5 (1), p. e8758.

Pasyukova, E. G. & Vaiserman, A. M. (2017). HDAC inhibitors: A new promising drug class in anti-aging research. *Mechanisms of Ageing and Development*, 166, pp. 6-15.

Pérez-Balibrea, S., Moreno, D. and García-Viguera, C. (2011). Improving the phytochemical composition of broccoli sprouts by elicitation. *Food Chemistry*, 129 (1), pp: 35-44.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research*, 29 (9), p. e45.

Pfaffl, M.W., Horgan, G.W. and Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in realtime PCR. *Nucleic acids research*, 30, e36.

Piantadosi, C. A., Carraway, M. S., Babiker, A. & Suliman, H. B. (2008). Heme Oxygenase-1 Regulates Cardiac Mitochondrial Biogenesis via Nrf2-Mediated Transcriptional Control of Nuclear Respiratory Factor-1. *Circulation Research*, 103 (11), pp. 1232-1240.

Picca, A. et al. (2019). Mitochondrial Dysfunction and Aging: Insights from the Analysis of Extracellular Vesicles. *International journal of molecular sciences,* 20 (4).

Pircs, K. et al. (2012). Advantages and Limitations of Different p62-Based Assays for Estimating Autophagic Activity in *Drosophila*. *PLoS ONE*, 7 (8), p. e44214.

Plafker, S. M. (2010). Oxidative stress and the ubiquitin proteolytic system in age-related macular degeneration. *Advances in experimental medicine and biology,* 664, pp. 447-456.

Pomatto, L. C. & Davies, K. J. (2018). Adaptive homeostasis and the free radical theory of ageing. *Free Radical Biology and Medicine*, 124, pp. 420-430.

Pomatto, L. C. D. et al. (2017). The age- and sex-specific decline of the 20s proteasome and the Nrf2/CncC signal transduction pathway in adaption and resistance to oxidative stress in *Drosophila melanogaster*. *Aging,* 9 (4), pp. 1153-1185.

Ponton, F., et al. (2011). Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in Drosophila melanogaster. *J. Insect Physiology,* 57, pp: 840-850.

Prasain, J., Carlson, S. and Wyss, J. Flavonoids ad age-related disease: Risk, benefits and critical windows. *Maturitas*, 66 (2), pp: 163-171.

Qin, S. et al. (2018). Sulforaphane attenuates microglia-mediated neuronal necroptosis through down-regulation of MAPK/NF-κB signaling pathways in LPS-activated BV-2 microglia. *Pharmacological Research*, 133, pp. 218-235.

Queen, B. L. and Tollefsbol, T. O. (2010). Polyphenols and aging. *Curr. Aging Sci*, 3 (1), pp: 34-42.

Raffaello, A. & Rizzuto, R. (2011). Mitochondrial longevity pathways. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1813 (1), pp. 260-268.

Ragnauth, C. D. et al. (2010). Prelamin A Acts to Accelerate Smooth Muscle Cell Senescence and Is a Novel Biomarker of Human Vascular Aging. *Circulation*, 121 (20), pp. 2200-2210.

Rajendran, P. et al. (2013). HDAC turnover, CtIP acetylation and dysregulated DNA damage signaling in colon cancer cells treated with sulforaphane and related dietary isothiocyanates. *Epigenetics*, 8 (6), pp. 612-623.

Repiso, A., Bergantiños, C., Corominas, M. & Serras, F. (2011). Tissue repair and regeneration in *Drosophila* imaginal discs. *Development, Growth & Differentiation*, 53 (2), pp. 177-185.

Rera, M. et al. (2011). Modulation of Longevity and Tissue Homeostasis by the *Drosophila* PGC-1 Homolog. *Cell Metabolism*, 14 (5), pp. 623-634.

Riso, P. et al. (2009). Effect of Broccoli Intake on Markers Related to Oxidative Stress and Cancer Risk in Healthy Smokers and Nonsmokers. *Nutrition and Cancer*, 61 (2), pp. 232-237.

Ross, C. A. & Poirier, M. A. (2004). Protein aggregation and neurodegenerative disease. *Nature Medicine*, 10 (S7), pp. S10-S17.

Rovillain, E. et al. (2011). Activation of nuclear factor-kappa B signalling promotes cellular senescence. *Oncogene*, 30 (20), pp. 2356-66.

Roy, S. K., Srivastava, R. K. & Shankar, S. (2010). Inhibition of PI3K/AKT and MAPK/ERK pathways causes activation of FOXO transcription factor, leading to cell cycle arrest and apoptosis in pancreatic cancer. *Journal of molecular signaling*, 5, p. 10.

Samieri, C., et al. (2014). Dietary flavonoid intake at midlife and healthy aging in women. *Am. J. Clin. Nutrition,* 100 (6), pp: 1489-1497.

Schumacher, B., et al. (2008). Delayed and accelerated aging share common longevity assurance mechanisms. *PLoS Genetics*, 4 (8).

Schwartz, S. & Rhiner, C. (2018). Reservoirs for repair? Damage-responsive stem cells and adult tissue regeneration in *Drosophila*. *The International Journal of Developmental Biology*, 62 (6-7-8), pp. 465-471.

Shankar, S., Ganapathy, S. & Srivastava, R. K. (2008). Sulforaphane Enhances the Therapeutic Potential of TRAIL in Prostate Cancer Orthotopic Model through Regulation of Apoptosis, Metastasis, and Angiogenesis. 14 (21), pp. 6855-6866.

Shell, B. C. et al. (2018). Measurement of solid food intake in Drosophila via consumption-excretion of a dye tracer. *Scientific Reports*, 8 (1), p. 11536.

Sheng, Y. et al. (2017). Opposing effects on cardiac function by calorie restriction in different-aged mice. *Aging Cell*, 16 (5), pp. 1155-1167.

Sivandzade, F., Prasad, S., Bhalerao, A. & Cucullo, L. (2019). NRF2 and NF-κB interplay in cerebrovascular and neurodegenerative disorders: Molecular mechanisms and possible therapeutic approaches. *Redox biology,* 21, p. 101059.

Speakman, J. R. et al. (2015). Oxidative stress and life histories: unresolved issues and current needs. *Ecology and Evolution*, 5 (24), pp. 5745-5757.

Spencer, C. C., Howell, C. E., Wright, A. R. & Promislow, D. E. L. (2003). Testing an 'aging gene' in long-lived *Drosophila* strains: increased longevity depends on sex and genetic background. *Aging Cell,* 2 (2), pp. 123-130.

Subedi, L. et al. (2019). Anti-Inflammatory Effect of Sulforaphane on LPS-Activated Microglia Potentially through JNK/AP-1/NF-κB Inhibition and Nrf2/HO-1 Activation. *Cells,* 8 (2).

Sun, Z., Huang, Z. & Zhang, D. D. (2009). Phosphorylation of Nrf2 at Multiple Sites by MAP Kinases Has a Limited Contribution in Modulating the Nrf2-Dependent Antioxidant Response. *PLoS ONE*, 4 (8), p. e6588.

Su, Z. Y. et al. (2013). Epigenetic Reactivation of Nrf2 in Murine Prostate Cancer TRAMP C1 Cells by Natural Phytochemicals Z-Ligustilide and Radix Angelica Sinensis via Promoter CpG Demethylation. *Chemical Research in Toxicology*, 26 (3), pp. 477-485.

Sykiotis, G. P. & Bohmann, D. (2008). Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. *Developmental cell*, 14 (1), pp. 76-85.

Thornalley, P. J. (2005). Dicarbonyl Intermediates in the Maillard Reaction. *Annals of the New York Academy of Sciences*, 1043 (1), pp. 111-117.

Thurmond J, et al. (2019) FlyBase 2.0: the next generation. *Nucleic Acids Res*. 47(D1), D759–D765.

Tower, J. (2015). Programmed cell death in aging. *Ageing research reviews*, 23 (Pt A), pp. 90-100.

Trinh, K. et al. (2008). Induction of the Phase II Detoxification Pathway Suppresses Neuron Loss in *Drosophila* Models of Parkinson's Disease. *Journal of Neuroscience*, 28 (2), pp. 465-472.

Ulgherait, M. et al. (2014). AMPK modulates tissue and organismal aging in a non-cell-autonomous manner. *Cell reports*, 8 (6), pp. 1767-1780.

Ullah, M. F. (2015). Sulforaphane (SFN): An Isothiocyanate in a Cancer Chemoprevention Paradigm. *Medicines (Basel, Switzerland)*, 2 (3), pp. 141-156.

Van Deursen, J. M. (2014). The role of senescent cells in ageing. *Nature*, 509 (7501), pp. 439-446.

Van Hiel, M. B., et al. (2009). Identification and validation of housekeeping genes in brains of the desert locust Schistocerca gregaria under different developmental conditions. *BMC Molecular Biology,* 10 (56).

Van Raamsdonk, J. M. & Hekimi, S. (2012). Superoxide dismutase is dispensable for normal animal lifespan. *Proceedings of the National Academy of Sciences of the United States of America*, 109 (15), pp. 5785-5790.

Vasiliou, V., Ross, D. & Nebert, D. W. (2006). Update of the NAD(P)H:quinone oxidoreductase (NQO) gene family. *Human genomics*, 2 (5), pp. 329-335.

Vázquez-Gómez, G. et al. (2010). Sulforaphane modulates the expression of Cyp6a2 and Cyp6g1 in larvae of the ST and HB crosses of the *Drosophila* wing spot test and is genotoxic in the ST cross. *Food and Chemical Toxicology*, 48 (12), pp. 3333-3339.

Villatoro-Pulido, M. et al. (2012). In vivo biological activity of rocket extracts (*Eruca vesicaria* subsp. *sativa* (Miller) Thell) and sulforaphane. *Food and Chemical Toxicology*, 50 (5), pp. 1384-1392.

Walters, H. E. & Cox, L. S. (2018). mTORC Inhibitors as Broad-Spectrum Therapeutics for Age-Related Diseases. *International journal of molecular sciences,* 19 (8).

Wang, K. & Klionsky, D. J. (2011). Mitochondria removal by autophagy. *Autophagy*, 7 (3), pp. 297-300.

Wang, Y. et al. (2018). Roles of ROS, Nrf2, and autophagy in cadmium-carcinogenesis and its prevention by sulforaphane. *Toxicology and Applied Pharmacology,* 353, pp. 23-30.

Wehr, N. B. & Levine, R. L. (2012). Quantitation of protein carbonylation by dot blot.. *Analytical biochemistry*, 423 (2), pp. 241-245.

Wong, R., Piper, M. D. W., Wertheim, B. & Partridge, L. (2009). Quantification of Food Intake in *Drosophila*. *PLoS ONE*, 4 (6), p. e6063.

WWW Cayman Chem. 2020. Cayman Chemical. [ONLINE] Available at: https://www.caymanchem.com/pdfs/14797.pdf. [Accessed 18 January 2020].

WWW Evgen Pharma. 2019. Evgen Pharma Technology. [ONLINE] Available at: http://evgen.com/technology/. [Accessed 12 January 2019].

WWW Flybase. 2020. Flybase Gene Report: Dmel\αTub84B. [ONLINE] Available at: http:// flybase.org/reports/FBgn0003884. [Accessed 20 January 2020].

WWW Flybase. 2020. Flybase Gene Report: Dmel\eEF1γ. [ONLINE] Available at: http:// flybase.org/reports/FBgn0029176. [Accessed 20 January 2020].

WWW Flybase. 2019. Flybase Gene Report: Dmel\Glo1. [ONLINE] Available at: http://flybase.org/reports/FBgn0283450. [Accessed 25 May 2019].

WWW Flybase. 2019. Flybase Gene Report: Dmel\GstD1. [ONLINE] Available at: http://flybase.org/reports/FBgn0001149. [Accessed 25 May 2019].

WWW Flybase. 2019. Flybase Gene Report: Dmel\Keap1. [ONLINE] Available at: http://flybase.org/reports/FBgn0038475. [Accessed 25 May 2019].

WWW Flybase. 2020. Flybase Gene Report: Dmel\ RpL32. [ONLINE] Available at: http:// flybase.org/reports/FBgn0002626. [Accessed 20 January 2020].

WWW Gene-quantification. 2020. Gene Quantification. [ONLINE] Available at: https://www.gene-quantification.de/REST_2009_Software_User_Guide.pdf. [Accessed 15 January 2020].

Xie, J., Méndez, J. D., Méndez-Valenzuela, V. & Aguilar-Hernández, M. M. (2013). Cellular signalling of the receptor for advanced glycation end products (RAGE). *Cellular Signalling*, 25 (11), pp. 2185-2197.

Xu, C. et al. (2005). Suppression of NF-κB and NF-κB-regulated gene expression by sulforaphane and PEITC through IκBα, IKK pathway in human prostate cancer PC-3 cells. *Oncogene*, 24 (28), pp. 4486-4495.

Yang, F. et al. (2018). Sulforaphane induces autophagy by inhibition of HDAC6-mediated PTEN activation in triple negative breast cancer cells. *Life Sciences,* 213, pp. 149-157.

Yang, W., Li, J. & Hekimi, S. (2007). A Measurable increase in oxidative damage due to reduction in superoxide detoxification fails to shorten the life span of long-lived mitochondrial mutants of *Caenorhabditis elegans*. *Genetics,* 177 (4), pp. 2063-2074.

Yuan Z., Rezai-Zadeh N., Zhang X., Seto E. (2009) Histone Deacetylase Activity Assay. In: Chellappan S. (2nd edn) Chromatin Protocols. Methods in Molecular Biology (Methods and Protocols), vol 523. Humana Press.

Yu, R., et al. (1998). Chemopreventive isothiocyanates induce apoptosis ad caspase-3-like protease activity. *Cancer Research*, 58 (3), pp. 402-408.

Yu, S. et al. (2010). Nrf2 Expression Is Regulated by Epigenetic Mechanisms in Prostate Cancer of TRAMP Mice. *PLoS ONE*, 5 (1), p. e8579.

Yu, W., Srivastava, R. & Shankar, S. (2015). *Abstract A121: Suppression of orthotopic pancreatic cancer growth by sulforaphane is associated with activation of FOXO transcription factors.* s.l., American Association for Cancer Research, pp. A121-A121.

Zhang, M. et al. (2013). Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. *Progress in neurobiology*, 100, pp. 30-47.

Zhang, Y. et al. (2009). Mice deficient in both Mn superoxide dismutase and glutathione peroxidase-1 have increased oxidative damage and a greater incidence of pathology but no reduction in longevity. *The journals of gerontology. Series A, Biological sciences and medical sciences*, 64 (12), pp. 1212-1220.

Zhang, Y. & Tang, L. (2007). Discovery and development of sulforaphane as a cancer chemopreventive phytochemical. *Acta Pharmacologica Sinica*, 28 (9), pp. 1343-1354.

Zhang, Z. et al. (2014). Sulforaphane prevents the development of cardiomyopathy in type 2 diabetic mice probably by reversing oxidative stress-induced inhibition of LKB1/AMPK pathway. *Journal of Molecular and Cellular Cardiology,* 77, pp. 42-52.

Zhao, F., Zhang, J. & Chang, N. (2018). Epigenetic modification of Nrf2 by sulforaphane increases the antioxidative and anti-inflammatory capacity in a cellular model of Alzheimer's disease. *European Journal of Pharmacology*, 824, pp. 1-10.

Zhou, Q. et al. (2016). Sulforaphane protects against rotenone-induced neurotoxicity in vivo: Involvement of the mTOR, Nrf2, and autophagy pathways. *Scientific reports,* 6, p. 32206.

Zhu, Y. et al. (2015). The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. *Aging cell*, 14 (4), pp. 644-658.