Investigating the Influence of Dietary Restriction and Omega-3 Fatty Acid Supplementation on Lifespan and Sarcopenia in *C. elegans*

by

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Abbreviations

DR	Dietary Restriction
ELC	Essential Light Chain
RLC	Regulatory Light Chain
EWGSOP	European Working Group on Sarcopenia in Older People
RT	Resistance Training
DXA	Dual-Energy X-Ray
MRI	Magnetic Resonance Imaging
VAT	Visceral Adipose Tissue
IMAT	Intermuscular adipose tissue
CSA	Cross-sectional area
EDL	Extensor Digitorum Longus
ROS	Reactive oxygen species
ETC	Electron Transport Chain
IL-6	Interleukin 6
SCFA	Short chain fatty acids
LPS	Liposaccharide
GFP	Green Fluorescent Protein
CGC	Caenorhabditis Genetics Center
MPS	Muscle Protein Synthesis
FOXO	Forkhead Box Protein O
mTOR	Mechanistic Target of Rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
AMPK	AMP-activated protein kinase
PAR-1	Protease-activated receptor 1
MPB	Muscle protein breakdown
MUFA	Mono-unsaturated fatty acid
PUFA	Polyunsaturated fatty acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
NHE	National Health Expenditures
MHC B	Myosin Heavy Chain B
FUDR	5-fluoro-2’deoxyuridine
LB	Luria Broth
NGM	Nutrient Growth Media
CFU	Colony Forming Unit
FAME	Fatty acid methyl esters
SMM	Skeletal muscle mass

sDR	Solid Dietary Restriction
TGFβ	Transforming Growth Factor-β
ALA	Alpha linoleic acid
AA	Arachidonic acid
GC-MS	Gas Chromatography Mass Spectrometry
NIST	National Institute of Standards and Technology
2PAC	2 phases of ageing in *C. elegans*
SIR	Smurf increase rate

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Abstract

Sarcopenia is an age-related condition defined by a progressive loss of muscle mass and strength over time. It is currently recognized as one of the major public health problems in the elderly populations worldwide with people over the age of 65 experiencing some form or symptoms of sarcopenia. Despite the ever-increasing prevalence of sarcopenia, the underlying mechanism behind sarcopenia is still an area of ongoing research. Intervention studies incorporating dietary supplementation and resistance training have been shown to limit muscle function decline. The resulting need to understand and alleviate the problems associated with sarcopenia has led to an increased use of animal models, including the nematode *Caenorhabditis elegans* which also undergoes sarcopenia with ageing.

The main aim of this thesis was to study the potential effect of Dietary Restriction and Omega-3 PUFA supplements on sarcopenia and to quantify sarcopenia rates using a novel fluorescence technique. Using an internal standard for quantification, a transgenic *C. elegans* strain (*unc-54:gfp*) expressing GFP in one of its muscle myosin isoforms (MHC B) was utilised to measure myosin density throughout its lifespan. *C. elegans* treated with different regimens of DR showed that lifespan increased significantly under a mildly restricted diet, consistent with the findings in previous studies. Healthspan was also significantly improved, as indicated by a delay in the age-related decrease in motility and reduction in sarcopenia rates. Additionally, long-term algae-oil supplementation resulted in significantly lower rates of sarcopenia and a corresponding increase in motility and lifespan. It has been proposed that the microbiota regulates skeletal muscle growth and function via a gut-muscle axis. In this study, algae-oil supplementation delayed the onset of sarcopenia with reduced decline of muscle density, which correlated with delayed onset of age-related intestinal barrier dysfunction. These results support the hypothesis of an association between microbiota and sarcopenia (gut-muscle

axis), indicating a novel role for algae-oil whose clinical relevance should be investigated in future studies.

Chapter 1: General Introduction

1.1 Skeletal muscle – Structure and Function

Human skeletal muscles are composed of bundles of muscle fibers called fascicles which in turn are made of repetitive functional units called sarcomeres (Mukund and Subramaniam, 2020). Each sarcomere is composed of parallel, overlapping thin (actin) and thick (myosin) filaments (Frontera and Ochala, 2015) (Figure 1.1). The myosin protein is a hexamer composed of two heavy chains, organized into three structurally and functionally different domains (Girón-Pérez, Piedra-Quintero and Santos-Argumedo, 2019). The head or motor domain is globular with ATP and actin binding sites, responsible for generating force; the neck region adjacent to the head domain is α-helical region of variable length and binds two pairs of myosin light chains (MLCs): essential light chain (ELC) and regulatory light chain (RLC) (Girón-Pérez, Piedra-Quintero and Santos-Argumedo, 2019). Whilst the tail domain consists of binding sites that are believed to be involved in determining the function of the particular myosin (Girón-Pérez, Piedra-Quintero and Santos-Argumedo, 2019).

When the actin and myosin filaments slide past each other, the muscle contracts, resulting in a shortening of the sarcomere and thus the muscle (Frontera and Ochala, 2015). This mechanism of contraction is known as the sliding filament theory (Herzog, 2017) (Figure 1. 1). Skeletal muscles are divided into two main types of muscle fibers, type I and type II (Talbot and Maves, 2016). Type I (slow) muscle fibers are composed of the slow-twitch muscle fibers and are employed during endurance activities such as long-distance running while the type II (fast) muscle fibers are composed of fast-twitch muscle fibers and are employed for a quick burst of energy such as sprinting (Yin *et al.*, 2021).

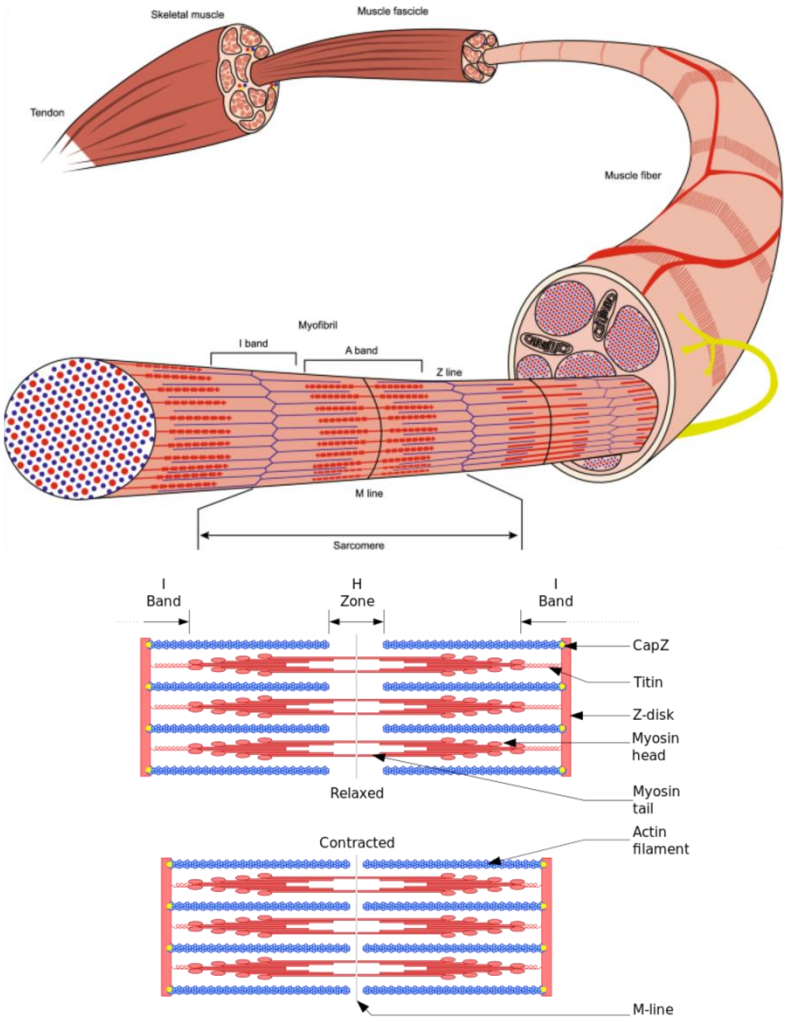

Figure 1. 1 Schematic representation of a skeletal muscle fiber structure. During relaxation, the thin filaments do not completely cover the thick filaments of myosin, resulting in a pronounced I band. During contraction, the thin filaments move toward the centre of the sarcomere and since they are tethered to the Z discs, their movement causes the sarcomere to shorten. Image reproduced from (Lim *et al.*, 2021) and (Goodman and Zimmer, 2008).

1.2 Sarcopenia: Ageing of skeletal muscle

Sarcopenia is an age-related decrease of skeletal muscle mass and strength (Tournadre *et al.*, 2019). Sarcopenia impairs mobility and independence in the elderly while also increasing the risk of developing other diseases and death (Tournadre *et al.*, 2019). Although the mechanisms underlying sarcopenia are unclear, it has been recognised as a complex condition involving changes in several critical pathways, all of which contribute to structural and functional decline in skeletal muscle (Wiedmer *et al.*, 2021).

The term, sarcopenia was first coined by Irwin Rosenberg in 1989 (Rolland *et al.*, 2008). The term's roots derive from the greek, where "sarx" refers to flesh and "penia" refers to loss, describing a decline in lean body mass (Rosenberg, 2011). Due to the complex nature of the condition, there are numerous definitions of sarcopenia till date (Cruz-Jentoft *et al.*, 2019). The prevalence of sarcopenia differs significantly, depending on the definition, setting, gender and age group (Therakomen, Petchlorlian and Lakananurak, 2020). For instance, in a community dwelling setting, the prevalence of sarcopenia was reported to be 11% in men and 9% in women, in a nursing home, 51% in men and 31% in women while the rates in hospitalised setting were 23% in men and 24% in women (Papadopoulou *et al.*, 2020). Despite the variations in prevalence of sarcopenia between the different settings, it is evident that a large proportion of the elderly have sarcopenia, including in stable populations (Wallengren *et al.*, 2021).

The most recent definition of sarcopenia as defined by the European Working Group on Sarcopenia in Older People (EWGSOP) is the progressive loss of skeletal muscle mass and function over time (Cruz-Jentoft *et al.*, 2019). The EWGSOP also includes a functional algorithm for detecting sarcopenia through the use of a performance-based questionnaire and exercises (Cruz-Jentoft *et al.*, 2019). The questionnaire is designed to assess the subject's

physical capabilities and allows for quick diagnosis of sarcopenia; however, this method is not always effective as sometimes, and the subjects might over or underestimate their capabilities. Performance-based exercises usually prove more effective as parameters such as muscle mass, strength and gait speed of the subjects are measured and compared against a young, healthy population of adults (Cruz-Jentoft *et al.*, 2019). However, this method is also not without problems as there is a natural variability of these parameters within different populations. Moreover, this screening tool does not take consideration into monitoring the improvement or deterioration of subjects in the future. This means that in clinical practice, doctors are not able to accurately diagnose sarcopenia or sometimes, may run the risk of misdiagnosing sarcopenia for some other age-related conditions (Avgerinou, 2020). As sarcopenia is a multifactorial disease, numerous targets exist for drug discovery, yet the lack of a clear consensus definition of sarcopenia has slowed down the development of drugs against sarcopenia (Beaudart *et al.*, 2014). Much of the sarcopenia research to date has been focused on defining a universally accepted definition of sarcopenia as well as approaches to manage sarcopenia through exercise and nutrition (Cesari *et al.*, 2012). Amongst the numerous interventional strategies developed against sarcopenia, only long-term resistance training (RT) has proven to be effective against sarcopenia (Chen *et al.*, 2021). However, the beneficial effect of RT is usually delayed or limited in older subjects in comparison to the younger subjects (Del Peral and Josa, 2018). This further reinforces the need to conduct more in-depth research on the molecular differences between young and old muscles and hopefully understand how these differences result in such different outcomes.

1.2.1 Age-related changes associated with Sarcopenia

1.2.1.1 Decreased Muscle Mass and Strength

Studies have shown that from the age of 20 to 80 years, there is a staggering 30% decline in muscle mass (Maliszewska, Adamska-Patruno and Krętowski, 2019). As with most age-related conditions, the aetiology of sarcopenia also changes with age (Ogawa, Yakabe and Akishita, 2016). After the age of 50, muscle mass loss occurs at a rate of 1-2% per year (Trevisan *et al.*, 2022). Many studies have reported that the age-related loss of skeletal muscle mass is not uniform across the body with greater loss observed in the lower body than in upper body, suggesting that the rate of sarcopenia differs between the upper and lower extremities (Frontera *et al.*, 2017).

Interestingly, many studies have shown that muscle mass and muscle strength do not decline at a similar rate (Schaap, Koster and Visser, 2013). Muscle strength has been shown to decline at a faster rate than muscle mass with advancing age (Sui *et al.*, 2020). This rapid decline in muscle strength is thought to result from a combination of decreased muscle fibers and reduced muscle protein synthesis (Mitchell *et al.*, 2012). Decreased muscle strength has been reported to place an increased risk to an individual's mobility and mortality as numerous studies have shown a strong association between reduced muscle strength and poor physical performance (Manini and Clark, 2012). As a result, muscle strength appears to be a better predictor of the adverse effects associated with sarcopenia (Menant *et al.*, 2017).

As the number of muscles fibers decreases, the resulting "empty space" between the muscle cells is thought to be inhabited by fat cells, reducing muscle strength and impairing mobility (Forsberg *et al.*, 2015). Dual-energy X-ray (DXA) and Magnetic resonance imaging (MRI) of healthy elderly women showed a significant reduction in total body skeletal muscle and an

increase in visceral adipose tissue (VAT) and intermuscular adipose tissue (IMAT) (Song *et al.*, 2004). While the presence of fat tissues in healthy elderly individual is a common occurrence with ageing, some studies have linked lack of muscle use with the significant increase and accumulation of fat tissues (Hamrick, McGee-Lawrence and Frechette, 2016). In healthy adult males, short periods of inactivity were demonstrated to be sufficient to initiate the formation of IMAT and reduce myofiber cross-sectional area (CSA)(Pagano *et al.*, 2018). High level athletes who trained 4-5 times a week, did not exhibit the characteristic loss of lean muscle mass and increased fat infiltration, associated with ageing in a sedentary population (Wroblewski *et al.*, 2011). This further reinforces the effect of chronic muscle disuse and suggests that the functional decline observed in ageing elderly populations is a consequence of their sedentary lifestyle.

1.2.1.2 Changes in Muscle Fibre Type

With age, the number of type II muscle fiber decreases and the remaining fibers also reduces in size as a result of muscle atrophy (Wall *et al.*, 2013). Using mass-spectrometry-based proteomics, Murgia et al. (2017) found a significant decrease in type II muscle fibers of the elderly subjects in comparison to the young subjects. This significant decrease in type II muscle fibers (Murgia *et al.*, 2017) is in accordance with previous findings where selective atrophy of type II fibers in ageing muscle is frequently observed (Larsson, Grimby and Karlsson, 2017). Moreover, the study also found that the mitochondrial content in both type I and II decreased with increasing age, however, the expression of glycolytic enzymes was significantly higher in older type I muscle fibres compared to younger type I muscle fibres (Murgia *et al.*, 2017). Altogether, these findings suggest that there are clear proteomic differences between type I and II muscle fibres, with type I muscle fibres being better equipped to sustain any changes

associated with ageing. This could possibly explain why over time, a fast to slow muscle fibre transition is observed in ageing muscles (Gannon *et al.*, 2009).

1.2.1.3 Decrease in Satellite Cells

Muscle stem cells known as satellite cells play a critical role in muscle fibre regeneration (Yin, Price and Rudnicki, 2013). Satellite cells in normal adult muscles are usually dormant however following injury, the satellite cells are activated and begin proliferating and differentiating into myoblasts, which then fuse to each other or to nearby pre-existing muscle fibers to repair and replace damaged muscle (Parker, 2015). In addition to their ability to repair damaged muscles, the activated satellite cells also have the ability to reverse to their quiescent state in order to replenish the satellite cell pool (Yin, Price and Rudnicki, 2013). It is important to establish a fine balance between satellite cells that differentiate into new muscle fibres and self-renewing satellite cells that sustain the satellite cell pool (Su *et al.*, 2020). Previous studies have shown that satellite cells undergoing premature differentiation result in muscle regeneration failure and a decrease in the number of satellite cells (Yue *et al.*, 2017). This in effect results in depletion of the satellite cell pool and affects the muscular regenerative capacity (Yue *et al.*, 2017). Many studies have shown that skeletal muscle's regenerative capacity decreases with age, which in turn is thought to lead to muscle mass loss in elderly people (Schüler *et al.*, 2021). Moreover, some studies have found that the extent of the age-related decline in satellite cells differs between muscles (Verdijk *et al.*, 2014). In line with the selective loss and atrophy of fast twitch muscle in sarcopenia, Shefer et al. (2010) reported that the number of satellite cells in slow twitch soleus muscle was higher compared to fast twitch extensor digitorum longus (EDL) at all age groups of mice. Similarly, in humans, Verdijk et al. (2014) found that compared with slow twitch muscle fibre, the size of fast twitch muscle fibre was significantly reduced with age, along with a significant decline in the number of satellite cells. Depletion of

healthy satellite cells is therefore likely to contribute to the decreased capacity of sarcopenic people to regenerate damaged muscle (Alway, Myers and Mohamed, 2014).

1.3 Factors associated with Sarcopenia

Sarcopenia is considered to be a multifactorial disease (Hollingworth *et al.*, 2020). As a result, various factors such as oxidative stress (Nishikawa *et al.*, 2021), chronic inflammation (Chhetri *et al.*, 2018) and changes in gut microbiota (Kang *et al.*, 2021) are all considered to contribute to sarcopenia.

1.3.1 Oxidative Stress

Accumulation of reactive oxygen species (ROS) has generally been known to damage the activity of cellular processes however its effect in ageing muscles is still an ongoing debate (Thoma *et al.*, 2020). Previous studies have shown that oxidative stress increases drastically with age, potentially as a result of altered electron transport chain (ETC) or reduced production of antioxidants (Zhao *et al.*, 2019). Carbonylation, in particular, is reported to be a major marker of oxidative stress in ageing skeletal muscles (Barreiro, 2016). Fast-twitch muscles are shown to be more susceptible to carbonylation with ageing than slow twitch muscles (Thompson, 2009). Moreover, the concentration of ROS is more pronounced in skeletal muscle since oxygen consumption is significantly higher in skeletal muscles in comparison to other muscle (Thompson, 2009). Some studies have suggested that the difference in susceptibility to oxidative stress between fast and slow twitch fibers might be responsible for the fiber-type transition we see in ageing muscles (Smith *et al.*, 2018).

Slow twitch fibres also have a higher concentration of myoglobin than fast twitch fibres, resulting in a higher concentration of antioxidants to counteract the effect of ROS (Kanatous

and Mammen, 2010). The subsequent difference in the concentration of antioxidants is thought to increase susceptibility to ROS in the fast twitch fibers (Loureiro *et al.*, 2016)**.** Additionally, as the slow twitch fiber employs the mitochondrial oxidative phosphorylation pathway to generate energy, it is able to produce a greater concentration of ROS in comparison to fast twitch fibers which use a glycolytic pathway to generate energy (Mishra *et al.*, 2015). This again results in an increased concentration of antioxidants being produced by the slow twitch fibers than the fast twitch fibers(Min *et al.*, 2011). Hence, even though the fast twitch fibers produce less amount of ROS, it might still be more susceptible to oxidative stress since its total antioxidants capacity is less robust (Koutakis *et al.*, 2013).

1.3.2 Chronic Inflammation

Although acute inflammation is important as a transitory response to injury or infections (Sugimoto *et al.*, 2016), chronic inflammation has been linked to numerous age-related conditions such as sarcopenia, Alzheimer's disease and diabetes (Chung *et al.*, 2019). The term inflammaging is often used to refer to chronic low-grade inflammation that contributes to the acceleration of human ageing (Franceschi *et al.*, 2018). Inflammaging is characterised by high levels of circulating pro-inflammatory markers in blood and tissues, associated with age-related diseases (Rea *et al.*, 2018). Consequently, a number of studies have explored the link between pro-inflammatory cytokines and declining muscle mass, strength and physical activity in elderly people (Wang *et al.*, 2017). Elderly women with high levels of inflammatory cytokines such as interleukin 6 (IL-6) had a higher risk of developing physical impairments and a sharper decline in walking compared to women with low levels of IL-6 (Ferrucci *et al.*, 2002). In line with these findings, circulating pro-inflammatory cytokines have been found to be significantly elevated in older sarcopenic patients (Bian *et al.*, 2017). For instance, increased levels of IL-6 and IL-10 were reported in elderly sarcopenia patients compared to non-sarcopenic subjects

(Rong *et al.*, 2018). Since most age-related diseases have an inflammatory origin, inflammaging is a major risk factor for both morbidity and mortality in the elderly (Furman *et al.*, 2019). Many studies suggest that changes in the gut microbiota in ageing contributes to systemic inflammaging (Wang, Chen and Wang, 2020). However, the precise pathophysiology of inflammaging and its causative role in contributing to negative health consequences are still unclear (Neves and Sousa-Victor, 2020).

1.3.3 Gut Microbiota

The human body is home to over 100 trillion microorganisms (Valdes *et al.*, 2018). These include bacteria, viruses, fungi, and other microorganisms which collectively make up the gut microbiome (Valdes *et al.*, 2018). Over the course of evolution, microbes have evolved alongside humans and have an important role in the human body (Huitzil *et al.*, 2018). There are bacteria all over the body, but they are most abundant and diverse in the gastrointestinal tract (Martinez-Guryn, Leone and Chang, 2019). The development of a symbiotic relationship between humans and microorganisms has resulted in the gut microbiome being essential for digestion and the breakdown of complex carbohydrates such as starch and fibre (Malard *et al.*, 2021). In addition to assisting with digestion, it is believed that the gut microbiome plays a significant role in disease prevention and protection (Durack and Lynch, 2019). There is increasing evidence to suggest that the gut microbiota may be linked with sarcopenia (Zhao, Huang and Yu, 2021). Many studies have proposed that the microbiome regulates skeletal muscle growth and function via a so-called gut-muscle axis (Ticinesi *et al.*, 2019). Colonization of germ-free mice with faeces from age-matched pathogen-free mice resulted in an increase in skeletal muscle mass (Lahiri *et al.*, 2019). *Lactobacillus casei*, *Bifidobacterium longum*, *Clostridium coccoides*, and *Barnesiella intestinihominis* are some examples of bacteria that have been found to aid in the growth and development of skeletal muscles (Fielding *et al.*,

2019). These bacterial species are believed to promote skeletal muscle function by producing short chain fatty acids (SCFAs) like acetate, propionate, and butyrate (Lustgarten, 2019). Human studies have also discovered a link between the composition of the elderly gut microbiota and skeletal muscle function (Casati *et al.*, 2019). Sarcopenic patients show a significant reduction in beneficial bacteria that have a high metabolic ability for generating SCFA (Ticinesi *et al.*, 2019). 16s RNA sequencing of human faecal samples revealed a significant reduction in butyrate-producing bacteria in sarcopenic patients compared to control (Kang *et al.*, 2021). In terms of bacterial species that negatively impact skeletal muscle mass, increased abundance of microbial taxa such as *Oscillospira* and *Ruminococcus* have been reported in sarcopenic populations (Picca *et al.*, 2019).

The gut microbiota has been demonstrated to influence gut barrier function, which is critical for maintaining the balance of proinflammatory and anti-inflammatory responses (Picca *et al.*, 2018). An intact intestinal barrier inhibits pathogens and other pro-inflammatory compounds from entering the human body, however disruption of the intestinal barrier enables their entry, generating systemic inflammation (Vancamelbeke and Vermeire, 2017). Intestinal barrier disruption in conjunction with age-related microbial dysbiosis has been associated with intestinal permeability (Grosicki, Fielding and Lustgarten, 2018). Increased intestinal permeability allows microbial by-products such as lipopolysaccharide (LPS) to enter into the bloodstream, causing systemic chronic inflammation and skeletal muscle alterations (Grosicki, Fielding and Lustgarten, 2018). Low levels of SCFA-producing bacteria in the gut microbiota have been linked to increased chronic inflammation and, in the long run, sarcopenia (Ticinesi *et al.*, 2017). Taken together, these studies indicate that structural and functional alterations in the gut microbiota may contribute to the loss of skeletal muscle mass and function in sarcopenic individuals.

1.4 Introduction to the Model Organism *C. elegans*

Nematodes, commonly known as roundworms, are important model organisms in ageing research (Tissenbaum, 2015). Some nematodes are parasitic on plants and animals and can grow to be extremely long; for example, the parasitic sperm whale nematode, *Placentonema gigantissima* can reach a length of 9 meters (Hermosilla *et al.*, 2015). *C. elegans*, on the other hand, is a small, non-parasitic soil nematode that can grow to be 1mm long (Meneely, Dahlberg and Rose, 2019). The name *Caenorhabditis elegans* is derived from the unified greek words caeno- which means recent, rhabditis- which means rod-like, and the latin word elegan- which means elegant (Frézal and Félix, 2015). When *C. elegans* larvae hatch, they are around 0.25 mm long and develop through four larval stages before reaching an adult length of 1mm (Schindler, Baugh and Sherwood, 2014). In its natural habitat, *C. elegans* are found in decomposing organic matter such as rotting fruits and plant stems where it feeds on a variety of bacteria (Guisnet *et al.*, 2021).

C. elegans is a diploid organism with male (XO) and hermaphrodite (XX) sexes (Clarke *et al.*, 2018). Both male and hermaphrodites *C. elegans* have five pairs of somatic chromosomes, however the hermaphrodites have an additional two sex chromosomes while males only have one sex chromosomes (Clarke *et al.*, 2018). The majority of *C. elegans* are hermaphrodites as self-fertilization allows homozygous worms to produce genetically identical offspring (Chasnov, 2013). During its reproductive period, a single hermaphrodite can lay up to 300 eggs (Chasnov, 2013). Male mating, on the other hand, allows for the isolation and maintenance of mutant strains as well as the transfer of mutations across strains (Barr and Garcia, 2006). Male *C. elegans* are rare, with a frequency of 0.02% arising from non-disjunction in hermaphrodites (Barr and Garcia, 2006). The two sexes have distinct anatomy and behaviour that can be differentiated as early as larval development (Meneely, Dahlberg and Rose, 2019). Males and

hermaphrodites can be distinguished by their morphological differences, as adult male *C. elegans* are more slender and shorter than hermaphrodites (Meneely, Dahlberg and Rose, 2019).

1.4.1 *C. elegans* as a Model Organism for Sarcopenia

A large volume of literature on sarcopenia comes from studies involving model organisms of sarcopenia (Christian and Benian, 2020). These studies have provided vital insights into the mechanism governing the development and progression of sarcopenia in humans (Rollins *et al.*, 2017). Monkeys and rodents present themselves as a perfect biological model to study sarcopenia, given the size of their muscles and high genetic similarity to humans (Christian and Benian, 2020). However, at the same time, the relatively long lifespan of these animals along with the inability to conduct large-scale genetic screens makes it difficult to sustain the study (Börsch *et al.*, 2021). Under these circumstances, it is more beneficial to conduct studies on sarcopenia using simpler model organism with a shorter lifespan (Olsen, 2006).

More recently, the nematode *C. elegans* has been presented as an excellent model for studying sarcopenia (Kashyap, 2012). *C. elegans* have a relatively short lifespan, that can be altered by changing the diet or temperature (Klass, 1977). Just like humans, *C. elegans* also develop sarcopenia during ageing, as with increasing age, they show increased muscle loss and a significant reduction in mobility (Herndon *et al.*, 2002). Body wall muscles of young *C. elegans* are structured in a compact, parallel fashion, however as the worm ages, the muscles become disorganised and disoriented (Herndon *et al.*, 2002). Furthermore, the lack of muscle stem cells in *C. elegans* allows for a focus on muscle deterioration during ageing without the complication of muscle regeneration (Christian and Benian, 2020). Additionally, the structural similarity of body wall muscle to humans, as well as the simplicity with which muscle structure

can be observed *in vivo*, makes *C. elegans* an ideal model organism for studying sarcopenia (Lesanpezeshki *et al.*, 2021).

Additionally, previous studies in *C. elegans* have revealed that the intestine plays a key role in a variety of lifespan models (Portal-Celhay, Bradley and Blaser, 2012). Modulation of the expression of key genetic factors in the intestine, in particular, has been shown to affect longevity (Ulgherait *et al.*, 2014). In *C. elegans*, the intestinal barrier has a similar role to that of humans in forming a selective permeability barrier that allows essential solutes to be absorbed while providing defence against potentially hazardous chemicals (Poupet *et al.*, 2020). Similarly, the intestinal barrier function of *C. elegans* has been shown to decline with age (Gelino *et al.*, 2016). However, the intestinal health in the context of sarcopenia has not been studied in *C. elegans* yet. To address this gap, the effect of algae-oil supplementation on *C. elegans'* intestinal health was investigated in conjunction with sarcopenia in this study.

1.4.2 *C. elegans* Anatomy

C. elegans have a simple anatomy consisting of an exterior tube containing a fluid-filled body chamber known as the pseudocoelum and two inner tubes (Schafer, 2016). The outer tube is made up of the cuticle, hypodermis, muscles, neurons, and the excretory system (Schafer, 2016). While the gonad and the alimentary canal, which includes the pharynx and the intestine, form the two inner tubes (Rasmussen *et al.*, 2013). Food ingestion in *C. elegans* is mediated by the pharynx, a neuromuscular pump that joins the mouth to the intestine (Trojanowski, Raizen and Fang-Yen, 2016). The body wall is composed of a strong, yet malleable collagenous cuticle produced by the underlying hypodermis (Gieseler, 2017) . By functioning as an exterior skeleton, the cuticle protects the worms from environmental hazards while also maintaining the worm's body form and allowing movement (Gieseler, 2017).

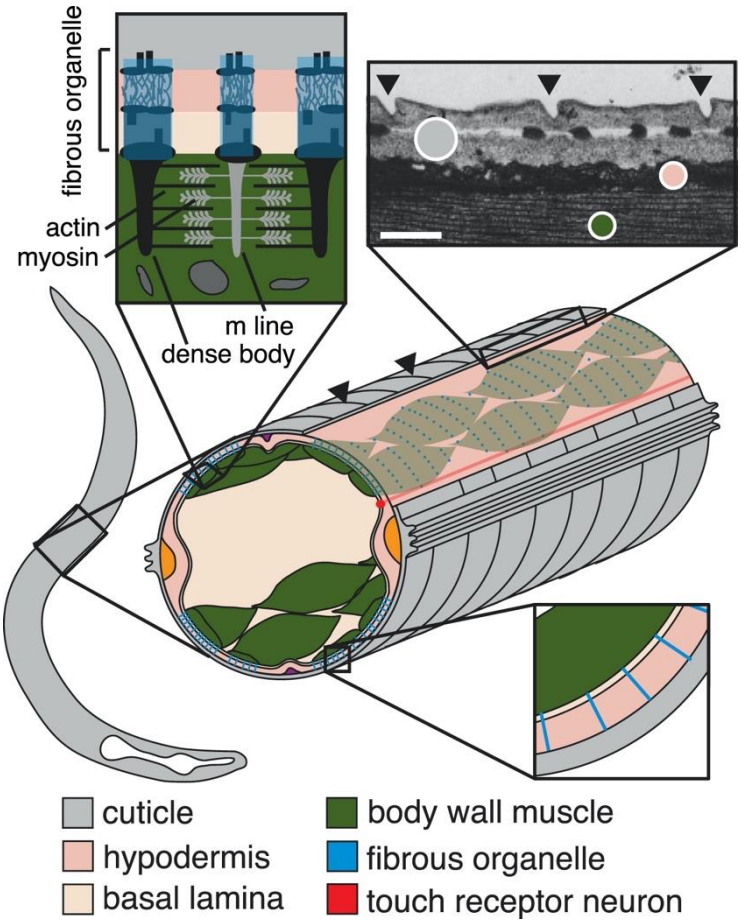

Figure 1. 2 Schematic representation of the *C. elegans* anatomy. The location of the body wall muscles (top left), hypodermis (middle), and cuticle (top right) are shown in the *C. elegans* body plan. Image reproduced from (Petzold *et al.*, 2011).

There are two types of muscle in *C. elegans*: striated and nonstriated muscle, the former for movement and the latter for feeding (Ono, 2014). *C. elegans'* primary striated muscle is situated in the body wall and functions similarly to vertebrate skeletal muscles (Benian and Epstein, 2011) (Figure 1. 2). The body wall muscles of *C. elegans* consists of four quadrants that run the length of the animal (Altun and Hall, 2005). The sinusoidal movement of *C. elegans* is enabled by alternating contraction and relaxation of the ventral and dorsal quadrants (Benian

and Epstein, 2011). In older *C. elegans*, the sinusoidal body movement slows and becomes less coordinated, eventually ceasing entirely (Collins *et al.*, 2008).

Age-related changes in motility of *C. elegans* can be assessed in two ways. Firstly, movement can be measured in terms of waves per minute using a dissecting microscope (Collins *et al.*, 2008). Secondly, *C. elegans* can be classed on the level of motility where class I worms move in a sinusoidal manner, class II worms show reduced uncoordinated movement and class III worms only show movement in the head and tail region (Herndon *et al.*, 2002). This motility classification system has been used to demonstrate sarcopenia in *C. elegans*, since the worms displayed an age-related shift from class I to class III, indicating an age-related reduction in muscle structure and function (Herndon *et al.*, 2002). Due to the transparent nature of *C. elegans*, the muscle structure can be easily visualised under a dissecting microscopy (Collins *et al.*, 2008). Alternatively, green fluorescent protein (GFP) localized to the body wall muscles can be used to visualize muscle structure using a fluorescence microscope (Glenn *et al.*, 2004).

1.4.3 *C. elegans* Life cycle

The life cycle of *C. elegans* is similar to that of other worms in that it consists of a succession of larval stages that develop through moults in which the cuticle is lost, and the hypodermis synthesizes a new stage-specific cuticle (Lažetić and Fay, 2017) (Figure 1. 3). Under favourable conditions, *C. elegans* larvae develop through four stages, L1-L4, before reaching adulthood (Fielenbach and Antebi, 2008). At 20°C, the transition from egg to adult takes around three days, however this varies depending on temperature (Porta-de-la-Riva *et al.*, 2012). Under stressful conditions, such as the absence of food, overcrowding or high temperature, *C. elegans* can enter an alternative stage called the dauer stage (Jeong *et al.*, 2009). Dauers differ from other larval stages in both morphology and behaviour (Wang *et al.*, 2009).

Dauers lack pharyngeal pumping, have reduced motility, and are resistant to desiccation (Androwski, Flatt and Schroeder, 2017). The nematode can withstand severe circumstances in this form by relying on its pre-formed energy reserves, such as glycogen and lipids (Fielenbach and Antebi, 2008).

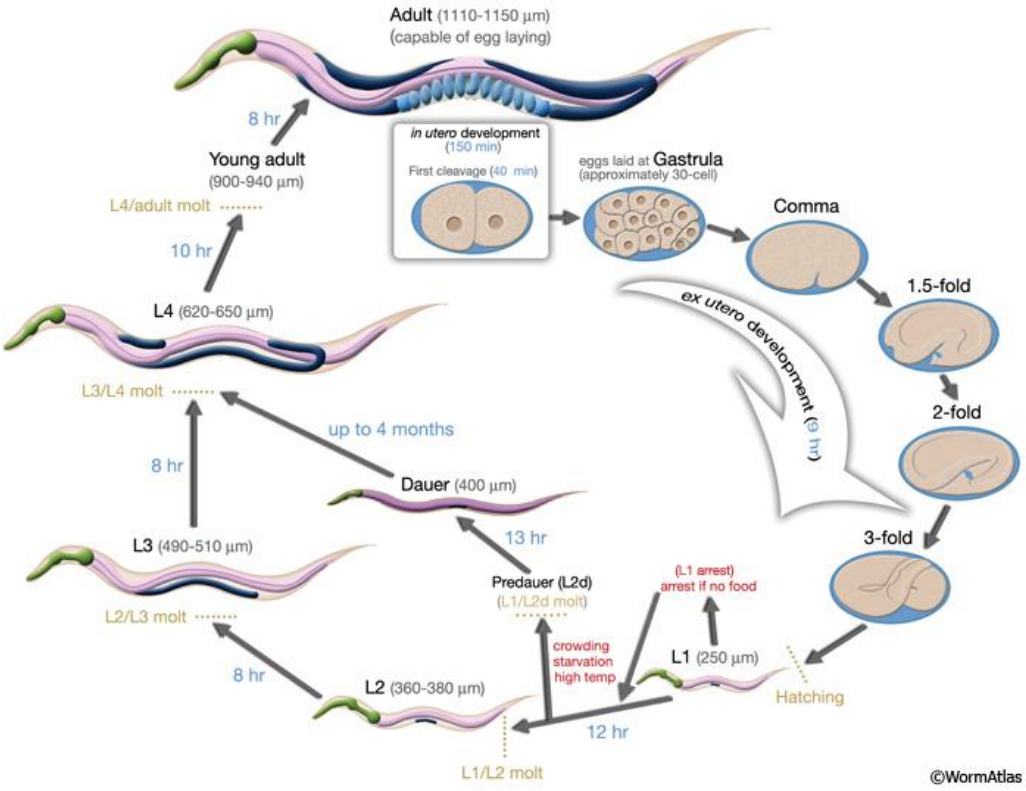

Figure 1. 3 Schematic representation of *C. elegans'* life cycle. The blue numbers along the arrows indicate the period of time the worm spends at each stage. Image reproduced from (Altun and Hall, 2009).

1.4.4 *C. elegans* Genetics

C. elegans is a particularly appealing model organism due to the vast range of genetic techniques accessible for research (Frézal and Félix, 2015). Since *C. elegans* hermaphrodites can self-fertilize, mutants with severe behavioural or morphological phenotypes can still survive and reproduce as homozygous populations (Yin and Haag, 2019). This makes screening for mutations considerably easier because no particular crossings are required to create homozygotes (Carvalho *et al.*, 2014). The Caenorhabditis Genetics Center (CGC) has a large collection of *C. elegans* strains and also allows users to customise deletion mutations that are not yet accessible (Daul, Andersen and Rougvie, 2019).

Furthermore, the availability of an RNAi library as well as the efficiency with which transgenic strains can be generated, make *C. elegans* an attractive tool for genetic study (Howe, 2019). As *C. elegans* is transparent throughout its life, non-intrusive *in vivo* imaging of fluorescently labelled organelles can be accomplished by attaching a fluorescent-protein reporter, such as GFP to the gene of interest (Corsi, Wightman and Chalfie, 2015). In this study, a transgenic *C. elegans* strain expressing a myosin-GFP hybrid protein called UNC-54::GFP was used to study the age-related decline in muscle mass.

1.4.5 *C. elegans* Nomenclature

The *C. elegans* scientific community has developed a worm-specific gene nomenclature consisting of three or four italicized lower-case letters, a hyphen and a number, such as *unc*-54. Gene names in *C. elegans* nomenclature frequently reflect the phenotype of gene mutations. An *unc* gene, for example, is a gene characterized by a mutation that results in a paralyzed or uncoordinated phenotype. Similarly, if a gene mutation causes a short or "dumpy" phenotype, the gene is called *dpy*, and the phenotype is called Dpy. The gene name for those genes characterized by sequence similarity will correspond to the predicted protein, such as *mlc*-2 for

myosin light chain 2. Genes with similar characteristics are often assigned the same three-letter name but distinct numbers. For example, the three known myosin light chain genes are denoted as *mlc-1*, *mlc-2*, and *mlc-3*. In terms of mutations, mutants are given names that consist of one or two italics letters followed by an italicized number, such as *e1092*. The letter prefix refers to the isolation laboratory as registered with the CGC. For example, 'e' stands for the MRC Laboratory of Molecular Biology (Cambridge, UK). When using gene and mutation names together, the mutation name is included in parentheses following the gene name, for example, *unc*-54::*gfp* (*e1092*). *C. elegans* proteins have the same letter-number designation as the gene that encodes them, but they are written in non-italicized capitals to distinguish them. Thus, the protein UNC-54 is encoded by the gene *unc-54*. This nomenclature will be used throughout this thesis.

1.5 Ageing pathways in C. elegans

1.5.1 Insulin-like growth factor 1 (IGF-1)

The IGF-1 signalling pathway is one of the major pathways involved in regulating muscle protein synthesis (MPS) in skeletal muscle (Lapierre and Hansen, 2012). The IGF-1 is an anabolic hormone that enhances the growth and repair of skeletal muscle (Locatelli and Bianchi, 2014). Along with skeletal muscle growth, the IGF-1 pathway is also involved in muscle repair, muscle metabolism and muscle hypertrophy in response to exercise (Velloso, 2008) (Figure 1. 4). In *C. elegans*, the IGF-1 signalling pathway plays an important role in the regulation of ageing. The daf-2 gene encodes the *C. elegans* homolog of the IGF-1 receptor (Pan and Finkel, 2017). Mutations in the *daf-2* gene along with other genes that code for the various components of the IGF-1 signalling cascade has been shown to downregulate this pathway and increase the lifespan of *C. elegans* (Kenyon *et al.*, 1993). Surprisingly, studies have shown that in *C. elegans*, the degree of lifespan extension caused by reduced IGF-1

signalling is specific to the tissue type (Libina, Berman and Kenyon, 2003). Libina et al. (2003) found significant difference in lifespan when *daf-2* mutant worms were expressed with DAF-16 (a transcription factor of the IGF-1 pathway) in different tissues. The study found that when *daf-16* is expressed in the neurons, there is only a 5-20% increase in lifespan whereas when the *daf-16* was expressed in the intestine, they found a significant increase in lifespan by 50-60% (Libina, Berman and Kenyon, 2003).

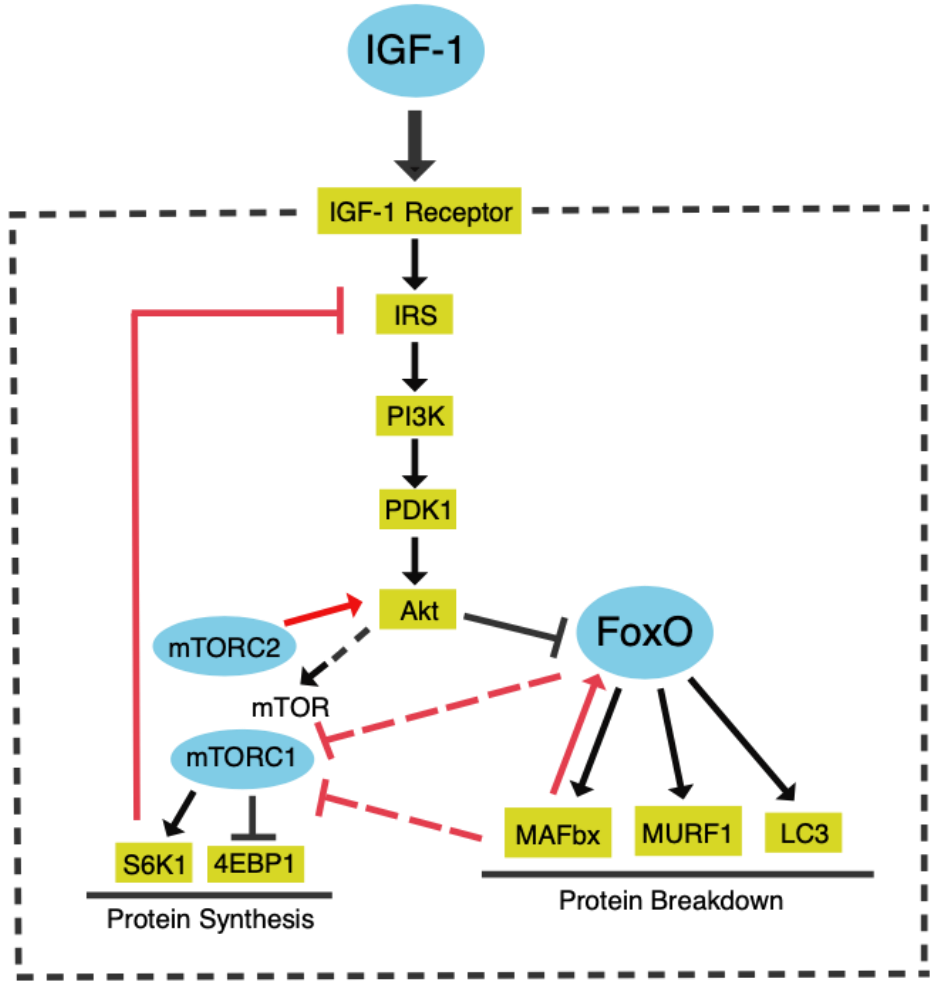

Figure 1. 4 Schematic representation of the IGF-1 signalling pathway. The IGF-1 signalling pathway controls muscle growth via mechanistic target of rapamycin (mTOR) and Forkhead box O (FoxO) (Schiaffino and Mammucari, 2011).

Previous studies have shown that with the exception of the nervous system, all the other tissues in *C. elegans* appear to decline at a similar rate during ageing (Garigan *et al.*, 2002a). This suggests that a complex signalling network is responsible for coordinating the ageing of different tissues which in turn might be influenced by some epigenetic factors. Some studies have shown that mutations in FOXO3a, human homolog of the *daf-16* gene is also associated with increased longevity (Willcox *et al.*, 2008). Wilcox et al. (2008) first reported a higher frequency of the FOXO3a3 alleles in the long-lived Japanese centurions (mean lifespan of 97.9yrs) compared to controls (mean lifespan of 78.5yrs). The study also found that long-lived men with the FOXO3a genotype exhibited phenotypes indicative of healthy ageing and increased insulin sensitivity (Willcox *et al.*, 2008). Further studies conducted on Italian and German centurions confirmed these findings, reinforcing the strong association between the FOXO3a gene and longevity (Anselmi *et al.*, 2009). Similar findings on the effect of IGF-1 signalling on longevity between nematodes, flies, mice and humans suggests that the IGF-1 signalling pathway is highly conserved across species and is crucial to our understanding of ageing (Vitale *et al.*, 2019).

1.5.2 Mechanistic Target of Rapamycin (mTOR)

The mechanistic target of rapamycin (mTOR) is another major signalling pathway involved in maintaining skeletal muscle mass (Papadopoli *et al.*, 2019) (Figure 1.4). The compound rapamycin was first discovered in 1964 on the island of Rapa nui (Easter Island) where the name is derived from (Arriola Apelo and Lamming, 2016). Upon its discovery, rapamycin was found to have immunosuppressive and anti-tumour properties (Blagosklonny, 2013). However, it was not until 1994, that researchers discovered the mTOR pathway and its targets within the cell (Laplante and Sabatini, 2009). mTOR exists as two complexes: mTOR complex 1 (mTORC1) and and mTOR complex 2 (mTORC2) (Laplante and Sabatini, 2009). The mTOR

pathway senses two different types of state at the organismal level: the fasting state when an organism does not have enough nutrients available or the fed state where the nutrients are abundant (Saxton and Sabatini, 2017). When nutrient is limited, mTORC1 is inhibited, enabling the induction of autophagy where the cell autoeats and breaks down to release nutrient and energy however when the nutrients are abundant, mTORC1 inhibits autophagy, making it a negative regulator of autophagy (Deleyto-Seldas and Efeyan, 2021). mTORC1 was found to be activated in aged skeletal muscle fibers of both mouse and human and its overexpression was associated with degenerative changes in muscle fibers (Markofski *et al.*, 2015). Inhibiting mTORC1 in old mice lowered the expression of growth differentiation factors in skeletal muscle, decreasing oxidative stress as well as muscle fibre damage and loss (Tang *et al.*, 2019). This suggests that maintaining a low level of mTOR signalling in aged muscle is critical for sustaining muscle performance (Ham *et al.*, 2020).

The mTOR pathway has several downstream outputs that can either be grouped into a pro-anabolism process such as protein synthesis, ribosome biogenesis and glycolysis or pro-cabalosim pathways such as autophagy, apoptosis and RNA degradation (Sabatini, 2017). Following its role in multiple processes and pathways, the mTOR signalling pathway has naturally been implicated in the ageing process (Papadopoli *et al.*, 2019). Decreased activity of the mTOR signalling pathway has been frequently linked with increased lifespan in a number of different organisms (Johnson, Rabinovitch and Kaeberlein, 2013). In *C. elegans*, inhibition of the mTOR pathway resulted in a significant increase in lifespan with the mTOR-deficient worms having a mean lifespan of 25 days in comparison to just 10 days in wildtype worms (Vellai *et al.*, 2003). This extension in *C. elegans* lifespan has been suggested to result, in part, from an increase in autophagy following the inhibition of the mTOR pathway in mTOR-deficient worms (Tóth *et al.*, 2008). Inhibition of the mTORC1 has also been shown to increase both lifespan and stress resistance in *C. elegans* (Li *et al.*, 2018).

Moreover, studies using rapamycin, an inhibitor of the mTOR pathway has shown to prolong lifespan in a variety of animals including fruitfly, mice and nematodes (Lind *et al.*, 2017). For instance, mice fed on a rapamycin-based diet increased the median and maximal lifespan in both male and females in comparison to control mice (Harrison *et al.*, 2009). Similarly, in *C. elegans*, rapamycin has also been shown to increase *C. elegans* lifespan by inhibiting both mTORC1 and mTORC2 (Robida-Stubbs *et al.*, 2012). It has long been hypothesised that long-lived worms might have reduced mTOR-signalling which in turn results in their extended lifespan (Kapahi *et al.*, 2010). However a study investigating the effect of rapamycin on the lifespan of short and long-lived *C. elegans* found that both short and long-lived worms responded equally to rapamycin and both of their lifespans were equally extended by rapamycin (Lind *et al.*, 2017). This suggests that the natural variation frequently observed in *C. elegans* lifespan is not just down to mTOR signalling and other factors might be involved (Lucanic *et al.*, 2017).

1.5.3 AMP-activated protein kinase (AMPK)

The AMP-activated protein kinase (AMPK) is an energy sensor that is activated when the cell's AMP/ATP ratio is high (Herzig and Shaw, 2018). It regulates several cellular pathways, including mTOR, and therefore acts as a switch for anabolic and catabolic pathways regulating skeletal muscle mass (Thomson, 2018) (Figure 1. 5). AMPK is comprised of three subunits consisting of an α catalytic subunit and two regulatory subunits, β and γ (Kim *et al.*, 2016). In mammals, there are three isoforms of the γ subunit (γ1, γ2 and γ3) and two isoforms of the α (α1 and α2) and β subunits (β1 and β2) (Willows *et al.*, 2017). *C. elegans* possess two catalytic subunits, AAK-1 and AAK-2, that are homologous to the α-catalytic subunit in mammalian AMPK (Lee *et al.*, 2008). aak-2(ok524) knockout mutants exhibit a 13% decrease in lifespan

compared to wild-type *C. elegans* (Apfeld *et al.*, 2004), whereas aak-2 overexpression increases life span by 13% relative to wildtype (Greer *et al.*, 2007). Weimer et al. (2014) showed that an endogenous glycolysis inhibitor, glucosamine, can increase the lifespan of *C. elegans* by impairing glucose metabolism and decreasing ATP levels, which in turn activates the AAK-2 (Weimer *et al.*, 2014). Similarly, Metformin, a first-line medication for type 2 diabetes and a well-known AMPK activator, has been shown to increase longevity in both *C. elegans* (Chen *et al.*, 2017). Chen et al. (2017) showed that metformin prolongs *C. elegans* lifespan and delays age-related motility loss by activating AMPK via the lysosomal pathway and inhibiting the mTORC1 pathway. Inhibiting the protease-activated receptor (PAR-1) was shown to increase *C. elegan's* lifespan by inducing an age-dependent activation of AMPK in the intestine (Wu *et al.*, 2020).

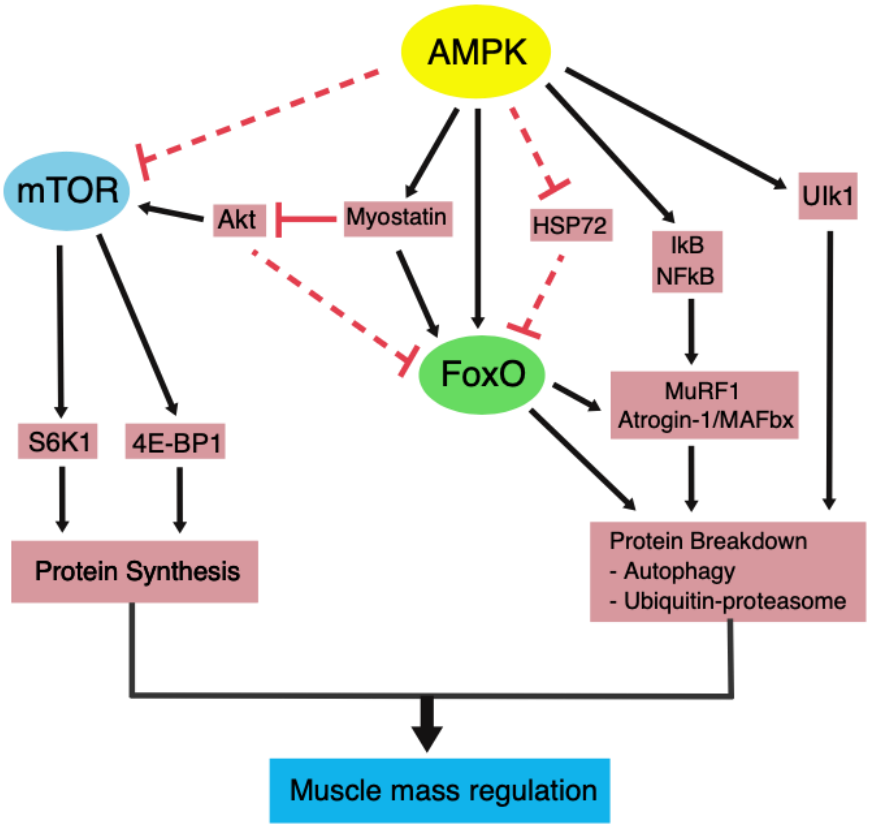

Figure 1. 5 Schematic representation of the AMPK signalling pathway in muscle mass regulation. Image reproduced from (Egawa, 2017).

Alternative methods of activating AMPK, such as aerobic exercise (Richter and Ruderman, 2009) and DR (Weir *et al.*, 2017) have also been shown to increase lifespan and healthspan. Although the exact mechanisms through which AMPK exerts these beneficial effects are unclear, emerging evidence shows that AMPK-mediated autophagy may play a key role (Bareja, Lee and White, 2019). In many animal models, autophagy has been demonstrated to diminish with age (Reznick *et al.*, 2007). Chang et al. (2017) found an age-dependent decrease in autophagy in several tissues of wildtype and long-lived *C. elegans* mutants (*daf-2* and *glp-1*), including the neurons, pharynx, muscle, and intestine. The long-lived mutants also displayed significant age and tissue-specific differences in autophagic activity, when compared to wildtype (Chang *et al.*, 2017).

1.5.4 Dietary Restriction (DR)

DR or reduction in total food intake without malnutrition, is a dietary intervention that has been shown to reproducibly increase lifespan and improve healthspan in a variety of species including *C. elegans* (Greer and Brunet, 2009). The lifespan extending effects of DR was first discovered by McCay and his team in 1935. They found that mice maintained on DR lived 50% longer in comparison to mice on standard diets (McCay, Crowell and Maynard, 1935). Following this discovery, similar findings were reported on a variety of organisms including fruit flies, nematodes and monkeys, suggesting a common evolutionary origin and a conserved mechanism behind these anti-ageing effect (Kapahi, Kaeberlein and Hansen, 2017).

Inhibiting the nutrient sensing pathways is believed to be one of the fundamental mechanisms by which DR delivers its positive benefits of enhanced lifespan and healthspan in a range of species (Regan *et al.*, 2020) (Figure 1. 6). As nutrient availability decreases, DR causes the nutrient sensing pathways to become less active (Regan *et al.*, 2020). Long-term DR in rats was shown to attenuate muscle protein content by downregulating mTORC1 signalling

(Margolis *et al.*, 2016). *C. elegans* mutants with reduced IGF-1 signalling have many similarities to DR worms, including decreased body size and increased stress tolerance (Houthoofd *et al.*, 2007). Investigating the effects of both DR and IGF-1 signalling pathway in *C. elegans* showed that in response to DR, there is an inhibition of protein synthesis and a decrease in IFG-1 signalling pathway (Depuydt *et al.*, 2013) (Depuydt, 2013). The study found that the resulting low IFG-1 signalling leads to preservation of muscle mass in long-lived *C. elegans* (Depuydt *et al.*, 2013).

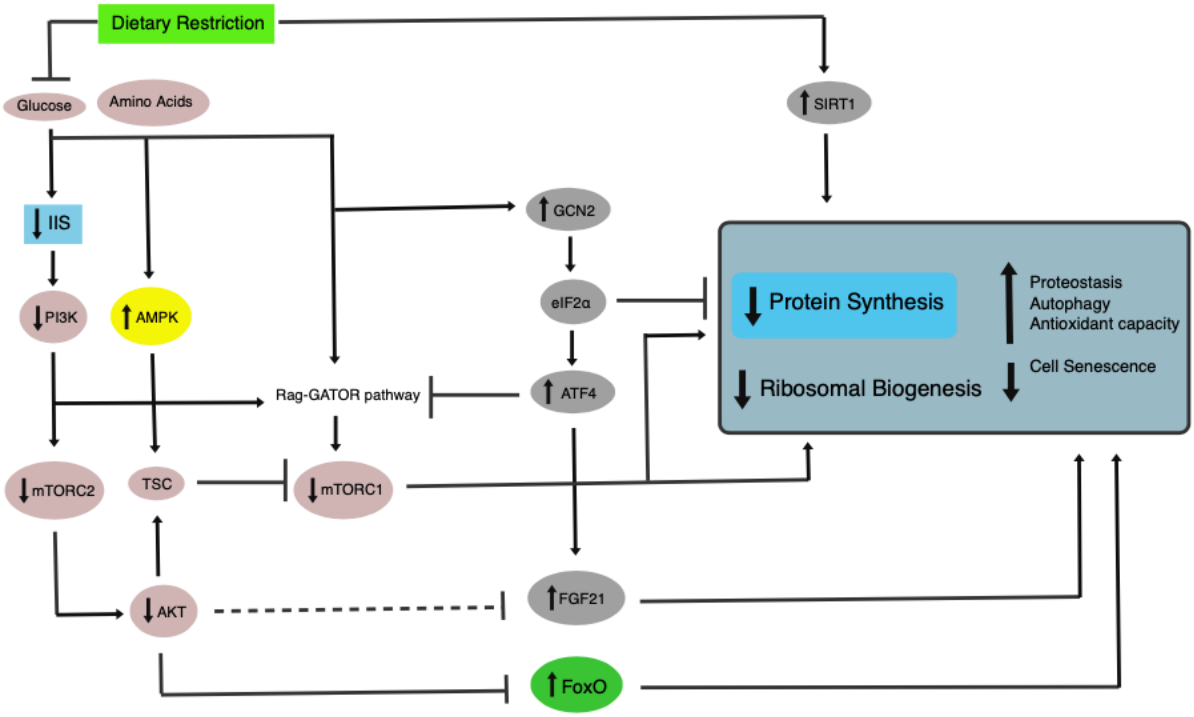

Figure 1. 6 Schematic representation of the DR pathway in muscle mass regulation. DR influences the activity of several signalling pathways including IIS, mTOR and AMPK, all of which are important nutrient-sensing signalling pathways that promote health and longevity. Image adapted from (Green, Lamming and Fontana, 2022).

1.6 Oxidative Damage Theory of Ageing in C. elegans

Long-lived *C. elegans* mutants have been demonstrated to be resistant to oxidative stress (Zhou, Pincus and Slack, 2011). The significance of decreased oxidative stress in increasing longevity is further reinforced by the fact that overexpression of the *clk-1* gene in wild-type *C. elegans* enhanced mitochondrial activity and lowered lifespan (Felkai *et al.*, 1999). There is evidence to suggest that a mild increase in ROS levels can increase lifespan and delay ageing in *C. elegans* (Schulz *et al.*, 2007). Schulz *et al.* demonstrated that treating *C. elegans* with 2-deoxy-D-glucose extends lifespan by inhibiting glycolysis, leading to increased mitochondrial respiration and ROS production. Interestingly, the rise in ROS levels was revealed to be essential for the lifespan extension, since treatment with antioxidants suppressed the increase in longevity (Schulz *et al.*, 2007). Similarly, low-dose exposure to arsenite in *C. elegans* has been shown to increase the ROS levels and increase lifespan while treatment to antioxidants reverses the lifespan extension (Schmeisser, Schmeisser, *et al.*, 2013). Moreover, rotenone treatment of *C. elegans* inhibits the function of mitochondrial ETC complex I, resulting in an increase in ROS and a longer lifespan (Schmeisser, Priebe, *et al.*, 2013). Treatment of *C. elegans* with metformin, juglone, and paraquat also resulted in a comparable increase in longevity via a similar mechanism (Shields, Traa and Van Raamsdonk, 2021).

In *C. elegans,* the effect of antioxidant supplementation on reducing oxidative damage has been studied extensively by employing compounds and mutations to increase *in vivo* antioxidant activity (Sadowska-Bartosz and Bartosz, 2014). When compared to age-matched control worms, treatment with Ginkgo biloba and Wisconsin ginseng extracts slowed the rate of sarcopenia in *C. elegans* (Cao *et al.*, 2007). Furthermore, both extracts improved age-related declines in motor functions such as locomotion, body bend, and pharyngeal pumping, demonstrating their anti-ageing properties (Cao *et al.*, 2007). Blueberry supplementation in *C.*

elegans enhanced longevity and motility in a dose-dependent way (Wang *et al.*, 2018). Similarly, raspberry extract was shown to reduce oxidative stress in *C. elegans* by decreasing the expression of ROS while promoting the expression of antioxidant-related genes via the skinhead 1 (SKN-1) pathway (Song *et al.*, 2020).

Supplementation of *C. elegans* growth media with a probiotic strain, *Bifidobacterium longum*, increased lifespan, maintained the gut-barrier integrity and protected against oxidative stress damage (Sugawara and Sakamoto, 2018). In a more recent study, endogenous glycolate was shown to inhibit hydrogen peroxide-induced oxidative stress on mitochondrial function and development in *C. elegans* (Diez *et al.*, 2021). While there is no conclusive evidence linking ROS directly to the ageing process, taken together, these studies suggest that antioxidants can extend lifespan by reducing oxidative stress via different signalling pathways.

1.7 Bacterial accumulation in *C. elegans*

During early adulthood, a symbiotic relationship is established between the *C. elegans* and *E. coli* within the intestine (Haçariz *et al.*, 2021). However, as the worm grows older, it loses its ability to regulate the proportion of live bacteria that enters the gut (Portal-Celhay, Bradley and Blaser, 2012). This loss is associated with a decrease in feeding and excretion, as well as a reduction in innate immunity (Cheng Huang, Xiong and Kornfeld, 2004). In *C. elegans*, the rate of pharyngeal pumping declines steadily with age, which corresponds to an age-related shift in feeding behaviour (Collins *et al.*, 2008). A shift occurs at this point, and the symbiotic relationship that existed between *C. elegans* and *E. coli* is transformed into an opportunistic one (Kim and Flavell, 2020). Accumulation of live bacteria in *C. elegans* is related with pathological symptoms, such as increased variability in intestinal shape and size, distention of the pharynx and intestinal lumen (Viri *et al.*, 2021). A negative correlation was observed between bacterial accumulation and *C. elegans* lifespan, suggesting that bacteria accumulation

contributes to ageing (Kim, 2013). Increased mortality rates have been linked to bacterial accumulation in the gut, as evidence suggests that *C. elegans* maintained on dead bacteria have a significantly longer lifespan (Garigan *et al.*, 2002b). Gomez *et al.* found that *C. elegans* fed respiration-deficient *E. coli* has increased lifespan and delayed bacterial accumulation, suggesting that changes in bacteria's capacity to proliferate in the gut affects *C. elegans* lifespan. Furthermore, it was shown that worms that were fed non-proliferating bacteria had a decreased frequency of bacterial accumulation in the pharyngeal lumen (Garigan *et al.*, 2002b).

However, it has been demonstrated that bacterial accumulation is not the only factor contributing to the high mortality rate in *C. elegans* (Chow *et al.*, 2006). Age-related declines in *C. elegans* pharyngeal pumping were observed in worms fed on non-proliferating bacteria, indicating that, while bacterial accumulation may have exacerbated functional decline in the pharynx during ageing, it was not the sole cause of decreased pumping rates in old worms (Zhao *et al.*, 2017). Recent research suggests that accumulation of non-pathogenic bacteria such as *E. coli* may only increase mortality when the immune system deteriorates as a result of ageing (Singh and Aballay, 2019). During early adulthood, bacterial accumulation appears to be controlled by gut immunity; however, as worms age, there is a decline in immune response, as well as a decrease in the regulation of bacterial proliferation, both of which are negatively associated with longevity (Portal-Celhay, Bradley and Blaser, 2012).

1.8 Therapeutic Interventions against Sarcopenia

1.8.1 Resistance Training (RT)

Currently, RT is the most effective treatment against sarcopenia (Law, Clark and Clark, 2016). RT helps build muscle strength by gradually increasing the amount of weight, allowing the muscles to adapt and become stronger (Mayer *et al.*, 2011). The beneficial effect of RT on ageing muscle was first established in 1988 by Frontera et al. (1988) who found that, following a 12-week RT, elderly men demonstrated a significant increase in both the size and strength of their quadriceps muscle. These gains in size and strength were associated with increased muscle hypertrophy and increased synthesis of myofibrillar proteins (Frontera *et al.*, 1988). Since then, several studies have confirmed the benefits of RT on counteracting the detrimental effects of sarcopenia in older people (Murphy *et al.*, 2018). With non-stop 16 weeks training, older subjects (60-75yrs) were able to restore their type II muscle fibres to the same size as the younger subjects (20-35yrs) (Kosek *et al.*, 2006). When subjected to RT, muscles undergo a wide range of neuromuscular and physiological adaptations (Folland and Williams, 2007). In terms of the neuromuscular adaptations, there is an increased recruitment of motor units and fast-glycolytic muscle fibres which results in increased force of contraction (Jenkins *et al.*, 2017). Physiological adaptations involve hypertrophy of muscle CSA and the number of muscle fibres (hyperplasia) leading to increased force production (Murlasits, Reed and Wells, 2012).

Consequently, RT focused on lower limbs has been shown to significantly improve gait speed in elderly people (Naczk, Marszalek and Naczk, 2020)(Santos *et al.*, 2017). Santos et al. (2017) showed that RT significantly increased lower limb strength and improved walking speed in elderly women. Similarly, increased muscle strength and fast walking speed were reported in resistance trained-elderly women compared to control group (Nunes *et al.*, 2017). People with

sarcopenia are reported to be three times more likely to fall compared to non-sarcopenic people regardless of age, gender and other contributing factors (Landi *et al.*, 2012). Many studies have shown that the prevalence of falls in elderly people is higher for those with low handgrip strength (Arvandi *et al.*, 2018). Maintaining good handgrip strength is important because during falls since people generally use their hand to grip and maintain balance (Maki and McIlroy, 2006). Research based on fall prevention has shown that RT increases muscle strength and balance in the elderly, minimising the likelihood of falling (Hewitt *et al.*, 2018).

Additionally, MPS and muscle protein breakdown (MPB) have been demonstrated to be affected by long-term physical inactivity due to a lack of exercise or bedrest following an illness or injury (Paddon-Jones *et al.*, 2006). Following a 10-day bed rest period, older adults showed a 6% loss in leg lean muscle and a 30% decrease in MPS (Kortebein *et al.*, 2007). However, it has been demonstrated that incorporating an RT-based exercise during the bedrest period can effectively maintain the MPS rates (Ferrando *et al.*, 1997)(Dirks, Wall and Van Loon, 2018) . Similarly, low-intensity RT exercise has been shown to attenuate the decline in strength and muscle mass associated with immobilization (Oates *et al.*, 2010).

1.8.2 DR

Along with extending lifespan, DR has also been found to delay the onset of age-related diseases including sarcopenia (Omodei and Fontana, 2011). The risk of sarcopenia and other age-related conditions was significantly lower in the DR group of rhesus monkeys than in the control group (Colman *et al.*, 2009). A more recent study showed that DR preserved metabolic efficiency in DR rhesus monkeys which, in turn was linked to a decrease in age-related muscle mass and physical activity (Rhoads *et al.*, 2020). Short-term DR in mice increased the number of satellite cells in skeletal muscle, indicating that DR has a protective effect against muscular

atrophy in rodents (Boldrin *et al.*, 2017). Similarly, resveratrol, which mimics DR, was shown to prevent muscle atrophy in aged rats (Bennett, Mohamed and Alway, 2013). When compared to controls, DR was shown to preserve the number and fibre type composition of lateral thigh muscle in aged rats (Aspnes *et al.*, 1997). DR has also been demonstrated to improve the activity of adult stem cells in elderly mice, particularly the ability of skeletal muscle stem cells to regenerate (Sato *et al.*, 2017).

In humans, long-term DR has been shown to enhance muscle protein quality by increasing autophagy and decreasing inflammation and allowing muscular homeostasis to be maintained (Yang *et al.*, 2016). The study also found that inflammatory factors were significantly lower in the skeletal muscle of DR individuals compared to age-matched controls (Yang *et al.*, 2016). In *C. elegans*, DR and *daf-2* mutants have been demonstrated to preserve more muscle mass despite their lower body size (Depuydt *et al.*, 2013). More specifically, proteomic analysis revealed that DR and *daf-2* mutants had elevated levels of muscle proteins including the four isoforms of myosin class II heavy chain (MYO-1, MYO-2, MYO-3, and UNC-54) (Depuydt *et al.*, 2013). This increase in muscle protein levels has been suggested to aid in the preservation of muscle mass and function during DR stress (Depuydt *et al.*, 2013). Apart from this study (Depuydt *et al.*, 2013), the effect of DR on muscle mass and function has not been thoroughly investigated in *C. elegans*. Due to the similarities in muscle structure and function between humans and *C. elegans*, muscle degeneration in *C. elegans* can be studied to better understand sarcopenia. In this study, the UNC-54-GFP levels were used to measure the sarcopenia rates of *C. elegans* subjected to DR and algae-oil supplementation.

1.8.3 Omega-3 Fatty Acids

Improving dietary intake is another approach that can be used to minimise and delay the loss in muscle mass and function in the elderly. Fatty acids are a major component of the muscle cell membrane (sarcolemma) and serve as the main source of energy during physical activity (Gerling *et al.*, 2019). The fatty acid is said to be saturated when all of its carbons are fully bound to hydrogens (Lund and Rustan, 2020) (Figure 1. 7). Butter, red meat, and coconut oil are all high in saturated fatty acids (Gershuni, 2018). On the other hand, when the hydrocarbon chain has fewer hydrogens, it is said to be unsaturated (Lu *et al.*, 2019). Instead of binding to a maximum number of hydrogens, some carbon atoms bind to each other via a double bond (Lu *et al.*, 2019). The presence of double bonds bends the hydrocarbon chain, creating gaps between molecules and making them less compact (Lunn and Theobald, 2006).

A fat molecule with a carbon-caron double bond is known as a monounsaturated fatty acid (MUFA), whereas one with several double bonds is known as a polyunsaturated fatty acid (PUFA) (Pinheiro and Wilson, 2017) (Figure 1. 7). Fatty acids are a major component of the muscle cell membrane (sarcolemma) and serve as the main source of energy during physical activity (Gerling *et al.*, 2019). While both saturated and unsaturated fatty acids are required for a functioning cell membrane, increased saturated fat, as is the case in a typical western diet, can cause the cell membrane to become rigid and impair cellular permeability (Figueiredo *et al.*, 2017). In contrast, the gaps in unsaturated fatty acids provide membrane fluidity, facilitating membrane transport and cellular signalling (Ballweg and Ernst, 2017). As a result, unsaturated fatty acids are considered to be healthier than saturated fatty acids (Figueiredo *et al.*, 2017).

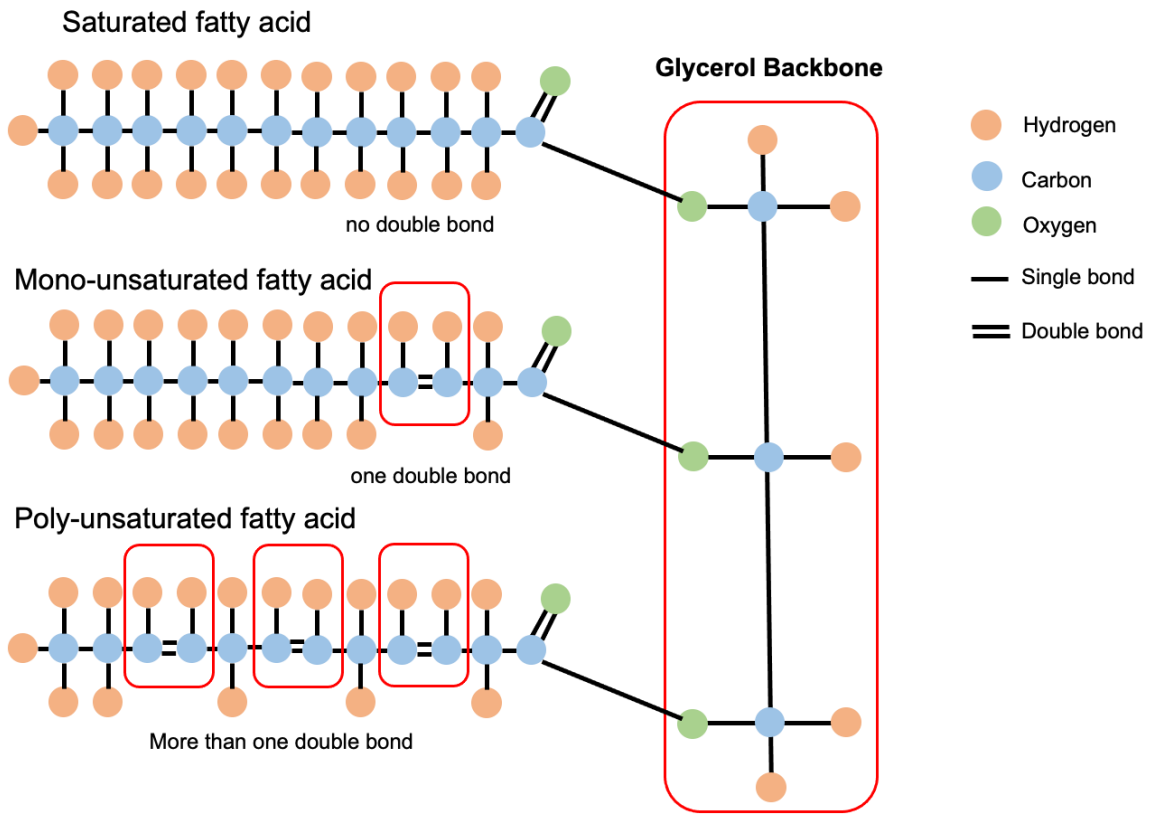

Figure 1. 7 Schematic representation of the saturated, monounsaturated and polyunsaturated fatty acid structure.

The human body is capable of synthesising all the fatty acids it needs, with the exception of PUFA, which must be obtained from the diet, making them an essential fatty acid (Saini and Keum, 2018). PUFAs are generally classified by their omega numbering; the omega carbon is the carbon atom at the end of the hydrocarbon chain (Jónasdóttir, 2019). Omega PUFAs are classified into four categories (3, 6, 7 and 9) based on where the first double bond carbon is found in the chain (Jónasdóttir, 2019). Omega-3 and Omega-6 fatty acids, in particular, play a crucial role in musculoskeletal health because they promote antioxidant-oxidant equilibrium, which prevents oxidative stress and skeletal muscle atrophy (Calvani *et al.*, 2015) (Figure 1. 8). Omega-3 PUFA can be found in fish, vegetable oils and nutritional supplements (Řezanka, Kolouchová and Mat'átková, 2021) whereas omega-6 PUFA can be found in processed

vegetable oils, nuts and certain meat (Saini and Keum, 2018). The main omega-3 fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Saini *et al.*, 2021).

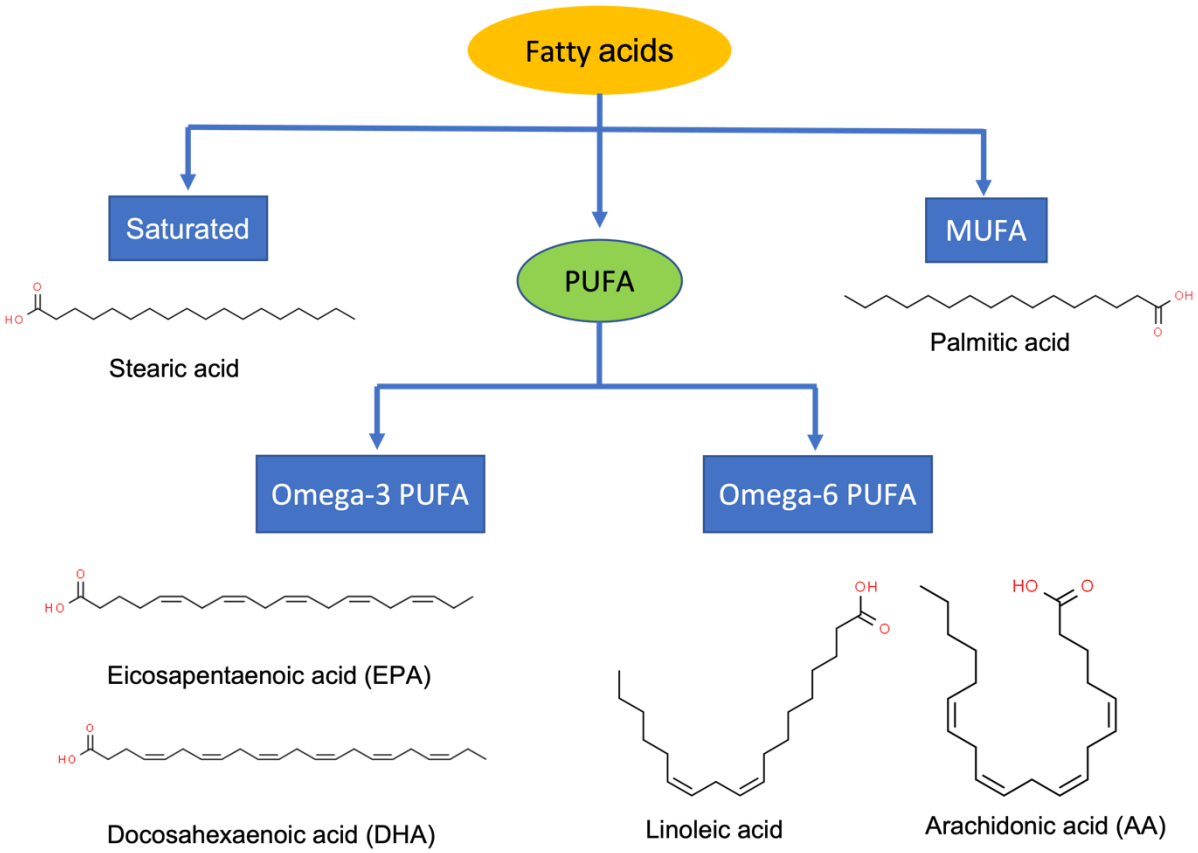

Figure 1. 8 Schematic representation of the saturated and unsaturated fatty acids. Omega-3 fatty acids differ from omega-6 fatty acids by the location of their first double bond from the methyl end of the fatty acid.

Omega-3 fatty acids are originally synthesised by microalgae, which are consumed by phytoplankton and subsequently consumed by fish, accumulating EPA and DHA in their tissues (Barta, Coman and Vodnar, 2021). Recent studies have found a positive link between omega-3 fatty acid consumption and skeletal muscle hypertrophy (McKee and Morley, 2019). In older people, higher omega-3 PUFA concentrations were associated with increased muscle mass and knee extension strength (Reinders *et al.*, 2015). Similarly, in community-dwelling older adults, higher serum concentrations of omega-3 PUFA were cross-sectionally linked to increased gait speed (Frison *et al.*, 2017). Omega-3 fatty acids have been demonstrated to significantly impact skeletal muscle mass by incorporating EPA and DHA into the membrane phospholipids of the sarcolemma (McGlory, Calder and Nunes, 2019). The presence of EPA and DHA in these membrane phospholipids has been associated to increased MPS and decreased MPB (McGlory, Calder and Nunes, 2019). A healthy balance between MPS and MPB is important to retain and maintain lean muscle mass (Tipton, Hamilton and Gallagher, 2018). Failure to maintain this balance has been suggested to promote the loss of lean muscle mass observed with ageing (S. Fry and B. Rasmussen, 2011).

Research suggests that omega-3 PUFA have anti-inflammatory effects whereas omega-6 PUFA have pro-inflammatory effects with negative implications for musculoskeletal health (Djuricic and Calder, 2021). As a result, maintaining the ratio of omega-3 and omega-6 fatty acids is essential for reducing inflammation (Dinicolantonio and O'Keefe, 2018). A higher omega-3 to omega-6 ratio has been shown to decrease inflammation and the risk of other inflammatory diseases (Balić *et al.*, 2020). Omega-3 PUFAs exert their anti-inflammatory effects by integrating into the cell membranes of immune cells and skeletal muscle fibres (Gutiérrez, Svahn and Johansson, 2019) which alters the expression of genes involved in inflammation (Heshmati, 2021). Additionally, omega-3 PUFAs also function as a precursor

for anti-inflammatory cytokines, inhibiting the development of pro-inflammatory cytokines derived from omega-6 PUFAs (González-Hedström *et al.*, 2021). Smith et al. (2015) investigated the effects of omega-3 PUFA on older adults for six months and found increased thigh muscle volume and strength in comparison to the control group. Meta-analysis studies found that omega-3 supplementation is associated with increased lean body mass and skeletal muscle mass, further implicating the use of omega-3 supplements in sarcopenia prevention (Huang *et al.*, 2020)(Bird *et al.*, 2021). Currently, the effect of omega-3 PUFA on *C. elegans* has only been studied in the context of lifespan (Sugawara *et al.*, 2013)(Qi *et al.*, 2017). However, considering the multiple health benefits of omega-3 PUFA, one of the main hypotheses of this thesis is that omega-3 may also be beneficial in sarcopenia. For the first time, transgenic worms expressing the UNC-54::GFP protein were employed in this thesis to explore the effects of algae-derived omega-3 PUFA in sarcopenia. The myosin UNC54-GFP was measured during the entire lifespan of *C. elegans* in order to determine the sarcopenia rates.

1.9 Consequences of Sarcopenia

Most of the effects of sarcopenia are prognostic measures of the burden on public health, such as the incidence of physical disability, admission to the nursing home, depression, hospitalisation and even death (Guralnik *et al.*, 2000). In particular, sarcopenia is linked to poor physical health, functional deterioration, and physical impairment, which in turn leads to a loss of independence among elderly (Walston, 2012). Sarcopenia co-exists with osteoporosis and can increase the risk of fracture, possibly directly through muscle-to-bone crosstalks and indirectly through increased risk of falling (Cianferotti and Brandi, 2014).

Sarcopenia also poses a higher risk of hospitalisation and is particularly prevalent among elderly people admitted to intensive care or nursing homes (Vetrano *et al.*, 2014). Moreover,

sarcopenia is considered to be a good predictor of patient's outcomes after a major surgery or serious illness (Miyamoto *et al.*, 2015). Trotter et al. (2018) reported increased mortality in sarcopenic patients undergoing an emergency laparotomy compared to patients without sarcopenia. Additionally, in-hospital mortality rate and post-operative complications in sarcopenic patients is reported to be higher among patients undergoing emergency surgery than non-sarcopenic patients (Du *et al.*, 2014). Collectively, all these health-related consequences illustrate how sarcopenia affects a person's quality of life with loss of independence, high impairment rate, adverse outcomes, institutionalisation and death (Beaudart *et al.*, 2014). At the same time, this results in significant healthcare and dependency costs for sarcopenic elderly people with disabilities (Pinedo-Villanueva *et al.*, 2019). Annual hospitalisation costs due to sarcopenia in the United States in 2018 were estimated at about $40.4 billion, reflecting a significant share of overall National Health Expenditures (NHE) on hospital treatment (Goates *et al.*, 2019). In the UK, Pinedo-Villanueva et al. (2019) attempted to measure sarcopenia's economic costs by measuring the excess economic burden of providing extra health and social services for people with and without sarcopenia. The study estimated an annual excess cost of £2.5 billion at £2707 per individual per annum, contributing to an increased economic burden (Pinedo-Villanueva *et al.*, 2019). Since sarcopenia-related costs account for a major fraction of UK health and social care expenditures, the findings of this thesis have important implications as they can be utilised to better understand the underlying molecular and cellular mechanisms of sarcopenia.

1.10 Aims and Objectives

The main aim of this study was to investigate the effects of dietary interventions on longevity and sarcopenia in *C. elegans*. This study differs from previous DR and supplementation studies in *C. elegans* by using a transgenic strain with a fluorescently labelled body wall muscle (UNC54::GFP). In *C. elegans*, *unc-54* codes for the myosin heavy chain B (MHC B) protein, which is a fundamental component of the body-wall muscle and is primarily responsible for inducing movement (Apfeld and Alper, 2018). Given its role in locomotion, *C. elegans* mutants lacking UNC-54 exhibit an uncoordinated phenotype and move at 20% the speed of wild-type worms (Brouilly *et al.*, 2015). MYH7, the human homologue of the *unc-54* gene, is expressed in the cardiac and slow skeletal muscle (Tajsharghi and Oldfors, 2013). Interestingly, the *unc-54::gfp* strain was originally developed to explore *in vivo* changes in muscle contraction induced by a decrease in a novel actin-binding region in myosin (Várkuti *et al.*, 2012). Briefly, the *unc-54* gene was cloned from wildtype N2 Bristol strain into the expression vector pPD95.77, which contained the GFP fusion protein, resulting in the plasmid punc-54::UNC-54::GFP. This vector was inserted into Δ*unc-54* via microparticle bombardment, resulting in the strain expressing a myosin-GFP hybrid protein called UNC-54::GFP. This transgenic *unc-54::gfp* strain was comparable to wildtype worms in terms of body size, morphology, and lifespan (Várkuti *et al.*, 2012). Due to its GFP-labelled body-wall muscle and a strong sequence similarity with MYH7 (Gil-Gálvez *et al.*, 2020), the *unc-54::gfp* strain was used in this study to study the effects of dietary interventions on sarcopenia. The fluorescence intensity emitted from the *unc-54::gfp* strain was used as an indicator of the myosin density. As the myosin density decreased with age due to sarcopenia, the rate for different populations was measured using a fluorescence microscope as an additional method for quantifying muscle mass loss. This strain was used to measure the effect of DR and omega-3 supplementation on myosin UNC54-GFP levels as a function of age to determine sarcopenia rates. The main contribution

of this work is that it expands on previous studies by developing a new technique of measuring sarcopenia rates in *C. elegans* and testing the gut-muscle axis hypothesis by demonstrating for the first time a direct link between sarcopenia and intestinal barrier function. By carrying out this research, contributions will be made to the fundamental knowledge base regarding muscle ageing; specifically, that relating to sarcopenia.

- Determine the effects of DR on lifespan and motility in *C. elegans* and quantify sarcopenia rates using a novel fluorescent method that determines myosin isoform UNC54 density in body wall muscle.
- Investigate the effects of dietary supplementation with algae-derived omega-3 EPA on the longevity, motility, and sarcopenia in *C. elegans*.
- Determine effects of algae-derived omega-3 PUFA supplementation on *C. elegans'* intestinal dysfunction associated with ageing and increased oxidative stress.
- Evaluate the impact of algae-derived omega-3 supplementation on the fatty acid composition of *C. elegans* using GC-MS.

Chapter 2: General Materials and Methods

Further chapter-specific information can be found in the Materials and Methods sections of each chapter (sections 3.3, 4.3 and 5.3).

2.1 Materials

2.1.1 Reagents

M9 Buffer (1litre in dH_2O)

3g KH_2PO_4 (Fisher Scientific), 6g Na_2HPO_4 (Sigma Aldrich), 5g NaCl (Sigma Aldrich) and 1ml 1M $MgSO_4$ (Sigma Aldrich) were dissolved in dH_2O and autoclaved at 121°C.

NaOH (4M)

16g of sodium hydroxide (Sigma Aldrich) was dissolved in 100ml dH_2O and stored at room temperature until use.

Hypochlorite solution (10mL)

4ml of 4M NaOH and 6ml household bleach were mixed in a 15ml falcon tube. Stored at 4°C until use.

Nematode Freezing solution

3ml of glycerol (Merck) was dissolved in 7ml of dH_2O to make 30% (v/v) freezing solution.

Sodium azide (10mM)

6.5 mg of sodium azide powder (Sigma Aldrich) was dissolved in 10ml dH_2O. Stored at 4 °C until use.

Ampicillin (100 mg/mL)

0.1g of Ampicillin (Sigma Aldrich) was dissolved in 1ml of dH_2O. Stored at 4 °C until use.

10% Tergitol (100ml in dH_2O)

70% Tergitol (Sigma Aldrich) was diluted with dH_2O to make 10% Tergitol. Stored at room temperature until use.

10mg/ml Palmitic acid

0.5g of palmitic acid powder (Sigma Aldrich) was dissolved in 50ml dH_2O. Stored at room temperature until use.

100mM FUDR stock

30mg of FUDR (Fisher Scientific) was dissolved in dH_2O to make a final volume of 1ml. Stored at 4°C until use.

Ethanol+M9 solution (Buffer A)

1ml of ethanol was mixed with 3.5ml of M9 buffer in a 10ml falcon tube. The mixture was vortexed and stored at 4 °C until use.

GFP-microbead solution (Buffer B)

40µl of buffer A was mixed with 10µl of standard solution D (InSpeck Green 505/515nm, ThermoFisher) to make the premixed GFP-microbead solution. This solution was made fresh for every fluorescence imaging session.

2.5% H_2SO_4 in Methanol

2.5ml of sulphuric acid (Merck) was dissolved in methanol (Sigma Aldrich) to a final volume of 100ml.

2.1.2 Bacterial Growth Media

Luria Broth (LB)

20g of premixed LB powder (Fisher Scientific) was dissolved in 1L of dH_2O and autoclaved at 121°C. Ampicillin was added to a final concentration of 100µg/ml after LB was cooled to 55°C. The media was thoroughly mixed to ensure even distribution of the antibiotic.

LB agar plates

40g in pre-mixed LB agar powder (Fisher Scientific) was dissolved in 1L of dH_2O and autoclaved at 121°C. Ampicillin was added to a final concentration of 100µg/ml after LB was cooled to 55°C. Under sterile conditions, equal volume of media was poured onto petri dish. The plates were left to dry overnight in the laminar hood. The plates were stored at 4°C until use.

2.1.3 Nematode Growth Media (NGM)

The standard NGM prepared without any supplements is depicted in Table 2. 1. Briefly, the agar, peptone, sodium chloride and cholesterol were added to 975ml of dH_2O and the mixture was autoclaved at 121°C. The remaining ingredients were added after the solution cooled to 55°C. The standard NGM was used for nematode stock maintenance. A peristaltic pump was used to pour equal volume of NGM into sterile petri dishes (60 x15mm). Plates were left at room temperature to dry for two to three days, after which they were stored at 4°C until use (up to two weeks).

Table 2. 1 Standard NGM without any supplements.

Name	Stock (MW or Concentration)	Final Concentration	Weight or Volume*
Agar			17g
Peptone			2.5g
NaCl			3g
Cholesterol	5mg/ml	5mg/l	1ml
KH_2PO_4	1M		25ml
$CaCl_2$	1M		1ml
$MgSO_4$	1M		1ml
dH_2O			975ml
* For a final volume of 1L NGM			

NGM without peptone (NGM(-Pep))

NGM prepared without any peptone was referred as NGM(-Pep) throughout the study. The NGM(-Pep) was prepared as above, however the peptone was excluded from the media.

NGM containing Algae-oil (NGM + AO)

The algal oil was kindly provided by the AlgaeCytes company (Discovery Park, Sandwich). The algal oil is obtained from their omega-3 enriched biomass via their proprietary microalgal Eustigmatophyceae strain, ALG01. The ALG01 strain was up-scaled from petri dish to 1000 L using AlgaeCytes in-house proprietary upstream pyramid process. Once the culture reached late exponential phase, it was transferred into AlgaeCytes pilot plant production module (approx. 12,000 L). After reaching an appropriate density, it undergoes harvesting to provide material for spray drying. On each harvesting day, an appropriate

amount of algal culture is dewatered using a centrifuge to produce an algal slurry. The algal slurry was subsequently dried using a spray dryer to produce a dried algal powder.
The omega-3 oils were then extracted and refined from spray dried algal powder to produce AVEPA^{TM} Range of EPA vegan friendly enriched algal oil, a sample of which was provided for this study.

Algae oil is prone to oxidation so extra caution was taken when preparing the working stock and supplemented NGM plates. To prepare the working stock, one aliquot of algae-oil was retrieved from the -80°C and thawed at room temperature. 0.3g of algae oil was dissolved in 10ml of ethanol to prepare the 100mM working stock. This stock was stored at 4°C until use. The algae-oil supplemented NGM was prepared as above (Table 2. 1) however, 10ml of 10% tergitol was added to the media before autoclaving. This ensures a uniform distribution of the algae-oil across the NGM plate (Deline, Vrablik and Watts, 2013). The solution was cooled to 55 °C in a water bath and 25ml of KH_2PO_4 (1M), 1ml of $CaCl_2$ (1M) and 1M $MgSO_4$ (1M) was added to the media. 1ml of 100mM working stock of algae-oil was diluted into the media to achieve the final concentration of 0.1mM and 3ml of 100mM working stock of algae-oil was diluted into the media to achieve the final concentration of 0.3mM. The media was stirred until the algae-oil was thoroughly mixed (approximately 5 min). Then a peristaltic pump was used to pour an equal volume of supplemented media onto sterile petri dish (60 x15mm). Throughout this study, NGM supplemented with algae-oil is referred to as NGM+AO. To prevent light oxidation, the plates were covered with a tray and left to dry overnight at room temperature in the laminar hood. The algae-oil supplemented plates were seeded with 100µl of *E. coli* OP50 two days prior to the start of the assay (See section 2.2.1.1 for further details). The OP50 seeded plates were covered with a tray and left to dry at room temperature until the start of the assay.

NGM containing 160μM EPA (NGM+EPA)

NGM supplemented with 160μM EPA is referred to as NGM+EPA throughout this study. A 25mM working stock was prepared by dissolving 0.08g of EPA into 10ml of ethanol. 1ml aliquots were made and stored at -80°C until use. The NGM containing 160μM EPA was prepared as described above (Table 2. 1) by adding 10ml of 10% tergitol to the media before autoclaving. The media was cooled to 55 °C in a water bath and 25ml of KH_2PO_4 (1M), 1ml of $CaCl_2$ (1M) and 1M $MgSO_4$ (1M) was added to the media. 6.4ml of the working EPA stock was diluted into the media to achieve the final concentration of 160μM. The media was stirred until the EPA was thoroughly mixed (approximately 5 min). Then a peristaltic pump was used to pour an equal volume of EPA supplemented media onto sterile petri dish (60 x15mm). The plates were covered with a tray to prevent oxidation and left overnight at room temperature in the laminar hood. Two days before the start of the assay, the 160μM EPA-supplemented plates were seeded with 100μl of *E. coli* OP50 (See section 2.2.1.1 for further details). The OP50 seeded plates were covered with a tray and left to dry at room temperature until the assay began.

2.1.4 Nematode strain

All experiments in this study were performed using the transgenic *C. elegans* strain, *unc-54::gfp* (Table 2. 2). The *unc-54::gfp* strain was kindly gifted by Dr András Málnási-Csizmadia (Eötvös Loránd University, Budapest, Hungary).

Table 2. 2 Transgenic *C. elegans* strain used in this study.

Strain	Genotype	Phenotype	Described in	Source	Chapter (s)
unc-54::gfp	unc-54 (*e1092*)	UNC54::GFP expressed in body wall muscles.	Várkuti *et al.* (2012)	Dr András Málnási-Csizmadia	3, 4 and 5

2.1.5 *E. coli* strain

The *E. coli* strain, OP50 was used as bacterial food source throughout the study. The OP50 *E. coli* strain is an uracil auxotroph that produces a small bacterial lawn on NGM plates, making it easier to monitor worms (Brenner, 1974). As described in chapter 3, *E. coli* OP50 culture was serially diluted in LB to achieve the different DR regimens (mild, medium and severe).

2.2 Methods

2.2.1 Bacterial Growth and Storage

2.2.1.1 *E. coli* OP50 culture

100ml of fresh LB broth was prepared (see section 2.1.2). The LB broth was inoculated with a single colony of *E. coli OP50* from a fresh OP50 streak plate (see section 2.2.1.3). The *E. coli* OP50 culture was left overnight in a shaking incubator at 37°C. The OP50 culture was stored at 4°C until use.

2.2.1.2 Preparation of Glycerol Stocks

Glycerol stocks were prepared by mixing 1ml of 50% glycerol with 1ml of OP50 bacterial culture in a cryovial. The cryovial was labelled and stored at -80°C for long-term storage of bacterial stocks.

2.2.1.3 *E. coli* OP50 Streak plate

Glycerol stocks were used to streak *E. coli* OP50 onto LB agar plates. Plates were incubated at 37 °C overnight. The plates were wrapped with parafilm and stored at -4°C until use.

2.2.1.4 Serial Dilution and Bacterial Cell Count

This section outlines the method used to estimate the concentration of OP50 *E. coli*, which was used for the DR assay in this study. Ten sterile culture tubes were labelled from 1 to 10 and 4.5ml of LB broth was added to each test tube. Then 0.5ml of fresh OP50 *E. coli* culture (see above) was added to tube number 1 and vortexed to mix thoroughly. Using a sterile pipette, 0.5ml of this diluted bacterial suspension was aliquoted and added to tube number 2. This process of aliquoting and resuspending continued until the final tube was reached,

diluting the stock concentration by a factor of 10 each with each step. Next, ten LB agar plates were labelled with the date and dilution factor corresponding to the tubes. Using a sterile pipette, 0.5ml of the diluted bacterial suspension was dispensed on the corresponding plates. A sterile cell spreader was used to evenly spread the bacterial suspension on the plate. The plates were incubated at 37°C overnight. The number of colony forming unit (CFU) were counted from each plate. If the number of CFUs were too numerous to count, the plates were divided into 4 sections and the number of CFU in one section was counted and multiplied by four to calculate the total CFU. The number of CFU was counted in triplicate and an average CFU was calculated for each plate. Finally, the number of bacteria per ml of each bacterial dilution was calculated using the following formula:

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL) x total dilution used}} \longrightarrow \frac{\text{Number of CFU}}{\text{mL}}$$

2.2.1.5 Seeding of standard NGM plates with *E. coli* OP50 (for nematode maintenance)

The NGM plates were seeded with 100µl of fresh *E. coli* OP50 under sterile conditions (laminar hood). The plates were left to dry overnight at room temperature and stored at -4°C until use.

2.2.2 Nematode Handling and Maintenance

2.2.2.1 Maintenance of *C. elegans* stocks

Unless otherwise specified, *C. elegans* were cultured on *E. coli* OP50 seeded standard NGM plates kept at 20 °C. To avoid starvation, *C. elegans* were moved onto fresh plates every three days. Unless otherwise stated, all experiments were carried out at 20 °C. Depending on the stage in the study, *C. elegans* were transferred to a fresh plate using the following two methods:

A large population of age-synchronised worms were required at the outset of each assay. Chunking is an efficient method for simultaneous transfer of many worms from one plate to another. To begin, two to three NGM plates containing densely packed worms were selected. The plates were examined under the microscope for any mold or bacterial contamination and not used if any contamination was detected. A metal spatula was sterilized by briefly passing it through the bunsen burner flame and a 1cm agar was cut around the densely packed region of worms. To allow the worms to transfer to the new plate, the chunk of agar was put upside down on a freshly seeded NGM plate. Worms should emerge from the agar chunk onto the bacterial lawn in a few seconds. Along with moving a large number of worms, the chunking method is also beneficial for moving worms that have burrowed into the agar or are difficult to pick individually. However, the chunking method is not recommended for moving a heterozygous worm population.

When age-synchronised worms needed to be transferred to experimental plates, individual worms were relocated using a worm pick. The plates were put face up on the microscope stage, and the focus was adjusted to allow the worms to be viewed clearly through the objectives. The wormpick was briefly passed through the bunsen burner flame to sterilise it. The wormpick was allowed to cool for a few seconds before picking up a small clump of

bacteria. Then, with a gentle touch of the wormpick, a single worm was picked up from the plate. Care was taken not to pierce the agar with the wormpick since this causes the worms to burrow into the agar. The worm was carefully placed on the experimental plates and the whole process was repeated until the required number of worms per plate was reached. It is important to sterilize the wormpick before and after each worm transfer to avoid cross-contamination between worms under different experimental groups.

The worm pick was fashioned out of a platinum wire and pasteur glass pipette. Briefly, 1-2 inch of 32-gauge platinum wire was cut and inserted into the tapered end of the glass pipette using tweezers. The platinum wire was secured in place by gradually melting the glass pipette over the blue flame. Next, the tip of the wire was slightly flattened to form a curved shape.

2.2.2.2 Age synchronisation of *C. elegans*

All assays in this study were performed using age-synchronized *C. elegans*, which were obtained by treating gravid worms with hypochlorite solution (see section 2.1.1). Except for the eggs, which are protected by their shell, this procedure eliminates all worms. The chunking method was used to grow two to three NGM plates with gravid worms. The gravid worms were washed off the plates using 2ml M9 buffer and collected in a 15ml falcon tube. This step was repeated with the remaining plates. The tubes were filled with M9 buffer up to 10ml and centrifuged for 1min at 1500rpm. The excess M9 were carefully discarded without disturbing the worm pellet. This wash step was repeated at least two times to remove any contaminants. Worms were re-suspended in 0.5mL of freshly prepared hypochlorite solution. The lid was secured firmly, and the tube was agitated vigorously for 3-5 minutes, while monitoring the dissolution of worms under a microscope. Once all the worm cuticles were dissolved, the bleaching process was quickly halted by adding M9 up to 10ml. The tube was centrifuged at 2000rpm for two minutes to pellet the eggs. The M9/bleach solution was

discarded leaving ~200µl of the egg suspension behind. Using a sterile pipette, the egg suspension was evenly distributed across two to three unseeded NGM plates. The plates were incubated overnight at 20ºC to allow the eggs to hatch. The next day, the plates were seeded with 100µl of OP50 and incubated at 20°C for two days to allow the worms to reach the L4 stage before moving them to the experimental plates.

2.2.2.3 Freezing and Recovery of *C. elegans* stocks

One of the most appealing aspects of adopting *C. elegans* as a model organism is that the worms can be preserved as frozen glycerol stocks indefinitely at -80°C. The ideal stage for preparing frozen stocks is freshly starved L1-L2 larva since they are least affected by the gradual freeze. The freezing method was followed as described by Stiernagle (2006) except that the M9 buffer was used instead of S buffer. Two to three plates containing age synchronised L1-L2 larvae were washed off the plates with 2ml of M9 buffer and collected in a 15ml falcon tube. The worms were suspended in an equal volume of nematode freezing solution (see section 2.1.1) and aliquoted into cryovials. The cryovials were labelled with the strain name and date and stored inside a styrofoam box at -80°C to ensure a slower freezing rate. To recover the frozen stocks, the cryovials were retrieved from -80 and thawed at room temperature. Once thawed, the contents of the vials were transferred to a freshly seeded NGM plate.

2.2.2.4 Decontamination of *C. elegans* stocks

To prevent bacterial and fungal infection, *C. elegans* were decontaminated prior to the start of each assay described in this thesis. Small mold contaminants from *C. elegans* stock plates can be removed by chunking worms away from the fungal growth and serially moving them to new plates. Briefly, a sterilized scalpel or pipette tip was used to pick a chunk of agar with worms from the infected plate. The agar chunk was placed on the edge of an OP50 seeded

NGM plate and the worms were allowed to crawl out onto the bacterial lawn. Bacterial and yeast contamination can be removed using the hypochlorite solution (bleaching) protocol which kills and dissolves the contaminated worms whilst leaving the eggs intact.

2.2.2.5 Palmitic acid ring in NGM plates

To avoid worms escaping from the plates, a ring of palmitic acid (see section 2.1.1) was applied around the edge of the plates (Fawcett, Horsman and Miller, 2012). As the ethanol evaporates, the palmitic acid precipitates out of the solution, forming a physical barrier to prevent the worms from escaping. It has been demonstrated that palmitic acid barriers have no effect on the rate of reproduction or longevity of *C. elegans*(Miller and Roth, 2007).

2.2.3 Lifespan Assay

All lifespan assays were carried out at 20°C. Synchronized populations were obtained by using the hypochlorite solution (see section 2.1.1). Worms that crawled off the plate were censored from survival analysis. Day 1 of adulthood was defined as the day the L4/young adult worms were moved to the experimental plates. Worms were moved to fresh treatment plates every other day, and the number of dead and living nematodes was counted daily until all the worms died. Dead worms were extracted from the agar plates if they did not respond to gentle contact with the wormpick. All statistical analyses were carried out using the GraphPad Prism software (version 9). Briefly, the elapsed time until death (X values) was entered for each group (Y values) and each dead worm was scored as 1 and each censored worm was scored as O. Then Kaplan-Meier survival curves were generated to illustrate the percentages of live worms in populations over time, and statistical significance of the survival curves were determined using the Log-rank test (Park, Jung and Lee, 2017).

2.2.4 Motility Assay

Worms were subjected to DR and algae oil supplementation as in the lifespan assay described above. The worm's motility was monitored daily using a stereo microscope. The phenotypes of worm locomotion were determined using a sterile wormpick and a mechanical stimulus to identify the motility classes as described by Herndon *et al.* Worms that exhibited spontaneous movement without external stimulus were graded as Class I. Worms that showed movement but required a mechanical stimulus from a wormpick were graded as Class II. Lastly, worms that only showed movement in head and tail region after mechanical stimulus were graded as class III. For data analysis, the motility data was divided into the respective motility classes and the data was normalised with the maximum number of individuals at the start of the assay set at 100%. The data was then fitted with a non-linear regression model (GraphPad Prism 9) to establish the mid-point ($_{K0.5}$) when 50% of the population was in each motility class.

2.2.5 Intestinal Barrier Function Assay (Smurf assay)

The smurf assay was adapted from the protocol described by Gelino et al. (2016). Worms were raised as described above for lifespan assays. The OP50+blue dye solution was made by dissolving 0.5g of erigolaucine disodium salt in 9.5ml of standard OP50 liquid culture (grown overnight). Five worms were randomly selected and suspended in 1ml of OP50+blue dye solution for 3 hours at 20°C. After three hours, dH_2O was added up to 10ml and centrifuged for 1min at 2000rpm. The excess bluedye+OP50 solution was discarded, and the wash step was repeated until the blue colour was cleared. The excess water was discarded and 100µl of 10mM sodium azide solution was added to immobilise the worms. After two minutes, the worms were transferred to an unseeded NGM plate using a sterile glass Pasteur pipette. Once the plates were dried (approximately 2-3min), the worms were imaged at 4x and 10x magnifications using a compound microscope (Leica ICC50 W) to determine the presence or

absence of blue dye in the body cavity. For each time point, three independent experiments were conducted.

2.2.6 Quantitative Measurement of GFP fluorescence in *C. elegans*

The fluorescence microscope (Olympus IX83, U-HGIGPS) was calibrated using calibration beads (InSpeck Green (505/515 nm) microscopy image intensity calibration kit (ThermoFisher). A standard calibration curve was constructed using GFP-labelled calibration beads. Briefly, each standard solution was prepared by mixing 40 μL of buffer A with 10μL of each calibration bead solution. 10μL of this solution was placed on a microscope slide and the beads were visualised with a fluorescence microscope. For each fluorescence concentration, ten individual beads were measured, and the average values were plotted against the microspheres (0.1-100%).

Fluorescence images of *C. elegans* were captured on fluorescence microscope driven by the CellSens imaging software using a 795.6ms exposure time at a magnification of 4x for whole body image and 20x for head and tail region of the worms. Fluorescence intensity images of *C. elegans* were measured by taking a random sample of five specimens from each population every other day. The microscope slides were prepared by mounting the worms on 10μl of buffer B and imaging the worms under the fluorescence microscope. Since buffer B is subject to drying, fluorescence images were taken within 20-30 minutes of placing the coverslip. Worms that had been damaged during handling were not included in the experiments. Whole-body fluorescence images of *C. elegans* were determined for quantification of GFP fluorescence using ImageJ and GraphPad Prism 9. All quantifications were calibrated using the GFP-labelled beads as an internal standard and corrected for background fluorescence (measured individually for each image) (Patterson *et al.*, 1997).

2.2.7 Techniques for Omega-3 PUFA Analysis

2.2.7.1 EPA Standards and Calibration Curve

1mg/ml working stock of EPA was made by dissolving in hexane. This stock solution was diluted with hexane to make six mixtures of standards with concentrations ranging from 1.25-50 µg/ml (see table 2.3).

Table 2. 3 Standard EPA stock solution concentrations.

Standard working solution (µl)	Hexane (µl)	EPA concentration (µg/ml)
1	199	5µg/ml
2	198	10µg/ml
4	196	20µg/ml
6	194	30µg/ml
8	192	40µg/ml
10	190	50µg/ml

To prepare the FAMEs, the same method as described by Deline et al. (2013) was followed. The EPA standards were mixed with 1ml of 2.5 % H_2SO_4 in methanol and heated at 70°C for 1 hr in a water bath. The standards were removed from the water bath and cooled at room temperature for 1min. FAMEs were extracted by adding 1.5ml of sterile water and 0.25ml of hexane. The vials were agitated vigorously for 1-2 minutes and centrifuged for 1min at maximum speed to separate hexane from the aqueous solvent. The hexane (top layer) was transferred to a GC vial, being careful not to transfer any of the aqueous phase. 1µl of the extracted hexane was injected and analysed by GC-MS as described above. Linear regression was used to construct the calibration curves (GraphPad Prism).

2.2.7.2 Generating FAMEs in E. coli OP50

The total fatty acid composition of *E. coli* and *C. elegans* can be evaluated by synthesising fatty acid methyl esters (FAMEs), which are extracted and quantified using gas chromatography-mass spectroscopy (GC-MS)(Deline, Vrablik and Watts, 2013). Algae-oil supplementation in *C. elegans* is dependent on the capacity of OP50 *E. coli* to uptake and incorporate fatty acid into its membrane. 100µL of *E. coli* OP50 was seeded on NGM plates with no supplement (control), 0.1 mM, and 0.3 mM concentrations of algae oil to determine its capacity to incorporate FAME into its membranes. Plates were dried at room temperature in the dark for two days before being incubated at 20°C for five days. After five days, *E. coli* was harvested into a silanized glass screw top vials using a flame-sterilized spatula. The FAMEs were generated by adding 1ml of 2.5% H_2SO_4 in methanol and heating the vial in a water bath for 1 hour at 70 °C. The vials were cooled for 1min at room temperature and FAMEs were extracted by adding 1.5ml dH_2O and 0.25 ml hexane. The mixture was agitated vigorously for 1-2 minute and centrifuged at maximum speed for 1 minute to separate the hexane from the aqueous solution. Then, the hexane (top layer) was transferred into a GC vial, taking care not to transfer any of the aqueous solution.

2.2.7.3 Generating FAMEs in *C. elegans*

Synchronized populations of *C. elegans* were obtained using the methods described at section 2.2.5. L4 adults were transferred to algae-oil supplemented plates and grown for five days at 20 °C. After five days, worms were washed off the plates with dH_2O and collected in silanized glass screw top vials. The worms were left to settle at the bottom of the vial for 5 minutes and excess water was removed. 1 ml of 2.5 % H_2SO_4 in methanol was added and heated at 70 °C for 1 hr in a water bath to induce the production of FAMEs. The vials were cooled for 1min at room temperature and the FAMEs were extracted by adding 1.5 ml water

and 0.25 ml of hexane. The vials were agitated vigorously for 1-2 minutes and centrifuged for 1 min at maximum speed to separate hexane from aqueous solvent. The hexane (top layer) was transferred to a GC vial with inserts, being careful not to transfer any of the aqueous phase.

2.2.7.4 Gas Chromatography and Mass Spectrometry (GC-MS)

For GC-MS analysis (Clarus SQ 8S, Perkin Elmer), 1-2 µl of FAMEs in hexane was injected onto a polar capillary gas chromatography column (VF-5MS) suitable for FAMEs analysis. The Agilent 7890 GC injector was set at 250 °C, with a flow rate of 1.4 ml/min, and the GC oven was programmed for an initial temperature of 130 °C, which was held for 1 min. Subsequently, the temperature was ramped 10 °C/min until 190 °C, and then ramped again at 5 °C/min until 210 °C and held for an additional 1 min. The components of FAMEs in the *E. coli* and *C. elegans* were identified by comparing their retention time and mass spectra against the National Institute of Standards and Technology (NIST) database.

Chapter 3: Effects of Dietary Restriction on Sarcopenia in Caenorhabditis elegans

3.1 Abstract

Sarcopenia leads to loss of functional mobility and independence in elderly people and is also a major component of frailty. With a growing ageing population, it is essential to understand the molecular mechanisms behind sarcopenia and possible therapies that can be used to address it. Here, using a novel fluorescence method, the transgenic, *unc-54::gfp C. elegans* strain which expresses the fluorescently-labelled muscle myosin UNC-54 was used to test the effect of three different dietary restrictions (mild, medium and severe) on the sarcopenia rates. Lifespan in mild and medium DR was significantly increased by 28% and 18%, respectively. In contrast, lifespan was significantly reduced by 16% in severe DR group compared to the control group. Motility data showed that the average time spent in class II motility was significantly increased in mild and medium DR (ΔC_2 = 6.8 and 5.8 days) compared to the control and severe DR groups (ΔC_2 = 5.1 and 3.1 days).

Data from the fluorescence intensity of the *C. elegans* body-wall muscle myosin UNC-54::GFP, displayed an age-related decrease in fluorescence in all groups, consistent with less overall myosin in the ageing worms. The fluorescence intensity in worms in mild and medium DR (k_F = 0.052 and 0.066 days, respectively) decreased at a slower rate in comparison to the severe DR and control group (k_F = 0.139 days). Mild and medium DR worms displayed a proportional increase in healthspan (28% and 19%, respectively) whereas healthspan was significantly decreased by 17% in severe DR compared to the control group.

Altogether, these results demonstrate that moderate DR significantly improved healthspan in *C. elegans* with increased motility and UNC54-GFP levels. Furthermore, the findings of this study confirmed the validity of the new approach for determining sarcopenia rates by using GFP fluorescence to measure myosin density.

3.2 Introduction

One of the changes associated with the ageing process is sarcopenia, the gradual loss of skeletal muscle mass and strength (Cruz-Jentoft and Sayer, 2019). Sarcopenia leads to the loss of functional mobility and independence and is linked to an increased risk of falls and fractures (Cederholm, Cruz-Jentoft and Maggi, 2013) (Senior *et al.*, 2015). Multiple factors have been implicated in development and progression of sarcopenia, such as hormonal changes (Melouane *et al.*, 2019), loss of neurons and satellite cells (Dao *et al.*, 2020), altered protein synthesis (Wiedmer *et al.*, 2021), inflammation and oxidative stress (Deepa *et al.*, 2019), but the molecular mechanisms are still not well understood. Lifestyle factors, such as physical inactivity and an unbalanced diet, can contribute to the loss of muscle mass and strength (Budui, Rossi and Zamboni, 2015). Currently there is no effective treatment to reverse sarcopenia, although dietary intervention and exercise can slow the rate of muscle loss (Domingues-Faria *et al.*, 2016)(Phu, Boersma and Duque, 2015). With a growing ageing population, it is essential to understand the molecular mechanisms behind sarcopenia in order to develop effective therapies (Han *et al.*, 2018).

Dietary restriction (DR), *i.e.* reducing calorie intake by 20-40%, has proven to be effective in attenuating and delaying the onset of sarcopenia in a wide range of organisms (Xie *et al.*, 2020). Although DR is not able to prevent the loss of muscle mass, it does slow down the process significantly. For instance, in both rats and rhesus monkeys, the dietary-restricted and control groups experienced skeletal muscle mass (SMM) loss with age however the percent loss was significantly greater in the control group compared to DR-treated groups (Rhoads *et al.*, 2020). The latter study also revealed that DR-monkeys had more mixed type 1 and type 2 myosin fibers compared to control (mainly type 1), a trait more commonly found in young monkeys (Rhoads *et al.*, 2020). The association between DR and ageing in general was first discovered

in 1935 in rats (McCay, Crowell and Maynard, 1935) and since then DR has been found to increase lifespan in many other organisms such as yeast, nematodes, flies, spiders, fish and primates, with conserved nutrient signaling pathways implicated in regulating longevity (Hwangbo *et al.*, 2020) .

The nematode *C. elegans* has proven to be an excellent model of human ageing and has played a major role in establishing lifespan regulatory pathways, including the IGF-1 and mTOR signaling pathways (Campisi *et al.*, 2019)(Daskalaki, Markaki and Tavernarakis, 2020). *C. elegans* have a relatively short lifespan (3-4 weeks) and also undergo sarcopenia with loss in muscle mass, sarcomere integrity and mobility (Park, Jung and Lee, 2017) (Ibáñez-Ventoso *et al.*, 2016). Mutations in the *daf-2* gene, that encodes for an IGF-1 receptor, increase lifespan by almost two-fold compared to wild type *C. elegans* (Kenyon, 2011)(Braeckman and Dhondt, 2017) and also delay the development of sarcopenia compared to wildtype (Glenn *et al.*, 2004).

Numerous studies in *C. elegans* have demonstrated the lifespan-extending benefits of DR (Moatt *et al.*, 2020), however the effect of DR on healthspan, and in particular sarcopenia, is less well documented (Bansal *et al.*, 2015). Long-lived *C. elegans* mutants were found to spent an increased proportion of time in a frail state (Bansal *et al.*, 2015). Instead of a genetic model of DR in *C. elegans* (*eat-2),* the work presented in this chapter uses the solid DR method (sDR) and a transgenic *C. elegans* strain (*unc-54::GFP),* to quantify myosin density in *C. elegans* using fluorescence spectroscopy. This strain has one of its myosin heavy chain isoforms (UNC-54) labelled with GFP (Várkuti *et al.*, 2012). The *unc-54* gene codes for the muscle myosin heavy chain B (MHC B), a major component of the body-wall muscle in *C. elegans* and essential for locomotion and maintaining structural stability in sarcomeres (Várkuti *et al*., 2012). To our knowledge, this is the first study documenting the detailed rate of the loss of the

major muscle myosin isoform UNC54 in *C. elegans*, exposed to different DR regimes, over the whole course of its lifespan. The results showed that worms in mild and medium DR groups maintained increased levels of UNC54 on day 10, 12 and 14 of adulthood compared to the control and severe DR groups. Additionally, the fluorescence results showed that the loss of UNC54 myosin was two-fold slower in the mild and medium DR groups (k_F =0.052 and 0.066 days) compared to the severe DR and control groups (k_F = 0.139 days). Lifespan and motility data were consistent with the observed sarcopenia rates. Collectively, these results suggested that different DR regimens result in different rates of sarcopenia, with mild/medium DR groups displaying a delayed onset of sarcopenia compared to control and severe DR groups.

3.3 Materials and Methods

3.3.1 *C. elegans* Strain

The transgenic *C. elegans* strain, *unc-54::gfp* was used unless otherwise indicated (See chapter 2, Table 2.2 for further details). *C. elegans* were maintained at 20°C on standard NGM with *E. coli* OP50 as a food source (Stiernagle, 2006).

3.3.2 Dietary Restriction Assay

The solid DR (sDR) method was adapted from Greer et al. (2009). Bacterial concentration was determined by plating serial dilution and subsequent counting of CFU (See chapter 2 for further details). Freshly grown *E. coli* OP50 were diluted to achieve DR concentrations of $8x10^{10}$ (mild), $8x10^{8}$ (medium) and $8x10^{6}$ (severe) cfu/ml. 100µl of the bacterial concentrations were seeded on NGM(-Pep) plates and FUDR was added on top of the bacterial lawn to prevent reproduction (Hosono, Nishimoto and Kuno, 1989). The peptone was excluded to prevent bacterial growth during the DR assay (Stastna *et al.*, 2015). For the control group, 100µl of *ad libitum* ($8x10^{10}$) was seeded on standard NGM plates. Synchronous populations of worms were generated by treating gravid adult worms with hypochlorite solution and the worms were allowed to grow to the L4 stage. DR was started at the L4 stage, with 15 worms per plate and 4 plates per group. Worms were moved to fresh plates every other day to prevent starvation and maintain the experimental conditions (Greer *et al.*, 2007). FUDR treatment was discontinued once reproduction had ceased (day 7).

3.3.3 Lifespan assays

All lifespan assays on *C. elegans* were performed at 20°C. For lifespan under DR conditions, L4 worms were moved to NGM(-Pep) plates with 15 worms per plate and 4 plates per group. Lifespan was measured by counting the number of dead and alive worms in each population

each day, and recording it as a percentage of the day's population (Lee *et al.*, 2006). Worms were considered dead when they no longer responded in any way to the stimulus of contact with the worm pick, and were removed from the plates (Lee *et al.*, 2006). Worms were censored if they crawled off the plate or died from vulvul bursting. Data from the lifespan assay were analysed using Kaplan-Meier Survival curves with the statistical significance determined by log-rank analysis in GraphPad Prism version 9. Differences were considered statistically significant at $p<0.05$.

3.3.4 Motility Assays

The behavioral criteria defined by Herndon et al. (2002) were used to determine age-dependent motility under DR conditions. 15 L4 stage worms were moved to NGM(-Pep) plates, again with 4 plates per group. Motility was assessed by subjecting each worm to gentle stroking with a wormpick and grading their response into three defined motility class: I, II and III (as detailed in Materials and Methods). Motility data was analysed by allocating the number of worms into the respective motility classes and the data was normalised with the maximum number of individuals at the start of the assay set at 100%. The data was then fitted with a non-linear regression model (GraphPad Prism 9) to establish the mid-point ($_{K0.5}$) when 50% of the population was in each motility class.

3.3.5 Fluorescence Microscopy

The fluorescence microscope (Olympus IX83) was calibrated using InSpeck Green microscope image intensity calibration kit. The calibration kit included 6.0 μm–diameter InSpeck fluorescent microspheres with fluorescence intensities ranging from very low (0.3%) to high intensity fluorescence (100%). Briefly, the calibration bead solutions were vortexed and each standard solution was prepared by mixing 40μL of buffer A with 10μL of each

calibration bead solution. 10µL of each calibration bead solution was placed on a microscope slide for imaging at 20X magnification. For each microsphere concentration, ten individual beads were measured, and the average values were plotted against the microspheres to generate a standard calibration curve (Appendix Figure A1). Image acquisition parameters were set at 12.8 dB (gain) and 1.8 sec (exposure time). All acquisition parameters and settings were controlled by CellSens imaging software.

C. elegans fluorescence intensity was measured by taking a random sample of five specimens from each DR population every other day. To normalize fluorescence intensity across experiments, 6µm polystyrene microspheres of 3% relative intensity fluorescence with excitation/emission wavelength of 505/515nm (Solution D) was used as an internal standard. Briefly, 40µL of buffer A was mixed with 10µL of solution D to prepare the GFP-microscope solution (buffer B). The worms were immobilised on microscope slides with 10µl of buffer B (See methods chapter) and whole-body fluorescence images were captured at 4X magnification. Mean fluorescence intensity of *C. elegans* expressing the myosin UNC-54::GFP was measured using ImageJ. The average background fluorescence was calculated by measuring several same-size regions of the image where no worms were present (Schneider, Rasband and Eliceiri, 2012). Then the background fluorescence was subtracted from the mean fluorescence intensity of *C. elegans* before normalising the data. The mean fluorescence intensity of *C. elegans* were normalised by the mean fluorescence intensity of microspheres from the same image (measured individually for each image) to calculate the final mean fluorescence intensity. Statistical analysis of the fluorescence intensity data was performed with GraphPad Prism (Version 9.3). Significances were calculated using two-way ANOVA. Fluorescence differences were considered statistically significant at $p<0.05$.

3.4 Results

3.4.1 Mild and medium DR increases Lifespan of *C. elegans*

The effect of various dietary restriction regimes on the lifespan of *C. elegans* (unc54::gfp), is depicted in Figure 3. 1 and summarized in Table 3. 1.

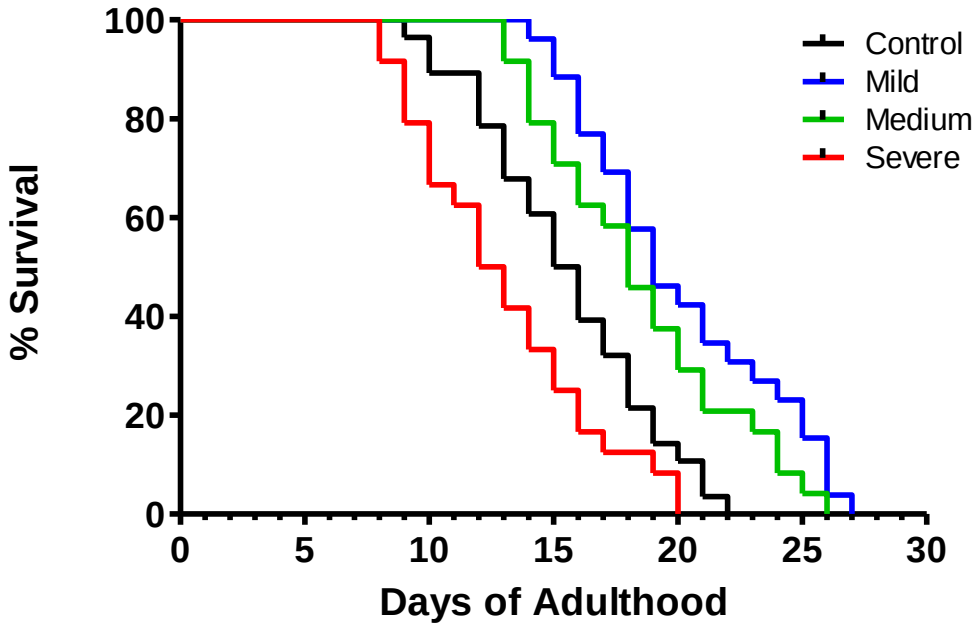

Figure 3. 1 Effect of different DR regimes on *C. elegans* lifespan. The mean life span of populations exposed to mild DR (19.9 days, $p<0.001$) and medium DR (18.3 days, $p = 0.011$) was significantly increased compared to control (15.5 days). Severe DR resulted in a reduction in mean lifespan (12 days, $p = 0.026$). Lifespan assays were done at 20°C. Survival plots were drawn by Kaplan-Meier survival assay. Shown are data from one experiment and is representative of two independently completed experiments.

The extension of mean lifespan was most effective under mild DR conditions with an increase of 28% whereas the medium DR conditions resulted in a moderate increase of 18 %. In contrast, the severe DR groups showed a reduction of the mean life span by 16% compared to the control. These findings are consistent with previous studies in which exposure of worms to moderate DR had a beneficial impact

on lifespan while severe DR resulted in detrimental effects on lifespan (Greer and Brunet, 2009)(Piper and Partridge, 2007)(Mair and Dillin, 2008). The median life span of the control group reported here for *C. elegans* (unc-54::gfp) agrees with previous studies that used wildtype (N2) worms (De Cuyper and Vanfleteran, 1982)(Hosono *et al.*, 1980) validating using the *unc54::gfp* strain.

Table 3. 1. Effect of different DR regimes on the mean lifespan of *C. elegans*. Shown is the mean and maximum lifespan and the % change compared with control.

Diet	Mean lifespan (days)	% Increase in Mean Lifespan	Maximum lifespan (days)	% Increase in Maximum Lifespan
Control	15.5±0.7		22.0±0	
Mild	19.9±0.8	28.4%	27.0±0	22.7%
Medium	18.3±0.8	18.1%	26.0±0	18.2%
Severe	13±0.8	-16.1%	20.0±0	-9.1%

3.4.2 Mild and medium DR increase Motility of *C. elegans*

Individuals in each DR group were allocated to one of three distinct motility classes (I, II and III) as described by Herndon et al. (2002). The results of the motility assay are shown in Figure 3. 2 and summarised in Table 3. 2. In line with previous findings (Greer and Brunet, 2009), differences in onset and progression of motility decline was observed across all groups. During the early stages of adulthood (day 1-3), 100% of the worm population exhibited spontaneous smooth movement (Class I) and there were no visible differences in spontaneous motility between all groups (Figure 3. 2A). The differences became more pronounced as the worms aged, as can be seen when individuals moved from class I to class II (Figure 3. 2A & B) or from class II to class III (Figure 3. 2B & C). When comparing the average number of days for 50% of the initial population to move from motility class I to class II (Table 3. 2, parameter $C_{1\text{-}2}$), non-linear regression analysis revealed that worms under mild and medium DR spend longer in class I ($C_{1\text{-}2}$ = 8.6 and 8.5 days respectively) compared to control and severe DR ($C_{1\text{-}2}$ = 6.9 days). Transition from class II to class III was also delayed for worms under mild and medium DR ($C_{2\text{-}3}$ = 15.4 and 14.3 days respectively) compared to control and severe DR ($C_{2\text{-}3}$ = 12.0 and 10.0 days). Thus, the average time spend in class II increased for the mild and medium DR groups (ΔC_2 = 6.8 and 5.8 days) compared to the control and severe DR populations (ΔC_2 = 5.1 and 3.1 days).

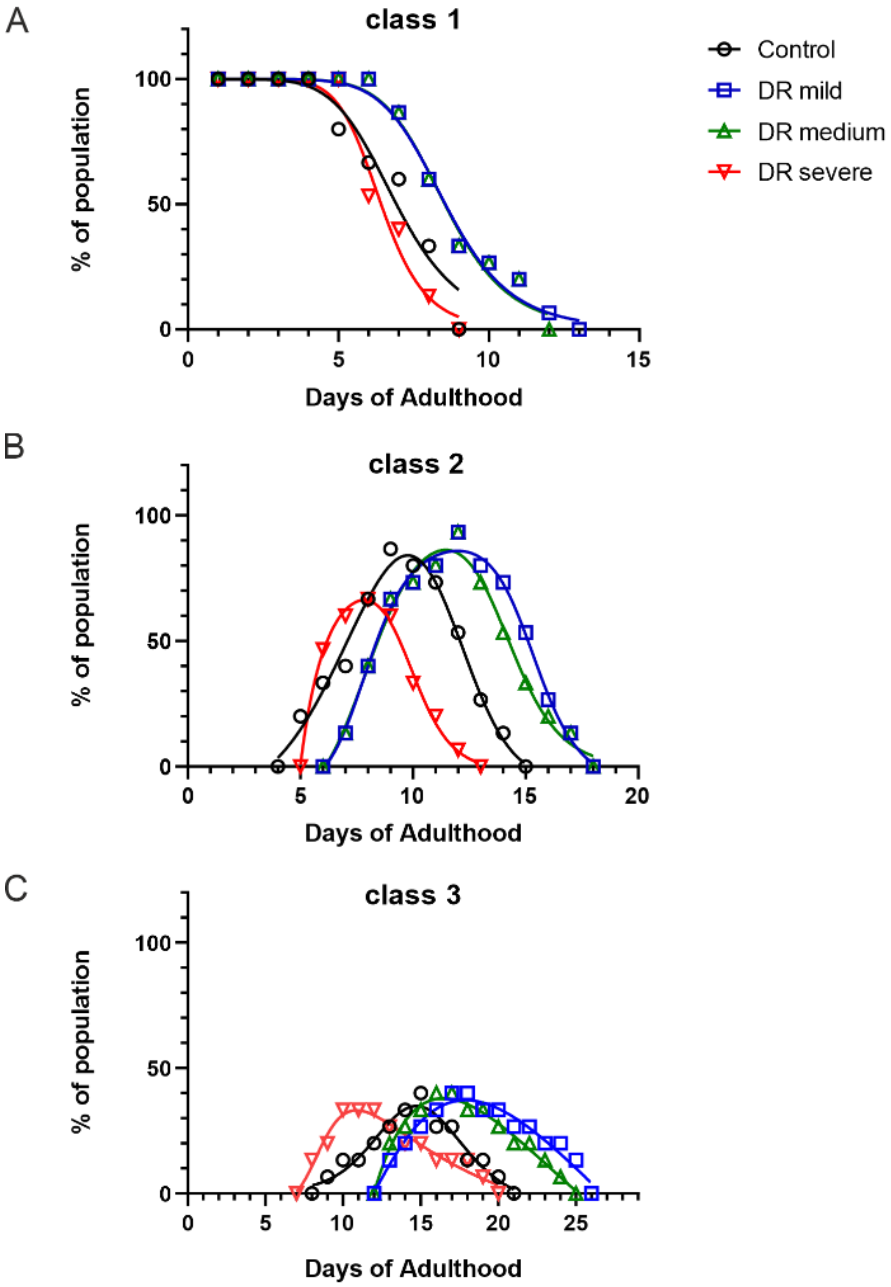

Figure 3. 2 Age-related changes in motility of DR worms. Classification of movement according to Herndon et al., (2002). (A) Class I – Continuous smooth movement, fast movement when stimulated. (B) Class II – Slow halting movement, smooth movement when stimulated. (C) Class III – Small movement of head or tail, very slow movement when stimulated. Data is normalised with maximum number of worms at the start of the assay set at 100%. Data graph shows the result of one experiment and is representative of two independent experiments.

Table 3. 2. Summary of age-related changes in motility under DR. Average number of days for 50% of the initial population to move from motility class I to class II, represented by $C_{1\text{-}2,}$ and average number of days for 50 % of the class II population (based on maximum % in class II) to move to class III (represented by $C_{2\text{-}3}$). The difference between the two mid-points, represented by parameter $\Delta C_{2,}$ is the average time spent in class II.

	$C_{1\text{-}2}$ (days)	**$C_{2\text{-}3}$ (days)**	**ΔC_2 (days)**	**Mean life span (days)**
Control	6.9 ±0.2	12 ±3	5.1	15.5±0.7
Mild	8.6 ±0.1	15.4 ±0.3	6.8	19.9±0.8
Medium	8.5 ±0.4	14.3 ±0.4	5.8	18.3±0.8
Severe	6.9 ±0.1	10.0 ±0.5	3.1	13±0.8

3.4.3 Mild and Medium DR improves Healthspan in *C. elegans*

Finding variables that promote healthy ageing (healthspan) in conjunction with lifespan is crucial for translating DR research into practical treatments. Previously, Bansal et al. (2015) showed that the extended lifespan of *daf-2* mutants comes at the expense of greater time spent in a fragile condition (gerospan). In light of these findings, the healthspan and gerospan of DR worms were examined at early and mid-adulthood to provide a more realistic assessment of *C. elegans* health. In early adulthood, health span was defined as the period when 50% of the worm population is categorised as motility class I, and gerospan was defined as the period when 50% of the worm population has lost class I motility. When measured chronologically, the healthspan period for control and severe DR group was just under seven days, however the healthspan period for mild and medium DR groups was extended to 8.6 and 8.5 days, respectively (Figure 3. 3A). When the data was normalized to maximal lifespan, both the DR and control groups showed similar healthspan to gerospan ratio (Figure 3. 3B). The severe DR

worms spent 35% of their lives in a healthy condition and 65% in a frail state (Figure 3. 3B), indicating a minor increase in healthspan when compared to control however, this improvement is attributed to a shorter lifetime in severe DR-treated worms.

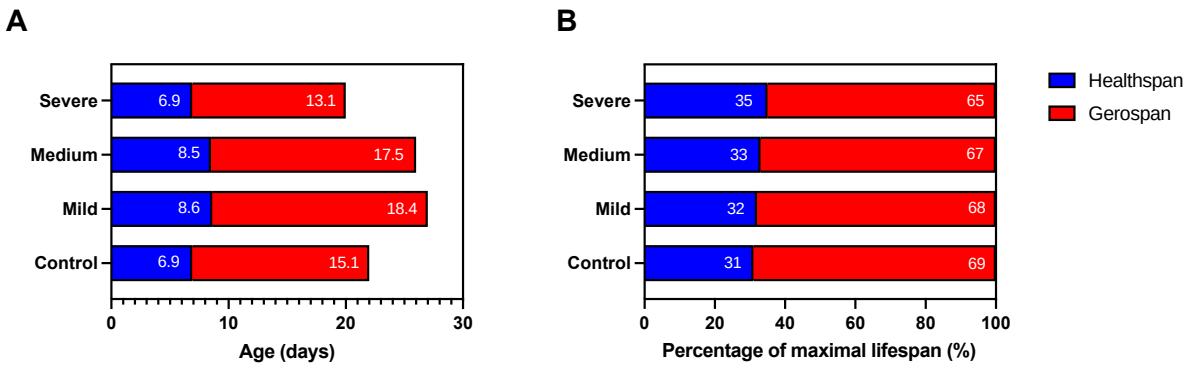

Figure 3. 3 Comparison of healthspan and gerospan in control and DR groups at early adulthood. A) Healthspan was defined as the period when > 50% of the initial nematode population show type-I motility. Gerospan was defined as the period when < 50% of the initial nematode population show < 50% of type-I motility. B) The ratio of healthspan to gerospan was normalized to their maximal lifespan in control and DR worms (Hahm *et al.*, 2015).

At mid-adulthood, the definition of healthspan was modified as the period when 50% of the worm population displayed class II motility, whereas gerospan was defined as the period when more than 50% of the worm population lost class II motility (Figure 3. 4). Mild and medium DR enhanced healthspan by 28% and 19%, respectively, when compared to controls (Figure 3. 4A), and the resulting normalised data showed a comparable healthspan-to-gerospan ratio (Figure 3. 4B). In contrast to Bansal *et al.*, these findings show that mild and medium DR extends lifespan and healthspan to a similar extent, without proportionally prolonging the frailty period.

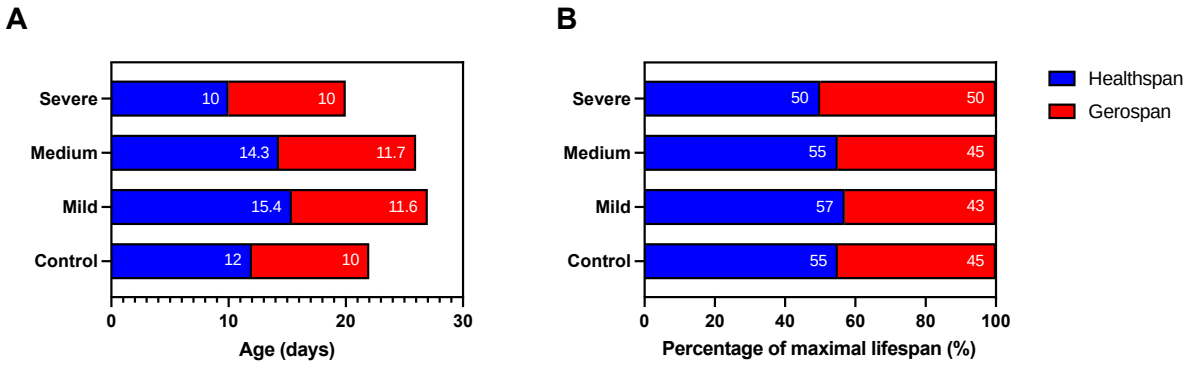

Figure 3. 4 Comparison of healthspan and gerospan in control and DR groups at mid-adulthood. A) Healthspan was defined as the period when > 50% of the initial nematode population show type-II motility. Gerospan was defined as the period when < 50% of the initial nematode population show < 50% of type-II motility. B) The ratio of healthspan to gerospan normalized to their maximal lifespan in control and DR worms.

3.4.4 Age-dependent decline in myosin levels is delayed under mild and medium DR conditions

To quantify the loss of muscle mass under various DR conditions, fluorescence microscopy was used to visualize the body wall muscles of transgenic worms expressing UNC54-GFP over the entire lifespan of *C. elegans*. Figure 3. 5 shows representative fluorescence images for all four DR groups at different time points, illustrating fluorescence intensity increased and decreased in an age-related manner. Boxplots of fluorescence intensity start to show significant differences in fluorescence for mild DR and medium DR groups compared to control from day 10 onwards (Figure 3.6). The fluorescence data in this study do not address heterogeneity in the fluorescence of individual worms. The individuals used for fluorescence measurements were randomly selected, based on age and diet, and their motility class was not taken into account. Therefore, at certain timepoints, one could expect to have

individuals from different motility classes and hence a wider data spread in fluorescence intensity (for a full data set of fluorescence intensity over lifespan see Appendix Figure A2).

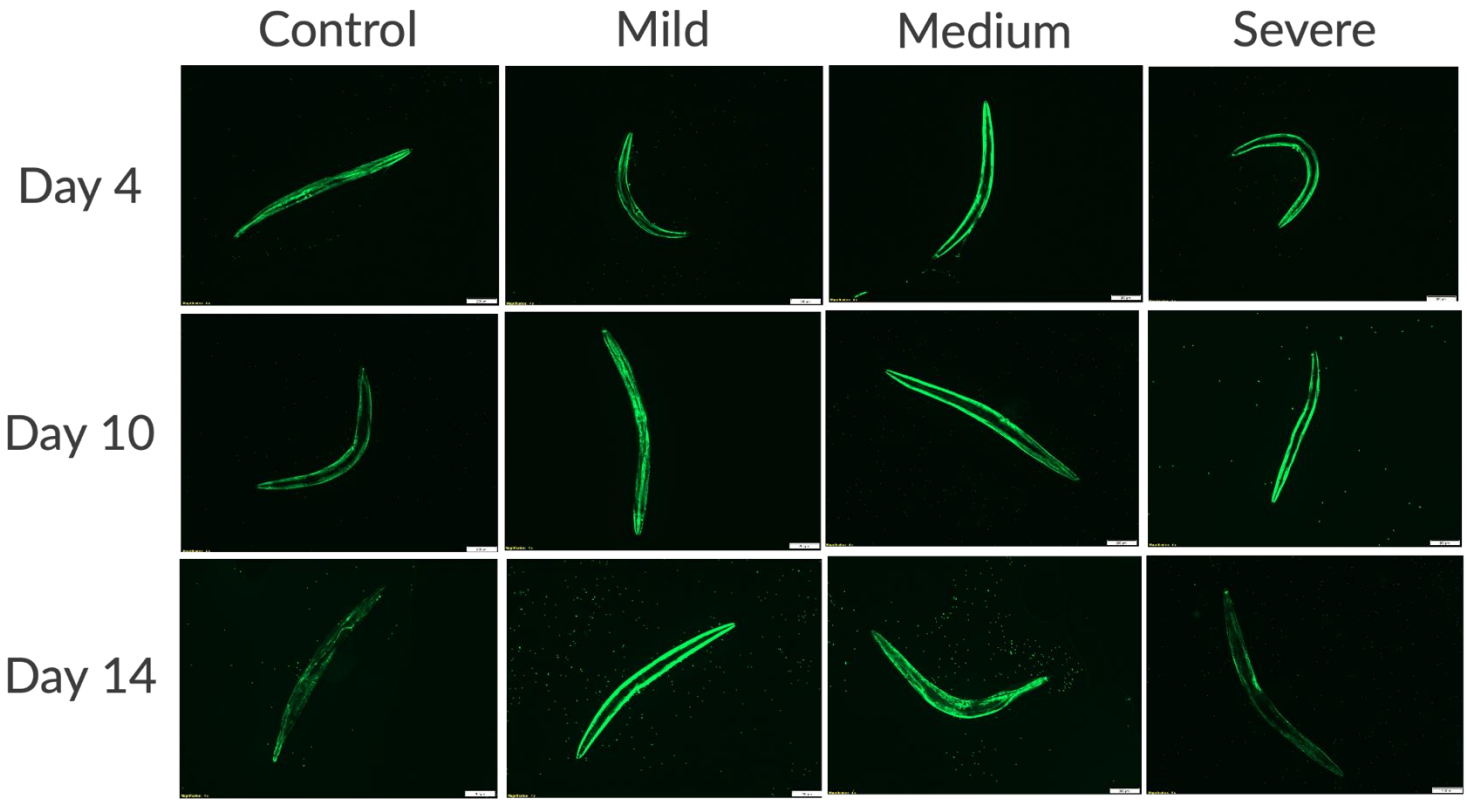

Figure 3. 5 Visualisation of muscle density over *C. elegans* lifespan using fluorescently labelled myosin (UNC54-GFP). Representative fluorescence microscopy images (4x magnification) of *C. elegans* grown under various DR conditions (mild, medium, severe) compared with control (no restriction) at day 4, 10, and 14 of adulthood.

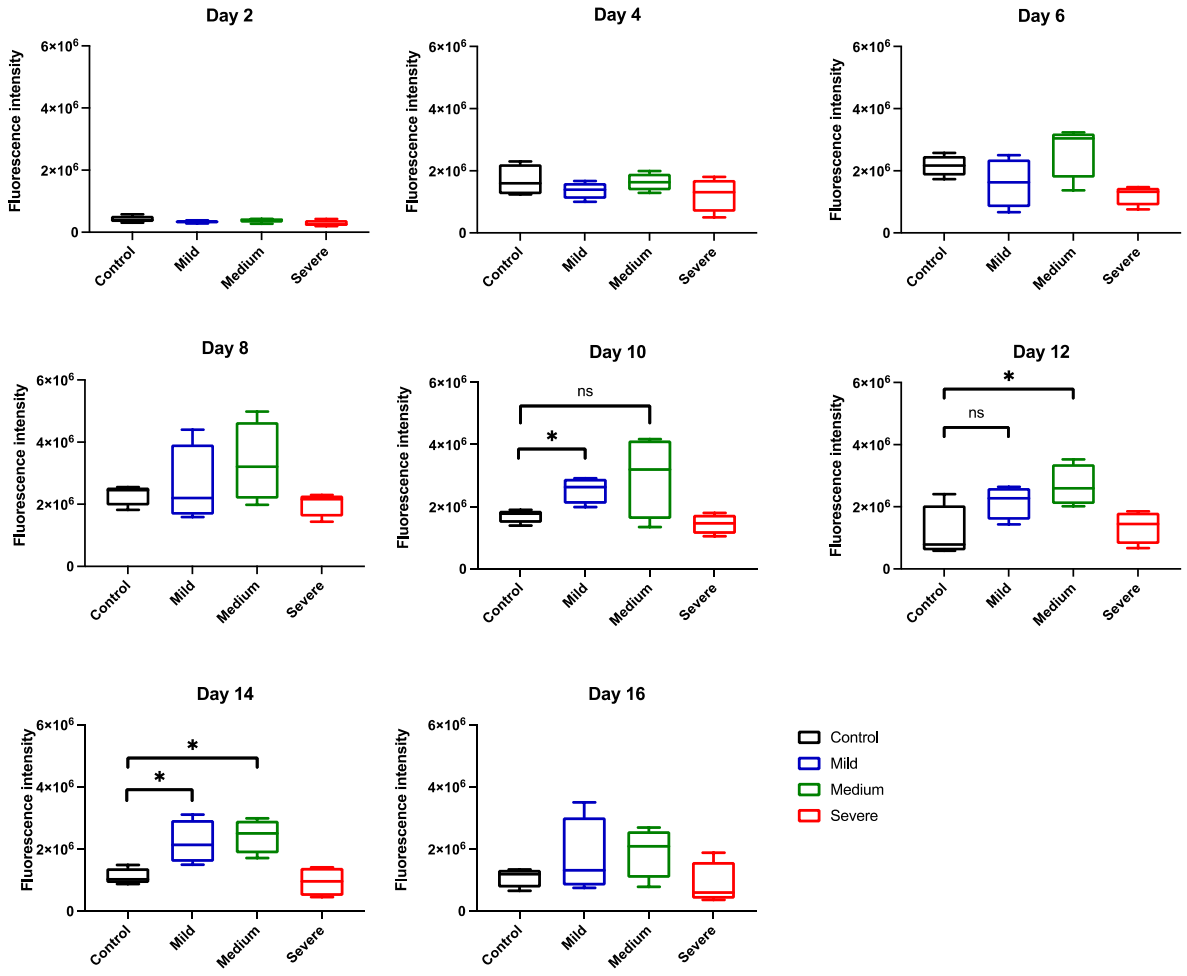

Figure 3. 6 Age-related loss in myosin density was delayed under mild and medium DR. Fluorescence intensity, representing myosin density of body-wall muscle at different time points for various DR conditions (mild, medium, severe) compared to control (no restriction). (Two-way ANOVA, *P < 0.05)

The increase in fluorescence, seen during the first eight days of adulthood, is indicative of the rate of muscle growth, with the medium DR group on average showing the largest increase in myosin density, followed by mild DR and control, whereas severe DR consistently showed the lowest myosin density increase (Figure 3. 7A). A decrease in fluorescence is seen after day 8 (Figure 3. 7B) with the amount of GFP-labelled myosin (UNC54-GFP) reducing for all groups. Single exponential fits of the average fluorescence intensity show that mild and medium DR groups lost 50% of their maximum UNC54-GFP myosin at 21.4 days and 18.6 days respectively (Table 3. 3), compared to control and severe DR groups which lost 50% of peak UNC54-GFP myosin at 13 days, respectively. The rate at which UNC54-GFP levels decrease, represented by the rate of fluorescence loss (k_F), is slower for mild and medium DR compared to control and severe DR (Table 3. 3), suggesting that the onset and rate of sarcopenia is delayed in worms under mild and medium DR, compared to the control and severe DR groups.

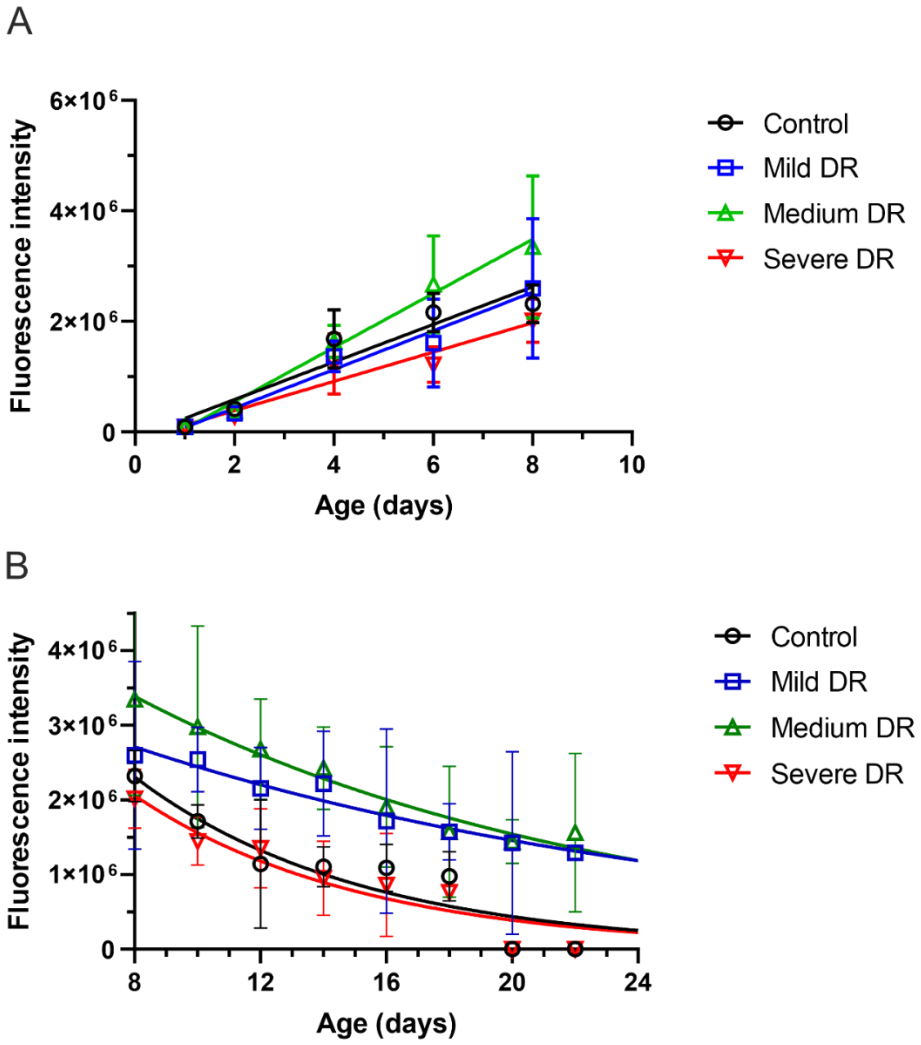

Figure 3. 7 Expression of myosin UNC54-GFP as a function of age. Average fluorescence intensity with standard deviation is shown (n=5). (A) Average fluorescence increased during day 1-8 for the various DR groups. Linear regression resulted in values for mild and medium DR with slope = $3.5\pm0.3\text{x}10^5$ and $4.9\pm0.3\text{x}10^5$ respectively (corresponding R^2-values of 0.97 and 0.99). Control and severe DR groups yielded slopes of $3.4\pm0.6\text{x}10^5$ and $2.6\pm0.4\text{x}10^5$ respectively (R^2-values of 0.91 and 0.93). (B) Average fluorescence decreases from day 8 towards end of lifespan for control, mild DR, medium DR and severe DR. Single exponential fits yielded a rate of fluorescence loss k_F of 0.139 $days^{-1}$ (control), 0.052 $days^{-1}$ (mild), 0.066 $days^{-1}$ (medium) and 0.139 $days^{-1}$ (severe). (R^2-values = 0.86 (control), 0.95 (mild), 0.97 (medium) and 0.89 (severe).

Table 3. 3 Summary of age-related changes in fluorescence and expression of myosin UNC54-GFP under DR.

	Fluorescence increase[1] (10^5 day^{-1})	**Fluorescence loss[2] k_F (day^{-1})**	**$t_{1/2}$[3] (days)**	**$T_{0.5}$ UNC54-GFP[4] (day)**
Control	3.4±0.6	0.139±0.029	5.0	13.0
Mild	3.5±0.3	0.052±0.005	13.4	21.4
Medium	4.9±0.3	0.066±0.005	10.6	18.6
Severe	2.6±0.4	0.139±0.026	5.0	13.0

[1]Measured from day 1- 8.
[2]Determined from day 8 (maximum fluorescence) until day 24.
[3]Determined using ln (2)/k_F
[4] Age at which 50% of maximum UNC-54-GFP myosin is lost.

3.5 Discussion

This work used the sDR and a transgenic *C. elegans* strain (*unc-54::gfp*), to determine myosin density in *C. elegans* body wall muscle in order to quantify sarcopenia rates. Based on the average rate of fluorescence decrease (k_F), loss of UNC54 myosin was about two-fold slower for the mild and medium DR groups compared to control and severe DR (Table 3. 3). Protein turnover studies in *C. elegans* using a pulse labelling method demonstrated low turnover of muscle-related proteins, suggesting that most muscle-specific proteins rarely turnover during the *C. elegans* lifespan (Dhondt *et al.*, 2016). In particular, Dhondt et al. (2017) reported an average half-life of 298hr (=12.4days) for UNC-54 protein which is very similar to the $t_{1/2}$ value (13 days) reported here for the UNC-54::GFP control (Table 3.3). Many studies have reported a significant decrease in protein turnover rates in ageing *C. elegans* (Dhondt *et al.*, 2017) (Visscher *et al.*, 2016). In elderly worms, lack of protein turnover may result in muscle protein degradation rates surpassing muscle protein synthesis, leading to sarcopenia (Dhondt *et al.*, 2017). Since protein loss follows an exponential decay function (Ross, Langer and Jovanovic, 2021), the muscle protein degradation rates were represented by the rate of myosin loss (k_F) in this study. Interestingly, the rate of myosin loss (k_F) was positively correlated with the motility parameter $C_{1\text{-}2}$ (R^2 = 0.99) with increased rate observed for control and severe DR compared to mild and medium DR (Appendix Figure A3. A). This suggests that mild and medium DR worms, with a longer lifespan, exhibited a later onset of protein degradation. This is consistent with previous studies which have shown that protein degradation is accelerated in short-lived and delayed in long-lived *C. elegans* (Gaffney *et al.*, 2018) due to long-lived *C. elegans* exhibiting a more stable proteome (Treaster *et al.*, 2014).

In contrast, the rate of myosin loss (k_F) did not correlate as well with the motility parameter $C_{2\text{-}3}$ (R^2 = 0.88) (Appendix Figure A3. B), consistent with the decreased turnover rates in ageing *C. elegans* (Dhondt *et al.*, 2017). Nevertheless, the rate of myosin loss (k_F) was still higher for control and severe DR groups compared to mild and medium DR (Appendix Figure A3. B),

indicating increased protein degradation and sarcopenia rates. Similar rates of decline between motility and body wall muscle suggests that these changes might be regulated by a common mechanism and /or the two changes share common signalling pathways. Numerous studies have demonstrated, that in *C. elegans,* the life-extending effect of DR is controlled by a number of overlapping and independent signaling pathways (Cypser, Kitzenberg and Park, 2013). For instance, sDR has been reported to extend lifespan via the AMPK pathway (Greer *et al.*, 2007). The study found that lifespan in mutant worms (*aak-2)* carrying a deletion in the gene that codes for AMPK showed a 48% decrease in lifespan in comparison to the wildtype N2 worms, suggesting that the AMPK pathway is crucial for DR-related lifespan extension (Greer *et al.*, 2007). Likewise, DR using mutants (*eat-2*) or complete bacterial deprivation (BD) has been shown to exert its lifespan extending effects by downregulating the TOR pathway in *C.elegans* (Houthoofd and Vanfleteren, 2006) (Houthoofd *et al.*, 2007). Inhibition of the TOR pathway has also been linked with the life-extending effects of DR in yeast and flies (Santos *et al.*, 2016). Similarly, intermittent fasting (IF) has been reported to extend lifespan by inhibiting the IGF-1 signalling pathway (Honjoh *et al.*, 2009). Given this plethora of signalling pathways, it is likely that the DR employed in this study could act on several distinct pathways to extend lifespan and motility in *C. elegans*. Moreover, the considerable difference in lifespan and motility between the different DR regimens, supports the idea that DR affects lifespan and motility through distinct pathways.

Loss of motility has been correlated with age-related muscle cell damage in *C. elegans* (Herndon *et al.*, 2002)(Glenn *et al.*, 2004). The motility differed significantly between the control and DR populations except for severe DR, suggesting that the body muscles aged differently between the groups (Figure 3. 3). Motility and lifespan in DR worms showed a fairly linear relationship with the motility parameter $C_{2\text{-}3}$ showing a positive correlation with the mean lifespan

(Appendix Figure A4. B), suggesting that mid-adulthood motility is a reliable predictor of lifespan. This is in agreement with a previous study that showed maximum velocity measured at mid-adulthood is a good predictor of maximal lifespan (Hahm *et al.*, 2015). Additionally, the motility data was combined with lifespan data to determine the healthspan to gerospan ratio in DR worms. Mild and medium DR increased healthspan at both early and mid-adulthood compared to severe DR and control groups. This demonstrates that mild and medium DR increased both lifespan and healthspan to a similar level, without extending the frail period of life. This is consistent with previous studies that showed increased healthspan in daf-2 mutants when compared to wildtype (Hahm *et al.*, 2015). Many studies have shown a decrease in skeletal muscle metabolism with ageing, mostly due to a decrease in mitochondria activity (Yamada *et al.*, 2013). There is growing evidence indicating that metabolic reprogramming, that occurs in response to DR, may underlie the beneficial effects of DR (Feng *et al.*, 2016). In *C. elegans*, *daf-2* mutants have reduced metabolic rate compared to wildtype, indicating that decreased metabolic rate in these worms may be responsible for prolonging their lifespan (Gallo and Riddle, 2010). Similarly, the preservation of muscle mass and physical function seen in dietary-restricted rhesus monkeys is also linked with metabolic pathways (Rhoads *et al.*, 2020).

Previous studies have shown that along with a decrease in mobility and feeding, ageing worms also lose the ability to respond to DR treatments that extend lifespan (Fletcher and Kim, 2017). In particular, Fletcher and Kim, (2017) showed that ageing worms show a marked decrease in the expression of *daf-7*, which is thought to promote lifespan extension in response to DR. *daf-7* encodes for a transforming growth factor-β (TGFβ) ligand which controls a diverse aspect of *C. elegans* development and physiology including metabolism, behaviour and Dauer formation (Li *et al.*, 2015). *daf-7* has also been implicated in regulating *C. elegans* lifespan and food-sensing behaviour as studies have shown that during periods of low food availability, dauer

inducing pheromones are released which in turn inhibits the expression of *daf-7*, leading the worms to undergo the dauer state (Shaw *et al.*, 2007). However, when food becomes available, the expression of *daf-7* increases and the worms recover from the dauer state (Ren *et al.*, 1996). Fletcher et al. (2017) found that in transgenic worms overexpressing *daf-7*, the worms retained the ability to respond to DR to extend lifespan in comparison to wildtype worms, suggesting that the decreased expression of *daf-7* in ageing worms is, in part, responsible for the reduced sensitivity to DR in ageing worms (Fletcher and Kim, 2017).

In conclusion, this study has shown that peptone-deprivation mediated DR delays the sarcopenia rates in *C. elegans*. These results indicate that moderate DR also improves healthspan, as shown by improved motility and maintenance of higher levels of UNC54 as the organism ages. This is in line with previous research in rhesus monkeys, which showed that moderate DR delayed the onset of sarcopenia and retained more muscle mass with age compared to control (Colman *et al.*, 2008)(McKiernan *et al.*, 2012). Severe DR results in detrimental effects to the lifespan and motility of *C. elegans*, indicating that the beneficial effects of DR can only be elicited at certain bacterial concentrations. These findings underscore the importance of diet on the lifespan and health span of *C. elegans*. This is the first study in *C. elegans* that focused on the effects of DR on muscle mass in the setting of sarcopenia by measuring myosin density directly. Subtle changes in sarcopenia rates can be quantified din C. elegans using this method and can be used as a starting point to develop a screening method to determine sarcopenia rates in response to various interventions. If these findings could be applied to humans, they might have a major impact on elderly people' functionality and quality of life while also lowering the global burden and health-care costs associated with sarcopenia.

Chapter 4: Effect of Algae Oil Supplementation on the Lifespan and Healthspan of *C. elegans*

4.1 Abstract

Sarcopenia is a multifactorial condition caused by age, chronic inflammation, poor diet, and lack of physical activity. The effects of dietary supplementation on age-related deterioration of muscle mass and function were investigated in this study. Transgenic *C. elegans* strain (*unc-54::gfp*) expressing UNC-54::GFP, localized in body wall muscles, were supplemented with 0.1mM and 0.3mM concentrations of algae-oil and muscle integrity was determined by quantifying GFP fluorescence regularly as the nematodes age. Along with increased lifespan, both concentrations of algae-oil attenuated age-related decline in motility and myosin UNC-54 loss compared to the control group. These findings demonstrate that algae-oil supplementation can extend not only lifespan but also health span in *C. elegans*, as demonstrated by preserving muscle function and myosin density. The data also confirm that *C. elegans* is a promising model for evaluating therapeutic interventions for delaying sarcopenia development.

4.2 Introduction

Sarcopenia is an age-related condition characterized by loss of skeletal muscle mass and function (Sanchez-Rodriguez, Marco and Cruz-Jentoft, 2020). Sarcopenia can affect individuals from as early as age 30 and becomes more severe with age (Sanchez-Rodriguez, Marco and Cruz-Jentoft, 2020). Sarcopenia is associated with many factors such as type II fibre atrophy, alterations in fibre type distribution, loss of motoneurons, and muscle fat infiltration (Gilligan *et al.*, 2020). Loss of muscle mass can affect an individual's ability to maintain balance, increase muscle fatigue and heighten the risk of other diseases or exacerbate existing conditions (Schneider and Correia, 2020). These can all lead to problems in performing normal everyday activities, falls and fractures, diminished quality of life, frequent hospitalisations, higher risk of post-surgical complications and eventually, the need for long-term care and institutionalisations (Schneider and Correia, 2020).

Ageing is associated with a decline in the production of key hormones such as testosterone, oestrogen, and insulin-like growth factor (IGF) that promote skeletal muscle mass and function (Priego *et al.*, 2021). This, in turn, is suggested to play a role in the progression of sarcopenia (Priego *et al.*, 2021). Elevated serum levels of proinflammatory cytokines have been linked to the development and progression of sarcopenia, since these cytokines directly alter muscle catabolic and anabolic signalling pathways (Nelke *et al.*, 2019). Environmental and lifestyle changes can also contribute to the development of sarcopenia such as lack of exercise, poor nutrition and obesity (Marcos-Pardo *et al.*, 2021). Moreover, chronic diseases such as gastrointestinal disorder (Hollingworth *et al.*, 2020), rheumatoid arthritis (Torii *et al.*, 2019) and osteoporosis (Greco, Pietschmann and Migliaccio, 2019) can also contribute to the development of sarcopenia. These diseases may affect some of the cellular changes that lead to sarcopenia, or have been shown to interfere with the individual's ability to maintain a

balanced diet and exercise (Hardee and Carson, 2017). Past research has demonstrated that exercise primarily focused on resistance training and dietary interventions can delay sarcopenia-related muscle loss and enhance physical performance (Sgrò *et al.*, 2019). This chapter will focus on the effect of dietary interventions on sarcopenia rates in *C. elegans*.

Okinawa, a small island off mainland Japan, is renowned for having the lowest risk of age-related illnesses such as cardiovascular disease, diabetes and cognitive health loss (Willcox, Scapagnini and Willcox, 2014). The island is also one of the five globally recognised "blue zones", known for having the highest number of centurions in the world (Houston, 2020). Research in the Okinawa centurions have identified diet as a significant factor in their longevity(Houston, 2020). The traditional Okinawa diet is low in calories and consists mainly of nutrient-rich vegetables, with moderate amount of meat and fish (Ricker and Haas, 2017) (Houston, 2020). Despite the various health benefits reported so far, no studies evaluating the Okinawa diet's potential influence on sarcopenia have been published.

Some aspects of the Okinawan diet are comparable with other healthy diets, including the Mediterranean diet (Shao *et al.*, 2017). The Mediterranean diet has been shown to provide many health benefits such as the prevention of heart diseases, stroke, diabetes and has also been linked to improving cognitive health (Critselis and Panagiotakos, 2020). A traditional Mediterranean diet is generally rich in fruits, vegetables, whole grains, nuts and locally sourced seafood (Tosti, Bertozzi and Fontana, 2018). Many studies have found that increased adherence to a Mediterranean diet is associated with decreased risk of sarcopenia. In particular, higher adherence to a Mediterranean diet has been related to increased muscle mass and a lower prevalence of sarcopenia in the elderly (Karlsson *et al.*, 2020).

Fish-derived PUFA are key nutritional components of the Mediterranean diet that are believed to contribute to longevity (Román *et al.*, 2019). Consequently, diets rich in PUFAs are frequently associated with several health benefits including improved cardiovascular health, reduced inflammation and lowered cholesterol levels (Shahidi and Ambigaipalan, 2018). Omega-3 fatty acids and omega-6 fatty acids are the two major groups of PUFAs but since humans cannot produce these essential fatty acids, they must be obtained from the diet (Saini and Keum, 2018). The main dietary sources of omega-3 PUFAs are fatty fish such as salmon, mackerel and tuna while omega-6 PUFAs can be obtained from plant-based oils such as olive, sunflower and corn (Saini and Keum, 2018). Omega-3 and Omega-6 PUFAs are precursors of eicosanoids, a family of biologically active signalling lipids that play an important role in a variety of physiological and pathological responses, including the inflammatory response (Wall *et al.*, 2010)(Calder, 2008). There is mounting evidence to suggest that omega-3 PUFA supplementation has a favourable impact on total body muscle mass and function, with a particular emphasis on skeletal muscle mass (Bird *et al.*, 2021). Omega-3 supplementation has been shown to improve muscle strength and performance in the elderly, leading to enhanced muscle mass and walking speed (Huang *et al.*, 2020). Chronic inflammation, as previously indicated, is believed to be involved in the development of sarcopenia (Dalle, Rossmeislova and Koppo, 2017). As a result, the reduction of pro-inflammatory cytokines is widely assumed to be one method by which omega-3 PUFAs may reverse sarcopenia (Dupont *et al.*, 2019). Along with fish oil, algae-oil is an equally rich and sustainable source of omega-3 fatty acids (Lane *et al.*, 2014). However, no research has been published on the impact of PUFAs from algal oil on age-related muscle degeneration or sarcopenia.

In the present study, the effects on lifespan and healthspan of lifelong supplementation of algae-oil were examined in a transgenic *C. elegans* strain, *unc-54::gfp*. Since the muscle myosin

isoform protein (UNC-54) is fluorescently labelled with GFP and the change in fluorescence intensity can be easily monitored under a fluorescence microscope, *unc-54::gfp* serves as an ideal model to study the effects of supplementation in sarcopenia. The primary goal of this study was to explore the potential of algae-oil for delaying age-associated muscle degeneration such as sarcopenia in the *C. elegans* model, as well as to test the hypothesis that age-related sarcopenia can be modulated by omega-3 fatty acids. For the first time, the effect of algae-oil supplementation on lifespan and healthspan, specifically the mobility and sarcopenia rates, were investigated in *C. elegans*. The NGM was supplemented with different concentration of algae oil (0.1mM and 0.3mM) and compared with a control group. The results presented here show that both algae-oil concentrations significantly increased lifespan and delayed age-dependent muscle degeneration in *C. elegans*. These findings may provide a basis for developing therapeutic interventions for sarcopenia in humans.

4.3 Materials and Methods

4.3.1 Preparation of Algae-oil Supplemented NGM plates

The Algae oil was kindly provided by the AlgaeCytes company (Discovery Park, Sandwich) (See chapter 2, section 2.1.3 for further details). The fatty acid composition of algae-oil used in this assay was assessed with GC-MS using the methods described in chapter 2 (see Appendix Figure B1). The NGM dietary supplementation method was adapted from Deline et al. (2013). The NGM+AO plates were prepared as described in methods chapter 2 (Section 2.1.3). The NGM+AO plates were seeded with 100µl of *E. coli* OP50 cultures and FUDR was added on top of the bacterial lawn to prevent reproduction (Hosono, Nishimoto and Kuno, 1989). The plates were left to dry overnight at room temperature and covered with a tray until the start of the assay. FUDR treatment was discontinued once reproduction had ceased (day 7).

4.3.2 Lifespan Assay

All lifespan assays on *C. elegans* were performed at 20°C. Age-synchronised L4 worms were moved to NGM+AO plates, with 20 worms per plate and three plates per group. Worms were moved to fresh plates every other day to prevent starvation and maintain the experimental conditions (Greer *et al.*, 2007). Worms were scored as dead if they did not respond to touch stimulus with a wormpick. Lifespan data was analysed using Kaplan-Meier Survival curves and the statistical significance was determined by log-rank test in GraphPad Prism software.

4.3.4 Motility Assay

Worms were treated as per the lifespan assay described above. 20 L4 stage worms were transferred to NGM+AO plates, with 3 plates per group. Motility was measured by gently stroking each worm with a wormpick and scoring their response into one of three categories: I, II and III (as detailed in Materials and Methods). Motility data was analysed in GraphPad

Prism by allocating the number of worms into the three motility classes and normalising the data with the maximum number of individuals at the start of the assay set at 100%. The data was fitted with a non-linear regression model to determine the mid-point ($K_{0.5}$) when 50% of the population was in each motility class.

4.3.5 Fluorescence Microscopy

Fluorescence intensity images of *C. elegans* were measured using the methodology outlined in chapter 2 (section 2.2.6) and chapter 3 (section 3.3.5). Briefly, a random sample of five worms were selected from each population every other day. The worms were immobilized on slides with 10µl of buffer B (See chapter 2, section 2.1.1). The image acquisition settings were controlled by CellSens imaging software with 795.6ms exposure time and 12.2 dB gain set for all fluorescence images in this study. The final mean fluorescence intensity was analysed in ImageJ using the methods described in chapter 3. The fluorescence data was analysed using Two-way ANOVA (GraphPad Prism 9) and the average fluorescence was plotted as a function of age. The average fluorescence increase was calculated using a linear fit, whereas the average fluorescence decrease was best fitted to a single exponential (control group) or a linear fit (algae-oil supplemented groups). The age at which 50% of maximum UNC54-GFP myosin is lost was directly measured for the control group but had to be extrapolated for the algae-oil supplemented groups, presuming a linear fit.

4.4 Results

4.4.1 Algae oil extends the Lifespan of *C. elegans*

The synchronised populations of *C. elegans* cultured on different concentrations of algae oil (0.1, 0.3mM) were monitored to investigate whether algae oil influenced the lifespan of *C. elegans*. The survival curves and summarized results are shown in Figure 4. 1. and Table 4. 1. Under 0.1mM and 0.3mM algae oil supplementation, the mean lifespan of *C. elegans* increased to from 13.7 days (control) to 18.3 and 18.5 days, respectively. This expanded the mean lifespan of supplemented worms by 33.6% and 35%, respectively, compared to the control group (13.7 days). These results indicated that algae-oil supplementation increases the mean lifespan in the *C. elegans*, consistent with previous studies (Qi *et al.*, 2017).

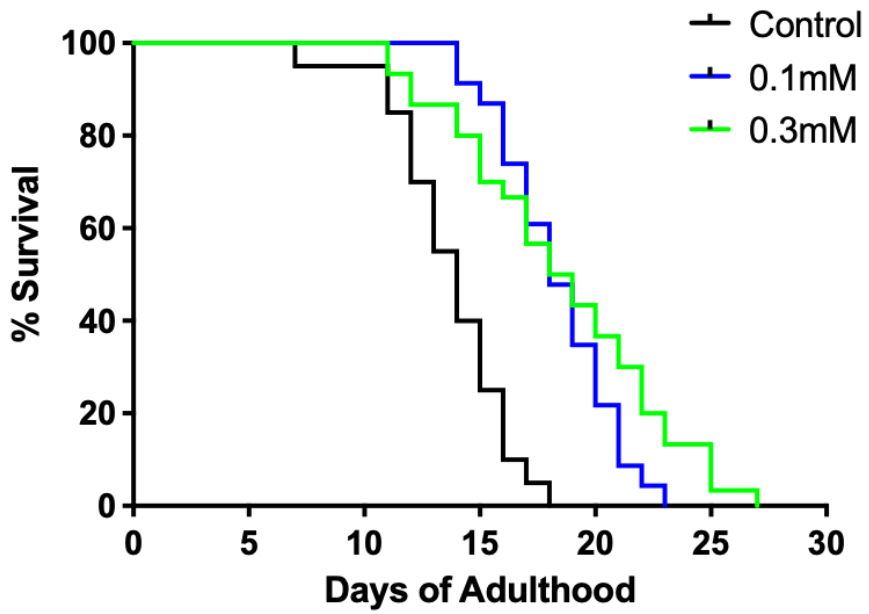

Figure 4. 1 Effect of algae oil supplementation on *C. elegans* lifespan. Unc-54 worms were supplemented with different concentrations of algae oil starting from day 1 of adulthood (L4) to death at 20°C. The mean life span of 0.1mM (18.3 days, $p<0.0001$) and 0.3mM (18.5 days, $p = 0.0001$) was significantly increased compared to control (13.7 days). Survival plots were

drawn by Kaplan-Meier survival assay. Data shown is from one experiment and is representative of two independently completed experiments.

Table 4. 1 Effect of different algae oil concentrations on the mean lifespan of *C. elegans*. Shown is the mean and maximum lifespan and the % change compared with control.

Diet	Mean lifespan (days)	% Increase in Mean Lifespan	Maximum lifespan (days)	% Increase in Maximum Lifespan
Control	13.7±0.6		18.0±0	
0.1mM	18.3±0.5	33.6%	23.0±0	27.8%
0.3mM	18.5±0.8	35.0%	27.0±0	50.0%

4.4.2 Algae oil supplementation delays age-related functional decline in *C. elegans* motility

The worms were monitored regularly from day 1 of adulthood to the end of their lifespan to get an accurate assessment of their motility at the early, mid, and late stages of the worm's life. As shown in figure 4. 2, the age-related decline in motility was attenuated by supplementation with algae oil. During the first week of adulthood, 100% of the worms were still classed as class I on all groups (Figure 4. 2A). On day 11 of adulthood, more than 50% of worm population under control group were classed as class II (Figure 2B) while almost all the worm population in 0.1mM and 0.3mM were still classed as class I (Figure 4. 2A). On day 16 of adulthood, all control worms were classed as class III (Figure 4. 2C) in comparison to 0.1mM and 0.3mM algae oil groups where more than 50% of the population was still classed as class II (Figure 4. 2B).

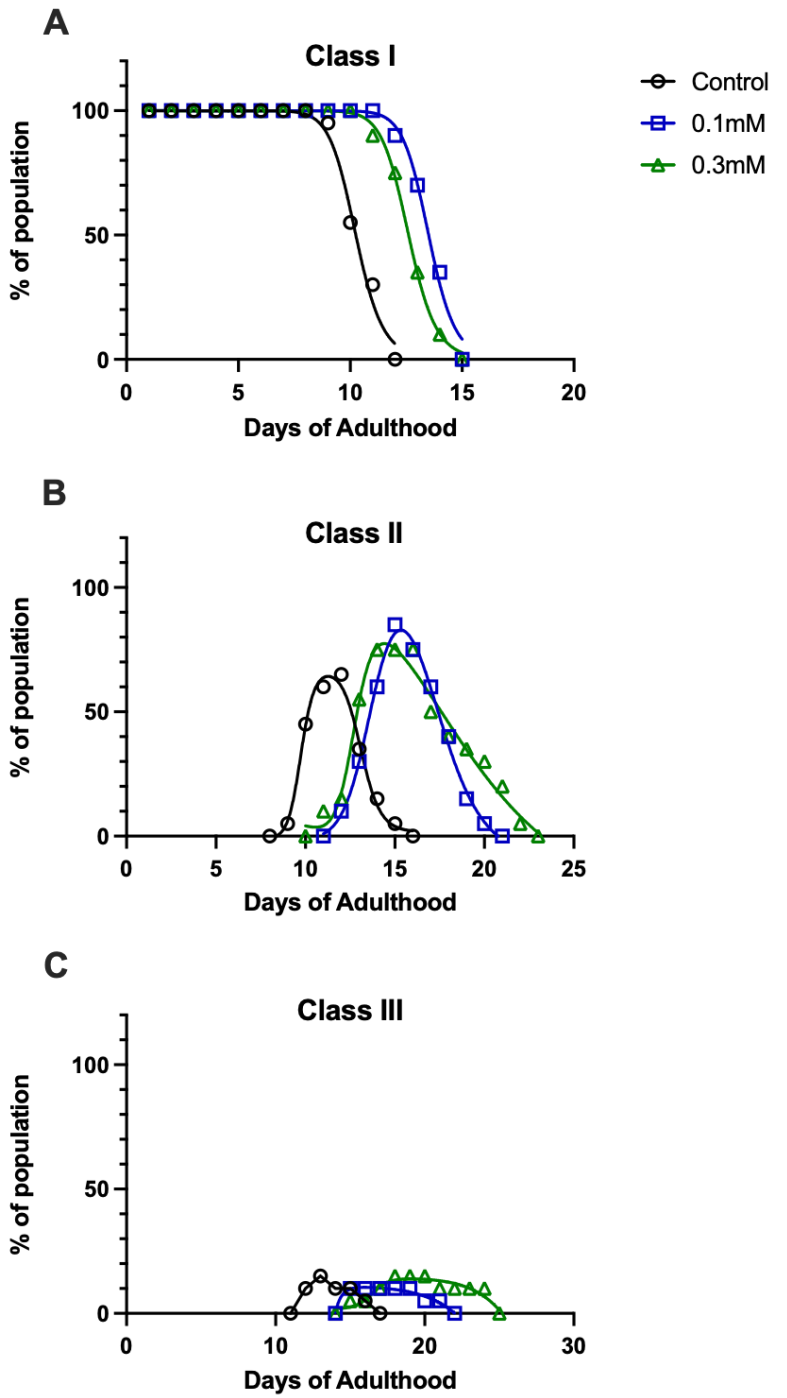

Figure 4. 2 Age-related changes in the motility of algae oil supplemented worms. Classification of movement according to Herndon et al. (2002). (A) Class I – Continuous smooth movement, fast movement when stimulated. (B) Class II – Slow halting movement, smooth movement when stimulated. (C) Class III – Small movement of head or tail, very slow movement when stimulated.

Table 4. 2 Summary of age-related changes in motility under algae oil supplementation. Shown is the average number of days it takes 50% of the initial population to move from class I to class II, represented by $C_{1\text{-}2}$, or to move from class II to class III (represented by C2-3. The difference between the two mid-points, represented by the parameter ΔC_2, is the average time spent in class II.

	$C_{1\text{-}2}$ (days)	**$C_{2\text{-}3}$ (days)**	**ΔC_2 (days)**	**Mean life span (days)**
Control	10.3 ±0.07	13.1 ±0.2	2.8	13.7±0.6
0.1mM	13.5 ±0.05	17.3 ±1.0	3.8	18.3±0.5
0.3mM	12.6 ±0.03	18.5 ±2.4	5.9	18.5±0.8

When comparing the average number of days for 50% of the initial population to move from motility class I to class II (Table 2, parameter $C_{1\text{-}2}$), non-linear regression analysis revealed that worms in 0.1mM and 0.3mM algae-oil groups spend longer in class I ($C_{1\text{-}2}$ = 13.5 and 12.6 days respectively) compared to control ($C_{1\text{-}2}$ = 10.3 days) (Table 4. 2.). Transition from class II to class III was also delayed for worms in 0.1mM and 0.3mM conditions ($C_{2\text{-}3}$ = 17.3 and 18.5 days respectively) compared to control ($C_{2\text{-}3}$ = 13.1 days). The average time spend in class II increased as well for the 0.1mM and 0.3mM algae-oil supplementation group (ΔC_2 = 3.8 and 5.9 days respectively) compared to the control population (ΔC_2 = 2.8 days).

4.4.3 Algae oil supplementation improves Healthspan in *C. elegans*

Additionally, *C. elegans'* lifespan was divided into healthspan and gerospan to assess their quality of life under algae-oil supplementation. More specifically, the quantity of time spent in a healthy and frail condition was measured at early and mid-adulthood. In early adulthood,

healthspan (period where >50% of the worm population is classified as motility class I) and gerospan (period where >50% of the worm population has lost its class I motility) ratios of control and algae-oil supplemented worms were determined (Figure 4. 3A), in the same manner as Bansal et al. (2015) When compared to control worms, 0.1mM and 0.3mM algae-oil supplementation increased healthspan by 31% and 22%, respectively (Figure 4. 3A). The healthspan to gerospan ratio was then normalised to their maximum lifespan (with the maximum lifespan set to 100%) and the physiological differences between the groups were analysed. As shown in Figure 4. 3B, the worms in algae-oil supplementation group or more specifically the 0.1mM group showed a proportional increase in healthspan with a reduction in gerospan compared to the control group. For control, the healthspan rises somewhat when compared to 0.3mM, however this is attributed to a shorter lifespan (Figure 4. 3B).

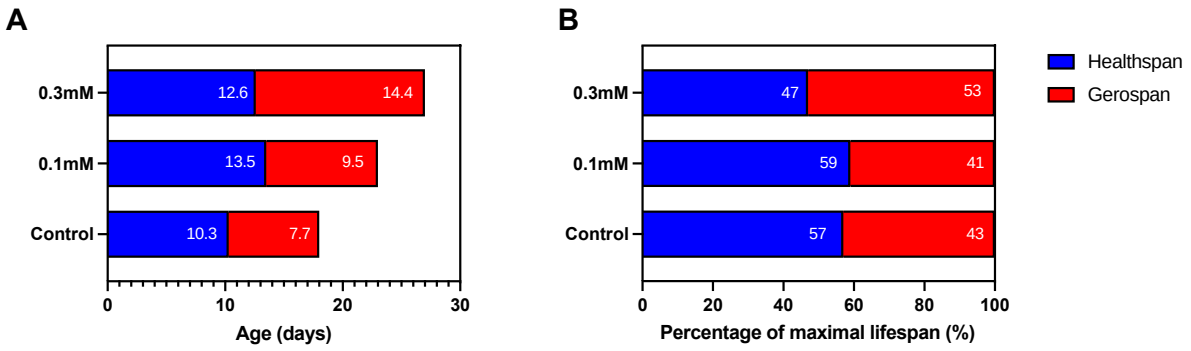

Figure 4. 3 Comparison of healthspan and gerospan in control and algae-oil supplemented groups at early adulthood. A) Healthspan was defined as the period when > 50% of the initial nematode population show type-I motility. Gerospan was defined as the period when < 50% of the initial nematode population show < 50% of type-I motility. B) The ratio of healthspan to gerospan was normalized to their maximal lifespan in control and DR worms (Hahm et al., 2015).

Subsequently, in mid-adulthood, healthspan was defined as the period during which 50% of the worm population showed class II motility, while gerospan was defined as the period during which more than 50% of the worm population lost class II motility (Figure 4. 4A). The healthspan increased in 0.1mM and 0.3mM algae-oil supplementation by 32% and 41%, respectively when compared to the control group (Figure 4. 4A). Similar to the observations in early adulthood, worms in the 0.1mM algae-oil supplemented group showed a proportional increase in healthspan with a decrease in gerospan compared to the control group (Figure 4. 4B). The healthspan of the control group increased slightly when compared to the 0.3mM group, however this is attributed to a significant difference in maximum lifespan between the control and 0.3mM groups (Table 4.1B).

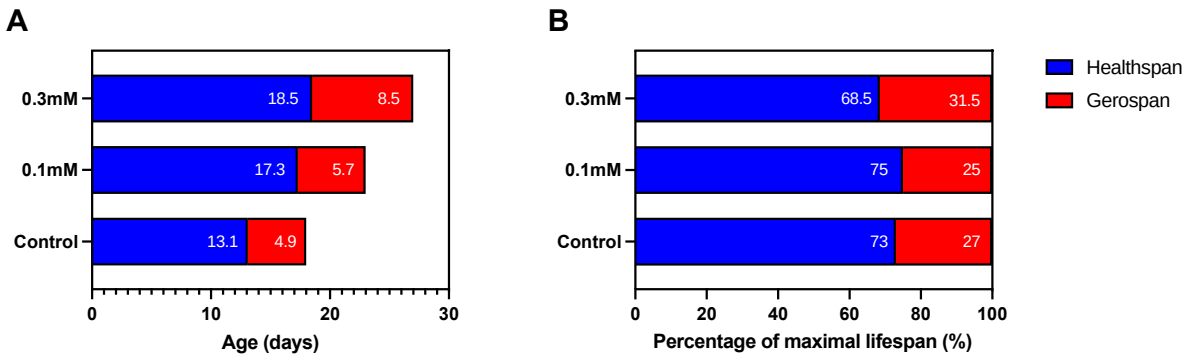

Figure 4. 4 Comparison of healthspan and gerospan in control and algae-oil supplemented groups at mid-adulthood. A) Healthspan was defined as the period when > 50% of the initial nematode population show type-II motility. Gerospan was defined as the period when < 50% of the initial nematode population show < 50% of type-II motility. B) The ratio of healthspan to gerospan normalized to their maximal lifespan in control and DR worms.

4.4.4 Effect of Algae oil supplementation on age-related muscle loss of *C. elegans*

Fluorescence microscopy was used to image the body wall muscles of the *unc-54*-GFP transgenic worms and measure the rate of muscle mass loss under algae oil supplementation. Low levels of UNC54-GFP fluorescence indicate a physiologically aged state; thus, fluorescence intensity is a valid reporter of nematode healthspan. Figure 4. 5 shows representative fluorescence images of *C. elegans* under control and algae-oil supplementation.

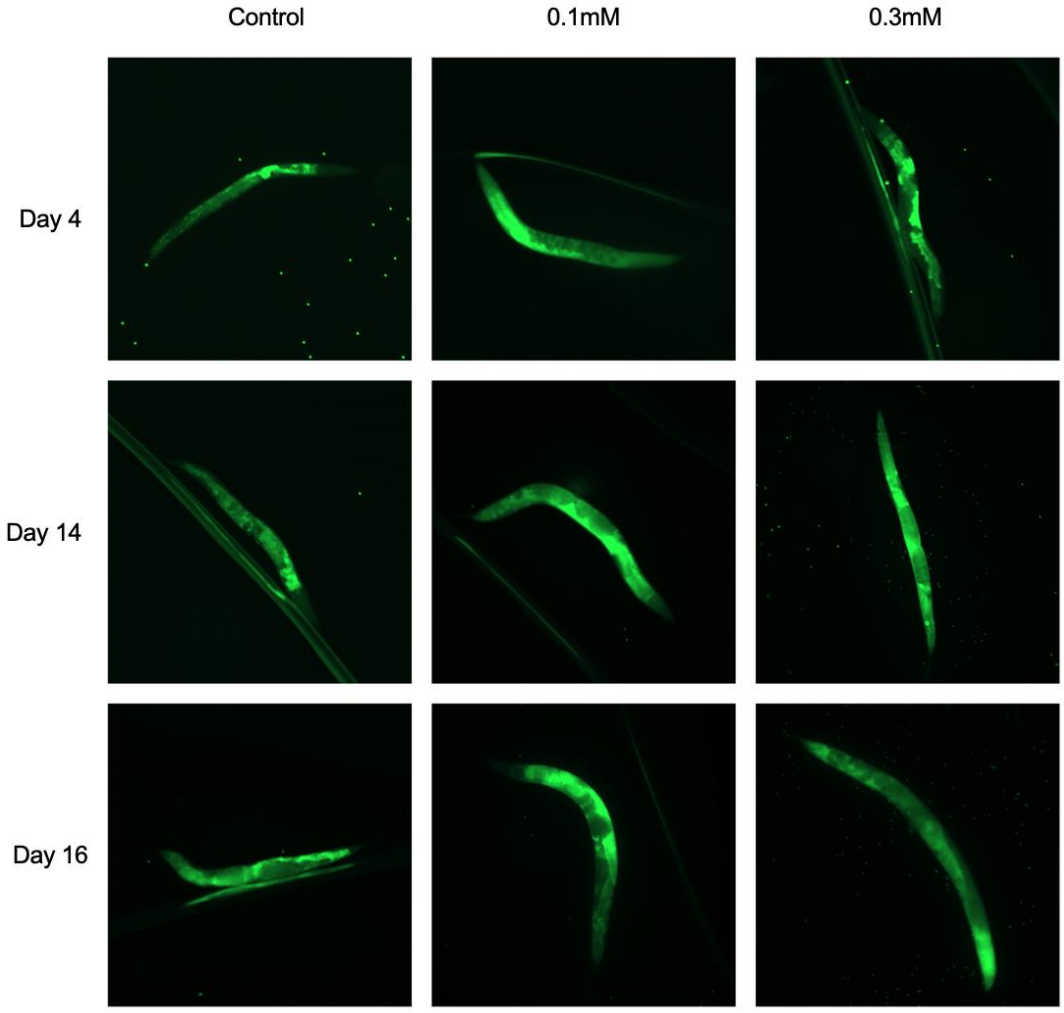

Figure 4. 5 Algae oil supplementation delays muscle mass loss in *C. elegans*.

Representative fluorescence microscopy images (4x magnification) showing *C. elegans* grown under control and two concentrations of algae-oil supplementation, expressing UNC54:GFP.

Figure 4. 6 shows boxplots of fluorescence intensity corresponding to the various time points. The fluorescence intensity of worms in control and algae-oil supplemented groups were not significantly different until day 10 of adulthood (Figure 4. 6). However, on day 12 of adulthood, the fluorescence intensity of worms in the control group was significantly reduced compared to the algae-oil supplemented groups (Figure 4. 6), reflecting an age-related muscle deterioration, with reduced UNC-54 levels which agrees with the observed decreased motility (Table 4. 2). When compared to control group, 0.1mM and 0.3mM algae-oil groups showed significantly higher fluorescence intensity at day 12 (P = 0.032 and 0.005), day 14 (P = 0.027 and 0.003) and day 16 (P = 0.042 and 0.003), respectively.

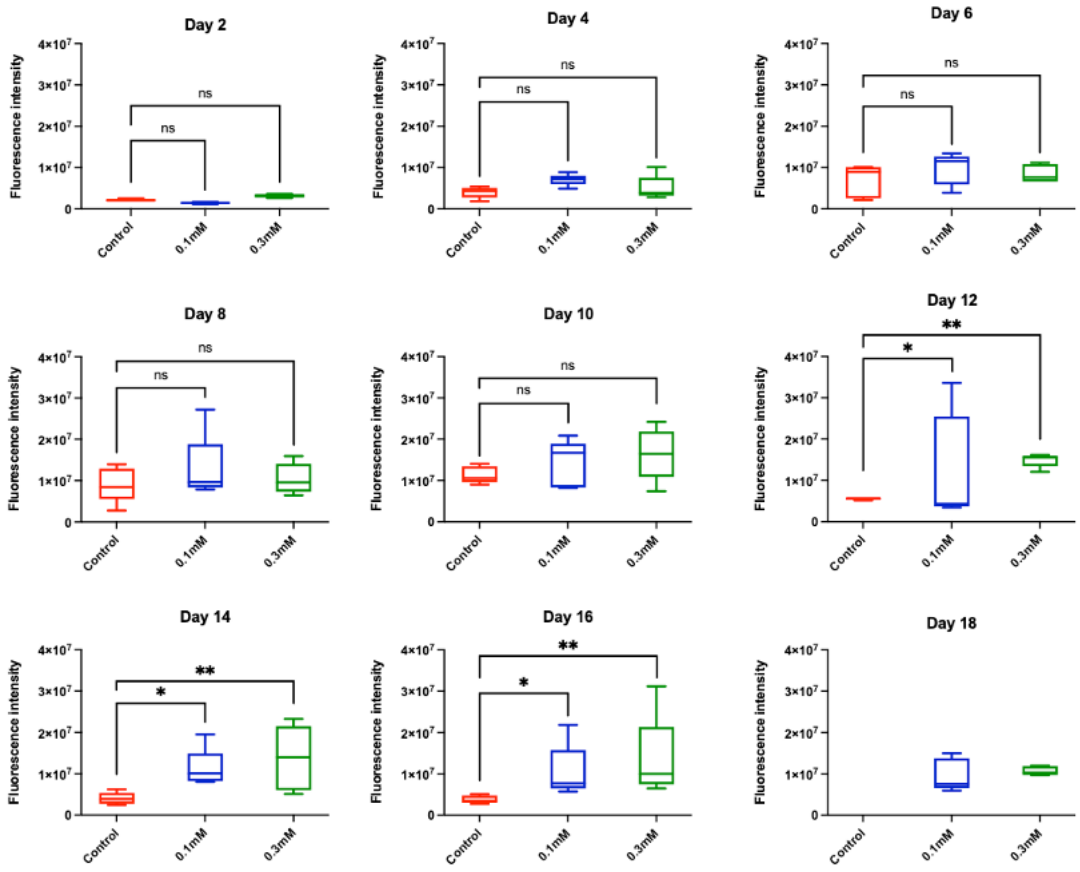

Figure 4. 6 Quantification of unc-54 GFP fluorescence in *C. elegans* during algae-oil supplementation. Boxplots showing the average fluorescence intensity of *C. elegans* under control and with algae-oil supplementation (Two-way ANOVA, *P<0.05; **P<0.01). The graph is representative of two independent experiments.

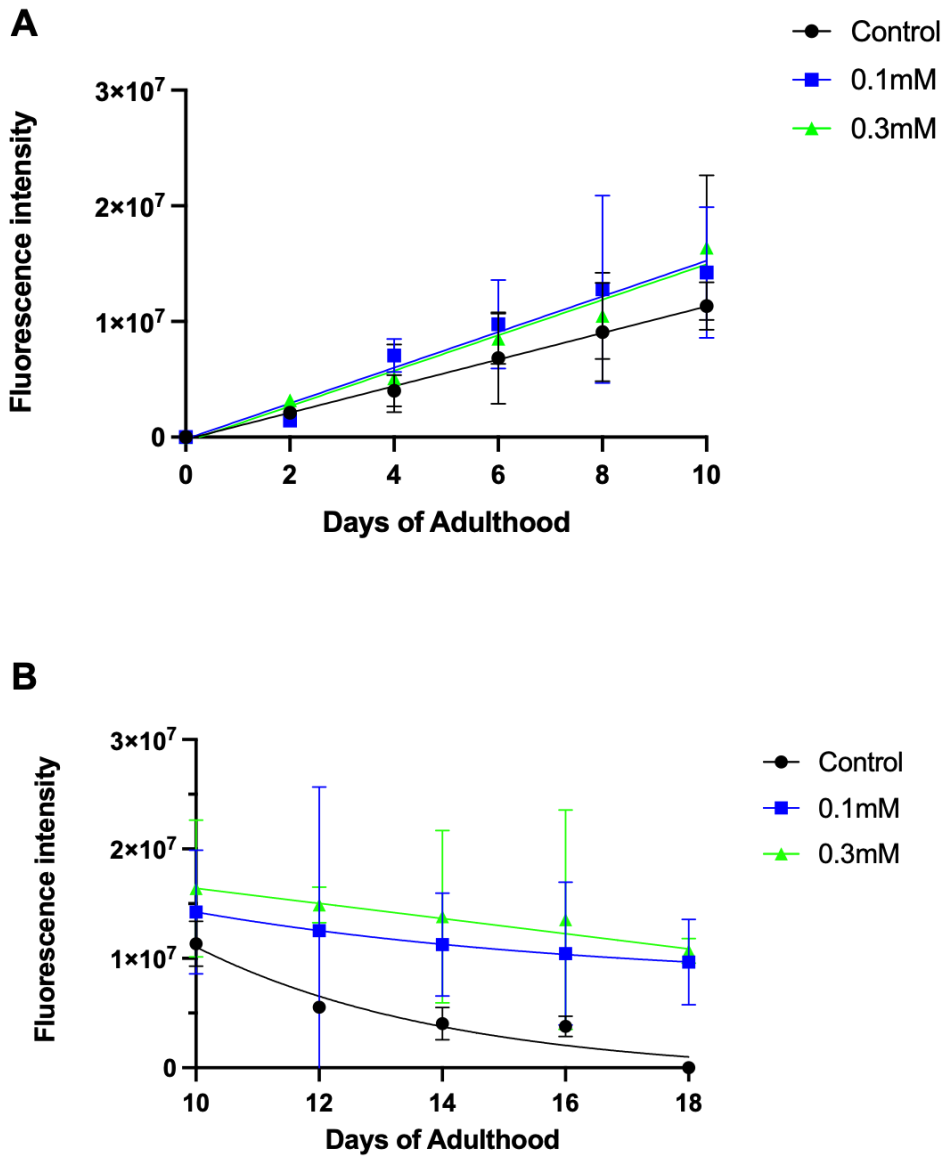

Figure 4. 7 Expression of UNC54-GFP as a function of age. (A) Average fluorescence increases during day 1-10 for the algae-oil supplemented and control populations. Linear fits resulted in slope values for 0.1mM and 0.3mM algae-oil groups with slope = $1.5\pm0.1x10^6$ and $1.5\pm0.1x10^6$ respectively (corresponding R^2-values of 0.97 and 0.97). Control group yielded slope value of $1.2\pm0.2x10^6$ (R^2-values of 0.99). (B) Average fluorescence decreases from day 10 towards end of lifespan for 0.1mM, 0.3mM and control groups. Single exponential fit was used to calculate $t_{1/2}$ values of 2.9 days (control) and linear fits were used to calculate $t_{1/2}$ values of 14.2 days (0.1mM) and 14.7 days (0.3mM).

The fluorescence increase represents the rate of muscle growth as the amount of GFP-labelled myosin increased from day 1 until day 10 for the control and algae-oil supplementation groups (Figure 4. 7A). Fluorescence intensity started to decline from day 10 for all groups (Figure 4. 7B) consistent with the age-related decrease in myosin density (UNC54-GFP). Since the worms began to die at the end of the assay, the number of worms were not sufficient to calculate the average fluorescence intensity towards the end of C. elegans lifespan. As a result, the age at which 50% of maximum UNC-54-GFP myosin is lost was calculated by extrapolating the average fluorescence intensity data. The control data was best fitted to a single exponential model whereas the algae-oil supplementation data were best fitted to a linear model (Figure 4. 7B). The fitted data was extrapolated to determine the t1/2 values for the 0.1mM and 0.3mM algae-oil groups, assuming the linearity continued. Based on the extrapolation, 0.1mM and 0.3mM algae-oil supplementation groups lost 50% of their maximum UNC54-GFP myosin at 24.2 days and 24.7 days respectively, compared to control group which lost 50% of peak UNC54-GFP myosin at 12.9 days (Table 4. 3).

Table 4. 3 Summary of age-related changes in fluorescence and expression of myosin UNC54-GFP under algae oil supplementation.

	Fluorescence Increase[1] (10^6 day^{-1})	**Fluorescence loss[2] k_F (day^{-1})**	**$t_{1/2}$[3] (days)**	**$K_{0.5}$ UNC54-GFP[4] (day)**
Control	1.2±0.2	0.241	2.9	12.9
0.1mM	1.5±0.1	0.049	14.2	24.2
0.3mM	1.5±0.1	0.047	14.7	24.7

[1]Measured from day 1-10.
[2]Determined from day 10 (maximum fluorescence) until day 18.
[3]Determined using ln (2)/k_F
[4] Age at which 50% of maximum UNC-54-GFP myosin is lost.

4.5 Discussion

Life expectancy in developed countries has increased dramatically over the last century as a result of technological advancements, improved healthcare systems, and better living standards (Vaupel, Villavicencio and Bergeron-Boucher, 2021). However, the fact that healthspan has not risen at the same rate as life expectancy is a cause for concern (Kennedy, 2019). Numerous human and model organism studies have shown that omega-3 fatty acids provide a wide range of health benefits, including reducing inflammation and delaying the onset of age-related functional decline (Gammone *et al.*, 2019). In this study, algae-oil's potential as an effective supplement for promoting healthy ageing was assessed in *C. elegans* as this has not been previously explored in *C. elegans*. For the first time, EPA-rich algae-oil has been demonstrated to significantly increase longevity and healthspan in *C. elegans.* Algae-oil supplementation significantly delayed the onset of sarcopenia with increased motility and UNC-54 levels maintained till late adulthood. The mean lifespan of *C. elegans* was extended by 33.6% and 35% under 0.1mM and 0.3mM algae oil supplementation. This finding is consistent with a previous study in *C. elegans* where omega-3 PUFA, alpha-linoleic acid (ALA) derived from a different source significantly increased the mean lifespan by 30% compared to the control group (Qi *et al.*, 2017).

While both omega-3 and omega-6 PUFAs are essential components of cell membranes, their regulatory roles as eicosanoids precursors are significantly different (Russo, 2009). Eicosanoids derived from omega-3 PUFAs such as EPA have anti-inflammatory properties whereas eicosanoids derived from omega-6 PUFAs such as arachidonic acid (AA) have pro-inflammatory properties (Nelson and Raskin, 2019). As a result, the EPA:AA ratio has been proposed to be a marker of chronic inflammation, with a lower ratio indicating a higher level of inflammation (Nelson and Raskin, 2019). Lower levels of circulating EPA in the elderly

have been linked to diminished physical capacity, most notably reduced gait speed (I. Reinders *et al.*, 2015). In a recent meta-analysis study, the level of omega-3 PUFAs in sarcopenia patients was found to be significantly lower compared to control subjects (Zhang *et al.*, 2022).

Diet has a strong influence on the ratio of EPA:AA (Simopoulos and DiNicolantonio, 2016). The western diet, which is characterized by refined foods and heavy sugar intake, has resulted in both a low intake of EPA and an overconsumption of AA (Simopoulos, 2016). Since western diets have a lower ratio of EPA:AA, AA-derived eicosanoids are produced in greater concentrations than those derived from EPA (Mariamenatu and Abdu, 2021). Consequently, several pro-inflammatory cytokines have been linked to muscle atrophy (Wang *et al.*, 2017); hence, omega-3 PUFAs' anti-inflammatory properties may be advantageous in preventing sarcopenia-related muscle loss and strength (Dupont *et al.*, 2019). Many studies have shown that increasing EPA consumption can decrease the concentration of AA-derived eicosanoids, since EPA acts as a competitive inhibitor to AA, resulting in increased level of anti-inflammatory cytokines (Nelson and Raskin, 2019). In the elderly, supplementation with EPA and DHA for 8 weeks was demonstrated to lower circulating levels of pro-inflammatory cytokines (Tan *et al.*, 2018).

Muscle atrophy and dysfunction are linked to a reduction in motility with ageing (Son *et al.*, 2019). Omega-3 PUFA supplementation has been shown to increase lean muscle mass and muscle strength in elderly people (Smith *et al.*, 2011a)(Smith *et al.*, 2011b). Supplementation with omega-3 PUFA derived from fish oil has been shown to delay age-related loss of muscle mass and function in elderly people (Smith *et al.*, 2015). Given that omega-3 PUFAs can integrate into sarcolemma membrane phospholipids, omega-3 PUFAs could very well influence muscle protein synthesis and increase muscular strength and function (McGlory, Calder and Nunes, 2019). Motility is one of the most essential characteristics that mirrors *C. elegans'* physical capabilities and stage of ageing (Son *et al.*, 2019). In this study,

supplementation with EPA-rich algae-oil was found to delay age-related motility decline in *C. elegans*. At several stages of life, worms supplemented with algae-oil demonstrated more energetic and active motility than control worms (Figure 4. 2). In comparison to control worms, algae oil supplementation resulted in a significant increase in the number of days spent in motility classes I and II of middle aged and elderly *C. elegans* (Table 4. 2). Since *C. elegans* motility diminishes with age, enhanced motility has been observed as a sign of restored youthful vigour (C. Huang, Xiong and Kornfeld, 2004). As a result, improved motility in algae-oil supplemented worms indicates a delay in age-related muscle degeneration, demonstrating for the first time, the therapeutic potential of EPA-rich algae-oil in sarcopenia.

Furthermore, algae oil supplementation improved additional age-related parameters such as myosin density in *C. elegans*. Body wall muscle serves as a significant marker of ageing in *C. elegans* since it is involved in both movement and structural support (Gieseler, 2017). Using the same experimental conditions as lifespan, the myosin density, defined by the average fluorescence emitted by an UNC-54-localized GFP, was measured over time to see how ageing and algae-oil supplementation affected muscle mass. There was a gradual increase in fluorescence followed by a gradual decrease in fluorescence intensity for all groups, suggesting that increased fluorescence is inversely correlated with age-related decline in muscle mass (Figure 4. 7). The rate of myosin loss (k_F) was found to be positively correlated with the motility parameters $C_{1\text{-}2}$ (R^2 = 0.92) and $C_{2\text{-}3}$ (R^2 = 0.96), with a lower rate observed for algae-oil supplemented groups compared to the control group (Appendix Figure B2), indicating that reduced motility is potentially due to a decrease in UNC-54 levels. When compared to the control group, the relative fluorescence observed with fluorescence microscopy was significantly increased on day 12,14 and 16 following supplementation with algae-oil, indicating an increased preservation of muscle mass (Figure 4. 6). Moreover, the $t_{1/2}$ values for the 0.1mM and 0.3mM algae-oil supplemented worms were greater than those for the control

worms, indicating that algae-oil supplementation maintained higher UNC54 levels for a longer period throughout the ageing period, resulting in less pronounced sarcopenia when compared to the control worms, respectively (Table 4. 3). Interestingly, the predicted $t_{1/2}$ values roughly correspond to the maximum lifespan (Table 4. 1) which could indicate that the worms die off before the sarcopenia process is fully initiated. This is also suggested by the motility data which shows that many worms die off while still in class II motility. However, since the $t_{1/2}$ values for the 0.1mM and 0.3mM algae-oil supplemented worms are based on extrapolation, average fluorescence intensity data during the end of *C. elegans* lifespan is needed to confirm the validity of these results.

As muscle tissue is one of the most energy-demanding tissues and has numerous mitochondria that produce ROS as a consequence of ATP production, the impact of omega-3 fatty acids on muscle ageing perhaps relates to their antioxidant activity. Many studies have documented an increase in antioxidant activity following PUFAs treatment (Gutiérrez-Pliego *et al.*, 2020). The accumulation of ROS has long been recognized to impair the activity of cellular processes, but its impact on ageing muscles remains unknown (Shields, Traa and Van Raamsdonk, 2021). Some studies have suggested that the lifetime accumulation of ROS might be responsible for the onset of sarcopenia (Damiano *et al.*, 2019). Previous research has found that oxidative stress rises dramatically with age, possibly due to lower antioxidant synthesis (Fulle *et al.*, 2004). Given the role of oxidative stress in driving mitochondrial dysfunction, the anti-oxidative properties of omega-3 fatty acids may be able to prevent muscle deterioration in *C. elegans.*

As with most studies, there are some limitations to this study. Even though most ageing processes and pathways in *C. elegans* have mammalian counterparts, effects in *C. elegans* do not always generalise to other species due to the absence of some essential organs and a circulatory system (Zhang *et al.*, 2020). Since omega-3 PUFAs are prone to oxidation, it is

important to examine the oxidation levels in algae-oil before evaluating its impact, as oxidised algae-oils may have altered biological activity, rendering them ineffective or detrimental. It is also worth noting that omega-3 PUFAs used in previous studies might have been oxidised. As a result, it is probable that the inclusion of oxidised oils may have obscured the results, particularly in studies where omega-3 PUFAs and mortality are found to be inversely correlated (Harris *et al.*, 2018). Therefore, future studies evaluating the health benefits of algae-oil should be aimed at measuring and reporting its oxidative status.

This study demonstrates the impact of algae-oil on the longevity and various health parameters of *C. elegans* for the first time. Many studies have shown that omega-3 fatty acids extend life span in *C. elegans*; however, the influence on age-related health indicators such as motility and muscle mass has not been studied. Algae oil supplementation improved *C. elegans* lifespan and overall healthspan by influencing key physiological parameters. Worms under algae oil supplementation showed improved motility and muscle preservation until late adulthood compared to control worms. Moreover, algae-oil supplementation enhanced both lifespan and healthspan to a similar extent, without proportionately increasing the frailty period of life. Overall, the results presented in this chapter show for the first time, that algae oil delays ageing by slowing the rate of muscle loss and motility in *C. elegans*. These findings reveal protective anti-ageing effects of algae oil *in vivo* with translational potential in humans due to the feasibility of incorporating algae oil into a typical human diet. Additionally, these findings offer important evidence for the future use of algae oil in the development of omega-3 PUFA health supplements for the prevention and treatment of sarcopenia. Future studies will be focused on examining the molecular pathways involved in this delayed onset of sarcopenia in order to develop effective compounds that can be screened for anti-sarcopenia properties.

Chapter 5: Algae oil Supplementation improves Intestinal Barrier Function in *C. elegans*

5.1 Abstract

There is emerging evidence to suggest that increased intestinal permeability contributes to the development of sarcopenia in the elderly. Based on the observed delay in sarcopenia, the present study aimed to investigate if intestinal barrier degradation is also delayed in algae-oil supplemented groups. The relationship between intestinal permeability and sarcopenia was investigated in *C. elegans* by using an intestinal barrier assay. In this study, age-induced disruption of intestinal barrier function was significantly delayed in algae-oil supplemented worms. In addition, gas chromatography coupled with mass spectrometry (GC-MS) showed that the level of omega-3 PUFA, EPA in the supplemented worms was significantly higher compared to the control group. These findings corroborate the gut-muscle axis hypothesis by demonstrating a beneficial effect of algae-oil PUFA in delaying sarcopenia and confirming the link between muscle and intestinal health.

5.2 Introduction

The global population of older adults is growing, and with it comes an increased risk of chronic conditions like obesity, diabetes, cancer, and cardiovascular disease (Sato and Yanagita, 2019). Chronic inflammation, in particular, is a significant risk factor for mortality in the elderly since it is a major hallmark of age-related conditions including sarcopenia (Ferrucci and Fabbri, 2018). Inflammation can be classified into two types: acute and chronic (Ferrucci and Fabbri, 2018). Acute inflammation is characterized by a localized, rapid response to tissue damage (Chen *et al.*, 2018). In contrast, chronic inflammation occurs for an extended period of time and can have long-term consequences for the entire body (Furman *et al.*, 2019). During chronic inflammation, damaged tissues continue to emit signals that attract white blood cells from the bloodstream. When the white blood cells circulate from the bloodstream into the tissue, the inflammatory response is further amplified (Furman *et al.*, 2019). Hence, in an effort to repair and restore damaged cells, the chronic inflammatory response can end up destroying healthy tissues (Furman *et al.*, 2019). Controlling chronic inflammatory responses is crucial for reducing further complications in chronic disorders exacerbated by chronic inflammation, such as autoimmune diseases, arthritis, diabetes, and atherosclerosis (Greten and Grivennikov, 2019).

There is increasing evidence to suggest that chronic inflammation contributes to the advancement of sarcopenia in the elderly (Pan *et al.*, 2021). Consequently, several studies have shown that pro-inflammatory cytokines are chronically elevated in elderly sarcopenia patients (Rong *et al.*, 2018). Rong et al. (2018) discovered a significant increase in pro-inflammatory cytokine concentrations in elderly people with sarcopenia as opposed to a control group. Similarly, a cross-sectional study found that higher inflammatory cytokine levels were associated with increased prevalence of sarcopenia in older people (Zhao *et al.*, 2021).

An increasing number of studies have linked intestinal permeability with increased chronic inflammation in older adults (Nagpal *et al.*, 2018). Increased intestinal permeability allows undigested food particles, toxins, and bacteria from the gut to enter the bloodstream, resulting in chronic inflammation (Fukui, 2016). Increased inflammation disrupts the intestinal barrier, inducing intestinal permeability (Fukui, 2016). The gut microbiota changes with age, and there is emerging evidence that the gut microbiota of older people is distinct from that of young people and significantly correlates with measure of frailty, morbidity and nutritional status (Odamaki *et al.*, 2016)(Xu, Zhu and Qiu, 2019). Dysbiosis is a condition characterised by an imbalance of gut bacteria which leads to inflammation of the intestinal membrane, promoting intestinal permeability (Carding *et al.*, 2015). Factors such as weakened immune system, reduced mobility and malnutrition may all contribute to gut dysbiosis in older adults (Spychala *et al.*, 2018). While it is uncertain if gut dysbiosis is a cause or an effect of ageing, it has been proposed that maintaining a healthy gut microbiota can improve the health of elderly people by reducing leaky gut and inflammation (Salazar *et al.*, 2017).

The emerging relationship between gut dysbiosis and age-related loss of muscle mass and function has led to the hypothesis that a gut-muscle axis exists, in which the microbiome regulates skeletal muscle mass and physical function (Kang *et al.*, 2021). The gut-muscle axis has recently been identified as a potential target for overcoming anabolic resistance and lowering the risk of sarcopenia (Prokopidis *et al.*, 2021). Increased chronic inflammation appears to facilitate a mechanistic relationship between the gut microbiome and muscle atrophy (Nay *et al.*, 2019). For instance, in mice models of chronic intestinal inflammation, reinstating commensal *E. coli* levels in the gut significantly reduced skeletal muscle atrophy (Schieber *et al.*, 2015). Additional studies in mice suggest that gut dysbiosis via bacterial depletion and faecal transplantation, may directly increase the risk of sarcopenia (Liu *et al.*, 2021).

The intestinal microbiota can be influenced by the type of food consumed, which in turn influences the body's susceptibility to diseases (Singh *et al.*, 2017)(Conlon and Bird, 2015). Claesson et al. (2012) showed that the elderly people living in community setting consumed a more varied diet, resulting in a more diverse gut microbiota compared to elderly people living in assisted living facilities. Diet rich in saturated fat is linked with higher levels of pro-inflammatory cytokines, notably in obese and diabetic patients as compared to healthy individuals (Zhou, Urso and Jadeja, 2020). In contrast, diets rich in PUFAs have been shown to have anti-inflammatory effects by promoting the expression of various inflammatory mediators, thus supressing the expression of pro-inflammatory cytokines (Mildenberger *et al.*, 2017). The key components of fish oil are the two omega-3 fatty acids: EPA and DHA (Yi *et al.*, 2014). Interestingly, these two components are originally synthesized by microalgae rather than fish (Tocher *et al.*, 2019). These microalgae are consumed by phytoplankton, which in turn is consumed by fish and the omega-3 fatty acids are accumulated in their tissues (Tocher *et al.*, 2019). EPA and DHA supplementation in older adults showed a significant reduction in pro-inflammatory cytokine levels compared to the control group (Tan *et al.*, 2018). Moreover, many studies have shown that omega-3 fatty acid supplementation significantly extends the lifespan of *C. elegans* (Qi *et al.*, 2017) (O'Rourke *et al.*, 2013). For instance, Sugawara et al. (2013) investigated the effect of varying concentrations of fish oil on the lifespan of *C. elegans* and found that small doses of fish oil significantly increased the lifespan of *C. elegans* while higher doses of fish oil decreased the lifespan (Sugawara *et al.*, 2013).

Chronic inflammation, as previously described, has been implicated in the development of sarcopenia (Chhetri *et al.*, 2018). However, there is a lack of well-defined studies on algae-oil exhibiting beneficial effects on ageing-related intestinal permeability, so further research on this subject is needed to evaluate the use of algae oil supplements on humans. Similar to

humans, *C. elegans'* intestinal barrier structure deteriorates with age, resulting in an intestinal permeability (Gelino *et al.*, 2016). The smurf assay, comprising of a non-absorbable blue dye, is a non-invasive method for studying the age-related decline in intestinal barrier function (Rera, Clark and Walker, 2012). The smurf assay has been tested in various model organisms including nematodes that exhibited the smurf phenotype with age (Martins *et al.*, 2018). According to the 2 phases of ageing in *C. elegans* (2PAC) model established by Tricoire and Rera (2015), ageing is defined as two successive phases separated by the smurf phenotype transition, instead of a continuous process. Phase 1 is characterised by a time-dependent rise in the possibility of an individual becoming a smurf (non-smurfs). Phase 2 is the penultimate stage of life, during which a range of age-related phenotypes, most notably increased intestinal permeability, occur simultaneously (Smurfs) (Dambroise *et al.*, 2016).

In chapter 4, algae-oil supplementation significantly delayed the onset of sarcopenia in *C. elegans*. Given the role of gut microbiota on muscle atrophy (gut-muscle axis), the delay in sarcopenia observed in chapter 4 may indicate a delay in in intestinal permeability breakdown. Hence, the study presented in this chapter is focused on investigating the effect of algae-derived omega-3 fatty acids on age-induced intestinal permeability. Additionally, GC-MS was used to confirm the uptake of EPA and other PUFAs from the algae-oil in *C. elegans*. The application of the SA in the context of algae-oil supplementation could potentially reveal whether there is variation in intestinal permeability between control and supplemented worms. It will also provide the first set of evidence to demonstrate omega-3 fatty acid's role in reducing inflammation and restoring the intestinal barrier in *C. elegans*.

5.3 Materials and Methods

5.3.1 *C. elegans* Strain

The transgenic *C. elegans* strain, *unc-54::gfp* was used unless otherwise indicated.

5.2.2 Preparation of NGM+AO plates

The Algae oil was kindly provided by the AlgaeCytes company (See chapter 2, section 2.1.3 for further details). The NGM+AO plates were prepared as described in chapter 4. Unless otherwise indicated, algae oil was used at a final concentration of 0.1mM and 0.3mM and the NGM with 10% tergitol was used as control. The NGM+AO plates were seeded with 100µl of *E. coli* OP50 cultures and FUDR was added on top of the bacterial lawn to prevent reproduction (Hosono, Nishimoto and Kuno, 1989). The plates were left to dry overnight at room temperature and covered with a tray until the start of the assay. FUDR treatment was discontinued once reproduction had ceased (day 7 of adulthood). Additionally, a day prior to the assay, a palmitic acid (10mg/ml in ethanol) ring was applied around the edge of the plate to prevent the worms from crawling out the plates and desiccating (Miller and Roth, 2009). This inhibits the worms from crawling out of the plates because the palmitic acid precipitates and produces a gritty barrier (Fawcett, Horsman and Miller, 2012). It has been demonstrated that the palmitic acid ring has no effect on worm's behaviour, reproduction and lifespan (Miller and Roth, 2007).

5.3.3 Lifespan Assay

Age-synchronised L4 worms were moved to NGM+AO plates, with 20 worms per plate and three plates per group. Worms were moved to fresh plates every other day to maintain the experimental conditions (Greer *et al.*, 2007). Worms were scored as dead if they did not respond to touch stimulus with a wormpick. Kaplan-Meier survival curves were used to analyse

life-span data, and the statistical significance was assessed using the log-rank test in GraphPad Prism software.

5.3.4 Motility Assay

Worms were treated in accordance with the above-mentioned lifespan assay. 20 L4 stage worms were transferred to NGM+AO plates, with 3 plates per group. Motility was measured by gently stroking each worm with a wormpick and scoring their response into one of three classes: I, II and III as detailed in chapter 2 (Materials and Methods). Motility data was analysed in GraphPad Prism by dividing the number of worms into the three motility classes and normalising the data with the maximum number of individuals at the start of the assay set at 100%. The data was fitted with a non-linear regression model to determine the mid-point ($K_{0.5}$) when 50% of the population was in each motility class.

5.3.5 Intestinal Barrier Function assay (Smurf Assay)

The SA was carried out according to the protocol defined by Gelino et al. (2016). Briefly, worms were raised as described for lifespan assays with varying concentration of algae oil in comparison with non-treated worms as control. The OP50+blue dye solution was made by dissolving 0.5g of Erigolaucine disodium salt (Fisher, UK) in 9.5ml of standard OP50 liquid culture (grown overnight). Worms were suspended in 10ml of this OP50+blue dye solution for 3 hours. The worms were washed with dH_2O, centrifuged at 2000rpm for 1min and 50µl of 10mM sodium azide was added to paralyze the worms for imaging. The worms were then transferred to an unseeded NGM plate. Once the plates were dry, the worms were imaged at 10x magnification using an Olympus IX83 microscope to determine the presence or absence of blue dye in the body cavity. A worm was counted as a smurf phenotype when the blue dye could be observed outside of the digestive tract. As described in Martins et al. (2018), the proportion of smurf phenotypes in each group was calculated by dividing the total number of

smurf phenotypes within the population. Comparison of smurf phenotype proportion per time point was carried out using one-way ANOVA with Dunnett's multiple comparison test (GraphPad Prism, version 9). To calculate the smurf phenotype increase rate (SIR), the average proportion of smurfs was plotted as a function of chronological age. A simple linear regression model was fitted to the data and the SIR was defined as the slope of the calculated regression line (Dambroise *et al.*, 2016). For each time point, three independent experiments were carried out, each with 10 worms per treatment.

5.3.6 Determination of EPA Uptake by GC-MS

The effect of algae oil supplementation in *C. elegans* is limited by the OP50 *E. coli's* ability to absorb the algae oil into its bacterial membrane(Zečić, Dhondt and Braeckman, 2019). To confirm the uptake of algae oil, OP50 *E. coli* was grown on NGM plates with no supplements (control), 0.1mM and 0.3mM concentrations of algae oil. The seeded plates were left to dry for two days in the dark at room temperature before being incubated for three days at 20 °C. Bacteria were collected by scraping the lawn gently with a flame-sterilized spatula. The bacteria were treated with 2.5% H_2SO_4 in methanol and heated at 70 °C for 1 hr in a water bath to generate fatty acid methyl esters (FAMEs). The resulting FAMEs were extracted by adding 1.5 ml water and 0.25 ml of hexane. The mixture was vortexed vigorously for 1 minute followed by centrifuging for 1 minutes at 5000rpm to separate the hexane layer from the aqueous solvent. The hexane layer at the top was then transferred to a GC vial insert for GC-MS analysis using the methods described by Deline et al. (2013). Briefly, 1µl of FAMEs in hexane is injected onto a polar capillary gas chromatography column suitable for FAMEs analysis. The Agilent 7890 GC injector is set at 250 °C, with a flow rate of 1.4 ml/min, and the GC oven is programmed for an initial temperature of 130 °C, which is held for 1 min. Subsequently, the temperature is ramped 10 °C/min until 190 °C, and then ramped again at 5

°C/min until 210 °C and held for an additional 1 min. The total run time was 40 minutes with helium as the carrier gas.

Similarly, to confirm the uptake of EPA in *C. elegans*, synchronised L4 stage larvae were grown on NGM+AO plates as described above and harvested after five days growth at 20 °C. The worms were collected off the plates and FAMEs were analyzed by GC-MS using the same methods described above. The fatty acid composition of *C. elegans* were identified by matching the unknown mass spectrum against a mass spectral database and the EPA was quantified by using the EPA calibration curve. For each GC-MS analysis, the same number of worms were used in the control and algae-oil supplemented groups.

5.3.7 Generation of EPA Calibration Curve

EPA was dissolved in hexane to make a stock concentration of 1mg/ml. The stock solution was than diluted to make six mixtures of standards with concentrations ranging from 5μg/ml to 50μg/ml. Standard mixtures were treated with 2.5% H_2SO_4 in methanol and analyzed by GC-MS as described above. The calibration curves were calculated by linear regression (GraphPad Prism 9).

5.4 Results

5.4.1 Algae-oil supplemented worms maintained Intestinal Barrier Function with age

In young *C. elegans*, the intestine acts as a robust barrier against intestinal leakage, however this function deteriorates with age (Gelino *et al.*, 2016). Using the intestinal barrier assay, it was confirmed that *C. elegans* with intact intestinal integrity maintained the blue dye inside the intestinal tract (Figure 5. 1, day 2), whereas *C. elegans* with a damaged intestinal barrier leaked the dye into their body cavities from day 5 onwards. The number of smurf individuals was used to calculate the proportion of smurfs as a function of age (Rera, Clark and Walker, 2012). The proportion of smurf worms in all groups were low during early adulthood (day 5, Figure 5. 2), which is consistent with previous research showing that this component of gut deterioration occurs later in adulthood (Gelino *et al.*, 2016). Dye leakage was first observed in control worms at day five of adulthood and increased in frequency as the worms became older (Figure 5. 2), showing an age-dependent deterioration in intestinal integrity similar to what has been found in *Drosophila* (Martins *et al.*, 2018) and zebrafish (Dambroise *et al.*, 2016). When compared to control worms, the intestinal integrity of algae-oil supplemented worms was significantly enhanced, as significantly fewer algae-oil supplemented worms displayed the smurf phenotype at mid (day 8-11) and late adulthood (day 14-17).

To further elucidate the specificity of these effects, the effect of EPA supplementation, which is a major component of algae-oil, was examined separately alongside the other groups. Supplementing the worms with 160μM EPA led to an age-related decrease in intestinal integrity, comparable to the findings in algae-oil supplemented worms (Appendix Figure C1). The worms under 160μM EPA supplementation showed intact intestinal integrity until day 5 of adulthood and dye leakage occurred from day 8 onwards (Appendix Figure C2).

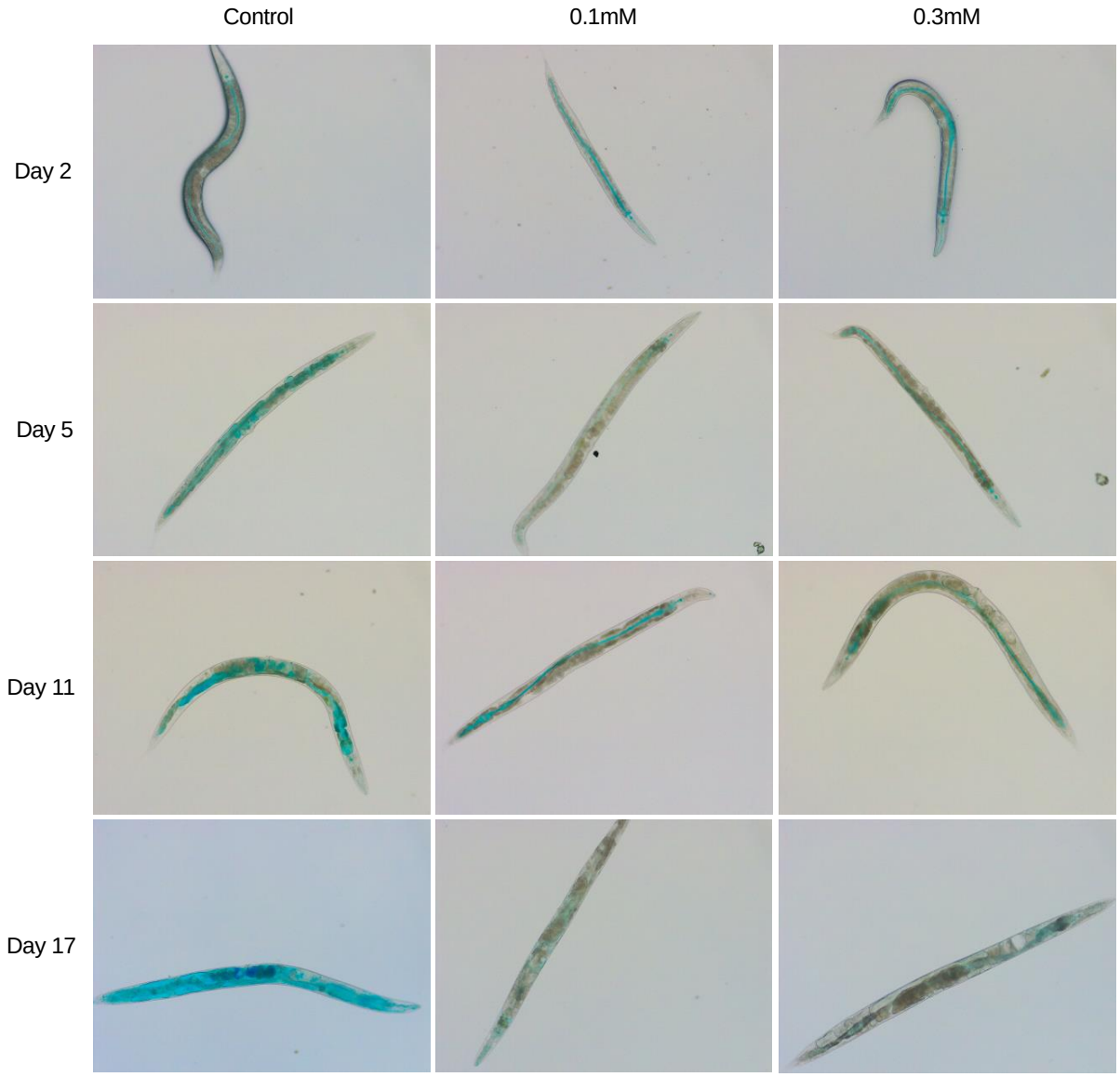

Figure 5. 1 Algae oil supplementation delays age-related intestinal permeability in *C. elegans*. Representative microscopy images showing loss of intestinal integrity over time in nematodes grown under control and algae oil supplementation. During early adulthood, dye is restricted to the intestine but during middle age, dye is seen throughout the body cavity.

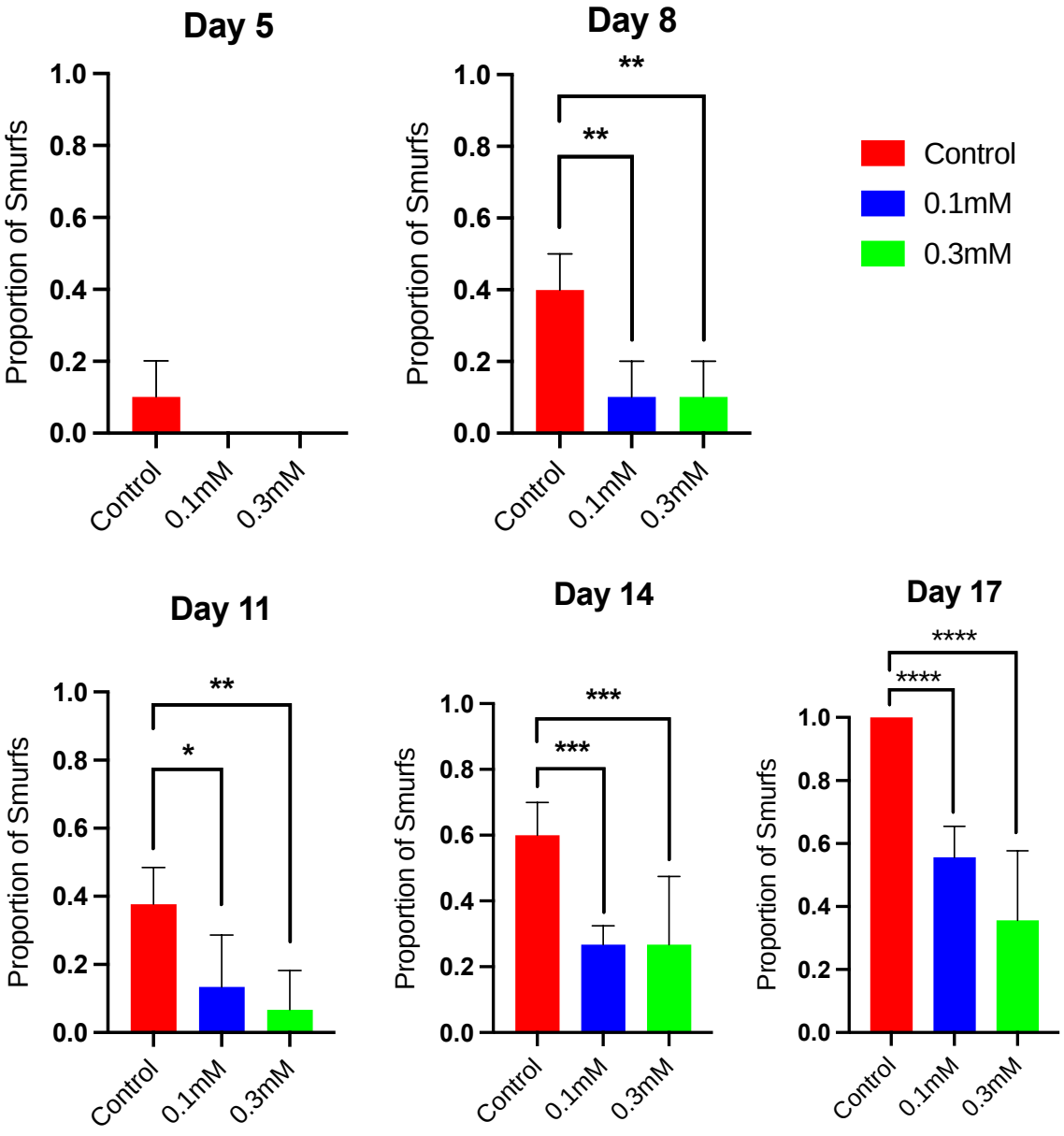

Figure 5. 2 Age-related breakdown of intestinal barrier function is reduced in algae-oil supplemented worms. Proportion of worms showing loss of intestinal integrity as a function of age, assayed using the Intestinal barrier assay. Data are the mean ± SD of three independent assays (Two-way ANOVA, **P<0.05, **P<0.01, ***P<0.001, ***P<0.0001*).

As per the 2PAC model, the proportion of smurf individuals in a synchronised population increases linearly as a function of chronological time (Tricoire and Rera, 2015). To compare the rate of increase in smurfs between the supplementation and control groups, the average proportion of smurfs was plotted as a function of chronological age with the slope of the calculated line defined as the smurf increase rate (SIR).

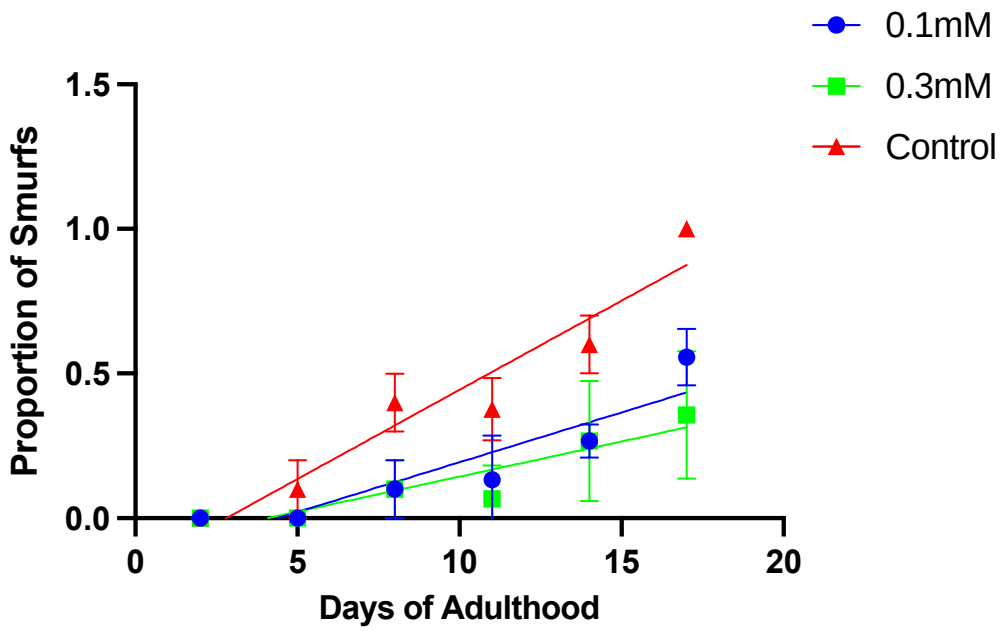

Figure 5. 3 Proportion of worms showing an age-dependent increase in smurf phenotype. The smurf increase rate (SIR) of the longer-lived population of 0.1mM (SIR = 0.034 ± 0.005, $R^2 = 0.7370$) and 0.3mM (SIR = 0.024± 0.006, $R^2 = 0.5005$) is significantly lower ($P < 0.0001$) than the SIR of the short-lived control population (SIR = 0.062± 0.006, $R^2 = 0.8847$). Statistical significance was determined via unpaired, two-tailed t-tests, calculated by GraphPad Prism (Version 9).

An age-dependent linear increase ($p<0.001$) in the proportion of smurfs was identified in synchronised populations of control and algae-oil supplemented worms (Fig. 5. 3). In addition, there was a two to three fold decrease in SIR for algae-oil supplemented groups compared to the control group (Figure 5. 3). Collectively, these findings suggest that algae-oil supplementation from early adulthood enhances intestine integrity and function in *C. elegans*, which is visible during a specific time frame later in life. Remarkably, aged worms supplemented with algae-oil retained their intestinal integrity well into late adulthood (Day 17; Figure. 5. 2), indicating a wider relationship between extended lifespan and enhanced intestinal integrity.

5.4.2 Algae- oil supplementation increases the Lifespan of *C. elegans*

The lifespan data confirmed the findings in chapter 4 when performed in parallel with the smurf assay. Under control conditions, the *C. elegans* had a mean lifespan of 14 days. Whereas, under 0.1mM and 0.3mM algae oil supplementation, the mean lifespan increased to 18.6 days and 19.4 days, respectively (Fig. 5. 4). Compared with the control group, the 0.1mM and 0.3mM treatment groups showed a 32.9% and 38.6% increase in mean lifespan, respectively (Table 5. 1).

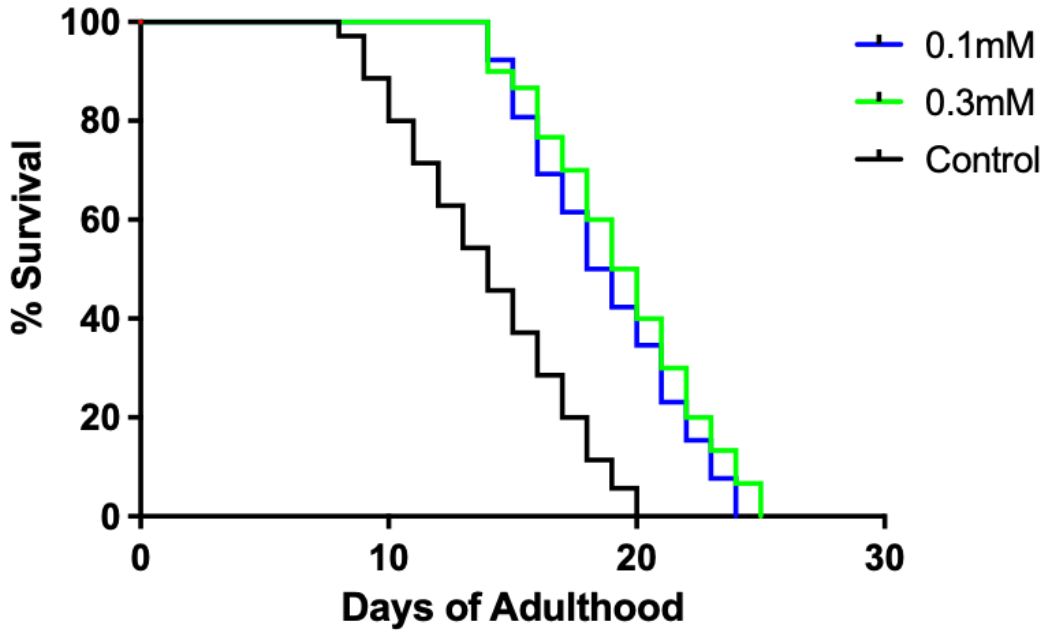

Figure 5. 4 Effect of algae oil supplementation on *C. elegans* lifespan. Unc-54 worms were supplemented with different concentrations of algae oil starting from day 1 of adulthood (L4) to death at 20°C. The mean life span of 0.1mM (18.6 days, $p<0.0001$) and 0.3mM (19.4 days, $p<0.0001$) were significantly higher compared to control (14.0 days). Survival plots were drawn by Kaplan-Meier survival assay. Data graph shows the result of one experiment and is representative of three independently completed experiments. Detailed parameters are presented in Table 5. 2.

Table 5. 1. Effect of different algae oil concentrations on the mean lifespan of *C. elegans*. Shown is the mean and maximum lifespan and the % change compared with control.

Diet	**Mean lifespan (days)**	**% Increase in Mean Lifespan**	**Maximum lifespan (days)**	**% Increase in Maximum Lifespan**
Control	14.0±0.6		20.0±0	
0.1mM	18.6±0.6	32.9%	24.0±0	20.0%
0.3mM	19.4±0.6	38.6%	25.0±0	25.0%

5.4.3 Algae oil supplementation improved Motility in *C. elegans*

The motility data recorded here confirmed the observations in chapter 4. Worms raised on algae-oil supplemented plates retained increased motility at all ages compared to control (Figure 5. 5). On day 11 of adulthood, all worms in control group, exhibited mid-life motility (class II), reflecting a decline in muscle mass (see chapter 4). In contrast, 50% of the worm population in algae-oil supplemented groups still retained increased motility on day 11. Similarly, non-linear regression analysis revealed that worms in the 0.1mM and 0.3mM algae-oil groups spend longer in class I ($C_{1\text{-}2}$ = 11.1 and 11.2 days, respectively) than in the control ($C_{1\text{-}2}$ = 9.3 days) (Table 5. 2). The average time spent in class II increased for the 0.1mM and 0.3mM algae-oil supplementation groups (ΔC_2 = 5.3 and 5.9 days, respectively) in comparison to the control population (ΔC_2 = 4.5 days) (Table 5. 2).

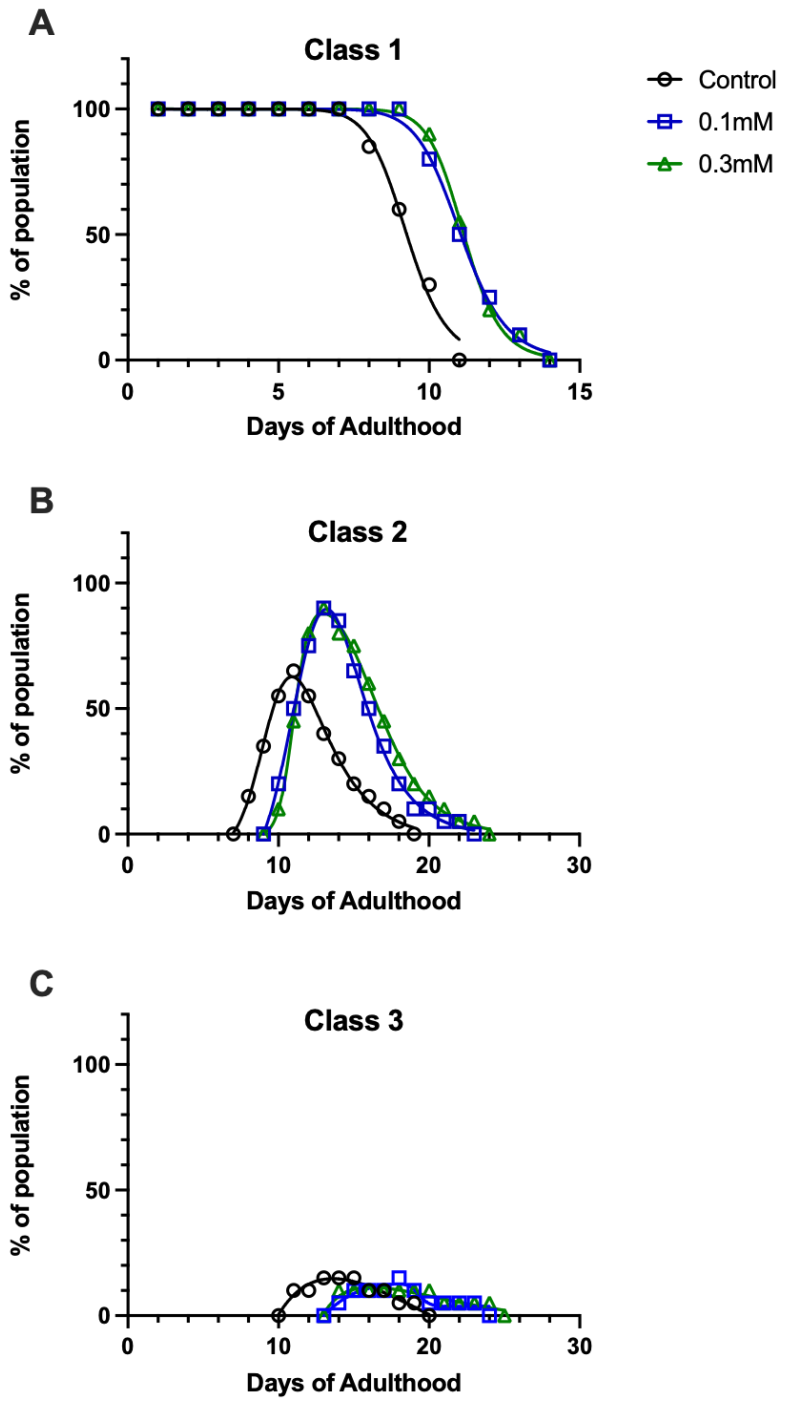

Figure 5. 5 Age-related changes in the motility of algae-oil supplemented worms. Classification of movement according to Herndon et al. (2002). (A) Class I – Continuous smooth movement, fast movement when stimulated. (B) Class II – Slow halting movement, smooth movement when stimulated. (C) Class III – Small movement of head or tail, very slow movement when stimulated.

Table 5. 2. Summary of age-related changes in motility under algae oil supplementation. Average number of days it takes for 50% of the initial population to move from class I to class II, represented by $C_{1\text{-}2}$, and average number of days for 50% of the class II population (based on maximum % in class II) to move to class III (represented by $C_{2\text{-}3}$). The difference between the two mid-points, represented by parameter ΔC_2, is the average time spent in class II.

	$C_{1\text{-}2}$ (days)	$C_{2\text{-}3}$ (days)	ΔC_2 (days)	Mean life span (days)
Control	9.26 ±0.07	13.8±0.1	4.5	14.0±0.6
0.1mM	11.05 ±0.04	16.3±0.2	5.3	18.6±0.6
0.3mM	11.15 ±0.03	17.0±0.1	5.9	19.4±0.6

Additionally, lifespan and motility data were combined to evaluate the effect of algae-oil supplementation on the healthspan and gerospan of *C. elegans*. Similar to chapter 4, the healthspan and gerospan of worms in early and mid-adulthood were evaluated to present a detailed overview of the effect of algae-oil supplementation. During early adulthood, healthspan was defined as the period when 50% of the worm population retained motility class I, while gerospan was defined as the period when 50% of the worm population lost motility class I. (Fig. 5. 6A). Analysing the motility data in table 5. 2 (parameter $C_{1\text{-}2}$), revealed that the healthspan period for the control group was just under nine days, whereas the healthspan period for the 0.1mM and 0.3mM algae-oil groups was extended to 11 days.

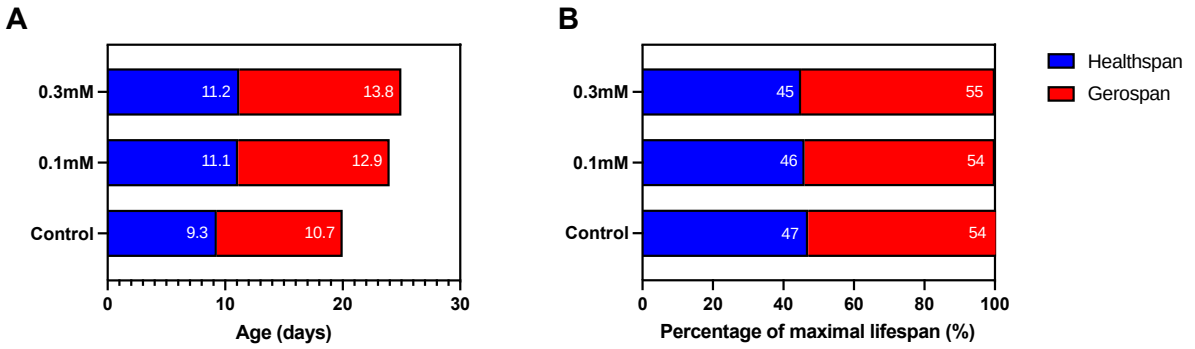

Figure 5. 6 Comparison of healthspan and gerospan in control and algae-oil supplemented groups at early adulthood. A) Healthspan was defined as the period when > 50% of the initial nematode population show type-I motility. Gerospan was defined as the period when < 50% of the initial nematode population show < 50% of type-I motility. B) The ratio of healthspan to gerospan was normalized to their maximal lifespan in control and algae-oil supplemented worms (Hahm *et al.*, 2015).

During mid-adulthood, healthspan was defined as the period during which 50% of the worm population retained motility class II, while gerospan was defined as the period during which 50% of the worm population lost motility class II (Fig. 5. 7A). When compared to control, healthspan in 0.1mM and 0.3mM algae-oil supplemented groups increased by 18% and 23%, respectively (Fig. 5. 7A). The normalised healthspan and gerospan ratios were fairly similar between all groups (Fig. 5. 7B).

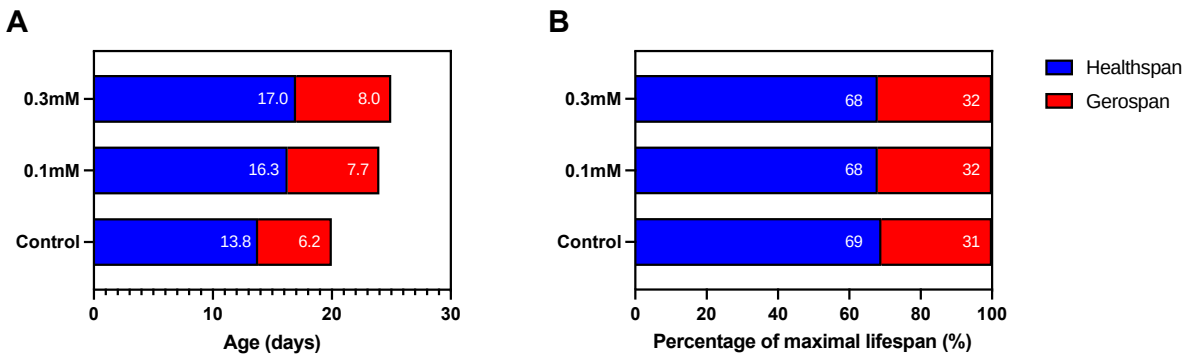

Figure 5. 7 Comparison of healthspan and gerospan in control and algae-oil supplemented groups at mid-adulthood. A) Healthspan was defined as the period when > 50% of the initial nematode population show type-II motility. Gerospan was defined as the period when < 50% of the initial nematode population show < 50% of type-II motility. B) The ratio of healthspan to gerospan normalized to their maximal lifespan in control and algae-oil supplemented worms.

5.4.4 Quantification of EPA in *E. coli* and *C. elegans*

The fatty acid composition of *C. elegans* supplemented with 0.1mM and 0.3mM algae oil were examined to determine whether the algae oils were absorbed by the worms. Since the worms obtain the fatty acids from their diet, EPA, which constitutes 25% of algae oil in this study, was quantified in both the *E. coli* (Figure 5. 8) and *C. elegans* (Figure 5. 9). The EPA standards were analysed as FAMEs by GC-MS (Appendix Figure C3). With co-efficients of correlation more than 0.99 and R^2 values of 0.99, the EPA standards calibration curve demonstrated strong linearity, suggesting that the GC-MS quantification method is exact and reproducible (Appendix Figure C4).

The EPA was quantified from *E. coli* and *C. elegans* after five days incubation period at 20°C with supplemented diets (Figure 5. 10). As a control, the EPA uptake was also measured on populations exposed to 160μm EPA only (Appendix Figure C5). The EPA levels were significantly higher in the algae-oil groups than in the control worms, with a 4.5 and 3.6-fold increase in the 0.1mM and 0.3mM algae-oil groups, respectively (Figure. 5. 10B). Taken together, these findings show that the relative accumulation of supplemented EPA in *C. elegans* may account for the significant improvement in longevity and healthspan.

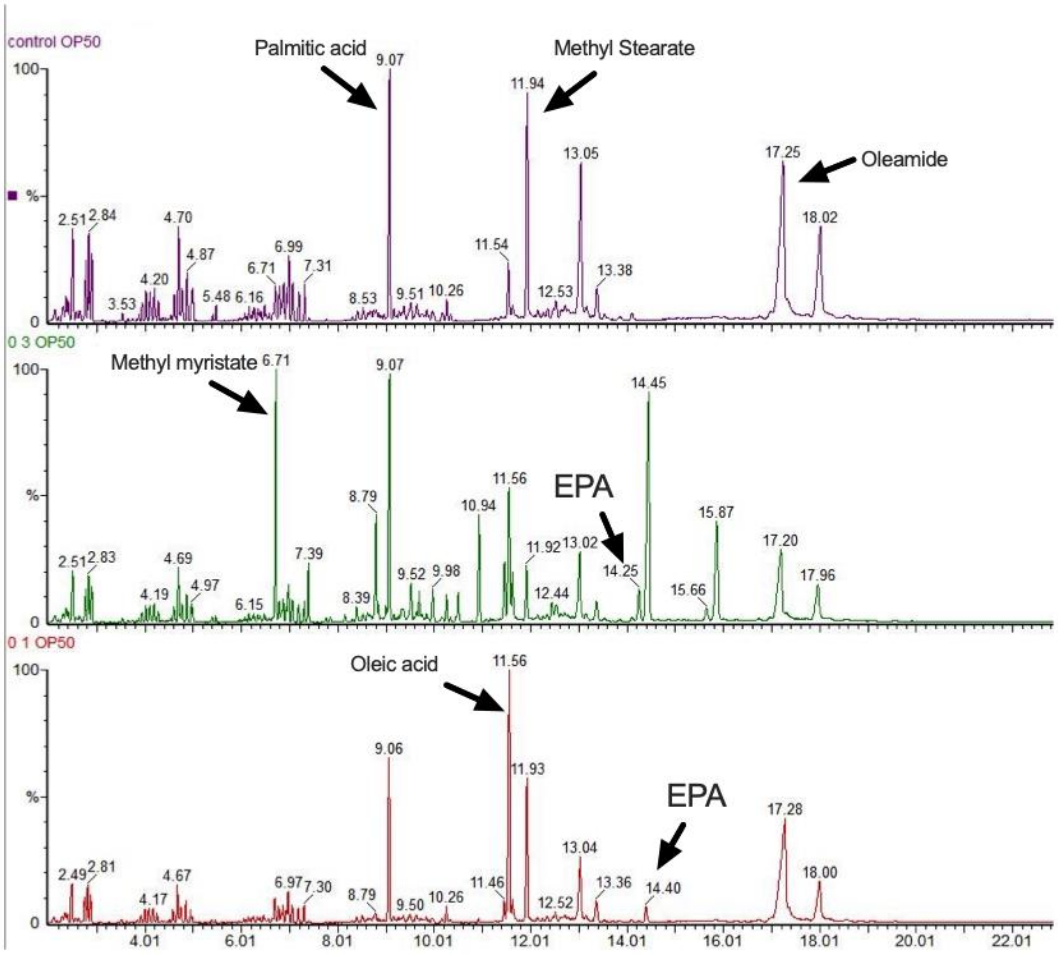

Figure 5. 8 Representative GC-MS chromatogram of *E. coli* OP50 grown on control (top), 0.3mM (middle) and 0.1mM (bottom) algae-oil supplemented NGM plates at 20°.

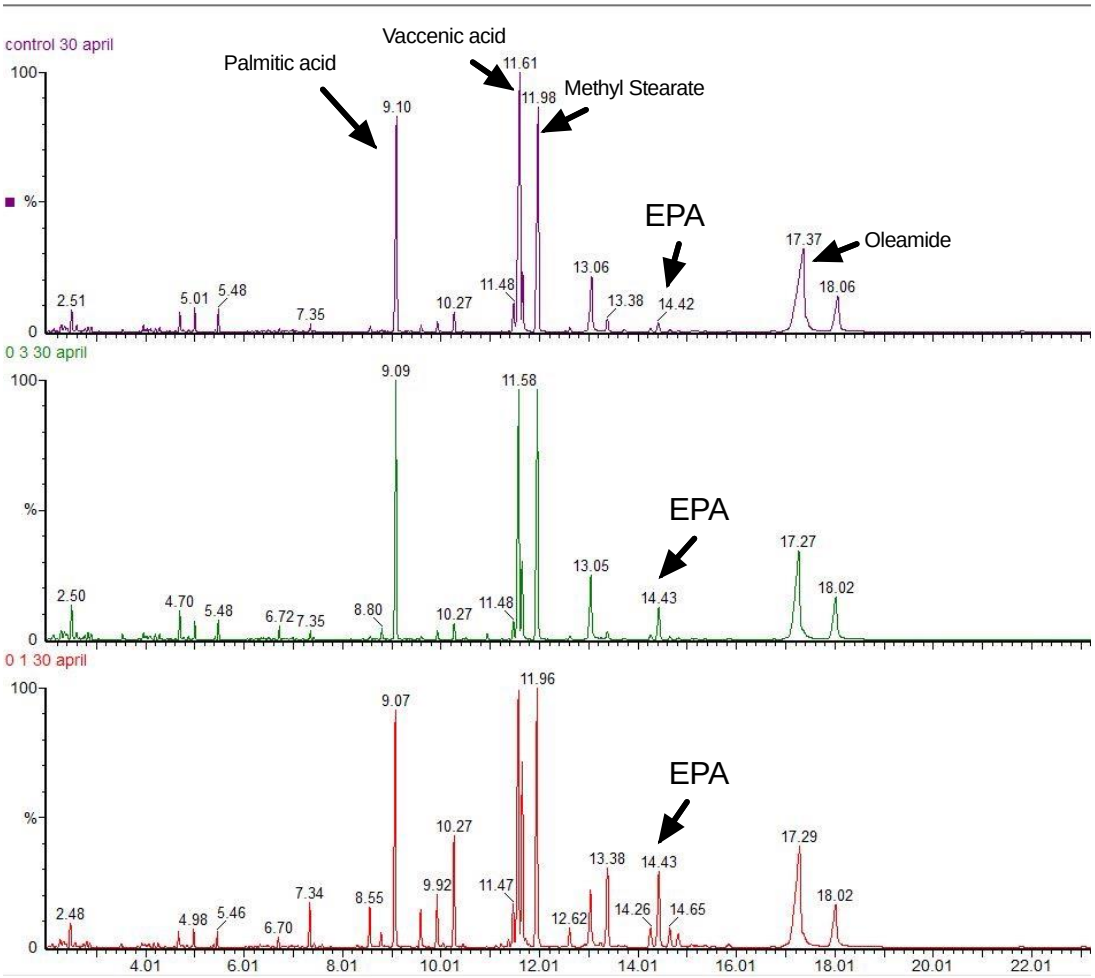

Figure 5. 9 Representative GC-MS chromatogram of *C. elegans* grown on control (top), 0.3mM (middle) and 0.1mM (bottom) algae-oil supplemented NGM plates at 20°.

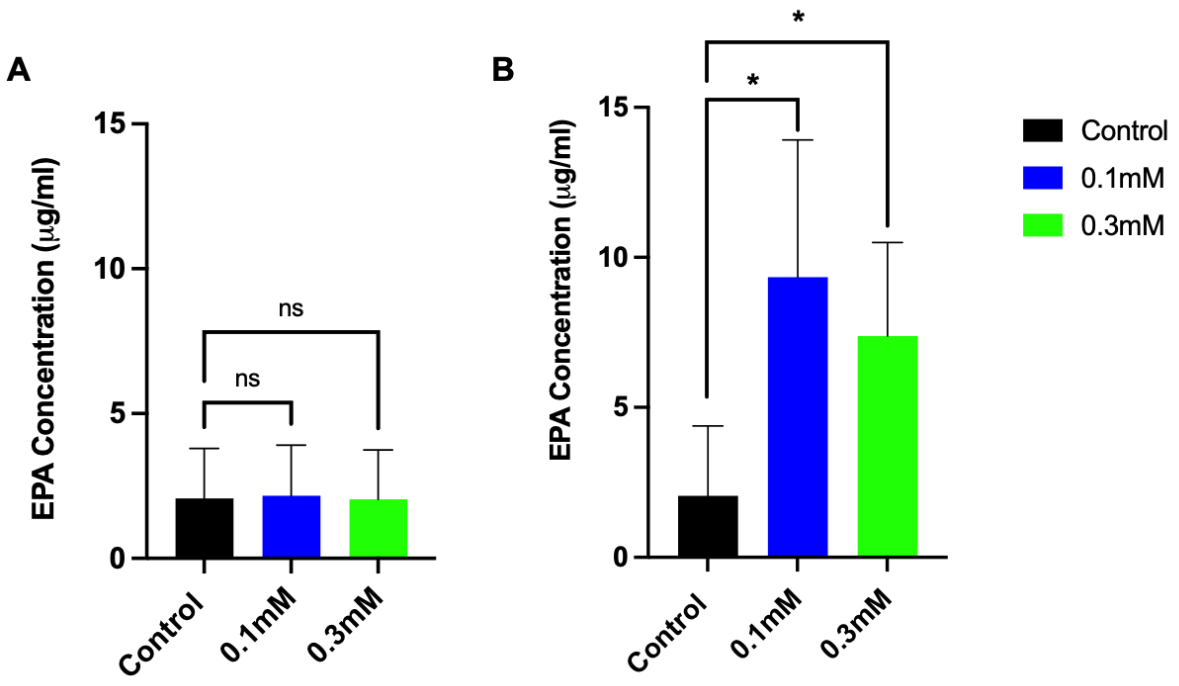

Figure 5. 10 Uptake and Incorporation of supplemented EPA by *E. coli* OP50 and *C. elegans*. The FAMEs extracted from A) *E. coli* and B) *C. elegans* were analysed with GC-MS to determine their fatty acid composition. The EPA content was calculated using the EPA calibration curve generated through GC-MS. Data are the mean ± SD of three independent assays (One-way ANOVA, *P<0.05).

5.5 Discussion

Recent studies have discovered a link between the gut microbiome and sarcopenia, since ageing is accompanied with increased dysbiosis and intestinal permeability (Liu *et al.*, 2021). The gut microbiota of elderly people differs from that of younger people, suggesting that the gut microbiota may play a role in the development of sarcopenia (Ragonnaud and Biragyn, 2021). A "gut-muscle axis" hypothesis has been proposed, according to which changes in the gut microbiome contributes to the loss of skeletal muscle mass and function in sarcopenia (Prokopidis *et al.*, 2021). In chapter 4, a quantitative fluorescence method was used to show that algae-oil improves healthspan by delaying age-related muscle loss in *C. elegans*. Given that the gut–muscle axis hypothesis is based on the correlation between gut microbiota and muscle function, the delayed sarcopenia observed in algae-oil supplemented worms (chapter 4) may be attributed to a delay in age-related breakdown of the intestinal barrier function. In order to test this hypothesis, an intestinal barrier function assay was used to investigate the effect of algae-oil supplementation on age-induced intestinal permeability of *C. elegans*. Similar to chapter 4, algae-oil supplementation enhanced the lifespan and motility of *C. elegans* compared to the control group.

Moreover, algae-oil supplementation was shown to delay the onset of age-related intestinal barrier dysfunction when compared to control confirming the original hypothesis of this study. In the algae-oil supplemented groups, the proportion of worms exhibiting the smurf phenotype was significantly lower in day 11 of adulthood compared to the control group. This result is consistent with the motility findings in Chapter 4, which showed that on day 11, most of the algae-oil supplemented populations were still in class I motility, whereas more than half of the worm population in the control group had already progressed to class II motility. Similarly, on day 14, the proportion of worms exhibiting the smurf phenotype was significantly lower in

algae-oil supplemented groups compared to the control group. Again, this finding is consistent with the fluorescence intensity data presented in Chapter 4, which showed that on day 14, the algae-oil supplemented worms had significantly higher fluorescence intensity, reflecting increased UNC-54 levels and increased muscle mass preservation when compared to the control group. Moreover, the SIR of algae-oil supplemented groups (Figure 5. 3) showed a good correlation (Appendix Figure C6) with the rates of myosin loss (k_F) in chapter 4 (Figure 4. 6). The beneficial effects of algae-oil supplementation on the intestinal barrier presented in this study is consistent with previous studies linking omega-3 fatty acids with decreased intestinal permeability (Chen *et al.*, 2016) (Kaliannan *et al.*, 2015).

Previous studies have shown that omega-3 fatty acids such as EPA and DHA, are integrated into the membranes of intestinal epithelial cells, limiting the production of pro-inflammatory cytokines while promoting the production of anti-inflammatory cytokines (Calder, 2020). In this study, the incorporation of algae-derived EPA in *C. elegans* was confirmed using GC-MS. A significant increase in EPA levels were observed in algae-oil supplemented worms compared to control (Figure 5. 10B), suggesting that supplementation with algae-oil changes the relative amount of EPA in worm tissues. Since many studies have shown that omega-3 PUFA treatment increases antioxidant capacity (Sakai *et al.*, 2017), it was also hypothesised that algae-oil supplementation in *C. elegans* could enhance the antioxidant properties *in vivo* by reducing ROS-induced cellular damage and thus extending *C. elegans'* healthspan. However, due to the high lipid peroxidation levels associated with PUFAs (Sugawara *et al.*, 2013), many studies have indicated that the omega-3 PUFA must be administered at low concentrations to have a beneficial effect (Beaudoin-Chabot *et al.*, 2019). In the present study, the use of a relatively low concentration (0.1mM and 0.3mM) of algae-oil significantly extended the lifespan and

healthspan of *C. elegans*, potentially due to decreased lipid peroxide levels in algae-oil supplemented worms.

The impact of diet in relation to the gut microbiota and sarcopenia has been demonstrated in studies of elderly people with varied diet (Liao *et al.*, 2020). Beneficial gut bacteria have been shown to lower chronic inflammation by promoting the secretion of anti-inflammatory markers (Lobionda *et al.*, 2019). Claesson et al. (2012) showed a that community dwellers who consumed 'low-moderate fat' diet had a higher proportion of beneficial gut bacteria compared to those in long-term residential care who consumed a ‘moderate-high fat' diet. Similarly, studies comparing the skeletal muscle of germ-free (GF) mice to pathogen-free (PF) mice showed that GF mice had decreased skeletal muscle mass and strength compared to PF mice (Lahiri *et al.*, 2019).

While our findings imply that supplementing with algae-oil delays sarcopenia by improving intestinal barrier integrity, it's crucial to note that the study's limitations preclude us from determining a causative mechanism. The motility class of individual worms was not taken into account since the worms used in the intestinal barrier assay were selected at random. Since each motility class represents a different stage of ageing, worms in motility class III are more likely to display the smurf phenotype than worms in motility class I and II. Thus, further research incorporating the motility class into the intestinal barrier assay will aid in understanding how algae-oil influences the intestinal barrier function in *C. elegans*. The C. elegans used in this study were only fed one strain of bacteria (OP50), which is a limited model for gut microbiota given the wide range of bacterial species found in gut microbiota. As a result, future studies will focus on the impacts of various bacterial strains in order to validate the C. elegans model. Other fatty acids found in algae-oil such as palmitic acid and linoleic acid (See

Appendix B1) may alter intestinal barrier function by a similar method, and therefore additional research is needed to address this question. Lastly, since the smurf phenotype described in this study using *C. elegans* cannot be translated directly to humans, additional research into the effects of algae-oil on the healthspan of different model species is needed.

With population ageing becoming a rising worldwide problem, further research into how dietary components could preserve intestinal barrier integrity and delay sarcopenia is required. This study showed that algae-oil supplementation can extend *C. elegans* healthspan through improved intestinal barrier integrity. The increased levels of EPA in *C. elegans'* tissues might explain the improved motility and delayed onset of sarcopenia. These findings add to our understanding of intestinal health and may contribute to development of prophylactic interventions to mitigate the impacts of ageing. *E. coli* has been found to accumulate in the pharynx and gut of ageing *C. elegans* which may affect how worms respond to new dietary interventions (Kumar *et al.*, 2019). Consequently, worms fed on UV-irradiated *E. coli* OP50 showed increased lifespan compared to worms fed on live bacteria (Gomez *et al.*, 2012). For future research, it would be interesting to evaluate the effects of algae-oil supplementation on *C. elegans* grown on dead bacteria as the observed physiological changes may differ.

Chapter 6: General Discussion and Conclusions

6.1 Summary of Key Findings

In recent years, it has become increasingly evident that the gut microbiota has a significant impact on muscle growth and function, giving rise to the gut-muscle axis hypothesis (Zhao, Huang and Yu, 2021). Intestinal microbiota has been demonstrated to play critical roles in muscle ageing, with gut dysbiosis contributing to increased intestinal permeability (Kinashi and Hase, 2021). Increased intestinal permeability facilitates the movement of pro-inflammatory cytokines, resulting in systemic inflammation that compromises skeletal muscle integrity (Gizard, Fernandez and De Vadder, 2020). The most significant approaches to counteract sarcopenia in the elderly are resistance exercise and nutrition; which have been shown to delay the age-related changes associated with sarcopenia (Yoshimura *et al.*, 2017).

Here, the effect of DR and omega-3 PUFAs from algae-oil on sarcopenia was investigated using a transgenic *C. elegans* model with fluorescently labelled myosin (UNC54-GFP). As previous research on DR and omega-3 supplements has mostly focused on lifespan, this thesis is focused on the effects on sarcopenia in the context of the gut-muscle axis and measures myosin density in response to dietary intervention. Mild and medium DR delayed the onset of sarcopenia in *C. elegans* with improved motility and increased myosin UNC-54 levels compared to control and severe DR groups. The rate of myosin loss represented by k_F (Table 3. 3) showed a good correlation with motility parameter $C_{1\text{-}2}$ (Appendix Figure A3.A). For the mild and medium DR treated groups, the reported $T_{0.5}$ values were significantly higher than for the control and severe DR groups, indicating that mild and medium DR worms maintained higher UNC54 levels for longer through the ageing period, resulting in less pronounced sarcopenia compared to control and severe DR worms (Table 3. 2). These results also highlight the improved sensitivity of the new fluorescence method used in this study as it can determine different rates of myosin loss in response to different DR regimes.

In chapter 4, algae-oil supplementation was shown to delay the onset of sarcopenia with enhanced motility and myosin UNC-54 levels compared to control group. The rate of myosin loss (represented by k_F) (Figure 4. 6, day 10) corresponded with the time when worms transitioned from class I to class II (Table 4.2, parameter $C_{1\text{-}2}$), suggesting that reduced motility is linked with decreased UNC-54 levels. When compared to the control group, the algae-oil supplemented groups showed significantly higher fluorescence intensity on day 12, 14 and 16, indicating increased muscle preservation at old age. Given the link between gut dysbiosis and sarcopenia (gut-muscle axis), it was hypothesised that the delayed sarcopenia rates observed in chapter 4 might be associated with a delay in intestinal permeability. As a result, the effect of algae-oil supplementation on age-related intestinal permeability was investigated in Chapter 5. The SIR was significantly lower in algae-oil supplemented groups compared to the control group (Figure 5. 3). These data correlates well (Appendix Figure C6) with the rate of myosin loss (k_F) in chapter 5 (Figure 4. 6). Altogether, these results confirmed the primary hypothesis that a delay in sarcopenia is associated with a delay in intestinal permeability, in accordance with the gut-muscle axis theory.

6.2 Implications of Key Findings on Sarcopenia

The findings of this thesis have significant implications for sarcopenia research, particularly in the context of gut-muscle axis theory. The nematode *C. elegans* proved to be an effective model organism for studying sarcopenia due to the similarities in body wall muscle to vertebrate muscle, as well as its transparent body, which allowed for *in vivo* imaging of GFP-labelled muscular components. Moreover, a novel quantitative fluorescence method was devised to determine sarcopenia rates in DR and algae-oil supplemented worms. In response to different DR interventions, the rate of myosin loss (kF) correlated well with health span (motility parameter C1-2). This demonstrates that the novel fluorescence approach is particularly sensitive since it can directly measure myosin loss (sarcopenia), which will be useful in future studies for initial screening of interventions to treat sarcopenia.

In both DR and algae-oil studies, a positive correlation was observed between motility parameter $C_{1\text{-}2}$ and myosin density (UNC-54:GFP), suggesting that this quantitative fluorescence method can accurately quantify the myosin density in *C. elegans*. This method can be used to rapidly screen the potential of new drugs or dietary interventions to treat sarcopenia. The characteristics sinusoidal movement of *C. elegans* is controlled by its body wall muscles (UNC-54) and previous studies have shown that the level of unc-54 expression decreases gradually with age (Adamla and Ignatova, 2015). Although the mRNA levels of *unc-54* was not measured directly in this study, a significant variability was found in the UNC-54 fluorescence intensity of worms at different ages for all groups, indicating a difference in the UNC-54 levels with ageing.

Previous studies have shown that moderate DR is the most effective at increasing the lifespan of *C. elegans*. Likewise, the findings in this study revealed that the beneficial effects of DR on sarcopenia rates were best achieved at moderate concentrations. Various experimental studies

have shown that DR can mitigate the effects of a variety of age-related conditions, including sarcopenia (Balasubramanian, Howell and Anderson, 2017). Previous studies have shown that C. elegans preserve their muscle during periods of nutritional scarcity (DR) to facilitate the capacity to scavenge for food and maintain its survival (Howard *et al.*, 2021). In particular, DR has been shown to exert its beneficial effect in lifespan and healthspan by attenuating signalling through the nutrient sensing mTOR pathway (Papadopoli *et al.*, 2019). The current findings in chapter 3 expand previous research on the impact of CR on longevity by examining the influence of DR on muscle mass modulation *in vivo*. Previous studies evaluating the role of DR in skeletal muscles have shown that DR preserves muscle mass in middle aged rats but not younger rats (Chen *et al.*, 2015), suggesting an age-specific response. This is consistent with the findings in chapter 3 where UNC54-GFP (representing muscle density) in mild and medium DR groups were not significantly different to control group. However, in mid-adulthood, UNC-54 levels were found to be significantly increased in mild and medium DR compared to control. This suggests that the age-dependent effects of DR on mTOR signalling (Chen *et al.*, 2019) accounts for the age-related effect of DR on muscle mass preservation.

There is growing evidence to suggest that omega-3 PUFAs have inherent anabolic effects in skeletal muscle (McGlory, Calder and Nunes, 2019). It has been shown that high levels of pro-inflammatory cytokines in the blood of the elderly causes impaired muscle regeneration, which leads to muscle mass loss (Kim *et al.*, 2011). Consequently, supplementation with EPA and DHA was shown to significantly lower the circulating levels of pro-inflammatory cytokines in elderly people compared to the control group (Tan et al., 2018). Many studies have suggested that omega-3 PUFAs can reduce gut dysbiosis by integrating into the phospholipid membranes on intestinal cells and exerting their anti-inflammatory effects (Fu *et al.*, 2021). As a result, the delay in sarcopenia observed in chapter 4 and the corresponding delay in intestinal permeability

shown in chapter 5 have significant implications for algae-oil supplementation in sarcopenia. Previous animal studies show that the interplay between gut microbiota and omega-3 PUFAs helps maintain the intestinal barrier integrity (Durkin, Childs and Calder, 2021). *E. coli* has been shown to accumulate in the intestinal lumen of old *C. elegans* (Kumar et al., 2019). This increase in bacterial accumulation as the worms get older might be attributed to the age-related loss of intestinal integrity, which makes them more susceptible to bacterial accumulation (Virk et al., 2016). Given the role of omega-3 PUFAs in maintaining intestinal integrity, the findings in chapter 5 suggests that the algae-oil supplementation may have reduced bacterial accumulation with age, resulting in lower intestinal permeability. This is the first study to provide evidence for the effects of algae-oil on the onset and progression of sarcopenia via the gut–muscle axis. The delay in intestinal permeability corresponded well with the delay in sarcopenia rates, highlighting the potential of PUFA from algae-oil in influencing the gut-muscle axis. Interestingly, omega-3 PUFAs and gut microbiota have been revealed to share key immune system activation and inhibition pathways that govern pro-inflammatory patterns (Cândido *et al.*, 2018). Some studies have also shown that omega-3 PUFAs can alter the composition of the gut microbiome by increasing the number of bacteria that reduce gut permeability and decreasing the number of bacteria that enhance gut permeability (Lam *et al.*, 2012). In humans, the number of butyrate-producing bacterial species was shown to increase after omega-3 PUFA supplementation (Watson *et al.*, 2018). Since that the concept of the gut-muscle axis is based on the correlation between gut microbiota and muscle function, the findings from this thesis suggest that supplementing omega-3 PUFAs from algae oil may be a viable method for promoting gut-muscle axis–induced prevention of sarcopenia.

Sarcopenia-related declines in muscle function impose a considerable burden on health-care expenditures since muscle mass, and specifically muscle strength is inversely correlated with mortality (Bachettini *et al.*, 2020). Measuring characteristics of decreasing muscle function,

such as motility and intestinal permeability, can lead to a better understanding of the mechanisms behind sarcopenia and ways to mitigate its effects in humans. Although this study is only a first step towards understanding the effects of algae-oil supplementation on sarcopenia, the findings from this study can be expanded to other model organisms.

6.3 Limitations and Future Research

Previous studies have demonstrated that different strains of bacteria have distinct effects on the longevity and healthspan of *C. elegans* (Stuhr and Curran, 2020). In this study, only one strain of *E. coli* (OP50) was used which is a limiting model for testing the gut-muscle axis theory since the gut microbiome is highly diverse. Hence, it is necessary to repeat the dietary intervention assays using different strains of bacteria and determine the sarcopenia rates and SIR. In addition, live *E. coli* OP50 is capable of metabolising algae-oil, which results in the production of secondary metabolites (Janßen and Steinbüchel, 2014). It would be interesting to analyse the secondary metabolites generated by different *E. coli* strains and determine the ones beneficial for sarcopenia. Furthermore, since the *C. elegans* in this thesis were assayed at the L4 adult stage, it would be useful to examine the muscle density (represented by UNC54-GFP) present in young (L1-L3) *C. elegans* using the novel fluorescence method to demonstrate the gradual increase and decrease in muscle mass with age.

The goal of this study was to uncover biological insights in the areas of dietary intervention. In this study, many beneficial effects were observed that might have implications for higher species. It is therefore worthwhile to verify these findings using other model organisms such as fruitflies and mice. Clinical significance of algae-oil supplementation can only be determined in higher organisms once preliminary investigations on various model organisms have been completed. Finally, for future studies, RNA-sequencing on algae-oil supplemented and control worms might be useful in determining which pathways are involved in the sarcopenia delaying process.

Appendices

Appendix A

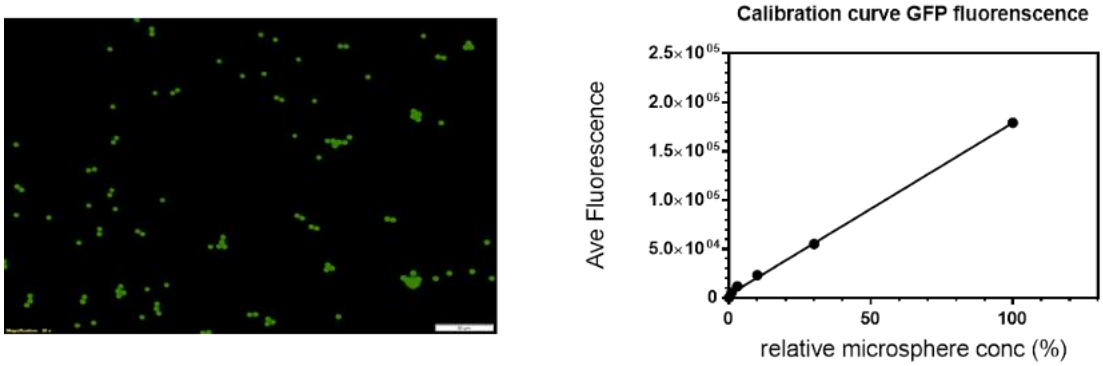

Figure A1. Calibration curve of GFP-labelled microspheres using the fluorescence microscope. Representative image of microbeads (3%) labelled with GFP (left) and the correlation between microsphere concentration and average fluorescence intensity (right). The best linear fit is described by y = 1761 x + 3004 with R^2 = 0.9986.

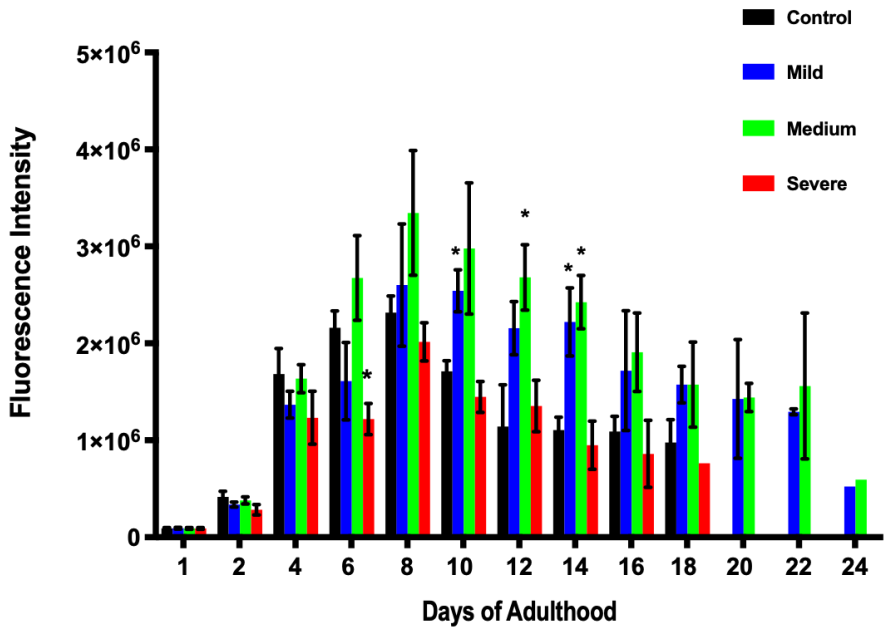

Figure A2. Average fluorescence as a function of age for the various DR groups (Two-way ANOVA, *P < 0.05).

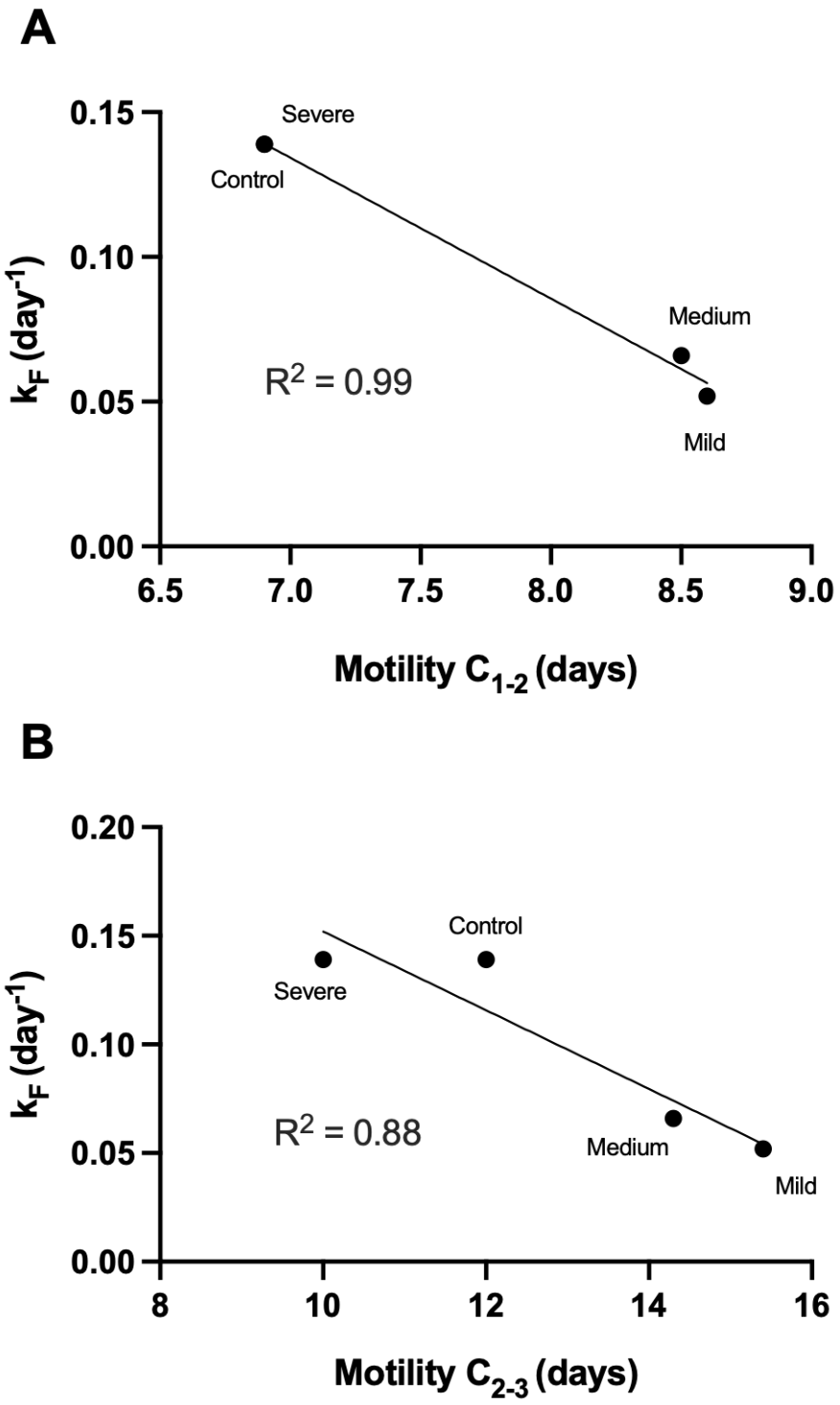

Figure A3. Rate of myosin loss (k_F) is correlated with healthspan in *C. elegans*. (A) Correlation of rate of myosin loss (k_F) with motility parameter C_{1-2} (B) Correlation of rate of myosin loss (k_F) with motility parameter C_{2-3}.

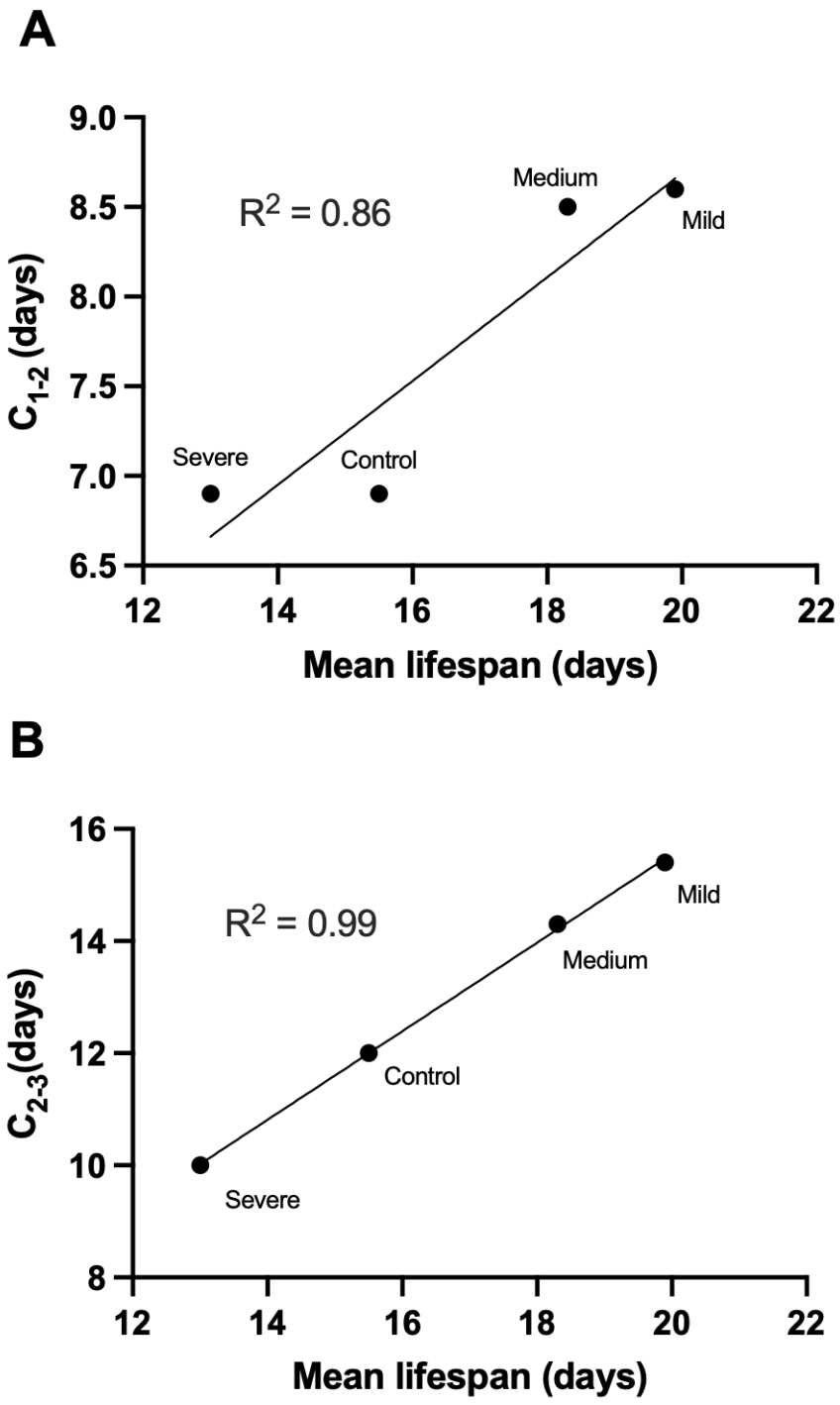

Figure A4. Motility transition correlates with mean lifespan in *C. elegans*. (A) Correlation of motility parameter C_{1-2} and mean lifespan. (B) Correlation of motility parameter C_{2-3} and mean lifespan.

Appendix B

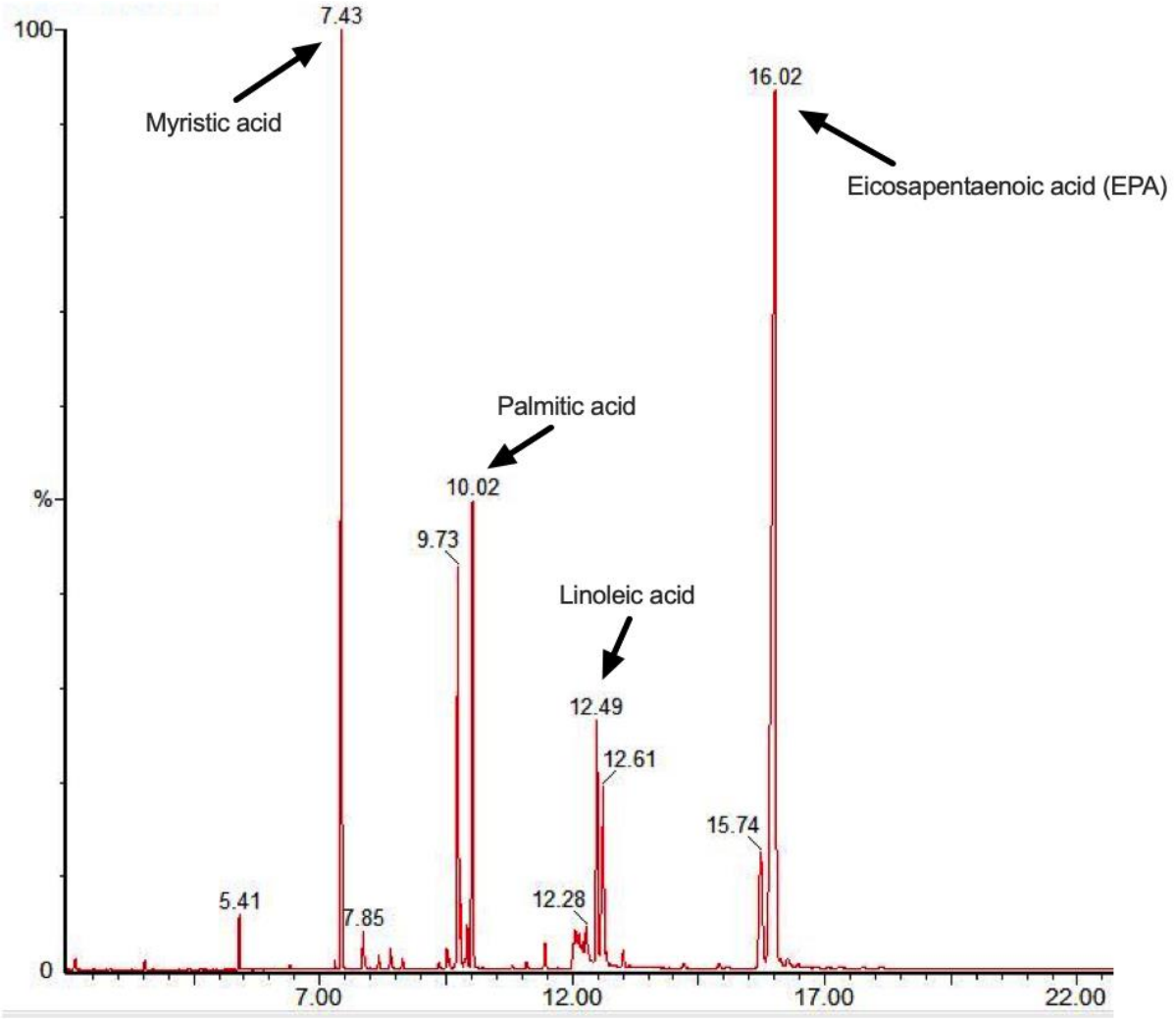

Figure B1. Representative GC-MS chromatogram of showing the fatty acid composition of algae-oil used in chapter 4 and 5.

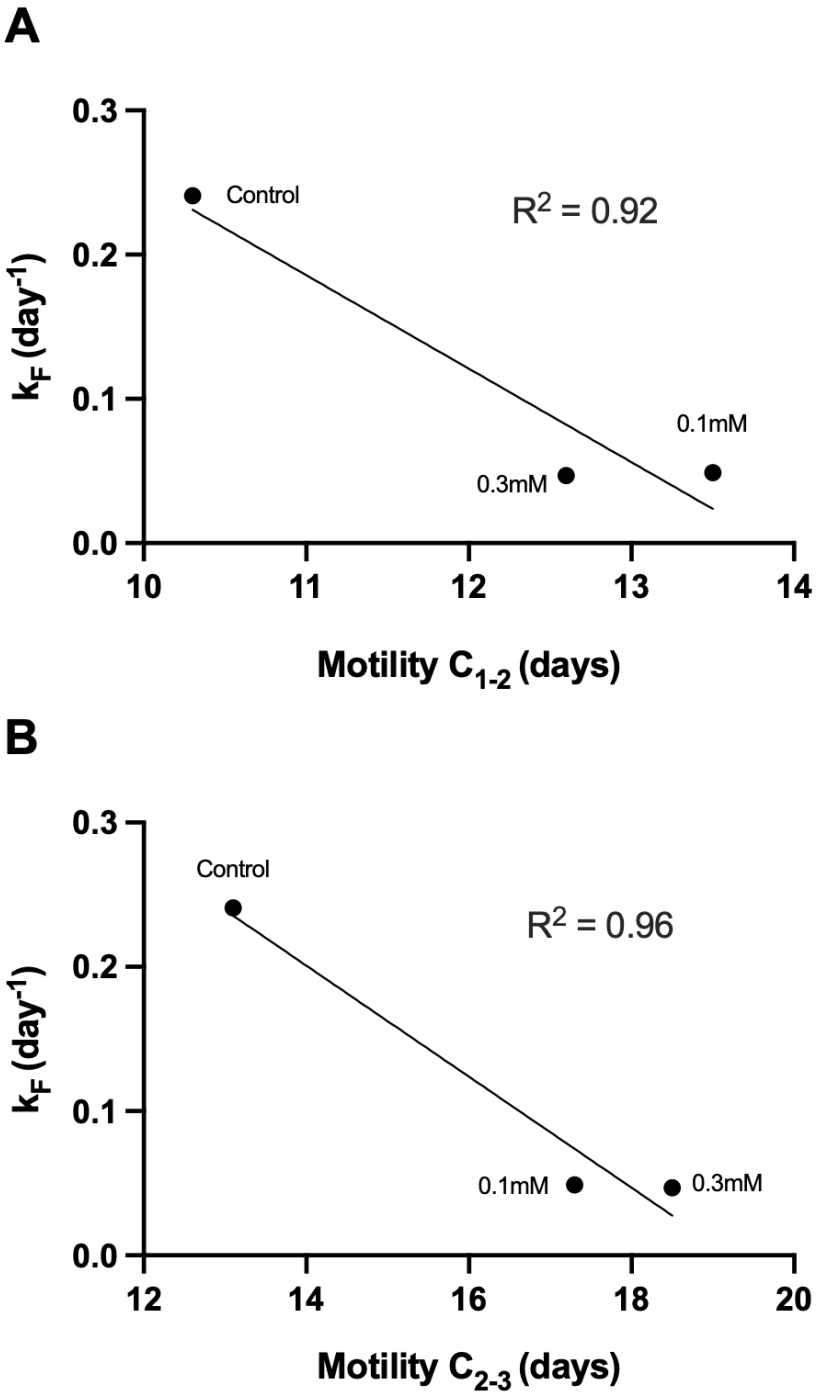

Figure B2. Motility transition correlates with fluorescence loss (k_F) in *C. elegans*.

Correlation of fluorescence loss (k_F) with motility parameters, A) C_{1-2} and B) C_{2-3}.

Appendix C

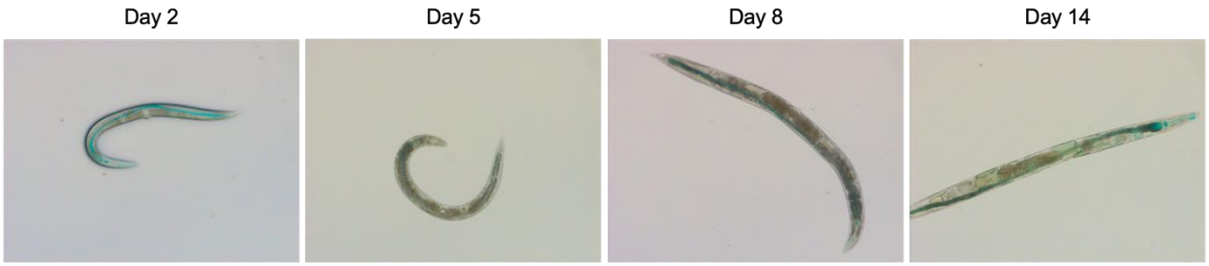

Figure C1. Representative microscopy images showing loss of intestinal integrity over time in *C. elegans* grown under 160µM EPA supplementation.

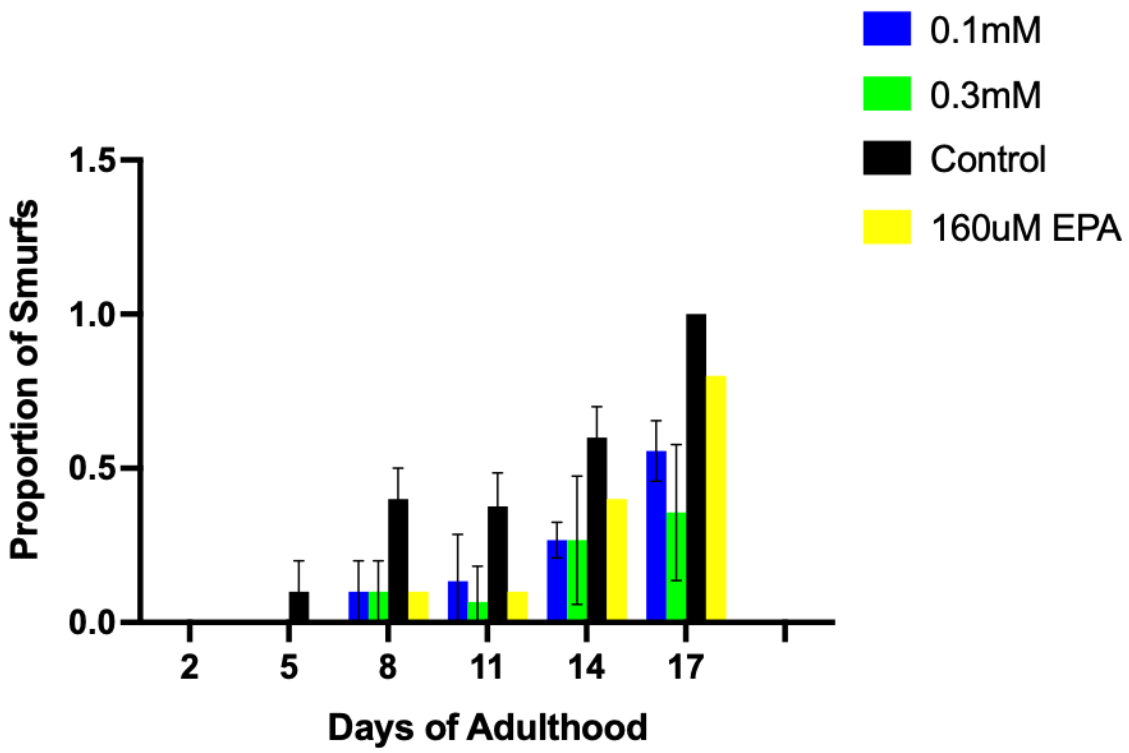

Figure C2. Proportion of worms showing loss of intestinal integrity as a function of age, assayed using intestinal barrier assay.

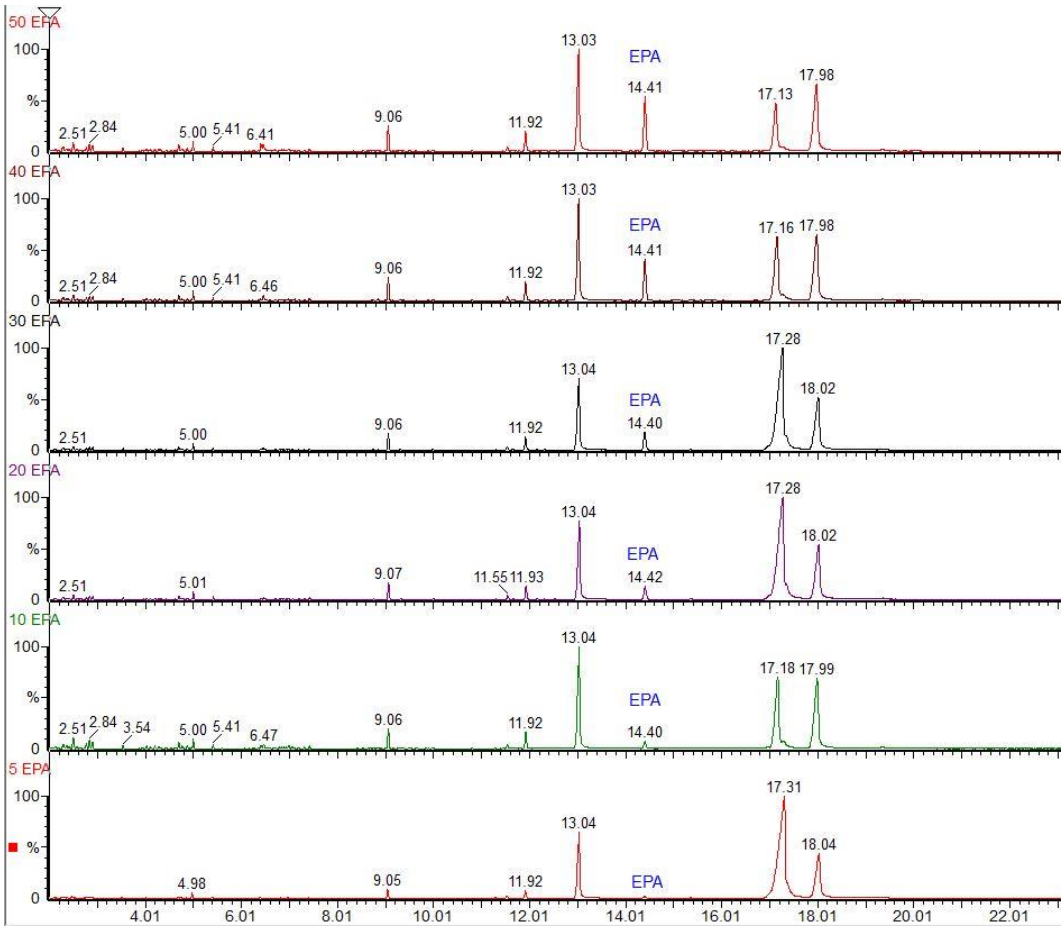

Figure C3. GC-MS chromatograph of EPA standards ranging from 5 to 50μg/ml. The x-axis is the retention time, taken from the time the sample was injected into the GC to the end of the GC run. The y-axis is the measured response of the analyte peak in the detector.

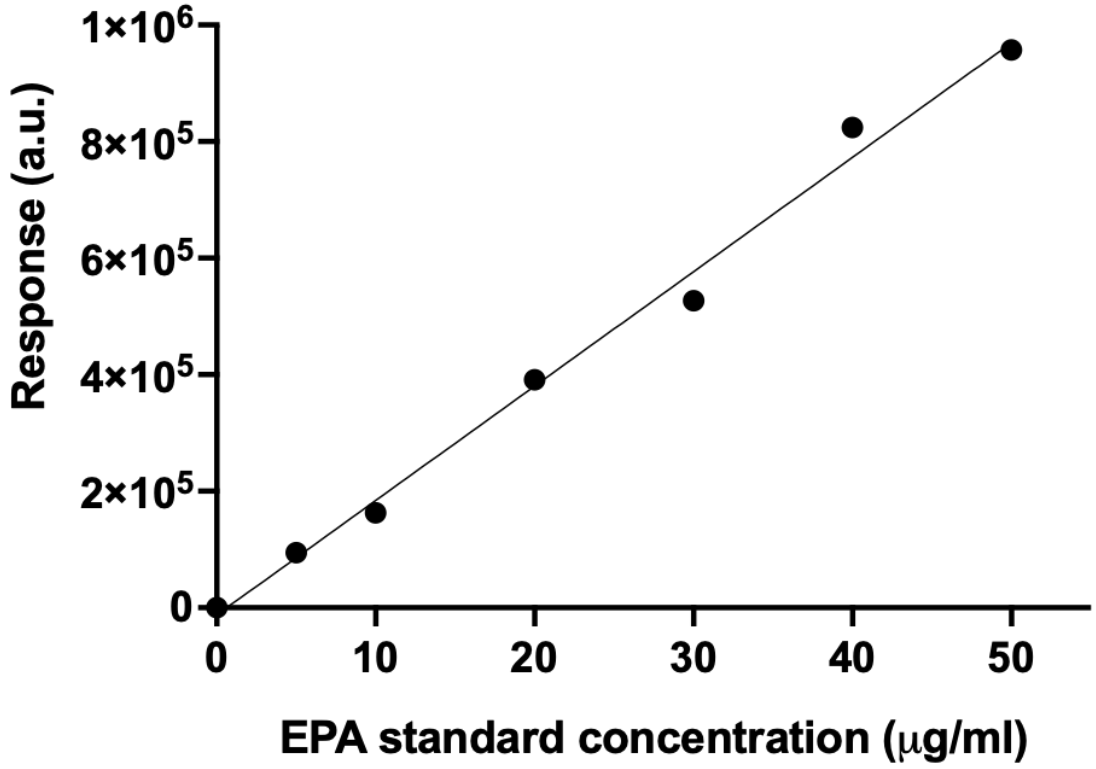

Figure C4. Calibration curve used for the quantification of EPA. Concentrations ranging from 5-50μg/ml of FAMEs standard were used for GC-MS analysis. A positive linear association was found between EPA and intensity (R^2 = 0.99).

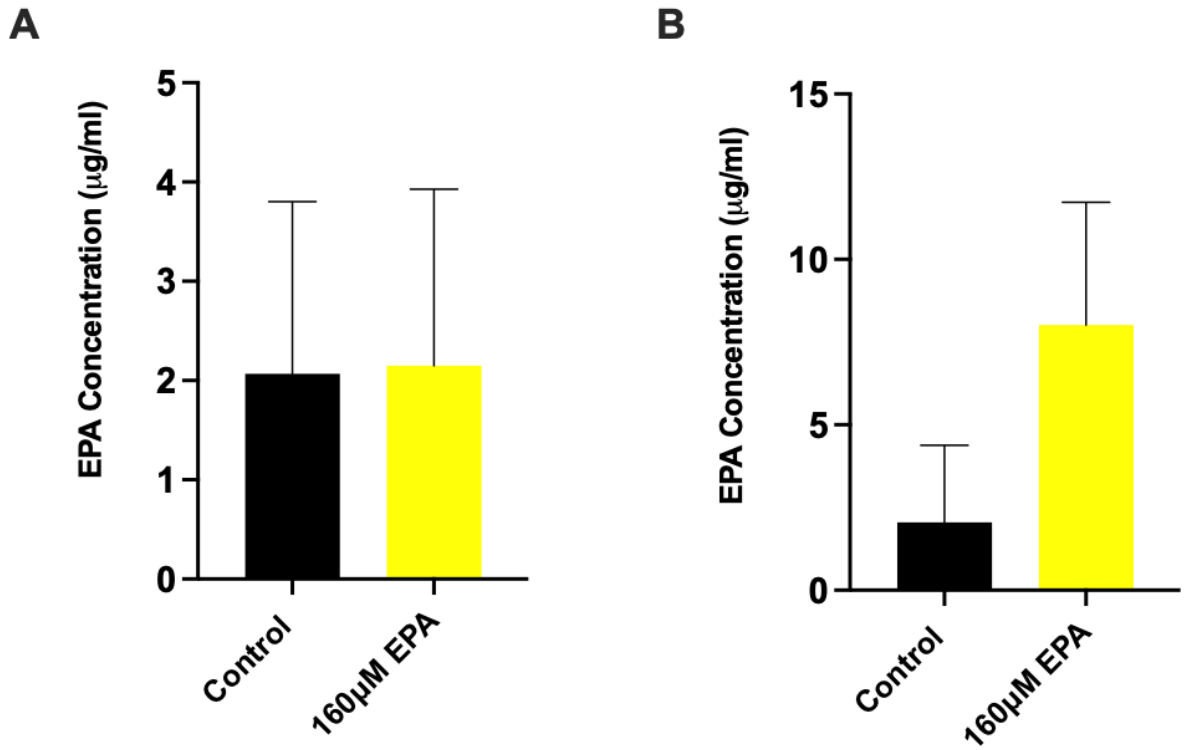

Figure C5. Uptake and Incorporation of supplemented 160µM EPA by *E. coli* and *C. elegans*. A) *E. coli* OP50 was cultured on NGM plates supplemented with 160µM EPA. The bacterial lawn was harvested after five days of growth at 20°C, and FAMEs were synthesized for GC-MS analysis and EPA quantification.

B) L4 stage C. elegans were grown on NGM plates supplemented with 160µM EPA for five days at 20°C. The worms were harvested, and the extracted FAMEs were analysed with GC-MS to determine their fatty acid composition. The EPA content was calculated using the EPA calibration curve generated through GC-MS.

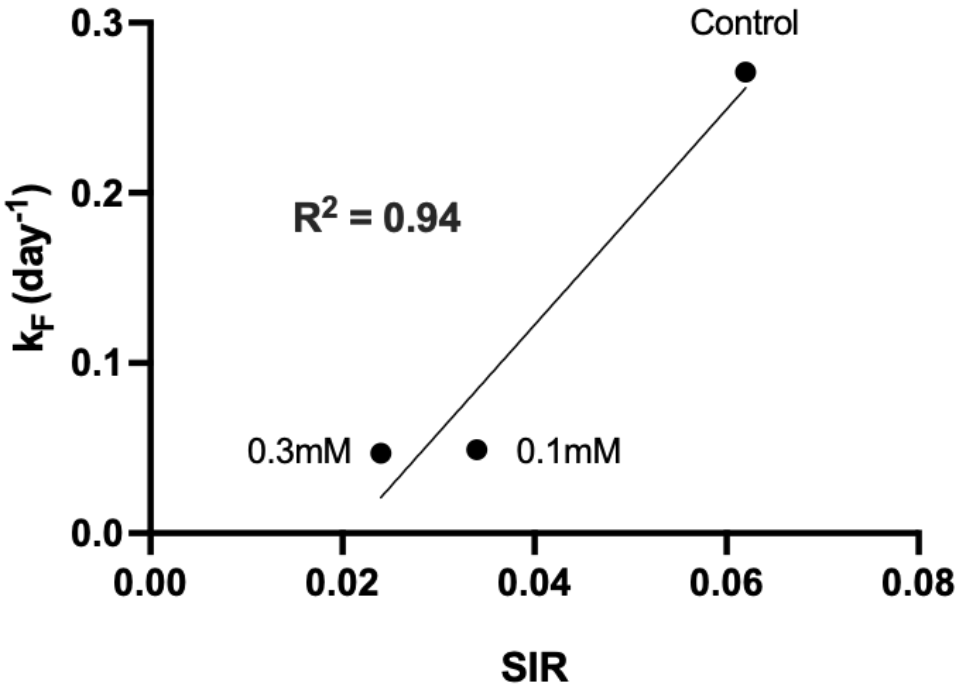

Figure C6. Rate of myosin loss (k_F) is correlated with the smurf increase rate (SIR) in algae oil supplemented groups.

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